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Glassy carbon electrode modified with Nafion–Au colloids for clenbuterol electroanalysis

Lijing Liu[a,c], Haibo Pan[a,b,c,*], Min Du[a], Wenqin Xie[a,c], Jian Wang[c]

[a] Fujian Key Lab of Medical Instrument & Pharmaceutical Technology, Yishan Campus, Fuzhou University, Fuzhou, Fujian 350002, PR China
[b] State Key Laboratory Breeding Base of Photocatalysis, Yishan Campus, Fuzhou University, Fuzhou, Fujian 350002, PR China
[c] College of Chemistry and Chemical Engineering, Qishan Campus, Fuzhou University, Fuzhou, Fujian 350108, PR China

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ABSTRACT

Stable Nafion–Au colloids were immobilized on a glassy carbon electrode (GCE) for detection of β-agonist clenbuterol by electroanalysis. The Au colloids were prepared by a one-step electrodeposition onto GCE, with obvious electrocatalytic activity present. The negatively charged Nafion film was an efficient barrier to negatively charged interfering compounds, resulting in accumulation of positively charged clenbuterol at the Nafion film. The electrochemical characters of the electrode during various modified steps in a redox probe system of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ were confirmed by cyclic voltammetry (CV) and AC-impedance. In Britton–Robinson (B–R) buffer solution (pH = 2.0) and the potential range of −0.2 to 1.2 V, the Nafion–Au colloid modified electrode, compared to a bare GCE, exhibits obvious electrocatalytic activity towards the redox of clenbuterol by greatly enhancing the peak current with a linear calibration curve from 8.0×10^{-7} to 1.0×10^{-5} mol/L and a detection limit of (1.0×10^{-7} mol/L) ($R = 0.996$). The modified electrode shows high sensitivity, selectivity and reproducibility. The recovery for detecting clenbuterol ($\sim 10^{-6}$ mol/L) in human serum is up to 98.19%.

1. Introduction

Clenbuterol ($C_{12}H_{18}C_{12}N_2O$) is a β-agonist. It stimulates β-receptors, reducing stress symptoms and asthma and leading to its widespread use in the treatment of human depression and pulmonary diseases. However, because it promotes muscle growth and lipid degradation, use of clenbuterol has been forbidden in meat-producing animals [1]. Moreover, due to its long half-life and stability, clenbuterol residues could present a potential risk for human health [2]. To date, various analytical methods have been reported for the determination of clenbuterol, including gas chromatography–mass spectrometry (GC–MS) [3], high-performance liquid chromatography (HPLC) [4–6], nuclear magnetic resonance spectrometry (NMR), capillary electrophoresis (CE) [7,8] and enzyme-linked immunosorbent assay (ELISA) [9]. Although the determination of clenbuterol by these methods is promising because of their high selectivity and sensitivity, these instruments are inherently expensive, pretreatment for samples is involved and these operating processes are complicated and longer. Thus, it is crucial to develop a simple and fast method for analysis of clenbuterol. Electroanalysis is an appropriate route for detection of β-agonist clenbuterol because clenbuterol contains an electroactive aromatic amino group.

Gold nanoparticles (GNPs), with large surface area, good bio-compatibility, high conductivity and electrocatalysis characteristics, have been used to improve the detection limit for biomolecules in electrochemical studies [10–16]. Additionally, Nafion is a perfluorinated sulfonated cation exchanger and consists of a linear backbone of fluorocarbon chains and ethyl ether pendant groups with sulfonic cation exchange sites (Fig. S1). To date, Nafion is still used as the best performing cation exchange membrane because of its high chemical and thermal stability and its mechanical strength. Some examples of Nafion film modified electrodes used to detect organic compounds and metal cations have been demonstrated in the literature [17–19]. Although Nafion has been used for clenbuterol electroanalysis with a high limit (1.0×10^{-9} mol/L), the linear range (4×10^{-9} to 1.5×10^{-8} mol/L) is sharp and these reports did not study its selectivity [20,21].

This paper presents an application of Nafion–Au colloid modified electrodes to detect trace clenbuterol by CV and differential pulse voltammetry (DPV). GNPs were electrochemically deposited on the bare GCE, and a Nafion film was then coated onto the Au colloid modified electrode. In this way, we can combine the advantages of Nafion (good ion-exchange and preconcentration features towards clenbuterol molecules) and the high electrocatalytic activity of Au colloids. Next, the redox behavior of clenbuterol

* Corresponding author at: Fujian Key Lab of Medical Instrument & Pharmaceutical Technology, Yishan Campus, Fuzhou University, Gongye Road No. 523, Fuzhou, Fujian 350002, PR China. Tel.: +86 591 83759450/22866127; fax: +86 591 22866127.
E-mail address: hbpan@fzu.edu.cn (H. Pan).

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on Nafion–Au colloid modified electrodes was researched. Finally, the experimental conditions were optimized, and performance features such as reproducibility and stability were evaluated. The biosensor was also been applied in real sample (human serum) determinations. The aim of this work is to develop an alternative electrochemical method to detect clenbuterol with wide linear range and high selectivity.

2. Materials and methods

2.1. Materials

Hydrogen tetrachloroaurate tetrahydrate ($HAuCl_4{\cdot}4H_2O$) and Nafion (5% ethanol solution) were purchased from Aldrich, and clenbuterol was purchased from Sigma. All other chemicals (analytical grade) were purchased from Beijing Chemical Reagent Company (Beijing, China) and were used without further purification. Ultra-pure water was obtained with a Milli-Q Plus water purification system (Millipore Co. Ltd., USA) (18 MΩ). The clenbuterol stock solution was prepared by pH = 2.0 B–R buffer solution as described in the literature [20], *i.e.*, 2.875 mL acetic acid, 3.38 mL phosphoric acid, 3.11 g boric acid and deionized water, and sodium hydroxide (2 mol/L) was used to adjust the pH of solution. All experimental solutions were stored at 4 °C and then deoxygenated by bubbling pure nitrogen for 15 min before determination.

All the electrochemical measurements were performed on a CHI 660B electrochemical workstation (CH Instrument Co., Shanghai, China). A conventional three-electrode system was chosen with working electrodes of glassy carbon electrodes modified by different layers. A Pt wire and an Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively. All potentials were versus Ag/AgCl, and all experiments were carried out at room temperature.

2.2. Preparation of colloidal gold nanoparticles

GNPs with an approximate average diameter of 20 nm were prepared by citrate reduction of $HAuCl_4$ according to the literature [22]. All glassware was thoroughly cleaned with aqua regia ($HCl:HNO_3$ = 3:1), rinsed with deionized water and dried before using. An aqueous solution of sodium citrate (2.0 mL, 1 wt.%) was rapidly added to a boiling $HAuCl_4$ solution (50 mL, 0.01 wt.%) under vigorous stirring. The color of the solution changed slowly from purple to blue and finally to red, indicating formation of GNPs. The solution was refluxed for an additional 15 min under vigorous stirring. After the heat source was removed, the solution was stirred continuously until it reached room temperature. Finally, the cooled solution was stored in the refrigerator at 4 °C.

2.3. Preparation of Nafion–Au colloid modified electrode

The surface of the GCE was polished with 0.3 and 0.05 μm alumina slurries, and sonicated with deionized water and ethanol. Then the electrode was dried under pure N_2. The smoothed GCE was treated by applying +1.5 V for immobilization of GNPs as a film on the surface of the working electrode and dried under infrared light. 10 μL 0.5% Nafion ethanol solution was dispersed uniformly on the surface of the Au colloid modified electrode and the resulting sensing interface was obtained by evaporating the solvent under infrared light.

2.4. Measurements and clenbuterol electroanalysis

UV–vis spectra were recorded with a spectrophotometer (Perkin-Elmer Lambda 900, USA). The size, size distribution, and morphology of the GNPs were measured by high-resolution transmission electron microscopy (HRTEM, JEOL model JEM 2010 EX instrument, 200 kV). For HRTEM analysis, one drop of gold colloidal solution without sonication was deposited on a HRTEM copper grid and dried under vacuum.

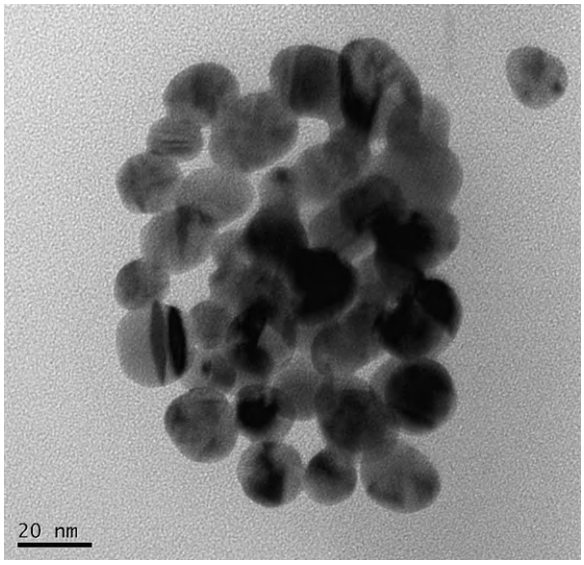


Fig. 1. TEM image of Au colloids.

The working electrode was dipped into a B–R buffer solution containing different concentrations of clenbuterol and then accumulated at −0.2 V for 360 s under stirring. Prior to detection, the electrode was cycled only in B–R buffer solution in the potential range of −0.2 to 1.2 V (scan rate 0.1 V/s) until the current became stable. The human serum samples for detecting clenbuterol were obtained from a local hospital (Fujian Maternal and Child Health Hospital, China). These samples were diluted using the buffer solution and then analyzed with the Nafion–Au colloid modified electrode in our laboratory by the standard addition method.

3. Results and discussion

3.1. Electrochemical characteristics of Nafion–Au colloid modified GCE

The UV–vis spectra of Au colloids show a sharp and characteristic plasmon band at 521 nm (not shown), and the HRTEM image for the GNPs displays a 20 nm sphere particle with dispersive state, shown in Fig. 1.

CV and AC-impedance were used to study the surface properties of the electrodes during different modified steps in a redox probe system of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$. Fig. 2 shows the CV of a bare GCE (a), an Au colloid modified GCE (b) and a Nafion–Au colloid modified GCE (c) in 1 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl. From Fig. 2(b), the deposition of GNPs on a GCE generates a small barrier to interfacial electron transfer, decreasing the current, and increases the separation between the cathodic and anodic peaks, implying that Au colloids have been immobilized on the electrode surface. When Nafion is coated on the Au colloid modified GCE, the redox peak currents dramatically decrease and the peak shape is not well-defined (Fig. 2(c)), suggesting that the presence of the Nafion film acts as an inert layer and blocks the diffusion of ferricyanide to the electrode.

Among the various transduction techniques, impedance spectroscopy is an effective method to probe the interfacial properties of the modified electrode. By using a $[Fe(CN)_6]^{3-/4-}$ redox couple as the electrochemical probe, the Nyquist plots of different modi-

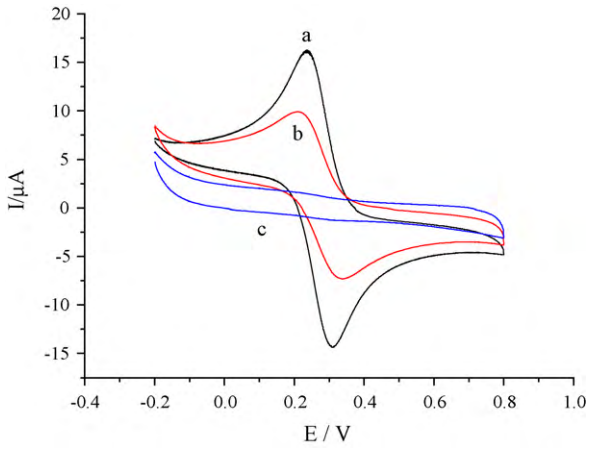


Fig. 2. Cyclic voltammograms of bare GCE (a), Au/GCE (b) and Nafion/Au/GCE (c) in a mixture solution of 1.0 mmol/L $[Fe(CN)_6]^{3-}$ and 0.1 mol/L KCl with the scan rate as 0.1 V s^{-1}.

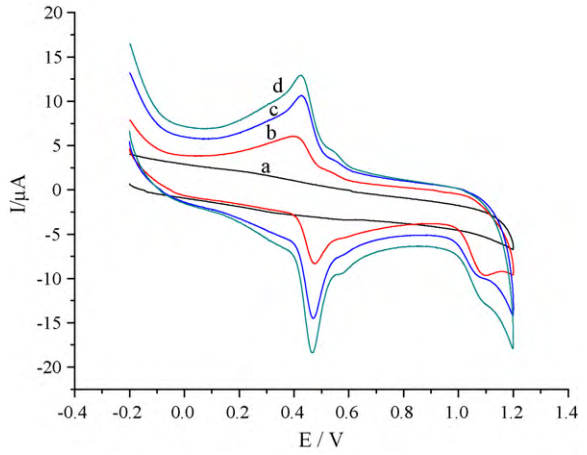


Fig. 4. Cyclic voltammograms at the bare GCE (a), Nafion/GCE (b), Au/GCE (c) and Nafion/Au/GCE (d) in pH 2.0 B–R solution for 4×10^{-6} mol/L clenbuterol at the scan rate of 0.1 V s^{-1}.

fied electrodes are shown in Fig. 3, where the semicircle diameters at high frequency and straight lines at low frequency correspond to the electron transfer resistance at the electrode surface and the diffusion of a redox couple, respectively. The electron transfer resistance of a bare GCE (Fig. 3(a)) is very small compared to the modified electrode. After Nafion coating on the Au colloid electrode surface (Fig. 3(c)), the semicircle diameter increases, indicating that the film formed on the surface hinders electron transfer. Thus the impedance is larger than that at Au colloid modified electrode (Fig. 3(b)). The results obtained are completely consistent with those from CV (Fig. 2), further confirming the success of the modifying procedure.

3.2. Electrochemical behavior of clenbuterol at the Nafion–Au colloid modified GCE

From Fig. 4, the polished bare GCE gives practically no response to clenbuterol (Fig. 4(a)), but the peak current of the Nafion modified GCE (Fig. 4(b)) is much larger than that of bare GCE (Fig. 4(a)), suggesting that clenbuterol molecules are prone to adsorption on the Nafion surface due to the electrostatic interaction between the sulfonic group of Nafion (Fig. S1) and the clenbuterol protonated amino group (Scheme 1). The peaks are also well-defined for the Au colloid modified GCE (Fig. 4(c)), indicating that the gold colloids immobilized on the electrode surface plays important roles as an active surface with a small, 20 nm spherical particle size and dispersive state (Fig. 1) and also as a conducting wire or electron-conducting tunnel for clenbuterol electron transfer. The current intensities for the oxidative and reductive peaks at 1.1 and 0.4 V by the Nafion–Au colloid modified GCE (Fig. 4(d)) are much greater than those of other electrodes (Fig. 4(a)–(c)), attributed to the excellent electrocatalytic activity carried out synergistically by both Au colloids and Nafion. Note that the open circuit potentials (OCP) for the different electrodes in Fig. 4, *i.e.*, the bare GCE (a), Nafion/GCE (b), Au/GCE (c) and Nafion/Au/GCE (d) in the B–R solution, are 0.591, 0.897, 1.02 and 1.02 V, respectively.

The CV curve without clenbuterol (Fig. 5(c)) shows essentially no response at the same scanning rate. However, the CV curves (a) and (b) in Fig. 5 exhibit typical reduction/oxidation behavior at the electrode surface with clenbuterol in the solution. Also, clenbuterol at the Nafion–Au colloid modified GCE undergoes an ECE process. In the first scan (Fig. 5(a)), an anodic peak I_a is observed near 1.1 V and then the I_a drops sharply in the next scan, suggesting that an adsorbed process existed on the electrode. The return scan for the first cycle shows a peak at 0.38 V (II_b). In the second cycle, the CV shows two obvious anodic peaks and one cathodic peak (Fig. 5(b)), *i.e.*, I_a (1.1 V), II_a (0.42 V) and II_b (0.38 V), respectively.

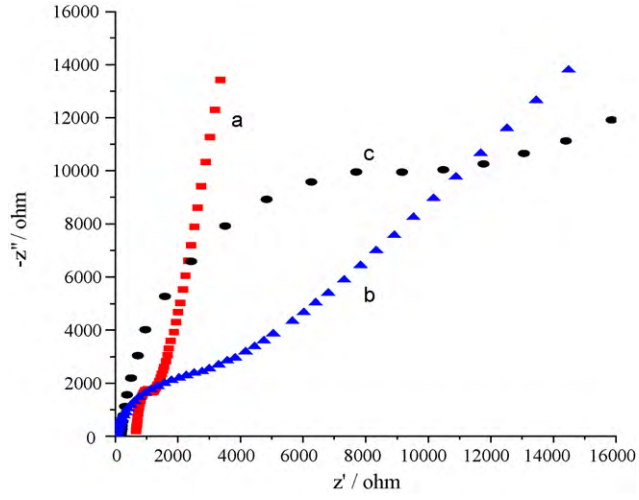


Fig. 3. Impedance plots (Z' versus Z'' at 270 mV versus Ag/AgCl) in 1 mM $Fe(CN)_6^{4-/3-}$ + 0.1 M KCl solution at bare GCE (a), Au/GCE (b) and Nafion/Au/GCE(c). The frequency range is between 0.1 and 100 kHz with signal amplitude of 5 mV.

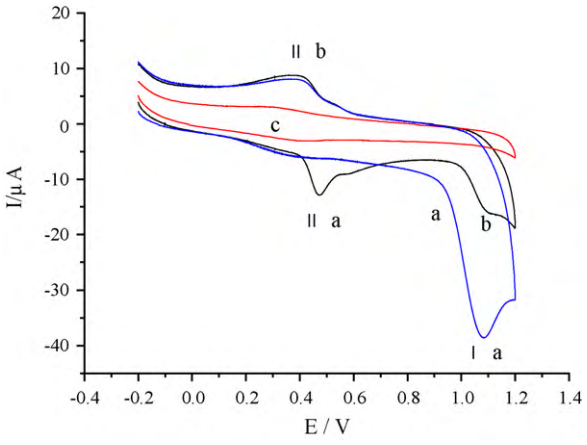
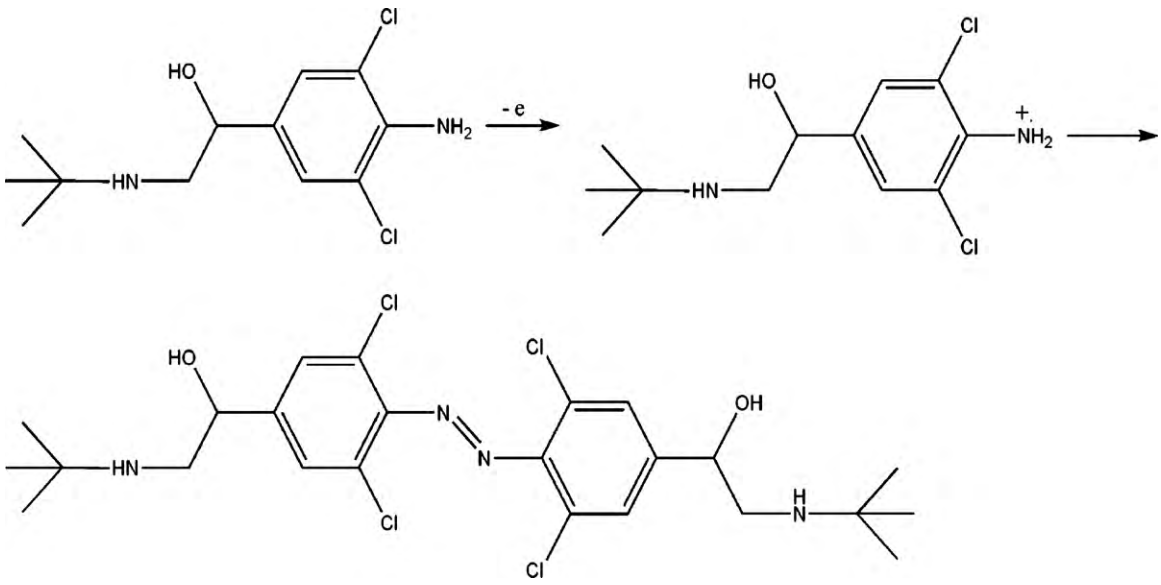


Fig. 5. Cyclic voltammograms based on a Nafion–Au colloids modified GCE with the presence at first cycle (a), second cycle (b), and without presence (c) of clenbuterol (4×10^{-6} mol/L) in the B–R solution (pH = 2.0).



Scheme 1. The formation of a clenbuterol dimer.

II_a and II_b currents gradually increase to a stable value with an increase in cycle time. This mechanism involves the electrochemical generation of a product at high positive potential (~1.1 V) with a chemical reaction following, giving rise to a product that exhibits a quasi-reversible couple at ~0.4 V. Additionally, when a new modified electrode was scanned between 0 and 0.8 V, a potential that was lower than the oxidation potential of clenbuterol (1.1 V), the reduction peak on the reverse scan and the subsequent reoxidation peak on the forward scan were absent (not shown), also suggesting that the quasi-reversible couple is due to a product formed at higher potentials. The proposed mechanism is that the nitrogens are sp^2 hybridized, and this results in a dimer of clenbuterol molecules with an azo bond as seen in Scheme 1 [23]. For further analysis of the dimer in the clenbuterol solution, the UV–vis spectra of fresh clenbuterol (Fig. 6(a)) and clenbuterol (Fig. 6(b)) oxidized for 2 h at 1.15 V (greater than 1.1 V, the oxidation potential of clenbuterol), were obtained. The absorption peaks at 243 and 295 nm (Fig. 6(b)) after oxidation are almost unchanged, though the dimer peak at 295 nm is higher and wider than that of the fresh electrode (Fig. 6(a)). Generally, the dimer peak should be blue-shifted due to the conjugation across the N=N bond (Scheme 1). However, the steric hindrance induced by the ortho-substituted chlorines hampers the conjugation, and the monomer clenbuterol molecules near the modified electrode were finally oxidized into dimers (295 nm) after 2 h of the oxidation of the clenbuterol on the modified electrode, inducing the drop in the 243 nm peak and the broadening of the 295 nm peak.

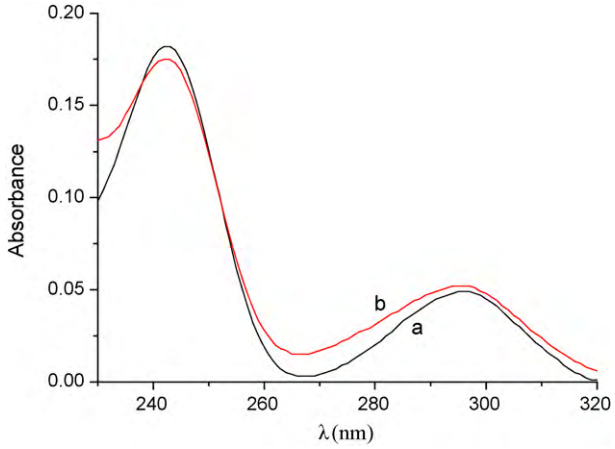


Fig. 6. UV–vis spectra for fresh clenbuterol ($c = 2 \times 10^{-5}$ M) (a) and oxidized clenbuterol ($c = 2 \times 10^{-5}$ M) at certain 1.15 V, 2 h (b) in the B–R solution (pH = 2.0).

The redox of clenbuterol is strongly pH dependent; thus, the effect of the pH of the buffer solution on Nafion–Au colloid modified GCE was studied by CV in the pH range of 1.4–4.0 of B–R buffer solutions. In the acidic solution, clenbuterol molecules exist predominantly in their cationic form and can therefore be readily incorporated into anionic Nafion film. Thus, the clenbuterol molecules are oxidized at 1.1 V, losing an electron and forming radical cations. According to the lack of shift with pH of the peak I_a potential (not shown), it is inferred that no proton participates in the oxidation of clenbuterol at 1.1 V. The negative shift of peak II_a occurs with pH value according to the following equation: E_p (II_a) = 0.50–0.049 pH (R = 0.998) (Fig. S2). This mechanism may also be expressed as two radical cations reacting via head to head coupling to form a dimer with an azo bond. The dimer is strongly dependent on pH value and is more easily oxidized than the radical cation, resulting in a lower redox potential of ~0.4 V [20,23].

Fig. S3 shows the relationship between the peak currents (I_a and II_a) and the scan rates in B–R solution (pH = 2.0) containing 4×10^{-6} M clenbuterol. A plot of the peak current (I_a) versus the square root of the scan rate is linear, indicating the rate controlling process is the diffusion of clenbuterol through the Nafion film. The regression equation for this relationship is as follows: $i_p = -77.14v^{1/2} - 6.56$ (R = 0.997) (Fig. S3A). The peak current II_a is proportional to the scan rate (Fig. S3B), revealing that the oxidation of clenbuterol at the modified electrode is a surface (adsorption) controlled process ($i_p = -18.14v - 4.40$ $(R = 0.997)$).

3.3. Optimization of experimental conditions

Because experimental conditions play a key role in detecting clenbuterol, their optimization is very important. At first, the effects of pH on the peak current of clenbuterol were studied in B–R buffer solutions in pH = 1.4–12 (Fig. S4). Note that the peak II_a current (Fig. 5) was recorded as a specific signal for clenbuterol detection in the following experiment due to its high oxidized current. A maximum value for II_a current existed at pH = 2.0, and the pH = 2.0 B–R buffer solution was chosen as the medium.

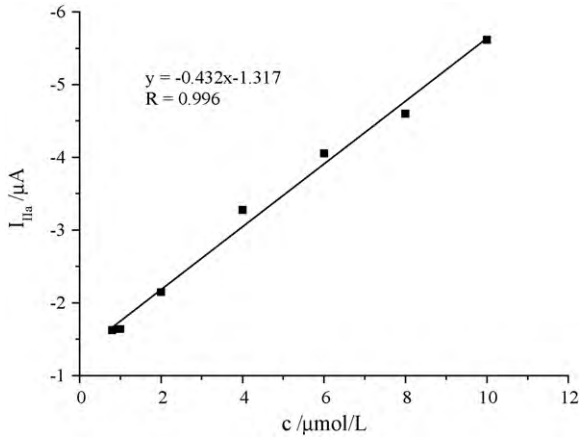


Fig. 7. Calibration curve between clenbuterol concentration and the current of peak II_a based on DPV.

Electrodeposition times for Au colloids on GCE were also studied from 200 to 1000 s using a potential of +1.5 V (Fig. S5). From these experiments, an electrodeposition time of at most 800 s was selected for all subsequent experiments. Fig. S6 shows the peak (II_a) current response curve of clenbuterol ($c = 4 \times 10^{-6}$ mol/L) in B–R solution (pH = 2) with different concentrations of Nafion. The response increased rapidly with Nafion concentration in the range of 0.1–0.5% and subsequently decreased. Because of hindrance of the electron transfer in a thick membrane at high concentrations, the optimal Nafion concentration of 0.5% was selected.

Based on the clenbuterol accumulation on the electrode, which is essential for detection sensitivity [2], the accumulation potential of the peak II_a current was performed in the range of −0.2 to 0.4 V with a specific accumulation time (AT = 360 s), as shown in Fig. S7. No clenbuterol accumulation of peak II_a existed on the bare GCE (not shown). For the Nafion–Au colloid modified electrode, however, the peak II_a current increased as the applied potential shifted negatively from 0.2 V. Preliminary results show that the peak current does not significantly vary with a more negative potential than −0.2 V (Fig. S7). At potentials more negative than −0.2 V, reactions such as the reduction of H_2O caused a large quantity of gas bubbles to appear at the electrode surface, which interfered with the CV signal [2]. Therefore, −0.2 V is the best choice for accumulation potential in clenbuterol detection. Similarly, the optimal accumulation time is 360 s under this accumulation potential (−0.2 V) (not shown). Finally, these optimized accumulation parameters were applied as test conditions for clenbuterol microanalysis.

3.4. Linear range, repeatability and stability of clenbuterol detection

Because it is a more sensitive method than CV, the determination of clenbuterol concentration at the Nafion–Au colloid modified GCE was performed by DPV. Calibration data were obtained for clenbuterol solutions under the optimum experimental conditions described in Fig. 7, and the plot demonstrates linear behavior in the range of 8×10^{-7}–1×10^{-5} mol/L ($R = 0.996$) with a limit of 1.0×10^{-7} mol/L and a signal-to-noise ratio of 3.

The stability of the modified electrode was tested by scanning the electrode continuously in 4×10^{-6} mol/L clenbuterol at a scan rate of 0.1 V s^{-1}. There was no apparent decrease in the current response for 100 consecutive cycles, indicating that the modified electrode is relatively stable. To study durability, the modified electrode was stored at 4 °C and measured intermittently for clenbuterol (4×10^{-6} mol/L). The current (II_a) retained 89% of its initial response after 30 days. A relative standard deviation of 3.9% was obtained for six successive measurements of 4×10^{-6} mol/L clenbuterol, suggesting good repeatability. Note that due to strong adsorption of clenbuterol onto the surface of the modified electrode, the electrode must be renewed each time.

Table 1
Result of clenbuterol recovery test in human serum.

Added (mol/L)	Measured (mol/L)	Recovery (%)	Average recovery (%)
1.0×10^{-6}	1.02×10^{-6}	101.8	98.19
4.0×10^{-6}	3.93×10^{-6}	98.15	
6.0×10^{-6}	5.85×10^{-6}	97.45	
8.0×10^{-6}	7.63×10^{-6}	95.35	

3.5. Interference for detecting clenbuterol

The interference factors for detecting clenbuterol using the Nafion–Au colloid modified electrode were also studied in the presence of normal anions or cations, such as 600-fold amounts of Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO^{3-}, SO_4^{2-}, urea, hydroxylamine hydrochloride and glucose, and 100-fold amounts of L-cysteine and uric acid. These samples did not interfere with detection.

3.6. Determination of clenbuterol in human serum

The proposed method with the above optimized conditions was applied to determine trace amounts of clenbuterol in human serum. The determination was performed by the standard addition method. The results and recoveries of known amounts of clenbuterol added to the serum sample are given in Table 1.

4. Conclusions

In this work, the use of a GCE modified with Au colloids and Nafion for electrochemical measurements was demonstrated as an effective alternative method to highly sensitive electrodes for clenbuterol detection. The synthesis procedure includes preparation of stable and reproducible modified films, leading to a considerable enhancement in the response to the clenbuterol electrochemical character. During the detection, GNPs with large specific surface area and Nafion films with high electron exchange capabilities catalyzed clenbuterol efficiently. Meanwhile, selectivity and sensitivity were greatly improved by applying the Nafion film. The modified electrode possesses such advantages as simple fabrication, wide linear range, fast response, excellent reproducibility and long stability. The recovery for detecting clenbuterol in human serum is up to 98.19%. Thus, the Nafion–Au colloid modified electrode could be very promising in the field of sensors and detection for clenbuterol microanalysis.

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.electacta.2010.06.078.

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