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Minireview

Biosensors: Frontier Techniques and their Recent Applications

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Abstract

Instant analysis of the desired analyte by biosensors is a burgeoning field in the world of chemical analysis. The present review provides an overview of the fundamental of biosensors including immobilization of sensing materials for recognition and transduction of the analyte of interest. Biosensors recently developed for detection of harmful micro-organisms, such as *E.coli, Salmonella typhii, dengue virus,* human immunodeficiency virus (HIV), biological warfare (BW) agents (*Bacillus anthracis, Burkholderia pseudomallei,Burkholderia mallei, Brucella spp., Fransicella tularensis,* and *Yersinia pestis),* explosives (TNT, RDX, HMX, etc.), environmental organic pollutants, organomercurials (MeHg), harmful ingredients in food and beverage industries, such as aspartame, saccharin, glycilic acid etc. have been discussed. Present requirements and future challenges with regard to biosensors are reviewed.

Abbreviations Used: AIDS, Acquired immunodeficiency syndrome; AMV, avian myeloblastosis virus; BHC, benzene hexa chloride; BW, biological warfare; DDT, dichloro diphenyl trichloro ethane; DHF, dengue hemorrhagic fever; DSS, denque shock syndrome; ELISA, enzyme linked immunosorbent assay; GC-MS, gas chromatography-mass spectrometer; HPCL, high performance liquid chromatography; NADPH, nicotinamide adenin dinucleotide phosphate; MMLV, maloney murine leukemia; PCR, polymerase chain reaction; PVA, polyvinylacrylate.

Introduction

Sensitive and selective determination of a large number of compounds is a great relevance for scientific research and industries (i.e. for process development in chemical, pharmaceutical and food industries). Analytical techniques, such as modern gas chromatography, high-performance liquid chromatography (HPLC), mass spectrometry (MS), hyphenated technique such as gas chromatograph-mass spectrometry (GC-MS), and atomic absorption spectroscopy (AAS) etc., though have high selectivity but, these powerful instrumentation techniques are costly and are used in specific laboratories with high skilled operators. Also they are not suitable for on-line operation. Therefore, sensors are becoming popular to analyze components than the conventional techniques. Sensors are devices, which convert physical and chemical quantities into measurable electrical signals. According to the International Electrotechnical Committee[1], "The sensor is the primary part of a measuring chain which converts the input variables into a signal suitable for measurement". Therefore, function of sensor is more or less similar to our sense organs. Chemical sensors involve a chemical process between the recognition element and analyte (quantity being measured), whereas the biosensor involves biological entity either as the recognition element or the analyte.

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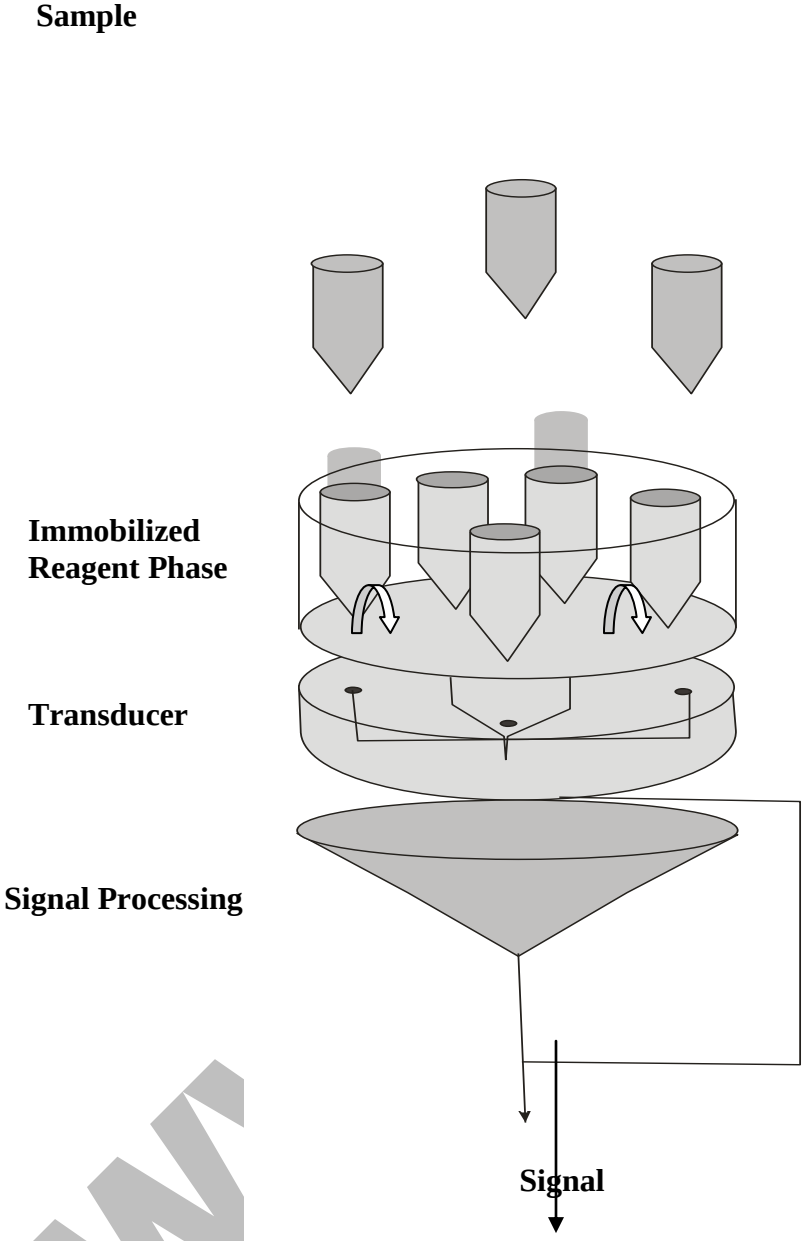

Fig. 1. Schematic diagram of a biosensor (Ref.2)

Biosensor utilizes high sensitivity and selectivity of biological sensing for analytical purposes in various fields of research and technology. This apart, biosensors possess high response rate high accuracy, broad range of measurement, reproducibility, simple calibration, high reliability, durability, portability (small weight, small dimension), low

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cost and safety for being a superior technique. They consist of an analyte selective interface in close proximity to, or integrated with a transducer, which relays the interaction between analyte and surface directly or through a chemical mediator [2, 3]. Schematic diagram of biosensor is shown in Fig. 1. It is applied in the field of medicine, environmental monitoring, pollution control, pesticide monitoring, on-line and off- line monitoring in food and drink industries, pharmaceutical and chemical processes, mines and explosives detection, biological warfare agent detections etc. In a biosensor, the immobilized biological sensing materials having specificity for the analyte of interest may be an enzyme, cell, organelles, tissue, cell membrane, antibody, nucleic acid, receptor, organic acid and molecules etc. The species recognition reagent in a biosensor is a macromolecule, immobilized into a membrane or chemically bound to a surface in contact with the analyte solution. The recognition reagent selectively reacts with analyte and produces a signal, such as color change, emission of fluorescent light, and change in oscillation frequency of a crystal. The transducer responds the signal and translates the magnitude of signal corresponding to the concentration of analytes. The transducer (i.e. the microelectronic part of the biosensor) depending on its type as shown in Fig.2, converts biochemical signals into a measurable response such as current, potential, thermal change, absorption of light and mass increase through optical and piezoelectric means thus may be called amperometric, potentiometric, calorimetric, fiber-optic and piezoelectric respectively.

Types of Biosensors

Biosensors may be differentiated[4] into bioactivity and bioaffinity sensor according to the biorecognition components present. Bioactivity sensor acts as a biocatalyst. Commonly used biocatalalysts are enzymes, whole cells (bacteria, fungi, yeast or eukaryotic cells) and tissues (plant or animal tissue slice). The products or adducts (O_2, H_2O_2, protons, heat, photons and nicotinamide adenine dinucleotide phosphonate (NAD (P)H) of the substrate-biocatalyst reaction is detected.

In enzymatic alcohol measurement, alcohol dehydrogenase (ADH, EC 1.1.3.13) and alcohol oxidase (AOD, EC 1.11.1.6) are used from various sources:

$$R\ CH_2OH + NAD^+ \longrightarrow RCHO + NADH + H^+$$

$$R\ CH_2OH + O_2 \longrightarrow RCHO + H_2O_2$$

Glucose can be converted to produce an easily detectable compound, H_2O_2 by utilizing the enzyme glucose oxidase (GOx):

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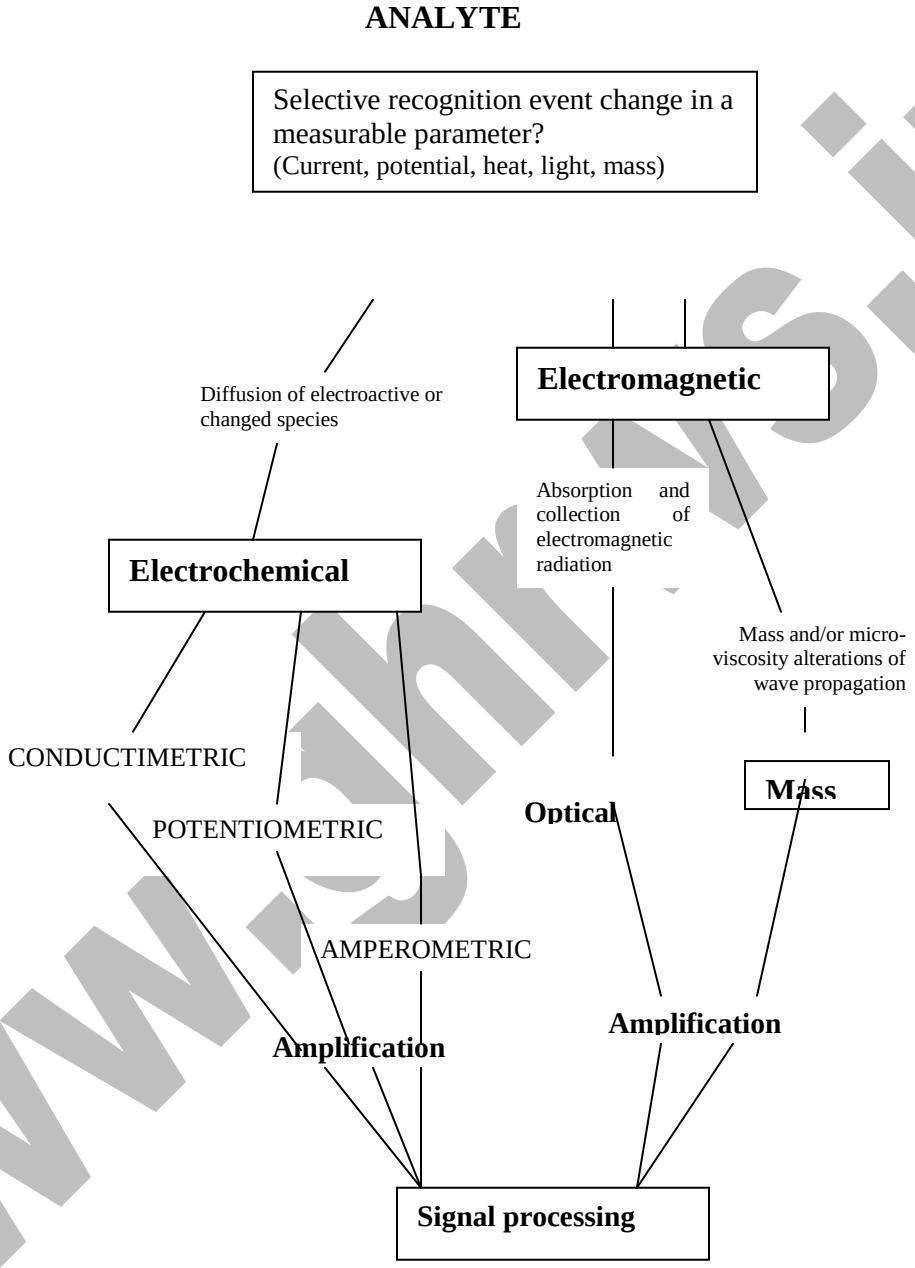

Fig.2 -Transduction parameters and device type (Ref.2)

$Glucose + O_2 \longrightarrow gluconolactone + H_2O_2$

It requires O_2 as co-substrate. The production of H_2O_2 is measured at a charged platinum electrode surface[5].

$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$

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In bioaffinity sensors, stable complexes are formed between the analyte and biorecognition element. The physico-chemical changes such as of layer thickness, refractive index, light absorption, or electrical changes caused by complex formation may be indicated by means of optoelectronic sensors, potentiometric electrodes or field-effect transistors. The biorecognition elements generally used in bioaffinity sensors are antibodies, antigens, enzymes, receptors, endogenous binding proteins, lectins, organelles, membrane bound chemoreceptors, etc. and the respective analytes are antigens, antibodies, hormones, neurotransmitters, aminoacids, drugs, steroids, glycoprotein's and glucose.

Biosensor participation in bioprocess is approached in two ways[6]: (i) the in-situ (on-line) sensor, which is inserted in bioreactor like pH or pO_2 electrode and should be easily sterilizable, insensitive towards protein adsorption and surface growth, and have an extended dynamic range and (ii) flow injection (FI) approach, which functions in automatic analytical means and superior in flexibility and reliability, multicomponent analysis and flexible adaptation of dynamic measuring range than *in-situ* sensor.

According to level of integration, biosensors can be classified into: 1st generation, 2nd generation and 3rd generation biosensors [1, 4] (Fig.3). In first generation type, the biocatalyst is entrapped between or bound to the membrane and this arrangement is fixed on the surface of transducer. In the second-generation, the biologically active components undergo immediate adsorption or covalent fixation on transducer surface by permitting elimination of semi-permeable membrane, but in third generation, direct binding of the biocatalyst to an electronic device occurs that transduces and amplifies the signal.

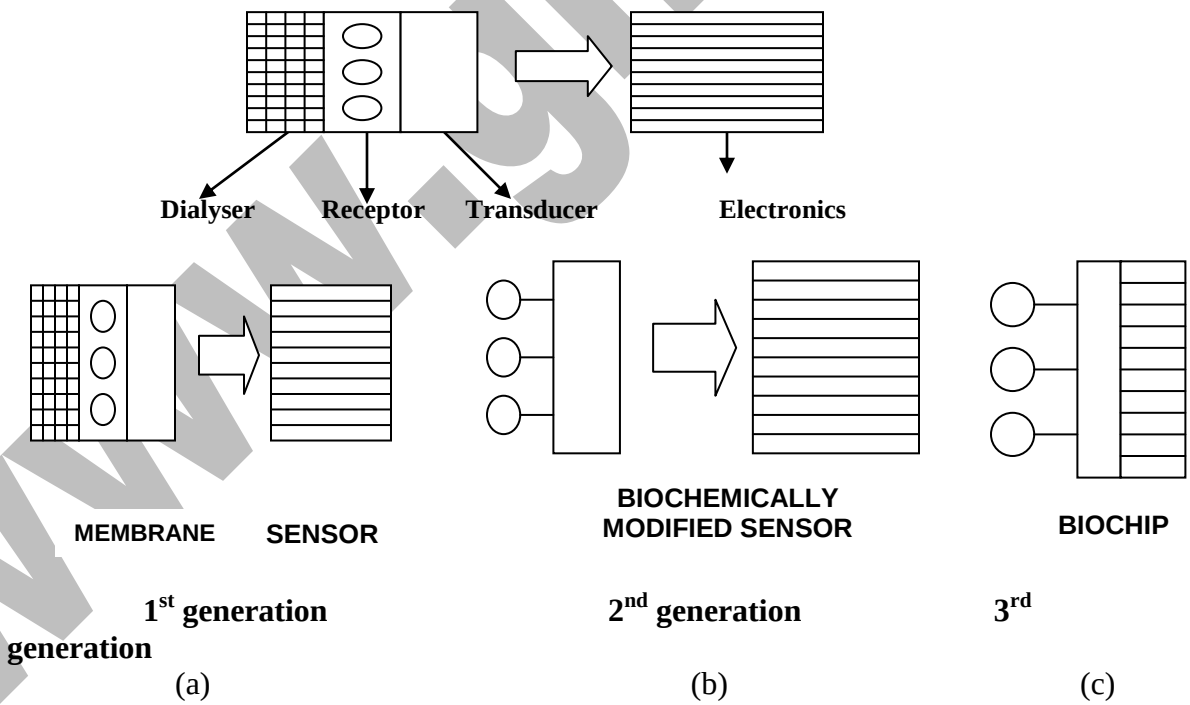

Fig. 3- Different generations of biosensors (Ref.1)

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Principles

The sequences of reactions that occur in biosensor[1] are: (a) specific recognition of analysts; (b) transduction of the physico-chemical effect caused by interaction with the receptor into an electrical signal; and (c) signal processing and amplification**.** Sequences (a) and (b) are essential for biosensor's high sensitivity and functional stability for which immobilization methods having high activity yield are desirable.

Immobilization

Immobilization which refers to a loss of movement of biorecognition element while retaining the catalytical activity and yielding long-term stability, is key to the developments of enzyme-based biosensor. IUPAC has recommended some important immobilization procedure[3] for biosensor development and also defined that the system should have a biological recognition element, which retains direct spatial contact with electrochemical transduction elements. Several methods like physical (adsorption, entrapment and encapsulation) or chemical methods (covalent attachment and cross linking) or combination of both have been investigated to fix biological receptors i.e., enzymes, antibodies, cells or tissues with high biological activities on to or within different materials and matrices.

(i) Physical Immobilization

(a) Adsorption

Adsorption of biorecognition molecules onto space of transducer is the simplest of immobilization processes [1, 3] (Fig.4a.). Substances, like ion exchange resins (anionic, cationic, polystyrene, silica gel, alumina, activated carbons, clay, porous glass and ceramics) are known to adsorb a variety of biological substances like enzymes. Adsorption may occur through ionic, hydrogen bonding or hydrophobic interactions. Adsorption of biomolecules on to the carriers that are insoluble in water is the simplest method of immobilization.

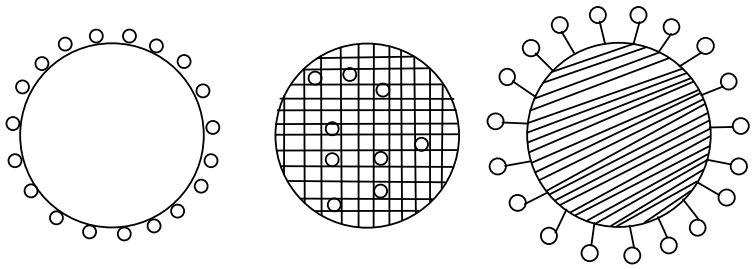

Fig. 4- Schematic representation of immobilization methods used for biosensor construction: (Ref.1)

a. adsorption b. entrapment c. covalent bonding d. cross linking

○ enzvme molecules ● cross linker molecules

(b) Entrapment

Some polymers, such as polyacrylamide are known to entrap biological compounds[6]. The entire enzyme or a whole cell can be entrapped in pores of a polymer (Fig4b). Collagens, agar, alginates, silicone rubber, cellulose triacetate, polyvinyl acrylate (PVA) and

conducting polymers i.e. polypyrrole[7,] polyaniline[8] and polythiophene, [9] etc are used to entrap enzymes. Enzyme entrapment in polymer membrane is a general immobilization process for a variety of transducers. Formation of membrane from the polymer solution in organic solvent on any surface is more simple and reproducible, compared to chemical polymerization. Polyelectrolyte nafion used for development of the enzyme containing membranes provides a biocompatible interface with a mammalian tissue and hence offers potential for use with implantable sensors[10]. The method of entrapment involves simple dipping of the electrode into polyelectrolyte solution or casting a small volume of the solution on to electrode surface and allowing the surface to evaporate. The resulting membrane possesses a high adhesion to the surface and a low swelling in aqueous media. Enzyme-Nafion membranes may be formed using Nafion solutions[11], excessively diluting with water to prevent denaturation of protein with organic solvent. But Nafion membranes deposited from the water organic mixture with low content of organic solvent seemed to be non-uniform. This type of study of enzymes in water miscible organic solvents is called nonaqueous enzymology.

(c) Encapsulation **[1, 3]**

Enzymes can also be encapsulated in nylon or other materials by placing the enzymatic solution in a medium, which upon reaction, with enzyme results in the formation of capsules**.** The enzyme-immobilized products obtained by physical methods are not stable. Chemical methods produces more stable immobilized products compared to physical methods, since the enzyme is tightly held to the solid support or a part of it**.**

(ii) Chemical Immobilization

(a) Covalent coupling

Covalent bonding of receptors on membranes or surfaces activated by means of bifunctional groups or spacers such as gluteraldehyde, carbodiimide, and self assembled monolayer (SAMs) or multilayers etc. is generally employed for stable immobilization. Biomolecules, such as enzymes or antibodies can be covalently coupled with carriers by treating dissolved protein either with an activated water-insoluble carrier or co-polymerizing with a reactive monomer[1] (Fig4c). The reaction should occur only with groups of biomolecule that are not essentially biologically active group. Chemically reactive site of a protein may be $-NH_2$ groups, -OH groups, phenol residue of tyrosine or immidazole derivatives of histidine. Immobilization is acheived by three steps [1, 3] (i) activation of carrier; (ii) coupling of biomolecule; and (iii) removal of adsorbed biomolecule. The carriers are water insoluble polysaccharides (e.g., cellulose, dextrin, agarose derivative etc.), high molecular weight proteins[12] (e.g., collagen, albumin, gelatin etc.), synthetic polymers (PVC and ion-exchange resins) and inorganic materials (porous glass).

(b) Cross-linking

Biopolymer may be intermolecularly cross-linked by bi- and multifunctional reagents (Fig.4d). Protein molecules may be cross-linked with each other or other biopolymers. Biomacromolecules can also be adsorbed to a water insoluble carrier or entrapped in a gel and then cross-linked. The choice of degree of cross-linking influences the physical properties and particle size. The main drawback of crosslinking is the possible loss of activity due to chemical alternation of the catalytically essential sites of the protein.

Application of Biosensors

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(i) Detection of micro-organisms

(a) *Salmonella*

A simple, specific, sensitive and rapid but effective method for detection of bacterial contamination of drinking water, food and dairy products is of public importance for health point of view. *Eshcerichia coli* are a common causative agent of intestinal and extra-intestinal infections and *Salmonella* is of food poisoning. Conventional immunoassay methods for their detection include enzyme-linked immunosorbent assay (ELISA), radio immunoassay and fluorescent-labelled antibody assays. However, these methods are expensive, time consuming and involve complex transducer and skilled labours.

Immunological methods using specific antigen-antibody reactions have been used in the construction of immunosensor by immobilizing the antibody on to a suitable transducer. The piezoimmunosensors, which use quartz piezoelectric crystal detector as transducer, have been developed for detection of *Salmonella* spp. in biological samples, clinical samples and food industries[13, 14], for the monitoring of environmental pollutants[15] and for clinical diagnostics[16]. The use of bimolecules, such as antibodies as an adsorbent that can selectively interact with the targeted analyte at the surface of electrode of the highly sensitive piezoelectric crystal led to a successful development of the specific biosensors[17]. Development of *Salmonella* piezoimmunosensor is focused on finding of a suitable adsorbent for immobilization of antibodies on to the electrode surface. Fung and Wong[18] developed a piezoimmunosensor, which is specific to differentiate *S. paratyphoid A* against *E. coli* and other serogroups of *Salmonella.*

(b) Escherichia coli

Enterohemorhasic *E. coli* serotype 0157:H7 contaminates milk, poultry products, vegetables and drinking water supplies and is frequently transmitted from person-to-person[19]. A sensitive, inexpensive amperometric enzyme biosensor based on the electrochemical detection of β-galactosidase activity, using *p*-amino-phenyl-β-D-galactopyranoside as substrate has been developed[20] for determining the density of coli-forms represented by *E. coli* and *Klebsiella pneumoniae.* Specific detection of *E.coli* is achieved using antibody-coated electrode that specifically binds the target bacteria. Amperometric detection helped determination of 1000 colony forming units/ml within 60-75 minutes. Quantitative determination of total and fecal coliforms is essential for monitoring microbiological water quality. The presence of fecal coliform, *Escherichia coli* conveys the potential presence of pathogens originating from humans and warm-blooded animals. Conventional microbiological plate counts and other cultivation methods for determination of the number of coliforms in drinking water are time-consuming. Also, the cultivation-based methods tends to underestimate the number of fecal bacteria, because they rapidly lose their colony-forming ability after their release into fresh or seawaters, while preserving certain metabolic activity and certain virulence properties[21, 22].

Most rapid enzymatic assays used for total coliform quantification are based on chemiluminometric or fluorometric procedures. Chemiluminometric methods allow the detection of very low concentration of 1 coliform/100ml of water after a 6-9 hour propagation phase, while a fluorometric method[23] could detect 1 fecal coliform /100ml of

water within 6 hour. The specific detection of *E.coli* is essential for water quality control because its presence directly indicates the presence of enteric disease causing bacteria[24].

(c) Dengue Virus Detection

Dengue virus exists as four antigenically distinct serotypes (Dengue 1-4) and is transmitted in humans by the *Aedes agypti* mosquito[25]. Dengue related disease is manifested as (i)dengue fever (DF), which is self limiting, acute feverish illness characterized by fever, headache, nausea and joint pain; (ii) dengue hemorrhagic fever (DHF), characterized by extremely high fever, hemorrhagic phenomena hepatomegaly, circulatory failure, and (iii) dengue Shock Syndrome (DSS), a hypovolaemic shock condition brought on by severe plasma leakage. A distinct subtype increases risks of DHF and DSS with the incidence of secondary infection. Dengue virus infection lacks specific treatment and its preventive measure has been mainly mosquito-eradication strategy. Initial symptoms of dengue virus infection are similar to those of influenza, measles, malaria, typhus, yellow fever, and other viral infections, which make the diagnosis, based on presenting symptoms problematic. ELISA assay[26] for the detection of IgG and IgM antibodies of dengue virus is available. Other conventional approaches in Dengue virus diagnostics, such as tissue culture and immunofluorescence[27], have limitations in terms of specificity, sensitivity, simplicity and rapidity.

Biosensors based on liposome technology have played a key role for the development of rapid, inexpensive, and field usable detection systems[28, 29]. Recently[30] a field usable, serotype- specific RNA Biosensor for rapid detection of dengue virus (serotype 1-4) in blood samples has been developed. These biosensors are membrane based DNA / RNA hybridization system that use liposome amplification. The generic DNA probe (reporter probe) is coupled to outside dye-encapsulating liposome and dengue serotype specific probe (capture probe) is immobilized on a polyethersulfane membrane strip. Liposomes are mixed with amplified target sequence and are applied to the membrane. The mixture was allowed to migrate along the test strip, and the liposome target sequence complexes are immobilized in the capture zone via hybridization of the capture probe with target sequence. The amount of liposome present in the immobilized complex is directly proportional to the amount of target sequences present in the sample and can be quantified, using a portable reflectometer. Analysis of clinical samples showed that dengue serotypes 1, 2, and 4 were identical, but serotype 3 interferes the analysis of 1 and 4.

(d) Human Immunodeficiency Virus (HIV)

HIV-1 RT (human immunodeficiency virus type-1 reverse transcriptase) is a key component in the life cycle of HIV-1 virus, which is the etiological agent of the acquired immunodeficiency syndrome (AIDS) [31]. RT is a marker for the HIV-1 virus and its activity is periodically used to titer (determine concentration) stocks of virus. Correlating HIV-1 RT activity from virus stocks of the known concentration with RT activity from viral cell lines of unknown concentration is routinely used for determination of HIV viral loads in cell culture for *in vitro* studies[32]. The determination of viral load in HIV positive individuals is important in the course of therapy. Although PCR based capillary electrophoresis/ laser induced florescence (CE/LIF) [33, 34] techniques, a calorimetric (RT) assay [35] and its chemiluminescent [36] and fluorescent [37] versions have been used in HIV 1 detection, however these are time-consuming and not specific for HIV 1 RT.

Direct and specific detection of HIV-1 RT by affinity capillary electrophoresis/ laser induced florescence (CE/LIF) using florescent labeled single stranded DNA aptamers, synthetic DNA and RNA oligonucleotides produced *in vitro* form a process termed as SELEX [38-41] (systematic evolution of ligands by exponential enrichment) has been reported. Single stranded DNA aptamers bind to HIV-1 RT [31].Two such oligonucleotides (aptamers), a 81-mer (RT 26) and 84-mer (RT 12) having binding constants of 1 *n*M and 2 *n*M, respectively with HIV-1 RT, represent a 1000 fold increase in ability over binding of RT with native DNA. RT 26 is specific for HIV-1 RT and exhibits no cross-reactivity with RTs of enhanced avian myeloblastosis virus (AMV), moloney murine leukemia virus (MMLV) or denatured HIV-1 RT. An affinity complex of RT 26-HIV 1 RT is readily formed. This non-competitive affinity assay has been developed for the direct and selective determination of HIV-1 RT in less than 5 min and is capable of quantifying up to 50nM (6µg/m^2) HIV-1 RT, not interfering with the presence of RTs from AMV, MMLV or denatured HIV-1.

(e) Biological warfare (BW) agents

The threat from biological warfare (BW) agents is a matter of concern both in the battlefield and for general public safety. Weapons of BW may be used by terrorists, and have potential to cause mass destruction, as they can be easily produced and are difficult to detect. Among BW agents[42], synthetic chemicals, toxins of plant and animal origin and biological materials (pathogens) and bacterial cells pose serious threat. Bacteria are considered to be potentially most prevalent type of BW agent. The Center for Disease

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Control, USA has catagorised several infectious agents including the bacteria *Bacillus*

Table 1-- Different types of biosensors for detection of warfare agents (BWs)

Toxic materials / BW	Category of Biosensor	Detection Limit	Matrix	Ref.
Yersinia pestis	Acoustic	10^6 cells/ml	Aqueous suspension	43
Yersinia pestis	Optical fiber	5ng/ml	Aqueous solution	44
Francisella tularensis (G-)	Light addressable potentiometric sensor (LAPS)	5100 cells/ml	Aqueous suspension	45
Brucella militensis (G-)	LAPS (antigen-antibody, enzyme labeled, biotinavidin, urease)	6000 cells/ml	Aqueous suspension	46
Bacillus anthrasis (G +) (Spores)	Optical fiber (Evanescent wave-fluorescent dye)	$3x10^3$ cells / ml	Aqueous suspension	47
Salmonella typhimurium (G-)	Optical fiber	10^5 cells/ml	Aqueous suspension	48

anthracis, Fransicella tularensis and Yersinia pestis having potential to be used as BW agents under 'Category A'; and *Brucella spp., Burkholderia pseudomallei,* and *Burkholderia mallei* as 'Category B'. The former is more dangerous and can be transmitted or spread more easily from person-to-person and causes high mortality. Some BW agents are listed in the Table 1.

Only a few methods are known for the detection of microorganisms as BW agent[49-51]. The main difficulty arises due to strict requirements for the sensitivity, specificity, response time and adaptability of the conventional instruments. DNA probe for detection of pathogenic microorganisms in water[52], and immunoelectrochemical and surface

enhanced infrared sensor[53] (SEIS) for detection of food borne pathogens have been reported. Detection of bacterial cells, using PCR is a recent approach. Semi selective bacterial sensor utilizing the SYTO13 (a green fluorescent cell strain) fluorophore immobilized in optical substrate has been also demonstrated[54]. However, it lacks species/strain specificity, such as distinguishing bacteria/viruses, bacteria/fungi, bacteria/spores, living and dead, and Gram positive and negative bacterial cells, which is essential to expose biological threats.

(ii) Detection of explosives

Field detection[55] of explosives such as TNT (2,4,6-trinitrotoluene), RDX (1,3,5-trinitro-1,3,5-triazacyclohexane),NG(Nitroglycerin), Tetryl(2,4,6,N-Tetranitro-N-ethylaniline),RDX(1,3,5-trinitro-1,3,5-triazacyclohexane), NG (nitroglycerin), tetryl (2,4,6,N-tetranitr-N-methylaniline), HMX (1,3,5,7-Tetranitro-1,3,5,7-tetraazacyclooctane), Composition C4 (RDX+Plasticizer) and Composition B (RDX + TNT+ wax) etc., is an important analytical issue in law enforcement and environmental applications. Onsite environmental detection and monitoring of traces of explosives in prone areas is essential. Commonly used methods for detection of explosives are x-ray, neutron analysis, nuclear quadruple resonance (NQR), mass spectrometer (MS), gas chromatography (GC) with electron capture detector (ECD), ion mobility spectrometer (IMS) and GC/MS etc.

The current standard protocol for TNT quantification in contaminated soil and ground water sample is off-site laboratory analysis by reverse phase HPLC[56]. Onsite methods including calorimetric tests and immunoassay kits based on a reaction between a target analyte and a specific antibody are enzyme linked immunosorbent assay[57] (ELISA), fiber-optic biosensor, displacement flow immunosensor and more recently a sol-gel based biosensor[58]. Conventionally dogs, pigs, bees, and birds act as biological sensors to detect criminals, explosives and presignalling of natural disasters [59].

The reduction of nitro aromatic compounds, which are widely used in agrochemicals and explosives, has environmental pollution effects. Enzymatic assay[60] of nitrite and nitrate is based on the following reaction catalyzed by nitrate reductase (EC 1. 9.6. 1) and nitrite reductase (EC 1.6. 6.4) as shown in Equation 1 and 2, respectively.

$$NO_3^- + 2H^+ + 2MV^+ \longrightarrow NO_2^- + 2MV^{2+} + H_2O \quad \ldots 1$$

$$NO_2^- + 8H^+ + 6MV^+ \longrightarrow NH_4^- + 6\,MV^{2+} + 2H_2O \quad \ldots 2$$

Where MV^{2+} represent oxidized methyl viologen.

Nitrate is reduced to ammonia via intermediate nitrite with the participation of reduced methyl viologen as electron donor. Enzyme reactor incorporating immobilized reductases, (nitrite and nitrate), in two separate columns detect liberated NH_3 gas and thereby enable measurement of nitrite and nitrate [61].

Various methods for detection of the aromatic compounds have been reported including a membrane based continuous flow displacement immunoassay for determination of nanomolar quantities of explosives[62]. A miniaturized field portable immunosensor (Fast 2000), for detection and quantification of TNT and RDX in ground water[63] and a fiber optic immunosensor[64] for simultaneous detection of TNT and RDX have been developed. Nitroreductases of enteric bacteria are flavoproteins that analyze reduction of a variety of nitro aromatic compounds to toxic, mutagenic or carcinogenic metabolites. Bryant *et al*[65]

studied cloning, nucleotide sequences and expression of the nitroreductase gene from *Enterobacter cloacae*. They also studied the mechanism of activity of *E.cloaqenitroreductase* and observed that 2, 4-dinitrotoluene DNT was the most efficient oxidizing substrate than *p*-nitrobenzoate, flavine adenine dinucleotide (FAD) or riboflavin[66]

An amperometric TNT biosensor based on the surface immobilization of a maltose binding protein (MBP) nitroreductase fusion (MBP-NR) on to an electrode modified with an electropolymerized film of N-(3-pyrol-1-yl-propyl)-4, 4-bipyridine (PPB) [67]. MBP domain of MBP-NR exhibits a high and specific affinity towards electropolymerised film of PPB with the immobilized enzyme retaining all of its enzymatic activity. The kinetics of catalytic reaction between the biosensor and TNT and 2,4-dinitrotoluene (DNT), using rotated disc electrode and cyclic voltametry techniques gave the values of 1.4×10^4 and 7.1×10^4 $M^{-1}S^{-1}$ for TNT and DNT, respectively. The detection limit for TNT and DNT were estimated to be 2µm, while sensitivities were 205 and 222 nA/ µM, respectively.

Simultaneous detection of multianayte explosives (RDX and TNT) has been achieved by using a fiber optic biosensor [68]. To achieve dual explosive detection, two α-TNT fiber and two α-RDX fiber probes are connected in series. The sample is mixed with fluorescent analogs, cy5-etylenediamine-trinitrobenzene (cy 5-EDA-TNB) and cy5-ethylnediamine RDX hapten (cy5-EDA-RDH). Inbition of the maximum signal in the presence of the sample is proportional to the concentration of the explosive. The multianalyte fiber-optic sensor is capable of detecting TNT and RDX simultaneously thus provides a means of simple and precise quantifications.

(iii) Organic Pollutants

Pollutants affect health of aquatic and terrestrial ecosystems. Some common contaminants of ground water are fluorinated compounds (freons), chlorinated compounds, pesticides, fertilizers, nitrates, aromatic solvents and their derivatives like benzene, toluene, xylene, DDT, BHC, polycyclic aromatic hydrocarbons (i.e. naphthalene, pyrene etc), and some toxic ions, such as Pb^{2+}, Hg^{2+} and As^{2+}etc. Mostly, these pollutants are irritant, toxic, carcinogenic or mutagenic. Conventional analytical techniques, though, can detect concentration of the pollutants in a contaminated sample, but their biodegradability remains unreported.

Biosensors can monitor pollutants in the environment by measuring the interaction of specific compounds with biological species through highly sensitive biorecognition processes. The whole-cell biosensors[69] are constructed by fusing a reporter gene to a promoter element that is induced by the presence of a target compound. Reporter genes (proteins) used in whole-cell biosensors are chloroamphenicol acetyltransferase, β-glycosidase, bacterial luciferase[70] (lux), firefly luciferase (luc), aequorin, green fluorescent protein, uroporphyrinogen III methyltransferase etc. As the biosensor is exposed to an inducing compound, reporter gene system is activated and the cell produces a measurable signal, such as emission of light. Since bacterial bioluminescence is tied directly to cellular respiration, any inhibition of cellular metabolism due to toxicity results in a decrease in light emission of affected cells. Commercially available Microtox™ assay measures toxicity of environmental samples by monitoring light production of the reconstituted freeze-dried cells of the naturally bioluminescent marine bacteria *Photobacterium phosphoreum*[71]. Another type of nonspecific biosensor[38] is

based on the response of the *E. coli* to environment stress, with lux genes fused to heat shock promoters so that the exposure of host cells to toxic agents such as heavy metals and organic solvents rapidly induces light production.

An optical biosensor for continuous on-line monitoring of naphthalene and salicylate bioavoilability in waste streams has been reported[73]. King *et al*[74] developed *P. fluorescens* HK44, a prototype bioluminescent catabolic reporter strain that can degrade naphthalene and its degradation intermediate salicylate. Exposure of this strain to either naphthalene or salicylate results in a bioluminescence intensity proportional to the metabolism rate. This strain has also been used for a bioassay for the quantitative assessment of naphthalene and salicylate biodegradation in aqueous samples, soil extracts, and soil slurries[75].

The biosensor based on *P. putida* B2 has been developed to monitor toluene and trichloroethylene[76]. A third generation biosensor, a novel system consisting of biosensor cells interfaced with an integrated circuit, termed as bioluminescent bioreporter integrated circuit (BBIC), using immobilized living cells as sensing component of a circuit has been developed recently[77]. Biosensor based on the bacterial cell of *Ralstonia eutropha*[78], strain JMP143-32 for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its degradation intermediate 2,4-dichlorophenol, and an amperometric biosensor for benzene[79] with *P. putida* ML2, can aerobically degrade benzene and utilize it as a source of carbon and energy have been also developed.

(iv) Organomercurials

Detection of mercury and its organic derivatives, especially methyl mercury (MeHg), in the environment is important, because of their high toxicity[80]**.** They concentrate in biota via biomagnifications and cause neurological disorders in animals. MeHg, present in seawater in nanogram / L, is accumulated by plankton, which in term is consumed by fish [81, 82] .GLC combined with spectroscopic detection is used for analysis of the organomercurial species[83]. Although the detection limits of MeHg by these methods are in the range of nanograms/L[84], they are labor intensive and require relatively expensive instrumentation and trained personnel.

The whole bacterial cells or sensor bacteria have been used for analyzing different compounds such as inorganic Hg[85], naphthalene, and arsenate[86]. In bacterial sensor, expression of receptor gene is controlled by a genetic regulatory unit which responds to the given analyte (receptor-reporter concept) [87]. Sensitivity and specificity of bacteria as sensor towards given analyte are mainly defined by the regulatory unit consisting of regulatory protein that recognizes the analytes. The bacterial sensors, which measure biological response (bioavailability), are inexpensive, and highly stable as compared to enzyme-based sensors. A new whole cell bacterial sensor has been constructed for detection of organic compounds of Hg, using receptor-reporter concept[88]. The whole cell bacteria sensor was constructed by fusing reporter gene of firefly luciferase (lucFF) and a regulatory region merR (regulatory part of the mer operon) and operated/promoter part of mer operon of the same board spectrum mer operon from the plastid pDU1358 (*Serratia marcescens*) [89]. It is based on the natural bacterial resistance mechanism towards Hg and organomercurial compounds. The resistance is achieved due to organomercurial lyase (product of merB gene), an enzyme produced by broard spectrum mer operon and catalyzes the breakdown of mercury-carbon bond of organomercurials[90]. The released

Hg^{2+} ions from complex with the regulatory protein of mer operon, Mer R (product of mer R gene) and also change the conformation of Hg^{2+}-Mer R complex. The Hg^{2+}ions are later detoxified by mercuric reductase (product of mer A gene) and metallic Hg volatize from the cell[91].

Many ligands, like 1-nitroso-2-naphthol (NN), 4-(2-pyridylazo) resorcinol (PAR), 2,4-dinitrosoresorcinol (DNR) and 1-(2-pyridylazo) naphthol (PAN) have been studied for the development of optical sensors for heavy metal ions such as Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, Cd^{2+}, Zn^{2+}, Pb^{2+} and Hg^{2+}. The ligands may be immobilized by physical adsorption on to polymeric materials, such as XAD-4 (cross-linked co-polymers of styrene and divinylbenzene), XAD-7 (cross-linked polymer of methylmethacrylate) and Dowex ion exchange resins exhibiting chromic characteristic irrespective of the presence or absence of metal ions in the solution.

(v) Food Contents

(a) Artificial sweetener

Artificial sweeteners are staple in the diet of many people and are suspected to cause cancer. Among artificial sweeteners, aspartame (N-L-α-aspartyl-L-phenylalanine-1-ethyl ester) is rapidly replacing saccharin and cyclamate in consumer market as a low calory sweetener. Although many analytical methods, such as spectrophotometry[93], capillary electrophoresis[94], thin layer chromatography (TLC), gas chromatography (GC), and HPLC[95] are available for determination of asparmate but they are time consuming and tedious. Some whole cell and enzyme-based biosensors have been reported for detection of aspartame. A potentiometric aspartame sensor [96, 97] and amperometric aspartame biosensor[98] have been developed recently. Microbial biosensors, using *Bacillus subtilis* cells[99] are non-specific and respond to glucose and amino acids. The enzyme electrode, using carboxypeptidase A and aspartase in combination with ammonia electrode is interfered by amines present in the food samples.[100] An enzymeatic assay technique for aspartame determination using a crude peptidase to cleave aspartame peptide bond and release aspartic acid and phenylalanine/phenylalanine methyl ester is also developed.[101] The aspartic acid is then transaminated to glutamic acid by aspartate aminotransferase. The resulting glutamic acid is monitored by measuring oxygen consumption during oxidation of aspartic acid by glutamate oxidase. Though this technique is successful for determination of aspartame in dietary product, but enzymes can't be re-used. A flow injection analysis biosensor incorporating immobilized enzymes (glutamate oxidase and aspartate aminotransferase) and an amperometric H_2O_2 electrode for aspartame determination is also reported [102.] The enzymes, peptidase and aspartate aminotransferase were immobilized on amino proppyl glass beads via glutaraldehyde activation, which were then packed into separate columns. Glutamate oxidase was immobilized on a membrane and attached to the tip of H_2O_2 electrode and inserted in a flow through the cell. The minimum detectable concentration of aspartame was 20 µM, which was slightly better than for enzyme assay[101] (25 µM) and significantly better than microbial[99] (70µM) and enzyme electrodes[100] (425µM) respectively.

An aspartame optical biosensor has been developed [103] by employing a bienzyme system composed of α-chymotrypsin and alcohol oxidase immobilized on to an eggshell membrane and an oxygen–sensitive optode membrane as the transducer. The rate of

oxygen consumption in the enzymatic reaction of aspartame and methanol is measured by oxygen sensitive optode membrane[104]:

$H_2NCH\ (CH_2COOH)\ CONHCH\ (CH_2C_6H_5)\ CO_2CH_3 + H_2O$

$\downarrow$ α-Chymotrypsin

$H_2NCH\ (CHCOOH)\ CONHCH\ (CH_2C_6H_5)\ COOH + CH_3OH$

Alcohol oxidase

$CH_3OH + O_2 \longrightarrow HCHO + H_2O_2$

α-Chymotrypsin hydrolyzes aspartame to yield methanol which is oxidized to formaldehyde by alcohol oxidase with the consumption of oxygen. Depletion of oxygen level is detected by oxygen- sensitive optrode membrane. The optical oxygen sensing is based on collision quenching of fluorcscence of $[Ru\ (dpp)_3]\ [4\text{-}Clph)_4B]_2$ molecules by oxygen molecules [105,106]. Depletion in oxygen level of the medium results in the concomitant increase of fluorescence intensity of oxygen sensitivity membrane. The aspartame biosensor was found to have no response towards potential interferences present in the food stuffs such as citric acid, cyclamic acid, ethanol, D-fructose, D-galactose, D-glucose, hydrogen peroxide, DL-malic acid etc. Aspartame biosensor has been used to determine aspartame contents in commercial food products, viz., Diet Coke, Diet Pepsi, Diet Seven-Up and Diet Sprite and the result was in good agreement with result of HPLC and spectrophotometric methods [104].

(b) Glycolic acid

Glycolic acid, a constituent of sugarcane juice, cosmetics, fruits, instant coffee etc. find wide application in several industries, such as processing of textiles, leather, metals, in manufacturing of adhesives, in copper brightening, decontaminant cleaning, dyeing, electroplating, cleaning and chemical milling of metals. It is also used as a solvent for intercorneocyte matrix, reducing excessive epidermal keratinization, and has a beneficial action for renewal of epidermis and reduction of wrinkles[107]. It efficiently increases the skin elasticity as a result of direct stimulation during the production of collagen elastin and mucopolysaccharides. α-hydroxy acids, (glyoxalic acid) are used in cosmetic products as exfoliants and moisturizers. Among α-hydroxy acids, glycolic and lactic acid are the most effective, with respect to their potential to sensitive skin, their ability to increase skin cell renewal, improvement of the moisture content and to reduce lines and wrinkles [108]. Commercial cosmetics containing 8.35% w/w glycolic acid are available in different forms (cream, lotion, gel, oil etc.) and also used for different therapeutical targets[109] such as skin smoothening, face and body care, exfoliation process, moisturizing, sun protection etc.

A limited number of methods are available for the detection of the glycolic acid. These include gas chromatography, HPLC, ion-exchange HPLC[110], and ion-exchange chromatography. These methods have their own inherent advantages (multianalyte analyzer) and disadvantages (require complex isolation, derivatisation and expensive instruments), however, these are complicated and do not exhibit the simplicity of the biosensors. Turner and co-workers[111] immobilized glycolate oxidase on to ferrocene

modified carbon paste electrodes, thus revealing the suitability of specific mediators in a number of oxidases. A plant tissue electrode for the assay of glycolate in urine samples [112] and an amperometric glycolate sensor based on glycolate oxidase and electron transfer mediator[113] have been developed recently. A chemiluminescence's flow method has been proposed for glycolate, based on the concept of plant tissue biosensor[114], but, the selectivity is limited because of the multienzyme systems present in the tissue.

An amperometric biosensor (enzyme-based) capable of determining glycolic acid in various complex matrixes i.e., cosmetics, instant coffee and urine have been developed[115]. Two separate designs–both based on three component membrane configurations consisting of an inner cellulose acetate membrane and outer polycarbonate membrane, which sandwich a membrane bearing biomolecule(s) have also been proposed [115]. Glycolate oxidase is immobilized onto a modified polyether sulfonate membrane by means of chemical bonding, and glycolate oxidase catalyzed enzyme mixture was immobilized into a mixed ester cellulose acetate membrane through physical adsorption. The proposed biosensors are interference-free to common electroactive species, fast, reliable, easy to use, cost effective, and were successfully applied for the determination of glycolic acid in various samples.

Conclusions

Advancement in detection strategies and the rapid identification of target analytes by biosensors has made it an indespancible analytical tool for the benefit and welfare of the mankind, but only a few of the biosensors are commercially available. It requires the novel immobilization schemes and sensor materials, promising transducers, micro fabrication and miniaturization technique to fabricate biochip-based microsensors containing nanostructured recognition materials for online and in vivo measurement of the desired processes. The goal can be achieved by accumulation and integration of the interdisciplinary knowledge from industries and academic institutions**.**

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