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*REVIEW PAPER*

# Biological Warfare Agents and their Detection and Monitoring Techniques

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**ABSTRACT**

Recently, threat from biological warfare agents (BWAs) has emerged as the foremost national and global security challenge because of their simple and cheap production, easy dispersal, complicated detection, expensive protection and psychological, economical and social impact. Early detection and identification of BWAs during intentional biological event is essential to initiate corrective emergency responses for management of such incidents. Efforts are being made across the globe for development of state of the art technologies and systems for detection and identification of BWAs. However, till date there is no single system which can detect all the bio-threat agents. In the present review, we describe the currently available techniques and systems for detection and identification of these agents. The basic identification techniques including biological culture, immunological methods, nucleic acid based detection, MALDI-TOF MS, cellular fatty acid profiling and flow cytometry based detection are presented. Detection of BWAs with bio-sensors, surface plasmon resonance, biological detectors, and stand-off detection systems is also summarized. However, despite of availability of several techniques and tools, no full proof system is available for detection/identification of all the BWAs.

**Keywords:** Biological warfare agents, bio-threat, bio-sensor, detection, PCR

## 1. INTRODUCTION

Though, microorganisms are essential for human and environment, yet disease outbreaks due to pathogenic microorganisms have killed far more people than the war itself in history. Scientifically, the existence of microorganisms was established during the period 1665-83 by two Fellows of the Royal Society, Robert Hooke and Antoni van Leeuwenhoek. However, there has been a long history of use of microorganisms as biological warfare agents (BWAs). Around 300 B.C., Greeks, Romans and Persians used animal cadavers to contaminate water wells of their enemies. In 1155, Emperor Barbarossa's troops during the battle of Tortona contaminated the water wells with dead bodies of soldiers and animals. An epidemic of plague was spread in 14th century during the siege of Kaffa[1]. In 1763, smallpox infected blankets were distributed to the Indian tribes hostile to the British during French-Indian war[2]. Several incidents of use of BWAs were reported during World War I, World War II and post World War era. The fears and predictions of biological attack were converted into reality just one week after September 11, 2001 terrorist attacks on the World Trade Center when letters harbouring anthrax spores were received by news media houses and two senators. Further, it proved how devastating these agents can be if come in the hands of terrorists or non-state actors.

Biological warfare is a planned and deliberate use of pathogenic strains of microorganisms such as bacteria, viruses, or their toxins to spread life-threatening diseases on a mass scale in order to devastate the population of an area. The term 'bioterrorism' is used when it is primarily directed against civilians on a small scale, whereas biological warfare is primarily directed against military on a large scale. Biological warfare agents are defined as living organisms, whatever their nature, or infected material derived from them, which are used for hostile purposes and intended to cause disease or death in man, animals and plants, and which depend for their efforts on the ability to multiply in the person, animal or plant attacked[3]. Biological weapon convention (BWC), 1972 has defined bio-weapons as 'microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes'. North Atlantic Treaty Organisation (NATO) has defined biological warfare as 'the employment of microorganism (or a toxin derived from it) to produce casualties in man or animals and damage to plants or material, or defence against such employment'.

As per BWC, 1972, there is no specific list of pathogens to be used as biological warfare agents. However, NATO has listed 31 pathogens and 8 toxins as potential BWAs (Table 1). Likewise, Center for Disease Control and Prevention (CDC), Atlanta has classified various microorganisms in 3 different categories based upon the mode of dispersal, mortality and morbidity, public health perception and public health preparedness. Toxins in the list are included on the basis of their toxicities, ability to damage human tissues, ability to disrupt normal human homeostatic mechanisms, capacity for delivery to humans and potential availability. Category A agents can

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**Table 1. List of biological warfare agents defined by NATO**

| Agent | Disease |
|---|---|
| **Bacteria** | |
| *Bacillus anthracis* | Anthrax |
| *Brucella melitensis* | Brucellosis |
| *Vibrio cholerae* | Cholera |
| *Burkholderia pseudomallei* | Melioidosis |
| *Yersinia pestis* | Plague (pneumonic) |
| *Shigella dysenteriae* | Shigella |
| *Francisella tularensis* | Tularemia |
| *Salmonella typhi* | Typhoid fever |
| **Rickettsia** | |
| *Rickettsia prowazekii* | Epidemic typhus |
| *Coxiella burnetii* | Q fever |
| *Rickettsia rickettsii* | Rocky Mountain spotted fever |
| *Orientia tsutsugamushi* | Scrub typhus |
| **Chlamydia** | |
| *Chlamydophila psittaci* | Psittacosis |
| **Fungi** | |
| *Coccidioides immitis* | Coccidioidomycosis |
| *Histoplasma capsulatum* | Histoplasmosis |
| **Viruses** | |
| Junin virus | Argentina hemorrhagic fever |
| Machupo virus | Bolivian hemorrhagic fever (Black typhus) |
| Chikungunya virus | Chikungunya fever |
| CCHF virus | Crimean-Congo hemorrhagic fever |
| **Viruses** | |
| Dengue virus | Dengue fever |
| Ebolaviruses | Ebola |
| Eastern equine encephalitis virus (EEEV) | Eastern equine encephalitis |
| Influenza virus | Influenza |
| Hantavirus | Korean hemorrhagic fever |
| Lassa virus | Lassa |
| Omsk hemorrhagic fever virus | Omsk hemorrhagic fever |
| Rift Valley Fever virus | Rift valley fever |
| Tick-borne encephalitis virus | Russian spring-summer encephalitis |
| *Variola major* | Smallpox |
| Venezuelan equine encephalitis virus | Venezuelan equine encephalitis |
| Yellow fever virus | Yellow fever |
| **Toxins** | |
| *Clostridium botulinum* | Botulinum toxins |
| *Clostridium perfringens* | Perfringens toxins |
| Members of family Trichothecenes | Mycotoxins of trichothecene group |
| Seaweed | Palytoxin |
| *Ricinus communis* | Ricin |
| Marine algae | Saxitoxin |
| *Staphylococcus aureus* | Staphylococcal enterotoxins |
| Marine fishes | Tetrodotoxin |

be easily spread or transmitted from person to person, have high mortality rate, cause public panic and social disruption and require special attention for public health preparedness. Category B agents are moderately easy to spread, cause moderate illness rates and low death rates, and require specific enhancements of laboratory capacity and enhanced disease monitoring. Category C agents include emerging pathogens that could be modified in laboratory for mass spread in the future because these are easily available, can be easily produced and spread, and have potential for high morbidity and mortality rates and major health impact.

Biological warfare agents are the appealing weapons in wars and for terrorists because of their ease of availability, low production costs, easy transportation and non-detection by routine security systems[4]. Further, BWAs (except toxins) can multiply in the host organism and get transmitted to others individuals causing unpredictable consequences in terms of victims and geographical spread. In several countries, diseases caused by BWAs are not very common and therefore, population is quite susceptible for the rapid infection. Use of BWAs causes fear and anxiety among the people and disrupts the functioning of the administration as demonstrated by the 2001 anthrax attack in United States. Therefore, effective protection against BWAs is quite difficult because of complicated detection and expensive protection measures.

Now, almost all countries have their biodefence programmes to strengthen the capability of detection, protection and decontamination of relevant bio-threat agents. Biological defence comprises the methods, plans and procedures involved in establishing and executing defensive measures (detection, protection, decontamination and medical management) against biological attack[5]. Early detection of BWAs is most important for timely management of any biological attack, whether intentional or natural. Therefore, rapid detection systems for BWAs are essentially required to countermeasure the effects of a biological attack. However, prompt detection and identification of a biological attack is hampered by the easy dissemination and high lethality of some of the BWAs[6]. Symptoms of biological attack in the population may appear in hours, days or even weeks. Further, it is very difficult to distinguish these symptoms from infections from more benign and common biological agents prevailing in the society in absence of definitive diagnosis. Therefore, medical management of the victims of BWAs may be ineffective. Traditional methods for identification of BWAs consume a lot of time and can't provide the results in real-time[7]. Despite of continuous efforts at global level, till date, the detection

systems for BWAs are not at par with those for chemical and radiological incidents. Basically two types of detection systems are required for a biological event, detect to treat and detect to protect. In case of a biological event, BWAs should be detected and/or identified by a monitoring system rapidly or in real-time. Detection systems for BWAs should be highly sensitive because as low as 100 particles/L of *B. anthracis* and 10 particles/L of *F. tularensis* are good enough to cause infection in humans[8,9]. Moreover, the system should be specific enough to discriminate BWA from other biological and non-biological components in order to get low/least false-positive rate. Though detection of a biological agent is complicated process, yet several efforts have been made globally to develop the tools for detection of BWAs. In this paper some of the techniques for detection and identification for BWAs have been presented.

## 2. BIOLOGICAL CULTURE

Isolation and identification of bio-threat agents by conventional culture techniques is considered as the gold standard method. Bacterial agents generally propagate in a variety of culture media while viral agents essentially require cell culture for their propagation. Selective culture media don't allow the non target bacteria to grow and hence differentiate the target agent from others. Culturing provides an additional advantage of enrichment of the agent for its further characterisation and also ascertains the viability of the organism. The bio-threat agent can be identified by a battery of tests including staining, colony morphology, motility, biochemical and metabolic characterisation etc.

Biological culture is a reliable and well understood method which generates ample information for identification of any agent. However, this method is laborious and time consuming. It often takes days to arrive at conclusive results. Automation of culture technique, however, has addressed the shortcomings of conventional method to some extent.

## 3. IMMUNOLOGICAL METHODS

Antigen-antibody interactions have been exploited in development of immunoassays for identification of bio-threat agents. Antibodies are the most critical reagents in the immunoassays, which bind to specific regions of the antigens and form a detectable complex. Production and purification of antibodies is a well established process. Polyclonal and monoclonal antibodies are most commonly employed in the traditional immunoassays. Immunoassays in different formats have been developed for detection of BWAs and a few are being described here.

### 3.1 Enzyme Linked Immunosorbant Assay

Enzyme linked immunosorbant assay (ELISA), a sensitive and solid-phase enzyme coupled assay for quantitative detection of antigen and antibody was developed in early 1970s[10]. In ELISA, an enzyme conjugated to either antibody or antigen is used to detect the formation of antigen-antibody complex on a solid phase. The unbound reagents are removed by washing and the enzyme is allowed to interact with its substrate to yield a coloured product that is quantitatively measured by optical density or can be visualised directly. ELISA is a simple, economical, robust and reliable technique widely being used for screening of large number of samples in low cost laboratory settings for diagnosis of various diseases. Many formats of ELISAs have been developed to detect antigens of biological warfare agents, *viz. B. anthracis*[11], *Y. pestis*[12], *B. pseudomallei*[13], and to detect host antibodies produced in response to the infection of *B. anthracis*[14-17], *F. tularensis*[18], *B. pseudomallei*[19], *B. mallei*[20,21], *Brucella abortus*[22], Ebola and Marburg viruses[23] or to detect bio-threat toxins[24]. Besides, some other variants of immunological assays like fluorescent microscopy[25,26] are also used for detection of BWAs.

### 3.2 Hand-held Immuno-Chromatographic Assay

Hand-held immuno-chromatographic assays (HHIAs) are inexpensive, rapid, simple to use and require minimal training. These assays are based on lateral flow immunoassay technique and are designed on nitrocellulose or nylon membranes packed in a plastic case. The device looks similar to common home pregnancy test kit. In such assays a colloidal gold (or others such as carbon/paramagnetic/coloured latex beads) labeled antibody is placed onto a sample application pad affixed to a nitrocellulose strip and capture antibody is immobilised in a line on the nitrocellulose strip. The liquid sample containing the analyte is applied onto the sample port and the analyte binds with the labeled antibody forming an immune complex. This analyte-labeled antibody complex migrates down the nitrocellulose strip by capillary action and gets captured by the immobilised antibody leading to formation of a visible coloured line, indicative of a positive result. These assays are designed to be qualitative, however, intensity of colour of the line can be correlated for semi-quantitative measurement. Modified HHIAs with fluorescent microspheres permit the quantitative assessment of the results with the help of a compatible reader[27]. HHIAs do have their own limitations. HHIAs generally provide a presumptive identification of unknown sample. Sensitivity and specificity of these assays is comparably lesser than the other immunological methods[28]. Another disadvantage of the HHIAs is that in presence of large excess of the analyte, these assays may exhibit false negative results due to 'hook effect'. HHIAs are useful for initial screening of suspected samples and any positive result must be confirmed with other tests such as PCR. HHIAs have been developed for detection of biological warfare agents and toxins viz. *B. anthracis, Y. pestis, F. tularensis, B. abortus, B. pseudomallei,* Small pox, Variola virus, botulinum and SEB, ricin and abrin toxins. Commercially available HHIA devices include ENVI assay system gold and FL (Environics), SMART® (new horizons diagnostics corporation), bio-threat alert and redline alert (Tetracore), RAMP (response biomedical corp.), Prime alert™ biodetection (GenPrime, Inc.), NIDS® Handheld bio-threat assay (ANP technologies), BADD™ bio-warfare agent detection devices (ADVNT Biotechnologie), UPTtest® bio-defence kits.

## 4. NUCLEIC ACID BASED DETECTION

Molecular biology techniques can be employed for more rapid identification of biological warfare agent than conventional microbiological methods. These techniques are highly sensitive and specific, and can yield results with 10-100

copies of the target nucleic acid in the sample. Disadvantage with nucleic acid based detection assays is that they are unable to detect proteins hence toxins can't be detected by these assays.

Polymerase chain reaction (PCR) based assays identify an organism on the basis of presence of specific DNA sequence(s) in the organism. The organism specific DNA sequence is exponentially amplified by PCR and millions of copies of specific DNA sequence are generated. PCR is based on thermal cycling, consisting of cycles of repeated heating and cooling. Typical PCR cycle consist of three steps *viz.* denaturation of nucleic acid, primers annealling and extension/ elongation which are performed at 2-3 temperatures, commonly three. Basic PCR is a qualitative assay. Amplification with simultaneous detection of amplicons can be achieved with quantitative real-time PCR (Q-PCR). Q-PCR is based on specific and non-specific detection. In non-specific detection, DNA-intercalating dyes (e.g. SYBR Green) emit fluorescence when bound to DNA and amplification in real-time is monitored by measuring the fluorescence. Melting curves analysis of the amplicons provides specificity. Specific-detection is based on use of fluorogenic labeled probes containing both fluorescent dyes and sometimes a quencher. Probes specifically bind to the target and real-time amplification can be detected by measurement of the fluorescence. PCR assays may demonstrate false negative results due to presence of PCR inhibitors (e.g. humic acids, chelating agents) in the test sample. An internal positive control is thus included in the PCR assays to detect the PCR failures. PCR-based assays have been reported for identification of various biological warfare agents such as *B. anthracis*[29], *F. tularensis*[29], *Y. pestis*[29], *C. burnetii*[30], Filo viruses, Arena viruses, and New World Hanta viruses[31]. Commercially available PCR and real-time PCR based systems/assays for detection of biological warfare agents include FilmArray®, RAPID®, RAZOR® EX, POCKIT™, Bio-Seeq™ PLUS and T-COR 4™.

There are other variants of DNA amplification techniques which allow the DNA amplification isothermally in absence of thermal cycling. Loop mediated isothermal amplification (LAMP) is a method of isothermal DNA amplification using an enzyme with strand displacement and polymerase activity. DNA polymerase and a set of four specially designed primers recognise a total of six distinct sequences on the target DNA. LAMP recognises the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity[32]. LAMP is a good molecular technique for detection of various pathogens because the assay is simple, rapid, sensitive and specific. LAMP assays can be performed in heating block or water bath and does not require thermal cycler. Primer designing is a crucial step in development of LAMP assays. The major problem of LAMP assays is carryover contamination leading to false positive results; however, this can be sorted out using dUTP-UNG approach[33,34]. LAMP assays have been used to detect various biological warfare agents including *B. anthracis*[35], *Y. pestis*[36], *Brucella* spp.[37], *B. pseudomallei*[38], *C. burnetii*[39], Ebola virus[40], Marburg virus[41], and Lassa virus[42].

Recombinase polymerase amplification (RPA) is another variant of DNA amplification techniques[43]. RPA does not employ thermal denaturation of template and works at a low and constant temperature (37 °C - 40 °C). Here, a phage recombinase forms nucleoprotein complexes with the primers and scans the double stranded template for homologous sequences. Once homologous sequences are found, the recombinase-primer complexes invade the double stranded DNA to let the primers hybridise with the homologous sequences. At this stage, single-stranded DNA binding proteins interact with the displaced template strand to stabilise the same and prevent ejection of primers by branch migration. Recombinase disassembly leaves the 3′ends of the primers accessible to strand displacing DNA polymerase for primer extension. The newly synthesised duplexes then act as secondary templates for the next cycle. Cyclic repetition of this process leads to exponential amplification of the target. Amplification products of RPA can be detected by agarose gel electrophoresis or by 'sandwich assay' employing lateral-flow strips making it an instrument free assay[43]. Instrument free RPA can be very useful in laboratories with low resource settings. RPA finds a place among the fastest amplification methods as it can be accomplished in ~ 20 min. RPA is a very sensitive assay and can detect as low as a single copy of the target in the sample. RPA assays and reverse transcriptase RPA (RT-RPA) assays have been developed for detection of BWAs such as *B. anthracis*, *Y. pestis*, *F. tularensis*, Variola virus, Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus and *Brucella* sps.

## 5. NEXT GENERATION SEQUENCING

Biological warfare agents can be unambiguously identified using the DNA sequencing techniques. Recently, next-generation sequencing (NGS) technologies have revolutionised the way DNA is sequenced and opened new perspectives for detection of bacterial and viral pathogens from clinical and environmental samples. NGS technologies have displaced traditional Sanger sequencing as these are capable of providing massive throughput at a modest cost employing minimum time. NGS has now become a routine step in characterisation of a microbe of interest. The first NGS system was made available commercially by 454 Life Sciences, a subsidiary of Roche Applied Sciences. However, now-a-days several systems based on NGS technologies are available for sequencing. NGS uses parallel sequencing of multiple small fragments of DNA to determine the sequence and is usually performed in three steps comprising of (i) library preparation, (ii) DNA capture and enrichment, and (iii) sequencing/detection. Technical features and principles of various NGS platforms have been reviewed recently[44,45].

NGS platforms have become important components of an effective biodefense strategy because of their capability to detect and identify BWAs. NGS analysis has been utilised for detection of *B. anthracis* from soil and air samples and it could detect as few as 10 genomic equivalents of *B. anthracis* DNA per nanogram of background nucleic acid[46]. NGS has also been used to detect strain-specific polymorphism in *B. anthracis* and *Y. pestis*[47]. Next-generation direct DNA sequencing could detect *F. tularensis* in a human abscess sample of unknown

etiology[48]. Recently, targeted amplification approach has been described for detection of bio-threat agents by NGS[49]. Use of NGS technologies is enormously increasing in diagnosis and monitoring of infectious diseases[50]. Further, NGS can be an important tool for identification of new or emerging infectious agent for which diagnostics are not currently available.

## 6. CELLULAR FATTY BASED PROFILING

Bacterial identification based on cellular fatty acid profiling was first reported in 1963 by Abel[51], *et al.* and Kaneda[52]. Fatty acids are primarily the components of phospholipids, Lipid A (Gram negative bacteria), lipoteichoic acid (Gram positive bacteria) and mycolic acid of aerobic actinomycetes. Variability of fatty acids structures in different bacterial species has been exploited for their identification and characterisation. In this method, the cellular fatty acids are first converted to fatty acid methyl esters and analysed by gas liquid chromatography. Methodology of CFAP generally involves harvesting of the bacterial cells, saponification of the fatty acids, methylation of free fatty acids, extraction into organic phase, washing of extracts and final analysis by gas liquid chromatography. Chromatographic readings are analysed with the help of pattern recognition software for identification of the bacterial sample.

Sherlock Microbial ID System, a product of MIDI Inc., Newark, DE is a commercially available system for identification of BWAs. The system identifies the BWAs by cellular fatty acid profiling employing fatty acid methyl ester analysis by gas chromatography (GC-FAME). CFAP has been used for identification and differentiation of *Y. pestis*, *B. anthracis*, *F. tularensis*, *B. pseudomallei*, *B. mallei*, and *Brucella*.

## 7. MATRIX-ASSISTED LASER DESORPTION/ IONISATION-TIME OF FLIGHT MASS SPECTROMETRY

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry **(**MALDI-TOF MS) has become a widely accepted technique for rapid, accurate and cost-effective identification of cultured microbes. Mass spectrometry was first time used for identification of bacteria in 1975 and MALDI-TOF MS reported in late 1980s. The technique is being routinely used in clinical microbiology laboratories for disease diagnosis. Sample preparation for MALDI-TOF MS involves mixing or coating of the analyte (e.g. proteins) with an energy absorbent organic compound called matrix. MALDI-TOF mass spectrometer comprises of three component *viz.* ion source, mass analyser and detector. The ion source ionises and transfers sample molecule ions into a gas phase. The mass analyser separates molecules according to their mass-to-charge (m/z) ratio and the detector monitors the separated ions. Finally, a characteristic spectrum known as peptide mass fingerprint (PMF) is generated based on the time of flight (TOF) information. MALDI-TOF MS utilise the library of reference spectra for identification hence it allows direct identification of multiple pathogens simultaneously. However, simultaneous detection of multiple agents by other identification techniques such as PCR and immunoassays requires lots of optimisation. MALDI-TOF MS based protocols have been successfully optimised for identification of BWAs and toxins such as *B. anthracis* , *Y. pestis*, *Brucella* , *F. tularensis*, *Vibrio* spp., *B. pseudomallei*, *B. mallei*, *C. burnetii*, botulinum and ricin.

## 8. FLOW CYTOMETRY

Flow cytometry makes use of laser light scattering and fluorescence excitation of fluorescent dyes associated with bacterial cells. Here a continuous single cell stream passes through a laser beam and each cell scatters laser light and emits fluorescence due to excitation by laser light. It measures cell size and counts the cell in liquid suspension by laser light scattering. Fluorescently labeled monoclonal antibodies can be used for immunological detection and identification of pathogens. Flow cytometry has been used as an effective platform for detection of BWAs. Multi-parameter flow cytometry has been demonstrated for immunological detection of *B. anthracis*[53,54]. Immunoassays employing bead-based, suspension arrays and the Luminex system or other flow cytometer platforms have been established for the detection of *Y. pestis*, *B. anthracis* and botulinum toxin. A Luminex flow cytometer operates as the detector in the autonomous pathogen detection system (APDS), developed at Lawrence Livermore National Laboratory for detection of BWAs[55-57]. APDS, Luminex system performs immunoassay for initial detection of multiple BWAs and a positive detection event is confirmed by a multiplexed PCR-based microsphere microarray assay. This approach has been demonstrated for simultaneous detection of *B. anthracis*, *Y. pestis*, *F. tularensis*, and *B. melitensis*.

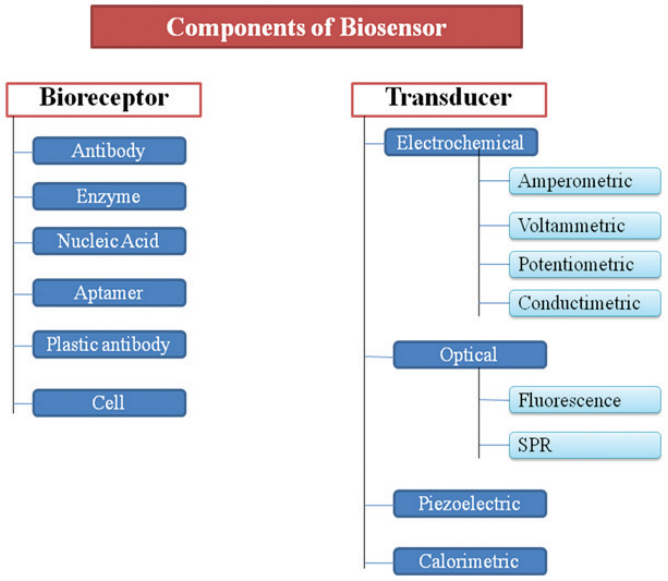
## 9. BIO-SENSORS

Bio-sensors are analytical devices that convert a biological recognition event into an electrical signal. IUPAC defines bio-sensor as 'a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is in direct spatial contact with a transducer'. Bio-sensor combines a biological component with physico-chemical detection of signal (luminescence, electrochemical or optical signal). It comprises of a biological recognition element that is coupled to a transducer (Scheme 1). The most common type of recognition elements for bio-sensors are antibodies and nucleic acid probes. Other types of recognition elements include aptamers, whole cells, and bacteriophages etc.. Identification of an agent is based on the interaction of an analyte (component of biological warfare agent, toxin) with biological recognition element and this biological response is converted to a detectable readout by the transducer. Bio-sensors can be categorised according to the transducer (acoustic, potentiometric and piezoelectric) or according to type of bioreceptor i.e. catalytic (enzyme based) or affinity based (antibody, aptamer, lectin, bacteriophage, etc.). Having considerable advantages over conventional detection approaches in terms of sensitive and selective identification of organisms, bacteria, toxins or viruses the bio-sensors may play a critical role in case of any biological emergencies.

### 9.1 Electrochemical Bio-sensors

The electrochemical bio-sensors can be divided into amperometric, potentiometric, impedometric and

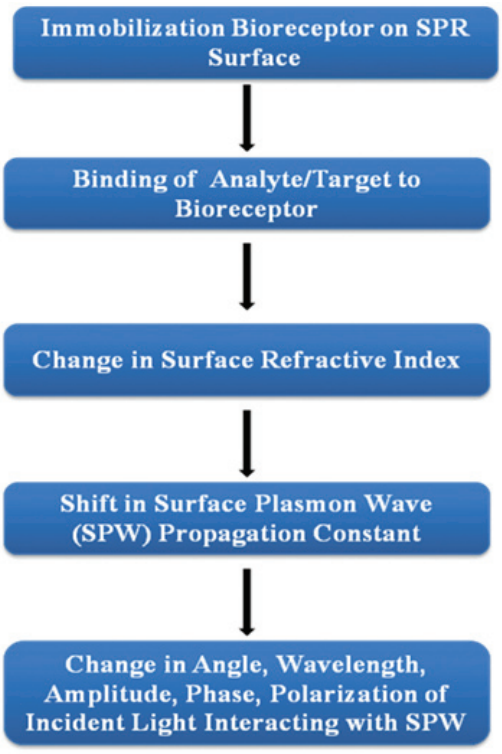
conductometric based on output electrochemical signal. Typically, an amperometric bio-sensor requires a three electrode system (i.e. sensing/working electrode, reference electrode and counter electrode) immersed in a suitable electrolyte solution. The resulting amperometric current which corresponds to analyte concentration is recorded as output signal. Other, electrochemical transduction techniques are cyclic voltammetry, differential pulse voltammetry and square wave voltammetry. In recent years, nanomaterials possessing high surface area, excellent biocompatibility, unique mechanical, electrochemical, electronic and chemical properties are widely used for the development of highly sensitive and specific electrochemical bio-sensors. Nanomaterials can either be explored as electrode matrix for signal amplification or non-enzymatic labels for highly sensitive and simple detection of BW agents. Nanomaterials ranging from carbon-based materials (graphite, graphene, and carbon nanotubes), quantum dots, metal nanoparticles (gold, platinum, copper, silver, etc.) are being widely utilised for electrochemical bio-sensing. A highly sensitive and specific electrochemical immunosensor consisting of bismuth nanoparticles (BiNPs) nanocomposites modified glassy carbon electrode and cadmium ion loaded titanium phosphate signal tag has been developed for detection of anthrax PA toxin[58]. Gold and palladium bimetallic nanoparticles loaded on boron-nitride nanosheets were explored as catalytic label for the development of electrochemical immunosensor for detection of *B. anthracis* with the minimum detection limit of 1 pg/mL[59]. An electrochemical genosensor was fabricated using gold nanoparticle deposited glassy carbon electrode for the detection of PCR amplicons of different base pairs of *B. anthracis*. The genosensor exhibited the linear range from $1x10^{-11}$ to $1.0 \times 10^{-9}$ M with the detection limit of 1.0 pM[60]. An electrochemical immunosensor for botulinum neurotoxin type-E (BoNTs/E) using diazotised graphene transducer and gold nanoparticles bio-conjugate particle signal amplifier was reported[61]. Wu[62], *et al*. reported an impedometric immunosensor for the rapid detection of *B. melitensis* based on gold nanoparticle-modified screen-printed carbon electrodes. The gold nano-ribbon nanostructures were utilised as transducer for the electrochemical genosensing of *Brucella* genome with a detection limit of 1.71 zmol $dm^{-3}$. Array bio-sensor, Bio-sensor™ 2200R and RAPTOR are among a few commercially available bio-sensors for detection of bio-threat agents.




**Scheme 1. Bioreceptors and transducer for biosensor**

### 9.2 Surface Plasmon Resonance

Several methods for label-free assay based detection of pathogens/microorganisms have been reported. The label-free detection approach offers the simple and quick response without need of secondary labeled biomolecules/bioreagents. The most popular are surface plasmon resonance (SPR), and quartz crystal microbalance (QCM) sensors. SPR, a physical phenomenon and label-free detection method has provided a reliable platform in analysis of biomolecular interactions. The interactions are measured in real-time with high sensitivity and without use of labels. SPR was first introduced in 1990s and has emerged as a powerful technique to determine specificity, affinity and kinetic parameters during macromolecular interactions. It determines the refractive index changes near the thin metal layers (gold, silver or aluminum films) in response to biomolecular interactions (scheme 2). The change in the SPR angle is determined by changing the incidence angle and recording the intensity of reflected light during biological interactions between various biomolecules. Typical SPR instrument consists of a monochromatic polarised light source, a glass prism, a thin metal film in contact with the prism base, and a photodetector. Recognised as prism-coupled SPR, this design is the most commonly used platform for SPR instrumentation. Other systems designs typically use either waveguide or grating coupled SPR. SPR has become a widely accepted method for




**Scheme 2. Principle of SPR biosensor.**

disease diagnosis, drug discovery, pathogen detection and has expanded to environmental and industrial area too. One of the potential drawbacks of SPR technology is that the ligand upon immobilisation on the sensor chip surface may lose its native configuration. Also, the orientation of the ligand may sterically hinder the binding of the analyte. SPR technique has been used for detection of *Brucella*[63], *B. anthracis*[64,65] and *Y. pestis*[66]. Researchers have exploited SPR technology to detect botulinum neurotoxin[67], Staphylococcal enterotoxin B[68] (SEB) and Staphylococcal enterotoxin A (SEA)[69].

### 9.3 Piezoelectric Bio-sensors

The piezoelectric bio-sensors based on the use of quartz crystal microbalances (QCM) are considered as cheaper alternative to SPR. The QCM has advantage of widespread use for field detection. QCM is a simple and sensitive device formed by thin quartz plate with gold electrode pads on the opposite sides. The antibodies/DNA/proteins/aptamers immobilised on gold pad surface make QCM highly sensitive to the target antigen/pathogen. The antigens interact with immobilised biomolecule on the surface and increase the mass loaded on the surface which helps to decrease the frequency of the quartz crystal or resonator. Piezoelectric Immunosensor has been reported for *F. tularensis* with detection sensitivity of 5 x $10^6$ cells[70]. An immunosensor with QCM detection operating in flow-through mode was developed for detection of staphylococcal enterotoxin A (SEA) in milk[71].

## 10. BIOLOGICAL DETECTORS

Biological detectors generally reveal the presence of biological agents in a particular environment without identifying the agents. However, sometimes these detectors can be integrated with identifier also for identification of a specific agent. Sample collection and detection/identification may be independent units or integrated in a single system.

### 10.1 Samplers/Collectors

Sampling is a very critical and sometime very difficult step in the process of detection of BWAs and therefore, sample collector is an important component of biological detectors. These collectors are available separately also which help in collection of the air sample and subsequently the sample can be processed by various methods for identification. Sample collectors enable the capturing of BWA into solid or liquid medium for their identification. Air is considered to be the most feasible route for releasing the BWAs to expose large number of people. A sample collector is essentially required to concentrate the aerosols/particles as extremely low concentrations of BWAs in the air stream may not be detected but may still cause severe effects. Today, several types of sample collection methods have been evaluated for collection of airborne and surface settled BWAs. Some examples of commercially available sample collectors include viable particle size samplers (impactors), virtual impactors, cyclones, and bubblers/impingers. Selection of samplers for BWA detection is very important as inappropriate selection could result in failure to collect particles of interest[72]. Sampling methods like wipe, wet/dry swab vacuum shock and composite-based sampling have been validated for collection of BWA samples from porous and non-porous surfaces

#### *10.1.1 Viable Particle Size Samplers (Impactors)*

In impactors an air stream of particles/aerosols is accelerated through a nozzle and is diverted against an impaction plate maintained at a fixed distance from the nozzle. The large particles get separated from the smaller one because of their large inertia and smaller particles move along fluid streamline to exit the sampler. Smaller particles pass through multiple stages of impactor and each stage contains a number of constant size orifices. Particles get directed towards the collection surfaces by the jet orifices and the particles not collected at specific stages move with the air stream to the next stage. The collection plate contains selective agar for growth of BWAs. Growth of the BWAs on the collection plate is monitored after 24 h - 48 h of incubation.

#### *10.1.2 Virtual Impactors*

Virtual impactors are similar to viable particle size samplers with exception of impaction surface. In virtual impactors, collection plate is replaced by a collection probe. Air flow in the impactor is controlled to collect particles of specific size. Additionally, in the final stage the particles flow into a liquid to yield a highly concentrated liquid sample. The Liquid Sampler (PEM-0020) uses virtual impaction for collection and concentration of particles into a liquid. Aerosol collector BioVIC™ is a front-end air sampler for biological detection systems. It pre-concentrates the air stream by confining large number of particles either into a small volume of liquid, into a small air stream or onto a solid surface for delivery into sensor. BioCapture BT 500 air sampler is a modified version of BioVIC™ wherein airborne biological material is captured and concentrated into a liquid for subsequent analysis[73].

#### *10.1.3 Cyclone Samplers*

In cyclone samplers, the airborne particles enter the cyclone body to form an outer spiral moving towards the bottom. Centrifugal force make the large particles settle on the outer wall whereas the smaller particles move with airstream in the inner spiral to the exit tube. Particles settled on the outer walls of the cyclone are collected by spraying water. The Interim Biological Agent Detector System (IBADS), personal cyclone sampler, the Smart Air Sampler System (SASS 2000) and SASS 2300 are a few examples of cyclone samplers[73,74].

### 10.2 Detectors

#### *10.2.1 Particle Size-based Detectors*

Particle sizers count the relative number of in predefined size ranges (typically from 0.5 µm to 30 µm). Different techniques are being used for monitoring and/or counting the particles. In aerodynamic particle sizing (APS) particulate air is drawn into the APS device through its nozzle thereby generating a controlled high-speed aerosol jet. The air velocity remains constant and the particles accelerate at rates proportional to their sizes. The particles pass through a laser beam which measures the time of flight of the particles. High volume aerodynamic particle sizer (HVAPS) uses accelerated

and concentrated air stream to obtain particle size distribution and concentration, through a laser-based particle counter[75]. Any change in the background aerosol concentration indicates the possibility of intentional release of a biological warfare agent. These devices can not differentiate biological aerosols from non-biological aerosols.

### *10.2.2 Fluorescence-based Detectors*

The fundamental mechanism behind fluorescence based method is the use of fluorescently labeled bacterial/viral pathogens to get an excitation by the laser wave of approximately 630 nm. The fluorescent signal is generated and detected by fluorescent detector. Commonly used fluorescent markers are fluorescein isothiocyanate (FITC) and lanthanide. Typically, fluorescence based methods utilise light beam (usually in UV spertrum) to excite the molecular components of the biological material (such as tryptophan) and the excited component reverts to normal state emitting light at different wavelengths. The emission spectra being specific to the molecular component and excitation wavelength, can be exploited for detection of biological warfare agents (biofluorescence)[76]. Fluorescence measurement approach can be of two types, primary and secondary. In primary approach, natural fluorescent component of the biological material (such as the aromatic amino acid tryptophan) is measured. The secondary approach involves introduction of a special fluorophore to the biological material before excitation with UV light.

Fluorescent Aerodynamic Particle Sizer (FLAPS) is the most prominent device among the fluorescence based detection devices. It is a modified version of an Aerodynamic Particle Sizer (APS) with an additional laser (blue or UV wavelength) and along with standard particle size information it generates specific fluorescence for detection of biological material[77]. Ultra Violet Aerodynamic Particle Sizer (UVAPS) a variant of FLAPS also detects biological agents nonspecifically by making use of time-of-flight particle sizing, light scattering, and UV fluorescence intensity[78,79]. The biological aerosol warning system (BAWS) is a micro-laser based device to detect an unusual biological event by analysing two biological fluorescence wavelengths. It can detect in real time, discriminate biological material from others but does not provide particle counts[79].

## 11. STANDOFF DETECTION TECHNOLOGIES

Standoff detection technologies are able to detect a biological warfare event remotely without sampling. These systems employ a light source such as a laser for BWA detection. Light detection and ranging (LIDAR) system is suitable for this purpose. LIDAR transmits short laser pulse through atmospheres and a distant target (such as aerosol, dust, pollen, cloud) scatters a part of this radiation back to laser receiver. LIDARs can detect the biological particles as far as tens of kilometers depending on the wavelength of light, laser power, optical configuration and ambient conditions. The light wavelength for the LIDAR is selected in accordance with the particle size of the target particles to be detected. LIDAR systems employ light signals at short wavelengths for detection of small aerosol particles (predominantly less than 20 μm in diameter) of BWAs. Infrared LIDARS (IR LIDARs) use wavelengths in IR spectrum and can detect the target particles distant at several kilometers. IR LIDARs are unable to discriminate biological particles from non-biological ones and therefore, exhibit an undesirably high false alarm rate in presence of interfering aerosols of pollens, industrial pollution, diesel exhaust, road dust and burning vegetation[80]. Differentiation of biological particles from non-biological particles can be achieved by Laser induced fluorescence-LIDAR (LIF-LIDAR). Biological particles (having endogenous fluorophores such as tryptophan, tyrosine, NAD-NADH, riboflavins, dipicolinic acid, etc) are illuminated by UV laser radiation (laser-induced fluorescence) and the fluorescence signals are detected by LIDAR receiving channel(s) in standard range-resolved regimes[81]. UV wavelength is an important factor in determining the range and efficiency of UV LIF LIDAR. Presently, UV lights of 266 nm, 294 nm, and 355 nm are used in most LIF LIDARs. UV light of 266 nm excites fluorescence primarily from tryptophan and tyrosine (also NADH and flavins to a lesser extent) and 355 nm excites fluorescence primarily from NADH and flavins but not tryptophan. The 266 nm wavelength is most appropriate for tryptophan excitation and 355 nm wavelength for NADH excitation related to spore viability. However LIDARs employing 355 nm light have a longer detection range as the attenuation of 266 nm UV by atmospheric ozone is 10 fold higher than that of 355 nm UV light. A set of different excitation wavelengths in UV spectral range (i.e. 266 nm, 273 nm, 280 nm, 294 nm, 300 nm, 340 nm, 355 nm) have also been tried to exploit the differences in the fluorescence signatures of biological agents[82]. Continuous efforts are being made by several government and private organisations across the globe for development of a reliable system for standoff detection of BWAs. Fluorescence measurement based remote detection of BWAs is usually complicated by interference of non-biological particles (harbouring aromatic hydrocarbons) and environmental background of biological particles such as pollen, fungi and other bacterial species. Therefore, a reliable and satisfactory stand-off detection system is yet to be developed.

Compact LIDAR, Hybrid LIDAR, MIRELA, MPL 1000, MPL 2000, JBSDS and SIMBAHD are some of the systems developed/under development for standoff BWAs detection. MPL 1000 (micro pulse LIDAR), a commercially available IR LIDAR is closest to become a reliable tool for remote detection of BWAs. Hybrid LIDAR, with both IR and UV component, has been developed for its deployment on an unmanned aerial vehicle (UAV), to autonomously detect and analyse suspicious aerosol cloud.

## 12. CONCLUSION

Biological warfare agents are the most suitable weapons for poor countries and terrorist groups. In view of emerging threats of bioterrorist attacks, there is urgent demand of technologically advanced systems for rapid and specific detection of BWAs. Early detection and identification of BWAs in the event of intentional release is essential to take necessary countermeasures. Innovative and sophisticated tools have either been developed or under development for detection of these

agents. Presently, several laboratory based methods are available for identification of BWAs but their remote detection is still a challenging task. PCR-based assays for rapid identification of these agents have been developed and the methods based on isothermal amplification of nucleic acids can be of great help in identification in laboratories with limited resource settings. Advanced genome sequencing techniques are going to be an important platform for detection and identification of BWAs in the upcoming years. Nanomaterials due to their high surface to volume ratio greatly enhance the biomolecular interactions and have enormous potential for use in optical, electrical and electrochemical bio-sensors for enhancing sensitivity, specificity and miniaturisation of detection systems. New nanomaterials and structures such as nanowires, nanotubes and graphenes are being developed and nanodiagnostic assays are expected to arrive for rapid detection and identification of BWAs. Several research organisations around the world are working on development of UV-LIF based systems for standoff detection of BWAs and these systems are in the preliminary phase of development. Analytical sensitivity, specificity and response time are the three major challenges in development of a successful detection system. Infectious dose of some of the BWAs is as low as < 10 viable organisms therefore detection systems with high sensitivity are desirable for detection of these agents. Presence of biological background in the environment affects the performance of standoff detection systems. Hence, more innovative standoff technologies are desired to detect the BWAs in presence of other biological and non-biological aerosol contaminations in the environment. Research should be focused on development of automated detection systems with integrated sample preparation and identification technologies with the ability to discriminate the potential agent in multi-analyte environment. Automated system will also be helpful in reducing the human error thereby increasing the accuracy of results.

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