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Review article

Impedimetric immunosensors—A review

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ABSTRACT

This review outlines the theoretical background of impedimetric biosensors and presents different types of impedimetric immunosensors along with the instrumental approaches that have been so far proposed in the literature for the evaluation of their performance. The electrode assemblies have been classified in four main categories with respect to the electrode material, the type of the insulating layer and the immobilization platform that have been used for their construction. Additionally, some selected works on recent developments in immunosensors, which are based on polymer degradation phenomena, magnetic nanobeads, etc. as well as strategies for the amplification of the measuring signals, are also presented.

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1. Introduction

The development of methods targeting the direct monitoring of antibody–antigen interactions is particularly attractive. The design of label-free affinity-based probing concepts is the objective of much current research, at both academic and industrial levels, towards establishing alternative methods to the already existing ELISA-based immunoassays. Among the different types of immunosensors that enable the direct monitoring of such interactions, impedimetric immunosensors have recently received particular attention since they possess a number of attractive characteristics associated with the use of electrochemical transducers, namely, low cost of electrode mass production, cost effective instrumentation, the ability to be miniaturized and to be integrated into multi-array or microprocessor-controlled diagnostic tools, remote control of implanted sensors, etc. Indeed, due to the above-mentioned characteristics, electrochemical impedance spectroscopy (EIS)-based sensors are considered as promising candidates for use at on-site applications.

Since pioneering works of Newman [1] and Martelet [2] on the concept of capacitive, or impedimetric based immunosensors, a lot of work has been done in this specific area. During the last decade, impedance spectroscopy has been widely used for probing various types of biomolecular interactions (immunosensors, DNA hybridization, rapid biomolecular screening, cell culture monitoring) and relevant literature has been comprehensively reviewed [3–7]. So far, impedimetric immunosensors have been successfully applied at the academic level. However, no prototypes have been released into the market and this fact has brought the reliability of them into question. Fundamental analytical issues, mostly as regards their reproducibility, have impeded so far their application in routine quantitative analysis. On the other hand, due to the plethora of their inherent advantages, impedimetric immunosensors could potentially be used for qualitative purposes, such as the detection of bacteria, pregnancy tests, allergy screening tests, etc.

2. Theoretical background and general description

EIS is an ac method that describes the response of an electrochemical cell to a small amplitude sinusoidal voltage signal as a function of frequency. The resulting current sine wave dif-

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fers in time (phase shift) with respect to the perturbing (voltage) wave, and the ratio $V(t)/I(t)$ is defined as the impedance (Z), and accounts for the combined opposition of all the components within the electrochemical cell (resistors, capacitors, inductors) to the flow of electrons and ions. In an electrochemical cell, electrode kinetics, redox reactions, diffusion phenomena and molecular interactions at the electrode surface can be considered analogous to the above-mentioned components that impede the flow of electrons in an ac circuit [8–10]. However, specifically with impedimetric immunosensors, the contribution from inductance is of minor importance.

Impedance is usually expressed as a complex number, where the ohmic resistance is the real component and the capacitive reactance is the imaginary one. The most popular formats for evaluating electrochemical impedance data are the Nyquist and Bode plots. In the former format, the imaginary impedance component (Z'' , out-of-phase) is plotted against the real impedance component (Z' , in-phase) at each excitation frequency, whereas in the latter format, both the logarithm of the absolute impedance, $|Z|$ and the phase shift, θ , are plotted against the logarithm of the excitation frequency [10].

Capacitive immunosensors exploit the change in dielectric properties and/or thickness of the dielectric layer at the electrolyte–electrode interfaces, due to the antibody–antigen (Ab–Ag) interaction, for monitoring this process. An electrolytic capacitor (working electrode/dielectric/electrolyte; the second plate is represented by the electrolyte) allows the detection of an analyte specific to the receptor that has been immobilized on the insulating dielectric layer, which has previously been deposited on the surface of the working electrode (Fig. 1a) [1–6]. Ideally, this configuration resembles a capacitor in its ability to store charge and thus, the electric capacitance between the working electrode and the electrolyte is given by Eq. (1):

$$C = \frac{\epsilon_0 \epsilon A}{d} \quad (1)$$

where ϵ , is the dielectric constant of the medium between the plates, ϵ_0 , is the permittivity of free space (8.85419 pF/m), A , is the surface area of the plates (m^2), and, d , is the thickness of the insulating layer (m).

A decrease of the total capacitance, due to the increase of the distance between the plates is thus expected upon the binding of the analyte to its specific receptor. Plausibly, this phenomenon can be represented by two capacitors in series; the inner one corresponds to the dielectric (C_{dl}) layer and the outer one corresponds to the biomolecule layer (C_{bm}) and consequently to the interactions of this layer with its specific ligand. Since the current must pass through the uncompensated resistance of the electrolyte solution, its resistance is inserted as a series element in the circuit (Fig. 1b). In this case, the total capacitance C_t , can be described by Eq. (2)

$$\frac{1}{C_t} = \frac{1}{C_{dl}} + \frac{1}{C_{bm}} \quad (2)$$

As can be seen from Eq. (2), in order to design a sensitive sensor with a wide dynamic range, the insulating layer should be thin enough and/or have a high dielectric constant. Otherwise, capacitance changes originating in the binding of the analyte to the receptor might not dominate the total capacitance. In addition, the insulating layer should be complete (pin-holes free), stable with the time, and provide functional groups for the immobilization of the receptor [11]. Signal changes in capacitive immunosensors can be also induced by changes in dielectric properties, charge distribution, or even conformational changes of the immobilized biomolecule layer upon its interaction with the analyte.

Commonly, the above-mentioned electrochemical capacitors are described as CPE (constant phase element). Mathematically,

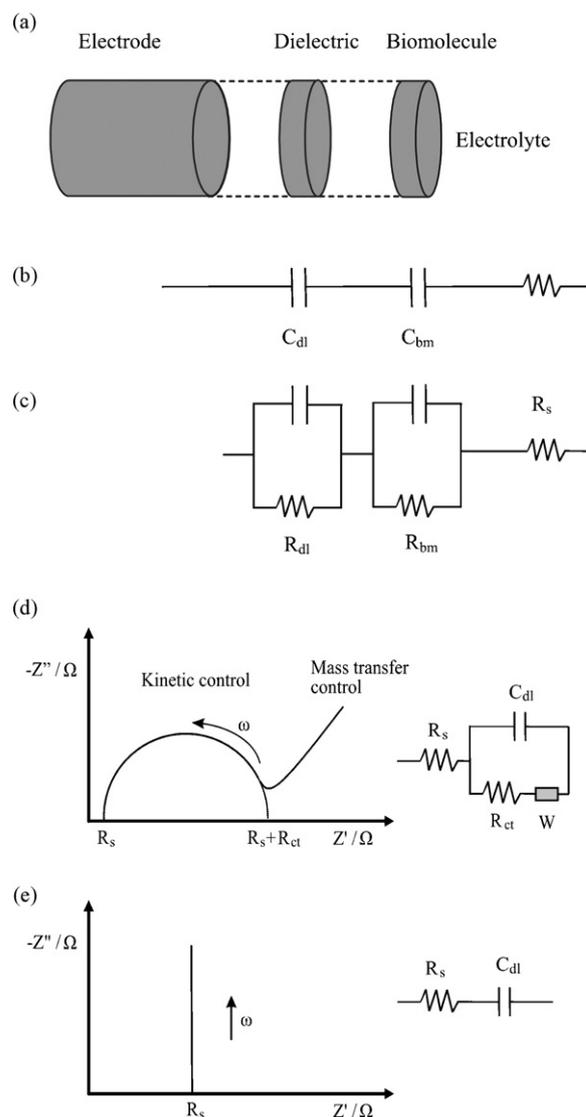


Fig. 1. (a) Representation of a capacitive biosensor. (b), (c) Electrical circuit models used to represent this (d) Nyquist plot arising from a Randles circuit (showing in the side panel) (e) Nyquist plot arising from an RC circuit in series, showing in the side panel. The terms shown are described within the text.

$CPE = (1/Q)^{-1}(j\omega)^{-n}$, where $j = (-1)^{1/2}$, and in fact, it counts as a more generalized electric element [12], consisting of a group of parallel or branched resistive-capacitive transmission lines, for adjusting deviations originating in surface heterogeneities; the extent of this deviation being controlled by the parameter n ($n \leq 1$). Although the exact physical basis of CPE has to be elucidated, CPE can serve as a very flexible tool for fitting experimental data. In practice, the presence of CPE instead of C means that the observed capacitance of the system is frequency dependent.

The dielectric behaviour of the immobilized biomolecule is also an important issue in the development of impedimetric immunosensors. A physical model proposed by Pethig and Kell [13] describes the dielectric behaviour of proteins being under an electric field with a dielectric constant around 20. However, it is well known that bilayers do not have a suitable dielectric character, since ions are moving through, or around them, causing “shorting” of the system. To overcome this problem, Berney et al. [14] proposed covering the bilayer with a non-conductive polymer. However, the polyethylene glycol that was used for this purpose resulted in practically inactive electrode assemblies.

The non-ideal dielectric behaviour of the insulating and biomolecule layers can be represented by a resistance as a parallel element to each of the corresponding capacitors (Fig. 1c). Defects in the construction of the insulating layer (pin-holes) and the existence of ions and water molecules within the protein structure are the major reasons for the non-ideal dielectric behaviour of a tested assembly. In this case, the dynamic range and the sensitivity of the sensors are decreased.

While no equivalent model can be guaranteed to be unique, simulation of the recorded impedimetric data to an equivalent electric circuit is a common strategy for understanding the physical origin of the observed response. The simplest, and in fact the most frequently used equivalent circuit for modelling of EIS experimental data is the so called Randles circuit (Fig. 1d), which comprises the uncompensated resistance of the electrolyte (R_s), in series with the capacitance of the dielectric layer (C_{dl}) and the charge-transfer resistance (R_{ct}), if a redox probe is present in the electrochemical cell. The latter two components are connected in parallel. An additional component, connected in series with R_{ct} , the Warburg impedance (Z_w) accounts for the diffusion of ions from bulk electrolyte to the electrode interface. A typical shape of the impedance spectrum of this circuit presented in a Nyquist plot (Fig. 1d) includes a semicircle region lying on the real axis followed by a straight line. The linear part ($\varphi = \pi/4$), observed at the low frequency range, implies a mass-transfer limited process [3,10], whereas the semicircle portion, observed at high frequency range, implies a charge-transfer limited process.

In the absence of a redox probe, and if the electrode surface has been covered with a non-porous, pin-hole free dielectric coating, the equivalent circuit includes a resistor (due primarily to the electrolyte) and the capacitance of the dielectric coating in series. The Nyquist plot for this model appears a straight line parallel to the imaginary impedance axis (Fig. 1e). The intercept of the line gives an estimate of the solution resistance, whereas the capacitance can be calculated by Eq. (3) (where radial frequency ω is expressed in rad s^{-1})

$$Z = \frac{1}{\omega C} \quad (3)$$

The model illustrated in Fig. 1e, implies that all of the measured current is capacitive (90° component). However, this situation is not common for electrolytic capacitors. The impedimetric response of the latter, in the format of a Nyquist plot, includes a curvature in the low frequency range, which represents the resistance of the insulating and biomolecule layers to species moving through the collapsed sites and pinholes within the film structure, and reflects the protective properties of these layers [3,4]. In some cases, the whole sensor assembly behaves as an electric circuit with two time-constants ($\tau = RC$) [15,16].

Based on the nature of the measuring signal, impedance immunosensors can be classified into two main categories:

- (a) *Capacitive*, where the surface of the electrode is completely covered by a dielectric layer and the whole electrode assembly behaves as an insulator. In this type of sensor, no redox probe is present in the measuring solution and the capacitive current is measured under a small amplitude sinusoidal voltage signal, at low excitation frequencies (typically 10–1000 Hz). According to the above discussion, Ab–Ag interactions are expected to cause a decrease of the measuring capacitance.
- (b) *Faradic or Faradaic impedimetric*, where the surface of the electrode, which is partially or wholly covered by a non-insulating layer, or partially covered by an insulating layer is able to catalyze a redox probe, which exists in the measuring solution. In this case, the measured parameter is the charge transfer resistance (the real component of impedance at low

frequency values, typically 0.1–1.0 Hz), and Ab–Ag interactions are expected to cause an increase in its value as the faradic reaction becomes increasingly hindered. In general, faradic impedimetric immunosensors exhibit a higher sensitivity to Ab–Ag interactions. However, the redox species may have an effect on both the stability and the activity of the electrode assembly.

As the disassociation constant for the antigen:antibody complex is low, the reusability of these sensors is difficult to be achieved reproducibly. Regeneration procedures include the use of low (<3), or high (>9) pH buffer solutions, detergents, high ionic strength solutions or non-polar water soluble solvents that normally have an influence on both the activity of the biorecognition layer and the stability of the inner dielectric layer.

3. Systematic presentation of different electrode assemblies

The numerous electrode assemblies that have so far been developed can be classified in the following main categories with respect to the electrode material, the type of the insulating layer and the immobilization platform that have been used for their construction. The following classification aims to cover the most popular electrode surface modification strategies. The use of special electrode designs (interdigitated electrodes, microlithographically fabricated electrodes architectures, nanoelectrode arrays), or works on integrated microfluidics-impedimetric biosensors-based devices are not included, as in those cases as well the chemical strategies employed in sensor's buildup are the same.

- Gold electrodes covered with thiol-based self-assembled monolayers (SAM).
- Oxide-based [indium-tin oxide (ITO), semiconductive (Si/SiO₂), and metal oxide (M/MO_xH_y)] electrodes functionalized with a "silane" layer.
- Entrapped or covalent bonded biomolecules onto conductive polymer coated electrodes.
- Covalent bonded biomolecules onto electropolymerized non-conductive films.

Thiol-based SAMs have been prepared on gold [17], mercury [18], platinum [19] and silver [20] electrodes; their dielectric constant is about 2.8 [11], and depending on their length, they provide conductive, semiconductive or insulating coatings. Long chain thiols provide stable coatings with low capacitance values, whereas shorter chains are more susceptible to desorption phenomena resulting in unstable background signals [21]. Stable and reproducible coatings based on SAMs can be achieved on perfectly clean and low roughness gold surfaces only, and thus the overall quality of the electrodes is largely determined by the treatment method of their surface. In this respect, various treatment protocols have been proposed including:

- (i) Chemical treatment in piranha solution (1:3, 30% H₂O₂:conc. H₂SO₄) [21].
- (ii) Chemical and electrochemical treatment in boiling 2 M KOH (in 30% v/v ethanol), followed by sonication in concentrated hot HNO₃ (30 min) and pure water, respectively. The electrochemical treatment includes cycling over the potential window –0.1 to +1.25 V versus Ag/AgCl in 0.1 M H₂SO₄ solution until a stable and reproducible gold oxidation peak at +1.1 V versus Ag/AgCl was obtained [22].
- (iii) Different electrochemical cleaning protocols by applying a linear voltage sweep from 0 to –2.0 V against a platinum counter electrode in 1 M potassium hydroxide [23], or potential pulses

(+1.6, 0.0, –0.8 V, each 0.1 s) for 30–120 min in 100 mM phosphate buffer [24].

- (iv) Thermal treatment. Steinberg et al. [25] have found that if the gold is thermally annealed at 400 °C the surface appears to improve in cleanliness and smoothness.

Using a lock-in amplifier and capacitance measurements at +0.3 V dc potential versus Ag/AgCl at 20 Hz, a comparative study on the performance of lithographic gold electrodes as immunosensors has been published by Mirsky et al. [21]. Electrodes were covered with different carboxyl- or amine-ended alkylthiols and various activation procedures were evaluated. Under optimum conditions, capacitive immunosensors were constructed, which were able to provide selective measurements of human serum albumin (HSA) in standard solutions in the range 1–20 mg mL⁻¹.

Immunosensors based on SAMs of thioctic acid and carbodiimide activation have been also reported. The resulting sensors were found to be quite sensitive to the target analyte. However, more than the half electrodes gave no response at all [26]. The same group also evaluated the performance between immunosensors based on SAMs of thioctic acid and cysteamine followed by post-blocking with dodecanethiol [27]. Epoxy-activated cysteamine-based electrodes had a much narrower dynamic range but a higher sensitivity than the electrodes activated with thioctic acid as a result of the high background capacitance of former. The reproducibility between different electrodes was only 30–40%.

Functionalization of gold surfaces with succinimidyl ester-terminated alkyldisulfides (SEAD) has also been proposed. SEAD can be used for the development of SAMs onto gold surfaces, through the spontaneous reduction of the –S–S– bond [28]. The use of SEAD-based SAMs as immobilization platforms offers certain advantages as such monolayers provide interfaces with specific hydrophobicity and desired chemical reactivity to amino-group containing biomolecules. In addition, there is no need to use coupling agents or activators, simplifying in this way, the experimental procedure for the construction of the immunosensors. Using dithio-bis(succinimidylundecanoate), Malamou and Prodromidis [28] was recently reported on the performance of a label-free faradic impedimetric immunosensor specific to blood group antigen A exists on membrane fragments of sonolysed red blood cells (RBS). The poor performance of the proposed immunosensors to intact RBC is a serious drawback for use in routine analysis.

Employing an amino-terminated thiol and a biotinylated activation step through a water soluble NHS-ester of biotin, Hammers and colleagues [15] reported on the mechanism of signal transduction and the physical factors that control the analytical sensitivity of the electrodes produced. Data was modelled according to an equivalent circuit containing two time constants. The results showed that binding of avidin to biotin changes both the inner (thiol-based SAM) and outer (biotin-avidin) layers and that the avidin-induced electrical changes could be sensed only at frequencies <1 Hz.

Hays et al. [29] reported on the construction of impedimetric immunosensors based on mixed 16-mercaptohexadecanoic acid and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-caproyl biotinyl self-assembled monolayers onto lithographic gold electrodes. Immobilization of IgG antibodies was achieved using the strong non-covalent bond formed between neutravidin and biotin to tether anti-human haemoglobin goat IgG to the mixed monolayers. Using ferricyanide as a redox probe, haemoglobin at a concentration level of 10⁻¹⁰ M was detected in standards solutions.

Label-free detection of *Escherichia coli* was proposed by Jaffrezic-Renault and colleagues [30] using nonfaradic EIS. Biotinylated polyclonal anti-*E. coli* was linked to a mixed SAM on a gold electrode through a strong biotin-neutravidin interaction. After modelling the impedimetric data, they concluded that the parameter that is modified after incubation of the immunosensors to whole or lysed

bacteria is the polarization resistance R_p . Limit of detection (LOD) as low as 10 colony forming units (cfu) mL⁻¹ whole bacteria in pure cultures of type strain is reported; however, for higher cfu values the specificity of the proposed immunosensors is relatively low.

Recently, our group has proposed impedimetric immunosensors for the direct detection of *Salmonella typhimurium* in pure cultures of type strains and inoculated milk samples, using faradic impedimetric measurements. To achieve the working simplicity expected by a biosensor, immunoreaction was performed directly in cultures. This resulted in the elimination of various centrifugation and washing steps, which are used for the isolation of bacteria cells from the culture, thus making the proposed immunosensors promising candidates for on-site applications. More specifically, polyclonal anti-*Salmonella* was cross-linked, in the presence of glutaraldehyde, on gold electrodes modified with a mixed SAM of 11-amino-1-undecanethiol and 6-mercapto-1-hexanol at a constant 1+3 proportion, respectively [31]. The mixed SAM was also deposited in the presence of triethylamine, which was used to prevent the formation of interplane hydrogen bonds among amine-terminated thiols. A meticulous study regarding the type and the concentration of different post-blocking agents, which used for blocking the non-specific binding sites of the sensors was made, and optimized immunosensors were successfully used for the direct detection of *S. typhimurium* in milk samples.

The most commonly used “silanes” are the tri-functional (3-aminopropyl)triethoxysilane [APTES, (C₂H₅O)₃-Si-(CH₂)₃-NH₂], (3-glycidoxypropyl)trimethoxysilane [GPMS, (CH₃O)₃-Si-(CH₂)₃-O-CH₂-CH(O)CH₂] and the mono-functional (3-glycidoxypropyl) dimethylethoxysilane [GPMS, (C₂H₅O)₂-Si-(CH₂)₃-O-CH₂-CH(O)CH₂]. APTES contains an amino end and protein immobilization can be achieved by means of a cross-linker such as glutaraldehyde or hexamethylene diisocyanate. In this respect, GPMS are superior, as the oxirane radical readily binds amino groups without an intermediate cross-linker being necessary.

Using Pt/Ti/TiO₂ electrodes and capacitance measurements at 2 kHz and 20 mV rms, Varlan et al. [32] proposed the direct assay of the allatostatin hormone in serum. A comparative study between different silanization procedures was made and best results were obtained with GPMS deposited from an ethanol solution containing 5% (v/v) distilled water. Mantzila and Prodromidis [16] reported on the impedimetric behaviour of anodically formed Ti/TiO₂ electrodes. The effect of the initial conductivity of electrodes, managed by controlling the hydroxylation process (duration of chemical etching), and the effect of “silane” layers deposited through liquid or gas-phase based protocols on the stability and the origin of the measuring signal were studied. Based on electrochemically growth tantalum/tantalum oxide electrodes, a flow-through cell for the real-time capacitance monitoring of IgG protein down to 0.2 ng/mL was proposed by Gebbert et al. [11]. These authors optimized the sensitivity of the immunosensors with respect to the thickness of the tantalum oxide layer obtained by formation at voltages between 0.1 and 250 V in acidic conditions. An immunosensor based on a discontinuous nanoscale platinum layer was also described by Pak et al. [33]. Monoclonal antibodies of alkaline phosphatase were covalently bound onto an epoxy-silane deposited followed a gas phase protocol. Binding of the target analyte to the prepared surface resulted in an impedance increase of 12%, whereas non-specific binding of HRP caused an impedance change <6%. Helali et al. [34] reported on the construction of immunobiosensors based on Nb/NbO_xH_y electrodes for the detection of atrazine in standard solutions. Niobium oxide was anodically formed onto niobium electrodes at 25 V in 1 M H₂SO₄ and hydrous oxide layers were silanized with APTES, and using glutaraldehyde as a cross-linker, Fab fragment K47 antibody was covalently immobilized onto the surface of the electrodes. Corry et al. [35] cleaned indium-tin oxide (ITO) surfaces with acetone and dichloromethane followed by hydrox-

ylation with a mixture of $\text{NH}_3 + \text{H}_2\text{O}_2 + \text{H}_2\text{O}$ (1 + 1 + 5) and then an aminosilane linker was directly reacted with anti-atrazine IgG antibodies (oligosaccharide moieties having been previously oxidised to aldehydes), as this procedure was determined to yield stable and homogeneous protein layers without loss of antigen binding capacity.

Considerable work onto semiconductive Si/SiO₂ heterostructures based capacitance immunosensors has been done by Martelet and his co-workers [36,37]. Measurements were conducted in an accumulation polarization regime (dc bias 2V versus SCE) thus making the silicon behaves as a metal. Anti- α -fetoprotein antibodies were immobilized through three different coupling reagents: aminosilane, cyanosilane and polysiloxane membranes. The latter approach gave the most promising sensor assembly able to detect α -fetoprotein in standard solutions in the range between 10 and 150 ng mL⁻¹.

Different types of affinity biosensors based on conductive electropolymerized polymer films are becoming an important class of analytical tools. Original works by Wallace and Sadik [38–40] as well as subsequent works on polypyrrole-based impedance immunosensors, were recently reviewed by Cosnier [41]. In these types of sensors, the immobilization of biomolecules can be accomplished either by direct adsorption [42] or by their incorporation into the polymer matrix during the growth of the latter [43]. In a third approach, properly functionalized polymer precursors can create readily reactive polymer films to biomolecules bearing amino or carboxy groups or to avidin-based conjugates [44–46].

Biomolecule interactions occurred on the redox polymer result in the exchange of the counter ions between the conducting polymer and the protein molecules thus altering the electrochemical properties of the polymer. The mechanism is not completely understood, and the recorded signal changes are strongly affected by the composition of the polymer, the nature of the counter ions, and the electrochemical waveform used in the electropolymerization process. Despite the broad applicability of this type of biosensors, preparation of reproducible polypyrrole layers and non-specific adsorption of proteins to polypyrrole layers remain the main problems in their use.

Owino et al. [42] reported on the electrostatic attachment of aflatoxin B1 antibodies on Pt electrodes modified with polyaniline and polystyrene sulfonic acid, while Gibson and colleagues [43] was proposed an immunosensor for the detection of bovine serum albumin (BSA) in standard solutions by incorporating anti-BSA into the conductive polymer at the surface of screen-printed carbon electrodes.

Electropolymerized biotinylated polypyrrole film was also used as an immobilization matrix for the development of impedimetric biosensors. Biotinylated anti-human IgG was attached to free biotin groups using avidin as a coupling reagent. The proposed assembly was found to be highly reproducible and stable, and only minor loss of the response was observed after two regeneration steps [44].

Another conducting polymer, a terthiophene monomer having a carboxylic acid group, 5,2':5',2''-terthiophene-3'-carboxyl acid was electropolymerized over a glassy carbon electrode and used as immobilization platform for the monoclonal anti-vitellogenin antibody. Linearity over the concentration range 1–8 $\mu\text{g L}^{-1}$ (LOD 0.42 $\mu\text{g L}^{-1}$) vitellogenin was observed and the sensors were successfully used on real male and female serum samples [45].

A new interesting research direction lies in chemical grafting of antibodies onto insulating electropolymerized films, such as polytyramine [46,47] and poly (*o*-phenylenediamine) [48].

Pournaras et al. [46] proposed a faradic impedimetric immunosensor for the direct detection of *S. typhimurium* in pure cultures of type strains and inoculated milk samples, by immobilizing polyclonal anti-*Salmonella*, in the presence of glutaraldehyde vapors, on electropolymerized poly-tyramine-modified gold elec-

trodes. Optimized immunosensors were successfully used for the direct detection of *S. typhimurium* in inoculated milk samples.

Fabrication of a capacitance immunosensor based on electropolymerized polytyramine for the detection of HSA in standard solutions was proposed by Wu et al. [47]. Specific antibodies were immobilized to the free amine groups of the polymer through glutaraldehyde cross-linking. The proposed immunosensors gave a linear response over the concentration range 1.84–368.6 ng mL⁻¹ HSA and an LOD of 1.6 ng mL⁻¹ HSA was calculated. The polytyramine films showed remarkable stability over a wide pH range and especially in acid region, thus making possible their reuse after treatment in a 0.1 M glycine-HCl pH 2.5 buffer solution.

Liu et al. [48] immobilized anti-transferrin antibodies onto a poly (*o*-phenylenediamine) film. The resulting immunosensors were evaluated using in a pulsed potentiometric interrogation and a linear calibration curve was obtained over the concentration range 0.1–45 ng mL⁻¹ of the target protein with an LOD of 61 pg mL⁻¹.

4. Recent developments in immunosensors

Various types of impedimetric immunosensors based on the electrodeposition of nanometer-sized hydroxyapatite [49], polymer degradation phenomena [50], molecular imprinted polymers [51], magnetic nanobeads [52] have also been proposed.

Impedance analysis of breakdown processes of polymer coatings on electrochemical transducers through the direct or indirect action of biomolecules constitutes a feasible detection protocol for the fabrication of generic integrated biosensors [50]. An interesting application of this concept was proposed for the development of a disposable non-competitive immunosensor for free and total prostate-specific antigen (PSA) using interdigitated carbon electrode coated with a pH-sensitive polymer layer (Eudragit S 100). PSA reacts with both immobilized anti-PSA and anti-PSA urease enzyme conjugate. Then, in the presence of urea, the pH of the reaction media is increased, due to the hydrolysis of urea, causing a breakdown of the polymer layer and a consequent measurable change in the capacitance of the system [53]. Using impedimetric measurements, Fredj et al. [52] presented an atrazine biosensor based on magnetic nanoparticles with an LOD of 5 ng mL⁻¹. Streptavidin labeled magnetic nanoparticles were immobilized over a polypyrrole film under appropriate magnetic field and through streptavidin–biotin interaction the whole electrode assembly was functionalized with a biotinylated Fab fragment K47 antibody.

Continuing this section, selected works, which are exploring different strategies for the amplification of the measuring signal are also presented. Aiming to achieve lower detection limits, the main interaction signal (for example, immobilized bioreceptor–target analyte interaction) may be enhanced by case-specific amplification schemes. In general, these schemes follow the main reaction step and include the application of an extra biotin–(strept)avidin complex, or enzyme-labels that catalyze the precipitation of an insoluble compound on the electrode surface building thus a rather insulating layer.

Ruan et al. [54] used alkaline phosphatase (AP) to amplify the antibody–antigen interaction signal, through the formation of an insoluble precipitation due to the action of AP to 5-bromo-4-chloro-3-indolyl phosphate. Similarly, Balkenhohl and Lisdat [55] has employed an extra incubation step with peroxidase-labeled immunoglobins in order to achieve, the peroxide-catalyzed conversion of the water soluble 3-amino-9-ethylcarbazole to the water insoluble 3-azo-9-ethylcarbazole.

An amplification strategy using a biotin labeled antibody–streptavidin complex was proposed by Pei et al. [56]. The redox couple hexacyanoferrate(II)/(III) was used as a probe

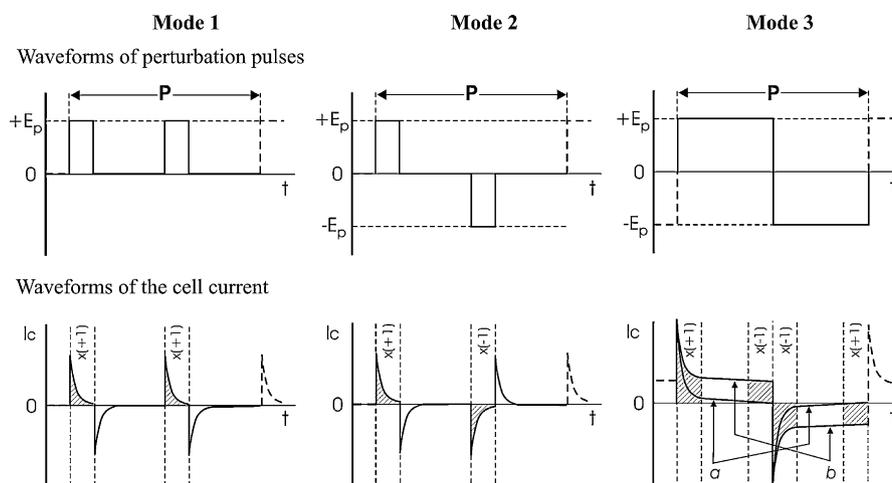


Fig. 2. Waveforms of perturbation pulses used in each operating mode of “Multipulser” and typical waveforms of the cell current corresponding to the perturbation of each mode.

to monitor the progressively increased insulating character of the tested assemblies, as it occurred upon binding of the specific antigen and the subsequent attachment of the specific biotinylated antibody and streptavidin.

Faradic impedance spectroscopy is usually considered to be more sensitive compared to capacitance measurements at electrically blocked electrodes. However, the hexacyanoferrate (II)/(III) system has been found to damage SAMs or to reduce the activity of the immobilized proteins according to the results published by Gopel and colleagues [23] in the development of a mixed self-assembled monolayer of a synthetic peptide and 11-hydroxyundecanethiol. Moreover, the observed signal changes are not always large as small inorganic redox probes can (freely) penetrate through pinholes of the sensing layer-target biomolecule complex. Targeting at the improvement of the sensitivity of faradic impedimetric immunosensors, bulky redox molecules, that is, NADH or even redox enzymes as glucose oxidase [57,58] have been also proposed instead of small redox species. A serious disadvantage in the use of the above bulky redox molecules is the increase of the cost of the analysis, whereas in the case of the protein-assisted probing, sensors also suffer in terms of working simplicity, since extra compound(s) should be added in the measuring cell. Alternatively, bulky inorganic redox compounds, that is, silicotungstic acid hydrate ($\text{H}_4\text{SiO}_4 \cdot 12\text{WO}_3 \cdot x\text{H}_2\text{O}$), have also been used as redox probes in faradic EIS experiments [59].

A two-stage amplification scheme has recently been described by Bonanni et al. [60]. Streptavidin-coated gold nanoparticles were used to amplify the impedimetric signal generated in a biosensor detecting a DNA hybridization event. A biotinylated complementary oligomer was used as target. The addition of streptavidin-coated gold nanoparticles was bound to the target due to the strong streptavidin–avidin interaction (first stage) and a further, remarkable increase of the signal was achieved after a silver enhancement treatment (second stage). Silver anions in solution nucleate around gold nanoparticles and precipitate as silver metal on gold nanoparticles.

It is important to mention a key application of impedance spectroscopy in the field of microbiology, where impedance microbiology is based on the measurement of changes in electrical impedance of a medium or a reaction solution resulting from the bacterial growth [4,5]. Even though the specific applications do not completely fit the concept of biosensors, they have found broad practical use in the rapid analysis of pathogenic bacteria [61,62], and more interestingly, commercial instruments based on this concept have been released onto the market. Using a lab-made experimen-

tal setup and employing impedance measurements Grossi et al. [63] has developed a method for the detection of microbial concentration in ice creams. As it claimed by the authors, the proposed method produced results within 10 h, instead of the 24–48 h needed by standards plate methods, while the whole design is suitable to be implemented as an embedded system for industrial machines. Impedance spectroscopy was also utilized for the assessment of cytotoxicity [64]. Immortalized mouse fibroblasts were cultured onto interdigitated electrodes and among the various parameters (impedance modulus, phase, real and imaginary components of the measured impedance), which were examined, impedance modulus was found to be the most sensitive for cytotoxicity testing of chemicals. The reader is referred to the review of Guan et al. [5] for a wider and more detailed description of impedance microbiology.

5. Instrumentation and quantification parameters

Several instruments based on small-signal ac admittance measurements such as LCR-meters [14,33], impedance analyzers [11], lock-in amplifiers [21,36,37,51,65] and frequency response analyzers (FRA) [15,16,60,23,34,35] have been used to monitor interactions between biomolecules. The last two approaches are the most widely used and bring both inherent advantages and disadvantages. Impedance systems based on lock-in amplifiers are very sensitive, can effectively remove background noise, minimize harmonic distortions and are relatively cost effective. On the other hand, it is difficult to use them for stand-alone measurements which are somewhat slow, and they cannot be used over a wide frequency range. Impedance systems based on FRA, provide fast analysis over a wide frequency range, remove harmonic distortions and dc components and can be easily fully automated. Limited sensitivity and background removal as well as their relatively high cost are disadvantages associated with FRA based measuring systems [66].

Berggren and Johansson [26] evaluated capacitance changes from the transient current response after the application of a potentiostatic step. In this approach, the capacitance of the sensor was extracted from the time constant of the exponential fit to the current *versus* time trace. Prasad and Lal [65] also developed a technique for measuring small-signal capacitance changes based on a computer-controlled two-phase lock-in amplifier and potentiostatic control by appropriate software. Efstathiou and colleagues [67] reported on the construction of a stand-alone, low-cost electronic device (Multipulser), for monitoring interactions between

biomolecules that may change the capacitance of an electrode. The operation of the Multipulser is based on the repetitive charging of the electrochemical cell capacitance by applying a predetermined number of short-duration, low-amplitude voltage pulses (perturbation pulses). All packets of charge are accumulated in an analog integrator whose output voltage is proportional to the cell capacitance. The Multipulser features three user-selectable operating modes, each one characterized by its own particular shape of the applied perturbation pulses (Fig. 2). Mode 3 seems particularly interesting for electrodes characterized by a dielectric layer of relatively low resistance. In these cases, contrary to what is expected with dielectric layers of near-infinite resistance, i_c never decays to zero, due to the constant (dc) component of non-capacitive current flowing through the cell. Mode 3 reduced or eliminates the contribution of this dc current component to the measured signal.

The physical parameters that have been used so far, as a measure of the analyte concentration include capacitance, charge-transfer or polarization resistance and impedance. Capacitance and impedance (Z , Z' or the modulus $|Z|$) measurements at a specific frequency are fast and provide the ability to monitor the kinetics of the coupling event. On the other hand, measurements based on charge transfer or polarization resistance are rather time consuming, as they require first a $Z'' = f(Z')$ spectrum over a wide frequency range to be obtained. Once the spectrum was obtained, resistance values can be calculated either graphically, or by fitting the equations associated with an equivalent circuit to the experimental data [68].

6. Conclusions

In general, impedimetric biosensors exhibit low detection limits and potentially can be used for on-site applications in combination with a proper metric device. Further research should be focused on the improvement of both their reproducibility and stability, and in addition, scientists still should increase efforts to optimize the proposed electrode assemblies for use in real samples, overcoming problems associated with the complexity of matrices in various natural or commercial samples. Fulfillment of these analytical parameters will accelerate their passage to routine use, and may even enable the construction of analytical devices based on this philosophy.

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