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Electrochemical immunoassay for CA125 based on cellulose acetate stabilized antigen/colloidal gold nanoparticles membrane

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Abstract

A novel separation-free electrochemical immunosensor for carcinoma antigen-125 (CA125) was proposed based on the immobilization of CA125 antigen on colloidal gold nanoparticles that was stabilized with cellulose acetate membrane on a glassy carbon electrode. A competitive immunoassay format was employed to detect CA125 antigen with horseradish peroxidase (HRP) labeled CA125 antibody as tracer, *o*-phenylenediamine and hydrogen peroxide as enzyme substrates. After the immunosensor was incubated with a mixture of HRP labeled CA125 antibody and CA125 sample at 35 °C for 50 min, the amperometric response decreased with an increasing CA125 concentration in the sample solution. The decreased percentage of the electrocatalytic current was proportional to CA125 concentration ranging from 0 to 30 U ml^{-1} with a detection limit of 1.73 U ml^{-1} (S/N = 3). The proposed immunosensor showed good stability, acceptable accuracy, and would be applicable to clinical immunoassay of CA125.
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Keywords: Immunosensor; Electrochemical sensors; Biosensors; CA125; Cellulose acetate; Colloidal gold nanoparticles; Immunoassay

1. Introduction

The electrochemical enzyme immunoassay has been an attractive subject for clinical diagnosis [1,2], biochemical [3,4] and environmental [5,6] analyses due to the innate amplification properties of enzymes and the high specificity of recognition between antigen and antibody [7]. A lot of enzymes such as horseradish peroxidase (HRP) [8–10], alkaline phosphatase [11,12], laccase [13], cholinesterase [14], and glucose oxidase [15,16] have been used to label the immunocomponents and improve the sensitivity of immunoassay. In particular, when HRP is used as the label an additional substrate such as hydroquinone [9], *o*-aminophenol [17] or thionine [18] is also added to the detection solution as the mediator to transfer the electron between hydrogen peroxide (H_2O_2) and the enzyme at an electrochemical immunosensor, at which the immunoreaction takes place on the transducer surface, and the electroactive product of the enzyme-catalyzed reaction is then formed to produce the electrochemical signal. As for the construction of an immunosensor, the crucial step is the immobilization of immunoreagent onto the electrode surface [19]. Here a novel immobilization method was proposed for preparation of separation-free immunosensor by the adsorption of carcinoma antigen-125 (CA125) on colloidal gold nanoparticles coated on an electrode, which was stabilized with cellulose acetate membrane.

Cellulose acetate membrane has been widely applied for the immobilization of biomolecules including enzyme [20–22], bacteria [23] and cell [24–26] due to its good biocompatibility and high stability. At the same time, colloidal gold nanoparticles have also been extensively used for cytochemical labeling and immobilization of proteins [27], enzymes [28], antibody [29] and cell [30] to retain efficiently their biological activity. Some works using colloidal gold nanoparticles modified chitosan membrane to immobilize immunoreagent have been reported [31]. However, cellulose

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acetate membrane has not been used for the immobilization of immunoreagent for immunosensor preparation. By combining with colloidal gold nanoparticles this work demonstrated that cellulose acetate membrane could efficiently stabilize immunoreagents on an immunosensor surface, a sensitive and selective immunosensor for CA125 was thus constructed.

CA125 is a useful tumor marker with a threshold value of 35 U ml^{-1} [32] expressed by more than 80% of patients with non-mucinous epithelial ovarian cancer [33]. The CA125 levels are often measured by immunoradiometric assay [34] and enzyme immunoassay [35–38]. These conventional immunoassays have some shortcomings such as time-consuming, high-cost and complicated operation with several separation steps [39]. So the development of fast, low-cost and easy-to-use methods for the determination of tumor markers such as CA125 is important for health protection and medical treatment. This work presented a separation-free competitive immunoassay for CA125 by using HRP as tracer, *o*-phenylenediamine (OPD) and H_2O_2 as substrates. The immobilized CA125 antigen competes with CA125 antigen in sample solution to bind HRP labeled CA125 antibody with a limited amount to form a HRP/CA125 modified surface, which was used for amperometric immunoassay of CA125. The presented CA125 immunosensor showed an acceptable accuracy and was practical, convenient, reliable and significant.

2. Experimental

2.1. Reagents

CA125 enzyme-linked immunoabsorbent assay (ELISA) and IRMA 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^{-1}, a stock solution and a diluent of HRP labeled CA125 monoclonal antibody. The diluent contained pH 7.2 phosphate buffered saline, bovine serum albumin, bovine globulin, Tween 20, sodium dodecylsulfate, a blue inert dye and 0.01% methylisothiazolone. OPD, cellulose acetate (CA, approximately 40% of acetate), and H_2O_2 were from the Shanghai Biochemical Reagent (China). Colloidal gold nanoparticles were prepared by adding 0.5 ml of 1% Na_3citrate solution to 50 ml boiling solution of 0.01% $HAuCl_4$. Prior to use, $HAuCl_4$ and Na_3citrate aqueous solutions were filtered through a 0.22 μm microporous membrane filter. All glassware used in this procedure was cleaned in freshly prepared 1:3 HNO_3–HCl and then rinsed thoroughly in doubly distilled water. The mixture was maintained at boiling point for 15 min and stirred for another 15 min after removing the heating source to produce 24 nm-diameter colloidal gold nanoparticles. The preparation was stored in a brown glass bottle at 4 °C. 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.

2.2. Apparatus

Electrochemical measurements were performed on a CHI 730 electrochemical analyzer (Co. CHI, USA) with a conventional three-electrode system comprised of platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference and HRP/CA125 modified glassy carbon electrode (GCE) as working electrode. IRMA procedure was carried out with a FMJ-182 Immunoradiometric Gamma Counter (China) according to the instruction and assay procedure in the operator's manual.

2.3. Preparation of CA125 immunosensor

The GCE (4 mm diameter) was polished to a mirror finish using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. After successive sonications in 1:1 nitric acid, acetone and doubly distilled water, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature. About 1% CA solution was prepared by dissolving 1.0 g CA in a mixture of 50 ml acetone and 50 ml of cyclohexanone. About 500 U ml^{-1} CA125 solution and colloidal gold nanoparticles with a volume ratio of 1:1 were mixed thoroughly. An amount of 3 μl of this mixture was dropped on the pretreated GCE and dried in a silica gel desiccator. Then 3 μl 1% CA solution was dropped on the electrode surface to form a cellulose acetate membrane for immobilizing CA125 modified colloidal gold nanoparticles (Fig. 1). The obtained immunosensor was stored in pH 7.0 phosphate buffer solution (PBS) at 4 °C.

2.4. Measurement procedure

The stock solution of HRP labeled CA125 antibody was first diluted with the diluent in a volume ratio of 3:2. About 10 μl CA125 standard solutions or serum sample with different concentrations were then mixed with 40 μl diluted HRP labeled CA125 antibody to obtain the incubation solutions, respectively. After the immunosensor for CA125 was incubated in 50 μl incubation solution at 35 °C for 50 min and then washed carefully with doubly distilled water, the electrochemical measurement was recorded in PBS solution containing 1.5 mM OPD and 1.0 mM H_2O_2. The differential pulse voltammetric measurements were from −300 to −800 mV with pulse amplitude of 50 mV and width of 50 ms.

All electrochemical measurements were done in an unstirred electrochemical cell at 20 ± 0.5 °C. The detection solution was bubbled thoroughly with high purity nitrogen for 5 min and maintained in nitrogen atmosphere.

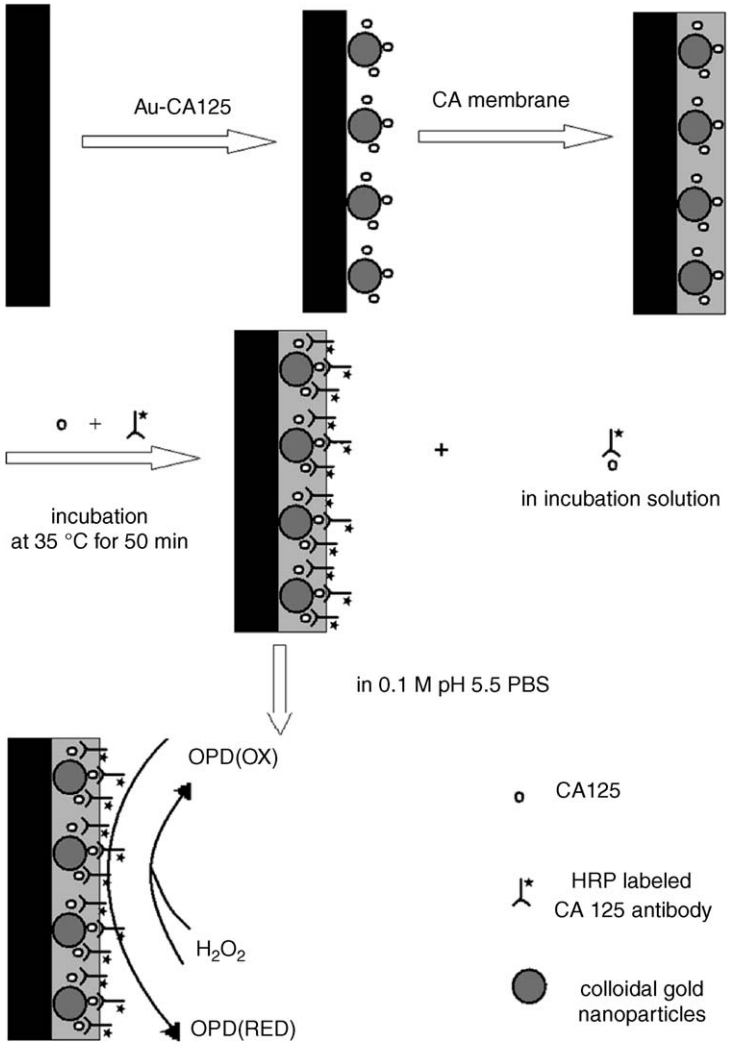

Fig. 1. Principle of CA125 immunosensor based on immobilized antigen, competitive immunoreaction and electrochemical detection.

3. Results and discussion

3.1. Cyclic voltammetric behavior of HRP/CA125 modified electrode

It is well known that HRP can catalyze the oxidation reaction of OPD by H_2O_2 [40]. Fig. 2a shows the cyclic voltammograms of HRP/CA125 modified electrode in different solutions. The HRP/CA125 modified electrode displays a low background current without observable electrochemical response of the immobilized HRP in 0.1 M pH 5.5 PBS (Fig. 2a, curve 1). When 1.5 mM OPD was added into PBS, the cyclic voltammogram shows an oxidation–reduction peak couple of OPD at −0.587 and −0.539 mV at 50 mV s^{-1} (Fig. 2a, curve 2). The peak separation of 38 mV is close to the theoretical value of 29 mV for a reversible diffusion-controlled electrode process with two-electron transfer. Upon addition of 1.0 mM H_2O_2 to the solution, the reduction peak current significantly increases, from 2.51 to 4.18 μA, the oxidation peak current decreases (Fig. 2a, curve 3), and the reduction peak potential of OPD shifts in a negative direction, from −0.587 to −0.601 mV, indicating an electrocatalytic process. Thus, the HRP immobilized in the immunosensor surface retains high enzymatic catalytic activity, and the OPD can effectively shuttle electrons between the redox center of HRP and GCE. The presence of OPD as an electron transfer mediator improved efficiently the sensitivity of ELISA [41]. As control, the cyclic voltammograms of the gold nanoparticles/CA modified electrode after incubation was shown in Fig. 2b, which also displayed a low background current and a couple of redox peaks of OPD. However, upon addition of 1.0 mM H_2O_2 to the solution, no obvious response was observed. When the electrode was modified with cellulose acetate membrane and CA125 antigen without the presence of colloidal gold nanoparticles, no obvious electrocatalytic process was observed, indicating that the colloidal

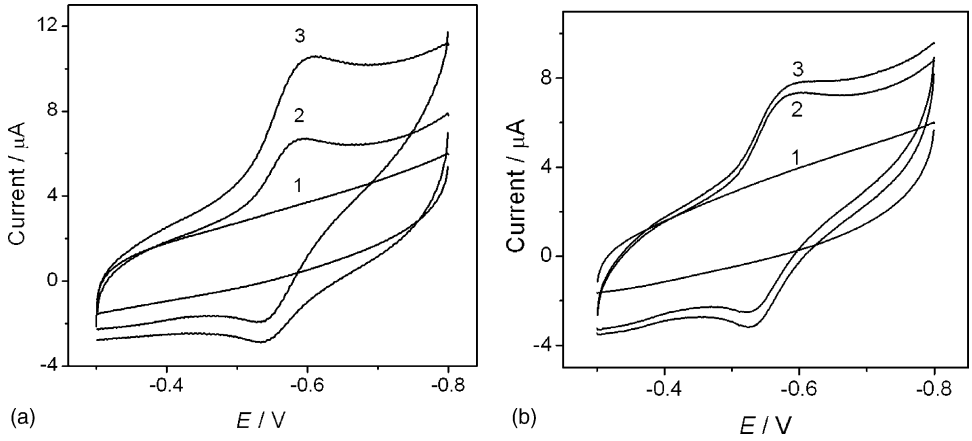

Fig. 2. Cyclic voltammograms of (a) CA125 immunosensor and (b) gold nanoparticles/CA modified electrode after incubated in 50 μl incubation solution containing HRP labeled CA125 antibody at 35 °C for 50 min in (1) 0.1 M pH 5.5 PBS, (2) (1) + 1.5 mM OPD and (3) (2) + 1.0 mM H_2O_2. Scan rate: 50 mV s^{-1}.

gold nanoparticles promoted the enzyme catalytic sites close to the substrate molecules.

3.2. Optimization of conditions for electrochemical detection

The analytical performance of the immunosensor was related to the concentrations of H_2O_2 and OPD in the detection solution and the pH of substrate solution. At a constant concentration of H_2O_2, the response current of the immunosensor after incubation in HRP labeled CA125 antibody increased sharply as the concentration of OPD increased from 0.1 to 1.5 mM and then kept at a maximum value at the concentrations larger than 1.5 mM (Fig. 3a). So 1.5 mM was the optimal concentration of OPD in this work. The effect of H_2O_2 concentration on enzymatic reaction was also examined as shown in Fig. 3b. With increasing H_2O_2 concentration, the differential pulse voltammetry (DPV) peak current of the obtained HRP/CA125 modified electrode in pH 5.5 PBS containing 1.5 mM OPD increased and reached a maximum response at the H_2O_2 concentration of 1.0 mM that could be used for electrochemical immunoassay of CA125. The acidity of the solution greatly affected the enzyme activity for catalytic reaction, thus changed the electrochemical response of the modified electrode. The immunosensor after incubation in HRP labeled CA125 antibody showed the maximum current response over the pH range of 5.0–6.0 in 0.1 M PBS containing 1.5 mM OPD and 1.0 mM H_2O_2, with the maximum response at pH 5.5 (Fig. 3c), So pH 5.5 PBS was selected for immunoassay.

3.3. Optimization of conditions for immunoreaction

At optimized detection conditions the conditions used for immunoreaction greatly affected the electrochemical response for immunoassay. These conditions included the concentration of HRP labeled CA125 antibody in the incubation solution, incubation time and incubation temperature. In order to determine the optimal concentration of the HRP labeled CA125, the immunosensor was incubated with varying concentrations of the HRP labeled CA125. As shown in Fig. 4a, the catalytic peak current of the obtained HRP/CA125 modified electrode increased and then tended to a constant value with an increasing volume ratio of HRP labeled CA125

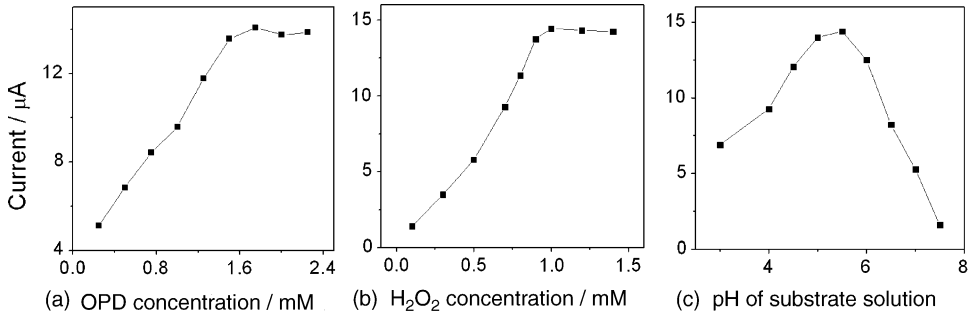

Fig. 3. Dependences of DPV response of the immunosensor after incubated in 50 μl incubation solution containing HRP labeled CA125 antibody at 35 °C for 50 min on (a) OPD concentration in 0.1 M pH 5.5 PBS containing 1.0 mM H_2O_2, (b) H_2O_2 concentration in 0.1 M pH 5.5 PBS containing 1.5 mM OPD and (c) pH of substrate solution in 0.1 M PBS containing 1.5 mM OPD and 1.0 mM H_2O_2.

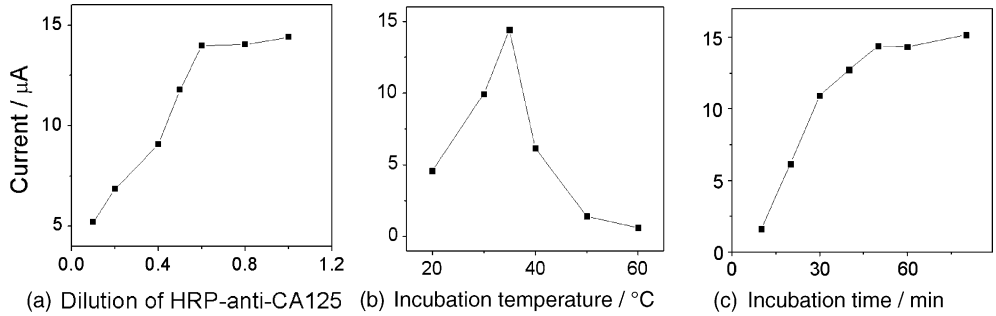

Fig. 4. Effects of (a) dilution of HRP labeled CA125 antibody solution for incubation at 35 °C for 50 min, (b) incubation temperature in 50 μl incubation solution containing HRP labeled CA125 antibody diluted with a volume ratio of 3:2 for 50 min and (c) incubation time in 50 μl incubation solution containing HRP labeled CA125 antibody diluted at a volume ratio of 3:2 at 35 °C on DPV peak current of the immunosensor in 0.1 M pH 5.5 PBS containing 1.5 mM OPD and 1.0 mM H_2O_2.

antibody solution to the dilution solution for preparation of the incubation solution. At a dilution of 3:5, the current reached a maximum value, indicating that the amount of the HRP labeled CA125 antibody in the incubation solution was enough to match the amount of the CA125 immobilized on the GCE.

At the optimized volume ratio, with an increasing incubation temperature from 20 to 60 °C the immunosensor after incubation for 50 min showed a maximum response at 35 °C as shown in Fig. 4b. Thus 35 °C was used for immunoreaction, at which the current response of the obtained HRP/CA125 modified electrode to H_2O_2 and OPD system increased with the increasing incubation time and reached a maximum at 50 min. Longer incubation time did not improve the response (Fig. 4c). Therefore, the incubation time of 50 min was chosen as the optimal incubation condition for the immunoassay of CA125 using the immunosensor.

3.4. Electrochemical response of the immunosensor to CA125 concentration

For the measurement of CA125, a competitive assay configuration was applied under optimized conditions. The standard solution of CA125 at a known concentration or one serum sample was added into the incubation solution containing HRP conjugate solution with a controlled volume ratio. The CA125 antigen in solution competed with the immobilized CA125 antigen in the membrane to bind the limited binding sites of the HRP labeled CA125 antibody. The DPV catalytic peak current of the obtained immobilized HRP to H_2O_2 and OPD system showed a decrease with an increasing CA125 concentration in the incubation solution (Fig. 5). As shown in Fig. 6, the decrease percentage of DPV peak current was proportional to CA125 concentration in the range of 0–30 U ml^{-1} with a correlation coefficient of 0.9975 and a linear slope of 3.2 (±0.1) ml U^{-1}. The detection limit was 1.73 U ml^{-1} (S/N = 3). The linear response range of the immunosensor to CA125 was wider than that of 2–14 U ml^{-1} reported previously [33]. Higher serum CA125 levels could be detected with an appropriate volume of the serum sample added to the incubation solution.

3.5. Detection of serum CA125 levels

The CA125 concentrations in seven serum samples were determined with the immunosensors and the working curve obtained above and compared with the results obtained from immunoradiometric assay. After 10 μl serum sample was added into the 40 μl incubation solution containing HRP labeled CA125 antibody, the immunosensor was incubated in the incubation solution at 35 °C for 50 min. After a washing step with doubly distilled water, the DPV measurement was carried out in PBS solution containing 1.5 mM OPD and 1.0 mM H_2O_2. The CA125 concentrations in these samples were 42, 48, 62, 123, 162, 189, and 218 U ml^{-1}, respectively, while those measured with immunoradiometric assay were 46, 48, 65, 131, 150, 199, and 210 U ml^{-1}, resulting in relative errors of −8.7, 0, −4.6, −6.1, 8.0, −5.0 and 3.8%, respectively. Results obtained with the two methods were

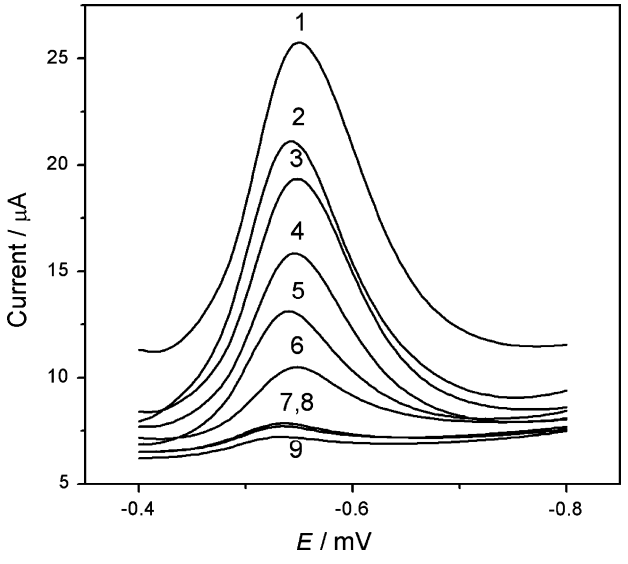

Fig. 5. Differential pulse voltammograms of the immunosensor in 0.1 M pH 5.5 PBS containing 1.5 mM OPD and 1.0 mM H_2O_2 after incubated in 50 μl incubation solutions containing HRP labeled CA125 antibody and (1) 0, (2) 5.0, (3) 10.0, (4) 15.0, (5) 20.0, (6) 25.0, (7) 30.0, (8) 35.0 and (9) 40.0 U ml^{-1} CA125 at 35 °C for 50 min.

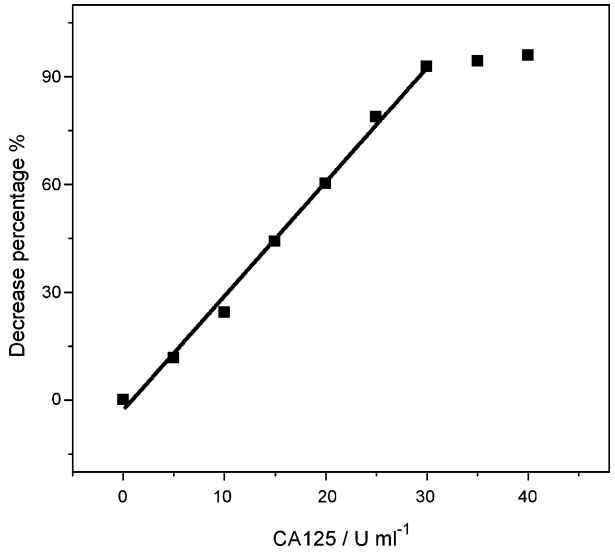

Fig. 6. Calibration curve for CA125 determination. Conditions as the same as in Fig. 5.

in acceptable agreement. Thus, the presented immunosensor could be satisfactorily applied to the clinical determination of CA125 levels in pancreatic cancer samples.

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 variation coefficients of intra-assay with this method were 5.8 and 4.4% at the CA125 concentrations of 10 and 20 U ml^{-1}, respectively, while the inter-assay variation coefficient on five immunosensors used independently was 7.2% at 15 U ml^{-1}, indicating acceptable precision and fabrication reproducibility. When the immunosensor was not in use, it was stored in pH 7.0 PBS at 4 °C. A decrease of 10.3% amperometric response was observed after a 7-day storage. The stability of this sensor was acceptable, thus it was suitable for the determination of CEA in human serum for routine clinical diagnosis.

4. Conclusions

This work describes a novel immunosensor based on CA125/colloidal gold nanoparticles/CA modified GCE. CA125 concentration in sample solution can be detected based on the oxidation reaction of OPD by H_2O_2 due to the formation of immunocomplex on the immunosensor surface after incubation in a solution containing sample and HRP labeled CA125 antibody. The immunosensor permits a reliable determination of CA125 of 0–30 U ml^{-1} with a detection limit of 1.73 U ml^{-1}. The immunosensor shows good accuracy, acceptable sensitivity, precision, reproducibility, and storage stability. Colloidal gold nanoparticles/CA efficiently immobilize antigen and could be used in the preparation of other amperometric immunosensors for the detection of important antigens.

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