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Review

The phenomenon of fluorescence in immunosensors

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The phenomenon of fluorescence in immunosensors is described in this paper. Both structure and characteristics of biosensors and immunosensors are presented. Types of immunosensors and the response of bioreceptor layers to the reaction with analytes as well as measurements of electrochemical, piezoelectric and optical parameters in immunosensors are also presented. In addition, detection techniques used in studies of optical immunosensors based on light-matter interactions (absorbance, reflectance, dispersion, emission) such as: UV/VIS spectroscopy, reflectometric interference spectroscopy (RIfs), surface plasmon resonance (SPR), optical waveguide light-mode spectroscopy (OWLS), fluorescence spectroscopy. The phenomenon of fluorescence in immunosensors and standard configurations of immunoreactions between an antigen and an antibody (direct, competitive, sandwich, displacement) is described. Fluorescence parameters taken into account in analyses and fluorescence detection techniques used in research of immunosensors are presented. Examples of immunosensor applications are given.

Key words: biosensor, immunosensor, fluorescence, absorbance, UV/Vis

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BIOSENSORS AND IMMUNOSENSORS

Biosensors are one of the greatest challenges in biotechnology, while simultaneously being one of the most promising fields in developing new generation sensors. They offer a fast, selective and sensitive detection of various components such as: pesticides (Meng *et al.*, 2013; Sassolas *et al.*, 2012; Mostafa, 2010), heavy metal ions (Bontidean *et al.*, 2003; Kamtekar *et al.*, 1995; Kulkarni *et al.,* 2011), chemical warfare agents (Burnwotrh *et al.*, 2007), bacteria (Sapsford *et al.*, 2004; Ivnitski *et al.*, 1999), viruses (Nidzworski *et al.,* 2014; Sapsford *et al.*, 2004; Xu *et al.*, 2007), tumor markers (Zhang *et al.*, 2013; Tothill 2009; Soper *et al.*, 2006), cardiac biomarkers (Mazher-Iqbal *et al.*, 2013).

Biosensors find wide applications in medical diagnostics, (Justino *et al.*, 2010), food industry (Leonard *et al.*, 2003), environmental protection (Kłos-Witkowska, 2015), arms industry (Bartoszcze, 2003), agricultural industry (Rana *et al.*, 2010) and biopharmaceutical research (Vo-Dinh & Cullum, 2000). According to the report (Thusu, 2010), biosensors are used in 47 applications and an increasing number of potential applications is recorded every year. These devices are most frequently used in clinical analyses and medical diagnoses. As defined by the International Union of Pure and Applied Chemistry (IUPAC 1999) biosensor is a self-contained integrated device capable of proving specific quantitative or semi-quantitative analytical information using elements retained in direct spatial contact with transduction element (Thevenot *et al.*, 1999).

Among a large family of biosensors there are immunosensors that in a sensitive selective receptor layer contain an immobilized biological component (antibody, antigen, hapten) being an immunological receptor for recognition of measurable analyte molecules.

The receptor layer directly contacts the transducer layer where the biological interaction is transformed into a measurable signal (Cruz *et al.*, 2002; Ramirez *et al.*, 2009).

Immunosensors are those biosensors that use a specific reaction between an antibody (Ab) and an antigen (Ag) or small molecules called haptens.

Haptens elicit the production of antibodies only when attached to larger molecules such as proteins.

Antibodies as immune-related proteins are very often referred to as immunoglobulins.

They consist of four polypeptide chains: two heavy and two light chains. They form Y-shaped molecules of molecular weight greater than 1.5 kDa. There are two types of light chains: $\varkappa$ and λ types and five types of heavy chains: α, δ, ε, γ, μ types.

Depending on the type of heavy chain present in an antibody, immunoglobulins are divided into five classes of different structure and immunological functions.

Immunoglobulins are used by the immune system to identify and neutralize foreign bodies, and show also binding properties to an antigen.

The types of antibodies and its function as well as references to its comprehensive description are listed in Table 1 and more deeply in the book (Goląb *et al.*, 2013).

The antibody-antigen bonds are the most compatible bonds for selectivity and affinity among all protein bonds (Leckband *et al.*, 2000). However, despite of a high affinity constant $K_a=10^{12}$–10^{14} (Borisov & Wolfbeis, 2008) they are not covalent bonds (Reverberi & Reverberi, 2007).

A high reaction rate, efficiency and affinity constant are a consequence of weak molecular interactions such as: Van der Waals forces, hydrogen bonds, ion-dipole bonds or hydrophobic bonds.

Knowledge of interaction mechanisms is a key for understanding antibody-antigen interactions (Reverberi &

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Abbreviations: UV/VIS, Ultraviolet–visible spectroscopy; RIfs, reflectometric interference spectroscopy; SPR, surface plasmon resonance; OWLS, optical waveguide light-mode spectroscopy; IUPAC, Union of Pure and Applied Chemistry; K_a, affinity constant; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; FRET, Fröster resonance energy transfer; ELISA, Enzyme Linked Immune Sorbent Assay)

Table 1. Types of antibodies and their function

No.	Antibodies	Functions	References
1	IgM	they are produced in the initial phase of the immune response to infection they recognize viral antigens and bacterial toxins	Quchida *et al*., 2012 Kaveri *et al*., 2012 Unzu *et al*., 2014 Ehrenstein & Notley, 2010 Grönwall *et al*., 2012
2	IgG	major antibody of secondary immune responses neutralizes the bacterial toxins and viruses	Hjelhold *et al*., 2013 Vidarson *et al*., 2014 Kulshrestha *et al*., 2013
3	IgA	acts as a first line defense against many invading pathogens key role in immune protection of mucous membranes	Woof & Kerr, 2006 Majkowska-Skrobek & Augustyniak, 2004 Snoeck *et al*., 2006
4	IgD	role is still incompletely understood function has been preserved since its ancient origins function is to signal the B cells to be activated and afterworld be ready to take part in the defense of the body in the immune system expression of IgD may rescue cells undergo programmed cell death	Edholm *et al*., 2011 Geisberger *et al*., 2006 Guo, 2010
5	IgE	is performed as part of an initial screen for allergies high *IgE* levels may indicate a parasitic infection	Amarasekera, 2011 Chang *et al*., 2015 Hamilton *et al*., 2010 Nepper-Christensen *et al*., 2003 Rosenwasser, 2011

Reverberi, 2007, Leckband *et al*., 2000). Such interaction is used in immunosensor design where the receptor layer can contain both antigens and antibodies.

Nevertheless, due to the loss of antibody affinity during the immobilization process, antigens are primarily used in the receptor layer, while antibodies perform the role of analytes (molecules to be detected) (Moina & Ybarra, 2012). During the detection process the binding of antibodies (Davies *et al*., 1988).

The mechanism of complex formation is commonly known as lock and key fit (Braden *et al*., 1995; Griffiths *et al*., 2014) and more vividly, it can be compared to fitting an appropriate key to the lock, where antibodies and antibodies perform the role of appropriate key and lock, accordingly.

During the complex formation process a signal is generated and the this signal is transformed to be then measured and analyzed with appropriate methods depending on its type. Depending on the type of an input signal, biosensors are divided into the following groups: optical, electrochemical, resistance, piezoelectric, thermometric (Monosik *et al*., 2012; Bhardwaj *et al*., 2014; Shruthi *et al*., 2014). According to the biological material used in the receptor layer we can distinguish enzymatic (containing an enzyme in the matrix), protein (using a protein in the receptor layer), nucleic (using DNA or RNA nucleic acids) biosensors (Korotkaya, 2014; Gabig-Cimińska *et al*., 2004; Cynk *et al*., 2012; Gabig-Cimińska *et all*, 2005; Suzuki *et al*., 1982) and immunosensors (where an antibody or antigen can be used) (Vo-Dinh *et al*., 2000). In immunosensors, analyte detection is based on an electrochemical, optical or mass change signal.

The types of immunosensors and receptor layer response to the interaction with analytes under investigation are presented in Fig. 1.

In electrochemical immunosensors the complex formation is indicated by parameter changes: amperage (amperometric immunosensors), potential difference (potentiometric immunosensor), resistance (conductometric immunosensor) (Moina & Ybarra, 2012). Electrochemical immunosensors were used for detecting protein tumor markers (Chikkaveeraiah *et al*., 2012; Chen *et al*., 2013; Yang *et al*., 2011).

Voltammetry and amperometry combined with immunosensors offer a fast, easy and low-cost detection of tumor markers due to a wide range of protein detectability.

A conductometric immunosensor based on magnetic nanoparticles was used for detecting *E. coli* (Hnaiein *et al*., 2008; Mujika *et al*., 2009).

However, in piezoelectric immunosensors the resonance frequency of oscillating piezoelectric crystals can be affected by its mass changes (Chen *et al*., 2011).

This type of sensors was used for detecting antigens of *Mycobacterium tuberculosis* (Kumar, 2000; Jaramillo *et al*., 2013).

Special attention should be paid to optical immunosensors that monitor optical changes resulting from the interactions with the analyte. These changes are

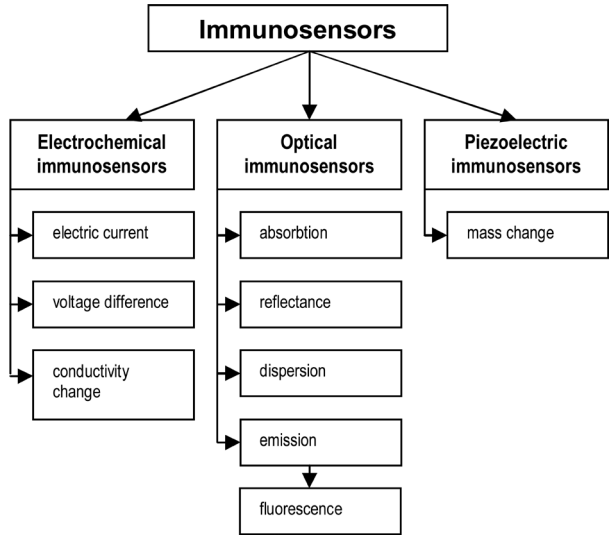

Figure 1. Types of immunosensors and response of bioreceptor layers to the interaction with the target analytes

Table 2. Mechanisms of light–matter interaction, detections techniques employed in optical immunosensors and its description

No.	Mechanism of light--matter interaction	Detection techniques	Description of methods	Literature
1	Absorbance	UV/VIS spectroscopy	This method uses electromagnetic radiation (200–1100 nm) Absorbance changes are caused by the interaction with an analyte Measurements are made in a transparent medium	Que *et al*., 2014 Ying *et al*., 2013 Gong *et al*., 2009
2	Reflectance	Reflectometric interference spectroscopy (RIfS) Surface plasmon resonance (SPR)	The technique based on white light interference in thin films Analyte binding changes the reflection coefficient, thus directly alters also the product Ln, where n- is reflection coefficient and L is film thickness The method uses the reflection coefficient of very thin films of materials absorbed in a metal	Alvarez *et al*., 2009 Oh *et al*., 2004 Oh *et al*., 2003
3	Dispersion	Optical waveguide light–mode spectroscopy (OWLS)	The technique based on measurements of the resonant polarization angle of laser light scattered from the grating and coupled with a thin film waveguide This methods allows the thickness of absorber to be measured	Szekacs *et al*., 2009
4	Emission	Fluorescence spectroscopy	The method for measuring fluorescence intensity, life time, energy transfer between donor and acceptor (FRET)	Anderson *et al*., 1988 Handbury *et al*., 1996 Grand *et al*., 2004

caused by light-matter interactions: absorption, reflectance, dispersion or emission (Podbierska, 2011).

Mechanisms of light-matter interactions, detection techniques and its description as well as references are presented in Table 2.

DETECTION METHODS USED IN OPTICAL IMMUNOSENSORS

Ultraviolet/Visible (UV/VIS) absorption spectroscopy – spectroscopy that enables imaging of electron transitions from the ground state to the excited state in a molecule, that are related to changes in vibrational and rotational energy. The range of electromagnetic spectrum used in this spectroscopy is 200–1100 nm. Measurements are usually made with a transparent medium, while absorbance changes are caused by an analyte.

Absorbance measurements made by employing UV/VIS spectroscopy were used for developing a new type of immunosensors designed for tracking antibiotic residues (Que *et al.,* 2014).

Ying and coworkers (2013) used this technique to modify amperometric immunosensors during carbofuran detection.

UV/VIS spectroscopy was also used by Gong and collaborators (Gong *et al*., 2009) in studies of the cyanidin-horseradish peroxidase-hydroperoxide reaction in terms of applications for enzyme-linked immunosensing assays.

Optical waveguide light-mode spectroscopy (OWLS) is a technique based on measurements of the resonant polarization angle of laser light scarred from the grating and coupled with a thin film waveguide.

With this technique it is possible to determine the thickness of the absorber layer (Szekacs *et al*., 2009).

This method was used to improve low-detection limit immunosensors (compared to traditional immunological tests for detecting: herbicide active ingredient triflualin, fusarium mycotoxin zearalenone, egg yolk protein and vitellogenim (Szekacs A *et al*., 2009).

One of very sensitive method that uses reflection of light is reflectometric interference spectroscopy (RIfS).

This method is based on white light interference in thin films. Through analyte binding the reflection coefficient from the film is changed, thus also the product nL, where n is reflection coefficient and L is film thickness. (Alvarez *et al*., 2009).

This technique was employed to determine the porous alumina (pAl_2O_3) film in immunosensors that use simple protein and two antibodies (IgG) (Alvarez et *al*., 2009).

Surface plasmon resonance (SPR) is an optical technique for measuring reflection coefficient of very thin films of materials absorbed by metals (Pattnaik, 2005).

This method was used in immunosensors by Oh and collaborators (Oh *et al*., 2003; Oh *et al.,* 2004) to detect sallmonella typhimurium and legionella pneumophila.

Fluorescence spectroscopy is one of the most common research methods in studies of immunosensors, although this method requires the presence of fluorophores that can be natural or artificial (created by fluorescent labeling of a non-fluorescent molecules).

This technique allows fluorescence intensity, life time, or energy transfer between donor and acceptor (FRET) to be measured.

According to Ramirez (Ramirez *et al*., 2009), optical immunosensors can be divided into three groups:

a) direct monitoring sensors (without immunoreaction labels),

b) sensors that detect compounds labeled in immunoreactions (fluorescent labeled compounds),

c) sensors that measure products of immunological reactions.

THE PHENOMENON OF FLUORESCENCE IN IMMUNOSENSORS

Among all detection methods described above, fluorescence is the most commonly used technique.

This results from a high single-molecule sensitivity and immediate response in most cases.

Table 3. Fluorescence detection methods used in immunosensor studies

No.	Fluorescence detection methods used in immunosensor study	Analyte	References
1	Fluorescent intensity	rothavirus, poliovirus, variola virus	Jung *et al*., 2010
2	Fluorescent intensity	immunoglobulin G (IgG)	Aoyagi & Kudo, 2005
3	Fluorescent intensity	coplanar polychlorinated biphenyls	Endo *et al*., 2005
4	Fluorescent intensity	cancer biomarker prostate specific antigen (PSA).	Zhu *et al*., 2014
5	Fluorescent intensity	cardiac biomarker	Matveeva *et al*., 2004
6	Fluorescent intensity	tumor marker	Lee *et al*., 2010
7	FRET	pathogenic bacteria	Hejduk & Hejduk, 2010
8	FRET	salmonella typhimurium	Ko & Grant, 2006
9	FRET	albumin concentrations in saliva, urine and serum	Wang *et al*., 2012
10	Time Resolved Fluorescence	ricin	Huang *et al*., 2015

This method is more often used than absorption methods because the detectable analyte concentrations measured through fluorescence are 10^6 times lower than those of absorption techniques (Liu S *et al*., 2013).

Fluorescent biosensors use the phenomenon of fluorescence that occurs when an analyte is recognized by biological fluorescent molecules (natural or fluorescent labeled) located in the receptor layer.

The phenomena of fluorescence in immunosensors occur in four typical immunoreaction configurations. They are called: direct, competitive, sandwich and displacement configurations.

In the direct format, an unlabeled antigen bonds with an unlabeled antibody (Borisov & Wolfbeis, 2008).

In the competitive configuration, an unlabeled antibody interacts with an unlabeled antibody present in the sample (Helder *et al*., 2002) and fluorescence intensity is proportional to analyte concentration.

In the sandwich format, an antibody is immobilized on the surface and bonds with an antigen present in the sample.

Another fluorescent labeled antibody binds with various antigen epitopes. This generates a fluorescence signal proportional to the amount of antigens in the sample (Cruz *et al*., 2002).

The displacement configuration is the least common one. It occurs between fluorescent labeled antigens previously bound to an immobilized antibody and an unlabeled antigen. The detection is based on changes in fluorescent intensity (Borisov & Wolfbeis, 2008).

The phenomenon of fluorescence reflects changes in fluorescent properties of the receptor layer.

This is an emission phenomenon and is a response of fluorophores to excitation by electromagnetic radiation in the visual light wavelength range.

Radiation may be emitted at a shorter, the same or longer wavelength than the excitation wavelength.

When the emission wavelength is longer than the excitation wavelength, it is said that an energy loss occurs and a spectral shift called Stokes shift (Das A *et al*., 2014).

In fluorescence-based immunosensors, measurements include: variations in fluorescent intensity, energy transfer between donor and acceptor (FRET) or life time.

The variation in fluorescent intensity is the most frequently measured parameter in immunosensors.

This measurement allows the activity of biosensor receptor layer under the effect of analyte binding to be determined (Li *et al*., 2014). Variations in fluorescent intensity was used for detecting viruses (Jung *et al*., 2010), disease biomarkers (Zhu *et al*., 2014; Matveeva *et al*., 2004; Lee *et al*., 2010, and immunoglobulins G (Aoyagi *et al*., 2005).

Energy transfer (FRET) occurs between a donor and acceptor if the distance between them does not exceed 10 nm and the dipole orientation of both molecules is appropriate (Liu *et al*., 2013 & Durick *et al*., 2001).

The studies of immunosensors by using fluorescent technique and based on measurements of energy transfer between donor and acceptor (Fröster Resonance Energy Transfer) were conducted by Anderson, Grant and Handbury. The measurement of phenytoin (Anderson *at al*., 1988); detection of troponin T and I (Grant *et al*., 2004) were made. This method was used to analyze immunosensor response to theophylline (Handbury *et al*., 1996). In addition, this method was employed by Heyduk & Hejduk (2010); Ko & Grant (2006); Wang *et al*. (2012) for detecting: pathogenic bacteria, salmonella typhimurium, concentration of albumin in saliva, urine and serum, respectively.

The life time is a fluorophore property that refers to the average duration of the excited state of a fluorophore (i.e. between photon absorption by stimulated emission of radiation and fluorescence emission) (Rae *et al*., 2009). The fluorescence technique based on this value is called Time Resolved Fluorescence. This technique was used, for example, for detecting ricin (Huang *et al*., 2015).

Examples of applications of the above fluorescence methods in studies of immunosensors along with analytes to be detected and references are presented in Table 3.

Taking into account scientific reports in the last two decades related to fluorescence detection methods commonly used in immunosensor studies, it is quite clear that the method based on variations in fluorescent intensity is the most common one, and then the method based on energy transfer (FRET). The method called Time Resolved Fluorescence occupies the last place.

APPLICATIONS OF IMMUNOSENSORS

When reviewing scientific reports on applications of immunosensors, the following two terms are most frequently used, namely: immunoassay (immunotests) and immunosensor. However, there is a difference between them. In biosensors the receptor layer can be used many times for detection purposes, thus enabling continuous monitoring. For immunoassay, after an immunological

reaction is completed, and before the next measurement is made, the receptor layer should be regenerated. (Ramirez *et al*., 2009).

Immunosensors and immunotests are commonly used in environmental protection where there is a need of monitoring of water quality (Long *et al*., 2008), toxic chemicals, pesticides, chemical weapons (Shankaran *et al*., 2007), and in food analysis (Tokarskyy & Marshall, 2008), but they are most often used in medicine (Medyanseva *et al*., 2001), where blood, urine or saliva samples enable concentrations of morphine, progesterone, estradiol, dopamine, insulin to be determined (Shankaran *et al*., 2007).

ELISA (Enzyme Linked Immune Sorbent Assay) is the oldest and also the most frequently used immunological test due its low cost and high repeatability. This test was used, among others, to detect malaria (Podder *et al*., 2015), HIV diagnosis (Igbal *et al*., 2012 or toxoplasmosis (Rahbari *et al*., 2012).

SUMMARY

Biosensors create a highly promising tool allowing fast, low-cost and accurate measurements.

They are commonly used in food analysis, environmental protection, arms industry as well as in medicine, especially in diagnosis.

Optical immunosensors, in particular those based on fluorescence, are frequently used due to their selectivity and sensitivity.

When considering immunosensor development, scientific challenges and future trends, biosensors, especially fluorescent immunosensors become very interesting and open huge opportunities for scientists, engineers and industries.

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