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Review article

A review of experimental aspects of electrochemical immunosensors

Francesco Ricci [a,b], Gianluca Adornetto [a], Giuseppe Palleschi [a,b,*]

[a] Dipartimento di Scienze e Tecnologie Chimiche, University of Rome, Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy
[b] Consorzio Interuniversitario Biostrutture e Biosistemi "INBB", Viale Medaglie d'Oro 305, 00136 Rome, Italy

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ABSTRACT

In this brief review we want to give a guidance to all the researchers who want to challenge themselves in the task of developing electrochemical immunosensors for the first time. We will focus here only on practical aspects trying to give the readers useful insights that should be considered such as the choice of the electrode to be used, the best electrochemical and immunological procedures, the immobilization procedures, the instruments to purchase and other basic aspects of electrochemical immunosensors. In doing so we offer a wide view of the research on electrochemical immunosensor applications that have appeared in the literature in the last 5 years.

Contents

1. Introduction

The detection of biological and chemical pathogens, contaminants and other important analytes plays a crucial role in the prevention of disease spread, infections and pathologies [1]. In order to ensure rapid appropriate counteractions in case of contamination and to improve food control, simple, inexpensive, and quantitative tools for the detection of these contaminants are urgently needed [2,3]. Since its introduction, more than 30 years ago, ELISA (enzyme linked immunosorbent assay) has become probably the most used technique for such task [4]. The availability of antibodies targeting potentially any molecule, the high sensitivity due to the easily assayed enzymatic amplification step and the easiness of use are among the most important reasons for this success. Moreover, the 96-well plate typically employed in ELISA tests is suitable for high-throughput and multianalyte tests. Because of the flexibility in format assay (i.e. competitive, sandwich or direct), ELISA is also equally useful for the detection of both antigens (i.e. usually small molecules) and antibodies [4]. For all these reasons ELISA is now one of the preferred tools for determining serum antibody concentrations (such as with the HIV test [5] or West Nile Virus [6]) and has found applications in the food industry in detecting potential food allergens [7]. Because of these advantages, during recent years, several efforts have been directed to the improvement of the ELISA method. For example, chromogenic reporters and substrates have been replaced by fluorogenic [8], electrochemiluminescent [9], and real-time PCR [10] reporters which may offer higher sensitivities and are more suitable for multiplexing. Many efforts have also been spent to find alternative measurement approaches. In this context simple and rapid lateral flow immunochromatographic assays with quick

* Corresponding author at: Dipartimento di Scienze e Tecnologie Chimiche, University of Rome, Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy.
E-mail address: palleschi@uniroma2.it (G. Palleschi).

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observation of results directly by the naked-eyes probably represent the best example [11]. ELISA is in fact an optical approach and, as such, can suffer of several drawabacks associated with this type of measurement. These include a requirement for generally bulky and power-intensive light sources, detectors, and monochromators, and potential false signals arising from complex colored samples. Moreover, because the sensitivity of optical methods follows the well-known Lambert–Beer law, a minimum sample volume and path length is required to achieve certain performances.

In this context, electrochemical methods appear as the most promising alternative to optical approaches. The possibility to miniaturize modern microelectronics allows to build microelectrodes which are useful for multiplexing and well suited for detection of very small volumes of samples (microliters to nanoliters), in fact the sensitivity of electrochemical methods is not affected by the volume used during measurements. The low cost and a large-scale production of electronic devices is another reason which makes the electrochemical approach more appealing for high-throughput analysis. Finally, the paucity of electrochemical interferents in real samples makes the electrochemical methods better suited for use with complex and colored samples. It is not a surprise, then, that during the last decade many research groups have devoted part of their efforts to the development, optimization and characterization of electrochemical immunosensors [12]. The adaptation of an ELISA assay to an electrochemical approach is not straightforward. In fact, despite the assay format and the overall analysis steps that remain usually the same, several factors should be taken in serious consideration to achieve performances comparable or even better than those of the original ELISA optical approach. The electrode surface to be used as support for the immobilization of the recognition element (i.e. antibody or antigen), the choice of the enzymatic substrate (which must lead to an electroactive product), the selection of the most appropriate electrochemical methods (instead of a simple optical absorbance) and of the adequate instrument are among the most important factors to be considered when adapting an optical ELISA to an electrochemical platform. In this short review we intend to give a practical guidance to all the researchers who want to challenge themselves in this delicate task. A detailed discussion on the background of ELISA is not within the scope of this paper and for this we send to more general reviews [4,13,14]. It is also important to note that there are different possible formats that can be used to develop an electrochemical immunosensor. Both competitive and sandwich formats can in fact be used. Two approaches could be considered when dealing with competitive immunosensors. A first one in which immobilized antibodies react with free antigens in competition with labeled antigens. A second one, using immobilized antigens and labeled antibodies, is generally preferred and prevents all the problems related to antibody immobilization (loss of affinity, orientation of the immobilized protein). In a sandwich assay, after interaction between immobilized antibodies and free antigens, labelled antibodies (directed toward a second binding site of the antigen) are added; at this point, antigen is "sandwiched" between two antibodies. Further details on the possible formats to be used for immunosensors can be found in three complete reviews [12,15,16]. We will focus here only on practical aspects which should be considered when an electrochemical immunosensor must be developed. We will give the readers useful insights which also take in consideration prices, commercial availability and other basic aspects of reagents and consumables needed to develop electrochemical immunosensors. In doing so we offer a wide view on the research on electrochemical immunosensor applications that have appeared in the literature in the last 5 years.

Electrochemical immunosensors are usually obtained through the immobilization of a recognition element (i.e. antigen or antibody) on the electrode surface. The assay steps often end with the injection of a secondary enzyme-labelled antibody which follows the addition of a proper enzymatic substrate (Fig. 1). In an electrochemical format the enzymatic reaction leads to the production of a molecule which is electroactive (that is, it gives an electrochemical signal in presence of certain inputs). The signal is recorded with the use of bench or portable instruments which are usually capable of applying different electrochemical techniques. We will begin our survey and this guidance with the first steps of an electrochemical immunosensor, the selection of the electrode surface, and the choice of the immobilization procedure of the recognition element.

2. Electrodes

The choice of the electrode to be used for an electrochemical immunosensor is of course crucial for several aspects including the sensitivity of the method, the cost of the assay and the possibility to adopt different immobilization procedures. Electrodes used for this task are commonly made of inert metals such as platinum [17], gold [18–27] and several forms of carbon including carbon fiber [28], epoxy graphite [29–33], graphene [34] or glassy carbon [35]. Many companies offer "conventional"-Teflon coated rods as electrodes where one end is the sensing surface and the other is for the connection to the instrument. Although these sensors are widely used in electrochemical laboratories they are not well suited for immunosensor applications. This is due to the fact that an electrochemical immunoassay usually requires several steps and many calibration points which would result in the need of a high number of such electrodes or in the reuse of the same immunosensor for several experiments. Moreover, although they behave very well from an electrochemical point of view, these sensors are usually quite expensive. We have recently reviewed the most important Companies that offer conventional rod electrodes and found that gold electrode prices vary from a minimum of 60 € to a maximum of ca. 300 €. Interestingly, the prices of rod glassy carbon electrodes span approximately the same range despite the cheaper nature of the electrode material. Moreover, these conventional electrodes are of course not intended for single use and often before each use they must be washed and cleaned very carefully, a procedure that it is usually time consuming and reagent intensive (we note, however, that some examples have been proposed where the classic gold electrode surface can be regenerated and used several times [25,26]). As an additional drawback, these sensors are not very amenable to high-throughput modification as they need to be immersed in a quite large volume of solution. Finally, the measurement step is also not too practical because of the need of an external reference electrode and, often, of a counter electrode. This usually requires a working solution of not less than 1 mL (unless special tricks are used) which is not suitable for large scale analysis. For these reasons, we believe that the use of conventional rod electrodes should be only limited to proof-of-principle applications of novel immunological approaches and should not regard the practical applications of optimized immunosensors. In this perspective, the use of disposable screen-printed electrodes (SPEs) which are characterized by low-cost fabrication and the possibility of mass-production seem much better suited. Screen-printing (thick-film) technology involves the printing of various inks on planar ceramic or plastic supports [36–38]. Of note, the planar nature of the SPE allows the easy modification of the surface and, through the help of an automatic dispenser, this can be done in a mass producible way. Due to the miniaturized dimensions of SPEs, all immunological steps can be performed in a drop, using only a few microliters of solution, thus reducing the reagent consumption. Gold [39–43], graphite [44–58] and silver screen printed electrodes are commercially available. The cost of each sensor usually spans 2–4 €/each for graphite and gold electrodes. Of note, the production of these

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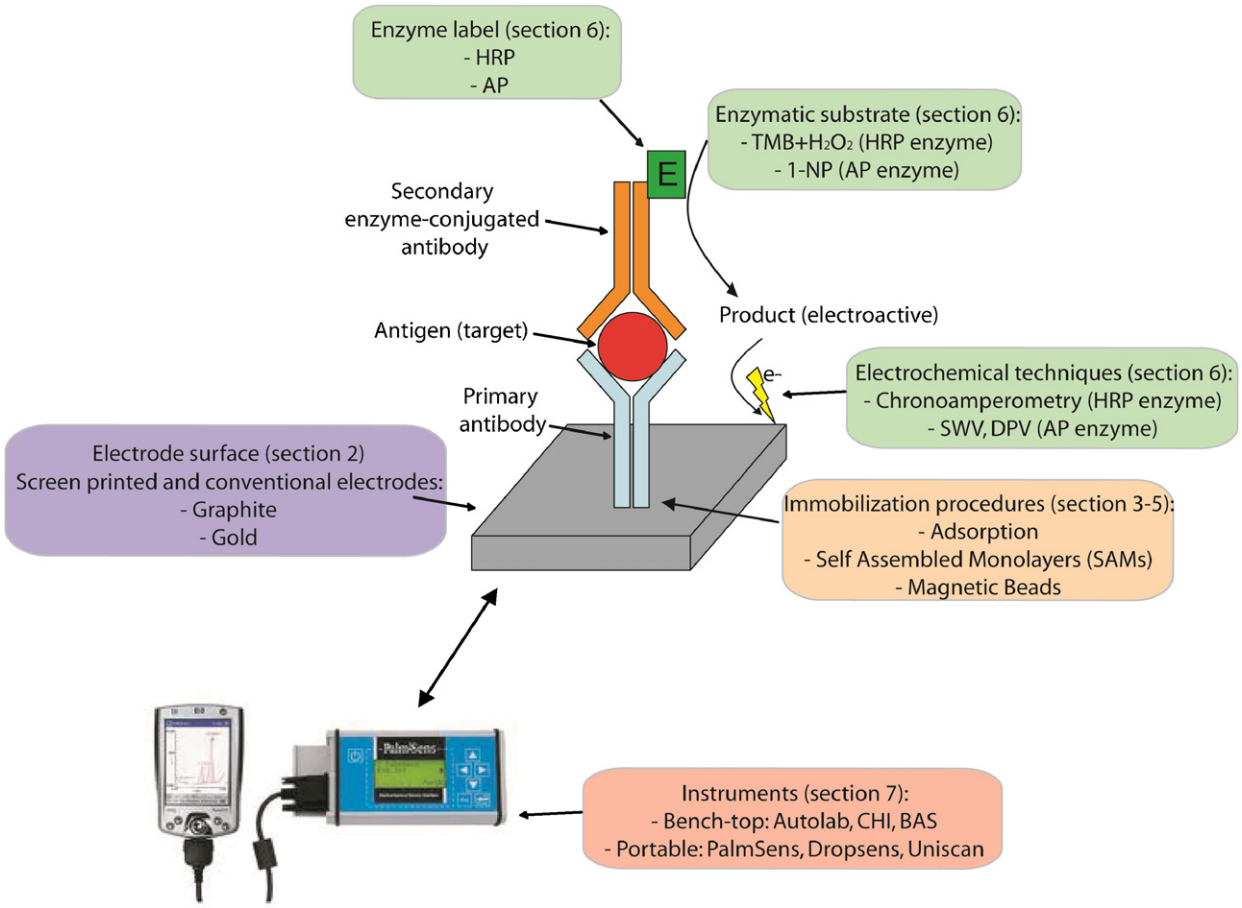

Fig. 1. General overview of the main components of an electrochemical immunosensor. In this scheme, for the sake of clarity, we have depicted a generic immunosensor based on a sandwich format. The components that should be considered in the development of an electrochemical immunosensor are briefly overviewed. In this review we highlight the reasons to choose among the multiple options that are available to the public in terms of electrodes, instruments, enzymatic labels and reagents.

screen printed electrodes is straightforward and, since the cost of a screen printer machine (i.e. DEK, Unitech) is not too high (prices start at ca. 20 k€), this instrument may be of utility if the use of a high number of electrodes is forecasted. Moreover, the possibility to customize the production of screen printed electrodes may be of utility in case of special requirements or to test novel electrode materials (Table 1). The consumables needed to print a batch of electrodes are quite cheap especially in the case that graphite electrodes are produced. Silver ink, used to print the pseudo-reference electrode of screen printed electrodes, is often the most expensive one (about 500–600 €/kg), while graphite ink costs less than 50 €/kg and the insulating ink (used to delimitate the surface area of the working electrode) is the most cost effective and can be found at less than 20 €/kg. Of note, 1 kg of ink is usually enough to print a huge number of electrodes (approximately 100,000 electrodes). Recently, arrays of eight screen printed electrodes [59–64] and of a 96-well plate [65–67] have been developed and this makes the use of these electrodes even more advantageous [68]. As an alternative to screen printed electrodes, the use of photolitography for the production of electrodes well suited for integration with microfluidic systems and for the construction of miniaturized multiplex arrays has been reported [69–73]. As an additional note, it has to be stressed here that electrodes are not necessarily used as an immobilization support of the recognition element. Often, in fact, the recognition element is immobilized on a separate support (magnetic- and nano- beads) and only at the end of the immunological chain it is placed onto the surface of an unmodified electrode for measurement of the enzymatic product. In these cases the electrode only acts as the "measuring device" and its properties to "hold" biomolecules are not too crucial.

3. Immobilization procedures

The procedure and method used to immobilize the recognition element either directly on the electrode surface or on other solid supports is a crucial step in the development of an electrochemical immunosensor. In this perspective a clear distinction with optical ELISA methods must be made. Generally, optical ELISA involves the non-specific adsorption of the recognition element on the bottom of polystyrene wells. The immunological chain (the series of reaction and washing steps that leads to the final signal) ends with the injection of a substrate of the label enzyme conjugated to the secondary antibody [4,13,14]. Of note, the enzymatic product produced by the enzyme label is detected in a homogeneous solution through the measurement of the absorbance at a selected wavelength. For this reason, in optical ELISA, the solution/well interface, and so the methods used to immobilize the recognition element on the solid support, is crucial for the optimal antibody/antigen interaction but does not directly affect the measurement step. This is not often the case of electrochemical immunosensors. In electrochemical methods the measurement step occurs in a heterogeneous phase, more specifically at the solution/electrode interface. To achieve a greater sensitivity it is thus preferable that the enzymatic product would be generated directly on the surface of the working electrode in order to favor the diffusion of the analyte to the electrode surface and to have a greater concentration of the analyte in the

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Table 1
Conventional and screen printed electrodes used for immunosensors applications.

Screen printed electrodes (SPE)

Material	Source/company, notes	References
Graphite/Carbon	DropSens	[44,47,58]
	Alderon Biosciences	[53]
	Home produced with DEK screen printer	[46,48–52,54,56,57]
	Home produced	[55]
	Home produced with DEK screen printer, Multi-8 electrodes	[59–63]
	Ecobioservices and researches, Multi-8 electrodes	[64]
	Alderon biosciences, 96 plate well	[65–67]
	DropSens, 2 Working electrodes	[40]
	Home produced with DEK screen printer, 4 Working electrodes	[99]
	Home produced, 8 Working electrodes	[74]
Gold	DropSens	[39–42]
	BVT technologies	[43]

Conventional electrodes

Material	Source/company, notes	References
Platinum	Wire electrode	[17]
Gold	Commercial	[18]
	BAS, Rod electrode (3 mm ø)	[19]
	Commercial, Rod electrode (3 mm ø)	[20,23]
	BAS, Rod electrode (2 mm ø)	[21,22]
	CHI, Rod electrode (2 mm ø)	[24]
	Commercial, Rod electrode (1.6 mm ø)	[25–27]
Graphite epoxy electrodes	Home produced	[33]
Glassy carbon	Commercial, Rod electrode (3 mm ø)	[34,35]
Carbon	Commercial, Microelectrode	[28]
Indium tin oxide (ITO)	Commercial, Modified with Amine-terminated G4 poly(amidoamine) dendrimer, ferrocenecarboxaldehyde	[98]

Other electrodes

Material	Source/company, notes	References
Gold	Home produced, Photolithographic chips of 16 working electrodes [1 mm × 1 mm]	[69–73]
Gold particles – Carbon nanotubes–Teflon powder	Home produced, Composite electrode	[92]
Graphite epoxy electrodes	Home produced, Magnetized electrodes	[29–32]

diffusion layer. Moreover, when the recognition element is immobilized directly on the electrode surface, the immobilization step does not only affect the antibody/antigen interaction but also modifies the sensing surface itself. For this reason, in order to have an optimal electrochemical behavior it is essential that the electrode surface is not passivated by the recognition element. These considerations explain the wide range of different procedures and methods used for the immobilization of the recognition element in the development of electrochemical immunosensors (Table 2). Traditionally, electrochemical immunosensors were obtained by immobilizing the recognition element (either antibody or antigen) directly on the surface of the working electrode. For the reasons we have explained in the previous section, screen printed electrodes are preferred over other types of electrodes and several groups have adopted a simple and straightforward adsorption procedure very similar to that adopted for optical ELISA [33,46,65,74,40]. Recently, to achieve a better presentation of the recognition element to the target analyte [75], the use of ordered layers on the surface of the electrode has been introduced. In this case the most used electrode material is gold (either as screen printed or traditional rod) which allows the formation of self assembled monolayers (SAMs) through thiol-gold chemistry in a very reproducible and ordered way. In this perspective the immobilization procedure usually involves two separate steps. The first one is the creation of the SAM with a linkage agent containing a thiol at one end and a functional group (carboxylic acid, amine etc.) for a convenient reaction with the recognition element at the other end. The second step involves the reaction of the recognition element with the functional group of the linkage agent usually through covalent links (such as amide bonds and Schiff's base formation) directly with the functionalized thiol monolayer or by the aid of bridging molecules such as glutardealdehyde [76,77]. The use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) [20,24,25,27,78] or of succinimide group by itself [41,72,73] has also found several applications. The resultant monolayer is well oriented on the surface of the electrode and this allows a better antibody/antigen interaction. Moreover, because SAMs are usually achieved through the use of a mixed solution containing both the recognition element and a coadsorbant (usually a short chain alkane-thiol), this leads to an optimal average spacing between the receptors and to a well-behaving electrochemical surface [79]. Of note, we have found this kind of immobilization especially with novel approaches which propose innovative immunosensing platforms. For further details on self assembled monolayers (SAM) for biosensor applications we send the readers to two very complete reviews on the argument [80,81]. The use of thiolated antibodies (Fig. 2) [23,26], or systems using thiolated scaffolds (Fig. 3) (e.g. DNA [21,22,82,83], protein G, and protein A or antibody binding peptides [28,84–90]) have also been reported with promising results. One of the limitations of SAMs is the preferential need of gold surfaces which can affect the costs of the immunosensor production. Alternative methods for the formation of SAMs are based on the use of diazonium salts which can be easily applied to graphite and carbon electrodes [49]. Another approach that has been recently proposed for the immobilization of recognition elements on the electrode surface involves the use of different polymeric matrices such as polysulfone (PSU) polymer [91] and chitosan (CS). These matrices form porous networks with

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high mechanical strength that allow the reagents to reach the electrode (Fig. 4) [52]. Moreover, the presence of reactive amino and hydroxyl functional groups is well suited for biomolecule immobilization [18,58].

4. Use of nanostructured materials for electrochemical immunosensors

We have discussed earlier how the electrode surface is not only a support for the immobilization of the recognition element but also acts as the sensing surface. For this reason, several attempts have been made in order to improve the electrochemical behavior of traditional or screen-printed electrodes with the use of different nanomaterials. In this perspective the use of both carbon nanotubes [92], gold nanoparticles [19,40,50,92–94], and graphene [34,58] has been proposed and previous papers have reviewed the use of nanomaterials with improved electrochemical performances [95–97]. However, the use of such nanomaterials does not seem yet suitable for mass-production of the sensors, so their use in prototypes or commercialized instruments appears not likely. Surface modification protocols are in fact often complicated long processes or, in the best cases, are difficult to be implemented as an industrial process. Moreover, a cost assessment should be done when using these materials since they are usually quite expensive especially if purchased from specialized companies. Despite this we note that a leading company in screen printed electrodes production (DropSens) offers SPE which are already modified with single and multi-walled carbon nanotubes. To the best of our knowledge these sensors have not been yet applied to the development of electrochemical immunosensors and for this reason we have no elements to suggest them to the readers. With regards to nanostructured materials it should be also noted that several interesting approaches have been reported to date for the immobilization of the recognition element through the use of nanoporous filter membranes in anodized aluminium oxide (AAO) to increase the surface area and the sensitivity of the sensor (Fig. 4) [52,56].

5. Use of magnetic beads for electrochemical immunosensors

As we have seen above, the immobilization of the recognition element directly onto the electrode surface may prove difficult or may require several steps that are not always suitable for mass-production. Another drawback in this perspective is that the electrode is used in the whole immunological chain thus leading to possible passivation or poisoning of the electrode surface through the non-specific adsorption of other species present in the sample. Moreover, since the immunological chain usually requires many washing steps, these can cause defects on the layer of the recognition elements that could compromise the reproducibility of the results. Finally, the confinement of the recognition element onto the surface of an electrode can be an obstacle to the kinetic of antibody/antigen reaction and can limit the number of biomolecules that can be immobilized on the electrode surface. All these drawbacks have been recently overcome by the use of magnetic beads (MBs), or magnetic nanobeads [48], as support for the immobilization of the recognition element (Table 3). Magnetic beads are particles constituted from a dispersion of magnetic material (Fe_2O_3 and Fe_3O_4) and then covered with a thin shell of polymer that also serves to define a surface area for the adsorption or coupling of a large variety of molecules. MBs can be easily functionalized with different linkage groups such as streptavidin (Fig. 5) [32,44,55], tosyl groups [29,31,51,55,61–63,98], amino groups [53], antibodies or proteins [30,35,39,45,47,59,60,64,66,99] for fast and specific immobilization of the recognition element. Moreover, because of their small size and spherical geometry, a large number of biomolecules can be immobilized on the surface of each MB. This improves the sensitivity of the assay and allows to reduce the reaction times and to use smaller volumes of solution on the working electrode surface. The whole immunological chain is performed in micro-tubes using a rotation sample mixer. After each incubation or washing step, the MBs are concentrated on the side wall of the micro-tubes by placing the tubes in a specially designed magnetic particle separator allowing the supernatant to be discarded. This allows fast immunoreactions between antigen and antibody and easy separation after washing and reaction steps. An additional advantage of the use of MBs is that the immobilization of the recognition element (coating step) can be performed in large quantities in a single step and the coated MBs can be stored for several weeks without loss of activity. This is crucial when a high number of samples is expected and could lead to an important reduction of the analysis time. Of note, in this case the electrode is used only as a sensing surface and so no passivation nor electrochemical interferents are expected. The only problem represented by the use of MBs is that the recognition element, and thus the secondary antibody at the end of the immunological chain, is not in direct contact with the electrode surface. This can pose some limitations in the sensitivity of the method. To avoid this it has been proposed the use of magnetized electrode surfaces either through the inclusion in the electrode material [29–32] of magnetic particles or through the placement of small magnets below the surface of the working electrode [59–64]. This allows to concentrate the MBs on the electrode surface for the final measurement at the end of the immunological chain. MBs are sold (functionalized or non-functionalized) by many companies (i.e. invitrogen, Pierce, Millipore, Promega etc.). The cost of the MBs depends on their size and their functionalitation. As an example the cost of tosylactivated MBs is around 250 € for 2 mL of solution. From our experience an average of 10 μL of MBs is enough to generate a complete calibration curve (8 replicated points) and 1 sample. It should be noted here that several accessories are also needed to implement this type of immunosensor. A magnetic separator (ca. 260 €), a rotation sample mixer (this can be easily constructed in-house or purchased for ca. 300 €) and strong magnets (neodinium magnets are preferable) are needed.

6. Enzymatic labels and electrochemical methods

A key step in the development of a new electrochemical immunosensor is the choice of the secondary antibody and of the procedure used to test its activity. Because of the easiness with which they can be found from commercial sources and their extended application with optical ELISA, the use of horse-radish peroxidase (HRP) and alkaline phosphatase (AP) conjugated antibodies has been the preferred route since the first examples of electrochemical immunosensors (Table 4). Of course, while with optical ELISA the enzyme label should catalyze the production of a colored species; in the case of electrochemical immunosensors it is essential that the enzymatic product is electroactive so that it can be easily measured through a voltammetric or amperometric technique. To this end several efforts have been devoted to find optimal enzymatic substrates which could achieve the required sensitivity [100,101]. We will discuss pros and cons of both these enzymatic labels and we will try to give valid reasons that should be considered when a choice is needed between these two options. The first practical consideration that must be made when developing a new electrochemical immunosensor is of course the commercial availability of the enzyme-conjugated antibody. In fact, not always the required antibody is available conjugated with both the enzymes. This is especially true when a sandwich format

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Table 2
Immobilization procedures of recognition element on the electrode surface.

Immobilization type	Electrode	Note	References
Self Assembled Monolayer (SAM)	Carbon SPE	Diazonium coupling reaction	[49]
	Gold	Thiol-functionalized gold electrodes + EDC/NHS	[20,24,25,27,78]
		Thiol-functionalized gold electrodes + succinimide reaction	[41,72,73]
		Thiol-functionalized gold electrodes + glutaraldehyde	[76,77]
		Thiolated DNA-scaffold	[21,22,83]
		Thiolated antibodies	[23,26]
Adsorbition	Graphite/carbon SPE	-	[46,65,74,40]
	Graphite-epoxy electrodes	-	[33]
Other	Gold	Chitosan-iron oxide-poly(amino amine) dendrimers-gold nanoparticles (CS-Fe_3O_4-PAMAM-GNPs) nanocomposites	[18]
		Adamantane-containing Carboxymethyl Cellulose Polymer (ADA-CMC)	[70,71]
		4-nitrophenol	[42]
	Carbon SPE	Chitosan-graphene	[58]

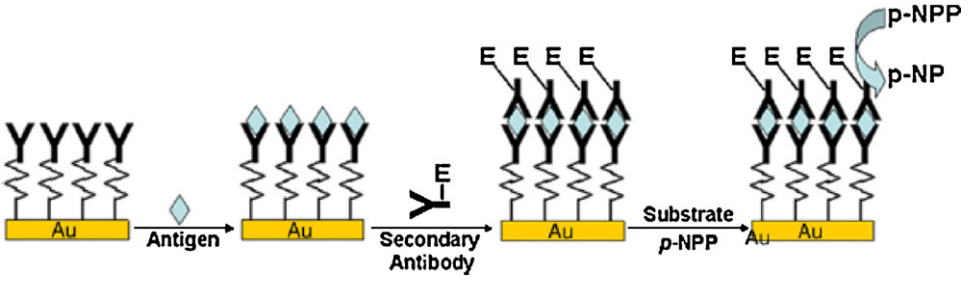

Fig. 2. Thiolated antibodies can be used to realize a reproducible and ordered self-assembled monolayer on a gold electrode surface. With this scheme, the thiolated antibody is the recognition element and allow to develop a sandwich assay with the target analyte (here Staphylococcal enterotoxin B) and AP-conjugated secondary antibodies. Reproduced with permission from [23]. Copyright (2007) from Elsevier B.V.

is employed and a primary monoclonal conjugated antibody is needed. In these cases it is usually easier to find commercially available HRP-conjugated antibodies. And even if conjugation protocols are straightforward, often it is preferable to avoid any waste of time and resources and entrust specialized Companies purchasing already conjugated antibodies. HRP enzyme label offers more options in terms of electrochemical methods and substrates. However, the great majority of examples found in literature [24,25,35,42,46,55,57,61,66,67] are based on the use of a mixture of H_2O_2 and 3,3′, 5,5′-tertramethyl benzidin (TMB) in a way similar to that used for optical ELISA [100]. The produced oxidized TMB is usually measured through amperometric reduction at a fixed potential in a range between −0.1 and +0.1 V. Of course, the selection of the right applied potential depends on the electrode used, the reference electrode and the specific conditions of the assay (pH, buffer used etc.). The most used technique to measure the produced TMB_{ox} is chronoamperometry which is usually performed on a time scale of few seconds (from 10 to 60 s) after the enzymatic reaction is let to proceed for several minutes (generally from 2 to 10 min). Since HRP is a non-specific enzyme it supports the use of many other substrates. In this perspective good results have been also obtained with catechol [92] and hydroquinone [26,29–31,33,45,69–73] (both coupled with H_2O_2). Also, the use of H_2O_2 [18,28,41,50] by itself has been applied and in this case the

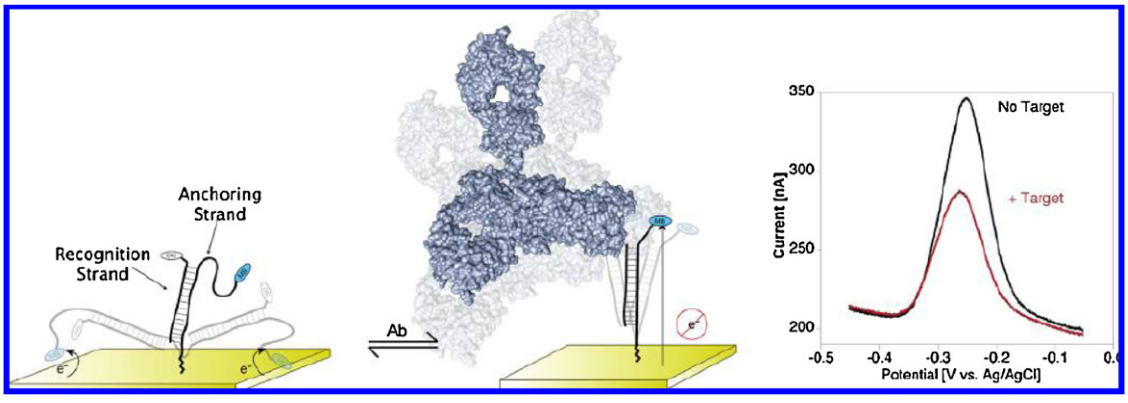

Fig. 3. Use of DNA-thiolated scaffold as a new strategy for the detection of antibodies. The new architecture utilizes a largely double-stranded DNA as a rigid-but-dynamic scaffold to support the recognition element. Left: one strand of the scaffold, the "anchoring strand", is attached to the electrode surface at its thiol-modified 5′ terminus and labeled with a redox tag (here methylene blue) at its 3′ terminus. The second strand, the "recognition strand", is modified at its 5′ terminus with a small-molecule recognition element (antigen). In the unbound state, the scaffold supports efficient electron transfer between the redox label and the electrode. The binding of the antibody target to this recognition element reduces the transfer efficiency, thus significantly reducing the observed faradaic current. Shown here are representative square wave voltammograms (right). Reproduced with permission from [21]. Copyright (2009) from American Chemical Society.

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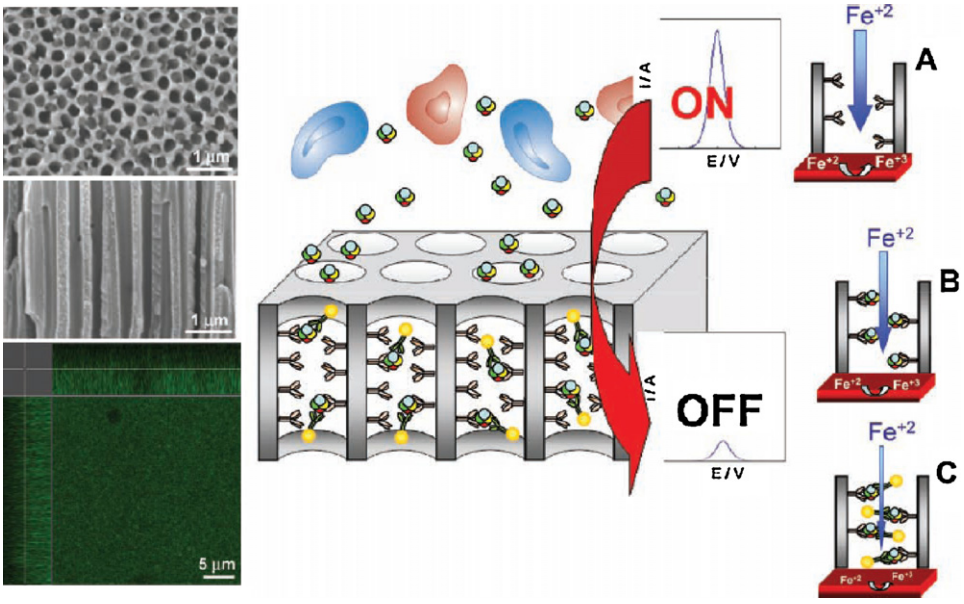

Fig. 4. Detection of proteins with anodized aluminum oxide (AAO) nanoporous membranes. The cells in the sample are excluded by the pores while the proteins enter inside and are recognized by specific antibodies. Right: sensing principle in the absence (A) and presence (B) of the specific protein in the sample and in the case of the sandwich assay using AuNP tags (C). Left: SEM images of a top view and cross-sectional view of the AAO nanoporous membrane containing 200 nm pores, and confocal microscopy image of a top and cross-sectional view 5 μm in depth from both sides of the membrane. Reproduced with permission from [52]. Copyright (2011) from Wiley/VCH.

use of differential pulse voltammetry (DPV) and cyclic voltammetry (CV) has been demonstrated in addition to chronoamperometry. We have to note that TMB (coupled with H_2O_2) is by far the most used substrate and this surely represents the safest and most optimal choice. It has to be considered that because both oxidized and reduced forms of TMB are electroactive, the use of pulsed voltammetric techniques is not possible with this substrate and the need to use chronoamperometric techniques has some costs in terms of sensitivity and measurement time. Recently we have demonstrated the possible use of pulsed amperometric methods (such as Intermittent Pulsed Amperometry, IPA) which led to very good results as this technique can allow the interrogation of 96 electrodes in few seconds [65–67]. Another practical aspect that should be considered in the use of HRP label is that the substrate solution is a mixture of two reagents and that the home-made preparation of a highly concentrated solution of TMB is usually a lengthy process because of the low solubility of this substrate. However, nowadays prepared solutions of TMB/H_2O_2 optimized for HRP reactions are commercially available and may represent a good choice. In conclusion, while for optical ELISA, HRP is the preferred choice because it allows a better sensitivity [102], with electrochemical immunosensors the high enzymatic activity is somehow hampered by the requirement of a technique which is not particularly sensitive. For this reason AP represents sometimes a valid alternative as enzymatic label and often represents the preferred choice. This enzyme in fact offers less flexibility than HRP but has its own advantages. The most used enzymatic substrate is 1-naphthyl phosphate (1-NPP) which is converted to the electroactive 1-napthol (1-NP) [23,44,47–49,57,59–61,63,64,99]. Unlike TMB, this product can be detected through pulsed voltammetric techniques such as DPV and Square Wave Voltammetry (SWV) at a potential of ca. +0.2/ + 0.3 V. This guarantees a better sensitivity of the method and a much faster measurement time (i.e. usually DPV and SWV can be completed in less than 3 s). This is mainly the reason that AP is a better choice than HRP for the development of an electrochemical immunosensor. Despite this, the use of AP presents also some limitations. The enzymatic reaction can only proceed at basic pH and this can be a drawback in case of special immunoassays where the antibody/antigen reaction and the measurement steps are performed in the same solution. It has to be stressed that the electrochemical reactions of both 1-NP and TMB_{ox} lead to the passivation/poisoning of the electrode surface. This requires the use of a new electrode for each measurement. This is another crucial reason why screen printed disposable electrodes are preferred over traditional ones in

Table 3
Immobilization on magnetic beads and nanomaterials.

Material	Type of functionalization or of nanomaterial	References
Functionalized Magnetic beads	Amino group	[53]
	Avidin/Streptavidin	[32,44,55]
	Protein A/Protein G	[39,45,47,59,60,99]
	Tosyl-activated	[29,31,51,55,61–63,98]
	With antigen	[64]
	With antibody	[30,35,66]
Nanoparticles and nanomaterials	Super paramagnetic nanobeads	[48]
	Carbon nanotubes	[92]
	Graphene	[58]
	Poly(dialyldimethylammonium chloride) graphene–gold nanoparticles	[34]
	Nanogold/gold nanoparticles	[18,40,50,92,93]
	Anodized aluminium oxide (AAO) nanoporous filter membranes	[52,56]

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Table 4
Enzyme and other catalytic labels.

Substrate	Electrochemical method	Measurement potentials (V)	References
Alkaline phosphatase			
1-NPP	DPV	From −0.15 to +0.60	[44,47–49,57,59–61,63–65,99]
1-NPP	SWV	From 0.0 to +0.45	[23]
p-APP	CA	+0.3	[17]
p-APP	DPV	From −0.2 to +0.6	[27]
3-indoxyl phosphate–silver nitrate	LSV	From 0.0 to +0.4	[40]
Horseradish peroxidase			
TMB – H_2O_2	CA	0.15	[24]
TMB – H_2O_2	CA	−0.1	[42,55,62]
TMB – H_2O_2	CA	+0.1	[46]
TMB – H_2O_2	CA	−0.2	[57]
TMB – H_2O_2	CV	From −0.5 to 0.8	[35]
TMB – H_2O_2	IPA	−0.1	[65–67]
Hydroquinone – H_2O_2	CA	ca. −0.15	[26,29–31,33,45,70–73]
Catechol – H_2O_2	CA	−0.05	[92]
Tetrathiafulvalene – H_2O_2	CA	−0.15	[39]
H_2O_2–Chitosan–Toluidin Blue O	CA	−0.5	[74]
5-methyl phenazinium methyl sulfate – H_2O_2	CA	−0.2	[54]
Thionine – H_2O_2	DPV	From −0.05 to −0.5	[58]
o-Phenylenediamine – H_2O_2	DPV	From −0.3 to −0.8	[34]
H_2O_2	DPV	From −0.4 to 0.0	[50]
H_2O_2	CV	From −0.2 to 0.6	[18]
H_2O_2	CA	0.0	[28,41]
Gold nanoparticles			
HCl	CA	−1.0	[51]
HCl	DPV	From +1.25 to 0.0	[32]
$[Fe(CN)_6]^{4-}$	DPV	From −0.4 to 0.3	[52]
4-nitrophenol + $NaBH_4$	DPV – CV	–	[98]

1-NPP: 1-Naphthyl phosphate; p-APP: p-Aminophenyl phosphate; TMB: 3,3′, 5,5′-Tetramethyl benzidin; $[Fe(CN)_6]^{4-}$: Ferrocyanide. DPV: Differential pulse voltammetry; SWV: Square wave voltammetry; CA: Chronoamperometry; LSV: Linear sweep voltammetry; CV: Cyclic voltammetry; IPA: Intermittent pulse amperometry.

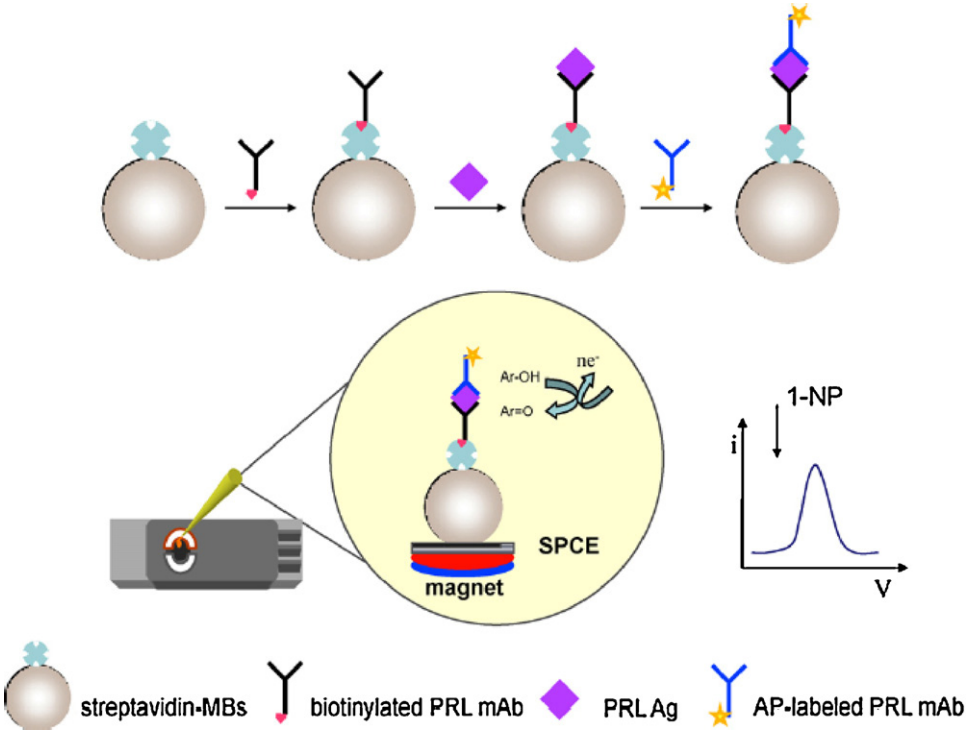

Fig. 5. Streptavidin-functionalized magnetic beads coupled with magnetized carbon SPE for immunosensor application. Biotinylated capture antibodies are immobilized onto streptavidin-modified magnetic particles. A sandwich-type immunoassay with the target analyte (prolactin, PRL) and anti-prolactin antibodies labelled with alkaline phosphatase (AP) was used. The resulting bioconjugate formed is then trapped on the surface of the carbon SPE with a small magnet. The quantification of PRL was accomplished by differential pulse voltammetric (DPV) determination of 1-naphtol formed upon 1-naphtyl phosphate additions. Reproduced with permission from [44]. Copyright (2011) from Elsevier B.V.

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electrochemical immunosensing. In few recent examples, enzyme labels have been also recently replaced by inorganic "signal amplification tags" such as catalytic gold nanoparticles [32,51,52,98] or quantum dots [19,53] that have been demonstrated to give interesting results in terms of sensitivity, analysis time and correlation with standard tests (Table 4). The use of amplification labels (either enzymatic or "inorganic") represents an attractive way to improve the sensitivity of the immunological method. However, this is also a source of higher costs and higher analysis times. In fact, the need of a conjugated antibody, the addition of substrates and the use of multiple reactions and washing steps is a waste of time and resources that should be avoided. In this perspective electrochemical methods offer probably more flexibility than the optical ones to develop methods that do not need enzymatic labels. Recently, several examples have appeared in the literature for the development of label-free and reagentless electrochemical immunosensors [20,82]. One of these is based on the use of scaffold DNA probes conjugated with an antigen and a redox tag [21,22,83]. Upon antibody binding, the DNA scaffold flexibility is reduced and this leads to a reduced electron transfer rate of the redox tag. This platform can then detect in real-time and without any washing step or added reagent the antibody/antigen binding event (Fig. 3). The sensitivity achieved with these sensors is of course not comparable with enzyme-conjugated methods, however, the fact that electrochemical immunosensors are less prone to interferences than the optical ones makes possible the use of such platforms in undiluted samples thus leading to detection limits that are often low enough to be useful for practical applications.

7. Instruments

In the development of electrochemical immunosensors an important choice is represented by the instrumentation to be used. We have detailed above the methods used and among these, chronoamperometry, DPV and SWV are the techniques mainly applied and, most of the commercially available electrochemical instruments, can perform these techniques. Electrochemical instruments can be divided in bench-top and portable. Bench-top instruments can usually perform a wider range of techniques and are more flexible. However, their cost is usually quite high (>20,000 €) and, from our experience, the performances achieved with these instruments are comparable to those portable, at least for the specific application of electrochemical immunosensors. In this context, three companies (DropSens, PalmSens, Uniscan) offer very precise, low-cost and friendly-user platforms that perform all the techniques usually required for electrochemical immunosensors. These instruments can be coupled with multi-array platforms and can be also customized quite easily. The prices of these portable instruments usually range between 2000 and 4000 € and they can be often customized for specific needs. They are all equipped with USB plugs for easy connection to PC, laptops or palm devices and, from our direct experience, they have friendly-user interfaces and are very reliable.

8. Conclusions

In this short survey we have given practical information for researchers that want to develop for the first time electrochemical immunosensors. We highlight the reasons to choose among the multiple options that are available to the public in terms of electrodes, instruments, enzymatic labels and reagents. We believe that electrochemical immunosensors may represent a valid alternative to optical ELISA. However, the most important advantages of electrochemical detection have not yet been completely exploited. The mere adaptation of the optical immunoassay to an electrochemical platform is not likely to give important advantages in terms of sensitivity, detection limit, analysis time and costs. Researchers should try to focus on the drawbacks of optical ELISA which can be overcome by electrochemical approaches and build, on these premises, immunoassays with better performances. For example, the possibility to use very small volumes and miniaturized array of electrochemical sensors has not been yet exhaustively employed for immunosensors development. Also, too few examples have been reported to date for the detection of antibodies in a reagentless and label-free format. In our opinion electrochemistry is the best technique to achieve such important results and more efforts should be dedicated to this goal. The low level of electrochemical interferences in complex clinical or food samples is also an important advantage over the optical approaches which makes this technique more suitable for point-of-care detection. An electrochemical sensor for antibody detection that does not need reaction or washing steps or the addition of any external reagents coupled with the low cost and easiness of use of electrochemical instrumentation would make such platform a perfect tool for the point-of-care diagnosis, prevention and treatment of many illnesses, including infectious and autoimmune diseases.

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