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Conductive three-dimensional ordered nano-gold film: Ultrasensitive electrochemical sensing platform for clinical immunoassay

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ABSTRACT

A simple, reusable and ultrasensitive electrochemical clinic immunoassay is proposed via developing a versatile CTDONG (conductive three-dimensional ordered nano-gold) film-modified gold electrode, in which ferrocene derivative and human immunoglobulin G (hIgG) are used as signaling probe and model molecule, respectively. A signal-on signaling mechanism is achieved by utilizing a sandwich format of the primary antibody/hIgG/the secondary antibody labeled with Ferrocene (PAb/Ag/SAb). Owing to the combination of the advantages of CTDONG film with the versatility of ferrocene derivatives, a substantially enhanced signal accompanied by a low background peak current is achieved. By this sensing scheme, target molecule can be readily quantified in a comparatively wide dynamic range (8.1×10^{-13}–6.2×10^{-10} M) with a relatively low detection limit (2.7×10^{-13} M). In addition to a greatly improved signal gain, this immunosensor gives a favourable reusability and good reproducibility. Moreover, the CTDONG film-based sensing interface shows excellent anti-interference ability to the coexistent proteins. Meanwhile, the recovery test and determination of target molecule in real samples have confirmed the feasibility of designed sensing system for clinic immunoassay of protein molecules, demonstrating the potential application of described CTDONG film in the development of biomolecule assay platforms.

1. Introduction

Scientists are faced with the great challenge of developing novel systems for effectively identifying and quantitating proteins since the research moves into the era of proteomics [1]. Although, the enzyme-linked immunosorbent assay (ELISA) has become a standard detection method used in clinical and environmental monitoring, it involves the relatively long analysis time, considerably complex assay procedure and high reagent consumption and often suffers from various influencing factors [2,3]. On the basis of specific antibody–antigen reactions, immunosensors provide a promising tool for the detection of immunoreagents and become an alternative tool to replace the traditional ELISA [4]. Among several detection methods, the electrochemical technique is of particular significance in the development of immunosensors.

Highly-sensitive detection of biomolecules could be typically achieved via sandwich immunoassay format where the primary antibody (PAb) is anchored to a substrate surface and the secondary antibody (SAb) is labeled with one or more signaling moieties [5]. The PAb is employed to capture and isolate target protein from the sample, and the labeled-SAb offers a specific signal upon sandwich immunoreaction event. For this assay format, the small amount of target molecules might trigger a detectable signal above background. Therefore, a very considerable proportion of protein sensing systems [6–13] were developed by the sandwich assay format.

The emergence and recent advance of nanoscience and nanotechnology have opened up a promising era in highly sensitive electrochemical bioassays. Various nanomaterials have been extensively employed to fabricate sensitive electrochemical immunoassays [7,14–20], and the recent advances in applications of nanomaterial labels and nanotechnology in electrochemical immunosensors and immunoassays have been reviewed [21]. Generally speaking, the applications of nanoscale materials in electrochemical immunosensors can be classified into the following three categories according to their functions: (1) nanoscale materials are used as electroactive reporters [14,16,22]; (2) nanomaterials are used as carriers to load a large amount of biomolecules or electroactive species for maximizing the ratio of signaling probes to immunoreaction event [23]; (3) nanomaterials are employed to prepare nanoscale film in order to immobilize biorecognition elements [24,25]. Despite the convenient detection, the third pro-

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tocol often offers a sparse nanoparticle-modified substrate due to the electrostatic repulsion between nanoparticles. To achieve improved biosensors, three-dimensional ordered nanostructured films have attracted increasing attention [26–28]. Although the improved analytical characteristics have been achieved compared with the traditional assay schemes, the templates are required for the electrodeposition of nanoscale materials and toxic reagents were generally involved during the removal of templates.

Among the nanomaterials, gold nanoparticles (GNPs) are particularly attractive for numerous biological investigations by virtue of their facile synthesis, the large specific surface area, high chemical stability, favorable biocompatibility, high surface free energy, good conductivity, optical properties, catalytic applications and high affinity for binding to amine/thiol-containing molecules [29,30]. The research [31] demonstrates the potential application of gold nanopartilces in utlrasensitive immunoassays for early disease screening and diagnosis. For the development of ultrasensitive electrochemical immunoassays, the biorecognition layer could preserve a sufficient binding capability, and the electroactive species conjugated to the secondary antibody should possess remarkable electrochemical property and be immunoadsorbed onto the sensing interface as many as possible. Ferrocene (Fc) and its derivatives are attractive components as electroactive probes, and several workers have confirmed that Fc is extremely useful for the construction of sensitive electrochemical biosensors [32]. Based on above facts, a sandwich-type electrochemical biosensor was developed via preparing a CTDONG (conductive three-dimensional ordered nano-gold) film by a seed-mediated growth method (a non-template strategy) and synthesizing ferrocenecarboxylic acid (FcA)-conjugated secondary antibodies as signaling reporters. The present sensing scheme was able to quantitatively detect subpicomolar protein hIgG, and the application and reliability were confirmed by comparing assay results obtained with those detected with (ELISA), opening new opportunities for ultrasensitive electrochemical bioassays.

2. Experimental

2.1. Reagents

Goat anti-human IgG antibody (a primary antibody), human immunoglobulin G (hIgG) (affinity purification), rabbit anti-human IgG, goat IgG and bovine serum albumin (BSA) were purchased from Beijing Dingguo Biotechnology Company (Beijing, China).

Ferrocenecarboxylic acid (FcA) was received from Maoji Fine Chemicals Co. (Shanghai, China), while 1,6-hexanedithiol (HDT) and mercaptoethanol was supplied by Alfa Aesar (USA). N-Hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), hexadecyltrimethylammonium bromide (CTAB) and nicotinamide adenine dinucleotide (NADH) were purchased from Aldrich, Sigma, Across Organics (New Jersey, USA) and Generay Biotech Co., Ltd., respectively. Chloroauric acid ($HAuCl_4$) and trisodium citrate were provided by Shanghai Chemical Reagents (Shanghai, China). Gold nanoparticle seeds were prepared by citrate reduction of $HAuCl_4$ according to the method reported in literature [33].

The blocking buffer solution was phosphate buffer solution (PBS, pH 7.4) containing 1% (w/v) BSA. Phosphate buffer solution (PBS, pH 7.4) consisting of 0.01 M phosphate-buffered saline, 0.137 M NaCl, and 3 mM KCl was used to prepare other protein solutions. The alternating current (AC) voltammetric measurements were carried out in 10 mM PBS (pH 7.4) containing 0.3 M NaCl and 3 mM KCl. AC impedances were performed in 10 mM PBS (pH 7.4) containing 0.1 M KCl and 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox couple. All other chemicals were of analytical grade and used as received. Deionized and sterilized water (resistance > 18 MΩ-cm) was used throughout the experiments.

2.2. Preparation of FcA-conjugated rabbit anti-human IgG (Fc-SAb)

FcA-conjugated rabbit anti-human IgG was performed utilizing the following procedure. The COOH group of ferrocenemonocarboyxylic acid was activated according to the reported method with a minor modification [32]. FcA (2 mg) was added to 1 mL of EDC/NHS activation aqueous solution (50 mM each). The reaction mixture was maintained under stirring conditions for 2 h. Then, 200 μL of 10 mg/mL rabbit anti-human IgG was added, and the mixture was incubated at room temperature overnight. The removal of unreacted ferrocene derivative was carried out by dialysis with three changes of fresh buffer (PBS, pH 7.4). The resulting mixture was brought to volume of 1 mL and stored in refrigerator at 4 °C until use.

2.3. Fabrication of conductive three-dimensional ordered nano-gold (CTDONG) film

Schematic representation of the construction of the electrochemical sensing interface and measurement procedure of the sandwich-type immunoassay are detailed in Scheme 1. A gold electrode was polished according to the reported method [34]. The cleaned electrode was immediately immersed in the 5 mM ethanolic solution of HDT for 4 h. Then, HDT-modified gold electrode was thoroughly rinsed with absolute ethanol, water, and dried with purified N_2. Afterward, gold nanoparticle self-assemblyed monolayer was prepared by immersing the HDT-modified electrode in a gold nanoparticle solution for 3 h. The resulting interface was rinsed with water under stirring conditions to remove the unbound GNPs. Finally, the GNP-immobilized electrode was immersed in the growth solution that contained 1.8×10^{-4} M $HAuCl_4$, 7.4×10^{-2} M CTAB, and 4×10^{-4} M NADH at 37 °C for 60 min [35], achieving a conductive three-dimensional ordered nano-gold film for amplifying immunoassay signal.

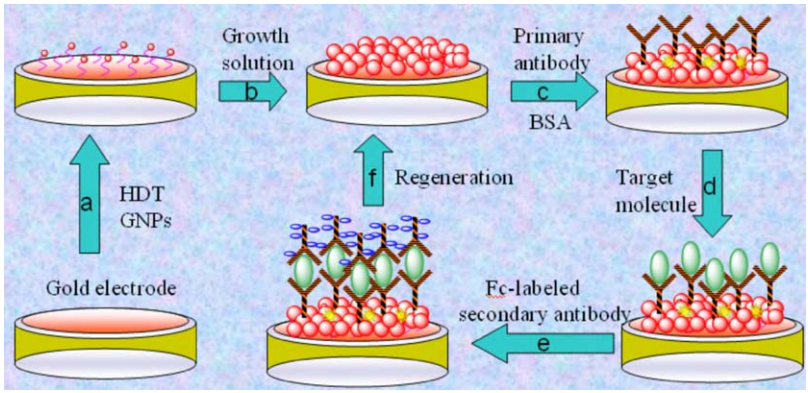
2.4. Preparation of biorecognition layer

The CTDONG film-modified electrode was immersed in PAb solution for 2 h at room temperature. Following the removal of the redundant protein species by rinsing with PBS, the primary antibody-adsorbed gold electrode was soaked in BSA solution for 30 min to block the remaining bare region. Then, the resulting interface was ready for the detection of target samples after washing with PBS.

2.5. Procedure of protein detection

In a typical experiment, a CTDONG film-based sensing interface that was held upside down was coated with 10 μL of target sample at specific concentration and kept in a water-saturated atmosphere for 50 min at room temperature to accomplish the immunoreaction (the first imunoreaction), followed by washing with PBS to remove the unbound hIgG. Then, 10-μL droplet of Fc-SAb conjugate solution was pipetted onto the resulting electrode surface. The reaction (the second immunoreaction) was allowed to maintain for 40 min in an identical environment, resulting in the specific immuno-adsorption of signaling species. After rinsing with PBS, electrochemical measurements of sandwich immunocomplex-modified electrode were carried out to estimate the amount of hIgG in sample.

To valuate the detection selectivity of the present assay system, protein interferents substituting hIgG were used for the immunore-



Scheme 1. Schematic representation of the construction of the sensing interface and measurement procedure of the electrochemical sandwich-type immunoassay. (a) Immobilization of GNPs on the electrode surface using HDT as cross-linking reagents via sulfur-gold affinity; (b) catalytic size enlargement of GNP seeds immersed in the growth solution; (c) the preparation of sensing interface in which primary antibody was adsorbed onto CTDONG film and BSA was used to block the remaining bare region; (d) the first imunoreaction; (e) the second immunoreaction; (f) the regeneration of used sensing interface. The detailed procedures are given in Section 2.

action, and detection experiments were conducted as described above.

2.6. Apparatus and electrochemical measurements

AC impedance and AC voltammetric measurements were performed using CHI 760B electrochemical workstation (Shanghai, China). The reference electrode was a saturated calomel electrode (SCE, saturated KCl) while a platinum foil was used as the auxiliary electrode. AC voltammetry was used to evaluate the response characteristics of the CTDONG film-based immunosensor. AC impedance spectra were collected at an applied potential of 240 mV with the AC voltage amplitude of 5 mV. The voltage frequency range involved was from 1 to 100,000 Hz.

The surface morphology of the modified substrate was investigated by scanning electron microscopy (SEM, Hitachi H8010, Hitachi, Tokyo, Japan).

2.7. Regeneration of used sensing interface

The regeneration of the sensing interface used is an important factor to consider for the development of practical immunosensors. In this contribution, a 5.0-mL mixture (regeneration solution) of 0.2 M NaOH and 0.5 M NaCl was employed to denature the immunocomplexes by immersing the used sensing interface in the regeneration solution for 10 min under gentle stirring conditions. After rinsing with PBS for another 10 min, the fresh primary antibodies were adsorbed onto the resulting electrode surface. The regenerated sensing interface could be employed to perform the next hIgG assay or stored in PBS at 4 °C.

3. Results and discussions

3.1. Fabrication of sensing system and conductive three-dimensional ordered nano-gold (CTDONG) film

High stability of sensing interface and function density of the surface-confined proteins are critical for the successful application of an immunoassay. Molecular self-assembly has become a popular surface derivatization technique, owing to its versatility, simplicity, and the establishment of a high level of order on a molecular scale as a means for the surface modification. In the present contribution, a CTDONG film-based sensing interface was prepared according to the seed-mediated growth method (see Section 2 for the details). The construction of the electrochemical immunosensing interface and the signal transduction procedure of the sandwich-type immunoassay are detailed in Section 2 (also shown in Scheme 1).

The surface morphology of the fabricated matrix is an important factor affecting the immobilization of biorecognition molecules. Therefore, the catalytically enlarged nano-gold film was investigated by scanning electron microscopy (SEM). A typical SEM image is shown in Fig. 1. One can see that the gold film obtained in this method mainly consists of multilayer gold nanoparticles with a narrow size distribution. These nanoparticles are linked to each other and result in a three-dimensional ordered structure with the abundant interstices. In the present contribution, the enlarged nano-gold layer achieved is defined as conductive three-dimensional ordered nano-gold (CTDONG) film. The average diameter of enlarged GNPs (about 16 nm) was slightly larger than that of GNP seeds, and the substantial increase of the surface coverage was achieved compared with GNP seed-modified electrode surface (not shown in this contribution). Even though prolonging incubation time for the assembly of GNP seeds to 12 h without the catalytic growth step, it is absolutely impossible to prepare a GNP-modified substrate surface with a high nanoparticle density owing to the electrostatic repulsion as reported in our another work [25]. To illustrate the role of GNP seeds bound to the substrate surface, the control matrixes without GNP seeds were treated with the same growth solution under identical conditions and also examined by

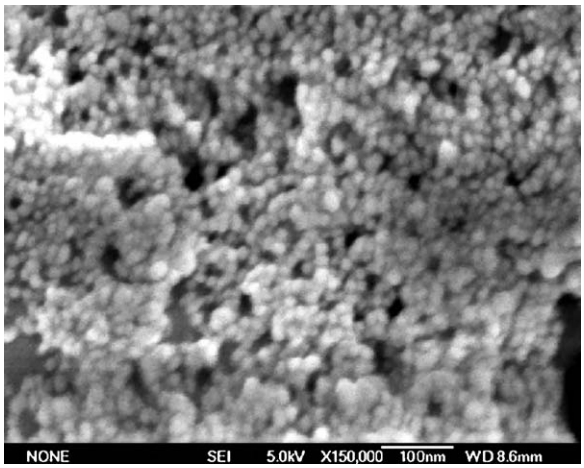


Fig. 1. Scanning electron microscopic images of the CTDONG film prepared by improved seed-mediated growth method. The average diameter of gold nanoparticles in the CTDONG film is about 16 nm. Catalytic growth was carried out for 60 min at 37 °C. For imaged surface the scale bar corresponds to 100 nm.

SEM. Control experiments reveal that there is no obvious change in surface morphology, demonstrating that GNP seeds are required for preparing the CTDONG film. Clearly, two steps are involved in the enlargement of the GNP seeds: (i) the rapid reduction of $AuCl_4^-$ by NADH to the colorless Au^I species and (ii) the slow catalyzed reduction of the Au^I species by the GNP seeds to the gold particles. They are described in Eqs. (1) and (2) [36], respectively.

$$AuCl_4^- + NADH \rightarrow Au^I + 4Cl^- + H^+ + NAD^+ \quad (1)$$

$$2Au^I + NADH \xrightarrow{\text{nano-gold seed}} Au^\circ + H^+ + NAD^+ \quad (2)$$

Taking into account a fact that, upon the enlargement of the parent nanoparticles, gold nanocrystalline flakes could be deposited on the seeds and catalytically enlarged at the sharp intersections [37], it was reasonable that catalytic size enlargement of GNP seeds was observed while the cascade generation and subsequent growth of new nanocrystallites on the surface of parent GNP seeds occurred smoothly, increasing considerably the number of enlarged GNPs. Although the seed-mediated growth method used to prepare nanoscale materials has attracted extensive researches because of its shape-controllability and reproducibility [38,39], we for the first time fabricated a conductive three-dimensional ordered nano-gold porous film composed of spherical nanoparticles. The different nanoscale materials obtained in our work should be attributed to the different GNP seeds and substrate, possibly as well as the slight change in growth solution. The successful preparation of three-dimensional ordered nano-gold film would promote the exploitation of desired biosensing systems as indicated in impressive researches [27,28].

3.2. Signal enhancement and experimental principle

To confirm that an enhanced signal can be given by the CTDONG film-based biosensor, the response signal of another biosensor (the HDT/GNPs-based biosensor) without catalytic enlargement procedure was investigated for the direct comparison. The experimental results are shown in Fig. 2. No detectable current response to the blank solution was observed for both biosensors. Assuming that the peak current induced by introduction of hIgG sample for the CTDONG film-based biosensor is assigned the value of 100%, the peak current observed for HDT/GNPs-biosensor is only 20%.

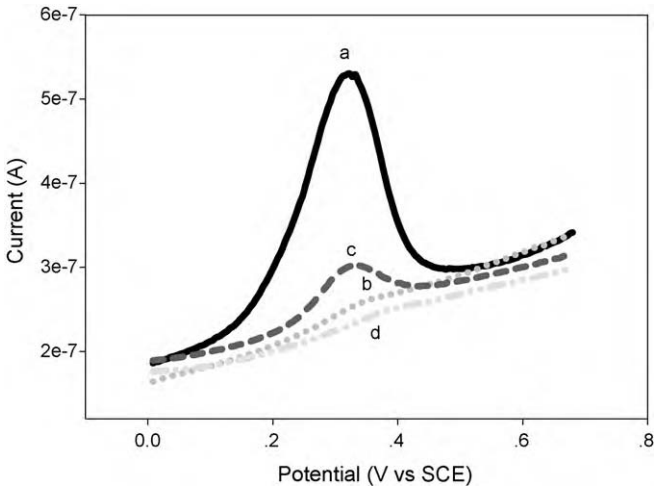


Fig. 2. The signal amplification obtained for the catalytic enlargement of GNPs. The AC voltammograms for the proposed CTDONG film-based immunosensor in the presence (a) and absence of (b) target molecules are compared with those collected for the HDT/GNPs-based biosensor without the catalytic enlargement process in the presence (c) and absence of (d) target molecules under identical conditions. The concentration of analyte is 100 ng/mL.

Namely, significantly enhanced signal, more than 5 times higher than that achieved by simply assemblying GNPs on the HDT-modified electrode, could be acquired. There are three possible reasons for the high detection capability of the developed electrochemical biosensor. First, GNPs might allow more freedom in orientation for anchored proteins and provide a mild microenvironment similar to that of these biomolecules in native system. Thus, the proteins immobilized onto GNP surfaces are capable of displaying high bioactivity [40]. Second, the CTDONG-modified electrode could provide a large surface area for immobilizing PAb and greatly increase the amount of adsorbed biomolecules, generating significantly enhanced current signal [41]. Finally, the CTDONG film consisting of interconnected nanoparticles exhibited a desired conductivity, facilitating the electron transfer between the electrode surface and the electroactive species conjugated to the SAb. The observations are consistent with those reported in a previous work [27].

3.3. Impedance characterization

As an effective method to investigate the properties of electrode surfaces, impedance spectroscopy was employed to evaluate the electronic conductivity of CTDONG film. Fig. 3 displays Faradaic impedance spectra (presented as Nyquist plot) of the same electrode with the CTDONG film enlarged for various incubation times. Compared with the bare electrode, the HDT/GNP seed layer inhibits the electron transfer to some extent as shown in Fig. 3A. This observation is also validated by literature work [25]. Presumably, the adsorption of species in the growth solution, for example, hexadecyltrimethylammonium bromide (CTAB) and nicotinamide adenine dinucleotide (NADH), onto the nano-gold modified electrode surface can change the surface characteristics and inhibit the electron transfer. On the other hand, the enlargement of surface-confined GNP seed can increase the conductivity of electrode. Thus, during the enlargement of GNP seeds, the impedance magnitude increases at an early stage and then decreases gradually with the increment of enlargement time within about 60 min. Finally, dramatic impedance decay could be observed implying an extremely high electronic conductivity. Possibly, the immoderate GNP enlargement tends to form a planar electrode surface similar to the integrated one. Such modified electrodes are not suitable to prepare a versatile biorecognition layer. Therefore, it is necessary to optimize the incubation time for the catalytic enlargement of GNP seeds to fabricate successfully a promising biosensor.

The optimized experimental conditions for improving the assay performance of sensing interface are detailed in Supplementary data.

3.4. Analytical characteristics of CTDONG film-based immunosensor

AC voltammetry as a signaling technique is extremely useful for the investigation of redox reactions occurred on electrode surfaces, particularly when the amount of electrochemically active moieties is very small [42]. Therefore, series of hIgG at the various concentrations ranging from 10.8 pg/mL to 400 ng/mL were detected by AC voltammetry, and the corresponding peak currents were used to evaluate the analytical characteristics of this sensor. Fig. 4 depicts a linear relationship between the peak current and the logarithm of the target concentration. The peak current increased monotonously with the augment of analyte concentration, and the hIgG might be accurately quantified over a concentration range of 0.13–100 ng/mL (8.1×10^{-13}–6.2×10^{-10} M). The regression equation is $Y = 8.287 \log X + 8.392$ with a correlation coefficient of 0.9965, where Y and X represent the peak current and the target concentration, respectively. The average relative standard

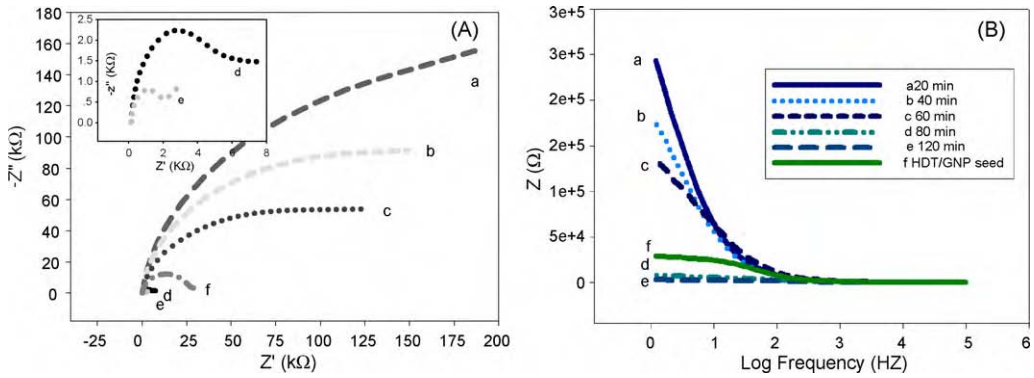


Fig. 3. (A) Typical Nyquist diagrams obtained in PBS (pH 7.4) containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox couple and 0.1 M KCl for the CTDONG film-modified electrode prepared by immersing the GNP seed-assemblyed surface in the growth solution for 20 (a), 40 (b), 60 (c), 80 (d) and 120 min (e). Line f represents the HDT/GNP seed-modified electrode. An applied potential of 240 mV was used. B) The relationship between the complex impedance and working frequency.

deviation is about 8.6%. Clearly, the developed CTDONG film-based immunosensor could offer a linear response range of nearly 3 orders of magnitude, indicating that the response range is improved by a factor of about 40 compared with the electrochemiluminescence immunoassay [43]. No substantial change of peak current was obtained when the target DNA concentration increased or decreased further. The detection limit of 43.4 pg/mL (about 2.7×10^{-13} M) could be achieved at which hIgG might trigger a small but appreciable current change. Clearly, this described electrochemical immunoassay can lower the detection limit by nearly 2 orders of magnitude compared with a previous electrochemical hIgG sensing system in which the multistep amplification of the immuno-binding event is involved [44]. Compared with a chemiluminescent immunoassay combining the advantage of a magnetic separation with the amplification feature of oxidative gold metal labels [45], the proposed electrochemical immunosensor could provide nearly a 3-order-of-magnitude increase in sensitivity. Even for a highly sensitive immunoassay, a sensing design based on the use of a double-codified nanolabel as well as an enzyme catalytic amplification, could offer an assay sensitivity only comparable to that offered by the present contribution [46]. These data demonstrate an ultrasensitive assay capability of the present sensing system. The high biological activity of antibody immobilized on the three-dimensional ordered nano-gold film and desirable conductivity should contribute to the improvement in the sensing performance. The three-dimensional ordered nano-gold film-based sensing interface could offer a mild microenvironment for the immunoreaction, facilitating the interaction between antibodies and antigens. Presumably, the high surface density of primary antibody on the basis of the three-dimensional nano-gold film can increase the collision frequency between the recognition molecule and target species, especially when target concentration is low. Consequently, an improved assay performance, including the detection limit, can be achieved. According to the results observed in other works [26], the high assay capability seems to be closely related to the formation of the three-dimensional nano-gold film.

In order to confirm that the peak current is indeed triggered by target protein, the nonspecific adsorption was investigated using BSA and commercially available goat IgG as model interferents. As seen in Fig. 5 (line b and c), virtually no obvious peak current is triggered by introduction of BSA, and the current change caused by the goat IgG samples had a negligible effect on the peak current (line a) albeit the concentration of interferents was much higher than that of target protein. These results verified that the peak current observed were caused specifically by the hIgG binding.

Reusability, reproducibility, stability and feasibility of the developed immunosensing system are separately evaluated as shown in Supplementary data.

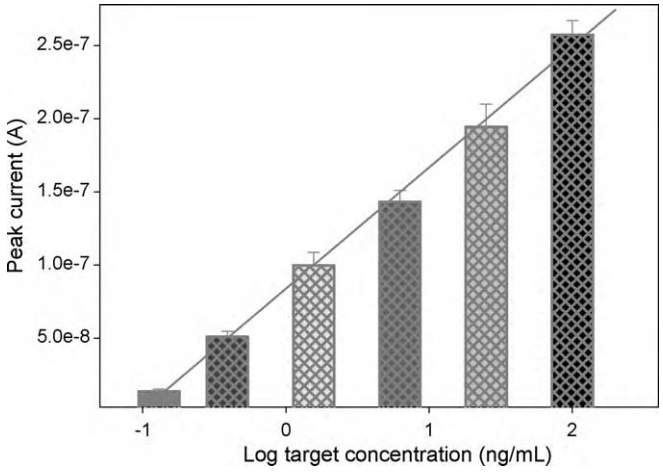


Fig. 4. The linear relationship between the peak current in AC voltammogram and the logarithm of the target concentration. The error bars indicated the standard deviation of triplicate determinations for each concentration of IgG. The regression equation was $Y = 8.287 \log X + 8.392$ with a correlation coefficient of 0.9965, where Y and X represented the peak current and the target concentration, respectively.

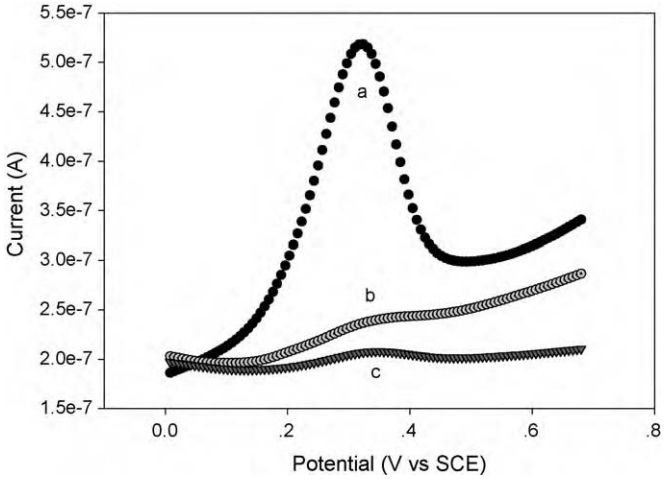


Fig. 5. The selectivity of the CTDONG film-based immunosensor. The AC voltammogram was collected for the sensing interface after exposure to 100 ng/mL hIgG (a), 100 g/mL BSA (b) or 500 ng/mL goat IgG (c).

4. Conclusions

This article presented a three-dimensional ordered nanoscale metal film and developed a simple, sensitive and reusable electrochemical immunosensor for hIgG detection. The distinct advantages of the CTDONG film and attractive electrochemical performance of Fc improve the analytical capabilities of the electrochemical sensing system. On the basis of the synergistic signal enhancement mechanism, the proposed electrochemical immunosensor exhibits the attractive assay features, including a wide linear response range, a relatively low detection limit, high reproducibility and reusability. Moreover, the recovery test and assay of human serum specimens give immediate evidence for the reliability and practicability of the immunoassay. Additionally, the present technique exhibits several advantages: a simple design of sensing scheme, low-cost and straightforward fabrication, and almost effortless detection procedure. Importantly, it was easy to extend the strategy to other immunoassay systems, allowing the detection of a broad spectrum of proteins. Given these improved characteristics, the CTDONG film seems to be desirable nanomaterials for developing high performance biosensors for biomedical sensing and application in diagnostics and proteomics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.06.033.

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