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ORIGINAL PAPER

A highly sensitive electrochemiluminescence immunosensor based on magnetic nanoparticles and its application in CA125 determination

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Abstract A novel electrogenerated chemiluminescence (ECL) immunoassay based on enzyme amplification and magnetic nanoparticle enrichment was developed, and carbohydrate antigen 125 (CA125) was chosen as the analyte. Fe_3O_4 magnetic nanoparticles loaded with anti-CA125 were synthesized. The sandwich-type immunoassay was performed on the magnetic force-controlled carbon paste electrode via the immunoreactions among glucose oxidase-labeled anti-CA125, CA125, and anti-CA125 on the surface of magnetic nanoparticles. ECL was generated by the reaction between luminol and hydrogen peroxide. Hydrogen peroxide was produced during the enzymatic reaction with glucose and markedly increased in the presence of CA125 antigen. The CA125 concentrations were determined within the range of 0–10 mU mL^{-1}, and the detection limit was 8.0 μU mL^{-1}. The CA125 immunosensor was more sensitive than those previously reported. The proposed ECL method also provided a simple selectivity immunoassay protocol, which was applied in the determination of CA125 in clinical serum samples.

Keywords Magnetic nanoparticles · Electrochemiluminescence · Immunosensor · CA125 · Enzyme amplifier

Introduction

In recent years, magnetic nanoparticles have drawn much attention in biological medicine, cytology, and biotechnology [1, 2]. They have been used extensively as carrier of biospecies for different purposes because of their good biocompatibility and large specific surface area that improves sensitivity. Among these nanomaterials, Fe_3O_4 nanoparticles [3, 4] are one of the most widely used magnetic nanoparticles because of their advantages, such as low cost, easy preparation, high saturation magnetization, as well as no adverse and toxic effect. Researchers have immobilized biological species such as enzymes, antibodies, and even cells on magnetic nanoparticles to construct biosensors for separation and detection in biochemistry, molecular biology, clinical medicine, and immunology [5–9].

Immunosensors with very high sensitivity and specific immune reactions have been developed [10]. Preparing magnetic nanoparticle-based magnet-controlled immunosensors [11–15] that can improve the simplicity, selectivity, and sensitivity of antigen detection has gained increased interest. The detection methods commonly used are electrochemical methods [16], chemiluminescence [17], fluorescence [18], and surface plasmon resonance [19].

Electrogenerated chemiluminescence (ECL) has been developed based on chemiluminescence. ECL has the advantage of low background, high sensitivity, high selectivity, good reproducibility, and easy controllability [20]. ECL immunosensors can improve not only the detection sensitivity but also the quantities of immunoreagents immobilized on the surface [21, 22]. ECL provides a novel method for bioassays [23–25]. Recently, ECL biosensors fabricated with enzyme and magnetic nanoparticles have attracted attention [26]. However, immunosensors fabricated with magnetic nanoparticles for ECL detection are rarely reported.

In this paper, a strategy for preparing a novel ECL immunoassay based on enzyme amplification and magnetic nanoparticle enrichment was proposed. Carbohydrate antigen 125

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(CA125) was selected as a substrate for the assay. CA125 [27], a glycoprotein, is a reliable index of ovarian function. Sensitive methods for CA125 determination in blood serum need to be developed. CA125 is used as a marker in the clinical diagnosis of several cancers, particularly for monitoring the treatment response in ovarian, lung, and stomach cancers. In recent years, a variety of immunoassays have been developed for CA125 detection, such as radioimmunoassay [28], enzyme-linked immunosorbent assay (ELISA) [29, 30], microparticle enzyme immunoassay [31], and chemiluminescence [32]. However, there is no report on a CA125 electrochemical biosensor based on magnetic particles and ECL. In the present study, we proposed a sandwich-type immunosensor in which CA125 antibody was immobilized on magnetic nanoparticles. Anti-CA125 was labeled with glucose oxidase (GOD), and hydrogen peroxide was produced in the presence of glucose. The ECL of luminol can be initiated by applying an appropriate positive potential to the working electrode in the presence of hydrogen peroxide [33]. Remarkably improved sensitivity was achieved due to the amplification effect of GOD. The sensor was highly sensitive, of low cost, renewable, and easy to use. The experimental results showed that the proposed method was a promising alternative tool for fabricating ECL biosensors for CA125.

Experimental

Apparatus and reagents

Cyclic voltammetric and ECL experiments were carried out using a model MPI-E ECL analyzer system (Xi'an Remex Instrument Co., Ltd, China) equipped with a three-electrode system comprising a platinum wire (auxiliary electrode), an Ag/AgCl electrode (reference electrode), and the CA125 immunosensor (working electrode). Scanning electron microscopy (SEM) was performed using a Hitachi S-4800 field emission SEM system. A pHS-2 C model pH meter (Shanghai Leici Instruments, China) and a DK-8B electrothermal constant-temperature incubator (Shanghai Jinghong Instruments, China) were also used.

Anti-CA125 (1 mg mL^{-1}), CA125, and GOD-labeled CA125 (GOD-CA125; 5 μg mL^{-1}) were purchased from Biosynthesis Biotechnology Company (Beijing, China). GOD (120 U mg^{-1}; from *Aspergillus niger*) was purchased from Sigma. 3-(Aminopropyl) triethoxysilane (APS, 98 %) was obtained from Johnson Matthey Company (Alfa Aesar, USA). Bovine serum albumin (BSA) and 25 % glutaraldehyde were obtained from Shanghai Biochemical Co. (Shanghai, China).

A 0.01-mol L^{-1} luminol stock solution was prepared by dissolving 0.0886 g of luminol (>98 %; Fluka) in 0.1 mol L^{-1} sodium hydroxide buffer. A Tris–HCl buffer solution was prepared by mixing 0.05 mol L^{-1} tris(hydroxymethyl)aminomethane and 0.1 mol L^{-1} HCl. Phosphate-buffered solution (PBS, pH 7.4) was prepared from 0.1 mol L^{-1} Na_2HPO_4, 0.1 mol L^{-1} NaH_2PO_4, and 0.1 mol L^{-1} KCl. The washing buffer solution consisted of PBS with 0.1 mol L^{-1} NaCl and 0.05 % (*v*/*v*) Tween 20 (PBST).

All other reagents were of analytical reagent grade and all solutions were prepared with doubly distilled water (18.2 MΩ cm^{-1}).

Preparation of immunosensor

Preparation of solid paraffin carbon paste electrode

Solid paraffin carbon paste electrode (SCPE) and magnetic nano-Fe_3O_4 particles were prepared according to previous methods [3].

An iron stick (ø 2.55 mm) about 3 cm in length and a glass tube (ø 3 mm, i.d.) were prepared. The ends were polished until smooth. Solid paraffin (mp 55 °C) and carbon powder (particle size<38 μm) were mixed at a 3:1 (*m*/*m*) ratio. The mixture was heated up to 60 °C until it melted thoroughly. After the mixture was filled into a glass tube, the iron stick was inserted. The residual mixtures were then removed. The SPCE was polished on a smooth duplicated microcloth (chamois leather) as well as 1.0, 0.3, and 0.05 μm aqueous slurry of alumina. Prior to modification, the SPCE was polished again, sonicated in 1:1 HNO_3/ethanol and doubly distilled water, and then finally dried at room temperature.

Preparation of Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were prepared by chemical coprecipitation of Fe(II) and Fe(III) ions (2:1 molar ratio) in alkaline medium. About 2 mol L^{-1} of NaOH solution was added to the ferric and ferrous chloride under vigorous agitation at 50 °C. During the reaction process, the pH value was maintained at about 10. The solution was then heated at 80 °C for 1 h under a N_2 atmosphere. Finally, the resulting precipitate was separated by magnetic decantation and washed with double-deionized water.

Immobilization of anti-CA125 on Fe_3O_4 nanoparticles

Anti-CA125 was immobilized covalently on Fe_3O_4 by a previously reported method [3]. Fe_3O_4 nanoparticles (48 mg) were dispersed in 20 mL of ethanol by sonication. Then, 0.2 mL of 3-APS was added and the solution was

mechanically stirred for 12 h under a N_2 atmosphere to ensure the amino functionalization of the magnetic nanoparticles. About 4 mL of glutaraldehyde solution (10 %) was added and the mixture was stirred for 3 h. Afterwards, the nanoparticles were separated by a magnet and washed with PBST. The obtained nanoparticles were then resuspended in 4 mL of PBS (pH 7.4). About 100 μL of anti-CA125 (1.0 mg mL^{-1}) was added to the solution, which was gently stirred for 12 h at 4 °C. The anti-CA125/Fe_3O_4 nanoparticles were separated by a magnet and resuspended in 2 mL of PBS.

Fabrication of the ECL immunosensor

The modified electrode was prepared following a published method [14]. The core of the electrode was attracted by a magnet. The fabrication processes of the ECL immunosensor are shown in Fig. 1. During the procedure, 10 μL of anti-CA125/Fe_3O_4 particle suspension was dropped on the surface and dispersed. The electrode was subsequently immersed in 1 % BSA to seal the nonspecific sites on the particle surface and then rinsed by PBST. The electrode was incubated for 30 min with CA125 with a concentration less than 10 mU mL^{-1}. Lastly, sandwich immunoconstruction was formed by incubation in 75 ng mL^{-1} GOD-labeled anti-CA125 for 30 min. The immunosensor was washed with PBST to remove excess antibody and then stored at 4 °C when not in use.

Experimental method

The ECL test was conducted in 10 mL of 0.05 mol L^{-1} Tris–HCl buffer (pH 8.5) containing 0.6 mmol L^{-1} luminol and 1 mmol L^{-1} glucose at room temperature. The ECL was measured from −0.3 to +0.6 V at the scan rate of 100 mV s^{-1}. The voltage of the photomultiplier tube was set at 600 V. The ECL signal–time curve under continuous potential scanning was performed for five cycles with the magnification of four. ECL signals related to the CA125 concentrations were then measured.

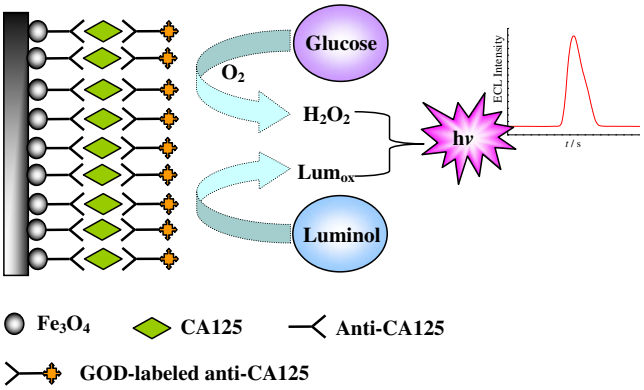

Fig. 1 Schematic illustration of CA125 determination based on the ECL immunoassay

Results and discussion

Characterization of Fe_3O_4 magnetic nanoparticles and Fe_3O_4/anti-CA125

Figure 2a, b shows the SEM in the secondary electron mode of Fe_3O_4 and Fe_3O_4/anti-CA125, respectively. The average diameter of the Fe_3O_4/anti-CA125 composite nanoparticles increased slightly compared with that of Fe_3O_4 nanoparticles.

Figure 3 shows the size distribution image of Fe_3O_4 nanoparticles. The particle diameters ranged approximately from 12 to 30 nm and were concentrated to about 20 nm. Hence, the Fe_3O_4 particles synthesized by the method are in nanometer size.

The magnetic hysteresis loop of the Fe_3O_4 magnetic particles is shown in Fig. 4. A saturation magnetization of 48 emu g^{-1} was determined for the Fe_3O_4 nanoparticles, indicating that the nanoparticles exhibited ideal magnetic properties. The superparamagnetic behavior of Fe_3O_4 was also clearly proven by zero coercivity and remanence on the magnetization loop.

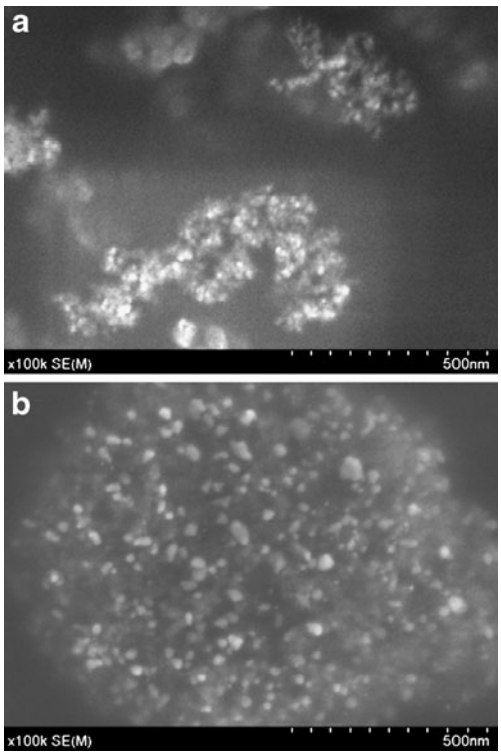

Fig. 2 SEM micrographs (secondary electron imaging) of Fe_3O_4 nanoparticles (**a**) and Fe_3O_4/anti-CA125 (**b**)

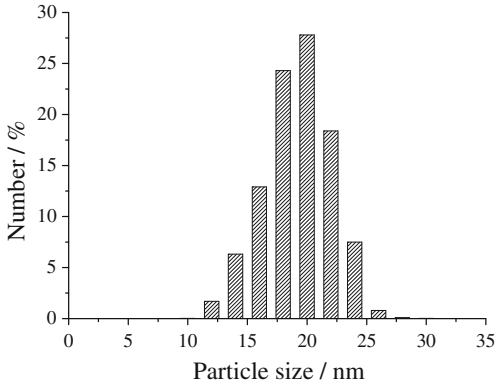

Fig. 3 Particle size distribution image of Fe_3O_4

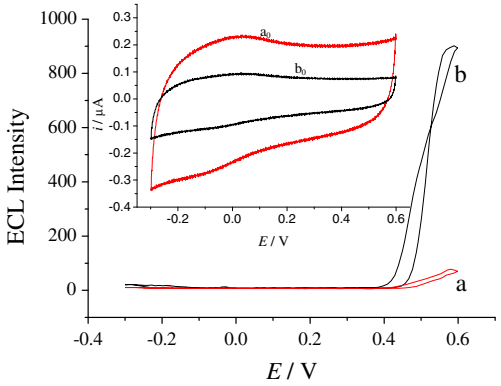

Fig. 5 ECL curves and cyclic voltammogram (*inset*). *a*, a_0: glucose, luminol, Tris–HCl buffer solution; *b*, b_0: a+anti-CA125+10 mU mL^{-1} CA125+75 ng mL^{-1} GOD-labeled anti-CA125

Electrochemical and ECL behaviors of enzyme-labeled anti-CA125 attached onto the immunosensor surface

The CV and corresponding ECL intensity curves are shown in Fig. 5. A high current response (a_0) and weak ECL intensity were observed at the bare electrode (point a). The current decreased (b_0) but the ECL intensity increased sharply (point b) when measured by the immunosensor, clearly demonstrating that GOD-labeled anti-CA125 was successfully bound onto the surface of the magnetic nanoparticles.

Optimization of the CA125 immunoassay

To obtain optimal ECL intensity, three buffer solutions were investigated, including 0.1 mol L^{-1} borax buffer solution, 0.05 mol L^{-1} Tris–HCl buffer, and 0.1 mol L^{-1} PBS (pH 8.0). The result demonstrated that the maximal ECL intensity can be obtained in Tris–HCl buffer. The effect of the pH values (6.5–9) of different Tris–HCl buffers on the ECL intensity was investigated, and the results are shown in Fig. 6. Luminol is known to have a strong intensity in alkaline solution [34]. The ECL signal increased with increased pH, reached the maximum at pH 8.5, and then decreased with the pH. On the other hand, the activity and affinity of antigen and antibody CA125 remained high in alkaline solution [35]. Thus, pH 8.5 was selected.

The effects of the incubation time and temperature on the ECL density were evaluated separately. The results are shown in Figs. 7 (points a and b). The ECL signal markedly increased (Fig. 7, point a) with increased incubation time (up to 30 min) and then changed slowly with increased incubation time. This finding indicated that the combination reaction of the antigen and antibody was completed and reached a maximum response after 30 min. Thus, 30 min of incubation time was selected.

ECL intensities were determined at varied temperatures from 15 to 55 °C (Fig. 7, point b), and the maximum response was obtained at 37 °C. Considering that the immunosensor life decreases at high temperatures, the incubation temperature was controlled at 25±1.0 °C.

The GOD-labeled anti-CA125 concentration was examined within the range of 0–175 ng mL^{-1}, and the results are

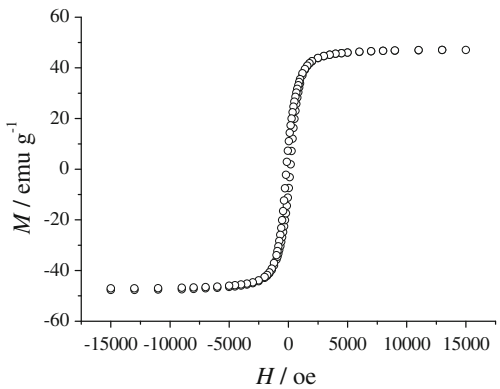

Fig. 4 Hysteresis curves of Fe_3O_4 magnetic particles

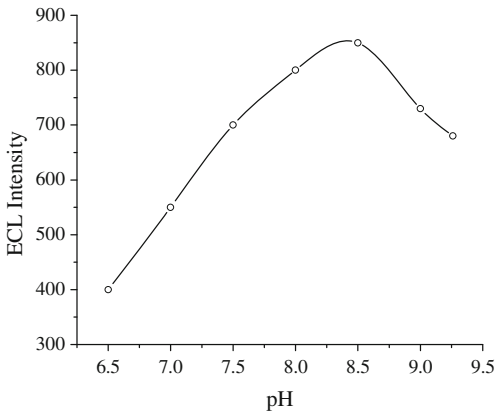

Fig. 6 Effect of pH on ECL intensity. Tris–HCl buffer (0.05 mol L^{-1}) containing 0.8 mmol L^{-1} luminol and 1 mmol L^{-1} glucose at 37±1.0 °C with an incubation time of 30 min and 75 ng mL^{-1} GOD-labeled anti-CA125

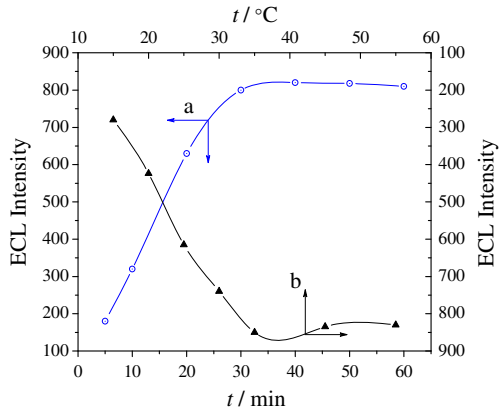

Fig. 7 Effect of incubation time (*a*) and incubation temperature (*b*) on ECL intensity: (*a*) 0.05 mol L^{-1} Tris–HCl buffer (pH 8.5) containing 0.6 mmol L^{-1} luminol and 1 mmol L^{-1} glucose at room temperature (25±1.0 °C), 75 ng mL^{-1} GOD-labeled anti-CA125, and 5 mU mL^{-1} CA125; (*b*) 0.05 mol L^{-1} Tris–HCl buffer (pH 8.5) containing 0.6 mmol L^{-1} luminol and 1 mmol L^{-1} glucose with an incubation time of 30 min, 75 ng mL^{-1} GOD-labeled anti-CA125, and 5 mU mL^{-1} CA125

shown in Fig. 8 (point a). The ECL intensity increased with the addition of GOD-labeled anti-CA125 and reached a plateau by 75 ng mL^{-1}. This result suggested that there were sufficient antibodies allowed to react with the antigens completely. Hence, 75 ng mL^{-1} of GOD-labeled anti-CA125 was selected.

The effect of luminol concentration within the range 0 to 1.1 mmol L^{-1} on ECL intensity was studied. Figure 8 (point b) shows that the ECL intensity increased with increased luminol and tended toward stability at 0.6 mmol L^{-1}. Hence, the optimal luminol concentration was 0.6 mmol L^{-1}.

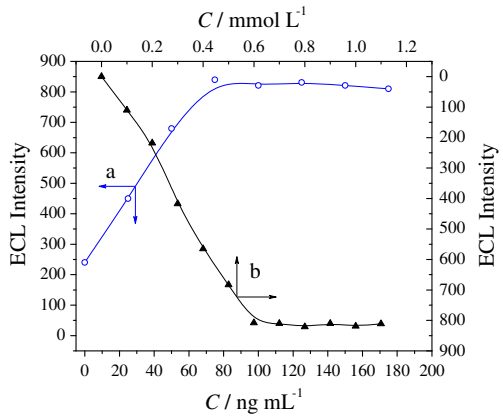

Fig. 8 Influence of the GOD-labeled anti-CA125 (*a*) and luminol concentration (*b*) on ECL intensity: (*a*) 0.05 mol L^{-1} Tris–HCl buffer (pH 8.5) containing 0.6 mmol L^{-1} luminol and 1 mmol L^{-1} glucose at room temperature (25±1.0 °C) with an incubation time of 30 min and 5 mU mL^{-1} CA125; (*b*) 0.05 mol L^{-1} Tris–HCl buffer (pH 8.5) containing 1 mmol L^{-1} glucose at room temperature (25±1.0 °C) with an incubation time of 30 min, 75 ng mL^{-1} GOD-labeled anti-CA125, and 5 mU mL^{-1} CA125

Immunosensor response to CA125

Under the optimized test conditions, there was a linear relationship between the ECL intensity (*I*) and CA125 concentration (*C*) within the range 0 to 10 mU mL^{-1} (Fig. 9). The regression equation was I=98.43 C (mU mL^{-1})+116.30 and the correlation coefficient was 0.9990. The detection limit of the designed immunosensor was 8.0 μU mL^{-1} according to 3σ/K, which indicated that it was a more sensitive CA125 immunosensor than any reported before.

Table 1 shows a comparison of analytical parameters such as the determination ranges and detection limits of the proposed Fe_3O_4 magnetic nanoparticle-based ECL immunosensor and others reported for CA125 determination. The immunoassay clearly exhibited a wide linear range and low detection limit, indicating that it is one of the most sensitive CA125 immunosensors compared with most electrochemical methods and the traditional method of solid phase ELISA [29]. Significantly, the method developed was capable of continuously carrying out all steps in less than 75 min for one sample, which is shorter than that using commercial ELISA.

Selectivity of CA125 immunosensor

The selectivity of the immunosensor was estimated by measuring the ECL responses of 5 mU mL^{-1} CA125 in the presence of some possible coexisting substances. When the relative deviation of the ECL intensities was less than ±5 %, the maximum allowable concentrations of the compounds were as follows: 200 ng mL^{-1} CEA, PSA, BSA, HSA, and IgG, as well as 4 μg mL^{-1} L-Cyss, L-Pro, L-His, L-Trp, and L-Ser. Most coexisting substances were not found to interfere with the detection of CA125.

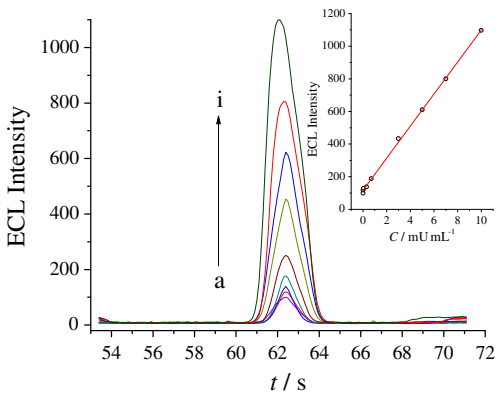

Fig. 9 Response curves of CA125 to the ECL immunosensor. The concentrations of CA125 (*a–i*) were 0, 0.01, 0.05, 0.3, 0.7, 3, 5, 7, and 10 mU mL^{-1}, respectively

Table 1 Analytical parameters reported for the determination of CA125

System	Method	Determination range (U $mL^{-1)}$	Detection limit (U mL^{-1})	Ref.
Fe_3O_4 nanoparticle membrane	EC[a]	0.1–450	0.1	[36]
Colloidal nano-gold membrane	EC	0–30	1.73	[37]
PPy nanowire biosensors	EC	1–1000	1	[38]
Carbon nanofiber membrane	EC	2–75	1.8	[39]
Nano-Au/$Co(bpy)_3^{3+}$/MWNTs[b]–Nafion film	EC	1.0–150	0.36	[40]
Thionine and gold nanoparticles-modified carbon paste interface	EC	10–30	1.8	[41]
Fe_3O_4 magnetic nanoparticles membrane	ECL	0–10 mU mL^{-1}	8.0 μU mL^{-1}	This work

[a] Electrochemical method

[b] Multi-wall carbon nanotube

Reproducibility and stability of the CA125 immunosensor

The reproducibility of the immunosensor was evaluated by determining the ECL response to 2 mU mL^{-1} CA125 using five immunosensors fabricated by the same electrode. A relative standard deviation (RSD) of 1.65 % was obtained. For four different electrodes, an RSD of 3.80 % was obtained for the same concentration of CA125, indicating a good reproducibility of the fabricated immunosensor.

To ensure stability, the bionanoparticles were stored in a refrigerator at 4 °C when not in use. The long-term stability of the immunosensor for CA125 was evaluated within a period of 60 days. About 2 mU mL^{-1} CA125 was tested periodically, and no obvious signal change was observed in 14 days. Over the next week, the ECL response decreased by about 10 %. After 42 days, a further decrease by about 15 % was observed compared with the initial response. This finding indicated that the proposed immunosensor had a good long-term stability, and the enzyme maintained its biological activity when immobilized on the electrode.

Application of immunosensor to serum samples

To further investigate the feasibility of the immunosensor for clinical applications, we analyzed several real samples freshly obtained from the Hospital of Guilin University of Technology. About 2 mL of serum was transferred to a centrifuge tube, and 500 μL of sodium citrate (2.29 gmL^{-1}) was added until complete blood coagulation within 1 h. The serum samples were stored at −20 °C until the assay. Before measurement, the serum samples were centrifuged at 3,000 rpm for 10 min, the supernatant was collected to be diluted stepwise with PBS (pH 7.4). The purpose of this step was to ensure that the unknown CA125 concentration was within the dynamic detection range of the proposed method. The ECL values were obtained, and the determination results as well as the recoveries are shown in Table 2. The RSD was from 1.14 to 4.31 %. The recoveries ranged from 93.4 to 109.5 %. Thus, the developed ECL immunosensor can be used to determine CA125 in human serum samples.

Conclusions

In the present work, a sensitive and easily renewable ECL sandwich immunosensor was constructed by immobilizing the antibody on the surface of Fe_3O_4 nanoparticles. The immunosensor exhibited high sensitivity, selectivity, specificity, and a wide linear range for CA125 detection. The proposed immunosensor can provide a new ultrasensitive device and approach for analyzing biologically active molecules. It meets the analytical requirements of biochemistry, molecular biology, clinical medicine, and immunology.

Table 2 Analysis of CA125 concentrations in clinical sera using the proposed ECL immunosensor

Sample[a]	Detected (U mL^{-1})	Mean (U mL^{-1})	RSD % (n=5)	Added (U mL^{-1})	Detected (U mL^{-1})	RSD % (n=5)	Recoveries (%)
1	2.78, 2.67, 2.99, 2.88, 3.09	2.84	4.31	10	11.99	1.19	93.4
2	14.58, 14.69, 14.48, 14.27, 14.16	14.44	1.51	10	23.33	2.26	95.5
3	20.80, 20.59, 20.49, 19.96, 19.54	20.28	2.54	10	33.15	2.93	109.5
4	34.61, 34.40, 33.87, 33.24, 33.71	33.77	2.35	10	41.01	2.17	93.7
5	45.15, 44.84, 45.15, 44.10, 44.20	44.69	1.14	10	56.21	3.34	102.8

[a] Samples underwent stepwise dilution 10^4 times

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