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Site-specific immobilization of a (His)6-tagged acetylcholinesterase on nickel nanoparticles for highly sensitive toxicity biosensors

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ABSTRACT

This paper reports site-specific affinity immobilization of (His)6-tagged acetylcholinesterase (AChE) onto Ni/NiO nanoparticles for the development of an electrochemical screen-printed biosensor for the detection of organophosphate pesticides. The method is based on the specific affinity binding of the His-tagged enzyme to oxidized nickel nanoparticle surfaces in the absence of metal chelators. This approach allows stable and oriented attachment of the enzyme onto the oxidized nickel through the external His residue in one-step procedure, allowing for fast and sensitive detection of paraoxon in the concentration range from 10^{-8} to 10^{-13} M. A detection limit of 10^{-12} M for paraoxon was obtained after 20 min incubation. This method can be used as a generic approach for the immobilization of other His-tagged enzymes for the development of biosensors.

1. Introduction

Successful development of electrochemical enzyme biosensors relies on the stable attachment of the enzyme onto the electrode surface while maintaining the catalytic activity and function as close as possible to the native state. Conventional immobilization methods involve adsorption, physical entrapment in polymers and sol–gels, covalent attachment or self-assembly onto a variety of materials including gold, graphite, silica and different nanocomposites, carbon nanotubes, nanoparticles and nanowires (Andreescu et al., 2002, 2008; Bonnet et al., 2003). These methods are difficult to control and usually yield randomly bound proteins. Ideally, immobilization should be achieved at a specific pre-determined location of the enzyme onto the electrode surface in order to provide a favorable orientation for biorecognition events while avoiding conformational changes.

Controlled and oriented immobilization of proteins has been achieved by creating (bio)affinity bonds between an activated electrode material and a specific group of the protein sequence. Examples include affinity interactions between lectins, streptavidin, sugars and metal chelates of an activated surface and an affinity tag such as a carbohydrate residue, biotin, histidine or cysteine present or genetically engineered at a specific location in the protein sequence. Streptavidin has been used as a molecular linker to immobilize biotinylated acetylcholinesterase (AChE) on CNTs (Gao et al., 2009) and construct a pesticide biosensor. The mannose residue, a sugar moiety used as a label or naturally present in enzymes has been used to create an affinity bond with concavalin A to fabricate catalytically active films for biosensor development (Anzai et al., 1998; Bucur et al., 2004). The affinity of cysteine for gold has been used to fabricate an electrochemical glucose biosensor (Andreescu and Luck, 2008). In other works, the principles of metal ion affinity chromatography have been adapted to construct biosensors with increased sensitivity and lower detection limits. We have used the affinity of histidine for nickel complexed with a nitrilotriacetic acid (NTA) chelate onto activated graphite (Andreescu et al., 2002, 2003, 2001) to immobilize a 6His-tagged (6His-AChE) and construct an electrochemical enzyme biosensor for the detection of pesticides.

Recent developments of this method involve immobilization of His-tagged proteins onto nanoparticle carriers after chemical modification of the particles with chelating agents, such as NTA and iminodiacetic acid (IDA). Genetically engineered proteins with accessible His residues have been immobilized with high specificity onto thionic-capped gold nanoparticles with a Co(II)-NTA terminated ligand (Abad et al., 2005) and onto gold electrodes via a self-assembled monolayer Cu(II)-NTA ligand (Balland et al., 2008). Xu et al. have fabricated NTA modified magnetic nanoparticles as substrate, carrier and anchor for the binding of His-tagged proteins via a transition metal terminated affinity ligand NTA group (Xu et al., 2004). We have used Ni-IDA functionalized magnetic nanoparticles to site specifically immobilize genetically engineered

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AChE for high sensitive detection of chlorpyriphos and chlorfenvinphos insecticides (Noguer et al., 2007). This "classical" approach of forming complex biofunctional nanoassemblies based on metal chelate affinity involves lengthy and elaborate synthesis procedures, surface activation and sequential modification steps. An alternative is to bind His-tagged proteins to bulk oxidized nickel surfaces onto nanoparticle or nanowire surfaces in the absence of metal chelators (Lee et al., 2004). This specific binding was demonstrated by fluorescent imaging using fluorescently labeled poly-His (Lee et al., 2004). Wang et al. reported the strong affinity between a hexa-His domain of a polypeptide and the nickel segment of nickel/gold/nickel nanowires (Awang et al., 2006). The binding mechanism was explained by a direct interaction between the Ni^{2+} ions of the nickel oxide present onto the surface of the nanowire by surface oxidation.

In the present work, we report the demonstration of this method for the development of electrochemical enzyme biosensors. We developed a one-step direct biofunctionalization procedure of a His-tagged enzyme on oxidized Ni nanoparticles for achieving oriented site-specific immobilization of the enzyme onto screen-printed electrode surfaces for developing highly sensitive toxicity biosensors. The advantages of this method are: (1) no diffusion barriers for enzyme substrate and inhibitors, thus lowering the response time and improving sensitivity of the biosensor and (2) binding through an external linker (His) preserves the active state of the enzyme. We demonstrate this principle for the immobilization of a (His)6-tagged AChE enzyme as a model and use this method to fabricate an electrochemical biosensor for the detection of an organophosphate pesticide, paraoxon, based on enzyme inhibition. AChE biosensors in various configurations have been developed as a rapid and sensitive method for the detection of pesticides (Andreescu and Marty, 2006). In addition to the oriented immobilization, the use of a nickel and nickel oxide particles as electrode material provides high conductivity and electrocatalytic activity, which have been valuable in electrochemical biosensors (Andreescu et al., 2010; Wang et al., 2005). Here, we demonstrate that nickel/nickel oxide nanoparticles (Ni/NiO) can be successfully used for site-specific attachment of His-tagged proteins. The biosensor described in this work shows great promise for ultrasensitive, rapid and cost-effective analysis of pesticides.

2. Materials and methods

2.1. Reagents and stock solutions

Genetically modified (His)6-tag AChE from *Drosophila melanogaster* was provided by Protein Bio Sensor (Toulouse, France). Acetylthiocholine chloride (ATCh), 5,5′-dithiobis (2′-nitrobenzoic acid) (DTNB), imidazole, paraoxon ethyl (O, O-diethyl O-4-nitrophenyl phosphate) were obtained from Sigma. All other reagents were of analytical grade. A pesticide stock solution of 0.1 M was prepared in methanol and preserved at 4 °C for no longer than 2 weeks. Working dilutions were prepared daily in distilled water. In order to overcome the risk of spontaneous chemical hydrolysis of the substrate, ATCh, 0.1 M solution was prepared daily in 0.9% NaCl (w/v) and stored in ice during measurements. Genetically modified AChE enzyme with His-tag was dissolved in 0.1 M phosphate buffer solution at pH 7.0 and stored in the refrigerator. Before immobilization, enzymatic activity was measured by spectrophotometry at 412 nm according to the procedure described by (Ellman et al., 1961) using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) from Sigma. All electrochemical experiments were carried out in 0.1 M phosphate buffer saline solution at pH 7.0. The washing buffer used in the affinity immobilization was 0.1 M phosphate buffer at pH 7.0 containing 0.1% Tween-20, and 0.2 M NaCl.

2.2. Instrumentation

Spectrophotometric measurements were performed using a Shimadzu P2041 spectrophotometer equipped with a 1 cm path length cell. Impedance measurements were performed with a PARSTAT 2263 electrochemical analyzer (Princeton Applied Research) equipped with Power Suite software. Electrochemical experiments were carried out using a three electrode system with a Ag/AgCl electrode (Ag/AgCl/3 M NaCl) as reference electrode, a platinum wire (BAS, MW-1032) as counter electrode and a graphite screen printing electrode (SPE) modified with an electrodeposited Ni/NiO layer as working electrode. All the potentials were referred to the Ag/AgCl reference electrode. Amperometric measurements were carried out in stirred solutions in a batch system with an Epsilon potentiostat (Bioanalytical Systems Inc., West Lafayette, IN, USA). The electrochemical cell, reference and auxiliary electrodes were provided by Bioanalytical Systems (West Lafayette, IN, USA). The screen-printed electrodes were produced using a DEK 248 (DEK, France) printing machine at the University of Perpignan Via Domitia, France using a previously described procedure (Andreescu et al., 2002). In brief, the working SPE electrodes were manufactured in groups of 32 sensors on clear plastic sheet by sequentially printing/drying at 60 °C of the following layers: a silver conducting film, a carbon pad, an insulating layer and a graphite/hydroxiethyl cellulose/7,7,8,8-tetracyanoquinodimethane (TCNQ) composite. The SPE graphite working electrodes had a geometrical surface area of 0.17 cm^2. A layer of Ni nanoparticles was then electrodeposited onto the working SPE electrode as described in the following section. Distribution and size of the nanoparticles deposited onto the screen-printed electrode surfaces were quantified with a JEOL JSM-7400F high resolution field emission scanning electron microscope (FE-SEM).

2.3. Electrochemical deposition of nickel nanoparticles

Nickel nanoparticles were electrodeposited onto the surface of the SPE electrode from a nickel solution using direct current potential amperometry according to a procedure described by Zach and Penner, 2000 (Zach and Penner, 2000). Before modification, the bare SPE was rinsed with water and dried using a nitrogen stream. Deposition of nickel nanoparticles was performed by applying a constant potential of −1.4 V for 40 s to the SPE electrode in a solution of 10 mM Ni $(NO_3)_2$, in 1 M NaCl and 1 M NH_4Cl. Prior to the immobilization of the (His)6-tagged enzyme, the nickel was oxidized to form nickel oxide onto the nanoparticle surface. Oxidation was achieved in a classical electrochemical cell with 3 mL phosphate buffer of pH 7.0 by scanning the potential between 0 and 0.9 V versus Ag/AgCl reference electrode at a scan rate of 50 mV/s.

2.4. Biosensor fabrication and enzyme immobilization

Immobilization of the (His)6-tagged AChE onto the surface on the nickel modified SPE electrode was performed via the oxidized nickel oxide layer, after blocking the non-specific adsorption sites using BSA and washing the electrode with a washing buffer to prevent non-specific binding. The Ni/NiO-SPE electrode was first immersed in BSA (0.1 mg/mL) for 10 min, washed with phosphate buffer and allowed to dry. To immobilize the enzyme, 2 μL of (His)6-AChE (17 mIU/μL) was uniformly deposited on the modified electrode surface and allowed to dry at room temperature for ∼30 min. Then, the electrode was washed with a phosphate buffer solution, containing 0.1% Tween-20 and 0.2 M NaCl at pH 7.0. The biosensor was washed with Milli-Q water and stored at +4 °C until use. To verify the specificity of the binding and quantify the amount of non-specific binding, the electrode was washed

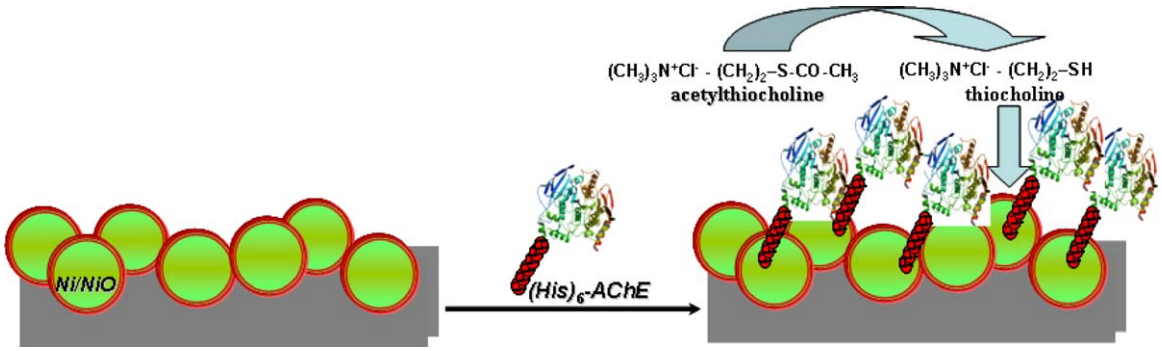

Fig. 1. Schematic representation of the oriented immobilization of the AChE enzyme onto the SPE-Ni/NiO modified electrode though the (His)6 residue.

with a solution of 0.1 M imidazole to competitively displace the (His)6-tag.

2.5. Electrochemical measurements and analytical performance characterization

Cyclic voltammetric measurements to verify the presence of the nickel layer were carried out in unstirred 0.1 m KNO_3 solution at pH 7.0 in the potential range from −0.5 to 1 V vs. Ag/AgCl, at a scan rate of 100 mV/s. All experiments were carried out at room temperature. Cyclic voltammetric experiments to determine the electrochemical active surface area of the bare and Ni modified electrode were performed in 2×10^{-2} M potassium hexacyanoferrate (III) in 0.2 M KCl at a scan rate of 100 mV/s. Amperometric measurements for substrate calibration and inhibition studies were performed in a batch system by measuring the current corresponding to the oxidation of thiocholine, as described previously (Andreescu et al., 2002). In brief, the biosensor was immersed in a 3 mL reaction cell containing phosphate buffer solution at pH 7.0 under constant stirring. Inhibition studies were performed by incubating the enzyme electrode in paraoxon solutions of concentrations ranging from 10^{-8} to 10^{-13} M for 30 min. After incubation, the electrode was washed with phosphate buffer and the residual response was measured. To evaluate the operational stability of the enzyme electrode, the response of the biosensor was measured repetitively by injecting the same amount of ATCh substrate at a final concentration of 1×10^{-3} M and rinsing the cell and the electrode in between measurements. The reproducibility of the biosensors was evaluated by measuring the response of $n = 9$ biosensors fabricated in identical conditions following the same procedure.

3. Results and discussion

3.1. Biosensor preparation and enzyme immobilization studies

Immobilization of the AChE enzyme relies on the formation of a direct affinity binding between the genetically engineered (His)6 residue and the Ni/NiO nanoparticles through the oxide layer in the absence of metal chelators. This strategy offers the advantage of achieving oriented and site-specific immobilization in a simple one-step procedure. To fabricate the biosensor and immobilize the enzyme, a layer of nickel nanoparticles was first deposited onto the surface of the SPE using an electrochemical procedure. Fig. 1 shows a schematic representation of the enzyme immobilization process onto the electrode surface. The presence of Ni was probed by cyclic voltammetry and scanning electron microscopy. Fig. 2 shows the SEM images of the graphite SPE electrode before and after deposition of the Ni nanoparticles. The SEM micrographs revealed uniform deposition of spherical Ni nanoparticles with an average diameter of 100 nm covering the entire working electrode surface. To determine the contribution of the Ni nanoparticles to the electrode surface, the active electrochemical surface area was determined for both bare and Ni modified SPE electrodes. The electrochemical surface area was calculated using the Randles–Sevcik equation for a reversible process monitored by cyclic voltammetry using potassium hexacyanoferrate (III) as redox probe. The electrochemical surface area increased by a factor of ∼4 after

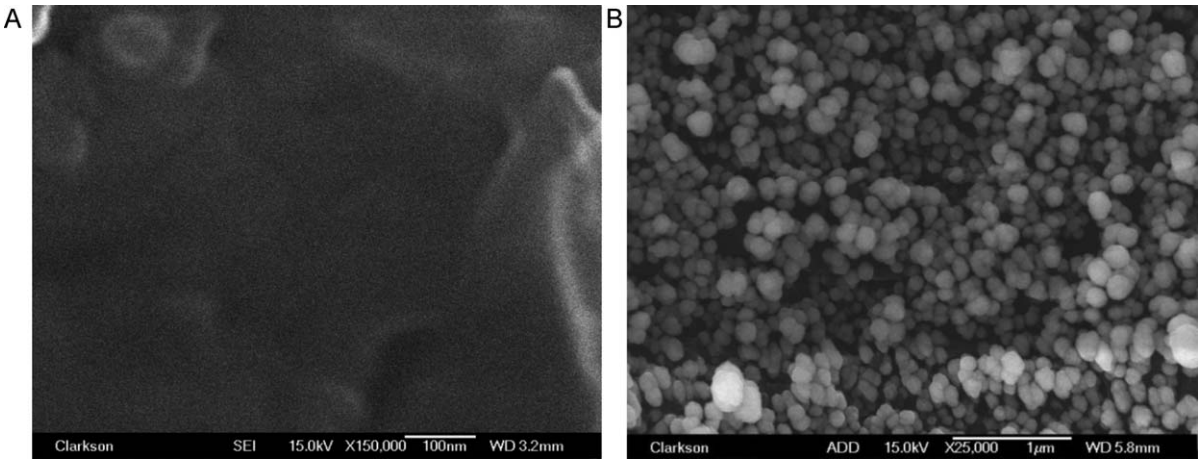

Fig. 2. Field-emission scanning electron microscopy (FE-SEM) of the bare SPE (A) and Ni (B) modified SPE electrode.

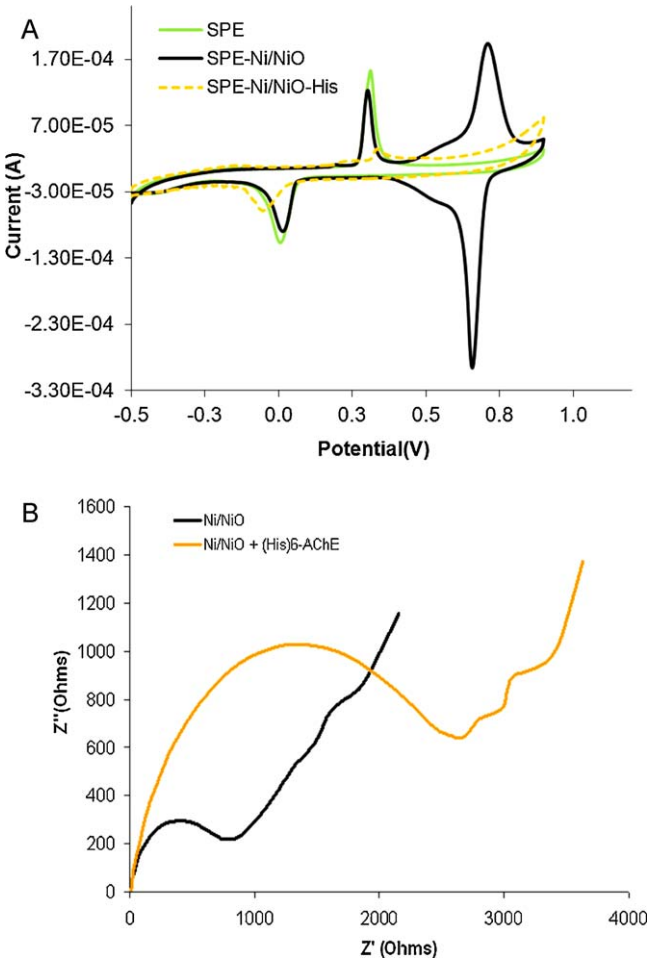

Fig. 3. A: Cyclic voltammograms of SPE-Ni/NiO electrode in 0.1 M KNO_3 before and after attachment of the (His)6-AChE enzyme at a scan rate of 100 mV/s. B: Nyquist plots for the electrochemical impedance spectroscopy measurements performed on enzyme modified (Ni/NiO-(His)6-AChE) and unmodified (Ni/NiO) SPE electrode in the presence of 2 mM ($Fe(CN)_6^{4-}$/$Fe(CN)_6^{3-}$) as soluble redox probe.

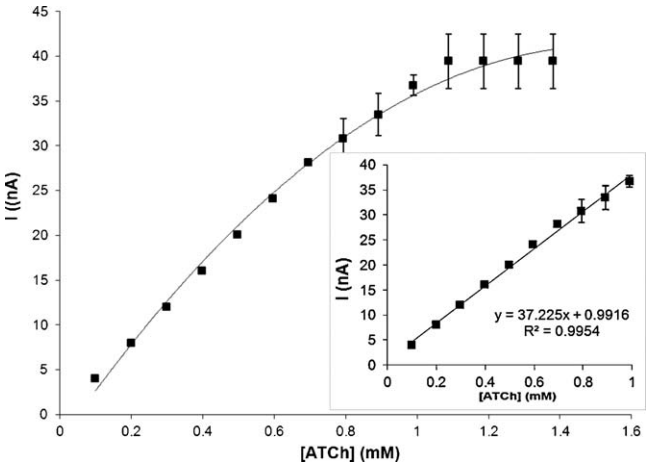

Fig. 4. Calibration curve of the (His)6-AChE biosensor to acetylthiocholine (ATCh) substrate.

deposition of the Ni nanoparticles, as compared to the bare graphite electrode. The enhancement is due to the presence of the highly conductive electrodeposited Ni nanoparticles, uniformly covering the electrode surface, as seen in the FE-SEM images. The high surface area of the modified electrode provided by the Ni/NiO nanoparticles facilitates effective enzyme binding. Fig. 3A shows the CV of the Ni/NiO modified SPE electrode in 0.1 M KNO_3 before and after attachment of the (His)6-AChE enzyme. The redox couple observed at 300 mV/50 mV corresponds to the electrochemical oxidation/reduction of the TCNQ mediator onto the electrode surface. The reversible redox couple observed at 680 mV/575 mV corresponds to the surface confined Ni(II)/oxyhydroxide species (Chen et al., 2007; Wang et al., 2005). The anodic peak at 680 mV corresponds to the oxidation of $Ni(OH)_2$ to NiO(OH). The cathodic peak at 575 mV corresponds to the reduction of NiO(OH) back to $Ni(OH)_2$. These peaks confirm the presence of nickel onto the electrode surface. On the other hand disappearance of the oxidation and reduction peaks corresponding to the oxidation/reduction of nickel species after enzyme binding indicates that binding occurs through the oxide layer of Ni. This observation suggests that these species are involved in the attachment of the enzyme.

Enzyme binding was further confirmed by impedance spectroscopy. The impedance measurements recorded in the presence of a soluble redox probe ($Fe(CN)_6^{4-}$/$Fe(CN)_6^{3-}$) plotted in the form of Nyquist diagram demonstrate the increase in the electron transfer resistance at the enzyme modified electrode as compared to the unmodified Ni/NiO based SPE electrode (Fig. 3B). This indicates attachment of an insulating layer of protein onto the working electrode surface, resulting in the partial inactivation of the electrochemical active surface, thus decreasing the rate of electron transfer of the redox probe at the enzyme modified electrode. The same behavior was observed in CVs performed in 2 mM $Fe^{II}(CN)_6^{4-}$/$Fe^{III}(CN)_6^{3-}$ in 0.1 M KNO_3 (Fig. 1S in the Supplementary Information); after incubation with enzyme, the Fe^{II}/Fe^{III} redox peak decreases as a result of enzyme binding, indicating partial inactivation of the electrode surface. Moreover, the CV exhibited the characteristic reversible redox peaks of the Fe^{II}/Fe^{III} both in the absence and presence of the enzyme, demonstrating good electron transfer of the redox probe onto the electrode surface even after enzyme attachment.

The pH plays an important role in achieving good analytical performance of this method, affecting both the enzyme activity and the affinity binding. The optimum pH for the AChE enzyme is between 7.0 and 8.0. At pH values <6.0, the histidine residues involved in the affinity binding are protonated, which prevents enzyme attachment through the His residue onto the Ni/NiO surface (Andreescu et al., 2003). Thus a pH of 7.0 was selected for analytical measurements with these biosensors.

Proteins have the tendency to adsorb easily onto electrode surfaces via non-specific electrostatic and/or hydrophobic interactions. To prevent the non-specific adsorption of the enzyme, the electrode was coated with BSA to inactivate the non-specific binding sites and washed with a binding buffer containing Tween-20 and a high salt concentration (0.2 M NaCl). To demonstrate the specificity of the affinity binding through the His residue, the enzyme electrode was incubated in a solution of imidazole for ~30 min, which selectively displaces His, thus removing the affinity bound enzyme. The process is schematically presented in Fig. 2S in the Supplementary Information, which shows the response of the electrode to two consecutive additions of 1 mM ATCh substrate before incubation with imidazole and one addition after incubation. Two identical amperometric responses of 8 nA were obtained upon addition of substrate before incubation with imidazole, indicating that the sensor is stable. After incubation with imidazole, no electrochemical response was obtained after injection of substrate, indicating the absence of the enzyme from the electrode surface due to displacement by imidazole.

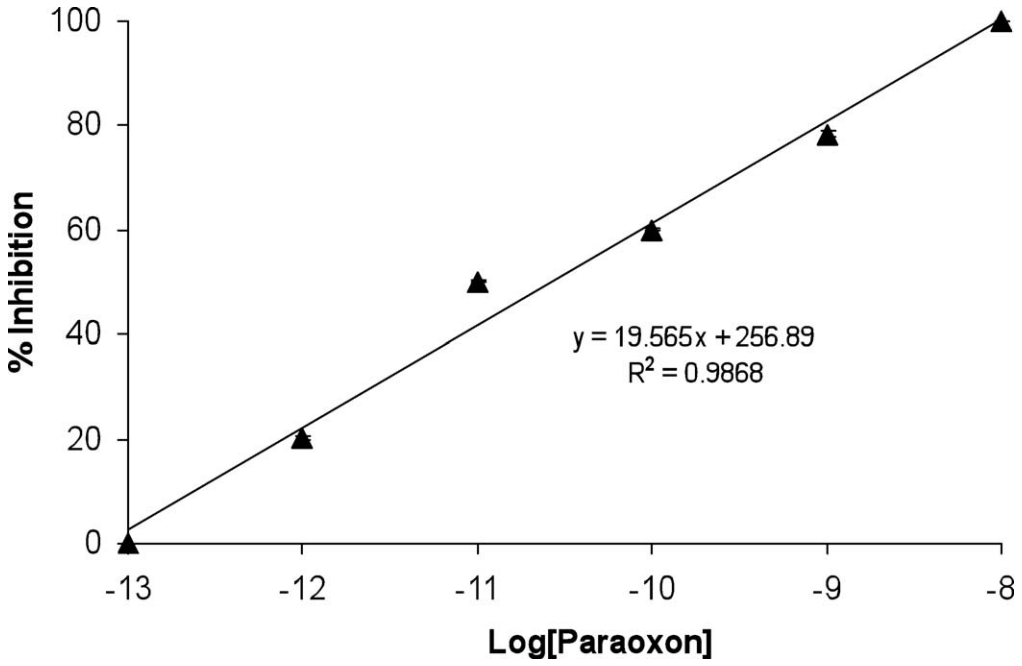

Fig. 5. Inhibition curves of the immobilized (His)6-AChE onto Ni/NiO modified screen-printed electrodes by paraoxon after 20 min incubation.

It is worth mentioning that the His-tagged enzyme does not bind to electrodes with un-oxidized nickel. No electrochemical response to the enzyme substrate was observed in this case. This demonstrates that binding occurs through the NiO layer and the oxidation of the nickel nanoparticle surface is necessary to immobilize the (His)6 tagged enzyme and fabricate the biosensor.

3.2. Analytical characterization

Fig. 4 shows the calibration curve and the linear range of the biosensor to addition of ATCh substrate of concentrations ranging from 0.1 to 1.4 mM. Using the optimized sensor, a sensitivity of 37.2 nA/mM with a linear range from 0.1 to 1 mM was obtained. The reproducibility of the AChE biosensors prepared in identical conditions following the same procedure was 7.4%. The average current value to injection of 0.6 mM ATCh for $n = 9$ biosensors was 25 (+/−1.86) nA. The stability of the enzyme onto the electrode was evaluated using ATCh substrate. The biosensor was stable for the first eight consecutive assays, followed by a rapid drop in activity. The decrease in response can be due to enzyme leaching or rapid deactivation, or to the passivation of the nickel surface by the product of the enzymatic reaction, thiocholine. We did not observe electrode passivation at low substrate concentrations; however, with increasing substrate concentrations and continuous use of the electrode, it is possible that thiocoline binds to the nickel surface due to the affinity of the thiol groups for nickel, and inactivates the electrode. An operational stability of eight assays is sufficient for inhibition assays since the electrode is disposable and it is not reused for consecutive measurements. The stability obtained with this method is comparable with that of the earlier reported (his)6-AChE biosensors in which the enzyme was immobilized using other affinity methods based on metal chelates such as Ni-NTA (Andreescu et al., 2001) and Ni-IDA (Noguer et al., 2007). The apparent Michaelis constants of the immobilized enzyme $K^{app}{}_m$, estimated from the Lineweaver–Burk representation using the electrode response was 0.9 mM. This value is higher than that of the soluble enzyme ($K_m = 0.38$ mM) but lower than that obtained with the enzyme immobilized by affinity onto magnetic IDA based iron oxide microbeads (1.04 mM) (Noguer et al., 2007).

3.3. Inhibition studies: determination of paraoxon

Fig. 5 shows the inhibition effect of paraoxon, an organophosphate pesticide commonly used as a reference pesticide, on the immobilized enzyme. Experiments were performed for pesticide concentrations ranging from 10^{-8} to 10^{-13} M using an incubation time of 20 min. The enzyme was completely inactivated by a paraoxon concentration of 10^{-8} M. The method allows detection of very low concentrations of pesticides, with detection limits (quantified as I20 – the concentration of pesticide corresponding to 20% inhibition) of 10^{-12} M for paraoxon. Biosensors prepared with this method are three orders of magnitude more sensitive than those based on a Ni-NTA chelate (Andreescu et al., 2003, 2001).

4. Conclusions

This study explores the affinity of (His)6 tagged proteins for Ni/NiO nanoparticle surface via the His residue to fabricate an electrochemical biosensor for the detection of paraoxon as a model organophosphate pesticides. The procedure provides a simple and elegant alternative to the commonly used Ni chelates such as NTA and IDA reported previously for the construction of (His)6 affinity based biosensors. The method allowed stable and effective attachment of a (His)6-AChE enzyme onto disposable screen-printed electrodes modified through electrochemically deposited Ni nanoparticles in a non-destructive, single-step procedure. The electrodes fabricated in this way allows highly sensitive detection of organophosphate pesticides, with detection limits of three orders of magnitude lower than previously reported AChE biosensors based on other immobilization methods. A detection limit of 10^{-12} M paraoxon was obtained. This study demonstrates the effectiveness of this approach to the immobilization of AChE onto screen-printed electrodes. The described technique can be applied to other His-tagged

enzymes and other types of Ni/NiO functionalized electrodes and surfaces.

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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.bios.2011.08.024.

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