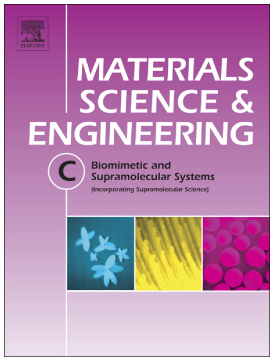

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Ultrasensitive cholesterol biosensor based on enzymatic silver deposition on gold nanoparticles modified screen-printed carbon electrode

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Abstract

Cholesterol is one of the essential structural constituents of cell membranes. Determination of cholesterol is of great importance in clinical analysis because the level of cholesterol in serum is an indicator in the diagnosis and prevention of heart diseases. In this work, a simple and ultrasensitive cholesterol biosensor based on enzymatic silver deposition was designed by immobilizing cholesterol oxidase (CHOD) and cholesterol esterase (CHER) onto the surface of gold nanoparticles (Au NPs) modified screen-printed carbon electrode (SPE). By the catalytic action of CHER and CHOD, the cholesterol was hydrolyzed to generate hydrogen peroxide (H_2O_2) which can reduced the silver (Ag) ions in the solution for the deposition of metallic Ag on the surface of Au NPs modified SPE. The ultrasensitive detection of cholesterol was achieved by anodic stripping voltammetry (ASV) measurement of the enzymatically deposited Ag. The influence of relevant experimental variables was optimized. The anodic stripping peak current of Ag depended linearly on the concentration of cholesterol in the range of 5-5000 μg/mL with the regression correlation coefficient of 0.9983. A detection limit of 3.0 μg/mL was attained by 3 sigma-rule. In addition, the ultrasensitive cholesterol biosensor exhibited higher specificity, acceptable reproducibility and excellent recoveries for cholesterol detection.

Key words: Cholesterol biosensor; Enzymatic silver deposition; Cholesterol oxidase; Cholesterol esterase; Screen-printed carbon electrode

1. Introduction

Cholesterol, an essential component of the mammalian cell membrane, is transported in the blood plasma in mammals and takes part in the production of hormones, bileacids, vitamin D and other vital molecules [1-4]. The level of cholesterol in serum is an indicator in the diagnosis and prevention of heart diseases [5-7], Desirable amount of cholesterol in healthy human serum is 200 mg/dL (5.17mM) [8]. Excessive cholesterol level in the blood serum causes poor cardiovascular conditions such as hypertension and arteriosclerosis which can lead to coronary heart disease, nephrosis and myocardial infarction. On the other hand, lower level of cholesterol concentration in blood causes anemia and wasting syndrome [9-11]. Thus, the level of cholesterol in blood serum is one of the major parameters for the diagnosis of a number of disorders [3], and the accurate detection of cholesterol is of great importance in clinical analysis [12].

So far, many techniques, such as gas chromatography/mass spectrometry, thin layer chromatography, liquid chromatography, spectrophotometry, fluorimetric method and so on, have been developed for the determination of cholesterol [12, 13]. However, these methods are labor-intensive, complex procedures, poor specificity and high cost [4]. Therefore, developing a sensitive, simple and cost effective method for cholesterol detection is needed [11]. Electrochemical procedures, especially the enzyme-based sensors, have been developed for the monitoring of cholesterol with more interest due to their high sensitivity, low cost, rapid quantification and excellent selectivity [13].

Enzymatic catalyzed silver deposition has been used as an effective way to amplify signals during the detection of various biomarkers because of high catalytic activity for biochemical reactions, highly sensitive and specific feature for a great enhancement in electrochemical signal [14-17]. da Silva Neves MMP, et al [17] had used hydroquinone diphosphate/Ag^{+} as an enzymatic substrate for alkaline phosphatase catalyzed silver deposition. Jiaul Haque AM, et al [18] had developed a novel enzymatic Ag-deposition scheme combined with chemical-chemical redox cycling

by reduced β-nicotinamide adenine dinucleotide and got good result for highly sensitive detection of creatine kinase-MB. Our group had developed an electrochemical immunosensor based on the enzymatic silver deposition reaction for the detection of prostate specific antigen (PSA), and the developed biosensor exhibited a linear response with PSA concentrations over a 6-decade range from 1.0 pg /L to 1.0 μg/L, with the detection limit of 0.9 pg/L[19].

Herein, a simple and ultrasensitive cholesterol biosensor based on enzymatic silver deposition is introduced. A screen-printed carbon electrode (SPE) modified with Au NPs was obtained by electrodeposition of Au on the surface of the SPEs (Scheme 1). The cholesterol oxidase (CHOD) and cholesterol esterase (CHER) were immobilized on the surface of Au NPs-modified SPE. Under the catalytic action of CHER and CHOD, the cholesterol was hydrolyzed to generate hydrogen peroxide (H_2O_2) with weak reducibility. The latter, in turn, reduced the Ag ions in the solution for the deposition of metallic Ag on the surface of Au NPs-modified SPE. The ultrasensitive detection of cholesterol was achieved by anodic stripping voltammetry (ASV) measurement of the enzymatically deposited Ag. The effect of silver deposition time, the concentration of silver ion, temperature, Au NPs deposition time, the deposition potential of Au NPs and pH were assessed as well as the specificity, reproducibility of the fabricated cholesterol biosensor were discussed.

2. Materials and methods

2.1 Chemicals and reagents

CHOD, CHER and cholesterol were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Glycine, $HAuCl_4$, Ascorbic acid, Glucose, and DL-cysteine were supplied by Sangon Biotech (Shanghai, China). All other reagents were of analytical grade and used without further purification. All solutions were prepared with ultrapure water of 18 MΩ purified from a Milli-Q purification system (Milli-Pore, Bedford, MA, USA).

Glycine buffer solution (0.05 mol/L, pH 8.6) was prepared by dissolving the glycine into

ultrapure water and the pH was adjusted to 8.6 using 0.1 mol/L NaOH.

A stock cholesterol solution (5.0 mg/mL) was prepared by dissolving 10mg cholesterol in a mixture solution of 0.2 mL isopropanol and 0.2 mL tritonX-100, heating until no precipitates. And then, the mixture solution was re-dispersed in 1.6 mL glycine buffer solution and stored at 4°C for future use. The other concentrations of cholesterol were prepared from stock solution.

2.2 Apparatus

All electrochemical measurements were performed on a CHI660D electrochemical workstation (Shanghai Chenhua Apparatus, Shanghai, China) at room temperature. The electrochemical system was screen-printed electrodes contained carbon electrodes as the working electrode and auxiliary electrode and Ag/AgCl electrode as reference electrode. The amperometric i-t curve was carried out in electrolyte solution under a constant potential of -0.5 V with a 100 mV/s scanning rate at room temperature with mild stirred. Cyclic voltammetry (CV) was carried out in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ from -0.3 to 0.6 V with a 100 mV/s scanning rate. Linear sweep voltammetry (LSV) was carried out in 0.1 mol/L HNO_3 solution containing 0.6 mol/L KNO_3 from -0.1 to 0.6 V with a 100 mV/s scanning rate.

The morphologies and surface structures of the prepared cholesterol biosensor were characterized with scanning electron microscope (SEM) and X-ray photoelectron spectroscopy (XPS). SEM was performed using a Quanta 200 field environmental scanning electron microscope (FEI COMPANY, USA). XPS was performed using an ESCALAB 250Xi spectrometer (ThermoFisher, USA). The Mg cathode radiation source of 1253.6 eV was used at the conditions of 10 mA and 15 keV with a pass energy of 40 eV. During the analysis the residual pressure in the chamber was 10^{-7} Pa.

2.3 Preparation of cholesterol biosensor

First, the SPE was scanned in 0.5 mol/L H_2SO_4 solution by cyclic voltammetry (CV) to activate the surface of the electrode. Then, the activated SPE was immersed in 0.01% $HAuCl_4$ solution and the Au NPs were electrodeposited on it by amperometric i-t curve for 120 s at room temperature with mild stirred under a constant potential of -0.5 V with a 100 mV/s scanning rate. After the Au NPs-modified SPE was cleaned carefully by ultrapure water, 1 μL CHER (1.0 mg/mL) and 1 μL CHOD (1.0 mg/mL) were immobilized on the SPE in 4 °C for 3 hours to obtain CHER&CHOD/Au NPs/SPE. The functionalized SPE were washed twice with glycine buffer solution. Thus, a cholesterol biosensor based on enzymatic silver deposition was come into being and stored at 4 °C in in refrigerator prior to use.

2.4 Electrochemical detection of cholesterol

Different concentrations of cholesterol (2 μL) and glycine buffer solution (1 μL) containing $AgNO_3$ (30 mmol/L) were dropped on the surface of CHER&CHOD/Au NPs/SPE. After incubated for 25 minutes at 37 °C, the SPEs were washed twice with glycine buffer solution and dried at room temperature. The electrochemical signals were obtained by using LSV at a potential range from -0.1 to 0.6 V with a 100 mV/s scanning rate in 6 mL HNO_3 (0.1 mol/L) solution containing KNO_3 (0.6 mol/L).

2.5 Exploration of the specificity of the cholesterol biosensor

In order to explore the specificity of the cholesterol biosensor, the sensing performance of the developed biosensor was performed with ascorbic acid, glucose or DL-cysteine (50 μg/mL) instead of the cholesterol under the optimal conditions. The electrochemical signals of the enzymatic silver deposition responses were recorded and compared.

3. Results and discussion

3.1 Analytical principle of electrochemical biosensor detection for cholesterol

In this work, a novel biosensor for the detection of cholesterol based on enzymatic silver deposition was fabricated and the analytical principle is schematically illustrated in Scheme 1. Firstly, the Au NPs-modified SPE was obtained by electrodeposition of Au on the surface of the SPE. Then, the CHOD and CHER were immobilized on the surface of Au NPs-modified SPE by electrostatic adsorption between the Au NPs and the enzyme. Lastly, cholesterol solution and $AgNO_3$ solution were dropped on the surface of CHER&CHOD/Au NPs/SPE. Under the catalytic action of CHER and CHOD, the cholesterol can be hydrolyzed to generate H_2O_2 with weak reducibility. The latter, in turn, reduced the Ag ions in the solution for the deposition of metallic Ag on the surface of Au NPs-modified SPE. The reaction can be explained by the following equations:

$$\text{Cholestery acetate} + H_2O \xrightarrow{\text{CHER}} \text{Cholesterol} + \text{Acetic acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \Delta\text{-Cholestenone} + H_2O_2$$

$$H_2O_2 + 2Ag^+ \longrightarrow 2Ag + 2H^+ + O_2$$

The metallic Ag deposited on the surface of Au NPs-modified SPE can be quantitatively determined by anodic stripping voltammetry (ASV). The electrochemical signal is directly proportional to the concentration of cholesterol.

(Here Scheme.1)

Fig. 1 illustrated the anodic stripping peak current, obtained by the LSV, the anodic stripping peak current, obtained by the LSV, for the Ag/CHER&CHOD/Au NPs/SPE in the presence of cholesterol (Fig.1, curve a), the Ag/CHER&CHOD/Au NPs/SPE without the presence of cholesterol (Fig.1, curve b), and the Ag/Au NPs/SPE in the presence of cholesterol (Fig.1, curve c). As can be seen in Fig. 1, there was a remarkable current response (502 μA) at 199 mV. As a control, there

appeared a mild current response (81.1 μ A) on the CHER&CHOD/Au NPs/SPE at 159 mV with the absence of cholesterol, which showed that the potential shift for CHER&CHOD on Au NPs/SPE was an enzyme-specific process. At the same time, only a weak current response (46.3 μ A) with a slight shift potential at 145 mV, indicating that Au NPs/SPE may be excellent substrate electrode with a wide dynamic response range for the developed biosensor. Hence, the biosensor showed that the analyte-dependent enzymatic deposition of silver on Au NPs/SPE provided a measure for determining the target protein.

(Here Fig.1)

3.2 Electrochemical characterization of cholesterol biosensor

Cyclic voltammetry was used to observe the electrochemical behavior of the electrodes in different stages of the sensor construction. Fig.2 showed the cyclic voltammetry responses of the various electrodes modification in 5 mmol/L $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution. As shown in Fig.2, a reversible redox peak appeared with bare SPE due to the equivalent amount of $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ ions in the reaction medium [20] (Fig.2, curve a). After the Au NPs was modified to the electrode surface by the constant potential deposition method, the redox peak current was increased with respect to the bare electrode (Fig.2, curve b), which is advantageous to the electron surface electron transfer for the conductivity of the Au NPs. Compared to curve b, the redox peak current began to decrease when the CHER and CHOD were immobilized on the surface of Au NPs-modified SPE (Fig.2, curve c), this is due to the enzyme which is a kind of the protein and has poor electro-conductibility. After the enzymatic silver deposition, the redox peak current increased clearly (Fig.2, curve d). Under the action of CHER and CHOD, the cholesterol dropped on the surface of the electrode was disintegrated and produced the weak reducing reagent, H_2O_2. At the same time, the

H_2O_2 reduced the silver ions to metallic silver and deposited on the surface of Au NPs, which has good conductivity and the redox peak current increased. The average value of the electroactive surface area could be calculated according to the Randles-Sevcik equation [5],

$$Ip=2.69\times10^5AD^{1/2}\,n^{3/2}\,\gamma^{1/2}C \qquad (1)$$

where Ip is the obtained current values, A represents the area of the electroactive surface area (cm^2), D is the diffusion coefficient of the molecule in solution (for $[Fe(CN)_6]^{3-/4-}$, $D=6.70\pm0.02\times10^{-6}$ cm^2/s) [21, 22], n represents the number of electrons participating in the redox reaction (for $[Fe(CN)_6]^{3-/4-}$, n=1), γ represents the scan rate of the potential perturbation ($V\cdot s^{-1}$), and C is the bulk concentration of the redox probe (mol/cm^3). The effective surface area of electrodes with various modifications were calculated in the trend of bare SPE (0.1644 cm^2) < CHER&CHOD/Au NPs/SPE (0.2203 cm^2) < Ag/CHER&CHOD/Au NPs/SPE (0.244 cm^2) < Au NPs/SPE (0.2623 cm^2), which indicated that Au NPs can enlarge the effective surface area of electrodes.

(Here Fig.2)

3.3 SEM characterization of cholesterol biosensor

The SEM images demonstrated the morphology of electrodes modified with various materials. The typical SEM micrographs of bare SPE (A), Au NPs/SPE (B), CHER&CHOD/Au NPs/SPE (C) and Ag/CHER&CHOD/Au NPs/SPE (D) were shown in Fig.3. One can see clearly from Fig.3A that the bare SPE gave a uniform dark image without particles on its surface, while there was a uniform distribution of spherical particles in the image of the Au NPs deposited on the surface of SPE (Fig.3B), the particle size of Au NPs deposited onto the SPE surface was about 120±10 nm. This implied that the Au NPs had been successfully generated with an electrodeposition procedure on SPE. Fig.3C was the SEM image of the SPE after enzymatic modification. Because of the smaller particle

size of enzyme, the difference between Fig.3C and Fig.3B was not significant. Seen from Fig.3D, there were some bright spots randomly distributed in the image of Ag/CHER&CHOD/Au NPs/SPE, which indicating that the silver ions was reduced to metallic silver and deposited on the surface of Au NPs-modified SPE in the presence of H_2O_2 generated by the hydrolyzing of cholesterol under the catalytic action of CHER and CHOD.

(Here Fig.3)

3.4 XPS characterization of cholesterol biosensor

The electrodeposition of Au NPs, immobilization of CHER&CHOD and the deposition of Ag on the surface of cholesterol biosensor were further evaluated by X-ray photoelectron spectroscopy (XPS). Five main features including C1s, N1s, O1s, Au4f and Ag3d peaks of the XPS spectrum were inspected and shown in Fig.4. The study of the Au4f and Ag3d spectra provided information on the surface coverage, while the C1s, O1s and N1s peaks provide information on the presence of enzyme, respectively. Fig.4A illustrated the full XPS spectra of the surface of SPE in different stages. And Fig.4B illustrated the core XPS spectra of O1s, C1s, N1s, and Au4f on the surface of SPE in different stages. As can be seen from Fig.4A, a higher intensity Au4f (83 eV) associated with lower intensity peak series of N1s (399 eV), O1s (531 eV). Moreover, the presence of Ag3d (367 eV) is related to lower intensity peak series of N1s (399 eV) and Au4f (83 eV). The above phenomenon represented a characteristic XPS spectrum of SPEs surface in different stages.

(Here Fig.4)

The Au4f (83 eV) signal increased sharply while C1s (284 eV), N1s (399 eV) and O1s (531 eV) signal decreased slightly due to the electrolytic deposition of Au NPs (Fig.4B, curve b).After immobilization of CHER and CHOD, the total intensity of O1s (531 eV), N1s (400 eV) increased,

meanwhile Au4f (83 eV) and C1s (284 eV) signal decreased slightly (Fig.4B, curve c). The CHER and CHOD were immobilized on the surface of SPE which contributed to the increase of C1s, O1s and N1s signal and reduction of Au4f signals further. Subsequently, the intensity of N1s (400 eV) and Au4f (83 eV) decreased slightly with the appearance of Ag3d (367 eV), the signal of N and gold decreased (Fig.4B, curve d).

A further insight on the reaction process can be obtained from the inspection of Table inset Fig.4A, where the relative abundance (% of atoms) of the various elements (C, O, N, Au, Ag) for reaction processes are summarized. The immobilization of CHER and CHOD caused a strong increase in the proportion of O which is above 25%. N presence in concentration of 7% or above is the final evidence of enzyme presence on the SPEs surface. The XPS data fully confirmed that the biological molecules were correctly immobilized on the SPEs surface.

3.5 Optimization of experimental conditions for detection cholesterol

The performance of Ag/CHER&CHOD/Au NPs/SPE biosensor for the detection of cholesterol was optimized by varying silver deposition time, the concentration of silver ion, temperature, Au NPs deposition time, the deposition potential of Au NPs and pH. Fig.5A showed the effect of silver deposition time on the current response of the cholesterol sensor. Seen from Fig.5A, the current response value increased with the increase of the deposition time when the deposition time was less than 25 minutes, when the deposition time extended to 25 minutes the current response value tended to be stable. Therefore, the time of enzyme catalyzed silver deposition in this study was selected for 25 minutes.

The activity of enzyme changes with the change of temperature. When the temperature is lower, the activity of biological molecules is decreased and the reaction rate is decreased. When the

temperature is higher, the enzyme will loss of biological activity, and is irreversible [23]. Therefore, the effect of different temperature (4-50 °C) on the stripping peak current of the sensor were studied and shown in Fig.5B. The response current of the sensor increased with the variation of incubation temperature. When the incubation temperature is 37 °C, the corresponding current of the sensor reached the maximum. As the temperature continued to rise, the corresponding current decreased. Therefore, the incubation temperature of 37 °C was selected for the further experiments.

Fig.5C depicted the effect of electrodeposition time of Au NPs on the electrocatalytic hydrolyzing of cholesterol. As shown in Fig.5C, with the increase of the deposition time, the particle size of Au NPs deposited onto the electrode surface increased while the number of Au NPs increased and the response current of the sensor is also increased. The response current of the sensor reached the maximum value when the deposition time was 120 s. However, increasing the deposition time of gold, the generated Au NPs size was too large, which may lead to the decrease of surface area and reduce biological catalytic activity, thereby reduced the electrochemical response signal. Therefore, the electrodeposition time of Au NPs of 120 s was the best time for the optimal gold deposition.

Fig.5D showed the effect of deposition potential of Au NPs on the perform of cholesterol sensor. With the increase of deposition potential, the response current of the sensor increased and reached a maximum value when the deposition potential reaches -0.5 V. However, the deposition potential continued to increase, resulting in a large particle diameter of the Au NPs, which may lead to a decrease in the specific surface area, reduced adsorption capacity and the activity of the catalyst, thus decreased the signal of the electrochemical response. In consequence, -0.5 V was selected as the best gold deposit potential to the further experiments.

The influence of the concentration of silver ion in solution to the current value of cholesterol

sensor was shown in Fig.5E. As can be seen from the Fig.5E, the current response value of the sensor increased with the increase of the concentration of silver ion when it was lower than 30 mmol/L. This is due to the low concentration of silver ions in the solution cannot meet the silver deposition reaction, and the quantity of silver ion which involve in the silver deposition reaction is more and more with the increase of concentration [24]. The stripping peak current reached the maximum value when the silver ion concentration was 30 mmol/L. The stripping peak current value declined sharply when the concentration of silver ion increased. This is due to the silver ions is a kind of heavy metals, the higher concentration of heavy metal ions cause inactivation and poisoning of enzyme, so that the peak current response of the sensor decrease.

pH has an important influence on the signal strength of the biosensor because the biological activity of the enzyme greatly depends on the pH of the reaction medium [9, 11]. The effect of pH on the current signal of the electrochemical cholesterol sensor was investigated over the range of pH 3.6 to 10.6 in 0.05 mol/L glycine buffer solution in the presence of cholesterol. The results were shown in Fig.5F. As can be seen from Fig.5F, the peak current of electrochemical sensor rapidly increased with augment of pH value from 3.6 to 8.6 and reached the maximum when the pH was 8.6 and then leveled off after over 8.6. Therefore, the pH 8.6 was chosen as the optimum working pH for the reaction medium.

(Here Fig.5)

3.6 Analytical performance of the cholesterol biosensor

Ag/CHER&CHOD/Au NPs/SPE biosensor was used to measure cholesterol at different concentrations under the optimum experimental conditions, and the amperometric responses was recorded at a steady state current to determine a calibration curve for the cholesterol. Different

oxidation peak currents obtained in the LSV response after transformed Ag^+ to Ag in the detection solution. As show in Fig.6, the current response of Ag was linearly proportional with the cholesterol concentration over the range of 5 μg/mL-5000 μg/mL. The regression equation was Y=0.0610X+138.9550 (Y was the peak current and X indicated the concentration of cholesterol) with a correlation coefficient of 0.9983. Moreover, the limit of detection (LOD) for the biosensor was calculated as 3.0 μg/mL with a signal/noise (S/N) ratio of 3. The results showed that the linearity of biosensor was very well and the proposed biosensor possessed a low detection limit and produced a fast response to the addition of cholesterol.

(Here Fig.6)

3.7 Specificity, reproducibility and stability of the cholesterol biosensor

In order to investigate the specificity of the cholesterol biosensor, the sensing performance of the developed biosensor was performed with ascorbic acid, glucose or DL-cysteine (50 μg/mL) instead of the cholesterol under the optimal conditions. The results were shown in Fig.7. When the test sample is ascorbic acid, glucose, DL-cysteine, the biosensor's current response value is 17, 28, 32 μA, accounting for 8.85%, 14.58%, 16.67% of the current response value of cholesterol, respectively,. The results showed that negligible response to the injection of interfering species at their physiological concentration level, which indicated that the proposed biosensor has a good specificity and can be used for the detection of cholesterol without any effect caused by possible interferents.

(Here Fig.7)

The reproducibility of the electrochemical biosensor was investigated by analyzing the same concentration of cholesterol (250 μg/mL) with intra-assay imprecision of six different sensors at one assay and inter-assay imprecision at six different assays. The relative standard deviation (RSD) of the intra-assay and inter-assay precisions of the cholesterol biosensor were 4.9% and 5.5%,

respectively, showing the good precision and acceptable fabrication reproducibility.

The operational stability of the fabricated electrodes was studied by measuring current responses of Ag/CHER&CHOD/Au NPs/SPE biosensor by 10 repeated measurements. Study was performed in 0.05 mol/L glycine buffer solution (pH 8.6) in the presence of cholesterol (250 μg/mL) and after each measurement the electrode was stored at 4 °C for 5 min. The response of Ag/CHER&CHOD/Au NPs/SPE biosensor was almost stable in first 4 measurements after that it began to decrease slowly, and eventually on the 10th measurements the biosensor showed 25% loss of its initial current response.

3.8 Recovery of the cholesterol biosensor

In order to verify the practical application of the fabricated sensor，we tested the recovery rate of the sensor by detecting different concentrations of cholesterol such as 3000 μg/mL, 2000 μg/mL, 1500 μg/mL, 800 μg/mL, 250 μg/mL. Results can be seen from Table 1 that the recovery rate of the sensor was in the range of 96.94% -105.69%. It can be known that the recovery results were high which could meet the needs of the actual sample detection.

(Here Table 1)

Conclusion

In summary, a cholesterol biosensor based on enzymatic silver deposition was developed by immobilizing CHOD and CHER onto the surface of Au NPs-modified SPE. The Au NPs were deposited by a constant potential which can increase the conductivity of the electrode surface. And the CHER and CHOD were immobilized on the surface of Au NPs to disintegrate the cholesterol to generate H_2O_2 which can reduced the Ag ions in the solution for the deposition of metallic Ag on the Au NPs surface. The signal was achieved by biocatalytic deposition reaction and detection by the electrochemistry detection system. The linear relationship between current and cholesterol

concentrations was very well over the range from 5.0 μg/mL to 5000 μg/mL with a readily achievable detection limit of 3.0 μg/mL at a signal/noise (S/N) ratio of 3. In addition, this sensor showed outstanding specificity, acceptable reproducibility and excellent recovery. The proposed biosensor was characterized by CV, XPS and SEM techniques. This new cholesterol biosensor can be very useful for detection of cholesterol and offer great application promises in providing a sensitive, specific, and potent method for detection of biomacromolecure in the clinical diagnostics.

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Figure Captions

Scheme. 1 The principle of the developed biosensor for cholesterol detection

Fig.1. LSV curves of the Ag/CHER&CHOD/Au NPs/SPE in the presence of cholesterol (a), the Ag/CHER&CHOD/Au NPs/SPE without the presence of cholesterol (b), and the Ag/Au NPs/SPE in the presence of cholesterol (c). The concentration of cholesterol was 5.0 mg/mL and the current signal was obtained by the LSV which was carried out in 0.1 mol/L HNO_3 solution containing 0.6 mol/L KNO_3 from -0.1 to 0.6 V with a 100 mV/s scanning rate.

Fig.2. Cyclic voltammetry response for bare SPE (a); Au NPs/SPE (b); CHER&CHOD/Au NPs/SPE (c) and Ag/CHER&CHOD/Au NPs/SPE (d) in 5 mmol/L $K_3Fe(CN)_6/K_4Fe(CN)_6$ from -0.3 to 0.6 V with a 100 mV/s scanning rate.

Fig.3. SEM images for bare SPE (A); Au NPs/SPE (B); CHER&CHOD/Au NPs/SPE (C) and Ag/CHER&CHOD/Au NPs/SPE (D) which was performed using a Quanta 200 field environmental scanning electron microscope.

Fig.4. XPS spectra of the surface of SPE in different stages: full spectra (A); XPS spectra of C1s, O1s, N1s and Au4f (B). Among them, bare SPE (a); Au NPs/SPE (b); CHER&CHOD/Au NPs/SPE (c) and Ag/CHER&CHOD/Au NPs/SPE (d).

Fig.5. Optimization of experimental conditions for detection cholesterol. Effect of silver deposition time on the current signal (A); Effect of incubation temperature on the current signal (B); Effect of Au NPs deposition time on the current signal (C); Effect of Au NPs deposition potential on the current signal (D); Effect of the concentration of silver ion on the current signal (E); Effect of pH on the current signal (F). The concentration of cholesterol was 5.0 mg/mL and the current signal was obtained by the LSV which was carried out in 0.1 mol/L HNO_3 solution containing 0.6 mol/L KNO_3

from -0.1 to 0.6 V with a 100 mV/s scanning rate. And the error bars represent the standard deviation of the mean (n=3 electrodes).

Fig.6. LSV curve of different level cholesterol including 5000 μg/mL, 2500 μg/mL, 1000 μg/mL, 500 μg/mL, 50 μg/mL, 5 μg/mL, 0 μg/mL (A); Working curve of the cholesterol biosensor (B). The current signal was obtained by the LSV which was carried out in 0.1 mol/L HNO_3 solution containing 0.6 mol/L KNO_3 from -0.1 to 0.6 V with a 100 mV/s scanning rate. And the error bars represented the standard deviation of the mean (n=3 electrodes).

Fig.7. Specificity of the proposed biosensor with ascorbic acid, glucose, DL-cysteine instead of cholesterol. The current signal was obtained by the LSV which was carried out in 0.1 mol/L HNO_3 solution containing 0.6 mol/L KNO_3 from -0.1 to 0.6 V with a 100 mV/s scanning rate. And the error bars represented the standard deviation of the mean (n=3 electrodes).

Table 1 Determination of cholesterol by the proposed electrochemical biosensor

Concentration Added (μg/mL)	Measured Current (μA)	Concentration Found (μg/mL)	Recovery (%)
3000	324.90 326.50 325.40	3057.57±14.69	102.0%
2000	250.10 258.80 262.70	1938.76±105.75	96.94%
1500	230.20 222.00 231.80	1459.99±86.19	97.33%
800	185.1 186.7 188.0	781.19±23.81	97.65%
250	155.6 156.3 154.5	264.22±14.87	105.7%

Figures

Scheme.1

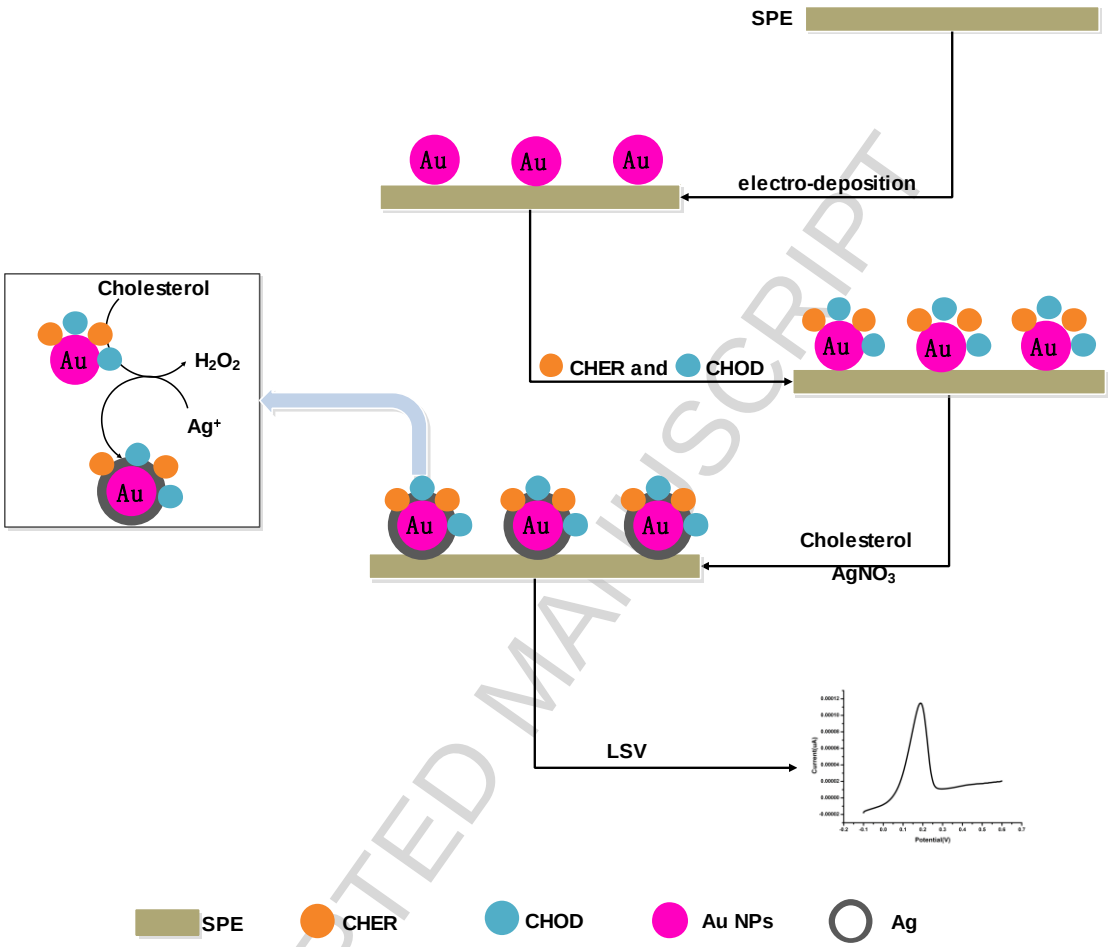


Fig.1

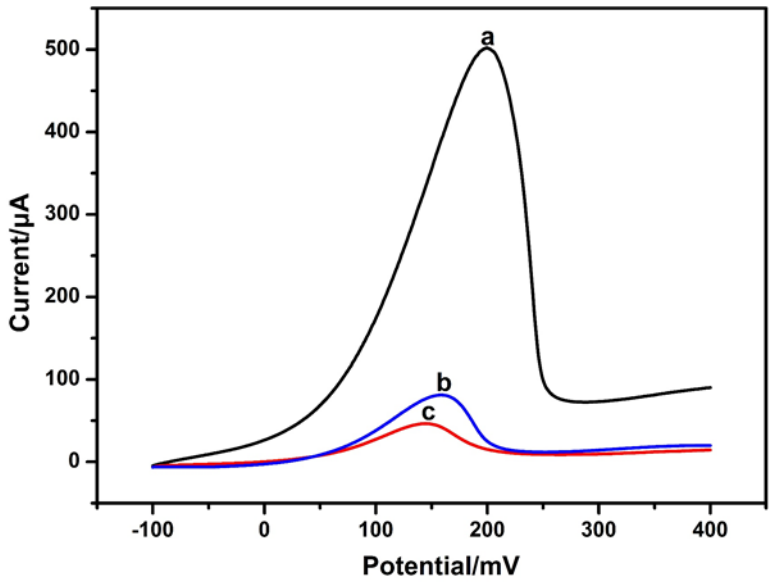


Fig.2

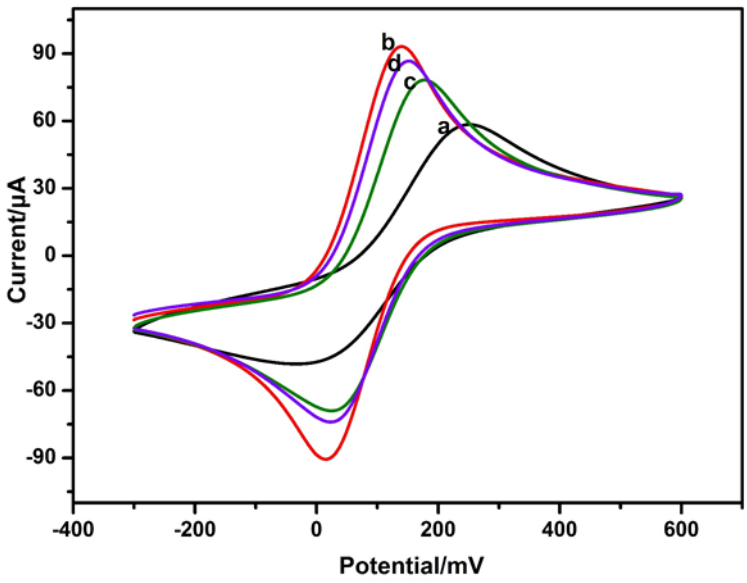


Fig.3

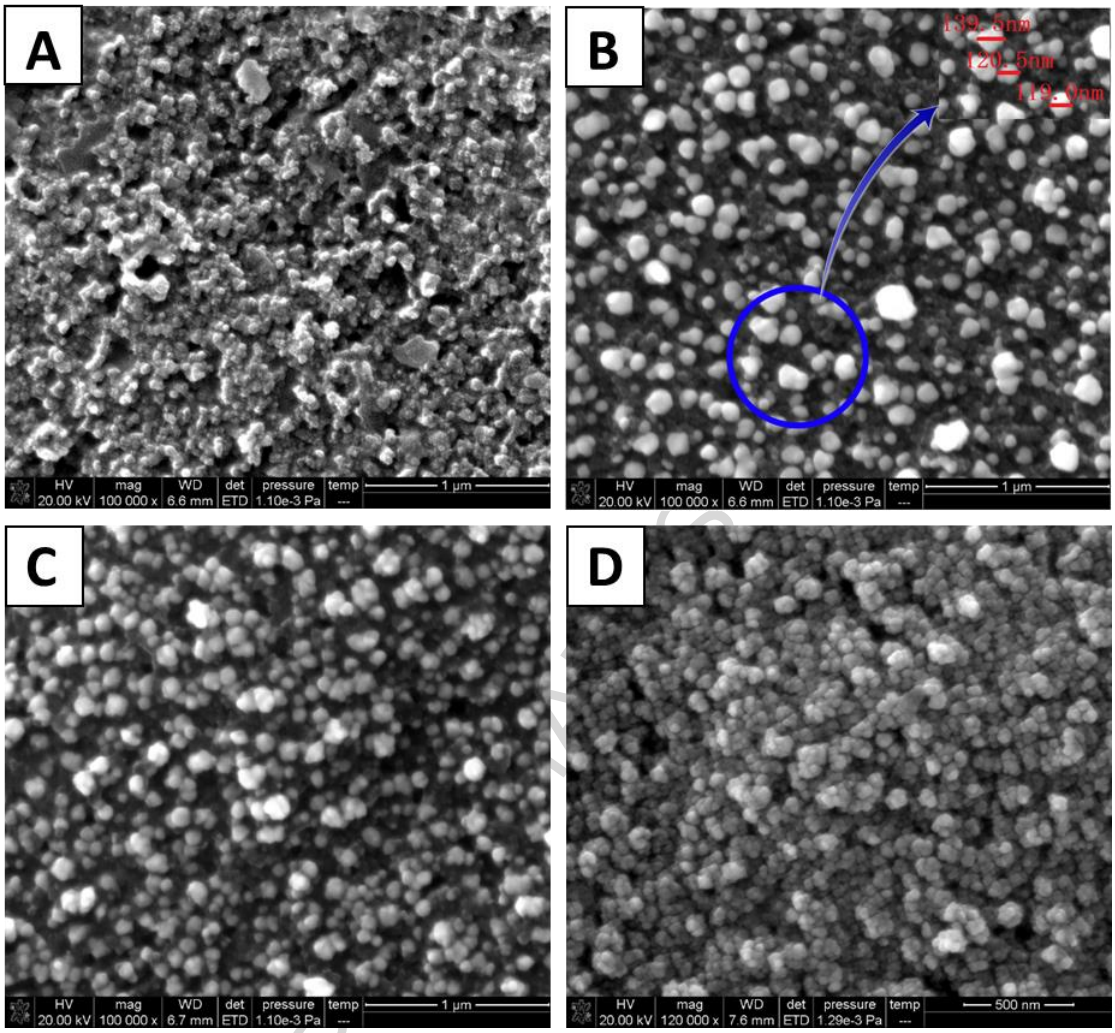
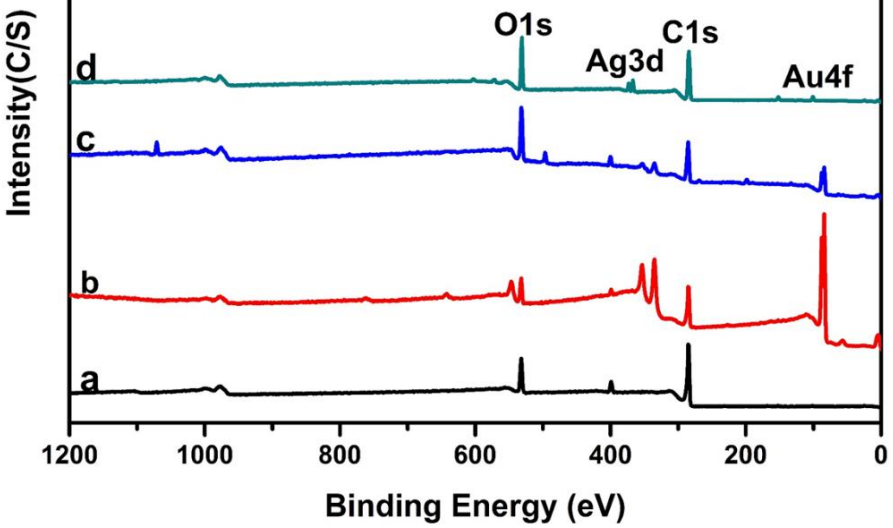


Fig.4

A

Name	Sample A		Sample B		Sample C		Sample D	
	Peak BE(eV)	Atomic(%)	Peak BE(eV)	Atomic(%)	Peak BE(eV)	Atomic(%)	Peak BE(eV)	Atomic(%)
O1s	532.04	14.94	532.11	13.49	531.87	27.94	531.76	24.5
C1S	284.32	77.48	284.3	68.62	284.83	62.35	283.77	73.89
N1s	399.2	7.58	399.3	6.03	399.72	7.56	400.23	0.77
Au4f	-	-	83.84	11.86	83.9	2.15	82.84	0.03
Ag3d	-	-	-	-	-	-	367.17	0.81



B

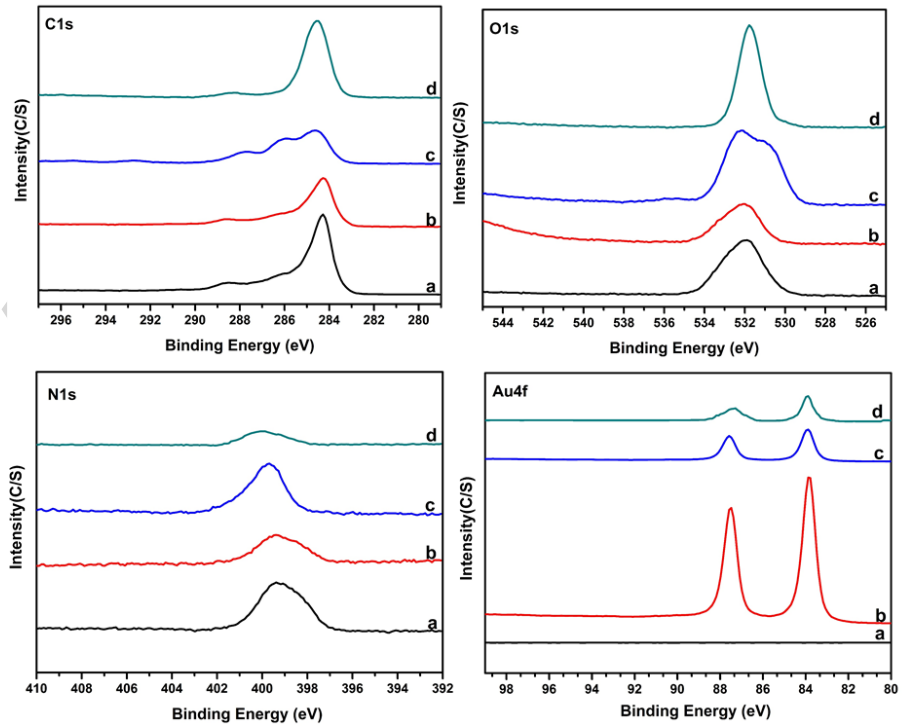


Fig.5

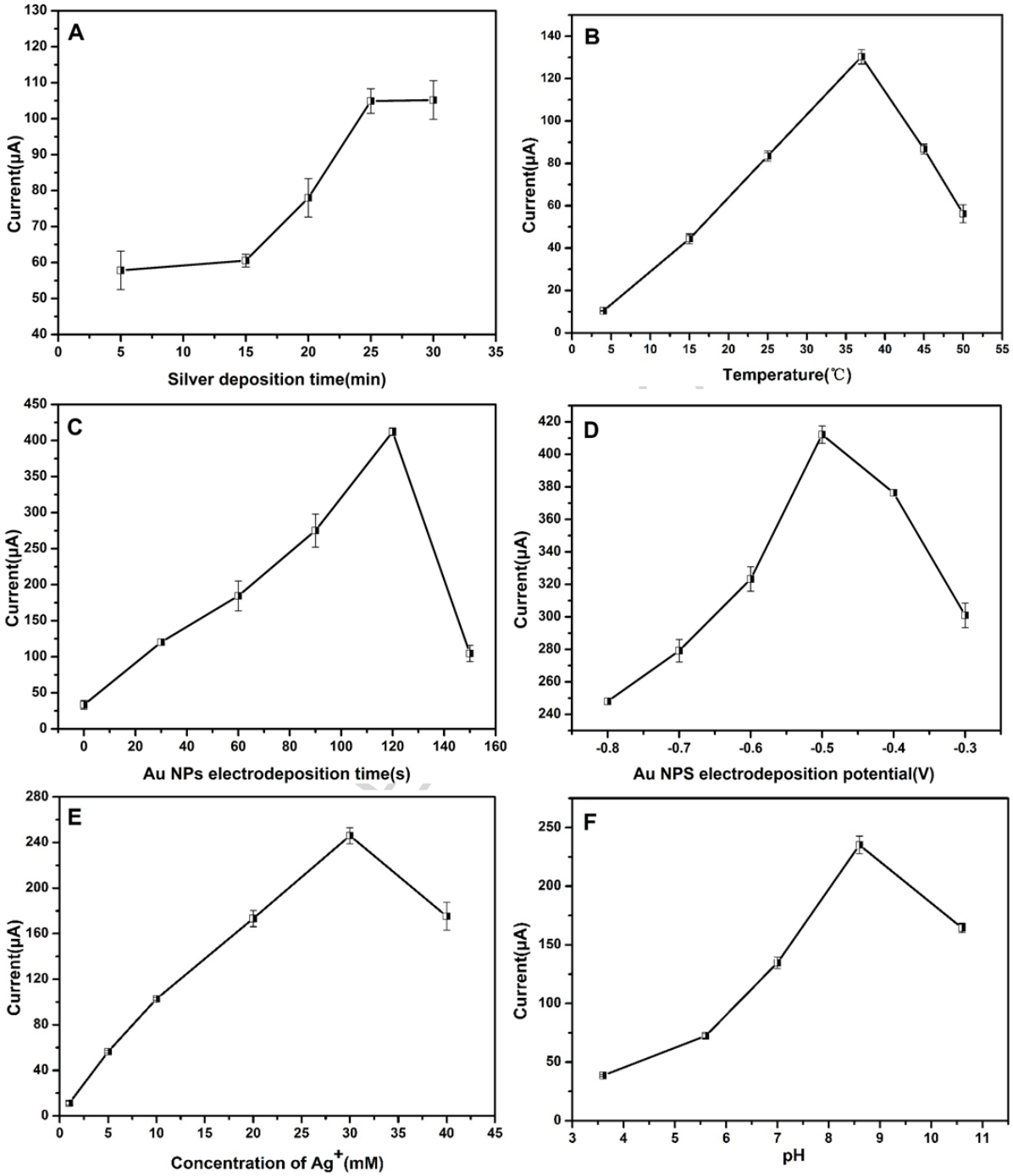


Fig.6

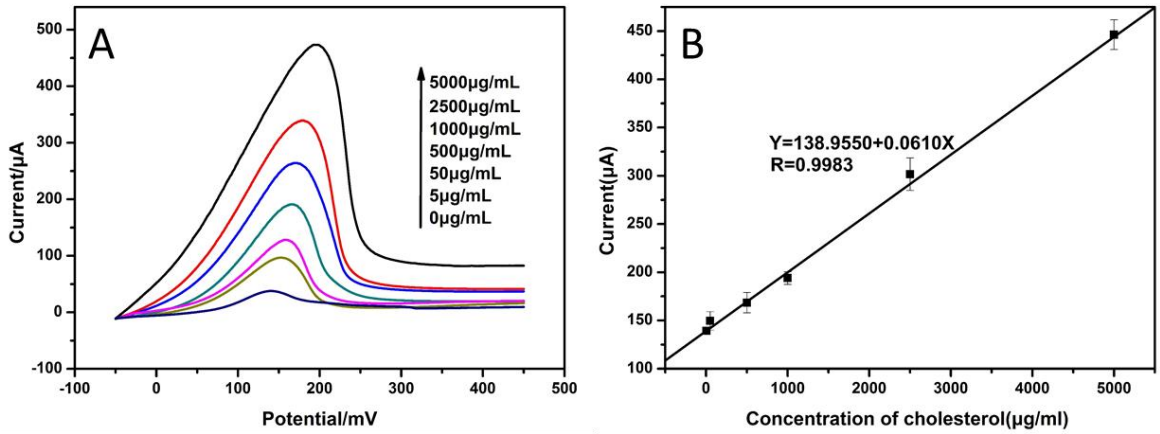
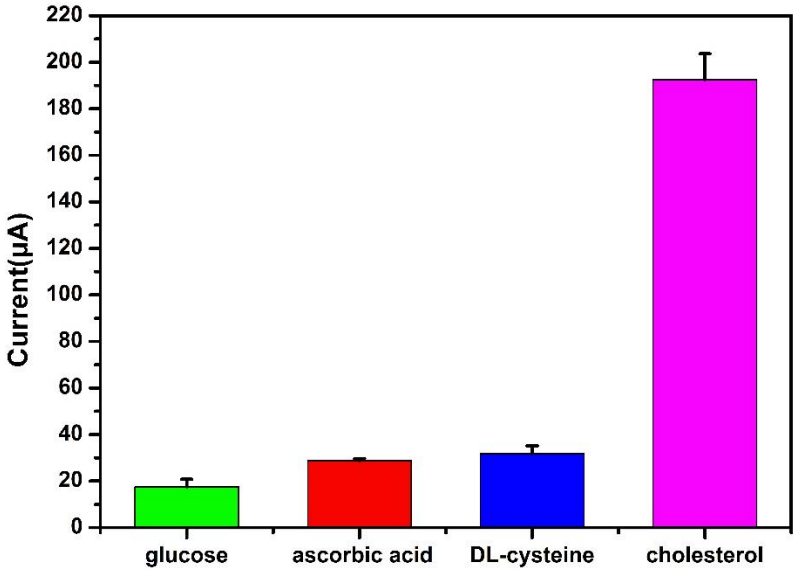
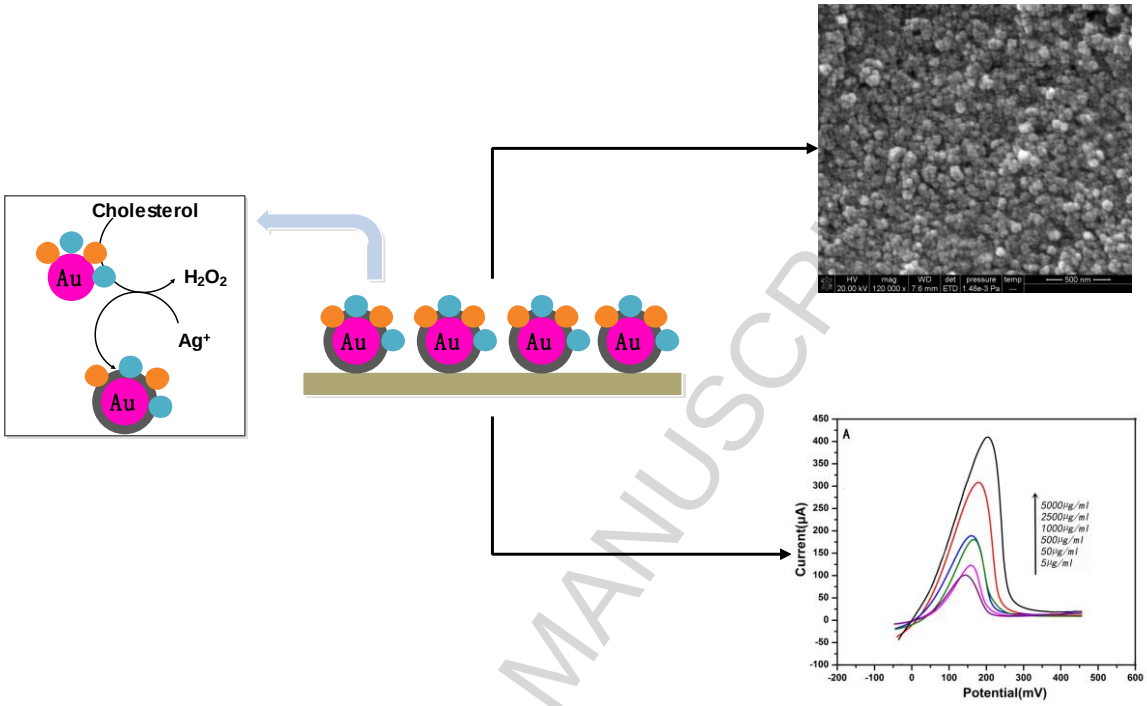


Fig.7



Graphical Abstract



Highlights

- A biosensor based on enzymatic Ag deposition on Au NPs/SPE was developed.
- The cholesterol biosensor was characterized by SEM, XPS and CV.
- The current of Ag depended linearly on the concentration of cholesterol.
- The biosensor showed outstanding specificity and acceptable reproducibility.