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Comparison between the performances of amperometric immunosensors for cholera antitoxin based on three enzyme markers☆

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Abstract

We developed a novel copolymer modified amperometric immunosensor for the detection of cholera antitoxin (anti-CT), by the electropolymerization of pyrrole–biotin and pyrrole–lactitobionamide monomers on platinum or glassy carbon electrodes. In the detection of cholera antitoxin we have used three enzymatic marker detection systems based on HRP-labeled rabbit IgG antibodies, biotinylated polyphenol-oxidase (PPO-B) and biotinylated glucose-oxidase (GOX-B). The comparison of the electro-enzymatic performances of these three configurations with different substrates, clearly shows that the more sensitive amperometric immunosensor was based on HRP with a lower limit of detection of 50 ng/ml anti-CT using hydroquinone/H_2O_2 system. The response time for this substrate was in range of 5–30 s. The HRP-amperometric immunosensor has thus proven to be a very sensitive tool to monitor nanomolar concentrations of anti-CT.

Keywords: Amperometric immunosensors; Poly(pyrrole–biotin; pyrrole–lactitobionamide); Anti-cholera toxin antibody; Permeability

1. Introduction

Vibrio cholerae microorganisms cause enteropathogenic cholera, a severe diarrhoeal disease. Cholera has afflicted humankind for centuries, occurring most frequently in epidemic form. When untreated, cholera becomes a disease of rapid onset and potentially high lethality. The enterotoxin, cholera toxin is very immunogenic and an ideal model biotoxin. It is monitored in epidemiological studies that require immunoassays for the evaluation of seroconversion of a vaccinated population, mainly the conventional enzyme-linked immunosorbent assay (ELISA) [1,2]. Besides this conventional approach, there has been, for three decades, a growing interest in the design of biosensors aimed at the detection, diagnosis and determination particularly in the fields of clinical and environmental analyses [3,4]. Owing to their adaptability, portability and potential for miniaturization, biosensors based on an electrochemical transduction constitute the main category. In particular, amperometric biosensors which combine the inherent specificity of enzymatic reactions with the sensitivity of amperometric detection, continue to be the subject of considerable research interest. In the same vein, amperometric immunosensors based on the determination of the antibody-antigen interaction via the use of an enzyme as marker, have received considerable attention [5–11]. These enzymatic markers, peroxidase, alkaline phosphatase, laccase or glucose oxidase were commonly attached to a secondary antibody. After their immobilization, the enzymes

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catalysed the formation of electroactive species which were amperometrically detected at the electrode surface allowing thus the quantification of the immunoreaction. The sensitivity of such an approach obviously depended on the specific activity of the enzyme as well as on the electrochemical properties of the products of the enzyme reaction. In addition, the immunosensor performances are mainly function of the accessibility to the electrode surface for the generated electroactive species. The latter point is controlled by the steric constraints due to the immobilization procedure of the immunoreagent used as probe and hence by the bulkiness of the diffusing species.

In that context, the concept of amperometric immunosensors for the detection of anti-cholera toxin was developed via the immobilization of immunogenic material (cholera toxin B subunit-biotin labeled) on a biotinylated electrode surface by affinity interactions. The analyte (anti-cholera toxin) antibodies, was detected through three alternative enzymatic markers: peroxidase-labeled rabbit IgG antibody (HRP) and biotinylated glucose oxidase (GOX-B) or biotinylated polyphenol oxidase (PPO-B) conjugated to avidin-linked rabbit IgG antibodies which was used, for the first time, to transduce an immunoreaction. Owing to the key role of the accessibility to the electrode surface and the required quality of the probe immobilization, the latter was achieved by the formation of avidin–biotin bridges with an electrogenerated biotinylated copolymer composed of pyrrole–biotin (for affinity interactions) and pyrrole–lactobionamide (for permeability improvement). The electropolymerization procedure ensures the reproducible and accurate formation of a fully active film of biotin as well as the absence of manufacturing defects, chemical stability and storage stability [12–16]. In addition, the high accessibility of the resulting immobilized antigen preserves the efficiency of the subsequent immunoreaction [15].

The aim of the present studies is the development then evaluation of the analytical performances of the three enzymatic amperometric immunosensors configurations based on copolymer modified electrodes for the detection of the model cholera antitoxin analyte.

2. Experimental

2.1. Materials

Cholera toxin B subunit-biotin labeled (lyophilized powder, biotin content 0.9 mol/mol protein), peroxidase-labeled IgG anti-rabbit antibody (from goat, protein content 0.8 mg/ml, affinity isolated antibody), anti-cholera toxin (from rabbit, protein content 48 mg/ml, purified toxin from *Vibrio cholerae*), biotin monoclonal anti-rabbit IgG -γ-chain specific (from mouse, protein content 4.2 mg/ml), glucose oxidase-biotinamidocaproyl labeled (GOX-B, from *Aspergillus niger*, lyophilized powder containing 40–70% protein, 137 U mg^{-1}), polyoxyethylene-sorbitan monolaurate (Tween® 20), bovine serum albumin (fraction V) and avidin (from egg white) were purchased from Sigma. Biotin-labeled polyphenol oxidase (PPO-B, from mushroom, 4800 U mg^{-1}) was prepared following the procedure described previously [14]. Catechol and hydroquinone (1,4-dihydroxy-benzene) were purchased from Fluka. Acetonitrile (Rathburn, HPLC grade) and lithium perchlorate (GSF Smith) were used as received. All chemicals were obtained commercially and were of the highest analytical purity available. Stock solutions of glucose were allowed to mutarotate at room temperature for 24 h before use, and were kept refrigerated.

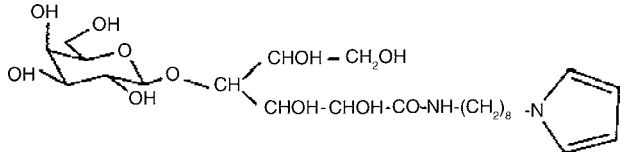

Fig. 1. Structure of pyrrole–lactobionamide.

Pyrrole–lactobionamide was prepared as described previously [17] (Fig. 1). The synthesis of pyrrole–biotin was adapted from the reported procedure [13]. Biotin (338 mg), 11-(1-pyrrolyl)dodecanol (327 mg), 1,3-dicyclohexylcarbodiimide (326 mg) and 4-(dimethylamino)pyridine (20 mg) were dissolved in dry CH_2Cl_2. The reaction mixture was stirred at room temperature for 5 days without argon atmosphere contrary to the previously described procedure [13].

After filtration and evaporation steps, a white precipitate (pyrrole–biotin) was obtained and purified by chromatography. ^{1}H NMR (200 MHz, DMSO) data: δ(ppm) 1.24–1.68 (m, 24H), 2.30 (t, 2H), 2.73 (m, 1H), 2.89 (m, 1H), 3.11 (m, 1H), 3.83 (t, 2H), 4.02 (t, 2H), 4.28 (m, 1H), 4.47 (m, 1H), 5.50 (s, 1H), 5.93(s, 1H), 6.10 (m, 2H), 6.62 (m, 2H). Until use pyrrole–biotin (250 mg) was kept under argon.

2.2. Apparatus

Electropolymerization and cyclic voltammetric experiments were performed with an EG&G PARC, Model 173 potentiostat equipped with a Model 175 universal programmer and a Model 179 digital coulometer in conjunction with a Kipp and Zonen BD 91 XY/t recorder. All experiments were carried out using a conventional three-electrode cell. The amperometric measurements were performed with a Tacussel PRG-DL potentiostat and an electrochemical cell thermostated at 23 °C under stirred conditions in 0.1 M phosphate buffer (pH 7). The working electrodes were glassy carbon or platinum discs (5 mm diameter) polished with 1 μm diamond paste (MECAPREX Press PM). An aqueous saturated calomel electrode (SCE) was used as reference in water media while a 10 mM Ag^+/Ag reference electrode was used in acetonitrile electrolyte.

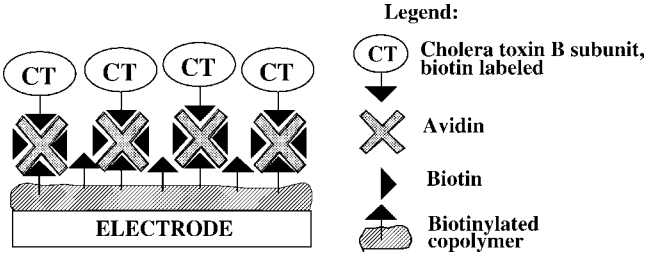

Fig. 2. Schematic representation of biotinylated cholera toxin B subunit-coated electrodes.

2.3. Preparation of amperometric immunosensors

The electropolymerization of the biotinylated copolymer was carried out by controlled potential electrolysis of a mixture of pyrrole–lactobioamide monomer (4 mM) and pyrrole–biotin monomer (4 mM) at 0.8 V in acetonitrile electrolyte. Taking into account the nature of the electroenzymatic reaction used for detecting cholera anti-toxin, platinum electrodes were used for GOX-B while glassy carbon electrodes were employed for HRP and PPO-B.

The modification of the working electrodes by the biotinylated copolymer was followed by a blocking treatment for 1 h at room temperature performed with fresh solution (20 μl) of 5% (w/v) bovine serum albumin (BSA) in PBST (0.1 M phosphate buffer saline containing 0.5% (w/v) Tween® 20, pH 7) to prevent non-specific binding of avidin onto the polypyrrole coating. Then, the deposition of avidin (20 μl, 1 mg/ml) dissolved in 1% (w/v) BSA/PBST for 20 min led to the formation of an avidin layer. The resulting electrodes were then incubated with biotinylated cholera toxin B subunit (15 μl, 0.3 mg/ml) dissolved in 1% (w/v) BSA/PBST for 20 min (Fig. 2). After each step the electrodes were rinsed several times with 0.1 M phosphate buffer saline pH 7 (PBS).

2.4. Amperometric transduction of the immunoreaction

The immunosensors were incubated for 20 min with anti-cholera toxin (anti-CT) antibody (20 μl) developed in a rabbit at concentrations ranging from 0.05 to 500 μg/ml. Different electroenzymatic transductions were then investigated.

For the HRP-immunosensor design, a secondary antibody, horseradish peroxidase-labeled goat anti-rabbit IgG immunoglobulin (20 μl) at a concentration of 0.5 mg/ml dissolved in antibody diluent was deposited onto the sample exposed electrode for 20 min. The electrodes were then rinsed extensively with PBS. Three different redox mediators (2 mM), hydroquinone, ferrocene di-carboxylic acid, and ferrocyanide were tested in the presence of H_2O_2 (2 μM) (Fig. 3). The HRP-immunosensors were potentiostated at −0.1, −0.01, and −0.1 V versus SCE to detect one of three amperometrically enzymatic products: ferricyanide, ferrocenium and quinone, respectively.

For GOX-B and PPO-B-immunosensor designs, biotin (30 μl, 10 mM) dissolved in 0.1 M phosphate buffer pH 7 was deposited onto electrodes and incubated for 20 min. This saturation of the binding sites of immobilized avidin was carried out before the immunoreaction in order to eliminate any possible immobilization of biotinylated enzymes on the first avidin layer. As previously described, anti -CT was deposited onto the electrodes and left to react for 20 min. Then, monoclonal anti-rabbit IgG biotin labeled 0.5 mg/ml suspended in buffer (1%, w/v BSA/PBST) was incubated for another 20 min. The resulting electrodes were then incubated with avidin (20 μl, 1 mg/ml) dissolved in 1% (w/v) BSA/PBST for 20 min. Finally, the avidin modified electrodes were incubated for 20 min with GOX-B or PPO-B (20 μl, 0.5 mg/ml) (Fig. 3). Between each step, the electrodes were rinsed with PBS. The GOX-B immunosensors with varying concentrations in anti-CT were potentiostated at +0.6 V versus SCE and the steady-current responses to 0.1 M glucose injections in 7 ml stirred phosphate buffer (0.1 M, pH 7) were recorded. For PPO-B immunosensors, the amperometric response was recorded at a reduction potential of −0.2 V versus SCE in the presence of catechol (10 mM) in 11 ml stirred 0.1 M phosphate buffer (pH 7).

3. Results and discussions

The detection of rabbit anti-cholera toxin antibody (anti-CT) was achieved using a sandwich immunoassay based on the immobilization of Cholera toxin B subunit protein (CTB), as the molecular bio-recognition layer (Fig. 2). Different immunosensor configurations based on three enzymatic markers, namely HRP-labeled rabbit IgG antibodies (HRP), biotinylated polyphenol-oxidase (PPO-B) and biotinylated glucose-oxidase (GOX-B) were developed and tested for the amperometric detection of anti CT (Fig. 3). In order to immobilize CT on the electrode surfaces by affinity interactions via the formation of avidin–biotin bridges, a novel biotinylated copolymer (poly(pyrrole–biotin), poly(pyrrole lactobionamide)) was used as the initial anchoring layer. This new polymerized film was designed to improve the transduction sensitivity of the enzymatic reactions based on the amperometric detection of the enzyme products at the underlying electrode surface. Catechol was used as an electrochemical probe to illustrate the difference of permeability between the biotinylated copolymer and the previously used poly(pyrrole–biotin). Fig. 4 shows a series of cyclic voltammograms collected at 100 mV s^{-1} for a bare electrode, a poly(pyrrole–biotin) and a copolymer modified electrodes.

For the poly(pyrrole–biotin) electrode, the electrochemical activity of the redox probe was markedly blocked by the presence of the polymer film. As expected, the oxidation peak decreased drastically while the peak separation increased indicating the low permeability of poly(pyrrole–biotin) film. In contrast, the copolymer appeared much more permeable since the catechol electroactivity remained almost identical.

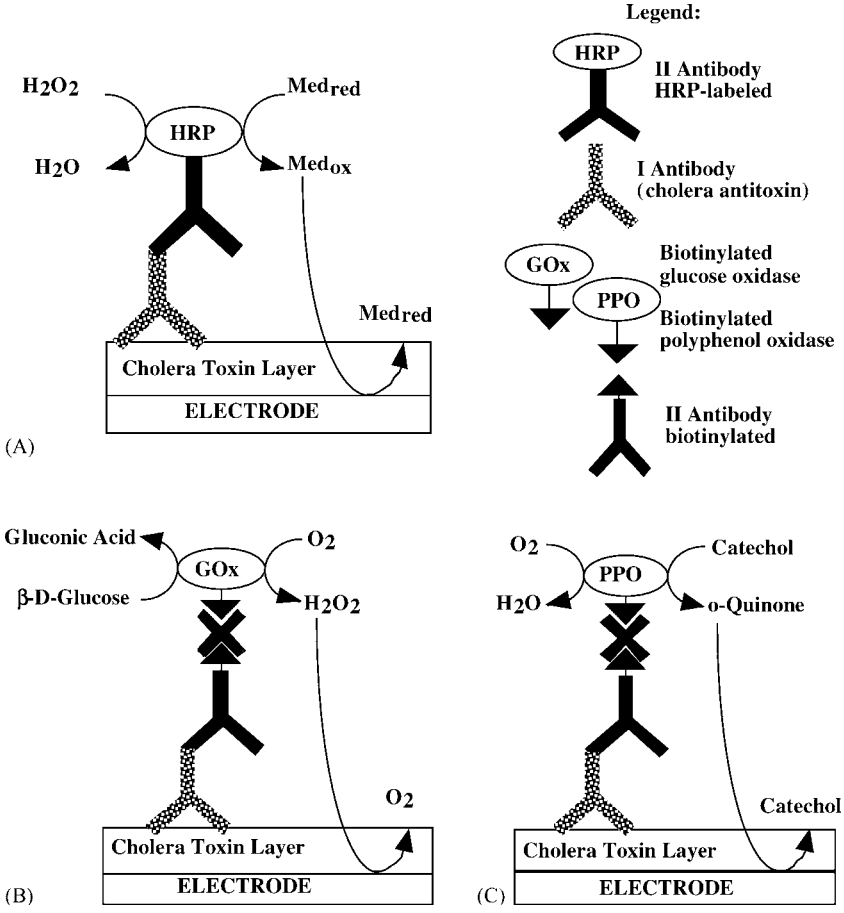

Fig. 3. Amperometric immunosensors set-up using a biotinylated copolymer (poly(pyrrole–biotin, pyrrole–lactitobionamide)) coated platinum or glassy carbon electrodes and three enzymatic markers (GOX-B, PPO-B, HRP) for the detection of cholera antitoxin. (A) HRP-immunosensor, (B) GOX-B-immunosensor, (C) PPO-B-immunosensor, M_{red}/M_{ox} = ferrocyanide/ferrycianide; hydroquinone/quinone; ferrocene/ferrocenium, GOx = biotinylated glucose oxidase; PPO = biotinylated polyphenol oxidase; HRP = peroxidase-labeled IgG anti-rabbit antibody.

The elaboration of the immunosensor was therefore performed with the biotinylated copolymer. The first step consisted then in the conjugation of avidin followed by the subsequent binding of biotinylated CTB via avidin–biotin bridges. The analyte, anti-CT, thereafter bound the corresponding immobilized CT subunit epitopes.

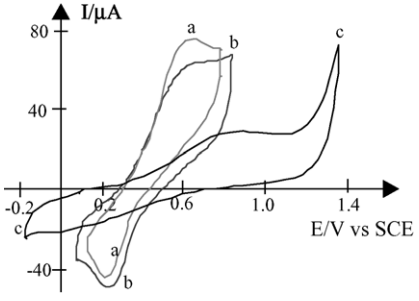

Fig. 4. Cyclic voltammograms of platinum electrodes (Ø = 5 mm): (a) bare, (b) modified with copolymer poly(pyrrole–biotin, pyrrrole-lactitobionamide), (c) modified with biotinylated polypyrrole in the presence of 1 mM catechol, 0.1 M $LiClO_4$ in water (pH 7). Polymer films were electrodeposited by controlled potential electrolysis at 0.8 V (charge: 1 mC). Scan rate at 100 mV s^{-1}.

3.1. Amperometric current response to anti-CT using an HRP-immunosensor

The simplest enzymatic configuration consists in the use of a secondary antibody conjugated with an enzyme, here HRP. Thanks to the wide range of enzyme substrates, three electroactive systems (ferrocyanide/H_2O_2, ferrocene di-carboxilic acid/H_2O_2 and hydroquinone/H_2O_2) were tested. By addition of hydrogen peroxide, HRP catalyzed the oxidaton of these redox probes. In order to detect anti-CT concentrations ranging from 0.05 to 500 μg/ml, HRP-immunosensors were potentiostated at −0.1, −0.01 and −0.1 V versus SCE to monitor the reduction of enzymatically generated ferricyanide, ferrocenium and quinone species respectively. The response time of all HRP-immunosensors was remarkably fast, ranging from 5 to 30 s illustrating the good permeability of the polymeric coating. Fig. 5 presents the resulting calibration curves of HRP-immunosensors for the amperometric detection of anti-CT via the three HRP substrates. An immunosensor sensitivity was determined as the slope of the initial linear part of the calibration curve. Table 1 summarizes the sensitivity and detection limit values, as well as, the

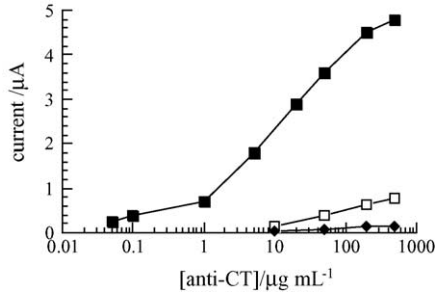

Fig. 5. Calibration curves for anti-CT at HRP-immunosensors using different redox mediator/H_2O_2 systems;(■) hydroquinone, (□) ferrocyanide, (♦) ferrocene.

values of maximum current (I_{max}) obtained at saturating anti-CT conditions. It clearly appears that the hydroquinone/H_2O_2 system led to the best analytical performances, namely the lowest detection limit (50 ng/ml recorded for a signal to noise ratio of 3) and the highest sensitivity (1.5 μA μg^{-1} ml cm^{-2}). In addition, the comparison of the I_{max} values recorded for the theoretical formation of an anti-CT monolayer and hence for a HRP monolayer unambiguously corroborates the preceding results. The better performances obtained with hydroquinone may be ascribed to the lower bulkiness of this redox probe compared to ferrocyanide and ferrocene, leading thus to a better permeation through the immunosensor coating.

The detection values obtained here (50 ng/ml) are similar to those obtained with optical fiber immunosensors, based on HRP-labelled secondary antibodies (160 ng/ml) [18–21].

3.2. Amperometric current response to anti-CT using GOX-B and PPO-B as enzyme markers of the immunosensors

With the aim to improve the detection limit, the HRP-labeled secondary antibody was replaced by a biotinylated antibody. The enzyme markers, GOX-B and PPO-B, were then attached to the secondary antibody by an avidin bridge. Although this procedure required two additional incubation steps, an amplification of the detection limit is expected. At very low anti-CT concentrations, only few immunoreactions occured at the electrode surface. As a consequence, the absence of steric hindrances between the scattered anti-CT over the electrode surface possibly lead, for each anti-CT molecule

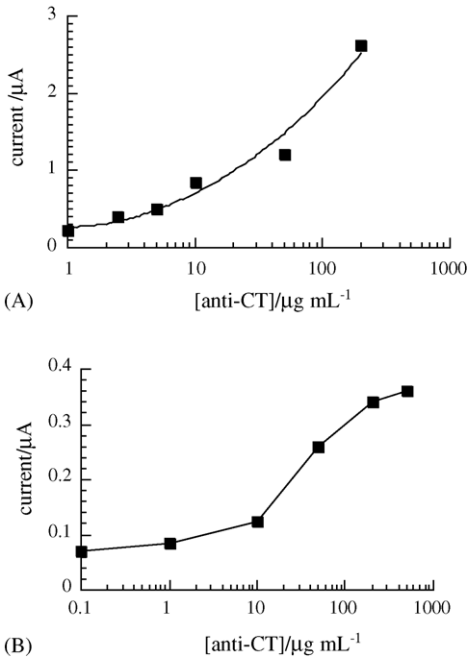

Fig. 6. Calibration curve for anti-CT at (A) GOX-B immunosensors in the presence of glucose; $E_{applied}$ = 0.6 V and at (B) PPO-B-immunosensors in the presence of catechol; $E_{applied}$ = −0.2 V.

to the subsequent anchoring of several enzyme molecules (up to 3) instead of one with HRP.

Since GOX-B catalyzes the aerobic oxidation of glucose in presence of oxygen with the concomitant production of H_2O_2, the amperometric detection of anti-CT was assayed by potentiostating the GOX-B-immunosensors at +0.6 V versus SCE. Very fast response times of the glucose substrate (3–6 s) were observed highlighting the good diffusion of H_2O_2 to the underlying platinum surface. Fig. 6A shows the calibration curve for GOX-B-immunosensors as a function of anti-CT concentrations. The comparison of the I_{max} values 24 and 13.1 μA cm^{-2} for HRP and GOX respectively seems to indicate a more efficient transduction by HRP. However, it should be noted that the immobilized amount of GOX-B is 3 times lower than that of HRP. Indeed, assuming that at saturating anti-CT conditions (500 μg/ml), the successive recognition by a biotinylated secondary antibody, an avidin and then GOX-B should provide a GOX-B monolayer, the maximum coverage of glucose oxidase for a compact monolayer corresponds to 2.96×10^{-12} mol cm^{-2} whereas a HRP monolayer was estimated to be 9×10^{-12} mol cm^{-2}

Table 1
Electroenzymatic performances of HRP, GOX-B and PPO-B-immunosensors towards the amperometric detection of anti-CT

Enzyme marker	Substrate	Detectionlimit (μg/ml)	I_{max} (μA cm^{-2})	Linear range (μg/ml)	R^2	Sensitivity (nA μg^{-1} ml cm^{-2})
HRP	Hydroquinone	0.05	24	0.05–5	0.990	1500
HRP	Ferrocene	10	0.8	10–50	0.995	6
HRP	Ferrocyanide	10	3.9	10–200	0.900	12
GOX-B	Glucose	1	13.1	1–10	0.981	344
PPO-B	Catechol	0.1	1.8	0.1–50	0.992	18.5

[22]. The sensitivity (determined from the linear part of the calibration curve) and the detection limit for anti-CT, respectively 344 nA μg^{-1} ml cm^{-2} and 1 μg/ml, were unfortunately less attractive than those previously observed with HRP. The bulkiness of the GOX molecule (MW 189000) must prevent the anchoring of several markers on one avidin molecule cancelling thus the expected transduction improvement. Concerning PPO-B, for the first time, a biotinylated polyphenol oxidase was used as an enzymatic marker for an immunosensor. The latter catalyzes in the presence of oxygen the oxidation of phenol and o-diphenols into o-quinone. As a consequence, the amperometric detection of anti-CT was carried out in the presence of catechol (10 mM), the main substrate of PPO, by potentiostating the PPO-B-immunosensors at −0.2 V versus SCE in order to reduce the generated *o*-quinone. Fig. 6B shows the calibration curve for PPO-B-immunosensors as a function of anti-CT concentrations.

The sensitivity and I_{max} values, 18.5 nA μg^{-1} ml cm^{-2} and 1.8 μA cm^{-2} respectively, remained markedly lower than those recorded with GOX-B. This may be attributed to the bulkiness of catechol compared to H_2O_2. Surprisingly the PPO/catechol system led to a more sensitive detection limit (0.1 μg/ml) than the preceding GOX/ glucose system. This effect may be ascribed to a recycling phenomenon providing an amplification of the amperometric immunosensor response [23,24]. In fact, the immobilized PPO-B catalyzes the oxidation of catechol to o-quinone while the amperometric detection regenerates by the reduction of catechol. The latter can undergo successive cycles of enzymatic oxidation–electrochemical reduction inducing an amplification of the immunosensor response.

4. Conclusions

We have demonstrated, herein, the possibility of developing immunosensors via the gentle and easy immobilization of the immunological material onto an electrode previously modified by a new electropolymerized copolymer film bearing biotin groups.

Thanks to the improved permeability of this initial anchoring layer, three electroenzymatic transducing systems were efficiently applied to the determination of Cholera toxin antibody. It is expected that these simple tranducing strategies of immunoreaction will be useful for the development of amperometric immunosensors.

Acknowledgements

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