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Electrochemical immunosensor for norethisterone based on signal amplification strategy of graphene sheets and multienzyme functionalized mesoporous silica nanoparticles

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ABSTRACT

Norethisterone is one kind of widely used anabolic steroid hormones which can help to promote livestock growth and sometime has been illegally used for livestock breeding. The residues of norethisterone in animal food will harm people's health, therefore, it has been banned to use for the growth promotion purposes in livestock. In this study, amino-group functionalized mesoporous silica nanoparticles (MSN) were prepared and used to immobilize Au nanoparticles, which was further utilized for the adsorption of horseradish peroxidase (HRP) and the secondary antibody (Ab_2). The resulting nanoparticles, Au-MSN-HRP-Ab_2 were used as labels for immunosensors to detect norethisterone antigen. A sandwich-type protocol was used to prepare the immunosensor with the primary antibody (Ab_1) immobilized onto thionine (TH) and graphene sheet (GS) modified glassy carbon electrode surface. The sensitivity of the sandwich-type immunosensor using Au-MSN-HRP-Ab_2 as labels for norethisterone antigen detection was much higher than that using either Au-MSN-Ab_2 or MSN-HRP-Ab_2 as labels. Within norethisterone concentration range (0.01–10 ng/mL), a linear calibration plot ($Y = 0.55671 + 8.27101X$, $r = 0.9993$) was obtained with a detection limit of 3.58 pg/mL under optimal conditions. The proposed immunosensor shows good reproducibility, selectivity, and acceptable stability. This new type of labels for immunosensors may provide many potential applications for the detection of growth hormone in animal derived food.

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1. Introduction

Norethisterone is one kind of anabolic steroid hormones, which has been used worldwide in oral contraceptive preparations for many years. It can also promote the rates of protein deposition in livestock. It had ever been used for livestock breeding for many years because it can bring good economic effects for livestock breeding process. However, it will remain in animal tissue with varying degree. The remained norethisterone in animal food will do harm to people's health, therefore, its usage has been prohibited for the growth promotion purposes in livestock in many countries, such as in China, in the European Union. (The Ministry of Agriculture Bulletin of PRC176, 2001; Commission of the European Communities. Council Directive 96/23/EC. Off. J. Eur. Commun. 1996:125.) But now, norethisterone could still be detectable in some animal derived food, which could affect the human health. The employed methods for the detection of norethisterone include radioimmunoassay methods (RIAs) (Stanczyk et al., 1983), high performance liquid chromatography (HPLC) (Li et al., 2005) and gas chromatography–mass spectroscopy (GC–MC) (Maria et al., 2001). However, RIAs require handling of radioactive materials which will bring environmental protection issues. HPLC often requires expensive equipment, long procedures and analysis times, standards (not always commercially available) and trained personnel. GC–MS method typically requires sample pretreatment, expensive apparatus, and also has to be performed by highly trained personnel. Herein, we would like to demonstrate the usefulness of an electrochemical method by developing a convenient biosensor for the sensitive detection of norethisterone.

Comparing to several other types of immunosensors based on fluorescence, chemiluminescence, surface-plasmon resonance, or quartz crystal microbalance (Liu et al., 2001; Ionescu et al., 2007; Cui et al., 2007), electrochemical immunosensors are bioanalytical tools characterized by the simplicity of construction, the possibility to be mass-produced, the cost-effectiveness, the easiness of use, the feasible miniaturization and the subsequent portability. These advantages make it attractive for its use as highly performing screening tool for detecting biomolecules (Lai et al., 2009; Yang

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et al., 2008; Sánchez et al., 2008). Electrochemical immunosensors with a sandwich-type structure are still the mostly widely used protocol for ultrasensitive immunosensors (Wang et al., 2008; Tang et al., 2010). In recent years, various types of labels have been used for such immunosensors, including enzymes, quantum dots, carbon nanotubes, and electroactive component-loaded nanoparticles in order to amplify the electrochemical signal (Mwilu et al., 2009; Wang et al., 2009; Tang et al., 2008; Deng et al., 2009; Mani et al., 2009; Yu et al., 2006). Nanostructured materials are very attractive due to their unique optical, electrical, catalytic and magnetic properties and have found wide applications in the fabrication of biosensors (Chen et al., 2007; Cheng et al., 2006).

Mesoporous silica nanoparticles (MSNs) have gained great interest for a wide arrange of applications such as catalyst, drug release, sensors and separation due to many unique structural features including a large surface area and high pore volume, ordered porous channels, a uniform and tunable pore structure, and a great diversity in surface functionalization (Ca et al., 2008; Slowing et al., 2006, 2007; Ren et al., 2009; Vivero-Escoto et al., 2009; Yang et al., 2010; Wu et al., 2009). Recently, we have used MSN as label for the preparation of immunosensor for the detection of human IgG, which exhibited high sensitivity and low detection limit (Yang et al., 2010). Graphene sheet (GS), a single layer of carbon atoms bonded together in a hexagonal lattice, has attracted considerable attention to fabricate immunosensors due to its excellent electronic properties and large surface area (Zhong et al., 2010; Du et al., 2010).

In the design and fabrication of highly sensitive electrochemical immunosensors, signal amplification and antibody immobilization are the crucial steps (Sánchez et al., 2008). In this study, amino-group functionalized MSN (MSN) were prepared and used to immobilize Au nanoparticles, which was further utilized for the adsorption of horseradish peroxidase (HRP) and the secondary antibody (Ab_2). The resulting multienzyme functionalized MSN, i.e., Au-MSN-HRP-Ab_2 were used as labels for immunosensors to detect norethisterone antigen. A sandwich-type protocol was used to prepare the immunosensor with the primary antibody (Ab_1) immobilized onto thionine (TH) and GS modified glassy carbon electrode. Enhanced sensitivity was achieved by using the large specific surface area of GS to increase Ab_1 loading, the large surface area of MSN to increase HRP loading and the high conductivity of GS and Au nanoparticles to promote electron transfer among HRP, TH and the electrode, which resulted in the high sensitivity of the immunosensor (Du et al., 2010). Based on signal amplification strategy of GS and multienzyme functionalized MSN, the fabricated immunosensors using Au-MSN-HRP-Ab_2 as labels showed a linear response within the wide range of 0.01–10 ng/mL of norethisterone antigen, low detection limit, good reproducibility and selectivity, as well as acceptable stability.

2. Materials and methods

2.1. Apparatus and reagents

Goat anti-rabbit norethisterone antibody, norethisterone antigen, horseradish peroxidase (HRP), cetyltrimethylammonium bromide (CTAB), tetraethoxysilane (TEOS), thionine (TH), glutaraldehyde and hydrogen peroxide were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd., China. Glassy carbon electrodes were obtained from Chenhua Instrument Shanghai Co., Ltd., China. All other reagents were of analytical grade and deionized water was used throughout the study.

All electrochemical measurements were performed on a CHI760D electrochemical workstation (Chenhua Instrument Shanghai Co., Ltd., China). Transmission electron microscope (TEM) images were obtained from a Hitachi H-800 microscope (Japan). Scanning electron microscope (SEM) images were obtained using field emission SEM (ZEISS, Germany). All SEM specimens were sputter-coated with a thin layer of gold palladium under vacuum in an argon atmosphere prior to examination. UV/VIS measurements were carried out using a Lambda 35 UV/VIS Spectrometer (PerkinElmer, USA).

2.2. Preparation of the amino-functionalized MSN

MSN were synthesized following the procedure reported by Zhao et al. (2009). Briefly, CTAB (0.5 g) was dissolved in 240 mL of water assisted by sonication. Then, sodium hydroxide aqueous solution (2.00 mol/L, 1.75 mL) was introduced to the CTAB solution and the temperature of the mixture was adjusted to 80 °C. Tetraethoxysilane (TEOS, 2.5 mL) was added dropwise to the surfactant solution within 5 min under vigorous stirring. The mixture was allowed to react for 2 h. The resulting white solid crude product was filtered, washed with water and methanol, and dried under high vacuum to yield the as-synthesized MSN. To remove the surfactant template (i.e., CTAB), 0.75 g of the as-synthesized MSN was refluxed for 6 h in a methanolic solution (the mixture of 0.75 mL HCl (37.2%) with 75 mL methanol). The resulting material was filtered and extensively washed with water and methanol. The surfactant-free MSN were heated to 60 °C under high vacuum to remove any remaining solvent from the MSN. Subsequently, MSN (1.00 g) was refluxed for 20 h in 80.0 mL of anhydrous toluene with 1.00 mL of 3-aminopropyltrimethoxysilane to yield the 3-aminopropyl-functionalized MSN (MSN) material.

2.3. Preparation of the Au-hybrid MSN

The Au-hybrid MSN (Au-MSN) sol was prepared according to literature report with some minor revisions (Bharathi et al., 1999). Briefly, the as-synthesized MSN was mixed with $HAuCl_4$ in an aqueous solution with the 1:1 ratio by mass of MSN to $HAuCl_4$. The mixture was stirred gently for 24 h. After centrifuge and re-dispersed in water, HCl was then added until the pH < 2 and kept stirring for at least 30 min. A 50 mmol/L solution $NaBH_4$ was added dropwise until all $HAuCl_4$ was reduced to metallic Au. The resulting solid fresh product was obtained by centrifuging, and was dried under high vacuum.

2.4. Preparation of the Au-MSN-HRP-Ab_2 labels

As shown in Fig. 1a, the Au-MSN (1 mg) was dispersed in 0.5 mL of phosphate buffer at pH 7.4. This dispersion was then mixed with 0.5 mL of 1 mg/mL HRP solution and 0.5 mL of 10 μg/mL of anti-norethisterone antibody. The mixture was allowed to react at room temperature under stirring for 60 min, followed by centrifuge. The resulting Au-MSN-HRP-Ab_2 were washed with buffer solution (pH 7.4) and then re-dispersed in 1 mL of buffer and stored at 4 °C before use.

2.5. Preparation of graphene sheet

GS was prepared from graphite oxide (GO) through a thermal exfoliation method (McAllister et al., 2007). At first, GO powders were produced from graphite by a modification of Hummer's method (Liu et al., 2008). In a typical experiment, 5 g of graphite was oxidized by reacting with 100 mL of concentrated H_2SO_4 under stirring for 12 h. Then, while immersing the reaction vessel in an ice bath, 30 g of $KMnO_4$ was added slowly. After the addition of $KMnO_4$, the solution was stirred at 100 °C for another 12 h to fully oxidize graphite GO. The obtained GO was then thoroughly washed and dried. Thermal exfoliation of GO was achieved by placing GO (100 mg) into a quartz tube under argon atmosphere. The quartz

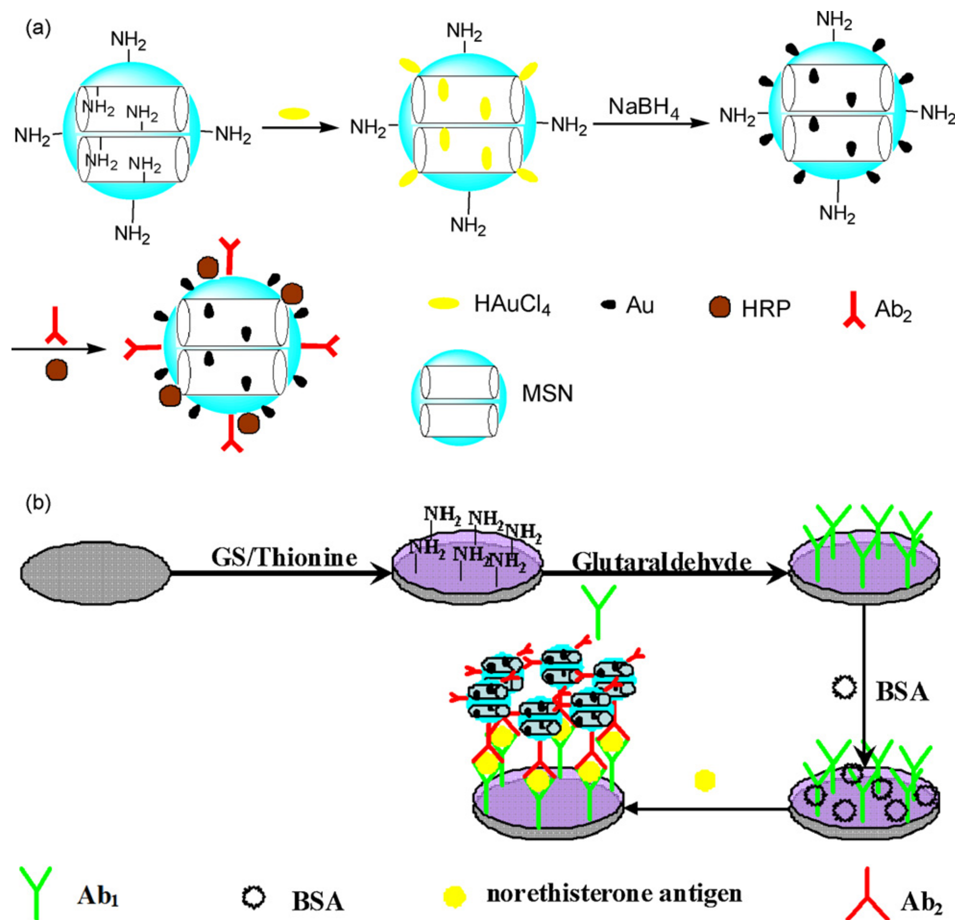

Fig. 1. Schematic representation of the preparation of the Au-MSN-HRP-Ab_2 (a) and immunosensor (b).

tube was flushed with argon for 10 min, and then quickly inserted into a furnace preheated to 1000 °C and held in the furnace for about 1 min.

2.6. Fabrication of the immunosensor

Fig. 1b shows the procedure to prepare the immunosensors. A mixture of 2 mg/mL thionine (TH) and 2 mg/mL GS was stirred for 24 h to get the TH-GS solution. A glassy carbon electrode (GC, 3 mm diameter) was polished repeatedly using alumina powder and then thoroughly cleaned before use. Onto the electrode, 5 μL of TH-GS solution was added. After the TH-GS coated electrode was dried and washed, 5 μL of glutaraldehyde was added onto electrode surface. After 1 h of incubation and washing, Ab_1 was added onto the electrode surface. After another 1 h of incubation, the electrode was washed with buffer and incubated in 1 wt% BSA solution for 1 h to eliminate nonspecific binding between the antigen and the electrode surface. Subsequently, norethisterone antigen buffer solution with a varying concentration was added onto the electrode surface and incubated for 1 h, and then the electrode was washed extensively to remove unbounded norethisterone antigen molecules. Finally, the prepared Au-MSN-HRP-Ab_2 buffer solution was dropped onto the electrode surface. After another 1 h, the electrode was washed and ready for measurement.

2.7. Characterization of the immunosensor

A conventional three-electrode system was used for all electrochemical measurements: a glassy carbon electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire electrode as the counter electrode. The pH 7.4 PBS buffer was used for all the electrochemical measurements. Cyclic voltammetry was recorded in PBS at 100 mV/s. For amperometric measurement of the immunosensor, a detection potential of −0.3 V was selected. After the background current was stabilized, 1.0 mmol/L H_2O_2 was added into the buffer and the current change was recorded.

3. Results and discussion

3.1. Characterization of the Au-MSN-HRP-Ab_2 nanoparticles

Fig. 2(a and b) shows the obtained rod like MSN with a uniform size of approximately 100 nm. The amino-group grafted onto MSN is 1.2 mg/g by ninhydrin test. Due to the high pore volume and large surface area of MSN, high loading level of guest molecules can be achieved using MSN for various biomedical applications (Chouyyok et al., 2009). So the $HAuCl_4$ can be adsorbed onto the surface of MSN or be encapsulated into the mesoporous pore of MSN effectively. With the reducing by $NaBH_4$, sufficient Au nanoparticles were immobilized on the surface and inside the mesoporous pore of MSN, which will be especially effective for the further adsorption of biomolecules (HRP, Ab_2). As shown in Fig. 2(c and d), a great amount of Au nanoparticles are immobilized onto MSN. The GS was characterized by TEM and SEM. As shown in Fig. 2(e and f), GS, with wrinkled paper-like structure was observed. They are transparent with irregular size.

The Au-MSN-HRP-Ab_2 nanoparticles were characterized with UV–vis spectroscopy (Fig. 3). All samples were dispersed in phosphate buffer at pH 7.4 and mixed thoroughly. After laying aside

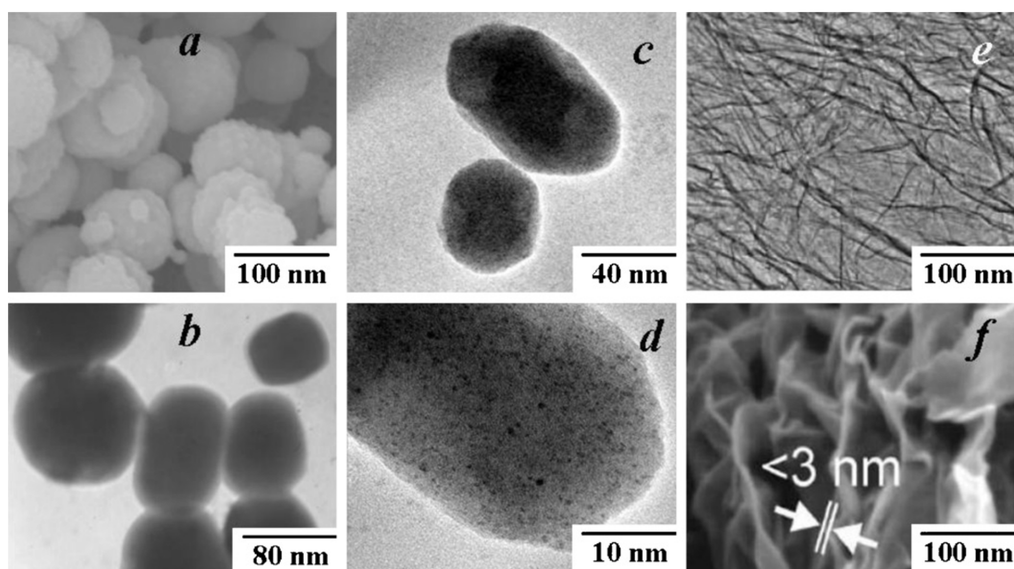

Fig. 2. Electronic microscope images of materials: (a) SEM image of MSN; (b) TEM of MSN; (c and d) TEM images of Au-MSN; (e) TEM image of GS; and (f) SEM image of GS.

for 2 min at room temperature, the UV–vis spectra were monitored at 200–800 nm. The result was shown in Fig. 3. No absorption peak was observed for the bare MSN (Fig. 3a). For pure Au solution, there was one major peak at approximately 530 nm (Fig. 3b). When Au was encapsulated into the MSN, an absorption peak around 530 nm was also observed, indicating the successful encapsulation of Au onto MSN (Fig. 3c). After HRP was adsorbed onto the MSN, an additional strong absorption peak appeared at 410 nm, which could be ascribed to the HRP attached onto the MSN (Tang et al., 2008). According to the UV–vis spectra analysis, both Au and HRP were successfully loaded onto the MSN. A distinct absorption peak from the antibody was not observed on the spectra of Au-MSN-HRP-Ab_2 (Fig. 3f), which may be attributed to (1) the relatively weak absorption peak from antibody itself (280 nm) and (2) peak overlap between the antibody and Au (around 280 nm).

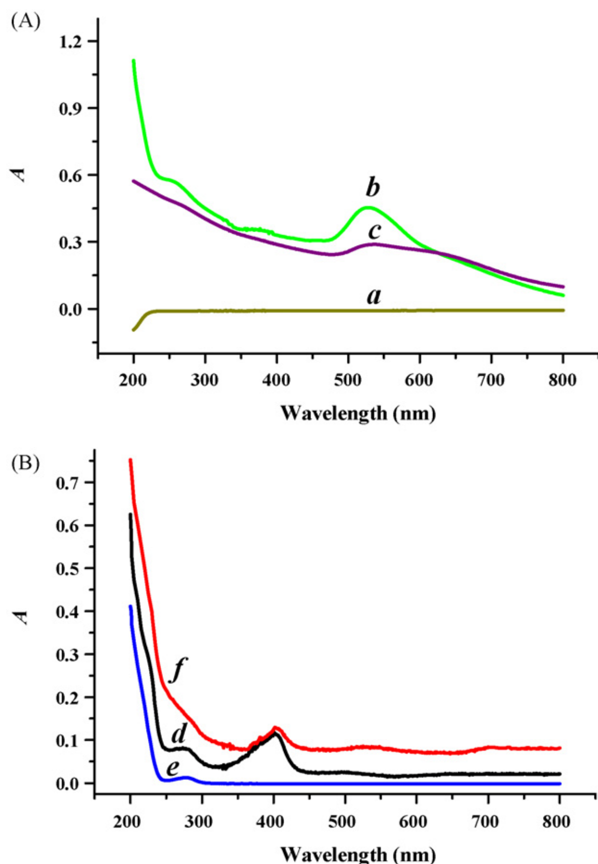

Fig. 3. UV–vis spectra of MSN (a), Au solution (b), Au-MSN (c), HRP (d), Ab_2 (e), and Au-MSN-HRP-Ab_2 (f).

3.2. Characterization of the immunosensor

Since we have proved the successfully immobilization of Au and HRP onto MSN, the sensitivity of the immunosensors using the modified MSN as label were investigated. In this study, Ab_1 was immobilized onto TH-GS modified electrode surface through glutaraldehyde based on the amino-groups on TH. The large specific surface area of GS can be used to increase Ab_1 loading and its good conductivity could enhance electron transfer among TH, enzyme HRP and electrode surface (Du et al., 2010). Aside from the Au nanoparticles and HRP molecules loaded onto the MSN-based labels, a relatively high amount of Ab_2 was also conjugated onto the MSN-based labels; thus, when antigen was present on the electrode surface, the Au-MSN-HRP-Ab_2 labels could be captured onto the electrode surface via the specific antibody–antigen interaction. In order to obtain the best analytical performance for norethisterone, experimental conditions have been optimized, and the optimal conditions have been described in supporting information and shown in Figure S1.

As controls, immunosensors using three different MSN-based nanoparticles, Au-MSN-HRP-Ab_2, MSN-HRP-Ab_2 and Au-MSN-Ab_2 as labels were also prepared and characterized. During the immunosensor preparation process, the same concentration of norethisterone antigen (10 ng/mL) was used in order to compare the performance of the immunosensors using the three different types of MSN-based labels. The judgment is based on the amperometric current change of the immunosensor toward detection of H_2O_2. The amperometric responses of the immunosensors are shown in Fig. 4(a). As expected, the immunosensor using Au-MSN-HRP-Ab_2 as labels displays the highest current change, which is about 100 times higher than those using MSN-HRP-Ab_2 and Au-MSN-Ab_2 as labels. In addition, the response is very fast, with the catalytic current reaching a steady-state value within 5 s. The high sensitivity can mainly be ascribed to the high amount of catalytic

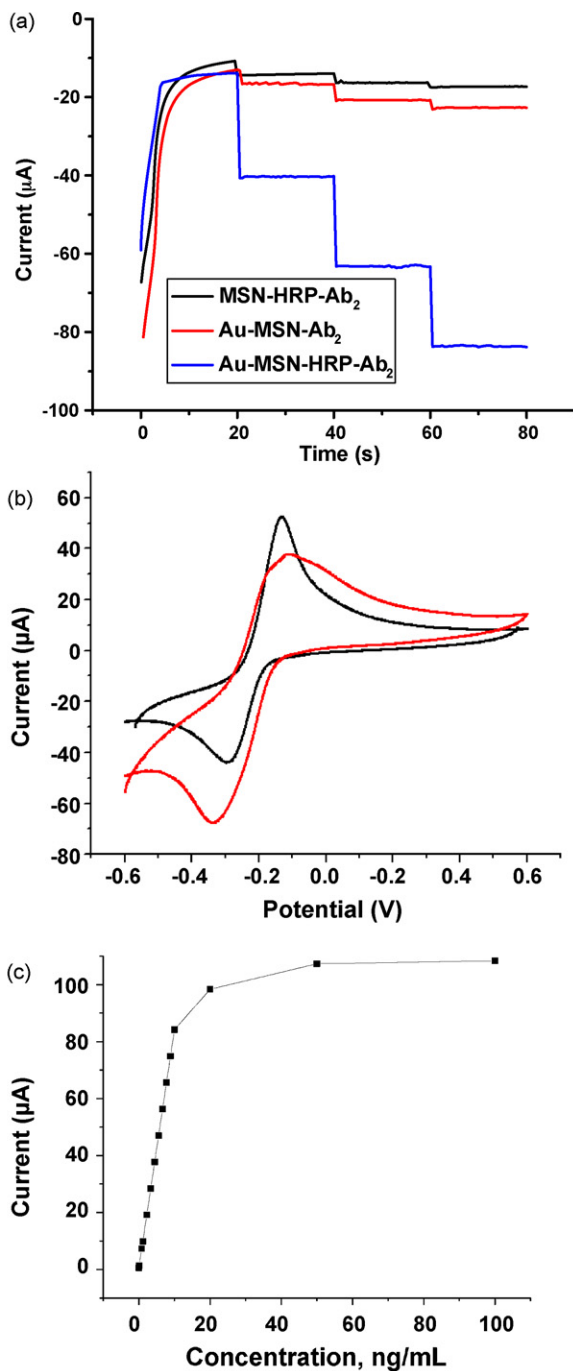

Fig. 4. (a) The amperometric response of the immunosensor for the detection of 10 ng/mL norethisterone antigen with different label at −0.3 V toward successive addition of 1.0 mmol/L H_2O_2. (b) Cyclic voltammograms of the immunosensor for the detection of 10 ng/mL norethisterone antigen using Au-MSN-HRP-Ab_2 as label before (a, black) and after (b, red) the addition of 1 mmol/L H_2O_2. Scan rate: 100 mV/s. (c) Calibration curve of the immunosensor toward different concentrations of norethisterone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

HRP enzyme molecules adsorbed onto MSN toward the catalytic reduction of H_2O_2 in the presence of mediator TH on the electrode surface. In addition, the good conductivity of Au and GS may also promote electron transfer and enhance the sensitivity.

Fig. 4(b) displays the cyclic voltammograms of the immunosensor for the detection of 10 ng/mL of norethisterone using Au-MSN-HRP-Ab_2 as label in PBS toward the response of 1 mM H_2O_2. In blank PBS, the immunosensor displays reduction and oxidation peaks at −300 and −130 mV, respectively, which is ascribed to the mediator TH on the electrode surface. After the addition of H_2O_2, a dramatic increase of the reduction current and a sharp decrease of the oxidation current were observed, again demonstrating the high sensitivity of the immunosensor.

Immunosensors using Au-MSN-HRP-Ab_2 as labels are used to detect different concentrations of norethisterone antigen. The relationship between the current response toward 1.0 mmol/L H_2O_2 and norethisterone antigen concentration is shown in Fig. 4(c). The value of current in the absence of northisterone at −0.3V is 43.74 nA. And the equation of the calibration curve: $Y = 0.55671 + 8.27101X$, $r = 0.9993$. The catalytic current increases linearly with the norethisterone antigen concentration within the range of 0.01–10 ng/mL. The low detection limit (3.58 pg/mL) may be attributed to two factors. First, a relatively large amount of Ab_2 has been conjugated onto the MSN-based labels. Generally, when the norethisterone antigen concentration is low, the amount of norethisterone antigen captured by the Ab_1 immobilized onto the electrode surface is also low; however, the relatively large amount of Ab_2 immobilized onto the labels can greatly increase the probability of Ab_2–antigen interactions thereby leading to higher sensitivity. Secondly, as discussed earlier, the large amounts of HRP and Au nanoparticles immobilized onto the labels facilitated electron transfer and catalytic reduction of H_2O_2.

3.3. Reproducibility, selectivity and stability

Intra- and interassay coefficients of variation (CVs) were employed to evaluate the reproducibility. By analyzing six concentration levels five times, the intra-assay precision of the used method was given. The CVs if intra-assay by the immunosensors were 3.8, 3.9, 3.5, 4.0, 4.6 and 5.4% at 0.1, 0.5, 2.0, 4.0, 6.0, 8.0 ng/mL of norethisterone antigen, respectively. And the interassay CVs in five immunosensors were 6.9, 6.3, 7.1, 5.2 and 5.6% at 0.1, 0.5, 2.0, 4.0, 6.0, 8.0 ng/mL of norethisterone antigen, respectively. The results indicated that the precision and reproducibility of the proposed immunosensor was quite good.

To investigate the specificity of the fabricated immunosensor, interferences study was performed using megestrol, medroxyprogesterone, stilbestrol and estradiol. The 1 ng/mL of norethisterone antigen solution containing 20 ng/mL of interfering substances was measured by the immunosensor and the results were shown in Fig. 5a. The current variation due to the interfering substances was less than 8% of that without interferences, indicating the selectivity of the immunosensor was acceptable.

In addition, the amperometric response of the immunosensor toward zeranol antigen, prostate specific antigen (PSA), BSA, glucose and vitamin C were also studied. The 1 ng/mL of norethisterone antigen solution containing 100 ng/mL of interfering substances was measured by the immunosensor and the results were shown in Fig. 5b. The current variation due to the interfering substances was less than 5% of that without interferences, indicating the selectivity of the immunosensor was acceptable.

The stability of the biosensor was also examined by checking periodically their current responses. When the biosensor was not in use, it was stored in air at 4 °C. After 3 weeks, the catalytic current of the immunosensor using Au-MSN-HRP-Ab_2 as labels decreased to about 87% of its initial value. The slow decrease in the current response may be due to the gradual denature of HRP (Yu and Ju, 2002).

3.4. Real sample analysis

In order to evaluate the feasibility of the immunosensor for possible applications, the proposed immunosensor was used for determining the recoveries of different concentrations of norethis-

Table 1
The result of norethisterone determination in chicken liver by immunosensor.

Chicken liver (μg/kg)	The addition content (μg/kg)	The detection content (μg/kg)	RSD (%)	Recovery (%)
0.0	5	5.11, 5.07, 4.95, 4.94, 5.05	1.50	100.5
	25	25.28, 24.73, 24.55, 24.37, 25.26	1.67	99.3
	50	52.23, 52.19, 49.78, 51.89, 49.45	2.74	102.2
	100	101.66, 102.32, 96.89, 96.38, 7.47	2.82	98.9

terone in chicken tissues by standard addition methods in healthy chicken liver. Samples with different norethisterone spiked concentrations of 0, 25, 50 and 100 μg/kg were analyzed by the proposed immunosensor. The result showed in Table 1 and the recovery was from 98.9 to 102.2% ($n = 5$) and the RSD was between 1.50 and 2.82%.

To further investigate the availability of this immunosensor for norethisterone detection, a comparison of the results obtained by the proposed immunosensor and HPLC method were made. Norethisterone contents determined by the two methods agreed well and the plot of norethisterone contents obtained by the two methods gave a straight line with a correlation coefficient of 0.9993 (Figure S2), indicating the proposed immunosensor might be used for the detection of norethisterone.

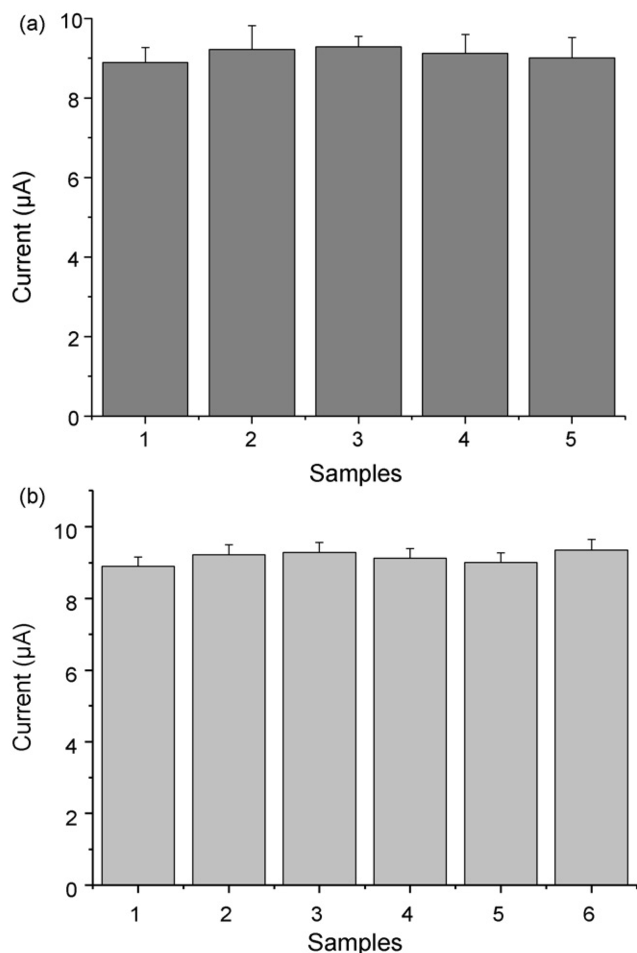

Fig. 5. (a) Amperometric response of the immunosensor to 1 ng/mL norethisterone antigen (1), 1 ng/mL norethisterone antigen + 20 ng/mL megestrol (2), 1 ng/mL norethisterone antigen + 20 ng/mL medroxyprogesterone (3), 1 ng/mL norethisterone antigen + 20 ng/mL stilbestrol (4), and 1 ng/mL norethisterone antigen + 20 ng/mL estradiol (5). Error bar = RSD ($n = 5$). (b) Amperometric response of the immunosensor to 1 ng/mL norethisterone antigen (1), 1 ng/mL norethisterone antigen + 100 ng/mL PSA (2), 1 ng/mL norethisterone antigen + 100 ng/mL BSA (3), 1 ng/mL norethisterone antigen + 100 ng/mL glucose (4), and 1 ng/mL norethisterone antigen + 100 ng/mL vitamin C (5). Error bar = RSD ($n = 5$).

4. Conclusions

This work develops a novel immunosensor using the Au-MSN nanoparticles as labels for norethisterone antigen detection. The immunosensor can be simply prepared based on a sandwich-type protocol with the primary antibody immobilized onto the surface of TH-GS modified electrode. The immunosensors using the Au-MSN-HRP-Ab_2 nanoparticles as labels displayed a linear response for detectin norethisterone antigen within a wide range (0.01–10 ng/mL). The proposed biosensor shows low detection limit (3.58 pg/mL), good reproducibility, selectivity and acceptable stability. The simple fabrication procedure required and the ultrasensitivity demonstrated by the immunosensor may provide many potential applications for the detection of growth hormone in animal derived food.

Acknowledgments

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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.bios.2010.06.052.

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