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REVIEW PAPER

Biosensors for Biological Warfare Agent Detection

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ABSTRACT

Biological warfare agents (BWA) such as *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis* or *butulotoxin* represent one of possibilities exploitable by military or terrorists. Rapid detection of BWA is one of the most important presumptions prerequisities for successful defence against them. The detection devices–biosensors–can be divided according to their physicochemical transducers to electrochemical, optical, and piezoelectric groups. A comparison of classical detection methods with biosensors is also given.

Keywords: Biosensors, optical biosensors, electrochemical biosensors, piezoelectric biosensors, biological warfare agents

1. INTRODUCTION

From military point of view, a number of pathogenic bacteria can be considered as possible biological warfare agents (BWAs). Highly prospective agents on the list of BWA are *Bacillus anthracis* (anthrax) and variola major virus (smallpox). The highly dangerous agents include botulinum toxin, *Francisella tularensis*, *Salmonella typhimurium*, and *Yersinia pestis*. Other bioagents, like Venezuelan equine encephalitis, Marburg, Ebola, and influenza viruses are of lesser importance, despite the fact, that infections with these viruses are serious and mortality is relatively high, but due to the difficulty in their preparation, their position on the list of BWA is lower. In short, every pathogenic organism can be abused for biological weapon construction but effectiveness might be very disparate. In comparison with chemical warfare agents (CWA), BWA production is much cheaper and terrorist or military attack with BWA is more effective in the range of hazard area and in the number of expected casualties. The infectious dose (ID) (amount of organism needed for infection outbreak) is different for every agent. The risk rate of every BWA is given not only by its ID, but also by the way of natural spreading, stability in either aerosol or water, and in the case of bacteria, also the possibility of spore formation. Usually the intake of aerosol (particles 1 - 10 μm) through lung is able to evocate disease with a lower ID for the given BWA. The effect of attack with BWA has been expressed by Kaufmann[1], *et al*. in a hypothetical model of aerosol attack with *Bacillus anthracis*, *Francisella tularensis* or *Brucella melitensis* on a model North American town with 100,000 inhabitants. In the case of *Bacillus anthracis*, 50,000 cases of anthrax inhalation are expected with 32,875 deaths. In the case of *Francisella tularensis*, 82,000 cases of pneumonic or typhoidal forms of tularemia can be expected with 6,188 deaths. *Bacillus melitensis* could cause 82,500 cases of brucellosis with 413 deaths. The expected economic impact varies from $ 478 million for *Bacillus melitensis*

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and $ 5.4 billion for *Francisella tularensis,* to finally $ 26.2 billion for *Bacillus anthracis*.

The history of biological weapons is reaching far into the past. Before adopting scientific approaches, the water poisoning or displacement of cadavers were typically used, e.g., Tatar Forces used dead bodies to spread plague[2] in the 14th century. In 1763, British troops under Commander Sir Jeffrey Amherst used smallpox in the war with Native Americans[3]. The first serious attempts to use pathogenic bacteria were undertaken in the late 19th century and intensive development of biological weapons became much more accelerated in the 20th century. In 1925, the International Protocol in Geneva, 'Protocol for the prohibition of the use in war of asphyxiating, poisonous or other gases, and of bacteriological methods of warfare', was signed by most of the countries. This Protocol solved only the use of BWA in war but not their production and storage. In the period between world war I and world war II, BWAs, were produced by many countries such as USSR, UK and Japan. The Japanese effort resulted in the formation of the 'Unit 731' in the occupied Chinese town Manchuria and criminal testing of BWAs on human beings with over 10,000 casualties[4]. The competition between powers after world war II resulted into implementation of missiles loaded with BWA and preparation of carriers for stabilisation of aerosols. This competition was formally terminated by the 'Biological and Toxins Weapons Convention' signed in 1972, but USSR secretly continued the development of biological weapons[5]. World was warned after the Sverdlovsk tragedy[6] in 1979 and the USSR Biological Offensive Programme was officially terminated in 1992.

Biological weapons have become the domain of interest for some terrorist organisations such as the Rajneeshee community which used *Salmonella species* to contaminate food in two restaurants in Oregon[7]. The unsuccessful attempts to use *Bacillus anthracis, Vibrio cholerae,* and butulinum toxin for biological weapon construction were made by the Aum Shinrikyo cult in Japan[8]. The *Bacillus anthracis* spores were spread by letters through the US Postal System in 2001 resulting in several deaths[9].

The defence against BWA military or terrorist abuse relies on the early detection of biological warfare agents, separation of infected individuals and evaluation of the contaminated area. For this reason fast, sensitive, and portable devices for BWA detection are required. In this review some examples of biosensors for detection of BWA are presented. These sensors types are divided according to the physicochemical transducer employed. Electrochemical, optical, and piezoelectric biosensors are mentioned. Selected examples of classical detection methods and their comparation with biosensors are also presented.

2. BIOSENSORS

Biosensor is an analytical device containing biological sensing - biorecognition - element (such as antibody, enzyme, receptor or antigen) in a close contact with a physicochemical transducer. For detection of BWA, sensors with immobilised antigen or antibody are used. The interaction between the biorecognition element and analyte changes the physical properties which are converted by the transducer into electric signal, and finally detected by some data processing unit. The most commonly used physicochemical transducers include electrochemical, optical and piezoelectric sensors.

2.1 Electrochemical Biosensors

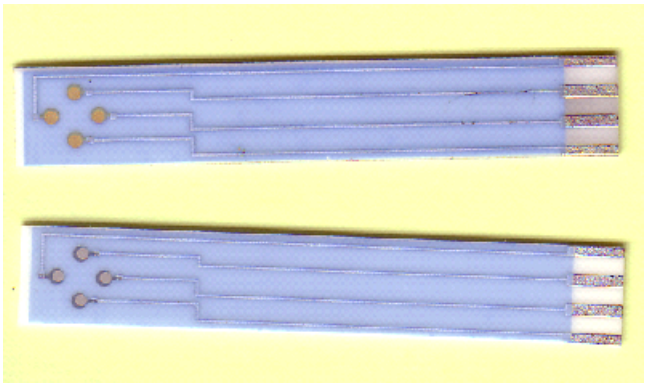
The original biosensors employed electrochemical measuring procedures. Due to their low cost and good sensitivity, these sensors are continuously upgraded, and presently, days commercial sets are available for various analytes. Several types of electrochemical biosensors are utilised: Potentiometric, amperometric, and impedimetric. Potentiometric biosensors are usually based on ion-selective electrodes. These devices measure the change in ion concentration during reaction taking place in the biorecognition layer. First sensors of this type used p*H* glass electrode with enzymes captured in a suitable membrane. Nowadays biosensors with ion-sensitive field effect transistors (ISFET) and other types of semiconductors are commercially available.

For potentiometric immunosensor construction, enzyme-labeled antibodies are usually chosen. The most convenient labeling enzymes are urease, glucose oxidase or alkaline phosphatase which are able to change either p*H* or ionic strength in the course of the detection. Very popular semiconductor-based biosensors are light-addressable potentiometric sensors (LAPS). Due to their small size and possible multi-channel arrangement, these devices seem to be very convenient for simultaneous analysis of several analytes; standard microelectronic procedures make mass production easy. Light-addressable potentiometric sensors (LAPS) biosensors are capable of signal amplification due to the incorporated field effect transistor (FET). Light-addressable potentiometric sensors (LAPS) are heterostructures consisting of a silicon base covered with silicon oxide and silicon nitride layers[10]. The backward illumination with light emitting diode (LED) induces a change of conductivity in the sensing area, and after formation of the immunocomplex labeled with urease, the change of p*H* is registered.

The LAPS immunosensors were used to detect *Francisella tularensis*[11] with a limit of detection (LOD) at 3.4×10^3 cells/ml and *Bacillus melitensis* with LOD equal to 6×10^3 cells/ml during the 1 h incubation time[12]. A better LOD was achieved for *Escherichia coli* DH5 α strain[13]; the secondary antibody specific against *Escherichia coli* labeled with urease was used and LOD of 10 cells/ml for 1.5 h assay time was claimed. The commercial system Bio-Detector based on 8-channel LAPS was developed by Smith Detection (Warrington, UK). This device intended for mobile laboratories is able to detect bacterial pathogens such as *Bacillus anthracis* with LOD 10^3 CFU/ml and toxins such as botulotoxin with LOD 0.1 ng/ml in 15 min.

The most common type of biosensors use amperometric measurements providing good sensitivity[14]. Large-scale production of screen-printed electrodes is cheap, resulting in low cost of the whole equipment. Some examples of amperometric sensors are presented in Fig. 1 (These sensors are produced by BVT Technologies (www.bvt.cz)..

As in the case of potentiometric immunosensors, amperometric ones usually utilise specific antibodies



(a)

(b)

Figure 1. Examples of multichannel screen-printed electrochemical sensors. (a) 4-electrode ver. with either gold (top) or platinum (bottom)-based working electrodes, sensor dimensions 50 x 8.5 mm, suitable for measurements in flow-through and stirred arrangements, (b) 8-channel ver. for measurements directly in the embedded miniwells, dimensions 25 x 50 mm.

labeled with enzymes such as peroxidase, alkaline phosphatase, and acetylcholinesterase. The chosen enzyme with a suitable substrate should provide sufficient electron transfer to the working electrode. Amperometric biosensor was for example constructed by Mirhabibollahi[15], LOD of 10^3 CFU/ml was reached for *Staphylococcus aureus* in beef and milk samples.

Simple biosensor using screen-printed electrodes was presented by Crowley[16], LOD for Listeria monocytogenes was 9×10^2 cells/ml in a 3.5 h assay. The amperometric biosensor for *Klebsiella pneumoniae* antigen as a marker for urea tract disease was recently developed[17]. It consisted of co-immobilised cholinesterase and antibodies against *Klebsiella pneumoniae* reached LOD 5 μg/ml. The biosensor using biolayer of polyclonal antibodies

attached through the covalently-immobilised Protein A was used by Skládal[18]. This flows though a 4-channel arrangement in sandwich format with peroxidase-labeled monoclonal antibody and was able to detect *Francisella tularensis* at 100 cells/ml. Amperometric biosensor was also used for serotypization of *Salmonella typhi* in patient sera[19]. This biosensor was able to interpret sera of all 28 patient positive when compared with normal health serum and one analysis took 75 min. Few commercial instruments are available in the market tuday such as Midas Pro with amperometric sensor as sensing element produced by Biosensori SpA (Milan, Italy). This device is able to detect 10^6 cells/ml within 20 min.

Impedimetric biosensors employ the change in impedance, conductance, capacitance or resistance characteristic of the immunosystem to provide measurable signal. Ehret[20], *et al*. used such biosensor to monitor cell density, cell growth and its long-term behaviour; Pless[21], *et al*. utilised impedance biosensor for detection of Salmonella species in food samples. The commercial device Malthus 2000 (Malthus Instruments, Crawley, West Sussex, UK) is constructed to estimate microbial populations including coliform and lactic acid bacteria or fungi causing a change in conductance of selective cultivation medium.

2.2 Optical Biosensors

Optical biosensors based on nonlinear optics systems such as surface plasmon resonance (SPR) and resonant mirror (RM) are convenient for direct detection of bacteria without any label as well as for serotypization. Classical photometric methods like fluorescence relay on labeled antibodies. In some rare cases, organic structures from cells or viruses can provide fluorescence[22] directly but this option is not typically used for their detection due to the limited sensitivity.

Surface plasmon resonance (SPR) devices [Fig. 2 shows the integrated surface plasmon resonance sensor Spreeta 2000 (3-channel ver. produced by Texas Instruments, www.ti.com) suitable for design of portable SPR biosensors.] can be used as label-free direct detector with immobilised antibody (antigen is chosen alternatively for serotypization) on the

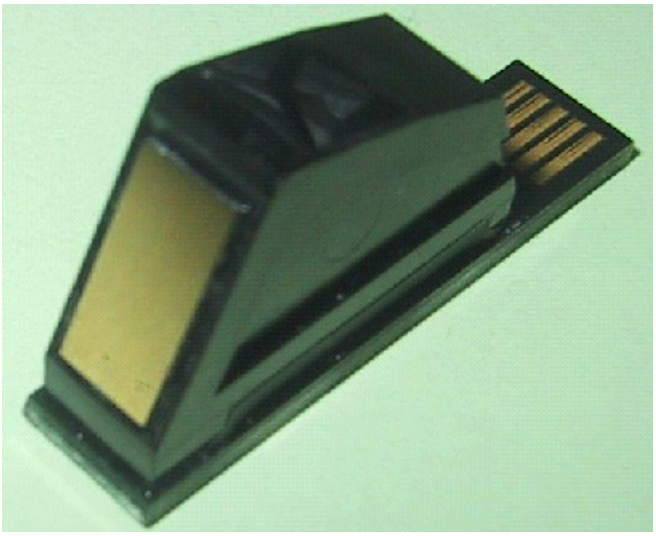


Figure 2. Integrated surface plasmon resonance sensor Spreeta 2000 suitable for design of portable SPR biosensors.

metal film for capturing and detecting either bacteria or viruses. The light beam incident under a defined angle through prism can interact with the delocalised electrons in the gold film providing signal observable as decrease of the light beam intensity (SPR).

On the other hand, the resonant mirror (RM) arrangement uses light beam which can interact with sensing coupling (TiO_2) and resonance layers and a sharp maximum of light profile is obtained with position depending on the bound mass. These devices can monitor interactions in real time. The inherent disadvantage of nonlinear optics arrangements is its higher price when compared with electrochemical and piezoelectric devices, however, highly integrated compact and miniaturised devices as Spreeta 2000 from Texas Instruments have become available. The SPR biosensor was used for detection of *Bacillus anthracis* with LOD $3.2 \cdot 10^2$ spores/ml in less than one hour[23], HIV[24] and *Clostridium perfringens* beta-toxin[25] as well as other pathogenic bacteria were detected: *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Legionella pneumophila* and *Yersinia enterocolitica*[26]. Watts[27], *et al*. applied RM for *Staphylococcus aureus* assay. They reached LOD 8×10^6 cells/ml in a detection time of 5 min. The evanescent wave interferometer was used by Schneider[28] to detect $5 \cdot 10^8$ cells/ml of *Salmonella typhimurium*. Some commercials devices working on the nonlinear

optics principle include: Bioward 1 (NBC-Sys, Saint Chamond, France) capable of detecting 10 ng/ml staphylococal enterotoxine B (SEB) in a 3 min assay, BIAcore (Pharmacia, Uppsala, Sweden) with LOD approximately 10^5 cells/ml.

Fluorochrome-labeled antibodies used for construction of fluorescent immunoassays (FIA) were consequently adapted for immunosensors. The sensitivity of fluorescence biosensors expressed as limit of detection for whole cells is similar to electrochemical biosensing systems. Cao,[29] *et al.* were able to detect 5 ng/ml of surface antigen from *Yersinia pestis* in 30 min; Wijesuriya[30], *et al.* detected *Bacillus anthracis* in the amount of $3 \cdot 10^3$ cells/ml. Aflatoxin the carcinogenic product of fungi– was detected in concentrations from 0.1 µg/l (ppb) using the fluorometric biosensor[31]. Other applications of fluorescence biosensing are based on the recognition of target nucleic acid sequence. Dengue fever virus serotype 3 DNA probes[32] were detected in concentration of 10 pM. Photometric biosensors can also be based on enzyme-labeled antibodies. This technology originally used in enzyme immunoassays (EIA), enzyme linked immunosrobent assay (ELISA), and electrochemical biosensors can be applied to the photometric biosensors too. The photometric detector including sandwich immunoformat with monoclonal antibodies labeled by peroxidase (HRP) was used by Koch[33] *et al.* for the detection of SEB toxin, M 13 virus and *Escherichia coli* as a bacterial agent. The detection limits were 10 ng/ml for SEB, 10^6 PFU/ml for M 13, and 10^7 CFU/ml for *Escherichia coli* and one analysis was completed in 15 min.

2.3 Piezoelectric Biosensors

Piezoelectric biosensors are convenient for label-free and real-time monitoring of interactions between antibody and antigen. Predominantly quartz crystal microbalance (QCM) is used. [Fig. 3 shows quartz crystal microbalance biosensor with two gold electrodes and basic frequency 10 MHz. This sensor is produced by International Crystal Manufacturing (www.icmfg.com)]. The change of resonance frequency recorded is proportional to the mass bound on the crystal surface. Commercials crystals have typically two gold electrodes on the opposite sides. Direct monitoring of cell concentration using monoclonal antibody immobilised through protein A to the gold electrode allows to detect 10^5 cells/ml of *Salmonella typhimurium*[34] and 10^6 cells/ml of *Escherichia coli* in drinking water[35]. This type of biosensor[36] used by Carter for V. cholerae was able to detect 10^5 cells/ml. Wong[37] developed the QCM sensor for Salmonella species with immobilised monoclonal antibody, it was possible to detect 10^4 cells/ml. The horse polyclonal antibody was used for the detection of SARS-associated corona virus[38] in the range of 0.6-4 g/ml of SARS antigen with a short analysis time reaching 2 min. QCM with immobilised antigen was used for *Francisella tularensis* assay as immunoprecipitate with monoclonal IgM antibody providing LOD of 5×10^5 cells/ml within 30 min[39].

3. CLASSICAL METHODS OF DETECTION

Numerous detection devices for bacteria, viruses or toxins have been developed. The conventional detection methods are based on selective cultivation,

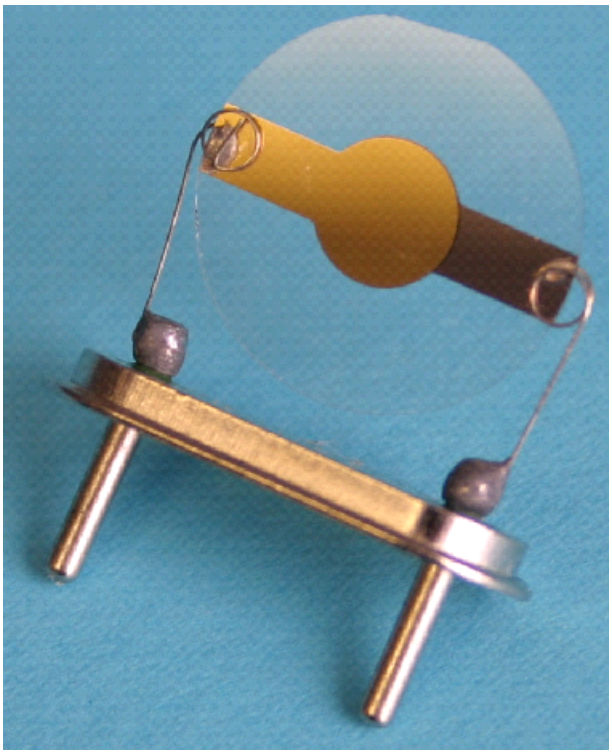


Figure 3. Quartz crystal microbalance biosensor with two gold electrodes and basic frequency 10 MHz. Quartz monocrystal dia. is 14 mm.

morphological evaluation, and microscopic techniques. For fast nonspecific aerosol detection, devices such as biological agent warning system (BAWS), fluorescence aerodynamic particle sizer (FLAP) or laser identification and ranging (LIDAR) are produced and employed. Specific detection can be accomplished by polymerase chain reaction (PCR) for a variety of organisms[40,41]. Grunow[42], *et al*. used PCR for *tul 4 gene* from *Francisella tularensis* and reached LOD 10^2 cells/ml for rabbit tissue samples within 4 h assay. Mass spectroscopy was used for the detection of *Bacillus cereus*[43] or *Bacillus anthracis*, *Yersinia pestis*, *V. cholerae*, *Bacillus melitensis* and *Francisella tularensis* fatty acids as markers[44]. Immunological routine methods for pathogen detection such as ELISA[45,46], dot immunobindig assay (DIA)[47], electrochemiluminiscence (ECL)[48], and time-resolved fluorescence (TRF)[49] were employed.

Considering portability of detection systems, hand-held assays are quite important. These immunochromatographic methods are convenient for fast, easy, and cost effective detection of different pathogens such as *Francisella tularensis*[50]. Commercials devices like Bio Threat Alert (BTA) from Alexeter Technologies (Wheeling, IL, USA) are able to detect *Francisella tularensis*, *Bacillus anthracis*, *Yersinia pestis*, *Bacillus melitensis*, SEB, ricin and botulotoxine. The expected LOD for bacteria is above 10^5 cells/ml in a 25 min assay.

4. CONCLUSION

Biosensor-based devices have become an important part of instrumental equipment of laboratories detecting biological agents. Biosensors provided similar sensitivity as provided by other conventional detection instruments and techniques. Some biosensors reached even detection limits similar to the PCR techniques. Typical time of immunodetection is 15 min, but some devices like resonant mirror, quartz crystal microbalance are able to provide signal within 5 min. Biosensors can be used for detection of bacteria and viruses as well as toxins. Due to their small size and low cost, biosensors are convenient not only for laboratory routine but also for mobile laboratories and portable systems in the field. In the future, one can expect development of new biosensors giving reliable detection results with amount of individual agents under their infectious doses, with multi-channel arrangement for simultaneous or consequent analysis of several agents, and with further miniaturised designs.

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