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Development of an immunosensor for the determination of rabbit IgG using streptavidin modified screen-printed carbon electrodes

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Abstract

Voltammetric enzyme immunosensors based on the employment of streptavidin modified screen-printed carbon electrodes (SPCEs) for the detection of rabbit IgG, as a model analyte, were described. Alkaline phosphatase (AP) and 3-indoxyl phosphate (3-IP) were used as the enzymatic label and substrate, respectively. The adsorption of streptavidin was performed by deposition of a drop of a streptavidin solution overnight at 4 °C on the pre-oxidized surface of the SPCEs. The analytical characteristics of these sensors were evaluated using biotin conjugated to AP.

The immunosensor devices were based on a specific reaction of rabbit IgG with its biotinylated antibodies, which were immobilised on the modified screen-printed carbon electrodes through the streptavidin:biotin reaction. The immunosensors were used for a direct determination of AP labelled rabbit IgG, and for free rabbit IgG detection using a sequential competitive immunoassay. A calibration curve in the range of 5×10^{-11} to 1×10^{-9} M of rabbit IgG was obtained with a estimated detection limit of 5×10^{-11} M (7.0 ng/ml). These immunosensors were stable for 5 months if they were stored at 4 °C.

Keywords: Alkaline phosphatase; 3-Indoxyl phosphate; Screen-printed carbon electrode; Streptavidin:biotin reaction; Rabbit IgG; Immunosensor

1. Introduction

Applications of immunoelectrochemical sensors for the determination of different biologically active compounds have increased significantly during the last 15 years [1–3]. These devices combine high specificity of traditional immunochemical methods with low detection limits of modern electrochemical systems. Other advantages of the electrochemical immunosensors are their simplicity and possibility to carry out continuous, fully automated assays [2]. Immunoenzyme electrodes constitute a significant portion of the elaborated immunosensors [2,4]. Operation of these instruments is based on the formation of enzyme-labelled immune complexes on the sensitive electrode surface and the subsequent electrochemical detection of the immobilised enzyme [4]. This approach allows applying standard ELISA formats and reactants for the immunosensors. The immunoenzyme electrodes retain the main advantages of solid-phase immunoassays, namely, high sensitivity and specificity, and furthermore, simplify and accelerate the analytical procedure.

For the design of an immunosensor, the crucial step is the immobilisation of immuno-reagents onto the electrode surface. The immobilisation method will determine the sensitivity and reproducibility of the immunosensor. General strategies for the immobilisation of immuno-reagents on solid surfaces include physical adsorption, entrapment in polymer matrix and covalent attachment [5–11]. Proteins have an amphiphilic nature and they therefore have a tendency to adsorb to a solid surface. So the direct adsorption of antibodies on the electrode surface seems to be a promising approach for immunosensor construction. It does not require the use of chemical linkers simplifying the fabrication procedure of the immunosensor [5–7]. The most important driving force for

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protein adsorption has been identified as hydrophobic interaction and electrostatic interaction. However, a drawback of physical adsorption is that immunosensing phases such obtained are disordered and consequently, a part of the binding sites are not accessible to analyte, implying a lack in sensitivity of the immunosensor device. The attachment of reactants to the support through the specific biotin:avidin (or streptavidin) interaction has been revealed as an effective and reliable approach for protein immobilisation [12–15]. This reaction is characterised by a high affinity constant, which is usually the basis for a very stable immobilisation of proteins. Other alternative for oriented immobilisation of antibodies is through protein A or protein G [16,17].

A wide variety of electrodes have been used as support to fabricate immunosensor devices including carbon paste electrodes, glassy carbon electrodes or gold electrodes. Recently, several immunosensor devices have been developed on screen-printed electrodes. The screen-printing microfabrication technology is nowadays well established for the production of thick-film electrochemical transducers. This technology allows the mass production of reproducible yet inexpensive and mechanically robust strip solid electrodes. Other important features that these electrodes exhibit are related to the miniaturisation of the corresponding device along with their ease of handling and manipulation in a disposable manner. However, as the exact formulations of the commercial inks are unknown, their functionalisation is difficult to control and depends on the ink used [18] and on the binder used [19]. As in the case of conventional electrode supports, the immobilisation of antibodies or antigens on screen-printed electrodes has been carried out by physical or electrostatic adsorption [20–25], by sol–gel entrapment [8] or through the affinity reactions as biotin:(strept)avidin [14,15] or protein A or protein G [17], obtaining different immunosensor devices.

In this work an enzyme-immunosensor device for the determination of a model analyte (rabbit IgG, RIgG) was fabricated on screen-printed carbon electrodes (SPCEs), which were previously modified with streptavidin by physical adsorption. The novelty of the methodology used to adsorb the streptavidin on the electrode surface is that the physical adsorption of streptavidin must be performed at a constant temperature above the room temperature. Moreover, the electrode surface must be previously electrochemically pretreated at an anodic potential in acidic media to improve its adsorptive properties. In this way, reproducible, sensitive and stable sensing phases are obtained. This methodology for coating the surface of a SPCE with streptavidin is very simple, in contrast to other works where the covalent attachment of biotin was performed on SPCEs followed by the reaction with streptavidin [14] or where the covalent attachment of streptavidin was carried out on conducting polymer modified SPCEs [15]. The attachment of an antibody on the electrode surface (goat anti-rabbit IgG) was carried out through the streptavidin:biotin interaction. The immunosensing phases obtained in this way are stable for several months. A sequential competitive assay between analyte and alkaline phosphatase (AP) labelled analyte was carried out on this immunosensor device, obtaining lower limits of detection than others reported in the literature [15].

On the other hand, 3-indoxyl phosphate (3-IP) was used as electrochemical substrate of AP. This substrate has been proposed by our group as a suitable electrochemical substrate for AP [26] and various immnunoanalytical devices with this substrate for the detection of different model analytes were performed on the surface of pretreated carbon paste electrodes [27,28]. The resulting enzymatic product is indigo blue, an aromatic heterocycle insoluble in aqueous solutions. Its sulfonation in acidic medium gives rise to indigo carmine (IC), an aqueous soluble compound which shows a similar electrochemical behaviour than indigo blue. Both 3-IP and IC have already been studied on SPCEs [29,30].

2. Experimental

2.1. Apparatus

Voltammetric studies were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6400 computer system and controlled by Autolab GPES software version 4.7 for Windows 98.

Screen-printed carbon electrodes were purchased from AndCare Inc. (Durham, NC, USA), together with and edge connector (Fig. 1). The AndCare electrodes incorporate a conventional three-electrode configuration, which comprises a disk-shaped working (4 mm diameter), counter and silver pseudoreference electrodes printed on polycarbonate substrates (4.5 cm × 1.5 cm). Both working and counter electrodes were made of heat-cured carbon composite inks. An insulating layer was printed over the electrode system, leaving uncovered a working electrode area of 7 mm × 5 mm and the electric contacts. A ring-shaped layer further printed around the working area constituted the reservoir of the electrochemical cell, with an actual volume of 50 μl.

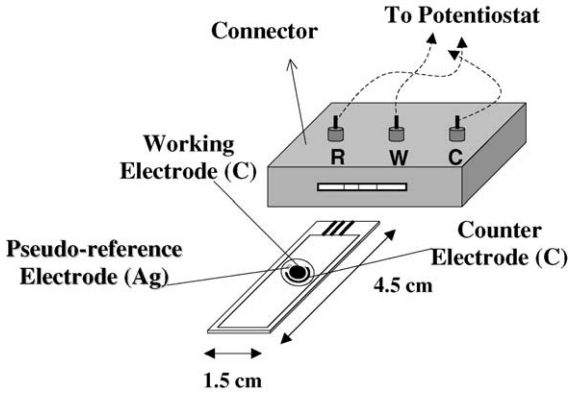

Fig. 1. Schematic diagram of the screen-printed carbon electrode and connector.

2.2. Materials

Tris(hydroxymethyl)aminomethane (Tris) and 3-indoxyl phosphate disodium salt were purchased from Sigma (Madrid). Six millimoles 3-IP solutions were prepared in 0.1 M Tris pH 9.8 and 20 mM $MgCl_2$ and stored in opaque tubes at 4 °C.

Bovine serum albumin fraction V (BSA), biotin conjugated to alkaline phosphatase (B-AP; dimmer, four units of B per molecule of AP, molecular weight = 160,000 g mol^{-1}), streptavidin (molecular weight = 66,000 g mol^{-1}), biotinylated goat anti-rabbit IgG (Ab-B), rabbit IgG conjugated to alkaline phosphatase (RIgG-AP) and rabbit IgG (RIgG molecular weight = 140,000 g mol^{-1}), were purchased from Sigma (Madrid). Working solutions of streptavidin, Ab-B and RIgG were made in 0.1 M Tris pH 7.2 buffer. Working solutions of B-AP and RIgG-AP were made in 0.1 M Tris pH 7.2 containing 2 mM of $MgCl_2$. BSA lyophilised powder was reconstituted in ultra-pure water.

Ultra-pure water obtained with a Milli-Q plus 185 from Millipore Ibérica S.A. (Spain) was used for all solutions.

2.3. Electrode pretreatment

An electrode pretreatment was carried out before each voltammetric experiment with the aim of improving the sensitivity and reproducibility of the results. Fifty microliters of 0.1 M H_2SO_4 was dropped on SPCEs and an anodic potential of +1.30 V was applied for 2 min. Then, the electrodes were washed using 0.1 M Tris buffer pH 7.2.

2.4. Adsorption of streptavidin on SPCEs and subsequent reaction with B-AP

Adsorption of streptavidin: A drop of 10 μl of streptavidin solution (different concentrations) was placed on the pretreated surface of the SPCE and left there for a fixed time at adequate temperature. Then, the electrode was washed with 0.1 M Tris buffer pH 7.2 to remove the excess of protein. Free surface sites were blocked with BSA by placing a drop of 40 μl of a 1.0 % (w/v) solution of this molecule for 15 min.

The response of these streptavidin modified SPCEs, was tested using the streptavidin:biotin interaction. Thus, the following steps were performed:

Reaction with B-AP: After another washing step with 0.1 M Tris buffer pH 7.2, an aliquot of 40 μl of B-AP (different concentrations) was dropped on the streptavidin modified electrode for a fixed time.

Enzymatic reaction: After a washing step with 0.1 M Tris buffer pH 9.8, the enzymatic reaction was carried out dropping an aliquot of 30 μl of 6 mM 3-IP on the electrode surface for 15 min. After that, the reaction was stopped adding 4 μl of fuming sulphuric acid and 10 μl of ultra-pure water. In this step, the corresponding indigo product is converted to its parent hydrosoluble compound indigo carmine.

Analytical signal recording: When cyclic voltammetry (CV) was used as electrochemical technique, the SPCEs were held at a potential of −0.25 V for 25 s. Then, a voltammogram was recorded from −0.25 V to +0.20 V at a scan rate of 50 mV/s.

When square-wave voltammetry (SWV) was used, the electrodes were held at a potential of −0.30 V for 45 s and a potential scan from −0.30 V to +0.20 V (frequency = 25 Hz, amplitude = 50 mV) was performed.

2.5. Design of the immunosensor device

The electrochemical pretreatment was carried out following the procedure explained in Section 2.3. The adsorption of streptavidin on the electrode surface was performed leaving an aliquot of 10 μl of a 1×10^{-5} M streptavidin solution overnight at 4 °C. The BSA blocking was carried out in a similar way than that explained in Section 2.4, but in this case a 2% BSA solution was employed to block the free sites.

The formation of the sensing phase was performed with 40 μl of an Ab-B solution (different concentrations) for 1.5 h. A washing step with 0.1 M Tris pH 7.2 buffer followed by a blocking step with BSA (a drop of 40 μl of a 2% BSA solution for 15 min) were carried out. Finally, the electrodes, so modified, were washed with 0.1 M Tris buffer pH 7.2 (Fig. 2A).

2.6. Analytical procedure for the detection of RIgG-AP

Detection of RIgG-AP was performed dropping aliquots of 40 μl of RIgG-AP solutions (different dilutions) on the immunosensor device for a fixed time. After a washing step with 0.1 M Tris buffer pH 7.2, the enzymatic reaction and the analytical signal recording were carried out following the procedure explained in Section 2.4 (Fig. 2B).

2.7. Sequential competitive imnunoassay protocol for the determination of rabbit IgG

RIgG was determined using a sequential competitive approach. Thus, an aliquot of 40 μl of RIgG solution (different concentrations) was dropped on the immunosensor (modified from a 4.28×10^{-9} M Ab-B solution) for 1.5 h. After a washing step with 0.1 M Tris pH 7.2 buffer, an aliquot of 40 μl of RIgG-AP solution (dilution 1/100,000) was placed on the immunosensor and left there for 1.5 h. For detection, the final steps, involving the enzymatic reaction and recording the analytical signal, were the same than those mentioned in Section 2.4 (Fig. 2C).

3. Results and discussion

3.1. Streptavidin coating and enzyme detection

The first studies developed in this work were focused on establishing the most adequate conditions for the

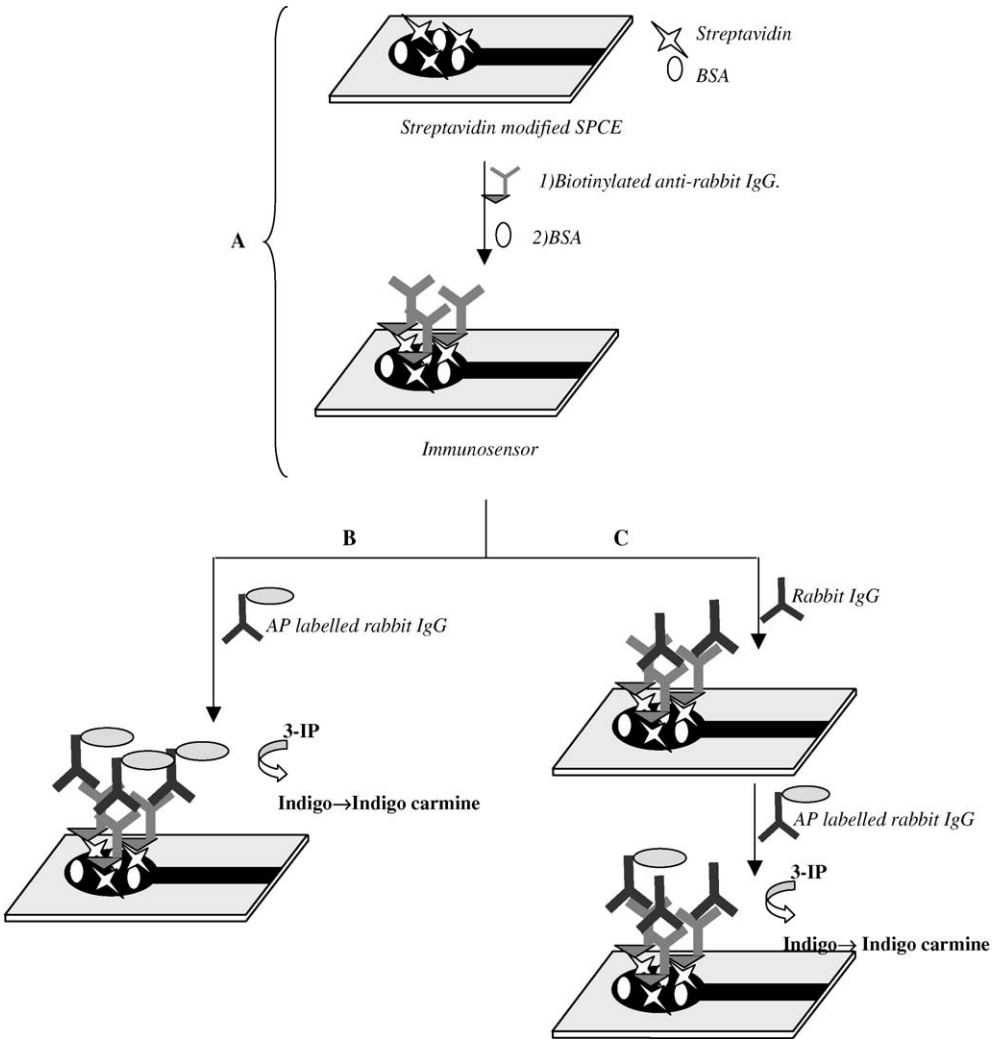

Fig. 2. Schematic representation of the steps performed for (A) the fabrication of the immunosensor device and its use for (B) the detection of RIgG-AP and (C) RIgG.

adsorption of streptavidin on the surface of SPCEs as well as on demonstrating that streptavidin:biotin interaction could be monitored on these electrodes (which would allow the orientation/enhanced adsorption of biotinylated antibodies). In order to reach this goal, 3-indoxyl phosphate (3-IP) was chosen as substrate of alkaline phosphatase.

Fig. 3 shows the cyclic (A) and square-wave (B) voltammograms obtained when 40 μl of a 6×10^{-11} M B-AP (in AP) solution were placed for 1.5 h on a pretreated electrode modified with streptavidin from 1×10^{-5} M solution at 4 °C (voltammograms (a)) and on a bare pretreated electrode (voltammograms (b)). The enzymatic product exhibits a reversible diffusion controlled process at a formal potential ($E_{1/2}$) of −0.15 V versus Ag-pseudoreference electrode. As it is shown in Fig. 3B, SWV offers several advantages over CV when working with reversible electrodic processes, which include higher sensitivity, better peak resolution as well as improved faradaic to capacitive current ratios.

This electrodic process measured for IC corresponds with the oxidation of the conjugated enol groups of the leuco-indigo carmine specie (generated by means of the application of the initial potential) to yield indigo carmine and its subsequent reduction [29]. The current measured by CV or SWV due to IC can be therefore directly related to the enzyme concentration.

Moreover, the absence of background signal demonstrates that the blocking with BSA avoids the non-specific adsorptions of B-AP on the electrode surface.

Several experimental conditions (accumulation time and temperature) to attach streptavidin on the electrode surface have been tested using the streptavidin:B-AP interaction. The concentrations of streptavidin and B-AP chosen in this study were 1×10^{-5} M and 6×10^{-11} M (in AP), respectively, and the reaction time was 1.5 h. Enzymatic reaction and analytical signal recording have been carried out as mentioned in Section 2.4. The results obtained are shown in Fig. 4 . When the adsorption of streptavidin was carried out for 15 min at

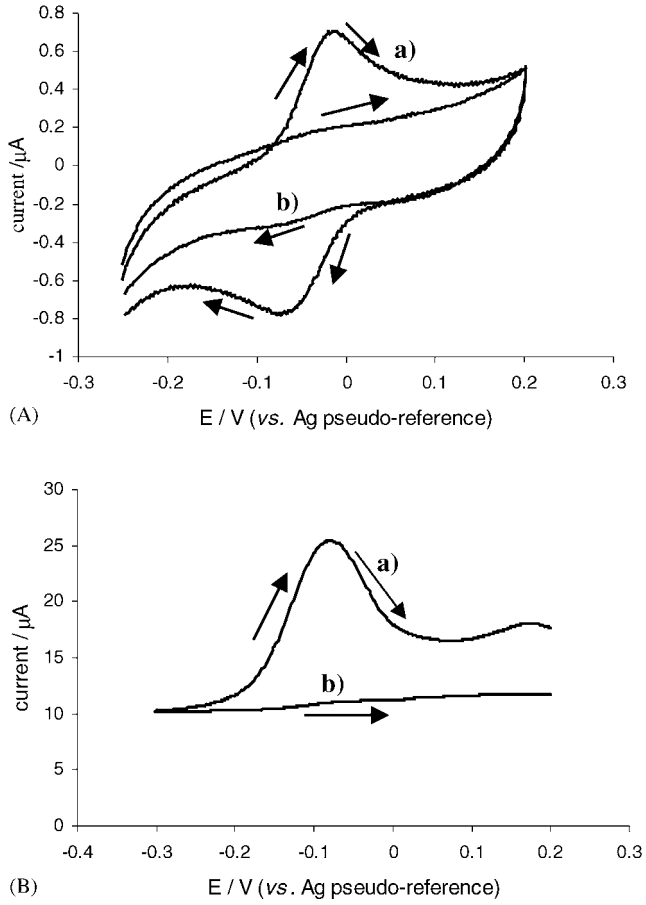

Fig. 3. Cyclic (A) and square-wave (B) voltammograms obtained when the streptavidin:B-AP interaction is carried out. Voltammograms (a) correspond to a pretreated electrode surface modified with streptavidin (from a 1×10^{-5} M solution at 4 °C overnight). Voltammograms (b) correspond to a bare pretreated electrode. [B-AP] = 6×10^{-11} M in AP; reaction time = 1.5 h.

room temperature, no analytical signals were obtained. It means that the surface of these SPCEs does not show the adequate adsorptive properties to attach proteins, in contrast to other conventional ones as carbon paste electrodes where these accumulation conditions of streptavidin are enough to achieve the attachment of the protein on the electrode surface [31].

Higher analytical signals were achieved when the modification of the SPCEs with streptavidin was carried out at 46 °C until dryness (approximately 20 min). In this case, the streptavidin was adsorbed on the electrodes. However, a lack of reproducibility of the analytical signal was observed in these conditions. The reproducibility of the peak current did not improve when the electrode pretreatment (described in Section 2.3) was performed (data not shown).

When the adsorption was carried out overnight at 4 °C on a non-pretreated SPCE, lower analytical signals were achieved but the reproducibility of peak current was improved. The use of pretreated SPCEs (Section 2.3) as well as these conditions of the adsorption of streptavidin allowed to obtain analytical signals three-fold higher than those with non-pretreated electrodes, without lost of reproducibility.

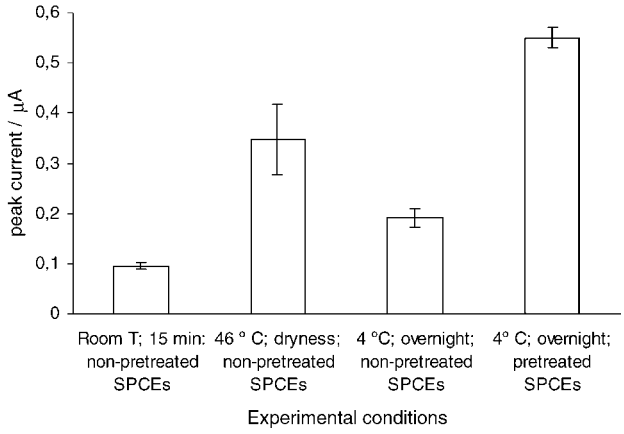

Fig. 4. Influence of streptavidin accumulation time and temperature on peak current. Peak [streptavidin] = 1×10^{-5} M; [B-AP] = 6×10^{-11} M; reaction time = 1.5 h. The rest of experimental conditions as in Fig. 3.

The electrode pretreatment improves the adsorptive conditions of the surface of SPCEs giving rise to an increase of the analytical signals.

Thus, the adsorption of streptavidin solution at 4 °C (overnight) on a pretreated electrode is chosen for further studies. Other advantage of the use of these conditions is that the response of these streptavidin coated SPCEs, so modified, is stable for several weeks if they are stored at 4 °C.

The effect of the concentration of streptavidin on the voltammetric responses was investigated. The peak current does not increase with increasing streptavidin concentration from 1×10^{-8} to 1×10^{-6} M and then increases rapidly up to 1×10^{-5} M. Beyond this point, the electrode response changes slightly indicating that an saturation state is reached on the surface. So electrodes were coated with 10 μl volumes of 1×10^{-5} M streptavidin solutions.

The influence of the B-AP reaction time with streptavidin coated SPCEs was also studied. The highest value of the peak current was obtained at 1.5 h of reaction time.

Under the above-mentioned experimental conditions, the relative standard deviation of five parallel experiments was 5.0% with a mean peak current of 559 nA for a 6×10^{-11} M B-AP (in AP), when CV was used as electrochemical technique. In the case of SWV, the relative standard deviation of five parallel experiments was 11.0% with a mean peak current of 2.83 μA for a 10^{-11} M B-AP solution (in AP).

The dose–CV response curve recorded under the above experimental conditions, and expressed in terms of alkaline phosphatase concentration in the incubation solution of B-AP, fits to the following equation:

$$\mathrm{Ip\,(nA)} = 7 \times 10^{9}[\mathrm{AP}]\,(\mathrm{M}) + 0.16; \quad r = 0.997;\ n = 5.$$

A linear range from 1×10^{-11} to 3×10^{-10} M was obtained.

In the case of SWV, a good linear relationship between peak current and concentration of AP in B-AP solution from

1×10^{-12} to 3×10^{-11} M was obtained with a correlation coefficient of 0.998 ($n = 5$), according to the following equation:

$$\text{Ip}\,(\mu\text{A}) = 2 \times 10^{11}[\text{AP}]\,(\text{M}) + 0.62$$

Although SWV results are less reproducible than those obtained when CV is used as electrochemical technique, a lower detection limit one order of magnitude is achieved, according to the usual better sensitivity of this electrochemical technique when reversible electrodic processes are recorded.

Therefore, these results showed an adequate and simple way of coating the surface of a SPCE with streptavidin, in contrast to other works where the covalent attachment of streptavidin was carried out on conducting polymer modified SPCEs [15]. Moreover, in this way the streptavidin:biotin reaction could take place, being the streptavidin modified electrode stable for several weeks at 4 °C, as above-mentioned.

3.2. Electrochemical characterization of the immunosensor device and direct detection of RIgG-AP

The next step was the fabrication of an immunosensor device to determine RIgG. As streptavidin modified SPCEs were employed, the sensing phase included a biotinylated antibody against RIgG (Ab-B).

Streptavidin coated SPCEs were modified in the same manner, using the experimental conditions optimised in last section. The blocking step was performed with 2% BSA buffer solution because the non-specific adsorption of the RIgG-AP on the electrode surface were higher than for signals recorded when B-AP.

The reaction time between streptavidin modified SPCEs and Ab-B was studied for a 1×10^{-10} M concentration of Ab-B solution. The reaction with RIgG-AP (1/10,000 dilution) was carried out for 1 h following the analytical procedure explained in Section 2.6. The peak current increases upon raising the reaction time whereas a plateau is reached at 1.5 h (data not shown). These results were expected because the attachment of biotinylated antibody only depends on the streptavidin:biotin interaction.

The influence of reaction time between Ab-B and RIgG-AP on the analytical signal was investigated. A 1×10^{-10} M concentration of Ab-B solution and a 1/10,000 dilution of stock RIgG-AP solution were employed for this study. The peak current increases upon raising the reaction time. For reaction times greater than 1.5 h, the slope of the curve decreases. A reaction time of 1.5 h, where a change in slope takes place, was chosen for further studies.

Different calibration plots were obtained with streptavidin coated SPCEs modified from 4.28×10^{-8}, 4.28×10^{-9} and 4.28×10^{-10} M Ab-B solutions (Table 1). Incubations of the resulting affinity probes were done in RIgG-AP solutions of decreasing dilutions for 1.5 h. Cyclic voltammetry was employed to record the analytical signal. The rest of the experimental conditions were kept constant.

For all concentrations of Ab-B assayed two linear ranges of dilutions of RIgG-AP were obtained with a change of the slope of the curve. The values of the slope of the equations for first linear ranges of RIgG-AP dilutions were similar for the three Ab-B concentrations assayed.

The equations of second linear ranges had lower values of slope than those obtained for first linear ranges of dilutions of stock RIgG-AP solution (approximately 2-fold, 6.6-fold and 7.5-fold lower for 4.28×10^{-8}, 4.28×10^{-9}, 4.28×10^{-10} M of Ab-B concentration, respectively). This fact was due to that the immunosensing phase started to reach the saturation. The incubation of streptavidin coated SPCEs with higher concentrations of Ab-B allowed larger amount of molecules of Ab-B in the immunosensing phase and consequently, more active sites to react with RIgG-AP. Thus, the rate of reaching the saturation is slower and the value of slope of linear equation for lower dilutions of labelled analyte is greater when higher concentrations of Ab-B were incubated. Although the modification of streptavidin coated electrodes with 4.28×10^{-9} and 4.28×10^{-10} M of Ab-B had similar values of slopes of the curves, the first linear range obtained for 4.28×10^{-10} M of Ab-B was shorter than that achieved for 4.28×10^{-9} M.

Although for a concentration of 4.28×10^{-8} M, the results are similar than those obtained for 4.28×10^{-9} M in the first linear range, a concentration of 4.28×10^{-9} M of Ab-B was considered enough to fabricate the immunosensing phase, based on the principle of adequate signal with minimal waste of protein. Moreover, the excess in the amount of the antibody in the sensing phase could be harmful to develop a competitive assay more sensitive to determine RIgG.

Under these experimental conditions, the reproducibility of the analytical signal has been studied for a dilution of

Table 1
Calibration plots of RIgG-AP

[Ab-B] (M)	Linear range (RIgG-AP dilution)	Linear equation[a]	r	n
4.28×10^{-8}	1/800000–1/100000	Ip (μA) = 6.12 [RIgG-AP] × 10^4 + 0.05	0.998	4
	1/100000–1/10000	Ip (μA) = 3.58 [RIgG-AP] × 10^4 + 0.25	0.994	5
4.28×10^{-9}	1/600000–1/80000	Ip (μA) = 7.42 [RIgG-AP] × 10^4 + 0.03	0.995	5
	1/40000–1/10000	Ip (μA) = 1.13 [RIgG-AP] × 10^4 + 1.09	0.995	3
4.28×10^{-10}	1/800000–1/200000	Ip (μA) = 5.98 [RIgG-AP] × 10^4 + 0.03	0.996	4
	1/100000–1/10000	Ip (μA) = 0.80 [RIgG-AP] × 10^4 + 0.14	0.999	4

For experimental conditions see text.

[a] [RIgG-AP] are expressed in terms of dilution of RIgG-AP stock solution.

1/100,000 of stock RIgG-AP. The relative standard deviation for four parallel experiments (inter-immunosensor reproducibility) was 7.0% with a mean peak current of 1.44 μA.

The stability of these immunosensors devices have been tested recording the analytical signal for a dilution of 1/100,000 RIgG-AP solution. The immunosensors were fabricated following the procedure described in Section 2.5 and using the Ab-B concentration optimised in previous studies, but after the attachment of Ab-B on the electrode and blocking with BSA, the immunosensors were stored at 4 °C until their use. Table 2 displays the results obtained. The immunosensor devices were stable for at least 5 months without lost of response, indicating effective retention of the specific binding ability.

Using SWV as electrochemical technique to record the analytical signal, a linear relationship between peak current and the dilution of RIgG-AP from 1/1,500,000 to 1/400,000 was achieved. The linear equation obtained was:

$$\mathrm{Ip}\,(\mu\mathrm{A}) = 5.0\,[\mathrm{RIgG\text{-}AP}] \times 10^{6} - 2.16;$$

$$r = 0.996;\ n = 5$$

where [RIgG-AP] are expressed in terms of dilutions. Although SWV detected dilutions of RIgG-AP greater than 1/1,500,000, the lack of reproducibility of peak current obtained with this electrochemical technique at these concentration levels of RIgG-AP did not allow to determine the labelled analyte with accuracy.

3.3. Significance of the attachment of Ab-B through streptavidin:biotin interaction

The streptavidin:biotin system is particularly suitable for use in competitive or sandwich immunoassays. The biotin molecule can be easily activated and coupled to either antigens or antibodies, with complete retention of activity. Subsequently, streptavidin can be conjugated with enzymes [32], radioisotopes [33] or colloidal gold [34] and used as a high affinity secondary reagent which can greatly increase the sensitivity of an assay. Moreover, the attachment of antibodies to the electrode surface through the streptavidin:biotin interaction has been revealed as an effective approach [12].

To test the influence of this interaction in the attachment of antibodies on the electrode surface, parallel experiments have been carried out using pretreated SPCEs modified with streptavidin and pre-treated SPCEs without streptavidin. Fig. 5 displays six cyclic voltammograms corresponding to the peak currents obtained for a 1/100,000 dilution of RIgG-AP stock solution. Voltammograms (a) have been recorded using immunosensor devices (1×10^{-5} M of streptavidin, 4.28×10^{-9} M of Ab-B, the rest of experimental conditions were described above). Voltammograms (b) correspond to pretreated SPCEs (without both streptavidin and BSA), where have been accumulated a drop of 40 μl of 4.28×10^{-9} M of Ab-B solution for 1.5 h. The other cyclic voltammograms (c) correspond to background signals (without Ab-B) obtained for both cases.

Table 2
Data of the stability of immunosensor devices

Time (weeks)	Mean peak current (μA)	Relative deviation standard (%)	*n*
0[a]	1.39	5.0	5
1	1.15	6.1	5
2	1.42	2.1	5
3	1.22	4.1	5
4	1.30	6.1	5
20	1.21	0.8	5

[a] Immunosensors devices fabricated 1 day ago.

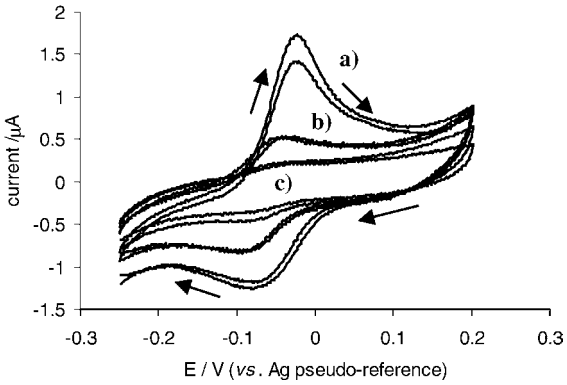

Fig. 5. Cyclic voltammograms recorded for a 1/100,000 dilution of RIgG-AP stock solution. Voltammograms (a) correspond to immunosensor devices (1×10^{-5} M of streptavidin, 4.28×10^{-9} M of Ab-B). Voltammograms (b) correspond to pretreated SPCEs without both streptavidin and BSA, where have been accumulated a drop of 40 μl of 4.28×10^{-9} M of Ab-B solution for 1.5 h. Voltammograms (c) correspond to background signals (without Ab-B) obtained for both cases.

When the Ab-B was attached to the electrode surface through the streptavidin:biotin interaction, the peak currents were approximately 3.7-fold higher than those obtained when Ab-B was attached to the electrode surface by physical adsorption. Two factors could be responsible of this behaviour. One of these is that streptavidin:biotin interaction allows to obtain a immunosensing phase with more molecules of Ab-B than its direct adsorption on the electrode surface. The other one is that the molecules of Ab against RIgG are oriented through the streptavidin:biotin reaction in the immunosensing phase, leaving most binding sites of antibody accessible for the reaction with RIgG-AP. In fact, the attachment of antibodies to a support (electrode, microtiter well, etc.) by physical adsorption gives rise to disordered sensing phases and therefore, a part of the affinity sites of antibodies are blocked to react with their antigens. The background signals obtained in both cases were similar. It means that the contribution of non-specific adsorption of RIgG-AP in the analytical signal only depends on the efficiency of the blocking step with BSA.

3.4. Indirect determination of RIgG

The two-step competitive assay developed in this work is based on the sequential interaction of RIgG and RIgG-AP

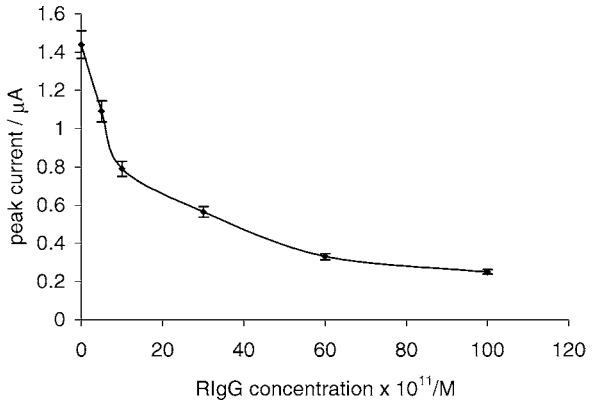

Fig. 6. Calibration plot for RIgG obtained in a sequential competitive assay. Experimental conditions in the text. Cyclic voltammetry is used as electrochemical technique.

with the antibody attached on the SPCEs. In the competitive assay, an analyte and a labelled analyte compete for a limiting number of immobilised antibodies binding sites. The amount of bound conjugate is inversely proportional to the amount of analyte in the sample.

Using the protocol explained in Section 2.7, it is feasible to quantify RIgG. The reaction time between the immunosensor devices and RIgG was 1.5 h, as in the case of the reaction with RIgG-AP. It was observed that increasing concentrations of RIgG provokes a decrease in the current response (Fig. 6). The calibration curve was linearly fitted on an linear $\log y$–$\log x$ from 5×10^{-11} to 1×10^{-9} M, according to the following equation:

$$\log \mathrm{Ip}\,(\mu\mathrm{A}) = -0.485 \times \log[\mathrm{RIgG}](\mathrm{M}) - 4.95;$$

$$r = -0.9999;\ n = 5$$

A limit of detection of 5×10^{-11} M of RIgG (7.0 ng/ml), estimated as the analyte concentration that gives an analytical signal three times the standard deviation of the signal obtained when no analyte is presented in solution. The value of this detection limit is lower than others reported in the literature [15,17].

The same study was carried out employing SWV as electrochemical technique to record the analytical signal but the results obtained did not improve those achieved when the analytical signals were recorded using cyclic voltammetry, due to a lack of the reproducibility of the analytical signal (data not shown).

4. Conclusion

A single-use immunosensor device for the determination of rabbit IgG has been designed in this work. The anchorage of the primary antibody on SPCEs is carried out using the streptavidin:biotin interaction. The attachment of streptavidin on the electrode surface is performed in a simple way by physical adsorption in contrast to other methodologies reported in the literature where a covalent bound is necessary. The physical adsorption of streptavidin on the surface of electrodes is performed at 4 °C overnight. This step is essential to guarantee good inter-electrode reproducibility of the analytical signal of the immunosensor.

The attachment of goat anti-rabbit IgG on the electrode surface through streptavidin:biotin interaction improves the sensitivity of the immunosensor devices (3.7-fold more sensitivity for the same antibody concentration assayed) due to the effect of orientation/enhance adsorption of antibodies on the immunosensing phase, which allows a major number of binding sites free to the subsequent reaction with the analyte. Other advantage of this methodology is that the immunosensor devices, so fabricated, are stable for months without lost of reproducibility of the analytical signal.

The immunosensor developed in this work allows detecting a concentration of 7.0 ng/ml of rabbit IgG, which is lower than other limits of detection reported in the literature.

The work is in progress to design different single-use immunoprobes for the detection of analytes of clinical interest where the streptavidin:biotin reaction is being used for the attachment of the primary antibody on SPCE supports.

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