

Biosensors – classification, characterization and new trends

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Abstract: Biosensors represent promising analytical tools applicable in areas such as clinical diagnosis, food industry, environment monitoring and in other fields, where rapid and reliable analyses are needed. Some biosensors were successfully implemented in the commercial sphere, but majority needs to be improved in order to overcome some imperfections. This review covers the basic types, principles, constructions and use of biosensors as well as new trends used for their fabrication.

Keywords: Biosensor, Transducer, Mediator, Bioreceptor, Carbon nanotubes, Nanoparticles.

Introduction

Nowadays, the importance of a monitoring and regulating many different parameters in areas such as a food industry (Monošík et al. 2012), clinical diagnoses, hygiene, environmental protection, drug development, or forensics is increasing. Therefore, there is a need to have reliable analytical devices available, which are able to perform quick and accurate analyses (Dzyadevych et al. 2008). One of the ways how to overcome many disadvantages of the conventional methods is to use proper designed biosensor. The main reason why biosensors are still rarely used in mentioned areas is their often impracticability for real samples, whereas a biosensor developed for standards is not automatically applicable for real samples. Hence, the challenge for scientist is to develop or improve some good existing concepts for constructing biosensors applicable on real samples and usable in commercial sphere.

The aim of this paper is to provide information on progress done during the period of last 5 years in relation to basic known functional principles of biorecognition elements and transducers in relation to specific biosensors application such as clinical diagnosis, food quality control and environmental screening. New trends including application of nanomaterials are also described.

Biosensors

According an IUPAC nomenclature, biosensor (Fig. 1) is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical

signals (McNaught and Wilkinson 1997). Beginning of biosensors may be dated to 1962, when Clark, known as the father of the biosensor concept, published an experiment in which glucose oxidase (GOX) was entrapped at a Clark oxygen electrode using dialysis membrane (Clark Jr. and Lyons 1962).

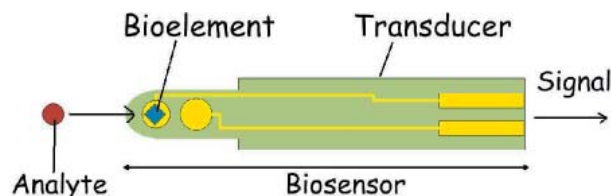


Fig. 1. Basic scheme of a biosensor.

As bio-components, an enzyme, antibody, nucleic acid, lectine, hormone, cell structure or tissue can be used. Its role is to interact specifically with the target analyte and the result of biochemical reaction is consequently transformed through transducer to measurable signal. The transducing systems can be electrochemical, optical, piezoelectric, thermometric, ion-sensitive, magnetic or acoustic one. Very important part of a biosensor fabrication is the immobilization of bio-component. Performance of biosensors with immobilized molecules depends also on factors such as the chemical and physical conditions (pH, temperature and contaminants), thickness and stability of the materials (Kissinger 2005).

Bioreceptors

Enzymes

Enzymes are often used as biomaterials for the development of biosensors. These biosensors utilize

enzymes (Table 1) which are specific for the desired molecules and catalyze generation of the product, which is then directly determined using one of the transducers mentioned above.

Tab. 1. Enzyme categories and their functions which are used for selective detection of their competent substrates as analytes by biosensor.

Enzyme category	Functions
Oxidoreductases	Oxidation/reduction reactions
Transferases	Transfer of molecular groups from one molecule to another
Hydrolases	Hydrolytic cleavage
Lyases	Cleavage of C—C, C—O, C—N bonds by other means than oxidation or hydrolysis
Isomerases	Intramolecular rearrangement
Ligases	Joining of two molecules

The most successful commercially available biosensors are those for measuring glucose in blood samples representing about 90 % of the global biosensor market utilizing glucose oxidase or glucose dehydrogenase (^bMonošík et al. 2012). Variety of enzymes were used for biosensor construction, for example oxidoreductase enzymes were used for lactate (Huang et al. 2009, Huang et al. 2008, Katrlík et al. 1999, Pereira et al. 2007), malate (Arif et al. 2002, ^cMonošík et al. 2012, Prodromidis et al. 1996, Wang et al. 2008), ascorbate (Vermeir et al. 2007, Wang et al. 2008), amino acids (Pollegioni et al. 2007, Sacchi et al. 1998), alcohol (Katrlík et al. 1998, Pena et al. 2002, Smutok et al. 2006, Tkáč et al. 2003), cholesterol (Lia and Gub 2006, Umar et al. 2009, Vidal et al. 2004), glycerol (Alvarez-Gonzalez et al. 2000, ^dMonošík et al. 2012, Niculescu et al. 2003), fructose (Tkáč et al. 2001, Tkáč et al. 2002), transferase can be utilized in biosensorial analysis of acetic acid (Mieliauskienė et al. 2006, Mizutani et al. 2003), determination of xenobiotics such as captan (Choi et al. 2003) or atrazine (Andreou and Clonis 2002), hydrolase in sucrose (Soldatkin et al. 2008, Surareungchai et al. 1999), lyase in citric acid analysis (Maines et al. 2000, Prodromidis et al. 1997), ligase in DNA point mutation detection (Pang et al. 2006), isomerase for 19-norandrostenedione (Sheu et al. 2008), etc. Many factors have influence on the performance of enzyme-based biosensors, such an enzyme loading, the use of a suitable pH, temperature and in some cases a cofactor can help to retain the abilities of the enzyme. Another factor that can affect the electrode performance is the type of immobilization method used to retain the enzyme as well as the thickness of the enzyme layer on the sensor.

Antibodies

An antibody is a complex biomolecule, made up of hundreds of individual amino acids arranged in a highly ordered sequence. An antigen-specific antibody fits its unique antigen in a highly specific way. This unique property of antibodies are crucial to their usefulness in immunosensors where only the specific analyte of interest, the antigen, fits into the antibody binding site (Vo-Dinh T and Cullum 2007) (Fig. 2). Biomolecular interactions can be divided in two categories, according to the test format performed (i.e., direct and indirect). Direct format is based on interaction between the immobilized target molecule and a ligand molecule or the immobilized ligand interacts with a target molecule directly. For immunosensors, the most basic situation involves *in situ* incubation followed by direct measurement of a naturally fluorescent analyte (Vo-Dinh et al. 1987). Oppositely, for non-fluorescent analyte systems, *in situ* incubation is followed by development of a fluorophore-labelled second antibody. The indirect immunosensors utilize a separate labelled species that is detected after binding by fluorescence or luminescence. In this case, the unlabeled analyte act as a competitor with the labelled analyte for a limited number of receptor binding sites. Principle of the assay is based on a change of the label signal that occurs when the analyte-label conjugate forms immunocomplex with antibody. Assay sensitivity increases with decreasing amounts of immobilized reagent (Tromberg et al. 1987). The reaction components are mixed with sample and the response is measured usually kinetically. Heterogeneous formats are studied more often since lower limits of detection are generally achieved. For example, the common enzyme-linked solid phase immunoassay (ELISA) is performed in microplates, tubes, capillaries or on glass strips, and some kind of electrochemical sensor is finally coupled to measure the label generated signal (Skládal 1997). Immunosensors can be designed for monitoring of cancer cells (Ehrhart et al. 2008, Malhotra et al. 2010) or their markers detection (Liu et al. 2008, Mani et al. 2009), for bacteria and virus determination assays (Carnes and Wilkins 2005, Konig and Gratzel 1993), for toxins (Kadir and Tothill 2010, Labib et al. 2009), etc.

Nucleic acids

Biosensors based on DNA, RNA and peptide nucleic acid gain their high sensitivity and selectivity from the very strong base pair affinity between complementary sections of lined – up nucleotide strands (Borgmann et al. 2011). Nucleic acid (NA) – based biosensors integrate an NA (natural and biomimetic forms of oligo- and polynucleotides) as the

biological recognition element. Nowadays, mainly synthetic oligodeoxyribonucleotides (ODNs) are used as probes in the DNA hybridization sensors. End-labels, such as thiols, disulfides, amines, or biotin, are incorporated to immobilize ODNs to transducer surfaces. A long flexible spacer is usually added by means of hydrocarbon linkers to provide sufficient accessibility for surface attachment (Labuda et al. 2010).

The electrochemical DNA biosensors, which rely on the conversion of the base-pair recognition event into a measurable electrical signal, are regarded to be suitable candidates for the rapid and inexpensive diagnosis of genetic diseases, the detection of pathogenic biological species of clinical interest, and for the compatibility with microfabrication technology (Lucarelli et al. 2004, Wang 1999, Wang et al. 2004). The complementarity of adenine-thymine and cytosine-guanosine pairing in DNA forms the basis for the specificity of biorecognition in DNA biosensors (Fig. 2).

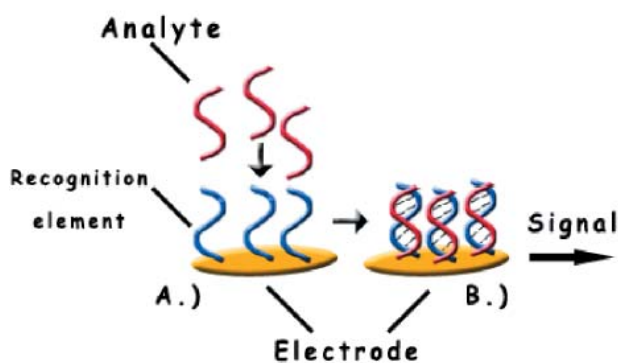


Fig. 2. General DNA biosensor scheme. Target DNA is captured at the recognition layer (A), and the resulting hybridization is transduced into a measurable electronic signal (B).

For the known sequence of bases in DNA molecule the complementary sequence, called a probe, can be synthesized and subsequently labelled with an optically detectable compound (e.g., a fluorescent label). The labelled probe will hybridize to its complementary sequence on the target molecule once the double-stranded DNA is unwound into single strands, then the probe is added, and finally the strands annealed (Vo-Dinh and Cullum 2000). The formation of the duplex may be considered as evidence that the target has the expected nucleotide sequence. Electrochemical (EC) detection of the formation of a DNA duplex, called hybridization event, is based on the EC signals due to NA electroactivity, labelling of the target or the probe with covalently bound electroactive species (e.g., nanoparticles), or changes in various electrochemically detectable

DNA properties related to changes in the DNA structure resulting from the hybridization step (Paleček and Bartošík 2011).

Aptamers, artificial single-stranded DNA or RNA oligonucleotides (typically <100mer) which are selected from randomized oligonucleotide libraries by SELEX (systematic evolution of ligands by exponential enrichment) are also used to specifically bind with various targets such as proteins, cells, viruses, bacteria, as well as small molecules such as organic dyes, metal ions, amino acids (Yan et al. 2011). Mainly, their considerable and modifiable stability promise the development of a new biosensor generation (Strehlitz et al. 2008). Aptamers are equal to monoclonal antibodies concerning their binding affinities and they are more resistant to denaturation and degradation. Moreover, by means of rational design or by techniques of molecular evolution, binding affinities and specificities, they can be also modified. For this purpose, many functional groups or tags that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers are used (Stadtherr et al. 2005). Aptamers may play role as a chiral selector and thus distinguish between chiral molecules and recognize a distinct epitope of a target molecule (Lin et al. 2009).

DNA probe immobilization is a key for proper working of a biosensor. Many different materials were successfully used for DNA immobilization, such a carbon paste (Girousi et al. 2004), pyrolytic graphite (Chen et al. 2000), glassy carbon (Pedano et al. 2003), carbon fiber (Tian et al. 2005) carbon nanotubes (Mani et al. 2009, Niu et al. 2008) etc. DNA biosensors were deeper reviewed for example by Drummond et al. (2003) or Sassolas et al. (2008). DNA-based biosensors were for example used for the determination of drug in blood serum matrix (Vaníčková et al. 2005), detection of the DNA damage and antioxidants protecting DNA from its damage (Bučková et al. 2002; Galandová et al. 2009; Labuda et al. 2009; Vyskočil et al. 2010), voltammetric determination of 1-aminopyrene and 1-hydroxypyrene (Ferancová et al. 2005), for detection of the effect of berberine on DNA from cancer cells (Ovádeková et al. 2006). Deep review devoted to the electrochemistry of DNA and RNA and to the development of sensors for detecting DNA damage and DNA hybridization can be found in the book *Electrochemistry of Nucleic Acids and Proteins – Towards Electrochemical Sensors for Genomics and Proteomics* (Fojta 2005).

Cells

These bioreceptors are either based on biorecognition by an entire cell/microorganism or a specific

cellular component that is capable of specific binding to certain species (Fig. 3).

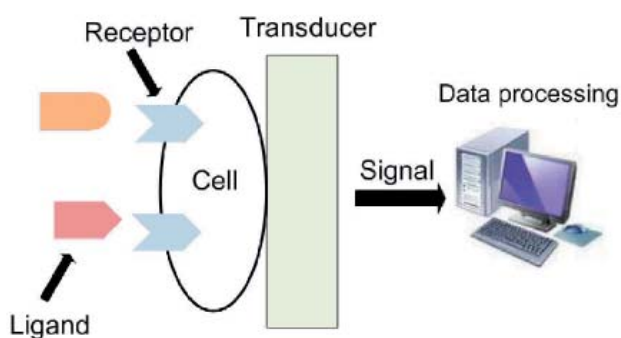


Fig. 3. Scheme of cell-based biosensor.

One of the major advantages resulting from using this class of bioreceptors is that the detection limits can be very low because of signal amplification. Many biosensors developed with these types of bioreceptors rely on their catalytic or pseudocatalytic properties (Vo-Dinh and Cullum 2000). For example in case of microbial biosensors viable or non-viable microbial cells are utilized. Non-viable cells obtained after permeabilisation or whole cells containing periplasmic enzymes have been used as a cheaper alternative for enzymes. Viable cells utilize the respiratory and metabolic functions of the cell, thus the analyte may be monitored being either a substrate or an inhibitor of these processes (D'Souza 2001). The sensitivity of the cell-based biosensors (CBBs) for certain agonist can be deduced by the receptor-ligand combination constant. CBBs may be applied to analyse the effect of pharmaceutical compound on a given physiological system (Xu et al. 2002). There are many complex obstacles when living cells were treated as the primary biosensor, including the selection, the culture and the maintenance of living cells. The coupling of living cells and the secondary sensor represents one of challenges (Wang et al. 2005). On the other hand, cell-based

biosensors can perform real-time bioassays dynamically and rapidly, and have numerous applications ranging from biomedicine to the environment, for example for the detection of pathogens, toxins or for agent classification (Aravanis et al. 2001, Banerjee et al. 2010, Dragone et al. 2009, Jacobs et al. 2009, Liu et al. 2007, Pancrazio et al. 1998).

Transducers

Transducer is an analytical tool which provides an output quantity having a given relationship to the input quantity (McNaught and Wilkinson 1997). Biosensors can be classified according the transduction methods they utilize (Fig. 4). Most forms of transduction can be categorized in one of five main classes: electrochemical, electrical, optical, piezoelectric (mass detection methods) and thermal detection.

Electrochemical

The basic principle for this class of biosensors is that chemical reactions between immobilized biomolecule and target analyte produce or consume ions or electrons, which affects measurable electrical properties of the solution, such an electric current or potential (Thevenot et al. 1999).

Amperometric

Amperometric biosensors are the most widespread class of biosensors. Most of biochemicals can now be detected and quantified amperometrically by their enzyme-catalyzed electro-oxidation or electroreduction, or their enzyme-catalyzed hydrolysis/phosphorylation followed by electro-oxidation/electroreduction, or their involvement in a bioaffinity reaction enabling electro-oxidation/electroreduction (Heller 1996). Amperometric biosensors are very sensitive and more suitable for mass production than the potentiometric ones (Ghindilis et al. 1998). The working electrode is usually a

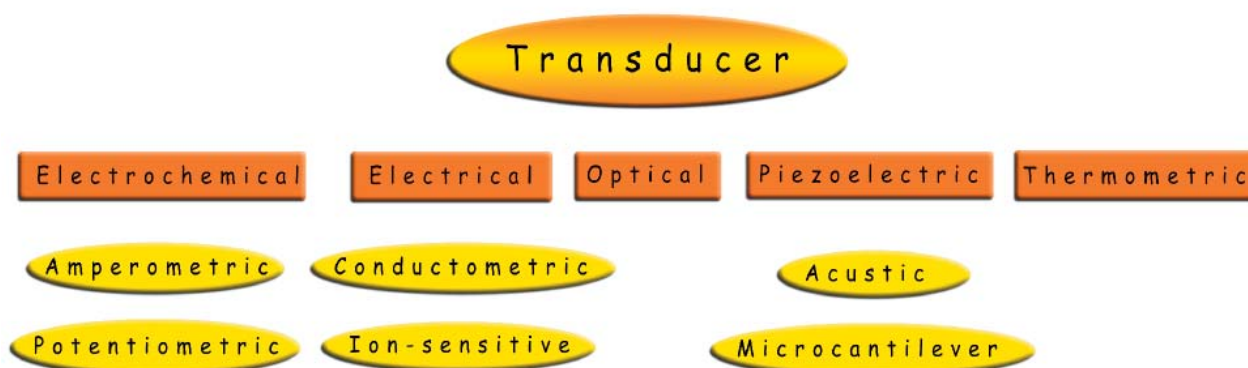


Fig. 4. Classification of transducers used in biosensors.

noble metal or screen-printed layer covered by the bioelement (Wang 1999). Modern option is to use carbon nanotubes (Jacobs et al. 2010). At the applied working potential, conversion of electroactive species generated in the enzyme layer occurs at the electrode and the resulting current (typically from nA to μ A range) is measured (Mohanty et al. 2006). Detailed examples of amperometric biosensors were reviewed by Dzyadevych et al. (2008). Amperometric biosensors utilize for their biochemical reaction mediators, i.e. molecules which are able to transfer electrons. They can participate in the redox reaction with the biological component and help in the faster electron transfer. According to Chaubey and Malhotra, they may be defined as a low molecular weight redox couple, which shuttles electrons from the redox centre of the enzyme to the surface of the indicator electrode (Chaubey and Malhotra 2002). As a result we can work with low potentials, thus the influences of oxygen (in case of oxidase) and of different interferants on response decrease.

An optimal mediator should be stable, able to react rapidly with target molecule, exhibit reversible heterogeneous kinetics, the overpotential for the regeneration of the oxidized mediator should be low and pH independent, and reduced form should not react with oxygen (Chaubey and Malhotra 2002). Mediators allow to measure at low working potentials and to avoid the interference with unwanted species. Measurements are thus less dependent on oxygen concentration and if the electrochemical reaction does not involve protons, the enzyme electrode becomes relatively pH insensitive. Very often used mediators are inorganic redox ions such as ferricyanide (Chen et al. 2010, Trivedi et al. 2009), organometallic compound ferrocene (Çevik et al. 2010) or organic dyes methylene blue (Wu et al. 1998), toluidine blue (Voštiar et al. 2002), or prussian blue (Wang et al. 2009).

Potentiometric

This transducer measures difference in potential that is generated across an ion-selective membrane separating two solutions at virtually zero current flow. Nearly all potentiometric sensors, including glass electrodes, metal oxide based sensors as well as ion-selective electrodes, are commercially available. Moreover, they can be easily mass-fabricated in the miniature formats using advanced modern silicon or thick-film technologies (Koncki 2007).

Electrical

Conductometric (Impedimetric)

When ions or electrons are produced during the course of biochemical reaction, the overall conduc-

tivity or resistivity of the solution is changing. The measured parameter when using this transducer is the electrical conductance/resistance of the solution. Conductance measurements have relatively low sensitivity. When using a sinusoidal voltage (AC) the electric field is generated which finally minimize undesirable effects such as Faradaic processes, double layer charging and concentration polarization (Mohanty and Kougiarios 2006). The inverse value of resistance is called conductance and thus the name conductometric has been used. The impedance biosensor is commonly a functional part of the Wheatstone bridge (Pohanka and Skládal 2008). Novel trends in case of impedimetric biosensors were reviewed by Guan et al. (Guan et al. 2004) and the use of conductometric biosensors for biosecurity by Muhammad-Tahir and Alocilja (2003).

Ion-sensitive

Biosensors based on ion-selective field-effect transistors (ISFETs) earlier considered as a category of potentiometric sensor, are now, according to the last IUPAC technical report on electrochemical biosensors, separated into the fourth class of electrochemical sensors (Thévenot et al. 1999). ISFET is a classical metal/oxide/semiconductor (MOS) field-effect transistor with a gate formed by a separated reference electrode and attached to the gate area via an aqueous solution (Dzyadevych et al. 2006). These semiconductor FETs have an ion-sensitive surface. The surface electrical potential changes due to the interaction between ions and the semiconductor. This change in the potential can be subsequently measured. ISFET can be constructed by covering the sensor electrode with a selectively permeable polymer layer, through which ions may diffuse and cause a change in the FET surface potential. This type of biosensor is also called an ENFET (Enzyme Field Effect Transistor) (Mohanty and Kougiarios 2006). Enzyme biosensors based on ISFETs were reviewed by Dzyadevych et al. (2006).

Optical

The output transduced signal that is measured is light. The biosensor can be based on fluorescence or optical diffraction. Fluorescence is often used for biosensing due to its selectivity and sensitivity. A fluorescence-based device detects the change in frequency of electromagnetic radiation emission which is caused by previous absorption of radiation and also by generation of an excited state lasting for a very short time. Single molecules may be repeatedly excited to produce a bright signal which can be measured even at single-cell level (Velasco-Garcia 2009). Optical diffraction based devices utilize

a silicon wafer coated with a protein via covalent bonds. The wafer is exposed to UV light through a photo-mask and the antibodies are thus inactivated in the exposed regions. Antigen-antibody bindings are formed in the active regions when wafer chips are incubated in an analyte. This allows creation of diffraction grating producing a diffraction signal when illuminated with a laser or other light source. Thus obtained signal can be further amplified or directly measured (Mohanty and Kougiianos 2006). Fiber-optic biosensors (FOBS) use optical fibers for signal transduction, and are dependable only on optical transduction mechanisms for detecting target biomolecules. Typical example of reliable and sensitive optical method is evanescent sensing. A majority of evanescent FOBS are tapered fiber-optic biosensors. For detailed information about fiber optic biosensors we recommend review from Leung et al. (2007). Surface plasmon resonance (SPR) biosensors are optical sensors using special electromagnetic waves – surface plasmon-polaritons – to monitor interactions between an analyte in solution and a bioelement immobilized on the SPR sensor surface. The main application of this type of biosensors is the detection of biological analytes and analysis of biomolecular interactions where SPR biosensors provide advantage of label-free real-time analytical technology (Homola 2003).

Piezoelectric (mass-sensitive)

These biosensors are based on the coupling of the bioelement with a piezoelectric component, usually a quartz-crystal coated with gold electrodes. Many types of materials (quartz, tourmaline, lithium niobate or tantalate, oriented zinc oxide or aluminium nitride) exhibit the piezoelectric effect. However, the properties of quartz are the main reason for its common usage for analytical applications (Cooper 2003). Piezoelectric transducers allow label-free detection of molecules (Janshoff et al. 2000). These crystals can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. Based on this, the frequency of oscillation is dependent on the electrical frequency applied to the crystal as well as the crystal's mass. With increasing of the mass due to binding of molecules, the oscillation frequency of the crystal is changed and the resulting change can be measured electrically and finally used to determine the additional mass (both positive or negative one) of the crystal (mass-sensitive techniques) (Vo-Dinh T and Cullum 2000). There is a high interest in the application of piezoelectric devices, since it was realized that many possibilities for molecular sensing can be opened up once a suitable recognition layer or molecule is coated on the crystal. Moreover, piezo-

electric biosensors showed potential applications in food, environmental and clinical analysis (Tombelli et al. 2005).

Other type of a mass-sensitive biosensor is a microcantilever. This sensor (physical, chemical or biological) detects the changes in cantilever bending or vibrational frequency. The principle of this detection is based on the transduction of molecular adsorption and specific molecular interactions on a cantilever surface into the mechanical response change of a cantilever. Viscosity, density, and flow rate can be measured by detecting changes in the vibrational frequency (Vashist 2007).

Calorimetric (thermometric)

These biosensors are constructed by immobilization of biomolecules onto temperature sensors. Once the analyte comes in contact with the biocomponent, the reaction heat which is proportional to the analyte concentration is measured. The total heat produced or absorbed is proportional to the molar enthalpy and the total number of molecules in the reaction. The measurement of the temperature is via a thermistor, and such devices are called as enzyme thermistors. Thermal biosensors do not require frequent recalibration and are insensitive to the optical and electrochemical properties of the sample (Mohanty and Kougiianos 2006). Calorimetric biosensors were used for food, cosmetics, pharmaceutical and other component analysis (Antonelli et al. 2008, Bhand et al. 2010, Ramanathan et al. 2001, Vermeir et al. 2007).

Immobilization methods

The most commonly used immobilization techniques for construction of biosensors are physical adsorption (Nanduri et al. 1997), covalent binding (Schuhmann et al. 1990), matrix entrapment (Gupta and Chaudhury 2007), inter molecular cross-linking (Nenkova et al. 2010) and membrane entrapment (Fig. 5) (Pancrazio et al. 1998, Scouten et al. 1995, Sharma et al. 2003).

1. *Adsorption*: The physical adsorption utilizes a combination of Van der Waals and hydrophobic forces, hydrogen bonds, and ionic forces to attach the biomaterial to the surface of the sensor. Many substrates such as cellulose, collodion, silica gel, glass, hydroxyapatite and collagen are well known to adsorb biocomponents. This method is very simple, however, employed forces are not very strong and biomolecules attached by this method may be released or not persist.
2. *Covalent binding*: The sensor surface is modified to acquire a reactive group to which the biological materials can be attached. In case of enzymatic

biosensors it is through the functional group in the enzyme which is not essential for its catalytic activity. Usually, nucleophilic functional groups present in amino acid side chains of proteins such as amino, carboxylic, imidazole, thiol, hydroxyl etc. are used for coupling. This method improves uniformity, density and distribution of the bioelements, as well as reproducibility and homogeneity of the surfaces. Covalent immobilization may decrease or eliminate some common problems such as instability, diffusion and aggregation, or inactivation of biomolecules. This occurs when biomolecules are immobilized on sensor surfaces by polymer matrices. For this purposes the reagents such as glutaraldehyde, carbodiimide, succinimide esters, maleinimides and periodate are often used for covalent immobilization (Collings and Caruso Frank 1997).

3. *Matrix entrapment*: In this case biomolecules are trapped within the polymeric gel matrix. For this method the polyacrylamide, starch, alginate, pectate, polyvinyl alcohol, polyvinyl chloride, polycarbonate, polyacrylamide, cellulose acetate and silica gel are often be used. Matrix entrapment has disadvantage of possible leakage of the biological species during use, resulting in a loss of activity (Collings and Caruso Frank 1997).
4. *Cross-linking*: For intermolecular cross-linking of biomolecules bi-functional or multi-functional reagents such as glutaraldehyde, hexamethylene di-isocyanate, 1,5-difluoro 2,4-dinitrobenzene and bisdiazobenzidine-2,2'-disulphonic acid, etc., are used. The most common cross-linking agent in biosensor applications is glutaraldehyde, which couples with the lysine amino groups of enzymes. This method has also some disadvantages, e.g. the enzyme layer formed is not rigid; there are higher demands for amount of biological material; cross-linking can cause the formation of multilayers of enzyme, which negatively affects the activity of the immobilized layers. Moreover larger diffusional barriers may delay interactions (Collings and Caruso Frank 1997).
5. *Encapsulation*: In this method a porous encapsulation matrix (e.g. lipid bilayers) is formed around the biological material and helps in binding it to the sensor. Other approach for encapsulation uses sol-gel method for the immobilization of biological molecules in ceramics, glasses, and other inorganic materials using. In the sol-gel procedure, biological molecules are entrapped in a porous matrix, such as a polymeric oxo-bridged SiO₂ network. These matrices allow optical monitoring of the chemical interactions since they are optically transparent. The sol-gel process can

be performed at room temperature and which protects biomolecules against denaturation. Biomolecules immobilized by this procedure are very stable, but achieving of sol-gels with reproducible pore sizes seems to be still an obstacle. Problems such as diffusional limitations inside the porous network, brittleness of the glassy matrix, reproducibility or discrepancies in the preparation procedures has to be solved before this procedure can be used for routine application (Collings and Caruso Frank 1997).

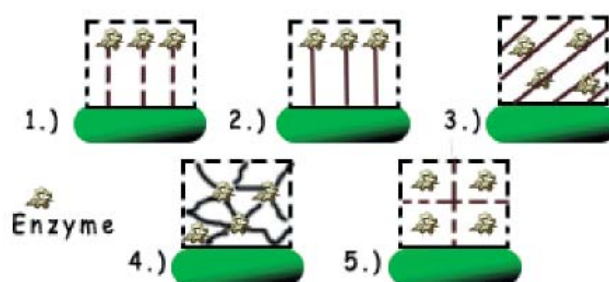


Fig. 5. Methods used for immobilization of enzymes and other bioreceptors in biosensors. 1.) Adsorption, 2.) Covalent binding, 3.) Matrix entrapment, 4.) Cross-linking, 5.) Encapsulation.

Nanotechnology innovations

Carbon nanotubes (CNTs) are new members of the carbon family providing unique mechanical and electronic properties with chemical stability (Luo et al. 2001). Possible applications of carbon nanotubes are now carefully investigated because of their very unusual properties (Tkáč and Ruzgas 2005). CNTs play an important role in nanotechnology including fields such as engineering, biology, chemistry, medicine, electronics and material science. CNTs can be nondestructively oxidized along their side-walls or ends and covalently functionalized with colloidal particles or polyamine dendrimers via carboxylate chemistry. Moreover, proteins may individually adsorb noncovalently and strongly along nanotube lengths. And next, electrical communication is possible between a redox-active biomolecule and the delocalized π system of its carbon nanotube support (Davis et al. 2003).

There are two groups of carbon nanotubes, multi-wall carbon nanotubes (MWCNTs) and single-wall carbon nanotubes (SWCNTs) (Laschi et al. 2008). Double-walled carbon nanotubes are sometimes considered as a separate group. MWCNTs can be described as concentric and closed graphite tubules with multiple layers of graphite sheet, defining a hole typically from 2 to 25 nm, separated by a distance of approximately 0.36 nm. SWCNTs consist

of a single rolled graphite sheet creating a cylinder of 1–2 nm diameter. Electrochemical properties of CNTs are connected with their pre-treatment before applying on an electrode surface. For the optimal electrochemical properties of nanotubes, the creation of open ends is important, so CNTs are usually purified in acids such as HNO₃ or H₂SO₄ for this purpose (Gooding 2005).

CNTs can behave as metals or semiconductors depending on the structure, mainly on the diameter and helicity (Rubianes and Rivas 2003). They have the ability to mediate electron-transfer reactions with electroactive species when used as an electrode (Britto et al. 1996). The ability of CNTs to allow electrochemistry of many compounds at low potential is promising for preparation of electrochemical biosensors, especially, for medical purposes (Gooding 2005). CNTs were successfully used for construction of biosensors for DNA (Wang et al. 2004), glucose (^cMonošík et al. 2012, Wang et al. 2003), lactate (^fMonošík et al. 2012), cholesterol (Li et al. 2005) detection and for others analytes (Wang 2005).

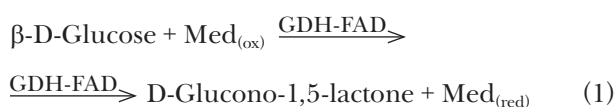
Nanoparticles also exhibit unique chemical, physical, and electronic properties. The main difference from bulk materials is their high surface-to-volume ratio which improves the performance of biosensors (Luo et al. 2006). Variety of nanoparticles such as metal, oxide, semiconductor or composite nanoparticles can be used in biosensors. Moreover, different kinds of nanoparticles may play different roles in different biosensor systems. For example, gold nanostructured thin-film electrodes were used as a surface for immobilization of DNA by double-stranded DNA absorption (Flickyngerova et al. 2008). Gold nanoparticles showed also potential to detect glucose in the micromolar concentration range. Amperometric biosensors modified by silver nanoparticles showed improved biocompatibility utilized in pesticide detection. Functional nanoparticles (electronic, optical, and magnetic) bound to biological molecules (e.g. peptides, proteins, nucleic acids) were developed in order to detect and amplify various signals (Huang et al. 2009, Chen et al. 2004, Galandová and Labuda 2009).

Biosensor applications

Clinical diagnosis

Although biosensor development made a huge progress in recent years, their application in clinical diagnosis is not very common, except for glucose biosensors representing about 90 % of the global biosensor market. Interferences with undesired molecules during measurements with real samples and also high selectivity and accuracy are still serious

issue. This is very important, since treatment is often dependent on individual levels of clinical markers. The most of the described biosensors are based on amperometric techniques what may indicate trends in biosensors development (Belluzo et al. 2008). Glucose concentration is one of the most monitored indicators in many diseases, such as diabetes and other endocrine metabolic disorders. Blood glucose is also the most common analyte measured after electrolytes and blood gases (Malhotra and Chaubey 2003). The most suitable concept for glucose determination is a biosensor utilizing the highly specific FAD – dependent glucose dehydrogenase (GDH-FAD) and oxidized form of a mediator (Med_(ox)) based on the reaction:



Reduced form of a mediator (Med_(red)) is re-oxidized on the working electrode at applied constant potential and resulting electric current is proportional to the glucose concentration. This model is now utilized in several commercial glucose biosensors. Extensive review of commercially available biosensors for glucose, cholesterol, lactate, triglycerides and creatinine determination can be found in the review by Monošík et al. (^bMonošík et al. 2012).

Food control

Food industry and biotechnology are the fields where biosensor applications are not as common as in the field of medical diagnostics (Dzyadevych et al. 2008). This can be explained that while in the medical area the main matrices are blood, serum or urine, in the food industry sector there are more types of samples with very variable composition. This makes the process of biosensor design, unification and optimization of measurement conditions more difficult. Company Biorealis Ltd together with Department of Nutrition and Food Assessment at Faculty of Chemical and Food Technology and with Institute of Measurement Science, Slovak Academy of Sciences developed the portable analytical device *Omnilab* utilizing biosensors (<http://www.biorealis.sk/index.php?content=intro&lan=en>). Amperometric biosensors designed for this device are based on oxidoreductase enzymes and analytes such as glucose, fructose, glycerol, lactic, malic or acetic acid can be measured in wines and beverages. The latest information regarding biosensor application for food processing, safety, and quality control can be found in the review from Monošík et al. (^aMonošík et al. 2012) and in the books from Mutlu (2010) or Orellana and Moreno-Bondi (2010).

Environmental screening

In environmental pollution monitoring, chemical analysis by itself may not provide sufficient information to assess the ecological risk of polluted waters and wastewaters (Castillo et al. 2001). In the European Union, along with more strict demands for water treatment (Council Directive 91/271/EEC), industrial and urban wastewater effluents have to conform certain limits of toxicity before the effluent can be discharged into the environment. Due to this, lot of bioassays and biosensors for toxicity evaluation were developed in recent years. For example, the toxicity assays Microtox® (Azure, Bucks, UK), is based on the use of luminescent bacteria, *Vibrio fischeri*, to measure toxicity from environmental samples. Other example is the Cellsense®, which is an amperometric sensor that incorporates *Escherichia coli* bacterial cells for rapid ecotoxicity analysis. It uses ferricyanide to divert electrons from the respiratory system of the immobilized bacteria of a suitable carbon electrode. The resulting current is proportional to a bacterial respiratory activity (Rodríguez-Mozaz et al. 2004).

It is known that endocrine disruptors may bind to the estrogen receptor (ER) as agonists or antagonists. Thus, several biosensors using estrogen receptors were developed and applied for screening or testing potential environmental toxicity providing useful information about estrogenic potency of the sample. Simplicity of these assays is their considerable advantage (Sara Rodríguez-Mozaz et al. 2004).

The presence of pesticides in natural waters is caused by their extensive use for agricultural purposes. Although HPLC/MS and GC/MS techniques provides satisfactory results for pesticide determination, new assays and biosensors represents cheaper and faster way for on-site analysis. Biosensors utilizing the inhibition of a selected enzyme are the most common biosensors used for the determination of pesticides. The principles of inhibition of acetyl cholinesterase (AChE) and choline oxidase were used for several biosensors fabricated for the detection of organophosphorous and carbamate pesticides (Mostafa 2010; Silvana and Jean-Louis 2006). Biosensors based on AChE inhibition are not selective, since the AChE is inhibited by neurotoxins, which include organophosphorous pesticides, carbamate pesticides, and many other compounds. For this reason they cannot be used for quantification of either an individual or a class of pesticides (Rodríguez-Mozaz et al. 2004). The biosensors mentioned above and also other types of biosensors designed for detection of environmental pollutants such as phenols, surfactants, alkanes, aromatic compounds, and polycyclic aromatic hydrocarbons, antibiotics etc., were thoroughly described and

discussed in the book Environmental Biosensors (Somerset 2011).

Conclusion

This review describes and characterizes different classes of biosensors according to utilized types of bioelements, transducer and methods of entrapment. Working principles, constructions, advantages, and applications of many biosensors are presented. Biosensors represent promising analytical tools applicable in areas such as clinical diagnosis, food industry, environment monitoring and in all fields, where rapid and reliable analyses are needed. Some biosensors were successfully implemented in the commercial sphere, but majority needs to be improved in order to overcome imperfections. The overall commercial status and acceptance will depend on their accuracy, reliability, cost of devices, price and time consumption of individual analysis, etc. There is also a real need to measure a group of analytes at once, which will complicate the process of biosensor development, because interferences with unwanted molecules are often a problem and a cause of failure of biosensors. The next generation of biosensors based on nanostructures could lead to a construction of devices able to markedly compete with other analytical methods used today.

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