Ultrasensitive Electrochemical Immunosensor for Clinical Immunoassay Using Thionine-Doped Magnetic Gold Nanospheres as Labels and Horseradish Peroxidase as Enhancer

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A new signal amplification strategy based on thionine (TH)-doped magnetic gold nanospheres as labels and horseradish peroxidase (HRP) as enhancer holds promise to improve the sensitivity and detection limit of the immunoassay for carcinoembryonic antigen (CEA), as a model protein. This immunoassay system was fabricated on a carbon fiber microelectrode (CFME) covered with a well-ordered anti-CEA/protein A/nanogold architecture. The reverse micelle method was initially used for the preparation of TH-doped magnetic gold nanospheres (nanospheres), and the synthesized nanospheres were then labeled on HRP-bound anti-CEA as a secondary antibody (bionanospheres). Sandwich-type protocol was successfully introduced to develop a new high-efficiency electrochemical immunoassay with the labeled bionanospheres toward the reduction of $H_2O_2$. Under optimized conditions, the linear range of the proposed immunoassay without HRP as enhancer was 1.2–125 ng/mL CEA, whereas the assay sensitivity by using HRP as enhancer could be further increased to 0.01 ng/mL with the linear range from 0.01 to 160 ng/mL CEA. The developed immunoassay method showed good precision, high sensitivity, acceptable stability and reproducibility, and could be used for the detection of real samples with consistent results in comparison with those obtained by the enzyme-linked immunosorbent assay (ELISA) method.

Immunassay, as a promising approach for selective and sensitive analysis, has recently gained increasing attention in different fields including environmental monitoring, food safety, and clinical diagnosis.1 Despite many advances in this field, it is still a challenge to find new approaches that could improve the simplicity, selectivity, and sensitivity of clinical immunoassays.2 Thus, considerable efforts have been made worldwide to develop and improve clinical immunoassays with the aim of making portable and affordable devices. Electrochemistry, with high sensitivity, low cost, low power requirements, and high compatibility with advanced micromachining technologies, has been applied for clinical immunoassays.3-4 Wilson and Nie reported two methods for simultaneously detecting multiple tumor markers by using an electrochemical enzyme-labeled immunoassay.5-6 The detection limit and sensitivity of the immunoassay were greatly improved due to the bioelectrocatalytic reaction of the labeled enzyme.

Signal amplification and noise reduction are crucial for obtaining low detection limits in clinical immunoassays.7-8 Sandwich-type immunoassay has the advantages of high specificity and sensitivity because of the use of a couple of match antibodies.9 The mode is performed using antibodies or antigens with different labels, including radioisotopes,10 enzymes,11 fluorescence compounds,12 and metal compounds.13 Cui et al.14 used CdTe quantum dots as labels for electrochemical determination of protein with a detection limit of 5 pg/mL. Lin et al.15 described a disposable electrochemical immunoassay for the detection of IgG by using quantum dots (CdS@ZnS) as labels with a low detection limit of 10 pg/mL. Other labels, such as bismuth ion,16 apoferritin nanoparticles,17 gold nanocatalyst,18 and CdSe quantum dots,19 have been developed for electrochemical immunoassays. Signifi-

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cantly, Yu et al.\textsuperscript{20} reported a greatly amplified sensitivity for electrochemical immunoassay of prostate-specific antigen with a detection limit of 4 pg/mL by using bioconjugates featuring horseradish peroxidase (HRP) labels and secondary antibodies linked to carbon nanotubes. These detection methods provided a rapid, clinically accurate, and quantitative tool for protein biomarker detection.

The gold nanoparticle label is an ideal one in biotechnological systems due to its inherent advantages, such as easy preparation, good biocompatibility, and so on.\textsuperscript{21,22} It has recently been extensively employed to label different biological receptors, such as protein A, immunoglobulin G, and glucose oxidase.\textsuperscript{13} Yang’s group\textsuperscript{18} developed an ultrasensitive and simple electrochemical method for the fabrication of a sandwich-type heterogeneous electrochemical immunosensor. An IgG-nanocatalyst conjugate was prepared via direct adsorption of IgG on 10 nm gold nanoparticles. The IgG-nanocatalyst conjugate and the immunosensing layer sandwiched the target protein. Signal amplification was achieved by using gold nanocatalyst labels for catalytic reduction of substrates with a very low detection limit of 1 fg/mL IgG.

For the development of sandwich-type electrochemical immunoassays, the captured amount for the secondary antibody labeled by using nanomaterials often influenced the sensitivity of the immunoassay.\textsuperscript{23} Carcinoembryonic antigen (CEA) is a glycoprotein most often associated with colorectal cancer and used to monitor patients with this type of cancer.\textsuperscript{24} Its most popular use is in early detection of relapse in individuals already treated for colorectal cancer.\textsuperscript{25} In view of the advantageous features of enzyme labels and nanogold labels, we synthesized thionine (TH)-doped magnetic gold nanoparticles for the labels of HRP-bound anti-CEA as secondary antibodies (denoted as bionanospheres) in this study (Scheme 1). The sandwich-type complex is formed in this protocol by the primary anti-CEA antibody immobilized on the surface of protein A/nanogold/CFME, the CEA antigen in the sample, and the secondary antibody labeled with bionanospheres. The magnetic gold nanoparticles amplified the surface coverage of HRP-bound anti-CEA, and the bound bionanospheres could catalyze the reduction of H\textsubscript{2}O\textsubscript{2} in the presence of the doped thionine, as a mediator, with amplified signal output. The performance and factors influencing the performance of the immunoassay were also investigated. Moreover, a number of clinical specimens were evaluated by the developed immunosensor with analytical results compared to those given by enzyme-linked immunosorbent assays (ELISAs). The attractive response performances of the developed electrochemical immunosensing strategy and potential merits for tumor marker detection are presented in detail.

**EXPERIMENTAL SECTION**

**Materials and Apparatus.** Mouse monoclonal anti-CEA, HRP-bound mouse monoclonal anti-CEA (HRP-anti-CEA), CEA, α-fetoprotein antigen (AFP), carcino antigen 125 (CA 125), and cancer antigen 19–9 (CA 19–9) were purchased from China Zhengzhou Biocell Biotechnol. Co. Ltd. (dispatched from U.S.A. Sigma). Chloroauric acid (HAuCl\textsubscript{4}), sodium citrate, bovine serum albumin (BSA, 96–99%), protein A (pA), and thionine (TH) were performed by Sigma (U.S.A.). Ammonium hydroxide (30 wt %) and bis(2-ethylhexyl) sodium sulfosuccinate (AOT) were the products of Tiantai Fine Chemical (Tianjing, China). Deionized and distilled water was used throughout the study. The 0.1 M acetic acid buffer solutions with various pH values were prepared by using acetic acid, sodium acetate, and K\textsubscript{2}HPO\textsubscript{4}. Clinical serum samples were provided by Chongqing Institute of Cancer Prevention and Cure, China. All other reagents were of the best grade available and used as received.

Electrochemical measurements were performed with an Autolab (Eco Chemie, The Netherlands) system. A three-electrode system comprising a prepared working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference was employed for all electrochemical experiments. Transmission electron microscopy (TEM) was performed with JEOL Model JEM 2000E × 200 electron microscope (Japan). Samples were prepared by placing small drops of dispersed particles in water on Formvar-coated copper grids and allowing the solvent to slowly evaporate at room temperature. Spectrophotometric study was carried out using an 8500 UV–vis spectrophotometer (Japan). While measuring the absorbance in reverse micelles, AOT reverse micelles of the same \( W_o \) value were used for reference.
Preparation of TH-Doped Magnetic Gold Nanospheres. Magnetic NiFe\textsubscript{2}O\textsubscript{4} nanoparticles (20 nm in diameter) were prepared according to our previous report.\textsuperscript{25} Briefly, Fe(NO\textsubscript{3})\textsubscript{3}, 9H\textsubscript{2}O, Ni(NO\textsubscript{3})\textsubscript{2}6H\textsubscript{2}O, and glycine (note: Fe\textsuperscript{3+}/Ni\textsuperscript{2+} = 2:1, glycine/nitrate = 4:1, in molar ratio) were dissolved in distilled water. After filtration, the transparent brown precursor solution was heated until a combustion reaction rapidly diffused. After combustion for several seconds, the loose pink powders formed were rinsed with deionized water and ethanol, filtered, dried at 150 °C for 2 h, and calcinated at 800 °C for 4 h to prepare the NiFe\textsubscript{2}O\textsubscript{4} nanoparticles.

TH-doped magnetic gold nanospheres were synthesized according to a modified method reported previously.\textsuperscript{26} Initially, 475 μL of 0.05 M NiFe\textsubscript{2}O\textsubscript{4} solution and 200 μL of 1.5 × 10\textsuperscript{-3} M aqueous solution of TH were added to 25 mL of 0.1 M AOT in isooctane to give a clear reverse micellar solution. After complete addition, the solution was stirred for 4–6 h at room temperature, and then an azury translucent solution was achieved due to the formation of TH-doped NiFe\textsubscript{2}O\textsubscript{4} nanoparticles in the aqueous core of the reverse micellar droplets. Following that, 200 μL of 10% (w/w) H\textsubscript{2}AuCl\textsubscript{4} solution was added drop by drop with constant stirring. After being stirred further for 2–3 h at room temperature, 300 μL of 0.05 M aqueous hydrazine hydrate was added, which reduced the Au\textsuperscript{3+} to Au\textsuperscript{0}. With the progressive addition of hydrazine hydrate to reverse micelles, the solution acquired an azury to red color due to the formation of gold colloids. After being stirred for 1 h at room temperature, 5 mL of absolute ethanol was added, and the mixture was stirred for 10 min, which resulted in the complete breakdown of reverse micelles with the formation of two immiscible layers of aqueous ethanol and isooctane. The ethanol was carefully removed using a separating funnel. The particles thus obtained were washed four times with isooctane and centrifuged to remove any residual AOT. The pelletized particles were then dispersed in 10 mL of water by vigorous stirring for 30 min, and the dispersed system was dialyzed against distilled water for 2 h using a 12 kDa cutoff dialysis bag. Afterward, the TH-doped magnetic gold nanospheres were obtained via magnetic separation (denoted as nanosphere, Scheme 1b, top).

The synthesized procedure of TH-doped magnetic gold nanospheres is illustrated in Figure 1 of the Supporting Information.

Magnetic Gold Nanospheres-Labeled HRP-anti-CEA. The procedure of magnetic gold nanosphere labeling was performed as follows: 500 μL of 500 ng/mL HRP-anti-CEA was added into 5 mL of 1% (w/w) TH-doped magnetic gold nanospheres suspension, and the mixture was continuously stirred for 2–3 h. After magnetic separation, 100 μL of 10 mg/mL BSA solution was added to cover the nonspecific sites. After stirring for at least 1 h, the solution was centrifuged at 4000 rpm for 15 min, the supernatant including the excess reagents was removed, and the red precipitation was redispersed in 5 mL of phosphate-buffered saline (PBS) (pH 7.4) solution. The magnetic gold nanospheres-labeled HRP-anti-CEA solution was stored at 4 °C until use (denoted as bionanosphere, Scheme 1b, bottom).

Immunosensor Fabrication. Carbon fiber microelectrodes (CFMEs) were fabricated according to the literature (Figure 2 of the Supporting Information).\textsuperscript{25} Briefly, a carbon fiber (1.0 mm in diameter), which was attached to copper wire with silver conducting epoxy, was carefully inserted into a glass capillary with a tip diameter of about 2.0 mm. The tip of glass capillary was sealed with epoxy resin and heat-cured at 100 °C for 2 h. The exposed carbon fiber was trimmed to a length of 10 mm with a scalpel under a microscope. After the prepared CFMEs were treated by ultrasonically rinsing with acetone, 1:1 HNO\textsubscript{3}, 1.0 M NaOH, and water sequentially, the electrodes were electrochemically activated by holding them at +2.0 V for 30 s and −1.0 V for 10 s in 0.10 M sulfuric acid solution and then pretreated by potential cycling in the same solution in the potential range from 0.0 to +1.0 V at 100 mV/s until a stable cyclic voltammogram was obtained.

Gold nanoparticles were electrochemically deposited on the pretreated CFMEs by a potential-step electrolysis from +1.1 to 0 V in 0.5 M H\textsubscript{2}SO\textsubscript{4} solution containing 1.0 mM HAuCl\textsubscript{4} with different pulse times, i.e., 10, 30, and 60 s.\textsuperscript{26} The gold-deposited CFMEs (denoted as Au/CFMEs) were taken out from the solution and thoroughly rinsed with water. Then, modification by protein A of the gold nanoparticles deposited on the CFMEs was performed by soaking the Au/CFMEs in 500 μL of 1 mg/mL

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\textsuperscript{26} Kumar, R.; Maïtra, A.; Pananjil, P.; Sharma, P. Biomaterials 2005, 26, 6743–6753.

protein A solution for 2 h. The electrodes (denoted as pA/Au/CFMEs) were then rinsed with water to remove the nonchemisorbed protein A prior to next modification of anti-CEA. Anti-CEA was immobilized on the pA/Au/CFMEs by immersing the electrodes in 0.10 M PBS (pH 7.4) containing 500 ng/mL anti-CEA for 2 h. The electrodes (denoted as anti-CEA/pA/Au/CFMEs) were rinsed with pH 7.4 PBS to remove the nonchemisorbed anti-CEA and stored at 4 °C while not in use. The fabrication process of the immunosensor is shown in Scheme 1a.

Immunoaassay Protocol. The detection process of CEA is depicted as follows. After incubating the immunosensor into incubation solution containing various concentrations of CEA for 20 min at room temperature and washing with pH 7.4 PBS, the resulting substrates were submerged in bionanosphere solution (i.e., TH-doped magnetic gold nanospheres labeled HRP—anti-CEA antibody) for 20 min at room temperature. After rinsing thoroughly with pH 7.4 PBS to remove the unbound bionanospheres, amperometric responses of the immunosensor were recorded in pH 6.8 acetic acid buffer containing 0.75 mM H2O2 due to the catalytic reduction of the bound HRP toward H2O2 in the presence of the doped TH (Scheme 1c). After each immunoaassay run, the immunosensors were regenerated by immersing into 0.1 M glycine—HCl (pH 2.0) for 5 min and washed with pH 7.4 PBS, and then carried out the following run cycle.29 The detection principle of the sandwich-type immunoaassay is illustrated in Scheme 1c.

Statistical Analysis. All measurements were carried out in triplicate. All current responses recorded in this study were subtracted from the corresponding primary current as the baseline unless specified otherwise. A statistical data analysis was performed using SAS ver. 9.0 and SPSS ver. 9.0 softwares. Comparisons between dependent variables were determined using analysis of variance (ANOVA), Duncan multiple range test, correlation analysis, and multiple regression analysis. Results are expressed as mean value ± standard deviation (SD) of three determinations, and statistical significance was defined at P ≤ 0.05.

RESULTS AND DISCUSSION
Characteristics of TH-Doped Magnetic Gold Nanospheres. Scheme 1 shows the schematic illustration of the immobilization procedures. The sandwich immunoaassay protocol based on TH-doped nanogold spheres as label and HRP as enhancer was allowed to improve the performance of the immunoaassay in this study. The amplification of the electrochemical signal could mainly be achieved from the bionanospheres toward the reduction of H2O2 in the presence of the doped TH. A well dispersion with a mean diameter of 40 nm could be observed on the TEM image of the TH-doped magnetic gold nanospheres (Figure 1a). The open structure provided a significant increment of the binding capacity and, the surface of gold nanoparticles exhibited good binding capacity to antibody molecules.30 The magnetic gold nanosphere included many gold nanoparticles, which could largely enhance the coverage of HRP—anti-CEA on the surface of nanospheres.

The magnetic gold nanospheres-labeled HRP—anti-CEA was characterized with UV—vis absorption spectra. No absorption peak was observed for pure NiFe2O4 nanoparticles (curve 1, Figure 1b). Two absorption peaks at 240 and 536 nm were obtained at TH-doped gold nanospheres (curve 3, Figure 1b). In comparison with those obtained at pure TH solution (curve 5, Figure 1b) and pure gold colloids (curve 6, Figure 1b), the slight deviation between absorption wavenumbers indicated an interaction between gold nanoparticles and thionine molecules. When HRP—anti-CEA was labeled to the surface of magnetic gold nanospheres, several adsorption peaks were simultaneously observed (curve 4, Figure 1b). The peak at 276 nm is mainly ascribed to anti-CEA antibody in contrast with that of pure anti-CEA (curve 2, Figure 1b). A small peak at 416 nm was derived from the bound HRP. The wide peak between 530 and 600 nm is due to the interaction between nangold—TH and HRP—anti-CEA. On the basis of the UV—vis results, we might make a conclusion that HRP—anti-CEA could be bound to the nanosphere surface.

Electrochemical Characteristics of Immunosensors. No peak was observed at bare anti-CEA/pA/Au/CFME at 50 mV/s in pH 6.8 acetic acid buffer (Figure 2a). After the immunosensor was incubated with CEA in the sample solution, a low background current was achieved (Figure 2b). This indicated that the formation of the antigen—antibody complex resulted in a great decrease in the background current. When the CEA/anti-CEA/pA/Au/CFME was reincubated with bionanospheres, however, a couple of redox peaks at +110 and +20 mV (ΔEp = 90 mV, ipc/ip0 ~ 1) was obtained in pH 6.8 acetic acid buffer (Figure 2c), indicating that thionine doped into bionanospheres is a good mediator and facilitates electron transfer. As shown in Figure 2d, upon the addition of hydrogen peroxide into the substrate solution, an obvious catalytic characteristic appeared with a dramatic increase of the reduction current and a sharp decrease of the oxidation current. This result indicated the immobilized HRP in the composite bionanospheres could retain high enzymatic catalytic activity and effectively shuttle electrons from the base electrode surface to the redox center of HRP. The electron-transfer pathway occurring at the immune-biosensor was described in a previous report.31 Moreover, the reduction peak currents increased with

![Figure 2. Cyclic voltammograms of (a) anti-CEA/pA/nanogold/CFME, (b) CEA/anti-CEA/pA/nanogold/CFME, and (c) bionanospheres/CEA/anti-CEA/pA/nanogold/CFME in pH 6.8 acetic acid buffer, and (d) electrode (c) in pH 6.8 acetic acid buffer containing 0.75 mM H2O2. Concentration of CEA: 35.3 ng/mL. Scan rate: 50 mV/s.](image-url)
the increment of CEA concentration in the sample solution (data not shown), which is in accordance with the antigen–antibody interaction of the sandwich-type immunoassay. During the incubation procedure, the bound bionanospheres increased with the increment of sample CEA concentration, and the increased HRP–anti-CEA enhanced toward the reduction of H$_2$O$_2$. Therefore, the reduction currents indirectly depended on the CEA concentrations.

**Comparison of Electrochemical Responses.** A biocomposite is formed by combination of two or more phases of different natures. It acts not only as a support for the immunologic material but also as a transducer. The binding material is essential in the biocomposite formation. Experimental results showed that the thionine and HRP doped into bionanospheres influenced the amperometric response of the immunosensor in different ways. A comparative study of the amperometric responses of immunoassay was carried out by using HRP–anti-CEA, gold nanoparticle-labeled HRP–anti-CEA, bionanospheres without TH, bionanospheres without HRP, and bionanospheres as a secondary conjugating antibody in the sandwich immunoassay, respectively (Figure 3).

Figure 3. Amperometric responses of the immunosensor with the various secondary labeled antibody toward different CEA concentrations: (a) bionanospheres, (b) bionanospheres without thionine, (c) nanogold particle-labeled HRP–anti-CEA, (d) HRP–anti-CEA, and (e) bionanospheres without HRP in pH 6.8 acetic acid buffer containing 0.75 mM H$_2$O$_2$.

As shown in Figure 3, one can find that use of bionanospheres with TH and HRP shows much greater amperometric changes than those obtained at the other label methods. Some possible explanations may contribute to these observations. First, the high surface-to-volume ratio of bionanospheres might greatly enhance the immobilization density of HRP–anti-CEA bound. Second, thionine doped into the bionanospheres acts as a good mediator, which could effectively shuttle electrons from the base electrode surface to the redox center of HRP. Third, the biocatalytic reaction of the immobilized HRP could amplify the amperometric signal output. The above results apparently suggest that use of the bionanospheres could generate better performances of detecting CEA.

**Optimization of Experimental Conditions.** The influence of pH value of the detection solution is an important parameter, because the acidity of the solution greatly affects the activity of the immobilized protein. Figure 4a shows the effect of pH value of the detection solution on the current responses of the immunosensors in acetic acid buffer containing 0.75 mM H$_2$O$_2$ after incubation with 30 ng/mL CEA for 20 min at room temperature. The analytical signal is based on the catalytic effect of the immobilized HRP on the H$_2$O$_2$ system. The current change was increased with the increment of pH value from pH 3.5 to 6.8 and then decreased. The optimal amperometric response was achieved at pH 6.8. Highly acidic or alkaline surroundings would damage the immobilized protein, especially in alkalinity. So pH 6.8 was selected as the optimum pH value for CEA detection.

In addition, the conditions used for immunoreaction greatly affected the amperometric response for CEA immunoassay. These conditions included incubation time and incubation temperature. With an increasing incubation temperature from 10 to 50 °C, the immunosensor after incubation with 30 ng/mL CEA for 20 min showed a maximum current response at 35 °C in pH 6.8 acetic acid buffer containing 0.75 mM H$_2$O$_2$ (Figure 4b). To simplify the analytical process, all the experiments were carried out at room temperature. At this temperature, the amperometric response of the immunosensor to 30 ng/mL CEA increased with the increment of incubation time and leveled off after 20 min (Figure 4c). Longer incubation time did not improve the response. Therefore, 20 min was chosen as the incubation time for the determination of CEA antigen in the following experiments.

**Dose–Response Curves.** Prior to the signal acquisition step with the developed immunosensor, an amperometric measurement was conducted to verify the biospecific interaction between the target antigens and the immobilized antibodies. When normal (negative) serum samples were analyzed using the developed immunosensor as the control tests, the anti-CEA-associated probes showed substantially low decrease in amperometric response (<1.7%) in contrast to the results obtained when the corresponding CEA positive serum was assayed. These results revealed the significant response difference between the lineage-specific recognition and the nonspecific adsorption.

To assess the sensitivity and the quantitative range of the proposed immunoassay, a cyclic voltammetric measurement with a sandwich immunoassay format was employed to detect CEA with bionanospheres-labeled HRP–anti-CEA molecules as tracer and H$_2$O$_2$ as enzyme substrate under optimized conditions. The curve was not a linear one, as is commonly observed for immunosassays, and a sigmoid relationship between the amperometric change and the CEA concentration was obtained (Figure 4 of the Supporting Information). The current responses increased with the increment of CEA concentration in the sample solution after the antigen–antibody interaction in pH 6.8 acetic acid buffer solution. For comparison, the current responses of the proposed immunosensor were recorded in pH 6.8 acetic acid buffer solution without the addition of H$_2$O$_2$, and the immunosensor exhibited a linear dependence on the CEA concentration from 1.2 to 125 ng/mL with a detection limit of 0.5 ng/mL (Figure 5a). The response is mainly due to the conjugation of the doped thionine. When 0.75 mM H$_2$O$_2$ was injected into the pH 6.8 acetic acid buffer, the reduction current rose sharply to reach a steady-state value within

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2 s (achieving 95% of the steady-state current), and the reduction current increased with the increment of sample CEA concentration. As shown in Figure 5b, the increase of reduction current was proportional to CEA concentration in the range of 0.01–160 ng/mL, and the linear regression equation is \( i_{pc} (\mu A) = 2.723 \times 10^{-5} + 0.758 \times [\text{CEA}] \) (ng/mL) with a detection of 5 pg/mL at a signal-to-noise ratio of 3 (where \( \sigma \) is the standard deviation of a blank solution, \( n = 11 \)). When the CEA concentration was higher than 160 ng/mL, an appropriate dilution of sample was necessary in the preincubation step.

**Repeatability, Reproducibility, Selectivity, and Stability of Immunosensors.** The reproducibility of the immunoassay system was evaluated by intra- and interassay coefficients of variation (CVs). The intra-assay precision of the analytical method was evaluated by analyzing four concentration levels five times per run in 5 h. The CVs of intra-assay with this method were 4.2%, 6.5%, 5.9%, and 7.1% at 0.5, 20, 80, and 140 ng/mL of CEA, respectively. Similarly, the interassay CVs on five immunosensors were 7.3%, 5.7%, 8.2%, and 4.8% at 0.5, 20, 80, and 140 ng/mL of CEA, respectively. Thus, the precision and reproducibility of the proposed immunoassay were acceptable.

To investigate the specificity of the proposed immunosensor, \( \alpha \)-fetoprotein (AFP), CA 19–9, CA 125, and BSA were used in this study. Amperometric responses of the proposed immunosensor in 0.5, 20, 80, and 140 ng/mL of CEA solutions containing interfering substances of different concentrations were assayed, and the RSD values were 3.2–10.3%, 5.6–8.9%, 4.6–8.3%, and 2.7–9.1%, respectively. Table 1 shows the experimental data in 20 ng/mL (as an example) of CEA solutions containing various interfering substrates. So the selectivity of the as-prepared immunosensor was acceptable. The stability of the immunoassay system was examined. When the immunosensor was dried and stored at 4 °C, it retained 89.7% of its initial response after a storage period of 19 days. The slow decrease of response seemed to be related to the gradual deactivation of the immobilized antibody incorporated in the composite.

**Preliminary Application of Immunosensors.** To monitor the feasibility of the newly developed immunoassay, 32 serum specimens, which were gifted from the Cancer and Tumor Hospital of Chongqing, China, were examined by the developed immunoassay and the ELISA method. The total assay time was about 55 min for one sample including 20 min of preincubation and regeneration of the reactor. The measurement method of the proposed immunosensor is as follows: (1) starting up operation, 2 min; (2) sample process, 2 min; (3) measurement before the antigen–antibody interaction, 2 min; (4) immunoreaction, 20 min; (5) immunoreaction with bionanoparticles, 20 min; (6) measurement after the antigen–antibody interaction, 2 min; (7) regeneration of sensor, 5 min.

Experimental results are described in Figure 6. The regression equation (linear) for these data is as follows: \( y = 1.5641 + 0.9628x \) \( (R^2 = 0.991) \) (\( x \)-axis, by the as-prepared immunosensor; \( y \)-axis, by ELISA). These data shows that this method was comparable and acceptable for CEA detection, that is, the developed immunoassay may provide a promising alternative tool for determining CEA in human serum in the clinical laboratory.

Additionally, the analytical performance of the developed CEA immunosensors has been compared with those of other CEA electrochemical immunosensors reported in the literatures.\(^\text{(37)}\)–\(^\text{(42)}\) Characteristics such as range of linearity of the corresponding

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**Figure 4.** Effects of (a) pH of acetic acid buffer, (b) incubation temperature, and (c) incubation time on amperometric response of the immunosensor toward 30 ng/mL CEA under optimal conditions.

**Figure 5.** Calibration curves of the immunosensor for CEA determination (a) before and (b) after the addition of H\(_2\)O\(_2\) in pH 6.8 acetic acid buffer. Inset: Amplification of the calibration curve in the low CEA concentration before and after the addition of H\(_2\)O\(_2\).

**Figure 6.** Assay results of clinical sera using the proposed and reference ELISA methods, where \( n = 5 \) for each point.
catalyze the reduction of H2O2 with the aid of the doped thionine. Paramagnetic nanospheres were modified with HRP anti-CEA, by which, the bound HRP could greatly catalyze the reduction of H2O2 in the presence of the doped thionine. The doped thionine was used not only as an immobilized substrate but also as an electron-transfer mediator in this paper. In comparison with other electrochemical immunoassay methods, the developed method could not only prevent the leak of hydrophilic thionine from the electrode but also avoid the addition of mediator to the solution. Significantly, the system was capable of continuously carrying out all steps in less than 60 min for one sample, including incubation, washing, enzymatic reaction, determination procedures, and regeneration of the sensor, which is shorter than that of the commercial ELISA (>4 h).

CONCLUSIONS
Signal amplification and noise reduction are crucial for obtaining low detection limits in biosensors. Herein, a new double-coded nanolabel based on TH-doped magnetic gold nanospheres modified with HRP anti-CEA is reported for electrochemical detection of CEA. The electrochemical signal is amplified both by magnetic biananosphere labels and by the bound HRP on the magnetic biananospheres toward the catalytic reduction of H2O2, and the noise is reduced by employing the CFME electrode and the hydrophilic immunosensing layer. Highlights of the developed immunoassay method are mainly focusing on the following properties: (i) the bound HRP activity toward the reduction of H2O2 that can be related to the analyte concentration and measured amperometrically; (ii) the intrinsic electrochemical properties of the biananosphere labels that being proportional to the protein concentration can be directly quantified by cyclic voltammetry. Moreover, a secondary indirect detection was applicable to this system, exploiting the high molar absorptivity of HRP anti-CEA, by which, the bound HRP could greatly catalyze the reduction of H2O2 in the presence of the doped TH. Paramagnetic nanoparticles were used as the label, which resulted in incubation and washing times shorter than those typically needed in classical ELISA tests by means of a rapid magnetic separation of the unbound components. Importantly, the developed immunoassay system could open a new avenue in double-coded nanolabel applications for immunosensing and provide a convenient platform to modify for clinical testing, drug screening, and discovery in the future.

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