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An electrochemical immunosensor based on covalent immobilization of okadaic acid onto screen printed carbon electrode via diazotization-coupling reaction

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ABSTRACT

In this work, an electrochemical method based on the diazonium-coupling reaction mechanism for the immobilization of okadaic acid (OA) on screen printed carbon electrode was developed. At first, 4-carboxyphenyl film was grafted by electrochemical reduction of 4-carboxyphenyl diazonium salt, followed by terminal carboxylic group activation by *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyle)-*N*′-ethyle-carbodiimide hydrochloride (EDC). Hexamethyldiamine was then covalently bound by one of its terminal amine group to the activated carboxylic group. The carboxyl group of okadaic acid was activated by EDC/NHS and then conjugated to the second terminal amine group on other side of the hexamethyldiamine through amide bond formation. After immobilization of OA, an indirect competitive immunoassay format was employed to detect OA. The immunosensor obtained using this novel approach allowed detection limit of 1.44 ng/L of OA, and was also validated with certified reference mussel samples.

1. Introduction

Recently, biosensors have gained growing attention as an attractive alternative to the conventional analytical approaches due to their low cost, small size and sharp response time. Biosensors are classified into enzymatic biosensors, cell biosensors, immunosensors and aptasensors depending on the bio-receptor [1–3]. In the past few years, much research work has been devoted to immunosensors, likely due to the high affinity interactions between antigens and antibodies, often involving higher sensitivity and lower limits of detection. Several immunoassay strategies, such as sandwich, direct, indirect and displacement assays, and detection methods have been used to develop improved devices. The electrochemical methods have attracted substantial attention because of their high sensitivity, their fast response time and simple equipment [4–6]. For the design of an electrochemical immunosensor, the key points are the choice of the basis electrode and the immobilization of the antibody or the analyte (analyte derivate) onto the electrode surface [7]. A wide variety of electrodes have been used as support to fabricate immunosensor devices, including carbon paste [8], glassy carbon [6], gold [9] and screen-printed electrodes [5,10]. The screen-printing microfabrication technology is nowadays well established for the production of thick-film electrochemical transducers. This technology allows the mass production of inexpensive and robust strip solid electrodes [11,12].

The immobilization strategies are identified either as chemical or physical depending on whether covalent bond is established or not. Among the physical methods, the immobilization by non-specific adsorption is a simple and an economic technique, which does not damage the activity of the biological material [13]. However, this method has low sensitivity and poor reproducibility due to elution of adsorbed component. The immobilization by entrapment has been preferentially used. In this technique, the biomolecules are entrapped into a three-dimensional network of the gel that could be natural or synthetic, such as sol–gel, and hydrogels [14–16]. It should be noted that small size molecules are difficult to immobilize by mean of entrapment due to leaching from the matrix.

Covalent coupling of the biomolecules to chemical activated surface has been shown an alternative to physical methods [17]. Various strategies involving covalent interaction such as thiols to gold [18,19], arylamides to silanized surface [20] and amines to aldehyde-treated surface [21,22] have been reported. Diazonium salts, firstly introduced in the early 1990s, have received substantial importance due to their simple preparation and flexibility of surface modification [23]. This approach has been demonstrated for a wide range of conducting materials such as carbon [24,25], carbon nanotubes [26], silicon [27], metals [28] and diamonds [29]. The basic principal of this technique is the electrochemical reduction of a diazonium salt leading to the formation of an aryl centered radical. The formed aryl diazonium forms a covalent binding to

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the surface by the spontaneous release of nitrogen. The diazonium salt molecules have been modified with various functional groups for immobilization of proteins, antibodies and other molecules. The electrodeposition of diazonium aryl salt for immobilization of biomolecules on carbon and gold surfaces has been demonstrated by numerous studies [30,31].

The direct immobilization of biomolecules via covalent coupling on transducer surface has been emerged as a powerful tool to improve the sensitivity of the electrochemical immunosensors. Direct assays involving antibody immobilization are used for the detection of high-molecular weight compounds, while indirect strategies via antigen immobilization are compatible and highly promising for detection of low-molecular weight analytes. Although, small size molecules are difficult to immobilize, but they have very little affect on electron transfer. In addition, an analyte immobilization format has the advantage of its stability during surface regeneration compared to an antibody immobilization. Moreover, immobilization of analyte can avoid functional loss of antibody that could happen due to the direct immobilization of antibody. Covalent immobilization of small molecules for the development of indirect immunosensors has been exploited by numerous studies [32–35].

Based on the above observations, we have investigated diazonium salt chemistry to immobilize okadaic acid (OA), a small toxin (MW 805) on the screen printed carbon electrode (SPCE) surface, in order to develop a highly sensitive electrochemical immunosensor. OA is a lipophilic marine biotoxin produced by *Dinophysis* and *Prorocentrum* dinoflagellates, mainly accumulated in the hepatopancreas of bivalves [36]. OA intoxication is considered as the most of concern diarrheic shellfish poisoning (DSP) for human health worldwide [37]. The European commission (EC) on the basis of toxicity to humans, has implemented regulation on the concentration of OA, and specified the maximum permitted level as 160 μg/kg of mussels (EC No.853/2004 15). Mouse bioassay has been used as the reference method for OA analysis until the 1st January 2011 [38]. This method is under consideration due to its poor selectivity and accuracy, in addition to ethical problems, and has been replaced with other techniques, such as liquid chromatography coupled to fluorescence (LC-FLD) or liquid chromatography combined with mass spectrometry (LC–MS) (EC No. 2074/2005 17) [39,40]. The chromatographic equipment, however, is very expensive, time consuming and also needs skilled persons to operate. Alternative to conventional methods, several electrochemical immunosensors have been reported for the detection of okadaic acid. Various linkers such as bovine serum albumin (BSA), ovalbumin (OVA), and magnetic beads have been used to immobilize OA on electrode surface, followed by indirect competitive assay. Similarly, electrochemical immunosensor based on immobilization of antibody or OA on CM5 sensor chips have also been reported [41–44]. To our knowledge, direct immobilization of OA via diazonium-coupling reaction on screen printed carbon electrode to develop competitive immunosensor has not been reported.

In the present work, a novel method of OA immobilization via diazonium salt was investigated. Taking account of the advantage of their low-cost and the convenience in manipulation, SPCEs were used as transducer surface. In the developed method, Differential Pulse Voltammetry (DPV) was employed to investigate the response of electrochemical sensor. Cyclic voltammetry and impedimetric studies were done to characterize electrode surface modification. After immobilization of OA, indirect competitive immunoassay format was used to detect the toxin and the method was validated with certified reference samples. The purposed method allowed very low limit of detection as compared to previously described methods for OA detection. Furthermore, immobilization technique could be extended for other small size biomolecules.

2. Experimental

2.1. Chemicals and materials

OA potassium salt, purchased from Sigma, was firstly dissolved in ethanol (0.1 g/L) and then diluted in phosphate buffer saline (PBS 1×). Buffer components, Tween 20, bovine serum albumin (BSA), diethanolamine (DEA), 1-naphthyl phosphate (1-NP), alkaline phosphatase (ALP)-labeled goat anti-mouse IgG antibody, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyle)-*N′*-ethyle-carbodiimide hydrochloride (EDC), potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$), 4-amino benzoic acid, hexamethyldiamine, sodium nitrate, ethanolamine were purchased from Sigma (France). Monoclonal antibody (anti-OA-MAb, developed in mouse) against OA was obtained from Novus Biologicals (UK).

2.2. Instrumentation

The electrochemical measurements were performed with an AUTOLAB PGSTAT100 potentiostat/galvanostat equipped with a frequency response analyzer system (Eco Chimie, Netherlands) controlled by two Autolab softwares; Frequency Response analyzer (4.9) for impedance and General purpose Electrochemical system (4.9) for voltammetry. SPCE systems, with graphite as working and counter electrode and Ag/AgCl as a reference electrode, were fabricated using a DEK 248 screen-printing system as reported for 2-electrode systems [45].

2.3. Activation of the carboxylic group of OA

To activate the carboxylic group of OA, 15 μL of a 26 mM solution of NHS and EDC were added to 25 μL of OA at 0.5 g/L in 0.1 M MES buffer, pH 5.5, in a test tube capped with a septum. The mixture was stirred for 1 h at room temperature. 100 μL of PBS (1×) was added to the reaction mixture. The resulting activated OA was stored at 4 °C.

2.4. Covalent immobilization of OA on the electrode surface

Prior to modification, SPCE was subjected to electrochemical pretreatment by 5 cyclic potential scans between 1.0 and −1.5 V in 0.5 M H_2SO_4. The electrode surface modification was carried out in the diazotation mixture by slightly modifying the method described by Baranton and Belanger [46]. In brief, the diazonium cation was synthesized by *in situ* reaction of 1 μL of 1 M $NaNO_2$ (final concentration, 10 mM) and 99 μL of 10 mM 4-aminobezoic acid prepared in 0.5 M HCl. The mixture was left to react for 5 min at room temperature. The electrochemical modification of SPCE with *in situ* generated 4-carboxyphenyl diazonium salt was performed by linear sweep voltammetry from 0.6 to −0.8 V. After modification, the electrode was rinsed with distilled water. The carboxylic group on the electrode surface was conjugated to the amine group of hexamethyldiamine by adding 100 μL of 10 mM hexamethyldiamine containing 25 mM NHS and 100 mM EDC for 1 h. After washing the electrode with water, 30 μL of activated OA at dilution of 1/10 were added onto the modified surface having amine group. After overnight incubation, the electrode was rinsed with water to remove the unbound OA and 100 μL of 1 M ethanolamine solution was dropped onto the electrode for 45 min to deactivate the remaining succinimide group and block unreacted sites. The modified electrodes can be used directly for immunosensor, or stored dry at 4 °C for several days without any decrease in the sensitivity.

2.5. Immunoassay protocol

Firstly, the blocking step was performed by adding 50 μL of PBS containing 1% BSA onto electrode surface. The competition step was performed using either 10 μL of OA standard solutions at different concentrations or 10 μL of spiked samples or 10 μL of certified reference samples, and 40 μL of anti-OA-MAb solution (dilution of 1/5000 in PBS 1×) for 1 h. Afterwards, 50 μL of ALP labeled goat anti-mouse IgG secondary antibody solution was added at dilution of 1/2500 in PBS (1×) for 1 h. 90 μL of 10% DEA buffer (pH 9.5) and 10 μL of 5 mg/mL 1-NP were added, with incubation period of 2 min at room temperature. Washing steps were performed by adding 100 μL of PBS (1×) containing 0.05% Tween 20 between each step. Electrochemical detection was performed by connecting the SPCEs to an AUTOLAB PGSTAT100 potentiostat. A DPV was applied (modulation time = 0.1 s, interval time = 0.2 s, initial potential = 0.1 V, end potential = 0.4 V, step potential = 0.01 V, modulation amplitude = 0.06 V, and stand by potential = 0 V). The height of the resulting peak waveform was recorded and plotted against OA concentration to give a calibration curve. Assays were performed in triplicate.

2.6. Preparation of sample and toxin extraction

Mussel samples without OA (Normandy, France) were blended with hand held homogenizer and extracted with methanol:water (80:20; 0.6 g/mL) for 5 min at 134,000 rpm. Crude extracts were centrifuged for 5 min at 3500 rpm. 1 mL of extract was evaporated in a speed VAC concentrator (Organomation Association, Inc.; USA) and the residue was resuspended in 1 mL of PBS (1×). The reconstituted solutions were passed through 0.45-μm cut-off Whatman nylon membrane filters. The samples were spiked with the stock solution of OA in the range from 12,500 to 0.025 ng/L after filtration, to study the matrix effect.

Certified reference mussel samples (CRM-DSP-Mus-b) containing toxin level for OA 10.1 μg/g and for DTX1 1.3 μ/g, were purchased from National Research Council Canada. The extraction was performed following a procedure similar to the one previously described. After extraction, a dilution step was performed to obtain a calibration curve with in range of 200 μg/kg to 0.012 ng/kg of OA.

2.7. Calibration curves for immunosensors

Standard calibration curve was performed by using OA standard solutions prepared in PBS (1×). Anti-OA-MAb binding was expressed as percentage of the control without OA. The matrix effects were assayed using blank samples without OA.

3. Results and discussion

3.1. Immobilization of OA onto SPCE

Schematic representation of the OA immobilization is given in Scheme 1b. As indicated, three steps were carried out for covalent immobilization of OA. Firstly, electrochemical reduction of the *in situ* generated 4-carboxyphenyl diazonium salt was carried out by linear sweep voltammetry (Fig. 1). The reduction of the diazonium species via one electron process lead to formation of 4-carboxyphenyl (4-CP) radicals that covalently attached to the surface. The terminal carboxylic group of 4-CP was activated to attach hexamethyldiamine via amine bond formation. OA was linked to the second terminal amine group of hexamethyldiamine via its activated carboxylic group. Hexamethyldiamine was used to provide a long spacer arm so that NH_2 group could be easily accessible to carboxylic group of OA (Scheme 1b).

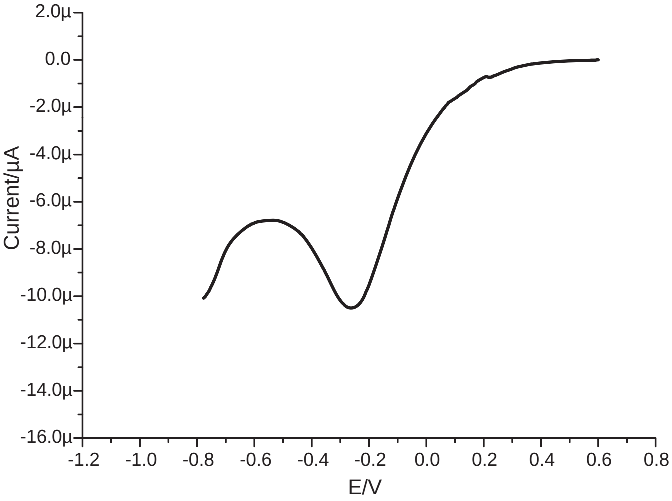


Fig. 1. Linear sweep voltammogram for the *in situ* generated 4-CDPS in the diazotation mixture ($NaNO_2$ + 4-aminobenzoic acid in 0.5 M HCl) at SPCE.

3.2. Electrochemical characterization of OA modified electrode

Cylic voltammetry in ferri/ferrocyanide solution is a useful and an important tool to monitor the behavior of modified electrode because electron transfer could take place either through the barrier or through the defects in the barrier. Therefore, this tool was used to investigate the blocking behavior and to follow the electrochemical modifications of the electrode surface. Cyclic voltammograms for each modification step were shown in Fig. 2. The characteristic anodic and cathodic peak potentials were observed in case of bare SPCE. The redox shape for 4-CP modified electrode disappeared, illustrating the successful covalent attachment of 4-CP with electrode surface that hindered the diffusion of the current towards the electrode surface. The negatively charged terminal carboxylic group COO^- acted as an electrostatic barrier that repelled $[Fe(CN)_6]^{3-/4-}$ anions and hindered the ability of the redox probe to access the layer and retarded the electron transfer kinetics between the redox probe and the electrode. There was an increase in peak current after OA immobilization onto 4-CP modified electrode. Only a slight difference in the voltammograms was observed in case of bare and OA modified electrodes.

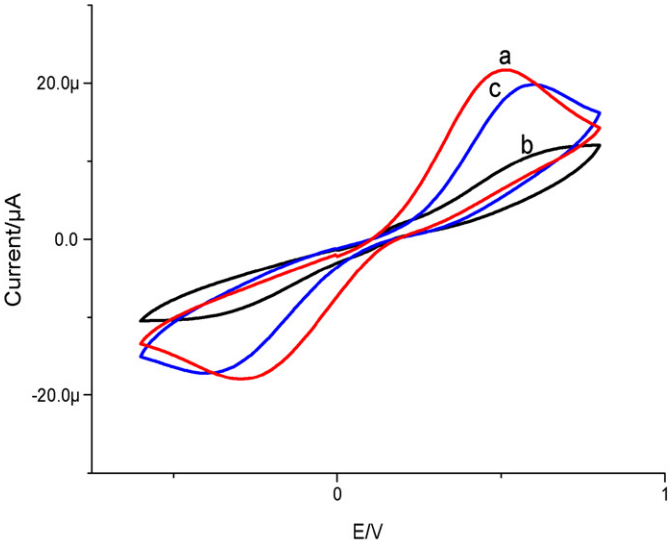
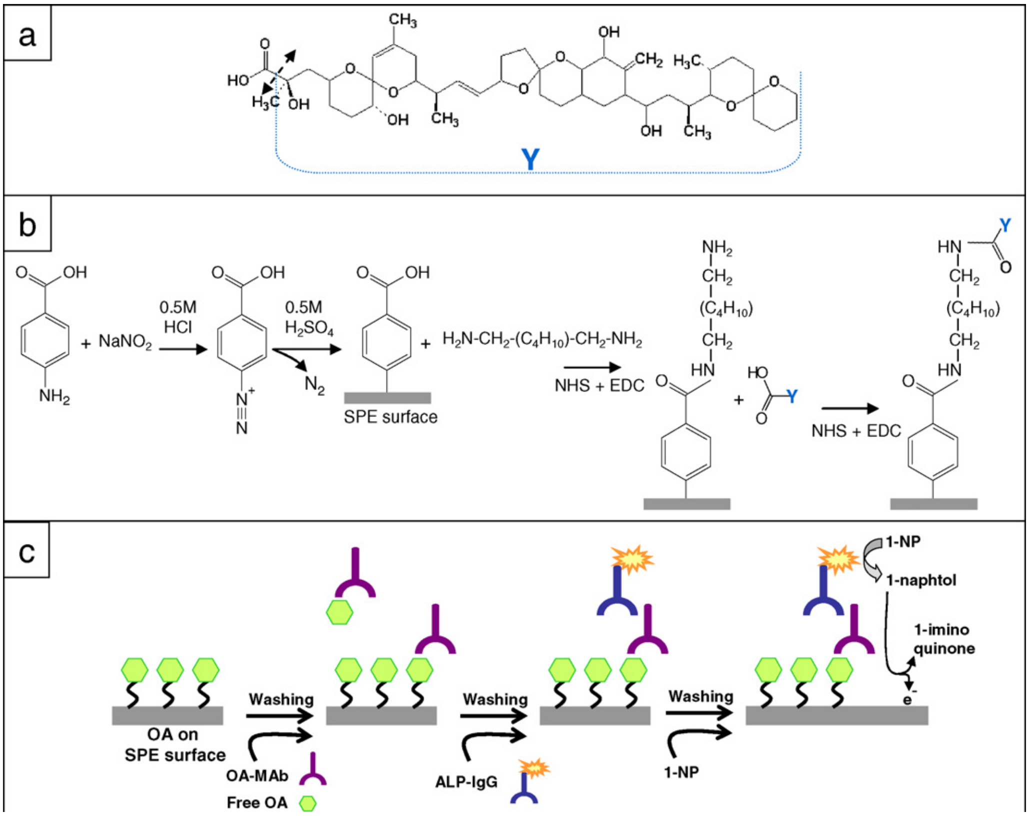


Fig. 2. Cyclic voltammograms of 1 mM $[Fe(CN)_6]^{3-/4-}$ at scan rate of 100 mV/s for; (a) bare SPCE, (b) 4-CP/SPC modified electrode, and (c) OA/4-CP/SPC modified electrode.



Scheme 1. (a) Chemical structure of OA, (b) schematic representation of the covalent immobilization of OA at SPCE, and (c) the working principal of the immunosensor with indirect competitive immunoassay.

It could be hypothesized that the covalent attachment of OA via hexamethyldiamine (pH 7) to the terminated carboxylic group has neutralized the negative charge of 4-CP modified electrode. Additionally, positive charge due to the second terminal amine group of hexamethyldiamine may facilitate the conductivity.

Electrochemical impedance spectroscopy can also provide detailed information on the barrier properties of the modified electrode. The values of the electron transfer resistance (R_{et}) obtained by fitting the curves to Randles equivalent circuit were shown in Table 1 for various steps of immunosensor elaboration. Faradic impedance spectra were shown in Fig. 3 for the stepwise modification process. The results were very similar to those obtained with CV shown in Fig. 2. The bare electrode showed a very small resistance (40.3 kΩ) to electron transfer. The resistance was significantly increased (~4 mΩ) after the deposition of 4-CP on the electrode surface due to the negatively charged carboxylic group. Following modifications with OA via hexamethyldiamine, the impedance spectra (44.2 kΩ) was very similar to that of bare electrode likely due to the factors explained for cyclic voltammogram. This behavior demonstrated that the conductivity is barely affected by the modification process, as cyclic voltammogram and impedance spectra were almost similar before and after modification. Such transducer surfaces are of little importance to be used for a protective barrier purpose, but they could be useful for the development of amperometric biosensors where easy electron transfer to the surface is highly desirable [47].

Table 1
Values of the electron transfer resistance (R_{et}) of the fitting curves for the various steps of the immunosensor fabrication.

Electrode	R_{et}
Bare SPCE	40.3 kΩ
4-CP/SCP modified electrode	~4 mΩ
OA/4-CP/SPE modified electrode	44.2 kΩ

3.3. Indirect competitive immunoassay for OA onto modified electrode

To validate the immobilization method, indirect competitive immunoassays were performed for OA analysis. The assays were relied on the competition between the free and the covalently immobilized OA for anti-OA-Mab in the solution. ALP labeled secondary antibody was used, as anti-OA-MAb was not labeled (Scheme 1c). The electrochemical detection was examined by DPV with signal generation based on the dephosphorylation of non-electroactive 1-NP by ALP, followed by the oxidation

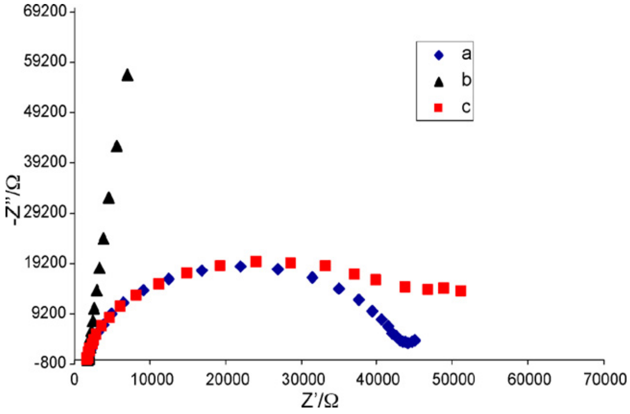


Fig. 3. Nyquist plots of 1 mM $[Fe(CN)_6]^{4-/3-}$ for; (a) bare SPCE, (b) 4-CP/SPC modified electrode, and (c) OA/4-CP/SPC modified electrode.

Table 2
Curve parameters derived from the non-linear four-parameter logistic regression fitting with electrochemical immunosensor.

Methods	LOD ($\mathrm{ng\,L^{-1}}$)	$\mathrm{IC_{50}}$ ($\mathrm{ng\,L^{-1}}$)	Sigmoidal logistic equation	R
PBS buffer	1.44	16.58	$\%\text{binding} = -6.901 + \frac{94.64}{1+(x/31.22)^{0.446}}$	0.995
Mussel sample spiked after filtration	1.99	26.08	$\%\text{binding} = -8.77 + \frac{94.57}{1+(x/54.77)^{0.451}}$	0.9802
Certified reference mussel sample	4.79	28.62	$\%\text{binding} = -3.29 + \frac{95.82}{1+(x/38.56)^{0.545}}$	0.9827

of electroactive 1-naphtol to 1-iminoquinone on the electrode surface.

Firstly, different experimental parameters were optimized without competition test. Antibodies concentration is an important factor in immunoassays because higher antibody concentrations decrease the sensitivity and lower antibody concentrations reduce the signal value. Based on the above facts, experiments with different dilutions of anti-OA-MAb and secondary antibody were performed. Best working dilutions were 1/5000 and 1/2500 for anti-OA-MAb and secondary antibody, respectively. To avoid the non-specific adsorption of antibodies on the electrode surface, three different blocking reagents were essayed. The use of 1% BSA resulted in lower adsorption than whole milk powder (5%) or casein (0.5%). When the system was tested without free OA by DPV, a current signal of 2.89 μA was obtained. This current was high enough to carry out the competition step and measure the consequently lower intensity currents. For stability study, the modified electrodes were kept at 4 °C. The sensors were found to have the same activity as freshly prepared one over a period of two weeks. This stability could be explained by the strong covalent attachment of OA with modified electrode. The chemical covalent bond could prevent the leaching of immobilized molecules as compared to the attachment via physical matrix or by adsorption.

After optimizing all the parameters, the calibrations curves were carried out by competition between the free and the immobilized OA. The curve obtained was shown in Fig. 4. The reproducibility of the immunosensor was accessed by analysis of each concentration of free OA using three equally prepared electrodes. A relative standard deviation of 5% showed a good reproducibility of the system. Due to the percentage error (5%), the LOD was defined as the toxin concentration corresponds to the 70% of anti-OA-MAb binding to be safe in screening. The calibration curves were fitted by the sigmoidal logistic four parameter-equation (Origin Pro 8 SR0) $y = y^0 + (a/1 + (x/x^0)^b)$, a and y^0 are the maximum and minimum values respectively, b is the slop of inflection point, and x^0 is the x value at the inflection point. Table 2 summarizes the results, consisting of correlation coefficient R, mid point ($\mathrm{IC_{50}}$) and LOD values derived from the regression equation for the assays.

Results obtained with immunosensor showed a very low LOD (1.44 ng/L) and $\mathrm{IC_{50}}$ (16.58 ng/L) values as compared to the already developed immunosensors for OA detection, which have the LOD in range of μg/L. This low LOD could be attributed to direct immobilization via covalent linking, which could increased the accessibility of OA to the anti-OA-MAb. Additionally, antibodies are positively charged proteins that could increase the conductivity of diazonium modified electrode [31,47,48]. Higher concentration of antibodies corresponds to higher value of conductivity. In case of the reported immunosensor, the competition of free OA with covalently immobilized OA could reduce the signal by decreasing the conductivity of modified electrode by decreasing the concentration of antibodies, leading to an increase of sensitivity.

3.4. *Matrix effect and OA detection in certified reference samples*

In order to investigate interferences from the mussel extracts, mussel samples without OA were spiked with known concentrations of OA. Correlation coefficient R, $\mathrm{IC_{50}}$ and LOD values derived from the regression equation were shown in Table 2 and the calibration curve was shown in Fig. 5a. The LOD (1.99 ng/L) and mid point (26.08 ng/L) values were comparable to those obtained using samples prepared in PBS (1×). The high number of washing steps and the specificity of antibody and antigen interaction might have reduced the matrix effect.

In order to validate the method with OA, a calibration curve was performed with certified reference mussel sample (Fig. 5b), and the data for correlation coefficient R, $\mathrm{IC_{50}}$ and LOD values derived were shown in Table 2. A slight increase in LOD (4.79 ng/L) and $\mathrm{IC_{50}}$ (28.62 ng/L) values was observed as compared to those obtained using samples preparation in PBS (1×). This increase in LOD might be due to partial lost of OA during extraction process. Further studies are required to improve the extraction efficiency. Despite this

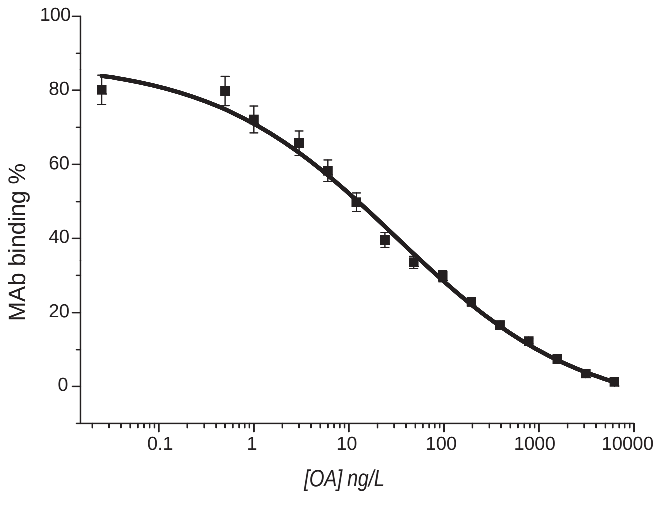


Fig. 4. Standard calibration curve obtained in PBS buffer in the presence of different concentrations of OA.

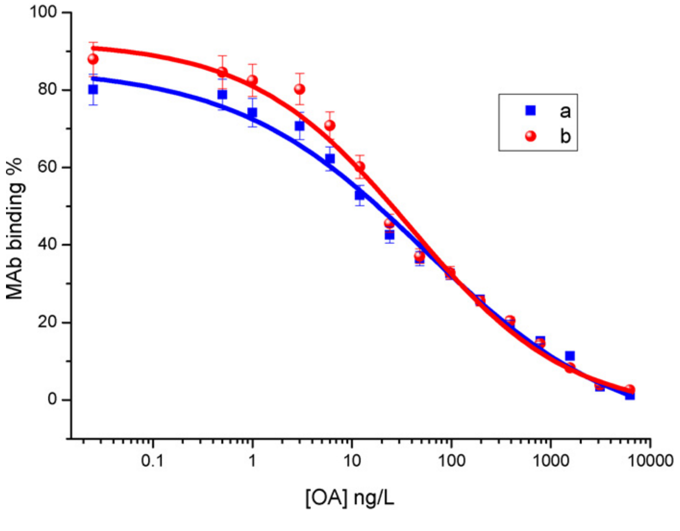


Fig. 5. Calibration curves obtained with. (a) Spiked mussel sample and (b) certified reference mussel sample.

underestimation, our system was able to detect much lower toxin concentration (2.4 ng/kg) in real samples as compared to already developed methods [40–43]. The results have demonstrated the suitability of the method even at extremely lower concentration of OA than the highest level allowed by the European Commission (160 μg/kg) (Commission Regulation (EC) No. 853/2004).

4. Conclusion

In current study, an approach involving diazonium-coupling reaction mechanism was developed for the immobilization of OA on the SPCE. The resulting OA/4-CP/SPC modified electrode showed an excellent electrical conductivity and displayed rapid and sensitive response towards the electrochemical oxidation. Our immobilization method could easily be extended for other small size biomolecules due to its simplicity and efficiency towards the electrochemical detection.

The current method was validated with certified reference mussel sample, and a very low limit of detection was achieved as compared to previously described methods for OA, thus illustrating successful fabrication of our system. The immobilization technique allowed detecting OA at nano-level without involvement of any expensive equipment. Moreover, the use of SPCEs could allow to miniaturize the system and to make the device portable.

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