

Enzyme-Based Fluorescent Biosensors and Their Environmental, Clinical and Industrial Applications

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Abstract

Enzyme-based fluorescence biosensors and their applications in environmental protection, medicine, and industry are described. Biosensors used in environmental protection measure toxicity effects. A chemical compound or group of compounds is detected by the recognition of molecules in the receptor layer and then by detecting a signal passing through the transducer layer. Biosensors are classified according to the transduction method. Special emphasis is placed on optical biosensors, especially fluorescent biosensors, and such measurement techniques as FRET (Fröster resonance energy transfer), FLIM (fluorescence lifetime imaging), FCS (fluorescence correlation spectroscopy), and changes in fluorescence intensity. The phenomenon of fluorescence in biosensors and the selection of appropriate methods are described. The use of enzymes in the receptor layer and enzyme classification according to its category and functions used for analyte detection are presented. The fluorescence properties of enzymes resulting from possessing such cofactors as flavin or heme (prosthetic) groups are discussed. Several methods for enzyme immobilization, namely entrapment, adsorption, covalent immobilization, cross linking, and affinity interaction are described, and the use of enzymatic fluorescence biosensors in the detection of analytes is presented.

Keywords: biosensor, enzyme, fluorescence, analyte, recognition

Introduction

The last two decades has seen a significant increase in interest in biosensors. According to the Web of Science database, the number of scientific publications over the past 20 years increased from 309 in 1993 up to 3,467 in 2013 (Fig. 1).

The demand for developing new detection methods, increasing interest in visualization techniques [1-3], emphasis placed on new drug discovery programs [4], diagnostic test development [5-7], and attempts to explain cellular action mechanisms and to trace a cell's metabolism path-

way [7, 8], and the possibility of using biosensors in numerous fields of application, (for example: medicine [9-11], environmental protection [12-14, 16-17], food industry [18], and defense industry [19]) has results in an increasing number of research projects targeted at biosensor design and fabrication. Biosensors used in environmental monitoring measure the toxicity effect based on the detection of a chemical compound or compound group by selective recognition of a biomolecule in the receptor layer and then by detection of a signal after passing through the transducer layer [16].

The application of fluorescence biosensors in environmental protection applications [20-25], medical diagnostics [26-35], and industries [36-45] also is growing.

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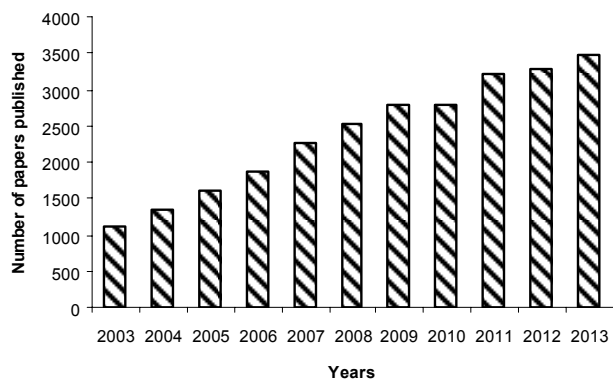


Fig. 1. Illustration of an increasing number of publications by year in Web of Science based on the “biosensor” keyword in 1993-2013.

An increasing interest in biosensors also is driven by a growing market that is estimated by Transparency Market Research to reach 18.9 billion USD in 2018 [46].

According to the definition recently propose by the International Union of Pure and Applied Chemistry (IUPAC 1999):

A biosensor is a self-contained integrated device capable of providing specific quantitative or semi-quantitative analytical information using elements retained in direct spatial contact with a transduction element [47].

A biosensor is a device consisting of two main elements: bioreceptor and transducer.

The transducer transforms the recognized element into a signal to be detected based on the phenomenon occurring in the receptor layer. The signal is generated from interaction between the analyte and the biosensor receptor layer. The intensity of the generated signal is proportional to the analyte concentration [48].

Based on the nature of the obtained signal, an appropriate detection technique is selected. Considering that fact, biosensors can be categorized into the following categories: electrochemical, including amperometric, potentiometric, and impedimetric techniques; mass detection, including magnetoelastic, piezoelectric and optical techniques, especially spectrofluorometric methods such as FRET (Fröster resonance energy transfer), FLIM (fluorescence lifetime imaging), and FCS (fluorescence correlation spectroscopy), and changes in fluorescence intensity. Classification of biosensors with respect to the transducer employed is shown in Fig. 2.

A bioreceptor contains an immobilized sensitive biological element (e.g. protein, enzyme, antibody, DNA) that recognizes the analyte that could be an ion enzyme substrate, or antigen, or complementary DNA.

Although antibodies, oligonucleotides, and proteins are widely used, enzymes belong to the most common biodection elements in biosensors [48].

Fluorescent Biosensors

Fluorescent biosensors are analytical devices for non-invasive detection of biomolecules present in a complex environment of biological samples.

Fluorescent biosensors work based on the phenomenon of fluorescence (Fig. 3). Fluorescence is an emission phenomenon that occurs when electromagnetic radiation is absorbed by fluorophores or fluorescently labeled molecules.

When the wavelength of emitted radiation is shorter or equal to the excitation wavelength, we speak about fluorescence resonance. If a loss of emitted energy occurs, the fluorescent emission wavelength is longer than the excitation wavelength and this is called the Stokes shift [49].

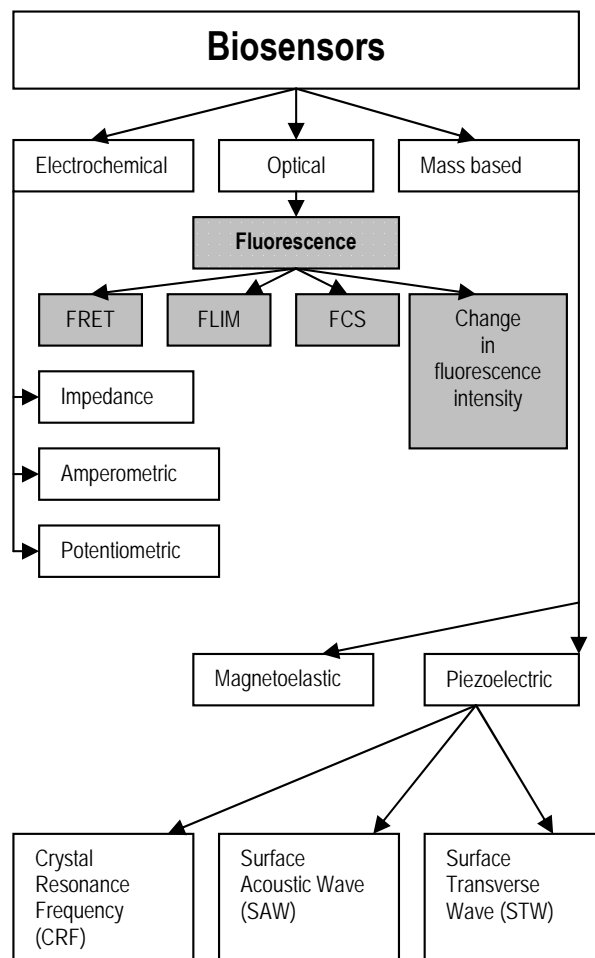


Fig. 2. Classification of biosensors based on type of transducer.

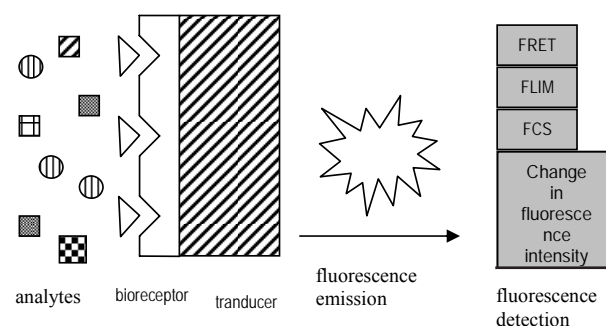


Fig. 3. Fluorescence in biosensors and fluorescence detection techniques.

When fluorescence occurs, the sample generates an optical measurable signal.

The three most common ways to generate a signal are:

- Analyte is recognized by a bioreceptor (signal reflects the presence of analytes and their concentrations)
- Conformational changes (signal reflects specific changes in bioreceptor array conformation)
- Activity changes (signal reflects enzyme activity)

The generated signal is detected by employing one of the following methods: FRET (Fröster resonance energy transfer), FLIM (fluorescence lifetime imaging), FCS (fluorescence correlation spectroscopy), and change in fluorescence intensity.

FRET (Fröster Resonance Energy Transfer)

This technique uses transfer of energy between the donor and a suitable acceptor. Energy transfer occurs when the distance between the donor and acceptor is not larger than 10 nm and the dipoles of both molecules are oriented appropriately. This method allows changes in bioreceptor surface activity induced by external factors to be determined [7, 50].

FLIM (Fluorescence Lifetime Imaging)

This technique is used for imaging biological tissues and reactions that take place in living cells. This method enables an average lifetime when a molecule remains in its excited state after absorbing a photon to be determined. By using this method it is possible to get information on changes in the fluorophore local environment or changes in its energy in response to interactions with the local environment [50, 52].

FCS (Fluorescence Correlation Spectroscopy)

This method is used for molecules in low concentrations. Small deviations from spontaneous fluorescence intensity of the sample are analyzed to gain information on kinetics of thermodynamic processes related to reversible fluorescence changes, e.g. diffusion coefficient, flow rate, molecular concentration [50, 52].

Change in Fluorescence Intensity

This technique is based on direct measurements of fluorescence intensity (fluorescence emission), i.e. the response of the system to excitation. A decrease or increase in fluorescence intensity or blue or red shifts may be connected with changes on the bioreceptor surface. This technique is commonly used for fluorescence investigations of biosensors containing an enzyme in its bio-sensitive layer [19, 53-56].

Enzymes Used in Biosensors

Enzymes used in biosensors are often chosen because of their binding properties and catalytic activity [6].

Table 1. Six classes of enzymes and their functions used in the detection of analytes.

No.	Enzyme category	Function
1	Oxidoreductases	Oxidation-reduction reactions
2	Transferases	Group transfer
3	Hydrolases	Hydrolysis reaction (transfer of functional group to water)
4	Lyases	Addition or removal of groups to form double bonds
5	Isomerases	Izomerization (intramolecular group transfer)
6	Ligases	Joining of two molecules

Enzymes are used for selective detection of analytes due to their special functions. Enzyme categories and functions used for analyte detection are listed in Table 1. [57, 58]. Oxidoreductases were used to detect glucose [59], fructose [60], amino acids [61], and lactates [62]. Transferases were used in biosensoric analysis of atrazine [63], and hydrolases to determine saccharose [64]. Lyases (phenylalanine ammonia lyases) were used to determine phenylalanine content in neonatal subjects [65]. Isomerases were used to interact with drug samples [66], and ligases were employed to detect non-nucleic acids [79].

Enzymatic biosensors are the main part of biosensor technology that currently is an analytical technique used in clinical diagnosis, environmental monitoring, and industrial applications. Enzyme-based fluorescent biosensors used in optical measurement enzyme properties result from various enzymatic reactions.

One of the most convenient and simple means for detecting chemical substances is generating an optical signal. An analysis is based on changes in the fluorescence and absorption spectra [19]. Enzymes find application in biosensor technology. Alkaline phosphatase (hydrolase enzyme) and creatine kinase isoenzyme (CK-MB) belong to the most frequently tested enzymes in clinical practice.

Creatine kinase isoenzyme (CK-MB) is widely used as a marker of myocardial infarction [67, 68], while changes in alkaline phosphatase (ALP) levels are linked to various diseases, for example bone diseases, carcinoma of the breast, and prostate [69, 70]. To develop quick and convenient ALP tests, fluorescence methods were used [69]. Hydrolases such as acetylcholinesterase (AChE) and organophosphorous hydrolase (OPH) and its fluorescence properties were used in the defense industry to detect toxic warfare agents [19]. The biosensor based on cellobiose dehydrogenase (CDH) is used both in industry and environmental protection [54]. Biosensors containing nitrate reductases (NR), phosphatases, and ureases [15] were used in environmental research, like tyrosinases for detecting phenols and catechols in food and pharmaceuticals, as well as in clinical and environmental samples [16]. Examples of potential applications of enzymes are presented in Table 2.

Table 2. Potential clinical, industrial, and environmental applications of enzyme-based biosensors.

No.	Enzyme category	Enzyme name	Application			References
			clinical	industrial	environmental	
1	Transferases	isoenzyme creatine- kinase (CK-MB)	x			[67]
2	Hydrolase	alkaline phosphatase (ALP)	x			[69]
3	Hydrolase	acetylcholinestase (AChE)		x		[19]
4	Hydrolase	organophosphorous hydrolaze (OPH)		x		[19]
5	Oxidoreductases	cellobiose dehydrogenase (CDH)		x	x	[54]
6	Oxidoreductase	nitrate reductase (NR)			x	[15]
7	Hydrolase	phosphatase			x	[15]
8	Oxidoreductase	tyrosinase			x	[17]
9	Oxidoreductase	urease			x	[15]

Fluorescence Properties of Enzymes

Enzyme molecules have a special pocket called the active site. The active site contains the amino acid side chain that forms a 3D surface complementary to the structure of the substrate. This binds the substrate and forms a substrate-enzyme complex. The complex decomposes to a product of reaction and the enzyme. Some enzymes connected with non-protein cofactors requiring metal ion cofactors (Zn^{2+} , Fe^{2+}) or organic molecules called the coenzymes (coenzyme A, NAD, FAD) to show biological activity [72]. These enzyme groups are referred to as cofactors. Another group contains holoenzymes. In the absence of a suitable cofactor, a typical apoenzyme shows no biological activity. They are able to bond a suitable substrate, but they are unable to convert the substrate. In addition, bond substrates may cause conformational changes that can be easily determined by fluorescence measurements [55].

Enzymes, mainly those containing flavin or heme groups, as cofactors have fluorescence properties that are widely used in research on fluorescent biosensors.

Flavoenzymes have intrinsic fluorescence (and absorption) in the visible region of the spectrum because of the presence of flavin groups [5]. It is commonly known that optical properties (molecular absorption and fluorescence) of flavin groups depend on the rate of oxidation (FAD , $FAD.H_2$) and its bonding (bound in a solution or bound to protein). Fluorescence of FAD solutions occurs in the visible spectrum, while $FAD.H_2$ is almost non-fluorescent.

Optical biosensors were designed based on optical properties of FAD , $FAD.H_2$.

During the enzymatic reaction, changes in fluorescence and absorption that can be assigned to the analyte are observed [71].

Proteins containing heme groups show molecular absorption and fluorescence due to the presence of heme groups. During the enzymatic reaction the central iron atom in oxidation state is replaced, thus causing changes in absorption [5].

Enzyme Immobilization in the Receptor Layer as a Factor Improving Biosensor Performance

Enzymes are very efficient biocatalysts capable of specific recognition of its substrates and accelerate its transformation. Such unique properties allow enzymes to be used as a powerful tool in the development of medical analysis. Enzyme-based biosensors work together with the biocatalyst contained in the sensitive surface of the transducer layer.

Enzyme immobilization is an important factor deciding on biosensor performance in the terms of its selectivity, repeatability, sensitivity, response time, and stability [67]. Enzymes in biosensors are normally immobilized in close vicinity to or on the transducer [72].

Depending on chemical and physical properties of the enzyme (enzyme nature), various enzyme immobilization methods are used [15, 57, 67, 72-74, 78].

- (i) *entrapment*: biosensors based on physical entrapment of enzymes are often characterized by an increase in storage stability. Enzyme activity is maintained during the immobilization process. This technique is relatively easy to perform [72]. The enzyme and its additives can be easily placed in the same envelope. To immobilize enzymes, photopolymerization matrices [73], electro-polymerization films [53], and sol-gel matrices [53] are used.
- (ii) *adsorption*: physical adsorption is the simplest way of enzyme immobilization and has the weakest effect on enzyme denaturation. The advantage of this method is its simplicity, low cost, and possible regeneration [72], while the disadvantage is low storage stability. The procedure consists in simple deposition of the enzyme on the electrode material and binding it by van der Waals forces [72,76, 77].
- (iii) *covalent immobilization*: is frequently used in optical biosensors [72]. The biosensor surface is modified to gain reactive groups to which biological material can be coupled.

It is thought that in enzymatic biosensors, enzyme functional groups are used for coupling. Covalent immobilization improves surface uniformity and increases measurement repeatability in the context of biosensors [57].

- (iv) *cross linking*: in this method glutaraldehyde is most frequently used in binding to lysine of enzyme amine groups [57]. Glutaraldehyde is used during cross-linking to create strong chemical bonds [72].

Both crosslinking and covalent binding strengthen the biosensor surface by binding the enzyme, although enzymes become less dynamic and their activity is reduced [74].

- (v) *affinity interaction*: enzyme immobilization also can be achieved by affinity interaction between functional groups such as lectin or metal chelates and markers (e.g. biotin, histidine, cysteine) [72].

Enzymatic Fluorescent Biosensors for Analyte Detection

The detection of analytes is important because an intervention of undesired molecules into the biosensor receptor layer may damage it [57]. Therefore, intensive scientific research is carried out to find the best fit between the analyte and biosensor receptor layer (Table 3).

An intensity decrease in fluorescent spectra of pyruvate kinase has led to detection of Na^+ , Mg^{2+} , Ca^{2+} , and K^+ [55]; in searching for 'nerve agent' the following two enzyme types were used: acetylcholinesterase (AChE) and organophosphorus hydrolase (OPH) [19].

To determine H_2O_2 , an enzymatic auto-indicator biosensor based on modified catalase was used [54].

The enzyme ChOx/HRP in combination with a water-soluble polymer was used for detecting choline [56]. A fluorescence method was also used for measuring signals of an oxygen-sensitive indicator to monitor enzymatic phenol oxidation when oxygen is consumed [53].

Conclusion

In this paper enzyme-based fluorescent biosensors are described based on recent scientific reports. This sensor type is commonly used in clinical diagnostics, environmental protection, and the defense industry, and also in those areas where rapid and reliable measurements are required.

Currently, the efforts of research groups are focused on enhancing biosensor selectivity, sensitivity, and repeatability to improve its operating parameters and detection quality.

Despite the quickly increasing number of publications, biosensors still provide room for research, and the obtained results will lead to a new generation of these devices in the future.

Table 3. List of enzymes and analytes detected on the basis of fluorescence spectral.

No.	Analyte	Enzymes	References
1	Na^+	Pyruvate kinase (PK)	[55]
	Mg^{2+}		
	Ca^{2+}		
	K^+		
2	Organophosphorus pesticide paraoxon	Acetylcholinesterase (AChE)	[19]
3	Paraoxon	Organophosphorus hydrolase (OPH)	[19]
4	H_2O_2	Catalase-ruthenium (Cat-Ru)	[54]
5	Choline	ChOx/HRP	[56]
6	Phenol	Tyrosinase (TYR)	[53]

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