Ferrocenyl-doped silica nanoparticles as an immobilized affinity support for electrochemical immunoassay of cancer antigen 15-3

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1. Introduction

CA 15-3, as a breast-associated mucin, is often elevated in the majority of breast carcinoma patients with distant metastases. Currently, the main application of this marker is to monitor and detect recurrences in patients with diagnosed breast carcinoma [1, 2]. The CA 15-3 levels are often measured by immunoradiometric assay [3], capillary electrophoretic (CE) analysis [4], chemiluminescence immunoassay [5], and optical immunoassay [6]. Conventional immunoassay systems have some shortcomings, e.g. time-consuming, high-costs and complicated operations in combination with several separation steps. Thus the development of fast, low-cost and easy-to-use methods for the determination of CA 15-3 is important for health protection and medical treatment.

In order to improve the sensitivity of direct immunoassay, electrochemical measurements at electrode interfaces have gained most attentions as its natural advantages such as simple, offer low detection limits and fast response times at relatively low-costs [7–12]. As a result, redox mediators [13] like ferrocene derivatives [14], ferrocyanide [15], or osmium complexes [16], which was an important element of electrochemical immunosensor fabrication, have attracted great interests. However, the major problem due to the leakage of the redox activities compounds with low-molecular weight is to remain the long-term stability of the artificial redox activities compounds [17–19].

Nanostructures have been used extensively as immobilized materials for the fabrication of biosensor [20–22]. The combination of nanotechnology and biotechnology for the detection of biological elements has brought new opportunities. Dye-doped silica nanoparticles [23–25] and Ru(bpy)32+-doped silica nanoparticles have been developed for immunochromatographic assays or electrogenerated chemiluminescence (ECL) [26]. In this work, a redox nanoparticle based on the FC-COOH-doped SNPs with a core–shell structure incorporated FC-COOH core in the silica matrix shell was synthesized for the first time. Three-dimensional network structure of silica shell protects the FC-COOH molecules from the leakage, increases electrostability and possesses redox activities. Furthermore, the facility of introducing functional groups, such as amino groups, mercaptos and carboxyls, to the surface of FC-COOH-doped SNPs makes it easily conjugate with biomolecules, so it was an ideal material for bioanalytical applications.

In the present paper, a simple and effective approach was developed to synthesize amino functionalized FC-COOH-doped SNPs. Based on its features of excellent biocompatibility and easy modification to couple with biomolecules, an amperometric immunosensor for fast detection of CA 15-3 was constructed. In our work, a water-in-oil (W/O) microemulsion method was used to develop FC-COOH-doped SNPs in which the FC-COOH were homogeneously dispersed and tightly captured in a three-dimensional cage of the silica network. Then, the surface of FC-COOH-doped SNPs were functionalized with amino groups by APTEOS through Si–O–Si bonding, and then FC-COOH-functionalized SiNPs were used as the immobilized support for couple with the target antigen. The FC-COOH-functionalized SiNPs was characterized by X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM), and the immobilization of the target antigen was demonstrated by using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques. Under optimal conditions, the developed immunosensor showed good linearity at the studied concentration range of 2.0–240 U mL−1 with a coefficient 0.9896 and a detection limit of 0.64 U mL−1 at S/N = 3.

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bonds [25]. The experimental results showed that the nanoparticles were uniform in size and the Fc-COOH did not leak out in aqueous solution, the reason may be the fact that strong hydrogen bonding forces between the Fc-COOH and silica. With amino groups on the surface, the nanoparticles were easily immobilized with CA 15-3 antibodies through covalent conjugation using glutaraldehyde. After CA 15-3 antibodies immobilization, BSA was used to block possible remaining active sites. The proposed electrode was then employed for detection CA 15-3. The experimental results indicated that this method was suitable for immunoassay.

2. Materials and methods

2.1. Chemicals and solutions

CA 15-3 (0–250 U mL\(^{-1}\)) and CA 15-3 monoclonal antibody (CA 15-3 antibodies) were purchased from Biocell Co. (Zhengzhou, China). Triton X-100 (TX-100), bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT, 99%) and bovine serum albumin (BSA, 96–99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tetraethyl orthosilicate (TEOS) was obtained from Beijing Yili Chemical Reagent Factory (Beijing, China); 3-aminopropyltriethoxysilane (APTEOS, 97%) was purchased from Aldrich. 1-Hexanol, cyclohexane, glutaraldehyde, and ammonium hydroxide (25 wt%) were purchased from Beijing Chemical Reagent Factory (Beijing, China). All chemicals and solvents that used are analytical grade. Double distilled water was used in the whole study. The serum samples were obtained from 22 clinically diagnosed patients with ovarian cancer. Blood samples were collected in plain tubes, centrifuged to separate the serum. The serum samples were stored at \(-20^\circ\)C until analysis.

2.2. Preparation of amino functionalized Fc-COOH-doped SNPs

Fc-COOH-doped SNPs were synthesized using a W/O or reverse microemulsion method [22,27]. First, 1.77 g of Triton X-100, 1.6 mL of hexanol, 7.5 mL of cyclohexane, 80 \(\mu\)L of a 0.1 M aqueous Fc-COOH solution, 400 \(\mu\)L of water, and 100 \(\mu\)L of aqueous ammonia was mixed and stirred for 30 min at room temperature, and then 100 \(\mu\)L of TEOS was added. The aqueous ammonia served as both a reactant (\(H_2O\)) and a catalyst (NH\(_3\)) for the hydrolysis of TEOS. The mixture was allowed to stir for 24 h, followed by addition of ethanol to break the microemulsion and recover the particles. After it, the Fc-COOH-doped SNPs were washed with ethanol and water several times to remove any surfactant molecules and physically adsorbed Fc-COOH on the surface of the particles.

The Fc-COOH-doped SNPs were functionalized with APTEOS by quickly adding 2.3 mL of APTEOS to 120 mL of a vigorously stirred dispersion of Fc-COOH-doped SNPs in ethanol, and the mixture was allowed to stir overnight at room temperature. The resultant amino-terminated spheres were washed with water five times by resuspension/centrifugation.

2.3. Preparation of the immunosensor

A gold electrode (4 mm in diameter) was firstly polished to a mirror-like surface repeatedly with 1.0 and 0.3 \(\mu\)m alumina slurry, followed by successive sonication in bi-distilled water and ethanol for 5 min and dried in air. Then, the electrode was placed in a solution of 0.5 M \(H_2SO_4\) and 10 voltammetric cycles were carried out between \(-0.4\) and 1.5 V at 50 mV s\(^{-1}\) (vs. SCE). Prior to modification, 50 mg amino functionalized Fc-COOH-doped SNPs resuspended in 1 mL ethanol and sonicated for 10 min. Afterwards, 10 \(\mu\)L of the suspension was added onto the cleaned gold electrode. After drying at room temperature, the modified electrode was immersed into 0.75% glutaraldehyde solution for 1 h and washed with 10 mmol L\(^{-1}\) PBS, then immersed into CA 15-3 antibodies solution for 1 h at 4°C. Finally, the proposed electrode was incubated in BSA solution for about 30 min at 4°C to block possible remaining active sites. The obtained electrode was stored at 4°C when not in use. The schematic diagram of the stepwise procedure of the immunosensor is shown in Scheme 1.

2.4. Experimental measurements

Cyclic voltammetric (CV) measurements were performed at a CHI 660A electrochemical workstation (Shanghai Chenhua Instrument Co., China) using a conventional three-electrode electrochemical cell (contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and a modified gold electrode as working electrode) in pH 7.0 PBS buffer solution with the potential between 0 and 0.6 V at 50 mV s\(^{-1}\). Electrochemical impedance spectroscopy (EIS) measurements were carried out in the presence of a 1.0 mM \(K_2[Fe(CN)_6]\)/\(K_4[Fe(CN)_6]\) (1:1) mixture as a redox probe in 0.1 M PBS buffer solution (pH 7.0) at a bias potential of 0.17 V. The alternative voltage was 5 mV and the frequency range is 50 mHz to 10 kHz. The pH measurements were made with a pH meter (MP 230, Mettler-Toledo, Switzerland) and a digital ion analyzer (Model PHS-3C, Dazhong Instruments, Shanghai, China).

X-ray photoelectron spectroscopy (XPS) measurements were carried out using a VGScientific ESCALAB 250 spectrometer, using Al K\(_\alpha\) X-ray (1486.6 eV) as the light source. Transmission electron micrograph (TEM) images were recorded on a JEOL-JEM 200CX.

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Scheme 1. Schematic illustration of the stepwise fabrication process of the immunosensor; (a) formation of amino functionalized Fc-COOH-doped SNPs layer; (b) glutaraldehyde cross-linkage; (c) anti-CA 15-3 loading; (d) BSA loading.
transmission electron microscope, using an accelerating voltage of 200 kV (Hitachi Instrument, Japan). The detection is based on the change of the amperometric response ($\Delta i_p$) before and after antigen–antibody reaction. Before the immuno-interaction happened, it facilitates electron-transfer pathway as the antibody molecular is relative small compared with antigen. After the antigen interacts with antibody, an insulation monolayer of the immunocomplexes would be formed which may hinder the electron-transfer pathway of Fc-COOH. As a result, $\Delta i_p$ was depended on the degree of the immuno-reaction. After incubated in CA 15-3 solution with various concentrations for 16 min at room temperature, the CVs of the immunosensor was recorded in 0.1 M pH 7.0 PBS buffer solution. After each immunoassay run, the contaminated immunosensor was regenerated by simply immersing in a stirred 0.2 M glycine–hydrochloric acid (Gly–HCl) buffer solution (pH 3.8) for about 5 min.

3. Results and discussion

3.1. XPS and TEM characteristics of the amino functionalized Fc-COOH-doped SNPs

In this paper, the biomolecules were immobilized onto the surface of amino functionalized Fc-COOH-doped SNPs. So the synthesis of nanoparticles is very important. The chemical composition of nanoparticles was studied with XPS analysis. A typical spectrum of amino functionalized Fc-COOH-doped SNPs was given in Fig. 1, showing the peaks of Si 2p (103.4 eV), O 1s (532.8 eV) and N 1s (399.2 eV). No characteristic peak of iron in Fe 2p region was observed (Fig. 1, inset), indicating that the metallic iron particles had been coated by silica but not simply mixed with Fc-COOH and SiO$_2$ particles [28,29].

The TEM was employed to characterize the amino functionalized Fc-COOH-doped SNPs. As shown in Fig. 2, the core–shell structural particles were rather spherical and uniform with a darksome fringe as the Fc-COOH was aggregated to the water pool in the microemulsion while the silica network formed outside. The Fc-COOH molecules can be observed as dark dots embedded inside the silica network as a result of the presence of the heavy metal iron atom. We have not observed such dark dots in the pure silica particles at the same TEM resolution.

3.2. Electrochemical characteristics of different electrodes

We carried out continuously scanning 100 cycles of CVs to test the validity of the electroactiivity stability. After the amino functionalized Fc-COOH-doped SNPs were dropped on the electrode and immersed into 0.75% glutaraldehyde solution for 1 h and washed with 10 mmol L$^{-1}$ PBS, the stable CV scans can be found (only 3.2% decrease of the initial redox peaks was observed after 100 cycles), and after 24 h, 3.7% decrease of the redox peaks can be found, which confirmed the Fc-COOH is not leached out from the SiO$_2$ network under this condition.

CVs of the different steps of modified electrodes were carried out in pH 7.0 PBS (Fig. 3). No obvious redox peaks were observed at bare gold electrode (Fig. 3, curve a). A pair of redox waves on the amino functionalized Fc-COOH-doped SNPs modified electrode was obviously exhibited in curve b of Fig. 3. Then a significant decrease in current could be measured after CA 15-3 antibodies anchor to electrode (Fig. 3, curve c), which indicated CA 15-3 antibodies had been successfully immobilized on the surface of the electrode. After BSA blocking, the peak current further decreased (Fig. 3, curve d) as the macromolecular protein BSA might retard the electrons transfer. EIS can give further information on the impedance changes of the immunosensor surface in the modification process. In EIS, the semicircle diameter of EIS equals the electron-transfer resistance, $R_{ct}$. Its value varies when different substances are adsorbed onto the electrode surface. So, EIS has been used to further confirm the assembly process of the electrode (Fig. 4). The semicircle of the electrode with amino functionalized Fc-COOH-doped SNPs (Fig. 4, curve b) increased in comparison to the bare gold electrode (Fig. 4, curve a). The reason might be the fact that the silica nanoparticles retard the electrochemical probe. After CA 15-3 antibodies were immobilized onto the electrode, the resistance shows an increase (Fig. 4, curve c), implying that the CA 15-3 antibodies were bound.
dependence of redox peak currents on the square root of potential sweep rates.

SNPs modified gold electrode; (d) BSA/anti-CA 15-3/Fc-COOH-doped electrode; (b) Fc-COOH-doped SNPs modified gold electrode; (c) anti-CA 15-3/Fc-COOH-doped SNPs modified gold electrode.

to electrode. The resistance was increased (Fig. 4, curve d) after the BSA was blocked onto the modified electrode surface, which may be ascribed to the inhibition of electron transfer by the BSA monolayer.

Fig. 5 shows CVs of the immunosensor after incubated in 100 U mL\(^{-1}\) CA 15-3 solution at various scan rates in 0.1 M PBS (pH 7.0). It can be observed that the peak currents are dependent on the scan rate. In addition, a plot of the peak current vs. the square root of the sweep rate exhibits a linear relationship (shown in the inset), suggesting a diffusion controllable process.

3.3. Optimization of experimental parameters

The effect of temperature on the immunoreaction was examined. It is found that the peak current responses decrease with the increasing temperature up to 35 °C. However, temperatures over 40 °C caused irreversible behavior (denaturation of proteins) involved in the process. As is well known, an optimal temperature of immunoreaction would be 37 °C. However, temperatures over 40 °C would cause irreversible behavior and deteriorate of response signals. Thus, the temperature of 25 °C (room temperature) was selected as a compromise.

The pH of the working buffer would influence the electrochemical response of the immunosensor. In order to optimize the pH, the immunosensors were tested by CVs in a series of PBS with the pH from 4.0 to 8.0. It was found that nearly reversible voltammograms can be observed for all the pH range tested from 4.0 to 8.0, with stable and well-defined peaks. The results indicate that the electron transport was independent from the pH value. Therefore, a pH 7.0 of PBS was chosen in the further study as it is close to the pH of body fluid.

The effect of the immunochemical incubation (i.e. when the antigen–antibody reaction occurs) time on the amperometric response signals was also investigated. The immunosensor was incubated with 100 U mL\(^{-1}\) CA 15-3 standard solution for 2, 4, 8, 12, 16, 20 and 25 min, then was washed with 10 mmol L\(^{-1}\) PBS and tested in 5 mL pH 7.0 PBS. When the antigens in the incubating solution reach the antibodies at the surface of the immunosensor, it takes times for the contacting species to form compact immunocomplexes. As shown in Fig. 6, the amperometric response signals decreases with the incubation time rapidly up to 16 min, and then the response signals tend to be stable after incubating 16 min, which was used as the optimal incubation time for the experiments. One would expect that most of the surface-exposed antibodies are binding with the antigens in the incubation solution, forming compact complexes on the surface of the immunosensor. Therefore, 16 min was used as the incubation time of the antigen–antibody reaction.

3.4. Performance of the immunosensor

3.4.1. CV response and calibration curve

To quantitatively detect the CA 15-3 concentration, a CV technique was employed to investigate the reaction between the immobilized anti-CA 15-3 and the CA 15-3 in the sample. After the antigen in the sample solution reacted with the immobilized antibody on the modified immunosensor, an insulating monolayer of the antigen–antibody complex can be formed which acts as an inert block layer to hinder the electron transfer toward the electrode surface. As a result, the peak current further decreased with the higher antigen concentration in the sample. Under the optimal conditions, the calibration graph for the current vs. the CA 15-3 concentration can be obtained with the immunosensor. As shown in Fig. 7, the peak current response is inversely proportional to the CA 15-3 concentration in the working buffer because the amount of the CA 15-3 bound to the antibody sites on the immunosensor surface increases, which acts as a definite kinetic barrier for the electron transfer. The linear range covers from 2.0 to 240 U mL\(^{-1}\) CA 15-3 with a detection limit of 0.64 U mL\(^{-1}\) (S/N = 3) CA 15-3. The linear regression equation was \(\Delta I_p (\mu A) = 0.2696x (U \text{ mL}^{-1}) + 7.8929\) with a correlation coefficient of 0.9986. Higher levels of CA 15-3 in serum could be detected by appropriate dilution of the sample.

3.4.2. Selectivity against interferences

To investigate the selectivity of the immunosensor, the immunosensor was incubated in the incubation solution contain-
ing 60 U mL\(^{-1}\) of CA 15-3 and various concentrations of interfering agents, such as α-fetoprotein (50 ng mL\(^{-1}\)), carcinoembryonic antigen (20 ng mL\(^{-1}\)), hepatitis B antigen (20 ng mL\(^{-1}\)), l-cysteine (50 μmol L\(^{-1}\)), L-glutamic acid (100 μmol L\(^{-1}\)), and BSA (40 ng mL\(^{-1}\)). The Δ\(\Delta p\) ratio (ratio of the Δ\(p\) of CA 15-3 in the presence of interfering to Δ\(p\) of CA 15-3) is 1.024, 1.008, 0.972, 0.984, 0.992, and 0.976, respectively. The results show that these interfering agents do not remarkably interfere with the immunosensor’s response (<2.8% difference). Thus, the selectivity of the immunosensor based on the highly specific antigen–antibody reaction was satisfactory.

### 3.4.3. Regeneration and stability of the immunosensors

Regeneration of immunosensors is of interest to the immunoanalysts. As well known, glycine could effectively dissociate the antigen–antibody complex, peeling the captured antigens off the immunosensor. In addition, NaCl would provide an appropriately high ionic-strength to facilitate the detachment of antibodies and antigens [30]. It might also aid to stabilize CA 15-3 antibodies bound to the regenerated probes through offering appropriate neutralization of charges to retard the possible dissociation of the isourea bonds formed [31,32]. So, in this experiment, after the immunosensor was incubated in 100 U mL\(^{-1}\) CA 15-3, the regeneration of the proposed immunosensors was performed by dipping into a solution consisting of glycine–HCl (0.2 mol L\(^{-1}\), pH 3.6) and NaCl (0.25 mol L\(^{-1}\)) for 5 min, to remove the CA 15-3 from the antigen–antibody immunocomplex, followed by rinsing with a phosphate buffer solution of pH 7.0. The decrease of amperometric signal is not remarkable. Only 8.2% decrease of the initial value after five measurements.

The stability of the sensors was evaluated over a period of 30 days by storing in phosphate buffered saline (PBS) (pH 7.0) at 4 °C and occasional running. No obvious amperometric changes are observed after storing. It retained 96.7% of its initial current after the first 10 days storage and 90.6% after 30 days storage. The stability of the immunosensor is acceptable.

### 3.4.4. Accuracy and clinical application

To evaluate the accuracy of the as-prepared immunosensor, several different concentrations of standard CA 15-3 solution were determined by using the developed immunoassay. A part of the experimental results were shown in Table 1. Seen from Table 1, the recovery was from 96.7% to 103.1%, so the proposed immunosensor represented good recovery.

In order to further investigate the possibility of the newly developed technique being used for clinical analysis, ten serum specimens, gifted from the Chongqing Institute of Cancer Prevention and Cure, have been examined by the developed immunosensor and the reference ELISA method. It can be seen that the relative error between the two methods was from −6.6% to 4.0% (Table 2). These data show that there is no significant difference between the results given by two methods which are in concordance with the results obtained using the standard methods proposed by ELISA, that is, the developed immunoassays may provide a promising alternative for determining the concentration of CA 15-3 in human serum in clinical immunoassays.

### 4. Conclusion

This communication describes a new electrochemical immunoassay for CA 15-3 by using antibody-functionalized silica nanocomposites with redox electroactivity as a disposable transducer. Highlights of the immunoassay system are direct, rapid and simple without multiple labeling and separation steps. Moreover, the use of the amino functionalized Fe-COOH-doped composite nanoparticles could enhance the surface coverage and improves the sensitivity of the assay. More significantly, the biomimetic interface provides a new clinic-applicable alternative for the determination of other antigens or tumor markers.

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