



NATO Science for Peace and Security Series - A:
Chemistry and Biology

Portable Chemical Sensors

Weapons Against Bioterrorism

Edited by
Dimitrios P. Nikolelis



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Portable Chemical Sensors

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Series A: Chemistry and Biology

Portable Chemical Sensors

Weapons Against Bioterrorism

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Preface

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. Chemical sensors usually contain two basic components connected in series: a chemical recognition element (“receptor”) and a physicochemical transducer. The biological recognition system translates the chemical information (i.e., concentration of the analyte) into a chemical or physical output signal. The transducer (i.e., a physical detection system) serves to transfer the signal from the output domain of the recognition element to the electrical, optical, or piezoelectric, etc. domain. A biosensor is a self-contained integrated device which is capable of providing specific quantitative analytical information using a biological recognition element (e.g., enzymes, antibodies, natural receptors, cells, etc.) which is retained in direct spatial contact with a transduction element. Recent advances in the technology of artificial receptors have prompted a clear distinction between chemical sensors and biosensors. The latter utilize a transduction element of biological origin; however, since there has not been much development in engineered molecules, both terms are and can be used in the literature for this class of devices. The chemical sensors should be clearly distinguished from an analytical system which incorporates additional separation steps, such as liquid chromatography (LC) or additional hardware and/or sample processing such as specific reagent introduction, e.g. flow injection analysis (FIA). Biosensors have not yet made a large impact in the area of environmental, food, biomedical etc. applications, but clearly offer advantages in comparison to standard analytical methods, such as minimal sample preparation and handling, real time detection, rapid detection of the analytes of concern, use of non-skilled personnel, etc. Because of the importance of the ability of biosensors to be repeatedly calibrated, the term multiple-use biosensor is limited to devices suitable for monitoring both the increase and decrease of the analyte concentrations. Thus, single-use devices which cannot rapidly and reproducibly be regenerated should be named single-use.

The new biosensor technology has significant technological advantages when compared to that of the traditional non-sensor based detection methods. Portable and handheld biosensors, for example, such as dynamic DNA and protein arrays for

rapid and accurate detection of pathogens are typical examples on how biosensor technology can contribute in the defence against bioterrorism. For example, vesicles for use in biosensors have both high specificity and sensitivity, where the vesicles include a receptor specific for the intended analyte and a signal generating component.

A large class of chemical and biological sensors were based on the physical characterization of interfaces. More specifically, electronic (bio)chemical sensing is often related to the characterization of interfaces between ion-based and electron-based conductive materials by means of electrical variables such as voltage, current and charge. Also recent trends of integrated electronics that have started a revolution in this field allowing the shrink of very complex electronic systems into millimetre square sizes were a follow-up in the literature. This would allow implementing complex and sophisticated instrumentation in cheap and portable devices for a fast detection of harmful and toxic agents.

The aim of this book is to bring into focus this important research area and advances of biosensors and more specifically to those related to the rapid detection of weapons of bioterrorism. The object is related to present advances in the development of portable chemical sensors for the rapid detection of chemical weapons of terrorism; the scope is related to provide a comprehensive review of the research topics most pertinent to the advances of devices that can be used for the rapid real-time detections of toxicants such as microbes, pathogens, toxins, nervous gases eg. as botulinium toxin, *Escherichia coli*, *K. Pneumoniae*, sarin, VX, *listeria monocytogenes*, salmonella, marine biotoxins (such as palitoxins, spirolides, etc.), staphylococcal enterotoxin B, saxitoxin, gonyautoxin (GTX5), francisella spore virus, *bacillus subtilis*, ochratoxin. Biosensors have found a large number of applications in the area of environmental, food and biomedical analysis. Recent advances include portable devices for the rapid detection of insecticides, pesticides, food hormones, toxins, carcinogenic compounds in environment, such as polycyclic biphenols, etc. Despite the public's anticipation that biosensors with real-time detection will be able to monitor biological and chemical weapons, the technology has not caught up with expectations. Presently, biosensors in environmental monitoring stations nationwide can detect compounds like anthrax—but detection can take 12–24 h. The best ones on the market take 20 min. The detection of explosives and especially of biomolecules (bacteria) is an important part of security and defence activities. Technology has enhanced the possibility of combining the functions of drugs and explosives through different bio-cells in the same analysis module. A testing platform has been developed that has built-in configuration flexibility, which allows it to be used for different applications such as chemical, explosives, drug and biological agents detection. The characteristics of detection are determined by the combination of bio-cells. It is a new biosensor technology and helps users combine a number of different detection tasks within the same test, without having to reprogram the instrument.

Biosensors have not yet a large impact in the area of rapid detection of chemical and biological agents and other weapons of terrorism. Terrorists may use biological agents because they are rather easy to prepare and can be extremely difficult to

detect. A biological weapon is useful to terrorists mainly as a method of creating mass panic and disruption to a society.

Types of agents include anthrax, smallpox, botulinum toxin, bubonic plague, epsilon toxin of *Clostridium perfringens*, food threats (e.g., *Salmonella species*, *E. coli* O157:H7, *Staphylococcus aureus*), staphylococcal enterotoxin B, typhus (*Rickettsia prowazekii*), viral encephalitis, water supply threats (e.g., *Cryptosporidium parvum*), etc. In fact these agents are emerging pathogens that might be engineered for mass dissemination because availability, easy to produce and disseminate, and possess high mortality or a major health impact.

Most reports in the literature suggested that biosensors were at a pre-competitive stage, but highlighted laboratory proof-of-concept. Presently, we are looking into portable and handheld biosensors, for example, such as for example dynamic DNA and protein arrays for rapid and accurate detection of pathogens. A few challenges for biothreat detection had a high sensitivity – *detect* very small amounts of pathogens, toxins, and chemical agents, high selectivity – *discriminate* targets from other materials, massively parallel to detect multiple pathogens, minimize false positive, have rapid response, without sample preparation, and inexpensive, To have high spatial resolution, time resolution, selectivity and sensitivity for chemical and biosensors nanowires and potentiometric measurements were used. Techniques which in principle give some nanometer resolution of the area where we want to measure were presented in the literature.

Electrochemical systems based on inhibition of acetylcholinesterase suggest that the detection of nerve agents can be accomplished with fast speed and sensitivity down to fmolar levels. Detection routes using antibodies and DNA provide many advantages such as high sensitivity of detection, selectivity and can be used by non-skilled personnel. A wealth of ideas for portability of the sensors was recently presented in the literature. Electroanalytical and optical strategies involving exploiting methods based on the use of immunosensors and genosensors were presented. The combination of screen-printed electrodes with functionalized magnetic beads constitutes a powerful and efficient strategy for the development of disposable magneto-biosensors for the rapid and ultrasensitive detection of many analytes of bioterrorism significance. Magnetic micro- and nanoparticles have a large active surface area which makes possible the immobilization of a high concentration of biomolecules onto the solid phase of the transducer as well as a decrease of matrix effects.

Protein and even cell detections methodologies with interest for various applications were based on nanotechnology (i.e. nanoparticles, nanochannels). Nanoparticles based immunosensing systems were offered as excellent screening alternatives to sophisticated and high cost equipments that require well prepared professionals for their use, including data treatment, prior obtaining of final results with interest for further decisions taken in analysis/screening scenarios.

Development of a sensitive and specific biosensor for rapid detection of microorganisms and protein toxins often requires the information on the identity of the analyte of interest. On the other hand, recent advances in microbiology and biotechnology have lead the possibility of creating new microorganisms as well as production

of new protein toxins with completely or partially unknown DNA or protein sequences. Thus, rapid identification of microorganisms and protein toxins not only enables the detection of the bio-agents but also facilitates the development of highly portable biosensors for field use.

Biosensors based on DNA aptamers are of growing interest due to their high sensitivity and selectivity comparable with that of antibodies. Aptamers can be chemically modified by biotin, thiol or amino groups, which allowing them to be immobilized on various solid supports. Aptamers can thus be considered as a valid alternative to antibodies or other bio-mimetic receptors, for the development of biosensors. The production of aptamers is commonly performed by the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process, which, starting from large libraries of oligonucleotides, allows the isolation of large amounts of functional nucleic acids by an iterative process of in vitro selection and subsequent amplification through polymerase chain reaction. Aptamers are suitable for applications based on molecular recognition as analytical, diagnostic and therapeutic tools.

Overall, the impact of biosensors for the rapid detection of chemical and biological agents and other weapons of terrorism can be realized. This will require focused further experimental work on the following challenges:

1. High sensitivity – i.e., detection of very small amounts on the order of fmolar range of pathogens, toxins, and other chemical agents.
2. Highly selective – *discriminate* target compound from similar materials
3. Simultaneous detection of multiple pathogens and multiple molecular signatures of the same pathogen to minimize false positive responses
4. Have rapid response, without sample preparation
5. Transportable or handheld, inexpensive, robust, simple to operate
6. Real-time monitoring of protein binding to aptamers at single molecule level.
7. Selectivity of protein detection, i.e., selectivity of human thrombin detection by anti-thrombin aptamers

The ongoing war on terrorism and the rising security concerns are driving the need for newer faster detection biosensors against bio-warfare agents for military and civil defence applications.

The volume brings together contributions from the most eminent international researchers in the field, covering various aspects work that has not published in any scientific journal and science beyond the “state of art” in this field or even commercial units that are available in the market. Very low detection limits e.g. 10^{-17} M are reported and novel detection schemes for existing or new pathogens, toxins and other bioterrorism weapons are presented. Inexpensive, robust portable miniaturized highly selective biosensing systems that were able to detect multi pathogens without sample preparation are the targets of the present review articles; this will give the opportunity to learn new technological schemes that will lead to the construction of devices against bioterrorism that will minimize the risk of weapons of terrorism. Classical detecting schemes such as electrochemical, surface plasmon resonance (SPR), field effect transistors, quartz piezoelectric acoustic method,

electrochemical impedance spectroscopy (EIS), and photoluminescence and other optical physical sensors are presented.

Work on rapid identification of microorganisms and protein toxins by proteomics is an issue that is well presented and the participants of the volume have the opportunity to present their techniques on how to bind the transduction element into the physical sensor unit. Novel techniques of transduction such as nanowiring, nanoparticles or molecular imprinted polymers are presented and mainly some of these transducers are able to be implanted in the human body. Target analytes detected include a wide range of microbes, pathogens, toxins, nervous gases such as botulinum toxin, *Escherichia coli*, *K. Pneumoniae*, sarin, VX, *listeria monocytogenes*, *salmonella*, marine biotoxins (such as palitoxins, spirolides, etc.), staphylococcal enterotoxin B, saxitoxin, gonyautoxin (GTX5), *francisella* spore virus, *bacillus subtilis*, ochratoxin A, cholera toxin, etc. Emphasis is given to the simultaneous analysis of multiple species screen printed low density microelectrode arrays that were used to develop genosensors to detect *Salmonella* SSP and *staphylococcus aureus*. Scanning electrochemical microscopy is used to monitor *listeria monocytogenes*. An approach of detection using chronoamperometry coupled to microfluidics is developed to monitor *legionella pneumonophila*. Chemical transduction biological elements acting as “receptor” such as antibodies, enzymes, DNA, RNA, lipids, natural and artificial receptors were immobilized on the physical sensor to recognize the target analyte.

In preparing the book, we have relied on the timely contribution of authors, without whose expert insight, motivation and commitment the publication of this volume would not have been possible. We, thus, extend appreciation to all the authors. We also convey our thanks to Springer for affording us the opportunity to publish this volume and to the editorial and publishing staff, in particular Wil Bruins, for their assistance, organization and efficiency in coordinating the timely preparation and production of the book. We are also grateful to the North Atlantic Treaty Organization for their valuable support of the Advanced Research Workshop on Portable chemical sensors for the rapid detection of chemical and biological agents and other weapons of terrorism, Lund, Sweden, which served as the original impetus for the publication of this volume.

Dimitrios P. Nikolelis

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Chapter 1

Portable Biosensors for the Rapid Detection of Biochemical Weapons of Terrorism

Georgia-Paraskevi Nikoleli, Dimitrios P. Nikolelis, and Nikolaos Tzamtzis

Abstract The present review article describes the weapons of bioterrorism, history, types of agents used and recent advances of chemical sensors for the rapid detection of chemical and biological agents and other weapons of terrorism. The release of viruses, bacteria, or other germs used to cause illness or death in people mainly, animals, or plants can be found in nature and mainly are prepared to cause diseases or death. These biological weapons are used by terrorists as a method of creating mass panic and disruption to a society. Therefore it is of primary concern to develop biosensors to detect these terrorism weapons prior to their use. Novel recent biosensor technology has significant technological advantages when compared to that of the traditional detection methods. However, biosensor technology still needs to construct a portable device for the rapid detection of bioterrorism weapons that can be used in airports and other cases.

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Keywords Biosensors • Rapid detection and quantification • Viruses • Bacteria • Toxins • Weapons of terrorism

1.1 Introduction

A *bioterrorism attack* is the deliberate release of viruses, bacteria, toxins, or other germs (agents) used to cause illness or death in people mainly, animals, or plants. These agents are found in nature, but it is possible that they could be prepared with increased ability to cause diseases or death. Biological agents can be spread through the air, through water supplies in cities, in airports, or in food. Terrorists may use biological agents because they can be extremely difficult to detect and sometimes do not cause illness for several hours to several days. Some bioterrorism agents, like the smallpox virus, can be spread from person to person and some, like anthrax, cannot [1].

Bioterrorism is an attractive weapon because biological agents are relatively easy and inexpensive to obtain or produce, can be easily disseminated, and can cause widespread fear and panic beyond the actual physical damage they can cause [2]. Military leaders, however, have learned that, as a military asset, biological warfare has some important limitations; it is difficult to employ a bioweapon in a way that only the enemy is affected and not friendly forces. A biological weapon is useful to terrorists mainly as a method of creating mass panic and disruption to a society. However, technologists have warned of the potential power which genetic engineering might place in the hands of future bio-terrorists [3].

Some typical examples of terrorism are the following:

1984 – USA – Rajneeshee bioterror attack

In 1984, followers of the Bhagwan Shree Rajneesh attempted to control a local election by incapacitating the local population. This was done by infecting salad bars in 11 restaurants, produce in grocery stores, doorknobs, and other public domains with *Salmonella typhimurium* bacteria in the city of The Dalles, Oregon. The attack infected 751 people with severe food poisoning. However, there were no fatalities. This incident was the first known bioterrorist attack in the United States in the twentieth century [4].

1993 – Japan – Aum Shinrikyo anthrax release in Kameido

In June 1993 the religious group Aum Shinrikyo released anthrax in Tokyo. Eye witnesses reported a foul odor. The attack was a total failure, infecting not even a single person. This case shows how difficult it is to aerosolize anthrax spores in high concentration [5].

2001 – USA – Anthrax Attacks

In September and October 2001, several cases of anthrax broke out in the United States in the 2001 anthrax attacks, caused deliberately. Letters laced with infectious anthrax were delivered to news media offices and the US Congress. The letters killed five people. Tests on the anthrax strain used in the attack pointed to a domestic source, possibly from the biological weapons program.

1.2 History

1.2.1 *Early Usage*

Biological terrorism dates as far back as ancient Roman civilization, when feces were thrown into faces of enemies. This early version of biological terrorism continued on into the fourteenth century where the bubonic plague was used to infiltrate enemy cities, both by instilling the fear of infection in residences, in hopes that they would evacuate, and also to destroy defending forces that would not yield to the attack.

Over time, biological warfare became more complex. Countries began to develop weapons which were much more effective, and much less likely to cause infection to the wrong party. One significant enhancement in biological weapon development was the first use of anthrax. Anthrax effectiveness was initially limited to victims of large dosages. This became a weapon of choice because it is easily transferred, has a high mortality rate, and could be easily obtained. Also, variants of the anthrax bacterium can be found all around the world making it the biological weapon of choice in the early nineteenth century. Another property of anthrax that helped fuel its use as a biological weapon is its poor ability to spread far beyond the targeted population.

1.2.2 *Twentieth Century*

By the time World War I began, attempts to use anthrax were directed at animal populations. This generally proved to be ineffective. Germany launched a biological sabotage campaign in the United States, Russia, Romania, and France shortly after the start of World War I. Germany and its allies infected French cavalry horses and many of Russia's mules and horses on the Eastern Front. These actions hindered artillery and troop movements, as well as supply convoys [6].

Accusations of the use of biological weapons against North Korea were spread during the Vietnam war, however it is believed that those accusations were propaganda developed by the North Korean regime to villainize US Armed Forces. US President Richard M. Nixon announced his new policy on biological warfare at a press conference in the Roosevelt Room of the White House on November 25, 1969. "Biological weapons have massive, unpredictable, and potentially uncontrollable consequences," he declared. "They may produce global epidemics and impair the health of future generations". He then stated that in recognition of these dangers, the United States had decided to destroy its entire stockpile of biological agents and confine its future biological research program to defensive measures, such as vaccines and field detectors [7]. As the 1970s passed, global efforts to prevent the development of biological weapons and their use were widespread. On August 10, 1972, President Richard M. Nixon formally transmitted the Biological Weapons

Convention to the United States Senate for ratification. In his transmittal, he states: “I am transmitting herewith, for the advice and consent of the Senate to ratification, the Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons, and on their Destruction”, that was opened for signature at Washington, London and Moscow on April 10, 1972. The text of this Convention is the result of some 3 years of intensive debate and negotiation at the Conference of the Committee on Disarmament at Geneva and at the United Nations. It provides that the Parties undertake not to develop, produce, stockpile, acquire or retain biological agents or toxins, of types and in quantities that have no justification for peaceful purposes, as well as weapons, equipment and means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict [8].

In the 1980s Iraq made substantial efforts to develop and stockpile large amounts of biological weapons. By the end of the 1980s Iraq allegedly had several sites dedicated to the research and development of biological warfare. Since that time, efforts to use biological warfare has been more apparent in small radical organizations attempting to create fear in the eyes of large groups. Some efforts have been partially effective in creating fear, due to the lack of visibility associated with modern biological weapon use by small organizations.

1.3 Types of Agents

1.3.1 Category A

These high-priority agents pose a risk to national security, can be easily transmitted and disseminated, result in high mortality, have potential major public health impact, may cause public panic, or require special action for public health preparedness.

Anthrax

Anthrax is a non-contagious disease caused by the spore-forming bacterium *Bacillus anthracis*. An anthrax vaccine does exist but requires many injections for stable use. When discovered early anthrax can be cured by administering antibiotics (such as ciprofloxacin) [9].

Smallpox

Smallpox is a highly contagious virus. It is transmitted easily through the atmosphere and has a high mortality rate (20–40%). Smallpox was eradicated in the world in the 1970s, thanks to a worldwide vaccination program. However, some virus samples are still available in Russian and American laboratories. Some believe that after the collapse of the Soviet Union, cultures of smallpox have become available in other countries. Although people born pre-1970 would have been vaccinated for smallpox under the WHO program, the effectiveness of vaccination is limited since the vaccine provides high level of immunity for only 3–5 years. Revaccination’s protection lasts longer [10]. As a biological weapon

smallpox is dangerous because of the highly contagious nature of both the infected and their pox. Also, the infrequency with which vaccines are administered among the general population since the eradication of the disease would leave most people unprotected in the event of an outbreak. Smallpox occurs only in humans, and has no external hosts or vectors.

Botulinum Toxin

Botulinum toxin is one of the deadliest toxins known, and is produced by the bacterium *Clostridium botulinum*. Botulism causes death by respiratory failure and paralysis [11]. Furthermore, the toxin is readily available worldwide due to its cosmetic applications in injections.

Bubonic Plague [12]

Plague is a disease caused by the *Yersinia pestis* bacterium. Rodents are the normal host of plague, and the disease is transmitted to humans by flea bites and occasionally by aerosol in the form of pneumonic plague [13]. The disease has a history of use in biological warfare dating back many centuries, and is considered a threat due to its ease of culture and ability to remain in circulation among local rodents for a long period of time. The weaponized threat comes mainly in the form of pneumonic plague (infection by inhalation).

Viral Hemorrhagic Fevers [14]

This includes hemorrhagic fevers caused by the Filoviridae (Marburg and Ebola), and by the Arenaviridae (for example the Lassa fever and the Bolivian hemorrhagic fever). Ebola has fatality rates ranging from 50% to 90%. No cure currently exists, although vaccines are in development. Death from Ebola is commonly due to multiple organ failure and hypovolemic shock. Marburg was first discovered in Marburg, Germany. No treatments currently exist aside from supportive care. The arenaviruses have a greatly reduced fatality rate, but a larger presence, chiefly in central Africa and South America.

Tularemia [15]

Tularemia, or rabbit fever, has a very low fatality rate if treated, but can severely incapacitate. The disease is caused by the *Francisella tularensis* bacterium, and can be contracted through contact with the fur, inhalation, ingestion of contaminated water or insect bites.

1.3.2 Category B

Category B agents are moderately easy to disseminate and have low mortality rates.

- Brucellosis (*Brucella* species) [16]
- Epsilon toxin of *Clostridium perfringens*
- Food safety threats (e.g., *Salmonella* species, *E coli* O157:H7, *Staphylococcus aureus*)
- Psittacosis

- Q fever [17]
- Staphylococcal enterotoxin B
- Typhus (*Rickettsia prowazekii*)
- Viral encephalitis
- Water supply threats (e.g., *Cryptosporidium parvum*)

1.3.3 Category C

Category C agents are emerging pathogens that might be engineered for mass dissemination because of availability, easy to produce and disseminate, or because of may possess high mortality or a major health impact.

1.4 Recent Advances of Biosensors for the Rapid Detection of Weapons of Terrorism

Recent biological terrorism threats and outbreaks of microbial pathogens clearly emphasize the need for biosensors that can quickly and accurately identify infectious agents. The majority of rapid biosensors generate detectable signals when a molecular probe in the detector interacts with an analyte of interest. Analytes may be whole bacterial or fungal cells, virus particles, or specific molecules, such as chemicals or protein toxins, produced by the infectious agent. Peptides and nucleic acids are most commonly used as probes in biosensors because of their versatility in forming various tertiary structures. The interaction between the probe and the analyte can be detected by various sensor platforms, including quartz crystal microbalances, surface acoustical waves, surface plasmon resonance, amperometrics, and magnetoelastics. The field of biosensors is constantly evolving to develop devices that have higher sensitivity and specificity, and are smaller, portable, and cost-effective. The present article describes recent advances in biosensors for applications in the rapid detection of bioterrorism weapons

Although biosensors are exhibiting double-digit growth rates, they still have to overcome a number of challenges, including the following:

- New research focus less into fundamental research due to impeding introduction of newer applications
- Development of a single biosensor platform with multi-purpose diagnostics capability has restricted biosensor applications
- Numerous problems encountered in successful commercialization of biosensors have encouraged conservative development strategies
- Competition from non-biosensor technologies has hindered revenue growths
- Low rate of technology transfer and lower level of development has deterred the development newer biosensors

Given the current zeitgeist, the market thrust has shifted to biosensors' security capabilities amid the hot topic of biowarfare. A recent report from market research firm In-Stat revealed that the media spotlight on this application may be premature: Despite the public's anticipation that biosensors with real-time detection will be able to monitor biological and chemical weapons, the technology hasn't caught up with expectations. Presently, biosensors in environmental monitoring stations nationwide can detect compounds like anthrax—but detection can take 12–24 h. The best ones on the market take 20 min.

Below we describe some typical examples of biosensors for bioterrorism that were recently given in the literature.

Prof. O. Hendrickson group has developed analytical techniques to control toxic nanoparticles. Carbon nanoparticles (CNP) have their unique structure appear to be unconventional immunogens, which do not have inducers of immune response typical of native biopolymers. This may be an impediment to the production of CNP-specific antibodies. In addition, obstacles to solubilization of hydrophobic CNP in aqueous media or at least in polar solvents compatible with immunoassay are expected to be additional problems in immunoassay realization. In her studies, she developed the ELISA for the detection of not only modified water-soluble fullerene derivatives but in the first use of free fullerenes C_{60} and C_{70} and carbon nanotubes in solutions and in multicomponent biological probes [18].

The immunization of rabbits and mice with the conjugate of fullerene C_{60} carboxylic derivative with thyroglobulin synthesized by carbodiimide activation led to the production of three polyclonal antisera and eight clones of anti-fullerene monoclonal antibodies, correspondingly. The specificity of the antibody–fullerene binding was confirmed. Indirect competitive ELISA was developed not only for the determination of water-soluble protein-conjugated fullerene, the fullerene aminocaproic acid and fullerenol but for the first time for pristine fullerenes C_{60} and C_{70} and multi-walled carbon nanotubes (MWCNT) in solution. To solubilize extremely hydrophobic free fullerenes a specially selected water-organic mixture compatible with immunoassay was proposed. To obtain stable dispersion of MWCNT the latter were treated by inorganic acid and redissolved in ethanol. The detection limit of the proposed ELISA varied from 0.04 to 5.5 ng/mL for different CNP forms. Fullerene C_{60} was also detected by ELISA in organ homogenates of rats intraperitoneally or intragastrically administered with fullerene. To reduce the influence of biomatrices on the assay results a technique was developed for the biological sample pretreatment by the extraction of C_{60} from bioprobe by toluene followed by the evaporation of toluene and dissolution of the fullerene-containing extract in the selected water-organic media. The ELISA procedure in the first use allowed the detection of fullerene C_{60} in different tissues.

University California San Diego, La Jolla, USA (Prof. J. Wang group) has developed a nanomotor-based biosensing device that was able to perform: nanoscale motion transduction and isolation with applications in the detection of bioterrorism [19, 20]. Their developments include new motion-based bioassays based on changes in the speed or distance of nanomotors induced by biomolecular interactions or on the selective capture, sorting and transport of target biomolecules. Different motion

transduction schemes were illustrated for monitoring a wide range of biomolecular interactions of nucleic acids, proteins or cancer cells including key factors governing motion-based sensing. The resulting assays add new and rich dimensions of analytical information and offer remarkable sensitivity, coupled with simplicity, speed and low costs. The challenges of implementing molecular recognition into the nanomotor movement and for generating well-defined distance signals were investigated. Motion-based biosensing was able to detect 1.8×10^5 CFU/ μ L of *K. Pneumoniae*. Gold electrode was used as cathode and Pt electrode as anode to monitor hydrogen peroxide decomposition.

Università degli Studi di Firenze, Italy has recently exploited nucleic acid based electrochemical biosensors for the detection of bioagents. Emphasis was given to the simultaneous analysis of multiple species. Screen printed low density micro-electrode arrays were used to develop genosensors to detect *Salmonella* SSP and *Staphylococcus aureus* [21].

Prof. Włodzimierz Kutner, Institute of Physical Chemistry, Warsaw, Poland, has developed techniques that use molecularly imprinted polymers (MIPs) for the development of chemical sensors for the determination of amine contamination [22]. The chemosensors were based on recognizing units of MIP films and the 10-MHz quartz crystal resonators with Pt electrodes serving as piezoelectric micro-gravimetry (PM) transducers of analytical signals [22, 23]. The target amines initially played the role of templates for imprinting. For that, first, the amine templates were complexed in solution by suitably selected functional monomers bearing either the benzo-(18-crown-6) [22–25] or 3,4-dihydroxyphenyl [25], or dioxaborinane [22, 24] substituent. Advantageously, all these monomers contained electrochemically polymerizing bis(2,2'-bithienyl)methane moieties. Therefore, the complexes were electrochemically polymerized under simultaneous potentiodynamic and PM conditions to form amine-templated MIP films directly on the Pt/quartz electrodes. Next, the templates were extracted from MIPs. Sensitivity and selectivity of the MIP films were largely increased if MIPs were networked with a bis(bithiophene-benzothiophene) cross-linking monomer. Additionally, an ionic liquid, used as a porogenic solvent for electropolymerization [23], enhanced the sensitivity. Analytical performance of the chemosensors was assessed under FIA conditions with the PM detection at QCM. The linear concentration ranges extended over several decades. The lower limits of detection were at the nanomole concentration levels. From kinetic studies, stability constants of complexes of the analytes or interferants with MIPs were determined. The chemosensors well discriminated the amine analytes from their common interferants.

University of Bucharest, Romania (Prof. Camelia Bala's group) has challenged the design of nano and biosensing materials as sensors for toxic compounds. Research was made on imidazolium-based ionic liquids carbon nanotubes (IL-CNTs) modified electrodes oriented towards biosensing applications involving the acetylcholinesterase [26]. The sol-gel matrix, having excellent properties of physical rigidity and chemical inertness, was used as stable and biocompatible matrix for the immobilization of the enzyme [27]. The ILs-CNT biosensors were demonstrated to offer the lowest potential reported and significant signal

enhancement effect [26]. Their bioelectroanalytical application of biosensors modified with IL-CNTs gel and the strategies for enhancing sensing performance was discussed. Emphasis is given to the application of enzyme-modified composite electrodes in direct detection of substances that disrupt the chemical communications through the nervous system by blocking the acetylcholinesterase, an enzyme that normally destroys and stops the activity of acetylcholine, a neurotransmitter [28]. AChE-based biosensors were developed for parathion and other toxic gas agents. Also biosensor devices for endocrine disruptors were developed using the ILs-CNT electrodes [29].

Prof. Ana Maria Oliveira-Brett, Coimbra University, Portugal, has investigated DNA-electrochemical biosensors for investigating DNA oxidative damage for the rapid detection of cyanobacterial hepatotoxins [30]. The electrochemical behaviour of microcystin-LR (MC-LR) and nodularin (NOD) at a glassy carbon electrode was investigated using cyclic, square-wave and differential pulse voltammetry [31, 32]. The oxidation is an irreversible, diffusion-controlled and pH-independent process that occurs with the transfer of only one electron. Chemical degradation of MC-LR and NOD, upon incubation in different pH electrolytes, was electrochemically detected by the appearance of a new oxidation peak at a lower potential, mainly in mild acid media. The MC-LR and NOD chemical degradation product, formed homogeneously in the buffer solution, undergoes an irreversible pH-dependent oxidation. The *in situ* evaluation of the MC-LR and NOD interaction with dsDNA using a DNA-electrochemical biosensor [33] showed that both MC-LR and NOD caused dsDNA structural modifications in a time-dependent manner.

Electroanalytical strategies involving methods exploiting methods based on the use of immunosensors and genosensors were developed by J. Pingarrón's group (of Complutense University of Madrid). The combination of screen-printed electrodes with functionalized magnetic beads constitutes a powerful and efficient strategy for the development of disposable magneto-biosensors for the rapid and ultrasensitive detection of many analytes of biochemical and clinical significance. Magnetic micro- and nanoparticles have a large active surface area which makes possible the immobilization of a high concentration of biomolecules onto the solid phase of the electrochemical transducer as well as a decrease of matrix effects.

In particular, disposable amperometric magnetoimmunosensors for the specific detection of *Streptococcus pneumoniae* was recently described in the literature [34]. Furthermore, the preparation of disposable DNA magnetosensors was recently discussed. In these designs, the use of electrochemical DNA biosensors with magnetic beads, PCR amplification and the use of disposable screen-printed electrodes were implemented. This type of design allowed the development of amperometric magnetogenosensors for the specific detection of a gene related to the *Enterobacteriaceae* bacterial family, based on the coupling of streptavidine-peroxidase to biotinylated *lacZ* gene target sequences [35]. As low as 2.5 aM asymmetric PCR product could be detected with the developed methodology. A further approach involved the use of direct asymmetric PCR amplified products for *E. coli* detection at a concentration level of 1 cfu/100 mL with no need for culture preconcentration steps [36]. Similar

strategies were employed to develop DNA sensors for the detection of a characteristic 235-bp region of the gene coding for autolysin (*lytA*), a specific *pneumococcus* virulent factor [37].

Comenius University, Slovakia (Prof. T. Hianik's group), has investigated DNA aptamer biosensor [38] sensitive to mycotoxin – ochratoxin A (OTA). The label-free detection of OTA was based on a thickness shear mode acoustic method (TSM) and on electrochemical impedance spectroscopy (EIS). Addition of OTA to the TSM sensor surface with immobilized biotinylated DNA aptamers resulted in decrease of frequency, f_s and increase of resistance, R_m . OTA was detected with limit of detection of 30 nM [39]. Subnanomolar sensitivity of detection OTA was obtained by the EIS method. The thiolated DNA aptamers specific to OTA were immobilized by chemisorption to a surface of gold electrode. The sensor revealed high selectivity and was regenerable by washing with 1 mM HCl. The biosensor for ochratoxin was applied for the determination of this toxicant in real samples of foods that mimic those contaminated by bioterrorists.

University of “Tor Vergata” Rome has investigated electrochemical biosensors for nerve agent detection. Most of the toxic common chemical weapons—the nerve agents—can be prepared using relatively unsophisticated facilities and in quantities sufficient for terrorist attacks. Due to the high toxicity of some organophosphates the detection system has to be rapid and easy to use. The developed biosensor was based on the use of cholinesterase enzyme coupled with an electrochemical device. When the cholinesterase biosensor is exposed to organophosphate compounds, an inhibition due to the nerve agent will occur and the resulting decrease of the signal will be related to the amount of the toxic agent. Butyrylcholinesterase (BChE) was immobilized on screen printed electrodes modified with Prussian Blue (PB-SPE). The electrochemical measurement was carried out using a portable PalmSens instrument. The optimised biosensor was tested with Sarin and VX standard solutions showing detection limits of 12 and 14 ppb (10% of inhibition), respectively. The enzymatic inhibition was also used to the rapid detection to Sarin in gas phase. Two different concentrations of Sarin gas (0.1 and 0.5 mg/m³) at different incubation times (from 30 s up to 10 min) were tested. It is possible to detect Sarin at the concentration of 0.1 mg/m³ after 30 s incubation time, with a degree of inhibition of 34% that matches the legal limits (IDLH, immediate danger to life and health) [40].

The development of a system able to detect the nerve agent and able to be assembled in a soldier was also reported from the same group. This miniaturised electronic system in which the biosensor was developed and tested with the pesticide paraoxon as the nerve agent. The system is able to give an alarm in few minutes demonstrating the possibility to develop a miniaturised and easy to use analytical system that allows the nerve agent detection using biosensor approach.

Immunobioassays for toxin and bacteria detection were recently reported from Prof. G. Palleschi group. Electrochemical immunosensors based on thick film technology were presented. Mass production and miniaturisable electrochemical transducers (screen printed electrodes, SPE) coupled with a portable potentiostat (Palm Sens instrument) were prepared using through this technique. The SPEs were

employed to develop an enzyme-linked immunosorbent assay (ELISA) for the detection and quantification *Staphylococcus aureus* [41]) a well-known air-borne organism and Saxitoxin [42] one of the most lethal non-protein toxins and known as one of the “Paralytic Shellfish Poisons” (PSP). In the case of saxitoxin, the characterisation and optimisation of electrochemical immunoprobes for its detection and their application using portable instrumentation were studied. Using the SPEs as signal transducers and as supports for the biocomponent immobilisation, a linear range between 1 and 1,000 ng/mL for saxitoxin was attained.

Ochratoxin A (OTA), a mycotoxin produced by several species of *Aspergillus* and *Penicillium* fungi and Aflatoxins (AF), (produced as secondary metabolites by several species of *Aspergillus* fungi) can be also detected using the previous biosensor constructed by Prof. G. Palleschi [43]. A fiber optic system coupled to portable and miniaturised spectrophotometer/fluorometer was developed to allow the detection of both OTA and AFB [43–46]. A linear range of 2–80 ng/mL was obtained for OTA, while in the case of AFB 10–60 ng/mL was assessed as linear range. These novel analytical methods have the advantage to use the same portable instrument and the same immunoaffinity columns (able to bind both AFB and OTA, commercial available) for OTA and AFB detection.

Prof. G.A. Evtugyn group from Kazan University, Russia has developed electrochemical biosensors for nerve agent detection based on nanostructured biorecognition layers. In his work, a new approach to the development of electrochemical cholinesterase sensors involving application of multifunctional macrocyclic ligands for nanostructuring the biorecognition layers has been examined for the detection of organophosphates. The detection of acetylcholinesterase activity was performed by Co phtalocyanine mediated oxidation of thiocholine formed in enzymatic hydrolysis of acetylthiocholine, an artificial substrate. The screen-printed electrode covered with polycarboxylated thiacaalix[4]arene derivatives was used as a sensor transducer.

As was shown by AFM on highly oriented pyrographite support, the polycarboxylated thiacaalix[4]arenes formed flattened round particles of 80–120 nm in size separated by an even film 2–4 nm thick [47]. The acetylcholinesterase was immobilized by carbodiimide binding. This morphology of the surface provides high accessibility of the reactants to the enzyme active site and diffusional permeability of the surface layer for charged species. As a result, the acetylcholinesterase sensor showed fast and reversible response toward acetylthiocholine at low working potential (100–250 mV). The working conditions of the surface layer assembling were established to reach maximal sensitivity toward irreversible inhibitors. The effect of the number of carboxylic terminal groups of thiacaalix[4]arene moiety and of the conformation of the macrocyclic ligand on the signal characteristics toward enzyme substrate and inhibitors was established. The acetylcholinesterase sensors developed make it possible to detect down to 0.1 ppb of paraoxon and 1 ppb of malaoxon as model anticholinesterase nerve agents [48]. In comparison with similar sensors prepared with no macrocyclic ligands in the sensing layer and those containing carbon nanotubes as enzyme and mediator carriers, significant sensitivity improvement and limit of detection decrease have been achieved [49]. The acetylcholinesterase sensors

developed were tested using spiked samples of tap waters. Possible influence of electrostatic interactions and steric factors on the sensitivity of inhibition detection is considered and the potentialities of nanostructured biorecognition layers discussed.

Bengt Danielsson from Lund University has developed fluorimetric bioprobes for genotoxicity based on DNA-Dye intercalation [50–52]. The biosensor that was developed employs long-wavelength intercalating fluorophores like TO-PRO-3 (TP3). Compounds that interact with the TP3-DNA complex are indirectly detected by a decrease in the fluorescence intensity. A fiber-optic capillary fluorescence system was finally developed as a biosensing device to analyze toxic aromatic amines and several kinds of antitumor toxic compounds, using small amounts of sample, down to 10 μL , with high sensitivity and detection limit as low as a few ppb. This approach is useful for routine broad range screening for monitoring of bioterrorism weapons or for detecting cytotoxic compounds. The ease of operation and the rapid response allow high-throughput screening. To enable sufficient high sensitivity to detect substances that may be used for bioterrorism, concentration enhancing methods such as solid phase extraction (SPE) can be used for sample collection.

1.5 Conclusions

Security and Biodefense is emerging as a strong market for new applications. The new biosensor technology has significant technological advantages when compared to that of the traditional detection methods; for example, vesicles for use in biosensors have both high specificity and high sensitivity, where the vesicles include a receptor specific for the intended analyte and a signal generating component.

We are looking into portable and handheld biosensors, for example, such as dynamic DNA and Protein Arrays for rapid and accurate detection of Pathogens. Bioweapons are extremely damaging and efficient. Progress of biotechnology opens also new ways for weaponizing pathogens.

Challenges for biothreat detection are:

1. High sensitive – detect very small amounts of pathogens, toxins, and chemical agents.
2. Highly selective – discriminate targets from other materials
3. Massively parallel to detect multiple pathogens, minimize false positive, have rapid response, without sample preparation
4. Transportable or handheld, robust, simple to operate
5. Inexpensive
6. Adaptable to new biothreats, integrated chemical-biosensor
7. and finally allow the detection of single molecules

Targets to achieve are:

1. Single RNA molecule detection
2. Real-time monitoring of RNA hybridization at single molecule level

3. Single DNA molecule detection
4. Real-time monitoring of protein binding to aptamers at single molecule level
5. Hybridization of synthetic target DNA with anti-anthrax
6. Selectivity of protein detection, i.e., Selectivity of human thrombin detection by anti-thrombin aptamers
7. Simultaneous detection of multiple pathogens and simultaneous detection of multiple molecular signatures of the same pathogen to minimize false positive responses
8. Kinetics of DNA hybridization. Ratiometric measurements

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Chapter 2

New Challenges in the Design of Bio(Sensors) for Biological Warfare Agents

Camelia Bala

Abstract The chapter highlights new concepts for the design of biosensing material in order to develop sensors for different biological warfare agents. Biosensing technologies that use electrochemical, piezoelectric, optical, acoustic and thermal transducers for detection of pathogenic bacteria are highlighted. Special attention is paid to methods for improving the sensitivity and analysis time of biosensors. Recent developments in physical transducers in biosensors for bacterial detection are overviewed.

Keywords Sensor • Sensing • Biological warfare agents

2.1 Introduction

The rapid and sensitive detection of toxic compounds is of great importance not only for human health but also for the establishment of homeland security against bioterrorism. Many biological and chemical agents such as bacteria, algae, fungi, viruses and toxins are capable of extensively affecting humans and animals. In terrorist attack actions or during military operations with biological warfare agents (BWA) food or water supplies can be contaminated [1].

The most conventional detection methods of toxic compounds based on bacteriology, serology–immunology and molecular biology, are usually insensitive, cross-reactive, and cost-intensive [2]. Because bioterrorism is difficult to predict or

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prevent, reliable platforms to rapidly detect and identify biothreat agents and to meet new challenge as fast and on-site detection of toxic compounds.

The biosensors have been developed as devices in which a biochemical recognition element (e.g. enzymes, antibodies, microorganisms or deoxyribonucleic acid (DNA)) called bioreceptor is integrated or are in direct contact with a physico-chemical transducer (e.g. electrochemical, optical, thermal, piezoelectric) which converts the biological signal in a measurable analytical signal. This usually obtained as a continuous or semi-digital electrical signal is proportional to the concentration of a chemical compound (analyte of interest).

Research in the field of chemical and biochemical sensors are one of the most dynamic areas of research in the last 30 years, manifested a keen interest in the production of new chemical and biological detection devices. During this period, investigations were orientated towards the characterization and modification of physical transducers, such as field effect transistors, semiconductor devices based on metal oxides, piezoelectric crystals and devices, surface acoustic wave (SAW). Also, classical electrochemical transducers have been developed and used both for chemical applications as well as biological ones. Recently, the construction of optical biosensors and optical methods apply techniques (i.e., plasmon surface resonance) has been extensively developed [3].

Biosensors have several potential advantages over other methods of analysis, including sensitivity in the range of ng/mL for microbial toxins and <100 colony-forming units (CFU)/mL for bacteria. Fast or real-time detection can provide almost immediate interactive information about the sample tested, enabling to take corrective measures before further contamination can occur. Miniaturization of biosensors enables biosensor integration into various equipment and device [4].

One of the important components of a biosensor is the selective membrane bioreceptor. This usually positioned on the edge sensor, interacts with the sample providing useful analytical information for a particular component of the sample. For this to be achieved membrane interaction with the sample must be selective and this is assured by the bioreceptor. The mechanisms of recognition in biosensors devices are:

- Electrostatic interactions;
- Acid-base reactions;
- Redox reactions;
- Antibody-antigen interactions;
- Enzyme-substrate interactions;
- Ion exchange reactions.

In order for biosensor technology to be effective in laboratory or field settings, it should analyze unknown samples in less than 15 min, with small sample volume and have sensitivity and specificity comparable to Enzyme-linked immunosorbent assay (ELISA) [5].

Biosensors platforms for BWA must not only be sensitive and specific, but must also be able to accurately detect a variety of pathogens, including modified or previously uncharacterized agents, directly from complex sample matrices [6].

The bioterrorism attacks have made public health officials acutely aware of the importance of rapidly and accurately detecting acts of bioterrorism. To address these considerations, the sensitive, easy usage and cost effective portable biosensors which are capable of providing continuous monitoring and rapid detection of various BWA need to be developed.

Furthermore, the discussion will be focused on performance of the biosensors for biological warfare agents underlying the biosensor configurations as well as the materials used to build up those devices.

2.2 Biosensor for Biological Warfare Agents

The most reported biosensors for detection of biological warfare agents have been constructed with electrochemical transducers, but a number of other types have been used, and optical transducers in particular are growing in importance [7]. Considering the number of published papers on biosensors as well as the type of commercialized sensors, electrochemical systems appears to be the most favored transducers [8, 9]. Electrochemical transduction means that the biological recognition or conversion event is transferred to an electrical signal, which might be a current or a potential. Thus, two types of electrode systems can be distinguished: amperometric and potentiometric. A third group are impedimetric transducers, which detect impedance changes resulting from the analyte interaction with the receptor molecule.

Today, impressive achievements have been made at the cross-section of nanotechnology and biotechnology [10]. The emergence of nanotechnology has opened new horizons for electrochemical biosensors for detection of biological warfare agents [11]. New materials such as nanotubes, nanowires, nanofibers, or nanoparticles has revolutionized the biosensors field for pathogen detection [12].

Carbon nanotubes (CNTs) are recently used for modifying the electrode surface in order to enhance the electrochemical properties. Various chemical sensors and biosensors based on CNTs have been developed to detect some important species that are related to human health or food monitoring [10, 11, 13–16]. Thanks to their extraordinary properties CNTs have a high electrocatalytic effect, a fast electron transfer rate, and a large working surface area.

These days screen-printed electrodes are widely used because these are cheap and therefore, can be produced at large scale [17–21]. Disposable screen-printed biosensors have been successfully employed in the early detection of microorganisms, which plays an important role in the prevention of human health problems [22].

Thick film technology using a screen-printing procedure is a simple and fast method for mass production of disposable electrochemical sensors. Single-use sensors and biosensors have several advantages such as avoidance of contamination, elimination of problems of loss of response due to fouling of the electrode and of enzyme denaturation.

This technique consists of depositing inks of different electrochemical properties on an inert substrate in a film of controlled pattern and thickness. One of the most attractive characteristics of screen-printing technology is its versatility in being able to print large number of inks on different substrates and the possibility in mixing modifiers to the base ink.

The use of magnetic particles or magnetic beads as labels in biosensing has become a very important topic in analytical research in the last years [23–29]. Magnetic particles show advantages in the process of immobilization by increasing stability of the surface-bound antibody, improving orientation of the immobilized antibody as well as increase in the protein loading. Another advantage is the possibility of modifying the surface of the particle with functional groups useful in various processes of binding [30].

Magnetic particles or magnetic beads are micro/nano size spheres of mixed iron oxide covered with a polymeric material, which allows physical/covalent/affinity attachment, usually of the antibody (Ab), onto the particle surface. Despite their name *magnetic particles*, many of the commercial beads are superparamagnetic, meaning that these can immediately be magnetised with an external magnetic field and redispensed immediately once the magnet is removed [28]. Therefore, the Ab-bead can be easily located avoiding the kinetic limitations of the process due to reagent diffusion. Also, the magnetic beads compared with the conventional solid supports used in immunoassays (e.g. ELISA microtitre plate polymer particles, etc.) have advantages of easy manipulation, good fluid-solid contact, high mass transfer rate, perspectives for system automation and miniaturization.

Recent advancements in surface plasmon resonance immunosensors for detection of small molecules of biomedical, food and environmental interest have been reported by several groups [31–37]. Surface plasmon resonance (SPR) is one of the new optical methods which enable rapid toxin detection. SPR is a quantum electro-optical phenomenon: energy carried by photons of light can be coupled or transferred to electrons in a metal. This coupling leads to a group of excited electrons on the surface of the metal, called plasmon. The intensity of the plasmon is depending by the type of metal and the environment of the metal surface. A protein interactions on the surface of metal cause changes in plasmon resonance. These changes can be measured as a change in the angle of incidence or shift in the wavelength of light absorbed [38, 39]. Built on the principle of specific biological-recognition measurements coupled with a signal transducer, SPR-biosensor technology has developed over the past several decades, resulting in commercial production of versatile tools for many applications that greatly impact disciplines from the life sciences to the medical, pharmaceutical, biotechnology and agri-food industries [31]. Moreover, this method was already included in portable sensors for on-site determinations of toxins [7, 37].

Most prominent BWA includes mycotoxins, exotoxins and enterotoxins from *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Bacillus anthracis*, *botulinum*, *Listeria monocytogenes*, *Vibrio cholera* and *Yersinia enterocolitica*. This chapter article tends to discuss the bottleneck achievements in the fabrication and application of specifically biosensors for BWA detection.

2.2.1 Anthrax

Bacillus anthracis is the causative agent of anthrax disease and the need for fast detection methods has gained attention since 2001 when letters containing the spores were mailed in the United States [40]. Some biosensors were reported to meet this challenge in terms of sensitivity, specificity, time and cost-efficiency [41–47]. A label-free system was reported for *B. anthracis* detection based on DNA probe functionalized quartz crystal microbalance (QCM) biosensor which specifically recognize the target sequences within 30 min, with a detection limit of 3.5×10^2 CFU/mL of vegetative cells [42]. The signal amplification was done by using gold nanoparticle functionalized DNA probe.

Aptamer based analytical methods provide a new alternative way for the detection of virulent toxins. Systematic evolution of ligands by exponential enrichment technique (SELEX) was applied successfully to find single stranded DNA aptamers for detection of the PA 63, the key protein for the early detection of anthrax. Based on the principle of aptamer-based ELISA, an oligonucleotide-based biosensor was developed, to detect the target protein and consequently for *B. anthracis* detection [43].

Huan et al. report an electrochemical biosensor for protective antigen (PA) detection, a protein biomarker for the diagnosis of anthrax [45]. Instead of an antibody they use, as biorecognition element, a short chain peptide (26mer) which has the advantage to be biological stable and easy synthesizability, leading to a biosensor with a detection limit of 5.2 pM. The immobilization steps on gold electrode are presented in Fig. 2.1.

In order to improve the electron transfer through the sensing layer the Tran Ngoc et al. proposed a new type of biosensor using multi-wall carbon nanotubes covalently immobilized onto a polyaniline modified glassy carbon electrode [41]. The biosensor limit of detection was 0.4 pM and it proves good selectivity.

A portable optical biosensor for rapid (35 min), label-free, specific, and quantitative detection of 34 spores of *B. anthracis* spores was developed by Acharya et al. [47].

An inexpensive biosensor based on an oligonucleotide sandwich-hybridization assay format with reflectometer as detection system was reported by Baeumner et al. [48]. The biosensor measurement requires 15 min assay time and is inexpensive, providing on-site analysis with no cross-reactivity for 11 organisms tested.

Magnetoelastic materials as transducers for biosensors for *B. anthracis* spores in liquids have been investigated [49]. The magnetoelastic sensors are acoustic wave sensors that when an alternating magnetic field is applied a corresponding oscillating shape is generated giving rise to a mechanical vibration with a characteristic resonant frequency. This frequency changes are caused by the mass of attached compounds on the sensor platform. Filamentous phage immobilizing on the surfaces of magnetoelastic micro-particles were employed for the detection of *B. anthracis*. Filamentous phage has several key advantages over antibodies: three-dimensional recognition surface of phage provide multiple binding sites and can be

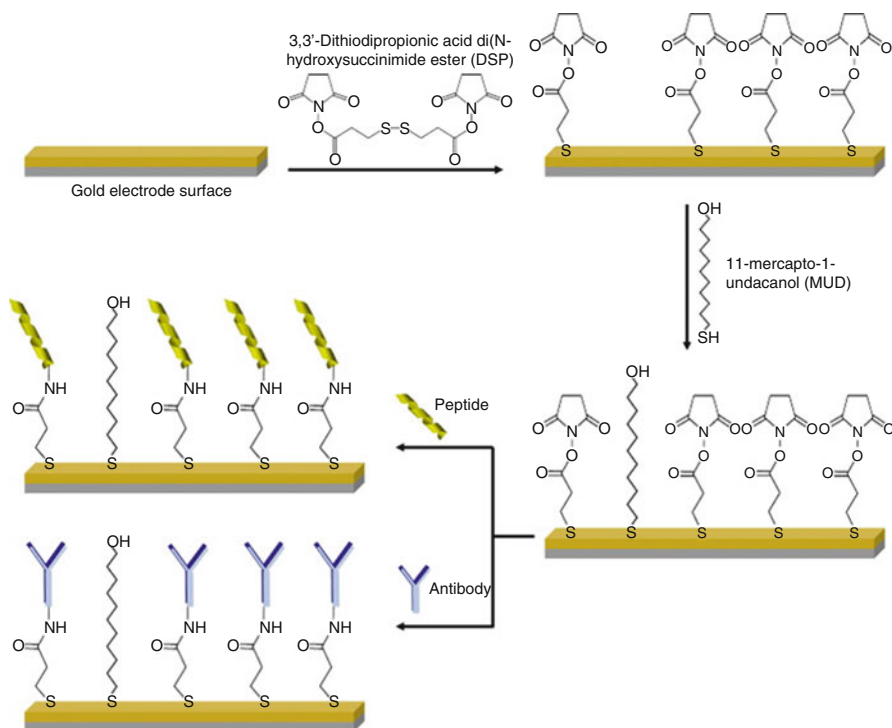


Fig. 2.1 Graphical descriptions of the preparation of the sensing layer using both peptide and antibody (With permission from Elsevier [45])

produced in large quantity at relatively low costs. The magnetoelastic iron–boron particles were fabricated using microelectronic processes and the magnetoelastic platform, has the following dimensions $500\ \mu\text{m} \times 100\ \mu\text{m} \times 4\ \mu\text{m}$. The biosensors could be used for testing the *B. anthracis* spore solutions with concentrations from 10^3 to 10^8 CFU/mL with a sensitivity of 6.5 kHz/decade.

2.2.2 *Botulinum Toxin*

Botulinum toxin is one of the deadliest toxins known, and is produced by the gram-positive bacterium species *Clostridium botulinum*, *C. barati*, and *C. Butyrium* [50]. Each from the seven Botulinum toxin serotypes (A–G) comprises a 100 kDa heavy chain and a 50 kDa light chain. The heavy chain facilitates entry into neurons via cell surface binding and receptor mediated endocytosis, whereas the light chain is the catalytic component that, once inside the cell, cleaves neuronal proteins necessary for neurotransmitter release [51]. The standard detection method is the mouse bioassay in which infected animals are observed for 2–4 days for signs of botulism

or death [52]. Alternate methods are based on sandwich immunoassays that require two specific antibodies – one for toxin capture, one for recognition after capture – as well as a detection antibody, thus adding to the cost and complexity of the assay [53–55]. Thus a high demand for rapid and sensitive detection of this toxin at low concentration in complex matrices is an important challenge. One of the major goals is to develop screening test requiring an hour or less to process the sample and to obtain the result.

The Ligler group reported in 1992 [56] the first immunoassay for botulinum toxin based on an optical fiber with capture antibodies immobilized on the surface. The modified surface was dipped into a solution containing the labeled detection antibody to create a sandwich complex. This approach achieved a limit of detection of 30 pM botulinum toxin A and an immunoassay took less than 20 min.

Ferracci et al. describe a label-free biosensor chip assay for botulinum neurotoxin B based on real-time surface plasmon resonance [57]. The biosensor detect the protease activity of botulinum neurotoxin B. Endoproteolytic cleavage of botulinum neurotoxin B substrate, vesicle-associated membrane protein 2 (VAMP2), was detected in 10 min with 2 pM native holotoxin. The sensor could be used for food safety applications and serological diagnosis of human botulism.

In 2009, a rapid screening approach based on fluorescence biosensor (sandwich immunoassay) with renewable surface was developed and it can detected 10 pM concentrations of botulinum neurotoxin in less than 20 min [58].

A new approach in biosensor design for botulinum neurotoxin A detection was a fluorescence detection platform using spatial electroluminescent excitation in order to develop a simple portable system [59]. The phosphor-based electroluminescent semiconductor strips were used for fluorescence excitation and biodetection. The lab-on-a-chip platform described in this study was designed with the aim of producing a portable, simple and inexpensive modular-based fluorescence detection system, capable of measuring botulinum neurotoxin A between 31 and 62 ng/mL, after only 2 h of incubation.

An antibody free solid phase to recognize Botulinum toxin type B from various matrices was described by Frisk et al. [25]. Micrometer-sized magnetic beads were conjugated to a synthetic 22 amino acid peptide derived from the synaptotagmin II (Syt II) neuronal protein that is specific for Botulinum toxin binding (Fig. 2.2).

Exposure to Botulinum toxin type B in buffer, whole milk and fruit juices resulted in toxin capture, which was confirmed using fluorescence detection method.

Peptide-modified beads were integrated into arrayed, polymeric microfluidic channels, and all assay steps, from capture to detection, were performed directly in the microchannels, thereby simplifying assay utility and increasing throughput relative to existing detection methodologies. The microscale sensor required only 7 μ L sample without any pre-processing steps. The total time of one assay is 1 h and it can detect 16.6 pg of Botulinum toxin type B in whole milk [25].

Botulinum toxin activity can be detected in vitro by utilizing the ability of the toxin to cleave a fluorophore-tagged peptide substrate specific for Botulinum toxin by the toxin light chain. Sun et al. designed a lab-on-a-chip biosensor platform which monitor the toxin light chain cleavage by Forster Resonance Energy Transfer (FRET) fluorescence [60]. The system enables detection of concentrations as low as

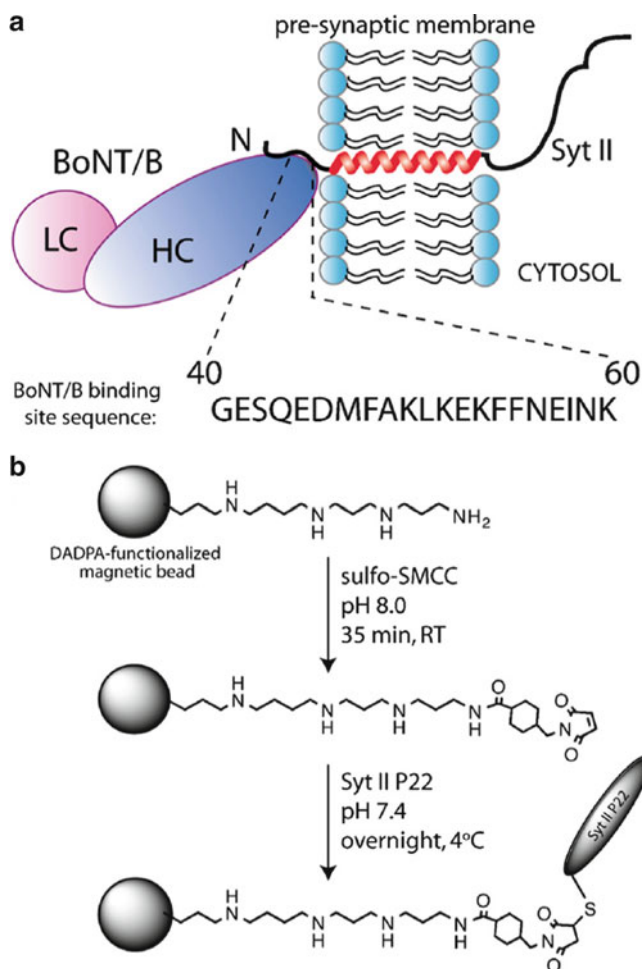


Fig. 2.2 (a) Botulinum neurotoxin type B (BoNT/B) recognizes and binds extracellular residues 40–60 of the synaptotagmin II (*Syt II*) transmembrane neuronal protein for translocation into the cell in vivo. (b) The Syt II 22mer peptide (P22) was synthesized to contain Syt II residues 40–60, in addition to a C-terminal cysteine for thiol-specific conjugation to magnetic beads via the hetero-bifunctional crosslinker sulfo-SMCC (With permission from Elsevier [25])

0.5 nM of toxin light chain in a FRET cleavage assay. Eight samples could be analysed simultaneously in 2 h with minimal sample handling or manipulation. Another advantage of the platform is that can be used for on field applications.

A custom built SPR biosensor could detect the three botulinum neurotoxin serotypes, A, B and F in less than 1 h with limits of detection between 0.5 and 1 ng/mL [61]. The biotinylated polyclonal antibodies for neurotoxin serotypes A, B, E and F were immobilized on the gold sensor chip modified with thiols. The signal amplification was done with a second antibody specific to the serotype being detected.

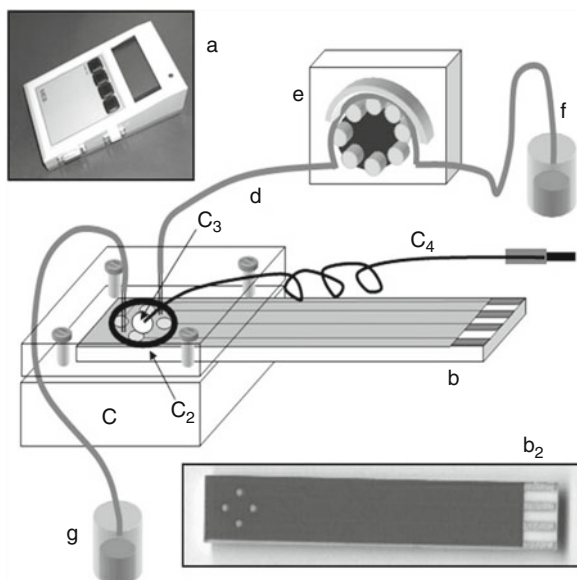


Fig. 2.3 Schematic view (not to scale) of the measuring set-up. The four-channel detector MEB (a) is connected to the screenprinted sensor (b, photo shown as b2) which is inserted to the transparent Plexiglas flow-through cell (c). Silicone tubings (d) is linked to the peristaltic pump (e) carrying the working solutions (f) through the cell to the waste (g). Inside the cell (c), the sensor (b) is fixed using a rubber o-ring (c2). In the top part of the flow cell, a silver pseudoreference electrode (c3) is embedded and linked (c4) to the detector (With permission from John Wiley & Sons [63])

2.2.3 *Francisella tularensis*

Infection with *Francisella tularensis* cause tularemia, a disease, result in a variety of symptoms as swollen ulcer or fever, chills, malaise, headaches and a sore throat. As few as 10–50 g negative coccobacillus *F. tularensis* are sufficient to cause disease if inhaled or inoculated into the skin. Discovered in the early twentieth century, the disease has caused multiple sporadic outbreaks. Tularemia is considered by the Centers for Disease Control and Prevention to be a Category A bioterrorism agent posing the greatest threat to US health and security. Pneumonic tularemia is considered one of the diseases most likely to be encountered in a bioterrorism event and epidemics of any form of tularemia should be rapidly investigated to rule out a bioterrorism event.

Pohanka and Skladal developed an immunosensor device based on a piezoelectric sensor for direct detection of *F. tularensis* [62]. The mouse polyclonal antibody was immobilized via protein A covalently linked to the gold surface of the piezoelectric sensor. The immunosensor was able to detect a limit of 10^5 CFU/mL of *F. tularensis* and was successfully tested for rapid detection of toxin in spikes in drinking water and milk. A four-channel amperometric immunosensor based on screen-printed gold electrodes was proposed by the same group [63]. The experimental set-up is presented in Fig. 2.3. The electrode surface was modified with

lipopolysaccharide surface antigen as a target for the anti *Francisella* antibodies. The bound antibodies were visualized using the antibody – horseradish peroxidase conjugate. The immunosensor was able to work with small volumes of serum samples (0.05 mL per assay). The portable sensing probes described can measure 40 samples per 1 h and are well suited for field applications. Each probe may consist of a number of fibers multiplexed together, each targeting a different DNA sequence or antigen.

Cooper et al. [64] reported a portable sensing probes that incorporate label-free, specific antibodies and single-stranded oligonucleotides for detection of *F. tularensis*, and differentiation of subspecies types A and B. Each probe may consist of a number of fibers multiplexed together, each targeting a different DNA sequence or antigen immobilized by layer-by-layer electrostatic self-assembly process. The optical fiber biosensor was tested using a standard transmission mode long period fiber grating of length 15 mm and period 260 μm , and coated with the Immunoglobulin (IgG) fraction of antiserum to *F. tularensis*. The sensor is very sensitive detecting nanogram quantities of the target DNA.

A new way for the antibodies against *F. tularensis* immobilization was developed by Meyer et al. [29]. They use polyethylene filters and magnetic beads with streptavidin surface on which unmodified anti-Ft antibody and biotinylated antibody were coated. The detection of *F. tularensis* was done with a biosensors based on sandwich immuno-magnetic assay that uses resonant coils with a special frequency-mixing technique as detector. The linear detection range of this biosensor was found to be 104–106 CFU/mL *F. tularensis* comparable to the detection limits of ELISA systems.

2.2.4 *Staphylococcus aureus*

Staphylococcus aureus, an important pathogen, is a gram-positive coccal bacterium, causing diseases to human and animal such as pneumonia, mastitis, food poisoning, and toxic shock syndrome and other. The detection of toxic shock syndrome toxin-1 (TSST-1)-producing *S. aureus* strains is very important in screening virulent strains in food, medical research and clinical diagnosis.

Wang et al. [65] reported a simple, rapid, sensitive and specific sensor with carboxyl-modified CdSe/ZnS quantum dots and oligonucleotide probe complexes, to detect specific (TSS T-1) toxin producing *S. aureus*. The detection is based on fluorescence resonance energy transfer occurring between CdSe/ZnS quantum dots as donors and black hole quencher (BHQ) as acceptors. The photoluminescence was detected in the presence of target DNA by BHQ-DNA detached from quantum dots – DNA probe because of the different affinities. The sensor has high specificity to *S. aureus* proved by the fact that the quenching of quantum dots fluorescence was found to be reversed on addition of target DNA but not upon the additions of DNA of other bacteria. The sensor can be used to detect target DNA at 0.2 μM .

A rapid sensor which can directly detect *S. aureus* in 10 min was reported by Tang et al. [66]. The sensing area is based on interdigitated array microelectrode-oxide-silicon with a surface of $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ on which are immobilized the target molecules. The new type of sensor is composed of Al interdigitated array microelectrodes capacitively coupled to a lowly doped p-type Si substrate by a layer of SiO_2 . The Al surface is protected by a layer of Al_2O_3 . The sensitivity of the sensor is increased both by the fact that the charged target molecules deposited on the surface can modify the interdigitated array microelectrodes capacitance but also the SiO_2/Si substrate interface impedance change. The sensor was coated with a rat monoclonal antibody anti protein A, which is specifically expressed by *S. aureus*. The measurements are made after drying the sensor surface to avoid the parasitic capacitive signal of the liquid medium and require only $100\text{ }\mu\text{L}$ of test sample. The detection time of is about 10 min, which is 10–20 times faster than the conventional plate count.

Recently a miniaturized sensor platform based on functionalized magnetic nanoparticles has been designed as a versatile tool to quantitatively and rapidly detect *S. aureus* in unprocessed biological samples [67]. The iron oxide nanoparticles are covered with a layer of dextran cross-linked with epichlorohydrin, to stabilize the coating, and aminated to provide primary amine groups. Then the modified nanoparticles were mixed with succinic anhydride to convert the amine groups into carboxylate groups. In the next step the activation was done with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N hydroxysulfosuccinimide (sulfo-NHS) in 2-(N-morpholino)ethanesulfonic acid (MES) buffer solution. For *S. aureus* detection vancomycin was added, an antibiotic that recognizes D-alanyl-D-alanine moieties in the bacterial cell wall. The new system is able to detect a small number of pathogens, around 10 colony-forming units of *S. aureus*, in less than 30 min. Moreover, the platform has an interface to facilitate system control and data sharing over wireless networks. All this features combined with automated feedback system to track and compensate the temperature drift allow the sensor platform for on-site detection.

In order to directly detect low concentrations of DNA without amplification, the electrochemical signal-to-noise ratio associated with DNA hybridization has to be maximized. Conducting polymers could be a great possibility to amplify the electrochemical signal. Spain et al. obtained a novel nanocomposite material based on polyaniline nanofibres modified with chemically grown gold nanoparticles [68]. The nanocomposite, deposited on gold electrodes, has a very large surface area, good conductivity and excellent porosity. The single stranded capture DNA was bound to the gold nanoparticles and allowed to hybridize with a complementary target strand, a horseradish peroxidase labeled DNA that is uniquely associated with the pathogen, *S. aureus*. The concentration of the target DNA was measured by monitoring the reduction of a hydroquinone that mediates electron transfer to the bound horseradish peroxidase. The developed sensor has a wide dynamic range, excellent ability to discriminate DNA mismatches and a high sensitivity. Picomolar concentrations could be detected in clean buffer and nanomolar concentrations of a

DNA sequence uniquely associate with *S. aureus* has been detected in whole milk, without the need for molecular amplification.

A magnetoelastic sensor platform for direct detection and monitoring of *S. aureus* in milk was reported by Huang et al. [69]. The sensor is a free standing ribbon-like magnetoelastic thick-film which responds to mass loading due to bacterial adhesion, and changes in solution viscosity. When an externally time-varying magnetic field is applied the magnetoelastic sensor mechanically vibrates at a characteristic resonance frequency. The low material cost facilitates use of the sensors on a disposable basis. *S. aureus* was quantified based on its growth curve and the time dependent change in viscosity curve is recorded. The biosensor is able to directly quantify *S. aureus* concentrations of 10^3 – 10^7 cells mL⁻¹ in milk. The material low cost enables the sensor platform use *in situ*, remote detection of spoiled milk samples.

A similar sensing configuration was modified by means of the biotin–avidin system in order to amplified the detection of *Staphylococcal enterotoxin B* [70]. For an 1 h incubation time the biosensor achieved a detection limit of 0.5 ng/mL.

Alegret S. group developed a genosensor based on a new conducting graphite-polymer composites as transducing material [71]. DNA was directly immobilised by dry-adsorption on the rigid composite material and hybridisation was achieved with biotinylated probes. Horseradish peroxidase-streptavidin conjugate was used as enzyme label. The hybridisation was detected by monitoring the amount of peroxidase bound to the DNA duplex. The major advantages of this transducer are both the ability to regenerate the surface by simple polishing and the possibility to be implemented for screen-printing procedures for the mass production. The entire analytical procedure takes 150 min and the level of detection for a detection level in the fmol range.

Due to the fact that *S. aureus* enterotoxin B (SEB) (one of seven enterotoxins produced by strains of *S. aureus*) is a small protein toxin (28.4 kDa) its detection at low concentrations was a continue concern of researchers. Several sensing platforms have been developed to identify and detect SEB signatures. One of the first attempt was on a piezoelectric immunosensors for the direct and label-free detection of staphylococcal enterotoxin B (SEB) [72]. Thus piezoelectric biosensor allowed specific SEB determination in the range of 2.5–60 µg/mL and can be reused three times with retention of 66% of the initial signal.

One sensing platform which could detect level of picogram/mL SEB, under flow conditions, using label-free antibody immobilized on the piezoelectric-excited millimeter-sized cantilever sensor, was recently reported [73]. The piezoelectric-excited millimeter-sized cantilever sensors were fabricated from lead zirconate titanate, piezoelectric material, bonded to a glass layer. The resonance frequency of the sensor is a function of its mass. The sensor glass surface was functionalized with 3-aminopropyl-triethoxysilane solution. The antibodies activated with EDC and sulfo-NHS were then immobilized on the modified surface leading to the decrease of the resonance frequency. The achieved detection limit of biosensor to SEB was between 12.5 and 50 pg/mL.

A piezoelectric-excited, millimeter-sized cantilever sensor was used for sensitive detection of SEB with concentrations levels of 2.5–25 fg/mL in apple juice and milk [74]. The anti-SEB was immobilized via protein G on the sensing surface coated with gold. One disadvantage of the biosensors is due to the presence of lipids, protein, and high molecular weight polysaccharides that could reduce the sensitivity of sensor response by 20–25%.

A horseradish peroxidase labeled anti-SEB was immobilized on a gold electrode for flow-injection capacitive biosensor development [75]. The capacitive biosensor could detect SEB concentrations as low as 0.3 pg/mL, with a linearity ranging from 2.8 pg/mL to 2.8 ng/mL and a response time about 10 min. The developed label-free biosensor has the benefits of simplicity, high sensitivity, and multiple use capability.

Different cross-linking agents for immobilization of thiolated SEB antibodies onto the gold electrode were used to develop an electrochemical enzymatic immunosensor [76]. An α -naphthyl phosphate substrate was added into the solution in order to generate α -naphthol an electroactive product. Low detection limit of 1 ng/mL in a 5- μ L sample, corresponding to 5-pg was achieved. Nevertheless, the regeneration of thiolated surfaces was generally poor (averaging 36%) showing that the proposed sensing technology could enable the development of single-use disposable devices.

Another alternative is to use an enzyme labelling immunosensor for determining the pathogenic microorganisms *S. aureus* [77]. The amperometric immunosensor was obtained by simultaneously immobilized cholinesterase and antibodies against corresponding antigens, in a nitrocellulose matrix. The bioactive film was fixed at the surface of an electrode shell with the use of O rings. Cholinesterase catalyzes the hydrolysis of butyrylthiocholine iodide, which is a specific substrate for this enzyme. The resulting immune complex, antigen–antibody, acted as an effector of immobilized cholinesterase. The binding constants of the immune complexes of *S. aureus* are $K_{a1} = (7.8 \pm 0.3) \times 10^9$ and $K_{a2} = (4.8 \pm 0.2) \times 10^6$ (mg/mL)⁻¹. The resulting values are indicative of the strong binding of antigens to the corresponding antibodies.

Bilayer Lipid Membranes (BLMs) are the most biomembrane-like, self-assembling and capable of the ligand-receptor recognition system [78]. Supported BLMs can be deposited on the surface of different electrodes in order to develop biosensors. Liu et al. reported a novel BLMs nucleic acid biosensor supported by modified patch-clamp pipette electrode for the detection of SEB [79]. The biosensor reacted to SEB gene and caused decreases of ion current by specific hybridization, and did not response to the other genes that do not have complementary sequence to the single-stranded DNA (ssDNA) probe of biosensor. The biosensor has a linear range between 20 and 5,000 ng/mL with the detection limit of 20 ng/mL.

Recently, single walled carbon nanotubes percolation-based sensing has been carried out using non-porous material in which single walled carbon nanotubes–antibody complex was used to form a bio-nanocomposite network [80, 81].

Yang et al. described a biosensor for SEB based on electrical percolation-based biosensing technology [82]. The passage of current between the conductive ends of each single walled carbon nanotubes provides a continuity and conductivity to the

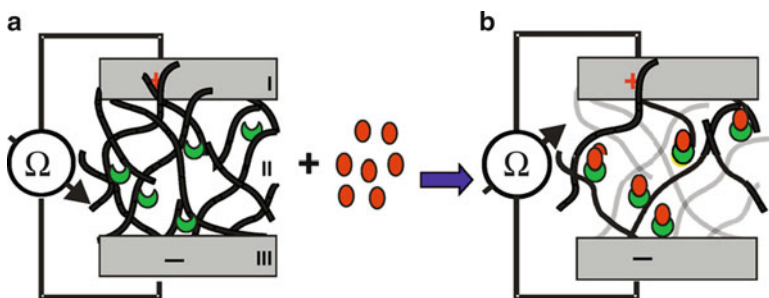


Fig. 2.4 A model of the electrical percolation-based biosensor. Biological semiconductor (BSC) with two electrodes (I and III) and a network (II) of randomly distributed SWNTs (black lines) with embedded antibodies (crescents). BSC is shown in low resistance mode (a) with no antigen present. In the presence of a specific antigen (b) which binds to the antibody (circles), the contacts between the SWNTs in the network (grey lines) are disturbed, resulting in a higher resistance (With permission from Elsevier [82])

system. Binding of specific antigens to the single walled carbon nanotubes–antibody complex disrupts the continuity by displacing the connected ends of the single walled carbon nanotubes, resulting in increased tunnelling distance and subsequent resistance. So the conductivity of the single walled carbon nanotubes–antibody network increases with the increase in concentration of single walled carbon nanotubes. At a specific single walled carbon nanotubes concentration (the percolation transition point), the change in resistance begins to level off. Below this point, there is a still relatively low statistical distribution of “contacts” between the single walled carbon nanotubes–antibody complexes in the network. Therefore, small changes in the single walled carbon nanotubes – antibody complexes can lead to dramatic changes in conductivity. The label-free biosensor is based on electrical percolation through a single-walled carbon nanotubes-antibody complex that forms a network functioning as a “Biological Semiconductor”. To create the bio-nanocomposite material, the single walled carbon nanotubes were functionalized with anti-SEB antibodies. Then, the pre-functionalized single walled carbon nanotubes-antibody complex were immobilized directly on the 16 “Biological Semiconductor” array surface made by poly(methyl methacrylate) and polycarbonate. Each “Biological Semiconductor” is connected via silver electrodes to a computerized ohmmeter, thereby enabling a continuous electronic measurement of molecular interactions. The model of the “Biological Semiconductor” operation is shown in Fig. 2.4. The biosensor was able to detect SEB with a limit of detection of 5 ng/mL. Assembly of biosensors are simple and capable of processing multiple analytes simultaneously [82].

SPR sensors offer several advantages over other detection methods, that it requires no labelling of the analyte and protein-protein interactions can be monitored in real time [83]. A SPR biosensor based on Biacore 3000, to measure the label-free biosensor is based on electrical percolation through a single-walled

carbon nanotubes-antibody complex that forms a network functioning as a “Biological Semiconductor”. To create the bio-nanocomposite material, the single walled carbon nanotubes were functionalized with anti-SEB antibodies. Then, the pre-functionalized single walled carbon nanotubes-antibody complex were immobilized directly on the “biological semiconductor” array surface made by poly(methyl methacrylate) and polycarbonate. Each “biological semiconductor” is connected via silver electrodes to a computerized ohmmeter, thereby enabling a continuous electronic measurement of molecular interactions. The model of the “Biological Semiconductor” operation is shown in Fig. 2.4. The biosensor was able to detect SEB with a limit of detection of 5 ng/mL. Assembly of biosensors are simple and capable of processing multiple analytes simultaneously [82].

A new type of biosensor, was developed based on SPR chip onto the anti-SEB antibodies were covalently cross-linked [84]. The signal was amplify with a second antibody which binds to the captured antigen. One entire measurement cycle takes 5 min using one antibody or 8 min using two antibodies with a detection limit of 10 ng/mL. The miniaturization of SPR sensing devices for developing portable instruments for field applications showed intense interest. Thus, a miniaturized fiber optic SPR biosensor for specific detection of SEB able to detect ng/ml concentrations of SEB in less than 10 min was developed by Slavik et al. [85].

Tsai et al. reported a SPR gold sensor with the surface functionalized with a self-assembled monolayer fabricated with carboxylic- and hydroxyl-terminated thiols mixture [33]. The system allow the immobilization of biomolecules in a manner which avoids steric hindrance between these molecules and their binding partners. The SPR sensor could detect *Staphylococcal enterotoxin A* (SEA) in buffer in a linear range from 100 to 1,000 ng/mL.

The increasing of the sensibility of a SPR biosensors towards SEB) was done by using antibody-coated superparamagnetic nanobeads [34]. Moreover, the immunomagnetic beads can be used to purify and concentrate the SEB cells allowing picogram levels of detection. Multiple detections of SEB in stool could be performed using the same sensor surface by regenerating the sensor surfaces with a pH 2.2 buffer.

Naimushin et al. reported a prototype SPR sensor based on a two-channel system for field use [86]. The gold-coated thin glass sensors are pre-functionalized providing a disposable and renewable sensor. *S. aureus* enterotoxin B detection was done directly at sub-nanomolar levels and with amplification, at femtomolar levels. The amplification was done with second goat anti-rabbit antibodies. The mouse monoclonal anti-SEB antibodies were used as capture antibodies. A regeneration procedure for the sensor surface allowed for over 60 direct detection cycles in a 1 month period.

The localized surface plasmon resonance based nano-biosensor is a refractive index-based sensing device which relies on the optical properties of noble metal. Zhu et al. developed such a biosensors for the detection of SEB [32]. Nanosphere lithography technique was employed to create the surface-confined hybrid Au–Ag triangular nanoparticles array which is supported on a glass substrate. The mouse anti-SEB IgG antibodies were immobilized by EDC/NHS chemistry.

One of the main advantage of this approach including is the good selectivity and sensitivity without labeling. The biosensor can detect the toxin at approximately 1 ng/mL within 1 min without needing temperature control. Taking into account that the commercial SPR instruments are expensive, the proposed biosensor is inexpensive.

Detection of SEB below 1 ng/mL in fresh milk was done with a SPR biosensor based on a competitive immunoassay [87]. First the anti-SEB was allowed to bind with the SEB in samples off line and then the excess and unbound anti-SEB was captured by SEB sensor. The biosensor analysis including the sensor regeneration was 15 min per sample in a fully automated system.

2.2.5 *Escherichia coli*

Escherichia coli (*E. coli*) is one of the most common bacteria types and is a normal inhabitant of the large intestine of warm blooded animals. From the hundred types of *E. coli* types, only *E. coli* O157:H7 produces a toxin that can cause a wide spectrum of human diseases, ranging from some types of hemorrhagic and nonhemorrhagic diarrhea, to occasional kidney failure or hemolytic uremic syndrome and death due to ingestion of contaminated food. Therefore, rapid, simple, and sensitive methods for detecting this bacterium are urgently required.

A QCM immunosensors were used for the detection of *E. coli* O157:H7 in foods. In order to improve the detection limit of QCM biosensors, Shen et al. use beacon immunomagnetic nanoparticles with *E. coli* O157:H7 [24].

The designer of the sensors and experimental set-up is presented in Fig. 2.5. The magnetic nanoparticles lead to the amplification of the QCM immunosensor for 3 times with high sensitivity. The detection limits of 23 CFU/mL in buffer and 53 CFU/mL in milk were achieved. Moreover, the total analysis time is approximately 4 h. The crystal is regenerated without difficulty, and can be used at least 10 times.

The microorganisms, proteins and viruses have carbohydrates on their surface and these structures can be recognised using lectins, offering an alternative for the specific detection. Therefore Serra et al. [88] proposed the use of lectin concanavalin A for a label-free bacteria piezoelectric biosensor. The lectins are immobilised onto a quartz crystal sensor. The electrochemical quartz crystal microbalance monitoring simultaneous both the frequency changes and the metabolic activity of bound bacteria by amperometric measurement of hydrogen peroxide consumption by bacterial catalase immobilized onto the gold-plated quartz crystal surface. In this way, it is possible to discriminate between living and dead organisms. A detection limit of approximately 1.0×10^4 CFU mL⁻¹ *E. coli* was achieved. Moreover, nonspecific adsorptions were minimised.

Shen et al. use a functional mannose self-assembled monolayer in combination with lectin concanavalin A as molecular recognition elements for the detection of *E. coli* W1485 [89]. Oligosaccharides are smaller than antibodies and consequently,

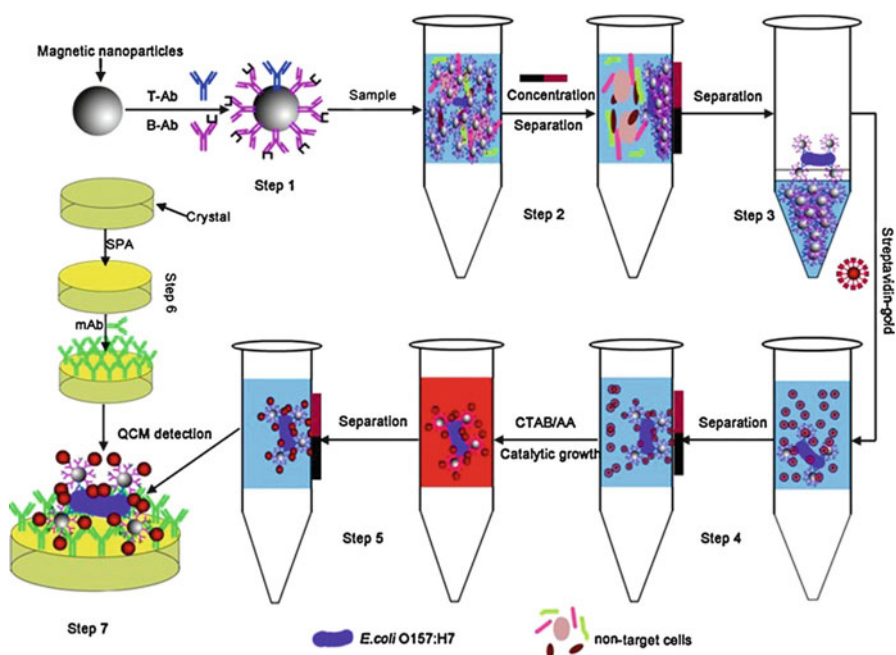


Fig. 2.5 Schematic illustration of the experimental strategy: preparation of *E. coli* O157-BIMPs (step 1); magnetic separation of target cells (step 2); centrifugation of free O157-BIMPs (step 3); conjugation of streptavidin-gold (step 4); catalytic growth of gold particles (step 5); fabrication of crystals (step 6); detection with the QCM immunosensor (step 7) (with permission from Elsevier [24])

higher densities of carbohydrate-sensing elements could lead to higher sensitivity and less nonspecific adsorption. Thus a nonlabeled QCM biosensor has been developed with a significant improvement of the sensitivity and specificity with an experimental detection limit of a few hundred bacterial cells. The linear range was from 7.5×10^2 to 7.5×10^7 cells/mL, which is four decades wider than the mannose-alone QCM sensor.

E. coli O157:H7 cells labeled with biofunctionalized electroactive polyaniline and deposited onto a disposable screen-printed carbon electrode show great sensitivity detecting as few as 70 CFU/mL of *E. coli* O157:H7 with a linear range of 10 – 10^5 CFU/mL [90]. The antibodies to *E. coli* O157 were immobilized onto magnetic beads. An external magnetic field was employed both for separation of immunomagnetic complexes and to keep the labeled cells to the electrode surface. Thus the electrochemical signal generated by the polyaniline could be amplified. The total time for one assay is 70 min giving a major advantage over standard culture methods. The sensor can be used with portable, handheld instrumentation without requiring biological modification of the sensor surface. Potential applications include field-based pathogen detection for food and water safety, environmental monitoring, healthcare, and biodefense.

E. coli CECT 675 cells were chosen as a model organism for pathogenic *E. coli* O157:H7 to detect the living bacteria in real samples with a label-free potentiometric aptamer-based biosensor [91]. The aptamers are chemically linked into a layer of previously carboxylated single walled carbon nanotubes (SWCNTs) for improving the electron transfer at the glassy carbon electrode. The carboxylic groups on the sidewalls of the SWCNTs were activated using the well-known carbodiimide-mediated chemistry (EDC and NHS) in order to form stable amide bonds between the carboxylic moieties and the primary amine spacer of the aptamers. Selectivity was tested by exposing the aptasensor to the same range of concentrations of *E. coli* CECT 4558, *L. casei*, and *S. enterica*. The results demonstrated that there are no cross-reactivity meaning that the biosensor is highly selective and could be used to analyze real samples. The detection of target bacteria in real samples can be carried out in a couple of minutes, with the minimum number of pretreatment steps, in close-to-real-time and almost zero tolerance conditions. Concentration levels as low as 6 CFU/mL in complex matrices such as milk or 26 CFU/mL in apple juice were identified. The biosensor can be easily built and regenerated without difficulty, and can be used at least five times with no loss in the minimum amount of detected bacteria.

Label-free biosensor for detection of *E. coli* use also electrochemical impedance transducers. Pingarron J.M. group developed a label-free electrochemical impedance immunosensors for the detection and quantification of *Escherichia coli* using self-assembled monolayers modified gold screen-printed electrodes were reported [19]. The immobilization of antibodies on the electrode surface could affect the biosensor analytical performance. Thus, two different immobilization strategies were reported and compared. In the first one, the covalent immobilization of the specific antibodies on gold screen-printed electrodes was done via a bifunctional cross-linker, 3,3'-dithiobis [sulfo succinimidylpropionate]. The other configuration was based on the immobilization of thiolated antibodies onto the electrodes surface. The thiolated antibodies configuration gave a better analytical performance and was applied to the analysis of inoculated river and tap water samples, without preconcentration, the detection of 3.3 CFU/mL. The total analysis time, from sampling to measurement, was 1 h representing an important practical advantages for *E. coli* tests.

Shriver-Lake et al. [92] reported an array biosensor for the detection of *E. coli* O157:H7 in a variety of matrices and in the presence of high levels of extraneous bacteria. The detection could be done in less than 30 min with minimal sample preparation. Capture antibodies were immobilized onto avidin-coated sensor surfaces by an avidin–biotin bridge yielding surfaces with improved functionality [93]. Upon binding of a fluorescent analyte the pattern of fluorescent spots was detected using an imaging camera. The image intensity was then converted to average fluorescence intensity per square of capture antibody using a special software. The location of the spot and its mean fluorescence value were used to determine the toxin identity and concentration. The limit of detection without sample concentration or enrichment is 5×10^3 cells/mL in buffer and $1\text{--}5 \times 10^4$ cells/mL in other matrices. To achieve the one cell/mL level desired by regulatory agencies, preconcentration with immunomagnetic separation is recommended.

The antibodies against *E. coli* O157:H7 were immobilized on alkanethiol self-assembled monolayer sensor chip in order to develop a surface plasmon resonance biosensors for *E. coli* O157:H7 detection in apple juice [94]. Using the sandwich assay, 106 CFU/mL of *E. coli* O157:H7 was detected, in less than 1 h, in apple juice in the presence of *S. aureus* proving the potential of SPR biosensors in rapid and label-free detection of pathogens.

Liposomes are small spherical vesicles composed of natural nontoxic phospholipids and cholesterol and they have been used by Ho et al. as signal amplifiers for a liposome-based microcapillary immunosensor for detection of *E. coli* O157:H7 [95]. The anti-*E. coli* O157:H7 antibodies were immobilized via protein A on the inner surface a microcapillary containing immunoliposomes with sulforhodamine B as fluorochrome. *E. coli* O157:H7 was detected by measuring the fluorescence intensity of sulforhodamine B after lysis of the bound liposomes. The immunosensor permits detection of around 360 heat-killed bacteria/mL (equivalent to ~53 heat-killed bacteria in the 150 μ L sample volume) at room temperature. A single assay can be performed within 45 min. Compared to current batch-type immunoassays such as enzyme-linked immunosorbent assay provides enhanced sensitivity and speed over traditional microbiological methods.

A sensor for pathogenic *E. coli* O157:H7 detection was developed using magnetic bead – quantum dots nanoparticle complexes [96]. The particle complex was subsequently conjugated with probe DNA, which hybridized with the signalling probe DNA via the target DNA sequence. Both the magnetic bead – quantum DNA complex and signalling probe labeled with a fluorophore were added into the sample. The magnetic beads allow the manipulation of DNA-conjugated quantum dots nanoparticles via magnetic field. A spectrofluorometer was used to quantify the amount of target DNA based on the fluorescence intensity of the magnetically-separated hybridized complexes. The limit of detection was determined to be of 9.72 fM.

2.2.6 Multiplex Assay

Measurement of multiple analytes in a single assay was a challenge for researchers especially for pathogen detections. In biosecurity area, rapid identification and differentiation of pathogenic from non-pathogenic species, viable from non-viable cells or active from inactive toxins is important.

The antibody microarray reported by Wojciechowski et al. is a versatile, sensitive detection platform for biological pathogens *Yersinia pestis*, *B. anthracis*, and the bacterial toxin SEB [97]. The sensor chip is comprised of microelectrodes coupled to a matrix coated with antibodies against the biological pathogens. The sensors array was able to detect SEB and inactivated *Y. pestis* individually at concentrations as low as 5 pg/mL and 10⁶ CFU/mL, respectively.

Bioterrorism agents as ricin, cholera toxin, and SEB, in milk, apple cider, and blood samples, were simultaneously detected using a sensitive antibody microarray

with fluorescent nanoparticle [98]. The microarray uses a sandwich format that consists of capture polyclonal antibodies, toxins, biotinylated detection antibodies, and avidin-conjugated nanoparticles. The ricin was detected at 100 pg/mL in buffer and at 1 ng/mL in spiked apple cider or milk, whereas cholera toxin and SEB were detected at 10 pg/mL in buffer and 100 pg/mL in spiked apple cider or milk. High specificities were also demonstrated in the detection of mixed toxin samples with similar sensitivities. The matrix effect of blood samples on the detection of mixed toxins seems to be minimal when the toxin concentration is at or above 100 ng/mL.

Recently a cell-based detection system was developed by Banerjee and Bhunia [99]. The principle of work is based on the sensing the functionality or biological activity of an analyte by monitoring perturbations in 'normal' physiological activities of mammalian cells following exposure to toxic compounds. The biosensing element consists in a B lymphocyte Ped-2E9 cell-line, encapsulated in collagen matrix in 3D scaffold. The limit of detection for a broad range of bacterial pathogens (*Listeria monocytogenes*, enterotoxigenic *Bacillus* and toxins, alpha-hemolysin from *S. aureus*, phospholipase C from *Clostridium perfringens*, cytolysin from sea anemone *Stoichactis helianthus*, lysin O from *I. monocytogenes* and enterotoxin from *Bacillus*) was 10–40 ng in 2 h.

Piliarik et al. reported a high-throughput surface plasmon resonance (SPR) biosensor for rapid and parallelized detection of nucleic acids identifying specific bacterial pathogens such as *B. abortus*, *E. coli* and *Staphylococcus aureus* at 100 pM levels [35]. The biosensor consists of a high-performance SPR imaging sensor with polarization contrast and internal referencing and an array of DNA probes microspotted on the surface of the SPR sensor. Detection of specific DNA or RNA sequences can be performed in less than 15 min.

B. anthracis spores and *Salmonella typhimurium* were detected simultaneously with multiple phage-based magnetoelastic biosensors [100]. The biosensors were formed by immobilizing phage onto the magnetoelastic resonator's surface. As the cells/spores were captured by the specific phage-coated sensor, the mass of the sensor increased, resulting in a decrease in the sensor's resonance frequency. A reference sensor (devoid of phage) is used to calibrate the system for environmental changes (e.g., temperature, pressure, viscosity, non-specific binding) that may influence the measured resonance frequencies, thus improving the accuracy of the technique. The detection limit for *S. typhimurium* is estimated to be 5×10^3 CFU/mL, and saturation occurring at greater than 5×10^8 CFU/mL. For *B. anthracis* spores the detection limit was estimated to be 5×10^3 CFU/mL.

An indirect quantification assay for *S. aureus* and *Salmonella choleraesuis* was developed based on glucose consumption [101]. The amperometric detection of glucose consumption was done with a graphite – Teflon – glucose oxidase-peroxidase – ferrocene composite biosensor under flow injection conditions at 0 V applied potential. The proposed methodology for screening of bacteria achieved limits of detection around 6.5×10^2 CFU/mL after 3 h of incubation with no pre-concentration step.

An eight-channel SPR biosensor based on wavelength division multiplexing to simultaneously and quantitatively detect four foodborne pathogens, four species

of bacteria, *E. coli* O157:H7, *Salmonella choleraesuis* serotype typhimurium, *Listeria monocytogenes*, and *Campylobacter jejuni* was reported [102]. The limit of detection for each of the four species of bacteria in the tested matrices ranges from 3.4×10^3 to 1.2×10^5 CFU/mL.

Development of multiplexed high density DNA arrays for the identification of biological warfare agents were reported by Song et al. [103]. The array format was composed of an etched coherent optical fiber bundle containing 6,000 individual 3.1- μ m-diameter fibers loaded with probe-functionalized microspheres. Microspheres were functionalized with eighteen different 50-mer oligonucleotide probes and optical encoding indicators. Probe sequences were designed for *B. anthracis*, *Y. pestis*, *F. tularensis*, *Brucella melitensis*, *Clostridium botulinum*, *Vaccinia virus* and one biological warfare agent simulant, *Bacillus thuringiensis kurstaki*. A detection limit of 10 fM was achieved within 30 min of hybridization for *B. anthracis*, *Y. pestis*, *Vaccinia virus*, and *B. thuringiensis kurstaki*.

Two different detection schemes were proposed in order to develop an antibody-based microarray for the detection of cholera toxin, diphtheria toxin, anthrax lethal factor and protective antigen, SAB and tetanus toxin C fragment [104]. In one strategy the fluorescently labeled toxins were captured directly by the antibody array and in the other was a competition assay that employed unlabeled toxins as reporters for the quantification of native toxin in solution. A significant advantage of the competition assay is the minimal sample preparation required prior to analysis.

The detection of low levels of *Bacillus cereus*, *Enterobacter cloacae*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *Streptococcus pyogenes* was done with a spore-based biosensor [105]. The system consists of microbial spores suspended in buffer containing a group of enzyme substrates, that are not themselves germinants, but produce a germinant upon enzymatic catalysis and a fluorogenic compound. The biosensor exploits spore's ability to produce fluorescence when sensing bacterial cells.

The microarray deposition equipment could be too costly for many laboratories, therefore was an increasing interest to design alternative method for increasing the number of analytes that can be detected on a single sensor substrate without increasing production costs. Taitt et al. demonstrate that by simply mixing different assay components it is possible to detect nine analytes (SEB, ricin, cholera toxin, *B. anthracis* Sterne, *Bacillus globigii*, *F. tularensis* LVS, *Y. pestis* F1 antigen, MS2 coliphage, and *Salmonella typhimurium*) in a single array [106].

The magnetic nanoparticles and antibody-conjugated semiconductor quantum dots were used to develop biosensors that can detect simultaneously three food-borne pathogenic bacteria, *Salmonella typhimurium* and *E. coli* O157:H7 [26, 107]. In this system the antibodies were immobilized on the magnetic nanoparticles modified with amino silane and glutaraldehyde. The complete procedures is takes 2 h and could detect food-borne pathogen bacteria in a food matrix at 10^{-3} CFU/mL [26].

Ngundi et al. reported an array biosensor, able to detect cholera toxin and tetanus toxin simultaneously [108]. The array surface modified with two sugars, N-acetyl galactosamine and N-acetylneuraminic acid, was used as receptors for protein toxins. These arrays were tested with different fluorescently labelled bacterial cells

and protein toxins. The *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli* and SE did not bind to either of the monosaccharides. Only the target toxins, cholera toxin and tetanus toxin, bound to modified surface array and could be detected at concentrations as low as 100 ng/mL.

An liquid arrays biosensor for use in domestic applications has been developed for specifically and simultaneous detection of multiple simulants of biological warfare agents, such botulinum toxin, staphylococcal enterotoxins, anthrax or ricin, [109]. The liquid arrays utilize polystyrene microbeads embedded with precise ratios of fluorescent dyes each with a unique spectral address. A sandwich immunoassay format was used: specific antibodies are immobilized on the polystyrene beads, the antigen is allowed to bind the beads, and the bound analyte is subsequently detected using secondary antibodies indirectly labelled with the phycoerythrin, a fluorescent compound. The assays are rapid; every sample could be analyzed in less than 1 h.

A protein chip has been developed by Huelseweh et al. for simultaneous detection of SEB, ricin, Venezuelan equine encephalitis virus, St. Louis encephalitis virus, West Nile virus, Yellow fever virus, Orthopox virus species, *F. tularensis*, *Y. pestis*, *Brucella melitensis*, *Burkholderia mallei* and *E. coli* EHEC O157:H7 [110]. The detection platform combines successfully the array technology with sandwich-enzyme-linked immunosorbent assay amplified by streptavidin-poly-horseradish peroxidase and can detect the biological warfare agents in 1 h.

2.3 Conclusions

Research in the field of biosensors for biological warfare agents is continually improving and its evolution is focused on ways of producing biosensors for small portable devices allowing fast, accurate and on site detection. The limiting step for commercialization is mainly due to the bioreceptor molecules that are not suitable, in most cases, to mass production. The future research in this area will exploit both new sensing materials delivered by nanotechnologies and new immobilization procedure in order to amplify the signal and improve the immobilization of biological elements. Thus a new generation of biosensors with as simple as possible configuration and as improved as possible analytical performances, will be designed.

New commercially available hand-held instruments will occur on the market characterized of as few steps as possible in the technological procedure of the screen-printed electrode preparation, direct request of the research area interested in this direction. All those will allow for more analyses to be decentralized and performed at the point-of-need and in monitoring in the field.

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Chapter 3

Nanomaterials-Based (Bio)Sensing Systems for Safety and Security Applications

Briza Pérez-López and Arben Merkoçi

Abstract The development of new nanomaterials and nanotechnologies has provided many new opportunities for (bio)sensing systems. The introduction of nanomaterials, such as magnetic nanoparticles, gold nanoparticles, graphene, quantum dots, etc. is bringing advantages in terms of improving the selectivity and sensitivity of these systems. These nanomaterials also offer advantages in biosensors owing to their nanometric size, shape, composition, physical properties, ability to manipulate their surface chemistry and the property that they have in terms of adsorbing biological molecules and the change of their physical properties. In recent years, several bacterial pathogens, toxins, viruses, parasites and explosives have been considered as potential threats for bioterrorism, among which can find *Escherichia coli*, *Salmonella*, *Bacillus anthracis*, *Clostridium botulinum*, *Botulinum Neurotoxin*, *Vaccinia*, *Plasmodium falciparum*, Trinitrotoluene, etc. Bioterrorism is extremely complex to tackle but the science and technology are fundamental elements to reduce its threat. For this reason, monitoring systems for quick identification of biomolecules are the core of much of the basic research activities in combating bioterrorism. In this chapter we discuss the research efforts by using nanobiotechnologies with the aim of developing accurate, easy, cheap, portable and ultrasensitive assays for

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agents that pose a biologic threat. Some nanomaterial-based (bio)sensing systems used to detect agents related with bioterrorism for safety and security applications in agriculture, food, forensic, biomedical are also given.

Keywords Nanomaterials • Magnetic nanoparticles • Gold nanoparticles • Graphene • Quantum dots • Nanotechnology • (Bio)sensing • Bacteria • Spores • Toxins • Virus • Parasites • Explosives • Bioterrorism • Biosystems

3.1 General Introduction. Nanomaterials and Bioterrorism

The discovery and study of nanomaterials has enabled the development of ultra-sensitive (bio)sensing systems. This is due to their high surface area, favorable electronic and optical properties and electrocatalytic activity as well as good biocompatibility induced by nanometer size and specific physicochemical characteristics [1–5].

In recent years, the advent of nanomaterial-based (bio)sensing systems for safety and security applications is offering key researches and developments. In this context, of special interest are the ‘nanosized’ and nanomaterials based biosensors, called also nanobiosensors – a modern and efficient class of detection systems [1, 3, 6–10]. The application of these devices in food industry, environmental monitoring and clinic diagnostics could lead great improvements in safety and security against bioterror agents. Nowadays, laboratories and institutions related with the nano-biotechnology are working together to increase the capabilities to detect and respond to an attack by biological or chemical weapons [11].

Examples of nanomaterial-based bioterrorist agents for safety and security applications are given in the Table 3.1. The magnetic nanoparticles (M NPs), graphene (G), quantum dots (QDs), and more extensively gold nanoparticles (Au NPs) are being invaluable nanomaterials to detect bioterrorist analytes in macro- to nano-scale, including bacterial pathogens, toxins, viruses, parasites, explosives, etc.

Biological agents such as bacteria, viruses, biological toxins, and genetically altered organisms are contagious, and during this lag time, infected persons could continue to spread the disease, further increasing its reach. Hundreds or even thousands of people could become sick or die if a biological attack were to occur in a major metropolitan area [11]. For this reason, problems related to the risks of human health and environmental need to be carefully considered, mainly assays that involve a variety of safety and security applications.

In this chapter will be given some important strategies used in (bio)sensing systems with different nanomaterials to detect bioterrorist agents. In addition, this chapter will give opinions about the importance that these systems have for safety and security applications in food, environmental and other related fields.

Table 3.1 Examples of nanomaterial-based bioterrorist agents for safety and security applications

Agent	Analyte	Nanomaterial	Technique	Sample	LOD	Ref.
Bacteria &	<i>Escherichia coli</i> O157:H7	Au NPs (13 nm diameter)	Electrochemical	Milk	50 CFU/strip	[12]
	<i>Escherichia coli</i>	Cu@Au NPs (ca. 15 nm)	Electrochemical	Surface water	3 CFU/10 mL	[13]
		Glyco-NPs	Optical	PBS	10 ⁴ cells/mL	[14]
		Silica particles (200 nm) coated with silver shells (ca. 20 nm)	Optical	Water	3–5 cells	[15]
Spores	<i>Salmonella</i> spp.	Au NPs (ca. 25 nm)	Electrochemical	Pork	1.0 × 10 ² CFU/mL	[16]
	<i>Salmonella typhi</i>	Au NPs (ca. 15 nm)	Electrochemical	PBS	98.9 CFU/mL	[17]
Toxins	<i>Bacillus anthracis</i>	EAM	Electrochemical	DNA	0.01 ng/μL	[18]
	Anthrax	Eu ³⁺ NPs	Optical	PBS	10 pg/mL	[19]
	Botulinum Neurotoxin B	Colloidal gold (25 nm in diameter)	IC	PBS	50 ng/mL	[20]
	Botulinum neurotoxin D	Colloidal gold (40 nm in diameter)	IC	Horse faeces	50 ng/L	[21]
	Botulinum neurotoxin A	QDs (655 nm)	Fluorescence	PBS	5 pM	[22]
Viruses	H9 AIVs	Colloidal gold	IC	Chicken eggs	0.25 units of HA	[23]
	H1N1 flu	Colloidal gold	Electrochemical	PBS	577 pM L ⁻¹	[24]
Parasites	<i>Plasmodium falciparum</i>	M NPs (1 μm diameter)	Electrochemical	Spiked serum	0.36 ng mL ⁻¹	[25]
		Au NPs	Electrochemical	Serum	–	[26]
Explosives	TNT	CdTe QDs	Fluorescence	Soil	1.1 nM	[27]
		Graphene	Electrochemical	0.5 M NaCl solution	0.5 ppb	[28]
	2,4-DNT	Graphene	Electrochemical	0.5 M NaCl solution	42 nmol L ⁻¹	[29]

PBS phosphate buffer solution, EAM electrically active magnetic nanoparticles, QDs quantum dots, M NPs magnetic nanoparticles, 2,4-DNT 2,4-Dinitrotoluene, TNT trinitrotoluene, Cu@Au NPs gold nanoparticles coated Cu, CFU colony forming units, NPs nanoparticles, CdTe QD cadmium telluride quantum dot nanoparticle, H9 AIVs H9 subtype avian influenza viruses, H1N1 flu Influenza A, IC Immunochromatography, LOD limit of detection, Eu³⁺ NPs Europium nanoparticles

3.2 Monitoring Systems for Bioterrorist Agents

Numerous laboratories and public and private research institutions during decades have been designing and developing technologies to safeguard from biological attacks provoked by terrorists. Nowadays, they are developing monitoring technologies for bioterrorism agents with fast analysis, low limit of detection, and high accuracy to prevent false negatives and positives and maintain confidence in the monitoring system [30].

Among the highest-risk threat agents in bioterrorism can be considered: (1) Bacteria, they are prokaryotes, and their distribution is ubiquitous (humans, animals, and environment). They are the oldest living organisms in the history of this planet and play an important role in maintaining the ecosystem [11]. *Escherichia coli* (*E. coli*) is a family of naturally occurring bacteria. Some of these cause sickness and even death. Most *E. coli* infections come from eating undercooked (i.e. contaminated ground beef). Terrorists could use *E. coli* 0157:H7 bacteria as a weapon to strike many people at one time, also the *Bacillus anthracis* (*B. anthracis*) is considered a high pathogenic agent that could deliberate contamination events causing anthrax [31]; (2) Neurotoxins produced by *Clostridium botulinum* are among the most known poisonous substances [32]. For example, *botulinum* toxin is the most toxic substance known and is extremely poisonous by the oral route (estimated lethal oral dose, 10–70 µg for a 70 kg human) and potentially toxic by inhalation [33], for this reason it is used as bioterrorism agent (3) *Influenza A* (H1N1) virus also could be considered a bioterrorist agent, it belongs to the *Orthomyxoviridae* family and corresponds to the specific combination of glycol-protein hemagglutinin (HA) and neuraminidase (NA) variants, which are present on the surface of the enveloped RNA virus [24]. (4) Malaria is the most prevalent parasitic disease in the world caused by the apicomplex protozoan of the *Plasmodium* genus. Malaria is present over the tropics, where four species, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* are transmitted to humans by the bites of the female mosquito vector of the *Anopheles* genus [34] and although the use of parasites as bioterrorism agents has not received so much attention. Parasites could contribute to the installation of fear in human population upon intentional addition to their food and water supplies, which makes malaria suitable for being used by terrorists (5).

In recent years, the detection and quantification of nitroaromatic explosives such as 2,4,6-trinitrotoluene (TNT) have also received considerable attention due to their environmental, security against terrorists and health related concerns [35, 36].

Various methods have been developed for the detection of these bioterrorist agents and many more still are in development phase. Most of the assays are based on detecting the (a) whole organism, (b) bacterial antigens, and/or (c) the related nucleic acid. In the Table 3.2 are described some detection methods more commonly used for bioterrorist agents with safety and security applications.

Conventional methods such as the culture and colony counting methods that involve counting of bacteria, immunology-based methods that use antigen-antibody

Table 3.2 Detection methods commonly used in detection of bioterrorist agents

Bioterrorist agents	Detection methods	References
Pathogens	Culture and colony counting	[37–41]
	Immunology-based methods	
	Polymerase chain reaction (PCR)	
	Lateral flow	
	Enzyme-linked immunoassays	
	Biosensors	
	Fluorescence immunoassay	
	Chemiluminescence assay	
	Electrochemical immunoassay	
	Surface plasmon resonance sensor	
	Fiber optic sensor	
	Microfluidic biochip	
Viruses	Cell culturing	[42–44]
	Polymerase chain reaction (PCR)	
	Enzyme linked immunosorbent assay (ELISA)	
	Flow cytometry	
	Lateral flow	
	Biosensors	
Explosives	Fluorescence	[27, 45]
	Raman and mass spectroscopy	
	Nuclear magnetic resonance spectroscopy	
	Surface plasmon resonance spectroscopy	
	Electrochemistry	
	High performance liquid chromatography (HPLC)	
	Gas chromatography–mass spectrometry (GC–MS)	
	Electrogenerated chemiluminescence (ECL)	
	Enzyme- linked immunosorbent assays (ELISA)	
Spores	Culture counting	[18, 46]
	Immunological detection	
	Nucleic-acid based assays	
	Ligand-based (Aptamers and Peptides) detection	
Toxins	Biosensors	[20, 47, 48]
	Radioimmunoassay	
	Enzyme-linked immunosorbent assay	
	Lateral flow	
	Electrochemiluminescence (ECL)	
	Biosensors	
Parasites	Fluorescence	[25, 26]
	Förster resonance energy transfer (FRET)	
	Fluorescent microscopy	
	Flow cytometry	
	Automated blood cell analysers	
	Serology antibody detection	
	Molecular methods	
	Laser desorption mass spectrometry	
	Enzyme-linked immunosorbent assays (ELISA)	
	Indirect fluorescence antibody test (IFAT)	

interactions and the polymerase chain reaction (PCR) method which involves DNA analysis are being used. These methods can be sensitive, inexpensive and give qualitative and quantitative information, however, a pre-treatment of the samples is needed; furthermore they are greatly restricted by assay time [37]. The electrochemical detection methods possess several advantages such as easy operation, low cost, high sensitivity, simple instrument and suitability for portable devices. However, to improve their performance one of the most popular ways is to use nanomaterials with a high surface area to functionalize the electrode [4, 5, 10]. In this context, nanomaterial-based methods have demonstrated improvement of the sensitivity, but due to reproducibility problems, as well as interferences, their application in real samples is still limited.

3.2.1 *Bacteria and Spores*

The prospect of nanomaterials is promising for rapid and sensitive pathogen detection [12, 14–16]. Current literature shows numerous applications of different nanostructures in biosensor devices for the detection of pathogenic microorganisms that are of importance to food and environmental safety, biosecurity, and medical diagnostics.

In this section, some aspects related to the detection of *Salmonella*, *E. Coli*, *B. anthracis* and *Clostridium botulinum* by using metal nanoparticles such as Au NPs, Cu@Au NPs, M NPs and QDs will be described.

Nanotechnology gives new approaches in order to detect microorganisms through the use of nanomaterials. This field has been explored by Lin et al. [12] using screen-printed carbon electrodes (SPCEs) modified with Au NPs (13-nm diameter) and ferrocenedicarboxylic acid (FeDC). The detection method consists of a sensitive detection of horseradish peroxidase (HRP) activity coupled with Au NPs and FeDC to amplify the amperometric effect. This has the potential for further applications in the rapid pathogen detection. One important advantage of these amperometric immunosensing strips is that approximately 50 CFU of *E. coli* O157:H7 in milk samples can be detected in 1 h. In this same context, Zhang and co-workers [13] described a sensitive electrochemical immunoassay for rapid detection of *E. coli* by ASV based on core-shell Cu@Au nanoparticles (NPs) as anti-*E. coli* antibody labels.

M NPs due to their high surface/volume ratio offer more contact surface area for attaching carbohydrates and for capturing pathogens. Based on this, *E. coli* detection using epifluorescent microscopy has been performed by functionalizing the surface of M NPs with D-mannose sugar (man-M NPs) through an amide linkage [14], subsequently incubations with fluorescein-labeled concanavalin A (Con A) and *E. coli* cells in phosphate buffer solution (PBS). After that, a magnetic field was applied for separating M NPs/*E. coli* aggregates (see Fig. 3.1a). The supernatants were removed and the remaining aggregates were washed thoroughly, stained with a fluorescent dye (PicoGreen), transferred to a glass slide, and imaged. Fluorescent

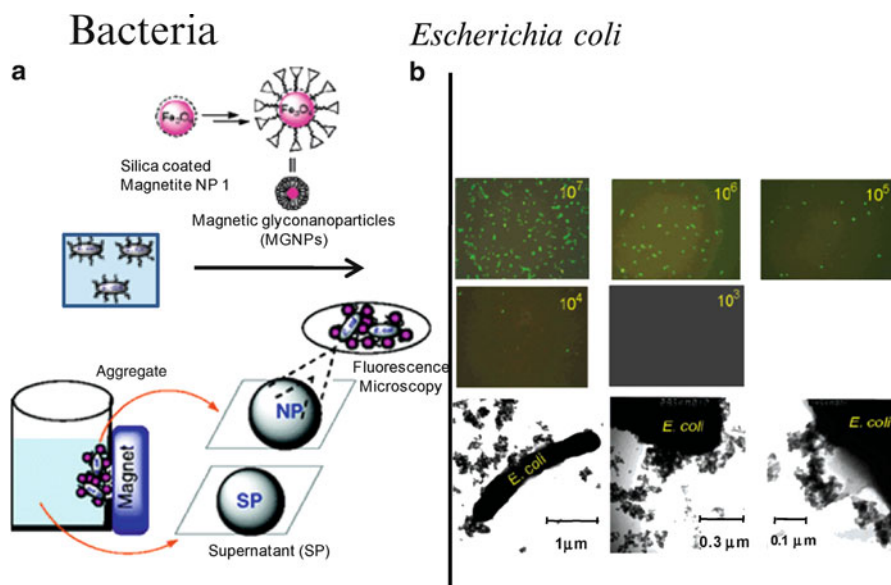


Fig. 3.1 *E. coli* detection using epi-fluorescent microscopy. (a) Schematic representation based on the functionalization of the silica coated magnetite nanoparticles, (b) (above) Increase in the fluorescent emission spectra for increasing concentrations (cells/mL) of *E. coli*., (below) TEM images of MGNP 3/*E. coli* complexes (Adapted from [14] with permission)

microscopic imaging showed that *E. coli* can be detected (see Fig. 3.1b) with a limit of detection 10⁴ cells/mL by obtaining a high capture of bacteria.

Metal nanoshells based nanomaterials that exhibit a surface plasmon resonance (SPR) are also used for *E. coli* detection. Metal nanoshells are thin coatings (a few tens of nanometers thick) on large particles (a few hundreds of nanometers in diameter) which form the cores. Researchers have demonstrated a rapid and reliable test for the detection of *E. coli*, by using the SPR band associated with the coupling of the antibodies to the silver nanoshells [15]. This detection method has shown that the *E. coli* antibody interaction is extremely specific and that the presence of other microorganisms could not produce changes in the SPR band. Furthermore, it could help to shorten the testing time of drinking water used as sample with interest in possible terrorist attacks beside other applications related to the human health.

Electrochemical immunoassays based on Au NPs have also attracted considerable interest for *Salmonella* determination due to its simplicity, high sensitivity, inexpensive instrumentation, and miniaturization. For example, a highly sensitive strategy based on Au NPs for detecting *Salmonella typhi* (*S. typhi*) has been studied by Dungchaia et al. [17]. They immobilized monoclonal antibodies (McAbs) on polystyrene microwells and captured *S. typhi* bacteria by using a copper-enhanced Au NPs label coupled with anodic stripping voltammetry (ASV). The amount of

deposited copper was related to the amount of Au NP tag present, which was controlled by the amount of *S. typhi* attached to the polyclonal antibody–colloidal gold conjugate. Therefore, the anodic stripping peak current was linearly dependent on the *S. typhi* concentration with a detection limit as low as 98.9 CFU/mL with interest for real samples analysis with a low detection limit, high accuracy, and fast analysis time.

Au NPs modified with *Salmonella* spp McAbs can also be evaluated using the electrochemical impedance spectroscopy (EIS) technique as an efficient method for fabricating a capacitive immunosensor for the detection of *Salmonella* spp. in real samples [16].

B. anthracis is another of the biggest threats special because of its potential use in bioterrorism. *B. anthracis* spores can be transmitted easily to humans. These spores are highly resistant to normally destructive environmental factors to living cells, such as heat, toxic chemicals, desiccation, and physical damages. These properties make them suitable for a potential biological warfare [49]. For this reason, the rapid and accurate detection of *B. anthracis* spores in the environment prior to infection is very important for human safety and national security. However, few technologies have been widely evaluated under field conditions [46].

Accordingly, nanomaterial-based biosensors are evolving as promising alternatives to meet this challenge in terms of sensitivity, specificity, time- and cost-efficiency [30]. For this, label-free (bio)sensing systems based on nanomaterials present certain advantages in detection. Among label-free assays, the quartz crystal microbalance (QCM) piezoelectric sensor has proved to be a useful platform in the efficient detection of pathogens including *B. anthracis*. Based on this platform, electrically active magnetic (EAM) nanoparticles are being used as concentrator of DNA targets as well as electrochemical transducers for detection of the *B. anthracis* protective antigen A (pag A) gene [18]. More details can be seen in the Fig. 3.2.

Another biosensor based on QCM has been developed by R. Hao and coworkers for *B. anthracis* spores detection by an anti-*B. anthracis* monoclonal antibody designated to 8 G3 (mAb 8 G3, IgG) functionalized QCM sensor [50].

3.2.2 Toxins

Neurotoxins produced by *Clostridium botulinum*, serotypes A through G (BoNT A-G), are considered the most potent toxins known to humans who cause neuro-paralytic disease [51]. The “Class A agents” are listed as one of the six highest risk threat agents for bioterrorism [22]. Food-borne botulism is the most common intoxication form due to the ingestion of pre-formed Botulinum Neurotoxin (BoNT) in food. For this reason, the development of nanomaterial-based rapid methods that can help to detect terrorist agents, such as anthrax, BoNT, etc. is very important for safety and security purposes.

Colloidal gold has emerged as the preferred label [52] for toxin detection. Alternative rapid methods, as colloidal gold-based immunochromatographic assays

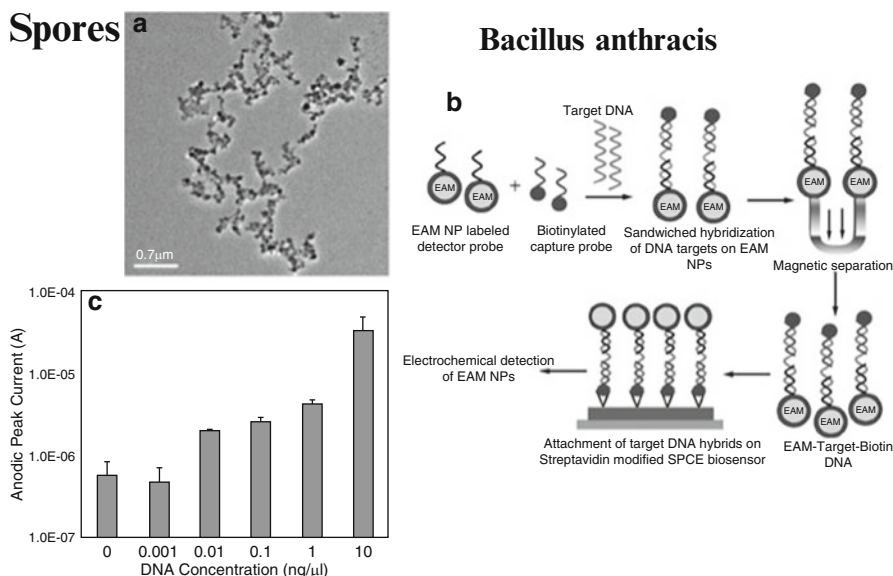


Fig. 3.2 Electrically active magnetic (EAM) nanoparticles in *B. anthracis* DNA detection. (a) TEM image of EAM nanoparticles, (b) Schematic representation of the EAM based electrochemical DNA biosensor detection principle, (c) Electrochemical response of the biosensor to different target DNA concentrations (mean current $\pm$ SD, $n=3$) (Adapted from [18] with permission)

(ICA) (also called lateral flow (LF) or strip assay) have been developed for detection of botulinum neurotoxin type B (BoNT/B). This class of assays was based on the sandwich format using polyclonal antibody (Pab). A thiophilic gel purified anti-BoNT/B Pab was immobilized in a defined detection zone on a porous nitrocellulose membrane and conjugated to colloidal gold particles as a detection reagent. The BoNT/B-containing sample was added to the membrane to react with Pab-coated particles, thus, a change of colour was given in the detection zone with an intensity of red colour proportional to BoNT/B concentration.

The strip assay exhibits potential as a rapid and simple assay method for the detection of toxin in biological fluid that requires no separation steps. Moreover, in function of these characteristics the assay can be considered superior to other immunoassay, such as radioimmunoassay and enzyme-linked immunosorbent assay [20].

Another test based on ICA has been reported by Klewitz et al. [21] to detect botulism neurotoxin D (BoNT/D). The test was based on double sandwich format using a gold-anti BoNT/D monoclonal antibody conjugates (detector reagent) and an anti-BoNT/D chicken polyclonal antibody. Faecal samples or standard samples spiked with various concentrations of BoNT/D were treated in gelatine-phosphate buffer, vortexed and stored at temperature ranging 5–8°C overnight. Polyclonal chicken anti-BoNT/D IgG and gold conjugated monoclonal antibody were added to the sample extracts and incubated at 37°C for 3 h. Then, 50 μ L of pre-treated

Toxins Botulinum Neurotoxin

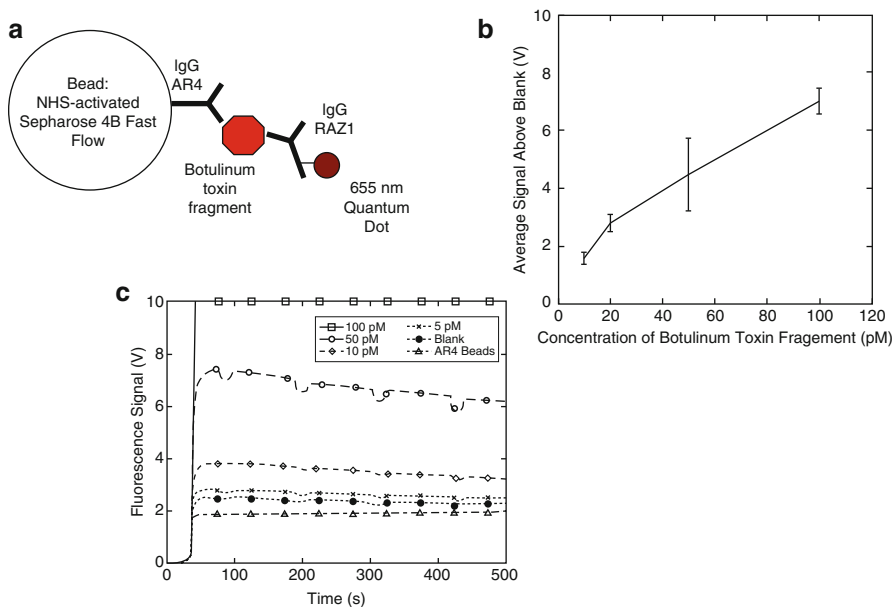


Fig. 3.3 Detection of BoNT/A-HC-fragment with bead-based immunoassay. **(a)** Scheme of the sandwich immunoassay: AR4 primary antibody specific for BoNT attached to a solid support as Sepharose bead; RAZ1 secondary antibody attached to the reporter 655 nm quantum dot and bound to the toxin fragment-primary antibody complex, **(b)** Calibration curve for the detection of BoNT/A-HC-fragment in FBS and **(c)** Detection from BoNT/A-HC-fragment in buffer using 655 nm QDs and detection on a flow system (Adapted from [22] with permission)

samples were applied to the test strip. The intensity of colour, of the red test line (signal intensity), is directly related with the concentration of BoNT/D in the standard or spiked horse faecal samples. Thus, toxin concentrations were determined within 3.5 h down to 50 pg/mL.

In the last years, several researchers have used luminescence methods instead of colorimetric methods for (bio)sensing applications. A novel class of luminescence nanomaterial is emerging for botulinum toxin assays [22]. This is the case of colloidal semiconductor QDs, which are single crystals of few nanometers of diameter whose size and shape can be precisely controlled by the duration, temperature, and ligand molecules used in their synthesis [53]. These nanomaterials have unique optical properties such as high extinction coefficients over a wide wavelength range, size dependent optical emission (due to quantum confinement effects on the electronic structure of the QDs) and relatively high quantum yields, which makes them suitable for biological applications such as fluorescence immunoassays, DNA array technology, fluorescence labeling of cells and tissues, and in the detection of chemical and biological agents. In Fig. 3.3 is shown an example of a fluorescence sandwich immunoassay using high-affinity antibodies and quantum dot (QD) reporters for detection of BoNT serotype A (BoNT/A) using a nontoxic recombinant fragment of

the holotoxin (BoNT/A-H_C-fragment) as a structurally valid simulant for the full toxin molecule [22].

Recently, a detection method based on the use of semiconductor QD-peptide Förster resonance energy transfer (FRET) assemblies to monitor the activity of the BoNT serotype A light chain protease (LcA) has been reported by Sapsford et al. [48]. They evaluated the ability to self-assemble dye-labeled LcA peptide substrates by using a number of different QD materials displaying charged or PEGylated surface-coatings by monitoring FRET interactions. Furthermore, Grate and co-authors [47] have developed for botulinum toxin detection an electrochemiluminescence (ECL) method based on a sandwich complex with paramagnetic beads and capture of antibodies bound to the beads by a streptavidin-biotin linkage, and the detection of antibodies labeled with a ruthenium chelate.

Historically, nanoparticles have been used as label biomolecules. Their properties coupled with the ability to attach nearly any biologic recognition element to the particle surfaces, facilitates their application in multi-target assays. These nanomaterials can be conjugated to DNA sequences or proteins so that, using size-dependent properties as just one example, fluorescence or light scattering can be used as an output signal, respectively. For example, a method for analyzing combinatorial DNA arrays using oligonucleotide-modified gold nanoparticle probes and a conventional flatbed scanner has been studied by the Mirkin's group [54]. Labeling oligonucleotide targets (based on the anthrax lethal factor sequence) with nanoparticles rather than fluorophore probes substantially alters the melting profiles of the targets from an array substrate. This difference permits the discrimination of an oligonucleotide sequence from targets with single nucleotide mismatches with a selectivity that is over three times that observed for fluorophore-labeled targets. In addition, when coupled with a signal amplification method in which silver ions are reduced by hydroquinone to silver metal at the surfaces of the Au NPs, the sensitivity of this scanometric array detection system exceeds that of the analogous fluorophore system by two orders of magnitude.

Labeling technology based on highly fluorescent europium (Eu⁺) NPs could provide a rapid and sensitive testing platform for sensing bioterrorist agents. Lately a europium-nanoparticle based immunoassay (ENIA) for the sensitive detection of anthrax protective antigen has been reported [19]. The use of Eu⁺ NPs further permits to the assay to be adapted to an ELISA format that is already in place in testing laboratories because the antibody-antigen sandwich complex bound to Eu⁺ NPs coated with streptavidin (SA) can be directly measured with a fluorescence reader. This ultrasensitive NP- based assay for the detection of anthrax toxin could provide a useful new tool for infectious agents and chemical contaminants.

3.2.3 Viruses

The detection of infectious viral diseases is very important for the public health. In recent years, a number of viral outbreaks, such as severe acute respiratory syndrome (SARS), influenza A (H1N1 flu) and avian influenza A (H5N1 flu) are

emerging. These have raised significant fears due to that could rapidly spread and turn into a pandemic similar to 1918 Spanish flu that killed more than 50 million people [55]. For this, rapid and sensitive diagnostic techniques using different nanomaterials are being developed for recognizing and controlling future epidemics. An example of this is the recent development of immunochromatographic strip for rapid detection of H9 subtype avian influenza viruses (H9 AIVs) [23]. The assay is based on a colloidal gold anti-hemagglutinin monoclonal antibody conjugate (detection antibody) and an anti-Nucleocapsid protein monoclonal antibody used as a precipitation reagent on the test line of a nitrocellulose membrane. This detection method is rapid and easy to operate without the requirement of special skills and equipment, which makes it a strip suitable for field detection. In addition, this generation of ICA allows doing multiple assays that can help to diagnostic of common poultry diseases, such as Newcastle disease, avian infectious bronchitis, avian infectious laryngotracheitis, etc. Another similar study has been reported by the same authors to detect IgG antibodies against the nucleocapsid protein of AIV subtypes (H5, H7 and H9) in chicken sera [56].

The use of immunosensors based on the identification of virus glycoproteins, [57, 58] or genosensors based on the detection of specific DNA sequences correlated to the virus RNA [59, 60], has been performed as an alternative to more expensive classical methods that consume time. In Mexico and USA, in March-April 2009, there was an outbreak of human H1N1 flu virus that created a pandemic concern [61]. At the time more than 207 countries worldwide reported cases of pandemic H1N1, including at least 8,768 deaths [62]. For this reason developing of accurate, rapid and low cost sensing methods for the early detection of this kind of virus is of great importance.

An impedimetric detection method of a DNA sequence correlated to H1N1 virus using carbon nanotubes platform and Au NPs amplification could be considered a good possibility for improving the sensitivity and rapidity of analysis [24]. Such systems are based on the use of colloidal gold for the labelling of DNA oligonucleotides, and the electrochemical signal of Au NPs onto screen printed carbon nanotubes electrode is measured and correlated to the DNA target concentration.

3.2.4 Parasites

The use of parasites as bioterrorism agents has not received so much attention. Parasites could contribute to the installation of fear in human population upon intentional addition to their food and water supplies. In the last years, biosecurity issues are gaining importance as a consequence of globalization. Surveillance is critical in maintaining biosecurity and early detection of infectious disease agents is essential [63].

Infectious diseases, like malaria, are being one of the greatest health challenges worldwide. Nanotechnology is one of the promising strategies for malaria treatment. The identification of new *Plasmodium* or infected cell targets can be used to modify existing drug delivery systems employing nanotechnology to more efficiently

deliver antimalarial drug molecules to the newly-targeted sites of action. Electrochemical immunosensors offer several advantages compared to alternative detection methods, including the ability to analyze the direct blood samples, high sensitivity, require low sample volume and can be used in field application [64]. For example, nanosized carriers are receiving special attention with the goal of minimizing the side effects of drug therapy, such as poor bioavailability and the selectivity of drugs. Several nanosized delivery systems have already proved their effectiveness in animal models for the treatment and prophylaxis of malaria [34].

A number of strategies for the detection of *Plasmodium falciparum* are being developed by using rapid diagnostic tests based on nanomaterials. Magneto immunoassay-based strategies for the detection of Plasmodium falciparum histidine-rich protein 2 (HRP2) related to malaria have been described by using M NPs [25]. The immunological reaction for the protein HRP2 was performed in a sandwich assay on M NPs by using a second monoclonal antibody labeled with horseradish peroxidase (HRP) enzyme. The modified M NPs were captured on the graphite-epoxy composite electrode surface using a magnet inside of the composite electrode which was used as transducer in the electrochemical detection. This magneto immunoassay based on magnetic nanoparticles has shown a limit of detection (LOD) of 0.36 ng mL^{-1} , which makes it a suitable method for *Plasmodium falciparum* histidine-rich Protein 2 detection related to malaria.

Recently, electrochemical immunosensors based on screen printed electrodes are (SPEs) attracting great interest [65] due to ease of fabrication, ability to mass produce, disposability and portability. Sharma et al. [26] have developed an amperometric immunosensor based on Au NPs/alumina sol-gel modified SPEs for antibodies to *Plasmodium falciparum* histidine rich protein-2 (*Pf*HRP-2) by dripping Al_2O_3 sol-gel on SPE followed by electrochemical deposition of Au NPs. The antibodies in rabbit serum sample were allowed to react with the *Pf*HRP-2 protein which was immobilized on the modified SPE to form antigen-antibody immune complex (*Pf*HRP-2/anti-*Pf*HRP-2). The bound antibodies were quantified by alkaline phosphatase (AP) enzyme labeled secondary antibodies (anti-rabbit immunoglobulins-AP conjugate). Enzymatic substrate, 1-naphthyl phosphate was converted to 1-naphthol by AP and an electroactive product was quantified using amperometric technique. Figure 3.4 shows some results of this electrochemical detection.

Although the electrochemical immunosensors together with the nanotechnology are providing certain advantages, in terms of improving the selectivity and sensitivity of the detection systems in the field applied to parasites detection, it is a domain that is still under development.

3.2.5 Explosives

The conventional methods to detect explosives are restricted by disadvantages such as expensive instrument usage and time-consuming processes. Therefore, it is important to develop methods for trinitrotoluene (TNT) assay with simplicity,

Viruses

Plasmodium falciparum

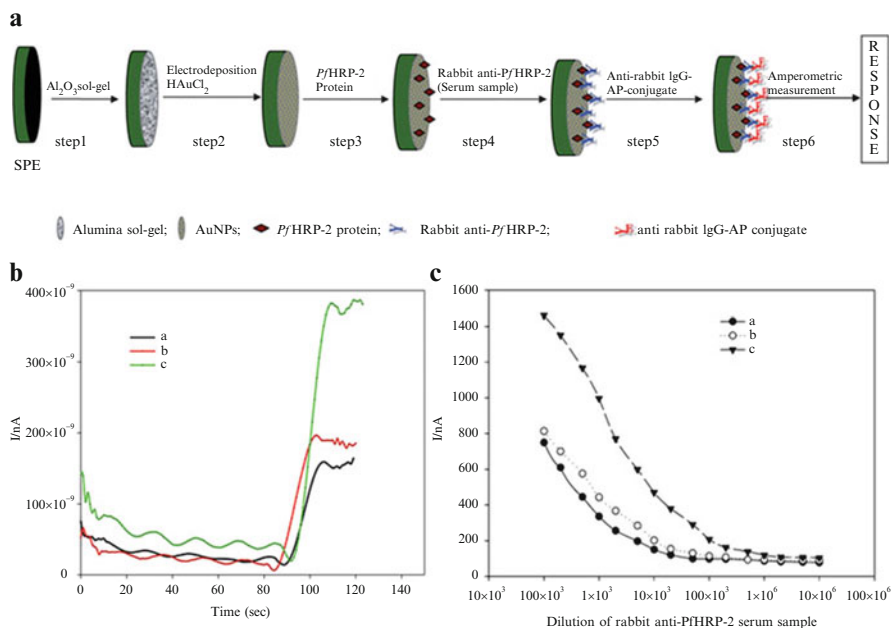


Fig. 3.4 Detection of *Plasmodium falciparum* histidine rich protein-2. (a) Schematic representation of the modification and immobilization of biomolecules on screen-printed electrode (SPEs), (b) Amperometric signals for rabbit-anti PfHRP-2 (1:20,000 dilution) on (a) Bare SPE, (b) Al_2O_3 -sol-gel/SPE, and (c) AuNPs/ Al_2O_3 -sol-gel/SPE immunosensor in DEA buffer solution at pH 9.8, applied potential 400 mV vs. Ag/AgCl/sat.KCl reference electrode (Adapted from [26] with permission)

sensitivity, rapidity and cost-efficiency. Lately, methods for TNT assay have been developed by using Au NPs and QDs. For example, Jiang and co-workers [66] reported a simple and sensitive method for colorimetric visualization of TNT at picomolar levels based on color changes of Au NPs in the presence of TNT via the electron-donor–acceptor interaction between TNT and primary amines.

New methods have been tested for the determination of TNT based on fluorescence quenching of QDs. Chen et al. [27] have reported a sensitive method with high selectivity for TNT detection by using water-soluble L-cysteine-capped CdTe QDs as fluorescence probe. L-cysteine is used as stabilizer of QDs and as primary amine provider. Intermediate complexes between TNT and cysteine are formed, resulting in the fluorescence quenching of the QDs. This method can be adapted for the detection of TNT. The LOD was 1.1 nM and specificity detection was achieved. More details are observed in the Fig. 3.5.

Other methods based on fluorescence detection have been reported by Wang and co-authors where the fluorescence of oleic-acid-covered CdSe QDs

Explosives

Trinitrotoluene

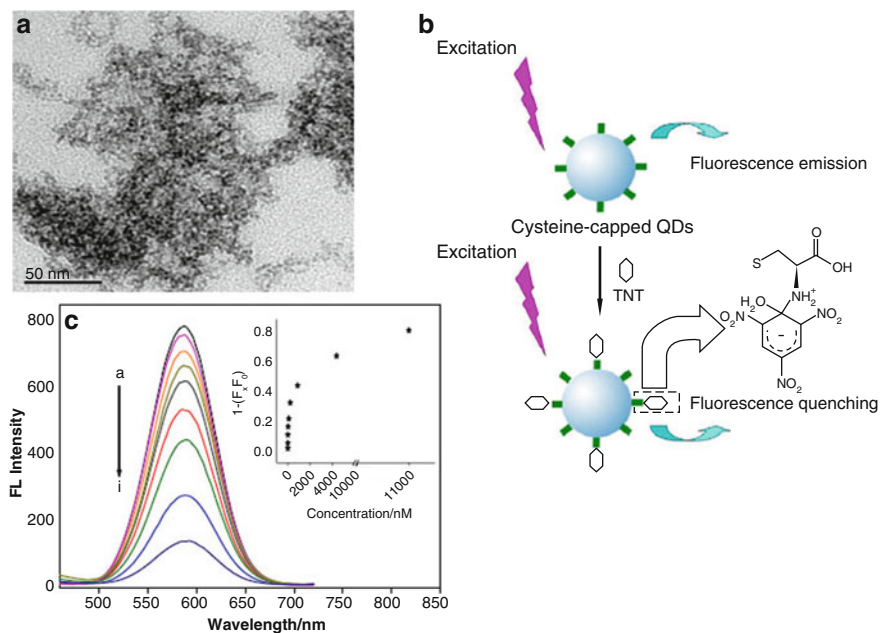


Fig. 3.5 L-cysteine-capped CdTe QD-based sensor for trinitrotoluene (TNT) detection. (a) HRTEM image of CdTe QDs, (b) Schematic representation for the L-cysteine-capped CdTe QD-based sensor for TNT detection, (c) FL spectra and the quenching efficiency (inset) of L-cysteine-capped CdTe QDs in the presence of different concentrations of TNT (from (a) to (i) 1.1×10^{-9} , 5.5×10^{-9} , 2.2×10^{-8} , 8.8×10^{-8} , 2.2×10^{-7} , 8.8×10^{-7} , 4.4×10^{-6} and 1.1×10^{-5} M) (Adapted from [27] with permission)

could be efficiently quenched by nitro aromatic analytes [67]. Zhang et al. found that aminoligand-modified ZnS-Mn²⁺ nanocrystals can be introduced to detect TNT [68].

Recently, graphene owing to its unique properties, such as remarkable electronic conductivity, incredibly large electroactive surface area, high affinity and electrocatalytic activity [69] is considered another of the interesting nanomaterials to be used for nitroaromatic explosives detection. Guo et al. [28] have evaluated a new ionic liquid (IL)-graphene composite prepared by combining IL and graphene with large specific surface area and pronounced mesoporosity for ultratrace explosive trinitrotoluene detection. On the other hand, the determination of 2,4-Dinitrotoluene (2,4-DNT) by means of electrochemically reduced graphene on glassy carbon electrode (GCE) has been analyzed by using electrochemical detection, which gave a low detection limit of 42 nmol L⁻¹ (S/N=3) [29].

3.3 Conclusions

Nanotechnology could provide unlimited opportunities for improving the efficacy of (bio)sensing systems for bioterrorist agents. In this chapter are presented some of the detection methods more commonly used for bioterrorist agents as well as for other safety and security applications. However, the domain that nanotechnology has in the detection of bacteria, toxins, parasites, viruses and explosives is still in development phase. To overcome the challenges of nanomaterial-based (bio)sensing strategies for safety and security applications a more detailed study related to interferences for real sample analysis as well as technological aspects related to final application need to be addressed.

Point strategies to overcome the challenges should be (a) *In-field applications*. *In-field* applications of nano-biosensing still need a big effort so as to overcome problems related to applications in real samples. Avoidance of interferences coming from sample matrix is the key point for success; (b) *Low detection limits*. Reaching of low detection limits (detection of few molecules, bacteria, cells, etc.) in a relatively high volume of samples (i.e. 1 molecule or 1 cell in 1 mL food sample) needs the development of fast and efficient preconcentration tools/routes based on nano & microfabrication.

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Chapter 4

Molecular Imprinting for Selective Sensing of Explosives, Warfare Agents, and Toxins

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Abstract The explosive, warfare agent, and toxin worldwide threats are important hazardous determinants for a safe living environment. The need for better methods of determinations of these compounds generates a strong demand for sensitive, selective, and fast sensing systems. Typically, biorecognition receptors, like enzymes or antibodies, are used to devise biosensors for selective determinations of these compounds. However, these biosensors suffer from several deficiencies. Therefore, synthetic receptors, and particularly those using the concept of molecular imprinting, are more and more often being devised as alternatives of the bioreceptors. Among them, the most popular now are molecularly imprinted materials. The present chapter critically evaluates achievements in the development of chemosensing using the imprinting idea for selective determination of biohazardous compounds. Different methods of preparation of synthetic imprinted recognition receptors are scrutinized and various platforms of analytical signal transduction are assessed.

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Abbreviations

AAm	acrylamide
ACN	acetonitrile
ACCN	EGDMA/1,1'-azobis(cyclohexane carbonitrile)
AgNP	silver nanoparticle
AIBN	azobis isobutyronitrile
APBA	3-aminophenylboronic acid
APh	2-aminophenol
APTMS	3-aminopropyl trimethoxysilane
2-AThPh	2-aminothiophenol
4-AThPh	4-aminothiophenol
ATR	attenuated total reflectance spectroscopy
AuNP	gold nanoparticle
BTEB	bis(trimethoxysilylethyl)benzene
BWA	biological warfare agent
CP	conducting polymer
CV	cyclic voltammetry
DMF	<i>N,N</i> -dimethylformamide
DPV	differential pulse voltammetry
ECP	electronically conducting polymer
EGDMA	ethylene glycol dimethacrylate
ELISA	enzyme-linked immunosorbent assay
GA	glutaraldehyde
GCE	glassy carbon electrode
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMPA	hexamethylphosphoramide
IA	itaconic acid
ITMS	iodotrimethylsilane
ITO	indium-tin oxide
IOW	integrated optical waveguide
LBL	layer-by-layer (transfer)
LOD	limit of detection
LSV	linear sweep voltammetry
MA	methacrylic acid
MIECP	molecularly imprinted electronically conducting polymer
MIP	molecularly imprinted polymer
MIPAPBA	molecularly imprinted poly(aminophenylboronic acid)
MIPEDOT	molecularly imprinted poly ethylenedioxythiophene

MIPPy	molecularly imprinted polypyrrole
NIP	non-imprinted polymer
NOPE	2-nitrophenyloctyl ether
OTS	octadecylsiloxane
PANI	polyaniline
PBS	phosphate buffer saline
PM	piezoelectric microgravimetry
PPV	poly(4-phenylene vinylene)
PVC	poly(vinyl chloride)
Py	pyrrole
SAM	self-assembled monolayer
SCE	saturated calomel electrode
SDS	sodium dodecyl sulfate
SERS	surface enhanced Raman scattering
SPE	solid phase extraction
SPME	solid phase microextraction
SPR	surface plasmon resonance
SWV	square wave voltammetry
TEOS	tetraethoxysilane
TMSEPy	2-(trimethoxysilylethyl)pyridine

Symbols

G	Gibbs free energy
H	enthalpy
KMIP-A	stability constant of a complex of the analyte A and the molecular cavity of MIP
S	entropy
q	resonance angle

4.1 Introduction

Chemical sensing is a part of an information acquisition, in which some insight is gained into chemical composition of the studied system in real time [1]. Chemical sensing involves selective recognition and selective signal amplification. The former is the ability to recognize primarily only one analyte in the presence of interferants. It generates a response, which requires amplification in order to produce an analytical signal in the acceptable, undistorted form. Thus, the recognition is provided by some selective interactions while the amplification by some physical means. The latter can be realized, for instance, by selective analyte accumulation or selective catalysis. The essential component of any chemical sensing system is a recognition unit,

which is responsible for selective binding of a target analyte in the presence of interferants. This binding can be irreversible in case of disposable, i.e., single-use chemosensors, and have to be reversible in case of multiple-use chemosensors. The recognition unit can be either artificial or natural. The former includes synthetic organic and inorganic materials whereas the latter embraces immobilized proteins (antibodies, enzymes, and histons), nucleic acids (DNA, RNA, and aptamers), intracellular receptors, cell receptors, microorganisms, living plants, and even whole animal tissues. Generally, biological recognition systems reveal higher affinity and selectivity than their synthetic counterparts. Moreover, in many systems they are even specific. However, these systems suffer from several drawbacks including limited stability (because of poor tolerance of extreme solution acidity or basicity, temperature, some organic solvents, and exposure to external fields, like the electromagnetic or ultrasonic field), short lifetime, low availability, and high cost because of the necessity of tedious purification as well as inadequate reproducibility. For instance, the same enzyme, from the same manufacturer but of different batch, may reveal different activity. Therefore, there is a growing demand for synthetic biomimetic materials as reusable alternatives of those expensive natural materials.

Over the last two decades, the introduction of specific binding sites within synthetic materials by template-directed synthesis, and preparation of molecularly imprinted polymers (MIPs) in particular, according to the well established supramolecular chemistry concepts [2] has attracted considerable attention manifested in numerous applications. MIPs are prepared by co-polymerizing functional and cross-linking monomers in the presence of a template and a porogen. This template can be either similar to the target analyte (dummy template) [3] or be the target substance itself, i.e., the analyte that the polymer should subsequently recognize selectively. During MIP synthesis, first, a template molecule and that of the functional monomer bearing complementary recognition sites are pre-organized in solution, by self assembly, to yield a complex by virtue of either covalent or non-covalent bonds formed between their complementary binding functionalities. Importantly, only these templates can be imprinted selectively, which form sufficiently stable complexes with functional monomers and stay intact during subsequent extensive polymerization. Moreover, structure of these complexes should be preserved during this polymerization in the presence of cross-linking monomers and porogens, such as appropriate solvents or ionic liquids. After subsequent removal of the template, molecular cavities (imprints) complementary in size, shape, and orientation of their recognition sites to those of the template are left in the polymer. These cavities are then capable of selective binding of analyte molecules even in the presence of interfering substances of similar structure [4]. However, if the analyte to be determined is not available in its pure form to serve first as a template or it is chemically transformed during imprinting then a dummy template is used.

The analogy between MIPs and biologically originating receptors as affinity reagents is straightforward. MIPs imitate the receptor-ligand, antibody-antigen, histone-DNA, or enzyme-substrate biorecognition [5]. Therefore, they can be used in applications relying on selective molecular binding (Fig. 4.1) [4, 6]. Advantages of the MIP-based biomimetic materials include (i) high binding affinities similar to

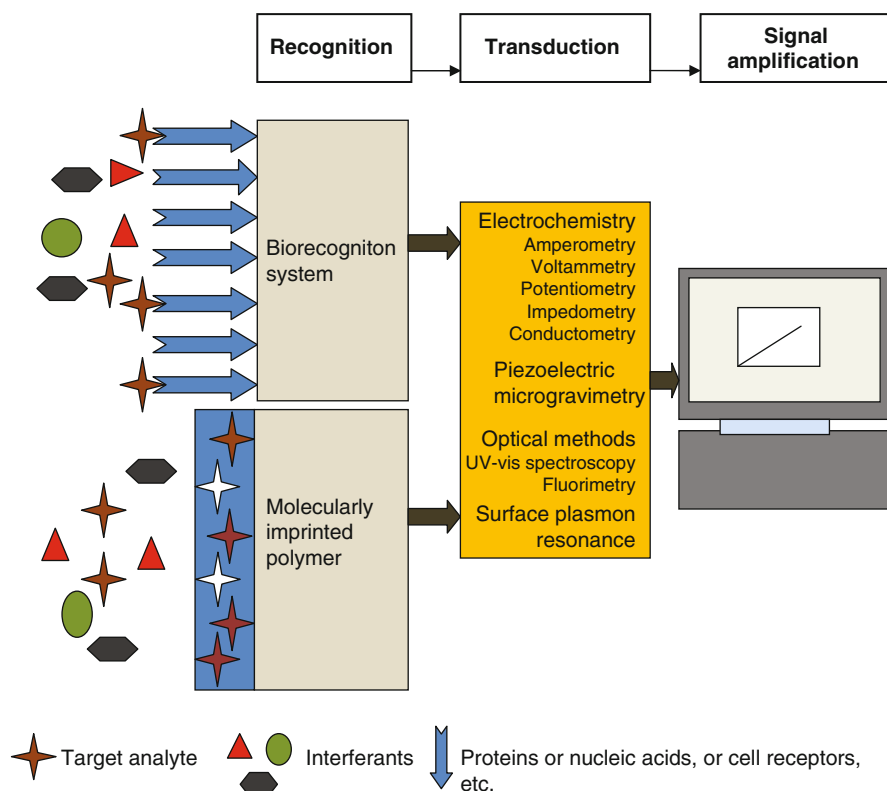


Fig. 4.1 Simplified representation of operation of a biosensor and a chemosensor with a synthetic receptor, such as a molecularly imprinted polymer film, using typical platforms of analytical signal transduction

those of biological recognition units, (ii) robustness and stability under a wide range of harsh chemical environments, such as the presence of oxidants or reductants, aggressive organic solvents, extreme pH or temperature values as well as the external electromagnetic or ultrasound field, and (iii) ability of easy designing of recognition sites for analytes that lack suitable biorecognition units. For instance, there is such a natural unit neither for the cyclosporine A immunosuppressive drug nor for the 2,4-dichlorophenoacetic acid pesticide both featuring small non-immunogenic molecules. Therefore, these analytes have to be coupled to some carrier molecules in order to bind to antibodies. However, these carrier molecules are not needed in molecular imprinting. Moreover, this imprinting does not require any animal tests. These advantages make MIPs attractive alternatives of biorecognition units for analytical applications, such as packings for column chromatographic separations [7], sorbents for solid phase microextraction (SPME) [8, 9], and recognition units for chemical sensors [10–15].

4.2 Preparation of Molecularly Imprinted Systems

There are three main approaches for preparation of molecular imprints, which include (i) covalent, (ii) non-covalent, and (iii) pseudo-covalent template binding (Fig. 4.2).

In covalent imprinting [16, 17], templates are covalently bound in a reversible way to one or more recognition sites of a monomer. After polymerization, the template is removed by bond cleavage and the recognition sites of the molecular cavities left are capable of binding the target analyte molecule by re-establishing the same covalent bonds. The advantage of this approach is that the recognition sites of the imprinted cavities are associated only with the template binding sites. However, just a limited number of compounds can be imprinted that way. These include diols, aldehydes, ketones, amines, and carboxylic acids.

In non-covalent imprinting [18–20], interactions between functional monomers and the template during polymerization, such as H-bonding, ion-pairing as well as

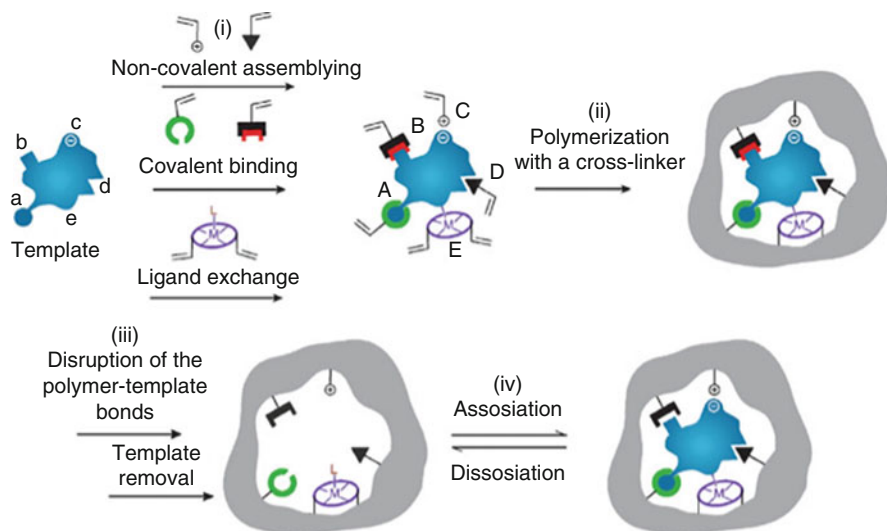


Fig. 4.2 Simplified representation of molecular imprinting. (i) Formation of reversible interactions between the template and the functional monomer may involve one or more of the following reciprocal actions (A) reversible covalent binding (B) covalent attachment of polymerizable binding groups that are activated for non-covalent interactions by template cleavage, (C) electrostatic interactions, (D) hydrophobic or van der Waals interactions, or (E) co-ordination by a metal centre; each formed with complementary functional groups or structural elements of the template (a–e), respectively. (ii) A subsequent polymerization in the presence of a cross-linking monomer, a cross-linking reaction, or other process results in formation of an insoluble matrix (which itself can contribute to recognition through steric, van der Waals and even electrostatic interactions), in which the template molecules reside. (iii) Template removal from the polymer through disruption of polymer-template bonds and extraction from the matrix. (iv) The analyte, or analogues thereof, selective binding in the cavities vacated by the template. While vinyl polymerization is presented here by way of example, the same basic scheme can equally well be applied to sol–gel polycondensation, electropolymerization, etc. (Adapted from [28])

the dipole-dipole and hydrophobic interactions, are reproduced in subsequent binding of the analyte by the template-free MIP [20]. Due to its simplicity, this procedure is most commonly used.

The pseudo-covalent strategy combines advantages of both the covalent and non-covalent procedures [21].

Initially, MIPs were mostly prepared by free-radical polymerization, resulting in non-conducting polymers [15]. Accordingly, the majority of early procedures of MIP syntheses refer to bulk chemical polymerization of vinylic or acrylic functional monomers, often derivatized with recognition sites. These sites allowed for non-covalent preassembly in solution of complexes of templates and these functional monomers [22]. Free-radical polymerization requires the presence of a polymerization initiator and light or heat to induce it.

Recently, electroactive monomers are used for preparation of electronically conducting (ECP), and non-conducting polymers for molecular imprinting and chemical sensing [23–27]. Imprinting of a template in these molecularly imprinted electronically conducting polymers (MIECPs) can be facilitated by formation of hydrogen bonds between atoms of the binding sites of template and atoms of recognition sites of the electroactive functional monomer or its derivative. Additionally, π - π interactions between MIECP and the template make the imprinted cavity more selective. That is, the presence of π electrons in ECP enhances stabilizing dispersive interactions between the aromatic rings of MIECP and the template.

For imprinting in polymers prepared of electroactive monomers, electrochemical polymerization is a method of choice for synthesis of a polymer film directly on a conducting transducer surface. This electropolymerization can be performed under galvanostatic, potentiostatic, and most commonly, under potentiodynamic conditions. This is mostly because the galvanostatic and potentiostatic electropolymerization lead to charged ECPs with counter ions entrapped to neutralize this charge while the potentiodynamic electropolymerization may lead either to the charged or uncharged polymer depending upon the final potential selected. Advantages of the MIP preparation by electropolymerization includes the possibility to control (i) the rate of polymer nucleation and growth by proper selection of electrochemical parameters, (ii) film thickness with the amount of charge passed through the electrochemical cell during film deposition, and (iii) film morphology, and porosity in particular, by selection of a suitable solvent and supporting electrolyte. Moreover, preparation of these polymer films does not require initiators, UV light, or heat for polymerization neither spin coating nor surface casting for film deposition. Notably, electropolymerization using electroactive functional monomers, such as aniline, pyrrole, thiophene, and their derivatives lead to conducting MIPs and those like phenol, thiophenol, and phenyldiamine, may lead to non-conducting MIPs [29].

In recent years, sol–gel polycondensation has emerged as an appealing alternative for preparation, under ambient conditions, of inorganic materials for molecular imprinting [13, 30]. These materials exhibit tunable porosity, high thermal stability, chemical inertness, and negligible swelling in aqueous solutions. For their syntheses, appropriate silica alkoxide precursors are selected as functional monomers. The resulting materials are applied as sensing films, and adsorbents [31–33]. In these syntheses, a template is directly added to the silica solution prior to its acid- or

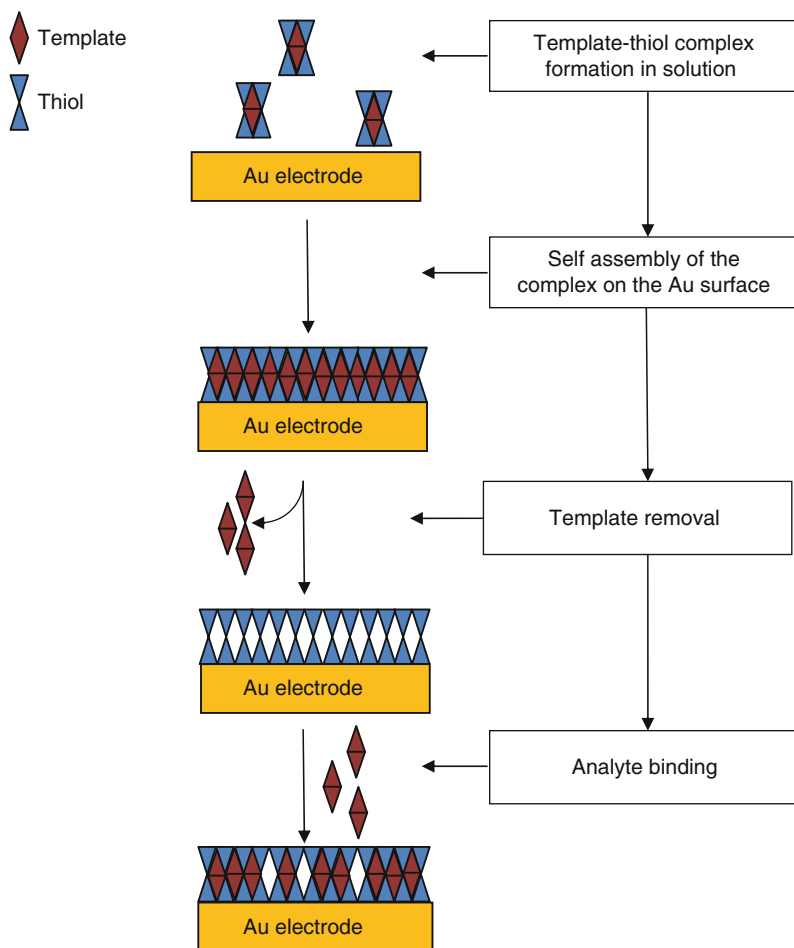


Fig. 4.3 Molecular imprinting by self-assembling of thiols and templates

base-catalyzed hydrolysis. Subsequent condensation involves electrostatic, π -stacking, and van der Waals interactions between silica functional monomers and the template. After drying, removal of the template with an appropriate solvent leaves the imprinted cavities with a favorable size, shape, and chemical environment capable of selective binding of the analyte. This cavity formation requires an adequate polar or nonpolar functional monomers and a solvent of medium polarity, like ethanol.

Other molecularly imprinted systems suitable for fabrication of artificial receptors include self-assembled monolayers (SAMs). These systems, prevalingly involving alkylthiols deposited on Au substrates, have recently attracted considerable attention due to the well described properties of the Au surface and ease of SAM processing [34, 35]. In these systems, recognition is based upon the formation of rigid molecular structures organized around the template molecules by SAMs at the Au electrode surface in a form of two-dimensional imprints (Fig. 4.3).

Preparation of these systems involves simultaneous adsorption of the template and alkylthiol molecules, fitted with recognition sites, at the Au surface followed by template removal. Subsequent recognition is possible if interactions between the analyte and the alkylthiol lead to the formation of a sufficiently stable surface-confined complex.

4.3 Application of Molecular Imprinting for Chemosensing

Typically, the recognition unit, containing artificial or natural receptor sites in a chemo- or biosensor, respectively, is integrated with a transducer surface to generate an output detection signal (Fig. 4.1). This principle is operational for chemosensors using molecularly imprinted recognition units as well. Preferential binding of the analyte by the receptor sites induces changes in the physicochemical properties of the chemosensing system [36]. For devising chemosensors of superior performance, several procedures were developed. They involve direct deposition of films of imprinted materials on transducer surfaces by (i) electropolymerization of an electroactive functional monomer to form MIECPs, (ii) drop-coating of a solution or suspension of a pre-prepared polymer, (iii) preparation of composite membranes containing a conducting material (e.g., metal nanoparticles, carbon nanotubes, graphite, or carbon black), MIP particles, and a binder (e.g., PVC), and (iv) in situ chemical polymerization of a complex of a functional monomer, cross-linking monomer and a template in solution then deposition of the resulting polymer on a transducer surface by spin-coating to form a thin MIP film. Compared to the drop coating, composite making, and spin coating procedures of MIP film preparation, electropolymerization is advantageous because of well adherence of the resulting MIP film to the transducer surface as well as the ease, simplicity, and rapidity of its preparation.

Although chemosensors devised by drop coating of their MIP films reveal excellent electrochemical characteristics, they endure poor reproducibility and low stability [37, 38]. These deficiencies hinder the use of drop coating for fabrication of disposable chemosensors. Chemosensors prepared by drop coating of composite films containing particulate MIPs and conducting materials embedded in PVC matrices can be used for single measurements only. This is due to swelling of MIP particles. Consequently, as the electrochemically active area increases so does the electrochemical capacitance. In addition, powder processing is time consuming, MIP-analyte equilibration time is long, non-specific adsorption is strong, and reproducibility of the measurements is low [37, 38]. Hence, fabrication of MIP chemosensors by using this approach is discouraged.

Spin coating of a transducer surface with a layer of a solution for polymerization, followed by in situ polymerization, is a superior alternative with respect to the use of acrylic formulas [39]. It enables direct control over the film thickness, tuning of the electrode features (like hydrophobicity and permeability of the film), and rapid screening of the performance of the recognition unit. Unfortunately, common procedures of renewal of these MIP chemosensors are limited because of the restricted

access of the washing solution through the pores of the polymer, which results in hindrance of outward diffusion of the electrochemically oxidized or reduced products through the MIP film [40]. However, single-use screen-printed electrodes can be prepared using this procedure.

The selectivity, robustness, ease of handling, and possibility of direct preparation as recognition units on transducer surfaces make MIPs to be a preferred choice for fabrication of sensing devices. Now-a-days, MIPs are successfully used as artificial recognition units associated to various transducers, especially optical, electrochemical, and microgravimetric being the most common among them. The present chapter critically evaluates the achievements in development of procedures using chemosensors based on the molecular imprinting concept for one of their most important applications, namely, for selective determination of biohazardous compounds. These include explosives, warfare agents, and toxins. Herein, different methods of MIP preparation are scrutinized and various platforms of analytical signal transduction assessed. In Tables 4.1 and 4.2, analytical properties of these chemosensors are compiled, including linear concentration range and limit of detection (LOD), along with the functional and cross-linking monomers used as well as conditions and solutions for template extraction applied, methods and conditions of signal transduction indicated, and composition of solutions for analyte binding given.

4.3.1 Determination of Explosives

Rapid detection of explosives (Fig. 4.4), and their determination in environmental samples in particular, is crucial because of the health, ecology, and security risks caused by their use in concealed weapons. Large efforts have been paid to devise innovative and effective MIP-based chemosensors, capable of determining the 2,4,6-trinitrotoluene **1**, 2,4-dinitrotoluene **2**, 2,4,6-trinitrophenol **3**, 1,3,5-trinitrope-
rhydro-1,3,5-triazine **4**, ethylene glycol dinitrate **5**, pentaerythritol tetranitrate **6**, 1,2,3-trinitroxypropane **7**, 2,4-dinitrophenol **8**, and 1,3-dinitrobenzene **9** explosives (Fig. 4.4) rapidly, at low concentration, and in complex matrices. These chemosensors predominately use electrochemical [41, 42], microgravimetric [43, 44], and optical spectroscopy [45–49] transduction platforms (Table 4.1).

The most commonly used highly explosive compound is **1** (Fig. 4.4). In response to great need of its selective determination, both in gas phase and solutions, several different chemosensors have been devised (Table 4.1).

In order to detect **1** in a gas phase, spectrophotometric MIP chemosensors utilizing the planar integrated optical waveguide (IOW) for attenuated total reflectance spectroscopy (ATR) transduction is applied [45, 46]. For MIP preparation, a pseudo-covalent procedure was adopted. For that, bis(trimethoxysilylethyl)benzene (BTEB) and 2-(trimethoxysilylethyl)pyridine (TMSEPy) silane were used as functional monomers. During acid-catalyzed sol–gel polycondensation, a silane derivative of **1**, namely **10** (used as the template), was covalently incorporated in the sol–gel

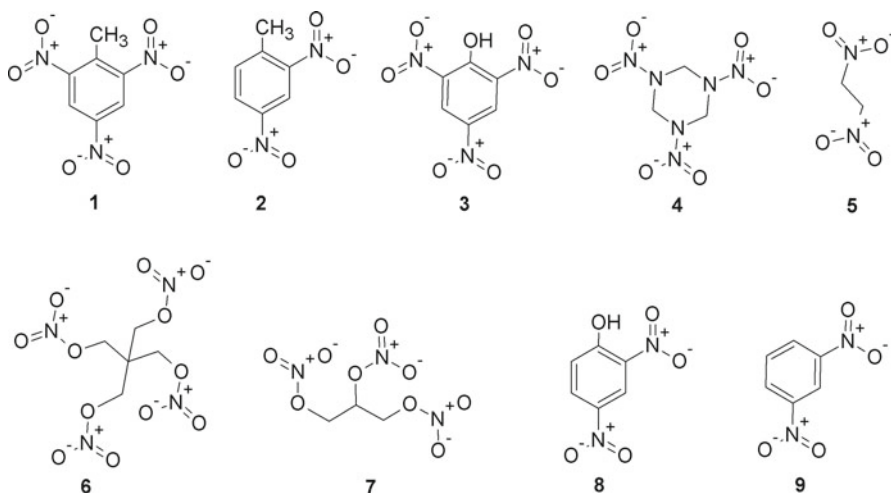


Fig. 4.4 Structural formulas of different explosives, their interferants and dummy templates: 2,4,6-trinitrotoluene **1**, 2,4-dinitrotoluene **2**, 2,4,6-trinitrophenol **3**, 1,3,5-trinitroperhydro-1,3,5-triazine **4**, ethylene glycol dinitrate **5**, pentaerythritol tetranitrate **6**, 1,2,3-trinitroxypropane **7**, 2,4-dinitrophenol **8**, 1,3-dinitrobenzene **9**

matrix (Fig. 4.5). The resulting recognition film of organically modified sol–gel film was deposited directly on the IOW surface. Then, the template was removed by cleavage of the template–polymer covalent bonds with 5% (v:v) iodotrimethylsilane (ITMS) in ACN yielding the shape-selective molecular cavities featuring recognition sites of primary amine groups. Subsequent binding of the **1** analyte and its conversion to the corresponding anion resulted in absorption of visible light at 540 nm propagating through the waveguide. Although sensitivity of the chemosensor was quite satisfactory, it unfortunately decreased markedly (>50%) at high relative humidity, if preventive measures were not taken.

Other studies resulted in chemosensors for **1** that integrated MIP recognition with signal transduction involving the surface enhanced Raman scattering (SERS) [47, 50]. SERS is an extremely sensitive and selective spectroscopic technique that involves enhancements in the Raman scattering intensities of analytes adsorbed on a roughened metal surface (typically, gold or silver) [51], when the incident light beam strikes the metal surface and excites localized surface plasmons. For preparation of a recognition unit, **1** was templated with the aminopropyl tetraethoxysilane, methyltriethoxysilane, and mercaptopropyltrimethoxysilane. This sol–gel mixture was spin coated on an SERS-active Au surface for deposition of a molecularly imprinted film. The **1** template was then removed from the resulting matrix with a mixed solution of ethanol, ACN, and acetic acid leaving in it the **1**-compatible molecular cavities. Subsequent binding of the **1** analyte within these cavities resulted in unique SERS bands at ~ 830 and ~ 1350 cm^{-1} . These bands, corresponding to the $-\text{NO}_2$ out-of-plane bending and stretching vibrations, respectively [52], were

Table 4.1 Properties of MIP-based chemosensors for determination of explosives

Analyte	Functional/cross-linking monomer/initiator	Porogen	Extraction solution or conditions	Transduction method	Solution of analyte binding	Linear concentration range	LOD	Ref.
1	(Methacrylic acid)/EGDMA/AIBN	CHCl ₃	Methanol	SWV	0.07 M HCl (pH = 1.1)	5 nM to 1 μ M	1.5 nM	[41]
	4-Aminothiophenol, 2-mercaptoethane sulfonic acid, AuNPs	0.1 M Phosphate buffer (pH = 7.4)	0.1 M Phosphate buffer (pH = 7.4)	LSV	0.1 M phosphate buffer (pH = 7.4)	–	46 ng L ⁻¹	[42]
	(Methacrylic acid)/EGDMA/AIBN	CHCl ₃	CHCl ₃	PM	Inert gas	–	150 μ g g ⁻¹ MIP	[43]
	(Methacrylic acid)/EGDMA/-	CHCl ₃	CHCl ₃	PM	Inert gas	–	150 pg μ g ⁻¹ MIP	[44]
	Bis(trimethoxysilyl)ethyl benzene, 2-(trimethoxysilyl)ethylpyridine	THF	Iodotrimethylsilane in ACN, 5% (v:v)	Optical spectroscopy	Air	–	5 μ g L ⁻¹	[45]
	Bis(triethoxysilyl)benzene, 2-(trimethoxysilyl)ethylpyridine	ACN	Iodotrimethylsilane in ACN, 5% (v:v)	Optical spectroscopy	Air	Up to 300 ng L ⁻¹	2.4 ng L ⁻¹	[46]
	3-Aminopropyl triethoxysilane, 3-mercaptopropyl triethoxysilane methyltriethoxysilane	–	Ethanol:ACN: (acetic acid) (8:2:1, v:v:v)	SERS	ACN	Up to 0.1 mM	3 μ M	[47]
	Poly(<i>para</i> -phenylenevinylene)	–	Lithium <i>n</i> -propyl mercaptide in HMPA	Fluorescence spectroscopy	–	–	–	[48]
	(Methacrylic acid)/EGDMA/1-hydroxycyclohexyl phenylketone	CHCl ₃	Methanol	Fluorescence spectroscopy	Phosphate buffer saline	Up to 0.5 mM	40.7 μ M	[49]

	4-Aminothiophenol	Ethanol:ACN (8:2, v:v)	Methanol: (acetic acid) (9:1, v:v)	SERS	–	0.1 μ M to 10 pM	1 pM	[50]
	1-Dodecanethiol	–	Potentiodynamic (0.1–0.8 V vs. SCE)	DPV	0.2 M Phosphate buffer (pH=7.0)	40 nM to 3.2 μ M	1.3 nM	[54]
	Gelatin	ACN:(phosphate buffer) (1:5, v:v) (pH=7.0)	Ethanol:ACN: (acetic acid) (8:2:2, v:v:v)	Optical spectroscopy	Aqueous	Up to 2.0 mM	–	[55]
	4-Aminothiophenol, 2-mercaptoethane sulfonic acid, AuNPs	0.1 M HEPES buffer (pH=7.2)	0.1 M HEPES buffer (pH=7.2)	SPR	0.1 M HEPES buffer (pH=7.2)	Up to 5 pM	10 fM	[56]
4	4-Aminothiophenol, 2-mercaptoethane sulfonic acid, AuNPs	–	0.1 M HEPES buffer (pH=7.2)	SPR	0.1 M HEPES buffer (pH=7.2)	Up to 1 pM	12 fM	[57]
5	4-Aminothiophenol, 2-mercaptoethane sulfonic acid, AuNPs	0.1 M HEPES buffer (pH=7.2)	0.1 M HEPES buffer (pH=7.2)	SPR	Ethanol	Up to 2 nM Up to 20 pM	400 fM 200 fM	[58]
7						Up to 2 nM	20 pM	
8	(Acrylamide, glycidyl methacrylate)/ (trimethylolpropane trimethacrylate)/AIBN	ACN	Methanol:(acetic acid)(9:1, v:v)	CV	–	0.7–30 μ g L ⁻¹	0.1 μ g L ⁻¹	[59]
9	Acrylamide/EGDMA/ AIBN	ACN	0.1 M HEPES buffer (pH=7.2) Methanol:(acetic acid) (9:1, v:v)	DPV	0.1 M Phosphate buffer (pH=7.0)	22 nM to 3.0 μ M	7.3 nM	[68]

2,4,6-Trinitrotoluene **1**, 1,3,5-trinitroperhydro-1,3,5-triazine **4**, ethylene glycol dinitrate **5**, pentaerythritol tetranitrate **6**, 1,2,3-trinitroxopropane **7**, 2,4-dinitrophenol **8**, 1,3-dinitrobenzene **9**

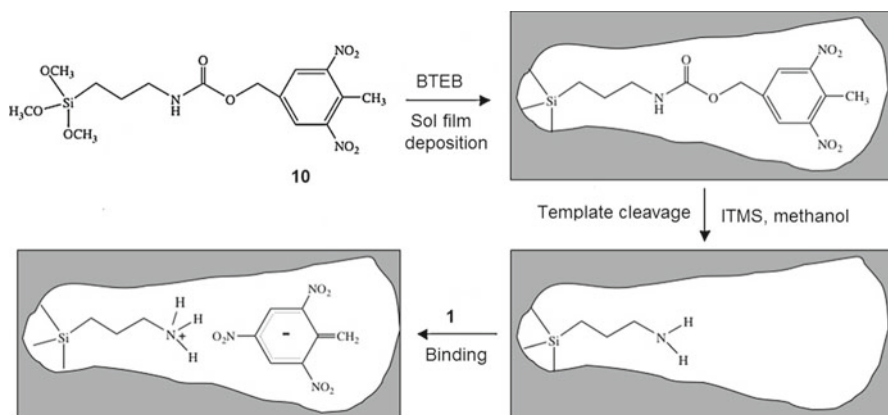


Fig. 4.5 Molecular imprinting of **1** in a sol-gel film. For that, the template **10** is co-polymerized with the bis(trimethoxysilyl)ethyl)benzene BTEB functional monomer and then **1** is removed from the film by iodotrimethylsilane (ITMS) (Adapted from [45])

used for the determination of **1**. This MIP-SERS chemosensor exhibited an apparent stability constant of the (MIP cavity)-**1** complex, $K_{\text{MIP-1}} \approx 4.3 \times 10^4 \text{ M}^{-1}$ [47].

In another application, **1** was imprinted with 4-aminothiophenol (4-ATHPh) as the functional monomer [50]. This MIP film was grown over silver molybdate nanowires decorated with silver nanoparticles (AgNPs). Photoinduced polymerization of 4-ATHPh generated a 4,4'-dimercaptoazobenzene matrix imprinted with **1**.

Piezoelectric microgravimetry (PM) chemosensors using MIP films as recognition units for determination of **1** were devised by coating quartz resonators with thin MIP films containing the **1**-selective cavities [43, 44]. In one such study, different functional monomers, like acrylamide (AAM), methacrylamide, methacrylic acid (MA), 2-hydroxypropyl methacrylate and butanediol monoacrylate, were tested for preparation of sensing MIP films of superior performance. These MIP films were directly deposited on resonator surfaces by spray coating followed by photopolymerization of the polymerization solution containing azobisisobutyronitrile (AIBN) as the photo-initiator [43]. The **1** template was extracted with chloroform from the resulting MIP films. Nevertheless, the MIP film prepared using AAM as the functional monomer in the CHCl_3 porogen resulted in a better performance. This film selectively bound **1** even in the presence of **2**, a structural analogue [43]. These mass-sensitive devices, coated with the explosive-selective MIP films, were promising to manufacture low-cost chemosensors capable of detecting traces of gaseous **1** explosive.

Moreover, a MIP-based square wave voltammetry (SWV) chemosensor was devised for determination of **1**. A great advantage of inherent sensitivity of this electroanalytical technique was exploited [41]. First, a MIP block was cast by thermal polymerization of a solution containing **1** as the template, MA as the functional, ethylene glycol dimethacrylate (EGDMA) as the cross-linking monomer, and AIBN as the polymer initiator. The resulting polymer was ground to a fine powder.

Then, a composite of a MIP powder, graphite powder, and icosane was used to fill a hole in the electrode body. The **1** template was removed from this MIP by Soxhlet extraction with methanol.

In another study, the nature of interactions between the functional monomer and the **1** template was modeled computationally [53]. During imprinting of **1** with acrylic acid and EGDMA, molecular cavities selective to **1** were impressed. Electrostatic interactions were operative during this imprinting. The three nitro groups of **1** were involved in hydrogen bonding with the -OH groups of the carboxy groups of the functional monomer. EGDMA preserved structural shape of the imprinted cavities. The analysis of the nature of bonding and solvent interactions as well as theoretical IR spectra derived for the simplest 1:1 complex showed that the applied approach was appropriate as a tool for the functional monomer selection for molecular imprinting.

In order to increase the total number of accessible imprinted sites and, therefore, the sensitivity of the DPV determination of **1**, a 2D film of MIP was prepared on a glassy carbon electrode (GCE) with its effective surface area enlarged by preliminary immobilization of the Au nanoparticles (AuNPs) [54]. For that, SAM of thiol was grown over the AuNPs modified GCE in the presence of the **1** template. Selectivity to **1** of this chemosensor was better than to other similar nitroaromatic compounds (Fig. 4.4).

In one of the recent studies, layer-by-layer (LBL) technique was combined with molecular imprinting to impress recognition sites at the surface of colloidal silica particles. For that, an assembly of the gelatin protein, covalently cross-linked with glutaraldehyde (GA), was used [55]. At the amine-modified silica particles, the **1** template molecules were synchronously imprinted forming gelatin shells by charge-transfer interactions between the electron-deficient aromatic rings of **1** and the electron-rich amine groups of the gelatin chains (Fig. 4.6). Then, the **1** template was extracted with a mixed solution of the ethanol-to-acetonitrile-to-(acetic acid) ratio of 8:2:2 (v:v:v). Selectivity of the imprinted cavities was investigated by testing the binding capability by the **1**-imprinted and non-imprinted particles to the **2**, **3**, and **4** structural analogues of **1** (Fig. 4.4). Unfortunately, **3** showed much higher binding affinity to imprinted particles than **1** due to its nonspecific binding by the gelatin shells.

Syntheses of MIP matrices assembled on metal NPs, such as AuNPs, demonstrated significant advantages in determination of **1**. Accordingly, AuNPs were modified with electropolymerizable thioaniline functional monomer, then electropolymerized onto the Au electrode surface in the presence of the **1** template or a dummy template. In effect, affinity to the thioaniline groups, and to the electrogenerated bis(aniline) bridges was incurred. On this basis, an electrochemical [42] and surface plasmon resonance (SPR) [56] chemosensor for **1** as well as those of SPR chemosensors for **4** [57] and **5–7** [58] were recently fabricated. These chemosensors featured enhanced sensitivity due to imprinting of structure-like **3** (Fig. 4.4) as the dummy template, thus substituting **1** in the composite film of AuNPs and ECPs [42, 56].

For the systems of low MIP coverage with the analytes or association of low-molecular-weight analyte with the MIP film, the generated dielectric changes at the

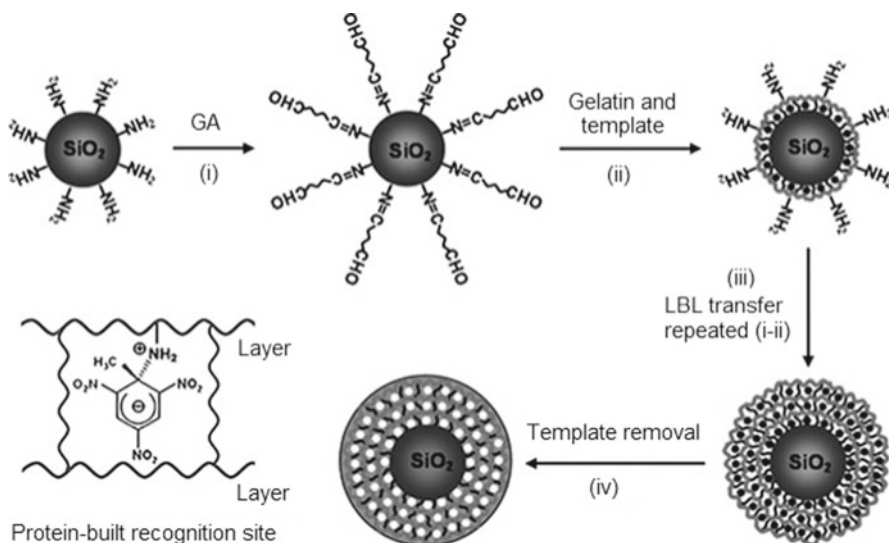


Fig. 4.6 Sketch of a procedure for LBL molecular imprinting at the surface of the amine modified silica nanoparticles. (1) Amine modified silica nanoparticles were first derivatized with aldehyde groups using glutaraldehyde (GA). (2) The resulting aldehyde-terminated silica particles were coated with gelatin and templates. (3) The GA and gelatin-template compounds were alternately assembled by LBL onto the silica particles until the desired number of layers was reached. (4) Finally, removal of templates generated the imprinted sites in gelatin multilayer shells. The bottom-left inset shows the charge-transfer complexing interactions between gelatin and the **1** template, and the formation of a protein-built recognition site between layers of gelatin (Adapted from [55])

Au surface were too small to allow for detectable SPR spectral changes. Conjugation of the MIP film with AuNPs served to amplify the SPR recognition signal of the **1** analyte. Two different complex stability constants of **1** were determined in the low and high concentration range, namely, $K_{\text{MIP-1}} = 6.4 \times 10^{12} \text{ M}^{-1}$ and $K_{\text{MIP-1}} = 3.9 \times 10^9 \text{ M}^{-1}$, respectively. The former signifies **1** binding by recognition sites of molecular cavities while the latter represents nonspecific **1** binding by MIP [56].

In another study, SPR sensing chips for determination of the **5–7** explosives were prepared [58]. For that, carboxylic acids, such as citric acid **11** (Fig. 4.7) and maleic or fumaric acid, of dimensions and structural features similar to those of these explosives served as dummy templates for imprinting (Fig. 4.7). In this imprinting, ionic and/or hydrogen bonds between the carboxy groups of the templates and the anilinium group of the functional monomer **12** (Fig. 4.7) provided strong template affinity to the MIP matrix.

Hydrophilic MIP filled with an Ni catalyst was used as the recognition unit of the electrochemical sensor for determination of **8** [59]. This MIP was synthesized using the **8** template, the AAm functional monomer, the glycidimethacrylate pro-hydrophilic co-monomer and ACN as the porogen. The hydrophilic modification enhanced accessibility of the imprinted cavities to **8** and improved selective recognition of

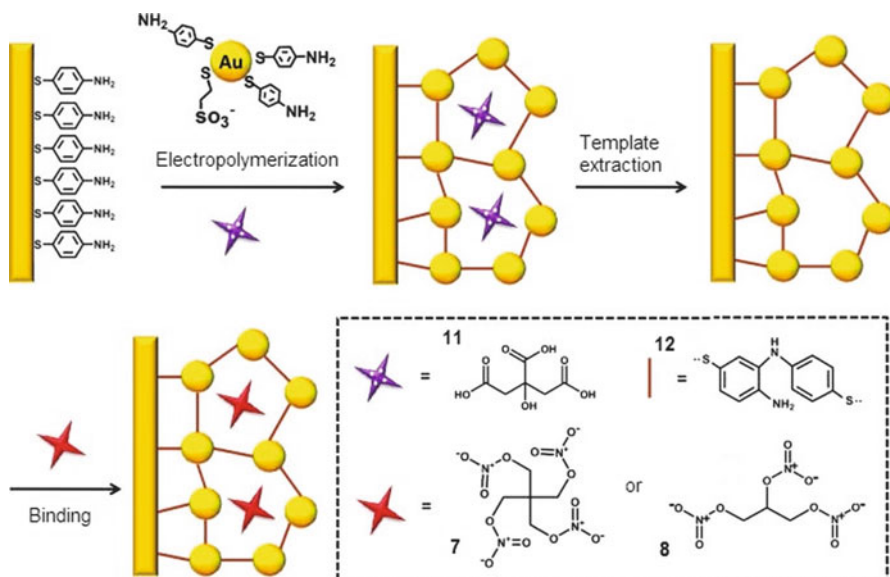


Fig. 4.7 Representation of the electropolymerization of a composite of bis(aniline) **12**-cross-linked AuNPs for determination of **7** or **8** using citric acid **11** as the dummy template (Adapted from [58])

8 in aqueous solutions. Therefore, a hydrophilic MIPs/Ni modified GCE could determine traces of **8** by CV.

Grafting a polymer on the surface of silica particles is most commonly performed by chemical immobilization of azo-initiators on this surface, followed by polymerization initiation [60–63]. However, surface immobilization of organic initiators in this system is complicated because of their chemical instability and difficulties in the formation of the uniform core-shell structures. Therefore, functionalized silica particles were successfully surface polymerized through modification of their surface with vinyl monomers, which were subsequently co-polymerized with appropriate functional monomers. The resulting functionalized silica particles were then used as substrates to grow MIP shells for selective determination of **1** [55, 64, 65].

Fluorescent ECPs appeared to be attractive alternatives for designing of the low LOD devices for **1** sensing due to their intrinsic ability of amplification of the detection signal. Toward that, a general procedure was developed for preparation of the cross-linked fluorescent MIECPs of improved selectivity and sensitivity, capable of signal transduction when incorporated in these sensors [48]. To study these capabilities, MIP was prepared for determination of **1** and related nitroaromatic compounds. Three-dimensionally cross-linked poly(*p*-phenylene vinylene)s (PPVs) were selected as functional monomers because of their useful conformational flexibility around the conjugated backbone. This flexibility is required for shaping the imprinted cavities. In addition, PPVs are highly emissive in the solid state and their fluorescent properties are strongly affected by the nature of the environment,

and the presence of quenchers in particular [66, 67]. For that, covalent imprinting was used for MIP preparation. In case of incomplete template removal, the residual template left in MIP may quench the fluorescence. To overcome this obstacle, a nonquenching surrogate of the shape similar to that of the **1** analyte, viz. 2,4,6-triisopropylbenzyl 2,5-diiodobenzoate, was chosen as the dummy template. The polymerization of a mixture of co-monomers diiodide and bis-vinylboronate, and the cross-linker 1,3,5-triiodobenzene was carried out in a mixed water, ethanol, and toluene solution at high speed stirring (~1,000 rpm) in the presence of sodium dodecyl sulfate (SDS) and the dummy template. In order to remove the template from the resulting MIP matrix, a solution of lithium *n*-propyl mercaptide in hexamethylphosphoramide (HMPA) was used. The synthesized fluorescent MIP featured remarkable both air and photostability.

Polyaniline (PANI) is the most favored ECP for various electrochemical and electronic applications due to its high and readily controlled electric conductivity as well as high environmental and thermal stability. In view of these advantages, MIP was prepared over a conducting PANI nanofiber [68]. First, this nanofiber was synthesized by chemical oxidation of aniline. After that, vinyl groups were introduced onto its surface to promote thermal polymerization. In this polymerization, AAm, EGDMA, AIBN and 1,3-dinitrobenzene **9** was used as the functional monomer, cross-linking monomer, initiator, and the template, respectively. The **9** template was then removed from the resulting MIP with the methanol-(acetic acid) (9:1, v:v) solution. Next, this MIP composite was suspended in chitosan. Finally, a GCE surface was drop coated with this suspension to devise a DPV chemosensor.

Fluorescent chemosensors based on MIPs have proven useful for identification of the explosive devices by recognizing vapors of explosive compounds that emanate from these devices and become airborne. For that, bulk MIPs were prepared by photopolymerization using **1** or **2** as templates, MA as the functional and EGDMA as the cross-linking monomer [49]. Next, these bulk MIPs were pulverized and the resulting microparticles dispersed in methanol in order to remove the template. The carboxylic acid functional groups of the MIPs served to label them with commercially available amine-functionalized quantum dots. Next, these fluorescent-labeled MIP microparticles were tested for their performance in detecting aqueous **2**, an analogue of **1**, as well as **1** itself. In effect, a highly robust fluorescent chemosensors were fabricated that were capable to operate under different environmental conditions.

As briefly presented above, several transduction techniques were successfully combined with MIP recognition for selective and sensitive determination of different explosives. For that, MIP films were prepared directly on the transducer surface by photo- or thermal polymerization, electropolymerization, or the sol-gel procedure (Table 4.1). Most of the techniques determined explosives only in solution [47, 49]. However, explosive determination in air is also needed. Fortunately, there are a few reports on determination of explosives in air as well [43, 45]. From the point of view of the high detectability and wide concentration range requirements, the SPR and electrochemical transductions are superior. In one of these studies, a very impressive detectability at the 10 fM as well as wide concentration range (fM-to-pM) was

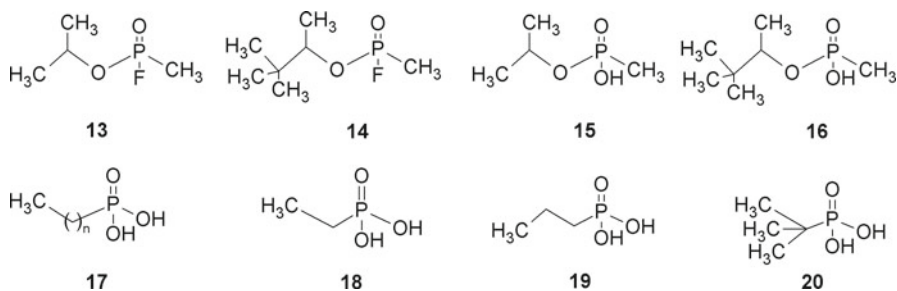


Fig. 4.8 Structural formulas of warfare agents and their hydrolysis products: isopropyl methylphosphonofluoridate (sarin) **13**, pinacolyl methylphosphonofluoridate (soman) **14**, methylphosphonic acid **15**, pinacolyl methylphosphonic acid **16**, alkylphosphonic acid **17**, ethylphosphonic acid **18**, propylphosphonic acid **19**, *tert*-butylphosphonic acid **20**

obtained [56]. Thin MIP films in combination with NPs served to amplify detection signals in these chemosensors. Fluorescent MIP chemosensors are selective but their detectability is sometimes insufficient [49] (Table 4.1).

4.3.2 Determination of Warfare Agents and Toxins

The isopropyl methylphosphonofluoridate (sarin) **13** and pinacolyl methylphosphonofluoridate (soman) **14** (Fig. 4.8) chemical warfare agents are highly toxic nerve gases. They are lethal at very low dosages [69–71]. Two sarin gas attacks in Matsumoto and Tokyo (Japan), in 1994–1995, confirmed this horrible threat. As these agents hydrolyze in the environment, detection of their decomposition product, viz. methylphosphonic acid **15**, has usually proved the use of these nerve agents [72]. Various biotoxins, such as T-2 toxin **21**, microcystin-LR **22**, ricin, ochratoxin **23**, aflatoxin **24**, and zearalenone **25** (Fig. 4.9), are also lethal at low levels for humans and animals. Therefore, determination of extremely low concentrations of these toxins in samples of, e.g., river water and food, is important. Different MIP-based sensing systems have been developed for these purposes (Table 4.2).

Product **15** is rapidly and selectively recognized with the potentiometric MIP chemosensor [73]. For preparation of this chemosensor, an ITO plate was soaked in a suspension of the octadecylsiloxane (OTS) functional monomer and **15** in a mixed CHCl_3 and CCl_4 solution. That way, OTS and **15** were allowed to self assemble on an indium-tin oxide (ITO) electrode surface. Then, the polysiloxane thin MIP film was formed. After subsequent extraction of the **15** template with CHCl_3 , the **15** analyte was determined selectively. This selectivity was satisfactory in the presence of interferants, such as alkylphosphonic acids **17**, ethylphosphonic acid **18**, propylphosphonic acid **19**, and *tert*-butylphosphonic acid **20**.

To improve detectability, another MIP-based potentiometric chemosensor for **15** was fabricated [74]. For that, bulk MIP was prepared by thermal polymerization

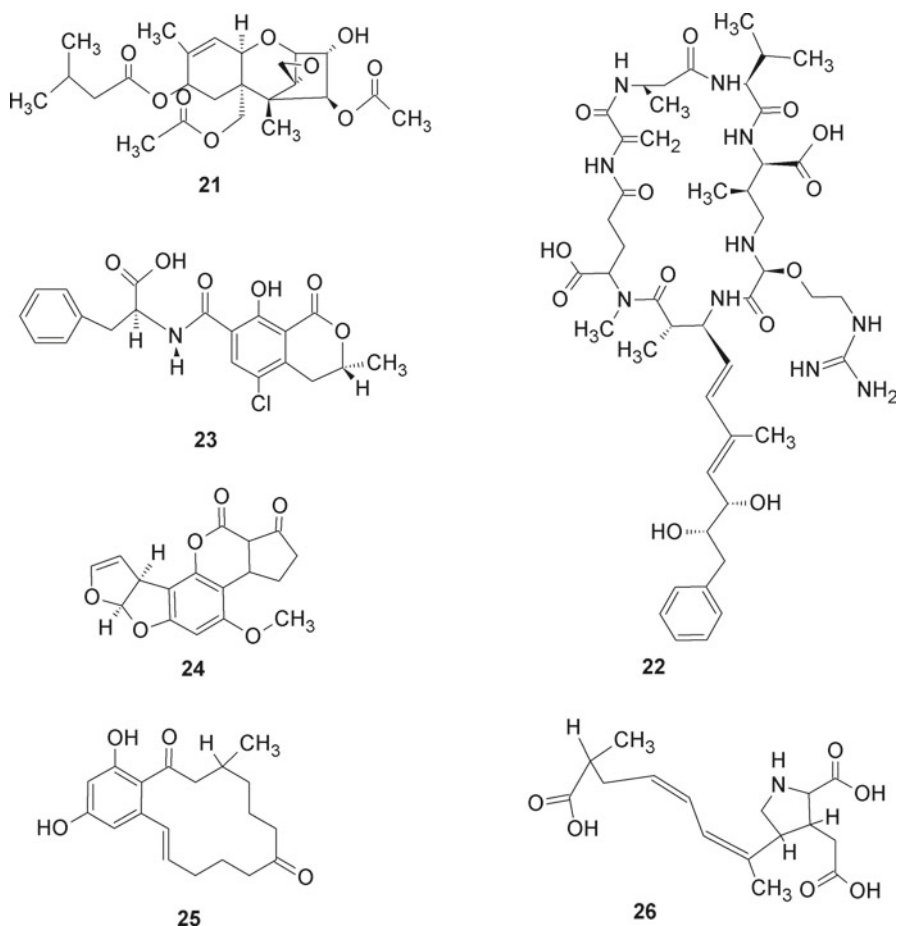


Fig. 4.9 Structural formulas of different toxins: T-2 toxin **21**, microcystin-LR **22**, ochratoxin A **23**, aflatoxin **24**, zearalenone **25**, domoic acid **26**

of the MA, EGDMA, and AIBN functional monomer, cross-linking monomer, and initiator, respectively, in the presence of **15**. After grinding and sieving the resulting bulk MIP, its particles were Soxhlet extracted with a mixed methanol-(acetic acid) (1:1, v:v) solution to remove the **15** template. Then, a poly(vinyl chloride) film was cast by using these **15** templated MIP particles suspended in solution of 2-nitrophenyloctyl ether (NPOE) and vinyl chloride. The potentiometric chemosensor using this recognition unit was selective over structurally or chemically related interferants, such as phosphoric acid and organophosphorus pesticides (OPPs).

MIP recognition has been combined with luminescence transduction in a chemosensor that selectively determined a phosphate hydrolysis product of **14**, i.e., pinacolyl methylphosphonic acid **16**, in water [75, 76]. This chemosensor

Table 4.2 Preparation MIP-based chemosensors for determination of warfare agents and toxins

Analyte	Functional/cross-linking monomer/initiator	Porogen	Extraction solution or conditions	Transduction method	Solution for analyte binding	Linear concentration range	LOD	Ref.
15	Octadecylsiloxane	$\text{CHCl}_3:\text{CCl}_4$ (2:3, v:v)	CHCl_3	Potentiometry	0.1 M Phosphate buffer (pH=7.2)	50 μM to 0.62 M	50 μM	[73]
	(Methacrylic acid)/EGDMA/AIBN	Methoxyethanol	Methanol/acetic acid (1:1, v:v)	Potentiometry	0.1 M Tris buffer (pH = 10.0)	50 nM to 0.1 mM	50 nM	[74]
16	(Eu^{3+} , divinyl methyl benzoate, template complex)/styrene, divinylbenzene/AIBN	Water:methanol (1:1, v:v)	1 M HNO_3	Luminescence spectroscopy	0.01 M NaOH	1 mM to 0.1 M	7 ng L^{-1}	[75]
	(Eu^{3+} , divinyl methyl benzoate template complex)/styrene/AIBN	Water:methanol (1:1, v:v)	1 M HNO_3	Luminescence spectroscopy	1 M NaOH	1 $\mu\text{g L}^{-1}$ to 150 mg L^{-1}	125 ng L^{-1}	[76]
21	(Methyl methacrylate)/EGDMA/AIBN	2-Methoxyethanol	Methanol: (acetic acid) (1:1, v:v)	Potentiometry	0.1 mM Phosphate buffer (pH=9.5), 0.1 mM KCl	40 nM to 10 μM	40 nM	[77]
	3-Aminophenyl boronic acid	0.05 M NaNO_3	80% Methanol, phosphate buffer (pH=7.4)(0.05% Tween 20)	SPR	Phosphate buffer (pH=7.5)	10 μM to 1 mM	0.1 fM	[83]
Ricin	Bis(2-hydroxy-ethyl-ethyl)-aminopropyltriethoxysilane, tetraethoxysilane	–	10 mM Tris buffer (pH=7.4), 0.05 M NaCl, water	Fluorescence spectroscopy	–	–	–	[84]

(continued)

Table 4.2 (continued)

Analyte	Functional/cross-linking monomer/initiator	Porogen	Extraction solution or conditions	Transduction method	Solution for analyte binding	Linear concentration range	LOD	Ref.
22	(2-Acrylamido-2-methyl-1-propanesulfonic acid)/imidazole-4-acrylic acid ester, EGDMA/ ACCN (3-Aminopropyl) trimethoxysilane, diphenyl-dimethylsialne tetraethoxysilane	DMSO	Methanol	PM	50 mM Phosphate buffer (pH=7.0)	0.5–50 nM	0.35 nM	[85]
23	Pyrrole	Methanol	Water	Optical spectroscopy	Water	0.3–1.4 $\mu\text{g L}^{-1}$	–	[86]
		Ethanol:water (1:9, v:v)	Methanol/water (1:9, v:v), 1% acetic acid	SPR	Ethanol:water (1:9, v:v)	0.05–0.5 mg L^{-1}	0.05 mg L^{-1}	[88]
	Diethylaminoethyl/methacrylate, itaconic acid/EGDMA/ ACCN	DMF	0.1 M NaOH : Methanol (5%), 0.1 M HCl, Methanol, water	Fluorimetry	60% ACN:water (1:4, v:v)	1–300 ng	1 ng	[89]
24	(<i>N,N'</i> -Bisacrylamide)/EGDMA/ACCN	DMF	Methanol, water	Fluorimetry	80% ACN:water (1:4, v:v)	1–200 ng	1 ng	[89]
25	Allylpiperazine/trimethylolpropane trimethacrylate	ACN	Pressurized liquid extraction	Fluorescence spectroscopy	ACN	50 μM to 50 mM	25 μM	[90]
	Pyrrole	ACN	ACN, methanol, CHCl_3	SPR	0.1% ethanol	0.3–3,000 ng mL^{-1}	0.3 ng g^{-1}	[91]
26	[2-(Diethylamino)ethyl methacrylate]/EGDMA/ 4,4'-azobis (4-cyanovaleic acid)	–	Methanol	SPR	10 mM HEPES buffer, HBS buffer, 150 mM NaCl 3.4 mM EDTA, 0.005% P 20 surfactant (v:v) (pH = 7.4)	5–100 $\mu\text{g mL}^{-1}$	5 $\mu\text{g mL}^{-1}$	[93]

T-2 Toxin **21**, microcystin-LR **22**, ochratoxin A **23**, aflatoxin **24**, zearalenone **25**, domoic acid **26**.

operated by selective and reversible binding of **16** to a Eu^{3+} luminescent lanthanide cation covalently immobilized in the imprinted molecular cavities of MIP. A narrow luminescence band at ~ 610 nm in the Eu^{3+} spectrum, which appeared when the **16** analyte was coordinated to this cation, served as the detection signal [75].

Analytical performance of potentiometric chemosensors fabricated by using **16**-templated MIPs were extensively studied [77]. Those MIPs were prepared by adopting different polymerization strategies, such as the bulk, precipitation, and suspension polymerization. For devising a potentiometric chemosensor, the resulting MIP particles were then dispersed in PVC matrices. Selectivity and detectability of the chemosensor fabricated by using MIP prepared by the bulk polymerization was higher than those prepared by the suspension and precipitation polymerization.

Moreover, various MIPs for determination of the warfare agent simulating compounds, such as diisopropyl methylphosphonate, diethylhydroxymethylphosphonate [78], and diethylchlorophosphate [79], were reported.

Toxin **21** belongs to the type-A trichothecene mycotoxin family. It is produced by various *Fusarium* species and mainly by *F. sporotrichoides*. Moreover, **21** is one of the most acutely immunosuppressive and cytotoxic trichothecene inhibiting both protein synthesis as well as in vivo and in vitro mitochondria functioning [80, 81]. It can be deployed as a biological warfare agent (BWA) for mass intoxication [82]. Toxin **21** was imprinted with ECPs for fabrication of an SPR chemosensor with high affinity and selectivity [83]. For that, a molecularly imprinted poly(aminophenylboronic acid), MIPAPBA, film was in situ deposited on an SPR chip by potentiodynamic electropolymerization of APBA, in the presence of **21**, by potential cycling between 0 and 1.1 V vs. Ag/AgCl . Next, the template was removed by washing the film first with 80% methanol and then with phosphate buffer saline (PBS) (pH=7.4) which was 0.05% in Tween 20. The SEM imaging of the **21**-templated MIPAPBA film exhibited nanopatterns formed due to the imprinting of **21**. The determined thermodynamic parameters of the MIPAPBA formation, such as the change of the Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS), revealed that the interaction between **21** and the MIPAPBA film was spontaneous, endothermic, and entropy driven. Super binding affinity of **21** by the MIPAPBA film was confirmed by a high value of the stability constant of the (MIPAPBA)-**21** complex, $K_{\text{MIP-21}} = 7.8 \times 10^{13} \text{ M}^{-1}$. The MIPAPBA film was much more selective to **21** than to the ricin, curcin, **22**, and abrin interferants due to nanopatterning of the MIPAPBA film.

Ricin, a potent toxin from castor beans (*Ricinus communis*), is a glycoprotein composed of two polypeptide chains, A and B, which are joined together by a disulphide bridge. This toxin as well as its 'A' and 'B' chains were molecularly imprinted. The respective complex stability constants were determined by steady-state fluorescence [84]. The Scatchard analysis for the ricin B chain binding to the ricin imprint lead to two complex stability constants, $K_{(\text{MIP ricin})-(\text{ricin B})} = 434 \times 10^7 \text{ M}^{-1}$ and $K_{(\text{MIP ricin})-(\text{ricin-B})} = 400 \times 10^7 \text{ M}^{-1}$, whereas the complex of the ricin B chain bound to the ricin A chain imprint exhibited just only one stability constant of lower value, $K_{(\text{MIP ricin A})-\text{ricin B}} = 11.2 \times 10^7 \text{ M}^{-1}$. During imprinting of the whole ricin protein,

molecular cavities were formed which could effectively bind whole ricin protein as well as its A and B chains alone. However, the situation was different in case of sole imprinting of the A or B chain. That was because molecular cavity impressed with each chain was selective either for A or B. Therefore, the ricin B chain showed lower binding affinity to the (ricin A)-templated molecular cavity.

Toxin **22** is one of the most widespread dangerous chemicals. It is produced by the blue-green algae, i.e., freshwater cyanobacteria. In addition to acute hepatotoxicity, the tumor promoting activity of microcystins can threaten human health by even low-level but chronic exposure to these toxins in drinking water. Toxin **22** destroys cellular control process by covalent binding to protein phosphatases. Therefore, an MIP receptor for **22** was synthesized [85]. This MIP played a dual analytical role. That is, it was used as a sorbent for solid-phase extraction (SPE) on the one hand and as the sensing film in a PM chemosensor on the other. Combination of the SPE preconcentration and subsequent detection with the PM chemosensing served to detect the toxin at an exceptionally low concentration (Table 4.2).

Moreover, a Fabry-Pérot optical sensing probe incorporating a thin MIP film selective to **22** was devised [86]. The imprinting was accomplished by curing a sol-gel film, prepared with (3-aminopropyl)trimethoxysilane (APTMS), diphenyldimethoxysilane, tetraethoxysilane (TEOS), in the presence of the **22** template. A single-mode fiber was dip coated with **22**-imprinted film. The **21** template was then removed by washing the film with distilled water. In determination of the **22** analyte, the template-free film acted as the shape-selective host (Table 4.2).

Toxin **23** is a carcinogenic mycotoxin of a wide natural abundance. It is produced by several species of *Aspergillus* and *Penicillium* fungi. It is a food and feed contaminant. Its toxicity in humans and animals is multiple. This include nephrotoxic, hepatotoxic, immunotoxic, teratogenic, and carcinogenic effects, which are serious health risks to humans and livestock [87]. Although most MIP-based procedures developed for determination of this toxin involve chromatography, an alternative strategy, such as an MIP-based chemosensing, was also proposed [88] (Table 4.2). For that, a **23**-templated MIP film was prepared by potentiostatic electropolymerization of pyrrole (Py) at 0.85 V vs. Ag/AgCl on the SPR chip from the ethanol-water (1:9, v:v) solution of **23** [88]. The film growth was manifested in situ by an increase in the SPR angle. The MIP film was regenerated by pulse elution with the methanol-water (1:9, v:v) solution of 1% acetic acid.

Two MIPs with affinity to two of the most abundant mycotoxins, viz., **23** and aflatoxin B1 **24** (Fig. 4.9), were computationally designed [89]. Based on this computational modeling, *N,N'*-methylene bisacrylamide and a mixture of diethylaminoethylmethacrylate and itaconic acid (IA) was chosen for preparation of MIP selective to **23** and **24**. That is, a bulk MIP was prepared by photopolymerization of suitable functional and cross-linking monomers in the presence of either **23** or **24** and the 1,1'-azobis(cyclohexane carbonitrile) initiator (Table 4.2). Then, ground MIPs particles were packed in plastic cartridges for SPE and used for selective fluorimetric determination of these toxins. Quantification of **23** and **24** comprised fluorimetric analysis of the mycotoxins adsorbed on MIPs upon exposure

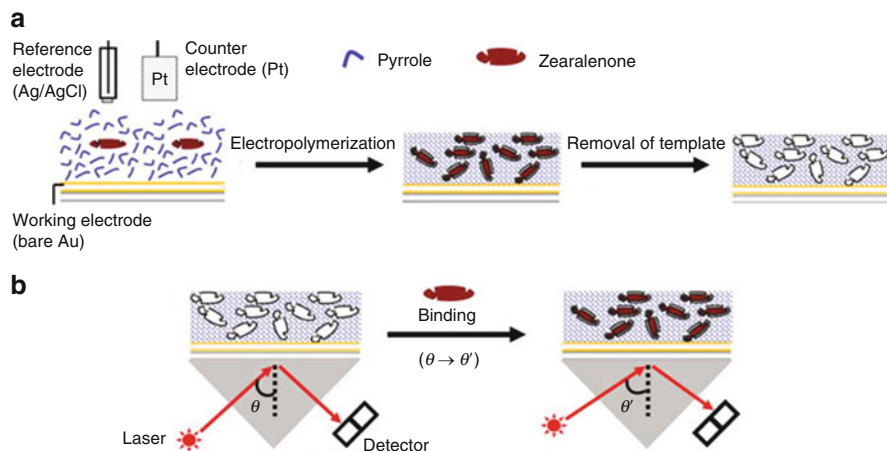


Fig. 4.10 Diagrams of the setup for MIP film preparation by electropolymerization and SPR detection of zearalenone **25**. **(a)** Preparation of a molecularly imprinted polypyrrole, MIPPy, film on bare Au electrode using a three-electrode electrochemical system. **(b)** The shift in the resonance angle θ of the SPR signal resulting from binding of **25** by the MIPPy film (Adapted from [91])

to UV light. High affinity of the developed MIP resins allowed for adsorption of both toxins as discrete bands on the top of the cartridges.

Toxin **25** (Fig. 4.9) is a mycotoxin of estrogenic activity produced by several species of fungi. These fungi belong to the genus *Fusarium* that colonizes cereals. An MIP-based fluorescent chemosensor was fabricated for sensitive determination of **25** [90]. The developed automated flow-through assay was based on the displacement from the MIP film of the tailor-made highly fluorescent tracers, such as pyrene or 7-nitrobenzen-2-oxa-1,3-diazol-4-yl, by **25**. For that, MIP was prepared by UV-light irradiation of a solution of a cyclododecyl 2,4-dihydroxybenzoate dummy template, 1-allyl piperazine functional monomer, and trimethylolpropane trimethacrylate cross-linking monomer in an ACN porogen. A dummy template was used instead of **25** itself as the template because of the presence of a styrene-type double bond in **25** that could polymerize resulting in undesired covalent incorporation of **25** in the polymer backbone. Although LOD for **25** in ACN of this fluorosensor was appreciably low (Table 4.2) the chemosensor revealed cross-reactivity for β -zearalenol but neither for resorcinol, resorcylic acid, 17 β -estradiol, estrone, nor bisphenol-A.

Moreover, **25** was templated in an MIP film, derived from ECP, deposited by potentiostatic electropolymerization of Py at 0.90 V vs. Ag/AgCl onto an Au electrode of the SPR chip (Fig. 4.10) [91]. Then, the **25** template was removed from the film by consecutive washing with ACN, methanol, and chloroform. Thickness, structural properties, and surface morphology of this film was characterized by the SPR, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), and AFM, respectively. Selectivity of the SPR chemosensor using MIPPy thus fabricated with respect to structurally related analogues, such as α -zearalenol,

β -zearalenol, zearalenone, and α -zearalenone, was 0.15, 0.21, 0.25, and 0.27, respectively. An average recovery of a blank corn matrix spiked with 30 ng g⁻¹ of **25** was 89%. Analytical parameters of this chemosensor (Table 4.2) well compared to those typical for the enzyme-linked immunosorbent assay (ELISA) of **25**.

Domoic acid **26** (Fig. 4.9) is a water-soluble natural excitatory and neurotoxic amino acid isolated from two red algae, namely, *Chondria armata* and *Alsidium coralliu*. On ingestion by humans, the **26**-contaminated shellfish may cause an intoxication syndrome known as amnesic shellfish poisoning. This syndrome is manifested by gastro-intestinal symptoms, such as vomiting, cramps, and diarrhea, as well as by neurological symptoms including severe headache, seizures, and either temporary or permanent memory loss [92]. For selective determination of **26**, a MIP based SPR chemosensor was fabricated (Table 4.2) [93]. The MIP film was prepared by photopolymerization. This reaction proceeded only near the Au surface of the SPR chip because a photo-labile radical initiator was immobilized onto this surface before reacting with the pre-polymerization solution. This solution contained 2-(diethylamino)ethyl methacrylate as the functional monomer, EGDMA as the cross-linking monomer, 4,4'-azobis(cyanovaleric acid) as the initiator, and **26** as the template. After the polymerization, this template was extracted with methanol. In effect, a MIP nano film covalently attached to the SPR chip resulted.

Optical techniques, and fluorescence spectroscopy in particular, were often used as detection tools for analyzing binding abilities of MIPs devised for chemosensing of warfare agents and toxins (Table 4.2). Introduction of a monomer with inherent fluorescence to the solution for polymerization allowed for quantitative determination of the toxins. Either background fluorescence decreased or the wavelength of maximum emission shifted after binding the analyte molecules in molecular cavities of MIPs. Combination of optical transduction with MIP recognition helped to determine these toxic analytes in a wide concentration range with a quite satisfactory detectability (Table 4.2). Advantageously, detectability of the SPR-based MIP chemosensor was in an fM range. Unfortunately, its linear concentration range was not wide [83]. Most of the potentiometric MIP chemosensors offer appreciable detectability at a nanomole range (Table 4.2).

4.4 Conclusions

Because of their inherent advantages, the number of chemosensors with synthetic molecularly imprinted receptors, used as recognition units, has dynamically been growing during recent years as attractive alternatives to biological recognition units. This is primarily due to the limited usage of biosensors in certain areas. For instance, an antibody-based fluorescence biosensor for determination of **1** is highly sensitive and specific. However, its operation is limited to aqueous solutions. Moreover, it is not suitable for detection of **1** in vapors [94]. Generally, antibody biosensors are delicate to extreme temperatures, which limits their scope of applications [95].

The major reasons limiting extensive development and broad use of biorecognition units include their high cost, low stability, and challenging transformation of the binding event into a processable signal. Moreover, there are difficulties in interpretation of the connection between signal formation and the biochemical function of the receptor. Although the availability of natural receptors has now significantly improved, their price remains too high to justify their commercial application in the field of sensing. In particular, stability and reproducibility are important factors limiting commercialization of biosensors based on bioreceptors. Bioreceptors often comprise several subunits, which degrade during immobilization under conditions different from those natural conditions.

Recognition units based on synthetic molecular imprinting appeared to be attractive alternatives to the biorecognition units because they are easy to prepare, reusable, and inexpensive. Moreover, their affinity is comparable to that encountered in natural receptors, for instance like in the antigen-antibody systems. They operate under harsh chemical conditions. In addition, preparation of the imprinting units is possible for analytes for which there are no biorecognition systems. These properties make imprinting based recognition units very useful for selective sensing. The future of these units as biomimetic receptors for sensor applications is very promising.

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Chapter 5

Biosensors for the Control of Some Toxins, Viral and Microbial Infections to Prevent Actions of Bioterrorists

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Abstract Foodborne toxins are very widespread and very dangerous for health of people. It is very important to control the level of T2 mycotoxin which has a high toxic effect and may serve as biological weapons and agent for bioterrorism. From other side Salmonellosis is the most common enteric infection. Retroviral bovine leucosis is very dangerous too since the level of ailing people correlates with the amount of sick animals in the area of habitat.

The most effective way to prevent not desirable results is in constant monitoring of the epidemiological situation. It may be done with the help of instrumental analytical approaches based on the principles of biosensorics. They may provide selective, high sensitive, simple and express analysis which may be fulfilled in “on line” regime and in field conditions. These approaches may be completed by the special GSM system for immediately transferring results of analysis in the specialized laboratory for appropriate action. In the article the development of authors of the immune biosensors for monitoring of toxins, bacteria and biochemical diagnostics of retroviral leukemia through analysis of blood and milk are analyzed. The main attention is paid the optical biosensors based on the surface plasmon resonance (SPR) and total internal reflection ellipsometry (TIRE) as well as photoluminescence (PhL) and photoconductivity (PhC) of nanostructured (sNPS) porous silicon. The characteristics of the immune biosensors may be changed by varying procedures of the preliminary preparation of the transducer surface. The special attention is paid to the developed procedures of the preparation of samples for analysis. At the final part of article in short form the preliminary results obtained at the application of some approaches for the simple and rapid decontamination of aquatic environments from number of toxic substances are considered.

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Keywords Mycotoxins • Viral leucosis • *Salmonella thyphymorium* • Control in environmental • Immune biosensors • Different types

5.1 Introduction

Despite the 1972 Biological Weapons Convention, it appears worldwide that some nations are continuing to develop and produce the biological warfare agents (BWA). Today the problem of the protection of environment from BWA which are applied not for the solving local conflicts only and for the psychological terror aimed purpose to arouse panic among people of the concrete country and to form permanent terror in the word is very current. The some information about countries which worked at the program of biological weapons is given early [1]. The investigations in frame of this program were directed on the induction of the acute diseases among people and animals, destroying sex status, prevention of antibiotic effects and others. BWA are divided into three main groups: toxins (the so called mid-spectrum agents); viruses; and bacteria (Table 5.1). Toxins are biochemicals of various origin and chemical structure. Mycotoxins may serve as one of characteristic example of BWA.

This micotoxin may be prepared by very simple way. Both circumstances (simplicity obtaining and high level of toxicity) form very serious problem since these toxic elements may be as instrument for bioterrorists. The dangerous characteristics are given in Tables 5.1 and 5.2.

Mycotoxins are presented by big group which include more than 300 individual toxins produced by different fungi strains [2]. T2, aflatoxins, searelenone, patulin and others cause a great interest since they are widespread and characterized by high level toxicity. T2 mycotoxin has more toxic effect (in 400 times) than mustard gas and lewisite. It is well-known that mycotoxin T2 was packed into rockets, bombs, cisterns, some explosive cylinders, hand-grenade and they were applied in Laos and Afghanistan as yellow rain [3, 4].

There is necessary to mention that the use of toxins in generally among others groups of BWA by terrorists is the most probably since viruses and bacteria present big danger not only sacrifices and executors of terrorist act too.

Table 5.1 Preferable substances for the bioterrorists and for the biowaffer

Type of substrate	Preference in application	Reasons which restricted or assisted application
Chemicals and Biochemical's	First	High level of toxicity, immediately action, accessibility
Pathogenic microorganisms and viruses	Middle	Insufficient accessibility, dangerous for persons which organize action, long latent time
Radioactive materials	Last	Difficult accessibility, long latent time, needed a high level doses

Table 5.2 Some characteristics of some biological substances (pathogenic microorganisms, viruses and toxins) which may be used as weapons for bioterrorism

Toxic agent	Illness	Active factor	Source	Mortality
<i>Bacillus anthracis</i>	Anthrax	Lung swelling	Foods	High level
<i>Brucella melitensis</i>	Brucellosis	“	“	Low level
<i>Vibrio cholerae</i>	Cholera	Enterotoxin	–	High level
<i>Staphylococcus aureus</i>	Pneumonia	Enterotoxin	–	“
<i>Staphylococcus pneumoniae</i>	“	Erithrogenic toxin	–	“
<i>Shigella dysenteriae</i>	Dysentery	Neurotoxin	–	“
<i>Rickettsia rickettsi</i>	–	–	Foods	“
<i>Yersinia pestis</i>	Plague	Toxin	–	High level
Ebola virus	Hemorrhagic fever	–	–	“
Yellow fever virus	Yellow fever	–	–	“
T2 mycotoxin	–	–	Water, foods	High level
Fumonisin	–	–	“	“
Ricin (<i>Ricinus communis</i>)	–	–	“	“
<i>Vibrio cholerae</i> O1 toxin	–	–	“	“
<i>Clostridium botulinum</i> neurotoxin	–	–	“	“

Among of the concrete biological substances for the biowaffer and for the bioterrorist activity there is necessary to distinguish some viruses, pathogenic microorganisms, mycotoxins, ricin from *Ricinus communis*, Toxin from *Vibrio cholerae* O1 and Neurotoxin from *Clostridium botulinum*.

The providing of appropriate services by simple, very selective and sensitive methods for express revealing of components BWA in environment is very important approach among other ones directed to prevent serious consequences.

Unfortunately the analytical methodologies for analysis of mycotoxins as well as other low molecular weight toxins include such instrumental analysis as high-performance liquid or gas chromatography with mass spectroscopy or liquid chromatography with mass spectroscopy. Due to the extremely high complication and cost of analysis fulfilled by these methods, the development of innovative approaches, such as immune analysis and particular chemo- and biosensors, is very urgent [5–13].

In this review the main attention will be paid to some mycotoxins, such pathogenic microorganisms as *Salmonella thyphymorium* and bovine viral infection. At first it will be observed the proposed instrumental analytical methods based on the biosensorics principles and intended for the express revealing of the above numerated dangerous substances in environmental objects. This aspect it will be analyzed from two points of view: estimation of total toxicity level at the screening analysis of environmental objects and then determination of the concentration of individual toxins in these objects.

5.2 Problem and Possibility to Its Solve

Today we can say that the problem with the biochemical diagnostics in medical and veterinary medicine as well as problems with the permanent and on line monitoring environment may be solved with the creation of family of modern instrumental devices based on the principles of the bio- and chemical sensorics. At the developed biosensor we used: planar electrodes, ion-sensitive field effect transistors (ISFETs), semiconductor structures, fiber optics (optrodes), sNPS, thermistors and microcalorimeters (based on the semiconductors and solid phase electrolytes) as physical trasducers. The principles of the biological signal measurements include: potentiometry, amperometry, electro capacity, luminescence, fluorescence, “evanescent wave”, total internal reflection ellipsometry (TIRE), non-emitting energy transfer, surface plasmon resonance (SPR), piezo crystal frequency and thermometry. As selective structures we use biological molecules (enzymes, nucleic acids and their components, antigens and antibodies), cells (microorganisms, viral components), small living organisms (*Daphnia magna*) as well as chemical substances (calyx-4-arenes and others).

The directions of our activity in field of biosensors includes: (1) fundamental aspects (choosing of sensitive biological material, selection of types of transducers, physical surfaces, development of effective methods for biological material immobilization and its directed orientation and selection of physical-chemical signals for the registration of the biological molecules interactions); (2) creation of working prototypes of biosensors intended for: biotechnology, ecology, human and veterinary medicine; (3) technological aspects: examination of the biosensor work in real conditions and the development of some elements of the technology of the biosensor production.

The strategy of our investigation and biosensors application are directed on: (1) at first, the control of total toxicity of environmental objects by usage of bioluminescent bacteria and optical registration of signal as well as the determination of the intensity of the chemiluminescence (ChL) of the *Daphnia* exametabolites; (2) then revealing of the groups of toxic elements with the help of enzymes and immune components and (3) at last, the determination of the nature of the concrete substances.

5.2.1 Analytical Devices for the Determination of Total Toxicity of Environmental Objects

The set of the developed analytical devices for control of total toxicity of the environmental objects is given in Fig. 5.1 [10]. It is stationary and portable chemiluminometers.

The use of these analytical devices it was demonstrated that the sensitivity of the analysis of the T2-mycotoxin toxicity by the ChL in several orders higher than with the

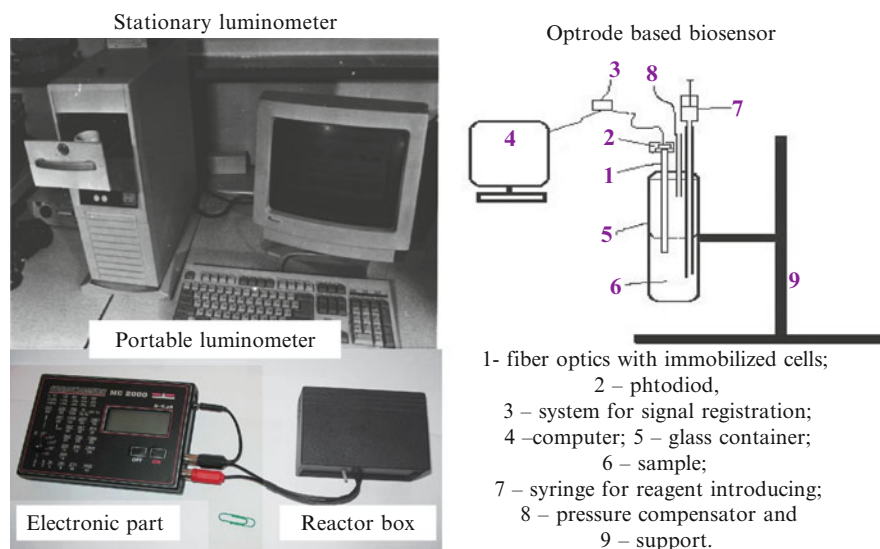


Fig. 5.1 Overall views of the analytical devices intended for the control of the level of ChL and bioluminescence (BL) of biological objects

help of the traditional methods when the some morphological and physico-chemical properties of *D. magna* are analyzed (Fig. 5.2) [9, 11–15]. The level of the sensitivity of analysis is much less in case of the application of bioluminescent (BL) bacteria. For all instrumental methods the overall time of analysis is much less than in case of the application of the traditional one. In particular, the time of analysis by the traditional method is equal one or several days. At the same time, the instrumental approaches allow registering results through several minutes, no more than 30 min only.

Diapason of the measurements of T2 mycotoxin by the traditional method is in frame of the concentration of 0.01–0.1 mg/L. At the same time in case of the use of the biosensor method this frame was from 0.001 to 1 mg/L. Moreover the level of sensitivity did not depend on time of *Daphnia* incubation in solution to be analysed. As far as patulin there was possibility to it quantitative determine by the biosensor method in frame of 0.001–1 mg/L [16].

Among of BL bacteria it was used *Photobacterium phosphoreum* K3 (IMB B-7071), *Vibrio fischeri* F1 (IMB B-7070) and *Vibrio fischeri* Sh1 purified from Black sea and Sea of Azov. The samples contented 0.8 mL of the tested substance in 2.5% solution of NaCl, 0.1 mL of 0.5 sodium phosphate (pH 7.0) or phosphate citrate (pH 5.5) buffers and 0.2 mL of bioluminescent bacterial suspension including 5×10^5 cells/mL. In other case bioluminescent bacteria (10^5 cells) were immobilized in sepharose gel (about 0.1 mL) deposited at the end of fiber optics. In both case the BL intensity (I) was registered through 30–120 min. The level of toxicity was presented as the concentration, which caused 50% decrease of the intensity of BL (EC_{50}).

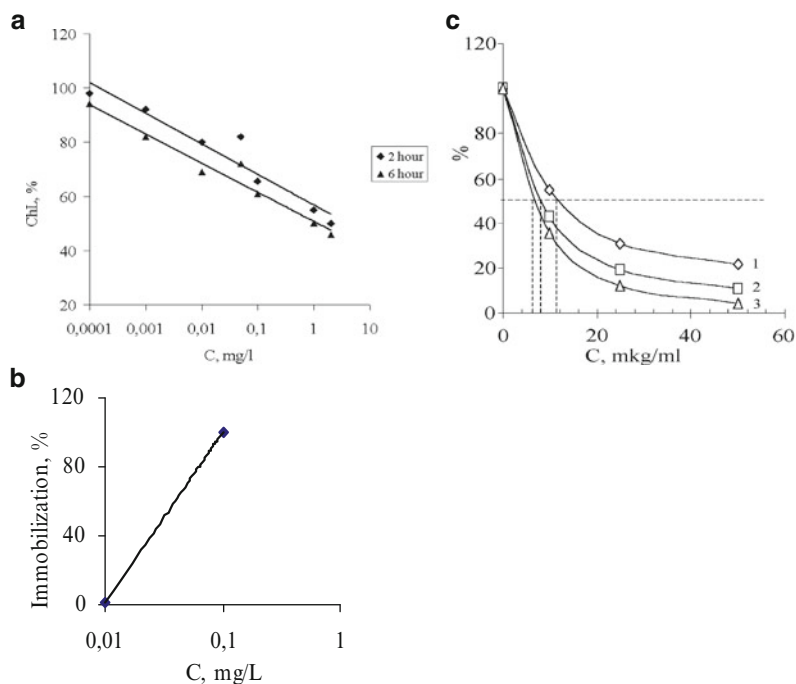


Fig. 5.2 The sensitivity of the T2-mycotoxin analysis by the traditional method (a), the determination of the ChL of *D. magna* metabolites (b) and (c) the registration of the BL of *V. fischeri* F1 (1–3 – 10, 20 and 30 min of exposition, respectively)

The sensitivity of *V. fischeri* F1 to mycotoxin T2 is much higher in comparison with the sensitivity of *Ph.phosphoreum* *Sq3*. The value of EC_{50} measured oscillated in range of 7–19 mg/L in dependence of time of incubation of bacteria in the T2 mycotoxin solution [12].

As far increasing of patulin concentration from 0.63 to 40 mg/L it caused sufficient decreasing of the BL intensity at the influence on *Ph.phosphoreum* *Sq3* during 12–60 min. The value of EC_{50} for patulin was in frame of 0.63–1.25 mg/L [15]. The dose-effect of patulin at the low concentration (low as 1 mg/L) may be confidently registered in case of three repeated measurements for each point. If it follows to this algorithm of analysis the toxic effect of patulin to bioluminescent bacteria may be revealed at the concentration less than 0.15 mg/L (Fig. 5.3).

Moreover with the prolongation of time of influence (up to 90 min) the toxic effect of patulin increased and value of EC_{50} was in frame of 0.15–0.63 mg/L. At the decreasing of medium pH to the lower physiological limit (5–5.5) the sensitivity increased up to one order. The value of EC_{50} has analogy with semi lethal dose established for animal and it correlates with other indexes of toxicity (cytotoxicity, irritation of mucouses, e.a.) [16]. It is necessary to mention that the intestinal barrier in animals is destroyed at the patulin concentration about 1 mg/L [17]. Taken into

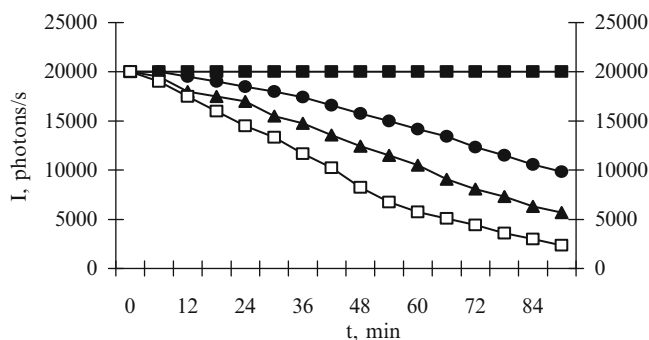
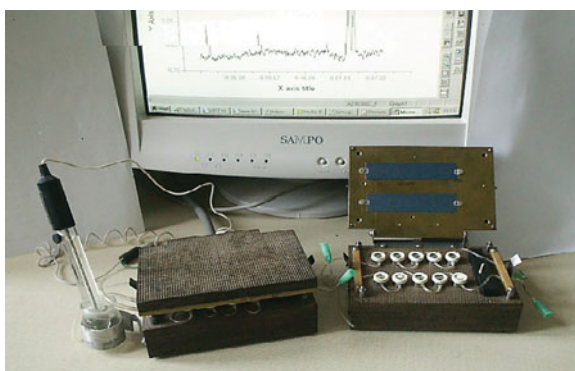


Fig. 5.3 Changes of BL intensity of *Ph.phosphoreum* at the effect of different concentration of patulin during 90 min, where: 1–4 – 0; 0.15; 0.63 и 1.25 mg/L, respectively

Fig. 5.4 Biosensor based on the EIS-structures (some details in the text)



consideration of this fact the above indicated data testify that the proposed biosensor analysis with the use of bioluminescent bacteria may be effective at the screening of samples of water, juice, foods and other environmental objects.

5.2.2 Biosensors for the Determination of the Groups of the Toxic Substances

To determine the group specific toxic substances, for example, phosphororganics, chlororganics, cyanides and others we have developed the multi-biosensor based on the electrolyte-insulator-semiconductor (EIS) structures [20]. The overall view of such biosensor, electronic part of which was developed in the Institute of Semiconductor Physics of the National Academy of Sciences of Ukraine, is given in Fig. 5.4. One of these biosensors is closed and reference electrode is situated near it. Another biosensor is opened and it can see two independent channels each of

which contains five measuring cells. The work of biosensor operates by the special computer program.

With the help of the enzymatic biosensors the level of such herbicides as simazine and 2,4 D was determined. The principles of design and work of biosensors were presented in [19]. Specific antibodies to herbicides were immobilised through the staphylococcal protein A. The analysis was fulfilled by the sequential saturation way when antibodies left unbound after their exposure to native herbicide in investigated sample, then have interacted with the labelled herbicide. The sensitivity of the EIS structures based sensor to simazine, when the horse radish peroxidase (HRP)-conjugates were used, was 5 $\mu\text{g/L}$ approximately. The linear plot of the sensor response lied in the range of the concentrations from 5 to 150 $\mu\text{g/L}$. This sensitivity of the EIS structures based sensor towards both herbicides was lower than it is requested in practice. We tried to elucidate the main reasons for such a situation. One of them may be connected with difficulties to register sensor output due to the formation of air bulbs, which appear as a result of high activity of the HRP. Use of high concentrations of ascorbic acid may be another reason for the lower sensitivity of this sensor. We changed HRP label to the β -glucose oxidase (GOD) one and obtained the sensitivity of the analysis approximately 5 times higher. The linear plots for simazine and 2,4-D were in the range of 1.0–150 and 0.25–150 $\mu\text{g/L}$, respectively [21, 22].

Immune biosensor based on the EIS structures attracts attention because of simple procedure of the analysis and existing possibility to fulfill multi-parametrical analysis. For the repeated analysis the replaceable membranes are very suitable. The overall time of the analysis is about 40 min. Therefore, the EIS structures based immune sensor may be used for wide screening of the environment for the presence of toxic substances. It gives possibility to carry out analysis of 8–10 samples simultaneously. For the verification of the results of analysis may be used others types of biosensors, for example based on the ion-sensitive field effect transistors (ISFETs) the sensitivity of which at the determination of the above mentioned herbicides is on the level of 0.1 $\mu\text{g/L}$ and less (up to 0.05 $\mu\text{g/L}$) that corresponds to practice demands [21–24].

If the membranes were prepared in advance the duration of the analysis may be shortened up to 10 min. Membranes are simple to prepare, they are very cheap and they can be stored for a long time in refrigerator. Since number of enzymes which have serine residuum in the active centre (first of all butyrylcholine esterase – BChE, acetylcholine esterase – AChE and total choline esterase – ChE) are very sensitive to phosphororganic pesticides (PhOrPe) and other ones (urease) with the thiol groups react with HMI there is possibility to simultaneous determine these classes of toxic elements [25, 26].

The sensitivity of the determination of heavy metal ions (HMI) and phosphororganic pesticides (PhOrPe) essentially depends on the incubation time of enzyme membranes in environment of these analytes. It was tested two different approaches: (1) registration of the sensor output signal in the mixture of a substrate and an analytes, (2) separation of the inhibition reaction from the following measurement of the residual enzyme activity. In the last case the threshold sensitivity of toxin analysis was about 10 times higher. The time of incubation was chosen experimentally and it was 15 min.

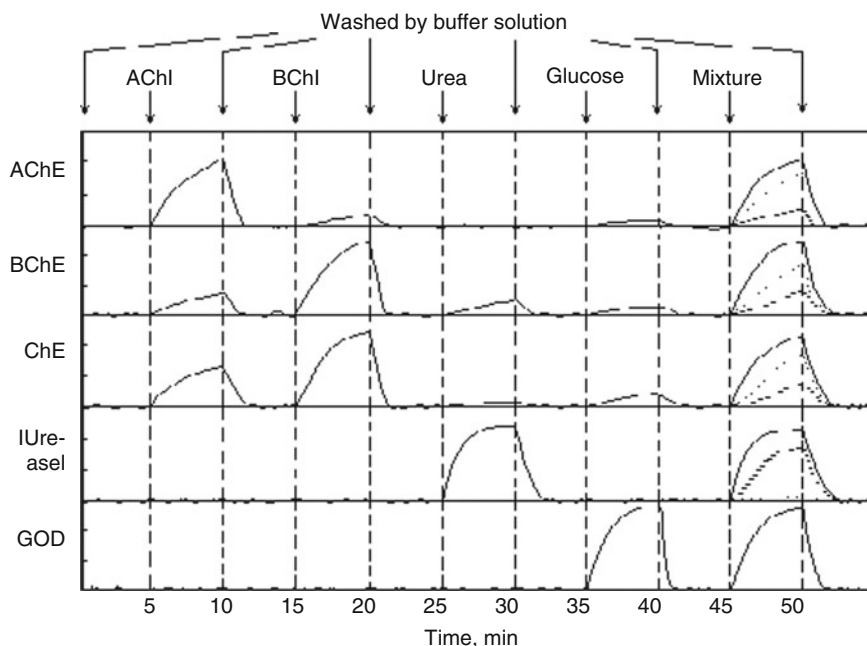


Fig. 5.5 Time diagram of the array operation of five different enzyme reactions. The mixture contained: $5 \cdot 10^{-4}$ mol/L of Co^{2+} -ions (point-lines); 10^{-5} mol/L of divinyl dipyrindine (dashed lines)

The concentration of HMI that could be determined by the urease channel of the sensor array lied within the range from 10^{-4} to 10^{-7} M, depending on the type of the metal used. The range of linear detection covered 2–3 orders of the concentration change. The effects of both pesticides are very similar. The limit of detection of pesticides indicated above was 10^{-7} M. The range of the linear response was from 10^{-5} to 10^{-7} M. At the same time the sensitivity of BChE to HMI was substantially lower than this of urease. The maximum sensitivity of BChE to HMI was for concentrations of more than 10^{-4} M. Activity of GOD depends on the presence of HMI for concentrations above 10^{-4} M. GOD was used as reference enzyme which has a minimal reaction in respect of both types of groups of toxins.

The diagram of the responses of channels with different enzymes (BChE, AChE, urease, ChE and glucose oxidase – GOD) is given in Fig. 5.5.

5.2.3 Biosensors for the Determination of the Individual Toxins

For the determination of the individual biochemical quantities it was developed immune biosensors based on the surface plasmon resonance (SPR) the general view of which was presented in Fig. 5.6. There are the devices which were created together with the collaborators from the Institute of the Semiconductors Physics

Fig. 5.6 General view of the biosensors a based on the SPR principle

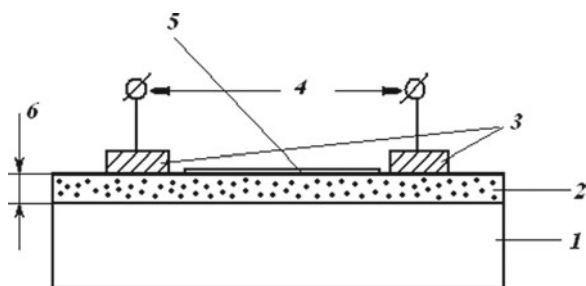
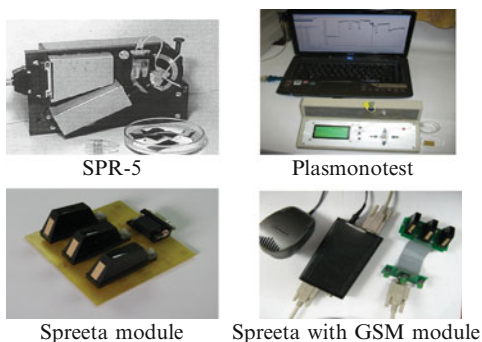


Fig. 5.7 Scheme of the photoresistor structure based on the sNPS and intended for the analysis of the interactions between biological structures. (1) the crystalline silicon, (2) the sNPS, (3) the electrical contacts (Al with the thickness of $\sim 3 \mu\text{m}$), (4) the applied voltage, (5) the biological object, (6) the thickness of the sNPS of 10–40 nm

(“SPR-5”) and V.M. Glushkov Institute of Cybernetics (“Plasmotest” and variant of “Spreeta module” with “GSM system”). Both are from the National Academy of Sciences of Ukraine.

Experiments with the biosensor based on the total reflection ellipsometry (TIRE) and with piezo crystal frequency were carried out in Sheffield Hallam University (Great Britain).

Scheme of the optical device based on the nano structured porous silicon (sNPS) for the registration of the signal about the formation the specific immune complexes is given in Fig. 5.7.

At the beginning of the measurement the specific Ab in the volume of $1 \mu\text{L}$ was placed on the photoresistor surface between the contacts. Then this solution was evaporated at the room temperature or at the air stream. The direct voltage (5 V) from the stabilized power supply was applied to the ohmic contacts and the current was measured by the digital voltmeter of B7-35 type at the absence of lighting (dark regime) as well as the photocurrent (the difference between the light and dark currents) was registered at the lightening of the sensitive surface by the white

spectrum light (source A, illumination of 7,000 lux). At the drawing of Ag layer on the sensitive plate and after its drying the measurements of the dark and light current were repeated. These measurements were made after the immune complex formation (interaction of Ag with specific Ab in the serum blood) too. The control of the reaching of the sensor initial state was done according to the reduction of the dark current value after washing of the sensitive surface by the buffer solution. The time of the single analysis was 5–10 min only.

Design of the prototype for the registration of the specific immune complex by the photoluminescence PhL of the sNPS includes the source of the ultraviolet (UV) radiation with the wavelength of 350 nm, two photodiodes (2 and 3) based on the mono crystalline silicon and placed at the angle of 20–25° relatively to the plate with the layer of the sNPS and the photo diode intended for the determination of the incident UV. At the adsorption of the biological molecules the level of the PhL of the sNPS and the output of the voltage of the consecutive connected photo registers are decreasing. Application of two photo registers of the PhL increases the sensitivity.

To take into attention the possible changing of the incident UV the additional photodiode is used. Photodiodes of the n-p-p⁺-structures work in the photo generative regime. Such construction is related to the systems of the differential type.

Besides of EIS-structures it is used the ISFETs with cerium oxide gate surface that gives to increase sensitivity of the analysis and its stability [27, 28] in comparison with that case when the transducer surface was covered by the silicon nitride [29]. Characteristics of such ISFETs and overall view of some parts of biosensors are presented in Fig. 5.8.

To achieve high density of the immobilization of the immune components on the transducer surfaces they were preliminary treated by one of some chemical substances among of which the most used are: (a) dextran sulphate; (b) dodecanthiol; (c) polyelectrolytes: polyalylamine hydrochloride (PPA) or/and polystyrene sulphat (PSS). After that the transducer surface was treated by some substances to achieve oriented immobilization of specific antibodies in advance, among such substances the most applied are: (a) protein A from *Staphylococcus aureus*; (b) protein G from *Staphylococcus*; (c) lectins.

The main algorithms of the analysis by the immune biosensors includes the next variants: (1) the “direct” way when the specific antibodies which were immobilized on the transducer surface interact with solution contented different concentration of antigen; (2) the “competitive” way-1 when antigen to be analyzed compete with its conjugate (covalent linked complex antigen with enzyme or some protein) for the specific antibodies which were immobilized on the transducer surface; (3) the “competitive” way-2 when antigen in form of conjugate (covalent linked complex antigen with enzyme or some protein) are immobilized on the transducer surface and its competes with free antigen to be analyzed for the specific antibodies in solution; (4) the “to saturate” way when the specific antibodies which were immobilized on the transducer surface interact at first with free antigen to be analyzed and then with antigen in form of conjugate (covalent linked complex antigen with the enzyme or some protein).

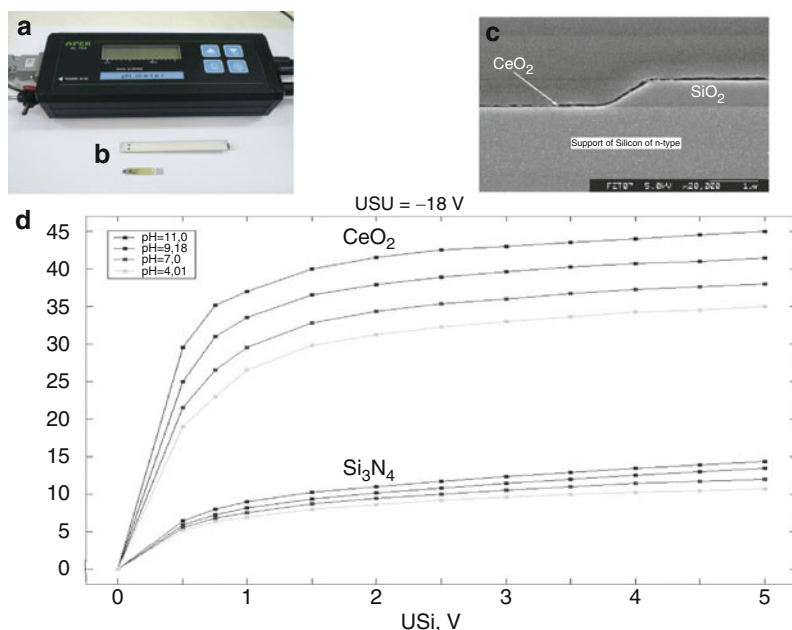


Fig. 5.8 Overall view and the physical characteristics of the biosensor based on the ISFETs with cerium oxide sensitive layer. Where: portable registering part (a) and chips (b); the scanning electron microscopy of ISFETs surface (c) and dependence of the output characteristics of ISFETs with the Si_3N_4 and CeO_2 dielectric structures on pH (d)

The detailed TIRE and piezo crystal spectra at the determination of the T2-mycotoxin are presented in (Fig. 5.9a, b) [30]. The calorimetric immune biosensor and that based on PhL of sNPS may be used for screening observation only (Fig. 5.10a, b). Their results of analysis should be verified with the help of other above-mentioned approaches [31–33].

The sensitivity of the determination of the T2-mycotoxin by the different types of the immune biosensors and with the application of some algorithms of analysis is given in Table 5.3. It can be seen that the most sensitive is the immune biosensor based on the TIRE or piezocrystal, which allows revealing this toxin at the concentration of 0.15 and 1.5 ng/mL, respectively, even at the “direct” way of the analysis. SPR or ISFETs-based immune biosensors may provide practical demands if the algorithm of the analysis will be as “competitive”, or “to saturate”.

The calibration curves at the determination of T2-mycotoxin, zearalenone and aflatoxin B1 are shown in Fig. 5.11 [34]. The most sensitive analysis was obtained for the T2-mycotoxin. The sensitivity of the determination of others is much less, that depends maybe on specificity and selectivity of the used antibodies. Nevertheless, the level of the revealing zearalenone and aflatoxin B1 may correspond to practical demands.

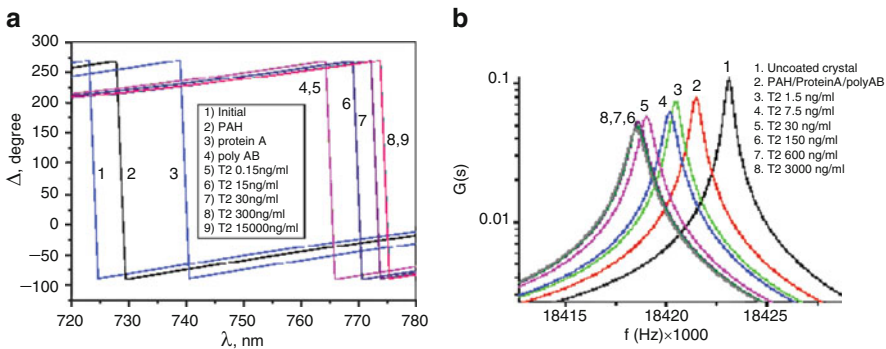


Fig. 5.9 TIRE spectra measured at different stages of adsorption and the “direct” way of analysis (a - left) and the admittance spectra of the QCM in the course of adsorption of T2-mycotoxin at the “direct” way of analysis (b - right)

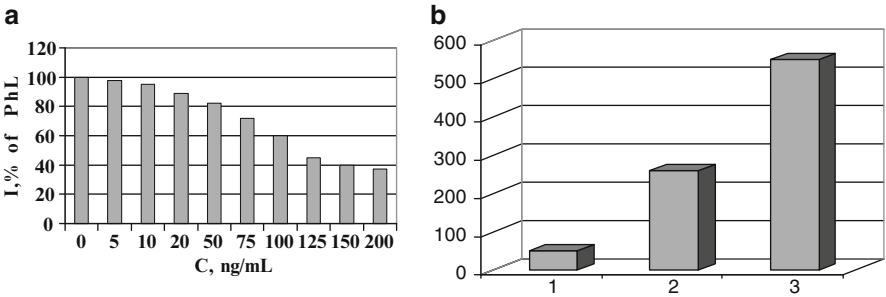


Fig. 5.10 Dependence of immune sensor signal (intensity of the PhL of sNPS) on the concentration of T2-mycotoxin in the solution to be analysed at the “direct” way of analysis (a - left) and dependence of PhC on the surface state, where: (1) (“direct” way of analysis). bare; (2) with antibody and three with the specific antibody and T2 mycotoxin (b - right)

Table 5.3 The sensitivity of T2 mycotoxin determination in the model solution by the different immune biosensors (according to own investigations)

N	Type of immune biosensors and algorithm of analysis (shown in brackets)	Sensitivity
1.	Immune biosensor based on the TIRE (“direct”)	0.15 ng/mL
2.	SPR based immune biosensor (“direct”)	~1.0 μ g/mL
3.	SPR based immune biosensor (“competitive”)	10.0 ng/mL
4.	SPR based immune biosensor (“to saturate”)	~5–10 ng/mL
5.	ELISA-method (“competitive” way of analysis)	~10.0 ng/mL
6.	Piezocrystal immune biosensor (“direct”)	1.5 ng/mL
7.	Cerium oxide ISFETs based immune biosensor (“competitive”)	~3.0 ng/mL
8.	Immune biosensor based on PhL of sNPS (“direct”)	~20 ng/mL
9.	Cerium oxide ISFETs based immune biosensor (“to saturate”)	0.5 ng/mL

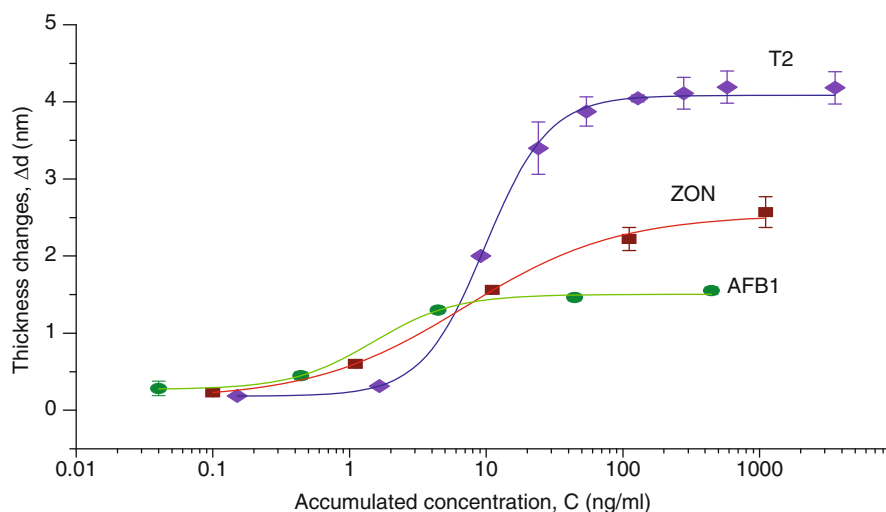


Fig. 5.11 Calibration curves of the determination of T2-mycotoxin, zearalenone and aflatoxin B1 by immune biosensor based on TIRE

Table 5.4 The evaluation of T2 mycotoxin contents in the grain products (TIRE based immune biosensor, the “direct” algorithm of the analysis and PAA with protein A as preliminary layer at the specific antibody immobilization)

Sample	δd (nm)	Estimated concentration
Mouldy fodder	4.900	>600 ng/mL
Mouldy bread	4.391	>600 ng/mL
Mouldy maize	4.369	>600 ng/mL
Mouldy buckwheat	5.274	>600 ng/mL
Stale bread	0.031	<1.5 ng/mL
Stale muesli	0.298	<1.5 ng/mL
Stale buckwheat	1.199	7.5–15 ng/mL
Fresh muesli	0.000	None

Since a most of environmental objects are presented as solid phases it was developed a effective method for extraction of mycotoxins from them. This approach included destroying object and its incubation with solvent and then the appropriate of which should be dissolved by buffer (50 mM, pH 7.3). Acetonitrile was more effective as solvent in the comparison with methanol [35]. Moreover, it may be presented in final sample in the concentration up to 30%. Using the developed TIRE based immune biosensor the level of the T2 mycotoxin was estimated in some grain products [30]. The results of the analysis are presented in Table 5.4. There can see that not fresh products may content this toxin in the quantities which are dangerous for people health.

The similar analysis was carried out at the determination of patulin in tomatoes with the different extent of damage of by rot. The content of this toxin increases with the increasing step of the effecting total square of tomatoes (Table 5.5).

Table 5.5 Determination of the patulin content in the tomatoes with the help of the immune biosensor based on the SPR (the “competitive” algorithm of the analysis and PAA with protein A as preliminary layer at the immobilization of the specific antibodies)

Item	Approximately step of affecting of tomatoes, % from total square	Content of patulin, μg/kg
1	0 (apparently not significantly)	0.1
2	10	100
3	30	400
4	50	800
5	100	1,800

At the control of the level of *S. typhimurium* the SPR (“Plasmotest”), the module “Spreta” (produced by TI, USA and modified in the V.M. Glushkov Institute of Cybernetics of National Academy of Sciences of Ukraine) and TIRE based immune biosensors were used. In both cases the transducer surfaces were successively treated by PAA, protein A and specific antibodies [36–38].

For detection using “Spreeta” it was prepared the model diluted solution of *S. typhimurium* with the number of concentrations (from 10^3 to 10^8 cells/mL) using 0.05 M Tris–HCl buffer (pH 7.3). The time of the sample incubation with the transducer surface was about 5 min and after that the last was washed by the above mentioned buffer. It has been defined that device sensitivity was on the level 10^3 – 10^7 cells/mL. For analysis using “Plasmonotest” model solution was prepared using 0.9% saline solution and dilution range of *S. typhimurium* was from 10^1 to 10^6 cells/mL. The time of the sample incubation with the transducer surface was about 10 min and after that the last was washed by the saline solution. The biosensor sensitivity was within 10^1 – 10^6 cells/mL. Transducer surface for TIRE based devise was prepared the same way as for SPR sensors. Range of dilution of initial *S. typhimurium* solutions was made in such way that 10 mL contended 1 and more cells (up to 10^5). The time of the sample incubation with the transducer surface was about 15 min. It was observed that received value almost did not change after 30 min incubation. Maximal level of sensitivity was on the level of several cells in 10 mL (up to the fact that less than five cells).

As we can conclude biosensor based on the TIRE has shown higher sensitivity than the SPR based. But infection dose of pathogenic microorganisms is less than ten cells per 100 mL [39] therefore it is necessary to find ways for the sensitivity increasing. Perhaps, satisfactory result can be obtained using monoclonal antibodies. Also it is necessary to pay attention to the effective methods of previous food samples preparation especially to the methods of bacteria cells concentration in the solution.

The comparison of our and literature data about the determination of *S. typhimurium* in model solution by the optical immune biosensors are presented in Table 5.6. There is necessary to underline that the main algorithm of the analysis is the “direct” way. The sensitivity of the analysis by the SPR immune biosensor is 10^1 cells/mL, or 5 cells/10 mL and linear field – in range 10^1 – 10^7 cells/mL. The overall time of

Table 5.6 Comparison the results of *S. typhimurium* determination in the solution obtained by optical immune biosensors [36–38]

Item	Type of immune biosensor and its producer	Sensitivity	Linear diapason
1.	“Spreta” module, intermediate layers – PPA, protein A	10^3 – 10^4 cells/mL	10^3 – 10^7 cells/mL
2.	“Spreta” module, intermediate layers – PPA, protein G	2×10^2 – 10^3 cells/mL	2×10^2 – 10^7 cells/mL
3.	“Spreta” module, intermediate layers – dodecanthiol, protein A)	5×10^3 – 10^4 cells/mL	5×10^3 – 10^7 cells/mL
4.	“Plasmonotest”, intermediate layers – PAA, protein A	10^1 cells/mL	10^1 – 10^6 cells/mL
5.	TIRE, intermediate layers – PPA, protein A)	5 cells/10 mL	–
6.	“Biacore”, intermediate layer – developed dextran [40]	1.7×10^3 FU/mL	–
7.	SPR, direct physical adsorption [41]	10^6 cells/mL	–

analysis is no more 30 min, In case of preliminary transducer preparation this time may be shortened up to 5 min. TIRE based biosensor allows having the sensitivity up to 5 cells per 10 mL. The above mentioned characteristics are in good correlation with other approaches published in the literature and complete respond to all practice demands.

The express control of the dispersion of the retroviral infection among peoples and animals is very urgent world problem. As it is known retroviruses are agents of acquired immunodeficiency in human which was classified as AIDs. They are pathogens a number of the diseases in bovines (leucosis), fishes (carcinomas) and others [42–45]. The main way to prevent these non-desirable effects in this situation is the permanent diagnostics and isolation of ill units. The most applicable approaches for the biochemical diagnostics are the ELISA-method, reaction immune diffusion (RID) and polymerase chain reaction (PLR) [45]. Unfortunately these approaches are very routine, demand expensive reagents, complicate, cannot provide analysis in field conditions and in online regime as well as have a number of others disadvantages. All these problems may be successfully solved at the application of new generation of the analytical devices based on the principles of biosensorics.

Early, we have developed the immune biosensor based on the SPR and intended for the bovine leucosis diagnostics [42–45]. This biosensor may provide direct (without any additional reagents), rapid (during 3–5 min), cheapness (less 1\$) and even without taken blood and using drop of milk. In the special experiments it was studied in what way may the preliminary treatment of the transducer surface effect on the results of analysis at the detection of the antiviral antibodies in the serum blood. As it was above mentioned for such treatment polyelectrolytes (PSS, or PAA), or dodecanthiol, or developed dextran may be used for the achievement of high density of the immobilized materials. Nevertheless, there is possible to block some active binding centers of the biological structures at the contact with the surface. It may be at high level in case of the immobilization of the biological material direct on the gold surface. To avoid

these disadvantages a different approaches are use among of them the oriented immobilization with the help of protein A, or G of *St. aureus*, or the including in the Lengmur-Blodgett layer are most applicable [42–47]. In our experiments it was shown that the modification of the biosensor sensor surface with polyelectrolytes is the most expedient and chip for the screening of the bovine leukosis [48].

The analysis of 20 of the standard positive and negative antisera by third different method (RID, ELISA-method, PLR and SPR based immune biosensor) has shown a good correlation in the obtained results [49]. Two and one differences were checked between data of the immune biosensor and the ELISA-method and PLR, respectively.

Nevertheless, the SPR based immune biosensor has some disadvantages and at first they connected with the high cost of the chips and the necessity to use not simple procedure of the preliminary transducer surface treatment from one site. From other site the SPR recorder has very high price and don't allow fulfilling a number repeated analysis. To overcome some of these disadvantages we try to apply others types of the optical biosensors for solving of the problem of the express biochemical diagnostics of BLV. Among others such biosensors that based on the sNPS attract an especial attention. Similar biosensors based on the sNPS were developed early by us for the control of the myoglobin level in the blood and for the monitoring of the bacterial protein in the air of environment [50–54]. The registration of the specific signal was made by the measurement of the changes of the PhL intensity. This approach met the practice requirements regarding the simplicity, sensitivity, selectivity and rapidity of the analysis fulfillment but the stability of the sNPS was very low and the signal registration demanded complicated device. To continue our investigations in this field we proposed a new variant of biosensor based on the sNPS.

At the determination of the retroviral specific Ab in the blood of the ill cow by the measurement of the sPNS photoconductivity it was stated that the photosensitivity of the sPNS is a little decreased after the immobilization of Ag (crude sample of the retroviral proteins) but at the addition of Ab (serum blood of ill cows) in the dilution of 1:5,000 and, particular, in 1:1,000 it sharply is decreased.

The deposition of the retroviral proteins on the sNPS a few increases the PhL level but at the formation of the specific immune complex it decreases. Moreover, the level of the PhL decreasing depends on the concentration of the specific antibodies in the blood. If we used the non-specific antibodies or the serum bovine albumin as antigen the level of the PhL does not change.

5.2.4 Perspective to Development of the Instrumental Analytical Devices on the Basis of the Artificial Sensitive Structures and Multi-parametrical, Multi-functional Principles

One of main among others approaches for the perspective development of the new generation of the instrumental analytical devices is the replacing biological sensitive biological materials by the artificial structures on the basis of calyx[4]arenas [55–58]. Certainly in this case the absolute selectivity will be absent but there is

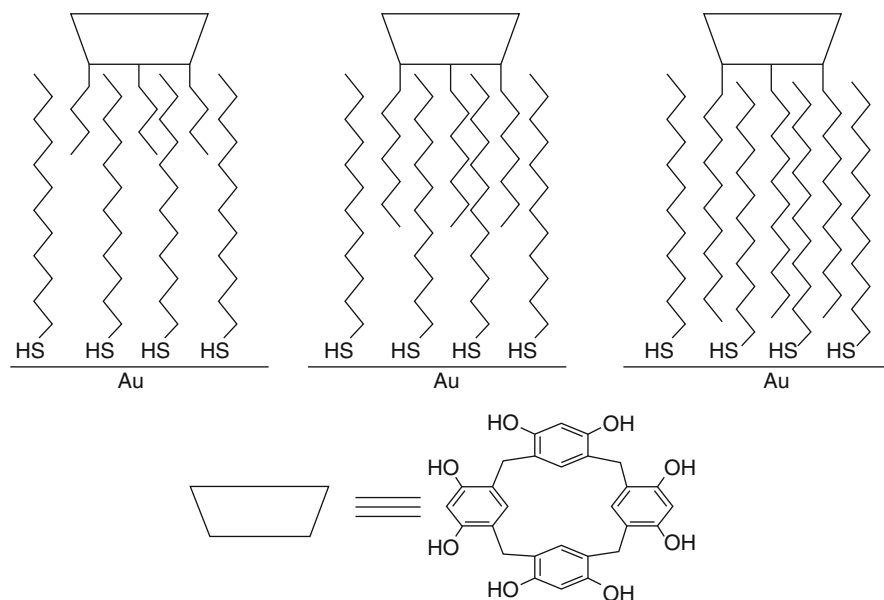


Fig. 5.12 Calyx[4]arenes (*below*) and possible type of binding of molecules on the surface covered by gold and modified by dodecanthiol (*above*)

Table 5.7 Constants (K_a) of “host-guest” complexes at the interaction of 2,4-D, atrazine and T2-mycotoxin with calix[4]arenes

„Guest”	$C_{\text{Calixarenes}} \times 10^{-4} \text{ M}$		
	1	2	3
2,4 D	3,657	772	5,077
Atrazine	6,785	2,513	6,483
T2-mycotoxin	550	8,850	1,020

possible to obtain specific signals by the application of the analysis of the number of the different step of the non-specific ones by the help of the special computer program. The overall view of the calyx[4]arenes and their immobilization of the gold surface of the transducer through dodecanthiol is shown in Fig. 5.12.

We have analyzed the binding constants of the calyx[4]arenes with some substances (Table 5.7). It was shown that they have strong differences which have possibility to discriminate the formatted complexes.

Of course this it is possible to synthesis a lot of variants of the calyx[4]arenes which will have much more differences in the affinity to the separate substances. For the realization of the proposed principle we tray creation of multi-parametrical and multi-functional analytical devices one of them used amperometric and thermometric registration of signal is presented in Fig. 5.13.

This device was tested at the simultaneous analysis of glucose according to parameters (exothermic reaction and registration of oxygen consumption) as well as the determination of anti-insulin antibodies [55–57].

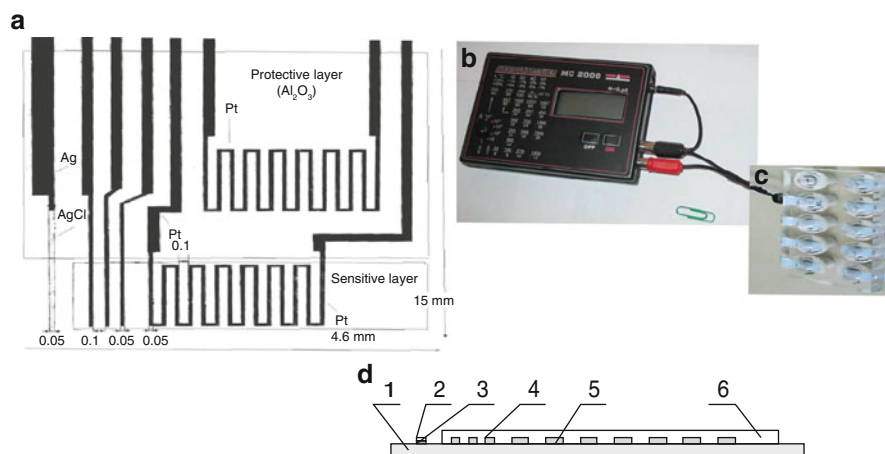


Fig. 5.13 Scheme of the microelectronic chip (with concrete materials and structure dimensions (a), overall view of two parts of the multi-parametrical biosensor: (b) electronic unit for the signal registration and (c) multi-channel block with the microelectronic registration of the ampero- and thermo metrical signals. (d) The section of the biosensor: (1) quartz basis; (2) layer of AgCl; (3) silver electrode; (4) measured platinum electrodes; (5) platinum thermo resistance; (6) biochemical structure

5.3 Conclusion

The developed immune biosensor based on the SPR may provide all practice demands in respect of the retroviral leucosis diagnostics by “direct” way using serum blood and milk samples. Moreover, such types of immune biosensors are able providing analysis of several mycotoxins with the needed sensitivity but in ‘competitive’, or “to saturate” ways. “Direct” way of the analysis of mycotoxins with high level of the sensitivity may be made with the help of the immune biosensors based on TIRE, ISFETs with cerium oxide gate and piezo crystal frequency. At the analysis of the *S. typhimurium* content in the model solution the immune biosensor based on the TIRE shown much more sensitivity than that based on the SPR. But both biosensors have the sensitivity much less that it is needed for practice. To achieve its demand there is necessary to develop a special procedure of the sample preparation. It was developed a most effective procedure for the sample preparation at the analysis of the level of mycotoxins in the real samples, in particular in the grain products and in the tomatoes. A special attention was paid the optimal procedure for the preliminary treatment of the transducer surface. The application of some polyelectrolyte’s and dedocanthiols as well as protein A or protein G increases the sensitivity of the analysis due to the orientation active binding sites towards solution and increase the density of the selective structures on the transducer surface. In generally it was demonstrated that the practical realization of the biosensorics

principles at the creation of the instrumental analytical devices may provide simple, cheapness, express, selective and sensitive control of the different toxins, viral and microbial infection sources in the environment. Further the development of such devices on the basis of sNPS is very perspective direction. The possibility to apply artificial substances, in particular, using calyx[4]arenas as selective sires and the development of multi-parametrical and multi-functional biosensors are considered as further perspective of the creation of the instrumental analytical devices. It was shown the possibility of application.

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Chapter 6

Electrochemical Biosensing of Pathogen Micro-Organisms

Susana Campuzano, María Pedrero, and José Manuel Pingarrón

Abstract In order to tackle the high demand existing for implementation of simple, low cost and rapid methodologies for routine monitoring of the presence or absence of pathogen bacteria, we have developed in the last years several electroanalytical strategies exhibiting improved analytical performance in terms of sensitivity, selectivity, easy availability and low cost for the ultrasensitive detection of relevant pathogenic bacteria. This chapter summarizes these works involving methods exploiting microbial metabolism, methods using lectins as bio-receptors and methods that imply the development of immunosensors, genosensors and the so-called magneto-biosensors.

Keywords Pathogen bacteria • Electroanalytical chemistry • Microbial metabolism • Lectins • Immunosensors • Genosensors • Magneto-biosensors

6.1 Introduction

Pathogenic bacteria are a major cause of human death and disease. The early detection of these micro-organisms is particularly relevant in a wide range of fields such as water quality management and food safety assurance, where efficient traceability systems are required. It is also very important in the prevention and defence against bioterrorism, and in medical diagnosis, where the correct treatment is determined by the bacterial species causing an infection. Therefore, the number of assays necessary

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to accomplish an efficient control is huge and the response time for remediation actions is limited. Consequently, there is a great demand for simple, cheap and rapid methods for routine in situ monitoring of bacteria presence or absence.

There are many available analytical methods that can be used for the determination of microorganisms. These include methods relying on cultural techniques that allow good selectivity and sensitivity but are time consuming and laboratory dependent. Methods based on electrical impedance or radiometry allow micro-organisms detection in a simpler way, although their sensitivity is low and they do not discriminate among species. Flow cytometry can achieve high sensitivity within a few minutes, although it cannot discriminate among organisms and between living and dead cells, and it is an expensive technique. Immunological and DNA techniques have provided means for selective detection of micro-organisms and in many cases with enough sensitivity, although they are still somewhat complex. Finally, methods exploiting microbial metabolism are simple, sensitive and they can provide some sort of selectivity.

In the research group of Electroanalysis and Electrochemical (Bio)sensors of the Department of Analytical Chemistry in the Complutense University of Madrid, we have been interested during the last years in the development of electrochemical biosensors which exhibited improved analytical performance in terms of sensitivity, selectivity, easy availability and low cost for the rapid and ultrasensitive detection of analytes with biochemical and clinical significance. In this context, the preparation of bioelectrodes combining immobilization methods for improving the stability of the biological recognition element with no significant loss of biological activity with sensitive electrochemical transducers constitutes a challenge.

This chapter summarizes some of the work carried out in our laboratory using different electroanalytical strategies for the detection of relevant pathogenic bacteria. These strategies involved methods exploiting microbial metabolism, methods using lectins as recognition systems and methods based on the development of immunosensors, genosensors and the so-called magneto-immunosensors and magneto-genosensors.

6.2 Methods Based on Metabolic Activity Monitoring

Methods based on micro-organisms metabolic activity monitoring provide important practical advantages such as (a) the possibility of discrimination between viable and non-viable cells, (b) the amplification that is usually achieved since a metabolic substrate or product is measured and, in general, there are millions of these molecules per cell, as well as (c) the possibility of performing the detection without a label and obtaining selective responses as some metabolic routes are specific for certain species.

Total coliform detection and quantification in drinking water was accomplished using a tyrosinase composite biosensor as electrochemical transducer [1]. Coliforms, and in particular *Escherichia coli* (*E. coli*), have been traditionally considered as an

indicator of the possible presence of pathogens in aquatic systems. Moreover, β -galactosidase, an enzyme that hydrolyzes lactose, is specifically found in coliform bacteria and, therefore, the detection of the galactosidase activity can be used as a method for coliforms detection. The measurement of a specific property of the hydrolysis product of a β -galactosidase substrate was used for such purpose. The employed methodology implied the addition of phenyl- β -D-galactopyranoside (PG) as the enzyme substrate which is hydrolysed to phenol and galactopyranoside by β -galactosidase. The phenol released was detected in the nanomolar concentration range by means of the tyrosinase composite biosensor. This enzyme reaction involves the catalytic oxidation of phenol to *o*-quinone at the expense of reducing oxygen to water [2] and the electrochemical reduction of this quinone, by transferring two electrons and two protons, at an applied potential of -0.15 V (vs Ag/AgCl) was employed to monitor the reaction. At this low potential value, the coliforms detection process is possible in complex media such as culture media without interferences.

The biosensor used was a graphite-Teflon composite biosensor in which tyrosinase was physically entrapped into the electrode matrix. This type of biosensor exhibited excellent performance for the detection of phenolic compounds, such as high sensitivity, great reproducibility of the amperometric responses, easy surface regeneration and long lifetime yielding reproducible responses. Moreover, they are very easy to fabricate and they can even be mass produced [3].

The methodology used for coliforms detection involved three steps. First, an enrichment step in which the sample was cultured in a medium where β -galactosidase expression was induced in order to achieve a high sensitivity. Second, as β -galactosidase is an intracellular enzyme, a permeabilizer agent was added to the medium in order to increase the diffusion of both the enzyme substrate and the enzyme reaction product and, therefore, reduce the assay time. Finally, the electrochemical detection of the released phenol was accomplished as commented above. Under the optimized conditions, it was possible to detect coliforms in drinking water in a concentration as low as one colony forming units (cfu)/100 mL in 6 h. One litre of water was filtered to preconcentrate the sample. The filter with the bacteria was placed in the culture medium (50 mL Luria-Bertani, LB) containing the β -galactosidase inducing agent isopropylthio- β -galactoside (IPTG), and the culture was incubated for 6 h. Thereafter, the sample was filtered again and the filter was introduced in the electrochemical cell containing 5 mL of phosphate buffer, pH 4.0, with the permeabilizer agent polymyxin B. After addition of the PG substrate, the phenol release by β -galactosidase was monitored amperometrically as a function of time. A clear difference in the biosensor response was observed when compared with a blank solution, showing fairly well the usefulness of the methodology for the ultrasensitive detection of *E. coli* in aquatic systems. Moreover, with this method, no interferences were produced by the presence of non-coliforms and its simplicity allows the possibility of automation for routine water quality control.

Another method relying on micro-organisms metabolic activity is the simultaneous detection and identification of different bacteria through the monitoring of metabolic glucose consumption using a graphite-Teflon electrode in which the enzymes

glucose oxidase (GOx) and horseradish peroxidase (HRP) together with the mediator ferrocene were coimmobilized in the composite matrix by simple physical inclusion [4]. Measurements were carried out in the flow injection mode, and the protocol for bacteria activity detection consisted of the following steps: (a) the current was allowed to stabilize at an applied potential of 0.00 V (vs Ag/AgCl); (b) injection of bacterial samples supplemented with glucose was carried out at different culture times; (c) flow injection amperometric responses were related to response at time 0. In this way, the construction of calibration graphs at fixed periods of time allowed quantification with different sensitivities. For example, it was possible to detect as low as 6 cfu/mL *E. coli* after 7 h of incubation.

Considering that different types of bacteria can be detected throughout their glucose consumption, it was also possible to identify bacteria by following their growth in culture media containing different antibiotics. Those microorganisms susceptible to certain antibiotics will not grow in that medium and therefore glucose level will remain constant. Conversely, if bacteria are resistant to the antibiotic, their glucose consumption will be the same as when no antibiotic is added. The assay involved testing of *E. coli*, *Staphylococcus aureus* (*S. aureus*) and *Salmonella choleraesuis* (*S. choleraesuis*) with a battery of seven antibiotics (polymyxin B, vancomycin, erythromycin, bacitracin, tetracycline, chloramphenicol and ciprofloxacin). Each microorganism exhibited a different antibiotic susceptibility profile, thus demonstrating the possibility of differentiation between them. Nevertheless, the response to chloramphenicol was the same for the three microorganisms and, therefore, this antibiotic would be discarded for the assay. In addition, vancomycin and erythromycin provided the same response and, consequently, only one of them needed to be used because no additional information is foreseen when employing both of them. Accordingly, when a real sample had to be evaluated, a series of culture media with different antibiotics and glucose would have to be prepared, and measurements of all of them would be carried out after fixed periods of incubation. As long as the specific antibiotic profile was established for a given pathogen, it can be detected and identified. Obviously, the more data accumulated, the more powerful the method becomes, and simultaneous detection and identification of several microorganisms could be performed using chemometric tools.

A different methodology for the rapid detection of microorganisms is that based on measuring bacterial catalase activity [5]. Bacteria can be classified as catalase-positive or negative, depending on whether they possess this enzyme or not. In general, catalase-positive bacteria are aerobic and facultative anaerobic. Bacterial catalase can catalyze the dismutation of hydrogen peroxide. Thus, if hydrogen peroxide is added to the bacterial sample, monitoring of hydrogen peroxide consumption by bacterial catalase with a HRP biosensor can be used for this type of microorganism detection. On the other hand, catalase-negative bacteria are aerotolerant or anaerobic bacteria, and many of them can generate hydrogen peroxide during their metabolic activity. Therefore, monitoring hydrogen peroxide formation with a HRP biosensor can be used for catalase-negative bacteria detection.

Examples of catalase-positive bacteria are *Escherichia*, *Salmonella*, *Staphylococcus* and *Mycobacterium*, among many others, and examples of catalase-negative

ones are *Streptococcus* and *Enterococcus*. The assay protocol was slightly different for catalase-positive or negative bacteria. For catalase-positive, the electrochemical cell was stabilized at an applied potential of 0.00 V (vs Ag/AgCl), and then, an appropriate amount of hydrogen peroxide was added. Next, the addition of the bacterial sample was carried out and hydrogen peroxide consumption was monitored. For catalase-negative bacteria, the protocol was even simpler. After stabilization of the current in the electrochemical cell, the bacterial sample was added and the generated hydrogen peroxide monitored.

Using this methodology it was possible to construct calibration plots allowing bacteria quantification. Taking *E. coli* as a model organism for catalase-positive bacteria, the detection of 10^6 cfu/mL was achieved directly in 10 min without any pre-enrichment or culture step. Lower limits of detection (LODs) can be easily obtained by introduction of any of these previous steps. In the case of catalase-negative bacteria, it was always necessary to incubate the sample for a period of time in order to generate hydrogen peroxide. Nevertheless, high hydrogen peroxide concentrations are generated in relatively short periods of time, being possible to detect a concentration as low as 10^3 cfu/mL after 3 h of incubation. In summary, this methodology allows the detection of both catalase-positive and catalase-negative in very short time and provides a mean for discrimination between them.

As overall conclusions for this section, it can be said that monitoring metabolic activity of bacteria by means of amperometric composite biosensors allows obtaining low cost, simple and sensitive methods for bacteria detection. Using selective growing media, such as ones composed by different antibiotic, permit achieving bacteria “fingerprints”. In this way, one single method is enough to identify a wide range of organisms, and therefore, a universal microorganisms detection method could be obtained. Protocol simplicity allows automation of the assays, making them suitable for routine, on-line monitoring.

6.3 Electrochemical Immunosensors

Different electrochemical immunosensor designs have been developed recently in our research group for the sensitive detection of bacteria. The two examples given in this section deal with immunosensors whose working protocols do not involve coupling with amplification strategies that imply the use of other materials apart from the electrode substrate.

The first example is an amperometric immunosensor for the quantification of *S. aureus*, one of the pathogenic microorganisms constituting a major cause of food poisoning [6]. The immunosensor design implied coimmobilization of tyrosinase and rabbit immunoglobulin G (RbIgG) by cross-linking with glutaraldehyde on a 3-mercaptopropionic acid (MPA)-modified gold electrode, giving rise to three-dimensional biosensing aggregates. The immunological reaction was based on binding of protein A-bearing *S. aureus* cells with the crystallisable fragment of RbIgG. The competitive immunosensing approach involved competition of

S. aureus cells with anti-RbIgG labelled with alkaline phosphatase for the binding sites of the RbIgG immobilized on the electrode surface. Monitoring of the affinity reaction was carried out by the amperometric detection at -0.15 V (vs Ag/AgCl) of phenol generated in the enzyme reaction of phenyl phosphate with alkaline phosphatase, at the tyrosinase-modified electrode.

Under the optimized conditions, a calibration curve for *S. aureus* was constructed with a dynamic range from 4.4×10^5 to 1.8×10^7 cells/mL, and a LOD of 1.7×10^5 cells/mL. This LOD could be remarkably improved by subjecting *S. aureus* cells to wall lyses by heat treatment. This process produced the breaking of bacteria and the availability of many more protein A-bearing cell portions. Moreover, their diffusion towards the immobilized antibodies was also improved. The calibration graph obtained with lysed cells allowed a LOD of 2.3×10^3 cells/mL to be achieved, two orders of magnitude lower than that obtained without cell lyses. This value is sufficient for the detection of contamination by *S. aureus* tolerated in many countries for most foodstuffs.

The immunosensor was applied to the determination of *S. aureus* in milk samples spiked at a 4.8×10^3 cells/mL level. The spiked milk sample was heated for 30 min to achieve bacteria cell lyses, and left to cool to room temperature. The calibration graph for *S. aureus* in milk was similar to that obtained with microorganism standards prepared in buffer solution, thus indicating that direct *S. aureus* determination in milk was feasible with no need for dilution or sample treatment. The estimated LOD in milk was 1.9×10^3 cells/mL. The analysis of three milk samples (inoculated at a 4.8×10^3 cells/mL concentration level) by triplicate yielded a mean value of $(5.0 \pm 0.9) \times 10^3$ cells/mL, with a mean recovery of $(105 \pm 19)\%$ which, considering the type of analysis carried out, could be considered as a very good result, thus showing fairly well the usefulness of the immunosensor for the analysis of real samples.

Another interesting strategy developed by our group concerns the development of label-free impedimetric immunosensors for detection and quantification of *E. coli* [7]. We immobilized anti-*E. coli* antibodies on gold screen-printed electrodes (AuSPEs) using two different approaches (Fig. 6.1). The first one implied the covalent immobilization of the specific antibodies on the electrode modified with a 3,3'-dithiobis[sulfosuccinimidyl]propionate] (DTSSP) self-assembled monolayer (SAM) (Fig. 6.1a). The second configuration involved the immobilization of thiolated antibodies using sulfo-LC-SPDP as thiolation reagent (Fig. 6.1b). In both cases, a specific binding event occurred between the immobilized antibodies and the antigen on the *E. coli* cell surface.

The stepwise assembly with both immunosensor designs was monitored by using electrochemical impedance spectroscopy (EIS). The Nyquist plots showed an increase in the electron transfer resistance (R_{et}) as a consequence of the antibodies immobilization. Nevertheless, blocking of the residual succinimidyl groups in the SAM with ethanolamine promoted the redox probe transfer to the electrode surface, and a decrease in the R_{et} was observed. This fact could be due to either electrostatic attractions or to the ability of ethanolamine to replace weakly immobilized antibodies. The further attachment of bacterial cells to the modified electrode surface gave

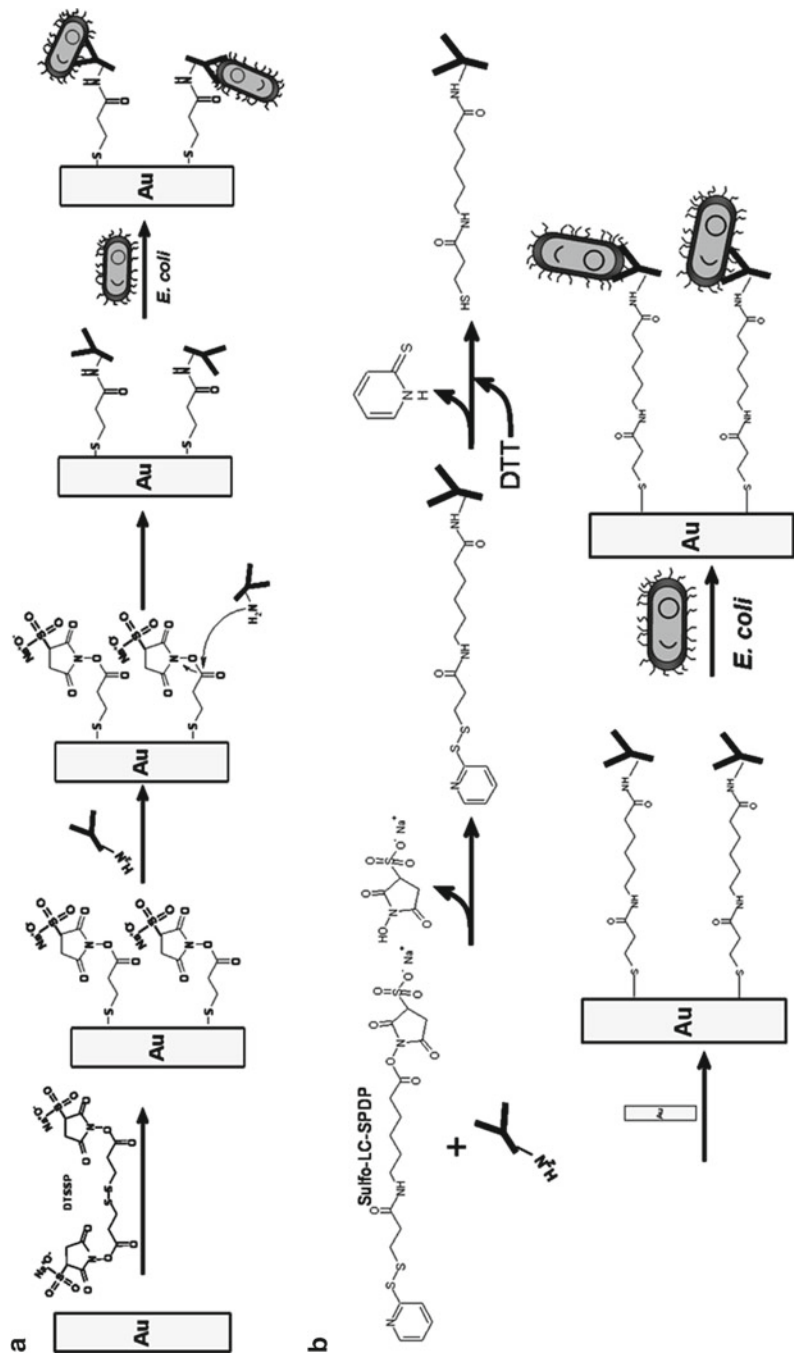


Fig. 6.1 Schemes of the both immunosensor configurations used: (a) covalent immobilization of anti-*E. coli* antibodies on the DTSSP-modified AuSPE; (b) immobilization of thiolated antibodies onto the AuSPE (Adapted from Escamilla et al. [7]. With permission, Copyright 2010 Elsevier)

rise to an additional barrier towards the access of the redox probe to the electrode, resulting in a further increase in the electron transfer resistance. Concerning the immunosensor configuration involving immobilized thiolated antibodies, it exhibited a much higher increase in the measured R_{et} (162%), which allowed the achievement of a much higher sensitivity for the assay. These results were likely due to a larger amount of immobilized antibody using this approach, as a consequence of the higher packing density of the SAM achieved with the thiolated antibodies. Furthermore, the 2-pyridyl-disulfide has been claimed to have a higher reactivity towards antibodies compared with DTSSP, thus giving rise to a larger amount of immobilized antibodies. In fact, a much lower LOD, as low as 3.3 cfu/mL, and a much wider range of linearity, covering almost eight logarithmic units ($5-1 \times 10^8$ cfu/mL), were obtained with the thiolated antibodies-based configuration, in spite that the binding efficiency of anti-bacteria antibodies to their antigen is normally low, and no pre-concentration or pre-enrichment steps were used. Negative controls without immobilized antibodies with both configurations confirmed that no significant electron transfer resistance changes were observed even for large bacteria concentration, thus ensuring that the measured electrochemical signal was specific and not partially due to non-specific adsorption of bacteria on the electrode surfaces.

The selectivity of the immunosensors was tested against two other bacteria (*S. aureus* and *S. choleraesuis*). Both configurations exhibited a good selectivity showing that there was not significant cross-reaction of the immunosensors with other bacterial species.

The thiolated antibodies-based immunosensor was applied to the detection and quantification of *E. coli* in water samples from a Spanish river and also in tap water. The response of the immunosensor to these samples was below the LOD, and also plate counting confirmed that there were no faecal coliforms in the analyzed samples. Therefore, they were inoculated with *E. coli* at a concentration level of 10 cfu/mL. The concentration of bacteria was calculated by interpolation of the electrochemical signal into the calibration plot prepared with standards, and the results obtained for six inoculated water samples in each case yielded *E. coli* mean contents of (11 ± 2) for tap water and (12 ± 2) cfu/mL for Eresma river water. The total time for the assay, once the immunosensor was prepared, was approximately 1 h. All these data showed fairly well the usefulness of the developed impedimetric immunosensor to be applied for the rapid and sensitive determination of this pathogen microorganism in water samples without pre-concentration or pre-enrichment steps.

6.4 Electrochemical Genosensors

Nucleic acid-based detection has shown to be more specific and sensitive for the detection of microbial pathogens than the detection based on immunological reactions [8]. DNA is also more resistant to denaturation than proteins and is able to survive for long periods under appropriate conditions [9]. Current developments in DNA-based sensing technologies aim to improve the specificity, sensitivity, rapidity,

user-friendliness and cost effectiveness of molecular diagnostics. In particular, direct means for bacteria detection and characterization are sought to replace tedious, hazardous, timely and uninformative conventional means of isolation, culture or characterization. DNA-biosensing methodologies are usually based on the detection of DNA hybridization. Very often, the amount of DNA to be detected (in the femtomolar or attomolar ranges) is lower than the LOD of conventional analytical techniques. In order to solve this problem, amplification of the sample and/or the signal is needed as it will be pointed out below [10].

Nevertheless, some electroanalytical strategies without amplification have demonstrated to be also useful to detect low concentrations of bacteria. For example, we described a sequence-specific electrochemical biosensor based on the immobilization of a 25 base single-stranded capture probe, specific to the *E. coli lacZ* gene, onto a gold disk electrode [11]. The capture probe was attached using a 3,3-dithiodipropionic acid di(N-succinimidyl ester) (DTSP) SAM without derivatization and the efficiency of the hybridization process was enhanced employing 6-mercapto-1-hexanol (MCH) for the displacement of non-specifically adsorbed oligonucleotide molecules. Moreover, hybridization of the immobilized probe with the target DNA at the electrode surface was monitored by square wave voltammetry (SWV), using methylene blue (MB) as electrochemical indicator.

A good stability of the single- and double-stranded oligonucleotides immobilized on the DTSP-modified electrode was demonstrated, and a target DNA LOD of 45 nM was achieved which meant an acceptable sensitivity taking into account that no signal amplification was employed. Hybridization specificity was checked with non-complementary and mismatch oligonucleotides. A single-base mismatch oligonucleotide gave a hybridization response only $(7 \pm 3)\%$, higher than the signal obtained for the capture probe before hybridization, which suggested the possibility of sensitive detection of single-base mutation.

This approach was extended to the use of disposable AuSPEs thus avoiding the need of surface regeneration. Moreover, a comparison with other DNA probes immobilization schemes was accomplished [12]. In the first configuration, direct coupling of thiolated probes to bare AuSPEs was carried out, MCH was employed as spacer for the displacement of non-specifically adsorbed oligonucleotide molecules and the hybridization event between the probe and target DNA sequences was detected by SWV using MB as electrochemical indicator. The second immobilization scheme consisted of the attachment of biotinylated *Enterobacteriaceae lacZ* gene specific capture probes to avidin-coated DTSP-modified AuSPEs. An enzyme amplified detection approach was employed, based on the coupling of streptavidin-HRP to the biotinylated complementary target, after the hybridization process, and immobilization of tetrathiafulvalene (TTF) as redox mediator atop the modified electrode. The amperometric response obtained at -0.15 V (vs a Ag pseudoreference electrode) after the addition of hydrogen peroxide was used to detect the hybridization process.

The biosensor designs exhibited good analytical performance in terms of repeatability and reproducibility with different biosensors, especially in the case of genosensors fabricated with biotinylated capture probes and high temperature cured

AuSPEs. Furthermore, a high sensitivity with no PCR amplification was obtained under the optimized experimental conditions, with a LOD of 0.002 ng/mL without any preconcentration step. It can be concluded that the combination of the suitability of this kind of SPEs to be modified with DTSP-SAMs offers the possibility of constructing robust and reliable disposable mass-produced genosensors with a short fabrication time (up to 20 sensors per day) for the detection of *Enterobacteriaceae* in real samples.

6.5 Lectins Adsorptive Affinity

An interesting alternative detection approach for the detection of micro-organisms is the one relying on carbohydrate recognition, which offers some potential advantages over antibody/nucleic acid detection. Most of microorganisms and viruses have carbohydrates at their surface and, therefore, the recognition of these carbohydrates can be used for their detection and identification. Lectins are glycoproteins that associate selectively and reversibly with mono- and oligosaccharide components of polysaccharide structures. Lectins are polyvalent and each lectin molecule possesses at least two carbohydrate binding sites to allow cross-linking between cells. Furthermore, these proteins are readily available and inexpensive, which is highly appropriate to combine with mass produced SPEs as transducer elements.

We investigated the potentialities of lectin adsorptive affinity impedance for the label free detection of bacteria [13]. In order to do that, pre-treated AuSPEs were modified with the bacteria-biotinylated lectin complex by successive deposition of the biotinylated-lectin solution and the bacteria culture, and allowing the immobilization to proceed for 1 h at room temperature (Fig. 6.2).

The formation of the lectin-bacteria complex at the electrode surface was evaluated by using biotinylated-Concanavalin A (Con A) and *E. coli* as a model system. As expected, the formation of the adsorbed biotinylated lectin layer produced an increase in the electron transfer resistance, and the further attachment of bacterial cells to the electrode surface gave rise to an additional barrier resulting in a further increase in the electron transfer resistance. The Randles modified equivalent circuit was used to fit the EIS data and to determine the electrical parameters for each step. The R_{et} value increased more than 400% upon binding of the biotin-lectin-*E. coli*

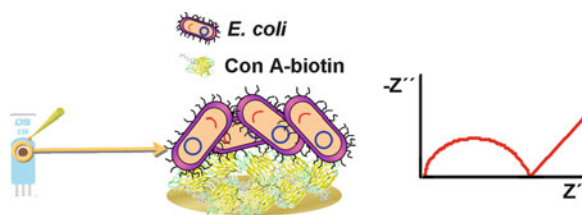


Fig. 6.2 Scheme for an *E. coli* impedimetric sensor based on the use of lectins

complex on the SPE surface, thus indicating that this technique was able to monitor the change in electron transfer resistance resulting from the immobilization of bacteria through the complex formed with an appropriate lectin without any amplification step. Cyclic voltammograms showed concordant results, with a decrease in the peak current and an increase in the separation of peak potentials in comparison with unmodified electrodes.

However, no change in resistance was measured with electrodes modified with non-specifically adsorbed *E. coli* cells (in the absence of Con A), indicating that no significant *E. coli* non-specific adsorption occurred on the electrode surface and that the bacterial cells immobilization was produced solely through the mannose and glucose residues existing in their structure. Similar experiments carried out with non-biotinylated Con A showed that no change of electron transfer resistance occurred after modification with the lectin-bacteria mixture, thus suggesting that the non-biotinylated lectin was not immobilized on the electrode surface. Therefore, we could conclude that the biotinylated lectin-bacteria complex was immobilized on the gold surface through biotin adsorption.

The electron transfer resistance change varied linearly with the logarithmic value of *E. coli* concentration over four orders of magnitude (5.0×10^3 – 5.0×10^7 cfu/mL) and the calculated LOD, without any pre-concentration step, was 5×10^3 cfu/mL. In addition, the reproducibility of the responses obtained with eight different sensors prepared in the same manner yielded a RSD value of 9.8%. The selectivity of the approach was evaluated by checking the impedimetric responses obtained with complexes formed between nine different lectins and three different bacteria (*E. coli*, *S. aureus* and *M. phlei*). In order to ascertain whether the impedimetric responses could be classified according to species patterns, principal components analysis (PCA) was applied to the set of results. This analysis was carried out in a semi-quantitative manner, and different bacteria response profiles were found when the different lectins were used as recognition elements. PCA analysis showed that three components explained more than 95% of the total variance across the matrix. The three-dimensional plot for the components loadings showed that a suitable clustering of the measurements obtained from each type of microorganism was achieved with this simple approach, thus allowing classification and discrimination among them.

In conclusion, the selective binding of lectins to oligosaccharides residues present on the surface of microorganisms can be exploited for their rapid label-free detection, identification and quantification, the impedimetric monitoring of this specific recognition enabling the detection of 5×10^3 cfu/mL *E. coli* within 1 h.

6.6 Electrochemical Magneto-Biosensors

The development of the so called magneto-biosensors implies the combination of the well-known characteristics of disposable SPEs with functionalized magnetic beads (MBs). These superparamagnetic micro- and nanoparticles possess a large

active surface area which allows the immobilization of high biomolecules loadings onto the solid phase of the electrochemical transducer through the application of a magnetic field. They have demonstrated to be extremely useful for purification and/or preconcentration purposes as well as for decreasing matrix effects [14]. This section summarizes some results obtained in our laboratory focused on the development of electroanalytical strategies involving magneto-immunosensors and magneto-genosensors for the ultrasensitive detection of relevant pathogenic bacteria.

The first example concerns the development of magneto-immunosensors for the specific detection of *Streptococcus pneumoniae* (*S. pneumoniae*). *Pneumococcus* is a major human pathogen and the leading cause of pneumonia, bacteremia and meningitis in adults and otitis media in children. Overall, the mortality rate of *pneumococcal* infections is higher than that originated by any other pathogenic bacteria. The increasing number of *pneumococci* found to be antibiotic-resistant highlights the importance of rapid identification of *pneumococcal* infections related to the adequate selection of antimicrobial agents for therapy. In order to tackle this important problem, we constructed disposable amperometric magneto-immunosensors [15].

A key aspect in the development of this immunosensor was the preparation of a specific antibody against the most frequently virulent serotypes found in clinical isolates. This selection was accomplished by flow cytometry among seven anticapsular and antisomatic antibodies. The product of percentage positive population by fluorescence intensity for all the bacteria strains tested in the study (*S. pneumoniae* R6, Dawn, TIGR4, *S. mitis*, *E. coli*, *S. oralis*, *S. pseudopneumoniae* and *S. sanguinis*) showed that the four anti-capsular antibodies (Ab20429, antiS37, antiR and PA1-7259) exhibited a good selectivity against other *Streptococci* and other non-phylogenetically related bacteria, with the exception of PA1-7259, which showed a high cross-reactivity to *E. coli*. On the contrary, the selectivity obtained with the antisomatic antibodies (antiPLY, antiTEPC15 and antiClytA) was much worse. Taking into account that the antibody antiS37 gave good signals towards all the *S. pneumoniae* virulent strains tested (Dawn, TIGR4, and the R6 type strain) and exhibited very low responses for other *Streptococci* and *E. coli*, it was selected as the antibody to be employed for implementing the magneto-immunosensor.

The disposable magnetoimmunosensors developed were based on a sandwich configuration, where capture antibodies were coupled to Protein A-coated MBs through the strong affinity of Protein A for the *Fc* part of IgG molecules. Then, the bacteria were captured by a 60-min conjugation step and a further conjugation of the HRP-labelled antibody to the bacteria-capture antibody-MB conjugate was performed. After each incubation or washing step, the MBs were separated from the supernatant by placing the tubes in a magnet separator until the beads migrated to the tube sides and the liquid was clear. In order to shuttle electrons between the oxidized enzyme and the working electrode, the MBs modified with the immunosensing complex were immobilized onto TTF-modified AuSPEs by means of a neodymium magnet placed under the electrode surface. The bio-recognition event was monitored upon addition of H_2O_2 , which was

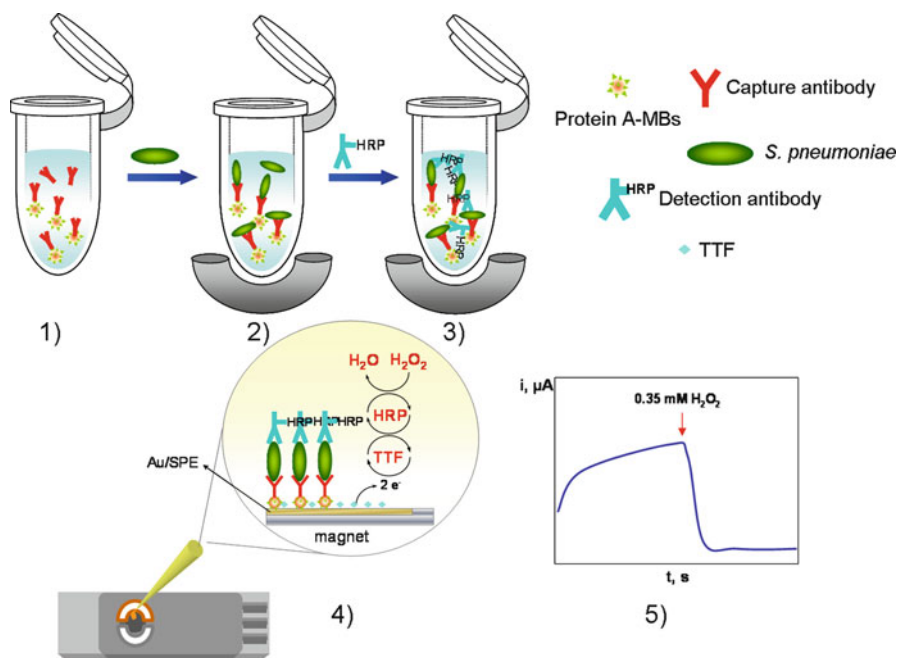


Fig. 6.3 Scheme displaying the different steps involved in the development of disposable magneto-immunosensors for the determination of *S. pneumoniae* (Reproduced from Campuzano et al. [15]. With permission, Copyright 2010 Elsevier)

reduced in the presence of HRP, the enzyme regeneration being mediated by TTF. Therefore, the generated TTF^+ was electrochemically reduced at an applied potential of -0.15 V (vs the Ag pseudoreference electrode) [16]. Using this protocol, immunoreactions occurred on the surface of MBs and not on the electrode surface, the TTF-Au/SPE acting only as a transducer of the recognition event. Figure 6.3 shows schematically the whole procedure.

Apart from the immunosensor configuration displayed in Fig. 6.3, two alternative formats were also assayed. One of them implied incubation of MBs modified with the antibody-bacteria conjugate in a solution of biotinylated antibody, which is marked subsequently with a streptavidin-HRP polymer. Another configuration made use of a two step signal amplification methodology. Biotinylated rabbit anti-streptavidin was added to the first layer of streptavidin-HRP polymer and the conjugate incubated again with the streptavidin-HRP polymer. When both the specific and non-specific signals obtained for the three formats were compared, it could be deduced that the specific responses were similar with the two former configurations. However, the signal obtained in the absence of bacteria was notably higher when using the streptavidin-HRP polymer, which was attributed to the non-specific adsorption of the polymer. Quite unexpectedly, the amperometric signal obtained

using the two-step amplification method were even weaker than the response for the format involving a single step, which was most likely due to the dissociation of the biotinylated anti-streptavidin antibody from the initial streptavidin-HRP layer as a result of the competition with the second streptavidin-HRP layer. According to these results, the configuration displayed in Fig. 6.3 was selected to explore the analytical performance of the immunosensor.

Calibration curves for both the Dawn and R6 strains were constructed. The ranges of linearity were 1.5×10^4 – 10^7 and 6.3×10^5 – 10^8 cfu/mL, with LODs of 1.5×10^4 cfu/mL (3.0×10^3 cfu) and 6.3×10^5 cfu/mL (1.3×10^5 cfu), respectively. These LODs can be qualified as quite good considering that no pre-concentration steps were performed out before quantification.

The selectivity of the magneto-immunosensor for *pneumococcus* detection was evaluated against other *streptococci* that may be present in clinical samples and also against phylogenetically unrelated bacteria such as *E. coli*. The non-encapsulated R6 strain produced almost a 70% of the signal provided by Dawn strain, while other *streptococci* like *S. pseudoneumoniae*, *S. oralis* and *S. sanguinis* yielded responses lower than 40%. This was an expectable and reasonable cross-reactivity between *pneumococci* and phylogenetically related *streptococci* since all of them form part of a very similar group of species.

The magneto-immunosensor was applied to the quantification of *S. pneumoniae* in urine. A statistical comparison of the slope values of calibration plots in urine and with standard solutions allowed us to deduce that no matrix effect existed. Urine samples were inoculated with 1×10^7 cfu/mL *S. pneumoniae* R6 or with *S. pneumoniae* Dawn at two different concentration levels, 5×10^4 and 1×10^6 cfu/mL. Analysis of five inoculated urine samples yielded mean contents of $(1.3 \pm 0.3) \times 10^7$, $(6.3 \pm 0.2) \times 10^4$ and $(1.0 \pm 0.2) \times 10^6$ cfu/mL, respectively. The total time for the assay, once the immunosensor was prepared, was of approximately 3.5 h. Taking into account that the diagnosis of urinary tract infection (UTI) is considered positive if $>10^5$ cfu/mL of a single bacterium is found in urine cultures, the obtained results showed fairly well the usefulness of the developed magneto-immunosensor to UTI detection without previous pre-concentration steps or sample pre-treatments.

The next examples of magneto-biosensor for the ultrasensitive detection of bacteria concern the preparation of disposable DNA magnetosensors. In these designs we integrated the use of electrochemical DNA biosensors, functionalized MBs, disposable SPEs and PCR amplification strategies. The first example is the design of amperometric magneto-genosensors with an enzyme-amplification approach for the specific detection of coliforms through a characteristic gene of this bacterial family, the *lacZ* gene, responsible for the beta-galactosidase activity [17].

The fundamentals of the developed methodology are displayed in Fig. 6.4. A biotinylated capture probe able to recognize a specific region of *lacZ* gene was attached to streptavidin-modified MBs and hybridization with a biotinylated target was allowed to proceed. Then, a streptavidin-HRP polymer was attached to the biotinylated hybrid and the resulting modified MBs were captured by a magnet on the surface of TTF-modified AuSPEs. Two successive magneto-separation and washing steps were performed after each modification stage which ensured the

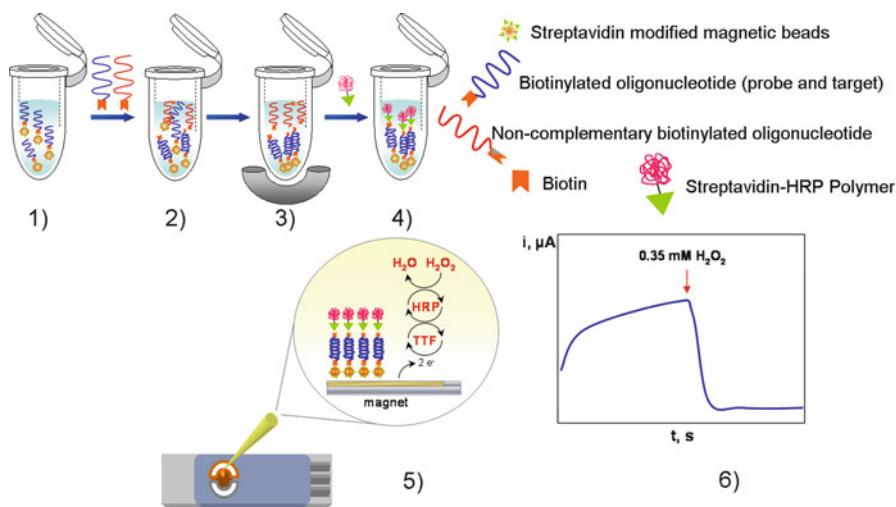


Fig. 6.4 Scheme displaying the different steps involved in the construction of magneto-genosensors for the specific detection of *Enterobacteriaceae* (Reproduced from Loaiza et al. [17]. With permission, Copyright 2008 ACS)

removal of weakly immobilized biological material on the surface of MBs. The amperometric response measured at -0.15 V (vs the Ag pseudoreference electrode) after the addition of hydrogen peroxide was employed to detect the hybridization event. With this methodology, the hybridization process was separated from the detection process, thereby avoiding undesirable and common non-specific adsorption of DNA on electrode surfaces.

The protocol was firstly checked with synthetic short-chain oligonucleotides. No response was observed with non-complementary oligonucleotides and, interestingly, the amperometric response for a single-base mismatch oligonucleotide was only 17% of that obtained for fully complementary target DNA, thus showing the ability to detect even a single-base mutation. Under the optimized working conditions a linear calibration plot was constructed for target DNA with a range of linearity between 0 and $2.4 \times 10^{-7} \text{ M}$, and a fairly low LOD of 0.11 nM without using PCR amplification.

In order to improve the sensitivity of the determination, asymmetric PCR (aPCR) was used to amplify a 326-b DNA fragment of *lacZ* gene containing the target sequence for the direct hybridization detection. Asymmetric PCR is carried out as conventional PCR, but with different concentration of the primers and, as a consequence, the predominant resulting amplification product is single-stranded DNA. Therefore, when asymmetric amplification was performed using a biotinylated reverse primer-to-forward primer concentration ratio of 10-to-1, a biotinylated single-stranded amplicon was generated. This amplicon could be directly hybridized with the capture probe immobilized on the MBs, without needing a previous

de-naturation step that is required when working with double-stranded amplicons obtained by conventional PCR, thus simplifying and reducing the time of the whole assay. The screening of the asymmetric PCR products by gel electrophoresis confirmed the successful amplification of PCR products with the right size.

The amplicons obtained by aPCR were purified, diluted and directly applied for the electrochemical detection following the same procedure used for synthetic oligonucleotides. An aPCR blank control did not yield a significant amperometric signal. Conversely, the presence of an amplicon concentration as low as 13.2 attomolar, gave rise to a remarkable amperometric response. The corresponding calibration plot allowed a LOD of 2.5 attomolar to be obtained, which meant a significant improvement compared with those provided by other electrochemical genosensors described in the literature. Furthermore, the results obtained for ten different measurements showed a quite good reproducibility with a relative standard deviation (RSD) value of 6.1% despite the low amplicon concentration used, 19.8 aM.

Another PCR type, direct PCR (DPCR) allows amplification and detection of specific target DNA sequences to be performed using whole cells without DNA extraction. Therefore, with the aim of reducing even more the complexity and the time for the assay, we carried out the detection of asymmetric amplified products obtained directly from *E. coli* cultures [18]. Gel electrophoresis confirmed that a DaPCR product could be obtained with only 1 cfu per 100 mL. The amperograms obtained for amplified products, corresponding to different bacterial cultures concentrations, exhibited similar amperometric responses, due to the fact that the final amplicon concentration was leveled off by PCR itself. Therefore, despite the high sensitivity achieved, it was not possible to quantify coliform density using DaPCR. However, taking into account that European Regulation establishes that drinking water should exhibit absence of bacteria in 100 mL of sample, and that the developed method allowed a clear discrimination between non-contaminated samples and samples contaminated with only 1 cfu per 100 mL, the developed methodology clearly demonstrated its usefulness to be potentially used as an alarm device for water quality control.

Another recent and interesting example of this kind of approaches is the one developed to achieve a specific and more sensitive detection of *S. pneumoniae* than that seen above using magneto-immunosensors. This was accomplished by designing magneto-genosensors based on the use of MBs, a specific capture probe and appropriate primers for asymmetric PCR amplification of a characteristic region of a gene encoding the *pneumococcal* major autolysin (LytA) [19].

In this case, the asymmetric PCR relied on the amplification of the *lytA pneumococcal* gene which possesses characteristic signatures that allow differentiation between the *lytA* allele of *S. pneumoniae* and those of other *streptococci* of the mitis group (SMG). To avoid any cross-recognition and to select capture probes and amplicons specific either for *S. pneumoniae* or SMG, a detailed analysis of all *lytA* alleles available in the databases was performed. The selected primers amplified a 235-bp fragment of *pneumococcal lytA* or a 259-bp fragment of the *lytA*-like SMG alleles.

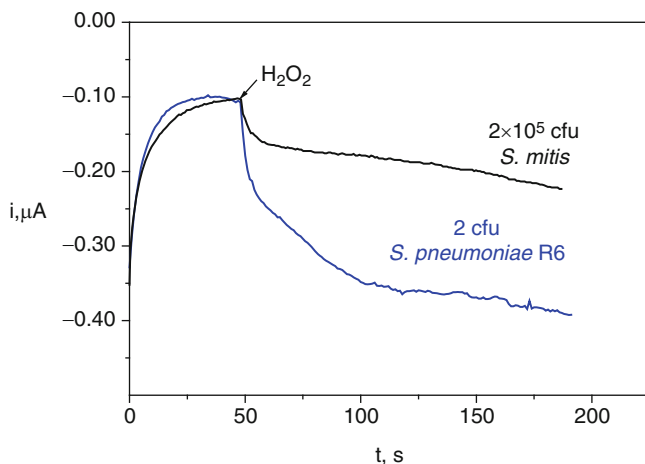


Fig. 6.5 Amperometric responses measured at the magneto-genosensor for DaPCR amplicons generated using as templates 2 cfu *S. pneumoniae* or 2.0×10^5 cfu *S. mitis*

The analytical characteristics of the calibration plots for both a 20-mer synthetic target and the aPCR amplicon showed LODs of 5.1 and 1.1 nM, respectively. Moreover, a RSD value of 6.9% was obtained for measurements carried out with seven different genosensors for 1.1 nM aPCR product.

Also, DaPCR was performed from cultures of *S. pneumoniae*. Gel electrophoresis showed that DaPCR products could be prepared with as few as 2 cfu in the PCR reaction mixture. Moreover, the resulting amperometric signal increased with bacterial concentration up to 1.3×10^7 cfu/mL.

The selectivity of the developed methodology was investigated against other *streptococci* that may be present in clinical samples and also against phylogenetically unrelated bacteria such as *E. coli*. Gel electrophoresis demonstrated that DaPCR for all the strains tested only produced the amplicon with *S. pneumoniae*, thus confirming the high specificity of the developed methodology. It is important to remark that DaPCR for three different capsulated strains of *S. pneumoniae* (TIGR4, D39 and Dawn) also provided the desired amplicon, demonstrating the usefulness of the methodology for the detection of both capsulated and non-encapsulated pneumococci. Figure 6.5 shows the amperometric signals obtained for amplicons generated using as a template 2 cfu of *S. pneumoniae* or 2.0×10^5 cfu of *S. mitis*, which proves the selective detection of concentrations of *S. pneumoniae* 10^5 times lower than those of *S. mitis*.

Current efforts in our laboratory aim at exploiting the applicability of the developed methodology for rapid determination of *pneumococcus* directly from clinical samples. Preliminary data in this direction testing on defibrinated sheep blood and human urine showed that this methodology is able to discriminate between non-inoculated blood and urine samples and samples inoculated with 10^3 cfu/mL (or 2 cfu in the PCR mixture) *S. pneumoniae*.

Table 6.1 Comparison of the analytical characteristics obtained with the magneto-immunosensing approach without PCR coupling and with the magneto-immuno-PCR strategy

	Strain	Linear range, cfu/mL	RSD ^a (n=5), %	LOD, cfu/mL
Magneto immuno without PCR coupling	Dawn	1.5×10^4 – 1.0×10^7		1.5×10^4 (3.0×10^3 cfu)
	R6	6.3×10^5 – 1.0×10^8		6.3×10^5 (1.3×10^5 cfu)
Magneto immuno PCR	Dawn	1.0×10^2 – 1.0×10^4	6.2	130 (26 cfu)
	R6	1.0×10^2 – 1.0×10^4	8.0	132 (26 cfu)

^aConcentration level, 1.0×10^3 cfu/mL

Finally, we also demonstrated that the coupling of the magneto-electrochemical immunoassay approach described above with DaPCR of the bacteria attached to the capture antibody-Protein A-MBs allowed LODs for *S. pneumoniae* to be obtained approximately three orders of magnitude lower than those achieved with magneto-immunosensors without PCR coupling [20]. The experimental strategy is similar to those commented above and implied, firstly, the attachment of antiS37 capture antibodies to the Protein A-coated MBs. The second step consisted of bacteria capturing on the antibodies-modified MBs. After appropriate washings of bacteria-antiS37-attached beads and their resuspension in water, DaPCR was accomplished to amplify the 235-b ssDNA fragment containing the target sequence (20-mer). Then, the purified amplicons were hybridized onto streptavidin-MBs modified with a specific biotinylated 20-mer capture probe. The biotinylated hybrid was labelled with the streptavidin-HRP polymer and the same process commented above was employed for monitoring the hybridization event.

Table 6.1 summarizes the analytical characteristics of the calibration graphs for Dawn and R6 serotypes using this immuno-PCR developed strategy. The LOD values obtained were 130 and 132 cfu/mL, respectively, which are approximately 100–1,000 times lower than those obtained with the disposable electrochemical magneto-immunosensors without PCR amplification. Furthermore, RSD values lower than 10% were obtained for five measurements of DaPCR products from 10^3 cfu/mL *S. pneumoniae* cultures, which can be considered as a quite good reproducibility taking into account that disposable sensors were used and the low bacterial cell concentration assayed.

6.7 Conclusions

As general conclusions, it can be stated that electroanalytical strategies involving biosensing approaches using enzyme electrodes, immunosensors and genosensors provide versatile and efficient analytical tools to be employed for rapid pathogen micro-organisms detection and quantification. Coupling of these approaches with different preconcentration-amplification methodologies (cell lyses, magnetic

microbeads, PCR techniques) allows ultrasensitive detection of bacteria as required to take urgent actions in many fields of economical and social significance. These electrochemical biosensing approaches compare advantageously with other available analytical methodologies in terms of simplicity, rapidity, portability and costs affordability and, therefore, can be easily transferred to the productive sector.

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Chapter 7

Nucleic Acid Based Electrochemical Biosensors for Multiplexed Investigation of Bioagents

Ilaria Palchetti and Marco Mascini

Abstract The need for rapid, reliable, specific, and sensitive methods of detecting pathogens, at low cost, is the focus of a great deal of research. To meet expectations of users, analytical methods for pathogen detection must have the specificity to distinguish between different bacteria, the adaptability to detect different analytes, and the sensitivity to detect bacteria on-line and directly in real samples without preenrichment. The device must also be simple and inexpensive to design and manufacture. Multiplexing, or simultaneous detection of multiple analytes, is another important prerequisites for pathogen detection. (Bio)sensor technology is claimed to satisfy these requirements. In recent years various kinds of electrochemical biosensors based on identification of the bacterial nucleic acid have been developed. The development of DNA-based biosensors for the detection of specific nucleic acid sequences consists in the immobilization, onto the surface of a chosen transducer, of an oligonucleotide with a specific base sequence called capture probe. The complementary sequence (target) present in the sample solution is recognized and captured by the probe through the hybridization reaction. The evaluation of the extent of the hybridization allows one to conclude whether the sample solution contains the complementary sequence of the probe or not. Electrochemical transducers have received considerable attention in connection with the detection of DNA hybridization. This paper will give an overview of two different multiplexed electrochemical approaches of pathogen detection using nucleic acid developed in author's lab, and based on screen-printed electrode array and on scanning electrochemical microscope respectively.

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Keywords Pathogen detection • DNA-based biosensors • Nucleic acid sequences • Screen-printed electrode array • Scanning electrochemical microscopy

7.1 Introduction

Natural outbreaks or the use of pathogenic organisms for acts of terror have had tremendous impact on human populations. Improving security depends on the ability to detect, identify and trace pathogens. The need for rapid, reliable, specific and sensitive method of detecting a target pathogen, at low cost, is the focus of a great deal of research.

As underlined by the huge amount of scientific literature on this field, there is an increased demand for reliable analytical method for the detection of pathogens and toxins [1–8]. Lazcka et al. [9], recently, give an overview of the literature of the field of pathogen detection. Among the 2005 scientific paper reported in literature over the last 20 years in the area of pathogen detection, 38% are related to food industry, 18% to clinical diagnosis, 16% to environmental monitoring, 26% to miscellaneous applications, 1% to defense purposes.

Most current microbiology methods rely on culturing following by biochemical metabolic identification of the pathogen or molecular techniques based on deoxyribonucleic acid (DNA) identification such as polymerase chain reaction (PCR), pulse gel electrophoresis, or DNA microarray. Immunological methods such as enzyme-linked immunosorbent assay (ELISA) and Western Blot are used for microbial toxin detection. Although robust and sensitive, these methods are slow (it may make more than a day to get results), labor-intensive, and, with some exceptions (ELISA and DNA microarray) difficult to automate and are not high-throughput methods.

Thus, the traditional detection technologies are often too slow and cannot provide effective real time monitoring.

To meet expectations of users, analytical methods for pathogen detection must have the specificity to distinguish between different bacteria, the adaptability to detect different analytes, the sensitivity to detect bacteria on-line and directly in real samples without pre-enrichment [3]. The device must also be simple and inexpensive to design and manufacture. Moreover, multiplexing, or simultaneous detection of multiple analytes, is another one of the most important prerequisites for bioagent detection. Biosensor technology is claimed to satisfy these requirements [3]. Biosensor technology, which has evolved rapidly in recent years, combines a biochemical recognition element with a physical transducer and has the potential to overcome some of the limitations of current technology. Although all biosensors are similar in that they comprise a biochemical recognition element and a physical transducer, numerous design variations in these two elements lead to a wide array of distinct biosensor designs. Biosensor technologies have the potential specificity and sensitivity, and enable high-throughput analysis [10–13].

Nevertheless, in the field of biosensors for pathogen detection, there is still the lack of portable, integrated systems. Electrochemical multiplexed biosensor systems,

which would also be sensitive, accurate, simpler and cheaper than other existing systems would be of great help in this kind of tasks. Speaking of electrochemical biosensors for pathogen detection, immediately we think at two possible formats: antibodies based system and nucleic acid based systems for evaluation of the hybridisation reaction. This chapter will deal on nucleic acid-based biosensor or genosensor. Genosensors have been extensively exploited in the detection of pathogenic microorganisms relevant to food, biodefense and environmental contamination applications. Mainly, genosensors have been coupled to PCR or NASBA as specific detection method of the amplified base sequence. Amplification of the DNA target is necessary due to the low abundance and extreme complexity of the non amplified targets. Some authors reported also the analysis of 16S rRNA [14, 15].

In this chapter we reported on the use of nucleic acid to detect pathogen, or better PCR amplicons of specific gene sequence of the pathogen, but having in mind the possibility of perform a multiplexed analysis. The method proposed were based on screen-printed low density array of electrodes [13] and SECM Imaging [16] and were recently optimized in the author's lab.

7.2 Disposable Electrochemical Genosensor for the Simultaneous Detection of Different Bacterial Contaminants

In our recent paper [13] we described the simultaneous detection of different food pathogenic bacteria by means of a disposable electrochemical low density genosensor array. The analytical method relied on the use of screen-printed arrays of gold electrodes, modified using thiol-tethered oligonucleotide probes (Fig. 7.1). The samples identifying the bacteria of interest were obtained from the corresponding genomic DNAs through PCR amplification. These unmodified PCR products were captured at the electrode interface via sandwich hybridisation with surface-tethered probes and biotinylated signalling probes. The resulting biotinylated hybrids were coupled with a streptavidin-alkaline phosphatase conjugate and then exposed to a α -naphthyl phosphate solution. Differential pulse voltammetry was finally used to detect the α -naphthol signal (Fig. 7.2).

The sequences of synthetic oligonucleotide capture probes and targets, as well as PCR primers and PCR amplicons are listed in Table 7.1.

The analytical strategy was based on the identification of toxins produced by the bacteria of interest. Thus, for each bacteria strain, the capture and signalling probes were selected within the sequence of a gene encoding a strain-specific toxin. This step was the most important in order to have a strain-specific assay. The genes were chosen considering the frequency of the presence of the expressed toxins in contaminated foods and the virulence of the different bacterial strains. As *Salmonella enterica* is the most important cause of salmonellosis infections [17], the gene coding for the invasion protein A (invA) was considered in the case of this bacteria. The gene of the enterlysin A (inlA) was used to design specific probe for *Lysteria*

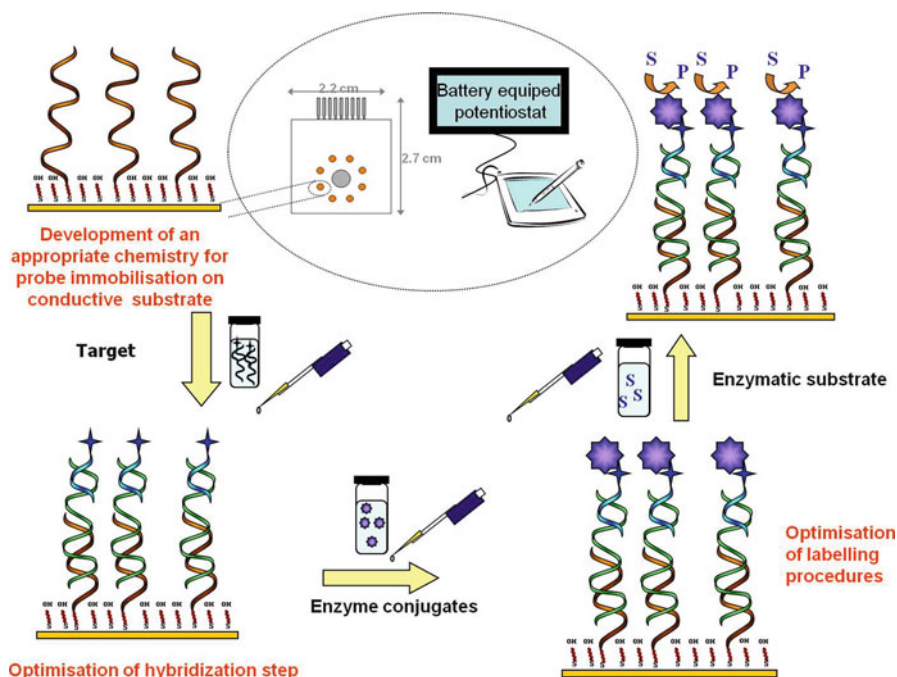


Fig. 7.1 Scheme of the detection method based on a gold screen-printed electrochemical transducer

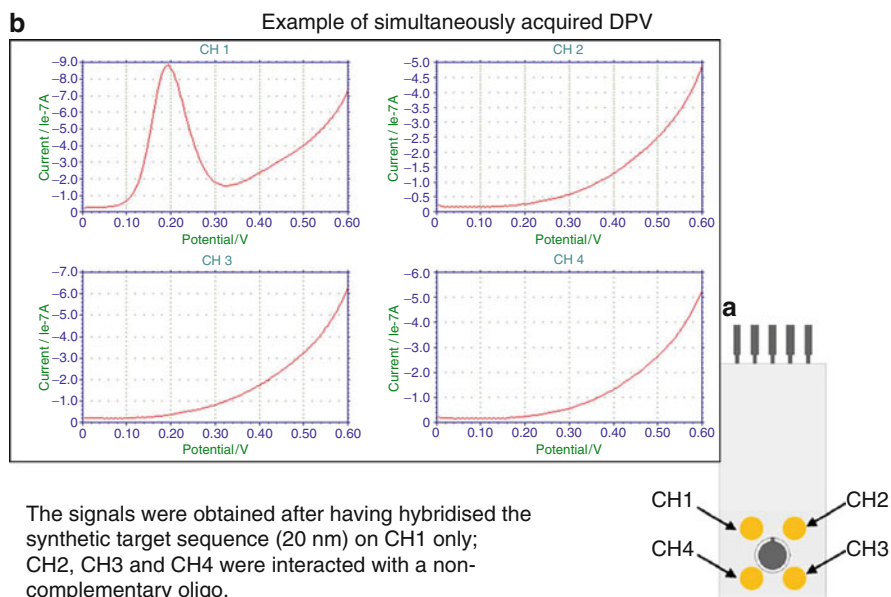


Fig. 7.2 (a) Scheme of the screen-printed array of electrodes comprising four gold working electrodes and a silver pseudo-reference electrode (in the *centre*). (b) Example of simultaneously acquired DPV signals, obtained after having hybridised the synthetic target sequence (20 nM) on CH1 only; CH2, CH3 and CH4 were interacted with a non-complementary oligo (Reprinted with permission from Ref. [13])

Table 7.1 PCR Amplicons and synthetic oligonucleotide probes and targets***Salmonella* spp.:** PCR amplified fragment of the InvA gene (388 bp)

5'-GCCGCGCGCGA**ACGGCGAAG**CGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCC
 TTTGACGGTGCGATGAAGTTTATCAAAGGTGAC**CGCTATTGCCGGC**ATCATTATTAT
 CTTTGTGAACTTATTGGCGGTATTTTCGGTGGGGATGACTCGCCATGGTATGGATT
 GTCCTCCGCCCTGTCTACTTATACCATGCTGACCATTGGTGATGGTCTTGTGCGCCCA
 GATCCCCGCATTGTTGATTGCGATTAGTGCCGGTTTTATCGTGACCCGCGTAAATGG
 CGATAGCGATAATATGGGGCGGAATATCATGACGCAGCTGTTGAACAACCCATTGT
 ATTGGTTGTTACGGCTATTTTGACCATTCAATGGGA**ACTCTGCCGGGAT**-3'

Capture probes (12-mer):

CP0: 5' - HS-(CH₂)₆ - GCG CGC GAA CGG - 3'CP1: 5' - HS-(CH₂)₆ - GGG AAA GCC AGC - 3'CP2: 5' - HS-(CH₂)₆ - GCT ATT GCC GGC - 3'

Signaling probe: 5' - TTT GTG AAC TTT ATT GGC GG -TEG -biotin - 3'

Synthetic target: 5' - CCG CCA ATA AAG TTC ACA AAACG CCG TTC GCG CGC - 3'

Lysteria monocytogenes: PCR amplified fragment of the InlA gene (255 bp)

5'-**CCTAGCAGGTCTA**ACCGCACTCACTAACTTAGAGCTAAATGAAAATCAGTTAGAAG
 ATATTAGCCCCAATTTCTAACCTGAAAAATCTCACATATTTAACGTTGTACTTT
 AATAATATAAGTGATATAAGCCCAGTTTCTAGTTTAACAAAGCTTCAA
 AGATTATTTTTCTATAATAACAAGGTAAGTGACGTAAGCTCGCTT
 GCGAATTTAACCAATATTAATTGGCTTTTCGGCTGGGCATA**ACCAAATTAGCGA** -3'

Capture probe (12 mer): 5' - HS-(CH₂)₆ - CCT AGC AGG TCT - 3'

Signaling probe: 5' - CGC TTG CGA ATT TAA CCA AT -TEG -biotin - 3'

Synthetic target: 5' - ATT GGT TAA ATT CGC AAG CGG TTA GAC CTG CTA GG - 3'

Staphylococcus aureusCapture probe (12 mer): 5' - HS-(CH₂)₆ - CAA TGT GCG GGT - 3'

Signaling probe: 5' - CGA TCA ATT TAT GGC TAG AC -TEG -biotin - 3'

Synthetic target: 5' - GTC TAG CCA TAA ATT GAT CGT TGA CCC GCA CAT TG - 3'

***E. Coli* O157:H7**Capture probe (12 mer): 5' - HS-(CH₂)₆ -CGC GAA CAG TTC - 3'

Signaling probe: 5' - ATC AAG CAT GCC TGA TA -TEG -biotin - 3'

Synthetic target: 5' - TAT CAG GCA TGG CTC TTG ATA ACG AAC TGT TCC GG - 3'

Within each amplicon the primers used for PCR amplification are underlined; the capture probes are indicated in bold-italics

monocytogenes [18], while the gene of the enterotoxin A was used for *Staphylococcus aureus* detection [19]. For *E. coli* O157:H7, the probe was designed from the gene codifying for Hemolysin A (hlyA) [20].

Since the same base sequence can be present in different genes [17], a detailed study on the specificity of the chosen base sequences was done, in order to minimise the number of false positive results. In the case of *Salmonella* and *Lysteria*, the specificity of PCR primers was controlled by using the BLAST (Basic Local Alignment Search Tool) [<http://www.ncbi.nlm.nih.gov/blast>] program. The sequences were aligned by means of MultAlin software [<http://prodes.toulouse.inra.fr/multalin/multalin.html>]. Sequences of highly specific regions were selected to design toxin-specific primers. Toxin-specific oligonucleotide probes were

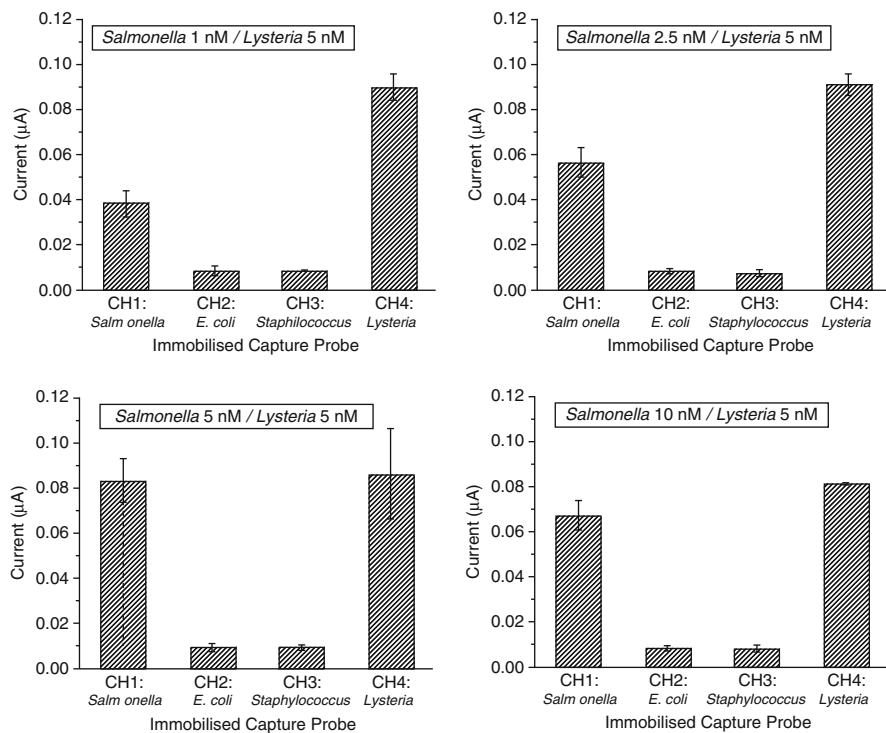


Fig. 7.3 Simultaneous analysis of diluted *Salmonella* and *Listeria* amplicons mixed in different ratios (1:5, 1:2, 1:1, 2:1). Each sample was analysed in triplicate. The error bars represent the standard deviation ($n=3$), (Reprinted with permission from Ref. [13])

designed within the region flanked by the primers. *E. coli* 0157:H7 and *Staphylococcus aureus* synthetic probes and targets were chosen accordingly to literature [20, 21].

Simultaneous detection of different samples was achieved analysing solutions of both *Salmonella* and *Listeria* amplicons mixed in different ratios (Fig. 7.3). As expected, the analytical signals were observed only at the positions modified with the corresponding capture probe, demonstrating the feasibility of the simultaneous detection of different analytes. The non specific signal observed at the WEs modified with *Staphylococcus* and *E. Coli* probes was comparably negligible, thus highlighting the selectivity of the procedure. Control experiments demonstrated that this undesired signal was essentially due to the direct non-specific adsorption of streptavidin-alkaline phosphatase conjugate onto the electrode surface, thus excluding cross-hybridisation phenomena. The dependence of the analytical signal on the concentration of the amplicon from *Salmonella* was also shown in Fig. 7.3. The electrochemical response rose with the sample concentration up to 5 nM and then is levelled off. Interestingly, the signal of 5 nM *Listeria* amplicon appeared to be unaffected by the presence of different amounts of the other samples, also demonstrating the absence of any cross-interference.

7.3 Electrochemical Imaging of Localized Sandwich DNA Hybridization Using Scanning Electrochemical Microscopy

Scanning Electrochemical Microscope (SECM), has demonstrated a high level of performance for studying immobilized biomolecules and biological processes [22], and some approaches in the use of SECM as read-outs of hybridization in DNA (micro)array were reported in literature [16].

Recently, we propose a SECM-based DNA array using a sandwich hybridization assay scheme with biotinylated signaling probe [16]. The analytical method relied on the use of gold conducting surface on which thiol-tethered ODN probes were spotted. Control over surface coverage and probe availability was achieved by creating mixed monolayers of the thiol-modified ODN probe and a spacer thiol, mercaptohexanol (MCH), by a two-step method, where first the gold substrate was exposed to a micromolar solution of HS-ssDNA, followed by exposure to a millimolar solution of MCH. Then, unmodified targets were captured at the sensor interface via sandwich hybridization with surface-tethered probes and biotinylated signaling probe. The resulting biotinylated hybrids were coupled with a streptavidin-alkaline phosphatase conjugate and then exposed to a proper substrate solution. SECM investigation of the hybridization process was performed in feedback-mode, after coupling of a streptavidin-alkaline phosphatase conjugate and biocatalysed precipitation of an insoluble and insulating product. Localized change in conductivity was sensitively detected by SECM tip and allowed high-quality imaging of DNA arrays. The proposed probe immobilization and hybrid labeling procedures were never used for hybridization detection using SECM. Thus, each step of the molecular assembly was characterized by SECM using ferrocene derivatives as neutral mediator. The effects of target concentration and of non-specific interactions were also studied.

7.3.1 Surface Development for SECM Imaging of *Lysteria*

SECM analysis of PCR samples were performed with 5' - HS-(CH₂)₆ - CCT AGC AGG TCT - 3' as CP and 5' - CGC TTG CGA ATT TAA CCA AT -TEG - biotin - 3' as signaling probe. The thiolated CP solution (1 μ M in 0.5 M sodium phosphate buffer, pH 7.0) was deposited by means of a nanodispenser (BJQ3000 BIODOT, UK) onto bare gold surface. The CPs were arrayed in spots in three rows of 10 nL at a distance of 1 mm or 1.5 mm center to center. Chemisorption was allowed to proceed overnight (16 h) with the chip stored in Petri dishes to protect the solutions from evaporation. DNA chips were post-assembled with MHC (1 mM in deionised water, 30 min, and at room temperature). A volume of 40 μ L was deposited onto the surface. The chips were then washed twice with 40 μ L of 0.5 M phosphate buffer, pH 7.0. Hybridization was performed in a Petri dish at ambient temperature by

exposing the CP spots for 30 min to 40 μL of a 0.5 M sodium phosphate buffer containing 0.15 μM of a biotinylated signaling probe and a proper concentration of target sequence. After hybridization, the chips were washed twice with 40 μL of DEA buffer, to remove non-specifically adsorbed sequences. After the hybridization step, the chip was reacted with 40 μL of a solution containing 0.8 U/mL of the streptavidin-alkaline phosphatase conjugate and 10 mg/mL of BSA (blocking agent) in DEA buffer. After 20 min, the chips were washed twice with 40 μL of DEA buffer. The enzyme modified chips were then incubated with 40 μL BCIP/NBT for 20 min. After precipitation of the insoluble and insulating product the chips were washed with 40 μL of 0.1 M phosphate buffer, NaCl 0.1 M pH 7.0.

7.3.2 SECM Imaging of *Lysteria Monocytogenes*

The scheme of the assay is reported in Fig. 7.4. The selectivity is guaranteed by the CP; whereas, as reported in the use of a signaling probe, preannealed to target DNA to form partially duplex target molecules, allows the labeling of the target and the prevention of secondary structure that can interfere with hybridization to the surface-tethered CP. In the proposed scheme the biotinylated hybrid was labeled with the streptavidin-alkaline phosphatase conjugate. After this molecular assembly the chip was incubated with the enzymatic substrate. The electrochemical transduction of the hybridization process was performed by means of feedback mode of SECM, taking advantage of the biocatalysed precipitation of an insulating indigo derivative which blocks the regeneration of the mediator at the substrate surface.

The effect of target concentration was examined in the range 40 pM to 400 nM. When the target concentration was high (≥ 40 nM) the approach curve becomes congruent with the theoretical curve over an insulating substrate. Thus SECM will not detect amounts greater than 40 nM because the deposition of more insulating substrate will not further decrease SECM current. On the other hand, target concentration lower than 4 nM alter the SECM approach curves from the behavior over insulating surfaces to that over surfaces with behaviors between completely conductive and insulating situations or to that over conductive surfaces. For this reason, further experiments were performed using 40 nM target. In imaging experiments, the SECM tip is scanned in constant height above the gold substrate after ODN hybridization and molecular assembly, while simultaneously monitoring the tip current as a function of the x/y tip position. The dependence of the microelectrode response on the tip to substrate distance and on the nature of the substrate allows revealing spatially resolved information about chemical properties and the morphology of the surface, with the imaging of array of spots of 2.5×2.0 mm of the same probe after hybridization with target oligonucleotides. High quality 3D SECM images of CP-target hybridized spots were reported in Fig. 7.5. The current values to the left and right of the spot are indicative for the positive feedback from the MCH modified gold surface. Drastically lowered current values above the spots signify reduced rates of redox recycling. The insulating precipitate is formed with

high precision around the spot, confirming the blocking of the gold surface to non-specific binding of biomolecules during hybridization and enzyme labeling from the MCH layer. As well discussed in literature the post-treatment with MCH displaced non-specifically adsorbed HS-ssDNA molecules, that interacted with the gold surface also or only through nitrogen-containing bases, thus dramatically increasing the availability of the surface-bound probes for specific hybridization. After treatment with MCH, probe molecules “stand up” consistently with the primary attachment through the thiol group becoming accessible for specific hybridization. Moreover, Fig. 7.5 clearly demonstrated that the MCH layer avoids non-specific adsorption of undesired biomolecules.

7.4 Conclusions

The results reported in this chapter demonstrate that multiplexed analysis of pathogen can be performed in different manners. Using the screen-printed platform, simultaneous detection of up to four different analytes is feasible in about 1 h. The analysis of non-specific amplicons and mixtures of different complementary sequences (after the modification of each addressable electrode within an array with a different capture probe) confirmed the selectivity of the proposed procedure and the absence of any cross-hybridisation. Moreover, imaging of localized DNA hybridization has also been demonstrated. The method relies on the inherent sensitivity of SECM for the detection of the small variation of surface conductivities. Target concentrations at nM level can be easily imaged. This concentration level is sufficient for most applications involving gene expression and sequence analysis. Moreover the reliability of the method is also demonstrated, with the low non-specific signal. Thus a highly sequence specific (single base mismatch) DNA analysis can be accomplished. Although the concept of using this SECM based method to image DNA array is demonstrated only with a low density chip, miniaturizing the assay by increasing the performance of the spotting system, visualization of a medium to high density DNA array should be possible. Moreover, immobilization of different CPs should be possible and thus multiplex analysis should be carried out.

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Chapter 8

Biosensing Systems Based on Metal Oxides Nanoparticles and Choline Oxidase for Environmental and Biomedical Monitoring of Neurotoxicants

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Abstract In this study we investigated the effects of monovalent anions in the series F^- , Cl^- , Br^- , I^- on the sensitivity of the biosensor assembled from polycations and ChO via their layer-by-layer deposition on the surface of carbon electrodes (CE) coated with MnO_2 . The efficiency of gamma-phase- MnO_2 nanoparticles, deposited on screen-printed electrodes, as a new transducer element of biosensing platforms, fabricated with “layer-by-layer” technology was demonstrated. The present report describes analytical possibilities of the biosensing platform. The hydrogen peroxide registration and subsequent application for neurotoxicants assay in the environmental field. The validation of peroxide-sensitive sensors, as a new transducer element based on MnO_2 nanoparticles, for fabrication of choline oxidase biosensor has been done. The revealed analytical characteristics of the choline oxidase biosensor are perspective for highly-sensitive monitoring of cholinesterases and their inhibitors in medical, biological and environmental samples.

Keywords Choline biosensor • Layer-by-layer assembly • Manganese dioxide nanoparticles mediating layer • Screen-printed carbon electrode • Cholinesterase inhibitor assay

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8.1 Introduction

Enzyme based amperometric biosensors are traditionally considered for a reliable, rapid, compact and sensitive detection of physiologically active compounds in aqueous medium. Special attention for choline biosensing is connected with the problem of cholinesterase inhibitors selective monitoring, as well as with prompt analysis of blood esterase activities in surveillance of individuals, exposed to cholinergic and neuropathic organophosphorus compounds [1]. The biosensor detection of choline is based on its biocatalytic oxidation by choline oxidase, immobilized on the electrode surface generating hydrogen peroxide. The following registration of hydrogen peroxide can be achieved by direct, mediated and horseradish peroxidase containing electrodes [2]. The recent application of mediating layers in hydrogen peroxide sensing electrodes fabrication resulted in the highest hydrogen peroxide detection limits ranging from 4 to 60 nM, ever reached before [3]. A number of mediator based biosensors containing hydrogen peroxide sensitive layers have been described previously, for instance, metal-organic complexes of Ni^{2+} [4], Cu^{2+} [5], Fe^{3+} (ferrocene) [6] and Co^{2+} (phthalocyanine) [7], complexes of transition metals and polymers (Os^{3+}) [8], Prussian blue [9–12], oxides of cobalt [13, 14], manganese [3, 15–18] iridium [19] and vanadium [20]. In a vast majority of already reported devices, the electrochemical/chemical deposition was used for mediator layer fabrication [16, 21]. Moreover, the variety of methods for choline oxidase immobilization on a surface of electrochemical sensors, described previously, include covalent immobilization [22–24], incorporation into membrane pores [25] and electropolymerized films [26, 27], cross-linking immobilization in thick films [11] and, recently, layer-by-layer (LBL) method [21, 28, 29]. In general, the modified electrodes show improved electrocatalytic responses with reduced overpotentials and increased voltammetric signals allowing low detection limits and high sensitivities.

The biosensor sensitivity also depends on the amount of active enzyme layer which is ensured by its effective adsorption on the pre-adsorbed polyelectrolyte. The ability of the pre-adsorbed polyelectrolyte layer to interact with an enzyme is expected to depend upon the nature of the counterion in the presence of which the adsorption occurred. Indeed, the nature of counterion has already been reported as a significant parameter for LBL assembling of multilayer polyelectrolyte films. A clear Hofmeister trend in the growth process of polyelectrolyte multilayers was described by Salomaki et al. [30]. Their studies revealed exponential growth of multilayer film thickness vs. hydration entropy of counterions. Therefore, the hydration entropy is assumed to govern polyelectrolyte-counterion interactions and the degree of counterion-induced charge screening of a polyelectrolyte. Strong screening of polyelectrolyte charges (provided by counterions with low hydration entropy) [31] induces deposition of the polyelectrolyte in the form of associates rather than single molecules [30]. Thus, in the first part of this chapter the effects of monovalent anions in the series F^- , Cl^- , Br^- , I^- on the sensitivity of the biosensor assembled from polycations and ChO via their layer-by-layer deposition on the surface of CE coated with MnO_2 [32] will be described.

On the other hand, some of the proposed immobilization techniques for the deposition of hydrogen peroxide oxidation mediating layers and choline oxidase suffer from low reproducibility, rather time-consuming, poor enzyme activity, and insufficient spatially controlled deposition. Thus, the later led to the development of new technological approaches for implementation of electrochemical biosensors, focusing on immobilization technique of the main sensor components: enzymes and mediators. At present, automatic methods based on mechanical plotting of small liquid volumes, seems to be most promising for electrochemical sensors fabrications. One of them – is the ink-jet printing, which is accurate, simple for control and applicable for wide range of sensor surfaces [33]. Such printing method requires the development of special ink – a composite water-based material, containing the panel of desired nanosized components for immobilization. It was shown [17], that MnO_2 nanoparticles has special physical and chemical properties, different from common MnO_2 powders, providing significant improvement of mediating properties on the one hand, and simplifying for the stable water mixtures on the other hand. Thus, the synthesis of the stable water suspension from MnO_2 nanoparticles is the essential step in the development of technology for high-throughput biosensor production.

A screen-printed amperometric sensor based on aqueous gamma-phase MnO_2 ‘mediator ink’ as hydrogen peroxide oxidation mediating reagent and electrostatic assembly of a choline oxidase on interpolyelectrolyte nanofilm has been developed in our laboratory [34] and described in the second part of this chapter. The developed approach for biosensor manufacturing is technological and highly productive; at the same time, it enables to achieve an excellent sensitive measurement of choline.

8.2 The Effect of Halide Anions on the LBL Formation of Bilayer Structures Consisting of PDDA and Choline Oxidase

PDDA/ChO bilayer was deposited onto the surface of graphite electrode which was electrochemically modified with hydrogen peroxide-sensitive manganese dioxide beforehand. Biocatalytic activity and topological features of these films were studied using electrochemical measurements, atomic force microscopy (AFM), and scanning electron microscopy (SEM).

Analytical response was determined by recording the current of electrooxidation of $\text{Mn}^{2+/3+}$ to Mn^{4+} on sensor surface (Fig. 8.1).

Variation of anion concentration from 5 to 100 mM revealed that for F^- , Cl^- and Br^- the sensitivity remained constant. In contrast to that, four-fold increase in sensitivity was observed in the presence of 20 and 30 mM KI (Fig. 8.2).

The observed effect is associated with the increase in the maximum rate (V_{MAX} (control) = $1,530 \pm 200$ nA, V_{MAX} (KI) = $5,670 \pm 340$ nA) of the enzymatic process

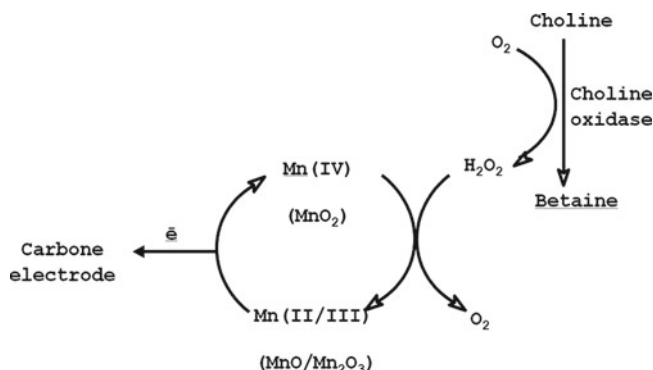


Fig. 8.1 The principle of amperometric measurement of biosensor sensitivity

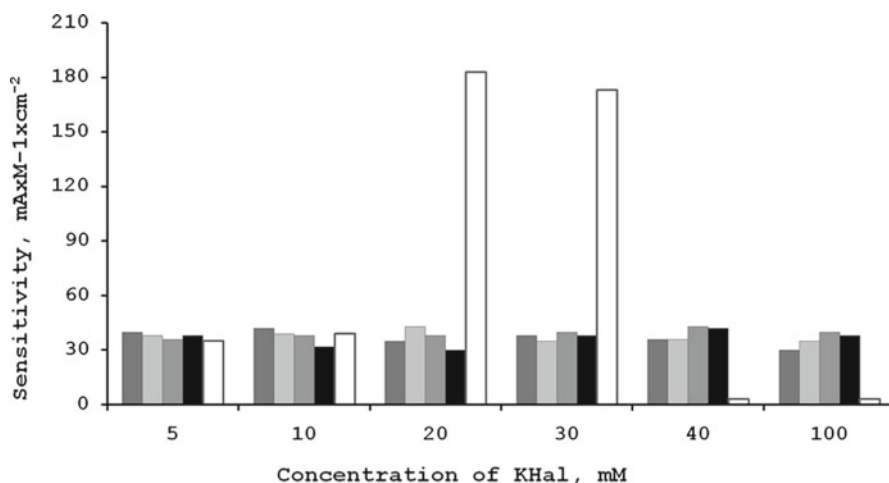
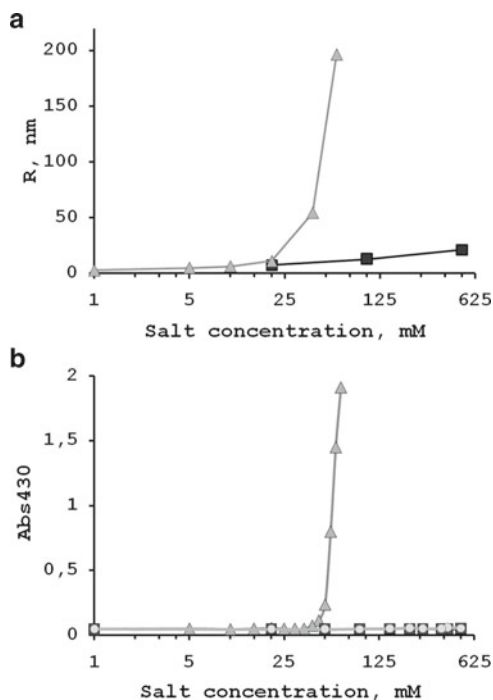


Fig. 8.2 Dependence of the biosensor sensitivity on potassium halide concentration in PDMA solution. (■) Control (without halide), (◐) KF, (▒) KCl, (■) KBr, (□) KI. Relative standard deviations for triplicate measurements were 30%

and does not affect the value of the Michaelis constant ($K_M(\text{control}) = 0.95 \pm 0.19$ mM, $K_M(\text{KI}) = 1.2 \pm 0.3$ mM) of the enzyme. The fact of Michaelis constant staying invariable indicates that the enzyme adsorption on the surface of the electrode does not affect the availability of the active center for the substrate. The observed effect of V_{MAX} growth could be caused by either the increase of catalytic constant (k_{cat}) or the increase of concentration of the enzyme active form on the surface of the electrode. Most probably, the V_{MAX} growth is due to the increase in the enzyme concentration.

Fig. 8.3 (a) Hydrodynamic radii of PDPA particles in the presence of increasing concentrations of (▲) KI and (■) KCl. (b) Turbidimetric titration curves of PDPA solution by (▲) KI, (■), KCl, and (○) KBr



Apparently, the effect is in connection with the characteristic features of the polymer adsorption in the presence of the mentioned KI concentration.

Dynamic light scattering and turbidimetric titration indicate a substantially different behavior of PDPA macromolecules in solution in the presence of iodide anions in comparison with other anions. Figure 8.3a shows the hydrodynamic radii of PDPA particles plotted against concentration of potassium halides. As follows from the plot, mean hydrodynamic radius of the particles in KCl solution is approximately 10 nm and weakly changes in the range of KCl concentrations from 20 to 500 mM. At the same time, sharp increase in the hydrodynamic radius of PDPA particles is observed from about 20 mM KI. Moreover, according to the data of turbidimetric titration (Fig. 8.3b), salting-out of PDPA occurred at the concentration of KI above ~70 mM, whereas PDPA solutions remained transparent at least in the whole range of KCl/KBr concentrations up to 500 mM.

Thus, in the presence of F^- , Cl^- and Br^- in the experimental range of concentrations (0–500 mM) there was no significant change in the PDPA radius or the polymer solution turbidity. Only iodide anions (with the lowest value of hydration entropy in the series of interest [31]) promote the association of PDPA macromolecules in solution even at low concentrations. However, the monotonous increase in particle size with the increase in KI concentration correlates poorly with the dome-shape dependence of the ChO sensor sensitivity on the KI concentration.

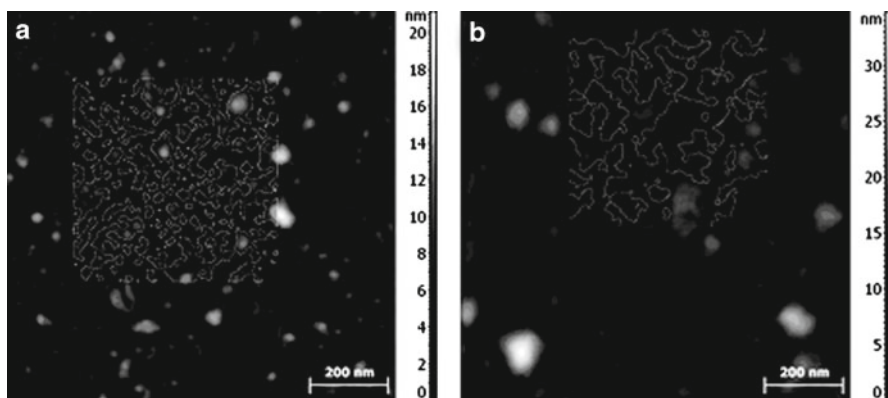


Fig. 8.4 AFM images of PDPA/ChO films on the surface of HOPG. Objects were resolved using cutting-plane method (see the *inset*). PDPA was deposited in the presence of (a) 20 mM KCl or (b) 20 mM KI. Image size is $1 \times 1 \mu\text{m}^2$

Apparently, the increase in the sensor sensitivity is to be ascribed to the characteristic features of the polymer adsorption under various conditions. According to AFM data the surface coverage in HOPG/PDPA_{KCl} and HOPG/PDPA_{KI} films is $33 \pm 6\%$ and $58 \pm 1\%$ respectively. Further ChO adsorption onto the produced surfaces resulted in the corresponding AFM images presented in Fig. 8.4.

It is worth noting that different patterns of ChO distribution were revealed for PDPA_{KCl}/ChO and PDPA_{KI}/ChO films deposited onto HOPG (Fig. 8.4). When PDPA adsorption takes place in the presence of KCl (Fig. 8.4a), the main structural element of PDPA_{KCl}/ChO film has the average lateral grain size of 50 nm (taking into account the convolution), which may correspond to individual molecules or small oligomers of the enzyme. On the contrary, when PDPA deposition is carried out in the presence of KI (Fig. 8.4b), it leads to the further integration of ChO into larger domains. Their structural details are indistinguishable using standard AFM probes. Objects extraction by the cutting-plane method for such a system yields a coherent object throughout the whole image area. For additional demonstration of different distribution of the oppositely charged particles binding sites on the surface of PDPA, gold nanoparticles (AuNP) were used. Negatively charged gold nanoparticles adsorbed on PDPA_{KHal} films deposited onto HOPG/MnO₂ surface instead of the enzyme behaved similarly. Figure 8.5 shows SEM images of PDPA/AuNP films simulating the biosensor surface. Uniform distribution of single AuNP nearly without aggregate formation was observed on the surface of PDPA pre-adsorbed in the presence of KCl (Fig. 8.5a). The formation of large aggregates of AuNP with unresolved internal structure was observed for the polycation deposited in the presence of KI (Fig. 8.5b). Thus, deposition of PDPA from solutions containing different halides induces formation of surfaces characterized by different ability to adsorb oppositely charged species.

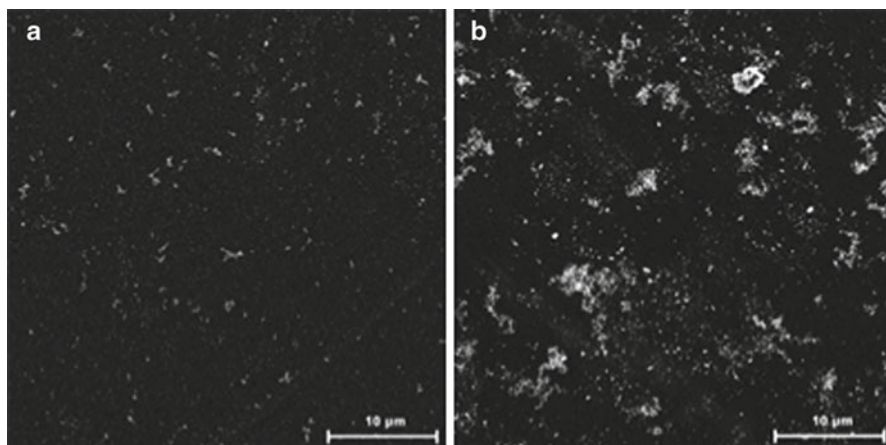
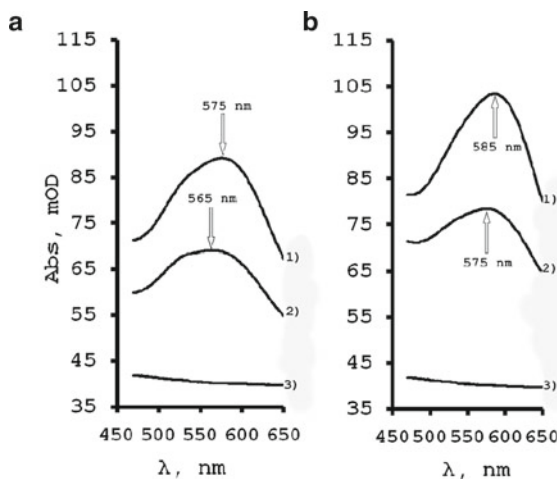


Fig. 8.5 SEM images of PDDA/AuNP films on the surface of HOPG/MnO₂; deposition of PDDA in the presence of (a) 20 mM KCl, (b) 20 mM KI. Image size is 40 × 40 μm²

Fig. 8.6 Absorption spectra of PDDA/AuNP films on the surface of (a) glass substrate, (b) polymethylmetacrylate substrate. (1) PDDA_{KI}/AuNP film, (2) PDDA_{KCl}/AuNP film, (3) substrate before AuNP adsorption



Absorption spectrometry of the metal nanoparticles is known to provide information on the particle size and aggregation [35]. Thus, we examined the absorption spectra of PDDA_{KCl}/AuNP and PDDA_{KI}/AuNP films prepared under the experimental conditions on the transparent surfaces of two kinds, namely, glass (Fig. 8.6a) and polymethylmetacrylate (Fig. 8.6b).

As a result, we can propose the following explanation for the assembling of oppositely charged species (namely, ChO molecules or AuNP) over the PDDA layer pre-adsorbed from KCl/KI solutions.

PDDA_{KCl}/ChO(or AuNP) – In the presence of chloride anions, PDDA stays in solution mostly in the form of individual molecules and its adsorption on the surface provides a homogeneous distribution of ionic groups (binding sites for the oppositely charged objects). Subsequent adsorption of the enzyme results in the formation of a dense film of the enzyme, characterized by a uniform distribution of small aggregates (Fig. 8.4a). The same situation occurs in the case of AuNP adsorption (Fig. 8.5a). Biosensor sensitivity for choline in this case is $35.3 \text{ mA} \times \text{M}^{-1} \times \text{cm}^{-2}$.

PDDA_{KI}/ChO(or AuNP) – Effective screening of PDDA charge by the iodide anion leads to association of polycation molecules. Under these conditions the polycation is adsorbed on the surface in the form of aggregates, thus providing an increased amount of ionic groups per unit of surface area exposed to the solution and available for interaction with the oppositely charged objects. This allows the ChO molecules (or AuNP) to be adsorbed with higher density forming large domains (Figs. 8.4b and 8.5b). Sensitivity of the biosensor in this case increases up to $183.3 \text{ mA} \times \text{M}^{-1} \times \text{cm}^{-2}$.

Thus, the effects of halides on the layer-by-layer deposition of PDDA and their influence on the subsequent assembly of the negatively charged species (molecules of choline oxidase or gold nanoparticles) have been demonstrated.

Deposition of PDDA from solutions containing iodide anion was found to form a surface allowing for a multifold increase in the $\text{MnO}_2/\text{PDDA}_{\text{KHal}}/\text{ChO}$ biosensor sensitivity. Exploitation of the observed effects provides for a simpler biosensor design and significantly improved sensitivity.

Here we report a $\text{PDDA}_{\text{KI}}/\text{ChO}$ biosensor twice as sensitive as the best choline oxidase biosensor described so far ($88.6 \text{ mA} \times \text{M}^{-1} \times \text{cm}^{-2}$) [36] fabricated *via* LBL deposition of ten layers of ChO/chitosan on a platinum electrode modified by Prussian Blue.

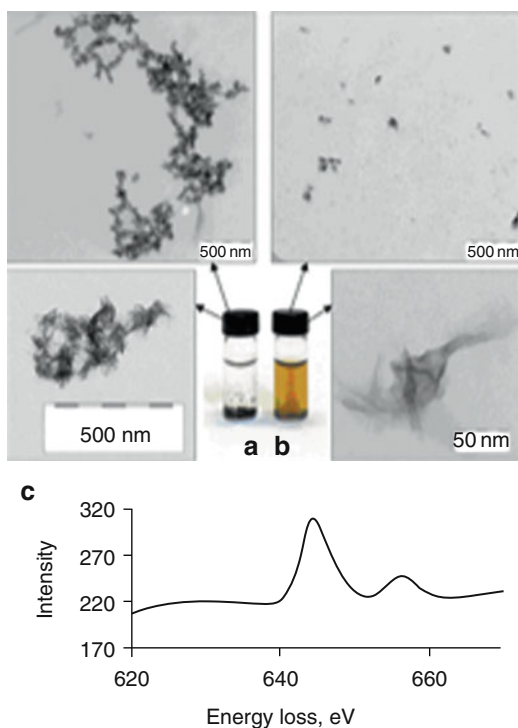
8.3 A New Transducer Element Based on MnO_2 Nanoparticles for Biosensing Platform

8.3.1 Synthesis and Characterization of MnO_2 Nanoparticles

Amorphous and beta-phase MnO_2 nanoparticles were prepared from 0.1 M solutions of KMnO_4 and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in a comproportionation reaction in a Teflon shell of an autoclave. The subsequent hydrothermal treatment (only for synthesis of beta-phase manganese dioxide) of the suspensions was performed in a 15-cm³ steel autoclave at 160°C during 2, 3 or 24 h. According to the X-ray diffraction data, samples obtained by hydrothermal synthesis were identified as amorphous MnO_2 , and as tetragonal MnO_2 modification of pyrolusite structure (beta- MnO_2).

For synthesis of gamma-phase MnO_2 nanoparticles a comproportionation reaction (using KMnO_4 as the oxidant and Mn^{2+} acetate as reducing agent) in aqueous

Fig. 8.7 Photographs and TEM images of different colloidal MnO_2 particles obtained in water according to comproportionation of potassium permanganate (0.250 mM) and (a) manganese (II) chloride (0.375 mM) (b) manganese (II) acetate (0.375 mM) resting for 14 days. (c) – electron energy loss spectrum for manganese dioxide sol solutions obtained in acetate



solutions at ambient temperature was applied. In dilute solutions the reaction leads to formation of manganese dioxide nanoparticles reddish brown dispersion. The stabilization of nanoparticles in the case of manganese acetate is provided by high degree of acetate-ion hydration in accordance with Hofmeister series [30]. Thus, in the presence of acetate charge screen on particle surface is lowered, protecting from aggregation. According to TEM data, most of chloride-based MnO_2 nanoparticles represent aggregates with dimensions from deciles of microns to several microns (Fig. 8.7a). MnO_2 nanoparticles based on acetate, represent wrinkled lamellar structures with estimated thickness 0,3–0,6 nm and characteristic dimensions 50–120 nm (Fig. 8.7b). Particles have polycrystalline nature; the crystalline sizes are about 2 nm. Electron energy loss spectrum was also obtained with TEM (Fig. 8.7c). The peaks observed at 645 and 655 eV corresponds to manganese (according to TEM database).

The crystal phase of the MnO_2 was analyzed by powder X-ray diffraction. The diffraction peaks can be indexed to orthorhombic symmetry and corresponding to gamma- MnO_2 (ICDD –JCPDS Card No. 72–1983). Spectrophotometrical data for hydrosol was also obtained (see Fig. 8.8). The maximum at 366 nm confirms, that hydrosol contains MnO_2 [37]. The hydrodynamic radius of manganese dioxide

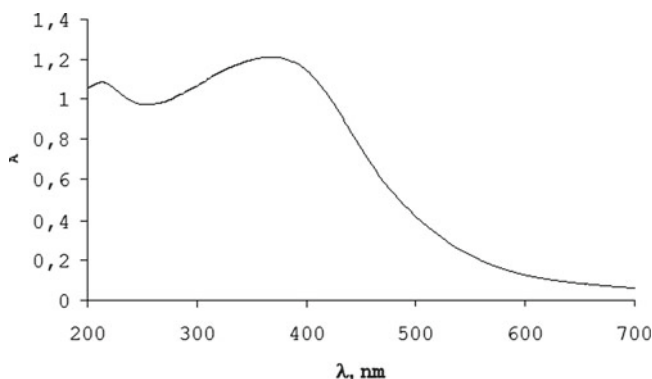


Fig. 8.8 Absorption spectra of MnO_2 sol solution

nanoparticles, estimated via light scattering was 64 ± 1 nm. The measurement of ζ -potential for manganese dioxide nanoparticles in hydrosol obtained using manganese acetate estimated -30 mV.

SEM images of the manganese dioxide hydrosols dried on HOPG surface are presented in Fig. 8.9. Amorphous MnO_2 (Fig. 8.9a) represented particles close to spherical in shape, 200–300 nm in diameter while betta- MnO_2 (Fig. 8.9b–e) different from the formed one by the presence of nano-size filamentous crystals of varying from 100 nm (b) to 50 nm (c) and 25 nm (d) and 15 nm (e) in diameter respectively. Figure 8.9f presents SEM photographs of gamma- MnO_2 with characteristic “wrinkled” structures on the surface of HOPG.

8.3.2 MnO_2 -Modified SPE for Hydrogen Peroxide Detection

Possibilities of SPEs covered with different crystalline modifications of manganese dioxide for hydrogen peroxide detection have been studied. Voltammograms obtained in the presence of hydrogen peroxide showed higher levels of oxidation current in comparison to similar data obtained in Hepes buffer for all samples. Oxidation of hydrogen peroxide lead to reduction of MnO_2 and formation of Mn (II) and (III) oxides and subsequent oxidation of these oxides with reiterated generation of MnO_2 on the electrode surface [16, 18]. Amperometric responses to $10 \mu\text{M}$ H_2O_2 do not change at working potential range from 250 to 500 mV.

Comparison of amperometric responses for electrodes coated with different MnO_2 nanoparticles is given in Table 8.1.

These data show that the electrodes that were manufactured on the basis of gamma MnO_2 are more sensitive at the determination of hydrogen peroxide than the electrodes prepared using other crystalline modifications.

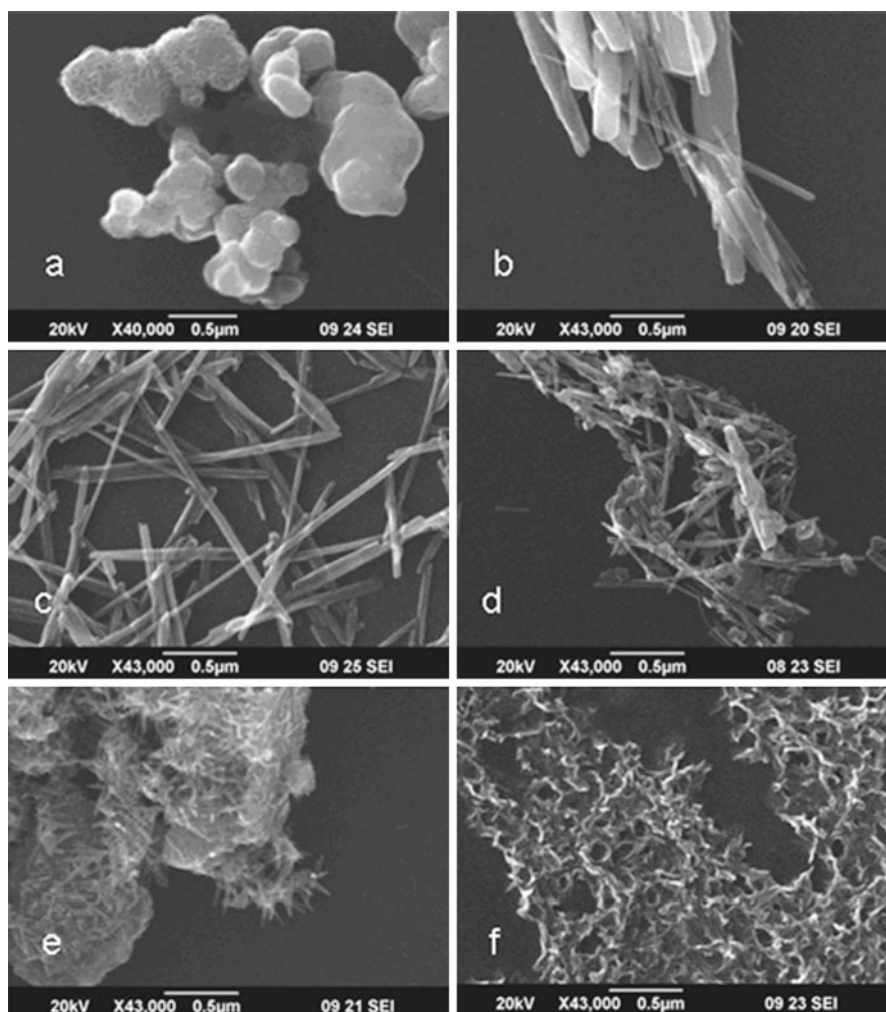


Fig. 8.9 SEM images for different crystalline modifications of MnO_2 nanoparticles. (a) *amorphous* MnO_2 , (b) *beta*- MnO_2 with average rod diameter – 100 nm, (c) *beta*- MnO_2 with average rod diameter – 50 nm, (d) *beta*- MnO_2 with average rod diameter – 25 nm, (e) *beta*- MnO_2 with average rod diameter – 15 nm, (f) *gamma*- MnO_2

8.3.3 Amperometric Choline Oxidase Based Biosensor

Choline oxidase was immobilized on the hydrogen peroxide sensitive electrode surface using “layer-by-layer” technology. Prior to enzyme immobilization, polycation (PDDA) was adsorbed on the surface of gamma-phase MnO_2 -modified graphite electrode and after then, choline oxidase was deposited to the same surface (Fig. 8.10a,b) from appropriate buffer solution. The general

Table 8.1 Amperometric responses to 100 nM hydrogen peroxide for electrodes based on different crystalline modifications of MnO_2

Type of MnO_2 nanoparticles	Electrode response, nA
Amorphous	100 ± 20
Beta-phase, rod diameter 100 nm	110 ± 22
Beta-phase, rod diameter 50 nm	115 ± 23
Beta-phase, rod diameter 25 nm	175 ± 35
Beta-phase, rod diameter 15 nm	210 ± 42
Gamma-phase	233 ± 36

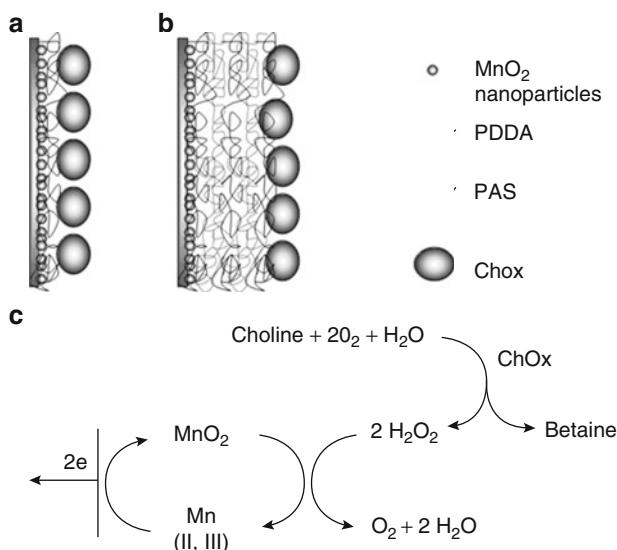


Fig. 8.10 Architectures of enzyme/polyelectrolyte layers of MnO_2 -based choline oxidase biosensor (a) PDPA/ChOx, (b) (PDPA/PAS)₂/PDPA/ChOx, (c) scheme of choline detection

scheme of choline detection by amperometric choline oxidase based biosensors is shown in Fig. 8.10c.

Initially, biosensor response at different concentrations of choline oxidase in solution for adsorption was evaluated (Table 8.2). The analytical response increases from 0.05 to 4.00 mg mL^{-1} of choline oxidase concentration in solution for adsorption. For validation of sensor fabrication reproducibility we have use R.S.D. % referred to data from five different sensor electrodes. It should be noted, that minimal R.S.D. (13–16%) was observed, when concentration of choline oxidase vary from 0.05–0.5 mg mL^{-1} . Thus, the selected optimum of enzyme concentration for effective biosensor functioning was 0.5 mg mL^{-1} .

Investigation of operational stability of the developed choline oxidase biosensors shows that there is a trend for decrease in analytical response from measurement to measurement.

Table 8.2 Dependence of analytical response (0.1 mM of choline) and reproducibility of MnO₂/PDDA/ChOx biosensor on choline oxidase concentration

ChO concentration, mg·ml ⁻¹	ΔI , nA	R.S.D. % of the response to choline for five different biosensors
0.05	58	16
0.10	98	13
0.50	239	13
1.00	276	24
2.00	318	29
4.00	331	37

Measurement conditions: 50 mM Hepes, pH 7.5, 30 mM KCl, 480 mV vs Ag/AgCl

In the selected fabrication scheme MnO₂/PDDA/ChOx, the decrement of sensor analytical response was $2.1 \pm 0.1\%$ per one measurement (R.S.D. was $7.0 \pm 0.3\%$ per ten measurements) at working potential 480 mV. The operation stability of MnO₂ layer was investigated at different hydrosol concentrations. Aiming the above reason, the concentrations of MnO₂ in hydrosol were chosen for linear (OD=0.17) and saturated (OD=1.5) response-concentration curve areas. The same sensor operation stability was estimated of named areas, i.e. the decrement of analytical response was estimated as $0.2 \pm 0.4\%$ (R.S.D.= $2.0 \pm 0.1\%$). Gave evidence, that hydrogen peroxide-sensitive layer remain intact. This indicating that observed decay in sensor response is connected with enzyme layer. Consequently, the improved stabilization of choline oxidase layers was carried out with two additional PDDA/PAS layer's variants (Fig. 8.10b). The decrement of analytical response in this case was $0.6 \pm 0.2\%$ (R.S.D. $1.9 \pm 0.4\%$) for MnO₂/(PDDA/PAS)₂/PDDA/ChOx electrodes, at working potential 480 mV.

The pH dependence values (in the range from 7.0 to 8.2.) of the electrochemical response for MnO₂/(PDDA/PAS)₂/PDDA/ChOx electrodes have shown the increase of response with increasing of pH. It is known, that pH-optimum of choline oxidase is about 8.0. However, the observed operation stability of choline oxidase biosensors was better at pH 7.5 (decrement of analytical response $0.6 \pm 0.2\%$ and R.S.D.= $1.9 \pm 0.4\%$), than at pH 8.2 (decrement of analytical response $3.2 \pm 0.0\%$ and R.S.D.= $11.1 \pm 0.4\%$). The biosensor response to 0.1 mM choline of the electrodes stored at 4°C and tested every week, kept 75% of initial activity for 3 weeks of storage.

The typical steady-state response of choline oxidase biosensor to 0.1 mM of choline is shown in Fig. 8.11a. The time needed to reach the 90% of the final response was about 10 s.

Figure 8.11b shows the dependence of choline oxidase biosensor sensitivity on number of PDDA/ChO layers. The electrode sensitivity increase at magnification of PDDA/ChO layers from 1 to 3, and reach upper limit.

Figure 8.11c, d illustrates the dependence of the MnO₂/(PDDA/PAS)₂/PDDA/ChOx sensors responses at the different concentration of choline. Obtained choline

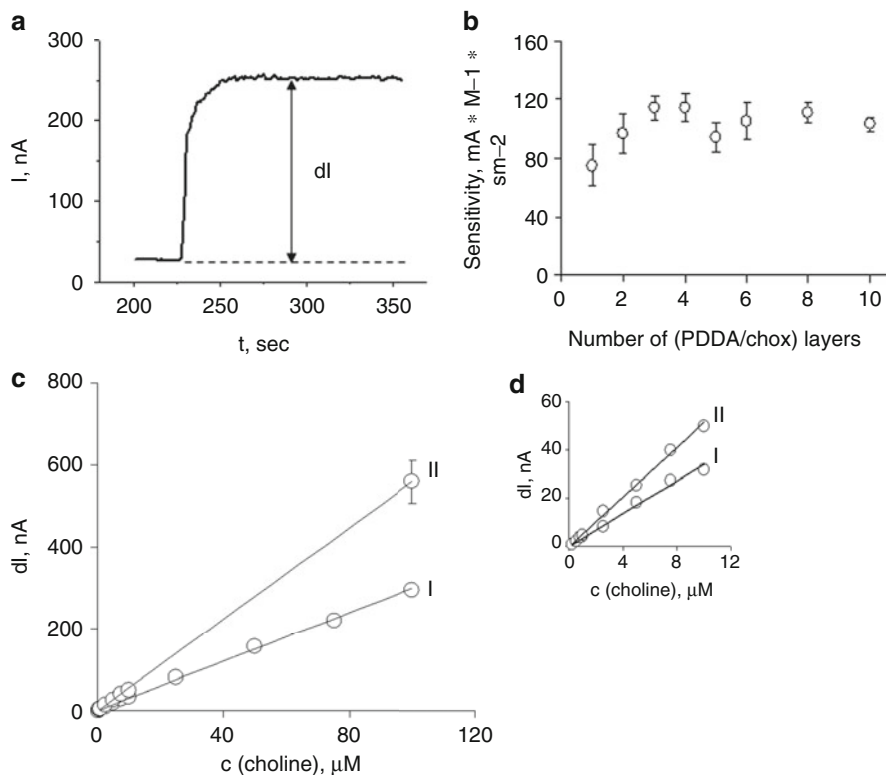


Fig. 8.11 (a) Analytical response to 0.1 mM of choline and (b) dependence of choline oxidase biosensor sensitivity on number of PDDA/ChO layers (c), (d) dependence of the $\text{MnO}_2/(\text{PDDA}/\text{PAS})_2/(\text{PDDA}/\text{ChOx})$ (line I) and $\text{MnO}_2/(\text{PDDA}/\text{PAS})_3/(\text{PDDA}/\text{ChOx})_3$ (line II) sensors responses at the different concentration of choline. Measurement conditions: 50 mM Hepes, 30 mM KCl, pH 7.5, 480 mV vs. Ag/AgCl

calibration curve showed a good linearity in a range between $3.0 \cdot 10^{-7}$ and $1.0 \cdot 10^{-4}$ M. The corresponding regression equation was: $y = 2.9 \cdot x + 3.5$, where y represents the current in nA and x the choline concentration in μM . The sensitivity $59 \pm 3 \text{ mA} \cdot \text{M}^{-1} \cdot \text{cm}^{-2}$ and the detection limit 300 nM (3σ) were calculated.

It should be noted that highest sensitivity of the developed biosensors was obtained, when three enzyme-containing layers were deposited on electrode surface: $\text{MnO}_2/(\text{PDDA}/\text{PAS})_2/(\text{PDDA}/\text{ChOx})_3$ (Fig. 8.11c,d).

In this case the detection limit was 130 nM (3σ) and sensitivity was $103 \pm 3 \text{ mA} \cdot \text{M}^{-1} \cdot \text{cm}^{-2}$. As to our concern, these analytical parameters are the best for amperometric choline oxidase – based biosensors.

An important factor for evaluating the analytical performance for medical and environmental applications of an electrochemical choline biosensor is the

Table 8.3 The effect of interfering compounds on analysis of choline. Electrodes: $\text{MnO}_2/(\text{PDDA}/\text{PAS})_2/\text{PDDA}/\text{ChOx}$

Components of biological liquids	Normal level in human blood serum [41]	Measured concentration	Response change, %
Ascorbic acid	50 μM	50 μM	394 ± 12
		5 μM	29 ± 3
		2 μM	13.7 ± 3.3
		0.5 μM	0.5 ± 4.5
Uric acid	500 μM	50 μM	129 ± 47
		5 μM	24 ± 11
		2 μM	-1.0 ± 2.4
		0.5 μM	15 ± 10
Ions of heavy metals	Maximum permissible concentration in fresh water		
Cd^{2+}	10 mg m^{-3} (90 nM)	1 mM	-40 ± 0
		0.1 mM	-19.5 ± 1.5
		10 μM	-4.5 ± 0.5
Co^{2+}	0.01 mg L^{-1} (170 nM)	1 mM	-26.5 ± 0.5
		0.1 mM	-10 ± 0
		10 μM	-5 ± 0
Cu^{2+}	2 mg L^{-1} (30 μM)	1 mM	-89 ± 4
		10 μM	-40.5 ± 1.5
		1 μM	-6 ± 2

Measurement conditions: 50 mM Hepes, pH 7.5, 30 mM KCl, 480 mV vs Ag/AgCl, choline concentration in the electrochemical cell 0.1 mM

interference of sampling “contaminating” compounds, easily oxidized at positive potentials. Therefore, these potential aberrations, introduced by interfering compounds, were additionally investigated, using couple of substances, commonly found in biological fluids (ascorbic and uric acids) and environmental objects (heavy metals like Cd^{2+} , Co^{2+} , Cu^{2+}). Two groups of samples were examined: “pure, non-contaminated” control samples, and those, spiked with an interfering compounds.

The summarized results are shown in Table 8.3. The interference for the normal physiological level of ascorbic acid (50 μM), increased the $\text{MnO}_2/(\text{PDDA}/\text{PAS})_2/\text{PDDA}/\text{ChOx}$ biosensor response to 394% and decreased to 0.5% at concentration 0.5 μM in the sample spiked with choline. Similar effect was found for uric acid (see Table 8.3). Thus, at least, 200-fold dilution of real blood samples is necessary to eliminate the interference of analogous concomitant compounds on studied electrode response.

Heavy metals (Cd^{2+} and Co^{2+}) are reversible inhibitors of choline oxidase [38].

The inhibition effect of Cd^{2+} and Co^{2+} ions is negligible at the concentrations, lower than 10 μM Cd^{2+} and 0.1 mM Co^{2+} , respectively (Table 3.3). Cu^{2+} does not show any influences on developed choline oxidase electrode function, in concentration $<1 \mu\text{M}$.

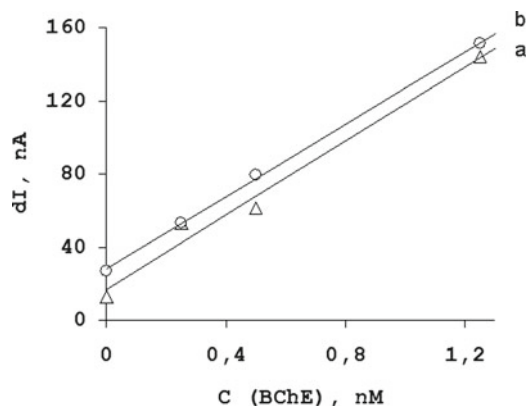


Fig. 8.12 Biosensor responses at different BChE concentration (a) in 50 mM Hepes, 30 mM KCl and 1 mg·mL⁻¹ BSA, pH 7.5; (b) in mouse blood sample diluted with Hepes buffer in 200 times after 30 min incubation with 0.01 M butyrylcholine. Measurement conditions: 50 mM Hepes, pH 7.5, 30 mM KCl, 480 mV vs Ag/AgCl. Slopes: (a) 101 ± 2 nA/nM, (b) 99 ± 1 nA/nM

8.3.4 Application of Choline Oxidase Based Biosensor for Environmental and Biomedical Monitoring

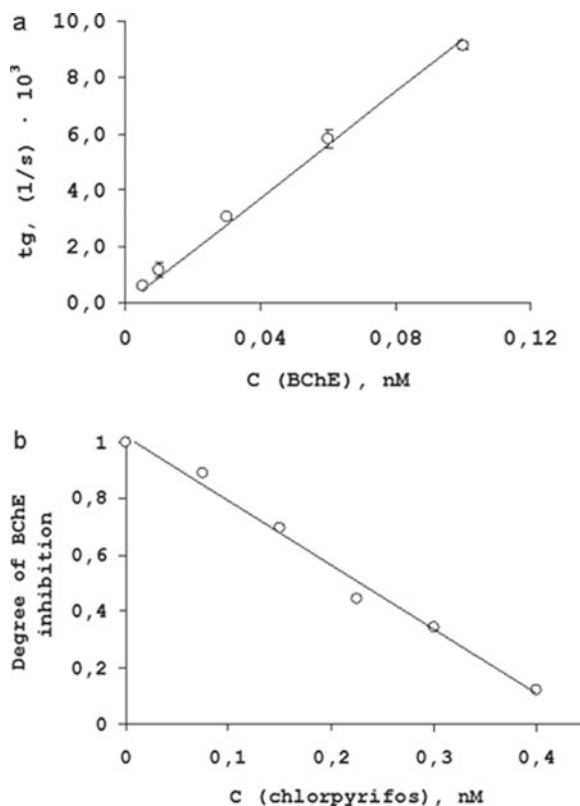
Two options of practical applications for the developed choline oxidase electrodes (MnO₂/(PDDA/PAS)₂/(PDDA/ChOx)₃) were demonstrated. Analysis of blood esterases in biological fluids have evident importance for monitoring of individuals, exposed to cholinergic and/or neuropathic organophosphorus compounds [39]. Figure 8.12 demonstrates the results of BChE detection in mice blood haemolysates (200-fold diluted and spiked with enzyme). The corresponding practically parallel straight lines presented in Fig. 8.12a,b), indicate the absence of matrix effect on biosensor signal. The ordinate shift of the dependence calibration line (see Fig. 8.12b), corresponds to intrinsic enzymatic activity in blood haemolysate samples.

The other important application of the named biosensor is the analysis of cholinesterase inhibitors for ecological monitoring and environmental surveillance [40].

In that case, it is essential to measure activity of cholinesterases before and after incubation with inhibitors. Figure 8.13a shows the calibration curve for BChE detection. Apparently, the biosensor response is linearly connected with enzyme concentration in the range 0.005–0.1 nM, and the detection limit for BChE, calculated according to the equation: $y = 2.35 \cdot x + 0.01$, (where y represents butyrylcholine enzymatic hydrolysis rate and x – the BChE concentration in nM), was estimated as $5 \cdot 10^{-12}$ M. This data demonstrates the evident capability of the developed biosensor to analyze the degree of BChE inhibition. Figure 8.13b presents the appropriate degree of BChE inhibition at different chlorpyrifos concentrations. The detection limit for chlorpyrifos was estimated as $5.0 \cdot 10^{-11}$ M.

Thus, the possibility for reliable application of the developed biosensor for detection of cholinesterases and their inhibitors in blood and environmental samples was demonstrated.

Fig. 8.13 (a) Biosensor responses at different BChE concentration measured by kinetic method. (b) Degree of BChE inhibition at different chlorpyrifos concentrations. Measurement conditions: Hepes 50 mM pH 7.5 buffer, containing 30 mM KCl 1 mg·mL⁻¹ BSA at working potential 480 mV (vs. Ag/AgCl reference electrode), 10 min incubation with inhibitor



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Chapter 9

Cyanobacterial Hepatotoxins Oxidation Mechanisms and Interaction with DNA

Ilanna C. Lopes, Paulina V.F. Santos, and Ana M. Oliveira-Brett

Abstract Cyanobacteria (blue-green algae) produce a variety of harmful compounds known as cyanotoxins. Microcystin-LR (MC-LR) and nodularin (NOD) are two potent cyanotoxins with strong hepatotoxic activity which pose a major risk to animals and humans, causing illness and death. Several methods for the detection and degradation of these toxins have been developed over the last years due to MC-LR and NOD strong toxic effects. An electrochemical investigation of MC-LR and NOD electrochemical behaviour, as well as their chemical degradation, is described and emerges as an innovation in the cyanotoxins detection field. The MC-LR and the NOD-DNA interactions were investigated using a DNA-electrochemical biosensor, adding more data to the studies of genotoxicity and carcinogenic potential associated to these toxins.

Keywords Cyanobacteria • Hepatotoxins • Microcystin-LR • Nodularin • dsDNA-electrochemical biosensor • Deoxyribonucleic acid damage • Electrochemical behaviour • Chemical degradation • Oxidation mechanisms

9.1 Introduction

Cyanobacteria (blue-green algae) produce a variety of harmful compounds known as cyanotoxins. Microcystin-LR (MC-LR) and nodularin (NOD) are two potent cyanotoxins with strong hepatotoxic activity which pose a major risk to animals and humans, causing illness and death.

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Due to MC-LR and NOD strong toxic effects, several methods for the detection and degradation of these toxins have been developed over the last years. However, none has focused on an electrochemical approach. In this context, the investigation of MC-LR and NOD electrochemical behaviours, which are described throughout this chapter, emerges as an innovation in the cyanotoxins detection field.

Furthermore, the MC-LR and the NOD-DNA interactions were investigated using a DNA-electrochemical biosensor, adding more data to the studies of genotoxicity and carcinogenic potential associated to these toxins.

9.2 Cyanotoxins

Cyanobacteria (formerly called blue-green algae) are Gram-negative autotrophic microorganisms found in many lakes, rivers and brackish waters throughout the world. Depending on climate conditions, these bacteria form water blooms and produce toxic compounds as secondary metabolites, known as cyanotoxins. These toxins have been implicated in illnesses and deaths [1], posing a major risk for animal and human health.

Among the 150 cyanobacteria genera, only 40 genera produce cyanotoxins and the most frequently reported are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nostoc* and *Oscillatoria* (*Planktothrix*) [2]. The cyanotoxins produced have different chemical structures, and therefore, different toxicities and different mechanisms of action. According to their chemical structure, the different cyanotoxins can be divided in three main groups: cyclic peptides, alkaloids and lipopolysaccharides. On the other hand, from a toxicological point of view, they can be divided into four groups: hepatotoxins, neurotoxins, cytotoxins and dermatotoxins (irritant toxins) [3].

9.2.1 Microcystins

Microcystins (MCs) are the cyanobacterial toxins most frequently found in freshwaters throughout the world, Fig. 9.1a. They were named after the cyanobacteria specie *Microcystis aeruginosa*, from which they were first isolated. Although there are several cyanobacteria genera capable of producing MCs, such as *Anabaena*, *Nostoc*, *Phormidium* and *Oscillatoria*, cyanobacteria which belong to the genus *Microcystis* remain the most frequent and widely distributed [1].

MCs are monocyclic heptapeptides with the chemical structure cyclo(D-alanine-R¹-D-MeAsp-R²-Adda-D-glutamate-Mdha), where R¹ and R² represent variable L-amino acids, D-MeAsp is D-erythro- β -methyl aspartic acid, Mdha is N-methyldehydroalanine, and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, Fig. 9.1a [4].

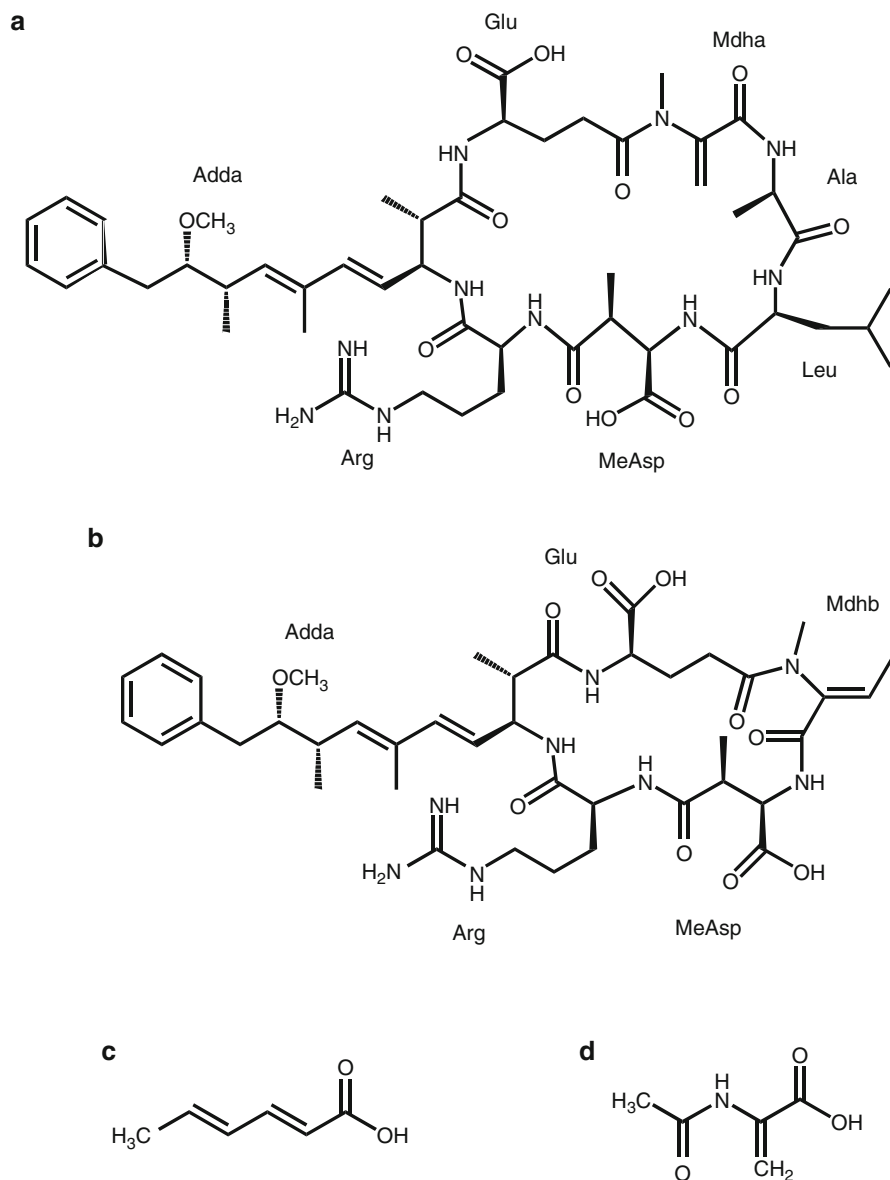


Fig. 9.1 Chemical structures: (a) microcystin-LR, (b) nodularin-R, (c) sorbic acid (SA) and (d) 2-acetamidoacrylic acid (2-AAA)

Among a group of more than 80 variants of MCs reported so far [5], microcystin-LR (MC-LR) stands out as the most frequent and the most toxic [6], in which the variable L-amino acid in position 2 is leucine (L) and in position 4 is arginine (R), Fig. 9.1a. The proper identification of each MC variant is achieved by adding the initials of the variable amino acids R¹ and R², like in the case of MC-LR.

9.2.2 *Nodularins*

Nodularins (NODs) are potent hepatotoxins produced by *Nodularia spumigena*, a cyanobacterial specie found in brackish waters (e.g. the Baltic Sea), like saline lakes and estuaries, throughout the world [7, 8].

NODs are monocyclic pentapeptides with a similar structure to MCs. The chemical structure of the most frequent variant of NODs is: Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), D-glutamate (D-Glu), *N*-methyldehydrobutyrine (Mdhb), D-erythro- β -methyaspartic acid (D-MeAsp), and L-arginine (L-Arg) as the variant amino acid [9], Fig. 9.1b.

The different forms of NODs vary not only by the variable amino acid, but also in the degree of methylation and stereochemistry [10]. Nevertheless, only nine different forms of this group of hepatotoxins have been documented to date [11].

Similarly to MCs variants nomenclature, the designation of NOD variants consists of adding the varying amino acid initial. For example, the NOD variant previously mentioned, which has arginine as its varying amino acid, is referred to as NOD-R, Fig. 9.1b. For simplicity, NOD-R will be mentioned as NOD throughout this chapter.

9.2.3 *Toxicity*

The problem of cyanotoxins toxicity is of increasing concern worldwide, as the incidence of harmful cyanobacteria blooms grows [11].

MCs toxicosis is known to affect animals and humans, causing illness and death [12–15]. Furthermore, the toxic effects of NOD have been reported since 1878, and are generally similar to those induced by MCs [1, 3].

MC-LR and NOD are considered as two potent cyanobacterial hepatotoxins owing to the fact that, in mammals, their primary target is the liver, where they may induce severe injury and even death.

MC-LR and NOD enter the hepatocytes through the bile acid multi-specific organic anion transporters located at the cellular membranes [16, 17]. However, it is thought that NOD penetrates these membranes more easily than MC-LR [18]. Once inside the liver cells, MC-LR and NOD inhibit the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A); as a consequence, a deregulation in protein

phosphorylation occurs, causing cytoskeleton disruption in hepatocytes, massive hepatic haemorrhage and, eventually, death [19].

The ability of MC-LR and NOD to inhibit PP1 and PP2A by binding covalently to them is attributed to the presence of the unusual Adda amino acid in the chemical structures of both toxins [20, 21], Fig. 9.1.

Lower doses of MC-LR inhibit PPs partially, leading to the phosphorylation of dynein, a mechanochemical protein responsible for intracellular vesicle movement along the microtubules. Therefore, dynein ATPase activity is inhibited and receptor mediated endocytosis cannot occur [22].

Beyond protein inhibition, other adverse toxicological effects have been reported concerning MC-LR and NOD exposure, such as intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation [23, 24].

Although MC-LR and NOD are known as hepatotoxins, there is also evidence that MC-LR might have cytotoxic effects on kidney cells, stretching the horizons of these toxins potential targets inside the organisms exposed to them [25].

9.2.4 Genotoxicity and Carcinogenic Potential

Beyond the main adverse toxicological effects commonly associated to MC-LR and NOD, these cyanotoxins have also been linked to genotoxic effects and might even be seen as carcinogenic agents under specific circumstances.

Tumour promotion [26] and induction of deoxyribonucleic acid (DNA) damage [27, 28] have been observed after exposure to MC-LR. On the other hand, NOD has been reported as a direct carcinogen in rat liver and not only as a tumour promoter like MC-LR. Thus, NOD is seen as a liver carcinogen possessing both initiating and tumour-promoting activities [29].

The induction of DNA damage by MC-LR and NOD has been observed *in vitro* and *in vivo* [27, 28]. Furthermore, the genotoxicity and the potential carcinogenicity of MC-LR and NOD have been extensively studied, also *in vitro* and *in vivo* [30]. However, some studies suggest that MC-LR and NOD genotoxicity and carcinogenicity arise mainly from the secondary effects of these toxins rather than direct toxin-DNA interactions [31–33].

The most frequent biomarker of DNA damage is 8-oxo-deoxyguanosine (8-oxoGua), which results from the addition of a hydroxyl radical to deoxyguanosine, causing misreading by DNA polymerase and G:C to T:A substitutions [34]. The detection of 8-oxoGua after exposure to MC-LR and NOD has been reported *in vitro* in cultured rat hepatocytes and *in vivo* in rat liver [35].

The DNA-electrochemical biosensor is a very good model for the evaluation of nucleic acid damage, taking advantage of the high sensitivity and selectivity of electrochemical detection and allowing the investigation of specific DNA-drug interactions [36], and has been applied for the study of cyanotoxins-DNA interaction.

9.2.5 *Cyanotoxins Detection and Degradation*

In general, both biological/biochemical and physicochemical methods are employed in the detection and identification of cyanobacterial hepatotoxins.

Among the biological/biochemical methods, the most common are mouse assay, enzyme linked immunosorbent assay (ELISA) and phosphatase assay. On the other hand, the most commonly used physicochemical methods are high performance liquid chromatography (HPLC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR) and mass spectrometry (MS) [20].

For the analysis and detection of MCs and NODs, the conventional method applied is the reverse-phase HPLC with UV detection at 238 nm [37]. Electrochemical approaches to the study of cyanobacterial toxins have been less frequently reported [38–40]. Nevertheless, electrochemistry is seen as one of the most sensitive detection techniques. Other advantages of the electrochemical methods consist in the possibility of electrodes and cells miniaturization, as well as in the equipment portability.

Due to MC-LR strong toxic effects, the World Health Organization (WHO) established a provisional guideline of $1 \mu\text{g L}^{-1}$ of drinking water [41], and various water treatment methods have been developed and proposed over the last years.

Degradation with immobilized TiO_2 photocatalytic films [42–44], microbial degradation [45] and advanced oxidation processes (AOPs) using O_3 , H_2O_2 , $\text{O}_3/\text{H}_2\text{O}_2$, $\text{O}_3/\text{Fe(II)}$, Fenton treatment and ultrasonic irradiation have been reported, being ozonation considered one of the most efficient oxidants in water treatment [46, 47].

In dense accumulations of cyanobacteria in water, very high concentrations (up to 25 mg L^{-1}) of these toxins have been reported [48]. The LD_{50} of NOD is approximately $50 \mu\text{g kg}^{-1}$ b.w. (body weight) in mice when administrated intraperitoneally. With such a low LD_{50} , NOD is one of the most potent natural toxins. NOD is considered to be relatively resistant to degradation owing to its ring structure and to the presence of several unusual amino acids in the molecule [8, 49].

Apart from the conventional analytical methods, new strategies such as biosensors have been developed for the detection of cyanotoxins in contaminated waters. In this context, the use of enzymes [6, 40], antibodies [50] and DNA probes [38, 51] has been reported.

However, the DNA-electrochemical biosensor on a glassy carbon electrode has never been applied for the study of cyanotoxins, until the studies described later in this chapter were performed.

9.3 *Cyanobacterial Hepatotoxins Oxidation Mechanisms*

In order to investigate the oxidation mechanisms of MC-LR and NOD cyanotoxins and their degradation products in aqueous solution, the electrochemical behaviour of these toxins was investigated at different pH's, using cyclic, differential pulse and square wave voltammetry, at a glassy carbon electrode.

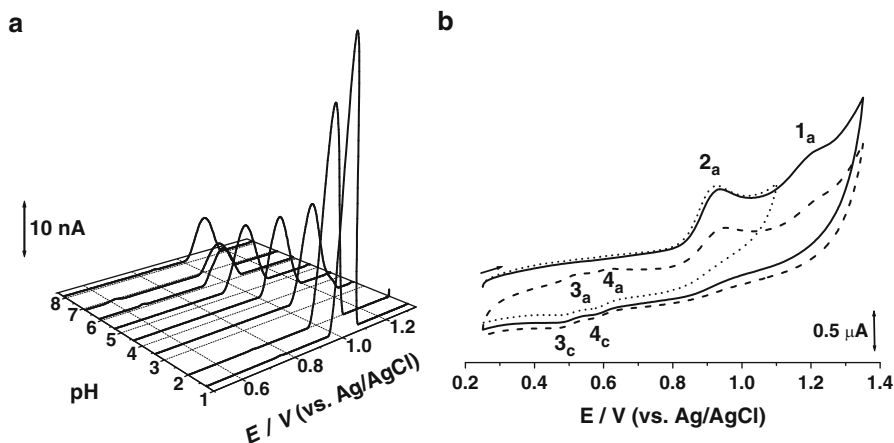


Fig. 9.2 (a) 3D-plot of differential pulse voltammograms background-corrected registered immediately after the addition of 30 μ M MC-LR to buffer as a function of pH; and (b) cyclic voltammograms in 50 μ M MC-LR after 96 h incubation in pH 3.4 0.1 M acetate buffer: (—) first and (---) second scans between +0.25 and +1.35 V, and (···) first scan between +0.25 and +1.10 V; $\nu = 100$ mV s $^{-1}$

9.3.1 MC-LR Electrochemical Oxidation Behaviour

MC-LR undergoes an irreversible, pH-independent electrochemical oxidation at a glassy carbon electrode, peak 1_a , that occurs with the transfer of only one electron and does not involve the formation of any electroactive oxidation product, as shown in the differential pulse voltammograms, Fig. 9.2a.

The electrochemical oxidation of MC-LR was studied over a wide pH range between 1.3 and 11.9 and the differential pulse voltammograms in different electrolytes, Fig. 9.2a, showed only pH-independent peak 1_a , at $E_{p1a} \approx +1.06$ V, with the peak width at half height $W_{1/2} \approx 90$ mV, indicating a mechanism involving only electron transfer [52, 53], MC-LR and/or MC-LR oxidation product undergoing chemical deprotonation after the rate-determining step. Peak 1_a current decreased with the increasing pH, showing maximum values in acid electrolytes and peak 1_a disappeared for $\text{pH} > 8.2$.

Successive differential pulse voltammograms were recorded in each solution without cleaning the electrode surface, and a progressive decrease of peak 1_a current was observed and its potential displaced to more positive values. This effect was caused by the decrease of the electrode surface area, owing to the adsorption of MC-LR and/or its non-electroactive oxidation products. However, no new peak was obtained on the second and third differential pulse voltammograms, showing that the oxidation mechanism of MC-LR does not involve the formation of any electroactive redox product.

The cyclic voltammetric behaviour of MC-LR was investigated at a glassy carbon electrode in a N_2 saturated solution, in several electrolytes, with different pH's, scanning the potential starting from 0.00 V and between the positive potential limit of +1.35 V and the negative potential limit of -1.00 V. Independently of the initial scan direction (towards positive or negative values), only one small anodic peak occurred in all cases, Fig. 9.2b, showing that MC-LR can only undergo oxidation at the glassy carbon electrode surface.

Recording successive scans in the same solution, the decrease of peak 1_a current due to the adsorption of MC-LR and/or its oxidation products on the electrode surface was observed.

The irreversibility of the redox reaction was confirmed by square wave voltammetry, plotting the forward and the backward components of the total current. Whereas the forward component showed the oxidation peak 1_a , at $E_{p1a} = +1.19$ V, at the same potential and with the same current as the total current obtained, on the backward component no cathodic peak occurred.

9.3.2 Chemical Degradation of MC-LR

Chemical degradation of MC-LR occurs with time in buffer solution, and was detected using electrochemical methods because the chemically degraded MC-LR (cdMC-LR) formed in solution after 96 h incubation in pH 3.4 0.1 M acetate buffer is electroactive, Fig. 9.2b.

The electrochemical oxidation behaviour of cdMC-LR after different incubation times of MC-LR in buffer was investigated, showing that cdMC-LR oxidation occurs at lower potential values, peak 2_a , than MC-LR, peak 1_a , Fig. 9.2b.

The oxidation current of peak 2_a of cdMC-LR increased with increasing incubation time, confirming that the MC-LR degradation process occurred homogeneously in solution. The variation of peak 2_a current with incubation time, in different buffer electrolytes, clearly showed that the degradation is faster in electrolytes with $5.3 < \text{pH} < 6.1$.

Cyclic voltammograms recorded in a cdMC-LR solution obtained after 96 h incubation in pH 3.4 0.1 M acetate buffer, Fig. 9.2b, showed a new anodic peak 2_a , at $E_{p2a} = +0.94$ V, on the positive-going scan due to the oxidation of cdMC-LR, and MC-LR oxidation peak 1_a , at $E_{p1a} = +1.21$ V. Changing the scan direction, two reduction peaks, peak 4_c , at $E_{p4c} = +0.58$ V and peak 3_c , at $E_{p3c} = +0.47$ V, occurred. These two peaks correspond to the reduction of cdMC-LR oxidation products formed at $E_{p2a} = +0.94$ V, peak 2_a , at the glassy carbon electrode surface during the anodic scan.

On the second cyclic voltammogram, in the same solution and without cleaning the glassy carbon electrode surface, the oxidation peaks 3_a , at $E_{p3a} = +0.52$ V, and 4_a , at $E_{p4a} = +0.62$ V, occurred, showing the reversible behaviour of these two products redox reactions, Fig. 9.2b. Moreover, it was found that these reactions are pH-dependent.

In order to clarify which peak, 1_a , from MC-LR, or peak 2_a , from cdMC-LR, led to the oxidation products corresponding to peaks 3_a and 4_a , cyclic voltammograms were recorded under the same conditions with a clean glassy carbon electrode surface, but the scan direction was inverted immediately after peak 2_a and before peak 1_a . In these experimental conditions both peak 3_a and peak 4_a occurred, confirming that these two peaks are only due to the cdMC-LR oxidation products formed at peak 2_a .

Square wave voltammograms were recorded in a cdMC-LR solution, obtained after 53 h incubation of a MC-LR solution in pH 5.3 0.1 M acetate buffer, in order to evaluate the reversible character of the electrochemical oxidation of cdMC-LR and its degradation products. The first square wave voltammogram showed the cdMC-LR oxidation peak 2_a , at $E_{p2a} = +0.78$ V, and the irreversibility of this reaction was confirmed. On the second square wave voltammogram recorded without cleaning the glassy carbon electrode surface, peak 3_a , at $E_{p3a} = +0.41$ V, and peak 4_a , at $E_{p4a} = +0.51$ V, appeared. The reversibility of these reactions was also confirmed plotting the forward and the backward components of the total current, and the oxidation and the reduction currents were equal.

The electrochemical oxidation behaviour of cdMC-LR and of its oxidation products was studied over a wide pH range, between 1.3 and 11.9, by differential pulse voltammetry after different incubation periods of time.

The differential pulse voltammograms registered after 5 h incubation in pH 5.3 showed peak 2_a , at $E_{p2a} = +0.76$ V, and a progressive increase of its current with increasing incubation times, 24 and 29 h, was observed, while the current of MC-LR peak 1_a decreased. The differential pulse voltammetry experiments were carried out in all buffer electrolytes and the higher cdMC-LR oxidation peak currents were found for $5.3 < \text{pH} < 6.1$.

These observations showed that upon incubation in buffer, structural modifications occurred in MC-LR. The decrease of peak 1_a current with incubation time corresponds to the decrease of MC-LR concentration, whereas the appearance and the increase of peak 2_a current correspond to an increase of cdMC-LR concentration.

The adsorption of cdMC-LR oxidation products at the glassy carbon electrode surface was confirmed when, after several differential pulse voltammograms in the cdMC-LR solution, the electrode was washed with a jet of deionized water and then transferred to the supporting electrolyte. The differential pulse voltammogram obtained in these conditions showed peaks 3_a and 4_a .

The pH-dependence of the cdMC-LR oxidation was studied in solutions obtained after 29 h incubation of 30 μM MC-LR solutions in different buffer electrolytes. The cdMC-LR oxidation peak 2_a appeared for all supporting electrolytes, except at pH 1.3. For $1.3 < \text{pH} < 9.2$, the potential was displaced to less positive values with increasing pH, following the relationship E_{p2a} (V) = $1.081 - 0.059 \text{ pH}$. The slope of the line, 59 mV per pH unit, shows that the oxidation mechanism involves the same number of electrons and protons [52]. Taking into consideration that the width at half height of peak 2_a was $W_{1/2} \approx 74$ mV, the oxidation process at peak 2_a involves the transfer of one electron, hence also one proton. For $\text{pH} > 9.2$, peak 2_a potential is

pH-independent, indicating a mechanism involving the transfer of only one electron and no proton, occurring if the cdMC-LR oxidation product undergoes chemical deprotonation in more alkaline electrolytes [54]. The value of $pK_a \approx 9.2$ for cdMC-LR was determined.

On the second differential pulse voltammogram, peak 3_a and peak 4_a occurred. Increasing the pH of the supporting electrolyte up to pH 9.2, a decrease on the potentials of peak 3_a and peak 4_a was observed. The currents of peak 3_a and peak 4_a varied with the pH, reaching a maximum at $5.3 < \text{pH} < 6.1$. The dependence was linear and the slope of the line, 59 mV per pH unit, showed that the oxidation mechanism for peak 3_a and peak 4_a involves the transfer of the same number of electrons and protons. Since the width at half height was $W_{1/2} \approx 46$ mV for both peaks, each of these oxidation processes involves the transfer of two electrons and two protons. For $\text{pH} > 9.2$, peak 3_a and peak 4_a potentials are pH-independent, indicating a mechanism involving the transfer of only two electrons and no protons.

9.3.3 MC-LR and cdMC-LR Oxidation Mechanisms

In order to determine the electroactive centres of MC-LR, the electrochemical oxidation of some MC-LR amino acids was investigated for the same incubation times studied for MC-LR. Electrochemical experiments carried out in different pH solutions containing MC-LR amino acids (Leu, Arg, Ala, Glu and Asp) showed no electrochemical response.

It was not possible to obtain Adda and Mdha amino acids present in MC-LR ring structure, so the oxidation mechanisms of sorbic acid (SA), Fig. 9.1c, and 2-acetamidoacrylic acid (2-AAA), Fig. 9.1d, were investigated. These compounds were chosen because sorbic acid (SA) mimics the Adda side chain of MC-LR, and 2-acetamidoacrylic acid (2-AAA) is an analogue of Mdha.

Differential pulse voltammograms recorded in SA and 2-AAA solutions showed that both compounds are electrochemically oxidized at a glassy carbon electrode, and their oxidation potentials are very similar to those of MC-LR.

Differential pulse voltammograms in 2-AAA solutions, in a pH range from 3.5 to 7.0, showed one peak at $E_{pa} = +1.30$ V. The 2-AAA oxidation is a pH-independent reaction, occurring with the transfer of one electron. After incubation in buffer for different incubation times no chemical degradation of 2-AAA was observed.

The differential pulse voltammograms recorded in SA solutions, in a pH range from 3.5 to 7.0, immediately after their preparation, showed that SA undergoes irreversible, pH-independent oxidation at $E_{p1a} \approx +1.36$ V, peak 1_a, involving the transfer of one electron, Fig. 9.3a.

After increasing the incubation times in buffer, peak 2_a, at $E_{p2a} = +0.83$ V, appeared and peak 1_a current decreased. On the second differential pulse voltammogram recorded in the same conditions, two new oxidation peaks, peak 3_a, at $E_{p3a} = +0.48$ V and peak 4_a, at $E_{p4a} = +0.57$ V, were observed, Fig. 9.3b.

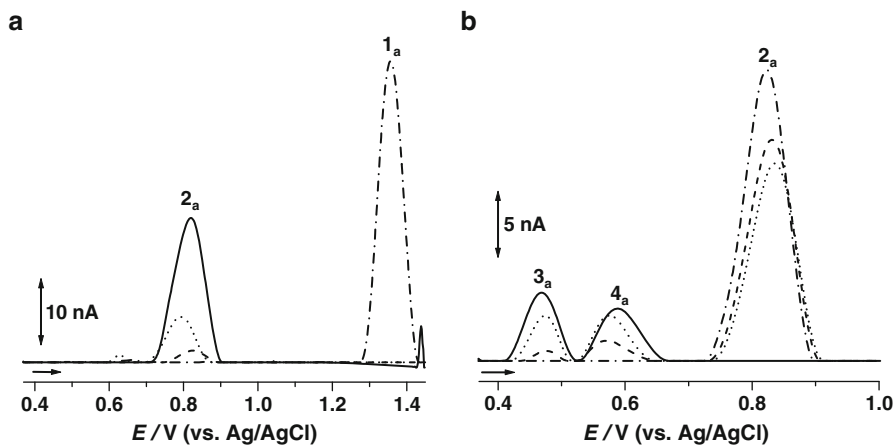


Fig. 9.3 Differential pulse voltammograms background-corrected in 3×10^{-5} M SA in pH 3.4 0.1 M acetate buffer: (a) after (---) 0 h, (----) 5 h, (— · —) 48 h and (—) 14 days incubation in buffer; and (b) after 14 days incubation: (---) first, (----) second and (— · —) third scans in solution and (—) first scan after transferring the electrode to buffer (From Ref. [55]. With permission)

Consequently, the electroactive centres responsible for MC-LR oxidation peak 1_a were attributed to the double bond at Mdha ($C=CH_2$), and/or to the double bonds between carbons C4-C5 and/or C6-C7 at Adda, Fig. 9.1a.

Moreover, differential pulse voltammetry performed in SA and 2-AAA solutions, after being in buffer solution for different time periods, showed that SA, as it is known [56–59], was chemically degraded, whereas 2-AAA was not chemically degraded.

SA underwent chemical degradation in buffer solution with increasing incubation time, Fig. 9.3. Upon chemical degradation, the main SA oxidation peak 1_a decreased while the new oxidation peak 2_a occurred at a lower potential, showing the homogenous formation of a degradation product. The oxidation of the SA degradation product led to the formation of two electroactive species that underwent reversible redox reactions, as in the case of MC-LR. The oxidation mechanism of SA was proposed, Fig. 9.4.

Given the similarity between the electrochemical behaviour of the two compounds, SA and MC-LR, a similar degradation and oxidation mechanisms of MC-LR and cdMC-LR is considered. Upon incubation of MC-LR in aqueous buffer solutions, the chemical degradation of MC-LR occurs in two different places of the Adda moiety, giving rise to two electroactive degradation products, each containing a hydroxyl group, and two non-electroactive degradation products, consisting of the MC-LR remaining peptide ring structure. Furthermore, the Adda side chain is separated from the peptide ring structure [55].

The oxidation of cdMC-LR, peak 2_a , corresponds to the oxidation mechanism of the two electroactive degradation products formed. The removal of one electron and one proton occurs and an unstable radical molecule is generated, which

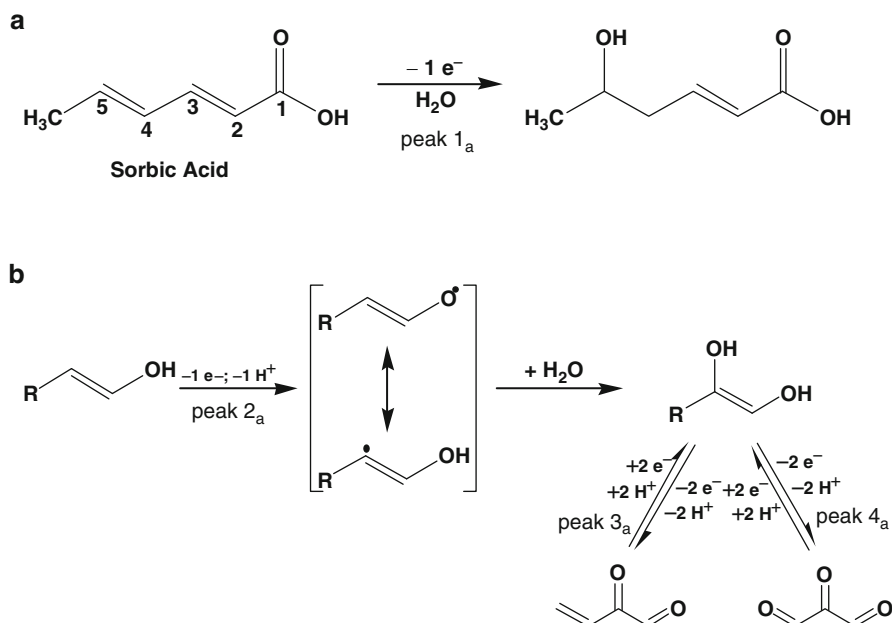


Fig. 9.4 Proposed oxidation mechanism of (a) sorbic acid and (b) sorbic acid degradation products: crotonaldehyde ($R = CH_2$), malonaldehyde ($R = O$) (From Ref. [55]. With permission)

incorporates another hydroxyl group, becoming dihydroxylated. The reversible redox reactions that lead to peaks 3_a and 4_a are attributed to the oxidation of the dihydroxylated products.

9.3.4 NOD Electrochemical Oxidation Behaviour

NOD undergoes an irreversible, pH-independent electrochemical oxidation at a glassy carbon electrode, peak 1_a, which occurs with the transfer of only one electron and does not involve the formation of any electroactive oxidation product.

The first square wave voltammogram in a fresh 50 μ M NOD solution, in pH 5.3 0.1 M acetate buffer, showed only peak 1_a, at $E_{p1a} = +1.09$ V. The deconvolution of the total current in forward and backward components confirmed the irreversibility of the NOD oxidation reaction. The forward component showed the oxidation peak at the same potential and with the same current as the total current, whereas on the backward component no peak occurred. The square wave voltammograms recorded in different buffer supporting electrolytes showed similar features.

The electrochemical behaviour of NOD by cyclic voltammetry, in pH 7.0 0.1 M phosphate buffer, scanning the potential starting from -1.00 V to $+1.35$ V, showed one anodic peak, at $E_{p1a} = +1.15$ V, meaning that NOD can only undergo oxidation.

A constant flux of N_2 was bubbled through the solutions during 10 min, and over the solution surface during the cyclic voltammetric measurements. The influence of the scan rate (v) on peak 1_a current was investigated in pH=7.0 0.1 M phosphate buffer. Peak 1_a current increased linearly with the square root of the scan rate, which is the characteristic behaviour of a diffusion-controlled process.

Successive scans recorded in the same solution, without cleaning the electrode surface, showed a decrease in peak 1_a current, due to the adsorption of NOD and/or NOD oxidation products onto the glassy carbon electrode surface. No other peaks were observed, suggesting that the oxidation of NOD does not involve the formation of electroactive products. A similar behaviour was observed in other electrolytes.

The electrochemical oxidation of NOD was investigated over a wide pH range, between 1.1 and 11.8, using differential pulse voltammetry. All differential pulse voltammograms were recorded in fresh 50 μ M NOD solutions, in different supporting electrolytes. The differential pulse voltammograms in $1.1 < \text{pH} < 9.2$, immediately after the addition of NOD to each buffer, showed peak 1_a , at $E_{p1a} = +1.06$ V, but for $\text{pH} > 9.2$, peak 1_a did not appear.

The potential of peak 1_a is pH-independent, which indicates an oxidation mechanism involving only electron transfer, possible if NOD and/or NOD oxidation product undergoes chemical deprotonation after the rate-determining step. The Nernst equation enables the determination of the ratio between the number of electrons and protons involved in the reaction. The peak width at half height, $W_{1/2} \approx 84$ mV, is close to the theoretical value of 90 mV for the transfer of one electron [53]. Therefore, peak 1_a involves the transfer of one electron, and its current decreased with the increasing pH, showing maximum values in acid electrolytes [60].

9.3.5 Chemical Degradation of NOD

The electrochemical oxidation behaviour of cdNOD after different incubation times of NOD was investigated [60]. It was observed that chemical degradation of NOD occurs with time in buffer solution, and was detected using electrochemical methods, because the chemically degraded NOD (cdNOD) formed in solution is electroactive, Fig. 9.5a. The chemical degradation of NOD (cdNOD) gave rise to electroactive degradation products and their electrochemical oxidation behaviour was investigated.

Square wave voltammetry was used to investigate the electrochemical oxidation behaviour of cdNOD in different electrolytes. The first square wave voltammogram in a NOD solution after 53 h incubation, in pH 5.3 0.1 M acetate buffer solution, showed the appearance of peak 2_a , at $E_{p2a} = +0.73$ V, Fig. 9.5a. The deconvolution of the total current showed that peak 2_a corresponds to an irreversible oxidation reaction.

On a second square wave voltammogram recorded without cleaning the electrode surface, peak 3_a , at $E_{p3a} = +0.37$ V, and peak 4_a , at $E_{p4a} = +0.47$ V, occurred. The reversibility of these reactions was shown by the two peaks on the forward and on

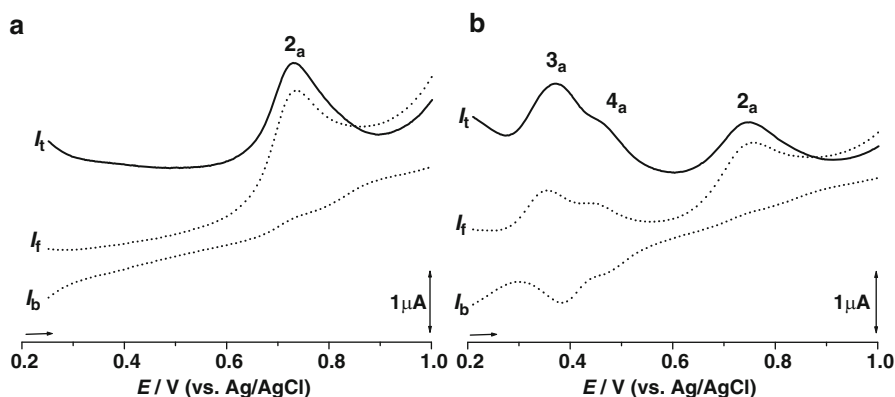


Fig. 9.5 Square wave voltammograms in 50 μM NOD after 53 h incubation in pH 5.3 0.1 M acetate buffer: (a) first and (b) second scans; $f=50$ Hz, $\Delta E_s=2$ mV, pulse amplitude 50 mV, $v_{\text{eff}}=100$ mV s^{-1} ; I_t – total, I_f – forward and I_b – backward currents

the backward components of the total current, where the oxidation and the reduction currents are equal, Fig. 9.5b.

The oxidation behaviour of cdNOD was investigated by cyclic voltammetry in NOD solutions incubated in different supporting electrolytes during 7 days, and a new anodic peak 2_a appeared. All cyclic voltammograms showed similar features, independently of the pH of the supporting electrolyte solution. Reversing the scan direction, two new reduction peaks were observed: peak 4_c , at $E_{p4c}=+0.47$ V, and peak 3_c , at $E_{p3c}=+0.36$ V. These two peaks correspond to the reduction of cdNOD oxidation products formed at the glassy carbon electrode surface during the anodic scan.

On the second cyclic voltammogram obtained in the same solution, without cleaning the electrode surface, the anodic peak 3_a , at $E_{p3a}=+0.39$ V, and peak 4_a , at $E_{p4a}=+0.49$ V, appeared, confirming the reversibility of these reactions. Moreover, on the second scan, peak 2_a current decreased due to the adsorption of cdNOD oxidation products onto the glassy carbon electrode surface.

The electrochemical oxidation behaviour of cdNOD and its oxidation products was also investigated using differential pulse voltammetry in different buffer electrolytes, with $1.1 < \text{pH} < 11.8$. Differential pulse voltammograms recorded in a NOD solution after 5 h incubation in pH 6.1, showed both NOD oxidation peak 1_a , at $E_{p1a}=+1.07$ V, and cdNOD oxidation peak 2_a , at $E_{p2a}=+0.70$ V. After longer incubation times (24, 29 and 53 h), a progressive increase of peak 2_a current with increasing incubation time, and the decrease of NOD oxidation peak 1_a current, was observed, in agreement with the decrease of NOD concentration and with the increase of cdNOD concentration, homogeneously formed in solution.

The first differential pulse voltammogram in a NOD solution after 53 h incubation in pH 6.1, showed peak 1_a , at $E_{p1a}=+1.07$ V, and peak 2_a , at $E_{p2a}=+0.70$ V. On the

second scan, the new oxidation peak 3_a, at $E_{p3a} = +0.34$ V, and peak 4_a, at $E_{p4a} = +0.44$ V, were observed, corresponding to the oxidation of cdNOD oxidation product formed at the electrode surface during the first potential scan. Increasing the number of scans in solution, both peak 3_a and peak 4_a currents increased due to the formation of more adsorbed cdNOD oxidation products at the glassy carbon electrode surface.

In the differential pulse voltammograms registered at pH 1.1 peak 2_a did not appear. For $1.1 < \text{pH} < 9.2$, its potential was shifted to less positive values with increasing pH, following the relationship $E_{p2a} \text{ (V)} = 1.081 - 0.059 \text{ pH}$. The slope of the line, 59 mV per pH unit, shows that the oxidation mechanism involves the transfer of the same number of electrons and protons [52]. Taking into consideration that the width at half height of peak 2_a was $W_{1/2} \approx 65$ mV, the oxidation process at peak 2_a involves the transfer of one electron and one proton. For $\text{pH} > 9.2$, peak 2_a potential is pH-independent, indicating a mechanism involving the transfer of only one electron and no proton, occurring because cdNOD oxidation product undergoes chemical deprotonation in more alkaline electrolytes. Thus, the value of $\text{pK}_a \approx 9.2$ for cdNOD was determined.

On the second differential pulse voltammograms recorded in different pH buffer electrolytes, without cleaning the electrode surface, peaks 3_a and 4_a occurred. For $1.1 < \text{pH} < 9.2$, the peak 3_a and peak 4_a potential variation was linear and the slope of the lines, 59 mV per pH unit, showed that the oxidation mechanisms involved the transfer of the same number of electrons and protons. The widths at half height of both peaks were $W_{1/2} \approx 45$ mV, which confirms that two electrons and two protons were transferred. For $\text{pH} > 9.2$, peak 3_a and peak 4_a potentials were pH-independent, indicating a reaction with the transfer of only two electrons, with no protons involved.

The variation of cdNOD oxidation peak 2_a current with incubation time and pH showed that NOD chemical degradation occurred at a higher rate in electrolytes with $5.3 < \text{pH} < 6.1$.

9.3.6 NOD and cdNOD Oxidation Mechanisms

The electrochemical study of NOD showed that oxidation and cyanotoxin chemical degradation in aqueous media follow a complex redox mechanism, and the electroanalytical determination of NOD and cdNOD is foreseen, which will provide very important and useful data for toxicity evaluation [60].

Upon incubation in different pH buffer supporting electrolytes, NOD chemical degradation was electrochemically detected by the appearance of a new peak at lower potential. The cdNOD, homogeneously formed in solution, undergoes oxidation in an irreversible, pH-dependent process, with the transfer of one electron and one proton, giving rise to two electroactive products with pH-dependent, two electron and two proton reversible reactions.

Since NOD has a similar structure to MC-LR, the electroactive centres are considered to be the same as in the case of MC-LR, with exception of Mdha, which is

not present in NOD chemical structure. Therefore, the mechanisms proposed for NOD degradation and for cdNOD oxidation, peak 2_a, and its oxidation products, peak 3_a and peak 4_a, were based on the mechanisms proposed for MC-LR. The proposed mechanisms for NOD electrochemical oxidation and chemical degradation were also based on the electrochemical results obtained for MC-LR, SA and 2-AAA.

The electrochemical oxidation of NOD, peak 1_a, corresponds to the oxidation of Adda's C7 in a process involving the irreversible pH-independent transfer of one electron and the formation of a radical cation in the rate-determining step. Subsequently, the addition of a hydroxyl group to position C7 occurs, and the radical is chemically deprotonated.

Upon incubation in aqueous acidic buffer solutions, the Adda side chain is separated from the peptide ring structure. This process may occur in two different places of the Adda moiety, giving rise to two electroactive degradation products containing a hydroxyl group and two other degradation products consisting of the NOD remaining peptide ring structure.

The cdNOD peak 2_a is attributed to the electrochemical oxidation of chemically degraded NOD, in a one electron and one proton reaction, followed by a chemical oxidation reaction where a second hydroxyl group is incorporated into the molecule. The reversible redox reactions that lead to peak 3_a and peak 4_a are attributed to the oxidation of the dihydroxylated products.

9.4 *In Situ* Electrochemical Study of DNA-MC-LR and DNA-NOD Interaction

The electrochemical study of the interaction of MC-LR and NOD with the double stranded DNA (dsDNA) was performed in order to investigate the ability of these toxins to induce conformational changes in DNA, cleavage of hydrogen bonds and/or oxidative damage to DNA bases. The mechanism of interaction of MC-LR and NOD with the dsDNA was investigated by differential pulse voltammetry at a dsDNA modified glassy carbon electrode, the so called dsDNA-electrochemical biosensor [61].

9.4.1 *Voltammetric In Situ Sensing with DNA-Electrochemical Biosensors*

The DNA-electrochemical biosensor incorporates immobilised DNA as molecular recognition element on the glassy carbon electrode surface, and measures specific binding processes with DNA, enabling the screening and evaluation of the effect caused to DNA by health hazardous compounds and oxidising substances. AFM imaging was used to characterize different procedures for immobilising nanoscale

double-stranded DNA surface films on carbon electrodes, in which a critical issue is the sensor material and the degree of surface coverage. The DNA-electrochemical biosensor gives very important mechanistic information because the mechanisms of DNA-hazard compound interaction at charged interfaces mimic the *in vivo* situation.

The dsDNA-electrochemical biosensor was prepared by successively covering the glassy carbon electrode surface with three drops of 5 μL each of a 50 $\mu\text{g mL}^{-1}$ dsDNA solution. After placing each drop on the electrode surface, the dsDNA-electrochemical biosensor was dried under a constant flux of N_2 .

The interaction of MC-LR and NOD with dsDNA was evaluated using a dsDNA-electrochemical biosensor.

The dsDNA-electrochemical biosensor was immersed in freshly prepared MC-LR or NOD solutions, and incubated for different periods of time. The interaction was evaluated comparing the purine bases oxidation peaks, deoxyguanosine (dGuo), at $E_{\text{pa}} = +1.00$ V and deoxyadenosine (dAdo), $E_{\text{pa}} = +1.25$ V, obtained for a control dsDNA-electrochemical biosensor in the absence of MC-LR and NOD toxins, with the peaks obtained with a dsDNA-electrochemical biosensor that was immersed in freshly prepared MC-LR or NOD solutions, incubated in buffer for the same periods of time.

The incubation of the dsDNA-electrochemical biosensors for 10 min in MC-LR or in NOD solutions resulted in the decrease of the dGuo and dAdo oxidation peak currents, compared with the control dsDNA-electrochemical biosensor peaks. This effect was also observed after 20 min of incubation, for which the voltammograms showed a significant decrease of dsDNA oxidation peak currents.

The occurrence of the peaks for guanine and/or adenine oxidation products, biomarkers 8-oxoguanine (8-oxoGua) and 2,8-dihydroxyadenine (2,8-DHA), $E_{\text{pa}} \approx +0.45$ V in pH=4.5 0.1 M acetate buffer, is an indication of oxidative damage caused to dsDNA. However, with the dsDNA-electrochemical biosensors incubated with MC-LR or with NOD, the peaks of these biomarkers were not detected.

This suggests that MC-LR and NOD interact with the dsDNA immobilised at the glassy carbon electrode surface. The decrease observed in the dGuo and dAdo peak currents might be due to conformational changes in the DNA double helix, such as condensation, which reduces the contact of the DNA bases with the electrode surface and eventually hinders the electron transfer but, as no peaks for the biomarkers 8-oxoGua or 2,8-DHA occurred, no DNA oxidative damage was detected.

9.4.2 Voltammetric Evaluation in Incubated Solutions

The interaction of dsDNA with the cyanotoxins MC-LR and NOD in incubated solutions was studied by differential pulse voltammetry for different incubation times in pH 4.5 0.1 M acetate buffer.

MC-LR-dsDNA and NOD-dsDNA interactions were evaluated by analyzing the changes of the dsDNA purine bases oxidation peaks, deoxyguanosine (dGuo), at

$E_{pa} = +1.00$ V and deoxyadenosine (dAdo), $E_{pa} = +1.25$ V, in the absence and in the presence of these toxins.

Solutions of dsDNA were incubated with MC-LR or with NOD, and the MC-LR-dsDNA and NOD-dsDNA interaction followed in time (up to 6 h). A control dsDNA solution was also prepared and analyzed after the same periods of time as the toxin-dsDNA incubated solutions.

The first differential pulse voltammograms recorded immediately after the addition of each toxin to a new dsDNA solution showed an increase of the peak current at $E_{pa} = +1.00$ V, when compared to the oxidation peaks obtained for the dsDNA control solution. This is due to the overlapping of two oxidation peaks, the oxidation of dGuo residues in DNA strands and the oxidation peak of MC-LR or NOD, both occurring at a similar potential. On the other hand, the dAdo peak current decreased, due to MC-LR-dsDNA or NOD-dsDNA interaction. This behaviour is similar to the previously described results using the dsDNA-electrochemical biosensor.

The differential pulse voltammograms recorded in MC-LR-dsDNA or NOD-dsDNA solution after 2 and 6 h of incubation showed a successive decrease in the dGuo and dAdo peak currents, with increasing incubation time, as it had been observed in the experiments with the dsDNA-electrochemical biosensor. Thus, the results obtained with the incubated solutions also confirm that the dsDNA double helix underwent condensation, making it a more compact structure after interacting with the toxins MC-LR or NOD.

Since no peak corresponding to the biomarkers 8-oxoGua or 2,8-DHA was observed in any of the differential pulse voltammograms recorded, it was concluded that, under the experimental conditions studied, no evidence of dsDNA oxidative damage induced by MC-LR or NOD was found.

9.5 Conclusion

The electrochemical behaviour of cyanobacteria produced hepatotoxins MC-LR and NOD was investigated over a wide pH range, using cyclic, square wave and differential pulse voltammetry, with a glassy carbon electrode. The results showed that they undergo oxidation in an irreversible, diffusion-controlled and pH-independent reaction, which occurs with the transfer of only one electron. Two electroactive compounds analogue to MC-LR and NOD, SA and 2-AAA, enabled the attribution of the electroactive centres of these hepatotoxins to Adda and Mdha amino acids, respectively.

NOD and MC-LR undergo chemical degradation in homogeneous solution upon incubation in buffer electrolytes, and the degradation products are electrochemically detectable by the occurrence of a new peak at lower potential.

The products of the chemical degradation of MC-LR and NOD are electrochemically oxidized in an irreversible and pH-dependent process, with the transfer of one electron and one proton, giving rise to two electroactive products with pH-dependent, two electron and two proton reversible reactions.

The electrochemical research of MC-LR and NOD revealed that their oxidation and chemical degradation in aqueous media follow complex mechanisms. Given the advantages associated to electrochemistry, the electroanalytical determination of MC-LR, NOD and of the correspondent degraded forms (cdMC-LR and cdNOD) is foreseen, which will provide very important and useful data for toxicity evaluation.

In situ evaluation of MC-LR-dsDNA and NOD-dsDNA interactions was investigated with DNA-electrochemical biosensors and with incubated solutions. Although no DNA oxidative damage evidence was detected, the results led to the conclusion that MC-LR and NOD induce conformational changes in the dsDNA structure, probably helix condensation, hindering the electron transfer between the purine bases and the electrode surface.

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Chapter 10

Biosensors for Detection of Ochratoxin A

Tibor Hianik

Abstract Mycotoxins such as ochratoxin A, aflatoxin B and others are dangerous food contaminants that usually occur in trace amounts from nanograms to micrograms per gram of food. Therefore high sensitive methods are necessary for their detection. The conventional methods such as high-performance liquid chromatography (HPLC), mass spectroscopy are rather expensive and time consuming, therefore biosensor technology is rather promising for rapid detection of toxicants in field conditions, far from specialized laboratories. Among biosensors based on affinity of monoclonal antibodies or DNA aptamers to mycotoxins are of special interest, because provide sensitivity of detection that is better than allowable quantities of toxicants in food. While antibodies are traditional receptors in biosensors, aptamers are novel biopolymers with the affinity comparable to that of antibodies. However in contrast with antibodies, aptamers are more stable and the biosensors based on DNA aptamers can be regenerated which allowing their multiple use. This contribution reviews recent achievements in development affinity biosensors for detection ochratoxin A.

Keywords Mycotoxin • Ochratoxin A • Antibodies • DNA aptamers • Biosensors

10.1 Introduction

The natural toxins, such as mycotoxins, abrin, ricin, saxitoxin, palytoxin, batrachotoxin, botulinum neurotoxin type A, mycrocystin–RC represent considerable hazard for health and could be considered as potential warfare agents [1]. Among

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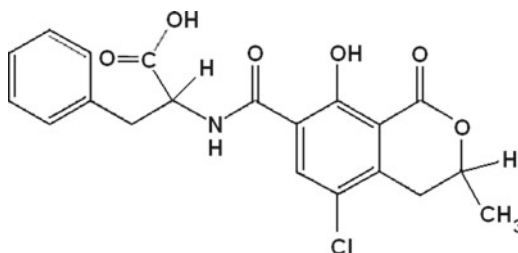
mycotoxins the ochratoxin A (OTA) is of special interest. OTA belongs to toxic fungal metabolites that can occur in primary food products. OTA is produced by *Aspergillus ochraceus* and *Penicillium verrucosum* and generally appears during improper storage of cereals, coffee, cocoa, dried fruit, pork etc. and occasionally in the field of grapes. It may also be present in blood and kidneys of animals that have been fed on contaminated feeds. In a blood OTA is bound to the serum proteins and is redistributed to various tissues. The most susceptible to OTA are kidneys. It has been shown, that accumulation of OTA in this organ causes acute and chronic lesions by affecting the anion transport [2]. This molecule causes also other toxicological effects including hepatotoxic, neurotoxic, teratogenic, immunotoxic. The toxicity of OTA is particularly connected with inhibition of protein synthesis because it competes with phenylalanine in the cells that utilize this amino acid. Animal studies indicated that OTA is carcinogenic [3]. This is connected with the genotoxicity of OTA because it induces oxidative stress in the cells [2, 4], oxidative base damage [5] and the cleavage of single stranded DNA [6, 7]. OTA interacts also with double stranded DNA (dsDNA), but the damage of double helix has not been observed [8]. The mechanism of OTA-induced cancer is not clear yet. Moreover the contradictory results were reported in OTA behavior *in vivo* and *in vitro* conditions. While *in vivo* OTA is poorly metabolized [9], *in vitro* investigations suggest that a hydroquinone/quinone redox couple and a carbon-bonded OTA-deoxyguanosine adducts are formed by electrochemical oxidation and photoreaction of OTA which may be the reason of OTA carcinogenicity [10].

Due to high toxicity of OTA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) already in 1991 evaluated a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight (b.w.) for this mycotoxin. This evaluation was based on the porcine nephropathies data [3]. Most recently, the European Commission has fixed maximum concentration of OTA in foodstuffs: 3 µg/kg (7.4 nM) for cereal products, 5 µg/kg (12.4 nM) for roasted coffee and up to 10 µg/kg (25 nM) for instant coffee. Similar contamination limit was fixed for dry grapes (10 µg/kg) (EC No. 466/2001, 1881/2006), but 2 µg/L (5 nM) contamination limit is valid for wine (EC No. 123/2005). Even stronger limit of contamination by OTA was fixed for all food preparation for babies (0.5 µg/kg, EC No. 466/2001). The cereals, especially in countries with hot climate are contaminated by OTA produced by *Aspergillus ochraceus*. But in countries with colder climate the contamination is due to *Penicillium verrucosum*. The studies performed by Pittet [11] suggest that 25–40% of cereals are contaminated by mycotoxins worldwide. Many works reports also on contamination of grape juices with approx 7 µg/L OTA (see [3] and references herein).

OTA is weak organic acid of a molar mass 403.8 g/mol. The chemical structure is presented on Fig. 10.1. It is a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine.

So far 15 naturally occurred ochratoxins were identified. Most frequently appeared are ochratoxins A, B, C, from which OTA is more prevalent. The differences between ochratoxins are in some cases minor. For example replacement of Cl on H yields in ochratoxin B [3]. At neutral pH (pH = 7) in a water OTA is negatively charged. This is due to ionization of carboxyl ($pK_a = 4.2\text{--}4.4$) and phenolic moieties

Fig. 10.1 Chemical structure of ochratoxin A



($pK_a = 7.0\text{--}7.3$) [12, 13]. Therefore both mono (OTA^-) and dianions of OTA (OTA^{2-}) are present at physiological pH.

OTA contamination is typically in trace amount from ng to μg per gram of food-stuff. Therefore sensitive analytical methods of detection should be applied. At present the OTA detection is performed mostly by high-performance liquid chromatography (HPLC) with fluorescence detection (OTA possesses natural fluorescence) [14], gas chromatography connected with mass spectrometry (GC-MS) [15], capillary electrophoresis [16], radioimmunoassay [17] or enzyme-linked immunosorbent assay (ELISA) [18, 19]. Association of Official Analytical Chemists (AOAC) official methods for determination of OTA in food are based on HPLC. However, traditional methods are rather expensive, time consuming and could be performed by qualified staffs only in specialized laboratories. In addition these methods usually require organic solvents for extraction of toxins from food, which represent additional pollution for environment. ELISA belongs to the rapid detection techniques, however the disadvantage is in necessity of using enzyme-labeling reagents, which are expensive. Therefore, there is urgent requirement for direct, rapid, and low cost methods for OTA detection.

The biosensor technology fulfills the above-mentioned requirements. The biosensor is a portable device consisting of a sensing element, which has usually a biological nature, for example antibody, enzymes, DNA, DNA aptamers, natural receptors. However, even the systems that mimic the biological structures, for example calixarenes incorporated into the lipid films can also be considered as belonging to these devices [20, 21]. The next part of the biosensor is the transducer that transforms usually a chemical signal to the electrical, optical or mass. This signal is analyzed by separate instruments, for example potentiostats, network analyzers, spectrometers, surface plasmon resonance (SPR) etc. The tendency however exists in the integration of sensing element and transducer into one chip, such as field effect transistor. The principles of biosensor construction have been explained in many books and reviews (see for example [22–24]).

The label free detection of OTA can be based on its redox properties which allow OTA detection at certain type of surfaces using electrical methods, such as cyclic, differential pulse or square wave voltammetry. However, more sensitive are the methods utilizing specific receptors, for example the enzymes, antibodies or DNA aptamers, immobilized at surfaces. This contribution reviews recent achievements in developing biosensors for detection of OTA and contains also results obtained in the author's laboratory.

10.2 The Biosensors for Detection OTA

10.2.1 OTA Oxidation and Its Detection by Amperometry

We already mentioned above that OTA carcinogenic effect may be connected with its oxidation, which causes appearance of reactive oxidation species in the cells. Oxidation of OTA is connected with its phenolic moiety [8, 25, 26]. It has been shown, that OTA can be oxidized at glassy carbon electrode (GCE) at specific conditions in organic solvents or aqueous media with pH between 6 and 8. Oxidation of OTA was studied also in presence of transition metal ions [10] and of a Fe-porphyrin system. In later case a hydroquinone species were detected by HPLC.

Detailed study of OTA oxidation at GCE at wide pH range (2–12) was performed by Oliveira et al. [8]. Using cyclic, differential pulse and square wave voltammetry they observed well resolved redox peaks. The peaks appeared at acidic conditions (pH=4.0) at potentials +1.05 and 0.45 V vs. Ag/AgCl reference electrode were used for analytical purposes. It has been shown that using square wave voltammetry it is possible to detect OTA with the limit of detection (LOD) 0.26 μM at optimal conditions. They also analyzed possible interferences with catechol, phenol and reweatrol, which significantly affected the OTA detection. Despite the fact that the LOD obtained by voltammetry at bare GCE is insufficient for practical applications, the method is useful for analysis of interaction of OTA with dsDNA. As mentioned above, authors observed interaction of OTA with dsDNA without causing its damage.

The electrochemical studies performed by Calcutt et al. [25] predicted that peroxidases could participate in OTA oxidation. This has been approved in work by Alonso-Lomillo et al. [27, 28] and used for detection OTA. Oxidation of horseradish peroxidase (HRP) at presence of hydrogen peroxide resulted in oxidation of OTA in aqueous solution. At the surface of screen printed carbon electrode (SPCE) at certain potential (around -0.3 V vs Ag/AgCl reference electrode) further oxidation of OTA took place. It is observed as an increase of anodic current in chronoamperometry experiment. It has been shown that immobilization of HRP has substantial effect on the sensor sensitivity. In Ref. [27] the HRP was immobilized at the surface of polypyrrol layer electropolymerized at SPCE. The OTA was in this case detected with LOD 0.25 nM (0.1 ng/mL). In most recent work the SPCE was prepared using carbon ink containing HRP [28]. However, this resulted worse detection properties of OTA (LOD 26.8 ± 3.6 nM). On the other hand in both cases the matrix effect of beer or roasted coffee was rather small and sensor revealed recovery 103% and 99%, respectively [27]. Rather high sensitivity in OTA detection was reported by Perrotta et al. [29]. They used square wave voltammetry for detection of OTA at the surface of gold electrode modified by cysteamine self-assembled monolayers. The detection and quantification limit was 4 ng/L and 12 ng/L, respectively. This is rather high sensitivity, but selectivity of this assay was not investigated,. They also performed test of the sensor in a red wine with a recovery ranging from 94% to 145%. However, without specific receptors the amperometric detection of OTA can

not be considered as a selective method due to various potential interferences from electroactive species that are present in large amount in a real samples, for example ascorbic acid, phenolic compounds etc. Large (145%) value of recovery in ref. [29] suggests that such interferences are very likely.

10.2.2 *Amperometry OTA Biosensor Based on ELISA*

Immunochemical methods of detection are of high sensitivity and allowing detection of OTA at concentrations below the EC regulatory values. The most popular immunochemical method is ELISA. The conventional method is based on ELISA optical microplate reader using spectrophotometric detection. The detection is performed in a small volume (around 100 μ L) in a well of microplate. Microplate containing usually large number of wells (typically 96), which allowing fast and simultaneous detection of several analytes. The product of enzyme reaction that posses absorbance or fluorescence signal is detected by optical reader. The detection can be indirect or direct and is based on competitive assay [30].

In indirect detection format usually 100 μ L solution of OTA conjugated with bovine serum albumin (BSA) is added into the well and kept at 4°C overnight. Then 100 μ L of 1% polyvinilalcohol (PVA) is added to block the microwells (1 h at 37°C). The anti-OTA IgG conjugated with enzyme alkaline phosphatase (AP) is added into the well in a sample containing unknown concentration of OTA. With increased amount of OTA in a sample less number of anti-OTA IgG is bounded to the OTA-BSA. AP transforms substrate 1-naphtyl phosphate into the product 1-naphtol (NP). The product absorbs light at wavelength 405 nm and thus can be detected by colorimetry. Therefore after addition of the substrate NP is detected. The amount of NP is indirectly proportional to the OTA.

In direct ELISA approx. 100 μ L solution of anti OTA IgG is added into the well and incubated overnight at 4°C. The well is then blocked by PVA like in indirect assay. The OTA-AP conjugate is then added in a sample containing unknown concentration of OTA. OTA-AP will compete with free OTA in a sample. Thus, with increased concentration of OTA, less number of OTA-AP complexes will bind to anti OTA IgG. The concentration of OTA-AP is detected by the same method like in an indirect assay.

In a biosensor format the ELISA approach can be easily adapted for amperometric detection of the enzyme reaction. NP is electroactive and at certain potential (approx. 0.3 V vs. Ag/AgCl) is oxidized at SCPE into 1-iminoquinone. The scheme of indirect and direct electrochemical detection in ELISA format is presented on Fig. 10.2.

The conventional colorimetry based ELISA has been used so far in a large number of works focused on detection various toxins, such are pesticides, marine toxins [31, 32], fungal toxins (aflatoxin, tricothecene) [33], OTA [34–38], ricin [39, 40] bacterial toxins [41–44]. See also [1, 45] for review. Simultaneous enzyme assay for screening the aflatoxin and OTA was also reported [46]. In this assay monoclonal

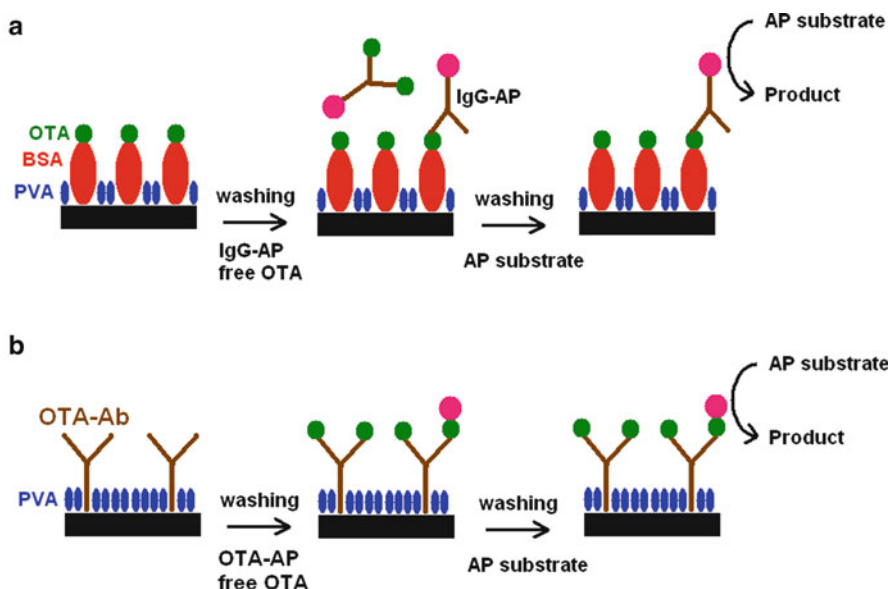


Fig. 10.2 The scheme of (a) indirect and (b) direct electrochemical ELISA assay (for explanation see the text)

antibodies were immobilized onto the nitrocellulose membranes. The method allowed detection of aflatoxin and OTA with LOD 2 and 10 $\mu\text{g/kg}$ for aflatoxin and OTA respectively and has been successfully used for determination these mycotoxin in chili samples. High sensitive indirect ELISA in a flow format with chemiluminescence detection was reported in Ref. [47]. In this assay the OTA was immobilized at the glass plate using peptide linker. The LOD 0.01 $\mu\text{g/L}$ (approx. 74 pM) belongs to the most sensitive reported so far. The method was useful for practical applications and has been approved for detection OTA in a roasted coffee.

The amperometric biosensors for detection OTA appeared only in 1987 when Aizawa [18] reported biosensor for detection OTA in ELISA like format. The sensor was composed of amperometric oxygen electrode and OTA covalently bound to a membrane that covered this electrode. The anti-OTA antibody labeled with enzyme catalase has been added in a fixed quantity to the sample solution and allowed to react competitively with the OTA immobilized at the membrane and with free OTA. The catalase activity (production of oxygen) was inversely proportional to the concentration of OTA in a sample. The sensor allowed OTA detection with LOD 0.1 $\mu\text{g/L}$ (0.25 nM, or 0.1 ppb).

In contrast with traditional methods such as HPLC in which the analyte to be detected is extracted from real sample using various solvents, the ELISA is different. The analyte is detected in a real sample. Therefore the matrix effect could affect the detection and should be therefore specially analyzed and detection assay optimized. The most sensitive methods detect OTA in real samples with sensitivity in

$\mu\text{g/kg}$ range in a batch [48, 49] or in a flow format [50]. Even $0.05 \mu\text{g/kg}$ LOD was reported for indirect immunoassay in microfluidic format with antibodies immobilised on magnetic nanoparticles. This assay was approved for detection OTA in apples [51]. High sensitivity in an indirect enzyme assay was reported in Ref. [52]. In this work the OTA-ovalbumin conjugates were immobilized on a gold colloid layer. The competitive amperometric detection was performed by addition of free OTA and specific antibodies conjugated by alkaline phosphatase with detection limit 8.2 ng/L and validated in a corn samples. The appearance of screen-printed electrodes that integrate the working electrode (carbon, gold, etc.) with reference Ag/AgCl and counter electrode resulted in further advantage of immobilization of the specific receptors [53, 54]. The recognition elements can be even entrapped into the ink during preparation of the sensing layer [28]. Optimization of immobilization and detection condition allowed detecting OTA with substantially improved sensitivity in a direct ELISA format with LOD up to $0.05 \mu\text{g/L}$ [30].

The enzyme based biosensors for detection OTA are rather useful, but still require enzyme conjugation and laborious preparation. Currently, only conventional ELISA kits are available at the market. But several hours are typically required for obtaining the result. Faster kits appeared recently (Veratox, Neogen, Lasing, USA) allowing detection of OTA within 30 min, but sensitivity is lower. Therefore there is attempt to develop another specific immunoassay that does not require application of antibody/enzyme conjugates. In this respect the focus is mostly on application of the surface plasmon resonance (SPR), quartz crystal microbalance (QCM) and electrochemical impedance spectroscopy (EIS) for detection OTA at surfaces modified by specific antibodies. The overview of achievements in development of SPR, QCM and EIS based immunosensors is presented in next section.

10.2.3 *Immunosensors Sensitive to OTA*

In this part we will analyze the works focused on development SPR, QCM and EIS based immunosensors for detection OTA. The main advantage of these devices is lack of enzymes and in some cases possibility of direct detection of OTA.

SPR method belongs currently to the standard one for detection affinity interactions at surfaces. This is due to high sensitivity, selectivity, possibility of surface regeneration and due to existence of powerful commercial instruments; such are Biacore (Sweden) or Spreeta (Texas Instruments, USA). The principles of SPR are described in many textbooks and reviews (see for example [23]). SPR is based on generation of plasmons in a thin layer adjacent to the glass prism by irradiation of laser beam at certain angle of incidence. The angle of reflected beam shifts with increasing the thickness of sensing layer. Thus, measurement of this angle allowing determination of the surface concentration of adsorbed molecules as well as the thickness of the layer [55]. This method has been used for multiple detection of mycotoxins, including OTA within 25 min with sensitivity approx. 0.5 ppb ($0.5 \mu\text{g/L}$) [56]. SPR device combined with molecularly imprinted polymer method (MIP)

allowed detection of OTA within a linear range from 0.05 to 0.5 mg/kg [57]. Yuan et al. [58] proposed interesting approach. They used IgG-gold nanoparticle conjugates for amplification the SPR signal following competitive interaction of OTA and monoclonal antibodies with the surface of specifically immobilized OTA. The indirect competitive assay was based on immobilization of OTA conjugated to BSA or OTA connected to polyethylene-based linker (PEG) to a Biacore chip. Simultaneous addition of OTA and monoclonal antibody specific to OTA (mAb) resulted in SPR signal due to binding of mAb to a surface. With increased concentration of OTA, more mAb bind to free OTA in comparison with that at the surface. Thus SPR signal was indirectly proportional to the OTA concentration. Approx. ten fold better detection limit was obtained for OTA immobilized to a surface through PEG linker (1.5 $\mu\text{g/L}$) in comparison with OTA-BSA conjugate. This has been attributed to higher density of OTA at surface. The PEG linker was also important in providing less nonspecific interactions of species with the sensor surface and allowed substantially improving surface regeneration (up to 600 binding cycles) using 5 M guanidine in 50 mM glycine (pH=2). The assay as well as surface regeneration was performed in a flow format (25 $\mu\text{L/min}$). Using gold nanoparticles of a diameter 40 nm conjugated to anti mouse IgG it was possible to amplify the SPR detection reaching LOD $0.042 \pm 0.004 \mu\text{g/L}$. The sensitivity is higher in comparison with requirements of allowable contamination of foodstuffs indicated by food and environmental agencies. The method was successfully applied for detection OTA in oat, corn, white and red wine, grape and apple juice spiked samples. Despite rather high detection limit there is still disadvantage consisting in indirect assay and in necessity to use OTA-mAb conjugates.

Most recently the direct SPR biosensor for OTA detection was reported by Zamfir et al. [59]. The monoclonal antibodies specific to OTA were immobilized at SPR chip surface using magnetic beads. The addition of OTA resulted in increase of SPR angle. The detection limit obtained (0.94 $\mu\text{g/L}$) is higher in comparison with previous indirect approach, but still sufficient for practical applications.

QCM method belongs to powerful tools for analyzing the affinity interactions at surfaces. The method is based on using specially cutting quartz crystal (AT cut) and modification of its one side by sensing layer. At certain frequency, typically between 5 and 10 MHz the shear oscillation of the crystal takes place. The resonant frequency of the oscillations is indirectly proportional to the mass of the sensing layer [60]. It should be, however, note that in a liquid the crystal oscillations are affected by viscosity forces, which resulted in damping of the acoustic wave amplitude [61, 62]. However, in certain cases the measurement of only oscillation frequency can be useful for analytical purposes. The advantage of QCM is in much lower price of instrument in comparison with SPR and in easy operation. QCM immunosensor for detection OTA by indirect assay was reported in paper by Tsai and Hsieh [63]. They immobilized anti OTA Ab at the surface of 16-mercaptohexadecanoic acid (16-MHDA). Then the OTA conjugated with BSA in a solution containing free OTA in different concentrations was added to a QCM surface. This competitive assay exhibited a working range of 50–1,000 $\mu\text{g/L}$ and a detection limit of 16.1 $\mu\text{g/L}$. The sensor was applied to several real samples with recovery in a range of 76–142%.

The detection limit is lower in comparison with SPR method. However, amplification by nanoparticles could improve this.

EIS method has been so far extensively used for detection affinity interactions in biosensors [64]. It is based on high sensitivity of impedance to the surface modification. The sensitivity is substantially enhanced at presence of redox probe, for example $[\text{Fe}(\text{CN})_6]^{-3/-4}$. At certain redox potential the probe provides enhanced electron exchange between the probe and the electrode surface. EIS allowing determination of the charge transfer resistance R_{ct} that is measure of the intensity of charge transfer. Higher electron transfer corresponds to lower R_{ct} and vice versa. The modification of the sensor surface resulted in changes of R_{ct} . Addition of analyte also yields in R_{ct} changes and may be affected by analyte charge. For example, because OTA is negatively charged at neutral pH, its adsorption to a sensor surface will result in repelling the redox probe. Thus, increased concentration of OTA will cause increase in R_{ct} . This approach has been used in recently reported OTA immunosensors, which differ mostly in the method of immobilization of OTA specific antibodies. Chronologically, the EIS sensor based on OTA specific Ab immobilized on a surface of chitosan-polyaniline conducting polymer prepared by electropolymerization on a indium–tin-oxide (ITO) electrode was reported by Khan and Dhayal [65, 66]. The highest sensitivity of 1 $\mu\text{g/L}$ was obtained, which is comparable with direct SPR assay. Shortly after these works Radi et al. [67] published paper in which Ab was immobilized on a gold surface using carbodiimide chemistry (LOD 0.5 $\mu\text{g/L}$). Rather high sensitivity (LOD 6 ng/L) was obtained with EIS immunosensor in which the OTA specific antibodies were immobilized on nanostructured zinc oxide deposited onto ITO covered glass plate [68] or using carbon nanotubes as immobilization matrix (LOD 2.5 nM/L) [69]. Comparable LOD (0.01 $\mu\text{g/L}$) was obtained in Ref. [58] in which Ab were immobilized by means of magnetic beads. Rather high sensitivity of EIS biosensor based on platinum electrode with electropolymerized sulfonated polyaniline with incorporated polyclonal anti OTA antibody was reported most recently [70]. The detection limit indicated 10 pg/kg , however seems to be overestimated considering the sensor response at the OTA concentrations studied 2–10 $\mu\text{g/L}$. The problem arises also with calibration curve presented in this paper as a plot of charge transfer resistance, R_{ct} , vs OTA concentrations. While Nyquist plot indicates the decrease of R_{ct} value, the calibration plot revealed opposite direction. From Nyquist plot it is also evident saturation of R_{ct} at higher OTA concentrations, but in a corresponding concentration range the R_{ct} value is presented as a linear plot vs. OTA content. In most works the analysis was performed also in real samples with minor matrix effect. However in certain cases the discrepancy between the concentrations of OTA in samples provided by vendor was substantially different in comparison with that determined by EIS biosensor and ELISA test. For example see [70] for detection OTA in roasted coffee and wheat. Thus, EIS immunosensors are rather useful and sensitive tools for detection OTA. Unfortunately in most cases the sensors were disposable without possibility of surface regeneration. Another disadvantage of immunosensors is necessity of rather laborious and expensive preparation of monoclonal antibodies and hapten conjugates. Antibodies are unstable in multiple uses. As an

alternative the DNA aptamers are of high promising receptors in biosensing applications that could replace antibodies. In a next section the biosensors based on these novel biopolymers sensitive to OTA are presented.

10.2.4 Aptamer Based Biosensors for OTA Detection

Biosensors based on DNA aptamers (aptasensors) are of growing interest due to their high sensitivity and selectivity [71–75]. This is particularly due to unique properties of DNA or RNA aptamers – the single stranded nucleic acids with high affinity to proteins or to other low and macromolecular compounds, which is comparable with that of antibodies. In contrast with antibodies, aptamers are synthesized *in vitro* by the SELEX procedure [76, 77]. Aptamers are thermally stable, reusable and once selected they can be produced by chemical synthesis in necessary quantity by means of conventional oligonucleotide chemistry. Aptamers can be chemically modified by biotin, thiol or amino groups, allowing them to be immobilized on various solid supports. In contrast with antibodies aptamers are more stable and the aptasensors can also be regenerated. This opens new routes for construction of biosensors for practical applications, for example for diagnosis purpose in medicine or for detection toxicants in food or in the environment. Recently the DNA aptamer sensitive to OTA has been developed [78]. This aptamer has the following oligonucleotide sequence: 5' GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA 3'. The analysis of this sequence using mfold program [79] shows two structures containing loops that slightly differ by Gibbs energy. Energetically more favorable structure ($\Delta G = -1.2$ kcal/mol) contains loop between 16 and 28 nucleotides. Larger loop was found for second structure ($\Delta G = -0.88$ kcal/mol). The analysis of the aptamer structure using the QGRS Mapper program predicting the existence of guanine quadruplexes [80] we showed that the OTA sensitive aptamer contains one guanine quadruplex connected by loops [81]. The existence of quadruplex is supported also by our recent data on the study of OTA aptamers thermodynamic properties [82]. The phase transition temperature for most stable aptamers at presence of 20 mM Ca^{2+} has been $48.3 \pm 0.5^\circ\text{C}$, which is close to the melting temperature of DNA aptamers sensitive to fibrinogen binding site of thrombin that also contain one guanine quadruplex. For this aptamers the quadruplex structure has been well established using various methods including circular dichroism (CD) [83]. Most recently the existence of quadruplex in OTA aptamer has been approved by CD method [84]. The changes in Gibbs energy for OTA aptamers determined from melting data was 3.8 kcal/mol, which is higher in comparison with that obtained from mfold program. However, this program does not taking into account the quadruplexes, which are rather stable also thanks to stabilizing role of K^+ ions.

The binding site for OTA in the aptamers is not known yet. However, as it has been shown in original work by Cruz-Aguado and Penner [78] this aptamer has rather high affinity and selectivity to OTA. The affinity substantially increases at presence of 20 mM Ca^{2+} (constant of dissociation $K_D = 49$ nM). At the same time,

no binding of OTA was observed without calcium or magnesium ions. However, we have recently shown that OTA sensitive aptamers modified by thiol groups and immobilized at gold surface by chemisorption can bind OTA even when no Ca^{2+} is present. Moreover, the constant of dissociation is lower in comparison with that in solution, which is evidence of improved affinity properties of the aptamers at the surface [81].

According to WOS database the first aptasensor for OTA was reported most recently and utilized electrochemiluminescence method of detection (LOD 17 pM) [85]. The sensitivity of chemiluminescence method of detection OTA by aptasensor was recently substantially improved by using Fe_3O_4 based magnetic nanoparticles (MNPs) and upconversion nanoparticles (UCNPs) as sensitive labels [86]. The assay was based on immobilization of aptamer DNA 1 sequence onto the surface of MNPs, which allowed capturing and concentrating OTA from bulk samples. The aptamer DNA 1 sequence then hybridized with UCNPs modified with DNA 2 sequence, which could dissociate from DNA 1 and result in a decreased luminescent signal when aptamer DNA 1 recognized and bound to OTA. Under the optimal conditions, the decreased luminescent intensity was proportional to the concentration of OTA in the range of 0.1 ng/L to 1 $\mu\text{g/L}$ with a detection limit of 0.1 ng/L (0.25 pM). This method allowed measurements of OTA in naturally contaminated maize samples.

Aptasensor utilizing amperometric detection based on methylene blue (MB) as a redox probe has also been reported [87]. In later case the effective detection range of OTA was 0.25–49.5 nM (sensitivity of detection: 74.3 pM). Such a high sensitivity has been achieved by signal amplification using gold nanoparticles. Three DNA oligonucleotides were used: 12-mer DNA 1 modified by amino group at 3' end was immobilized to the activated surface of glassy carbon electrode (GCE). The aptamer, 36 – mer DNA 2, containing complementary part to DNA 1 was then added and allowed to hybridize with DNA 1. Finally, 12-mer DNA 3 thiolated at 5' end containing complementary part at 3' end and modified by gold nanoparticles at 5' end was added and allowed to hybridize with DNA 2. Addition of MB, that selectively binds to guanine residues posses well resolved CV signal with two redox peaks at -0.23 and -0.18 V vs. saturated calomel electrode (SCE). Addition of OTA resulted in folding of the aptamers into 3D configuration and caused removing of the DNA 2 and DNA 3 from the sensor surface. Because MB was bounded mostly with DNA 2 this removal also caused decrease of the amplitude of redox peaks that served as analytical signal. The sensor selectively detected OTA in comparison with aflatoxin B. However slight interaction was observed with OTA analogue – ochratoxin B. Sensor was validated in a red wine with a good recovery in a range of 95–110%.

Further the electrochemical aptasensor based on indirect and direct competitive assay (LOD 0.27 nM) has been developed [88]. In an indirect assay the biotin-OTA conjugates were immobilized on a surface of magnetic beads coated by streptavidin. The magnetic beads were attached to the SPCE by magnet. The competitive assay was performed by addition of various concentration of OTA in a buffer containing fixed concentration of OTA-sensitive aptamers conjugated with HRP. The presence of HRP was detected chronoamperometrically at presence of the H_2O_2 , substrate of HRP at

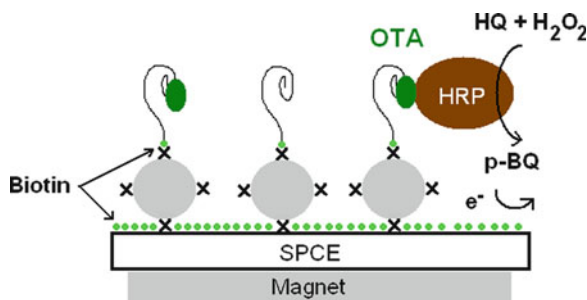


Fig. 10.3 The scheme of direct competitive assay based on biotinylated aptamers immobilized at magnetic beads covered by streptavidin. Competition between OTA and OTA-HRP was detected amperometrically by DVP method (for explanation see the text) [90]

potential -0.2 V vs. Ag/AgCl electrode. The amplitude of current was proportional to a surface density of HRP and indirectly proportional to the concentration of OTA in a solution. The LOD in an indirect assay ($1.1 \mu\text{g/L}$) was similar to that obtained in competitive indirect immunoassay [89]. In a direct assay the aminated aptamers were immobilized in a magnetic beads coated by carboxylic acid using carbodiimide chemistry. The beads were attached to a surface of SPCE by magnet. Free OTA was added in a solution containing fixed concentration of AP-OTA conjugates. The surface density of AP was measured by differential pulse voltammetry (DVP) at presence of the non-electroactive substrate 1-naphtyl phosphate (1-NP). 1-NP has been dephosphorylated by AP into electroactive 1-naphtol, which was oxidized at electrode to 1-iminoquinone. Oxidation current was measured at the range 0 – 0.4 V vs. Ag/AgCl. Similarly like for indirect assay the amplitude of current was indirectly proportional to the concentration of OTA. The indirect assay was ten fold more sensitive in comparison with indirect one (LOD $0.11 \mu\text{g/L}$ or 0.27 nM). The sensitivity of this sensor was validated in spiked wine with a good recovery in a range 94 – 97% .

Slightly improved sensitivity (LOD 0.17 nM) has been obtained with aptasensor based on the similar magnetic beads technology, but instead of aminated the biotinylated aptamers were used for modification of streptavidin coated magnetic beads and HRP-OTA conjugates were used in a direct DVP assay [90]. In this work indirect and direct competitive assay has been used. The best sensitivity was obtained using direct competitive assay using streptavidin coated magnetic beads with immobilized biotinylated aptamers. (biotin has high affinity to streptavidin, $K_D \sim 10^{-15} \text{ M}$). The competitive binding of OTA and OTA conjugated with HRP (OTA-HRP) to the aptamers adsorbed at the magnetic beads surface took place. By means of magnet the beads were separated from unbounded compounds. Then the beads were immobilized at the surface of SPCE with help of magnet placed at the bottom part of the electrode. The detection of OTA was performed by DVP at the potential -0.125 mV vs. Ag/AgCl. At this potential the maximal current was observed due to the electron transfer between the electrode surface and the p-benzoquinon (p-BQ) – the product of enzymatic degradation of hydroquinon (HQ) (at presence of H_2O_2) (Fig. 10.3).

The current was inversely proportional to the concentration of OTA. Authors also confirmed that at presence of 20 mM Ca^{2+} the signal increased by approx. 12%

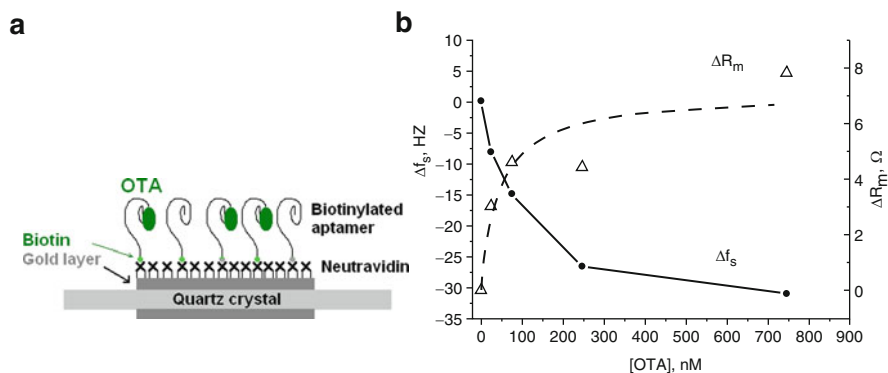


Fig. 10.4 (a) The scheme of immobilization biotinylated aptamers on a thin gold layer of the quartz crystal transducer covered by neutravidin. (b) The representative plot of the dependence of the changes of resonant frequency, Δf_s , and motional resistance, ΔR_m , as a function of OTA for TSM sensor. Dashed line is the fit according to Langmuir isotherm (Eq. 10.1) [82]

due to improved binding of OTA to the aptamers. At presence of Ca ions negatively charged OTA probably easier binds to the negatively charged aptamers. The binding of OTA was selective. Approx. 100 fold less binding took place for ochratoxin B and structural components of OTA – L-phenylalanine and warfarin. The sensor was validated in OTA containing wheat standard with recovery ranged from 102% to 104%. The sensor revealed higher sensitivity in comparison with immunosensor utilizing similar detection method [91]. The disadvantage of the sensor consisted in necessity of using enzyme conjugates as well as in possible non-specific interactions of conjugates with SPCE.

The direct, one step detection of OTA would be, however, more advantageous for practical applications. Most recently the simple colorimetric method of OTA detection has been reported [84]. In this work the gold nanoparticles were modified by OTA sensitive aptamers. Addition of OTA resulted in removal of the aptamers from the surface of nanoparticles and after addition of salts the changes in color has been observed due to nanoparticle aggregation. This method allowed detection of OTA with LOD of 20 nM.

In our recent work we reported acoustic aptasensor for detection OTA using biotinylated aptamers that have been adsorbed on a surface of a thin gold layer of quartz crystal transducer covered by neutravidin (neutravidin, similarly to streptavidin has very high affinity to biotin) (Fig. 10.4a). This sensor allowed direct detection of OTA with LOD 30 nM [82]. In this method we used network analyzer for measurement changes of series resonant frequency, Δf_s , and the so-called motional resistance, ΔR_m . The later value is sensitive to the viscosity contribution due to the friction between the sensing layer and the buffer. This approach is important especially for detection molecules with relatively low molecular mass like OTA. For such molecules changes of thickness in a sensing layer are negligible. However due to negative charge, OTA can alter the surface properties. This has been certainly confirmed. Addition of

OTA to the sensor surface resulted in increase of the R_m and decrease in resonant frequency (Fig. 10.4b). The shape of the frequency and resistance changes is typical for Langmuir adsorption isotherm. This means that the OTA binds to the aptamers independently. The binding of OTA to the aptamers can be quantitatively characterized by Langmuir equation [24]: For example for changes of R_m one can write:

$$-\Delta R_m = -(\Delta R_m)_{\max} \left[c / (K_D + c) \right] \quad (10.1)$$

where $(\Delta R_m)_{\max}$ are the maximal changes of the motional resistance. The K_D value is a measure of the affinity of OTA to the aptamers at the sensor surface. Using Eq. 10.1 and the least square method the K_D value has been determined as $K_D = 43.9 \pm 30$ nM. This value is in good agreement with that reported in Ref. [77] for free aptamers in a volume at presence 20 mM Ca^{2+} (fluorescence detection of OTA, $K_D = 49 \pm 3$ nM). The limit of detection (LOD) for acoustic sensor was 30 nM. It has been determined using common criteria of significant analyte determination at the level corresponding to signal to noise ratio, $S/N=3$. The obtained LOD was comparable with QCM immunosensor based on indirect competitive detection method using OTA specific antibodies [63].

Another approach for direct detection of OTA is based on EIS electrochemical aptasensors. The results in development of such aptasensor for detection OTA utilizing thiolated OTA specific aptamers chemisorbed on a gold surface have been presented by us in AISEM conference in February 2011 [92]. The approach is similar to those for EIS immunosensor at presence of redox probe $[\text{Fe}(\text{CN})_6]^{-3/-4}$. As we mentioned above, the EIS method can sensitively monitor the changes of charge transfer resistance, R_{ct} , due to the alterations at the sensor surface. The binding of negatively charged OTA to the aptamers resulted in increase of negative surface charge and in repealing of the redox probe from the surface. This causes increase in R_{ct} value. At the same time other parameters of the circuit such as capacitance and Warburg impedance changed only slightly. The changes in R_{ct} can serve as an analytical signal. This value sharply increased at relatively low OTA concentration range 0.1–3 nM with saturation at concentrations approx. 100 nM. This dependence had the shape of Langmuir isotherm and can be described by the equation analogical to that presented above (Eq. 10.1). The analysis of this dependence allowed to obtain $K_D = 8.3 \pm 0.8$ nM [81]. This value is lower in comparison with that determined by fluorescence method for free aptamers in a volume [78] as well as that determined by acoustic method [82]. But in EIS sensor the aptamers have been immobilized by different method, using chemisorption, which may affect the aptamers affinity properties. This biosensor exhibited comparable sensitivity with above-mentioned indirect assay (LOD 0.4 nM) and selectively detected OTA. The sensor was regenerable in 1 mM HCl and successfully validated in coffee and flour with recovery of 88% and 104%, respectively for spiked samples containing 10 nM OTA [81, 92]. Most recently the EIS biosensor based on DNA aptamer specific to OTA covalently immobilized onto mixed Langmuir–Blodgett monolayer composed of polyaniline–stearic acid and deposited on ITO coated glass plates has been reported [93]. This sensor revealed similar detection limit (0.24 nM), however the fabrication procedure has been more

complicated in comparison with simple chemisorption used in our work. The sensor discriminated between OTA and aflatoxin, however it has not been validated in real food samples. In addition the R_{ct} changes were of opposite direction, i.e. with increased OTA concentration the R_{ct} value decreased at presence of redox probe. This is in contradiction with already published papers.

Thus, the aptasensors are of high perspective even for detection small molecules such are OTA. The sensitivity of detection in most cases is similar to those of antibodies. Substantial advantage of aptasensors is possibility of surface regeneration which allowing their multiple use. Moreover, recent papers on application SPR, thickness shear mode acoustic method and EIS are evidence of possibility of direct detection OTA without using OTA-protein conjugates or other labels.

10.3 Conclusion

The biosensor technology is certainly powerful tool for detection food mycotoxins such as ochratoxin A. The achievements reported in this review revealed that most of the approaches allowing detect OTA with high sensitivity, which is better than allowable contamination of food by this toxin. Rather perspective direction in biosensor development consisting in application of DNA aptamers. Using these novel biopolymers even most sensitive biosensor assay was demonstrated, allowing detection of OTA with LOD 0.25 pM. Direct detection methods such are acoustic and electrochemical impedance spectroscopy are rather challenging, due to fast response and high sensitivity which is substantial advantage over traditional methods such are HPLC or mass spectroscopy. We believe that further effort should result in appearance of low cost, portable and easy to use biosensor for detection OTA and other toxins applicable in food factories and agricultural farms.

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Chapter 11

ATP Measurement in Bio-Contamination

Golnaz Borghei and Elizabeth A.H. Hall

Abstract One method to evaluate cell viability is by measuring mitochondrial activity and ATP cell content. Thus, ATP can either provide information on the presence of microbial contamination or be an indicator of cytotoxicity, since it declines very rapidly when cells undergo necrosis or apoptosis. Methods to measure the presence of ATP have been explored, based on different measurement techniques but ATP bioluminescence is presently the most sensitive rapid method for detection of bacterial contamination. Some efforts to improve the assay have adopted protein-engineering techniques to achieve better pH stability and longer wavelength emission. Fortuitously these mutants also show good spectral overlap with the fluorescence proteins that emit in the red region of the spectrum so that a BRET construct can be designed that shifts the emission further to longer wavelengths. With such a format, a ratiometric measurement based on the luciferase/luciferin: fluorescent protein emission can be correlated with ATP concentration. Typically a range of 10^{-7} to 10^{-10} M ATP can be measured under optimized conditions. Better sensitivity can be demonstrated by amplifying the ATP in an enzyme-recycling pathway, by using, for example, adenylate kinase and polyphosphate kinase. This can result in 10,000-fold amplification of ATP. To put this into context, the average cultivable microbial population in the samples from ordinary rooms is in the range 70–560 CFU ml⁻¹ or $40\text{--}3,400 \times 10^{-15}$ mol ml⁻¹ ATP.

Keywords Adenosine triphosphate • ATP • Measurement • Assay • Detection • Bioluminescence • Resonance energy transfer • RET • BRET • Fluorescent proteins • Microbial contamination • Protein engineering

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11.1 Introduction

Adenosine triphosphate (ATP) is the universal energy source for cellular function in almost every type of biological cell and plays a critical role in energy exchange [1]. Development of ATP biosensors has always been of high importance due to its central role in cellular metabolism and extracellular signaling in a number of tissues including blood and brain [2]. ATP concentration levels can provide valuable information in the analysis of cellular mechanism, enzymatic processes, sensory transduction (such as pain and thermosensory), biosynthesis, enzymatic processes, neurotransmission and cardiovascular performance [1–3]. In a different format, one of the most popular methods to evaluate cell viability is by measuring mitochondrial activity and ATP cell content [4]. ATP concentration declines very rapidly when cells undergo necrosis or apoptosis and this is used as a tool for identifying cytotoxicity [5, 6]. Such techniques are receiving considerable attention as possible substitutes for *in vivo* testing [7].

However, elevated ATP levels can also be used just to indicate the presence of microorganisms and somatic cells in environments where their presence would have a harmful effect. For example, in food and water safety, toxicity and contamination analysis, the presence and growth of microorganisms are widely analysed by an ATP test and it has been shown that measurement of ATP concentration is an effective technique to analyse the microbiological quality of water and therefore health safety [8]. Since water can be used as an ingredient or cleansing product, it may be used directly or indirectly in the food and pharmaceutical industry, or as part of the industrial and manufacturing process such as cooling and transport [9]. Even where its usage is seemingly indirect the evidence shows that there can be a significantly higher bacterial contamination where non-drinking water is used for washing. For example, Vilar et al. [10] showed that farms using private wells (~70%) for hosing down their milking parlors had significantly higher bacterial counts (as measured via ATP level) on the milk receivers than those using chlorinated water.

Microbial contamination may also be deliberate. According to the U.S. Center for Disease Control and Prevention (CDC): “A bioterrorism attack is the deliberate release of viruses, bacteria, or other germs (agents) used to cause illness or death in people, animals, or plants ...” [11]. Thus, methodology that is appropriate for routine hygiene control can also be used to warn of bioterrorism attack and because ATP is present in all active microorganisms, monitoring ATP levels can be useful for predicting the active biomass content. However, key factors, irrespective of the source (or purpose) of the contamination are speed and relevance of result. Thus, questions that arise are how ATP can be measured effectively and how the result correlates with the level of bacterial contamination (probably in terms of colony forming units, cfu). This review looks at ATP assay methodology and considers its relevance to measurement of bacterial contamination.

11.2 Methods of ATP Measurement

11.2.1 *Electrochemical Techniques*

Electrochemical biosensors have been investigated as simple and low cost detection techniques [2, 12, 13] showing a fast response and portability, sometimes without pretreatment of the sample [14, 15]. So far a number of electrochemical sensors for a polyphosphate anion species have been developed [3, 12, 16]. For example since the electrochemistry of the nucleotides can be distinguished, a method can be developed that exploits the selective determination of the purine adenine polyphosphate [12]. However, this technique still suffers from the main drawback of limit of detection, which is in the order of 70–200 nM for ATP.

In general, these electrochemical methods have suffered from high background signal and irreversible adsorption on the electrode, which results in decrease in sensitivity with use [17]. However, Singhal et al. demonstrated one of the first direct electrochemical measurements of purine nucleotides, with microelectrodes by sinusoidal voltammetry [13, 18]. Compared to some of the other electrochemical methods, this ATP detection technique presently shows relatively high sensitivity and selectivity.

An alternative approach to electrochemical measurement has been to employ a biosensor format with a dual or multi-enzyme structure [17]. In a number of reports, measurement of ATP has been based on phosphorylation of glucose to glucose 6-phosphate. The reaction is ATP dependent and thus the reduction in glucose is related to ATP concentration [2]. Schubert et al. have developed a dual enzyme layer which consists of glucose-6-phosphatase dehydrogenase and hexokinase (HEK) [19] and other enzyme combinations can also achieve the ATP dependence that leads to its measurement. For example, glucose oxidase and hexokinase [15, 17] or glucose-6-phosphate dehydrogenase, pyruvate kinase, and hexokinase [20]. Similarly, Llaudet et al. have introduced an ATP biosensor using a Pt microelectrode with a thin biolayer containing glycerol kinase and glycerol-3-phosphate oxidase. The enzymes have been entrapped in a robust and porous silicate layer around the microelectrode. Therefore, ATP can be detected in the presence of glycerol with a response time of 10 s and in the range of 200 nM to 50 μ M. Llaudet's sensor has rapid response, high temporal and spatial resolution and therefore could be suitable for ATP signaling during physiological activity [2].

A necessary control is the substrate concentration for the chosen enzyme (e.g. glucose, glycerol etc.), since this also effects ATP response [15, 17] and although these ATP measurement techniques are generally known as simple, rapid and low cost, the high limit of detection, of the order of 200–300 nM ATP, remains a drawback.

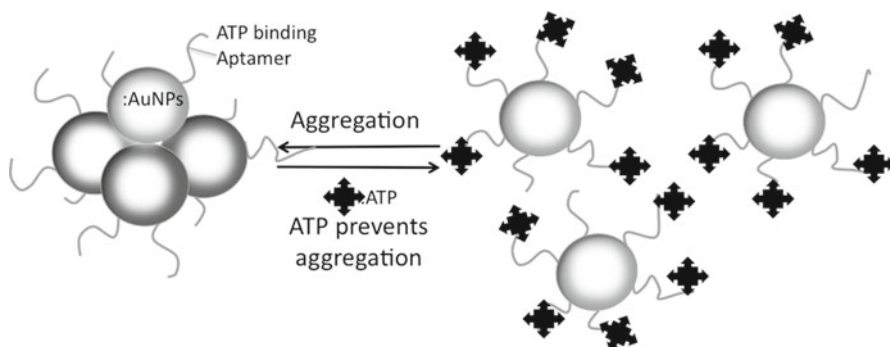


Fig. 11.1 Sensing mechanism of Apt-Au NPs for the colorimetric determination of ATP [24]

11.2.2 Optical Techniques

11.2.2.1 Chromogenic and Colorimetric Methods

One of the simplest (and oldest) analytical methods produces a colorimetric result that can often be read by eye [21]. Sancenon et al., have developed a family of easy-to-prepare chromogenic reagents for uv/vis spectrophotometric detection of ATP in an aqueous-organic environment. This can be used as a naked-eye, simple technique which results in a highly selective color response in the presence of ATP. The main drawback of this system is significant interference of sulfate anions with the ATP response [22]. However, in a recent work by Mahato et al., two nontoxic chromogenic complexes were used to bind to ATP selectively under controlled condition. These staining agents have been used for studying the cell growth and to mark out different strains of bacteria [1].

11.2.2.2 Agglutination Methods

Agglutination methods causing an optical response have become popular in immunoassay, but can also be configured for ATP. For example, the optical properties of gold nanoparticles have been used for ATP detection in several reports [23, 24]. Chen et al. introduced an aggregation-based aptamer-modified gold nanoparticle (Apt-Au NPs, Fig. 11.1) sensor, where the color of Apt-Au NPs solution changes as a result of salt-induced aggregation, whereas ATP binding to the analyte prevents the aggregation of the Au NPs. The limit of detection for ATP is 10 nM in urine samples.

11.2.2.3 Enzyme Linked Assays

Probably the most promising and most studied approach to ATP measurement has been enzyme linked. Since ATP is critical in numerous enzyme pathways, there are

many possibilities to exploit assays designed for a selective enzyme substrate, reformatted to be limited by the ATP concentration rather than the substrate concentration. For example:

NADH-Linked Assay

In a report by Karon et al., an assay for simultaneous measurement of calcium uptake and ATP hydrolysis has been developed. Visible light absorption has been used to see the rate of Ca uptake and ATP hydrolysis in sarcoplasmic reticulum (SR) vesicles. Absorption of Fura Red (a calcium indicator) and NADH at 490 and 340 nm, respectively, has been used to calculate ATP production rate [25]. A similar more direct assay uses ATP hydrolysis measured by NADH enzyme-linked ATPase assay [26]. This technique is based on ATP hydrolysis coupled to the oxidation of NADH, which can be monitored continuously. NADH has absorbance at 340 nm, and by consumption of NADH, the rate of ATP hydrolysis can be monitored in real time. However ATP hydrolysis can be measured only down to micromolar concentration [27].

5'-Nucleotidase Linked Assay

An alternative enzyme linked ATP dependent assay could be to use 5'-nucleotidase [28]. This is based on the change in UV absorbance following the enzymatic reaction between a phosphate donor and a nucleoside by 5'-nucleotidase. It is adaptable for other nucleoside analogs as substrates. However, it also suffers from low selectivity and poor limit of detection. Furthermore, separation of product and substrate by HPLC and then quantification by UV is needed. In the work done by Fujitaki et al., this technique has been successful for nucleoside monophosphates, however the adaptability to other nucleotides, including ATP, has been suggested.

Bioluminescence

The most widely used technique to measure ATP employs luciferin-luciferase due to its high sensitivity and selectivity. The firefly luciferase catalyses a two-step reaction that requires substrates firefly luciferin (LH2), Mg, ATP, and oxygen resulting in production of yellow-green light [29]. The luciferase enzyme reaction has fast response (milliseconds) and a broad range for ATP detection. Since other adenosine-containing nucleotides such as AMP or ADP do not react with the enzyme, this reaction is highly specific to ATP. The sensitivity is extremely high, because the background signal is theoretically zero [3]. However, although luciferase can be measured down to the attomole level using photomultiplier tubes (PMTs) or charge-coupled devices (CCDs) [30, 31], the luciferin-luciferase method still requires highly sensitive instrumentation to reach sufficient sensitivity and resolution [3]. Furthermore, the background signal of extraneous light needs to be precisely

monitored and eliminated [9]. Despite these drawbacks, bioluminescence seems to have become the method of choice for ATP measurement. Attention has thus been given to seeking approaches to improve this method.

11.3 Improvement of the Bioluminescent ATP Method

11.3.1 *Engineering the Luciferase*

The main weak points of the luciferase enzyme are: short wavelength and instability with pH and temperature. To overcome these drawbacks, the isolation of novel luciferases from newly discovered organisms is being carried out and new luciferases with red emission [32] or higher stability [33] have been discovered.

Genetic engineering and mutagenesis is an alternative tool to improve the shortcomings of firefly luciferase. However, the mutations on luciferase enzymes have not been as diverse and varied as other luminescent proteins like fluorescent proteins [34]. The main mutagenesis has been focused on shifting the emission to longer wavelength (Fig. 11.2). Emission above 600 nm has a lower background due to less light absorption and scattering, therefore is more suitable for imaging [30, 35, 36]. Other improvements have tried to alter the pH stability and thermostability of the protein [37–41].

Branchini et al. have made significant progress on shifting firefly luciferase emission. They have developed an enzyme emitting at 615 nm, with a narrower emission bandwidth [36] however lower enzyme activity. In other work reported by the same group, red emitting firefly luciferases have been introduced with satisfactory specific activity and thermostability [30]. Other groups have also tried to shift the luciferase emission wavelength through different mutations. For example, Fahmi et al., incorporated a glycosylated tyrosine into firefly luciferase, which resulted in 18 nm red shift of emission wavelength, but a drop in enzyme activity [42].

Among beetle luciferases, firefly luciferase shows a red-shift at acidic pH which accompanies a significant decrease in enzyme activity [37, 43]. pH stability, accompanied by red light emission, seems to be associated with thermal instability [40, 43] and in recent years several pH-insensitive firefly luciferases have been developed [44, 45] to overcome this. In addition to pH instability, thermo instability of firefly luciferase appears to be more problematic since the enzyme loses its activity in room temperature, and dramatically at 37°C. This has focused research effort to improve the thermostability of firefly luciferase through several rounds of mutagenesis [38, 39, 41].

Considering the low thermostability and pH tolerance of wild-type luciferase enzymes, the firefly luciferase engineered by Law et al., which replaced 5 solvent-exposed non-conserved hydrophobic amino acids by hydrophilic residues, has been particularly successful. The result is a novel mutant of luciferase with improved pH-tolerance and thermostability with the same activity of wild enzyme: x5-luc [37].

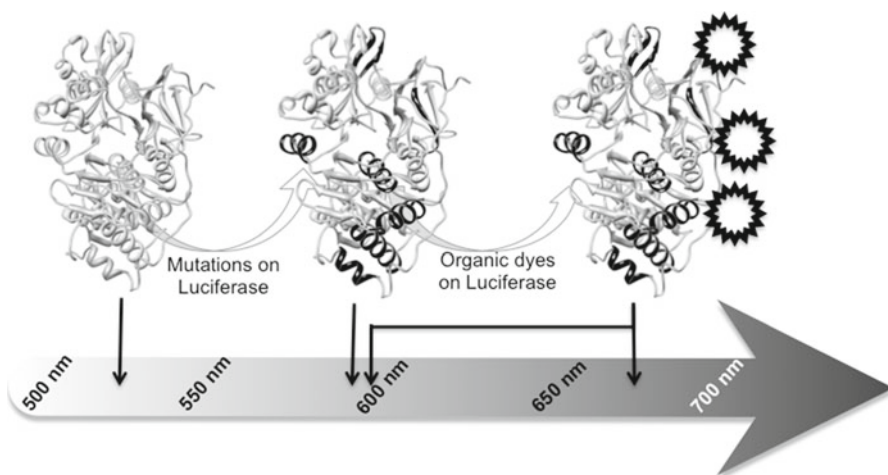


Fig. 11.2 The emission wavelength for the luciferase/luciferin reaction has been engineered by genetic manipulation through luciferase mutations and through chemical manipulation by attachment of organic dyes, permitting FRET

Despite the improvements achieved with engineering the luciferase, conventional luciferase-based ATP measurement methodology can suffer from drawbacks in that the wavelength of the luminescence of the product of the luciferase reaction is still mostly below 600 nm. Emission in the red to infrared (>600 nm) is preferred due to low absorption in tissues and in the presence of organic compounds [31, 46] (Fig. 11.2). Thus there is a high interest now in shifting the emission to higher near-infrared wavelengths.

11.3.2 *Bret-Based Bioluminescence*

For the last decade, Bioluminescence Resonance Energy Transfer (BRET) from a bioluminescence donor to a fluorescent acceptor has been widely used for a number of applications mainly *in-vivo* imaging and protein-protein interactions [46–49]. In 1999 the first BRET between *Renilla* Luciferase and EYFP was introduced for assaying protein interactions [50]. Shortly after the discovery of red fluorescent proteins, Arai et al. designed BRET between tetramer DsRed red fluorescent protein and firefly Luciferase for protein interactions [47] and other early examples used red fluorescent-tagged luciferase enzymes as the donor and receptor couple [46, 51]. However, low quantum yield of the fluorescent protein, resulted in a significant loss in the total emission of luciferase.

In 2007, Hoshino et al. introduced the use of a luciferase-fluorescent protein fusion as a probe. They used *Renilla* luciferase and an enhanced yellow fluorescent protein for real-time single-cell imaging [48]. These probes are of high interest due

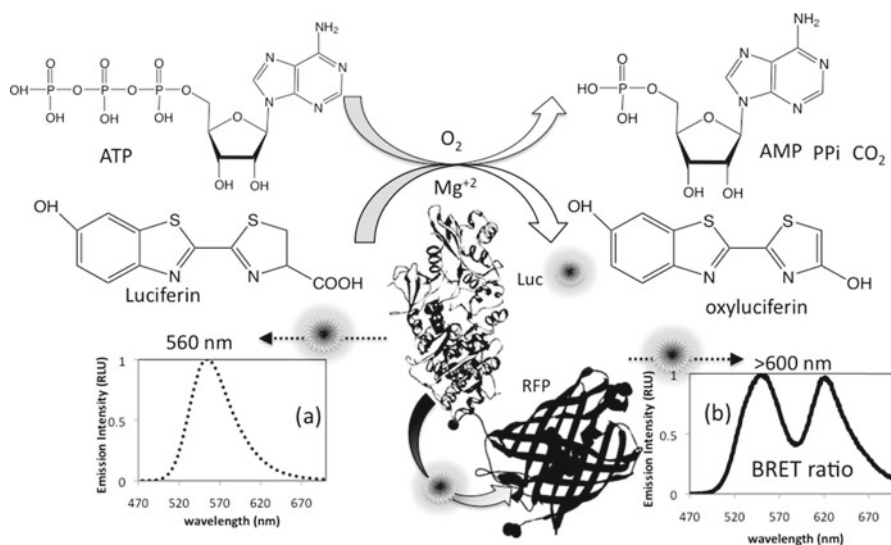


Fig. 11.3 BRET in Luciferase-Luciferin reaction: Luciferase acts as enzyme and donor, while RFP is the acceptor. ATP measurement via (a) the luciferase-luciferin signal; (b) the BRET ratio of signal due to the luciferase-luciferin donor and RFP acceptor

to their double functionality as both an auto-fluorescent protein and a fluorescent-tagged bioluminescent probe. They also lead to the idea of creating couples that bridge the protein-synthetic fluorophore barrier. For example, in a more complex combination Branchini et al. tried a ratiometric luminescent probe for protease activity, using sequential BRET-FRET. Their donor was a firefly luciferase variant that catalyzes yellow-green (560 nm maximum) bioluminescence and mKate fluorescent protein covalently labeled with a near-infrared fluorescent dye [52]. A family of BRET-based self-illuminating dyes and long wavelength emitting luciferase is an ongoing development using the conjugation of luciferase enzymes with organic dyes [53].

Another couple, with firefly luciferase covalently labeled with nIR fluorescence dyes, resulted in an efficient BRET and shift of spectral emission above 700 nm [54]. Although a number of near-infrared fluorescent proteins have been developed since 2002, mCherry (CH) remains one of the best monomeric red options due to its pH stability, high extinction coefficient, photostability and brightness [55, 56]. Fortuitously the mCherry provides a good acceptor for the more thermally stable luciferase engineered by Law et al. (see above) and work in our laboratory (unpublished) has coupled mCherry and x5-luc achieving a BRET-based probe to measure ATP (Fig. 11.3). In this technique ATP can be measured with more than 50% of the output light transferred to wavelengths above 600 nm. In addition, the BRET ratio (ratio of emission at secondary maximum peak to emission at first maximum peak) proves to be an alternative quantitative measurement of the substrate, independent

of accurate background determination and sensitivity. This is illustrated in Fig. 11.3: ATP is measured either in the conventional manner (Fig. 11.3a) or shifted to wavelengths above 600 nm and measurement can be achieved via the BRET ratio (Fig. 11.3b). This is particularly useful in media that absorb light with shorter wavelengths. Although BRET ratios result in a slightly higher detection limit, since the signal is divided between the two emission wavelengths, it can provide a more reliable measurement.

Furthermore, a fusion protein of mCherry and x5-luc (CL) shows greater pH stability than the individual proteins. This fusion protein, expressed at 37°C can also be used as a BRET-based ATP measurement probe. This suggests a number of potential applications for both *ex vivo* and *in-vivo* measurement of ATP with a dual color protein, possessing the properties of both bioluminescence of luciferase and fluorescence of fluorescent proteins. The designed probe has resulted in an emission wavelength shift, related to luciferase, of 70 nm. As discussed above, such a shift in wavelength is of high interest in application in complex media [30, 54]. Using this BRET-based fusion methodology, a range of 10^{-7} to 10^{-10} M ATP can be measured under optimized conditions.

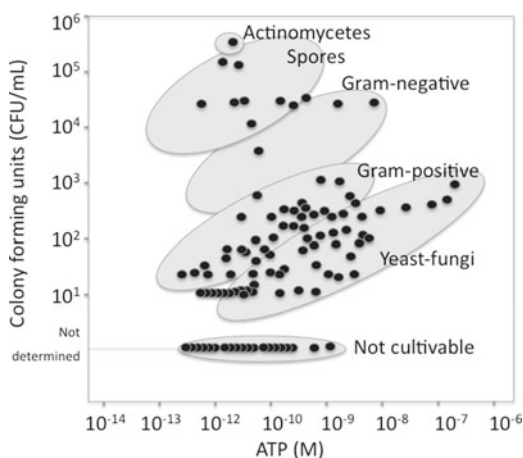
11.4 Correlation and Relevance

Conventional techniques to evaluate the microbiological contamination, include plate count agar, culturing microorganisms and the coliform test. For years the culture method has been the accepted technique for monitoring microorganism contamination. However, this method is slow, typically taking 12–72 h. In addition, there are some bacteria, which are difficult to culture and can yield false negatives under the wrong growth conditions. In comparison the ATP bioluminescence methods are an attractive alternative because they provide a real-time rapid assay [1, 8, 57–60].

However, what is the correlation between an ATP measurement and, say, viable colony forming units? This is an important question to enable benchmarking of the ATP assay and its proper use and interpretation [61]. In the first place ATP measurement may include both intracellular ATP and extracellular ATP, which has been produced by the living microorganism but released outside the cell due to stress or death. Properly configured, the ATP bioluminescence assay has the unique feature that it can distinguish extra- from intracellular ATP by the enzymatic hydrolysis (apyrase) of extracellular ATP prior to lysis of the cells. This hydrolysis step also reduces background signal and produces a more reproducible result. The distinction between extra- and intracellular ATP can be of great importance in looking at potential contamination in rooms, where notably the level of free extracellular ATP on floors, attributable to lysed, dead cells is typically 2 or 3 orders of magnitude greater than the intracellular values.

To put the ATP results further into context, the average cultivable microbial population in the samples from ordinary rooms is in the range 70–560 CFU ml⁻¹ or

Fig. 11.4 Comparing intracellular ATP measurement with colony count in different microorganisms (plot compiled with data from multiple sources)



$40\text{--}3,400 \times 10^{-15}$ mol ml^{-1} ATP. For the clean-room surfaces <70 CFU ml^{-1} are expected or $1\text{--}18 \times 10^{-15}$ mol ml^{-1} [62]. However, the ratio of ATP/CFU differs greatly from one sampling area to another, depending of the types of microorganisms that might be present. For example, a very low ATP/CFU ratio, might indicate Gram-negative bacteria or spores, whereas a high ATP/CFU ratio would point to gram-positive bacteria, yeasts and fungi (Fig. 11.4). As is clear from this figure, while the ATP method is sufficiently sensitive for contamination by certain microorganisms, it would be desirable to increase the sensitivity to target gram-negative bacteria. Certain approaches, that amplify the bioluminescent signal have been reported that may be adopted to achieve this [63]. For example Satoh et al. [64] have extended the assay by using adenylate kinase and polyphosphate kinase in an ATP enzyme recycling pathway, resulting in 10,000-fold amplification of ATP. This means that 10^2 CFU are easily identifiable (even for gram negative bacteria) and <10 cells are potentially detectable for other organisms.

In another approach Cheng et al. [65] have used antibody modified magnetic nanoparticles to selectively ‘collect’ *E. coli* cells prior to ATP bioluminescence measurement. This also achieves a second goal: the cells are collected and concentrated by the magnetic nanoparticles. The method combining the nanoparticles with ATP bioluminescence was applied to detect *E. coli* in pasteurized milk and apple juice, with a claimed detection limit of 20 cfu/ml.

11.5 Conclusions

ATP bioluminescence is presently the most sensitive rapid method for detection of bacterial contamination. Various formats for this ATP assay have emerged, all based on the luciferase enzyme and engineered variants. Possibly the most stable current luciferase enzyme is the x5-luc mutant produced by Law et al. In fusion with the

mCherry fluorescent protein the protein is more stable and produces an ATP signal that is partially shifted to longer wavelengths at the mCherry emission. This allows either a long wavelength ATP dependent assay or one based on BRET ratio. Taking this BRET protein combination a potentially powerful assay emerges that includes amplification methodology to determine microorganisms down to a few cfu, even for gram-positive bacteria.

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Chapter 12

Nanomaterials in the Cholinesterase Biosensors for Inhibitor Determination

Gennady Evtugyn, Ravil Younusov, and Alexey Ivanov

Abstract Cholinesterase biosensors based on different nanomaterials have been considered and discussed in terms of sensitivity and selectivity of irreversible inhibitor detection. The function and influence of various types of nanoparticles, i.e., carbon nanotubes, metal and metal oxide nanoparticles and polymeric materials are described and the future progress in the development of cholinesterase sensors is discussed. The principal attention is focused on the approaches to the increase of the sensitivity of inhibitor determination and improvement of the biosensor operation.

Keywords Acetylcholinesterase • Cholinesterase • Biosensor • Enzyme sensor • Inhibitor determination • Nanomaterials • Carbon nanotubes • Nanoparticles • Polyelectrolytes

12.1 Introduction

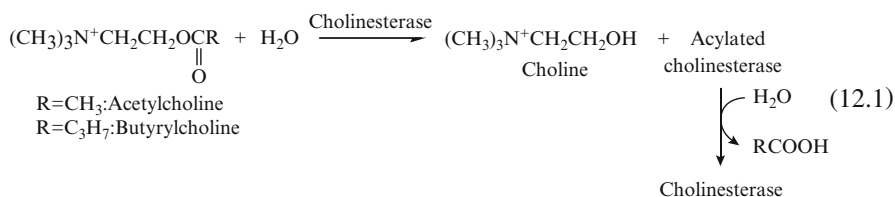
Cholinesterases are key enzymes with a significant role in the nerve impulse transduction. Damage of the enzymes, e.g., irreversible inhibition caused by chemical warfare or pesticides, may result in severe consequences for human beings including abdominal cramps, muscular tremor, hypotension, breathing difficulty, slow heartbeat and death. Synthesis of nerve gases with high acute toxicity caused by anticholinesterase effect at sub-ppb level called for the development of adequate analytical devices. They are devoted to the early detection of such compounds on seat of war as a part of individual bio/chem protection gears. Cholinesterases are

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rather similar for various vertebrates and show remarkable specific activity which simplifies their immobilization with no respect of the enzyme sources. Nevertheless, the development of appropriate biosensors applicable in field conditions with satisfactory operation stability and lifetime calls for further experiments directed both to the enhancement of their sensitivity toward irreversible inhibitors and amplification of the substrate signal. The use of nanomaterials as the immobilization matrices and signal amplifiers offers new prospects for the progress in the cholinesterase biosensors. In this review, the use of nanomaterials in the assembly of the electrochemical cholinesterase sensor since 2000 is summarized.

12.2 Biochemical Backgrounds

Cholinesterases are widely used not only in biosensor development but also in toxicology, neurobiology and pharmacology [1]. In accordance with the rate of the substrate hydrolysis, there are divided into two major groups, i.e. acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE, also called the true cholinesterase, is mainly present in the central neural system, and is bonded to the cellular membranes of excitable tissues and erythrocytes. BChE, a serum cholinesterase, is primarily found in plasma, liver and muscle tissues. Although both groups have a similar molecular structure, they differ in substrate and inhibitor specificity. AChE preferentially hydrolyzes acetylcholine (12.1), a natural neurotransmitter (1, R=CH₃) whereas BChE is more active in the hydrolysis of butyrylcholine (R=C₃H₇).

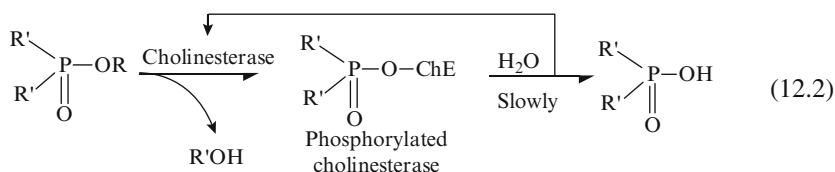


The active site of AChE involves anionic and esteratic centers which differ in their pH-dependent affinity toward the substrate, choline and H⁺ ions. The distance between the above centers does not change while free enzyme is acylated by acetylcholine. This follows rather small conformational changes of the enzyme and hence the high efficiency of the substrate conversion [2]. The anionic center of AChE lies at the bottom of a sterically restricted, hydrophobic cleft and remains open after acylation. The interaction of the anionic center with acetylcholine maximizes the rate of its hydrolysis whereas the interaction with the second substrate molecule suppresses the cleavage of the acetylated AChE and the enzyme recovery. For this reason, AChE exerts allosteric inhibition by a substrate excess. BChE differs from the AChE by the relative affinity of the active site toward the substrate/product and the pH dependence of the rates of the

reaction stages. Butyrylcholine is not involved in the allosteric regulation of the BChE activity, either.

Both AChE and BChE are widely used in biosensor assembly because they provide different sensitivity toward certain groups of irreversible inhibitors. First, BChE from horse and human serum was mainly applied in biosensor devices due to the high specific activity and the stability of preparations available at the moment [2, 3]. Later on, AChE from *Electric eel* and then from *Drosophila melanogaster* became dominating due to their higher sensitivity towards common organophosphate pesticides [4, 5]. In addition, genetically engineered ChEs have also been explored in biosensor applications [5–7]. Differences in the behavior of various groups of cholinesterases as well as the kinetic and thermodynamic aspects of their interaction with substrates and inhibitors have been reviewed [1, 6, 8–10]. However, it should be mentioned that some of the results especially those obtained with crude extracts or unpurified preparations can be affected by traces of carboxylesterases or other esterases. This might be one of the reasons for contradictions in the relative sensitivity of the inhibitor detection sometimes observed in the works with the enzymes manually isolated for biosensor assembling.

Potential nerve agents as well as carbamate and organophosphate insecticides irreversibly inhibit cholinesterase activity due to the formation of a covalent bond with the esteratic center of the enzyme (12.2).



Contrary to the acylated enzyme, the product of such an interaction is rather stable to hydrolysis and this leaves the enzyme out of the catalytic cycle. In some cases, spontaneous re-activation of the enzyme takes place. This can be accelerated by special reagents, e.g. oximes, called as antidotes and re-activators. However, even in this case the product of the reaction differs from the initial inhibitor. Along with a significant effect on enzyme activity, this fact differentiates between irreversible and reversible inhibitors, such as heavy metal ions, biogenic amines, and quaternary ammonia salts. The first stage of their reaction with cholinesterases assumes the reversible formation of an enzyme-inhibitor complex mainly aided by electrostatic interactions with the anionic center of the active site.

For quantification of irreversible inhibition, cholinesterase is first incubated in the inhibitor solution for a definite period of time called the incubation period. After that, the substrate is added to the same solution and the residual enzyme activity is measured by an appropriate method. If the concentration of an inhibitor exceeds that of the enzyme active site, Ellman equation (12.3) can be used [11]. Here, v_0 and v_t are the reaction rates of the enzymatic hydrolysis of a substrate prior to and after incubation, C_i the inhibitor concentration and τ the incubation period.

The bimolecular inhibition constant k_{ii} is a measure of the inhibition efficiency which depends on the nature of an inhibitor, the enzyme source and incubation conditions but not on the inhibitor concentration.

$$\ln \frac{v_0}{v_t} = k_{ii} C_i \tau \quad (12.3)$$

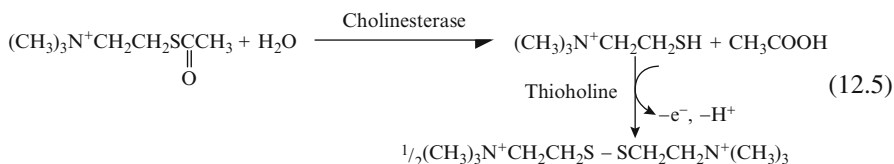
In some works related to the biosensor development, another form of Ellman equation (12.4) which expressed the dependence of the remained enzyme activity (a) on an inhibitor concentration is used for kinetic calculations.

$$\log a = \log 100 - \frac{k_{ii} \tau}{2.303} \quad (12.4)$$

However, the biosensor format of the inhibition measurement is commonly based on simplified schemes, assuming either the simultaneous addition of a substrate and an inhibitor or a single measurement of the enzyme activity after incubation stage. In the first case, inhibition kinetics corresponds to competitive inhibition which shows theoretically lower sensitivity toward an inhibitor. In the second case, the performance of the biosensor highly depends on the stability of the signal. For equal other measurement conditions, the same slope of calibration curve and somewhat higher limit of detection due to a worse signal-to-noise (S/N) ratio can be expected in comparison with the two-stage measurement protocol [12, 13].

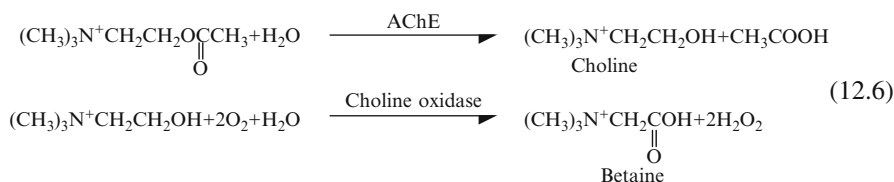
This should be taken into account when different biosensors are compared for the same inhibitor or the biosensor action specially discussed for field applications or semi-automatic control systems.

Prior to considering the nanomaterials used in electrochemical cholinesterase biosensors, let us briefly mention the approaches to the signal measurement. Two main protocols are most often used for the amperometric signal detection. The first one involves the registration of the oxidation current related to the thiocholine produced in the enzymatic hydrolysis (12.5) of an artificial substrate, the acetyl (or butyryl) thiocholine [14].



The electrode reaction is well characterized and commonly requires additional mediators of the electron transfer to decrease the overvoltage and omit difficulties related to the electrode poisoning.

The second way uses the signal related to the bi-enzymatic system with cholinesterase and choline oxidase [15]. The latter catalyzes the oxidation of choline yielding hydrogen peroxide and betaine (12.6).



For this purpose, both oxidation and reduction of the hydrogen peroxide either direct or mediated are described. Both approaches have some advantages and limitations. The use of thiocholine esters simplifies the biosensor assembly and the measurement protocol. However, the affinity of the artificial substrate to the enzyme active site can differ from that of acetylcholine. This may have an effect if the substrate and inhibitor contact the enzyme simultaneously. On the other hand, the introduction of choline oxidase decreases electrochemical interferences of the signal and the operation and stability of the biosensor. Both approaches impose different requirements on the measurement systems and the properties of immobilization matrices.

12.3 Nanomaterials in Cholinesterase Biosensors Assembly

Nanomaterials are intensively investigated in the assembly of electrochemical sensors and biosensors [16]. The interest was first initiated by the development of carbon nanomaterials, i.e. multi- and single-walled carbon nanotubes (MWCNTs and SWCNTs, respectively) which demonstrate both high electroconductivity and electrocatalytic activity.

For cholinesterase, the adsorption of enzyme molecules and their stabilization onto carbon nanotubes is more important. In addition, to obtain a higher rate of signaling, carbon nanoparticles approach mediators of electron transfer closer to the active site.

To some extent, this can be also referred to metal nanoparticles, especially gold able to form various sophisticated architectures via Au-S binding. Other nanodispersions of metals and their compounds are considered mainly as effective mediators necessary for amplification of amperometric signal with a lesser consideration of the specific features of the enzyme/substrate/inhibitor system.

Finally, polymeric low-conductive supports based on nanostructured polymers and hyper-branched structures lead to a significant improvement of the immobilization protocols due to the increase of the specific surface and the electrostatic control of immobilization. To some extent, polyelectrolytes can also affect the rate of the stages of the hydrolysis of the charged substrate. Meanwhile, there is only little evidence of such kind of influence which calls for a more detailed consideration. Although many polymeric supports traditionally used for enzyme immobilization have pores and structural elements formally belonging to nanoscience, in most cases they do not alter the performance of cholinesterase biosensors the behavior of which

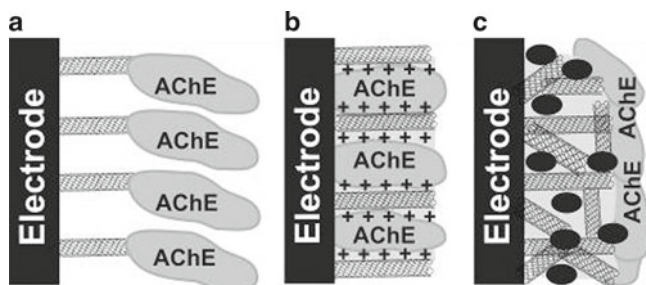


Fig. 12.1 Assemblies of the surface layers involving carbon nanotubes and AChE: (a) terminal immobilization of AChE onto aligned carbon nanotubes; (b) electrostatic self-assembling of AChE within the carbon nanotubes layer provided by polycationic substances; (c) non-specific cross-linking of AChE onto the layer of carbon nanotubes bearing other nanoparticles of mediators

is well described by models assuming a semipermeable homogeneous surface layer with an evenly distributed enzyme activity.

Below, examples of the use of various nanomaterials in the assembly of cholinesterase biosensors are summarized with particular emphasis on the analytical characteristics of the determination of irreversible inhibitors.

12.3.1 Carbon Nanotubes

The structure of carbon nanotubes can be described as a graphene sheet rolled up into a nanoscale tube, alone or with additional tubes. Depending on the number of layers, SWCNTs and MWCNTs are defined. Both their ends are normally capped with fullerene like semi-spheres. Common oxidative treatment often opens the ends by partial oxidation of the fullerene cups resulting in the formation of terminal carboxylic groups which can be then easily modified with mediators or cholinesterase. MWCNTs reduce the number of layers and the linear size in oxidation stages. Carboxylic groups can appear on the side walls of MWCNTs due to some defects in the graphene sheet. Both SWNTs and MWNTs are compatible with biopolymers. They can be used for immobilization and stabilization of enzyme preparations by electrostatic interactions or covalent binding. Coupling mediator systems with enzymes increases the signal of biosensors toward substrates and inhibitors. MWCNTs are also introduced in the carbon paste or ionic liquid gels to improve durability and electroconductivity of the surface layer. The common approaches to the manufacture of the surface layers containing carbon nanotubes are summarized in Fig. 12.1.

In some cases, the use of carbon nanotubes made it possible to reach the sub-ppb levels of inhibitor determination. Thus, the FIA biosensor has been developed for organophosphate detection on the base of glassy carbon electrode decorated with MWCNTs treated with polyelectrolyte and AChE [17]. Due to the electrocatalytic

properties of the carbon nanotubes, the working potential of thiocholine oxidation was lowered from about 700 mV (bare glassy carbon) to 150 mV. Meanwhile, the deviation of the signal toward the substrate was rather high (about 12%). The biosensor lost only 6% of the initial AChE activity in its 1 month storage at 6°C and 15% activity during the periodical operation in the FIA mode for 1 week. The limit of detection (LOD) estimated from the semi-logarithmic calibration curve of paraoxon was found to be 0.4 pM (incubation 6 min).

Thick film electrodes modified with MWCNTs were used as transducers for single-use AChE sensors [18]. The working potential of thiocholine oxidation was found to be 200 mV vs. Ag/AgCl. The calibration curve was linear in the plots of the inhibition degree against paraoxon concentration within 0.5–6.9 nM (LOD 0.4 nM for 30 min incubation). Spiked samples of lake water were used for the biosensor validation. A similar system was described for the detection of saline AChE activity as a biomarker of poisoning [19]. Screen-printed electrodes were covered with MWCNTs and the current of thiocholine oxidation was measured in the FIA mode at about 300 mV. Measurements were performed in a 50 mM phosphate buffer with 5 mM acetylthiocholine as a substrate. From 5 pM to 0.5 nM of AChE could be determined. The biosensor was successfully applied for the long-term investigation of the *in vitro* inhibitory effect of 0.7 nM paraoxon.

Vertically aligned SWCNTs were used as a matrix for the AChE immobilization followed by inhibition measurements [20]. Self-assembling on a gold electrode was used to reach the regular monolayer filling of the surface layer. SWCNTs were first wrapped with single-stranded oligonucleotides bearing terminal thiol groups. After deposition onto an Au electrode, a monolayer was self-assembled by Au-S binding. Then polyaniline was electrochemically deposited onto the side walls of the carbon nanotubes and the AChE cross-linked with glutaraldehyde onto the self-assembled layer. The enzymatic hydrolysis of acetylcholine was performed in the phosphate buffer at pH 7.4. The acetic acid produced in the enzymatic reaction (12.1) protonated the polyaniline molecules which became electrochemically active. This resulted in a redox peak which appeared on the square-wave voltammogram at about 300 mV. The peak current changed with the enzyme activity. The biosensor retained 86% of the initial AChE activity within 5 days. The inhibition effect was quantified by a non-linear logistic calibration curve of an inhibition degree against the inhibitor concentration. For methyl parathion and chlorpyrifos the concentration ranges from 10^{-11} to 10^{-6} M and LOD of 10^{-12} M were established.

MWCNTs coated with β -cyclodextrin were used in the assembly of the amperometric AChE sensor. For assembling the surface layer, glassy carbon electrode was first coated with chitosan required for electrostatic immobilization of AChE and then with homogenate obtained by the physical mixing of MWCNTs and β -cyclodextrin. Then the modified electrode was soaked in an enzyme solution. The biosensor retained 80% of AChE activity after 30 days storage. The working potential of the thiocholine oxidation was rather high (about 640 mV) in comparison with that of the biosensors mentioned above but the accuracy of the substrate detection (about 2%) allowed detecting low inhibitor concentrations. The biosensor detected from 0.01 to 2.44 μ M of dimethoate with the LOD of 2 nM (8 min incubation).

The calibration curve contained two linear pieces but the second one which appeared at higher pesticide concentration had the slope about eight times lower and could not be used for real inhibition measurements. The same authors suggested the AChE biosensor based on MWCNTs-chitosan composite [21]. In similar measurement conditions the biosensor allowed detecting 0.03–7.8 μM of triazophos with the LOD of 0.01 μM .

A composition of MWCNTs and ionic liquid (1-butyl-3-methylimidazolium tetrafluoroborate) was described for decreasing the potential of thiocholine oxidation to 50 mV vs. Ag/AgCl and improving the sensitivity of the AChE inhibition quantification [22]. After mixing the components, the excess of ionic liquid was removed by ultracentrifugation and the residue was ground and used as electrode material. AChE was immobilized in the organosilica matrix obtained by the sol–gel technology onto the surface of the MWCNTs-ionic liquid electrode. The biosensor retained 95% of the initial enzyme activity after 1 week storage in a phosphate buffer. In semi-logarithmic plots, the calibration curve of chlorpyrifos was linear between 10^{-8} and 10^{-6} M (incubation period 15 min). The following increase of the incubation time did not increase the inhibition degree. This is not typical for irreversible inhibition and can be considered an evidence of either spontaneous re-activation of phosphorylated cholinesterase or the non-enzymatic paths of acetylthiocholine hydrolysis. The LOD (4 nM) was estimated from the 10% inhibition level.

The use of modified poly-(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes for simultaneous immobilization of AChE and MWCNTs is another example of the miscellaneous techniques of enzyme immobilization [23]. The incorporation of AChE was achieved by three protocols, i.e. the random binding via glutaraldehyde cross-linking and the site specific affine interaction with concanavalin A performed with and without MWCNTs. The optimal design of the surface layer was established by factor design. The LOD of acetylthiocholine was found to be 0.34 μM . The inhibition measurement was examined for methyl paraoxon. The biosensor based on the polymer membrane coated with the concanavalin A – MWCNTs – AChE composite showed a linear response of the current to the logarithm of the pesticide concentration in the range from 10^{-11} to 10^{-8} g/L. The reported LOD ($1.39 \cdot 10^{-12}$ g/L) corresponded to the inhibition of about 5%. This seems too optimistic from the R.S.D. 2.43% obtained for the signal toward the substrate. Nevertheless, the site specific immobilization resulted in a very high sensitivity of inhibitor determination. To some extent, this can be referred to rather low amount of the AChE taken for immobilization.

Another approach to the site specific immobilization was realized in [24], with AChE covalently attached to the terminal carboxylic groups of pre-oxidized SWCNTs. Dispersion of the SWCNTs-AChE composite with Co phthalocyanine in DMF was spread onto the screen-printed carbon electrode. The biosensor showed an extremely low working potential of thiocholine oxidation (starting from –50 mV vs. Ag/AgCl). But, the most reproducible current was recorded at 150 mV. The biosensor made it possible to detect 5–50 ppb of paraoxon and 2–50 ppb of malaoxon with the LODs of 3 and 2 ppb, respectively (incubation 15 min). The R.S.D. of the signal was equal to 1% and 2.5%.

Among cholinesterases, some plant esterases can hydrolyze choline esters and are inhibited by organophosphates. Usually they are more active in the cleavage of acetate esters and are called acetyl esterases. The only example of the use of plant esterases in the assembly of biosensor for organophosphate determination is given in the work [25]. Plant esterase from maize seedlings was covalently attached to MWCNTs by carbodiimide binding. The reaction was performed on a polycrystalline Au electrode covered with the MWCNTs decorated with Fe_3O_4 nanoparticles. The enzymatic hydrolysis of acetylthiocholine was performed at 35°C in a phosphate buffer, pH 7.4. The oxidation peak current was observed at 270 mV vs. Ag/AgCl. The maximum inhibition of about 80–90% was reached for malathion, chlorpyrifos, monocrotophos and endosulfan with a very similar slope of the incubation period dependence. The LOD of the above pesticides corresponded to 20–30% inhibition varied in their nature and incubation period within 0.1–100 nM. The irreversible inhibition of plant esterase was found less sensitive toward metal cations than the AChE analogs. This might be due to the absence of anionic binding centers in the enzyme active site. For the same reason, the plant esterases show less selectivity of their response toward aliphatic and choline esters. The biosensor was successfully applied for the determination of pesticides in spiked milk and natural water samples. The recovery obtained for their nanomolar concentrations was in between 95% and 110%.

12.3.2 Nanoparticles of Metals and Their Compounds

Au nanoparticles have been predominantly applied in the assembly of enzymatic and DNA biosensors as a matrix for the immobilization of biopolymers and labels [26]. Spontaneous formation of Au-S bonds provides easy design of the surface monolayers and the multiplication of the redox centers in sandwich type mode of the signal measurement. The synthesis of Au nanoparticles is performed by controlled reduction of AuCl_4^- anions by chemical reactants in the conditions limiting the growth of the metal phase nuclei. Other metal nanodispersions are obtained in a similar manner. The electrochemical deposition of nanoparticles by the reduction of metal cations is an alternative of the electrode modification. Polymeric materials deposited or synthesized onto the electrode limit the size of the particles formed and affect their geometry and properties. Besides immobilization, metal and metal oxide nanoparticles are used as additives to electrode material or mediators to improve the electroconductivity of the surface layer and decrease the overpotential of the redox reactions.

The most common approaches to the introduction of metal nanoparticles in the assembly of cholinesterase biosensors are summarized in Fig. 12.2.

Thus, Au nanoparticles were deposited onto glassy carbon electrode modified with polypyrrole by cycling the potential in the solution of HAuCl_4 [27]. As was shown by SEM, a uniform distribution of metal nanoparticles 30 nm in average size along polypyrrole rods was reached. The potential of thiocholine oxidation

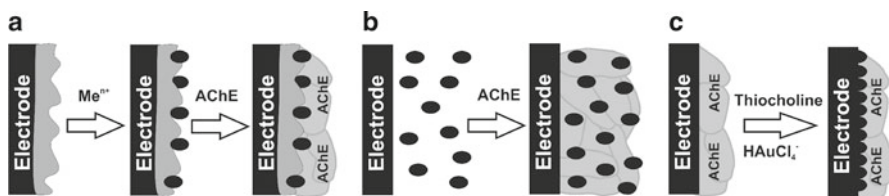


Fig. 12.2 Introduction of the metal nanoparticles in the assembly of cholinesterase biosensors: (a) electroplating on the electrode covered with a polymeric template; (b) chemical reduction and co-deposition of an enzyme and nanoparticles from the homogeneous solution followed by cross-linking of an enzyme molecules; (c) chemical reduction of $AuCl_4^-$ anions by thiocholine formed in enzymatic reaction of acetylcholine hydrolysis

measured in phosphate buffer at pH 7.0 was rather high (about 700 mV vs. Ag/AgCl). Also, the deposition of the Au nanodispersion resulted in an insignificant improvement of the measurement conditions (35 mV shift of the working potential). The biosensor retained 60% of the initial enzyme activity after 30 days storage. Inhibition measurements were performed with a standard solution of methyl parathion with 12 min incubation. The inhibition degree linearly depended on the pesticide concentration in the range from 0.005 to 0.12 $\mu\text{g/mL}$. After reaching 60% inhibition, the sensitivity of the response dramatically decayed though the linear character of the response was retained until 5 $\mu\text{g/mL}$. The LOD estimated from the calibration curve was equal to 2 ng/mL.

MWCNTs covered with chemically synthesized Au nanoparticles were used for the modification of glassy carbon electrode followed by the adsorptive immobilization of AChE [28]. The signal of thiocholine oxidation was measured at 0.25 V in potentiostatic regime. The biosensor made it possible to detect 0.1–7.0 nM of paraoxon (30 min incubation) with the LOD of 0.1 nM for $S/N=3$ criterion and 1 nM for 10% inhibition level.

One-dimensional Au nanoparticles were synthesized by electrochemical deposition in the pores of alumina template followed by its dissolution by the NaOH solution [29]. AChE was immobilized by entrapment in chitosan film. The oxidation current of thiocholine was recorded at 0.55 V vs. SCE. The inhibition caused by methamidophos increased with the incubation time up to 10 min and then stabilized. The R.S.D. of the inhibition degree was equal to 2.8% for 5 $\mu\text{g/mL}$ of the pesticide. The calibration curve was linear in the range from 0.010 to 0.56 $\mu\text{g/mL}$ with the LOD of 0.005 $\mu\text{g/mL}$ (10% inhibition level).

The composite of Au nanoparticles covered with poly(diallyl-dimethylammonium chloride) (PDDA) and Prussian Blue was successfully applied to improve the performance of amperometric AChE biosensor toward organophosphate insecticides [30]. The composite was obtained by the self-assembling of the oppositely charged components performed in homogeneous conditions under sonication. Although the potential of thiocholine oxidation was still high (680 mV) the biosensor showed remarkable sensitivity of monocrotophos detection within 1.0 pg/mL and 1.0 ng/mL with the LOD estimate of 0.8 pg/mL ($S/N=3$). As in the previous case,

the maximal detectable concentration of the inhibitor is limited to 60% inhibition level. The unusual pH maximum of the signal (pH=6.0) and the high sensitivity toward irreversible inhibitor were related to the hydrophobicity of the PDDA in the assembly of the AChE layer.

A similar approach was used for deposition of Au-PDDA nanoparticles onto the graphene sheet [31]. Contrary to Prussian Blue [29], the use of graphene as an Au nanoparticle carrier reduced the potential of the thiocholine oxidation to 0.47 V. The high durability of the surface layer and the stability of the response allowed the FIA mode of the measurement. In semi-logarithmic plots, the calibration curve of paraoxon was linear in the range from 10^{-8} to 10^{-13} M with the LOD of 0.1 pM (15 min incubation, phosphate buffer, pH 7.4).

Another ensemble obtained by layer-by-layer deposition was obtained from Au nanoparticles, a PAMAM dendrimer and MWCNTs onto the glassy carbon electrode [32]. Chemical reduction of AuCl_4^- anions *in situ* was performed in the presence of PAMAM and yielded ca. 13 nm particles uniformly distributed along the MWCNTs applied as a mechanical support and conductor to wire the enzymatic reaction with the electrode. The biosensor makes it possible to detect 4.8–900 nM of carbofuran with the LOD of 4.0 nM.

Bimetallic particles of Au and Pt were co-deposited from the $\text{HAuCl}_4/\text{H}_2\text{PtCl}_6$ solution onto a glassy carbon modified with 3-aminopropyltriethoxy silane [33]. After that, the mixture of AChE and choline oxidase was added and covalently linked to amino group of the modifier by glutaraldehyde. Due to the electrocatalytic properties of Pt observed in the reaction of the H_2O_2 oxidation, the biosensor showed a remarkable response toward acetylcholine. This made it possible to reduce the amount of AChE down to 0.004 U per electrode and hence to increase the sensitivity of inhibitor detection. For aldicarb and paraoxon ethyl, the inhibition gradually increased with the contact time up to 25 min incubation whereas sarin reached the plateau to the 7th min. The LOD was estimated in accordance with the inhibition level equal to 30–40% (150–200 nM, 40–50 nM and 46–60 μM for paraoxon ethyl, sarin and aldicarb, respectively).

Thiocholine formed in the enzymatic reaction can also be applied as a reducer to obtain Au nanoparticles on the electrode [34]. This approach makes it possible to eliminate many interferences of the signal measurement because of the high affinity of thiol groups toward Au. The growth of the Au layer can be monitored using the redox probe, $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as an example. The reaction of AChE is followed by a simultaneous increase of the redox current observed at about 0.2 V. AFM demonstrated the formation of a regular layer of about 1 nm height which grew with time and thiocholine concentration to a micrometer-size cluster at 4.8 mM thiocholine corresponding to the saturation of the enzymatic layer. The biosensor makes it possible to detect 0.1–500 ng/mL of malathion with the LOD of 0.03 ng/mL.

The sensitivity of inhibitor determination can be enhanced by specific processing of the voltammetric signal to increase its resolution in the presence of ultra-small amounts of inhibitors. Thus, Fourier transformation was used in combination with continuous cyclic voltammetry to process the thiocholine oxidation signal recorded on the electrode covered with Au-MWCHTs composite and AChE in the chitosan

matrix [35]. To record the signal, the potential was scanned between 0 and 1,000 mV, with the scan rate of 5 V/s. Although the EIS measurements indicated rather small resistance of the charge transfer, the thiocholine oxidation was observed at a rather high potential (800 mV) and the background current was subtracted to improve the peak resolution. The following integration resulted in the estimation of the charge required for thiocholine oxidation. Contrary to the peak current, this characteristic is less sensitive toward the measurement procedure and allows recording rather small changes in the enzyme activity. 92.3% of the enzyme activity was retained after 54 days of the storage. The concept was examined on the example of monocrotophos detection. The biosensor makes it possible to detect 0.1–100 μM of the inhibitor in the presence of 1.0 mM of acetylthiocholine (competitive inhibition scheme). More recently, the same biosensor was described for the determination of paraoxon with the LOD of $6.2 \cdot 10^{-13}$ M (12 min incubation) [36].

Besides other metal containing nanoparticles, Prussian Blue [30, 37–40] and quantum dots [41–43] are of most interest.

Prussian Blue is obtained by mixing a Fe(III) salt with $\text{K}_4[\text{Fe}(\text{CN})_6]$. In some cases, the first reagent is generated *in situ* by the oxidation of a Fe(II) salt. The insoluble crystal sediment contains $\text{Fe}^{2+/3+}$ and $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions with some amount of K^+ counter ions important for the reversibility of the electron transfer. Although the exact composition of Prussian Blue varies with the synthesis conditions and depends on many factors like the electrode nature, the surfactant presence, the redox potential etc., it is one of the most effective mediators in the electrocatalytic reduction of H_2O_2 . For this reason, the term “artificial peroxidase” was introduced for these compounds [37]. It lowers the potential of thiocholine oxidation to a degree comparable with that of carbon nanotubes and Co phthalocyanine. Thus, the deposition of Prussian Blue – Nafion composite onto the carbon screen printed electrode made it possible to detect the thiocholine signal at 200 mV vs. Ag/AgCl [38]. The biosensor made it possible to detect down to 30, 10 and 5 ppb of aldicarb, paraoxon and parathion-methyl, respectively, with 10 min incubation. The biosensor was applied for the estimation of degradation of organophosphate pesticides in red wine.

Similar biosensors were applied for the comparison of AChE and BChE immobilized onto a Prussian Blue by glutaraldehyde cross-linking in an albumin matrix. AChE based biosensors exerted a higher sensitivity towards aldicarb (50% inhibition with 50 ppb) and carbaryl (50% inhibition with 85 ppb) while BChE biosensors were more effective in the detection of paraoxon (50% inhibition with 4 ppb) and chlorpyrifos-methyl oxon (50% inhibition with 1 ppb) [39].

A disposable screen-printed electrode covered with two types of nanoparticles was used for the sensitive detection of anticholinesterase agents [40]. AChE from *Drosophila melanogaster* was immobilized on magnetic beads obtained from Au nanoparticles covered with a Fe_3O_4 layer. Prussian Blue was entrapped in the matrix obtained by sonication of the dimethylformamide solution of ZrO_2 nanorods, MWCNTs and Nafion. The reaction of the enzymatic hydrolysis of the substrate and inhibition was performed in a drop of solutions placed directly onto the working area. The linear calibration curve of the inhibition degree on the dimethoate concentration

from 0.001 to 10 ng/mL was obtained at pH=7.5 and 35°C. The detection limit was equal to $5.6 \cdot 10^{-4}$ ng/mL ($S/N=3$).

Quantum dots are semiconductor microcrystals exerting high luminescence yields depending on the size and structure of the particles. They are used in light emitting devices, e.g. LEDs, diode lasers, medical imaging systems etc. [41]. The use of quantum dots, preferably CdTe, in electrochemical sensors is based on their electron transduction properties even though the size of the particles is commonly much bigger than those used in microelectronics. In the work [42], CdTe particles were obtained from soluble salts directly on the Au electrode covered with a monolayer of cystamine. AChE was immobilized directly onto CdTe particles by carbodiimide binding. The oxidation peak of thiocholine was recorded at 0.86 V. The biosensor showed a maximum of the enzyme activity and the inhibition effect of carbaryl, a carbamate pesticide, at the same pH=7.0. The calibration curve was linear in the range from 1 to 50 ng/mL (LOD 0.6 ng/mL). The biosensor was successfully applied for the detection of carbaryl in spiked samples of garlic. The R.S.D. value of the inhibition was 3.4–7.8% and the recovery was 94.0–100.6% depending on the inhibitor content.

CdS quantum dots were synthesized from ionic components in the presence of polyvinyl pyrrolidone and then used for immobilization and stabilization of AChE preparations. The biosensor based on the CdS-AChE composite showed electrocatalytic activity toward thiocholine oxidation. Under optimal conditions, the biosensor made it possible to detect down to 12 ppb of trichlorfon with 5 min incubation [43].

Progress in nanotechnology offers new materials which can substantially extend the possibilities of conventional electrochemical techniques. Recently, choline biosensor has been developed both for the determination of choline, AChE activity and sensitive detection of irreversible inhibitors [44]. For this purpose, a novel mediator of electron transfer, i.e. nanorods of MnO_2 was synthesized and introduced together with choline oxidase in the assembly of the polyelectrolyte complex onto the carbon screen-printed electrode. The biosensor made it possible to detect $1.3 \cdot 10^{-7}$ to $1 \cdot 10^{-4}$ M choline. With native AChE added to the working solution, down to 50 pM of chlorpyrifos were detected.

ZnO semiconductor nanoparticles were co-immobilized with AChE in a chitosan film by layer-by-layer deposition techniques onto the Pt electrode [45]. The signal of thiocholine oxidation was measured under UV illumination. The biosensor was examined on the model inhibitor, dimethoate, which was quantified from 0.25 to 1.50 and from 1.75 to 10.00 μ M (LOD 10 nM for $S/N=3$).

12.4 Conclusion: Is *Nano* Important for Inhibitor Detection?

The use of nanomaterials offers many advantages of the biosensor manufacture and the detection of the biorecognition event. Effective cooperation of the components of a surface layer, the high regularity of its composition and the simplicity of manufacture based on self-assembling are often referred to. Indeed, the consideration of

the assemblies of cholinesterase biosensors including nanomaterials substantiates such a conclusion. In some cases, the detection limits of anticholinesterase agents are below the ppb level and seem sufficient for field applications and direct risk assessment.

However, the reasons for such an opinion are not obvious. Following the backgrounds of the biosensor development, the use of nanomaterials can be referred to as (i) conditions for electrochemical signal generation and (ii) as initiated by previous experience of the use of such systems in other electrochemical sensors. Meanwhile, the performance of cholinesterase biosensors is rather specific and does not allow a trivial transfer of methodology be it as attractive as nanochemistry and nanotechnology.

For inhibitor detection, the biosensor signal is measured twice in the same conditions, i.e. prior to and after the incubation stage. This means that the importance of the factors determining the signal generation becomes less important as compared to biochemical stages. This is true in particular when an enzyme is inhibited in the absence of a substrate as it is in the case of irreversible inhibition. The Ellman equation (12.2) predicts the dominant effect of the incubation time and the accessibility of the active site for an inhibitor (inhibition constant). The use of nanomaterials as mediators of electron transfer or enzyme immobilization cannot change these parameters. This is especially true for cholinesterases with a rather open active site which is rarely affected by steric limitations of substrate/inhibitor access. This calls for consideration of other factors which can improve the sensitivity of the signal within the kinetic approach discussed above. The following aspects of biosensor performance can be discussed:

Substrate/inhibitor distribution. Ellman equation as well as other relationships initiated by formal inhibition kinetics assume homogeneous conditions which are extended to the enzyme surface layer. Meanwhile, the latter assumption is based on an even and regular deposition of the reactants on the support providing equal access of biochemical reagents to the enzyme. Nanomaterials provide reproducible but not uniform morphology of the surface which can offer some predominance of the inhibitors in accordance with their size, charge, affine interactions etc. The inclusion of polyelectrolytes and hydrophilic polymers amplifies the possible influence of the distortions in the reactant distribution across the surface layer.

Metrological performance. The inhibition quantification assumes repeated measurement of the signal separated by the incubation stage. This results in an increase of the inhibition deviation as compared to that of a substrate measurement. Nanosized mediators amplify the signal so that the response toward the substrate becomes higher and its relative deviation decays. Therefore the LOD calculated from the $S/N=3$ ratio is significantly decreased against traditional transducer systems. The effect is less pronounced if we take into account that almost all inhibitor biosensors are intended for a disposable use and a series of biosensors should be tested for a reliable estimation of metrological parameters. However, some researchers calculate the degree of inhibition for a single biosensor from two signal measurements and assign the deviation estimated for the substrate detection to the result. Except

for a few works devoted to real sample testing, no evidence of the variation of the signals in the biosensor series has been presented. This is particularly true for non-linear and semilogarithmic calibration plots where the confidence interval is non-linearly changed from the median value of the argument. This does not mean all the advantages in the LODs can be assigned to the chemometric artifacts but this is a call to a more careful description of the metrological aspects of the investigations.

Enzyme loading. A higher signal means the possibility to decrease the amount of an enzyme taken for immobilization. This is especially important for strong irreversible inhibitors because the relative decay of enzyme activity caused by such poisons directly depends on the initial enzyme activity. Unfortunately, the common mistake of beginners is to optimize the immobilization protocol to reach the highest response toward the substrate. Actually, the lowest signal is most desirable to get highest sensitivity of the inhibitor determination. The high efficiency of nanosized mediators allows the significant decrease of the cholinesterase loading to have a rather high signal and this might be the most important advantage of nanosystems.

Non-enzymatic paths. The hydrophobicity of some supports like dendrimers was cited as a possible reason of the sensitivity improvement. From this point of view, the higher the granulation of the surface the better the inhibition signal. Unfortunately, there are no specific investigations estimating the distribution of the pesticides in the surface layer in the absence of an enzyme. To some extent, the effect of hydrophobic/hydrophilic properties of an enzyme support was mentioned for common plastic membranes as well [3, 46, 47]. Limitations in the linearity of the calibration graphs as well as the maximal inhibition degree can also be due to some side reactions unfavorable for biosensor performance. All the irreversible inhibitors should give a single calibration graph with 100% inhibition level reached at a high concentration. The same refers to an increase of the incubation time. In some works, only a 60–70% inhibition was reached even though highly toxic chemical warfare agents were tested. This does not follow from kinetics of inhibition and assumes either the non-enzymatic hydrolysis of a substrate or the recovery of enzyme activity within the incubation step. The second explanation is less probable because of the properties of phosphorylated cholinesterases but can be realized for some carbamate pesticides. Limitations of the inhibitor access caused by its adsorption on the matrix, steric reasons or chemical instability can occur as well.

Manufacture and operation. Self-assembling and regular composition of nanoparticles simplify their combination in an appropriate matrix and on the transducer system. Unfortunately, less attention was given to miniaturization prospects. Meanwhile, the synthesis of nanoparticles bearing enzymes or mediator groups plays a positive role in the improvement of the reproducibility of the signal and hence the LOD of appropriate biosensors. It should be mentioned that many of the biosensor concepts described above have not yet been tested in field conditions and with real samples. This complicates the estimation of the contribution of appropriate systems in the long term stability of inhibition measurements and biosensor life-time. The results of the substrate measurements do not contribute to the problem

because the sensitivity toward an inhibitor tends to decrease even with about a constant signal to a substrate. Some of the protocols described apply a separate manufacture of the nanomaterials followed by their combination at the last stage or direct synthesis of nanoparticles on the electrode by chemical or electrochemical treatment of their precursors. This seems attractive from the point of view of the future commercialization of the results but such technologies are far from their technological realization as yet.

Summarizing the data presented in the review it can be concluded that the use of nanomaterials affects all the aspects of inhibitor determination starting from enzyme immobilization and ending in side reactions interfering with the signal measurements. Many of the characteristics obtained with such nanoforms of biorecognition materials have hold great promise for their future practical application. Their future realization calls for extended attention to be focused on real sample analysis and the metrological characterization of the results.

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Chapter 13

Detection and Detoxification of Organophosphate Warfare Agents

Georges Istamboulie and Jean Louis Marty

Abstract Biosensors have been described for many years as good substitutive or complementary tools to conventional methods for the detection of pesticides, due to their ability to provide real-time qualitative information about the composition of a sample with minimum preparation. Biosensors based on acetylcholinesterase (AChE) inhibition have been intensively studied in the aim of detecting carbamate and organophosphorus insecticides. However, a problem related to these biosensors is due to their poor selectivity as AChE can be inhibited by various classes of neurotoxic compounds. This chapter presents the development of biosensors for the sensitive and selective detection of organophosphorus and carbamate insecticides. The high sensitivity of the devices is brought by the use of a genetically modified AChE, combined with soft immobilization methods. The selectivity of AChE-based sensors can be? by the use of phosphotriesterase, an enzyme hydrolyzing specifically some organophosphate compounds. This enzyme has been successfully coupled to AChE for designing selective sensors to two organophosphate compounds of great environmental concern: chlorpyrifos and chlorfenvinfos. Despite this progress, the main problem still remained the analysis of pesticide mixtures, which can be solved in some cases by the use of a sensor array coupled with a chemometric tool. In this sense, artificial neural networks (ANNs) have been found to be powerful tools, particularly suited for various tasks in information processing. ANNs are non-parametric calibration methods specially created to process non-linear information. It has been demonstrated that by combining native and recombinant variant of AChE with ANNs data processing, sensitive multianalyte detection is possible.

Keywords Cholinesterase • Phosphotriesterase • Electrochemical • Inhibition • Neuronal network

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13.1 Introduction

In the last three decades, research in the field of biosensors has strongly increased as a result of the need for cheap, fast and easy to use analytical tools that are able to provide real-time qualitative and quantitative information about the composition of a sample with minimum sample preparation. Biosensors are analytical devices which utilize the sensitivity and selectivity of a bio-receptor attached onto the surface of a physical transducer. The transducer is able to respond to and transform a bio-chemical and/or physico-chemical property into a measurable signal as a result of a bio-recognition event between the bio-receptor and its target analyte [1–3]. Chronologically, the first biosensor was an enzyme sensor developed by Clark and Lyons in 1962 [4]. This sensor utilized glucose oxidase attached onto the surface of an amperometric oxygen electrode and was used to directly quantify the amount of glucose in a sample. Since then, numerous enzyme sensors in different configurations have been developed.

Organophosphates (OPs) are a class of synthetic pesticides developed from the Second World War, which are used as insecticides and nerve agents [5, 6]. Since the removal of organochlorine insecticides from use, OPs have become the most widely used insecticides. They are normally used for agricultural, industrial, household and medical purposes. OPs poison insects and mammals by phosphorylation of the acetylcholinesterase (AChE) enzyme at nerve endings [7, 8]. Inactivation of this enzyme results in an accumulation of acetylcholine leading to an overstimulation of the effector organ [9, 10]. Biosensors based on cholinesterase enzymes (ChEs) have emerged as an ultra sensitive and selective technique for toxicity monitoring for environmental, agricultural, food or military applications.

The principal motivation for designing ChE biosensors for toxicity monitoring is to provide a reliable alternative to classical methods currently used in analytical chemistry laboratories (chromatographic and coupled chromatographic- spectrometric procedures: GC-MS, HPLC-MS). These techniques are very powerful tools for monitoring toxic analytes such as pesticides and heavy metals but, they are expensive, time-consuming (involve extensive preparation steps), are not adapted for *in situ* and real time detection and require highly trained personnel [11, 12]. In addition, they are not able to provide any information concerning the toxicity of the sample. ChE biosensors appear as a rapid and simple method for the detection of these pollutants.

A successful ChE biosensor for toxicity monitoring should offer comparable or even better analytical performances than the traditional chromatographic systems. Ideally, such sensors should be small, cheap, simple to handle and able to provide reliable information in real-time without or with a minimum sample preparation. The use of the enzyme should also provide increased sensitivity and selectivity for the analyte of interest [12].

In this chapter, we present an overview of significant advances in the research and development of ChE biosensors and we discuss the trends and challenges associated with designing a reliable ChE sensor for practical applications. Although these devices show a high sensitivity, they have been shown to be poorly adapted to the selective detection of OPs.

To overcome this problem, we have recently described a biosensor associating a highly sensitive genetically-modified AChE with a phosphotriesterase (PTE) [13]. This latter enzyme has the ability to hydrolyse organophosphate compounds. It has already been used in other works as a target enzyme for the detection of parathion [14].

This chapter will review the different configurations and fabrication techniques of mono or bi-enzymatic sensors with a special emphasis on the low-cost electrochemical sensors and the approaches used for enzyme immobilization.

13.2 Cholinesterase Enzymes and Their Use in Biosensors

Cholinesterases (ChEs) are important enzymes present in vertebrates and insects, which hydrolyze the neurotransmitter acetylcholine in the nervous system [15]. In the body, ChE is responsible for the transmission of nerve impulses to the cholinergic synapses and is connected with human memory and Alzheimer's disease. Two types of cholinesterases are known and have been used for biosensor fabrication: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). BuChE has a similar molecular structure to that of AChE but is characterized by different substrate specificity: AChE preferentially hydrolyses acetyl esters such as acetylcholine, while BuChE hydrolyzes butyrylcholine [16]. Another aspect that distinguishes AChE from BChE is the AChE inhibition by excess of substrate. This property is related to substrate binding and - catalytic mechanism. Apart from the natural substrate, ChEs also hydrolyze esters of thiocholine such as acetylthiocholine, butyrylthiocholine, propionylthiocholine, acetyl- β -methylthiocholine as well as *o*-nitrophenylacetate, indophenylacetate and α -naphthyl acetate. Many of these have been used in different ChE biosensor configurations. ChE enzymes extracted from the *Drosophila melanogaster* and the Electric Eel are commercially available and are the most widely used for biosensor fabrication.

The ChEs are inhibited by several toxic chemicals such as organophosphate and carbamate pesticides, heavy metals, nicotine, nerve gas and anatoxin-a(s). Depending on the extraction source, ChEs can have different substrate specificity and susceptibility to inhibitors. Studies of AChE as a target of pesticide toxicity have yielded several practical outcomes and are the basis for constructing ChE biosensors. These devices are primarily designed to determine and to quantify the inhibition of ChE by toxic chemicals.

13.2.1 Principles of Detection

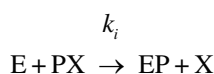
An alternative to the elaborated chromatographic methods is to determine pesticides using enzymatic assays, mostly based on AChE inhibition. Many detection kits have been successfully developed based on this principle. The most advanced systems

have been designed as low cost disposable biosensors [12, 17]. Most advanced configurations involve the use of recombinant AChEs that were specifically engineered to enhance sensitivity and selectivity towards inhibitors [17]. The mechanism of inhibition of AChE by OP and carbamate compounds is well-known [9]. The inhibitor phosphorylates or carbamoylates the active site serine and the inhibition can be considered as irreversible in the first 30 min [18].



Where: E =enzyme, PX=carbamate or OP and X=leaving group.

This scheme can be simplified using the bimolecular constant $k_i = k_2/K_d$:



13.2.2 Recombinant Enzymes

The enzyme used in a biosensor design should be sensitive enough to decrease the threshold detection as low as possible [19, 20]. In addition, it should be selective towards the target analyte or class analytes for which the biosensor is designed. As discussed previously, ChE biosensors are selective toward pesticides and heavy metals, but they are generally not able to differentiate between individual compounds. Enzymes extracted from different sources have different sensitivities and selectivities toward pesticides. For instance, the AChE extracted from the *Drosophila melanogaster* (Dm) is eight-fold more sensitive than the AChE from the Electric eel [21]. Advances in molecular biology have made possible engineering of more sensitive and selective ChE with individual sensitivity patterns towards a target inhibitor [22]. The use of these enzymes to construct biosensors is expected to increase both the selectivity and sensitivity of the sensor.

Table 13.1 shows a comparison between the inhibition constants of recombinant Dm, wild type Dm, and the commercial Electric eel AChE. The Inhibition constants (k_i) of the recombinant enzyme was 2.5 and 40 times higher than that of the wild type enzyme. The ultrasensitivity of mutant Dm-AChE was obvious when comparing it with the widely used Electric eel enzyme, the inhibition constant of the recombinant enzyme being 1,000-fold higher for both pesticides tested.

In addition to mutant enzymes, the use of multiarray sensors coupled with an artificial neural network for data treatment and analysis could substantially improve biosensor selectivity and allow exact identification of the inhibitor present in a sample.

This approach has been used by Bachmann *et al.*, who employed an array of multienzyme biosensors with four immobilized Dm AChEs (wild type and three

Table 13.1 Inhibition constants k_i ($\mu\text{mol}^{-1} \text{min}^{-1}$) for the *Drosophila* and Electric eel enzymes using six OPs as inhibitors [17, 19, 20]

OPs	Electric eel (EE) k_i	Wild Dm		Modified Dm	
		k_i	Factor/EE	k_i	Factor/EE
Paraoxon	0.29	1.55	5	19.15	66
Azinphos	0.36	33.00	92	691.90	1,922
Chlorpyrifos oxon	2.85	1135.00	398	2500.00	877
Dichlorvos	0.03	1.90	63	487.00	16,233
Chlorfenvinphos	0.25	4.80	19	208.30	833
Omethoate	0.001	0.042	42	2.32	2,320

mutants) [23, 24]. The system allowed discrimination of malaoxon and parathion in a binary composite mixture and enabled detection of 11 out of 14 organophosphate and carbamate pesticides.

13.2.3 ChE Immobilization

The most important step in the development of an enzyme biosensor is the stable attachment of the enzyme onto the surface of the working electrode. This process is governed by various interactions between the enzyme and the electrode material and strongly affects the performance of the biosensor in terms of sensitivity, stability, response time and reproducibility. The main methods used for depositing ChEs onto the working electrode surface are summarized in Table 13.2.

Apart from the natural substrates, ChEs are also able to hydrolyze esters of thiocholine such as acetylthiocholine, butyrylthiocholine, propionylthiocholine, acetyl- β -methylthiocholine as well as *o*-nitrophenylacetate, indophenylacetate and α -naphthyl acetate. Many of these substrates have been used in different ChE biosensor configurations. AChE enzymes extracted from the *Drosophila melanogaster* and electric eel are commercially available and are the most widely used for biosensor fabrication. Most AChE biosensors have been used in amperometric detection mode by measuring the product of the enzymatic reaction at a constant potential and evaluating the inhibition degree after exposure to pesticides.

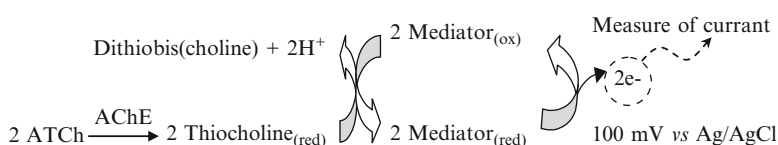
The first devices described were coupling a ChE with a choline oxidase, the detection being based on either the oxidation of H_2O_2 or the reduction of oxygen. This complicated system was further simplified using a synthetic substrate of AChE, acetylthiocholine, which produces an easily oxidizable compound, thiocholine, according to the reactions in Fig. 13.1:

The use of an appropriate mediator, like tetracyanoquinodimethane (TCNQ), cobalt phthalocyanine (CoPC) or poly(3,4-ethylenedioxythiophene) (PEDOT) [25] allows decreasing the detection potential to values lower than 100 mV vs. Ag/AgCl, thus minimizing interfering oxidations. The mediator can be used in solution or incorporated in the working electrode material, the latest being the preferred and

Table 13.2 Principal methods used for ChEs immobilization in biosensors [12]

Procedure	Electrode material/immobilization agents	Observations
Adsorption	Graphite; SPE; carbon nanotubes	Simple, short response time; poor operational and storage stability; sensitive to changes in pH, temperature, ionic strength
Covalent binding	Pre-activated or functionalizable electrode surfaces: noble metals, graphite, SPE, Glass Glutaraldehyde, carbodiimide/NHS	Poor reproducibility, high amount of enzyme, possible denaturation; Stable; Absence of diffusion barriers; short response time
Self-assembled monolayers	–	Complex and difficult to reproduce; possible electrode fouling
Physical entrapment	Noble metals; Graphite; SPE; Glassy carbon electrodes, photopolymers (PVA-SbQ), Electro-polymerizable, polymers (pyrrole, aniline); Sol–gel	One-step procedure at ambient or low temperature; Simple; Many biocompatible polymers available; Suitable for a large variety of bio-receptors; Problems of reproducibility and control of pore size; diffusion barriers
Affinity	Noble metals; SPE; Silica NTA-Ni, Carbohydrates	Need the presence of specific groups in the bio-receptor molecule (e.g. histidine, biotine, lectin ...) Reusable surface; Low amount of enzyme; controlled and orientated immobilization

SPE screen-printed electrodes; PVA photocrosslinkable poly(vinyl alcohol); NTA nitrilotriacetic acid; Con-A concanavalin A

**Fig. 13.1** Principle of Amperometric detection of thiocholine: ATCh (Acetylthiocholine), AChE (Acetylcholinesterase)

more convenient method. The most versatile method for manufacturing the electrode is the screen-printing method. This technology allows the production of screen-printed three-electrode system with a low cost and high reproducibility.

The detection principle of AChE biosensors is based on the inhibition of enzyme by OP insecticides, leading to reduced thiocholine production. Typically, amperometric measurements are performed in stirred PBS solution at pH values between 7 and 8. After applying the appropriate potential for mediator oxidation, the current

intensity is recorded in the presence of a saturating concentration of acetylthiocholine substrate. The time necessary to reach the plateau is 2–3 min. The measured signal corresponds to the difference of current intensity between the baseline and the plateau. The cell is washed with distilled water in between measurements. The pesticide detection is made in a three step procedure: first, the initial response of the electrode to acetylthiocholine (1 mM) is recorded two times, then the electrode is incubated in a solution containing a known concentration of insecticide, and finally the residual response of the electrode is recorded. The percentage of the inhibition is then correlated with the insecticide concentration. Based on this method, highly sensitive biosensors have been developed by our group using recombinant enzymes immobilized on magnetic micro-beads by nickel-histidine affinity. We have mainly focused our attention on two insecticides of interest: chlorpyrifos (CPO) and chlorfenvinfos (CFV). The developed sensors allowed the detection of pesticide concentrations as low as $1.3 \cdot 10^{-11}$ M ($0.004 \mu\text{g} \cdot \text{L}^{-1}$) [17].

13.3 Phosphotriesterase Enzymes and Their Use in Biosensors

Enzymes able to degrade OPs have been the focus of several research studies in the environmental decontamination/detoxification field [26]. OP-hydrolyzing enzymes have been found in bacteria, *Archaea* and *Eukarya*. The best-known and characterized enzyme is phosphotriesterase (PTE), firstly isolated from the soil bacterium *Pseudomonas diminuta*. However, its natural substrate and the main physiological function are still unknown [27]. PTE has the ability of hydrolysing most organophosphates, including chemical warfare agents like sarin or soman [6, 28]. Due to these properties, PTE has been also described as a potential catalytic scavenger for the treatment of organophosphate poisoning [29].

Another possible route for the detection of OP insecticides is to use phosphotriesterase (PTE) biosensors [30]. Several PTE-based amperometric, potentiometric, or optical biosensors have been described [31]. Deo and coworkers have reported an amperometric biosensor for organophosphate pesticides based on a carbon nanotube (CNT)-modified electrode and PTE as a biocatalyst. In this sensor design, the detection is based on the electrochemical oxidation of *p*-nitrophenol, which is produced upon hydrolysis of paraoxon or methyl-parathion by PTE [32]. However, it must be noticed that this method can only be applied to the detection of a few insecticides including parathion, methyl-parathion and their oxidation products (paraoxon and paraoxon-methyl), due to the fact that other organophosphates do not generate *p*-nitrophenol upon hydrolysis.

13.3.1 Catalytic Mechanism of PTE

The phosphotriesterase (PTE) (EC 3.1.8.1) is a member of the amidohydrolase superfamily [33]. Like other members of this superfamily PTE utilizes one or two

Table 13.3 Kinetic constants of PTE enzyme using eight different organophosphate insecticides (substrates)

PTE	CPO	CPO-methyl	Para-oxon	Paraoxon-methyl	CFV	Dichl-orvos	Omethoate, Fenamiphos
Km (mM)	0.04	0.06	0.25	1.2	0.017	0.31	Not substrate
Relative Vm (/paraoxon)	1.8	0.25	1	0.38	0.0002	0.003	

CPO chlorpyrifos-oxon; *CFV* chlorfenvinfos. (In order to facilitate the comparisons, the maximum velocity was expressed as paraoxon-ethyl-equivalent activity)

divalent metal ions to activate a hydrolytic water molecule for a nucleophilic attack at tetrahedral phosphorus or trigonal carbon centers. The active site of the native enzyme contains two zinc ions per monomer. The enzyme retains catalytic activity when the native Zn^{2+} is replaced by Co^{2+} , Cd^{2+} , Ni^{2+} , or Mn^{2+} . The cobalt-substituted enzyme is the most active form [34].

The catalytic mechanism for the hydrolysis of organophosphate triesters has been extensively studied using paraoxon as a reference substrate [28, 35]. For other substrates, the catalytic efficiency and the rate-limiting step are dependent on the pK_a of the leaving group [36].

The kinetic constants (K_m and V_m) of PTE for different organophosphate substrates are summarized in Table 13.3 [13].

It has been demonstrated that PTE displays a higher affinity for chlorpyrifos-oxon (CPO) derivatives than for paraoxon compounds (lower K_m), which are generally used in PTE catalysis studies. Both CPO and chlorfenvinfos (CFV) show a very good affinity for PTE, but even though CFV has a better affinity than CPO, its hydrolysis rate was 9,000-fold lower. The high affinity and the low degradation velocity of CFV indicate that this substrate is acting as a competitive inhibitor of PTE for the hydrolysis of other organophosphates [13].

The proposed mechanisms of CPO and CFV hydrolysis by bacterial PTE are presented in Fig. 13.2. The main products of hydrolysis of CPO and CFV were respectively O,O-diethyl phosphoric ester, 3,5,6-trichloro-2-pyridinol and 2-chloro-1-(2',4'-dichlorophenyl)vinyl alcohol.

It is important to mention that these detoxification products are not inhibitors of AChE; furthermore they are not mentioned in European norms for drinking water.

13.3.2 Effect of PTE Loading on AChE Inhibition by OPs

Our group has studied the combination of PTE with recombinant AChE for a selective and sensitive detection of OPs. This bi-enzymatic sensor based on the co-immobilization of both enzymes has been shown to allow the discriminative

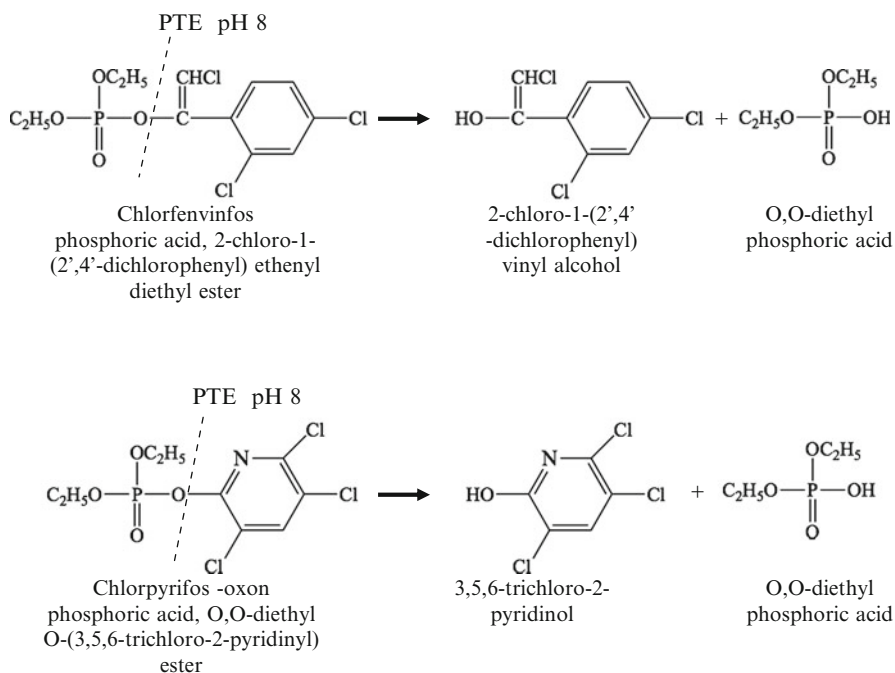


Fig. 13.2 Molecular structures of CPO and CFV and their hydrolysis reactions catalysed by phosphotriesterase (PTE) [37]

detection of CPO and CFV in a wide range of concentrations. Table 13.2 shows that the inhibitory effect of CPO was decreased when the amount of PTE immobilized on the electrode was increased. In each case, the limit of detection I_{10} , the I_0 , I_{50} , and I_{100} were calculated corresponding to the pesticide concentration inducing respectively 10%, 0%, 50% and 100% inhibition. These results are summarized in Table 13.4.

The inhibitory effect of CFV was maintained whatever the concentration of PTE immobilized on the electrode, this insecticide being not hydrolysed by PTE. Consequently, the response of a dual-sensor device incorporating AChE and AChE+PTE will be likely the same when the sample contains only CFV.

When both CPO and CFV were present, it was shown that CFV induced a competitive inhibition of PTE, which in turn was unable to react with CPO. Consequently, a dramatic increase of the sensor inhibition was observed due to the non-degradation of CPO (Fig. 13.3). This feature dramatically complicated the selective detection of these pesticides.

To overcome this problem, the combined response of these biosensors was modeled by means of artificial neural networks ANNs.

Table 13.4 Characteristics of the biosensors based on the co-immobilization of recombinant acetylcholinesterase (1 mIU/sensor) and PTE (0, 0.14 or 0.85 paraoxon-equivalent U/sensor) for the detection of CPO and CFV [13]

PTE U/sensor	Equation	I_0 (M)	I_{10} (M)	I_{50} (M)	I_{100} (M)
CPO					
0	$I = 25.085\text{Ln}(C) + 591.3$	$5.8 \cdot 10^{-11}$	$8.6 \cdot 10^{-11}$	$4.3 \cdot 10^{-10}$	$3.0 \cdot 10^{-9}$
0.14	$I = 17.928\text{Ln}(C) + 376.6$	$7.5 \cdot 10^{-10}$	$1.0 \cdot 10^{-9}$	$1.2 \cdot 10^{-8}$	$2.0 \cdot 10^{-7}$
0.85	$I = 19.501\text{Ln}(C) + 336.8$	$3.2 \cdot 10^{-8}$	$5.3 \cdot 10^{-8}$	$4.1 \cdot 10^{-7}$	$4.1 \cdot 10^{-6}$
CFV					
0 or 0.14 or 0.85	$I = 16.487\text{Ln}(C) + 383.8$	$7.7 \cdot 10^{-11}$	$1.4 \cdot 10^{-10}$	$2.0 \cdot 10^{-9}$	$3.3 \cdot 10^{-8}$

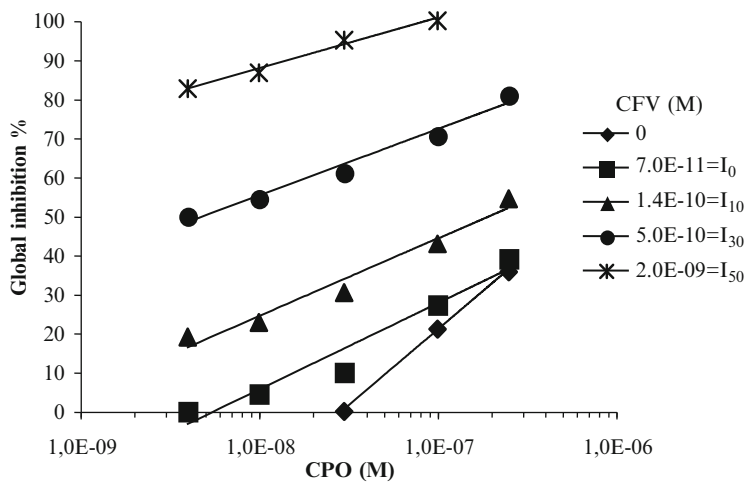


Fig. 13.3 Effect of CPO and CPO+CFV mixtures on the inhibition rate of a bienzymatic sensor incorporating 1 mIU of mutant AChE and 0.85U of PTE [13]. Equation of curves obtained: CFV = 0, $I = 16.877\text{Ln}(C) + 292.6$; CFV = $7.7 \cdot 10^{-11}$ M (I_0), $I = 9.587\text{Ln}(C) + 181.4$; CFV = $1.4 \cdot 10^{-10}$ M (I_{10}), $I = 8.636\text{Ln}(C) + 186.2$; CFV = $5 \cdot 10^{-10}$ M ($=I_{30}$), $I = 7.372\text{Ln}(C) + 190.8$; CFV = $2 \cdot 10^{-9}$ M ($=I_{50}$), $I = 5.622\text{Ln}(C) + 191.1$

13.4 Artificial Neural Networks and Their Use in Biosensors

One shortcoming in present stage of biosensors development using inhibition of AChE is the fact that various OP and carbamate pesticides inhibit this enzyme to a different extent, rendering calibration for an unknown mixture virtually impossible. A detection system capable of discriminating and quantifying several inhibitors in a mixture should provide a more reliable and robust biosensor analysis. In this sense, the use of a sensor array coupled with a chemometric tool, such as an Artificial Neural Network (ANN) employed for data treatment, could substantially improve biosensor selectivity and allow exact identification of the inhibitor present in a sample [23, 38].

Artificial Neural Networks (ANNs) have seen an explosion of interest over the last two decades and have been successfully applied in all fields of chemistry and particularly in analytical chemistry. Inspired from biological systems and originated from the perceptron, i.e. a program unit that learns concepts, ANNs are capable of gradual learning over time and modelling extremely complex functions [39].

In addition to the traditional multivariate chemometric techniques, ANNs are often applied for prediction, clustering, classification, modelling of a property, process control, procedural optimisation and/or regression of the obtained data.

Several intelligent biosensors for the resolution of mixtures of pesticides have been developed based on the principle of the AChE inhibition and chemometric data analysis using ANNs.

Bachmann and Schmid [37] developed a sensitive screen-printed amperometric multielectrode biosensor for the rapid discrimination of the insecticides paraoxon and carbofuran in mixtures. For this purpose, four types of native or recombinant AChEs (electric eel, bovine erythrocytes, rat brain and *Drosophila melanogaster*) were immobilized by screen printing on four-electrode thick film sensors in sets containing each AChE. The sensors registered a detection range for both analytes of 0.2–20 mg·L⁻¹ with an overall assay time of less than 60 min. The individual inhibition pattern of each AChE–analyte combination enabled the discrimination of both analytes by the use of ANNs. Thus, paraoxon and carbofuran in mixtures displaying a concentration range of 0–20 mg·L⁻¹ for each analyte could be analysed with prediction errors of 0.9 mg·L⁻¹ for paraoxon and 1.4 mg·L⁻¹ for carbofuran.

The same group improved the multianalyte detection by selecting different AChE mutants [23]. They developed two different multisensors: the first one included the wild-type *Drosophila* AChE and mutants Y408F, F368L and F368H; in the second one, the use of the mutant F368W instead of the F368H increased the sensor's capacity even further. Both multisensors were used for inhibition analysis of binary paraoxon and carbofuran mixtures in a concentration range 0–5 mg·L⁻¹, followed by data analysis using feedforward ANNs. The two analytes were determined with prediction errors of 0.4 mg·L⁻¹ for paraoxon and 0.5 mg·L⁻¹ for carbofuran. A complete biosensor assay and subsequent ANN evaluation was completed within 40 min. In addition, the second multisensor was also investigated for analyte discrimination in real water samples. Finally, the properties of the multisensors were confirmed by simultaneous detection of binary organophosphate mixtures. Malaoxon and paraoxon in composite solutions of 0–5 mg·L⁻¹ were discriminated with predication errors of 0.9 and 1.6 mg·L⁻¹, respectively.

Our group has developed different amperometric systems to resolve pesticide mixtures [40–42]. These systems have been termed as electronic tongues, since they combine a sensor array to generate multidimensional data and their proper processing to obtain more detailed information [43]. The combined response of these electrodes was always modelled by means of ANNs.

Firstly, an electronic tongue to quantify dichlorvos and carbofuran pesticide mixtures was developed [40]. In that case, the signal was generated from a three biosensors array that used different AChE enzymes: the wild type from Electric eel and two different Dm-AChE (wild type and recombinant). Mean values of concentration

of evaluated pesticides were 0.79 nM for dichlorvos and 4.1 nM for carbofuran. The developed electronic tongue was also applied to the determination of dichlorvos and carbofuran in real water samples. Both pesticides could be determined with low errors from a direct measurement step.

Secondly, a bioelectronic tongue was developed to resolve pesticide mixtures of two organophosphate pesticides: dichlorvos and methylparaoxon [42]. The biosensor array used three different AChE enzymes: and two different Dm-AChE (wild type and recombinant). In this case, the biosensor array was used in a flow injection system, permitting to perform automatically the inhibition assay of the pesticide mixture. The triplet inhibition response was trained with mixture solutions that contained dichlorvos from 10^{-4} to 0.1 mM and methylparaoxon from 10^{-3} to 2.5 mM. When applied to real samples, the two pesticides could be determined with low errors using an extremely simple procedure.

Finally, an amperometric AChE biosensor array was developed to resolve mixtures of two organophosphate insecticides: chlorpyrifos oxon (CPO) and chlorfenvinfos (CFV) [41]. Three different biosensors were built using the wild type from Electric eel, the mutant Dm-AChE and mutant Dm-AChE co-immobilized with a phosphotriesterase (PTE). Specifically two different ANNs were constructed. The first one was used to model the combined response of mutant Dm-AChE + PTE and Electric eel biosensors and was applied when the concentration of CPO was high and the other, modelling the combined response of mutant Dm-AChE + PTE and mutant Dm-AChE biosensors, was applied with low concentrations of CPO. In both cases, good prediction ability was obtained. The developed system was also applied to the determination of CPO and CFV pesticides in real water samples. Both pesticides could be quantified with low errors from a direct measurement step.

13.5 Conclusions

Acetyl- and butyl-cholinesterases have been described for many years as sensitive tools for the detection of many neurotoxic compounds such as insecticides (OPs and carbamates), chemical weapons and toxins (anatoxin-a(s)). They have been extensively used in biosensor configurations based on amperometric detection. Basically, the first devices described were coupling a cholinesterase with a choline oxidase, the detection being based on either the oxidation of H_2O_2 or the reduction of oxygen. This complicated system was further simplified using a synthetic substrate of AChE, acetylthiocholine, which produces under hydrolysis an easily oxidisable compound, thiocholine. Since then, the sensitivity of AChE-based sensors has been greatly improved, mainly due to the use of genetically modified AChEs, which were specifically modified for their sensitivity to special classes of inhibitors. However, the described devices often lack of selectivity and specificity, mainly due to the fact that AChE enzymes are globally sensitive to a class of inhibitors. The selectivity of AChE-based sensors can be tuned by the use of phosphotriesterase, an enzyme hydrolysing specifically some organophosphate compounds. This enzyme has been successfully

coupled to AChE for designing sensors selective to two organophosphate compounds of great environmental concern: chlorpyrifos and chlorfenvinfos. Despite this progress, the main problem still remained the analysis of pesticide mixtures, which can be solved in some cases by the use of a sensor array coupled with a chemometric tool. In this sense ANNs have been found to be powerful tools, particularly suited for various tasks in information processing. ANNs are non-parametric calibration methods specially created to process non-linear information. It has been demonstrated that by combining native and recombinant variant of AChE with ANNs data processing, a sensitive multianalyte detection is possible.

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Chapter 14

Disposable Electrochemical Biosensor Based on Cholinesterase Inhibition with Improved Shelf-Life and Working Stability for Nerve Agent Detection

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Abstract Organophosphonate compounds such as Sarin are potent toxic nerve agents. The development of fast, simple and miniaturisable analytical systems for the detection of these compounds is an important goal in analytical chemistry. In this chapter we present the results obtained in the developing cholinesterase biosensor for nerve agent detection characterised by high shelf and operational stability. Different types of immobilisation were tested immobilizing in different ways the acetylcholinesterase from *electric eel* and butyrylcholinesterase from *horse serum* on the screen printed electrodes chemically modified with the electrochemical mediator Prussian Blue (PB-SPEs). The enzymatic activity of the immobilized enzymes was thus measured in amperometric mode at +200 mV vs Ag/AgCl using as substrate the acetylthiocholine and butyrylthiocholine, respectively, quantifying the enzymatic product thiocholine by means of PB-SPEs. The biosensor with butyrylcholinesterase immobilized using glutaraldehyde, bovine serum albumin and Nafion® has demonstrated a shelf life higher than 6 months at RT in dry condition and an operational stability up to 10 h. The biosensor was challenged with Sarin gas. The enzymatic measurements were carried out also measuring the rate of enzymatic activity in the first 30 s by means of a new version of Palm Sens® instrument software. In this way, the time of analysis was lower than 2 min. Keeping in mind that the IDLH (Immediately Dangerous to Life or Health) is 0.1 mg/m³, this biosensor with kinetic measurements supported by the Palm Sens® software allows to detect 0.1 mg/m³ in 90 s, demonstrating that is an useful analytical system for environmental security.

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14.1 Introduction

The detection of pesticides in food, water and soil is one of the major issues for the analytical chemistry. Among the pesticides, organophosphorus insecticide species are one of the most used, due to their high insecticidal activity and relatively low persistence in environment. Its insecticidal activity is based on the fact that these compounds are able to irreversibly inhibit the acetylcholinesterase (AChE), a key enzyme of nervous transmission. The same principle is the base of the toxicity of nerve agent gases such as Sarin, Soman, Sarin, VX which are able to inhibit strongly the AChE present in human nervous system. The fact that nerve agents belong to organophosphorus compounds is due to the accidental discovery of these compounds in 1936 by Dr. Gerhard Schrader, working for IG Farben in order to develop new types of insecticides. Schrader experimented numerous fluorine-containing compounds that led to the preparation of Tabun. After, Sarin, Soman and CycloSarin were also synthesised (nerve agents G series). The G-series are named labelling Tabun as GA (German Agent A), Sarin as GB, Soman as GD, and CycloSarin as GF. The V-series is the second family of nerve agents: VE, VG, VM, VR and VX. Nerve agents were employed against the Kurdish opposition in Iraq and Sarin gas was utilised in the terrorist attack by the Aum Shinrikyo sect in the Tokyo subway (1995) [1]. Also the tragic events of September 11, 2001 have led to an increased awareness of threats regarding the use of chemical warfare agents; for this reason, analytical tools which combine reliability and rapidity of response for the detection of lethal chemicals are strongly required. Chromatographic methods (i.e. GC-MS) are the analytical instruments usually adopted for their quantification, however this type of instrumentation usually requires laboratory set-up, sample pretreatment and skilled personnel [2, 3]. Furthermore the instrumentation is not miniaturisable avoiding the analysis in field. In this context, the cholinesterase (ChE)-based biosensors have demonstrated the ability to be useful analytical tools able to give an alarm [4, 5]. The principle of analysis is based on the enzymatic activity measurement before and after the exposure of the biosensor to the nerve agent gas, thus by the calculation of the degree of inhibition it is possible to affirm if the air sample is polluted by nerve agent gas. In order to have a ChE biosensor suitable for market, a biosensor for nerve agent detection should be developed taking in consideration that it should be cost-effective, miniaturisable and characterized by satisfactory shelf and operational stability. In this context this chapter will present the results obtained for the development of a ChE electrochemical biosensor for nerve agent detection characterized by high sensitivity, working and shelf stability in order to be suitable for a future commercialization.

14.2 Experimental Section

14.2.1 Apparatus

Thiocholine amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to an *X-t* recorder (L250E, Linseis, Selb, Germany), while the enzymatic activity measurement of biosensors were performed by amperometric measurements using a portable PalmSens, (Palm Instruments®, The Netherlands). Cyclic voltammetry (CV) was performed using an Autolab/PGSTAT-12 electrochemical system (Eco Chemie, Utrecht, The Netherlands).

14.2.2 Electrodes

Screen-printed electrodes (SPEs) were home produced with a 245 DEK (Weymouth, England) screen printing machine and using different inks obtained from Acheson (Milan, Italy). Graphite-based ink (Electrodag 421), silver ink (Electrodag 477 SS), silver/silver chloride ink (Electrodag 4038 SS) and insulating ink (Carboflex 25.101.S) were used, the substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 48. The diameter of the working electrode was 0.3 cm resulting in an apparent geometric area of 0.07 cm². Before ChE measurements, in the case of SPEs in which the silver ink was used, the reference electrode was chlorinated. To do this, a potential of +0.6 V was applied between the silver ink and an external Ag/AgCl electrode for 20 s in a phosphate buffer solution in the presence of 0.1 M KCl [6].

14.2.3 Reagents

All chemicals from commercial sources were of analytical grade. Acetylcholinesterase (AChE) from *electric eel*, butyrylcholinesterase (BChE) from *horse serum*, bovine serum albumin (BSA), S-butyrylthiocholine chloride, acetylthiocholine chloride, glutaraldehyde and paraoxon were purchased from Sigma Chemical Company (St. Louis, USA). Nafion® (perfluorinated ion-exchange resin, 5% v/v solution in lower alcohols/water) was obtained from Aldrich (Steinheim, Germany). Sarin was provided by TNO defence, security and safety centre in Rijswijk, the Netherlands.

14.2.4 Modified Screen Printed Electrodes

14.2.4.1 Preparation of Prussian Blue Modified Screen Printed Electrodes

Prior to Prussian Blue modification, screen printed electrodes (SPEs) were pre-treated in a 0.05 M phosphate buffer +0.1 M KCl, pH 7.4 by applying an anodic potential of +1.7 V vs Ag pseudoreference for 3 min. Prussian Blue modification of SPEs was then accomplished by placing a drop (10 μ L total volume) of “precursor solution” onto the working electrode area. This solution is a mixture obtained by adding 5 μ L of 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$) in 10 M HCl to 5 μ L of 0.1 M ferric chloride in 10 mM HCl. The drop was carefully applied exclusively on the working electrode area. The electrodes were shaken gently on an orbital shaker for 10 min and then rinsed with a few millilitres of 10 mM HCl. The probes were then left 90 min in the oven at 100°C to obtain a more stable and active layer of Prussian Blue. The Prussian Blue modified electrodes were stored dry at room temperature in the dark [7].

14.2.4.2 Preparation of Cobalt Phthalocyanine Bulk Modified Screen Printed Electrodes

The preparation of cobalt phthalocyanine modified SPEs was obtained by following the procedure by Hart and co-workers [8]. Before the printing step, the graphite ink was carefully mixed with 5% of cobalt phthalocyanine powder in order to make the paste homogenous. After this, the ink was used to print the working electrode of SPE as for the other electrodes (same geometry and surface area as above).

14.2.4.3 Preparation of the SPEs with Gold Film

SPEs fabricated in our laboratory have been modified with a gold thin film. The first step was a pre-treatment of the working electrode surface in 0.05 M phosphate buffer +0.1 M KCl, pH 7.4, by applying positive potential of +1.7 V vs Ag pseudoreference for 3 min. The second step was the deposition of the gold film. This was accomplished by applying a positive potential +0.3 V vs Ag/AgCl in solution of $AuCl_3$, 1,000 ppm, for 6 min. Finally, the third step was the conditioning of the gold film surface. This was achieved by recording 10 cyclic voltammograms at 0.05 Vs^{-1} , using potential windows between +0.2 V and +0.6 V vs Ag/AgCl.

14.2.5 Thiocholine Measurement

Thiocholine was enzymatically produced by AChE using acetylthiocholine as substrate (because thiocholine is not commercially available). For this purpose, 1 mL of 1 M acetylthiocholine solution was prepared in phosphate buffer 0.1 M (pH=8), and 100 units of AChE were added to this solution. After 1 h, the concentration of

thiocholine produced by AChE was estimated spectrophotometrically by Ellman's method. For this purpose, 900 μL of phosphate buffer solution (0.1 M, pH=8), 100 μL of 0.1 M DTNB, and 5 μL thiocholine solution (diluted 1:100 in water) were put in a spectrophotometric cells. The absorbance was measured, and the real concentration was evaluated by using the Lambert–Beer law with the known molar extinction coefficient of TNB ($\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) [9]. After 1 h, the acetylthiocholine hydrolysis is completed, and 1 mL solution of 1 M thiocholine is obtained. The solution is stable for 1 day at 4°C.

In the case of PB modified SPE, thiocholine measurements were performed using amperometric batch analysis in a stirred phosphate buffer solution 0.05 M+0.1 M KCl, pH 7.4 (10 mL) with an applied potential of +200 mV vs Ag/AgCl.

Cobalt phthalocyanine modified SPEs were used with the same procedure, but the phosphate buffer solution was pH 8 (10 mL) and the applied potential +100 mV vs Ag/AgCl.

In both cases, when a stable baseline was reached (1 min), the analyte was added and the response was recorded.

In the case of gold modified SPE, they were immersed in phosphate buffer solution 0.05 M+0.1 M KCl, pH 7.4 (10 mL) with an applied potential of +850 mV vs Ag/AgCl. When a stable baseline was reached (20 min), the analyte was added and the response was recorded.

Thiocholine measurements were also carried out with unmodified SPE in a solution containing 1 mM of ferricyanide ions in a phosphate buffer solution 0.05 M+KCl 0.1 M, pH 8 (10 mL) with an applied potential of +300 mV vs Ag/AgCl. After 1 min, required for baseline stabilisation, the analyte was added and the response was recorded.

14.2.6 Acetylthiocholine and Butyrylthiocholine Solution Stability Measurement

The stability of acetylthiocholine and butyrylthiocholine solution was measured evaluating the thiocholine produced by the spontaneous hydrolysis. The concentration of thiocholine was estimated spectrophotometrically by Ellman's method as described in Sect. 14.2.5.

14.2.7 Cholinesterase Biosensor Based on Prussian Blue Modified SPE

Prussian Blue modified SPEs were used as substrate for enzyme immobilisation. Two different biosensors were produced immobilizing, in one case acetylcholinesterase to create an AChE biosensor, and in the other case butyrylcholinesterase to obtain a BChE biosensor. For this purpose several immobilisation procedures were used. In the

case of physical adsorption 4 μL of enzyme (AChE or BChE) solution were placed on the working electrode surface, then the electrode was maintained at 4°C overnight. After that, the surface of the working electrode was covered with 4 μL of stabiliser (trehalose, gelatine, lactitol, sucrose) solution prepared in aqueous solution with sodium azide 0.02% (w/v). The electrode was maintained at 4°C for 2 h, then at RT before the use. This type of procedure was also modified adding a final Nafion® coverage by simply addition of 2 μL of Nafion® on the working electrode surface and waiting to be dry. In the case of cross-linking method, two steps were adopted. 2 μL of a glutaraldehyde solution were applied with a syringe exclusively on the working electrode. For the AChE biosensor, a 1% v/v solution of glutaraldehyde (diluted in water) was used, while for the BChE biosensor a 0.25% v/v solution (diluted in water) was utilised. The solution was then left to evaporate. Then, 2 μL of a mixture of BSA, enzyme and Nafion® were placed on the working electrode. The mixture was obtained by mixing 25 μL of BSA (5% w/v prepared in water), 25 μL of Nafion (0.1% v/v diluted in water) and 25 μL of enzyme stock solution.

14.2.8 Acetylthiocholine and Butyrylthiocholine Measurement with AChE and BChE Biosensors

Acetylthiocholine measurements were performed using an amperometric “drop” or a batch procedure in phosphate buffer solution (0.05 M + 0.1 M KCl, pH 7.4) with an applied potential of +200 mV vs Ag/AgCl. In the case of “amperometric drop” procedure, a drop (50 μL) of buffer containing different amounts of acetylthiocholine was placed onto the AChE biosensor in such a way that the counter and reference electrodes were also covered. After applying the potential, the signal was recorded continuously and the current value at the steady state was measured. For butyrylthiocholine measurement the same procedure was carried out using a BChE biosensor. In the case of amperometric batch analysis, the biosensor was immersed in a stirred phosphate buffer solution 0.05 M + KCl 0.1 M, pH 7.4 (10 mL) and the potential was applied to +200 mV vs. Ag/AgCl. When a stable baseline current was reached, the acetylthiocholine was added and the response was recorded at the steady state. For butyrylthiocholine measurement the same procedure was carried out using a BChE biosensor.

14.2.9 Inhibition Measurement Using AChE and BChE Biosensors

The inhibitory effect of different pesticides on AChE and BChE biosensors was evaluated by determining the decrease in the current obtained for the oxidation of thiocholine that was produced by the enzymes. To do this the cholinesterase biosensor was first incubated in the pesticide solution for a certain period (incubation

time) and then rinsed three times with distilled water. After that, the response toward the substrate was measured as described above and the degree of inhibition was calculated as a relative decay of the biosensor response Eq. 14.1.

$$I\% = [(I_0 - I_i) / I_0] \times 100 \quad (14.1)$$

when I_0 and I_i represent the biosensor response before and after the incubation procedure, respectively.

14.3 Results and Discussion

14.3.1 *Thiocholine Sensor*

Electrochemical biosensors can be classified as bi-enzymatic in which the ChE is coupled to choline oxidase (ChOx) enzyme and mono-enzymatic system in which only ChE is used as biocomponent. The bi-enzymatic ChE biosensor is constructed using: AChE, that hydrolyses the substrate acetylcholine to choline and acetic acid (or butyrylcholine in the case of BChE) and ChOx that oxidises the choline to betaine with the production of H_2O_2 . The use of ChOx is necessary in the case of amperometric biosensors because the enzymatic products of the reaction (choline and acetic acid) are not electroactive. In order to produce monoenzymatic amperometric biosensors, a synthetic substrate must be used; in fact, acetylthiocholine was adopted instead of acetylcholine. The enzymatic reaction hydrolyses the acetylthiocholine to acetic acid and thiocholine and the thiocholine, being electrochemically active, can be measured. This biosensor has the advantage to use only one enzyme: AChE which is also characterized by the higher turnover than the one of choline oxidase enzyme [10]. Thus, in order to have a sensitive nerve agent biosensor, the first step in the construction of amperometric monoenzymatic biosensors is to choose a sensitive thiocholine (enzymatic substrate) sensor. In this work, we have investigated the response of four different thiocholine sensors. The comparison of their performances in terms of thiocholine signal, stability and response time would allow us to select the most suitable sensor for the successive ChE enzyme immobilisation. All the sensors investigated are based on the use of SPEs and applications of three of them have already been described in the literature. The Co-phthalocyanine modified sensor is well known and many examples of its use can be found in literature [11, 12]. This is also the case for the use of ferricyanide ions as mediator, even if its applications to real samples have not been so numerous [13]. In the case of Prussian Blue modified electrodes, their first application for this purpose was quite recently proposed by our group [14]. Thus for all these sensors, the measuring procedure needs only to be adapted and optimised. The use of gold electrodes for the amperometric measurement of thiols has also been amply reported, even if it is always hampered by the surface passivation of the electrode [15]. Moreover, gold SPEs are

more expensive than those based on graphite with a negative impact on the overall analysis cost. To overcome these problems, a gold based electrode is proposed in this work. A graphite SPE was modified with an electrochemically deposited gold film and its performance in terms of thiocholine measurement was evaluated. In this case a study of the applied potential for thiocholine measurement was performed: a current/potential plot was investigated in presence of thiocholine ($1.5 \cdot 10^{-6}$ M). The oxidation current increased rapidly at a potential near +800 mV vs Ag/AgCl, reaching a relative maximum at +850 mV vs Ag/AgCl (data not shown). This applied potential was then chosen as optimum applied potential for further measurements. For a summary of the relative performance, Table 14.1 shows the analytical parameters obtained for amperometric thiocholine detection using the four proposed sensors at their optimised conditions. In the case of SPE utilising hexacyanoferrate ions in solution, a low detection limit was achieved ($3 \cdot 10^{-7}$ M) with a high sensitivity and fast response time. However, a major drawback associated with this system is the fact that the mediator has to be used in soluble form and should be added to the working solution. In the new approach based on a SPE electrode modified with gold, the film electrode was generated by the reduction of AuCl_3 , Au being deposited on the surface of the SPE. In this case, at the concentration of thiocholine tested, no fouling problem was observed. In fact, three different calibration curves could be generated using the same electrode and demonstrating good reproducibility. This result can probably be attributed to the nanostructured of the gold film which does not allow thiol chemisorption. The investigation of the hypothesis is on going. Another positive aspect was the low (0.1 μM) LOD achieved with this sensor making it a sensitive sensor for thiocholine measurement. However, there are some major drawbacks to the real application of these probes. The process utilised to modify a single electrode is, in fact, time-consuming and still not amenable to mass production. Moreover, the long time needed to reach a stable background current (20 min) makes the overall time of analysis too long. Many research articles [8, 11, 12] have illustrated the advantages of cobalt phthalocyanine modified electrodes. In addition to their ease and speed of production, a good electroactivity and reproducibility of this mediator towards the thiocholine oxidation is observed. The sensors were able to detect thiocholine at a concentration of $5 \cdot 10^{-7}$ M with a linearity up to $7 \cdot 10^{-5}$ M. Moreover, the low applied potential and the fast response time make them, in theory to reach high selectivity and avoid the majority of the electrochemical interferences. However, under our operative conditions only a few measurements could be performed with the same sensor because of their limited working stability. The performance of Prussian Blue as a mediator of thiocholine oxidation has been investigated by our group in a previous work [14]. This mediator shows a good stability (when stored several months dry at room temperature in the dark) [7] and reproducibility (7%), and the modification of the electrode surface is easy to perform as well as being amenable to mass production. Moreover, the Prussian Blue modified sensors allow the measurement of thiocholine at μM levels, with high sensitivity and a broad linear range. The major advantage associated with the use of such a sensor is the very high stability even under drastic conditions, thus making it very useful for practical applications. Also, no effect due to thiol passivation has

Table 14.1 Analytical parameters for the various thiocholine sensors. For details about measurement conditions see text (Sect. 14.2.5)

Transducer	Modifier	Reagent	Applied Potential vs Ag/AgCl, mV	Time for background stabilisation	Sensitivity mAM ⁻¹ cm ⁻²	Noise nA	Detection limit, M	Linear range, M	Working stability
Screen printed graphite	Cobalt	–	+100	30 s	24	0.2	5·10 ⁻⁷	1·10 ⁻⁶ /7·10 ⁻⁵	Fair
	phthalocyanine								
	Prussian Blue	–	+200	30 s	143	4	5·10 ⁻⁶	5·10 ⁻⁶ /5·10 ⁻⁴	High
	Gold film	–	+850	20 min	378	1	1·10 ⁻⁷	1·10 ⁻⁷ /5·10 ⁻⁶	Fair
	–	Ferricyanide	+300	30 s	286	0.3	3·10 ⁻⁷	1·10 ⁻⁶ /4·10 ⁻⁵	Poor

been observed, thus allowing measurements without loss of sensitivity. For these reasons, and given our long experience in the use of Prussian Blue modified SPEs, these sensors were selected as probes for thiocholine detection to develop a biosensor for nerve agent detection. Table 14.1 shows the analytical parameters for the various thiocholine sensors.

14.3.2 Shelf Life Stability of Cholinesterase Biosensor

As well described by Gibson [16] the working stability may be defined as the retention of activity of an enzyme when in use and the shelf stability is defined as the enhancement or improvement of activity retention of an enzyme when stored under specified conditions after manufacture. Both shelf-stability and operating stability are important parameters but, in some cases, the shelf-stability is the most crucial parameter for the world market.

14.3.2.1 Acetylcholinesterase Biosensor

The AChE biosensors were produced using different types of immobilization here briefly summarised. In the first approach, the enzyme was adsorbed onto the working electrode surface, storage at 4°C overnight and after that some μL of several stabilizers were added to the working electrode surface; the biosensors were tested when the working electrodes of biosensors were completely dry. In the second approach a similar protocol aforementioned was adopted but followed by a final step of Nafion® coverage by simply addition of some μL of Nafion® on the working electrode surface and waiting to be dry. The last approach was made using the cross-linking method by means of glutaraldehyde, Nafion® and BSA. In the Fig. 14.1a the results obtained after 1 week of shelf stability at 37°C in dry conditions was reported. As shed light from the Fig. 14.1a the best result in terms of shelf stability was obtained in the case of AChE immobilized by gelatin in presence and absence of trehalose as additive. In this case, the measurement was performed in amperometric batch conditions because the layer of gelatin renders difficult the diffusion of the substrate into the membrane in absence of convective transport of the substrate. Thus, the biosensor constructed by immobilization of AChE with gelatin and gelatin+trehalose was tested after several days: we observed that the biosensor in which the AChE is immobilized by gelatin retains its activity up to 5 months when maintained at RT in dry conditions while the biosensor made with gelatin and trehalose up to 7 months, however a better reproducibility was obtained in the case of gelatin only, thus the biosensor with gelatin only was used to test its operational stability. In this case we observed the possibility to use this biosensor for not more than five times probably because after that, there is a probably solubilisation of the membrane, thus even if this biosensor is characterized by high shelf stability it is also characterized by low robustness that renders it no so much suitable for a future commercialization.

14.3.2.2 Butyrylcholinesterase Biosensor

In the case of BChE biosensors, they were produced using the same several types of immobilisation used in the case of AChE biosensors before mentioned. As inferred by the Fig. 14.1b, in this case the best shelf life stability was obtained using the biosensor constructed immobilizing the BChE by means of glutaraldehyde, Nafion® and BSA. Thus, this type of BChE biosensor was tested after several months, at least after 6 months the biosensor retains its activity. The biosensor, in this case, was tested in chronoamperometry observing a good reproducibility (RSD%) intra and inter-biosensor equal to 3% and 10% (n=5), respectively. The operational stability of this biosensor was also tested. In this case we have observed a decrease of signal

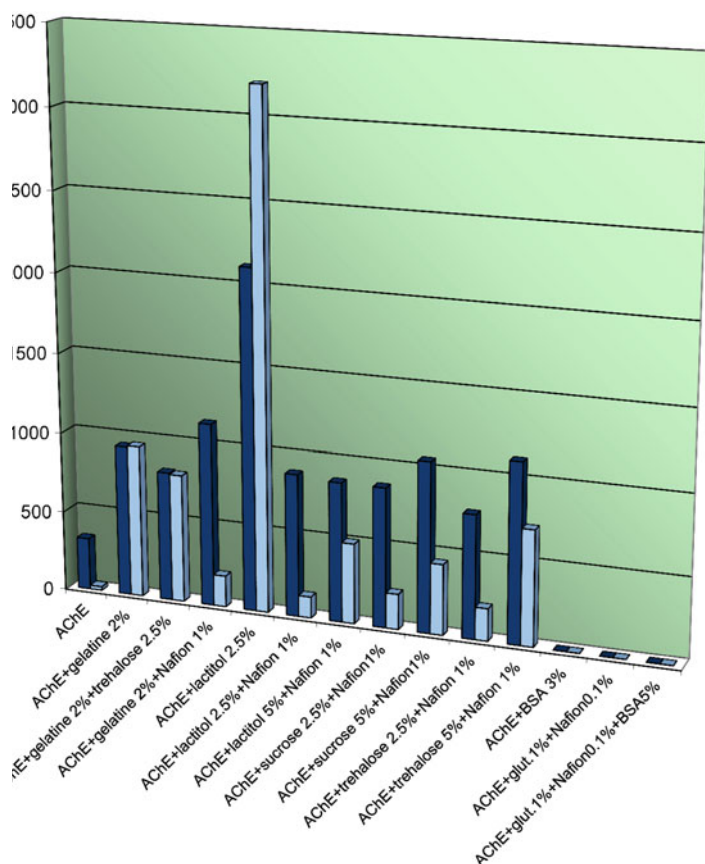


Fig. 14.1 Shelf life stability of AChE (**a**) and BChE (**b**) after 1 week at 37°C. (glut=glutaraldehyde). Applied potential: +200 mV vs Ag/AgCl. Acetylthiocholine 1 mM in the phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4 in the case of AChE biosensor. Butyrylthiocholine 5 mM in phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4 in the case of BChE biosensor

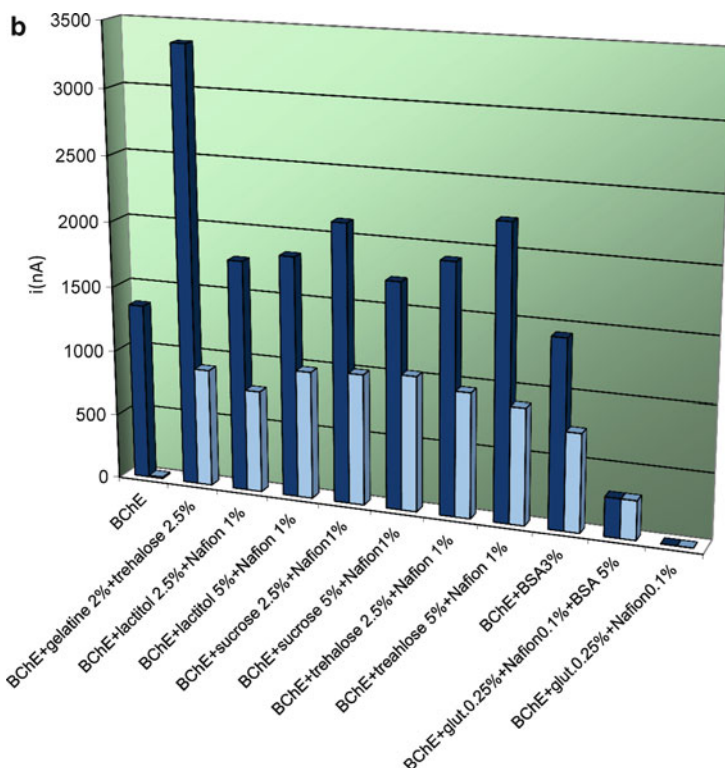


Fig. 14.1 (continued)

rapidly to zero after around 3 h, in our opinion this behavior is ascribed to the passivation of the silver reference electrode by thiocholine even if a silver chloride film was electrochemically applied to the silver ink electrode before the measurement [6]; this approach was previously used by us but in the case of time measurements lower than 30 min. In order to avoid this problem, the SPE was printed using the silver/silver chloride ink observing in this case a stability at least of 10 h (Fig. 14.2), confirming that this biosensor is characterized by high working and storage stability.

14.3.2.3 Storage Stability of Substrate

In order to have an analytical system characterized by high shelf and operation stability, also the enzymatic substrate should be characterized by high shelf stability. In this case, to our knowledge, a stability study of the substrate taking into consideration

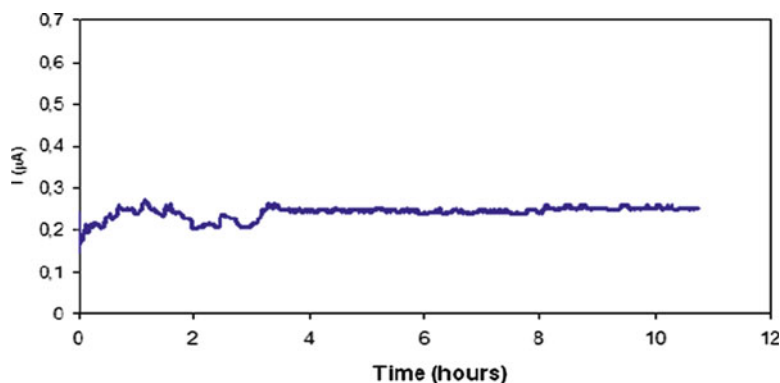


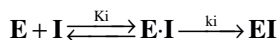
Fig. 14.2 Working stability of BChE biosensor prepared by cross-linking method by means of glutaraldehyde, Nafion® and BSA (for details see Sect. 14.2.7). Applied potential: +200 mV vs Ag/AgCl. BChE=0.0025 U and butyrylthiocholine 5 mM in phosphate buffer 0.05 M+KCl 0.1 M, pH 7.4

that the acetylthiocholine has to be stored at -20°C and the butyrylthiocholine at $+4^{\circ}\text{C}$ [17, 18] has never been reported, thus in the case of ChE biosensor in order to be competitive in the market also the enzymatic substrate need to be stable at RT. To this regard, we have studied, for instance, the spontaneous hydrolysis of acetylthiocholine solution 0.1 M prepared in phosphate buffer pH 3, 7.4, 9.5 and in distilled water observing a spontaneous hydrolysis of 0.4%, 3%, 5.5% and 0.26% if the solution is maintained at RT for 1 day, thus also the substrate needs a good storage in order to be stable for several days. In this case, we have carried out the stockpiling of the butyrylthiocholine and acetylthiocholine powder in eppendorf weighting a certain amount of powder, after that nitrogen was flown in the eppendorf for 1 min and the eppendorf closed with parafilm. In this case we have observed a storage stability at least of 6 months.

14.3.3 Analytical Applications of BChE Biosensor Characterized by High Shelf and Operational Stability

In order to be sure that the BChE biosensor is a more suitable biosensor for nerve agents, a sensitivity comparison of the analytical performances was performed using AChE and BChE enzyme immobilised using the same approach: glutaraldehyde, Nafion® and BSA. The efficiency of inactivation of AChE and BChE biosensor was quantified using paraoxon as nerve agent simulant and the enzymatic kinetic approach [19].

The irreversible inhibition of ChE by nerve agents could be described as follows:



where:

- **E** is the active enzyme;
- **I** is the inhibitor;
- **E·I** is the reversible enzyme-inhibitor complex;
- **EI** is the irreversible enzyme-inhibitor complex;
- K_i is *thermodynamic constant* that describes the affinity of the inhibitor for the active zone of the enzyme;
- k_i is *kinetic constant* that describes the rate of binding of inhibitor to the enzyme active zone.

When $[I] \gg [E]$, the decrease of enzyme activity versus time is expressed by the following equation: :

$$\ln([E]_t/[E]_0) = -K_{app} t$$

where:

$K_{app} = (k_i [I]) / (1 + K_i/[I])$ and t is incubation time.

when $K_i \gg I$ the equation of K_{app} becomes:

$K_{app} = (k_i/K_i)[I]$ where k_i/K_i represents the efficiency of inactivation.

Thus, plotting the $\ln([E]_t/[E]_0)$ vs incubation time, the k_i/K_i value, which represents the efficiency of inactivation, was calculated in the case of AChE (Fig. 14.3a) and BChE (Fig. 14.3b) biosensor which found equal to 2.9×10^{-4} and $8.4 \times 10^{-4} \text{ ppb}^{-1} \text{ min}^{-1}$ at fixed concentration of paraxon (200 ppb) [19]. The analytical performances of AChE and BChE biosensor was also investigated towards paraoxon (Table 14.2) confirming that the BChE biosensor has higher sensitivity towards paraoxon than AChE biosensor. In the light of these results, the BChE was chosen for the further study keeping in mind that respect AChE has showed superior sensitivity and shelf stability.

14.3.3.1 Nerve Agent Detection by Means of BChE Biosensor

Our purpose was the developing of a biosensor for Sarin gas measurement, given that in terrorist attacks the Sarin is used in gas phase. Sarin gas was produced with a sophisticated instrument realised in a specialised center (the TNO laboratory) [19]. Before the exposure of biosensor to Sarin gas, the working electrode was covered with 5 μL of phosphate buffer at $\text{pH} = 7.4$ in order to inhibit the BChE irreversibly by Sarin gas. It is well known, in fact, that the active site of ChE contains two sub-sites, the esteratic and anionic sub-sites, corresponding respectively, to the catalytic site and choline-binding pocket [20]. The esteratic site contains a serine resi-

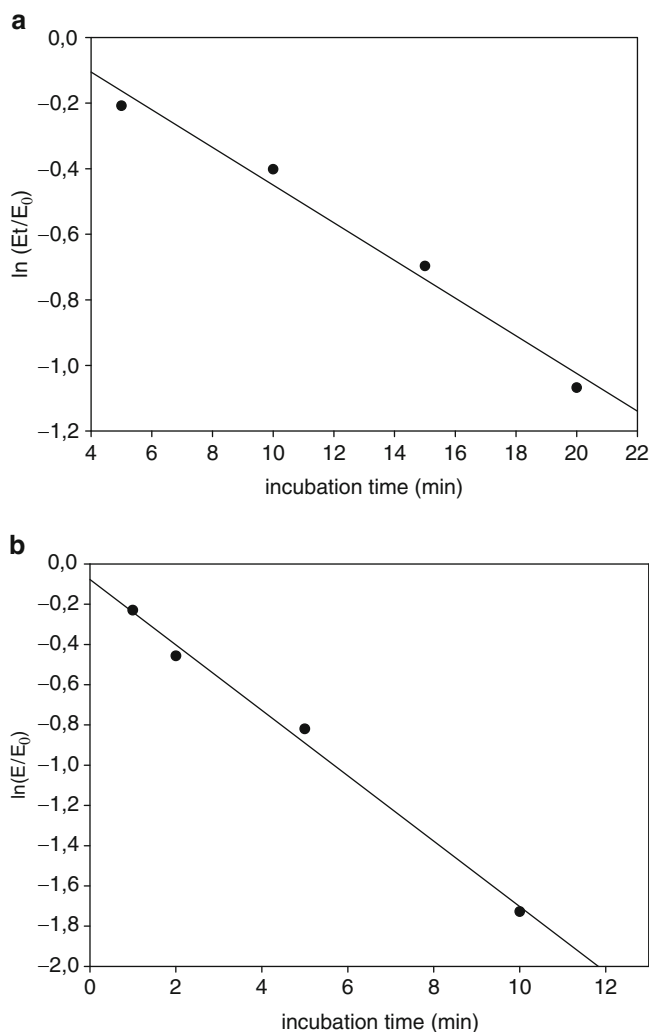


Fig. 14.3 Study of $\ln(E/E_0)$ vs incubation time using AChE (a) or BChE (b) biosensors. Paraoxon concentration 200 ppb. Applied potential +200 mV vs Ag/AgCl; 0.01 U AChE, 0.05 M phosphate buffer +0.1 M KCl, pH 7.4. In the case of the AChE biosensor 1 mM acetylthiocholine was used, while for the BChE biosensor 5 mM butyrylthiocholine was used

due which reacts in appropriate pH conditions (pH comprised between 7 and 8) with the substrate or the irreversible inhibitor (Sarin), thus in order to have the right pH, 5 μ L of phosphate buffer at pH=7.4 was used to cover the enzymatic membrane immobilised on the working electrode surface. The biosensor was exposed to the air stream in absence of the nerve agent in order to have the blank (control). After 10 min of exposure no change in biosensor activity was observed, showing that the air stream itself had no effect on the enzyme activity. Then, the system was

Table 14.2 Analytical parameters for AChE and BChE biosensors relative to nerve agents simulant paraoxon, acetylthiocholine (1 mM) and butyrylthiocholine (5 mM) were used as substrates for AChE and BChE, respectively

Nerve agents simulant	Type of biosensor	Linear range, ppb	50% of inhibition	LOD, ppb	RSD, %
Paraoxon	AChE	5–50	25	5	8
Paraoxon	BChE	2–5	4	2	7

Applied potential: +200 mV vs Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Incubation time = 30 min

challenged against the Sarin gas at two different concentrations 0.1 and 0.5 mg/m³ and different incubation times (from 30 s to 10 min). As reported in our previous paper 30 s of incubation time is sufficient to obtain a degree of inhibition equal (34 ± 1)% by Sarin gas 0.1 mg/m³ [19]. This demonstrated that the procedure of exposing the biosensor (with few μ l of phosphate buffer on the working electrode) to the nerve agent gas flow is an effective approach and also quite simple. It is likely that an equilibrium between the Sarin in gas phase and in aqueous phase (a drop on the working electrode) was achieved. This equilibrium is a dynamic one because the molecules of Sarin irreversibly bonded to BChE subtracted from aqueous phase, are restored from gas phase. A typical response to butyrylthiocholine using the BChE biosensor before and after the exposure to the Sarin gas is reported in Fig. 14.4. As we can see in this Fig. the time of measurement of the enzymatic activity was chosen equal to 5 min. However, to make a fast measurement of nerve agent, a shorter measurement time would be possible by measuring the rate of the enzymatic reaction in the first minute, instead of the current at steady state. In collaboration with Palm Sens company a dedicated version of software was developed that allows measuring the rate of enzymatic reaction. In Fig. 14.4 were reported the amperograms before and after the exposure of the biosensor to Sarin gas 0.1 mg/m³ with an incubation time of 1 min. Using the steady state approach that allows a total analysis time of 11 min a degree of inhibition equal to 51% was found, instead in the case of kinetic approach with a total time of analysis of 90 s a degree of inhibition of 71% was measured. In the case of the kinetic approach the Eq. 14.1 was used, where instead of the current at steady state the values of the slope were used for the inhibition degree calculation. It is well known that usually the kinetic approach is characterised by less precision, but to give an alarm this system have demonstrated to be efficient considering that IDLH (Immediately Dangerous to Life or Health) [21].

14.4 Conclusions

In this chapter the results obtained by our research group in the development of a biosensor for nerve agent detection were reported. The biosensor developed was based on the use of cholinesterase because the toxicity of nerve agent gases such as Sarin, Soman, Sarin, VX is due to the fact that they inhibit strongly the AChE, a key

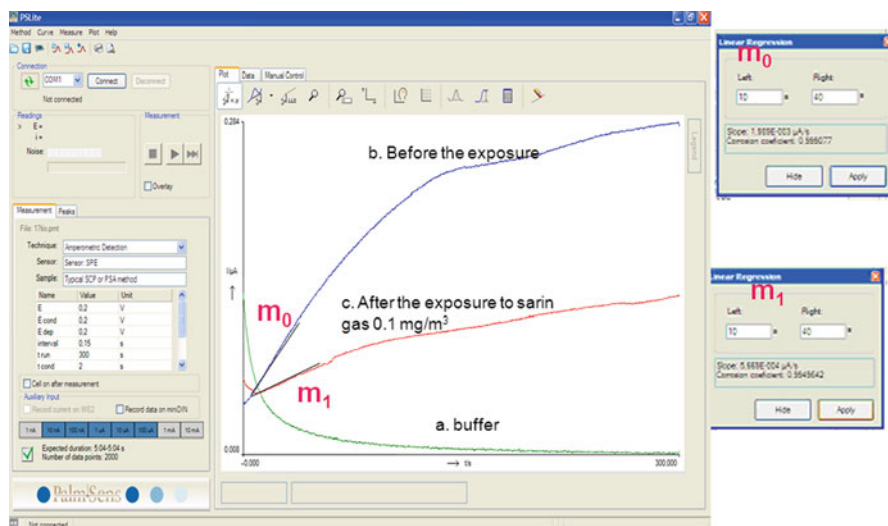


Fig. 14.4 Original recording obtained using biosensor in phosphate buffer solution 0.05 M + KCl 0.1 M, pH 7.4. Applied potential: +200 mV vs Ag/AgCl. Signal recorded in phosphate buffer (a) and in a solution of butyrylthiocholine (5 mM) before the exposure of biosensor to Sarin gas (b) and after 1 min exposure to 0.1 mg/m³ (c) of Sarin gas

enzyme of human nervous system. Two different enzymes of cholinesterase family commercially available (acetylcholinesterase from *electric eel* and butyrylcholinesterase from *horse serum*) were used to construct a biosensor for nerve agent detection. For this purpose different procedures of enzyme immobilization on the SPE modified with Prussian Blue was investigated founding that the butyrylcholinesterase immobilizing using glutaraldehyde, Nafion® and BSA allows to obtain a biosensor with high storage and working stability. The biosensor was then tested with Sarin gas with satisfactory results, in fact taking in consideration that the IDLH (Immediately Dangerous to Life or Health) is 0.1 mg/m³ and that the biosensor in kinetic measurements supported by the Palm Sens® software allows the detection of 0.1 mg/m³ in less of 2 min, it is indeed an useful analytical system for environmental security.

Acknowledgments Dr Kees van Velzen for the Palm Sens software. Aerosekur SpA Aprilia, Italy

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Chapter 15

Generic Preconcentration/Dilution Sequential Injection Manifold for the Automated Amperometric Determination of Free Cyanides from Sub-ppb to High ppm Levels

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and Demetrius G. Themelis

Abstract Hydrogen cyanide and its simple sodium and potassium salts are among the most rapidly acting poisons affecting the central nervous system and a potentially terrorism threat. The present study reports a generic flow manifold based on sequential injection analysis coupled to gas diffusion that allows both preconcentration and dilution of real samples in a practically single-channeled configuration. By selection of the appropriate sequence and instrumental variables (volumes, flow rates etc.) cyanides can be determined from the sub-ppb ($<1 \mu\text{g L}^{-1}$ e.g. in tap water samples) to high ppm ($>500 \text{ mg L}^{-1}$ e.g. in electroplating baths) levels at sampling rates between 3 and 22 h^{-1} .

Keywords Sequential injection • Cyanides • Amperometry • Automation

15.1 Introduction

Cyanide originates either from natural or human-made sources and can exist in gaseous, liquid, and solid forms. It is among the most rapidly acting of poisons and, when present in sufficient concentrations, one of the most lethal [1].

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In recent years there is a continuously growing research interest in cyanide poisoning [2]. Some critical factors that favoured this trend are: (i) the potential for domestic or international terrorism involving cyanides [3]; (ii) increased understanding of the important role of cyanide in smoke inhalation-associated deaths; and (iii) the availability of an antidote that can be administered as empiric therapy in the pre-hospital and hospital settings for the range of causes of cyanide poisoning [4].

From an analytical chemistry point of view there are numerous official (e.g. EPA-recommended) and un-official methods (published in the international literature) for the determination of cyanides in various matrices [5]. Selectivity requirements are more or less known and well-studied and several sample preparation and preservation procedures are recommended and applied extensively (e.g. distillation, UV irradiation, gas-diffusion – GD, use of ascorbic acid to destroy excess of chlorine, precipitation of sulphide with lead acetate, etc.). In terms of sensitivity however, cyanide levels may vary from a few micrograms-per-litre (e.g. in environmental or biological samples) to several hundreds milligrams-per-litre (e.g. in electroplating baths or wastewater effluents [6]). An analytical protocol capable of covering the above-mentioned demand would be useful for practical applications.

In this study we expand our previous work [7] and propose a generic automated flow manifold based on the sequential injection principle (SI) that is capable of analyzing free (or weak acid dissociable – WAD) cyanides at an extremely wide concentrations range. In principle this method is based on the replacement of the flow-injection (FI-GD) part of the EPA-approved amperometric method for cyanides (OIA-1677 [8]) with a more versatile SI setup that expands the possibilities of the method in terms of automated sample preparation (pre-concentration and dilution), computer-controlled operation and single-channeled configuration.

15.2 Experimental

15.2.1 Apparatus

Flow detection was carried out in the amperometric cell of a Cyanide Solution® 3000 Analyzer (Alpkem®, O.I. Analytical, College Station, TX, USA). It comprised an Ag working and a Pt counter electrode and a Ag/AgCl reference electrode. The detector was operated at a potential of 0.0 V and the current (pA) was monitored by the WinFLOW™ software (O.I. Analytical). Equilibration / maintenance of the electrode are described in details elsewhere [7]. The SI setup (Fig. 15.1) was consisted of: a micro-electrically actuated 10-port valve (Valco, Switzerland); a Gilson (Minipuls3, France) peristaltic pump; a Chemifold™ Type V gas diffusion unit (72×2 mm, Tecator, Sweden) equipped with PTFE membranes. All coils (holding,

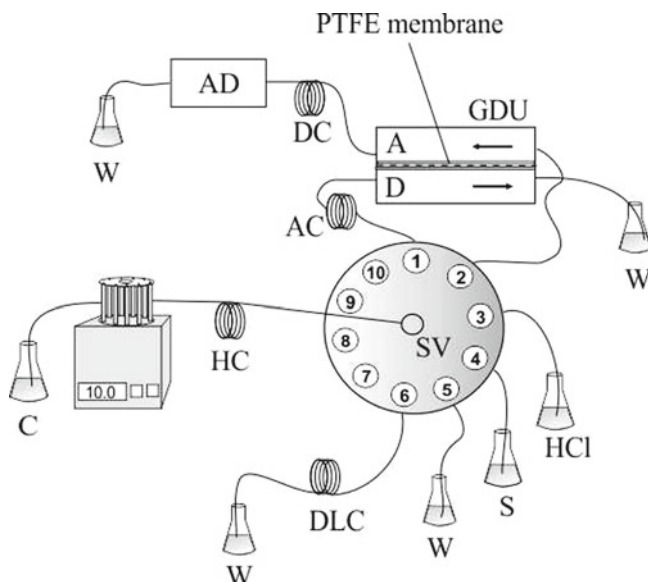


Fig. 15.1 Instrumentation setup of the SIA-GD, where *C* carrier ($0.2 \text{ mol L}^{-1} \text{ NaOH}$), *HC* holding coil ($3,000 \text{ }\mu\text{L}$), *SV* selection valve, *S* sample, *HCl* HCl solution at 1.0 mol L^{-1} , *AC* acidification coil ($60 \text{ cm}/0.5 \text{ mm i.d.}$), *DLC* dilution coil ($250 \text{ cm}/0.7 \text{ mm i.d.}$), *DC* delay coil ($60 \text{ cm}/0.5 \text{ mm i.d.}$), *W* waste, *A* acceptor, *D* donor, *GDU* gas-diffusion unit, *AD* amperometric detector

dilution, delay, mixing etc.) were made of PTFE tubing (0.5 mm i.d.). The control of the system was performed through a program developed in house using LabVIEW 5.1.1 (National Instruments, U.S.).

15.2.2 Chemicals and Solutions

All reagents (e.g. KCN, NaOH, HCl etc.) were of analytical grade and provided either by Merck or Sigma. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA) and used for preparing all solutions. Stock CN^- solutions ($\gamma\text{-CN}^- = 1,000 \text{ }\mu\text{g mL}^{-1}$) were prepared in $0.1 \text{ mol L}^{-1} \text{ NaOH}$ and kept refrigerated at 4°C for a week. Working standards were prepared by serial dilutions of the stock in $0.01 \text{ mol L}^{-1} \text{ NaOH}$. Carrier and reagent solutions were filtered through $0.45 \text{ }\mu\text{m}$ membrane filters (Schleicher & Schuell, Dassel, Germany) and degassed ultrasonically for 10 min prior to use.

Safety: Cyanide ions and salts are extremely poisonous and should be handled with great care. All solutions must be prepared in a well-ventilated hood, while the waste tank from SI experiments should contain NaOH in order to avoid any risk of HCN formation. Cyanide-containing wastes were disposed of by converting the cyanides to the less toxic cyanate ions via alkaline chlorination using NaOH and NaOCl.

15.2.3 SI-GD Procedure

The general SI procedure was consisted of the following steps: (i) washing of the donor channel with carrier ($C=0.2 \text{ mol L}^{-1} \text{ NaOH}$); (ii) loading the acceptor channel with $0.2 \text{ mol L}^{-1} \text{ NaOH}$; (iii) stacking of samples (S) and HCl (R, 1.0 mol L^{-1}) in the holding coil in a “sandwich” pattern (R-S-R-S-R); (iv) propulsion of the acidified mixture in the donor chamber of the GD unit and diffusion of the volatile HCN through the PTFE membrane; (v) propulsion of the acceptor stream to the flow-cell for the amperometric detection of the collected cyanides at a flow rate of 0.9 mL min^{-1} .

The potentials of the proposed SI system to determine cyanides in an extended concentrations range was based on controlling critical instrumental variables (sample volume and flow rate of donor) and applying preconcentration or dilution steps prior to the GD process.

15.2.4 Analysis of Real Samples

Tap water samples were collected from three different areas of the city of Thessaloniki and were treated as described in our previous report [7]. Samples from electroplating baths were purchased from domestic companies. The pH of these samples was brought to 12 by adding aliquots of a $6 \text{ mol L}^{-1} \text{ NaOH}$ solution and filtered through $0.45 \text{ }\mu\text{m}$ filters to remove suspended material.

15.3 Results and Discussion

Our experiments pointed out two factors that govern the sensitivity of the analytical procedure and therefore the determination range: (i) the sample volume that is well-known to affect the dispersion of the sample in the flow system [9] and (ii) the flow rate of the donor stream that directly influences the efficiency of the gas-diffusion process as it determines the contact time between the gaseous analyte and the semi-permeable membrane [10]. The starting values of these parameters were $V(S)=250 \text{ }\mu\text{L}$ ($2 \times 125 \text{ }\mu\text{L}$, see experimental section) and $Q_v(\text{donor})=0.2 \text{ mL min}^{-1}$ and allowed determination of cyanide in the range of $1\text{--}200 \text{ }\mu\text{g L}^{-1}$ (Table 15.1, P3). As can be seen in Table 15.1, by simply controlling the above-mentioned parameters via the SI software, the determination range could be extended ca. 12-fold (Table 15.1, P6).

In order to further extent the determination range for direct application of the method in more concentrated samples (e.g. electroplating baths) we applied an automated, on-line dilution step prior to gas-diffusion, based on the zone sampling technique. In brief, a defined sample volume (V_s) is aspirated in the holding coil of

Table 15.1 Developed protocols for the determination of cyanides at various levels

Protocol ^a	Sample volume (μL)	Q_s (donor) (mL min ⁻¹)	Preconcentration cycles (<i>N</i>)	Pre-dilution step ^b	Linear range (μg L ⁻¹)	Slope (pA ⁻¹)	Sampling rate (h ⁻¹)
P1	250 (2 × 125)	0.2	3	—	0.2–70	13,560 (±55)	3
P2	250 (2 × 125)	0.2	2	—	0.5–100	9,860 (±40)	5
P3	250 (2 × 125)	0.2	—	—	1–200	4,730 (±25)	9
P4	250 (2 × 125)	0.6	—	—	10–500	1,980 (±10)	18
P5	50 (2 × 25)	0.6	—	—	50–2,000	460 (±8)	19
P6	50 (2 × 25)	0.9	—	—	75–2,500	410 (±6)	22
P7	50 (2 × 25)	0.6	—	B	500–20,000	45.3 (±1.1)	15
P8	50 (2 × 25)	0.6	—	D	3,000–100,000	9.32 (±0.34)	14
P9	50 (2 × 25)	0.6	—	G	15,000–500,000	1.88 (±0.12)	13
P10	50 (2 × 25)	0.9	—	G	20,000–625,000	1.44 (±0.07)	15

^aFor more details on protocols P1–P3 see [7]

^bSee Table 15.2

Table 15.2 On-line dilution protocols

Pre-dilution step	V_S (μL)	V_T (μL)	V_A (μL)	Dilution factor
A	50	100	50 (2×25)	6
B	50	150	50 (2×25)	10
C	50	200	50 (2×25)	22
D	50	250	50 (2×25)	50
E	50	300	50 (2×25)	75
F	50	350	50 (2×25)	130
G	50	400	50 (2×25)	250

the SI system, followed by propulsion (V_T) in a suitable dilution coil (DLC). A fraction of the initial sample (V_A) is subsequently sampled-back for analysis (in our case gas-diffusion) [11, 12]. The advantages of this approach are: (i) simplicity; (ii) there is no need for re-configuration of the manifold since only a dilution coil is added in a port of the multi-position valve; and (iii) the desirable dilution factor can be achieved by controlling the three volumes mentioned above. On-line dilution experiments were performed by the typical bromothymol blue approach [13] and transferred to the amperometric SI-GD method for cyanide analysis. As can be seen in Table 15.2 dilution factors up to 250 were achieved, extending the determination range of cyanides at levels higher than 500 mg L^{-1} (Table 15.1, P9 & P10). The precision of the protocols was investigated by replicate injections ($n=8$) of various concentrations of cyanides. The relative standard deviations were better than 7% in all cases.

The developed generic approach was applied to the analysis of samples containing both low and high levels of cyanides. Tap water samples were spiked at cyanide concentrations lower than $70 \text{ } \mu\text{g L}^{-1}$ according to the European Union directives [14] and analyzed by the protocols P1-P3. Samples from electroplating baths were analyzed undiluted, after only a filtration step by the protocols P9 and P10 and after ten-fold manual dilution by the protocol P8. The results are summarized in Table 15.3. The recoveries ranged between 85% and 110% with precision in the range of 4–8%.

15.4 Conclusions

Some concluding remarks on this study: (i) the proposed method allows the determination of cyanides in the range of $0.2 \text{ } \mu\text{g L}^{-1}$ to 625 mg L^{-1} ; (ii) preconcentration and/or dilution of the samples is achieved by simple computer-controlled operations without reconfiguration of the practically single-channeled SI manifold; detection is based on a robust, EPA-approved amperometric method (OIA1677); (iii) the sampling rates were acceptable, ranging between 3 and 22 h^{-1} .

Table 15.3 Analysis of real samples by the developed SI-GD protocols^a

Sample	CN ⁻ added (μg L ⁻¹)	Recovery (%)		
		P1	P2	P3
Tap water (I)	—	ND ^b	ND	ND
	5	90	92	85
	10	95	94	104
	50	98	105	95
Tap water (II)	—	ND	ND	ND
	5	88	106	96
	10	92	98	95
	50	95	103	105
Tap water (III)	—	ND	ND	ND
	5	102	90	108
	10	97	94	110
	50	92	105	98
Sample	CN ⁻ added (mg L ⁻¹)	Free cyanides found (mg L ⁻¹)		
		P8 ^d	P9	P10
Electroplating bath (I)	—	350	335	340
Electroplating bath (II) ^c	—	ND	ND	ND
Electroplating bath (III)	—	420	440	425

^aFor details on the SI-GD protocols see Table 15.1^bN.D. = not detected^cCyanide-less Cu electroplating bath^dApplication of the P8 protocol after ten-fold manual dilution

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Chapter 16

General Platform for *In Vivo* Sensors for Oxygen, Glucose and Lactate Monitoring

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Dale Bickham, Hong Chang, and Pankaj Vadgama

Abstract Essential properties of biosensors for continuous monitoring in blood and tissue are selectivity, sensitivity and biocompatibility. A variety of membranes have been explored to achieve these properties. A good understanding of the diffusion properties of analytes and interferent species through these membranes is important for improvement of design and the understanding of response change in complex biological matrix. A simple analytical expression for the accurate determination of diffusion coefficient of analytes through biofilms and cylindrical granules is described and its application for biosensing presented. A general, needle-based, amperometric platform for the continuous monitoring of oxygen, glucose and lactate is also described, most notably a recess tip format, and some considerations on biosensor design and application are discussed.

Keywords Diffusion • Membranes • Oxygen • Glucose • Lactate • Recess disc electrode • *In vivo* monitoring

16.1 Introduction

Progress in practical biological sensing, essentially commenced with the report of a polarographic oxygen electrode for continuous blood oxygen monitoring by Clark et al. [1]. The key improvement compared with previous sensors was the placement of both sensing and reference electrodes behind a gas permeable membrane, thus

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isolating them from the measurement environment, allowing for stable electrochemistry. The first functional enzyme biosensor was reported 10 years later by Lyons and Clark [2]. It was based on a thin layer of glucose oxidase immobilised over an oxygen electrode retained *via* a semipermeable dialysis membrane. Response was obtained by monitoring the oxygen co-substrate consumption by the enzymatic reaction. Since those early days, a great of effort has been directed towards the development of enzyme-based biosensors for clinical, food industry and sports applications. A major driver for enzyme based biosensor research is still, primarily, the need for a truly reliable glucose sensor for diabetes monitoring and control, although lactate, oxygen, reactive oxygen and nitrogen species have also been investigated because of their pathophysiological importance [3, 4].

For short term *in vivo* use, the consensus is that the needle format offers the best option for ready, non-surgical, implantation and removal. The first construct of this type for continuous monitoring was the glucose electrode reported by Shichiri et al. [5]. Not only was this electrode semi-implantable, and clearly demonstrated to be readily inserted and removed without surgical procedure, but its use of glucose oxidase for classical hydrogen peroxide production and amperometric detection set the model for general reagentless deployment of oxidase chemistry *in vivo*.

Continuous *in vivo* monitoring can be undertaken in either blood or tissue. However, due to the risk of thromboembolism, blood monitoring is avoided and most research has focused on interstitial fluid monitoring. The challenge, admittedly, has been the lack of full understanding of the dynamic exchange of metabolites between the vascular and tissue compartments, and therefore of the pathological correlates of the latter.

Lactate is an important parameter of huge importance in gauging the hypoxic states of tissues in shock patients. However, it is also an invaluable indicator of the physical readiness and training status of the elite athletes, e.g. blood lactate is already known to correlate with the anaerobic threshold of an individual [6, 7]. Lactate levels increase considerably when oxygen supply to the muscles is insufficient to cover acute metabolic needs, as in the case of extreme training, or more generally in peripheral tissues during shock. For a long time it was considered as harmful metabolite that degrades the athlete's performance; however, compelling evidence exists today that lactate may have a protective effect as a non-toxic end metabolite, and in the case of the central nervous system it may actually be used as an alternative energy source [8].

The simultaneous measurement of lactate and glucose is of considerable interest for monitoring patients undergoing intensive care and during major surgical operations. There are also parallel needs in the process control of biotechnological plants, the quality control of food production, e.g. the wine and dairy industries, and in sport medicine [6, 9]. A potential niche application is in neurosurgical monitoring: rapid changes occur in both brain lactate and glucose level after traumatic brain injury, notably lactate is elevated and glucose is decreased.

Biosensors based on amperometric methods are the most popular due to their simplicity, low cost, possibility of miniaturisation, good sensitivity and easy maintenance [8]. Potential errors with amperometric sensors are likely to occur due to

the presence of competing electroactive species, oxygen deficit and sensor biofouling. Attempts to overcome these issues include use of solvent deposited and electropolymerised inner and outer polymers [10–12], use of mediators as electron shuttles in oxidoreductase based enzymatic electrodes [13] and entrapment of enzyme within protective sol–gel layers [14].

The rate of diffusive transport of molecules through different media is an extremely important parameter, relevant to many areas including clinical drug delivery, membrane covered biosensors and sample separation systems. As an example, oxygen transfer through the membrane of an artificial lung and through contact lens materials strongly influences clinical utility, and knowledge of the permeability (diffusion rate combined with partitioning) of such materials is central to their design and optimisation.

Electrochemical methods are particularly suitable for the determination of diffusion properties of electroactive species through different membrane materials, solutions and barrier phases. The understanding of diffusional resistance and the superimposed effects of biofouling on this as a further, adventitious barrier is of paramount importance for proper design of sensors which inevitably utilise a wide range of polymers as interfacing materials exposed to surface biofouling in complex biomatrixes. Accordingly, protein and cell accumulation on the implanted biosensor surface, and biofouling, can be treated as an additional membrane layer, thus allowing modelling of the biofouling effect upon implantation and augmenting traditional assessment based on structural characterisation.

16.2 Diffusion Through Membranes and Biofilms

The diffusion of electroactive species to the electrode surface can be studied using classical electrochemical principles. These methods can be separated into two groups based on the state of the system: dynamic and steady state methods. Steady state methods utilise a single experimental parameter for diffusion coefficient calculation; namely steady state current, which is proportional to the concentration gradient of the analyte at the working electrode surface. However, many parameters need to be known, notably current, working electrode area, bulk solute concentration and barrier layer thickness. Traditionally the diffusion coefficients of electrochemical species have been determined using disc electrode and voltammetric methods.

Diffusion to the electrode follows the Fick's 1st and 2nd Laws, Eqs. 16.1 and 16.2, respectively:

$$J = D \frac{\partial c(x,t)}{\partial x} \quad (16.1)$$

$$\frac{\partial c(x,t)}{\partial t} = D \frac{\partial^2 c(x,t)}{\partial x^2} \quad (16.2)$$

where D is a diffusion coefficient, c is a concentration, x is a spatial coordinate and t is time. Fick's Laws are partial differential equations, so initial and two boundary conditions need to be specified in order to obtain an exact solution in one dimension. For a planar disc electrode under amperometric conditions, the initial condition is typically $c(x, 0)=0$, and the two boundary conditions are $c(0, t)=0$ and $c(x, t)=c(\text{bulk})$.

To work with partial differential equations it is convenient to use dimensionless quantities. Thus, dimensionless time can be defined as $T=Dt/L^2$ and displacement $X=x/L$. Equation 16.2 can then be solved as

$$\frac{c}{c_0} = 1 - X - \frac{2}{\pi} \sum_{m=1}^{\infty} \frac{\sin(m\pi X)}{m} \exp(-\pi^2 m^2 T) \quad (16.3)$$

where m is an integer. Corresponding transient amperometric current, normalized to the steady state current $I_s = nFADc_0/L$, where n is the number of electrons transferred, F is the Faraday constant and A the effective area of a planar electrode, at $X=1$ is:

$$I = 1 + 2 \sum_{m=1}^{\infty} (-1)^m \exp(-\pi^2 m^2 T) \quad (16.4)$$

For small T Eq. 16.2 can be solved to give normalized current:

$$I = \frac{2}{\sqrt{\pi T}} \sum_{m=0}^{\infty} \exp\left(-\frac{(2m+1)^2}{4T}\right) \quad (16.5)$$

For practical computation, Eqs. 16.4 and 16.5 are not convenient because of the infinite terms involved. Furthermore, Eq. 16.4 converges slowly for small T and rapidly for large T , whilst Eq. 16.5 converges rapidly for small T and slowly for large T . Through analysis of the convergent properties of both Eqs. 16.4 and 16.5, a solution function was constructed by combining the rapidly convergent parts [15, 16]. The rapid convergent domains of the two expressions can thus be combined to obtain simple bipartite expressions for normalized amperometric current:

$$I = \frac{2}{\sqrt{\pi T}} \exp\left(\frac{-1}{4T}\right), T \leq 0.237 \quad (16.6a)$$

$$I = 1 - 2 \exp(-\pi^2 T), T \geq 0.237 \quad (16.6b)$$

When $T=0.237$, the two Eqs. 16.6a and 16.6b result in the same normalized current, $I=0.807$. In order to compare experimental current with the theoretical, the baseline current, which is different for each measurement, has to be accounted for. Thus, the experimental current I_{ex} needs to be expressed as [15]:

$$I_{ex} = \frac{I_m - I_b}{I_s - I_b} \quad (16.7)$$

Where I_m is the measured current, I_s steady state current and I_b the baseline current. The expression for the theoretical current also needs to be adjusted for conditioning time, thus, $T=D(t-t_0)/L^2$ is used, where t_0 is an adjustable parameter optimized from

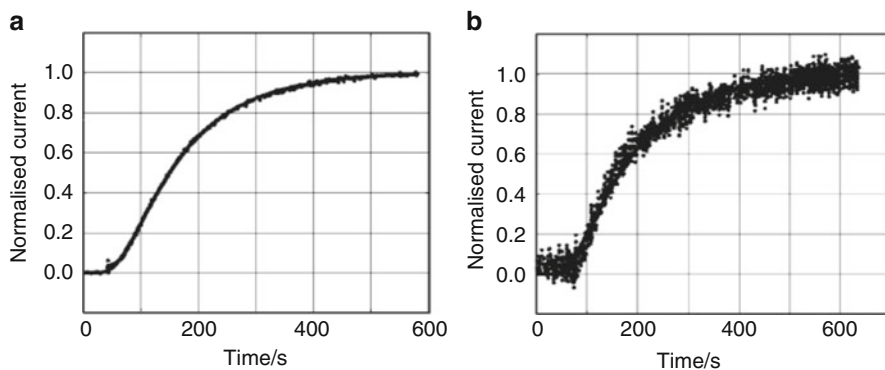


Fig. 16.1 The normalised observed (...) and simulated (-) currents for catechol diffusion through 0.025 μm pore diameter mixed cellulose esters membrane after 5 h BSA exposure (a). The normalised observed (...) and simulated (-) currents for 4-acetaminophen diffusion through 0.05 μm pore diameter mixed cellulose esters membrane after 5 h BSA exposure [15] (b). Just before refer [15]

the best fit. The best fit is obtained from simulation which minimizes the standard deviation between the experimental and simulated values:

$$\sigma = \sqrt{\frac{1}{m-1} \sum (I_{ex} - I_{sn})^2} \quad (16.8)$$

where $I_{sn} = ID(t-t_0)$ is a theoretical current from Eq. 16.6 corrected for stabilization time t_0 [15]. This model was validated by determination of diffusion coefficients of acetaminophen and catechol through cellulose ester membranes in phosphate buffer [15, 16], (Fig. 16.1) and of glucose through collagen gel [17]. It was further used to obtain information on the change of diffusion coefficient through a membrane following prolonged exposure to protein solution [18] and in assessing uniaxial solute transport through a column of solution at a recessed disc electrode [19].

These expressions were further modified for the determination of the diffusion coefficients of acetaniline, aniline, acetaminophen and phenol within cylindrical, porous granules, suitable as stationary chromatographic phases [20]. These expressions could also be useful, for example, for designing biodegradable polymeric carriers to provide controlled drug release.

16.3 *In Situ* Formation of Membranes in Microfluidic Channels

Microfluidics technology is becoming increasingly important for biosensor applications due to general advances in microfabrication technology, the imperative for reduced consumption of expensive and toxic reagents and the need to be able to

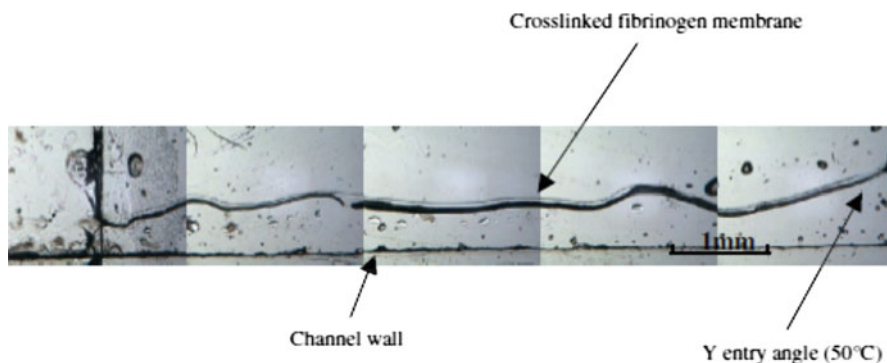


Fig. 16.2 Edge view of the crosslinked fibrinogen inside a microfluidic channel observed with optical microscopy. The thickness of the crosslinked fibrinogen was 60 μm , the reaction time was 5 min, after which the flow channel was perfused with PBS, pH 7.4 [21]

move small volumes of fluid to desired locations with high precision. Microfluidic devices, typically, are first fabricated to completion, thus any post-fabrication deposition of sensing layer or membrane is not possible.

Our work has focused on the development of a method for *in situ* membrane formation in the middle of microfluidic channels. The emphasis has been on the formation of crosslinked protein membranes. Two different protein models have been reported to date, namely globular bovine serum albumin (66 kDa) and linear fibrinogen (342 kDa) [21]. An ultrafast crosslinking agent was used, notably terephthaloyl chloride. Membranes were formed at the interface of two reagent carrier liquids; controlled, precise protein membrane formation was possible due to the dominance of laminar flow in the microchannels. A particular advantage of this method was that membrane growth was self-limiting. The permeability of both membrane types was compared for model analytes, namely hydrogen peroxide, acetaminophen and catechol. As all model analytes are electroactive, permeability studies were based on chronoamperometric measurement using method described above.

Membrane permeabilities were additionally determined optically for the dyes Ponceau S and Meldola Blue. Both dyes were able to diffuse through the fibrinogen membrane, while neither permeated through the albumin membrane. The reasons for this remain to be clarified; however, scanning electron microscope (SEM) images showed that whilst both membranes had pores, those in the fibrinogen membrane were larger, and consequently more likely to allow for easier pore-mediated diffusion. The diffusion coefficients through the membranes were determined using a model based on the bipartite expressions for transient currents as described above. Figure 16.2 shows a vertical image of a crosslinked membrane within a channel.

The biocompatibility and toxicity of the formed protein membranes was established by studying cell adhesion and growth on the membranes. The cells used were a B50 neuron hippocampal cell line. The study showed that cell adhesion and growth on the albumin and fibrinogen membranes was enhanced comparing to a

hydrophilic glass surface, studied in time period of 72 h. However, cell viability on fibrinogen was found to be better than that on albumin, established using cell morphology and density assessment. Crosslinked fibrinogen membranes did not have an effect on cell morphology or density, thus confirming the non-toxicity of the membranes studied. Thus, there may be advantages to growing cells on crosslinked fibrinogen, and under laminar flow conditions both the effect of controlled shear and nutrient conditions could be explored.

16.4 General Platform for *In Vivo* Monitoring

Needle-based sensors have the advantage of small size, ease of insertion and the potential for expanding to further analytes by choice of membranes and sensing layers. Figure 16.3 shows a schematic of a general sensing platform developed for the detection of oxygen, glucose and lactate and a scanning electron microscopy image taken of a sensing tip. This sensor design is based on a platinum wire inserted into a stainless steel tube which serves as a combined counter and reference electrode. A constant potential is applied between two electrodes, and the resulting current is recorded. In this way a compact sized, easily implantable electrode can be achieved.

A further sensor type utilised a relatively unused format where the working electrode, a gold disc, was recessed within a robust tube to give added protection to the sensitive sensor surface. The counter/reference was again a stainless steel tube and by deposition of the appropriate functionalising layers was developed for amperometric detection of hydrogen peroxide and glucose [19]. The key fabrication innovation was the use of a fused silica capillary, partially filled with molten gold to create a controlled recess.

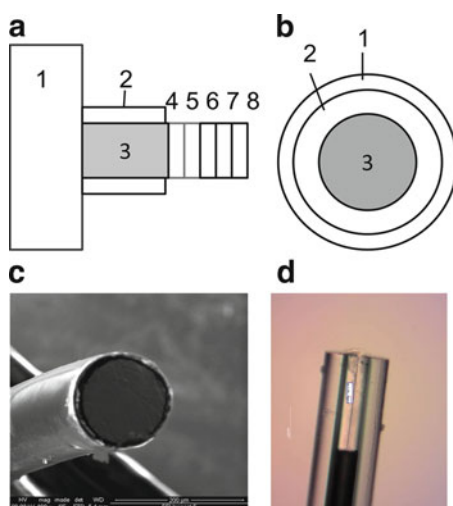


Fig. 16.3 Schematic of the general in vivo sensing platform (**a** and **b**), side view (**a**), top view (**b**), scanning electron microscope image on a Pt sensing tip, magnification 800 (**c**), an optical microscope image of a recessed tip electrode (**d**)

16.4.1 *Role of Membranes in Biosensor Applications*

Amperometric sensors have very high sensitivity, but lack selectivity in the presence of electroactive species. In order to achieve selectivity to the analyte of interest and to reduce interference of the other species present, a sensing layer needs to be covered with a membrane or a combination of membranes. Membranes used in biosensors have three different functions [3, 22]:

A protective layer. The outer membrane prevents large molecules such as proteins or biological sample cells from interfering with the reaction layer, whilst at the same time preventing leakage of the sensing layer into the sample solution. This is particularly important for implantable sensors, since any implantation antigenic component may cause an immunological reaction by human body. In addition, the outer membrane should be sufficiently permeable to analyte yet fairly impermeable to interfering substances.

Diffusional outer barrier. Enzymatic reactions follow Michaelis-Menten kinetics, and therefore they demonstrate non-linear behavior at high concentrations. By applying an outer diffusional barrier membrane, the response becomes controlled by substrate diffusion through the membrane instead of the enzymatic kinetic process. The outer diffusional barrier also makes the sensor response independent of the amount of enzyme present, thus improving the response stability.

Biocompatibility and biostability of the surface. Extensive research on biocompatibility in recent years has shown that biocompatibility does not necessarily have to mean that an implant is inert, as was originally specified. Rather, the concept should be defined as minimal perturbation of the *in vivo* environment and likewise the *in vivo* environment should not adversely affect sensor performance [3]. Biofouling or adsorption and infiltration of biomolecules into sensor membranes contribute to the reduction in sensitivity from the moment the sensor is implanted. There is evidence that smaller biomolecules (<15 kDa) are the greatest contributors to the decrease of sensitivity for glucose sensors [3].

Plasma is a very complex medium containing not only multiple electroactive species but also high concentrations of proteins, e.g. fibrinogen. When a sensor is implanted, tissue is disrupted and capillaries damaged. This converges to generate three overlapping developments influencing sensor performance: rapid sensitivity loss due to direct biofouling (minutes-hours), acute inflammatory response (days), and foreign-body capsule formation (weeks). These processes are partially reversible. The full explanation for the functional sensor loss is neither completely understood nor predictable, but simple protein adsorption on the sensor surface should not be considered as a prime problem. Because of unpredictability of signal loss, sensors require obligatory calibration *in vivo*.

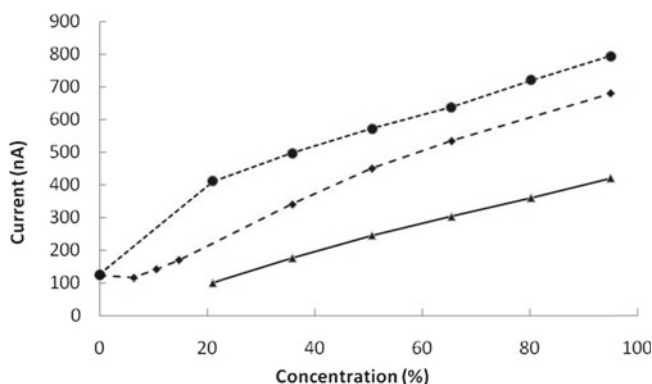


Fig. 16.4 Three different membrane combinations were tested for oxygen sensing: polyurethane containing 2% Triton X-100 and silicone elastomer MED-4211 as an outer membrane (*dotted line*), polyurethane containing 2% of Triton X-100 (*dashed line*) and polyurethane containing 2% Triton X-100 and silicone elastomer MED-6010 as an outer membrane (*solid line*). In all cases a linear range from 0% to 100% oxygen was achieved, but at low signal polyurethane membrane was the best at lower, physiological oxygen range. Measurement was done on Pt working electrode in two electrode mode by applying constant potential of -0.65 V vs. stainless steel reference electrode

16.4.2 Oxygen

The qualitative criteria for the measurement of tissue oxygen can be summarised as: minimal tissue damage caused by insertion of the probe, good spatial resolution of pO_2 profiles, good temporal resolution of pO_2 transients, minimal oxygen consumption of the cathode so that it does not seriously alter the environment it aims to measure, minimal artefacts introduced by either fluid convection or variation in the oxygen transport properties of the medium [23].

Tissue pO_2 is related to the oxygen concentration through solubility of oxygen in the tissue, i.e. the solubility coefficient of the oxygen in the tissue times the concentration of the dissolved oxygen is equal to the partial pressure of oxygen in the tissue [24]. The amperometric detection of oxygen is based on the reduction of dissolved oxygen at a cathode. Electrode performance depends on the applied potential, sensing cathode material, the resistance of the circuit, the surface area of the cathode, temperature, the solubility of oxygen in the electrolyte and the rate of diffusion of oxygen through the electrolyte and the membrane. Permeation of oxygen through the membrane is a function of gas partial pressure and thus the current readings reflect partial pressure rather than concentration.

Silicones are highly permeable to oxygen, which is why they are of interest for oxygen sensors. However, as the oxygen is also a co-reactant for enzymatic biosensors, it is important to identify membranes with high oxygen permeability, but only limited permeability to glucose or lactate, to enable wide linear range. Figure 16.4

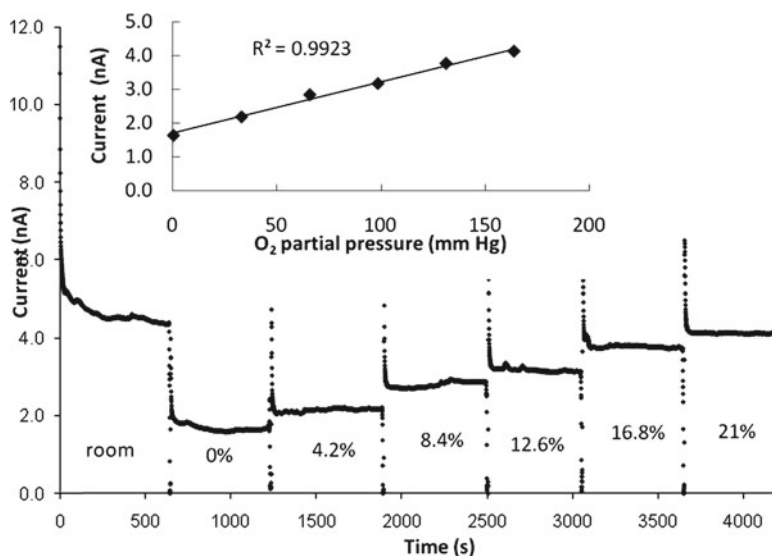


Fig. 16.5 Platinum based oxygen sensor with 20% polyurethane membrane. Oxygen concentration was measured between 0% and 20% oxygen, which covers physiological range. Measurement was done in two electrode mode by applying a constant potential of -0.65 V vs. stainless steel reference electrode

shows comparison of oxygen calibration curve obtained using three membranes/membrane combinations. All three membrane systems gave a linear range for oxygen concentration between 0% and 100%, however, the addition of silicon on polyurethane caused deviation from linearity at lower concentrations.

Therefore, subsequent experiments for physiological oxygen level monitoring were undertaken using 20% (w/v) solvent cast polyurethane membranes only. The polyurethane only coated gold electrodes show linear response between 0% and 100% oxygen and especially between 0% and 20% oxygen (Fig. 16.5).

Oxygen is detected by reduction at negative potential. In these conditions there are not many interfering substances that can contribute to the signal. However, biofouling is a potential problem for any implantable biosensors. Albumin, in particular, is a dominant constituent at the interface, and out-competes other proteins in plasma such as immunoglobulins, fibrinogen and kininogen. Its presence at a surface, however, helps to reduce the deposition of other surface fouling components including cells, and it is, therefore, regarded as a passivating protein. Figure 16.6 shows the results of biofouling tests performed in 40 mg/mL bovine serum albumin solution. No substantial decrease in response was observed after prolonged exposure (12 h).

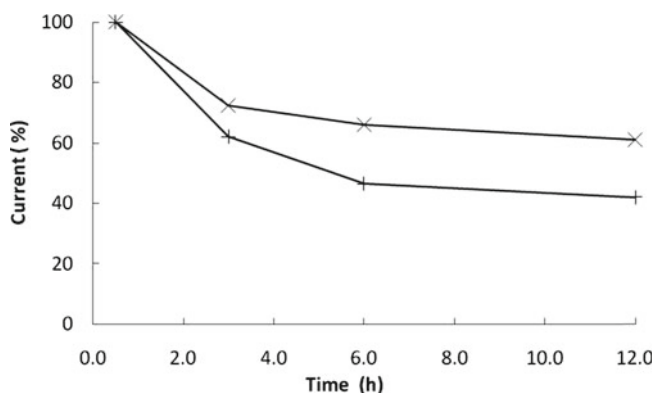


Fig. 16.6 Oxygen sensor resistance to biofouling was tested for 12 h in a PBS buffer (x) and a buffer containing 40 mg/mL bovine serum albumin (+). Measurement conditions were same as described in previous figure, but oxygen concentration was equilibrated with atmospheric conditions

16.4.3 Glucose and Lactate Sensors

Glucose and lactate are the most important analytes commonly detected by amperometric biosensors based on oxidase type enzymes. These types of sensors are based on the detection of hydrogen peroxide, generated at the working electrode surface following the reaction of glucose or lactate oxidase (GOX or LOX) with their respective substrates. Hydrogen peroxide is thereby amperometrically detected by the application of a positive potential at the sensing electrode. Regardless of the electrochemical elegance of mediator based systems and the independence they achieve from oxygen and the reduced interference conferred from working at low polarising voltages, the one aspect they fail on is the toxicity risk of mediator leaching. Accordingly, peroxide based sensing remains the only safe route to *in vivo* work.

At positive potential, several electroactive species present in plasma, such as ascorbic acid, urate and acetaminophen contribute to the response, thus giving too high signal output. Another common problem of peroxide based enzyme sensors is their dependence on ambient oxygen levels. The benefit for *in vitro* analysis using mediators, such as ferrocenes and ferricyanides [25] has accordingly been substantial; the mediators allow for both reduced oxidation potential of the system, lowering electrochemical interference, and minimise dependency on oxygen through highly efficient electron uptake from enzyme reduced during the reaction. Whilst mediator toxicity might be considered to be low in some cases, any presence of a leachable element that might accumulate locally must be considered a risk, so leaching into the system remains problematic.

Progress on implantable glucose sensors needs to remain centred on the non-mediated pathway; this is not an application where reduced leachable mediator is permissible; it is a matter of health care absolutes. One added risk to reliable operation of the hydrogen peroxide system is the diffusive loss of H_2O_2 from the enzyme layer away from the device and into the matrix; this is partly conditioned, and controlled, by fixed external membrane permeability, but variable external losses could, nevertheless, affect response sensitivity and linearity, quite independently of any phenomena affecting inward substrate transport [26]. A further variable that has received insufficient attention is the effect of natural catalase present in tissue, and likely to act as a powerful sink for the hydrogen peroxide generated by the biosensor.

Lactate and glucose are typically present in the brain and in interstitial fluid of subcutaneous tissue at concentrations that are at least of the same order of magnitude as those present in blood (mM), notwithstanding any second order differences. Thus detection limit is not an issue for most monitoring purposes. In oxidase based reactions, where oxygen plays the role of electron acceptor, oxygen supply may not be sufficient to support enzymatic reaction at higher substrate levels. Thus, tissue oxygen concentrations are an order of magnitude below those of glucose. The choice of outer polymer layer is therefore highly significant: it reduces the diffusion of glucose and lactate to the enzyme layer by a factor of nearly 100, whereas the flux of oxygen is relatively unaffected [27], hence oxygen limitation of the oxidase reaction is avoided.

Enzymatic sensors utilizing oxidases are based on amperometric detection of hydrogen peroxide. As constant potential is applied at the electrode, and as the concentration is stepped from c_i to c_{i+1} , hydrogen peroxide generated by enzymatic activity and diffused to the electrode surface is oxidized, and current rises until steady state is achieved. Hydrogen peroxide oxidation occurs at relatively high potential, +0.65 V vs. Ag/AgCl, and many common interferents present in serum and plasma are oxidized at the same time. Therefore, *in vivo* sensors have to have membrane with high selectivity towards the analyte of interest while excluding most of the interfering species.

Figure 16.7 shows SEM images of 10% (w/v) SPEES/PES and 20% (w/v) polyurethane membranes dip coated on to planar electrodes. SPEES/PES is a low molecular weight cut-off barrier against charged interferences in biological fluids. It consists of an uncharged chain containing aromatic rings and charged sulphonate groups. Due to its negatively charged nature, it is highly selective against anionic interferents such as ascorbate, whilst being very permeable to neutral, low molecular weight hydrogen peroxide.

Experiments in artificial serum demonstrated that the internal polymer, SPEES/PES, plays very significant role in achieving stable response of the biosensor in complex media. A particular, medical-equivalent, polyurethane, Trixene®, is a mixture of isocyanate terminated prepolymer and polycarbonate urethane. As an external membrane we use polyurethane (proprietary prepolymer Trixene SC7602), with addition of 2% (v/v) of the neutral surfactant Triton X-100.

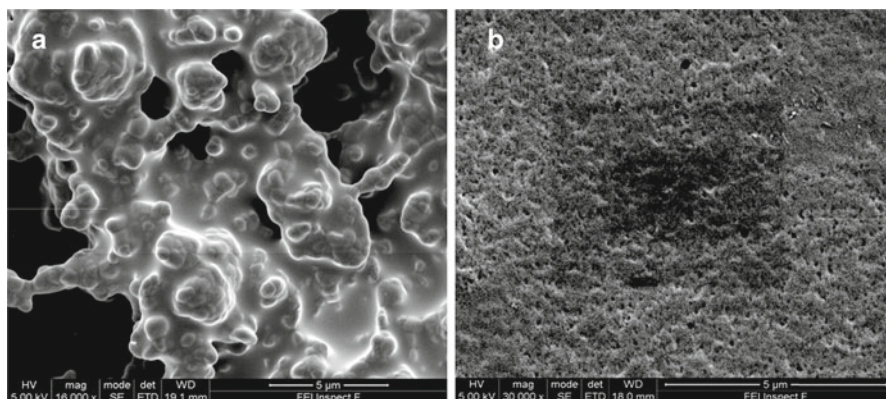


Fig. 16.7 SEM images of SPEES/PES (a) and polyurethane membrane (b). Magnification 16,000 (a) and 30,000 (b). Membranes were dip coated on planar Pt electrode and allowed to dry. For SEM imaging they were coated with a thin layer of gold

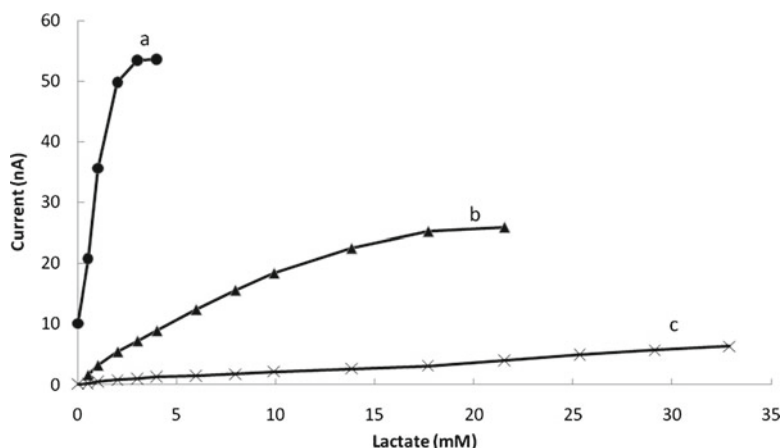


Fig. 16.8 Effect of an outer membrane (PU) on linear range of lactate sensor. No outer membrane (a), 20% PU (b) and 20%, 30%, 40% and 50% PU (c) membranes. Measurement conditions: a constant potential of 0.65 V vs. stainless steel reference was applied in phosphate buffered solution, pH 7.4

The role of the outer membrane is manifold. It slows down the diffusion rate of the analytes, namely glucose and lactate 100–1000 fold, while retaining high permeability to oxygen. This enables widening of linear range of glucose and lactate sensors from 2 mM for a bare electrode up to 25 mM for a polyurethane covered sensor. Figure 16.8 shows the effect of an outer membrane on the linear range of lactate. Similar results were obtained for glucose.

At rest, the human body produces on average of 15–20 mmol/kg/day of lactate, with primary consumption by the liver and kidneys. This results in a normal blood level of 0.5–2.0 mM [6]. Interstitial tissue lactate levels increase following tissue hypoxia. There is a correlation between the severity of injury or hemorrhage with lactate level. When the cellular oxygen supply becomes limited, as upon reaching anaerobic threshold in sports or in hemorrhagic shock, lactate production increases *via* anaerobic glycolysis.

16.4.4 *In Vivo Study*

Devices aimed for *in vivo* measurement in tissue need to provide reliable information of the analyte concentration in the localised tissue environment, though it has to be recognised that there will be true physiological differences in levels at different tissue locations. Despite this, artefactual differences in measurement due to reactive interaction of the sensor and the biological medium must be avoided. Any device implantation leads to a perturbation of the local environment and initiates a response, which often translates into ~50% loss of sensitivity *in vivo*, as compared to *in vitro* values, within a short period from implantation [3]. The exact causes of this decrease are not fully understood, but there is some evidence that small molecules, such as protein fragments produced by acute inflammatory response, rather than intact proteins are culprits [27]. This process results in physical masking of the available surface for analyte diffusion, though it may be reversed to a variable degree if the sensor is removed from the tissue and placed in purging buffer solution.

Another phase of sensor instability occurs after 3 days, coincident with the start of the chronic inflammatory response or the evolution of the healing process. The outcome is a foreign body response with a low permeability collagenous capsule formed around the sensor, isolating it from the rest of the tissue.

For this reason, although thorough evaluation of biosensors *in vitro* is necessary, it is not a sufficient condition for clinical deployment. With a prolonged period of implantation, the danger of adverse, local effects mount up, ranging from infection to inflammatory change. For this reason the tissue compartment is seen as the safer one in contrast to the vascular space, since it avoids dissemination of local adverse changes such as clotting or infection. The uncertainty problem with tissue, however, is the imprecise correlation of glucose or lactate levels in tissues *vs.* those in blood.

In evaluatory studies, lactate sensors were tested *in vivo* using Wistar rats [28]. Figure 16.9 shows difference in blood plasma and subcutaneous tissue lactate levels at low and high shock level. Lactate level in rats was changed by induction of a controlled haemorrhage shock, achieved by arterial blood withdrawal.

Results were validated by comparison with lactate values in blood, and the sensors were calibrated *in vitro* both pre- and post-implantation. Sensors showed a

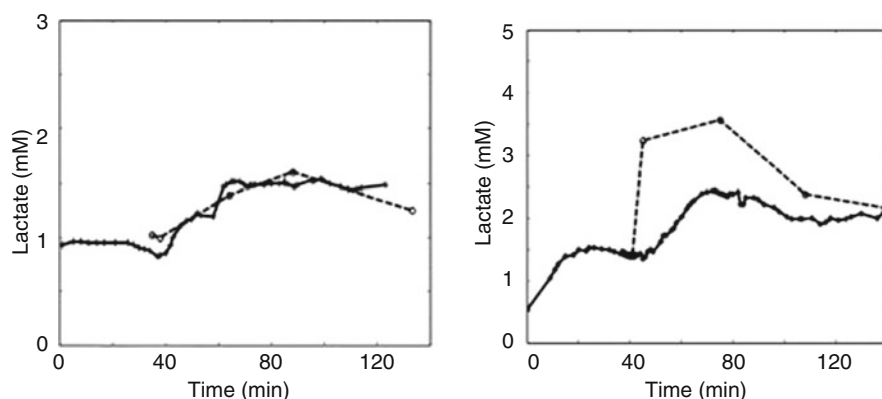


Fig. 16.9 Observed lactate concentrations measured subcutaneously in low back region of rat tissue (*) is compared with lactate concentrations in blood plasma (o) at low shock level (*left*) and high shock level (*right*) [28]

linear range up to 7 mM lactate and independence of pO_2 between 40 and 70 mmHg, which corresponds to the lower venous blood levels for pO_2 . Here, a high correlation between lactate concentration in tissue and in blood in normal conditions and in the state of low shock was found, however, in high shock, blood lactate was found to increase significantly more than tissue lactate. Sensor calibration before and after implantation indicated some drift in sensitivity (20%), but still permitted a valid *in vivo* assessment. This could be due to protein deposition on outside membrane, and could be assessed using the dynamic bipartite model of diffusion described above.

16.5 Conclusion

A flexible design of an implantable biosensor for continuous monitoring of oxygen, glucose and lactate is described. Selectivity for the desired analyte, wide linear range and biocompatibility can be achieved by the correct choice of membranes. Furthermore, understanding the diffusion properties of analytes and interferents under experimental conditions could improve the design and choice of membranes significantly. A simple, mathematical expression for diffusion coefficient determination at membrane covered electrodes and its applications to biosensor evaluation are discussed.

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Chapter 17

Zinc Oxide Nanostructures Based Bio- and Chemical Extra- and Intracellular Sensors

Magnus Willander, Omer Nur, and Syed M. Usman Ali

Abstract In this chapter we present sensors based on ZnO nanostructures suitable for sensing in small volumes and can be used for intracellular as well as extracellular environments. The principle of the measurement is based on the potentiometric effect. The measurement electrodes were constructed by growing ZnO nanostructures on the tip of sub-micrometer glass pipettes and were used versus Ag/AgCl reference electrodes. By functionalizing the ZnO nanostructures based electrodes, the selectivity of the sensors can be tuned to detect the ion or the analyte in question. The developed sensors were used to measure the concentration of most of the common ions like calcium, potassium, sodium and magnesium. In addition different biological analytes were also measured. These include glucose, urine, uric acid and cholesterol. The developed sensors showed good sensitivity and a wide dynamic range and represent a step towards developing efficient sensor of interest for human health diagnostic.

Keywords Nanosensors • Potentiometric • Intracellular sensing • Zinc oxide

17.1 Introduction

The innovation of using new measurement tools is being intensively explored in research and for some areas the demand is a never ending, e.g. for healthcare application. The most important impact of bio and chemical sensors based on

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nanostructures is for monitoring of chemical elements or biological analytes is their potential use for critically ill patients [1]. This is mainly due to the suitability of these sensors to provide rapid and continuous real time measurements and the possibility of inserting them inside the patient body or attaching them to the surface of the body [1]. Due to the important of human health demand there has been a steady evolution of sensors for biomedical measurements aimed for clinical measurements at hospitals [1]. The measurements of interest are diverse, ranging from pressure, displacement of organs, and to molecular species like gases, ions, proteins, bacteria, viruses and DNA, to mention few. Moreover, sensors based on nanostructures are having active elements of the same order of size as that of biological analytes and other chemical species and hence are considered as an excellent natural signal transduction elements. To probe the physics and chemistry in living cells using a real time robust scenario is one of the basic main goals of modern cellular biophysics and is a prerequisite for the progress nanomedicine [2]. Probing of this type has conventionally be achieved using different spectroscopic techniques, nevertheless, due to practicality, these techniques can not be considered as real time measurements. However such time domain probing can only be achieved by using nanostructures [3]. In addition, the relatively large surface area to volume ratio makes nanostructures sensitive to surface phenomena and lead to good immobilization properties with high sensitivity. On top of all these advantageous such nanostructures based sensors can efficiently deal with situations where only small volumes with low concentration of the species are available for the desired measurement [4].

As mentioned above, the determination of biological or biochemical processes is of utmost importance for medical, environmental and biotechnological applications. However, converting the biological signal to an easily processed electronic signal is challenging due to the complexity of connecting an electronic device directly to a biological environment. Electrochemical biosensors provide an attractive means to analyze the content of biological and chemical samples due to the direct conversion of a biological or chemical event to an electric signal. Over the past decades, several sensing concepts and related devices have been developed. The electrochemical sensors can be divided into amperometric, potentiometric, and conductometric. The potentiometric based sensors are of interest because they rely on measuring accumulated charges and no current passes through the biological environment. Hence no damage to the environment can be caused.

Among the different materials, zinc oxide (ZnO) a II-VI wide bandgap semiconductor, represents an attractive material due to the combination of many interesting properties. Beside being a semiconducting material ZnO, is bio-safe and biocompatible [5]. It has an ionicity of 60% and the surfaces of the material are both polar and non-polar in nature [5]. Adding to this the material is piezoelectric [6]. This piezoelectric property is also of strategic importance for sensor applications as it can be used to develop self powered implanted sensors that rely on transferring the mechanical energy in human body into electrical energy to power the sensor. This is in fact a very important concept as one of the problems of implanted sensors is the

supply of power. Moreover ZnO possesses the richest family of nanostructures and has a self organized growth property [5]. The self organized growth property is of important consequences as it allows the growth of ZnO nanostructures on substrates of interest to sensing, like e.g. submicrometer glass pipettes, thin metallic wires etc. [3]. The polar surfaces of ZnO and its high isoelectric point allow the easy functionalization of the surface of ZnO nanostructures by many different membranes, enzymes, ionophores etc. to tune the selectivity of the sensor to a specific element, ion or analyte.

In this chapter we will present a technique based on the use of the potentiometric effect to develop sensors suitable for medical diagnostics and human healthcare applications. The developed sensors are based on different ZnO nanostructures e.g. nanorods (NRs), to nanotubes (NTs), nano-walls (NWs) etc... Moreover, the developed sensors are used for both extra- as well as intracellular environments, i.e. it is also applied for single cell diagnostics. In addition to ions like e.g. calcium, potassium, magnesium, sodium, zinc, and iron ions and analytes like e.g. glucose, urine, cholesterol etc. are all sensed in single cells as well as in extra cellular environments with high accuracy. It is important to mention that these sensors can be applied when small volumes of the ion or analyte to be measured are available.

17.2 Experimental

The growth of the different ZnO nanostructures presented here and used for developing the potentiometric sensors were all grown using a low temperature (<100°C) approach, basically described in [7, 8]. Such low temperature growth regime allows the growth on non-conventional and soft substrates like thin glass capillary and metal wires etc.. Basically this method is a wet chemical approach and is often termed as the aqueous chemical growth (ACG). This wet chemical fabrication of ZnO nanostructures have further been developed by many researchers [4]. The growth solution mixture consists of analytic grade hexamethylenetetramine ($C_6H_{12}N_4$, HMT) and a grade reagent of zinc nitrate hydrate ($Zn(NO_3)_2 \cdot 6H_2O$). The concentration of both reactants (HMT and zinc nitrate) in the solution can be varied between 0.01 and 0.1 mol. In most cases a ratio 1:1 is used. However, in some special cases the ratio between the zinc nitrate and the HMT was varied between 3:1 and 1:3 to adjust the grown ZnO nanostructure morphology. The reaction process that leads to the growth of the ZnO nanostructures can be understood by the following reaction described below. Since zinc nitrate is water soluble salt and thus is dissociated in aqueous solution, we obtain Zn^{2+} ions in our solution. With the presence of OH^- ions, ZnO and water are formed after heating, as described by the following path [9]: $Zn^{2+} + 2OH^- \rightarrow Zn(OH)_2$ will lead to $ZnO + H_2O$. The OH^- ions are supplied by the HMT. In a first step the HMT and water react to form aldehyde (COH_2) and ammonia (NH_3). The NH_3 dissociates in water into NH_4^+ ions and finally the desired OH^- ions. These reactions are described by the following equations: $N_4(CH_2)_6 + 6H_2O$ will lead to $6COH_2 + 4NH_3$, and $NH_3 + H_2O$ will lead to $NH_4^+ + OH^-$. Two approaches

were investigated: the non-seeded and the seeded growth approaches. For the non-seeded growth approach the substrates were directly used after cleaning without any further surface preparation to perform the ACG process. For the second approach, the substrates were prepared with a thin layer of ZnO seed crystals prior to the actual chemical growth step. After the ZnO nanorods growth the samples were thoroughly purged with deionized water. We have found that when using the ACG for obtaining ZnO controlled nanorods, the substrate pre-treatment, the solution pH, and the angle between the substrate and beaker all play an important role in producing well aligned and size controlled nanorods [10, 11]. For more details the reader is directed to reference [5].

The ACG was used to grow different ZnO nanostructures on thin glass pipettes having a sub-micrometer tip and on thin silver wires. These ZnO were then functionalized for selectivity using the proper functionalization material depending on the element or analyte to be detected. The sensing electrode was used versus an Ag/AgCl reference electrode. This was followed by calibration of the two electrode system and then the sensing configuration was used being intra- or extracellular measurements and the concentration was extracted using the calibration curve. When dealing with cells mechanical manipulation was used to gently penetrate the cells and perform the measurements.

17.3 Results and Discussion

After the successful and controlled synthesis of ZnO nanostructures such as nanorods/nanowires on different substrates, they have been the target of numerous investigations due to their unique properties. The diameters of these one dimensional nanostructures are comparable to the size of the biological and chemical species being sensed, which intuitively makes them excellent primary transducers for producing electrical signals. We have successfully demonstrated the utilization of ZnO nanowires based electrochemical sensors for the selective determination of glucose (extra and intra cellular), metallic ions (K^+) and uric acid and urea etc. In addition to that we have successfully employed these developed sensors in extended gate MOSFET configuration and remotely monitoring of glucose using existing GSM infrastructures.

An electrochemical glucose sensor was fabricated using ZnO nanowires grown on thin silver wire by immobilization of glucose oxidase (enzyme) onto them. Glucose oxidase (GOD) was electrostatically immobilized on the surface of the well aligned zinc oxide nanowires resulting in sensitive, selective, stable and reproducible glucose biosensors. The potentiometric response vs. Ag/AgCl reference electrode was found as shown in Fig. 17.1 to be linear over a relatively wide logarithmic concentration range (0.5 μ M to 10 mM) suitable for extra and intracellular glucose detection. In Fig. 17.2, the set up and photographs of two different cells under intracellular measurements are all shown.

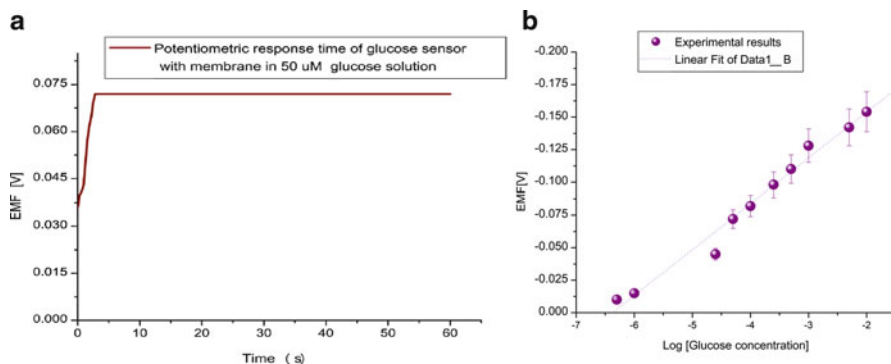


Fig. 17.1 (a) Calibration curve showing the time response of the sensor electrodes in 50 μM glucose solution and (b) calibration curve showing electrochemical voltage response versus logarithmic glucose concentration using ZnO nanowires sensor electrode with Ag/AgCl reference electrode

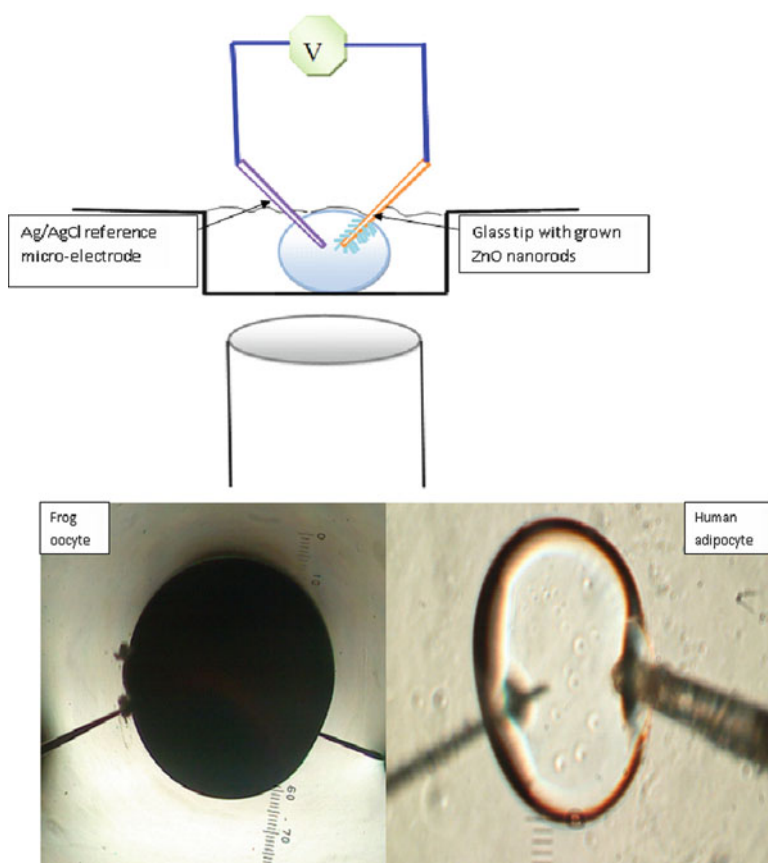


Fig. 17.2 (Top) Schematic diagram illustrating the selective intracellular glucose measurement setup, (Bottom left) microscope images of a single frog (*Xenopus laevis*) oocyte and in (Bottom right) single human fat cell (*adipocyte*) during measurements with a functionalized ZnO-nanorod-coated probe as a working electrode and with an Ag/AgCl reference microelectrode

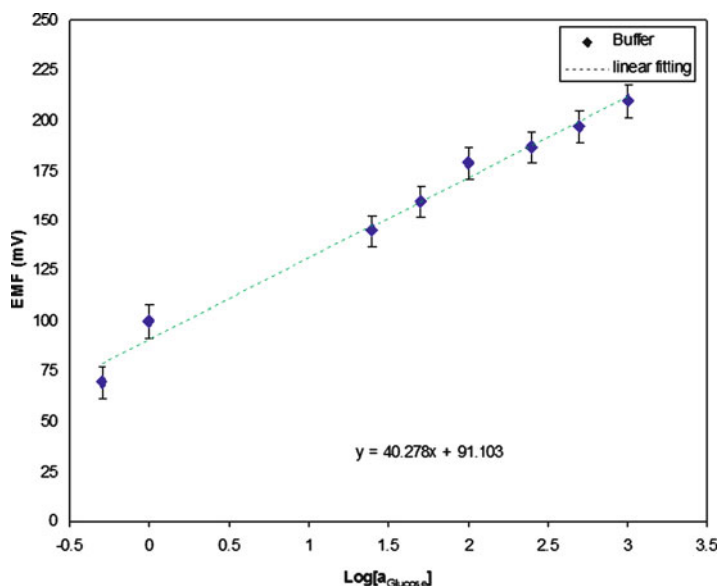
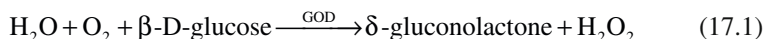
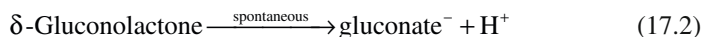


Fig. 17.3 A calibration curve showing the electrochemical potential difference versus the glucose concentration (0.5–1,000 μM) using functionalized ZnO-nanorod-coated probe as a working electrode and an Ag/AgCl microelectrode reference microelectrode

The sensing mechanism of most electrochemical glucose sensors is based on an enzymatic reaction catalyzed by glucose oxidase (GOD) according to the following reaction:



As a result of this reaction, δ -gluconolactone and hydrogen peroxide are produced. These two products and the oxygen consumption can be used for the glucose determination. With H_2O availability in the reaction, gluconolactone is spontaneously converted to gluconic acid, which at neutral pH, form the charged products of gluconate – and proton (H^+), according to the equation below:



This proteolytic reaction of the δ -gluconolactone to gluconic acid shown in Eq. 17.2, results in a decrease of the medium pH, and can be used for the determination of the glucose concentration [12]. Concentration changes of the ions surrounding the ZnO nanowires will change the electrode potential [13]. The potentiometric response of the sensor electrodes were studied in glucose solutions made in buffer (PBS pH 7.4) with concentration ranging from 0.5 μM to 10 mM. A very fast response time was noted over the whole concentration range with 95% of the steady state voltage achieved within 4 s for a sensor electrode with the membrane. As shown in Fig. 17.3, the tested sensor configuration showed large dynamic ranges

with an output response (EMF) that was linear vs. the logarithmic concentration of glucose going from -10 mV for 0.5 μM and -154 mV for 10 mM glucose. This corresponds to slope of around 35 mV/decade.

These obtained signals were reproducible and strong enough to open a gate channel in a commercial MOSFET having low threshold gate voltages. Moreover, these signals can be integrated in an electronic circuitry for remote monitoring the patient's diabetes level from remote locations and wireless sensor configurations. The excellent performance in sensitivity, stability, selectivity, reproducibility and freedom from interference was achieved when the sensor was exposed to glucose solution. All these advantageous features can make the proposed biosensor applicable in medical, food or other areas.

This method is simple and can be extended to immobilize other enzymes and other bioactive molecules with small isoelectric points for a variety of biosensor designs.

Based on all these advantageous features during extracellular investigations, we implied this proposed sensors for intracellular configurations for the determination of glucose and potassium (K^+) ions using micro-injection technique. First we demonstrate the functionalised ZnO-nanorod-based selective electrochemical sensor for determination of an intracellular glucose. To prepare the sensor suitable for an intracellular glucose measurement, we grew hexagonal ZnO nanorods on the tip of a silver-covered borosilicate glass capillary whose outer diameter was 0.7 μm and immobilized them with the enzyme glucose oxidase by employing simple physical adsorption method. This prepared nano-electrode device was successfully applied using micro-injection techniques to selectively determine the glucose concentrations in human adipocytes or frog oocytes (Fig. 17.2).

The electrochemical measurements were carried out using the immobilized ZnO nanorods as working electrode and the Ag/AgCl reference microelectrode (prepared on silver-coated borosilicate glass capillary, diameter was 0.7 μm as well) in an electrolyte drop. Initially, both microelectrodes were immersed inside a 30 μL drop of distilled water as a test sample. To test the response of the probe, a 30 μL drop of a 1 mM glucose solution was added to the drop of distilled water. The signal change from one level to another was recorded, giving the response behavior of the ZnO nanorods based glucose sensor without stirring (controlled by diffusion to the sensor). The experimental setup for the intracellular measurements is shown in Fig. 17.2. The extra cellular response of the electrochemical potential difference of the ZnO nanorods to the changes in buffer electrolyte glucose was measured in vitro model for the range of 500 nM to 1 mM and shows that this glucose dependence is linear and has sensitivity equal to 40.27 mV/decade at around 23°C (Fig. 17.3). This linear dependence implies that such sensor configuration can provide a large dynamic range.

To start the intracellular measurements, first we used the nanosensor to measure the free concentration of intracellular glucose in a single human adipocyte. The glucose selective nano-electrode, mounted on a micromanipulator, was carefully moved into position in the same plane as the cells. The ZnO nano-electrode and the reference microelectrode were then gently inserted inside the cell to a short distance using micro-manipulated system (Fig. 17.2). Once the ZnO-nanorod working

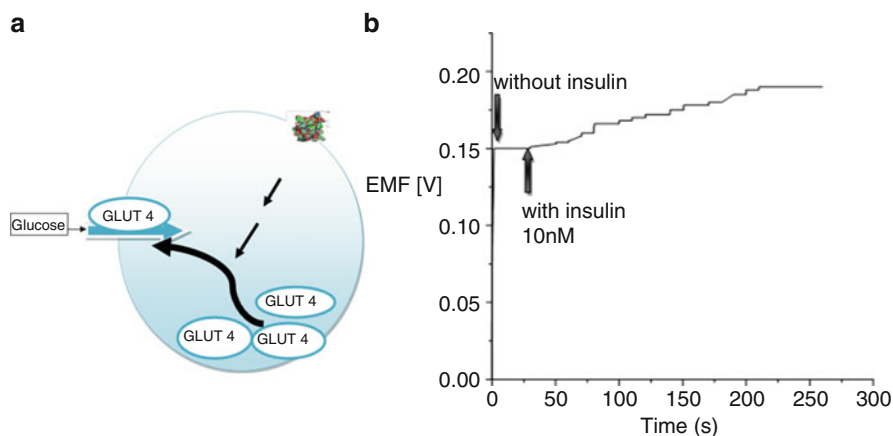


Fig. 17.4 (a) Intracellular mechanism for insulin-induced activation of glucose uptake. (b) Output response with respect to time for intracellularly positioned electrodes when insulin is applied to the extracellular solution

electrode and the Ag/AgCl reference microelectrode were inside the cell, that is, isolated from the buffer solution present in surroundings, an electrochemical response was detected and identified as the presence of glucose. The intracellular glucose concentration was estimated to be $50 \pm 15 \mu\text{M}$ for ($n=5$). The obtained results were in good agreement as compared with the $70 \mu\text{M}$ intracellular concentration determined by nuclear magnetic resonance spectroscopy in rat muscle tissue in the presence of a high, 10 mM extracellular glucose concentration as reported [14]. In order to investigate the impact of insulin, we added insulin to the cell medium to stimulate glucose uptake. Insulin stimulates glucose uptake by binding to its receptor at the cell surface, which initiates intracellular signal transduction, causing translocation of insulin sensitive glucose transporters (GLUT4, glucose transporter-4). After integration in the plasma membrane, GLUT4 allows glucose to enter the cell along a concentration gradient, as shown in Fig. 17.4a. Thus, when we achieved a stable potential for intracellular measurement, 10 nM insulin was added to the cell medium. After several minutes insulin increased the glucose concentration in the cell from $50 \pm 15 \mu\text{M}$ to $125 \pm 15 \mu\text{M}$ as shown in Fig. 17.4b.

In another set of experiments, we have applied the nanosensor to measure intracellular glucose concentration in single frog oocytes. The intracellular glucose concentration was $125 \pm 23 \mu\text{M}$ for ($n=5$). This is slightly higher than what has been reported earlier i.e. $<50 \mu\text{M}$ [15]. We do not know the reason for this difference, but one possibility is that the electrodes behave slightly differently inside the oocyte than outside, where they were calibrated. However, to test whether the electrode is measuring the glucose concentration inside the oocytes, we added 10 nM of insulin to the cell medium to stimulate glucose uptake. Indeed, the glucose concentration in the frog oocytes increased from 125 ± 23 to $250 \pm 19 \mu\text{M}$. The viability of the

penetrated cells strongly depends on the size of the ZnO nanorods. By reducing the size of ZnO nanorods, the total diameter of the tip will be reduced, which in turn increases the cell viability, and the sensitivity of the device is also expected to increase. The proposed intracellular biosensor showed a fast response time less than 1 s and has quite a wide linear range from 0.5 to 1,000 μM . The performance regarding sensitivity, selectivity, and freedom from interference when the sensor was exposed to intra- and extracellular glucose measurements were quite acceptable. The stability of the sensing ZnO layer was, however, limited and should be improved although the experiments described here have a short duration and could be performed without influence of this drawback. The effect of the hormone insulin, which increased the concentration of intracellular glucose, was also demonstrated. These results demonstrate the capability to perform biologically relevant measurements of glucose within living cells. The ZnO-nanorod glucose electrode thus holds promise for minimally invasive dynamic analyses of single cells.

Similarly, potassium ions (K^+) and other alkali metal ions play important roles in biological systems [16]. Only about 2% of the total body K^+ resides in the extracellular fluid and its levels are crucial to normal homeostasis [17]. Therefore it is important to monitor and regulate the K^+ concentrations in human body because K^+ not only plays a critical role in the extracellular solution but also is the main intracellular ion in the body where it participates in several vital functions, such as a cell growth, maintenance of cell volume, DNA and protein synthesis, enzymatic activity, acid base balance, and cellular apoptosis [18]. The purpose of the present investigation is to develop a simple method to determine the intracellular K^+ concentration. We have fabricated a highly K^+ selective sensor electrode capable of penetrating the cell membrane based on ZnO nanowires coated with a K^+ selective thin ionophore (Valinomycin) containing polyvinyl chloride (PVC) membrane. A ZnO thin film may also be used but we chose ZnO nanowires due to a higher surface-to-volume ratio thus capturing more ions on the ZnO structure also because the nano-surfaces have other chemical properties. These are the reasons why nanowires are more sensitive, have an extended detection limit, and have a fast response time as compared to a thin film [19, 20].

The K^+ -selective intracellular measurements were carried out by a potentiometric method using the two microelectrodes as described earlier. Initially, we performed extracellular measurements for calibration using a standard Ag/AgCl reference electrode. The Ag/AgCl reference microelectrode was calibrated against the standard Ag/AgCl reference electrode. The calibration results showed approximately the same potential difference in all K^+ solutions for concentrations ranging from 1 μM to 125 mM. This calibrated potential difference was added to the data obtained from intracellular measurements with the reference microelectrode.

For the intracellular measurements, we have prepared K^+ selective ionophore coated ZnO nanowire based microelectrodes and electrochemical experiments start after the 3 h of ionophore coating. First, we tested the fabricated microelectrodes inside a 100 μL drop of distilled water and did not obtain any signal from it. When the microelectrodes were inside a droplet containing 1 mM KCl in deionized water, a change of the signal was observed within 30 s. This response behavior indicates

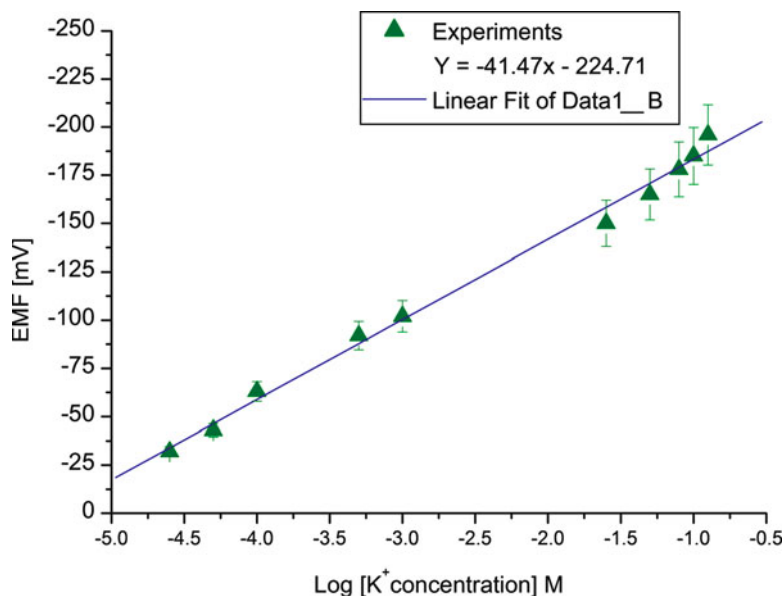


Fig. 17.5 Calibration curve showing the electrochemical potential difference between the K⁺-selective ZnO nanowire coated microelectrode and the Ag/AgCl reference microelectrode in solutions only containing KCl

that the fabricated sensor is sensitive to a specific metallic ion, i.e., K⁺. The latency for the detection of K⁺ depends on the number of ionophores located in PVC membrane. With a low density of ionophores, it will take a long time to reach equilibrium, while a high density increases the access to the ZnO nanowires hidden under the PVC cover, thus making the response time faster.

After calibration, the electrochemical response (EMF) was measured in the K⁺ solutions for the concentrations ranging from 1 μ M to 125 mM. The tested sensor configuration showed a large dynamic range with an EMF linearly related to the logarithm of K⁺ concentration between 25 μ M and 125 mM with a slope of 41.5 mV/decade (Fig. 17.5). The selectivity is a very important parameter, which describes the specificity toward the target ion in the presence of other interfering ions. To investigate common interferences normally present inside cells, we prepared two types of K⁺ solutions. One solution was prepared with 85-mM KCl in deionized water. The other one was prepared with 85-mM K⁺ in PBS in which we have introduced the possible interfering ions such as 235 mM Na⁺, 1 μ M Ca²⁺, and 500 μ M Mg²⁺. Good selectivity was observed from the output response curves with and without interfering ions. This can be clearly seen in Fig. 17.6a that shows that the response of the sensor is quite stable for K⁺ in deionized water without interfering ions, while Fig. 17.6b shows negligible noise in the response due to the mixing of interfering ions in the solutions. The pH effect on the sensor performance was

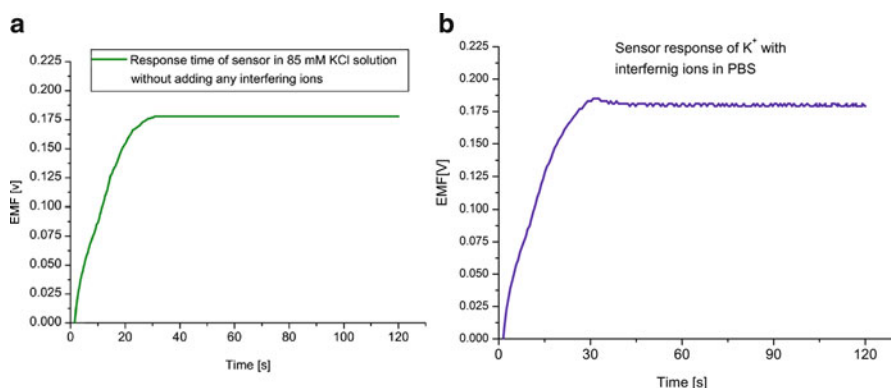


Fig. 17.6 (a, b) The time response of the measurement of the signal in two K^+ solutions of 85 mM KCl without and with interfering ions (Na^+ , Ca^{2+} and Mg^{2+})

investigated over the pH range from 2 to 10 for 1 μ M to 125 mM of K^+ . The corresponding potential values obtained were independent of pH in the range 4–8. Deviations in sensor potential were observed at pH values above and below this range due to dissolving phenomena of ZnO nanowires.

To measure the electrochemical potential in human adipocytes, we used micro-manipulators to move the two electrodes gently into the cell. When the K^+ -selective and the reference microelectrodes were inside the cell, the electrochemical potential difference could be recorded and analyzed. The intracellular K^+ concentration in a single human adipocyte was 83 ± 10 mM ($n=5$). The only other published data we have found for adipocytes are 177 mM in primary rat adipocytes determined from the steady-state accumulation of $86Rb^+$ [21]. In the second set of experiment, we measured the intracellular K^+ concentration in frog oocytes in a similar way. The intracellular concentration was 110 ± 20 mM ($n=5$), which was close to what has been reported before: 92 (large oocyte), 117 (mature oocyte), 120 (oocyte with the follicle cell layer), and 150 mM (large oocyte) [22]. In order to study the stability and reproducibility of the fabricated sensor, eight sensor electrodes were prepared independently: four electrodes were used to measure the K^+ concentration in human adipocytes and four other electrodes were used to measure the K^+ concentration in frog oocytes. Figure 17.7a, b shows the good reproducibility for the measurements. The insertion of thin K^+ -selective ZnO-nanowire-based microelectrodes into the cytoplasm of single living cells did not visibly seem to affect cellular viability. However, larger microelectrodes affected the viability of the penetrated cells. Thus, the viability depends strongly on the dimensions of the electrode tip. This study demonstrated that the small size of the sensing microelectrode made it a minimally invasive tool appropriate for monitoring K^+ inside living cells of the size used in the present investigation.

We have also successfully demonstrated the selective determination of uric acid using the zinc oxide (ZnO) nanowires fabricated on a gold coated flexible plastic substrate. Uricase was electrostatically immobilized on to the surface of well aligned

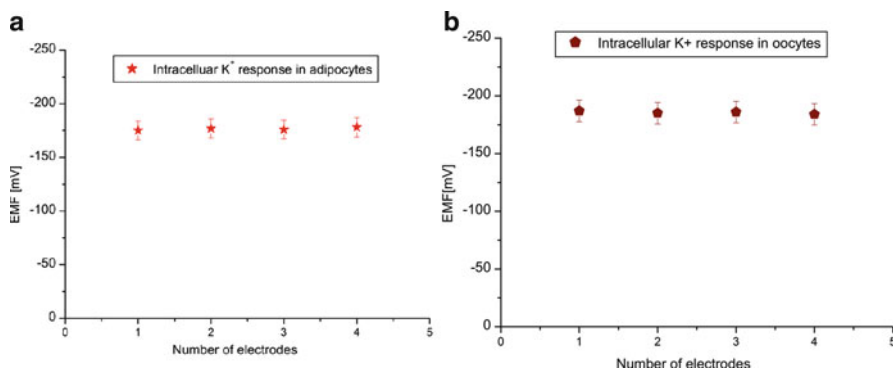


Fig. 17.7 (a, b) The sensor-to-sensor reproducibility of four K^+ -selective microelectrodes for human adipocytes and frog oocytes

ZnO nanowires in conjunction with the Nafion coating resulting in a sensitive, selective, stable and reproducible uric acid biosensor.

The electrochemical measurements has been carried out by utilizing two electrodes system and the resultant slope is drawn from calibrated values of electrochemical response (EMF) vs. the logarithmic concentrations of uric acid solution ranging from $1\ \mu\text{M}$ to $1,000\ \mu\text{M}$. During the experiments, a response of approximately 127 mV without membrane and 128 mV with membrane sensor was observed for $100\ \mu\text{M}$ uric acid solutions. The same experiment was repeated with different volumes ranging from 0.25 mL to 20 mL with the same length of 1 mm of the sensor electrode dipped into the $100\ \mu\text{M}$ of uric acid solution. The response remained within 127–128 mV for both types of sensors regardless of the sample volume.

Uric acid has oxidized in the presence of uricase into Allantoin along with carbon dioxide and hydrogen peroxide. Due to the presence of H_2O , the allantoin/decade at around is accepting proton from H_2O and converted to allantoinium ion, which in results interact with ZnO nanowires and producing a potential at the electrode. The concentration of ions change in surrounding the ZnO nanowires will change the electrode potential [17]. The potentiometric response of the sensor electrodes were studied in uric acid solutions made in buffer (PBS, pH 7.4) with concentration ranging from $1\ \mu\text{M}$ to $1,000\ \mu\text{M}$. A very fast response time was noted over the whole concentration range with 95% of the steady state voltage achieved within 6.25 s as shown in Fig. 17.8a for a sensor electrode without membrane and within 9 s for a sensor electrode with membrane Fig. 17.8b. The linearity, stability, and reproducibility of the uric acid sensor have been evaluated by performing three repeated experiments by utilizing a same sensor electrode. The results of these experiments reveal good consistency in the calibration traces as shown in Fig. 17.9a. The reproducibility and long term stability was evaluated by using five different uric acid sensor electrodes constructed independently under same conditions; the relative standard deviation of the fabricated sensor electrodes in standard uric acid solutions was less than 7%. The sensor to sensor reproducibility in $100\ \mu\text{M}$ uric acid

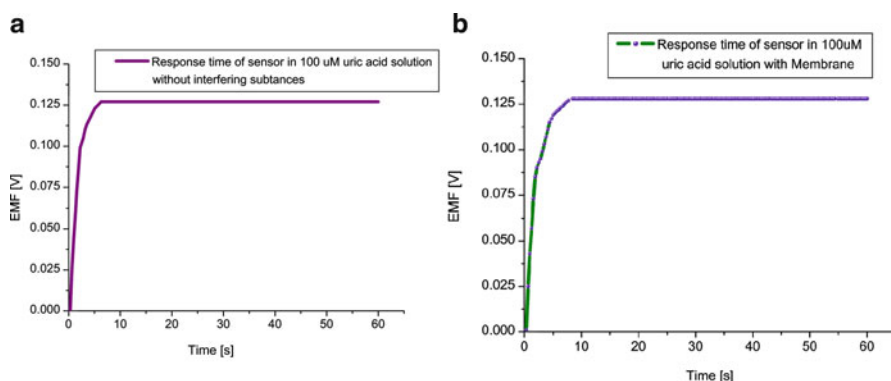


Fig. 17.8 (a, b) Time response of the uric acid sensor in 100 μM uric acid solution (a) without membrane and (b) with membrane

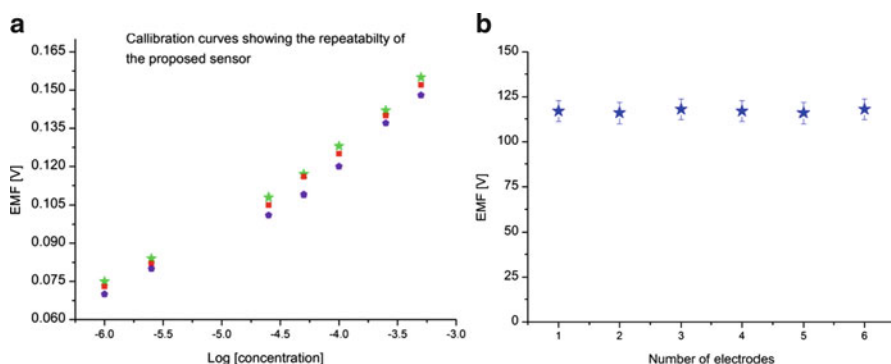
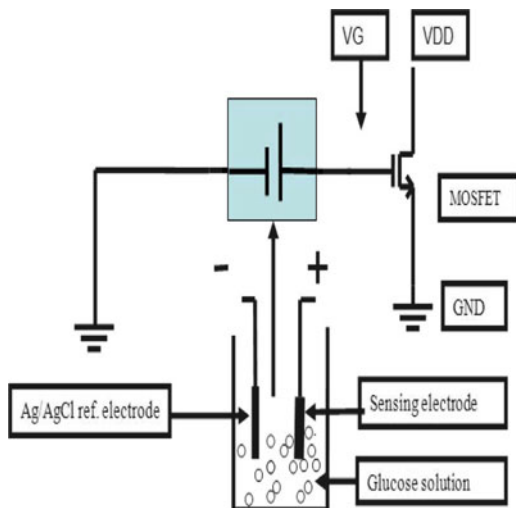


Fig. 17.9 (a) Calibration curves from three different experiments using the same sensor electrode and Ag/AgCl reference electrode, (b) the sensor to sensor reproducibility of five ($n=5$) ZnO nano-wires/uricase/Nafion® electrodes in 100 μM uric acid solution, (c) effect of potentially interfering substances on sensor response (emf) upon adding 5 mM glucose (GL), 100 μM ascorbic acid (AA) and urea (UR) into 100 μM uric acid solution

solution has shown in Fig. 17.9b. The selectivity of a uric acid sensor was also investigated. The enzyme analyte reaction is very specific due to the nature of the uricase functionality. The possible interferences that normally interfered with an amperometric uric acid biosensor included ascorbic acid (AA) urea (UR) and glucose (GL) [21]. Hence, ascorbic acid, urea and glucose are adopted to affirm the selectivity of potentiometric uric acid sensor in present work. After adding 5 mM (GL), 100 μM ascorbic acid (AA), and urea (UR) 1 mM in 100 μM of uric acid solution. The effect of interfering substances in output response was shown in Fig. 17.9c, which shows that the signal slightly changed as glucose, AA and urea are added to uric acid solution as interfering substances. However, no significant change

Fig. 17.10 Schematic diagram illustrating the configuration used for glucose detection with MOSFET using extended-gate functionalized ZnO nanowires as working electrode and Ag/AgCl as a reference electrode



in the out response was observed when these interfering substances were added, so slight interference can be neglected.

The sensing mechanism of most electrochemical uric acid sensors is based on an enzymatic reaction catalyzed by uricase according to the following:



Finally we have coupled/interfaced the fabricated glucose sensor with a commercial MOSFET having very low/zero threshold voltages in an extended gate configuration. The extended-gate MOSFET sensor approach demonstrates the possibility and potential of the use of nanostructures coupled to standard electronic components for biosensing applications.

In order to interface the output signal from the electrochemical measurement with a commercial MOSFET, we selected the highly sensitive n-channel zero threshold ($V_{\text{th}} = 0$ V) ALD/110900 commercial n-MOSFET (Advanced Linear Devices, Sunnyvale, CA), which can operate in precision zero threshold mode. This transistor was integrated with the extended-gate sensor together with an Ag/AgCl electrode and connected to a Keithley 2602 unit, as schematically shown in Fig. 17.10. In addition, a pH meter (Model 744, Metrohm) was used to measure the potentiometric output voltage of the different ZnO nanowires sensors presented here. Moreover, time response measurements were also performed to study the stability. For the time response measurements, a model 363 A potentiostat/galvanostat (EG and G, Inc., Idaho Falls, ID) was used. The working electrode (ZnO nanowires) is negatively charged due to oxidation [see Eqs. 17.1 and 17.2]. The gate voltage must be positive in order to invert carriers at the n-MOSFET channel and observe the drain current modulations. The output voltage can be made positive by interfacing instrumentation

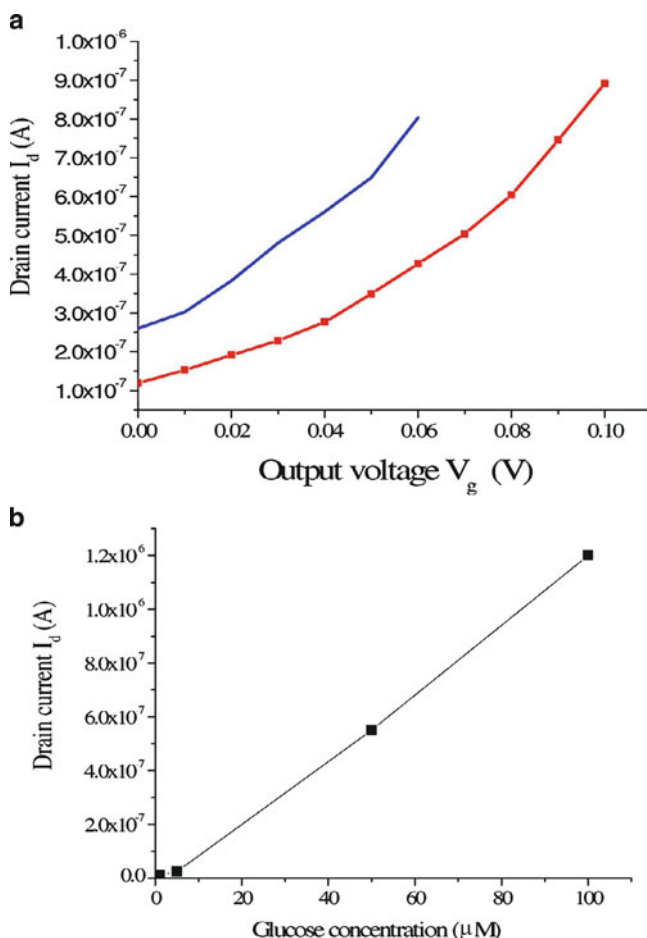


Fig. 17.11 (a) Typical drain current (I_D) versus gate voltage (V_G) for the extended-gate MOSFET, the upper curve (line) is for 50 μ M glucose solution while the lower curve (dotted line) is for the case of 100 μ M of glucose concentration. (b) Relation between the drain current and glucose concentration for a range of 1–100 μ M glucose concentration

amplifier in an inverting mode with unity gain between the sensor output and gate terminal of the MOSFET. If a p-channel MOSFET is used, then there is no need for an instrumentation amplifier interfacing.

When the extended gate was immersed in 50 μ M glucose solution, an induced voltage of about 60 mV was added to the gate. As a result, a strong modulation of the drain current was observed. In this case, when the extended-gate transistor was stable, 0.540 μ A was added to the drain current, as shown in Fig. 17.11a. This increment is due to the reaction between the glucose and GOD/ZnO/Ag electrode leading to an electron transfer to the ZnO nanowires. It is important to mention that on contrary to the non-stable behavior of both the GOD/Ag and bare Ag wire, the

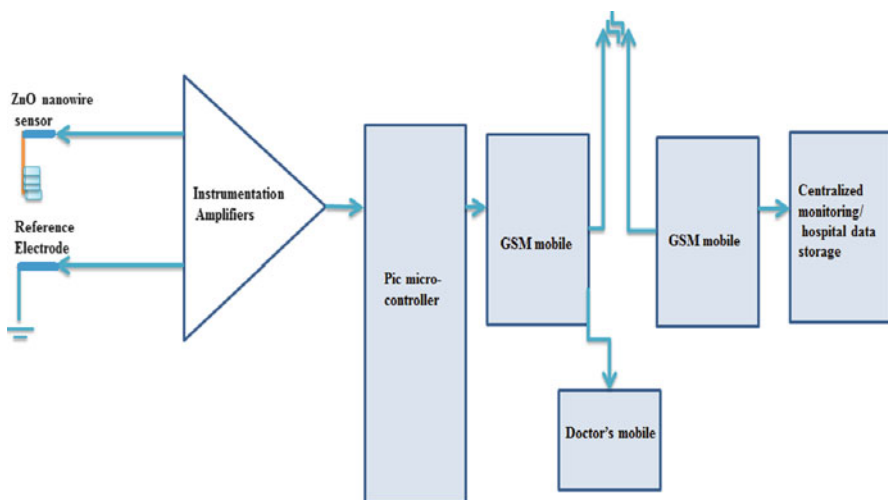


Fig. 17.12 The proposed system block diagram of wireless remote monitoring system for the functionalized ZnO *nanowire arrays* based glucose sensor

modulation observed here prevail for a long time with no observable signal decay. The dependence of ID on the glucose concentration is shown in Fig. 17.11b. As evident from Fig. 17.11b, the ID increased as the glucose concentration is increased, as expected. It is also clear that the dependence of the ID on the glucose concentration is showing a linear relationship. The reproducibility and long-term stability was evaluated by using five different sensor electrodes, all containing GOD/ZnO/Ag. After more than 20 successive measurements, the immobilized GOD/ZnO/Ag electrode has lost only 25% of its initial activity. Thus, the electrode retains 75% of the electro catalytic activity of GOD, and prevents it from leaking out of the sensor. Thus, the extended-gate MOSFET sensor concept presented here is robust, and opens the possibility of externally integrating nano-sensing element to commercial transistors giving the advantages of simplicity and low cost. In addition, the extended gate makes it easier and more practical to sense elements when the available sample volume is relatively small. This simple method of fabricating ZnO/GOD biosensor can also be extended to immobilize other enzymes and other bioactive molecules on various nanostructures, and form versatile electrodes for biosensor studies.

In another application, we have applied our fabricated glucose sensor for remotely monitoring the glucose levels from remote locations using existing GSM/GPRS mobile communication infra structures. As the GSM/GPRS infrastructures have proven to be reliable and cost effective, the services provided by these systems are inevitably used for data acquisition and monitoring applications. Thus, we have chosen this system in our present work due to its wide area coverage and can reach to doctor/caregiver at any time.

Figure 17.12 is showing the proposed system block diagram of wireless remote monitoring system for the ZnO nanowires based glucose sensor. The electrical signals

generated by our glucose sensor are stable and strong enough with ranging from 10 mV to hundreds of mV with varying glucose concentrations. These signals were firstly collected by electrodes, then pass through amplifier and filters to get rid of noises using flexible shielded cables. After that the signals are connected to the input port of built-in ADC of the PIC18F452 microcontroller. The PIC18F452 is a general purpose MCU from Microchip. It has several convenient built-in modules such as A/D, UART, SPI, PSP etc., which reduce development time. In particular, an on chip analog to digital converter (ADC) eliminates the need to design separate ADC circuitry. Also, on-chip universal asynchronous receivers transmit (UART) module makes asynchronous communication with the compatible transceiver module relatively simple. ADC and UART modules operate in parallel with the microcontroller's CPU. Operation of A/D and UART modules in parallel with CPU saves CPU instruction cycles. In other words, once ADC and UART are configured properly, they operate almost independently with the CPU inside PIC Microcontroller. The input signal from the sensor is first interfaced to an instrumentation amplifier using flexible coaxial cables whose gain was adjusted to an appropriate level accordingly and then this signal amplified by instrumentation amplifier (IA). The output from the amplifier is then fed to the input of the ADC built in with microcontroller which converts this signal to corresponding digital signals readable by the microcontroller. Software in the controller, which is written in C language, then reads the signal and compares it to a lookup table. This algorithm has the key responsibility to read the sugar level in terms of input electrical signal and from the lookup table convert it into corresponding molar concentrations. After conversion it generates instruction set for the GSM mobile device connected with RS 323 serial data cable to the circuit board. This instruction set when passed to the mobile using the serial port sends sugar levels as SMS messages to the physician mobile and medical data storage system for immediate consultation and medications. SMS message typically take 10–30 s to deliver but depending upon the network load, it may take longer than 30 s. In this study the developed communication protocol [23, 24] is used for the monitoring of sugar levels. The system detected the sugar levels in modeled glucose solutions and sent an SMS messages as designed. During the test, SMS delay was found to be varying between 8 and 30 s. The system is also tested in manual simulation mode and similar results were obtained.

17.4 Conclusion

In conclusion, we demonstrated new class of bio- and chemical sensors based on ZnO nanostructures suitable for efficient sensing in environments where small volumes are available. The developed sensors are based on nano-material grown using a low temperature regime and hence suitable for growing nanostructures on non-conventional substrates, like e.g. sub-micrometer pipettes etc. The developed sensors are based on the principle of potentiometric measurements and were used for intracellular as well as extracellular environments. The developed sensors showed wide dynamic range with excellent sensitivity.

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Chapter 18

Electrochemical Biosensors for Screening of Toxins and Pathogens

Arzum Erdem, Mihrican Muti, Hakan Karadeniz, Gulsah Congur, and Ece Canavar

Abstract Many important technological advances have been still progressed for the development of advanced sensors to monitor specific biointeractions and different recognition events of biomolecules in solution and at the solid substrates. Some of typical applications on screening of selected toxins and pathogens have been overviewed herein for environmental, agriculture, food monitoring and control with coming important features of electrochemical sensor strategies.

Keywords Electrochemical biosensor • Biointeractions • Toxins • Pathogens

18.1 Introduction

Detection of biological toxins and also food pathogens is on the practical frontier to keep public health and food safety from foodborne illness and bioterrorism [1]. Since the efficient protection is of concern to public health and food safety, the detection method has to be sensitive, rapid, and easily applicable to diverse sorts of diagnostic samples. Thus, there is an urgent need to speed up the recognition and monitoring scheme of numerous toxins and pathogens.

Many important technological advances have been progressed for the development of sensors to monitor interactions and recognition events of biomolecules.

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Table 18.1 Toxin detection technologies based on different devices

Toxin	Working electrode	Method	Detection limit	Ref.
OTA	SPE	DPV	0.07 ng/mL	[11]
OTA	GCE	CV	30 pg/mL	[12]
OA	SPE	DPV	0.38 g/L	[13]
DON	SPE	Chronoamperometry	63 ng/mL	[15]
SEB	SPE	CV	10 pg/mL	[16]
Cholera toxin	ITO	CV, Amperometry	80 ng/	[17]
OA, PbTX, DA, Ttx	SPE	Amperometry	2 ng/mL for DA, 1.5 ng/mL for OA, 0.016 ng/mL for Ttx	[18]
Ricin	MWCNTPE, CPE	Amperometry	562 pg/mL for MWCNTPE, 1.7 ng/mL for CPE	[19]
Zea	SPE	DPV	7.103 ng/mL	[20]
YTX	SPE	CV, DPV	0.6 μ M	[21]
SEB	Conductive support, polarized working electrode	CV, EIS	10 pg/mL	[22]
The toxic dinoflagellate <i>Alexandrium ostenfeldii</i>	CPE	CV	16 ng/mL	[23]
Cyanide	GCE	CV, EIS	0.6 mg/mL	[24]
Microcystin	SPE	Chronoamperometry	37 μ g/mL	[25]
<i>Microcystis</i> spp.	CPE	DPV, CV	1.34 ng/mL	[26]

Abbreviations: **Target molecules:** OTA Ochratoxin A; OA Okadaic acid *SEB* *Staphylococcal enterotoxin B*; DON deoxynivalenol; PbTX brevetoxin; DA Domoic acid; Zea Zeaalenone; YTX yessotoxin; Ttx tetrodotoxin. **Types of the electrodes:** SPE screen printed electrode; GCE glassy carbon electrode; ITO indium tin oxide; CPE carbon powder paste electrode; MWCNTPE multiwalled carbon nanotubes paste electrode. **Measurement techniques:** DPV Differential Pulse Voltammetry; CV Cyclic Voltammetry; EIS Electrochemical impedance spectroscopy

The growth of highly developed biosensors could effect significantly the areas of genomics, diagnostics, drug discovery and environmental monitoring [2–6].

Electrochemical biosensors offer great promise for exploring a difference in various biointeraction process occurred in solution and also at the sensor surface. These sensor platforms coupling the inherent specificity of nucleic acid recognition reactions with the high sensitivity of physical transducers by the advantages of various nanomaterials, hold great promise for sequence-specific detection for clinical, environmental, forensic investigations and other applications in security, since these nanomaterial based sensors can bring some important advantages; more sensitivity and selectivity by resulting in a greatly simplified protocol.

An overview is reported in the following sections, and also summarized in Tables 18.1 and 18.2 respectively about some typical applications on screening of selected toxins [7–26] and pathogens [27–40] for environmental, agriculture, food monitoring and control with coming important features of electrochemical sensor strategies based on label free systems and/or labeled ones (representatively shown in Fig. 18.1).

Table 18.2 Pathogens detection by electrochemical techniques

Pathogen	Working electrode	Method	Detection limit	Ref.
<i>Escherichia coli</i>	AuE	CV, Chronoamperometry	1 pM DNA & 250 CFU/ μ L	[27]
<i>Vibrio anguillarum</i>	GCE	AFM, FTIR, CV, EIS	10^2 CFU/mL	[29]
<i>Escherichia coli</i> O157:H7,	SPCEs	CV	70 CFU/mL	[30]
<i>Aeromonas hydrophila</i>	AuE	CV, SWV, CC	10 μ M tDNA	[31]
<i>Salmonella typhimurium</i>	PCB	EIS	500 CFU/mL	[32]
<i>Salmonella</i>	SPMM	CV, EIS, Chronoamperometry	10^2 CFU/mL	[33]
<i>Salmonella</i>	GEC	DPV	9.68 fmol/mL	[35]
<i>Legionella pneumophila</i>	AuE	DPV	10^2 genomes	[36]
<i>Escherichia coli</i> O157	SPE	SWV	0.75 amol	[37]
<i>Escherichia Coli</i> O157:H7	SPCEs	CV	6×10^2 CFU/mL	[39]
<i>Salmonella typhimurium</i>	PCBE	FCM, EIS	10 CFU in a 100 mL volume	[40]

Abbreviations: Types of the electrodes: AuE Gold Electrode; GCE glassy carbon electrode; SPCEs Screen-printed carbon electrodes; PCBE Printed Circuit Board Electrode; SPMM Screen-Printed Microchannel Microsystem; GEC Graphite Epoxy Composite; SPE Screen-Printed Electrode; **Measurement techniques:** DPV Differential Pulse Voltammetry; CV Cyclic Voltammetry; EIS Electrochemical impedance spectroscopy; SWV Square Wave Voltammetry; CC Chronocoulometry; AFM Atomic Force Microscopy; FTIR Fourier Transform Infrared Spectroscopy; FCM Flow cytometry; CFU colony-forming unit

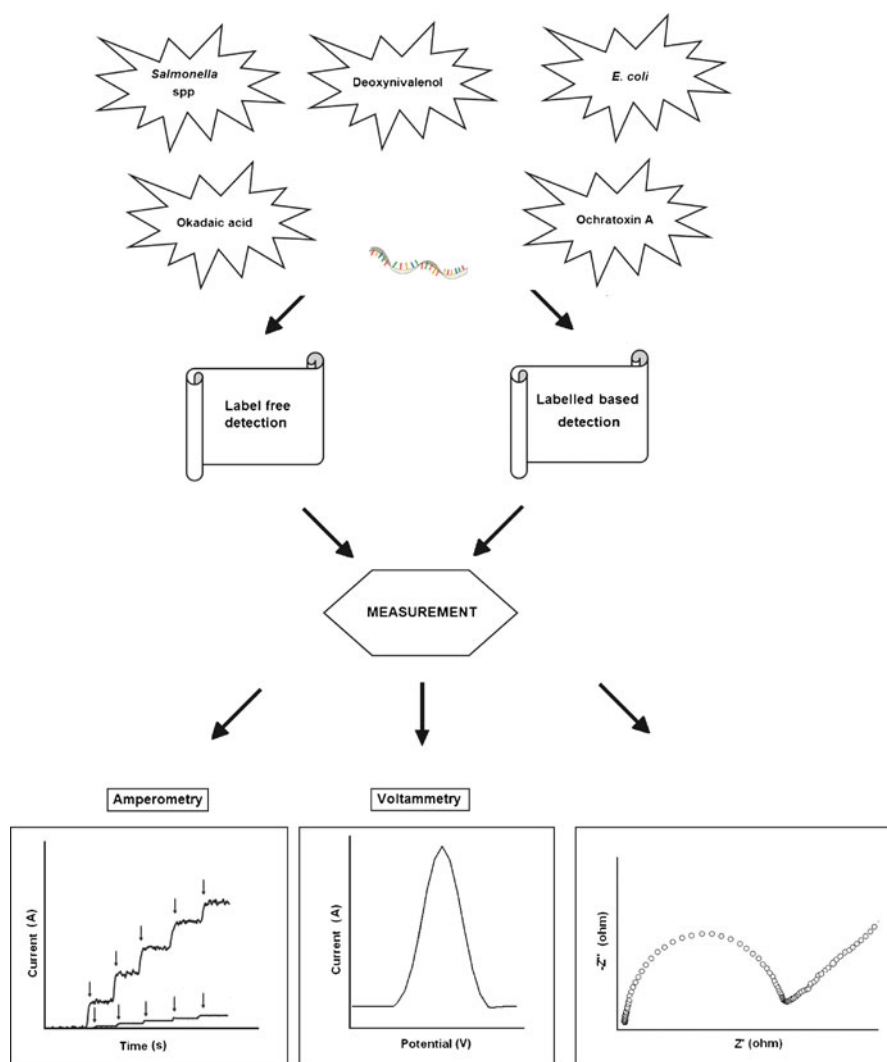


Fig. 18.1 Electrochemical screening strategies of toxins and pathogens for environmental, agriculture, food monitoring based on label free systems and/or labeled ones

18.2 Electrochemical Biosensors for Toxins and Pathogens

18.2.1 Toxin Detection by Electrochemical (Bio)Sensors

Toxins are produced by pathogenic bacteria, interact and damage the cell membrane of the host in different ways. This interaction causes forming heptameric pores in the cell membrane and destroying the structure of the membrane [41], disrupting intracellular signalling [42], inhibition the secreting neurotransmitters [43] or

activation the host immune system reactions [44]. To the present, approximately 300 bacterial protein toxins are identified as causative of various diseases in human and about one third are known to damage the cell membrane [45]. Recently, food poisoning events which were caused by microcystins from polluted water, aflatoxins from tainted corn, and ochratoxins from contaminated food have been reported increasingly. As an example, ochratoxins are contamination agents for foods and beverages and by-products of several fungal species, mainly in the *Aspergillus* and *Penicillium* genera [46, 47].

Ochratoxin A (OTA) is the most toxic member in this group and widely known to be hepatotoxic, nephrotoxic, teratogenic and mutagenic for mammalian species. It may also be found in human blood and breast milk [48]. Reactions of clinically important toxins such as cholera, tetanus and pertussis toxins with lipid bilayer have been examined via fluorescence microscopy [49] quartz crystal microbalance [50] and scanning probe microscopy [51] techniques. Compared to these conventional analytical methods, immunoassays such as the enzyme-linked immunosorbent assay (ELISA) seems to be more advantageous by virtue of their reliability, but it is time consuming, including expensive [52, 53]. Considering all of these disadvantages, new aptamer based electrochemical detection systems have been introduced in the literature by many of researchers to detect OTA [11, 12].

Bonel et al. [11] introduced a new detection system based on electrochemical competitive aptasensor. OTA specific aptamer was immobilized onto paramagnetic microparticle (MBs) surface. Mycotoxin coupled with horseradish peroxidase and free OTA were interacted these MBs. MBs were then localized SPEs under a magnetic field, enzymatic reaction product was consequently detected by using DPV. Limit of detection was found as 0.07 ± 0.01 ng/mL for magnetic assay. Because of OTA is one of the most important mycotoxin contaminants of foods, its detection is important of ppb levels. The development of an aptamer-based electrochemical sensor for the detection of OTA and the real application in practical samples was examined by also other group [12]. Kuang et al. developed an ultrasensitive and rapid electrochemical platform for the specific detection of OTA by indicator based system, and consequently, a sensitive detection of OTA was reported as low as 30 pg/mL, and the effective sensing range was varying from 0.1 to 20 ng/mL [12].

A competitive indirect enzyme-linked electrochemical immunosensor based on super paramagnetic nanobeads has been developed for the detection of okadaic acid (OA) by Hayat et al. [13]. The streptavidin-coated magnetic beads were used as support to immobilize the biotinylated OA and disposable graphite screen printed electrode was used as the working electrode. The limit of detection (LOD) was calculated as 0.38 g/L, by using these immunoassay [13]. In different study for the electrochemical detection of OA an enzyme sensor has been developed by Campas et al. [14]. The strategy was based on the inhibition of immobilised protein phosphatase (PP2A) by this toxin and the electrochemical measurement of the enzyme activity by the use of appropriate enzyme substrates, that were electrochemically active after dephosphorylation by the enzyme.

Romanazzo et al. [15] was introduced a novel electrochemical detection system to determine deoxynivalenol (DON) which was a member of group A mycotoxins produced by *Fusarium* fungi in cereals and cereal-based food samples. In their

study, anti-DON biotin conjugated Fab fragment was used that was linked with horseradish peroxidase (HRP) enzyme. For an analytical system, they used enzyme-linked-immunomagnetic-electrochemical (ELIME) assay, which was designed with both immunomagnetic beads (IMBs) and eight magnetized screen-printed electrodes (8-mScPEs) as electrochemical transducers to measure reaction product 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 via chronoamperometry technique and an 8-mScPEs strip. This sensor platform was then tested with real samples, cereals and cereal-based food samples (wheat, breakfast cereal and baby-food). Within-laboratory precision and recovery data were also calculated and their results showed that this electrochemical sensor system could be successfully employed for DON even if real samples [15].

Konry and collaborators [17] reported a study which compared both optical and electrochemical biosensor technologies for the detection of anti-cholera toxin (CTB) antibodies. The detection systems were based on singular conductive optic fibers coated with conductive indium tin oxide as biosensor platform. Pyrrole-benzophenone electropolymerization was carried out using this working electrode. Anti-CTB antibodies and secondary antibodies were labelled with HRP. As a result of catalytic activity of this enzyme, hydroquinone oxidized into quinone and the latter one electrochemically determined via amperometry. Limit of quantification of analyte concentration was as low as 80 ng/mL [17].

An amperometric disposable immunosensor based on screen-printed electrode (SPE) system coupled with amperometric detection of p-aminophenol for detection of seafood toxins such as okadaic acid, brevetoxin, domoic acid and tetrodotoxin was optimized by Kreuzer and co-workers [18]. It was reported that this biosensor system could adapted in-field measurements due to SPE system and reached short detection time as 30 min. They reported the detection limits as 2, 1.5 and 0.016 ng/mL for domoic acid, okadaic acid and tetrodotoxin, respectively [18].

Ricin is a toxic lectin and may cause allergic reactions. Suresh et al. performed a study based on ricin detection in water samples by utilizing amperometric immunosensors. Two different paste electrodes, graphite powder and multiwalled carbon nanotubes paste electrodes (MWCNTPE) were used in the study of Suresh et al. [19]. A sandwich ELISA system was used to detect ricin. The authors reported that the response of amperometric sensor is proportional to the ricin concentration in both the cases, and it is linear in the range 0.625–25 ng/mL for MWCNTPE and 2.5–25 ng/mL for CPE. LOD was found as 562 pg/mL for MWCNTPE and 1.7 ng/mL for CPE.

Yessotoxins (YTXs) are a group of lipophilic sulphated polyether compounds that are structurally related to brevetoxins and ciguatoxins [54]. Campas et al. [21] described two functional assays for the detection of YTX based on the phosphodiesterase (PDE) enzyme and the subsequent measurement of the enzyme activity by colorimetric and electrochemical methods. Electrochemical measurements; cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using a single-drop configuration on a horizontally supported screen-printed three-electrode system. LODs of 0.8 and 0.6 μM were obtained by colorimetry and electrochemistry, respectively.

A time-resolved electrochemical impedance spectroscopy and CV were utilized for the detection of trace concentrations of *Staphylococcus enterotoxin B* (SEB) by Chai and co-workers [22]. An anti-SEB antibody had been attached to the nanoporous aluminum surface using 3-aminopropyltriethoxysilane/glutaraldehyde coupling system. The authors reported that it was possible to determine the presence of SEB at the levels as low as 10 pg/mL in 15 min by using this biosensor platform [22].

A different study was reported by Wang et al. [24] based on electrochemical biosensor for the determination of cyanide through its inhibitory effect on HRP encapsulated by chitosan (CHIT) on the platform of hydroxyapatite nanowires array (HANWA). They presented that the CHIT–HRP/HANWA biosensor has the advantages of spatial resolution, high sensitivity, rapid regeneration, and fast response associated with individual nanowires.

The bloom-forming genera of cyanobacteria, *Microcystis* spp., produce toxins including hepatotoxic microcystins that are potential health hazards for livestock and humans [55].

In the study reporting the development and applications of electrochemical biosensors specific for *Microcystis* spp. [26], the probe has been designed as a 17-base single stranded (ss) oligonucleotide, was based on sequence polymorphism within the 16S ribosomal DNA (rDNA) of *Microcystis* spp [56]. The voltammetric electrochemical biosensor based on graphite electrodes was developed for the specific detection of short DNA sequences related to *Microcystis* spp. by using these external redox labels, methylene blue (MB) and $[\text{Ru}(\text{bpy})_3]^{2+}$, and the formed hybrids on the surface of CPE were evaluated by DPV [26].

18.2.2 Pathogen Detection by Electrochemical (Bio) Sensors

In clinical diagnosis, disease control, environmental monitoring and food safety, rapid, selective and sensitive detection technologies for pathogenic bacteria are very important. Conventional identification methods include polymerase chain reaction (PCR) [57], culture and colony counting [58], immunological techniques [59] and fluorescence-based assays using organic dye molecules [60]. Unfortunately, despite these approaches being powerful and error-proof, most of them are laborious, complex and time consuming, and they do not afford the necessary detectability and specificity towards the target.

Waltera et al. [27] introduced an electrochemical genosensor in which signal amplification is achieved using p-aminophenol (p-AP) redox cycling by nicotinamide adenine dinucleotide (NADH). An immobilized thiolated capture probe was combined with a sandwich-type hybridization assay, using biotin as a tracer in the detection probe, and streptavidin-alkaline phosphatase as reporter enzyme. The group reported that the convenient use of DNA biosensor arrays associated with a simple one electrode, one-enzyme format redox cycling strategy led to the specific

and sensitive detection of 1 pM DNA target, as well as 250 CFU/ μ L *E. coli* pathogenic bacteria, which shows great potential in clinical application.

In the study of Chen et al. [28] a label free and sensitive DNA/RNA silicon based electrochemical microsensor array was developed by using thin film of the conducting polymer polypyrrole doped with an oligonucleotide probe. The electrochemical potential pulse amperometry technique was used for a biowarfare pathogen target DNA detection.

Wan's research group [29] was developed a sensitive and reliable impedimetric immunosensor based on reduced graphene sheets (RGSSs) using a controlled electrodeposition technique for the selective detection of marine pathogenic sulphate-reducing bacteria (SRB).

A rapid, sensitive, and field-ready detection method for *Escherichia coli* O157:H7, a highly infectious and potentially fatal food and water borne pathogen, was developed in the study of Settingington et al. [30]. Cells are isolated by immunomagnetic separation, labeled with electroactive polyaniline, and detected by cyclic voltammetry on screen-printed carbon electrodes. The LOD of 70 CFU mL⁻¹ was found with a linear range of 10¹–10⁵ CFU/mL.

Tichoniuk et al. [31] described an electrochemical DNA biosensor used for the detection of *Aeromonas hydrophila* which was recognized as an emerging food-borne hazard and is associated with a variety of virulence factors including production of cytotoxic enterotoxin aerolysin. The presented genosensor enabled discrimination between the DNA specific one for the aerolysin gene of *A. hydrophila* and the DNA samples isolated from other bacterial microorganisms. Chronocoulometric quantitation of DNA and square wave voltammetry (SWV) measurements of electroactive indicator, methylene blue (MB) were performed to detect the influence of hybridization reaction time, concentration of target DNA fragments, and presence of non-complementary DNA on the electrochemical response of genosensor recognition interface.

In the study of Nandakumar et al. [32] a three-electrode system based sensor has been developed using printed circuit boards for detecting bacterial contamination by using electrochemical impedance spectroscopy (EIS). The sensor was tested on the common food-borne pathogen *Salmonella typhimurium* and it was found that it could detect bacterial concentrations of the order of 500 CFU/mL within 6 min.

A low-cost, fast, reliable, selective and high sensitive, easy-to-fabricate micro-system, capable of electrochemically detecting *Salmonella* has been manufactured with screen-printed electrodes by Mata et al. [33]. The electrochemical detection has been investigated by electrochemical impedance and amperometric methods. Mata et al. reported that the electrochemical sensor platform detected the concentrations of 10² and 10⁸ CFU/mL in less than 30-min time.

A label-free electrochemical approach using graphite epoxy composite electrodes (GECE) was developed as the first time for the detection of the specific sequence related with *Salmonella* spp. by using the oxidation signal of DNA base, guanine without any modifications in the native DNA bases, or using any external

labeling agent [34]. Erdem et al. [35] also presented a label-free genomagnetic assay for the electrochemical detection of *Salmonella* spp sequence by using GECE and magneto-GEC electrodes as electrochemical transducers. LOD was found as 9.68 fmol/mL target concentration in the 20 μ L samples using GEC electrode with dry-adsorption at 80°C.

Miranda-Castro et al. [36] presented an electrochemical method, different from real-time PCR approaches, for semiquantitative evaluation of *L. pneumophila*. A PCR assay targeting the 16 S-rRNA gene of *L. pneumophila* giving rise to a 95-mer amplicon was established. Amplicons were hybridized to a biotin-labeled reporter sequence and then to a thiolated stem-loop structure immobilized onto gold electrodes as a reporter molecule with 1-naphthyl phosphate as a substrate. 1-Naphthol enzymatically generated was finally determined by DPV.

Liao's research group [37], developed a novel electrochemical competitive genosensor, featuring gold-electrodeposited screen-printed electrodes (nanoAu/SPE) modified with a self-assembled monolayer of thiol-capped single-stranded DNA (capture probe), for the detection of the *rfbE* gene, which is specific to *E. coli* O157. The current signal of the released liposomal $\text{Ru}(\text{NH}_3)_6^{3+}$ was measured using square wave voltammetry. The LOD was reported as 0.75 amol of the target *rfbE* DNA.

Zuo et al. [38] introduced an electrochemical DNA biosensor based on a zirconia (ZrO_2) modified thin film screen-printed electrode (ZrO_2/SPE). Short DNA sequences from the *Escherichia coli* pathogen, modified with a phosphate group at the 5' end, were attached to the surface of the electrode through the affinity of the phosphate group for zirconia. DNA immobilization and hybridization were characterized using DPV by employing methylene blue as redox indicator.

Lin and coworkers [39] fabricated a disposable amperometric immunosensing electrode for rapid detection of *Escherichia coli* O157:H7. The LOD was found approximately 6×10^2 CFU/mL.

18.3 Conclusion

Biosensors offer several advantages over existing techniques such as limited hands-on time, high-throughput screening, improved detectability, realtime analysis and label-free detection methods and devices [61]. The biosensor field is evolving to develop cost-effective portable devices with higher sensitivity and easier usage, and to be capable of multiplexed and continuous monitoring. These challenges are of great importance for bacteria identification and detection since the achievement of ultrasensitive detection by pushing sensitivity towards single-molecule LOD, that is critical to prevent pathogenic infections and pandemics.

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Chapter 19

Fluorimetric Bioprobe for Genotoxicity Based on DNA-Dye Intercalation

Bengt Danielsson and Yang Liu

Abstract The potential of a nucleic acid-based optical bioprobe for environmental measurements and drug monitoring is described. The sensor employs long-wavelength intercalating fluorophores like TO-PRO-3 (TP3). Compounds that interact with the TP3-DNA complex are indirectly detected by a decrease in the fluorescence intensity. We found that the configuration and length of the DNA dramatically affected the intensity of the fluorescence emitted from the TP3-DNA complex. We have compared nucleic acids from different sources and optimized the system. In one proposed method, we attempt to combine broad-range detection with rapid and simple operation. A fiber-optic capillary fluorescence system was used to analyze toxic aromatic amines, antibiotics, and several kinds of antitumor drugs, using small amounts of sample, down to 10 μ L, with sensitivity comparable to that of current electrochemical methods. The detection limit can be as low as a few ppb or submicromolar. This approach is useful for routine screening in environmental monitoring or for controlling cytotoxic drug administration. The ease of operation and the rapid response allow high-throughput screening. To enable sufficiently high sensitivity to detect substances that may be used for bioterrorism concentration enhancing methods such as SPE can be used for sample collection.

Keywords Genotoxicity • Fluorometric screening • DNA-intercalating dyes • Sample arrays • Optical fibers • CCD camera

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19.1 Introduction

Recent years have seen an enormous increase in the use of nucleic acids as a tool in the recognition and monitoring of chemical compounds of environmental interest that may affect the genome. This is because nucleic acids possess many unique structural features offering reliable means of recognizing and monitoring compounds of medical or environmental importance. Therefore major research activities in these areas have been directed toward the design of sequence-selective biosensors especially those based on hybridization [1]. Commonly used methods for identifying carcinogens include epidemiology, mammalian bioassays, and rapid assays, such as low-temperature laser-induced fluorescence detection, have been suggested for the analysis of DNA-carcinogen interactions [2, 3]. A procedure using a simple fluorometric device for the detection of mutations in oligonucleotides using double-stranded DNA (dsDNA) and a specific nucleic acid intercalating dye was presented 1997 and a strategy was proposed for broad-range detection of compounds with affinity for nucleic acids [4]. At about the same time, electrochemical methods for the same purpose were introduced [5–7].

The studies reported here were carried out using a number of different formats to detect the interaction of various potentially genotoxic compounds with nucleic acids through changes in the fluorescence intensity of fluorescent intercalating dyes, such as TO-PRO-3 (TP3) and TOTO-3 (TT3) from Molecular Probes. The intercalation reflects the ability of these planar aromatic or heterocyclic aromatic systems to become inserted between contiguous base pairs of a nucleic acid molecule without disturbing the overall stacking pattern or requiring any covalent modification. Developments have considerably decreased the volume of sample/reagent required (total volume ca 10 μ L) compared to previous procedures, increased the sensitivity, and facilitated high-throughput microchip screening applications. TP3 and TT3 specifically bind with dsDNA and are frequently used in flow cytometric analyses. They have very high binding affinity to dsDNA, with dissociation constants in the micromolar range, but they lack sequence selectivity. Upon binding to dsDNA, their fluorescence increases. Simultaneous binding of certain other compounds to the polynucleotide may induce a partial block of the duplex in the DNA sequence, which leads to a decrease in the degree of intercalation of the dyes. The displacement of the dye by another compound with affinity for the nucleic acid can also be detected as a decrease in the fluorescence intensity of TP3 or TT3. Both DNA complexes emit long-wavelength emission after excitation (642/661 nm), in a wavelength range with low autofluorescence from natural compounds, which enhances the signal/noise ratio.

Our concept was developed as a complement to mutagenicity tests based on whole cell or bacterial assays in order to provide higher throughput. An early version was based on a simple and relatively portable fiber fluorometer with a light emitting diode as light source and a solid state photometric sensor [4]. The test mixture was contained in a small glass capillary into which a piece of the optical fiber was inserted. The exciting and emitting lights were efficiently coupled into

capillary wall and distributed over the total liquid volume in the capillary. This approach worked quite well and nanomolar sensitivity could be achieved. It was anticipated that this form of sensing could be used for broad-range environmental screening of genotoxic compounds, for instance in old industrial sites and gas stations where the grounds were considered for residential buildings. Other applications would be testing of food and drugs for mutagenic contaminations. The latter could be a realistic risk even with a highest allowable level of impurities at 0.1% considering that many drugs are taken in daily doses at gram quantities [8].

Bioterrorism is a newer risk scenario and even if chemical compounds can be carcinogenic at concentrations where they lack acute toxicity, it is not impossible that evil groups could contaminate food and drinking waters over times long enough to be harmful. We have, however, not yet investigated this area even if the sensitivity of the actual approaches would be adequate. In applications requiring very high sensitivity, samples can be upgraded by a number of methods including solid phase extraction. In the following examples will be given from studies on different types of genotoxic compounds that have tested with some instrumental concepts that could be used for high throughput testing also related to threats of bioterrorism.

19.2 Experimental Section

19.2.1 *Materials*

The fluorescent dyes TP3 and TT3 (Fig. 19.1) were obtained from Molecular Probes (Eugene, OR). Low-molecular-weight DNA from salmon sperm and calf thymus DNA and doxorubicin were from Fluka. Plasmid pBR322 and the *Hind*III restriction endonuclease were bought from Invitrogen Corporation (Carlsbad, CA) and Lambda DNA from Fermentas (Germany). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The glass end-to-end capillaries were purchased from Sarstedt AG (Numbrecht, Germany). The 384 wells FluoroNunc™ plates were obtained from Nunc A/S (Denmark). Serum samples were obtained from healthy volunteers and from the University Hospital in Lund.

19.2.2 *Instrumentation*

Three different arrangements for fluorescence measurements will be described here. In two of them we used a QuantaMaster model C-60/2000 spectrofluorometer from Photon Technology International (Lawrenceville, NJ, USA), with a 75-W xenon compact arc lamp and a bifurcated quartz fiber (060–8011) The glass imaging probe used in the first set-up (I) was obtained from Oriel Instruments (Stratford, CT, USA). See Fig. 19.2.

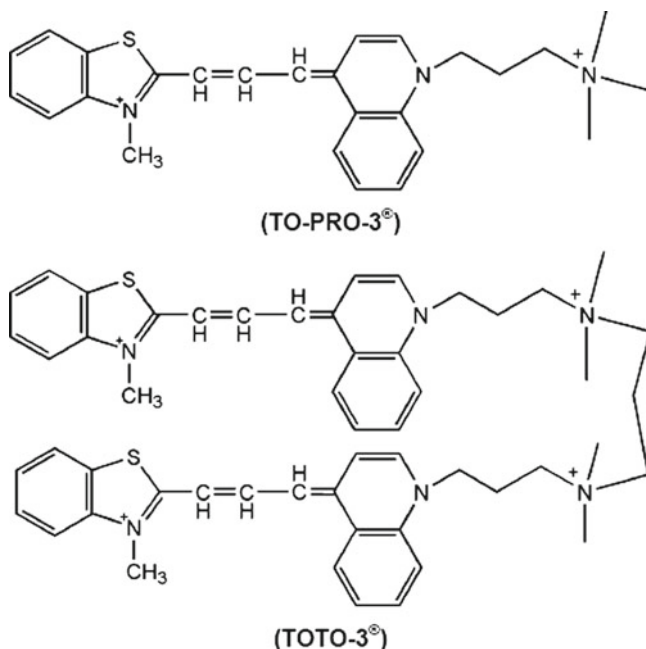


Fig. 19.1 Chemical structures of the fluorescent dyes used

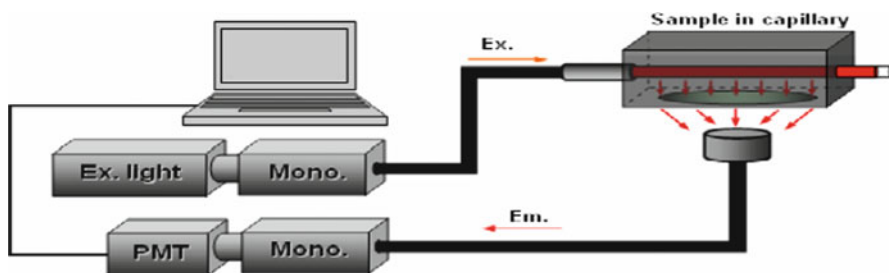


Fig. 19.2 Schematics of the fiber-optic fluorometer I

In the second arrangement (Fig. 19.3) a bifurcated fiber optic probe (R400-7) from Ocean Optics BV was used to monitor the fluorescence from the sample wells. Light from the excitation monochromator was focused into the central fiber of a “six-around-one” bifurcated fiber optic reflection probe for illumination of one well at a time on the plate. The probe was fixed to a motorized XYZ-stage that could be programmed to stop at each well. The peripheral fibers of the probe were connected to the emission monochromator coupled with the photomultiplier detection system. The wavelengths for maximum excitation/emission for TT3 are 642/660 nm and the slit width of the excitation monochromator was set to 2 mm, corresponding to a

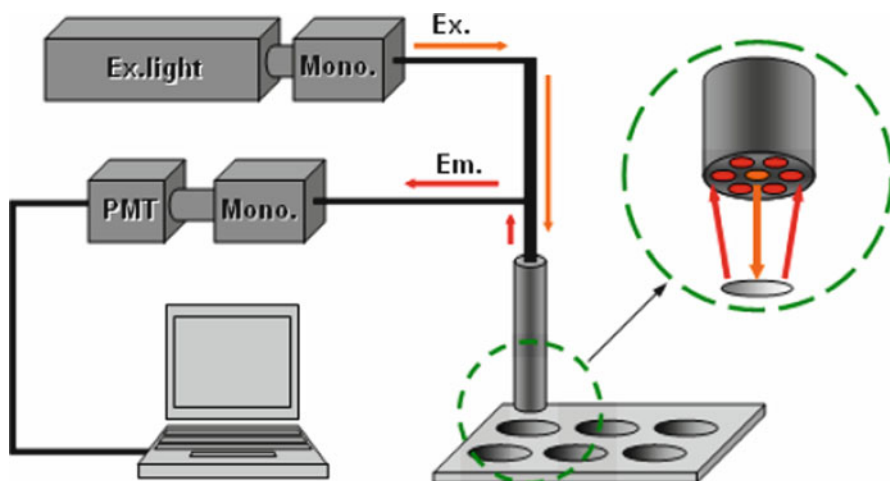


Fig. 19.3 Setup of the fluorometric screening system type II

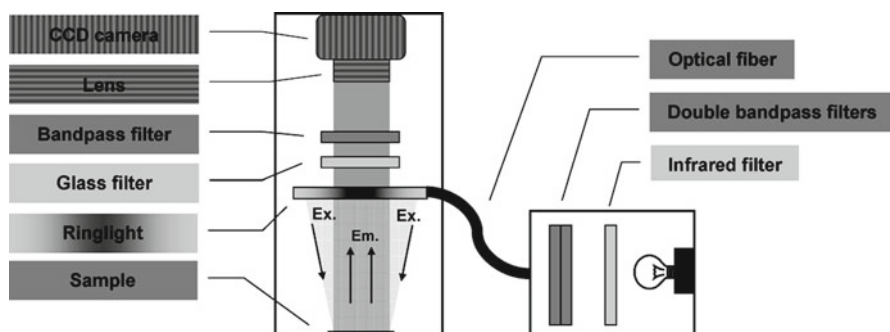


Fig. 19.4 Setup type III for fluorometric screening of micro titer plates

bandpass of 8 nm while the emission monochromator was set to 0.25 nm, corresponding to a bandpass of 1 nm.

With the third experimental arrangement (Fig. 19.4) the assay results could be directly read out from 384 well plates with a Hamamatsu ORCA-ER CCD camera equipped for fluorescence detection. A Dolan-Jenner Fiber-Lite® MI-150 illuminator (MA, USA) connected with an adjustable ringlight was used as the excitation light source. The wavelength for TT3 was selected to 640 nm with double interference filters with 10 nm bandwidth mounted together with an infrared filter on the illuminator. The camera used a Schneider Kreuznach Xenon 0.95/25 lens preceded with 664 nm bandpass filter with 12 nm bandwidth to pick up the emitted light from the plates.

19.3 Results

Initial studies were performed to see how different types of DNA affected the fluorescence intensity. DNA from different species were compared, including salmon sperm chromosomal DNA (low molecule weight), calf thymus chromosomal DNA, plasmid DNA pBR322, and plasmid DNA pBR322 digested by the restriction endonuclease *HindIII*. The fluorescence intensity was greatly influenced by the choice of DNA. It was lower when the salmon sperm chromosome DNA and the calf thymus chromosome DNA were used as substrate. For many of the following investigations, the well-defined nucleic acid material from plasmid DNA pBR322 was chosen. It is 4361 bp long and has a unique cleavage site for restriction endonuclease *HindIII*. A common observation is that the higher the molecular weight of the DNA used, the faster the binding and the stronger the fluorescence intensity is.

Another common observation is that the sensitivity of the assay strongly depends on the intercalating dye [9]. For instance, a comparison was made of SYBR Green I, which is by far the best high-sensitivity reagent for double-stranded DNA staining and TP3. It was seen from the binding curves that the fluorescence intensity of SYBR Green I was significantly higher than that of TP3, but the competition assay indicated that TP3 was more sensitive for fluorometric competition assays. This is because SYBR Green I has a much stronger binding affinity than TP3, which makes it difficult to be displaced by a compound competing for binding. These investigations were carried out with system I using glass capillaries.

There is a good potential for pharmaceutical applications of the proposed method. Chlorpromazine and clomipramine are tricyclic agents with antidepressant and antipsychotic properties, and both can intercalate/interact with dsDNA (Fig. 19.5). Another pharmaceutical that can interact in the same way is chloramphenicol (CAP), which is often used for treating infections caused by bacteria. As it has been shown to be harmful to humans, EU has established safe maximum residue limits for this substance for use as a veterinary drug in food-producing animals. Various analytical methods have therefore been developed for the determination of CAP, most of them unfortunately connected with high assay cost, which can be overcome by the proposed method. The linear detection range is 10–150 nM, which is sufficient to make this low-cost, easy-to-handle method a potential alternative for testing for CAP in seafood or other agricultural products.

Indeed a separate study was made to see if it was possible to develop a quantitative method for the determination of certain antibiotics at trace levels in aquaculture products. In this case a scanning fiber optic probe was used to measure interactions on a 96 well plate (system II), which dramatically shortened the analysis time and simplified the experimental procedure without loss of sensitivity [10]. The fluorescent intercalator dye used here was TOTO-3 (TT3), a symmetric dimer of a cyanine dye. The method was validated using extraction from shrimps spiked with chloramphenicol (CAP) at the range of ng per 100 g shrimp level.

The TOTO-3-DNA complex was found to produce a stronger fluorescence signal here than the TOPRO-3-DNA complex. The illumination conditions were chosen to minimize contributions from light directly reflected from the back of the plate

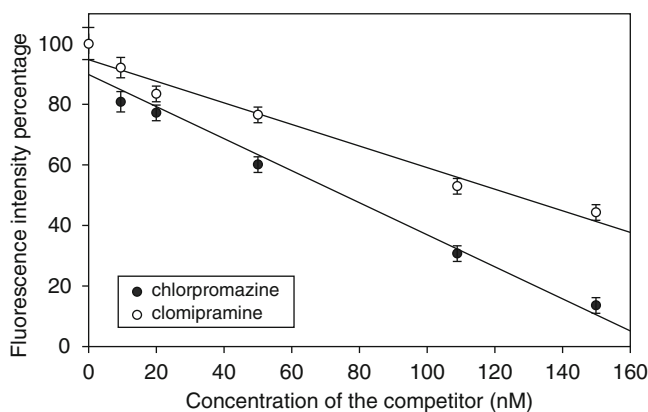


Fig. 19.5 Competition curves for chlorpromazine and clomipramine using a TP3-DNA complex (digested plasmid pBR322)

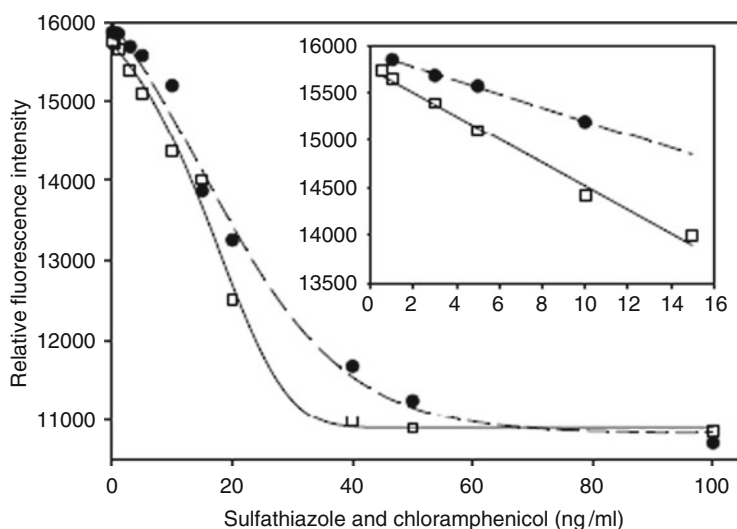


Fig. 19.6 Calibration curves for sulfathiazole (*open squares*) and chloramphenicol (*filled dots*)

into the probe and to collect only the emitted light. As the plate was black it efficiently absorbed the reflected light, thus increasing the fluorescence signal-noise ratio. Excitation/emission wavelengths were set at 640/642 nm on the monochromators. The DNA employed was Lambda DNA, a linear, double-stranded DNA, with a molecular weight of 31.5×10^6 Da containing 48502 base pairs. This DNA was chosen because the long-chain linear dsDNA-dye complex provides a better fluorescence signal than circular dsDNA or short-chain dsDNA complexes [9].

The results of the binding assay are shown in Fig. 19.6. A lambda DNA concentration of 10 mg/mL was chosen for the fluorometric assays. Sulfathiazole and related sulfonamide drugs are used against a wide variety of gram-positive and

gram-negative organisms, as well as *Chlamydia trachomatis*. They are also often used in veterinary practice and can therefore contaminate the resulting food products, i.e. milk and meat. The sulfathiazole calibration curve indicates that the linear range of detection is from about 0.5 to 15 ng/mL. This is comparable to other DNA-based biosensor assays reported in literature.

Chloramphenicol (CAP) is frequently found in animal-derived foods (mainly aquaculture products) originating from Asia. CAP is a broad-spectrum antibiotic, and its use in food-producing animals is prohibited in many countries such as the USA, Canada, Australia, and EU member states. Relatively low levels of CAP may give rise to an irreversible type of bone marrow depression, which may lead to aplastic anemia. No maximum residue limit has been established for CAP in animal-derived foods as its toxic effects are not dose dependent, but rather related to the hypersensitivity of certain individuals. Several methods for the determination of CAP in foods and biological fluids have been developed and the procedure reported in [10] is quite acceptable with an operating range of 1–10 ng/mL and a good sample throughput. The recovery of chloramphenicol in spiked shrimp extracts using the rapid fluorometric screening method was 96–98%.

Many effective anticancer drugs in clinical use interact with DNA and some of them do so through intercalation. The intercalation process involves the insertion of a planar aromatic or heteroaromatic system between adjacent base pairs of a DNA molecule. Doxorubicin hydrochloride is an anthracycline antibiotic with a wide spectrum of antitumor activity. Its use is, however, limited due to its toxic effect on different organs and tissues. A sensitive and rapid technique for determination of serum levels of anticancer drugs is needed also because of inter-patient heterogeneity for the same drug dosage. Many methods for the determination of anthracyclines and their respective metabolites in blood by chromatography have been reported, but they require complicated sample preparation and the time consuming measurement with expensive instrumentation. Procedures for high throughput, rapid on-the-spot tests are lacking. A simple DNA based bioprobe for fluorometric detection of anticancer agent, such as doxorubicin could be the answer.

A double stranded DNA based fluorescence bioprobe for anticancer agent (doxorubicin) detection was developed for sensitive DNA/drug interaction study by a homogeneous assay [11]. The anticancer agent interacts with the DNA-TT3 complex, and can be indirectly measured by the decrease in the fluorescence intensity. Of the various oligonucleotides with different sequences that were examined doxorubicin had highest preference for the oligonucleotide 5'AGCACG3'. A CCD camera setup III was applied along with 384 well plate assay format for high throughput fluorescence imaging. The detection range was 25 ng/mL to 100 µg/mL. The recovery test with spiked serum sample shows that this method can be a potential routine method for therapeutic drug monitoring.

In addition, metal ions, such as Cu^{2+} , Cr^{3+} , Fe^{2+} , Ni^{2+} , Co^{2+} have an effect on the fluorescence in the range 0.1–3 mM, which may not be sensitive enough for practical determination of the metal ions, but could be interesting as a general tool for chemical studies of interactions with DNA molecules.

19.4 Conclusions

The above described fluorescence-based optical sensing methods are able to detect a broad range of substances with affinity for nucleic acids. The sensing method is based on the decrease in the fluorescence intensity of the complex formed by the sensing DNA molecule and an intercalating dye upon interaction with the analyte. With respect to fluorescence intensity and sensitivity, TOTO-3® was found to be a more suitable dye to the presence of various analytes of interest than TO-PRO-3®. The advantages of this method are rapid analysis, simple experimental procedure and good sensitivity. The ability to measure many varieties of toxic compounds makes this technique useful in the screening and monitoring of environmental sites for contamination and this could also be of interest for investigations of bioterrorism.

This type of analysis is not intended for specific detection, but for screening and rapid quantification, which can be followed by traditional analytical techniques. For example, combined with the high throughput technique it can be used to initially characterize and possibly group a number of different compounds. Also, by choosing oligonucleotides with different sequences the sensitivity and the specificity to different analytes can be improved. Based on the features of nucleic acids and on the powerful techniques of nucleic acid engineering, further developments in the field can be expected.

The entire detection system is worthy of further development and integration. The development of integrated arrays of broad range recognition elements is an important step in the path towards creating artificial sensing devices. The use of optical fiber based detection opens a new window of possibility for wireless remote sensing from a central location. With the help of miniaturized detection device and further simplified sampling, even an untrained user can be instructed to perform the test and send the signal through wireless mode.

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