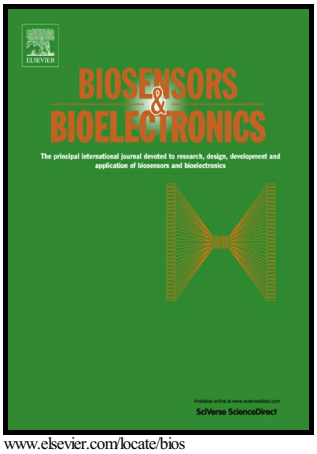

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Fabiana S. Felix, Lúcio Angnes

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ELECTROCHEMICAL IMMUNOSENSORS – A POWERFUL TOOL FOR ANALYTICAL APPLICATIONS

Fabiana S. Felix[1,2] and Lúcio Angnes[2*]

[1]Departamento de Química, Universidade Federal de Lavras (UFLA), CP 3037, Lavras, CEP 37200-000, MG - Brazil
[2]Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748 – CEP 05508-000, São Paulo, SP, Brazil
[*]Correspondence to: Tel.: +55 11 3091 3828. luangnes@iq.usp.br

Abstract

Immunosensors are biosensors based on interactions between an antibody and antigen on a transducer surface. Either antibody or antigen can be the species immobilized on the transducer to detect antigen or antibody, respectively. Because of the strong binding forces between these biomolecules, immunosensors present high selectivity and very high sensitivity, making them very attractive for many applications in different science fields. Electrochemical immunosensors explore measurements of an electrical signal produced on an electrochemical transductor. This signal can be voltammetric, potentiometric, conductometric or impedimetric. Immunosensors utilizing electrochemical detection have been explored in several analyses since they are specific, simple, portable, and generally disposable and can carry out *in situ* or automated detection. This review addresses the potential of immunosensors destined for application in food and environmental analysis, and cancer biomarker diagnosis. Emphasis is given to the approaches that have been used for construction of electrochemical immunosensors. Additionally, the fundamentals of immunosensors, technology of transducers and nanomaterials and a general overview of the possible

applications of electrochemical immunosensors to the food, environmental and diseases analysis fields are described.

Keywords

Immunosensors, electrochemical transducers, food, environmental, biomarkers, review

1. Introduction

Biosensors (catalytic or affinity type) offer a number of advantages over conventional analytical techniques, including portability, miniaturization and on-site monitoring as well as having good selectivity (or even specificity) and sensitivity depending on the transducer. Moreover, they are being developed as suitable tools for different applications, including bioprocess control, food quality and in agriculture, military and medical applications, generally with minimum sample pre-treatment. Currently, some research groups are devoted to designing biosensors for environmental monitoring (Bahadir and Sezginturk, 2015; Verma and Bhardwaj, 2015). However, the determination of acceptable concentration levels of emerging pollutants, such as pesticides, phenols, hormones and antibiotics, as well as drugs, proteins and hormones in clinical analysis, requires the development of fast and reliable methods to detect concentrations as low as 10^{-12} mol L^{-1} (*e.g.* atrazine analysis) (Belkhamssa et al., 2016). For this purpose, immunosensors have received major attention, since they combine the inherent specificity of an immunoreaction with the very high sensitivity of different detectors (Lin and Ju, 2005; Uliana et al., 2014; Hasanzadeh, M., Shadjou, 2017; Campuzano et al., 2017; Eivazzadeh-Keihan et al., 2017).

Immunosensors are analytical devices used to detect the binding event between antibody (*Ab*) and antigen (*Ag*) with formation of a stable complex (Lin and Ju, 2005).

Either *Ab* or *Ag* can be immobilized on the surface of different transducers, so producing a potential new immunosensor (Holford et al., 2012). More recently, the studies involving the aptasensors have received increasing attention. Aptamers are synthetic oligonucleotides that can bind to specific biomolecules, and are used for the construction of novel biosensors. They have presented attractive advantages in comparison to immunosensors based on the use of antibodies, such as longer shelf life, high specificity and generally are more stable than *Ab* or *Ag* (Eivazzadeh-Keihan et al., 2017; Xu et al., 2017; Nguyen et al., 2017).

The aim of this review is to cover the literature selectively and discuss many examples of electrochemical immunosensors based on use of *Ab*, highlighting the advantages of these biosensors for different applications (food analysis, environmental matrices and medical diagnosis). Furthermore, there will also be a focus on the main concepts to understand these important analytical devices.

2. Basic principles of immunoassays and immunosensors

Antibodies, also called immunoglobulins (Ig), are a large family of glycoproteins capable of recognizing antigens with high specificity. They are composed of one or more copies of a characteristic unit that can be visualized by its 'Y' shape (Figure 1). Each 'Y' contains four polypeptide chains – two identical heavy chains (molecular weight ~ 50 kDa each) and two identical light chains (molecular weight ~ 25 kDa each). A single disulfide bond connects each light and heavy chain pair (Gaudin, 2013; Wujcik, 2014; Stanley, 2002; Gil and Kubota, 1999).

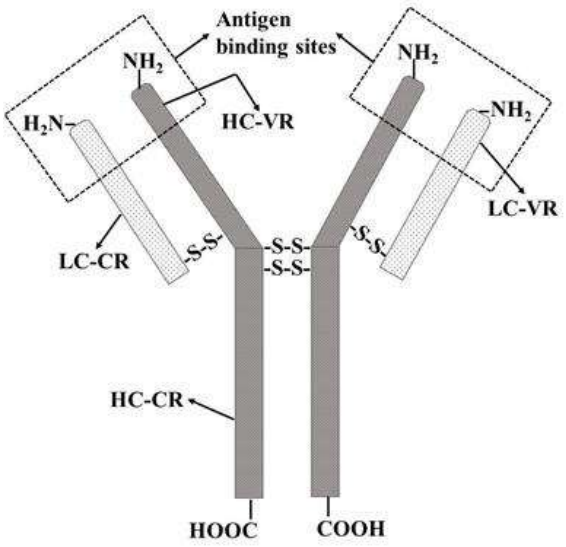

Figure 1 – Schematic representation of heavy and light chains combined to form the most common antibody (IgG). -SS- = disulfide bridges, LC-CR = light chain and constant region, HC-VR = heavy chain and variable region, LC-VR = light chain and variable region and HC-CR = heavy chain and constant region.

There are two antibody types, monoclonal and polyclonal *Ab*, depending on the epitope (region of an *Ag* that interacts with an *Ab*). Monoclonal *Ab* units (Figure 2A) are more specific for a single epitope, diminishing the chance of cross-reactivity. Polyclonal *Ab* (Figure 2B) can bind multiple epitopes on an *Ag*, showing a heterogeneous immunological response (Kumar et al., 2009; Omidfar et al., 2013). The choice of antibody will be determined by its analytical application. For example, in the case of environmental analysis, it can be interesting to use polyclonal antibodies for determination of pollutant classes (Sherry et al., 1992).

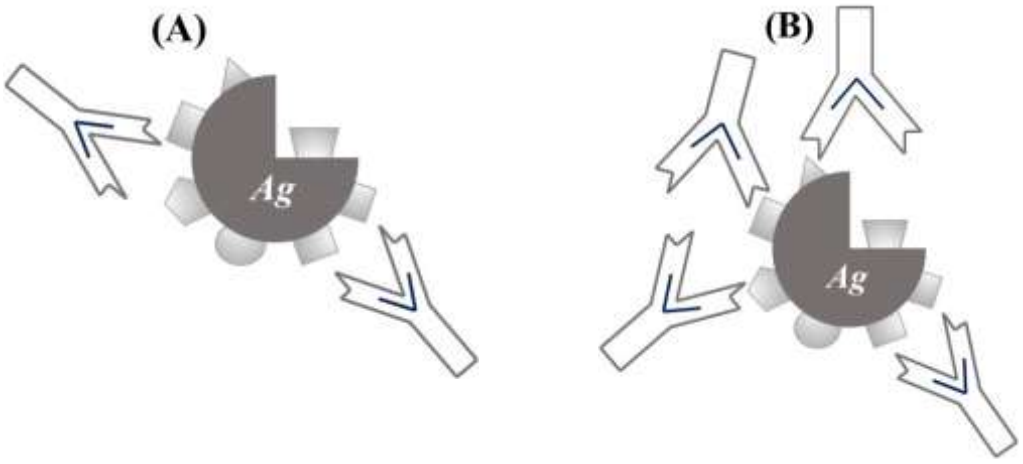

Figure 2 – Schematic representation of: (A) monoclonal *Ab* binding a specific epitope (it's represented by a square) on an *Ag* and (B) polyclonal *Ab* binding to several epitopes on an *Ag*.

An immunosensor is a type of bioaffinity biosensor in which the *Ab* binds to the *Ag* (it can be a biological agent such as toxins, bacteria, viruses, pollen, fungi, tissue cells and proteins, with a molecular weight larger than 1.5 kDa) and forms a stable complex (Figure 3). However, minor chemical modification of the molecular structure of the *Ag* can dramatically lower its affinity for the *Ab*. The affinity of the *Ag* and the *Ab* is a measure of the strength of the binding forces in the resultant *Ag–Ab* complex (affinity or association constant). For most *Ag–Ab* interactions under conventional pH, temperature and buffer solution, the association constant has a very high value; values up to 10^{15} have been related (Suri and Raje, 2002; Riccardi et al., 2002).

$$Ab + Ag \underset{K_D}{\overset{K_A}{\rightleftharpoons}} Ab\text{–}Ag$$

$$K_A = \frac{[Ab\text{–}Ag]}{[Ab][Ag]}$$

Figure 3 – Representation of equilibrium equation: K_A = affinity or association constant, K_D = dissociation constant, [*Ab*] = concentration of the unoccupied antibody binding sites, [*Ag*] = concentration of the unoccupied antigen binding sites and [*Ab-Ag*] = concentration of the antibody-antigen complex that will be found in equilibrium.

Interaction between *Ab* and *Ag in vitro* is not always readily observed as, for example, by precipitation and agglutination. Radioactive labels (radioimmunoassays) were initially used because of their high sensitivity. Later, the restriction of the use of radioisotopes led to the use of other labels, such as enzymes (enzyme immunoassays – EIA), ELISA, chemiluminescent compounds (chemiluminescent immunoassays) and fluorophore compounds (fluoroimmunoassays). Among these, ELISA is the most widely used and presents two major formats, sandwich and competitive binding methods, the first of which has the disadvantage that the analyte must have multiple antibody binding sites (Ramirez et al., 2009).

In the simplest format of ELISA, *Ag* is immobilized onto a solid support, and then a specific *Ab* is applied over the support to bind to the *Ag*. This *Ab* is previously linked to an enzyme (a type of marker) and, in the final step, a solution containing the enzyme's substrate is added. The further reaction produces a detectable signal, most commonly a colour change (optical detection). Figure 4 depicts a typical ELISA reaction, in which a specific antigen is sandwiched between two antibodies (primary *Ab* and secondary *Ab* linked to an enzyme label) (Mistry et al., 2014). For sandwich-type

immunosensors, the primary antibodies are often immobilized on a working electrode surface, and the sandwiched immunocomplex is formed between the immobilized primary antibodies and secondary or multiple detection antibodies (usually, secondary *Ab* and enzyme labels are loaded with appropriate labels, such as paramagnetic beads, for amplification of the electrochemical signal) (Pei et al., 2013).

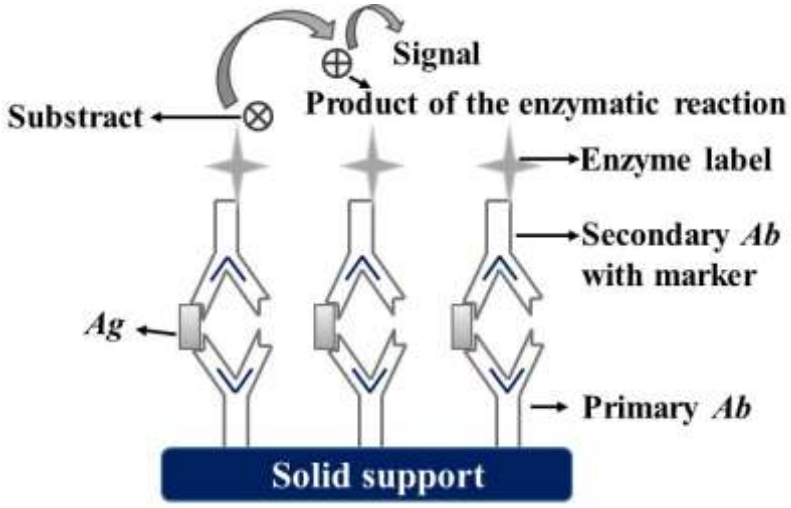

Figure 4 – Schematic representation of the immunodetection steps for sandwich-type ELISA.

Immunosensors combine the advantages of good sensitivity and high selectivity. Furthermore, they allow the progress of immunoreactions on detector surfaces to be followed in real time. Immunosensors can be classified as optical (luminescence, fluorescence, surface plasmon resonance – SPR, refractive index), electrochemical (voltammetric, potentiometric, conductimetric, capacitive or impedimetric), calorimetric (based on thermistors, thermopairs or thermoresistors) and mass variation (electrochemical quartz crystal microbalance) (Rogers, 2000). There are many reviews dealing with the development and application of immunosensors associated with different forms of detection, highlighting the main advantages of their construction

methods (Wujcik et al., 2014; Hasanzadeh et al., 2013; Ricci et al., 2007; Xu et al., 2016a; Liu et al., 2016; Speight and Cooper, 2012; Byrne et al., 2009).

The immunoreactions in electrochemical transducers can cause changes of potential, current, ion concentration, conductance, capacitance or impedance. Electrochemical transducers are generally compact, inexpensive, robust, have fast response times, can be mass-produced and require small analyte volumes and, as a consequence, are widely used as immunosensors for different applications (Timothy et al., 2012). The major drawbacks of immunoanalytical methods are based on the high molecular weight (several analytes of environmental, pharmaceutical and food interest have a molecular weight lower than 1 kDa). Therefore, it is essential to choose appropriate markers, which must present good sensitivity, stability, elevated binding affinity with antibodies and relatively low cost. In many cases, the *Ab–Ag* interactions are not promptly reversible due to high affinity constant values and, ideally, immunosensors should be capable of detecting the species of interest reversibly, continuously and selectively (Marco et al., 1995).

3. General considerations about electrochemical detection

Electrochemical techniques are powerful and versatile tools, which offer good precision, accuracy and sensitivity with relatively low cost and simple instrumentation. In addition, the requirement of small sample volumes and portability are advantages of these techniques (Ronkainen et al., 2010; Farghaly et al., 2014). These methods are classified as bulk methods (*e.g.* conductometry) and interfacial methods (*e.g.* potentiometry and voltammetry). Interfacial methods can occur in the static or dynamic mode (*e.g.* potentiometry and voltammetry, respectively). In the first group, the measurements are carried out under equilibrium conditions (no net current signal flows

across the electrochemical cell) and in the dynamic method, measurements are performed under conditions of current or potential control. Voltammetry is the electrochemical technique most frequently used under dynamic conditions. It is associated with a class of electroanalytical methods focused on studying the current responses (cyclic voltammetry, differential pulse voltammetry, square wave voltammetry and amperometry, among others). Generally, voltammetry is based on the voltage–current–time relationship in an electrochemical arrangement constituted by three electrodes: working, auxiliary and reference electrodes (Trojanowicz et al., 2003; Uslu and Ozkan, 2011; Rackus et al., 2015).

Voltammetric experiments can be carried out in quiescent solutions, but hydrodynamic conditions at rotating or vibrating electrodes, under solution stirring or flow, can be done too. In the latter group (hydrodynamic mode), mass transport to the working electrode is significantly improved, lowering by a similar proportion the detection limit. Additionally to the improved sensitivity, there is an extra advantage: the utilization of flow injection analysis (FIA), sequential injection analysis (SIA) or batch injection analysis (BIA) associated with voltammetric techniques (mainly amperometry) favours rapid analysis, resulting in elevated throughput of samples (Trojanowicz, 2016; Felix and Angnes, 2010).

Electrochemical impedance spectroscopy (EIS) is an electrochemical technique based on analysis of the linear response of the perturbation/response ratio (transfer function) when a low amplitude sinusoidal perturbation (typically 5–10 mV) is applied. It is a powerful tool for measurement of non-faradaic processes, in which the capacitive behaviour established by the separation of charges at the electrode–electrolyte interface predominates, while faradaic processes involve charge transfer between the electrode and some species to be oxidized or reduced in the solution (Prodromidis, 2010). Figure

5 shows a schematic representation of electrochemical methods with their classifications that includes the analytical signals of the main methods used for detection systems with immunosensors.

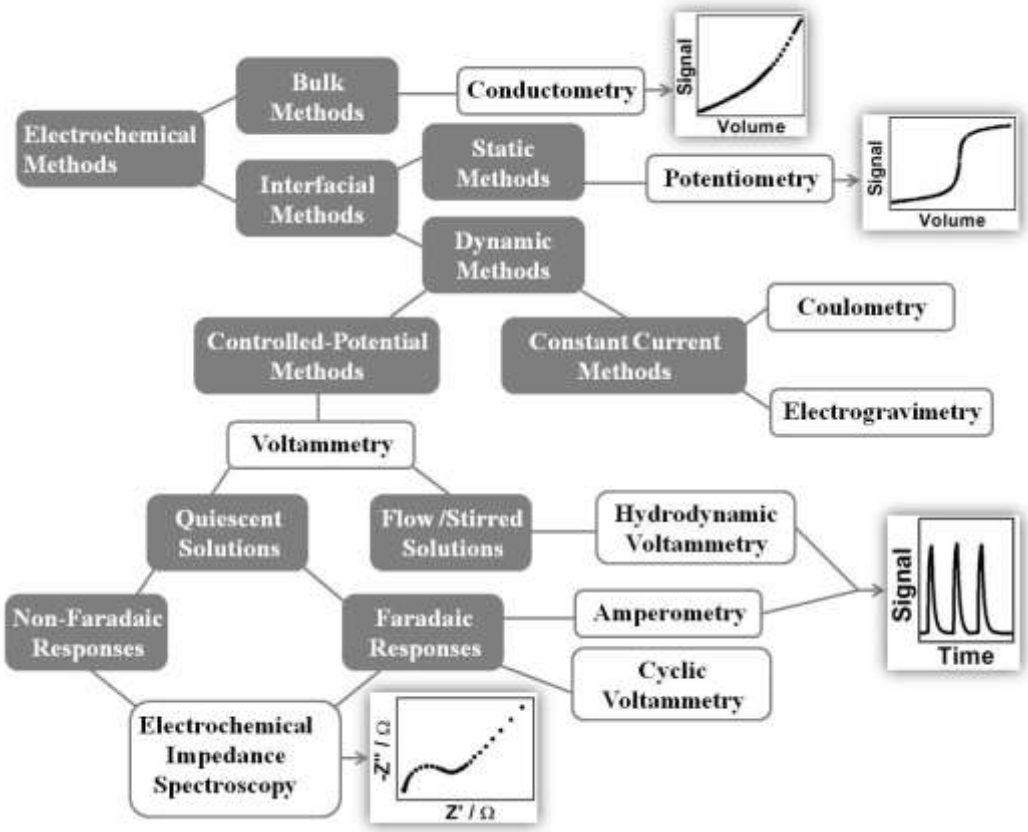

Figure 5 – General scheme of the main electrochemical methods, so as the distinct forms of electrochemical signals plots (conductometry, potentiometry, amperometry-FIA and electrochemical impedance spectroscopy) obtained during immunosensor analyses.

4. Working electrodes and nanomaterials in the construction of immunosensors

It is noteworthy that the performance of voltammetric analyses involving immunoreactions is strongly influenced by the characteristics of the working electrode. The application of microfabrication technologies to construct electrochemical cells and biosensors is becoming very common, as can be seen in many reviews (Suzuki, 2000;

Derkus, 2016; Whitesides, 2006). Three electrode arrangements can be easily built on a single substrate, enabling automation of their construction using extremely small amounts of reagents and allowing the analysis of a very low volume of samples (Derkus, 2016). The combination of microfluidic processes and the use of arrays with interdigitated detectors have provided great benefits in the biosensor field, including wide linear range, specificity and sensitivity (Jia, 2015; Couto, 2016). Screen-printed electrodes (SPEs) are especially attractive for the construction of immunosensors because they can be used as disposable devices (in some cases they can be reused), are mechanically robust, present low cost and are stable and reproducible for mass production (Derkus, 2016; Taleat et al., 2014). Disposable gold electrodes built from recordable compact discs (CD-Rs) were first reported by our group (Angnes, 2000) and have been used in the construction of DNA-based biosensors for the detection of the hepatitis C virus (Uliana et al., 2011) and amperometric immunosensors for quantification of Chagas disease with high sensitivity (Figure 6) (Foguel et al., 2011).

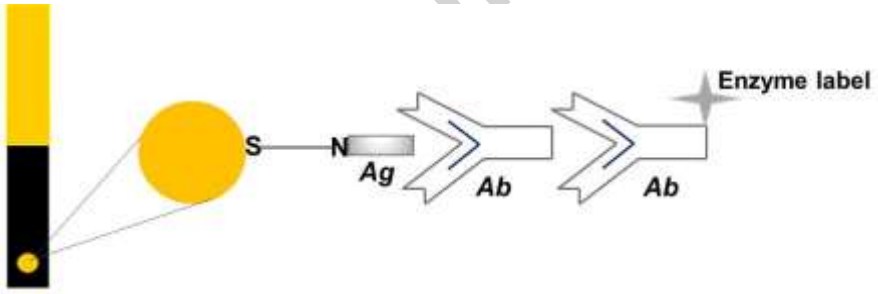

Figure 6 – Schematic illustration of electrochemical immunosensor proposed for determination of Chagas disease, which explores the use of a disposable gold electrode as working electrode. S-N corresponds to the self-assembled monolayer (SAM).

Another disposable electrode described in the literature is indium tin oxide (ITO), used in electrochemical platforms due to its (relatively wide) potential window and stable electrochemical properties (Wang et al., 2016a; Yu et al., 2014; Jia et al., 2014) allied to its transparency in the visible region.

The incorporation of nanomaterials in the development of immunosensors has been extensively explored in several biosensing systems in which they play different roles to enhance performance. Because of their elevated conductivity and electrocatalytic effect, nanomaterials have been shown to favour electron transfer, increasing electrochemical signals. Furthermore, modification of working electrodes with nanomaterials provides more roughened surfaces that facilitate intimate attachment to electrode surfaces, especially of the biological elements.

The increase of the electroactive surface area leads to high sensitivity and low limits of detection. Many nanomaterials provide biocompatible surfaces for immobilization of biological elements, helping to retain the biomolecules with high stability. Different nanomaterials have been used in immunosensors, including gold nanoparticles, carbon nanomaterials, magnetic nanoparticles or magnetic beads (MBs), silica nanoparticles, quantum dots (QDs) and hybrid nanostructures (Saxena and Das, 2016; Wang et al., 2016b; Ramnani et al., 2016, Kumar et al., 2015; Reverte et al., 2016).

Figure 7 shows an example of a sandwich immunosensor built from a disposable working electrode modified with nanomaterials and primary *Ab*. In the same study, bovine serum albumin (BSA) was used to block the electrode surface in order to avoid non-specific adsorption (Chikkaveeraiah et al., 2011). MBs loaded with polyclonal antibodies (secondary *Ab*) and enzyme labels are highlighted in the figure to capture antigen analytes (*Ag*) for later electrochemical detection.

Figure 7 – Schematic representation of sandwich immunosensor showing a strategy for its construction.

Different materials such as magnetic particles, QDs, gold or silver nanoparticles and organic dyes have been encapsulated into mesoporous silica for applications in the bioanalytical field (Wang et al., 2014a; Lai et al., 2014; Yin et al., 2014; Johari-Ahar et al., 2015; Liu et al., 2014a; Cincotto et al., 2016). Silica prevents the oxidation of MBs, has a large surface area, elevated pore volume, high mechanical and thermal stability and provides functional groups for biomolecule immobilization (Wang et al., 2016b).

5. Applications of electrochemical immunosensors

5.1. Food analysis

The contamination of foods with pathogenic microorganisms (*e.g.* bacteria and viruses) denotes a problem of global concern. The presence of these microorganisms and/or the products of their metabolism can cause serious infectious diseases in animals and humans, as well as fast spoilage of the food products. Bacteria, viruses, biological toxins and other microorganisms are resistant to environmental conditions. Humans are quite susceptible, and the incidence of most common diseases caused by foodborne

pathogens, such as *Salmonella* sp., *Campylobacter coli*, *Bacillus cereus* and *Escherichia coli*, is still elevated in many countries. While standard microbiological tests allow the detection of a single bacterium or biotoxin, the procedure is time-consuming and the interpretation of the results is often a complex mission (Teles et al., 2010; Swaminathan and Feng, 1994; Ivnitski et al., 1999; Turner et al., 2009). In response to this problem, electrochemical immunosensors arise as an interesting alternative for fast detection of different pathogens in food samples.

E. coli refers to a large group of Gram-negative, rod-shaped bacteria commonly found in the intestines of humans and animals. The *E. coli* O157:H7 bacterium is considered one of the most dangerous foodborne pathogens and can lead to haemorrhagic colitis, haemolytic–uremic syndrome (HUS) and other complications (Delannoy et al., 2016). In the literature, there are reports of many immunosensors built for electrochemical detection of the pathogenic bacterium *E. coli* O157:H7. Anti-*E. coli* antibodies have been immobilized onto different transducers such as a gold electrode modified with multi-walled carbon nanotubes (MWCNTs)/SiO_2 nanoparticles (Li et al., 2012-b) and a screen-printed carbon electrode (SPCE) modified with MWCNTs/natural biopolymer (Zhan et al., 2013) (Figure 8).

(A) **(B)**

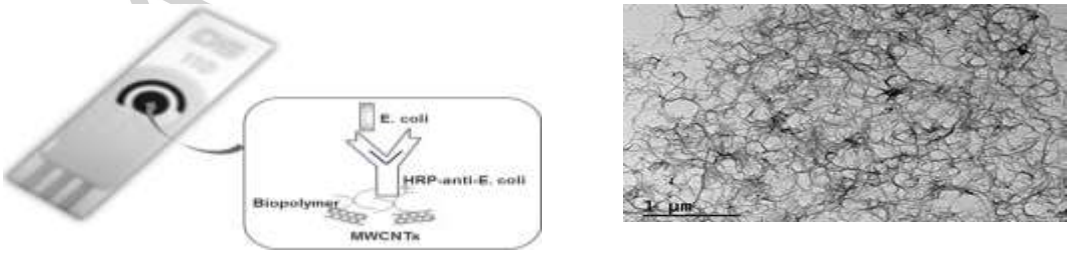

Figure 8 – (A) Schematic representation of electrochemical immunosensor based on the modification of SPCE with MWCNTs/natural biopolymer used for quantification of

bacterium *E. coli* O157:H7 and (B) Transmission electron microscopy (TEM) of MWCNTs.

Other examples include a glassy carbon electrode (GCE) modified with rGO/conducting polymer/AuNPs (Guo et al., 2015a), a tin oxide electrode (dos Santos et al., 2015), a nanoporous membrane modified with hydrophilic biopolymer (Joung et al., 2013) and rGO paper modified with AuNPs (Wang et al., 2013a). Additionally, a gold electrode modified with a hydrophilic biopolymer (Joung et al., 2012), an SPCE modified with polyallylamine/MWCNTs (Viswanathan et al., 2012), a gold electrode modified with cysteine (Zhang et al., 2015) and a screen-printed interdigitated gold microelectrode (Xu et al, 2016b) have been developed.

Salmonellosis is a disease caused by anaerobic, non-spore-forming and Gram-negative bacteria of the genus *Salmonella*. Generally, people infected with *Salmonella* develop fever, vomiting, diarrhoea and abdominal cramps. There are more than 2,500 *Salmonella* serotypes and only a limited set of them are associated with poultry diseases and/or human salmonellosis, such as *Salmonella enterica* serotype Gallinarum and *S. enterica* serotype Pullorum (Cosby et al., 2015). Fei et al. (2015) described a fast sandwich immunoassay method for voltammetric detection of *S.* Gallinarum and *S.* Pullorum in food samples (chickens). The authors modified the surface of an SPCE with Fe_3O_4/SiO_2/AuNPs for immobilization of anti-*S.* Gallinarum and *S.* Pullorum antibodies. Cyclic voltammograms were performed at a scan rate of 25 mV s^{-1} in the potential interval from −0.6 to −0.1 V for reduction of thionine/H_2O_2 in the presence of HRP. For this system, a linear response was obtained to *S.* Pullorum and *S.* Gallinarum in the concentration range from 10^2 to 10^6 CFU mL^{-1}.

Listeriosis is a bacterial infection usually caused by *Listeria monocytogenes*, which occurs in immunocompromised individuals, including HIV patients, infants,

pregnant women and the elderly. It can cause infections of the central nervous system, gastroenteritis and bacteraemia (Nyarko and Donnelly, 2015). Lately, an impedimetric immunosensor has been chosen for quantification of *L. monocytogenes* in lettuce samples (Chen et al., 2015). In this study, magnetic nanoparticles (MNPs) were modified with anti-*L. monocytogenes* monoclonal antibodies (Listeria-MAb) to separate *Listeria* cells from samples containing other cells. AuNPs modified with anti-*L. monocytogenes* polyclonal antibodies (Listeria-PAb) and urease were conjugated with MNPs/Listeria-MAb to form the sandwiched immunocomplexes. Urease catalyses the hydrolysis of urea, increasing the ionic strength of the solution, which was detected by an interdigitated array of microelectrodes. Impedance studies, operated in the 1–50 kHz range, demonstrated the occurrence of changes in the quantification of *L. monocytogenes*. Under the best condition (frequency of 10 kHz), there was a linear relationship between the impedance signal (ΔZ) and the concentration of analyte ranging from 3.0×10^2 to 3.0 x 10^4 CFU mL^{-1} (Chen et al., 2015).

Aflatoxins are toxic metabolites produced by certain fungi (*Aspergillus flavus* and *A. parasiticus*) during the harvest and storage of cereal grains, mainly maize, wheat, rice and barley. There are different types of aflatoxins, of which aflatoxin B_1 (AFB_1) is considered the most potent toxin. Aflatoxins can cause both acute and chronic toxicity in many animals and humans (Womack et al., 2016). The liver is the main organ affected, but high levels of aflatoxin have also been found in the lungs, brain, kidneys and heart.

Yu et al. (2015) proposed the use of an impedimetric immunosensor for sensitive quantification of AFB_1 based on a GCE modified with MWCNTs and room temperature ionic liquid (GCE/MWCNTs/RTIL). According to the authors, this combination (CNTs and IL) provides numerous conductive microcavities for *Ab* immobilization and the

formation of electrochemical biosensing layers. This immunosensor was used for analysis of AFB_1 in olive oil samples, with a detection limit calculated as 0.03 ng mL^{-1}. Different immunosensing platforms for determination of bacteria and toxins in food samples using electrochemical signals can be found in literature. Table S1 (Supplementary Material) described several examples of these immunosensors.

Electrochemical immunosensors have been proposed for the detection of different hormones, including testosterone (Conneely et al., 2007a), stanozolol (Conneely et al., 2007b), norethisterone (Wei et al., 2010), somatotropin (Lim et al., 2015), ghrelin (Martinez-Garcia et al., 2015), leptin (Ojeda et al., 2013) and progesterone (Arevalo et al., 2010). Hormones are chemical messengers that are secreted directly into the blood and are carried to tissues and organs of the body to exert their functions. Unfortunately, many of these hormones have been used incorrectly to promote rapid livestock growth, and residual hormones in the meat affect human health (Esquivel-Hernandez et al., 2016).

Other electrochemical immunosensors have been described for monitoring food allergens such as ovalbumin, β-lactoglobulin, shrimp tropomyosin and peanut protein (Cadkova et al., 2015; Cao et al., 2011; Eissa et al., 2012; Jiang et al., 2013; Alves et al., 2015a, Singh et al., 2010; Montiel et al., 2015; Alves et al., 2015b; Eissa et al., 2013) as well as residues of antibiotics (*e.g.* 3-amino-2-oxazolidone, tetracyclines, sulfonamides, kanamycin, chloramphenicol and benzylpenicillin, among others) (Yang et al., 2011; Conzuelo et al., 2012a; Conzuelo et al., 2012b; Conzuelo et al., 2013; Wei et al., 2012; Liu et al., 2014b; Zhao et al., 2011; Thavarungkul et al., 2007; Merola et al., 2015) and β-adrenergic agonists (*e.g.* clenbuterol, ractopamine and salbutamol) (Liu et al., 2011; Regiart et al., 2013; He et al., 2009; Zhou et al., 2014) which are often used as feed additives to stimulate growth in animal husbandry. Consequently, antibiotics and β-

agonists can accumulate in animals and from there are transferred to the human food chain, causing allergic reactions or even being potential carcinogenic molecules (Conzuelo et al., 2013; He et al., 2009).

5.2. Environmental and agricultural analysis

Although pesticides are necessary for extensive agriculture (indirectly, they can control the quality and quantity of farm products and food, and help to limit diseases in humans transmitted by pests) many of them are dangerous environmental contaminants due to their tendency to bioaccumulate, their mobility (in air, water and soils) and, in the long term, effects on living organisms. Usually, determination of these compounds is performed using chromatographic techniques that are relatively expensive and often require laborious sample treatment before analysis, as well as clean-up steps, which increases analysis time and the risk of errors (Mas et al., 2010). Electrochemical immunosensors arise as a very interesting alternative because they are able to determine pesticides at low concentrations quickly and in a simple way (Suri et al., 2002).

Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) is a pyridine herbicide used for general woody plant control. It is highly soluble in water, does not adhere to soil and, consequently, easily leaches into groundwater. This pesticide is persistent and may accumulate in living organisms (Janikova-Bandzuchova et al., 2016). Therefore, it is important to determine trace amounts of picloram in the environment. Chen et al. (2010) synthesized ordered three-dimensional gold nanoclusters used to modify the surface of a GCE, employed to construct an amperometry immunosensor based on competitive immunoreactions. This biosensor was used for the quantification of picloram in peach and in sludge. The stability of this voltammetric immunosensor was 15 days with a negligible decrease of signal.

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a substituted phenylurea herbicide, introduced by Bayer Company, which is generally persistent in water, groundwater and soil. This pesticide is described as slightly toxic to birds and mammals (Giacomazzi and Cochet, 2004). A biochip for the detection of Diuron was developed by gold sputtering on a polyester substrate, which was modified with Prussian blue. This surface was then used for construction of a competitive immunoassay by using a specific hapten–protein conjugate. During oxidation of 1-naphthol, the product formed in the enzymatic reaction, and using a square wave voltammetry technique, it was possible to observe a wide linear range (from 1.0 ng to 10 mg L^{-1}) and a coefficient of variation of 3.69% (n = 3) in a repeatability study (Sharma et al., 2011).

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) belongs to the triazine class (which includes atrazine, simazine, cyanazine, ametryn, prometryn, prometon and hexazinone) and is one of the most common herbicides employed in agriculture (*e.g.* plantations of sorghum, corn, pineapple and sugarcane). Atrazine can affect the endocrine system of animals and humans (Nwani et al., 2010). An electrochemical immunosensor was developed for the quantification of atrazine in low concentrations (about 10 pg mL^{-1}) (Ionescu et al., 2010). This immunosensor was built on a gold electrode that was coated with a polypyrrole film. In sequence, this film was modified with copper, on which an anti-atrazine antibody was immobilized by affinity binding. The impedimetric experiments showed that the proposed immunosensor is able to monitor atrazine in river and lake water, being an interesting tool to monitor water quality.

Table 1 presents examples of electrochemical immunosensors for quantification of pesticides, as well as additional information about transducers, limits of detection and linear range; respective references are shown.

Table 1 – Examples of electrochemical immunosensors used for the determination of pesticides in environmental and agricultural analysis.

Analyte	Transducer	Detection Limit	Linear Range	References
Picloram	[a]GCE modified with gold nanoclusters	0.5 ng mL^{-1}	0.001-10 µg mL^{-1}	Chen et al., 2010
Diuron	[a]Gold electrode modified with PB/AuNP	1.0 ng L^{-1}	from 1.0 ng L^{-1} to 10 mg L^{-1}	Sharma et al., 2011
Chlorpyrifos	[b]Chip modified with gold nanoparticles	0.5 ng mL^{-1}	0.5-500 ng mL^{-1}	Jia et al., 2015
Chlorpyrifos	[b]Gold interdigitated array microelectrode modified with protein A	0.01 ng mL^{-1}	10^{0}-10^{5} ng mL^{-1}	Guo et al., 2015b
Atrazine	[b]Gold electrode modified with polypyrrole film	not found	from 10 pg mL^{-1} to 1.0 µg mL^{-1}	Ionescu et al., 2010
2,4-D	[b]Gold electrode modified with thiocompounds	not found	from 45 nmol L^{-1} to 0.45 mmol L^{-1}	Navratilova and Skladal, 2004
Triazinic, organophos-phates and chlorurates	[a]Pt electrode covered with a membrane	10^{-2} µmol L^{-1}	from 10 nmol L^{-1} to 4.0 µmol L^{-1}	Martini et al., 2015
Paraoxon	[a]GCE	12 µg L^{-1}	up to 1920 µg L^{-1}	Hu et al., 2003
Paraoxon	[a]GCE	0.3 ng mL^{-1}	From 1.0 nmol L^{-1} to 100 µmol L^{-1} and 5.0-200 µmol L^{-1}	Sun et al., 2011
Diuron	[b]SPCE modified with AuNP	5.5 ng mL^{-1}	1.0-1000 ng mL^{-1}	Bhalla et al., 2012

Table 1 – Continued

Analyte	Transducer	Detection Limit	Linear Range	References
Atrazine	[b]Au/Cr interdigitated microelectrode	< 100 µg L^{-1}	not found	Rodriguez et al., 2008
Atrazine	[c]Au interdigitated microelectrode modified with N-acetylcysteamine	2.4 µg L^{-1} (+25 mVdc)	not found	Valera et al., 2010
Carbofuran	[b]Gold electrode modified with L-cysteine	0.1 ng mL^{-1}	0.1-1000 ng mL^{-1}	Liu et al., 2015
Endosulfan	[a]GCE modified with SWCNT	0.05 µg L^{-1}	0.05-100 µg L^{-1}	Liu et al., 2014
Paraoxon	[a]GCE modified with SWCNT	2.0 µg L^{-1}	2.0-2500 µg L^{-1}	Liu et al., 2014
Atrazine	[a]GCE modified with polymer film	0.1 pmol L^{-1}	from 0.1 pmol L^{-1} to 10 µmol L^{-1}	Tran et al., 2012
Atrazine	[a]GCE	4.0 µg L^{-1}	9.0-180 µg L^{-1}	Vianello et al., 1998
2,4-D	[a]GCE	not found	0.2-70 µg L^{-1}	Trau et al., 1997
2,6-Dichlo-robenzamide	[a]SPCE	not found	0.0008-62.5 µg L^{-1}	Uthuppu et al., 2015
Isoproturon	[a]SPCE	3.2 ng mL^{-1} or 0.2 ng mL^{-1}	1.0-2000 ng mL^{-1} or 0.9-3000 ng mL^{-1}	Baskeyfield et al., 2011
2,4-D	[a]Gold electrode	0.1 µg L^{-1}	not found	Kalab and Skladal, 1995

Abbreviations: SWCNT: single-walled carbon nanotubes, SPCE: screen-printed carbon electrode, GCE: glassy carbon electrode, 2,4-D: dichlorophenoxyacetic acid, AuNP: gold nanoparticles, PB: Prussian blue.

Forms of detection: [a]voltammetric, [b]impedimetric and [c]conductometric.

Besides pesticides, other biological elements (*e.g.* fungi, antibiotics, phytohormones and cyanotoxins) have been determined in agricultural and environmental samples using electrochemical immunosensors. Fernandez-Baldo *et al.* (Fernandez-Baldo et al., 2009) modified an SPCE with CNTs and immobilized specific monoclonal antibodies onto the surface for quantification of *Botrytis cinerea*, a necrotrophic fungus that affects many plant species, producing a disease known as grey mould. This competitive immunoreaction was utilized for the reduction of 4-tertbutyl o-benzoquinone (working potential: −0.15 V *vs.* Ag/AgCl), presenting a limit of detection for the fungus of 0.02 μg mL^{-1} in apple tissues.

Merola et al. (2014) developed a sensitive electrochemical immunosensor for determination of penicillin G and other β-lactam antibiotics in river wastewater samples. The amperometric immunosensor was assembled using a polymeric membrane in which the *Ab* or *Ag* was immobilized. After the optimization step, the authors obtained a linear response in a wide range of concentrations (from 5.0×10^{-10} to 5.0×10^{-5} mol L^{-1}), as well demonstrating the possibility to regenerate the polymeric membrane employing a solution containing glycine and $MgCl_2$.

5.3. Cancer biomarker diagnosis

Biomarkers receive distinct definitions according to different international organizations. The National Cancer Institute (NCI) defines a biomarker as 'a biological molecule found in blood, in other body fluids, or in tissues that is a sign of a normal or abnormal process, or of a condition or disease. It may be used to see how well the body responds to a treatment for a disease or condition.' Tumour biomarkers are possibly one of the most valuable tools for timely cancer detection and monitoring the progression of this disease. Most tumour markers can be found in the blood, urine, different tissues or

bodily fluids of patients with cancer. Examples of tumour biomarkers are: carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), α-1-fetoprotein (AFP), carbohydrate antigen 19-9 (CA 19-9), cancer antigen 125 (CA125), neuron-specific enolase (NSE), murine double minute 2 (MDM2), squamous cell carcinoma antigen (SCCA) and human epididymis specific protein 4 (HE4), among others (Li et al., 2012a; Chikkaveeraiah et al., 2012).

Methods based on immunoassays have been developed and used for the quantification of tumour markers in a sensitive, precise and accurate way. Recently, Bahadir and Sezginturk (2015) published a review about applications of electrochemical immunosensors for determination of tumour and cardiac markers. In this paper, the developed biosensors are compared in terms of reproducibility, stability, selectivity, precision and regeneration.

A sandwich-type electrochemical immunosensor was developed for the quantification of CA 72-4, a biomarker that can be used to diagnose and monitor gastric cancer. For this immunosensor, the primary CA 72-4 *Ab* was immobilized onto a GCE modified with rGO/tetraethylene pentamine and the secondary CA 72-4 *Ab* was adsorbed onto PtPd-Fe_3O_4 nanoparticles. The electrochemical immunosensor was evaluated for serum samples with a low detection limit (3.0×10^{-4} U mL^{-1}) (Wu et al., 2015).

Different transducers have been proposed for the construction of electrochemical immunosensors and determination of the lung, ovarian and breast cancer biomarker CEA in low concentrations.

Examples are:

- GCE modified with rGO/thionine/AuNPs (Kong et al., 2011),

- Ag-SPE modified with polypyrrole (Moreira et al., 2016),

- Gold electrode modified with thiol/cysteine (Yang et al., 2014),
- ITO modified with polydopamine/functionalized mesoporous silica nanoparticles (Wang et al., 2013b),
- GCE modified with GO/AuNPs (Feng et al., 2016),
- GCE modified with AuNPs decorated with 3-aminopropyltriethoxysilane-functionalized graphene sheets (Wang et al., 2014b),
- GCE modified with multilayer films of MWCNTs/polyethylenimine/AuNPs/Prussian blue (Zhang et al., 2012),
- AuNPs and Prussian blue film (Cao et al., 2013).

Zhao et al. (2015) developed a voltammetric immunosensor for the simultaneous quantification of AFP and PSA, biomarkers for liver and prostate cancer respectively, in clinical samples. Using a sandwich-type structure, the primary AFP and PSA antibodies (Ab_1) were immobilized onto a GCE modified with rGO/AuNPs. Enhanced sensitivity was achieved for this sensor due to the large surface area and high conductivity of rGO/AuNPs. The secondary antibodies (Ab_2) were adsorbed onto Si/AuNPs and the GCE decorated with the redox probes Azure (A) and ferrocenecarboxylic acid (Fc); the resulting Fc@Ab_2/Si@AuNPs and Azure A@Ab_2/Si@AuNPs were used as labels for the preparation of immunosensors to detect AFP and PSA at −0.48 V (*vs.* SCE) and +0.12 V (*vs.* SCE), respectively.

Representative examples of electrochemical immunosensors for determination of cancer biomarkers described in the literature are summarized in Table S2, which can be found in the Supplementary Material.

6. Conclusion

Electrochemical immunosensors have gained prominence in recent years. Advantages such as sensitivity, selectivity, portability, the possibility of simultaneous multi-target analysis and miniaturization are very favourable aspects that are making researchers look for them with increasing attention in the last few years. Additionally, the effective low cost of these devices allows them to be disposable, a decisive advantage in many situations. Immunosensors are of great value for use in clinical diagnosis, environmental monitoring and food analysis, because they are based on *Ab–Ag* reactions which are highly specific and proportionate rapid and reliable responses. The possibility of working with very small amounts of samples is another aspect that makes them attractive.

This review addresses recent advances in food samples, environmental analysis and cancer biomarker diagnosis. Examples of electrochemical immunosensors developed for the detection of pathogens (*E. coli*, *F. tularensis*, *Salmonella* and *Fusarium* fungi), pesticides (picloram, atrazine, carbofuran and chlorpyrifos) and tumour biomarkers (CEA, PSA and AFP) have been referred to, highlighting the main analytical characteristics of each immunosensor.

Immobilization of *Ab* plays a fundamental role in the development of electrochemical immunosensors, because it is the recognition element to form the immunocomplex *Ab–Ag*. Different methods including chemical and physical adsorption for immobilization of the antibody on the transducer have been discussed. The sensitivity and stability of immunosensors is improved many times by the association of nanostructured materials such as CNTs, graphene, metallic nanoparticles, QDs and MBs on the sensor.

The evolution of immunosensors verified in recent years suggests that they will play a fundamental role in various areas, especially in medicine, pharmaceuticals and in the food industry.

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HIGHLIGHTS

1 – Description of the basic principles of immunoassays and immunosensors.

2 – Explanation in a simple stile about electrochemical detection.

3 – Description of the main applications of electrochemical immunosensors

4 – Presentation of successful applications of immunosensors in different fields