

*Mini Review*

## **Electrochemical Hand-Held Biosensors for Biological Warfare Agents Assay**

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Biological warfare agents are infectious microorganisms and toxins that have lethal potential for military use or terrorist activities. Early and reliable detection of the specific biological warfare agent at the sites of their use is one of the most important countermeasures; Ideal analytical devices should be hand-held size or pocket size if possible and should allow covering all scenarios and situations where a biological incident can occur. The current handheld analyzers have many limitations such as being too large, being overweight, or having low sensitivity to particular viruses, microorganisms, and toxins. Biosensors, analytical devices combining a simple physical sensor with a recognition part, would represent a platform for the assay of biological warfare agents allowing detection with high sensitivity typical for advanced laboratory instruments but also a simple design for building the biosensor into a pocket-sized analyzer. This review focuses on summarizing facts about the detection of biological warfare agents with hand-held devices based on electrochemical biosensors and the progress of electrochemical biosensors to assay agents. The actual literature is surveyed for this purpose, and actual trends in the assays of biological warfare agents are described.

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**Keywords:** Bacillus anthracis; Bioassay; Biohazard; Biological Weapon; Botulinum Toxin; Detection; Francisella tularensis; Identification; Variola

### **1. INTRODUCTION**

Biosensors are analytical devices that combine two basic parts: a biological structure that gives the final selectivity called a biorecognition element, and a sensor called a physicochemical transducer. Various enzymes, antibodies, antigens, receptors, lectins, DNA, cells, cells' compartments, tissues, and chromosomes can be mentioned as possible biorecognition elements for biosensor manufacturing [1-6]. Conventional biorecognition elements are frequently replaced by new types of artificial materials such as aptamers, nanoparticles, nanomaterials with catalytical (pseudo-enzymatic) properties, nanomaterials

with optical properties, and molecularly imprinted polymers [7-13]. The physicochemical transducers used for biosensors construction belong to a wide group of analytical devices and sensors. Electrochemical (eg, potentiometry, conductometry, voltammetry), optical (e.g. spectral assay, colorimetry, fluorimetry), piezoelectric, and thermometry can be examples as common examples of physicochemical transducers [14-17].

Biosensors have gained popularity due to a simple concept that allows for the construction of the final analytical devices with low costs, and they also exert reliability and sensitivity comparable to those of much more expensive and larger apparatuses. The first biosensors for blood glucose levels in the late 1950s and early 1960s. Since their discovery, these biosensors have gained a great deal of popularity, and have been researched and improved since their discovery [18-20]. Currently, biosensors for detecting several analytes such as toxic substances [21,22], biochemical and immunochemical markers [23,24], drugs [25,26], pesticides [27,28], and food components [29,30] can be examples of areas where biosensors are applicable. Biosensors are also suitable for the detection of pathogenic microorganisms and toxic substances of biological origin [31,32]. The simplicity of the biosensor concept, in combination with portability and low cost of manufacturing processes, makes it attractive for use in conditions outside laboratories. This review focuses on portable electrochemical biosensors that can serve for the detection of biological warfare agents and represent an alternative to standard microbiological, genetic, and immunochemical approaches and handheld devices. The emerging concepts seem to provide pocket-size devices with good sensitivity to the biological warfare agents. Recent progress in this field is covered and discussed to summarize the main trends. The actual literature was used for this purpose.

## **2. BIOLOGICAL WEAPONS AND BIOLOGICAL WARFARE AGENTS**

Biological weapons belong to mass destruction weapons that are devices that cause a large scale of destruction and have an impact on a broader area compared to conventional weapons. They are a relevant part of the area known under the acronym CBRN, which comes from the first letters of the words Chemical, Biological, Radiological, and Nuclear representing the main groups of mass destruction weapons. In the current military and security environment, the importance of a biological weapon as a tool for a regular army is limited due to international agreements, however, biological weapons are a threat that can get into the hands of an unstable or metastable state or an organization that promotes their political request by violence based on bioterrorism or biocrime and does not obey international agreements or conventions [33-38].

In technical terms, the biological weapon is a device based on a biological agent: infectious organisms, viruses, or toxic substances coming from the biological agents and also containing other parts allowing application (spraying, running out, etc.), protection during storage and manipulation, and other parts making the biological weapon compatible with the other weapons systems. Biological weapons are internationally banned by Biological Weapons Convention since 1972 and the ban is targeted in multiple areas including their manufacturing, storage, use, etc. The Biological Weapons Convention has been entered into force under the proper name *The Convention on the Prohibition of the Development,*

*Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction*, and it is effective for most the countries over the world up today.

Under specific circumstances, any infectious agents or toxins can be used for military or terrorist activities. However, only a small number of the infectious agents and toxins are of strategic relevance or use in military tactics. They should be stable enough, applicable, lethal, and have other premises to be considered biological warfare agents [39]. The significance of biological warfare agents can be learned from categories from the Centers for Disease Control and Prevention (CDC, Atlanta, USA) which receive a letter A indication for the biological warfare agents representing the most serious threat and letter B for the moderate threat [40,41]. Letter C enables the less probable biological warfare agents or biological warfare agents with the lowest likelihood of being used for a biological attack. A bacterial microorganisms *Bacillus anthracis* (a causative agent of anthrax disease), a bacterial microorganism *Francisella tularensis* (a causative agent of tularemia disease), bacterium *Clostridium botulinum* producing botulinum toxins, viruses Variola major (a causative agent of smallpox), virus Marburg (a causative agent of Marburg hemorrhagic fever), virus Lassa (a causative agent of Lassa hemorrhagic fever), and virus Machupo (a causative agent of Bolivian hemorrhagic fever) represents the category A of biological warfare agents. The biological warfare agents in category A can be easily disseminated and cause diseases with serious progression and high mortality, and the diseases caused by them are not easily curable. Compared to category A, category B also causes serious diseases, but the expected mortality and burden for the healthcare system are lower and their overall impact can be scaled as moderate. Toxins such as ricin (a toxin of the plant *Ricinus communis*), bacterial microorganisms *Brucella melitensis* (a causative agent of brucellosis), *Burkholderia mallei* and *pseudomallei* (a causative agent of glanders and melioidosis), *Chlamydomphila psittaci* (a causative agent of chlamydiosis), *Escherichia coli* O157: H7 and its toxin Shiga (a causative agent of foodborne diseases and intoxications), *Rickettsia prowazekii* (a causative agent of typhus), *Vibrio cholerae* (a causative agent of cholera), *Staphylococcus aureus* and staphylococcal enterotoxins (a causative agent of staphylococcal infections and poisonings by staphylococcal enterotoxin), and viruses causing encephalitis can be examples as biological warfare agents of category Category C is the last. It covers emerging pathogens with future potential. Zoonotic viral diseases of the family *Paramyxoviridae* like Nipah and Hendra virus causing infections with a high mortality rate belong to the C category. A survey distinguishing biological warfare agents into individual categories according to the CDC system made by CDC is given in Table 1.

**Table 1.** Categories of biological warfare agents according to the CDC

Category	Description	Examples of biological warfare agents
A	Pathogens and toxins are easily disseminated, cause diseases with serious progression and high mortality, and problematic therapy	<i>Bacillus anthracis</i> , <i>Francisella tularensis</i> , <i>Clostridium botulinum</i> , botulinum toxins, Variola major, Marburg, Lassa, Machupo
B	Pathogens that cause serious diseases, but the expected mortality, burden on healthcare care and over impact are moderate ones	ricin, <i>Brucella melitensis</i> , <i>Burkholderia mallei</i> and <i>pseudomallei</i> , <i>Chlamydomphila</i>

		<i>psitaci</i> , shiga toxin, <i>Rickettsia prowazekii</i> , <i>Vibrio cholerae</i>
C	Emerging pathogens with future potential	Nipah virus, Hendra virus

### 3. HAND-HELD ANALYTICAL METHODS FOR BIOLOGICAL WARFARE AGENTS

Under laboratory condition, biological warfare agents are analyzed in the same way as any microorganism, virus, or toxin. Several standard protocols and analytical devices exist, such as microorganism cultivation tests, mass spectrometry, chromatography, and molecular biology methods for the recognition of specific sequences in genetic information using polymerase chain reaction (PCR). The standard methods have significant limitations when intended to use for the detection of biological warfare agents. Most of the equipment is too bulky to be used on sites where biological warfare agents are used for a biological attack. Some effort was taken to miniaturize these devices with promising results. However, such devices are still quite expensive and trained staff should operate them.

Portable ion mobility spectrometers are widely used for the detection of chemicals, drugs, explosives, and pollutants [42,43]. Some ion mobility spectrometers are suitable for the detection of toxins and microorganisms by assay of their marker [44,45]. These devices can easily detect analytes and markers with lower molecular weight; however, biological samples with high molecular weight analytes are not optimal for ion mobility spectrometers. However, these devices are a routine part of the equipment of CBRN teams as universal analyzers. RAID devices sold in various configurations by Bruker (Billerica, MA, United States) can be written as an example. A hand-held battery-powered variant RAID-M 100 weight 2.9 kg. AeroTracer by Airsense (Schwerin, Germany) is another example of an ion mobility analyzer.

Standard PCR is commonly used for the detection and identification of various pathogens under laboratory conditions [46-51]. Considering field use, PCR analyzers were also downsized and some devices weights under 5 kg in field modifications, are battery charged and have humidity protection to be used in harsh field conditions. A battery-powered PCR analyzer RAZOR by BioFire Diagnostics, Inc. (Salt Lake City, Utah, United States) can be written as an example. PCR analyzers were optimized for most biological warfare agents with quite high sensitivity and accuracy of an assay [52-54]. The development of new PCR methods was accelerated by the reaction to anthrax letters in 2001 [55,56]. At present, portable analyzers are accepted by CBRN teams as standard devices. They can be used for fingerprinting biological warfare agents and can be also easily adapted for analysis of new microorganisms when new PCR primers are developed. However, portable PCR analyzers are still quite expensive instruments. The PCR device-based assay can recognize viruses and microorganisms, but it is not suitable for the assay of toxins. Both ion mobility spectrometers and portable PCR analyzers should be carried by a designated person in a CBRN team; though they are portable, these devices are not pocket-size and the costs should be also considered as these devices are quite expensive compared to the further mentioned simple analytical devices.

Lateral flow tests are among the simplest devices that can be used for the detection of biological warfare agents. The lateral-flow tests represent a maximal simplicity of an assay that can be operated by

people without any training or education needed, which is uncommon to the more elaborative methods. These tests are typically pocket-sized with minimal weight around a few grams and a size of a few centimeters in the length and thickness of some few millimeters. The lateral flow tests were originally developed as pregnancy tests for the detection of human chorionic gonadotropin in the urine [57]. Currently, lateral flow tests are commercially available for several analytes and markers. Biological warfare agents, including the most relevant ones, can also be detected by lateral-flow tests and even multichannel strips analyzing contemporary more than one analyte can be achieved [58-60]. The advantage of lateral-flow tests represented by their simplicity is balanced by some disadvantages. The lateral flow tests are suitable for the detection of an analyte but quantification of the analyte is either impossible by the standard tests or the assay is semiquantitative only. Research on improved lateral-flow test strips is ongoing, and it can be expected that some quantification will be possible by the tests in the future, it is questionable how accurate such an assay will be. Problems with false positivity or false negativity and lack of sensitivity can occur as well. Price is another specification that should be taken into consideration. The lateral flow tests are quite cheap because no instrumentation is necessary and the formed color lines can be scaled with the naked eye. On the other hand, the price of one kit can be higher than the cost of reagents for one assay cycle by instrumental analysis. When a large number of analytes are processed, lateral flow tests may be less economically effective compared to the other methods. The same devices based on the lateral flow test can determine up to five biological warfare agents in a single step as the individual tests are combined. Lateral flow tests are often considered as a spared method or a method for emergency use in improvised sites of biological protection, point of care, or field conditions where no other analytical methods are available. Devices such as BioDetect by Alexeter Technologies (Wheeling, IL, United States) and Biowarfare Agent Detection Devices BADD by Advnt Biotechnologies (Phoenix, AZ, United States) can be examples.

Biosensors and biosensor-like devices are also available in the praxis of biological warfare agents assay, though they are only a minor part of the current market on protective means against biological warfare and biological terrorism. Three devices working on the fluorescence principle: Biosensor 2200R Biological Agent Detector by MSA (Pittsburgh, PA, United States), Raptor, and BioHawk by Research International, Inc. (Monroe, WA, United States) are biosensors that succeeded in the development processes and were introduced into the market. All the mentioned devices are portable, battery powered and can be operated by a single person, but they are not wearable electronics that can be placed into a pocket or a small pouch. Biosensor 2200R is the lightest with 2.7 kg, the second two instruments are significantly heavier: Biohawk has 12 kg and Raptor 6.5 kg. All biosensor devices are suitable for the assay of multiple biological warfare agents with quite low limits of detection. All of these biosensors use antibodies labeled with fluorescence dye. The test of the specific biological warfare agent depends on the used antibody. Biosensor 2200R detects one analyte; the detection of another biological warfare agent is possible after the exchange of used reagents. The Raptor device is suitable for the contemporary assay of four and Biohawk for the contemporary assay of eight analytes. A survey of the aforementioned devices is depicted in Table 2.

**Table 2.** Survey of handheld analytical devices for biological warfare agent assay

<i>Type of devices</i>	<i>Examples of specific instruments</i>	<i>Principle and use</i>	<i>Size and weight</i>
Ion mobility spectrometers	RAZOR by BioFire Diagnostics, Inc. (Salt Lake City, Utah, United States)	Standard ion mobility spectrometry, suitable for toxins and markers, not designed for whole microorganisms and virions	hand-held assay with a weight around 3 kg for field instruments
Polymerase chain reaction analyzer	RAZOR by BioFire Diagnostics, Inc. (Salt Lake City, Utah, United States)	standard polymerase chain reaction for specific areas of genetic information in microorganisms and viruses, not suitable for toxins	hand-held assay with a weight around 5 kg for field instruments
Lateral flow tests	BioDetect by Alexeter Technologies (Wheeling, IL, United States), Biowarfare Agent Detection Devices BADD by Advnt Biotechnologies (Phoenix, AZ, United States)	standard lateral flow immunochromatographic assay for semiquantitative detection of biological warfare agents by antigen-antibody interactions	pocket size, weight few grams
Optical biosensors	Biosensor 2200R Biological Agent Detector by MSA (Pittsburgh, PA, USA), Raptor, and BioHawk by Research International, Inc. (Monroe, WA, USA)	flow-through fluorometric devices for assay of various biological warfare agents by antigen-antibody interactions	hand-held assay with a weight between 2.7 and 12 kg

#### 4. ELECTROCHEMICAL BIOSENSORS

The current research of biosensor devices in the field of biological warfare agent detection is focused on the construction of simple and cheap devices that will exert high sensitivity combined with low limits of detection on the one hand and minimized the weight of resources for construction, low size and weight on the other. These newly constructed biosensors should be designed as handheld devices suitable for performance in harsh field conditions and accepting a wide number of samples without an elaborate pretreatment. The design of the aforementioned biosensors as wearable electronics is another challenge. Electrochemical biosensors would become a practical alternative to standard hand-held analytical devices used for the detection of biological warfare agents. Electrochemical biosensors have gained high popularity since the 1950s, when pioneering works on glucose biosensors were done [61,62]. Electrochemical biosensors on biological warfare agents arise from the opportunities arising from

progression in related technologies and experiences of constructing biosensors for the other analytes. Currently, many researchers recognize the electrochemical sensor platform for biosensor construction is recognized by many researchers as optimal for many purposes including control of food contaminants [63,64], pollutants in the environment [65,66], drugs [67], recognition of single nucleotide polymorphism [68], point of care diagnosis of various pathologies and diseases [69-72], detection and identification of toxins [73,74], microorganisms [75] and viral particles [76]. Biological warfare agents have also recognized as targets for electrochemical biosensors [77-79].

While prototypes of new electrochemical biosensors on biological warfare agents represent only a minor part of the total electrochemical biosensors, some relevant discoveries have been made and the area of research is gradually developing. Karadeniz et al. [80] prepared an electrochemical biosensor for of anthrax about the lethal factor genetic information as a marker of *B. anthracis* [80]. The researchers combined graphite electrodes with single- and multi-walled carbon nanotubes and the recognition capacity of DNA by its hybridization. Electrochemical impedance measurement resulted in a detection limit of 1 pmol/l in an assay lasting less than 20 minutes. An electrochemical biosensor for *B. subtilis* using a filter paper as a platform [81]. The authors chose *B. subtilis* as a safe simulant of *B. anthracis* with nearly the same physical specifications, but low virulence. The filter papers were cut and treated by wax printing, resulting in the formation of carbon paste working electrodes and Ag/AgCl reference electrodes. The working electrode was further modified with peptide specific to *B. subtilis*. The biosensor exerted good properties by differential pulse voltammetry and was suitable for the determination of *B. subtilis* with a detection limit of 690 CFU/ml in 30 minutes lasting assay. Mazzaracchio et al. developed an impedimetric aptasensor for another simulant of *B. anthracis*, spores of *B. cereus*, [82]. They prepared an aptasensor by binding aptamers specific to *Bacillus cereus* on the surface of gold screen-printed electrodes and performed an impedimetric assay of the spores of *B. cereus* in tested samples. The authors reported a limit of detection of  $3 \times 10^3$  CFU/ml, a linear range of the biosensor between  $10^4$  CFU/ml and  $5 \times 10^6$  CFU/ml for an assay lasting 3 hours.

An applicable biosensor for the *F. tularensis* was constructed by Dulay and coworkers [83]. The authors prepared an amperometric device composed of a fragmented antibody specific to *F. tularensis* and sensors containing gold working, Ag/AgCl reference, and Pt counter electrodes prepared by lithography. A secondary antibody labeled with peroxidase was responsible for forming an immunosandwich and catalyzing the conversion of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine, which is recorded by amperometry. The assay exerted a detection limit of 4.5 ng/ml for bacterial lipopolysaccharide and 31 cells/ml, the time for analysis was approximately 18 minutes. Electrochemical biosensors for toxins were also developed. Cartelli and co-workers made an electrochemical biosensor for botulotoxin serotypes A and C using a paper platform [84]. They chose a natural substrate for botulotoxins A and B: the protein SNAP-25 and labeled it with an electroactive molecule methylene blue. The labeled protein was bound to gold nanoparticles and placed on a paper-based electrode. The assay was performed as standard voltammetry using a portable potentiostat and the analytes were recognized in a detection label-free assay with a limit of 10 pmol/l, linearity of the assay was proven to be up to the botulotoxin level of 1 nmol/l. In another article, botulotoxin A was detected using an impedimetric immunosensor [85]. A glassy carbon electrode was modified with gold nanoparticles, chitosan, and anti-botulotoxin A antibody. Interaction of botulotoxin A with the surface of the biosensor

restricted the electron transfer from potassium ferrocyanide/ferricyanide. The assay had a detection limit of 0.11 pg/ml and a calibration range of 0.27 – 268 pg/ml.

As seen from the examples of electrochemical biosensors mentioned above, the newly constructed biosensors exert sensitivities, limits of detection, and other specifications approximately equal to those of the standard laboratory tools. However, they are miniaturized devices that can be developed into the shape and size of a pocket instrument. The authors did not report the exact size and weight of biosensors developed by them; the cited devices can be marked as pocket size. A survey of the electrochemical biosensors for biological warfare agents assay is written in table 3.

**Table 3.** Survey of the aforementioned electrochemical biosensors for biological warfare agents assay

Recognition part of the biosensor	Sensor part	Analyte	Principle of assay	Specifications	References
DNA with carbon nanotubes	graphite electrode	genetic information of the anthrax lethal factor genetic information	electrochemical impedance assay	limit of detection of 1 pmol/l, assay time under 20 minutes	[80]
specific peptide	graphite working electrodes and Ag/AgCl reference electrodes on filter paper	<i>Bacillus subtilis</i>	differential pulse voltammetry	limit of detection 690 CFU/ml in 30 minutes lasting assay	[81]
aptamer	screen printed gold electrodes	spores of <i>Bacillus cereus</i>	impedimetry	limit of detection $3 \times 10^3$ CFU/ml, linear range $10^4$ - $5 \times 10^6$ CFU/ml, time of an assay 3 hours	[82]
fragmented antibodies	sensors containing gold working, Ag / AgCl reference electrodes, and Pt counter electrodes	<i>Francisella tularensis</i>	amperometry	limit of detection 4.5 ng/ml for bacterial lipopolysaccharide, and 31 cells/ml, time of analysis 18 minutes	[83]



	prepared by lithography				
protein SNAP-25 labeled with methylene blue	paper electrodes, gold nanoparticles	botulotoxin A and C	voltammetry	limit of detection 10 pmol/l, linearity up to 1 nmol/l	[84]
anti-botulotoxin A antibody	glassy carbon electrode	botulotoxin A	impedimetry	limit of detection 0.11 pg/ml, calibration range 0.27 – 268 pg/ml	[85]

## 5. CONCLUSION

Recent discoveries on electrochemical biosensors for the detection of biological warfare agents make these devices highly competitive with standard analytical equipment. The biosensors can be developed into a shape and size that allows them to be used as a pocket device. The analytical specifications are nevertheless comparable with those of standard laboratory instruments. Further research and development on these biosensors would change current approaches in the assay of biological warfare agents.

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