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A disposable electrochemical immunosensor for flow injection immunoassay of carcinoembryonic antigen

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Abstract

A new simple immunoassay method for carcinoembryonic antigen (CEA) detection using a disposable immunosensor coupled with a flow injection system was developed. The immunosensor was prepared by coating CEA/colloid Au/chitosan membrane at a screen-printed carbon electrode (SPCE). Using a competitive immunoassay format, the immunosensor inserted in the flow system with an injection of sample and horseradish peroxidase (HRP)-labeled CEA antibody was used to trap the labeled antibody at room temperature for 35 min. The current response obtained from the labeled HRP to thionine–H_2O_2 system decreased proportionally to the CEA concentration in the range of 0.50–25 ng/ml with a correlation coefficient of 0.9981 and a detection limit of 0.22 ng/ml (S/N = 3). The immunoassay system could automatically control the incubation, washing and current measurement steps with good stability and acceptable accuracy. Thus, the proposed method proved its potential use in clinical immunoassay of CEA.

Keywords: Flow injection analysis; Biosensors; Immunoassay; Disposable immunosensor; Carcinoembryonic antigen; Chitosan; Screen-printed electrode

1. Introduction

The determination of tumor makers, the potential prognostic factors for tumors, plays an important role in clinical research and diagnosis (Faraggi and Kramar, 2000). The lever of tumor makes in human serum can give information of the disease stage, the grade of metastasis and the recidivism of tumor (Szilvas et al., 2001). Carcinoembryonic antigen (CEA) is an important antigen associated with colon cancer (Gold and Freedman, 1965; Schlageter et al., 1998; Duffy et al., 2003), lung cancer (Kleisbauer et al., 1996; Hernandez et al., 2002), ovarian carcinoma (Engelen et al., 2000) and breast cancer (Bremer et al., 1995; Jezersek et al., 1996; Sahin et al., 1996). A variety of methods and strategies have been reported for the determination of CEA, such as radioimmunoassay (Szturmowicz et al., 1995; Villena et al., 1996; Behera et al., 1997), enzyme immunoassay (Alumanda and Minoru, 1995; Pina et al., 2001) and fluoroimmunoassay (Yuan et al., 2001). However, these methods are relative to radiation hazards, time-consuming, qualified personnel and sophisticated instrumentation (Darain et al., 2003). Thus, alternative approaches are desirable. Here, a flow injection immunoassay (FIIA) method was developed as an alternative by combining a disposable electrochemical immunosensor with flow injection analysis (FIA).

FIA has the characteristics of rapidity, precision and accuracy due to the high degree of control and constancy of analytical parameters (Zhang et al., 2001). Combined with the high selective immunoassay, the methodology has been widely used in food (Marquette et al., 1999; Nandakumar et al., 2000; Badea et al., 2004), environmental (Liao, 1996; Wilson et al., 1997) and clinical assays (Bereczki and Horvath, 1999; Lin et al., 2004a,b). Recently, FIA has been combined with immunosensors based on screen-printed electrodes (SPCEs) for amperometric detection of mouse IgG (Valat et al., 2000; Gao et al., 2003), gentamicin (Van Es et al., 2001) and pneumolysin (Alvarez et al., 2002). The technology for preparation of SPCEs is sim-

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ple, inexpensive, versatile, friendly-used and mass-produced (Alvarez et al., 2002). The screen-printed biosensor challenges the conventional electrochemical biosensors for disposability and portability. Therefore, it had been widely used in the determination of biomolecules (Hart et al., 2004), pesticides (Ivanov et al., 2003; Touloupakis et al., 2005), antigens (Laschi et al., 2003; Yu et al., 2004) and nucleic acid (Hernandez-Santos et al., 2004). This work prepared one novel disposable electrochemical immunosensor based on SPCE by immobilizing antigen in a biocompatible colloidal Au/chitosan membrane.

In recent decades, chitosan, a kind of natural biopolymer, has been widely used as an immobilization matrix due to its attractive properties of biocompatibility, biodegradability, nontoxicity, high mechanical strength and excellent film-forming ability (Wang et al., 2003; Luo et al., 2004; Liu et al., 2005; Zhang and Gorski, 2005a,b). The colloidal Au nanoparticles can offer an environment similar to nature and retain the bioactivity of immobilized biomolecules (Tang et al., 2004), provide a high surface to volume ratio and enhance the electron transfer kinetics by giving more freedom to the immobilized biomolecules in orientation, which makes the active sites closer to the conducting electrode and permits the biomolecules to orient in conformations more favorable for direct electron transfer (Liu et al., 2003). In order to enhance the amperometric response of the immunosensor, a composite of colloidal Au nanoparticles doped chitosan was prepared for immobilization of immunoreagents.

The purpose of this paper is to describe the design, fabrication and application of the disposable immunosensor and develop a FIIA system based on the SPCE modified with a biocomposite film consisting of CEA, colloidal Au and chitosan. The system was capable of continuously carrying out all steps in less than 40 min for one sample, including incubation of 35 min, washing, enzymatic reaction and determination procedures. The quantitative detection of CEA showed excellent analytical performance and good consistency with the reference method. This method was advantageous in miniaturization, portability, programmable operation and no need of skilled operator, thus could further be used for clinical immunoassay of CEA.

2. Materials and methods

2.1. Reagents

CEA standard solutions from 0 to 500 ng/ml were purchased from Wallac Oy (Turku, Finland). HRP-labeled anti-CEA solution was from Everlong (California, USA). Chitosan (MW 1.9×10^5–3.1×10^5; 85–90% deacetylation) was obtained from Aldrich Inc. (USA). Thionine and H_2O_2 (analytical reagent grade) were from Shanghai Biochemical Reagent Company (China). All other reagents were of analytical reagent grade and used without further purification. 0.2 M phosphate buffer solutions (PBS) of various pHs were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M NaOH and H_3PO_4. Doubly distilled water was used in all experiments. Serum specimens provided by Jiangsu Institute of Cancer Prevention and Cure were stored at 4 °C.

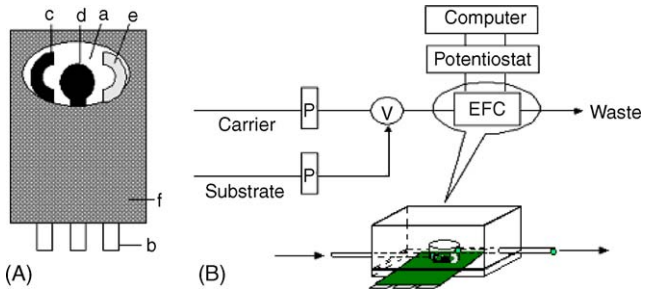

Fig. 1. Schematic diagrams of (A) three-electrode SPCE system and (B) the flow system. (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) graphite working electrode, (e) Ag/AgCl reference electrode and (f) insulating dielectric; (P) peristaltic pump, (V) eight-port rotary injection valve, (EFC) electrochemical flow-through cell.

2.2. Preparation of SPCE and immunosensor

Schematic diagram of the three-electrode SPCE system with graphite working electrode (2 mm in diameter), graphite auxiliary electrode and Ag/AgCl reference electrode is shown in Fig. 1A. The SPCE was fabricated according to the steps reported previously (Yu et al., 2004). Firstly the silver ink was screen-printed on a nylon sheet as conductive bands. Then the graphite ink was imprinted to cover the area that server as the working and auxiliary electrode. After that the silver chloride ink was applied onto the area of reference electrode. Finally, the conductive bands were insulated by overlaying an insulating dielectric material except on the electric connectors and the three-electrode areas.

Chitosan solution (1 wt%) was prepared by ultrasonically dissolving chitosan powder in 1% acetic acid. Twenty-four-nanometer-diameter colloidal gold nanoparticles was prepared according to literature (Liu and Ju, 2002). CEA (500 ng/ml), as-prepared colloidal gold nanoparticles and 1% chitosan solutions with volume ratios of 1:1:1 were mixed thoroughly. After the mixture was placed at 4 °C for 12 h, 1 μl of this mixture was dropped on the working electrode. After drying at room temperature for 5–6 h, the resulting CEA/colloidal Au/chitosan/SPCE was thoroughly rinsed with doubly distilled water prior to use.

2.3. Instrumentation

The disposable immunosensor was connected to the flow injection system illustrated in Fig. 1B. The flow injection system comprised of two peristaltic pumps (BT100-1J, Baoding Longer Precision Pump Co. Ltd., Baoding, China), an eight-port rotary injection valve and a homemade flow cell. Polytrafluoroethylene tubing (0.8 mm i.d.) was used to connect all components in the flow system. Amperometric and cyclic voltammetric experiments were performed with a CHI 730 electrochemical analyzer (CHI Co., USA). All experiments were carried out with the three-electrode SPCE system. The reference values of the serum were obtained with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche, Switzerland).

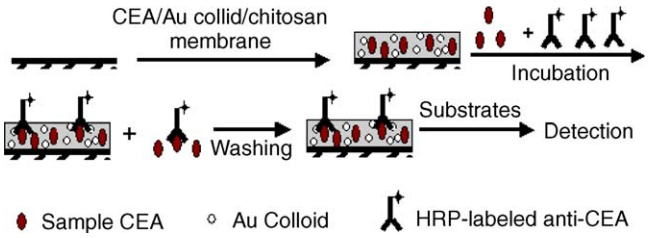

Fig. 2. Schematic diagram of the immunoassay procedure.

2.4. Analytical procedure

The immunoassay procedure was shown in Fig. 2. The CEA/colloidal Au/chitosan/SPCE was linked to the flow system and connected the potentiostat. The stock solution of HRP-labeled anti-CEA was first diluted with 0.2 M pH 7.0 PBS in a volume ratio of 1:25. Then, 100 μl of diluted HRP-labeled anti-CEA solution was mixed with CEA standard solution or serum sample to act as incubation solution. After the incubation solution was injected into flow cell and flow over the immunosensor, it was retained in the cell with a stop flow technique for a duration of 35 min at room temperature. The residual was removed with doubly distilled water. 0.2 M PBS (pH 7.0) was then delivered with a flow rate of 3.6 ml/min and a potential of −0.4 V (versus Ag/AgCl) was applied to obtain a stable chronoamperometric baseline. Finally 170 μl mixture containing 0.24 mM thionine and 6.0 mM H_2O_2 in 0.2 M PBS (pH 7.0) was injected into the flow cell with the aid of the valve and the chronoamperometric signals were recorded. The detection solution was bubbled thoroughly with high purity nitrogen for 5 min prior to injection.

The differential pulse voltammetric measurements of CEA were curried out from −100 to −550 mV with the pulse amplitude of 50 mV and the pulse width of 50 ms in 0.2 M PBS (pH 7.0) containing 0.24 mM thionine and 6.0 mM H_2O_2 after the immunosensor was incubated in incubation solutions containing different CEA concentrations.

3. Results and discussion

3.1. Morphologies of CEA/colloidal Au/chitosan film on SPCE

The surface morphology of the film is a vital factor affecting the immunosensor performance. Fig. 3 shows the morphologies of bare, colloidal Au nanoparticles doped chitosan composite and CEA/colloidal Au/chitosan membrane modified SPCEs by scanning electron microscope (SEM). The surface of bare SPCE was inhomogeneous and uneven (Fig. 3a), while the membrane of colloidal Au nanoparticles doped chitosan composite showed a relatively homogenous porous structure with a narrow particle size distribution resulted from the doped colloidal Au nanoparticles (Fig. 3b). This uniform open structure could significantly increase the effective electrode surface for CEA loading. When CEA was mixed with the composite, the formed membrane retained the uniform open structure and showed bright particles of CEA with an islandlike structure due to its aggregation (Fig. 3c). Because of the porous structure and the excellent properties of the colloidal Au doped chitosan membrane, it retained the bioactivity of CEA to produce good analytical performance of the immunosensors.

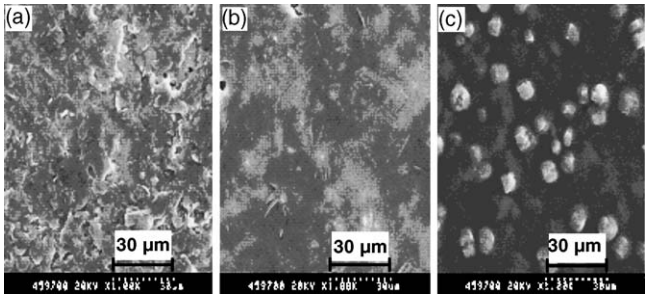

Fig. 3. SEM images of (a) bare, (b) colloidal Au doped chitosan membrane and (c) CEA/colloidal Au/chitosan composite membrane modified SPCEs.

3.2. Electrocatalysis of the immunosensor for oxidation of thionine by H_2O_2

For further electrochemical measurements, the immunosensor was inserted into 0.2 M pH 7.0 PBS with or without substrates. As shown in Fig. 4, no detectable cyclic voltammetric response of CEA/colloidal Au/chitosan modified SPCE was observed in 0.2 M pH 7.0 PBS (curve a). After the modified SPCE was incubated in 100 μl 1:25 diluted HRP-labeled anti-CEA solution at 24 °C for 35 min, the obtained immunosensor displayed a low background current without observable electrochemical response of the immobilized HRP in 0.2 M pH 7.0 PBS (curve b). When 0.24 mM of thionine was added into PBS, the cyclic voltammogram of the immunosensor showed a pair of stable and well-defined oxidation–reduction peaks at −362 and −281 mV for a scan rate of 50 mV/s (curve c). These values were in agreement with those reported for the redox of thionine (Yu et al., 2004). At CEA/chitosan modified SPCE without

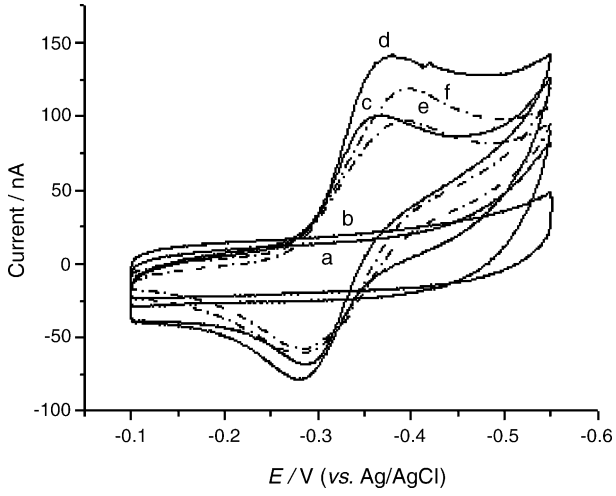

Fig. 4. Cyclic voltammograms of (a) CEA/colloidal Au/chitosan and (b) HRP-labeled anti-CEA/CEA/colloidal Au/chitosan modified SPCEs in pH 7.0 PBS, (c) (b) + 0.24 mM thionine, (d) (c) + 6.0 mM H_2O_2, (e) HRP-labeled anti-CEA/CEA/chitosan modified SPCEs in pH 7.0 PBS containing 0.24 mM thionine and (f) (e) + 6.0 mM H_2O_2 at 50 mV/s.

the presence of colloidal Au nanoparticles, the redox couple peaks occurred at −394 and −281 mV with a peak separation of 113 mV (curve e). Compared with the peak separation of 81 mV at CEA/colloidal Au/chitosan modified SPCE, the presence of colloidal Au nanoparticles in the immunosensor improved the electrochemical reversibility of thionine.

Upon addition of H_2O_2 to the solution containing thionine, the reduction peak currents at both CEA/colloidal Au/chitosan and CEA/chitosan modified SPCEs significantly increased, the oxidation peak currents decreased and the reduction peak potentials shifted slightly in negative direction (curves d and f in Fig. 4), indicating a typical electrocatalytic process, which was attributed to the enzymatic catalysis of the immobilized HRP to the oxidation reaction of thionine by H_2O_2. This result indicated the HRP immobilized in the immunosensor surface could retain high enzymatic catalytic activity. The mechanism of enzymatic catalysis was similar to that reported previously (Ruan et al., 1998; Dai et al., 2004). The electrocatalytic currents (the increase in reduction peak currents upon addition of H_2O_2) at two immunosensors with and without presence of colloidal Au nanoparticles were 40.7 and 17.2 nA, respectively. Obviously the presence of the nanoparticles increased the electrochemical response of the immunosensor.

From the peak potential of the enzymatic system, an applied potential of −0.4 V (versus Ag/AgCl) was chosen for amperometric FIIA of the immunosensor for CEA.

3.3. Optimization of detection system

FIIA performance depended not only on the immunoassay procedure, but also on the flow rate of the carrier solution and the concentration of the substrates. A too high flow rate would damage the membrane immobilized on the electrode and produce unstable detection signal, while the detection signal suffered from tailing at a slow flow rate. Here, a flow rate of 3.6 ml/min was used for immunoassay of CEA.

The substrates used for FIIA of CEA were thionine and H_2O_2. The effects of thionine and H_2O_2 concentrations on detection signal were studied in detail. As seen in Fig. 5, under optimal conditions the currents reached the maximum values at the thionine and H_2O_2 concentrations of 0.24 mM (Fig. 5A) and 6.0 mM (Fig. 5B), respectively. So, the optimal concentrations of thionine and H_2O_2 were selected at 0.24 and 6.0 mM, respectively.

The pH value of the detection solution is also an important parameter. The acidity of the solution greatly affects the catalytic activity of the enzyme. Most enzymes display good activity only in a limited range of pH. Fig. 5C shows effect of pH on the current response of the immunosensor in 0.2 M PBS containing 0.24 mM thionine and 6.0 mM H_2O_2. The maximum current response occurred at pH 7.0, which was chosen for the whole experiment.

3.4. Optimization of immunoassay procedure

Under optimal detection conditions, the electrochemical response depends on the formation of immunocomplex on the electrode surface. The effect factors included the incubation time, incubation temperature and the working concentration of HRP-labeled anti-CEA. In order to gain the optimal concentration of the HRP-labeled anti-CEA, the CEA/colloidal Au/chitosan modified SPCEs were incubated in HRP-labeled anti-CEA solutions with different dilutions. With the decreasing of dilution or the increasing of HRP-labeled anti-CEA concentration the FIIA response increased and then trended to a plateau at the dilution of 1:25, which indicated that all the specific sites of immobilized CEA were matched with the enzyme conjugate. Thus, 1:25 dilution of HRP-labeled anti-CEA was used for incubation step.

The effect of incubation temperature was studied in the temperature range from 14 to 34 °C. The catalytic peak current increased with an increasing incubation temperature, and reached the maximum response at a temperature of 24 °C. In order to simplify the analytical process, all the experiments were carried out at room temperature. At this temperature, the amperometric response of the immobilized HRP to thionine and H_2O_2 system increased with the increasing incubation time and reached a maximum at 35 min, which was chosen as the optimal incubation time for the immunoassay of CEA.

With a competitive immunoassay format, the differential pulse voltammetric peak current of the immunosensor in thionine and H_2O_2 system decreased with the increasing CEA concentration in incubation solution (Fig. 6a). The peak decrease was proportional to the CEA concentration in the range of

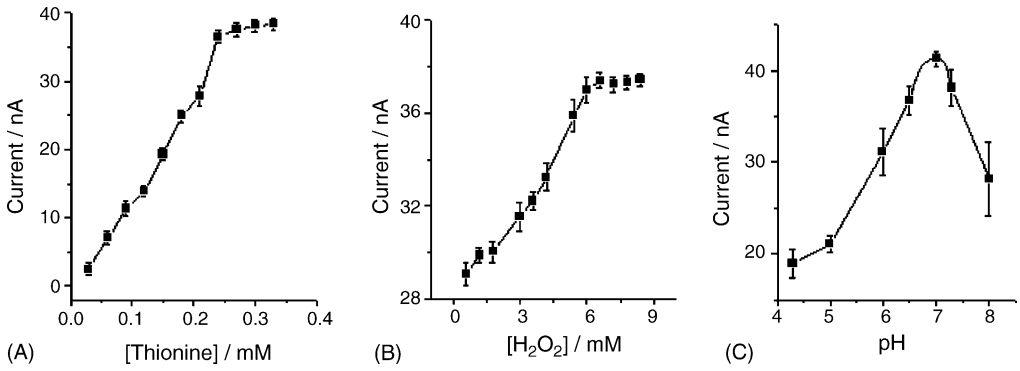

Fig. 5. Dependence of amperometric response of immunosensor on thionine concentration (A), H_2O_2 concentration (B) and pH (C) of detection solution at an applied potential of −0.4 V under optimal conditions.

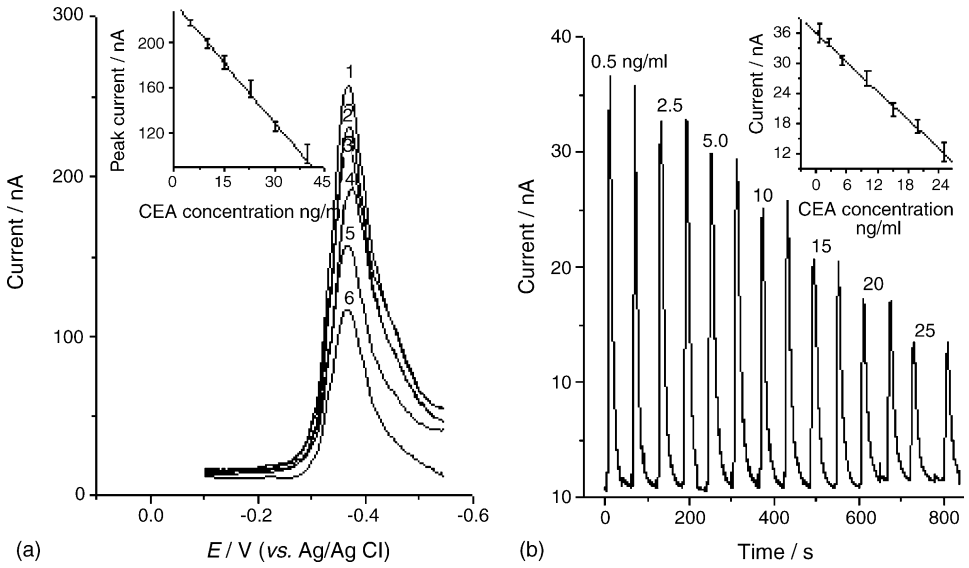

Fig. 6. Differential pulse voltammograms of immunosensor in stop flow system for (1) 5, (2) 10, (3) 15, (4) 20, (5) 30 and (6) 40 ng/ml CEA concentrations (a) and FIA diagram at −0.4 V at different CEA concentrations (b) from competitive immunoassay of CEA under optimal conditions. *Inset*: Linear relation between relative current response and CEA concentration.

5–40 ng/ml with a correlation coefficient of 0.9959 (inset in Fig. 6a). The detection limit was 0.45 ng/ml at a signal to noise ratio of 3.

3.5. Detection of CEA in flow injection system

Under the optimal conditions, the typical flow injection response was obtained with a competitive assay configuration. Any change of the variables from their optimal values would reduce the analytical performance of FIIA. 1:25 diluted HRP-labeled anti-CEA solutions containing different concentrations of CEA were injected into the flow system and flow over the CEA/colloidal Au/chitosan modified SPCEs, which were incubated with a stop flow mode for 35 min. The CEA in the solution competed with the immobilized CEA in the membrane to bind the limited binding sites of the HRP-labeled anti-CEA. The current response obtained from the enzymatic catalysis of immobilized HRP to thionine and H_2O_2 system decreased proportionally to the CEA concentration in the range of 0.50–25 ng/ml (Fig. 6b) with a correlation coefficient of 0.9981 and a linear slope of 0.95 nA/ng/ml (inset in Fig. 6b). The detection limit was 0.22 ng/ml at a signal to noise ratio of 3, lower than the result of differential pulse voltammetric measurement in a stop flow system and the threshold value of 2.5 ng/ml for clinical diagnosis.

3.6. Precision, reproducibility, repeatability, stability of the immunosensor

The inter-assay precision of the immunosensor was evaluated using five modified electrodes. The coefficient of variation for inter-assay on this method was 7.8% at 10 ng/ml and 6.3% at 20 ng/ml, indicating acceptable precision and fabrication reproducibility. The average intra-assay coefficient of variation of this method was 2.0% (Fig. 6b), and the residual deviation of the slopes of two calibration curves was 1.8%, showing a good repeatability. When the immunosensor was stored in air at 4 °C, it could keep its initial response for 15 days, and the amperometric response was 92% of initial response after a storage period of 25 days. Thus, the immunosensor had acceptable storage stability, and was suitable for the clinical diagnosis.

3.7. Accuracy and clinical application

The accuracy of CEA determination was examined by comparing the results obtained from five sera with this method and the commercial electrochemiluminescent analysis method. The CEA concentrations determined with this method were 3.8, 7.5, 10.2, 16.5 and 19.6 ng/ml, while the values obtained from a recognized and commercial electrochemiluminescent analysis performed in Jiangsu Institute of Cancer Prevention and Cure for clinical diagnosis were 3.1, 7.8, 11.0, 16.0 and 19.4 ng/ml, respectively. These results indicated the present method is in good agreement with the electrochemiluminescent method. Thus, the present method could satisfy the need of immunoassays of CEA in clinical diagnosis.

4. Conclusions

This paper presents a novel simple immunoassay method for CEA based on a disposable immunosensor integrated to a flow injection electrochemical system. The immunosensor is prepared by immobilization of antigen on a SPCE with colloidal gold nanoparticles doped chitosan composite. The colloidal gold nanoparticles improve the reversibility of the electrochemical reaction of substrate and increase the sensitivity of the immunosensor to CEA detection. The integration of the disposable immunosensor and the flow cell meets the demand of facil-

itating the electrochemical immunoassay process. This process includes programmable incubation step of 35 min, washing and current measurement steps of less than 5 min. The immunosensor shows good accuracy, acceptable precision, reproducibility and satisfiable storage stability. Summarily, the proposed method had potential to be further developed for practical clinic detection of serum CEA level.

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