

# Reagentless Amperometric Immunosensors Based on Direct Electrochemistry of Horseradish Peroxidase for Determination of Carcinoma Antigen-125

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**A novel strategy for immunoassay and the preparation of reagentless immunosensors was proposed. This strategy was based on the immobilization of antigen and the direct electrochemistry of horseradish peroxidase (HRP) that was labeled to an antibody. A reagentless immunosensor for carcinoma antigen-125 (CA 125) determination was developed. The immunosensor was prepared by immobilizing CA 125 with titania sol–gel on a glassy carbon electrode by the vapor deposition method. The incubation of the immunosensor in phosphate buffer solution (PBS) including HRP-labeled CA 125 antibody led to the formation of a HRP-modified surface. The immobilized HRP displayed its direct electrochemistry with a rate constant of  $3.04 \pm 1.21 \text{ s}^{-1}$ . With a competition mechanism, a differential pulse voltammetric determination method for CA 125 was established by the peak current decrease of the immobilized HRP. The current decrease resulted from the competitive binding of the CA 125 in sample solution and the immobilized CA 125 to the limited amount of HRP-labeled CA 125 antibody. Under optimal conditions, the current decrease was proportional to CA 125 concentration ranging from 2 to 14 units  $\text{mL}^{-1}$  with a detection limit of  $1.29 \text{ units mL}^{-1}$  at a current decrease by 10%. The CA 125 immunosensor showed good accuracy and acceptable precision and fabrication reproducibility with intraassay CVs of 8.7 and 5.5% at 8 and 14 units  $\text{mL}^{-1}$  CA 125 concentrations, respectively, and interassay CV of 19.8% at 8 units  $\text{mL}^{-1}$ . The storage stability was acceptable in a pH 7.0 PBS at 4 °C for 15 days. The proposed method provided a new promising platform for clinical immunoassay.**

In the past decades, immunoassay techniques, especially enzyme immunoassay and those based on immunosensors, have become the predominant analytical methods in clinical,<sup>1–3</sup> bio-

chemical,<sup>4,5</sup> and environmental<sup>6,7</sup> analyses. The immunosensors combine the selectivity of the immunological reaction with the sensitivity and convenience of electrochemical or spectral techniques and can operate effectively in untreated samples without the requirement for separation or washing steps; thus, they have received considerable attention. Many kinds of immunosensors such as electrochemical,<sup>8–10</sup> chemiluminescence,<sup>11,12</sup> fluorometric,<sup>13,14</sup> piezoelectric,<sup>15</sup> and surface plasmon resonance<sup>16</sup> immunosensors, impedance biosensing chips,<sup>17</sup> and even multi-channel microchips for multianalyte determination<sup>18,19</sup> have been developed.

So far most of the electrochemical immunosensors have been structured by immobilizing various kinds of antibody or antigen on the electrode surfaces. After being incubated in the sample and enzyme conjugate solutions, the electrochemical immunosensors can determine the levels of analytes by the potential change<sup>20–23</sup> or the oxidation and reduction of electrochemically active species, generated by the action of the enzyme conju-

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gate.<sup>23,24</sup> The enzyme-catalyzed potential change results from the bioelectrocatalysis of laccase or peroxidase enzyme as an electrocatalyst label to the oxygen<sup>25</sup> or H<sub>2</sub>O<sub>2</sub><sup>26</sup> electroreduction or the urease-catalyzed hydrolysis reaction of urea to produce NH<sub>3</sub> that is determined by a gaseous diffusion potentiometric electrode.<sup>23</sup> The enzymes are used to produce the electrochemically active species for amperometric immunosensor preparation and include horseradish peroxidase (HRP),<sup>27–29</sup> alkaline phosphatase,<sup>30,31</sup> laccase,<sup>32</sup> and glucose oxidase.<sup>23,24,33,34</sup> These enzymes are usually labeled to the immunoreagents. The immunoassay based on these amperometric immunosensors needs the presence of H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub> for HRP, naphthyl,<sup>30</sup> or *p*-aminophenol<sup>31</sup> phosphate for alkaline phosphatase, O<sub>2</sub> and ferrocene for laccase<sup>32</sup> and glucose, and O<sub>2</sub> and immobilized HRP for glucose oxidase. Sometimes, for the amperometric immunosensors based on HRP-labeled immunoreagents, one substrate such as hydroquinone<sup>28</sup> or *o*-aminophenol<sup>29</sup> is also added to the detection solution as a mediator to transfer the electron between HRP and H<sub>2</sub>O<sub>2</sub>. Although the enzyme conjugate amplifies the response of the immunosensor to the oxidation or reduction of the electrochemical active species,<sup>8–10,23,24,27–34</sup> the presence of dissolved O<sub>2</sub> or the addition of one or two nonimmunoreagents into the detection solution is necessary. The concentration of dissolved O<sub>2</sub> usually depends on the environment conditions such as temperature, pressure, and the solution components and thus may affect the detection results. The addition of nonimmunoreagents causes the immunoassay system to be more complex, increases the analytical time and expense, and thus limits the practical application of immunosensors, particularly in the clinical on-line diagnoses. To eliminate the addition of mediator or other reagents has become a highly desirable project.<sup>32</sup> This work reports the first attempt to develop a reagentless and mediatorless immunosensor based on the direct electrochemistry of HRP labeled to immunoreagent without addition of any nonimmunoreagents. Its aim is to establish an effective platform for the development and popularization of amperometric immunosensors.

The direct electron transfer of immobilized proteins such as HRP,<sup>35–42</sup> cytochrome *c*,<sup>43–45</sup> and hemoglobin<sup>46</sup> with regard to Fe(III) to Fe(II) conversion has been well studied and extensively

employed for biosensing and the preparation of mediator-free biosensors.<sup>35,41–43,45–47</sup> This strategy avoids the trouble of adding a mediator to the sample solution and the pollution of some mediators of the electrode systems. McNeil and co-workers designed two separation-free electrochemical immunosensors based on the direct electron transfer of HRP immobilized on activated carbon electrode.<sup>27,33</sup> In these works, the direct electrochemistry of HRP was used to electrocatalyze the reduction of hydrogen peroxide that was produced from other enzymatic catalytic reactions by glucose oxidase and alkaline phosphatase, respectively. At the same time, a method for immunoassay of anti-human immunoglobulin G by the electrocatalysis of HRP-anti-human immunoglobulin G bound to immobilized human immunoglobulin G to the reduction of hydrogen peroxide was also developed.<sup>27</sup> These methods involved the addition of enzymatic substrates or nonimmunoreagents, such as hydrogen peroxide, glucose, or 5-bromo-4-chloro-3-indolyl phosphate, into the detection solution.

Different materials have been used for the immobilization of proteins or enzymes.<sup>35–47</sup> In previous work, we developed a titania sol-gel thin film by the vapor deposition method for protein immobilization.<sup>48</sup> This film has been proved to be chemically clean, porous, and homogeneous and to have a very narrow particle size distribution. Thus, we here use this film to immobilize carcinoma antigen-125 (CA 125) on an electrode surface to prepare a new reagentless immunosensor for CA 125, a glycoprotein commonly produced by the endometrium. With silica sol-gel-derived thick-film amperometric immunosensors based on alkaline phosphatase-labeled anti-RIgG and naphthyl phosphate as the substrate,<sup>30</sup> the HRP-labeled CA 125 antibody can readily diffuse toward and bind with the encapsulated CA 125 to form an immunoconjugate. Furthermore, the association reaction can be easily detected by the direct electron transfer of the immobilized HRP without addition of enzymatic substrates or nonimmunoreagents. With a competition mechanism, the CA 125 concentration in sample solution can be determined by the current decrease of the direct electron transfer. The proposed CA 125 immunosensor has an acceptable precision, reproducibility, and storage stability. In comparison with the results obtained with IRMA, the immunosensor shows an acceptable accuracy. This method simplifies

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greatly the immunoassay procedure, shortens the analytical time, and thus provides a new promising platform for clinical immunoassay.

## EXPERIMENTAL SECTION

**Reagents.** CA 125-ELISA kits were purchased from Diagnostic Products (DPC). The ELISA kits consisted of a series of CA 125 standard solutions with different concentrations from 0 to 500 units  $\text{mL}^{-1}$  and a stock solution of HRP-labeled anti-CA 125 monoclonal antibody from mouse. Bovine serum albumin was a product of Sigma Chemical Co. (St. Louis, MO). The dilution solution of enzyme conjugate contained 0.04% BSA, 1.0 mM EDTA, and 0.1 M pH 7.0 phosphate buffer. Titanium isopropoxide was obtained from Aldrich. HRP (EC 1.11.1.7, RZ > 3.0, A > 250 units  $\text{mg}^{-1}$ ) was purchased from Sigma. All other reagents were of analytical grade. Doubly distilled water was used for all experiments. The serum samples were obtained from two clinically diagnosed patients with ovarian cancer. The sera were separated from the cell, without hemolysis.

**Preparation of CA 125 Immunosensor.** The glassy carbon electrode (GCE) (4-mm diameter) was polished to a mirror finish using 0.3- and 0.05- $\mu\text{m}$  alumina slurry (Beuhler) followed by thorough rinsing with doubly distilled water. After sonicated successively in 1:1 nitric acid, acetone, and doubly distilled water, the electrode was pretreated electrochemically by applying a potential of +1.75 V in 0.1 M pH 5.0 PBS for 300 s and scanned between +0.3 and +1.25 V and then +0.3 and -1.3 V until a steady-state current-voltage curve was observed.<sup>49</sup> The electrode was rinsed with doubly distilled water and allowed to dry at room temperature.<sup>48</sup> A 5- $\mu\text{L}$  aliquot of 500 units  $\text{mL}^{-1}$  CA 125 standard solution was dropped onto the treated electrode surface. The electrode was then suspended vertically above titanium isopropoxide in a sealed flask kept at a constant temperature of 25 °C for 4 h. This resulted in adsorption of saturate titanium isopropoxide vapor by the CA 125 solution and slow formation of a titania sol-gel membrane through hydrolysis of titanium isopropoxide on the surface, trapping the CA 125 in the membrane.<sup>48</sup> The obtained immunosensor was rinsed thoroughly with doubly distilled water and kept in pH 7.0 PBS at 4 °C prior to electrochemical experiments.

**Apparatus.** Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems Inc.) with a three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference, and a CA 125-modified glassy carbon electrode as working electrode. IRMA procedure was carried out with a FMJ-182 immunoradiometric  $\gamma$ -counter according to the instructions and assay procedure in the operator's manual.

**Measurement Procedure.** The electrochemical measurement was based on a competitive immunoassay method. The schematic diagram of the procedure is shown in Figure 1. The stock solution of HRP-labeled CA 125 antibody was first diluted with the dilution solution in a volume ratio of 5:1. Aliquots (10  $\mu\text{L}$ ) of CA 125 standard solutions with different concentrations were then added into the mixture of 40  $\mu\text{L}$ , respectively, to prepare the incubation solutions. After being incubated in an incubation solution at 33 °C for 40 min, the immunosensor was washed carefully with

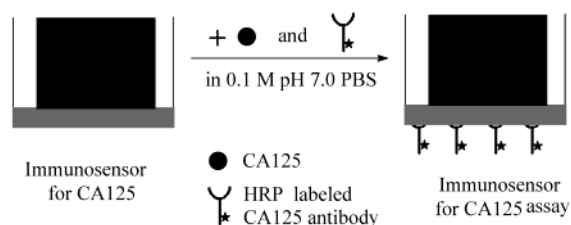


Figure 1. Principle of reagentless amperometric immunosensor based on immobilized antigen, competitive immunological reaction, and direct electrochemistry of HRP label.

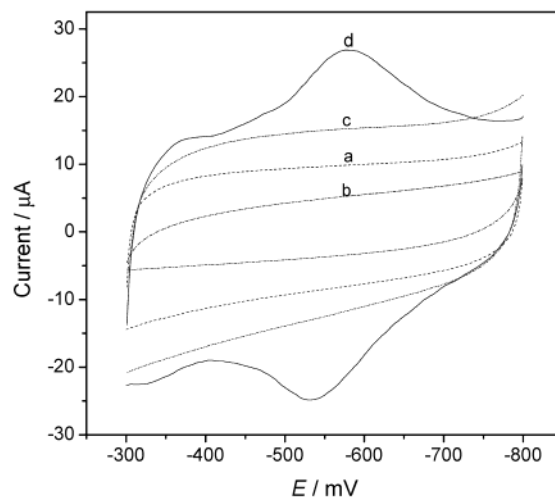


Figure 2. Cyclic voltammograms of different electrodes in pH 7.0 PBS at 50  $\text{mV s}^{-1}$ : (a) unpretreated bare GCE, (b) pretreated bare GCE, (c) CA 125 immunosensor, and (d) CA 125 immunosensor after incubation in HRP-labeled CA 125 antibody solution at 33 °C for 40 min.

doubly distilled water. It was then immersed in pH 7.0 PBS to study the direct electrochemistry of the immobilized HRP using cyclic voltammetry and to detect the amperometric response for CA 125 assay using differential pulse voltammetry.

All electrochemical measurements were done in an unstirred electrochemical cell at  $20 \pm 0.5$  °C. The detection solution was bubbled thoroughly with high-purity nitrogen for 5 min and maintained in nitrogen atmosphere. Differential pulse voltammetric (DPV) measurements were performed from -300 to -800 mV with a pulse amplitude of 50 mV and a pulse width of 50 ms.

## RESULTS AND DISCUSSION

### Electrochemical Behavior of the CA 125 Immunosensor.

The cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PBS are shown in Figure 2. No response was observed at unpretreated, pretreated, and CA 125-modified GCEs in the work potential window (curves a-c). The pretreated GCE showed a charging current half that of unpretreated GCE (curves a and b), indicating a cleaner and more smooth surface. The CA 125-modified electrode showed a larger charging current. After incubation, some CA 125 molecules encapsulated in the titania sol-gel membrane bound with HRP-labeled CA 125 antibody, leading to the immobilization of HRP. A pair of stable and well-defined redox peaks with regard to Fe(III) to Fe(II) conversion of the immobilized HRP were observed. The anodic and cathodic peak potentials were -0.534 and -0.575 V (vs SCE) at 50  $\text{mV s}^{-1}$ , respectively (curve d). Both the anodic and cathodic peak

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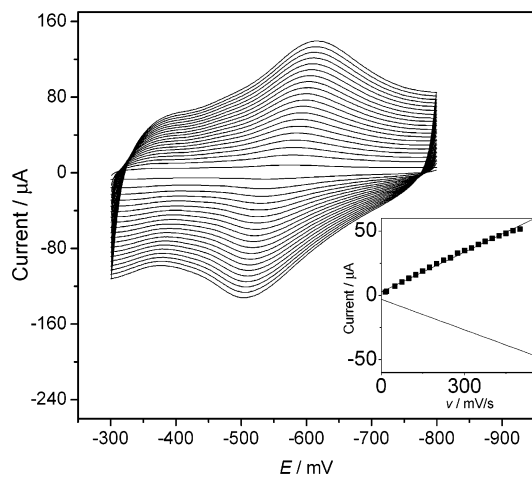


Figure 3. Cyclic voltammograms of CA 125 immunosensor after incubation in HRP-labeled CA 125 antibody solution at 33 °C for 40 min in pH 7.0 PBS at 15, 20, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500  $\text{mV s}^{-1}$  (from lowest to highest peak currents). Inset: plots of peak currents vs scan rate.

currents were proportional to the scan rate in the range from 15 to 500  $\text{mV s}^{-1}$  (Figure 3). From the peak-to-peak separations at different scan rates, an average electron-transfer rate constant of  $3.04 \pm 1.21 \text{ s}^{-1}$  was obtained using Laviron's model.<sup>50</sup> The average surface concentration of HRP calculated from the peak areas of cyclic voltammograms were  $(1.04 \pm 0.14) \times 10^{-9} \text{ mol/cm}^2$ .

When the CA 125-modified electrode was incubated in PBS containing HRP under the same conditions, no peak corresponding to the direct electrochemistry of HRP was observable. Furthermore, as reported in previous work,<sup>48</sup> these redox peaks also could not be observed at the HRP-encapsulated titania sol-gel membrane-modified electrode. When HRP-labeled CA 125 antibody was immobilized in titania sol-gel membrane without the presence of CA 125, the obtained electrode did not show any response relative to the direct electron transfer of HRP. No response was observed even after the electrode was incubated in an incubation solution without the presence of CA 125 at 33 °C for 40 min. Thus, the immunological reaction between immobilized CA 125 and HRP-labeled CA 125 antibody played an important role in the direct electrochemistry of the HRP. Binding of the labeled antibody to the immobilized antigen might result in a slight change of HRP conformation, which exposed the activity center of HRP.

The formal potential of the immobilized HRP was  $-0.550 \text{ V}$  (vs SCE), which was close to that of  $-0.58 \text{ V}$  (vs Ag/AgCl)<sup>36</sup> and more negative than those reported by other authors.<sup>39–41</sup> It was caused by the negatively changed microenvironment of the titania sol-gel membrane, which made the reduction of the immobilized HRP more difficult thermodynamically.

#### Optimization of CA 125 Immunosensor Preparation.

Preparation of the CA 125/titania sol-gel membrane was greatly affected by the surface character of the GCE. At an untreated electrode, direct electron transfer of the immunosensor after incubation in the HRP-labeled antibody solution was not observed during the cyclic voltammetric sweep, though a very small peak

was observable on its differential pulse voltammogram. It was probably due to the more hydrophobic electrode surface than that of the pretreated electrode, which resulted in the congregation of CA 125 molecules. The congregated CA 125 molecules were difficult to bind with HRP-labeled CA 125 antibody due to steric hindrance. After the GCE was pretreated, a blue thin film could be observed on the electrode surface. According to the results of Kepley and Bard,<sup>51</sup> the activation of GCE involved the formation of a new phase, which contained a significant amount of microcrystallinity and graphite oxide and thus increased the surface hydrophilicity.

The performance of the immunosensor also depended on the deposition temperature and membrane thickness. Titanium isopropoxide was reactive with water. The vapor deposition method greatly decreased the hydrolysis rate, resulting in the formation of titania sol-gel without  $\text{TiO}_2$  particulate. Meanwhile, the temperature affected directly the vapor pressure of titanium isopropoxide and thus controlled the hydrolysis rate. An optimal temperature was 25 °C.<sup>48</sup> The efficiency of the immunological reaction between the immobilized CA 125 and HRP-labeled CA 125 antibody in the incubation solution was related to the membrane thickness, which was related to both the volume of CA 125 solution dropped on GCE and the deposition time. Membrane thickness increased with increasing volume of the CA 125 solution. At a larger membrane thickness, the binding efficiency of the immobilized antigen with HRP-CA 125 decreased due to blocking of the membrane to the HRP-CA 125 molecules when they entered the membrane to react with inner CA 125 molecules. Thus, a thin membrane of titania sol-gel was suitable for the surface immunoreaction. Considering the fact that the formed titania sol-gel membrane could not cover the electrode surface completely when the volume of CA 125 solution was less than 4  $\mu\text{L}$ , we used 5  $\mu\text{L}$  of CA 125 solution for the preparation of CA 125 immunosensor. At this volume, the suitable deposition time was 4 h.

**Optimization Conditions of Immunoassay.** The effect factors of the immunological reaction between the immobilized CA 125 and HRP-labeled CA 125 antibody included incubation temperature and incubation time. The effect of incubation temperature on the DPV peak current for the direct electron transfer of the immobilized HRP was studied in the temperature range from 27 to 45 °C. From the results shown in Figure 4, the maximum response occurred at an incubation temperature of 33 °C. The effect of incubation time on the current response is shown in Figure 5. With increasing incubation time, the direct electrochemical response of the immobilized HRP increased and then reached a constant value when the incubation time was longer than 40 min. Thus, an incubation temperature of 33 °C and incubation time of 40 min were selected for the immunoassay of CA 125 using a HRP-labeled CA 125 antibody solution diluted with the dilution solution at a volume ratio of 5:1.

The concentration of HRP-labeled CA 125 antibody in the incubation solution was another important parameter. After the stock solution of HRP-labeled CA 125 antibody was diluted with the dilution solution in different volume ratios, the incubation was performed in the mixed solution of 50  $\mu\text{L}$  at 33 °C for 40 min. The obtained peak current increased with an increasing amount

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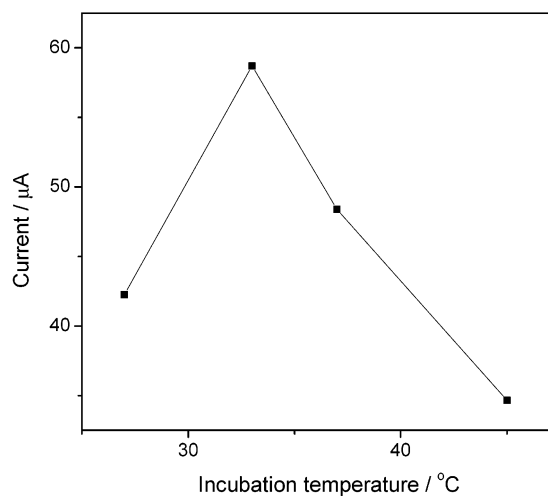


Figure 4. Effect of incubation temperature on immunological reaction at an immunosensor incubated in a HRP-labeled CA 125 antibody solution diluted with the dilution solution at a volume ratio of 5:1.

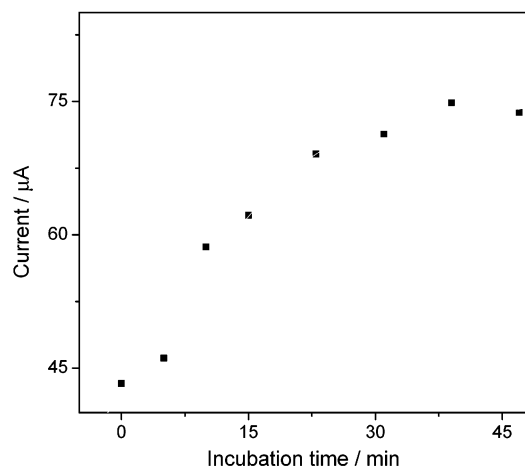


Figure 5. Dependence of DPV peak current for direct electrochemical reduction of the HRP immobilized by the immunological reaction on incubation time in a HRP-labeled CA 125 antibody solution diluted with the dilution solution at a volume ratio of 5:1.

of HRP-labeled CA 125 antibody (Figure 6). When the volume ratio of HRP-labeled CA 125 antibody stock solution to the dilution solution was larger than 5:1 (0.83), the response reached a maximum value and remained at this value at higher ratios, indicating that the amount of HRP-labeled CA 125 antibody in the incubation solution was enough to match the amount of the immobilized CA 125 in the immunosensor.

#### Electrochemical Response to CA 125 Concentration.

Under optimal conditions, the differential pulse voltammograms showed the direct electron transfer of the immobilized HRP at a reduction peak potential of around  $-0.558$  V (Figure 7). When the incubation solutions included CA 125 of different concentrations, the peak current decreased due to the competitive binding of soluble CA 125 and immobilized CA 125 with the limited binding sites of HRP-labeled CA 125 antibody. At the same time, the peak potential shifted slightly to more positive values. With an increasing CA 125 concentration in the incubation solution, the peak current decreased and trended to a minimum value. The peak current decrease was proportional to the CA 125 concentration

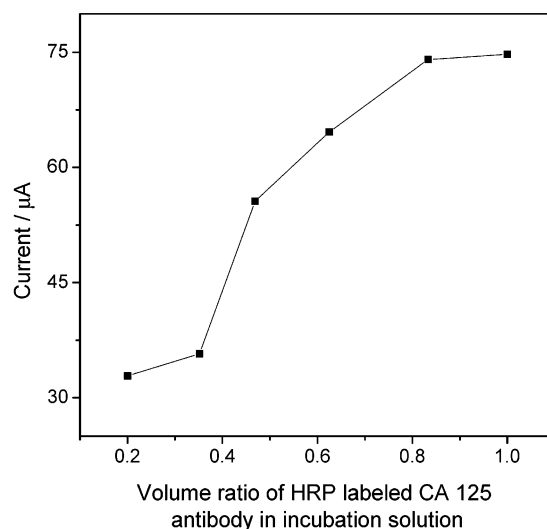


Figure 6. Effect of the concentration of HRP-labeled CA 125 antibody in incubation solution on DPV peak current for direct electrochemical reduction of the HRP label immobilized by the immunological reaction.

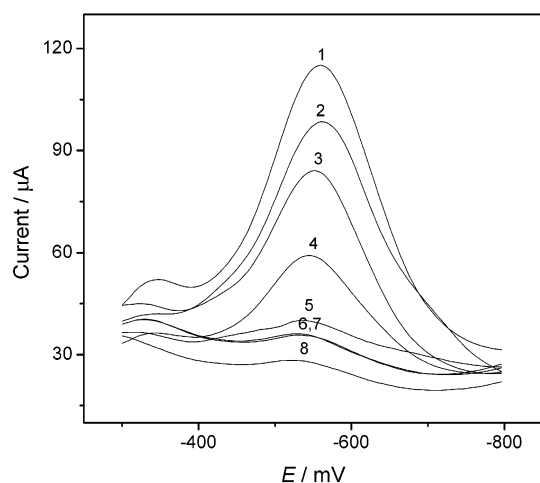


Figure 7. Differential pulse voltammograms for CA 125 immunosensors incubated in the incubation solutions containing HRP-labeled CA 125 antibody and (1) 0, (2) 2.0, (3) 5.0, (4) 8.0, (5) 14.0, (6) 20.0, (7) 30.0, and (8) 40.0 units  $\text{mL}^{-1}$  CA 125 at  $33$  °C for 40 min.

in the range of  $2\text{--}14$  units  $\text{mL}^{-1}$  with a correlation coefficient of  $0.9918$  ( $n = 5$ ) and a linear slope of  $4.63 \pm 0.34$   $\mu\text{A units}^{-1} \text{mL}$  (Figure 8). The detection limit, taken as the concentration equivalent to a 10% decrease in signal,<sup>52</sup> was calculated to be  $1.29$  units  $\text{mL}^{-1}$ . Considering the dilution of CA 125 sample after it was added to the incubation solution, the immunosensor could be used for serum CA 125 detection in the concentration range of  $10\text{--}60$  units  $\text{mL}^{-1}$ . Higher serum CA 125 levels could be detected with an appropriate dilution with pH 7.0 PBS.

**Detection of Serum CA 125 Levels.** The serum CA 125 levels in two samples were detected using the proposed CA 125 immunosensor. From the standard curve and the DPV peak currents of the CA 125 immunosensors incubated in the incubation solutions containing the obtained samples, the mean serum CA 125 concentrations were determined to be  $29.2$  and  $52.3$  units

(52) Gascón, J.; Durand, G.; Barcelo, D. *Environ. Sci. Technol.* **1995**, *29*, 1551–1556.

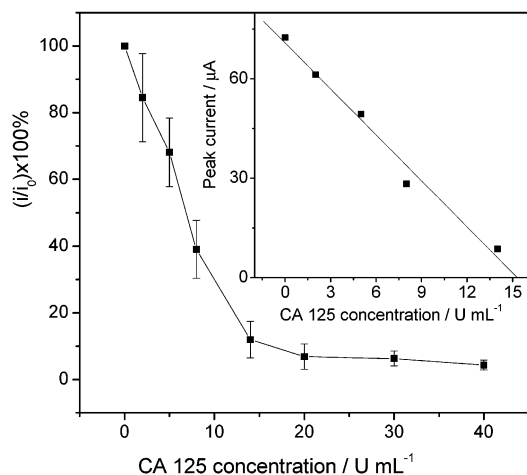


Figure 8. Calibration for CA 125 determination. Inset: plot of the decrease in DPV peak current vs CA 125 concentration.

mL<sup>-1</sup>, respectively, while the values obtained from IRMA were 24.8 and 55.5 units mL<sup>-1</sup>, respectively. They were in acceptable agreement. The relative errors of the results obtained with two methods were 17.7 and 5.8%, respectively. Thus, the proposed method could be satisfactorily applied to the clinical determination of the CA 125 level in human serum.

**Precision, Reproducibility, and Stability of the CA 125 Immunosensor.** The reproducibility of the immunosensors for CA 125 was investigated with intra- and interassay precision. The intraassay precision of the immunosensors was evaluated by assaying the CA 125 levels of three sera for five replicate measurements in the same run. The variation coefficients (CVs) of the intraassay for this method were 13.2, 8.7, and 5.5% at CA 125 concentrations of 2, 8, and 14 units mL<sup>-1</sup>, respectively. The interassay precision, or the fabrication reproducibility, was estimated by determining, in duplicate, the CA 125 level in one serum sample with three immunosensors made at the same electrode independently. The CV of the interassay for this method was 19.8% for the current determined at 8 units mL<sup>-1</sup>, showing an acceptable reproducibility. Thus, the vapor deposition technique could provide a simple method for reproducible preparation of the immunosensor in batches.

When the immunosensor was not in use, it was stored in pH 7.0 PBS at 4 °C. No obvious change was observed after a 15-day storage. This indicated that titania sol-gel prepared by a vapor deposition method, a mild process, provided a biocompatible

microenvironment around the antigen molecule to stabilize its biological activity to a large extent. The large quantities of hydroxyl groups in the sol-gel hybrid material could form strong hydrogen bonds. These hydrogen bonds and the intermolecular interactions between antigen molecules and specific sites of titania sol-gel prevented the immobilized antigen from leaking out of the film. On the other hand, the titania sol-gel could retain its porous structure upon the storage, which made the HRP-labeled antibody able to bind with the immobilized antigen.

## CONCLUSIONS

Serum CA 125 has been widely considered to be an especially valuable serum marker with a threshold value of 35 units mL<sup>-1</sup> expressed by >80% of patients with nonmucinous epithelial ovarian cancer. This work combines the immobilization of CA 125 in a titania sol-gel film by a vapor deposition method and the direct electron transfer of HRP bound by the immunological reaction between the immobilized CA 125 and HRP-labeled CA 125 antibody to develop a novel CA 125 immunosensor. The immunosensor has an acceptable precision, sensitivity, and storage stability and provides a reagentless and mediatorless immunoassay method for the rapid determination of CA 125 without the requirement for separation or washing steps by using a competitive immunoassay scheme. The detection results of CA 125 in serum samples show a good accuracy of this method for practical application in clinical serum CA 125 determination. The acceptable reproducibility indicates the vapor deposition method can fabricate the immunosensor in batches with a very small amount of antigen and at a low cost. Thus, this strategy could be readily extended toward the preparation of other amperometric immunosensors and the detection of other clinically important antigens.

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