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Research paper

An amperometric immunosensor for separation-free immunoassay of CA125 based on its covalent immobilization coupled with thionine on carbon nanofiber

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Abstract

A carbon nanomaterial, soluble carbon nanofiber, was used for the first time to construct an immunosensor for a rapid separation-free immunoassay. The acidic oxidation of the carbon nanofiber provided its solubility and wettability for convenient preparation of a porous carbon nanofiber membrane and a larger number of active sites for covalent binding of carcinoma antigen-125 (CA125) and thionine as electron transfer mediator. This matrix was a suitable environment for the immobilized protein. The immobilized HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of thionine by hydrogen peroxide. With a competitive mechanism, the differential pulse voltammetric peak current of this system decreased linearly with increasing CA125 concentration (from 2 to 75 U/ml) in the incubation solution. The CA125 immunosensor showed good precision, high sensitivity, acceptable stability and reproducibility with a detection limit of 1.8 U/ml. The soluble carbon nanofiber is a novel method for preparation of immunosensors.

Keywords: Biosensors; Electrochemical sensors; Immunosensor; Immunoassay; Carcinoma antigen-125; Carbon nanofiber

1. Introduction

The need to generate fast, reliable, and precise analytical information on biomedical, industrial and environmental processes has resulted in an intensive search for more selective systems of molecular recognition (Santandreu et al., 1997). Immunoassay is a very popular analytical method for samples of clinical, pharmaceutical, biochemical and environmental importance, utilizing the high specificity of recognition between antigen and antibody (Tang et al., 2002). Compared with conventional immunoassay techniques, immunosensors are of great interest because of their potential utility as specific, simple, label-free and direct detection techniques and the reduction in size, cost and time of analysis (Hianik et al., 1999). Many types of immunosensors have been developed: electrochemical (Cui et al., 2000; Wilson,

Abbreviations: CA125, carcinoma antigen-125; CNF, carbon nanofiber; DPV, differential pulse voltammetry; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EIS, electrochemical impedance spectroscopy; GCE, glassy carbon electrode; HRP, horseradish peroxidase; MWCNTs, multiwall carbon nanotubes; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffer saline; SCE, saturated calomel electrode; XPS, X-ray photoelectron spectra.

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2005), chemiluminescent (Kokado et al., 1997), fluorometric (Li and Gong, 2004), piezoelectric (Pan and Shih, 2004) and surface plasmon resonance immunosensors (Lyon et al., 1998), impedance biosensing chips (Oh et al., 2003) and multichannel microchips (Tang et al., 2002; Yakovleva et al., 2002). Electrochemical immunosensors have several advantages such as high sensitivity and low cost of the resulting sensors and instrumentation; they have attracted extensive interest as bioanalytical devices in recent years. Since most antibodies and antigens are intrinsically unable to act as redox partners, enzymes such as horseradish peroxidase (HRP) (Santandreu et al., 1999; Du et al., 2003), alkaline phosphatase (Wang et al., 1998; Crowley et al., 1999), laccase (Kuznetsov et al., 2001), cholinesterase (Babkina et al., 1996), and glucose oxidase (Keay and McNeil, 1998) have been used to label the immunocomponents and improve the sensitivity of the immunoassay. In particular, when HRP is used as the label, an additional substrate such as catechol (Du et al., 2003), hydroquinone (Santandreu et al., 1999), *o*-aminophenol (Liu et al., 2001), thionine (Yu et al., 2004) or *o*-phenylenediamine (Chen et al., 2006) is also added to the detection solution as mediator to transfer the electron between hydrogen peroxide (H_2O_2) and the enzyme. The electroactive product of the enzymatic reaction is then formed to produce the electrochemical signal. For detection convenience, the co-immobilization of the mediator and immunocomponent for preparation of immunosensors has attracted considerable attention.

Nanostructures have been used extensively as immobilized materials for different purposes, including immunosensing (Cunningham and Campbell, 2003). They provide a unique opportunity for the development of biosensors. The immobilization of antibody on the surface of carbon nanotubes for preparation of immunosensors has been reported previously (Wohlstadter et al., 2003; O'Connor et al., 2004; Yu et al., 2005; Viswanathan et al., 2006). This work used a novel carbon nanomaterial, soluble carbon nanofiber (CNF), for the first time to construct an immunosensor for rapid separation-free immunoassay.

CNF has been recognized as a very promising material based on its nanostructure and properties (Yoon et al., 2004) and has been used as a catalyst or catalyst support (van der Lee et al., 2005), probe tip (Cui et al., 2004), and a fuel cell (Hacker et al., 2005). Oxidation of CNF with nitric acid can produce carboxyl groups without degradation of the structural integrity of its backbone. Compared to carbon nanotubes CNF has a much larger functional surface area and higher ratio of surface active groups to volume (Vamvakaki et al., 2006). Thus, it can be used for covalent binding of proteins and mediators with the help of a cross-linking reagent. The covalent attachment of proteins to the CNF surface overcomes the problems of instability and inactivation. With carcinoma antigen-125 (CA125) as a model, this work co-immobilized CA125 and thionine as mediator on a CNF modified electrode to develop an immunosensor for CA125. Compared with the immunosensors for CA125 reported previously (Dai et al., 2003; Wu et al., 2006), it showed wider linear range and better stability. This method avoided the addition of an electron transfer mediator to the solution; thus, less incubation and washing steps were required. It is simpler than the traditional enzyme-linked immunoabsorbent assay (ELISA) methods.

2. Experimental

2.1. Reagents

CA125 ELISA kits were purchased from Everlong (USA). The ELISA kits consisted of a series of CA125 standard solutions with different concentrations from 0 to 500 U/ml, a stock solution, horseradish peroxidase (HRP) labeled CA125 monoclonal antibody and its diluent. The diluent contained phosphate buffered saline (PBS, pH 7.2), bovine serum albumin, bovine globulin, Tween 20, sodium dodecylsulfate, a blue inert dye and 0.01% methylisothiazolone. CNF was a gift from WPI (Sarasota, USA). Thionine and H_2O_2 were from Shanghai Biochemical Reagent (China). Multiwall carbon nanotubes (MWCNTs, >95%, 10–20 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Merck (Germany) and *N*-hydroxysuccinimide (NHS) from Aldrich. All other reagents were of analytical grade. Double-distilled water was used for all experiments. The serum samples were obtained from patients clinically diagnosed with ovarian cancer.

2.2. Apparatus

Electrochemical measurements were performed on a CHI 660 electrochemical analyzer (Co. CHI, USA) with a conventional three-electrode system comprised of platinum wire as the auxiliary electrode, saturated calomel electrode (SCE) as the reference and a modified glassy carbon electrode (GCE) as the working electrode. Electrochemical impedance spectroscopy (EIS) was performed in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1/1) mixture with 0.10 M KNO_3 as supporting electrolyte, using an alternating current voltage of 5 mV, within the

frequency range of $0.01–10^5$ Hz. The reference values of the sera were obtained with an automated electrochemiluminescent analyzer (Elecsys 2010, Roche, Switzerland). X-ray photoelectron spectra (XPS) were recorded on an ESCALAB MKII X-ray photoelectron spectrometer.

2.3. Preparation of soluble CNF

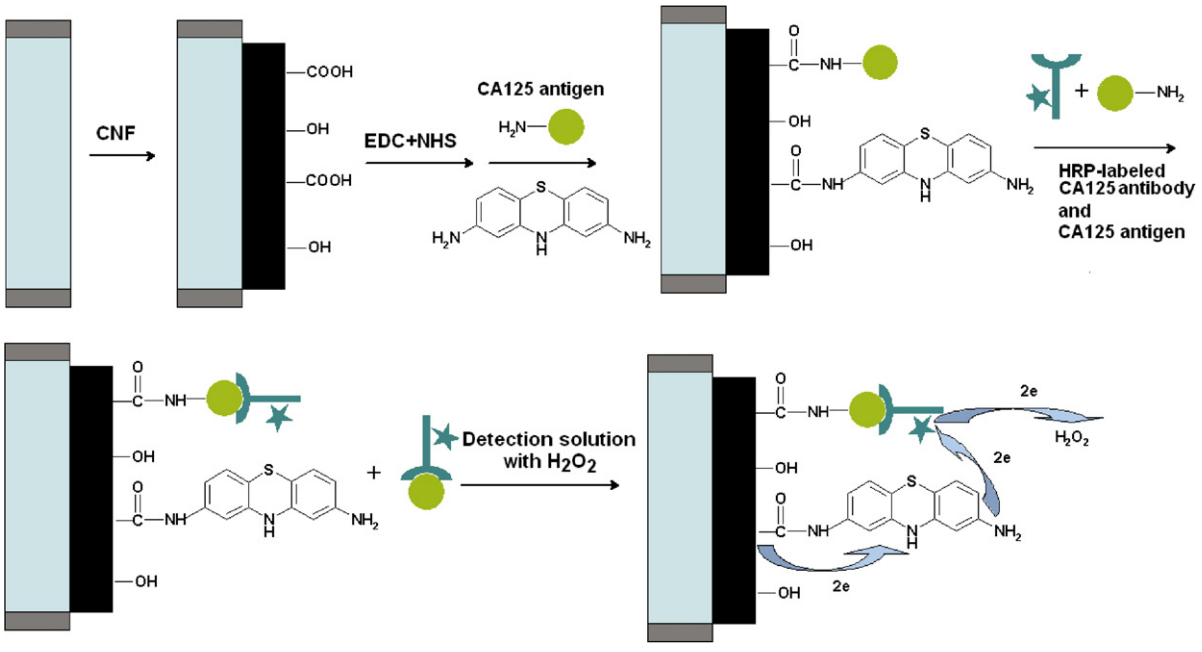
One hundred milligrams of CNF were dispersed in 30 ml of 30% HNO_3 and then refluxed for 24 h at 140 °C. The resulting suspension was centrifuged and the precipitate was washed with water to obtain carboxylic group functionalized CNF. The CNF could be completely dissolved in water at pH 1.0. The CNF solution was then neutralized to pH 7.0 with 0.1 M NaOH and diluted to a concentration of 5 mg/ml. MWCNTs underwent the same procedure as CNF and dispersed in neutral water.

2.4. Preparation of the CA125 immunosensor

The GCE (3 mm diameter) was polished to a mirror finish using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by thorough rinsing with double-distilled water. After successive sonications in 1/1 nitric acid, acetone and double-distilled water, the electrode was rinsed with double-distilled water and allowed to dry at room temperature. Three microliters of CNF solution (5 mg/ml) was dropped on the pretreated GCE and dried in a silica gel desiccator, then immersed in a solution containing 2 mM EDC and 5 mM NHS for 1 h. After the activated CNF/GCE was thoroughly rinsed with double-distilled water, it was immersed immediately in a mixture of 25 μl of 500 U/ml CA125 solution and 25 μl of 0.2 mM thionine solution for 2 h to yield a thionine/CA125/CNF modified GCE. The immunosensor obtained was stored in PBS (pH 7.0) at 4 °C.

2.5. Measurement procedure

Ten microliters of CA125 standard solutions or serum samples with different concentrations were mixed with 40 μl of diluted HRP-labeled CA125 antibody to obtain the incubation solutions. The dilution of the antibody was expressed as the ratio of the volume of commercial HRP-labeled CA125 antibody solution to the total volume. After the immunosensor for CA125 was incubated in 50 μl of incubation solution at 35 °C for 40 min and then washed carefully with double-distilled water, the electrochemical measurement was recorded in degassed PBS containing 4.0 mM H_2O_2. The differential pulse voltammetry (DPV) measurements ranged from 0.36 to −0.76 V with pulse amplitude of 50 mV and width of 50 ms. The preparation and detection procedures of the CA125 immunosensor are shown in Scheme 1. The data for



Scheme 1. Preparation and detection procedures of the CA125 immunosensor.

condition optimization and the calibration curve were the average of three measurements.

3. Results and discussion

3.1. Cyclic voltammetric behavior of the CA125 immunosensor

The cyclic voltammogram of thionine/CA125/CNF modified GCE in PBS (pH 7.0) showed two redox peaks at −0.257 and −0.280 V at 50 mV/s, which were attributed to the redox reactions of the immobilized thionine (Fig. 1, curve a). Upon addition of 4.0 mM H_2O_2 to the solution, the reduction peak current increased slightly due to the direct reduction of H_2O_2 at the CNF modified electrode (Fig. 1, curve c). After the immunosensor was incubated with HRP-labeled CA125 antibody, the pair of redox peaks of thionine decreased slightly (Fig. 1, curve b), while the reduction peak increased from 4.6 to 10.3 μA with a shift in peak potential from −0.280 to −0.308 V and an obvious decrease of the oxidation peak after 4.0 mM H_2O_2 was added to the detection solution, indicating electrocatalytic behavior (Fig. 1, curve d). Thus, the HRP immobilized on the immunosensor surface retained high enzymatic activity, and the thionine could effectively shuttle electrons between the redox center of HRP and GCE. However, after incubation

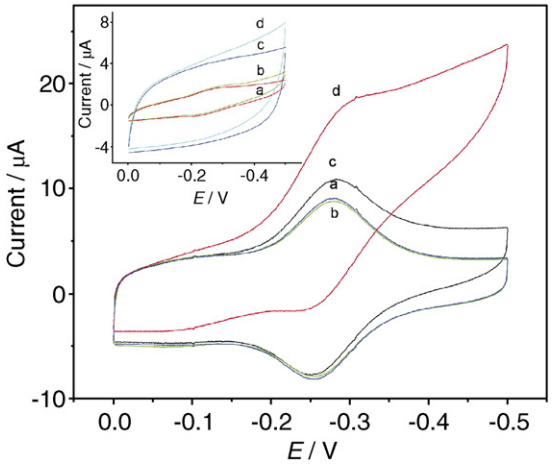


Fig. 1. Cyclic voltammograms of thionine/CA125/CNF modified GCE (a, c), thionine/CA125/CNF modified GCE (b, d) after incubation in 50 μl of incubation solution containing HRP-labeled CA125 antibody at 35 °C for 40 min in 0.2 M PBS (pH 7.0) (a, b) and 0.2 M PBS (pH 7.0)+4 mM H_2O_2 (c, d). Scan rate: 50 mV/s. Inset: cyclic voltammograms of thionine/CA125 (a, b) and CA125/CNF (c, d) modified GCE in 0.2 M PBS (pH 7.0) (a, c) and 0.2 M PBS (pH 7.0)+4 mM H_2O_2 (b, d) after incubation in 50 μl of incubation solution containing HRP-labeled CA125 antibody at 35 °C for 40 min.

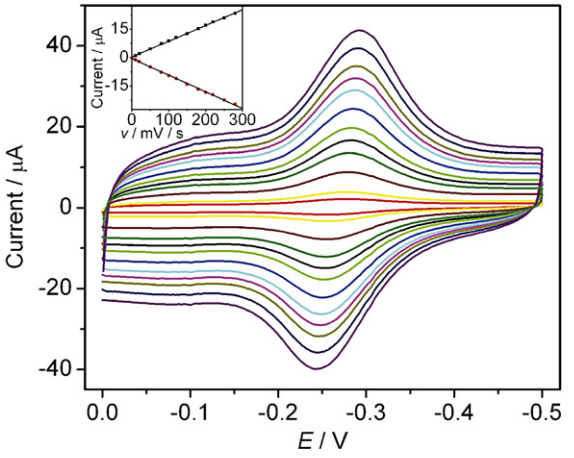


Fig. 2. Cyclic voltammograms of the incubated immunosensor in 0.2 M PBS (pH 7.0) at 10, 20, 50, 80, 100, 120, 150, 180, 200, 220, 250 and 280 mV/s (from lowest to highest peak current). Inset: plots of peak currents vs *v*.

the thionine/CA125 modified GCE, without the presence of CNF, showed only weak redox peaks (curve a, inset in Fig. 1) and a slight response to 4.0 mM H_2O_2 (curve b, inset in Fig. 1). In comparison with the response at the CA125/CNF modified GCE (curve c, inset in Fig. 1) these peaks were attributed to the redox of thionine, which was much lower than the response at the thionine/CA125/CNF modified GCE. Thus CNF provided a larger number of active sites for covalent binding of CA125 and thionine to improve sensitivity. At the CA125/CNF modified GCE the catalytic reaction of the entrapped HRP was very weak (curve d, inset in Fig. 1), indicating that thionine was a key factor in the enzymatic reaction to obtain a sensitive immunoassay.

Both the anodic and cathodic peak currents of the thionine/CA125/CNF modified GCE incubated in HRP-labeled CA125 antibody solution were proportional to the scan rate in the range from 10 to 280 mV/s (Fig. 2), indicating a surface-controlled electrode process. From the peak-to-peak separations at different scan rates, an average electron transfer rate constant of $4.82 \pm 0.702\ s^{-1}$ was obtained. The surface coverage of thionine was calculated from the peak areas to be $(2.51 \pm 0.23) \times 10^{-9}$ mol/cm^2, which was much larger than that of $(3.21 \pm 1.62) \times 10^{-11}$ mol/cm^2 for covalent co-immobilization of thionine and HRP-labeled CA125 antibody on a GCE (Dai et al., 2004) due to the more activated sites of nitric acid treated CNF. The functional groups and surface active groups-to-volume ratio of the CNF were higher than those of MWCNTs, as demonstrated by XPS analysis. The results showed that the treated MWCNTs and CNF contained 89.0 and

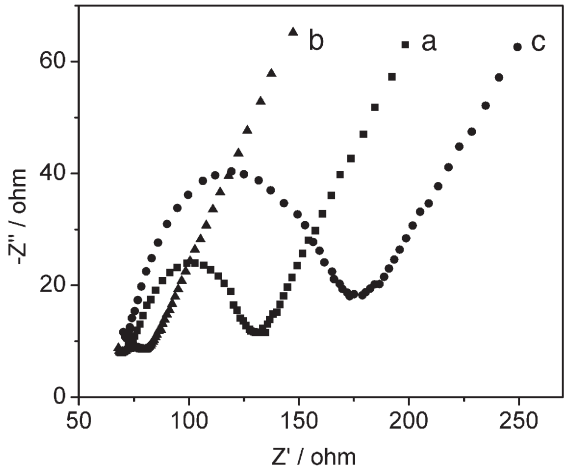


Fig. 3. EIS of bare (a), CNF (b), and thionine/CA125/CNF (c) modified GCEs in 0.10 M KNO_3 containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

85.1 at.% carbon and 11.0 and 14.9 at.% oxygen, respectively.

3.2. Impedance characterization of thionine/CA125/CNF/GCE

At a bare GCE, the redox process of the $[Fe(CN)_6^{3-/4-}]$ probe showed an electron transfer resistance of about 59 Ω (Fig. 3, curve a); the CNF modified GCE showed a much lower resistance for the redox probe (Fig. 3, curve b), implying that the CNF was an excellent electric conducting material and accelerated the electron transfer. When thionine and CA125 antigen were assembled on the CNF/GCE, the electron transfer resistance increased (Fig. 3, curve c), suggesting that thionine/CA125 was immobilized on the electrode and blocked the electron exchange between the redox probe and the electrode surface.

3.3. Condition optimization for electrochemical detection

The analytical performance of the immunosensor was related to the concentration of H_2O_2 and the pH of the detection solution. The response current of the immunosensor after incubation with HRP-labeled CA125 antibody increased with the increasing concentration of H_2O_2 from 1 to 4 mM and then maintained the maximum value at higher concentrations (Fig. 4a), at which the enzymatic reaction rate became dependent on the amount of the immobilized HRP. The amperometric response of the immobilized HRP to H_2O_2 displayed a curve characteristic of a Michaelis–Menten's mechanism. Therefore, the optimal H_2O_2 concentration for the immunoassay of CA125 levels was 4 mM. The acidity of the solution greatly affected the enzyme activity for the catalytic reaction and the electrochemical response of the modified electrode. The incubated immunosensor showed a maximum current response at pH 7.0, corresponding to maximum enzyme activity (Fig. 4b). Thus, the optimal pH value of the enzymatic reaction was 7.0.

3.4. Optimization of immunoreaction conditions

The immunoreaction conditions included the concentration of HRP-labeled CA125 antibody in the incubation solution, incubation temperature and time. The response of the immunosensor depended on the amount of HRP-labeled CA125 antibody bound to the immunosensor surface, which corresponded to the concentration of HRP-labeled CA125 antibody in the incubation solution. As shown in Fig. 5a, the catalytic peak current increased and then tended to a constant value with an increasing volume ratio of the commercial HRP-labeled CA125 antibody solution in the incubation solution. At a dilution of 0.7, the current reached a maximum value, indicating that the amount of HRP-labeled CA125

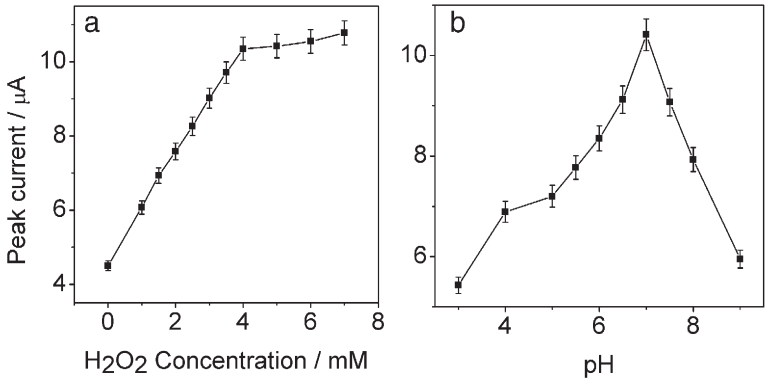


Fig. 4. Dependence of the cyclic voltammetric response of the incubated immunosensor on (a) the H_2O_2 concentration at pH 7.0 and (b) the pH of the detection solution at 4.0 mM H_2O_2.

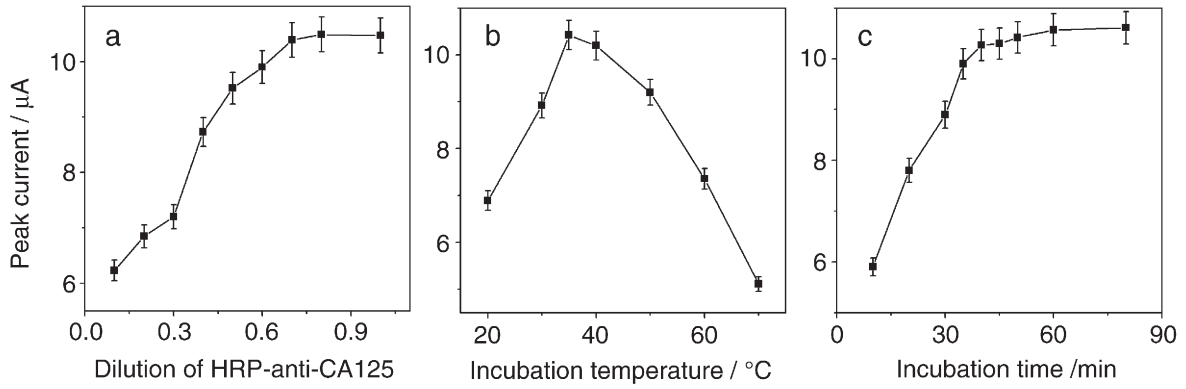


Fig. 5. Effects of (a) dilution of HRP-labeled CA125 antibody solution, (b) incubation temperature and (c) incubation time on the cyclic voltammetric response of the immunosensor under other optimal conditions.

antibody in the incubation solution was enough to match the amount of the CA125 antigen immobilized on the immunosensor.

At the optimized dilution, with increasing incubation temperature from 20 to 70 °C, the immunosensor showed a maximum response at 35 °C after incubation for 40 min (Fig. 5b). Thus, 35 °C was used for the immunoreaction. The current response of the thionine/CA125/CNF modified GCE to H_2O_2 increased with the increasing incubation time and reached a maximum at 40 min (Fig. 5c). Longer incubation time did not improve the response. Therefore, 40 min was chosen as the optimal incubation time for the immunoassay of CA125 using the proposed immunosensor.

3.5. Electrochemical response of the immunosensor to CA125 concentration

For the measurement of CA125, a competitive assay configuration was applied under optimized conditions. A standard solution of CA125 at a known concentration or one serum sample was added into the incubation solution containing HRP-labeled CA125 antibody with a controlled volume ratio. The CA125 antigen in the incubation solution competed with the immobilized CA125 antigen in the thionine/CA125/CNF membrane to bind the limited binding sites of the HRP-labeled CA125 antibody to form immunocomplex. The DPV catalytic peak current of the immobilized HRP to H_2O_2 showed a decrease with increasing CA125 concentration in the incubation solution (Fig. 6a). At higher CA125 concentrations, the amount of the bound antibody on the surface of the immunosensor was less, and therefore, the response was also lower. The electrochemical immunoassay displayed well-defined concentration dependence. As shown in Fig. 6b, the decrease in the percentage of DPV peak current was proportional to the CA125 concentration in the range of 2–75 U/ml with a correlation coefficient of 0.996 and a linear slope of 0.607 (±0.0152) μA/(U/ml). The detection limit was calculated to be 1.8 U/ml at a three times signal-to-noise ratio, which was estimated at the

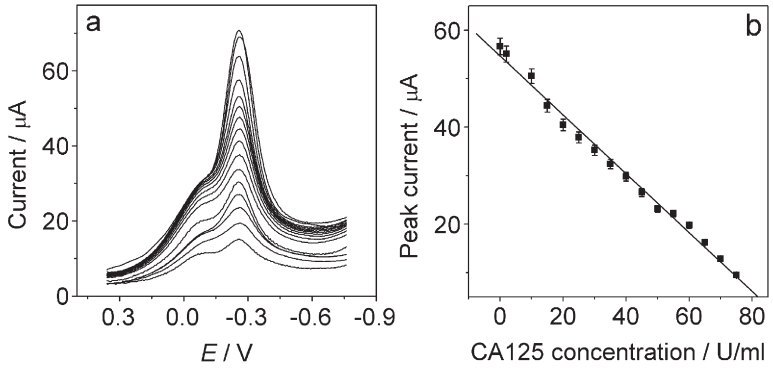


Fig. 6. (a) DPV of the immunosensor in 0.2 M PBS (pH 7.0) containing 4.0 mM H_2O_2 after incubation with 0, 2.0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75 U/ml CA125 (from highest to lowest peak current) and (b) calibration curve for the CA125 immunoassay.

Table 1
Comparison of serum CA125 levels determined using the two methods

Sample	Proposed method	Electrochemiluminescent method	Relative deviation (%)
1	8.2	8.8	−6.8
2	9.5	11.1	−5.4
3	17.1	16.8	+1.8
4	37.7	38.2	−1.3
5	46.9	46.8	+0.2
6	61.7	59.6	+3.5

immunosensor in 0.2 M PBS (pH 7.0) containing 4.0 mM H_2O_2 after incubation in the solution without CA125. The linear response range of the immunosensor to CA125 was wider than that of 2–14 U/ml reported previously (Dai et al., 2003). Higher serum CA125 levels could be detected with an appropriate volume of the serum sample added to the incubation solution.

3.6. Precision, reproducibility and stability of the CA125 immunosensor

The intra-assay precision of the immunosensor was evaluated by assaying the CA125 levels of two sera for five replicate measurements in the same run. The intra-assay variation coefficients with this method were 4.8% and 3.4% at CA125 concentrations of 10 and 50 U/ml, respectively, while the inter-assay variation coefficients at these concentrations on five immunosensors made independently were 5.9% and 7.7%, respectively, indicating acceptable precision and fabrication reproducibility. When the immunosensor was not in use, it was stored in PBS (pH 7.0) at 4 °C. A decrease of 5.6% amperometric response was observed after a 20 day storage, which indicated the retention of the specific binding ability of antigens and minimal microstructural changes. The stability was better than the 15 day (Dai et al., 2003) and 7 day (Wu et al., 2006) storage periods reported previously.

3.7. Detection of serum CA125 levels

The CA125 levels in six serum samples obtained using the proposed immunosensor and working curve are shown in Table 1, which compares the results with those obtained from a commercial electrochemiluminescent analysis. The results obtained with the two methods are in acceptable agreement. Thus, the immunosensor presented here was highly efficient for the clinical determination of CA125 levels in human samples.

4. Conclusions

This work describes a novel CA125 immunosensor prepared by co-immobilizing thionine and CA125 antigen on a CNF modified GCE. CNF has a large functionalized surface area and high surface active groups-to-volume ratio. Thus it can be used for covalent binding of proteins and mediators with the help of a cross-linking reagent. The covalent attachment of proteins to the CNF surface overcomes the problems of instability and inactivation. The CA125 concentration can be detected based on the electrocatalytic behavior of thionine to the reduction of H_2O_2 catalyzed by labeled HRP due to the formation of immunocomplex on the immunosensor surface after incubation. The CA125 immunosensor shows good precision, high sensitivity, acceptable stability and reproducibility. CNF can efficiently immobilize antigen and could be used in the preparation of other amperometric immunosensors for the detection of important antigens.

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