



Review

Investigation of SPR and electrochemical detection of antigen with polypyrrole functionalized by biotinylated single-chain antibody: A review

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ABSTRACT

An electrochemical label-free immunosensor based on a biotinylated single-chain variable fragment (Sc-Fv) antibody immobilized on copolypyrrole film is described. An efficient immunosensor device formed by immobilization of a biotinylated single-chain antibody on an electropolymerized copolymer film of polypyrrole using biotin/streptavidin system has been demonstrated for the first time. The response of the biosensor toward antigen detection was monitored by surface plasmon resonance (SPR) and electrochemical analysis of the polypyrrole response by differential pulse voltammetry (DPV). The composition of the copolymer formed from a mixture of pyrrole (py) as spacer and a pyrrole bearing a *N*-hydroxyphthalimidyl ester group on its 3-position (pyNHP), acting as agent linker for biomolecule immobilization, was optimized for an efficient immunosensor device. The ratio of py:pyNHP for copolymer formation was studied with respect to the antibody immobilization and antigen detection. SPR was employed to monitor in real time the electropolymerization process as well as the step-by-step construction of the biosensor. FT-IR demonstrates the chemical copolymer composition and the efficiency of the covalent attachment of biomolecules. The film morphology was analyzed by electron scanning microscopy (SEM).

Results show that a well organized layer is obtained after Sc-Fv antibody immobilization thanks to the copolymer composition defined with optimized pyrrole and functionalized pyrrole leading to high and intense redox signal of the polypyrrole layer obtained by the DPV method. Detection of specific antigen was demonstrated by both SPR and DPV, and a low concentration of 1 pg mL^{-1} was detected by measuring the variation of the redox signal of polypyrrole.

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1. Introduction

Electrochemical immunosensors have been widely developed for the detection of proteins in clinical diagnostics and drug discovery [1] due to their simplicity, low detection limit and their easy integration into miniaturized systems. In electrochemical immunosensors, the immunological compounds (antibody or antigen) are immobilized on an electrochemical transducer such as beads, nanocarbon nanotubes, nanoparticles [2], or a conducting polymer.

Conducting polymers (CPs) are largely used as transducers for biological interactions [3]. The success of CPs relies on their high electrical conductivity with their ability to monitor transfer of biological recognition processes, produced by probe/target interactions, to an electrochemically measured signal [4]. Furthermore, CPs also provide a suitable interface for grafting bioreceptors onto micron-sized surfaces [5], opening the way to electrochemical biochips [6]. The most commonly used CP in sensing applications is polypyrrole (Ppy), owing to its biocompatibility [7], high hydrophilic character and high stability in water [8]. Different strategies are investigated to immobilize biomolecules on Ppy [9], including direct adsorption [10], entrapment [11], and chemical grafting on N- [12] or 3-substituted [13] polypyrrole. 3-Substituted polypyrrole has demonstrated an advantage in maintaining its full intrinsic electrical properties during both the construction of immunosensors and the immunosensing reaction, and thus has allowed a direct measure of all these processes [14].

The control of antibody orientation and its accessibility constitute a real challenge for the development of an efficient immunosensor. Indeed, for effective detection of analyte by antibody, the variable region of the antibody and its active site should be exposed to the analyte in solution [15]. Furthermore, the streptavidin–biotin strategy has been extensively employed to immobilize biomolecules and has demonstrated its ability to control antibody orientation on the film and to be highly compatible with many biological functions without denaturation [16]. However, with such an approach, the electrochemical detection of the immunological recognition requires an indirect measurement.

Electrochemistry coupling with optical spectroscopy promises to generate novel and effective molecular recognition technologies, especially in the purpose for direct and sensitive investigation. Electrochemical-SPR measurements have been investigated to characterize the structural and optical properties of conducting polymer film on metal support [17]. Electrochemical-SPR experiments have also been used to sense the oxidation of glucose [18] and DNA [19] or receptor detection. Recently, Tharamani et al. [20] reported that electrochemical methods may be employed as a complement of SPR to monitor the interaction of papain with ferrocene-peptide immobilized on a gold surface. Among all the electrochemical-SPR biosensors described for protein detection, immunosensors based on conducting polymers are still rare. Li and co-workers [21] described a sandwich immunosensor based on a copoly(pyrrole–pyrrole propylic acid) film able to detect a mouse IgG by indirect electrochemical measurement.

Here we report, to the best of our knowledge, the first example of an in situ electrochemical surface plasmon resonance immunosensor based on a 3-substituted polypyrrole film. In this

work, immobilization of a biotinylated single-chain antibody fragment (Sc-Fv Ab) on a copoly(py–pyNHP) film functionalized with the biotin/streptavidin system was studied in detail using DPV and SPR techniques simultaneously. FT-IR was used to confirm the presence of the copolymer and the covalent bonding with biotin. Scanning electron microscopy (SEM) was applied to characterize layer-by-layer the morphologies of the modified films. The orientation control as well as the density of the biotinylated Sc-Fv Ab are demonstrated by SPR in regard of the copolymer formation. Finally, the sensing process is investigated by direct electrochemical and SPR measurement to demonstrate the high efficiency of the electrochemical surface plasmon resonance immunosensor.

2. Experimental

2.1. Reagents

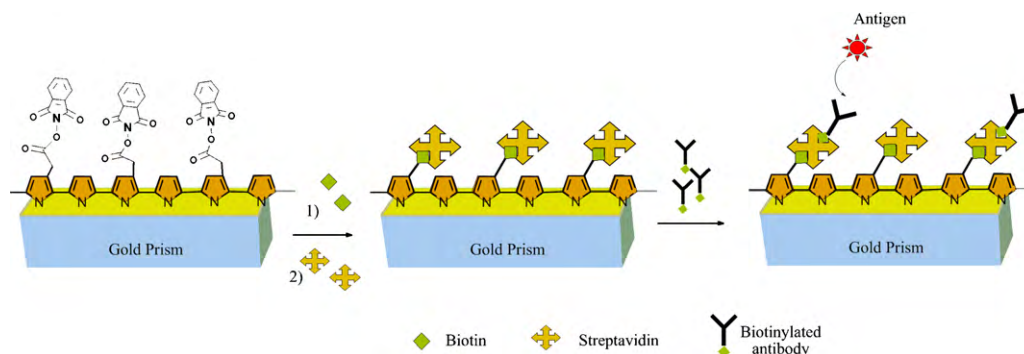
Pyrrole (py) was purchased from Sigma–Aldrich, and distilled under argon before use. Biotin hydrazide, streptavidin, anti-albumin biotinylated antibody, ovalbumin and phosphate buffer saline (PBS) tablets, were purchased from Sigma–Aldrich. The buffer solutions of 10 mM at pH 7.4 was prepared with doubly distilled water and stored in the freezer until use. The antibody is a recombinant single-chain fragment (Sc-Fv Ab) consisting of heavy-chain and light-chain domains covalently linked through a 16 amino-acid peptide. The monoclonal antibody was derived from an immunized goat by DBDx phage display technology and is expressed as an antibody Sc-Fv Ab fragment with a His tag biotin residue for binding. The antigen is a peptide sequence of 13 amino-acids conjugated to BSA. The masses of the single-chain antibody and the antigen, checked by MALDI-ToF mass spectroscopy, were 20 and 64 kDa, respectively. Antigen and Sc-Fv Ab were produced and purified by Wyeth Company (UK).

The 3-(*N*-hydroxyphthalimidyl ester) pyrrole was synthesized according to a strategy described previously [22]. The synthesis is available in supporting material.

2.2. Instrumentation

SPR measurement. An AUTOLAB ESPRIT double-channel instrument (Eco Chemie, Utrecht, the Netherlands) was used to perform optical measurements of the SPR angle and electrochemical measurements with an incorporated autosampler. A polarized laser light ($\lambda = 670$ nm) is directed to the bottom side of the sensor via a hemispheric lens placed on a prism (BK7 with a refractive index of 1.52) and the reflected light is detected using a photodiode. The standard electrochemical cuvette supplied allows measurements on a three-electrode system containing a fixed contact point to the gold layer of the sensor disk; the gold operates as working electrode, a replaceable Ag/AgCl reference electrode and a fixed platinum counter-electrode. The active electrode surface was 0.06 cm^2 .

Electrochemical measurement. Electrochemical polymerization and characterization was performed using an AUTOLAB PGSTAT 12 electrochemical analysis system with GPES software. The electrochemical cell consists of a three-electrode cell with platinum as counter-electrode, a saturated calomel reference electrode (SCE) and a gold surface as working electrode. The copolymer was analyzed by differential pulse voltammetry (DPV), by using different



Scheme 1. Schematic representation and synthetic procedure for the construction of the immunosensor: (a) electropolymerization of copoly(py-pyNHP) film at the electrode surface, (b) covalent grafting of biotin on copoly(py-pyNHP) layer and immobilization of streptavidin via biotin, (c) anchoring of biotinylated antibody on polypyrrole-biotin-streptavidin double layer.

values for the time and potential parameters defining the waveform, in order to seek for the set of values leading to the best results. DPV measurements were performed at 0.05 V s^{-1} , with a pulse height of 45 mV and 0.05 s pulse width.

FT-IR Fourier Transform infrared spectra were measured using a Bruker IFS66 FT-IR spectrometer equipped with a MCT detector and an attenuated total reflectance (ATR) crystal of germanium.

Scanning electron microscopy (SEM) images were acquired using a ZEISS SUPRATM 55VP GEMINI[®] apparatus. The copolymer film and different steps of the biosensor construction were prepared by electropolymerization on the gold disk electrode according to the method described in Section 2.3.

2.3. Electro-copolymerization

The copolymer film, poly[pyrrole, 3-*N*-hydroxyphthalimido pyrrole] (copoly(py-pyNHP)) was grown on a gold surface of the prism in the electrochemical cell containing 50 μL of a 10 mM solution of pyrrole (py) and 3-*N*-hydroxyphthalimido pyrrole (pyNHP) monomers and 0.1 M LiClO_4 in acetonitrile. The ratio of the two monomers py:pyNHP is varied from $1:10^{-4}$, $1:10^{-3}$, $1:10^{-2}$, $1:10^{-1}$ and 1:1. Electropolymerization was performed by applying a fixed potential of 0.8 V versus Ag/AgCl reference electrode for 100 s and the reaction was stopped when a charge of 450 μC was reached. The modified surface was rinsed with acetonitrile and three times with PBS in order to remove any trace of monomers. The SPR curve presenting the variation of angle versus time was recorded during the electropolymerization reaction. The sensogram presenting the variation of reflectivity versus angle was recorded in the same PBS-buffered solution before and after the polymerization step at open circuit potential.

2.4. Construction of immunosensors

Biotin was covalently bonded on copoly(py-pyNHP) by immersing the modified electrode with 50 μL of a 2 mg mL^{-1} solution of biotin hydrazide in PBS pH 7.4 for 10 min at 25°C . The resulting biotinylated pyrrole film was washed three times with 10 mM PBS followed by the addition of 50 μL of 100 $\mu\text{g mL}^{-1}$ streptavidin solution during 10 min. Afterwards, 50 μL of 8 $\mu\text{g mL}^{-1}$ biotinylated antibody solution in PBS was incubated for 10 min. Then the electrode was carefully washed three times with PBS.

Before interaction with the antigen, the electrode was blocked with casein to avoid non-specific interactions. Addition of 50 μL of 50 mg mL^{-1} casein solution in PBS was performed during 3 min, followed by thorough rinsing with PBS. Each step in the immunosensor's construction was directly monitored by SPR measurement in the same buffer solution at open circuit potential.

2.5. Antigen incubation

Antigen incubation was performed at 25°C by plunging the modified electrode for 10 min in buffer solution with different concentrations of antigen from 1 pg mL^{-1} to 100 ng mL^{-1} . The electrode was then washed three times with PBS solution. Before each new addition of antigen, the surface of the biosensor was regenerated with a buffered solution of 0.05 M glycine in 0.05 M HCl at pH 3. The concentration of glycine in HCl has previously been optimized by SPR and this reactant serves to completely remove antigen from the biosensor surface.

3. Results and discussion

The direct electrochemical and optical detection by SPR of the antigen-antibody interaction was achieved using an immunosensor based on a biotinylated single-chain antibody immobilized on a functionalized copolypyrrole film. The biotinylated Sc-Fv Ab was grafted onto the conducting polymer thanks to the high-affinity interaction of the streptavidin-biotin complex (association constant $K_a = 10^{15} \text{ M}^{-1}$) [23], leading to the control of antibody orientation and to improve the access of the antibody active site.

The first step requires the electrochemical polymerization of various mixtures of py and pyNHP on the electrode surface to form activated film. Then biotin is covalently grafted to the copolymer film with an amide link between the amino group of the biotin hydrazide and the activated ester of the pyNHP followed by the immobilization of streptavidin. Finally, biotinylated Sc-Fv Ab is anchored to the polypyrrole-biotin-streptavidin scaffolding to elaborate the bioactive surface (Scheme 1). Each step of biosensor construction is characterized by various techniques: FT-IR, scanning electron microscopy (SEM), SPR and electrochemical measurement.

3.1. Electrochemical deposition of the copoly(py-pyNHP) film

The copolymer formed by the mixture of functionalized pyrrole-bearing activated ester as linking agent and non-functionalized pyrrole as spacer easily undergoes electropolymerization in acetonitrile containing 0.1 M LiClO_4 at the gold electrode. The film was grown by electrolysis at a fixed potential of 0.8 V versus Ag/AgCl and the polymerization was stopped at a charge consume from 450 μC giving a stable adherent film [24]. The polymerization reaction was monitored by SPR experiments (Fig. 1). Fig. 1A shows the variation of resonance angle versus time. The SPR kinetic response shows an increase in the angle from -333 to 2420 m° within 100 s during the polymerization step followed by a small decrease during the washing step. The increase in the resonance angle deviation is due

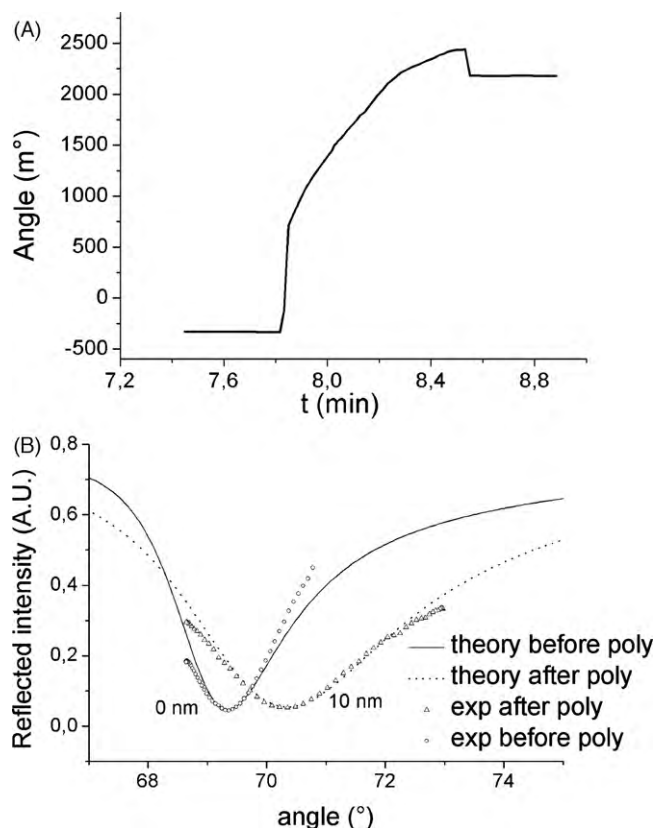


Fig. 1. Electropolymerization of py 10 mM–pyNHP 1 mM solution in $\text{LiClO}_4/\text{CH}_3\text{CN}$ 0.1 M. (a) SPR angle versus time, (b) reflectivity versus SPR angle in PBS detected before and after electropolymerization (solid line) theoretical curve; (dashed line) experimental curve.

to the mass of polypyrrole deposited on the gold electrode, leading to the variation of the refractive index of the gold layer during electropolymerization [25,26]. The washing step eliminates all the non-attached polymer from the gold surface, leading to a decrease in the SPR angle. The kinetics of the reaction shows a continuous increase in the angle demonstrating that the polymerization of the two monomers leads to homogenous copolymer formation.

The changes in kinetic curve are likely to account for the change in the shapes of the plasmon resonance curves [27] measured before and after polypyrrole deposition at open circuit potential in PBS buffer has shown in Fig. 1B. The angle of resonance ($\theta = 69.34^\circ$) shifts to higher value ($\theta = 70.38^\circ$) in the presence of the polymer layer [28]. The thickness of the copoly(py–pyNHP) film is estimated at 10 nm by fitting theoretical SPR curves to the experimental curves using Winspall's program and with fitting parameters: $n_{\text{prism}} = 1.52$; $n_{\text{titanium}} = 2.36 + i3.11$ with $d = 2.5$ nm, $n_{\text{gold}} = 0.09 + i3.82$ with $d = 49$ nm, $n_{\text{copoly(py–pyNHP)}} = 1.421 + 0.0915i$. The obtained thickness is in good agreement with electrodeposition thickness of polypyrrole [29] on the gold surface and formed table interfaces for electrochemical and SPR studies.

3.2. Construction of immunosensor and in situ EC-SPR characterization

3.2.1. Study of the py:pyNHP ratio

The composition of the copolymer film formed with functionalized pyrrole, bearing an activated ester as linking agent for further modification, and pyrrole as spacer should have a large effect on the immobilization capacity of the antibody and thus the biosensor sensitivity. For this purpose, various ratios of py and pyNHP were investigated during the polymerization step. The ratio of the two pyrrole monomers, py:pyNHP was varied from $1:10^{-4}$, $1:10^{-3}$, $1:10^{-2}$, $1:10^{-1}$ and 1:1 keeping 10 mM as a total concentration. Biotin hydrazide, streptavidin and biotinylated antibody are successively immobilized on each copolymer film to elaborate the bioactive surface. The antibody immobilized is a biotinylated anti-albumin and the antigen detected is the ovalbumin. Each step of biosensor construction is followed by SPR, which allows to measure the amount of biomolecule immobilized on the film using the relation 120 m° shift corresponds to 1 ng mm^{-2} [30]. Table 1 resumes the values for the biotinylated anti-albumin immobilized on each copolymer film prepared and the amount of antigen immobilized after incubation of two concentrations of ovalbumin, $1 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$. These results demonstrate firstly that the immobilized antibody increases with the ratio of PyNHP to py during film formation. Indeed the amount of immobilized antibody increases from 0.1 to $1.01 \text{ fmol mm}^{-2}$ for the ratio of py:pyNHP $1:10^{-4}$ to 1:1, respectively. By increasing the proportion of pyNHP used as linking agent, large amounts of biomolecule were attached to the polypyrrole layer. However, antigen recognition did not follow the same behaviour, as when large amounts of antibody were immobilized no antigen detection was measured (Table 1, line 5). This result may be explained by the loss of accessibility and orientation of antibodies due to steric hindrance for antigen–antibody reaction. Small proportions of pyNHP in the film, $1:10^{-4}$ and $1:10^{-3}$ did not lead to any detection of antigen, as the immobilized antibody is not sufficient for antigen detection (Table 1, lines 1 and 2). $1 \mu\text{g mL}^{-1}$ of ovalbumin is detected by the sensor as soon as the ratio of py:pyNHP in the solution is greater than $1:10^{-2}$. The maximum of sensitivity is obtained for a ratio of $1:10^{-1}$ for py:pyNHP, where the optimum quantity of antibodies is immobilized on the film and optimum pyrrole as spacer is obtained leading, to good accessibility for antigen interaction at the antibody's active site. This result demonstrates that the optimum ratio which should be chosen between functionalized pyrrole and a pyrrole as spacer for immunosensor construction is a crucial parameter to improve the immobilization of proteins and then the sensitivity of detection.

3.2.2. FT-IR spectroscopic studies and SEM pictures

Copoly(py–pyNHP) film was studied by FT-IR spectroscopy (Fig. 2, solid line) to demonstrate the covalent attachment of the biotin to copolymer film on the functionalized pyrrole(PyNHP) as linker. Polypyrrole is characterized by bands at 1631 , 1564 and 1475 cm^{-1} , corresponding to $\text{C}=\text{C}$ stretching vibrations, and broad bands at 1182 and 1134 cm^{-1} may be assigned to $\text{N}-\text{C}$ stretching [31]. The presence of the pyNHP in the copolymer is characterized

Table 1
Amounts of biotinylated anti-albumin antibody immobilized onto copolymers formed with different py to pyNHP ratios and amount of antigen immobilized on these biosensors.

	py:pyNHP	Anti-albumin 2 mg mL^{-1} (fmol mm^{-2})	Ova-1 $\mu\text{g mL}^{-1}$ (fmol mm^{-2})	Ova-10 $\mu\text{g mL}^{-1}$ (fmol mm^{-2})
1	$1:10^{-4}$	0.10	Indetectable	0.03
2	$1:10^{-3}$	0.24	Indetectable	0.05
3	$1:10^{-2}$	0.55	0.09	0.06
4	$1:10^{-1}$	0.67	0.19	1.05
5	1:1	1.01	Indetectable	Indetectable

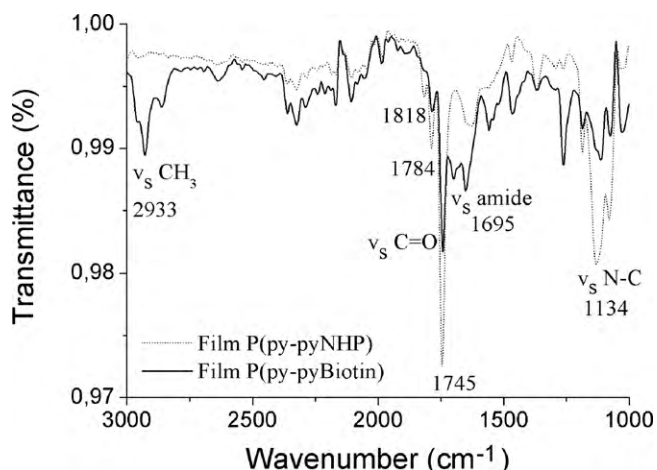


Fig. 2. FT-IR analysis of copoly(py-pyNHP) film (solid line) and covalent grafting of biotin on copolypyrrole layer (dashed line).

by frequencies at 1818, 1784 and 1745 cm^{-1} , corresponding to the C=O stretching of the activated ester group substituted to the pyrrole monomer. Covalent bonding of the activated polypyrrole with the amino group of the biotin hydrazide was also confirmed by FT-IR (Fig. 2, dashed line). The FT-IR spectra show the disappearance of the bands associated with pyrrolidinedione at 1818 and 1784 cm^{-1} , with the concomitant appearance of a new band at 1695 cm^{-1} characteristic of an amide function. The spectra of biotin exhibit peaks at 2933 and 1458 cm^{-1} attributed to CH_2 stretching [32].

Scanning electron microscopy (SEM) pictures show the morphology of the surface layer corresponding to different steps of the construction of the biosensor (Fig. 3). The copolymer deposited on the gold surface (image 3a) shows a compact morphology in agreement of the instantaneous nucleation mechanism observed generally during the formation of polypyrrole by potentiostatic electropolymerization [33]. Covalent grafting of biotin hydrazide (image 3b) leads to a stacked structure with the appearance of globular granules covering the entire surface of the electrode, demonstrating more structuring of the surface after biotin attachment. After complete immobilization of proteins (streptavidin, biotinylated Sc-Fv antibody) the morphology does not change (image 3c) and the same structure is observed with highly dispersed granules. This result demonstrates that the molecular recognition of the streptavidin with biotinylated single-chain antibody was specifically achieved, leading to a good dispersion of the Sc-Fv antibody over the polypyrrole surface.

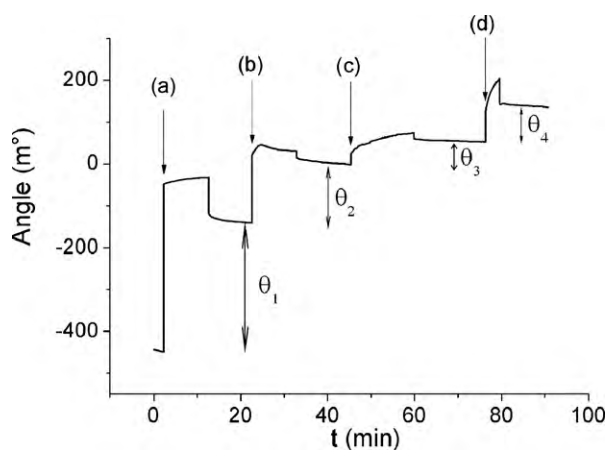


Fig. 3. SEM analysis of copoly(py-pyNHP) film (image a), copoly(py-pyBiotin) film (image b) and biosensor copoly(py-pyBiotin/streptavidin/Sc-Fv Ab) film (image c).

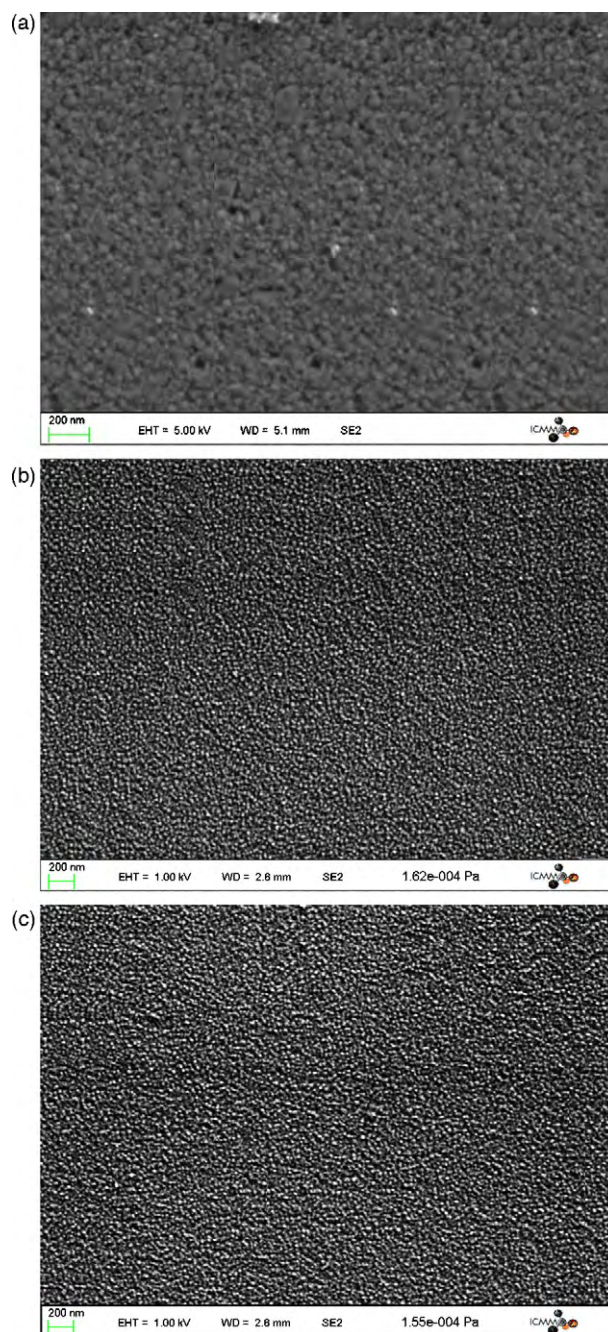


Fig. 4. SPR kinetic curve of different steps of biosensor construction: (a) immobilization of biotin hydrazide 2 mg mL^{-1} in PBS, (b) streptavidin 100 $\mu\text{g mL}^{-1}$ in PBS, (c) biotinylated single-chain antibody 8 $\mu\text{g mL}^{-1}$ in PBS and (d) casein 50 mg mL^{-1} in PBS 10 mM, pH 7.4.

3.2.3. Monitoring by SPR

Any modification of the functionalized film, such as the mass due to the binding of biomolecules, causes a change in the refractive index and leads to change in the resonance angle which can be monitored in real time. The sensorgram (Fig. 4) shows the real time SPR binding curve during the construction of the immunosensor. When biomolecules were injected into the cell, the SPR angle increased rapidly, corresponding both to the association phase and the modification of the refractive index of the solution due to the presence of biomolecules. This step was followed by attachment where the angle varies progressively. For each immobilization step the time of reaction was optimized and the experiment was stopped as soon the saturation was reached. Each immobilization step was

followed by washing the surface with PBS to remove non-attached molecules and a diminution of the SPR angle was observed. Stabilization was achieved before SPR measurement to avoid variation due to interference. Addition of 2 mg mL^{-1} of biotin hydrazide leads to a change in the SPR angle of ca. 306 m° ($\Delta\theta 1$) indicating a good coupling with the pyNHP unit and the formation of a stable layer. When $100 \mu\text{g mL}^{-1}$ of streptavidin is added, the SPR angle increases by 151 m° ($\Delta\theta 2$) indicating the whole adsorption process and the good affinity of biotin for streptavidin. Then the injection of $8 \mu\text{g mL}^{-1}$ biotinylated Sc-Fv antibody increases the SPR angle by 55 m° ($\Delta\theta 3$), confirming the immobilization of the antibody via the biotin–streptavidin interaction. Finally, casein is injected and an angle variation of ca. 189 m° is observed. This protein acts like conventional BSA, by blocking the free binding site of the polypyrrole layer and avoiding the non-specific interactions of the antigen during the subsequent recognition step.

The amount of immobilized biotinylated Sc-Fv antibody can be calculated as ca. 0.47 ng mm^{-2} according to the correlation of the SPR response with the surface protein concentration (120 m° for 1 ng mm^{-2}). Assuming the molecular weights of streptavidin and Sc-Fv Ab are 60 and 20 kDa, respectively, the surface concentration can be calculated to be around 0.021 and $0.023 \text{ pmol mm}^{-2}$, respectively. This result indicates that each Sc-Fv Ab interacts with one streptavidin, leading to a good dispersion on the modified polypyrrole films. It appears also that the immobilization of the Sc-Fv antibody is well controlled by the biotin–streptavidin strategy. The thickness of the immobilized Sc-Fv Ab layer is evaluated at 0.5 nm by fitting the experimental SPR curve (curve not shown) as previously done for the polypyrrole layer.

The strategy using a progressive step-by-step immobilization technique developed for the construction of the immunosensor and the use of the streptavidin–biotin complex improves considerably both the orientation and accessibility of the immobilized antibodies. Furthermore, this method is sufficiently sensitive to detect the streptavidin–biotinylated antibody interaction even with an antibody of low molecular weight (20 kDa).

3.2.4. Monitoring by differential pulse voltammetry (DPV)

The copoly(py–pyNHP) film is electrochemically characterized by DPV in phosphate buffer at pH 7. DPV measurement shows only the faradic current obtained from electron transfer behaviour directly at the electrode surface and not the capacitive current emanating from the diffusion of ions at the electrode/electrolyte interface. DPV has demonstrated advantages of high sensitivity and the lowest detection limit by amplification of the electrochemical signal of polypyrrole.

The voltammogram (Fig. 5a) shows a large oxidation peak at 0.16 V/SCE associated with the oxidation of the polypyrrole backbone. The presence of only one potential peak for the copolymer demonstrates a good distribution of both monomers in the film. In the case of the formation of a block of each monomer two separate electrochemical signals would be expected, as redox waves at 0.45 V/SCE for polypyrrole–NHP [34] and at -0.2 V/SCE for the non-functionalized polypyrrole as expected [35].

The modified electrode is incubated successively with biotin hydrazide, streptavidin, biotinylated Sc-Fv Ab and casein. Incubations lead to a significant modification of the voltammogram (Fig. 5b–e). Indeed it appears that the immobilization of biomolecules on the polypyrrole film induces a decrease in the current density. Similar results were previously observed after the immobilization of DNA in polypyrrole layers [36]. The decrease in current is due to the modification of the surface of the polypyrrole layer by biomolecules blocking charge transport and penetration of counter-ions to assure the doping process. Hence, these phenomena induce a decrease in the electroactivity of the polymer film.

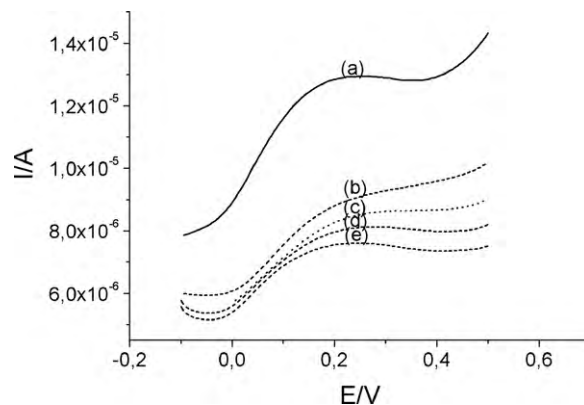


Fig. 5. Differential pulse voltammetry record of: (a) copoly(py–pyNHP) film, (b) copoly(py–pyBiotin) film, (c) copoly(py–pyBiotin/Streptavidin), (d) copoly(py–pyBiotin/Streptavidin/Biotinylated Sc-Fv Ab) and (e) copoly(py–pyBiotin/Streptavidin/Biotinylated Sc-Fv Ab/Casein) film at 0.1 V s^{-1} scan rate in PBS 10 mM, pH 7.4.

3.3. Detection of antigen by electrochemical and SPR methods

Antigen–antibody reactions can be followed directly by electrochemical techniques due to the high intrinsic electrochemical properties of polypyrrole films. SPR data support the electrochemical assays and confirm the strong and specific interaction of the antigen with the reduced form of the antibody [37].

Fig. 6 shows the binding curves of the Sc-Fv Ab immobilized on the polypyrrole film incubated with different concentrations of the specific antigen and the Human IgG in PBS. At low antigen concentration ($0.01 \mu\text{g mL}^{-1}$) the SPR kinetic response is slow (Fig. 6a) and reaches saturation after 8 min. Fast kinetic response is obtained at high antigen concentration ($1 \mu\text{g mL}^{-1}$) (Fig. 6c). A rapid increase in the resonance angle observed initially corresponds to the fast antibody–antigen recognition event, beside the variation of refractive index of the buffered solution due to the presence of a large amount of antigen. This step is followed by a continuous increase until the saturation corresponding to the immobilization of maximum antigen on the biosensor. The SPR response of the specific antigen suggests good accessibility and orientation of the immobilized Sc-Fv Ab. After washing with the regeneration buffer, the SPR signal returns to the original baseline which proves the good stability of the sensor (figure not shown). Reproducible responses are obtained between analyses. This specificity of the antibody–antigen complex is confirmed by the injection of a solution of Human IgG on the modified electrode. A rapid increase in the angle is observed

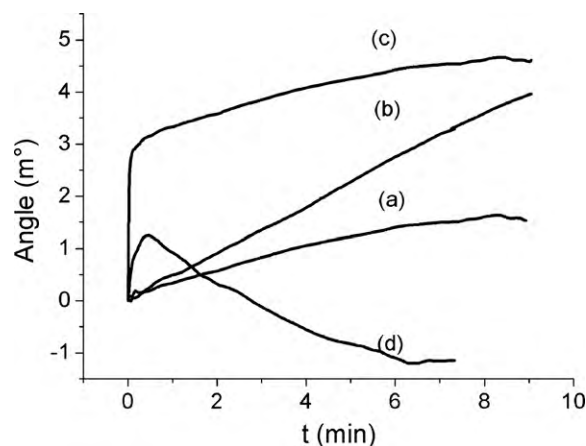


Fig. 6. SPR responses at various concentrations of specific antigen: (a) $0.01 \mu\text{g mL}^{-1}$, (b) $0.1 \mu\text{g mL}^{-1}$, (c) $1 \mu\text{g mL}^{-1}$ and (d) non-specific IgG $1 \mu\text{g mL}^{-1}$.

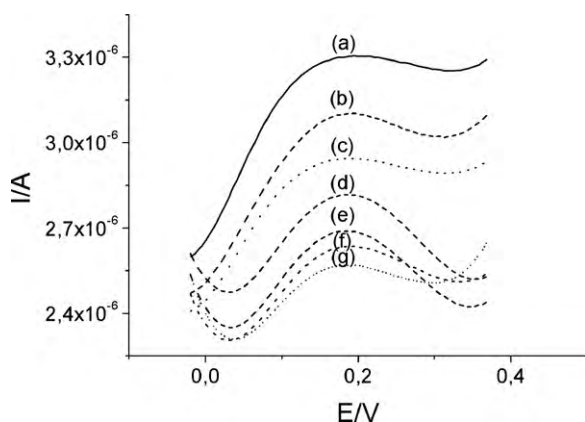


Fig. 7. DPV curves in PBS 10 mM, pH 7.4 of copoly(py-pyBiotin/Streptavidin/Biotinylated Sc-Fv Ab/Casein) film recorded after interaction with different antigen concentrations: (a) 0 pg mL⁻¹, (b) 1 pg mL⁻¹, (c) 10 pg mL⁻¹, (d) 100 pg mL⁻¹, (e) 1 ng mL⁻¹, (f) 10 ng mL⁻¹ and (g) 100 ng mL⁻¹. Scan rate 0.1 V s⁻¹.

after addition of 1 $\mu\text{g mL}^{-1}$ (Fig. 6d), corresponding to the variation of the refractive index of buffer solution, due to the presence of a large amount of the IgG in solution as observed with high concentration of specific antigen (1 $\mu\text{g mL}^{-1}$). However in this case, the angle decreases dramatically during incubation indicating the non-immobilization of the non-specific antigen Human IgG. These SPR experiments demonstrate that the increase in the angle depends directly on the interaction of the antigen to the reduced single-chain antibody immobilized on the polypyrrole layer with high specificity.

Analysis of the detection of the specific antigen was then achieved by electrochemical measurement using the DPV method. For this purpose, the biosensor is incubated with successive addition of various concentrations of antigen from 1 pg mL⁻¹ to 100 ng mL⁻¹ in PBS. These curves show (Fig. 7) a decrease in the oxidative current peak at 0.16 V with increasing antigen concentration, which is directly proportional to the antigen–antibody interaction. This diminution in current intensity is explained by the formation of the antibody–antigen complex, which decreases the penetration of dopant ions and then avoids the electron transfer from electrode to polypyrrole layer. The same behaviour was observed by electrochemical impedance spectroscopy after the formation of immuno-complex, based on bovine leukemia gp51 proteins immobilized on a polypyrrole and anti-gp51 antibodies, reducing the mobility of ions [38].

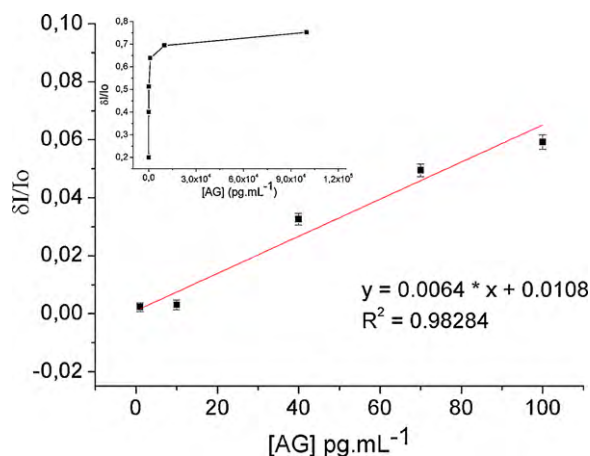


Fig. 8. Calibration curve of antigen recognition between 1 and 100 pg mL⁻¹. Inset: calibration curve between 1 and 100 $\times 10^3$ pg mL⁻¹.

In order to study the immunosensor response, a calibration curve corresponding to the variation of the current response at 0.16 V versus specific antigen concentrations, is presented in Fig. 8. From the experimental data, the immunosensor calibration curve exhibits a linear relation between current response and antigen concentration from 1 pg mL⁻¹ to 100 ng mL⁻¹ with a sensitivity of 17.6 nA (ng mL)⁻¹. Such a measurement is highly reproducible: 5% relative standard deviation for 3 measurements. These results demonstrate the high potentialities of this immunosensor configuration combining electrochemical transduction of the conducting polypyrrole signal and affinity immobilization with the streptavidin/biotin strategy.

4. Conclusion

In this paper, we describe for the first time the immobilization of a biotinylated single-chain antibody (Sc-Fv Ab) using a step-by-step construction on a copolypyrrole film consisting of pyrrole functionalized with *N*-hydroxyphthalimide acting as linker agent and pyrrole as spacer to prevent steric hindrance of the biomolecules. Sc-Fv Ab was immobilized on the surface using the biotin/streptavidin system to control the orientation and accessibility of the single-chain antibody. Firstly the composition of the copolypyrrole film was studied by varying the ratio of the two monomers in solution, py and pyNHP, during electropolymerization and then its effect on the immunosensor sensitivity was investigated by SPR. Results demonstrate that the immobilization of the antibody was influenced by the proportion of pyNHP in the film and, for effective immunodetection, at least 10% of pyNHP as linker was necessary in the preparation of the polypyrrole film. We demonstrated by SPR and SEM that an optimal amount of Sc-Fv Ab antibody is immobilized on copolymer film with good dispersion and organization on the surface layer. The electrochemical signals of the oxidation and doping processes of the polypyrrole were measured by the DPV method where only the faradic current was measured. The well defined oxidation peak allowed the monitoring of the immobilization of Sc-Fv Ab on the polypyrrole as well as the detection of antigen. Copolypyrrole film shows an oxidation peak at 0.16 V/SCE with a continuous decrease in the current density after biomolecule immobilization and recognition due to a lower electron transfer process. We demonstrated that the affinity interaction of the Sc-Fv Ab with the antigen could be measured with an antigen concentration as low as 1 pg mL⁻¹ by measuring the faradic current of polypyrrole oxidation. The non-specific interaction was tested with Human IgG antigen. The immunosensor described in this work by using an optimized conducting polypyrrole transducer and DPV as amplification method could be applied to any antibody and presents a versatile system for measuring antibody–antigen interaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2010.06.008.

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