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Analytical Methods

Development of a label-free electrochemical immunosensor based on carbon nanotube for rapid determination of clenbuterol

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ABSTRACT

We have fabricated a label-free electrochemical immunosensor for the detection of clenbuterol, a kind of β-agonist. Clenbuterol was covalently linked to multi-wall carbon nanotubes (MWCNTs) through a two-step process using 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide and *N*-hydroxysulfo-succinimide as crosslinkers. The clenbuterol-MWCNT conjugates were cast on a glassy carbon electrode. Cyclic voltammetry and differential pulse voltammetry were employed to monitor the fabrication steps of immunoreaction system using the redox probe of $K_3Fe(CN)_6$. In the presence of monoclonal antibody against clenbuterol, the redox peak current of $[Fe(CN)_6]^{3-/4-}$ was decreased, presumably due to that antibody in solution could adsorb on the electrode surface modified clenbuterol-MWCNT conjugates. The selected monoclonal antibody showed very high sensitivity and specificity for clenbuterol, and was used for the detection and quantitative determination of clenbuterol in solution with a competitive mechanism. This approach provided a detection limit of 0.32 ng mL^{-1}. Accurate detection of clenbuterol in spiked animal feeds was demonstrated by comparison with conventional ELISA assays and LC–MS method.

1. Introduction

β-Agonists are phenyl ethanolamines with different substituents on the aromatic ring and on the terminal amino group. The family of β-agonists include compounds such as clenbuterol, ractopamine, cimaterol, zilpaterol and salbutamol. In the livestock industry, β-agonists have been used as repartitioning agents and many of researches have been reported that β-agonists could improve growth rate and reduce carcass fat when fed to poultry (Wellenreiter & Tonkinson, 1990), pigs (Engeseth et al., 1992; Watkins, Jones, Mowrey, Andersom, & Veenhuizen, 1990), and calves (Andersom, Veenhuizen, Wagner, Wray, & Mowrey, 1989). As we know, clenbuterol is the most effective β-agonist as growth promoting agent (Blanca et al., 2005). However, the residues of clenbuterol which accumulate in animal tissues can cause symptoms of acute poisoning in human (Mitchell & Dunnavan, 1998). Symptoms from clenbuterol residue-induced food poisoning have been reported from investigations of separate events in several countries (Martinez-Navarro, 1990; Pulce, Lamaison, Keck, Bostvironnois, & Descotes, 1991). Moreover, drug residues may negatively impact the export trade of edible animal products and result in nearly incalculable economic loss. Therefore, the use of clenbuterol has been banned in most countries.

In an effort to combat the illicit use of β-agonists and related compounds, regulatory organisations worldwide are testing animal tissues and excreta for the presence of illicit drugs (Kuiper, Noordam, van Dooren-Flipsen, Schilt, & Roos, 1998). For example, various analytical methods have been reported for the determination of clenbuterol in feedstuff and animal tissues. These include liquid chromatography with electrochemical or mass spectrometric detection (Guy, Savoy, & Stadler, 1999; Lin, Tomlinson, & Satzger, 1997), gas chromatography with mass spectrometry (Abukhalaf et al., 2000; He, Su, Zeng, Liu, & Huang, 2007), enzyme-linked immunosorbent assays with polyclonal or monoclonal antibodies (Johansson & Hellenas, 2004; Posyniak, Zmudzki, & Niedzielska, 2003), capillary electrophoresis with amperometric detection (Chen, Wang, Duan, Chen, & Chen, 2005), elctrochemical method with differential-pulse voltammetry (Moane, Smyth, & Keeffe, 1996), and immunosensor with surface plasmon resonance (Johansson & Hellenäs, 2003; Traynor, Crooks, Bowers, & Elliott, 2003).

Recently, electrochemical immunosensors have gained growing attention since they combine the high specificity of traditional immunoassay methods with the low detection limits and low expenses of electrochemical measurement system. The method has been used by a number of investigators for the determination of various analytes with great sensitivity and specificity using different types of electrodes (Liu, Wu, Wang, Shen, & Yu, 2001; Piermarini, Micheli, Ammida, Palleschi, & Moscone, 2007; Wang & Pamidi, 1998). Various substances have been used as immobilisation

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matrix materials, such as polymers, sol–gels, nanoparticles and hydrogels (Shi, Yuan, Chai, & He, 2007). There are an increasing number of approaches to develop biosensors using carbon nanotubes (Wohlstadter et al., 2003; Yu, Kim, Papadimitrakopoulos, & Rusling, 2005), since electrochemical studies have shown the ability of carbon nanotubes (CNTs) to promote electron-transfer reactions, to minimise electrode surface fouling, and to enhance electrocatalytic activity (Banks, Moore, Davies, & Compton, 2004; Baughman et al., 1999). The immobilisation of antibody or antigen on the surface of CNT electrodes as biosensors has been reported (Okuno et al., 2007; Wu, Yan, & Ju, 2007; Yun et al., 2007). For example, viswanathan et al. reported the application of glassy carbon electrodes (GCEs) immobilised with Nafion-MWCNTs as a voltammetric sensor in an immunoassay for cholera toxin (Viswanathan, Wu, Huang, & Ho, 2006). Moreover, it has been reported that the crucial aspect of electrochemical immunosensor is the immobilisation of immunologically sensitive compounds on the electrodes (Gao, Lu, Cui, & Zhang, 2006). In previous literatures, two strategies were applied to immobilize immunological molecules (1) antibody immobilised on the electrode was applied to directly determine the antigen in solution by monitoring the response change of ferrocyanide as the redox marker on the electrode (Lei, Gong, Shen, & Yu, 2003; Zhang, Wang, Hu, & Xiao, 2006; Zhou et al., 2005). (2) Antigen immobilised on the electrode was used to compete with free antigen in solution for a specific antibody (Chen, Yan, Dai, & Ju, 2005). However, these methods almost suited to detect biomacromolecules, such as protein, virus, and cell factor. In addition, the direct chemical cross-linking of immunological molecules might lose part of their activities, and the regeneration of the sensor required complete removal of all immobilised materials from the electrode surface.

So far, only a few immunosensors are used to detect haptens which are difficult to immobilise and have little effect on electron transfer of the electrochemical mediator in solution, since haptens usually are small molecular compounds. For the detection of electroactive hapten, it has been reported that the antibody was immobilized on the electrode to capture the hapten, then the hapten adsorbed on the electrode showed well-shaped redox responses (Hu et al., 2003). However, it is difficult to detect non-electroactive small molecules. Our group has reported a label-free electrochemical immunosensor for rapid determination of ractopamine by incorporating ractopamine-bovine thyroglobulin (BTG) antigen in agarose hydrogel films modified on a GCE. A competitive immunoreaction system was applied to detect the free ractopamine in solution (Shen & He, 2007). This is a usual procedure to fabricate electrochemical immunosensor for the detection of small molecule hapten. Recently, Kong et al. constructed a highly enantioselective and sensitive immunosensor for the detection of chiral amino acids based on capacitive measurement (Zhang, Ding, Liu, Kong, & Hofstetter, 2006). The hapten was firstly covalently immobilized onto the gold electrode by diazotization. Using a competitive setup, quantitative detection of amino acid enantiomers was possible by monitoring the current response of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ on the gold electrode. To the best of our knowledge, it is the first report to directly detect hapten by covalently immobilizing hapten on electrode.

In this paper, clenbuterol was covalently linked to carboxylated multi-wall carbon nanotubes (MWCNTs) using the EDC/NHSS protocol. A novel approach for electrochemical immunoassay based on the immobilization of clenbuterol-MWCNT conjugates on the GCE is described. MWCNTs, with its unique and excellent properties such as large surface area and good electric conductivity, can absorb large numbers of hapten molecules, here is substantial amplification and clenbuterol can be detected at a very low level of detection. The electrochemical character of the immunosensor during different modified stages in a redox probe system of $K_3[Fe(CN)_6]$ was investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). In the presence of anti-clenbuterol antibody, significant decrease of the peak current and the voltammetric response of redox probe were both observed, indicating $K_3[Fe(CN)_6]$ electron transfer is inhibited. A competitive immunoreaction system, which was defined as clenbuterol-MWCNT conjugates immobilized on electrode compete with the free clenbuterol in the solution for specific antibody, was applied to determine the free clenbuterol in phosphate buffered saline and spiked animal feeds. In addition, in this paper we also obtained the results using ELISA and LC–MS from analyses of the same samples to assess the utility of electrochemical immunosensor analysis of clenbuterol. As we know, this is the first report that clenbuterol bound MWCNTs has been used as a voltammetric sensor in an immunoassay for clenbuterol detection.

2. Experimental

2.1. Materials and reagents

Clenbuterol, ractopamine, and salbutamol were obtained from Sigma/Aldirch Company (St Louis, MO) and were used as received. Goat anti-mouse IgG-horseradish peroxidae (HRP) was obtained from Jackson Immuno-Research Laboratories (West Grove, PA, USA). Mouse monoclonal antibodies to the clenbuterol were obtained from Laboratory of Space Cellular & Molecular Biology Institute of Space Medico-Engineering (Beijing, China). The high purity MWCNTs (diameter 10–20 nm) were gifts from Peking University (Beijing, China). 1-(3-(Dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfo-succinimide (NHSS) were purchased from Sigma/Aldirch and dissolved in water immediately before use. All other chemicals were analytical grade.

Each litre of phosphate buffered saline (PBS) contained 8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \cdot 12H_2O$, and 0.2 g KH_2PO_4 and the buffer was adjusted to pH 7.4 with 0.2 M Na_2HPO_4. Phosphoric acid–methanol extraction solution (0.2 M) was prepared by dissolving 3.92 g phosphate acid in 200 mL water and adding methanol to 1 L. All water used was double-deionized water (Milli-Q, Millipore Corporation, Bedford, MA).

2.2. Fabrication of clenbuterol-MWCNT conjugates

The procedure of covalent attachment process between clenbuterol and carbon nanotube was carried out according to the previous report (Yu et al., 2006). Briefly, multi-wall carbon nanotubes were sonicated in H_2SO_4:HNO_3 (3/1, V/V) at about 70 °C for 6 h. The resulting dispersion was washed with water and dried under vacuum overnight. Subsequently, 1.5 mg of the functionalized MWCNTs were dispersed in 2 mL pH 7.4 PBS buffer and sonicated for 10 min to obtain a homogeneous dispersion, which indicated that the MWCNTs were well activated with hydrophilic carboxylate groups. This dispersion was mixed with 1 mL of pH 6.0 MES buffer including a mixture of 400 mM EDC and 100 mM NHSS, then vortexed at room temperature for 20 min. The resulting mixture was centrifuged at 15,000 rpm for 15 min, and the supernatant was discarded. This centrifugation procedure was repeated using PBS to remove excessive EDC and NHSS. Clenbuterol at 2 mg mL^{-1} was added to the mixture (1:1, V:V) and vortexed overnight at room temperature. The reaction mixture was then centrifuged at 15000 rpm for 15 min, and the supernatant was removed. It was reported that inhibition of non-specific binding in biosensors using carbon nanotubes was critical to achieve the best sensitivity and detection limits (Chen et al., 2003; Shim, Kam, Chen, Li, & Dai, 2002). In order to keep the formed monolayer insulating and pin-free, and to ensure a high sensitivity, bovine serum albumin

(BSA) was used to block uncovered spaces on the MWCNT surface. Thus, the clenbuterol-MWCNTs conjugates was treated with 1 mL 5% BSA solution for 30 min to block non-specific binding. The final solution was centrifuged 3 more times using PBS to remove any free clenbuterol and BSA. The clenbuterol-functionalized MWCNTs were re-dispersed in 1 mL PBS, vortexed to form homogeneous dispersions, and stored in refrigerator at 4 °C before use.

2.3. Preparation of clenbuterol immunosensor

Prior to the surface modification, the bare GCE was sequentially polished with 1 μm and 0.05 μm alumina slurry, then washed ultrasonically in distilled water and ethanol for a few minutes, respectively. To obtain the best responses, the experimental conditions for film casting, such as the concentration of clenbuterol-MWCNTs, and the constructed total volume of coating solution, were optimized. Typically, 10 μL of clenbuterol-MWCNT dispersions was spread on the surface of the GC electrodes, and then dried at room temperature for 5 h. After rinsing thoroughly with PBS, the clenbuterol-MWCNT films were formed on GCE surface.

For the immunoassay, the modified electrode was immersed in 100 μL of PBS solution including anti-clenbuterol antibody and incubated, then washed thoroughly in PBS.

2.4. Preparation of animal feed samples

A 5 g sample of finely ground swine feed, spiked with clenbuterol, was accurately weighed into a 50 mL polytetrafluoroethylene tube and 40 mL of freshly prepared phosphate acid–methanol extraction solution (0.2 M) was added. The mixture was shaken vigorously for 30 min and was then centrifuged at 3000 rpm for 10 min. The supernatant was placed into a 100 mL volumetric flask. The residue was extracted two times using 40 mL and 20 mL of the same extraction solution, respectively. The supernatant was collected and made up to 100 mL by the addition of the extraction solution. A 1 mL aliquot of the supernatant was decanted into a 5 mL tube and the solution was evaporated under a stream of nitrogen in a water bath at 55 °C. The extract was reconstituted in PBS for enzyme-linked immunosorbent assays (ELISA) and electrochemical analysis. If necessary, the resulting solutions were diluted using PBS so that the sample concentrations were within the measurable range of ELISA and electrochemical method. However, the extract was reconsitituted in 1 mL 2% acetic acid solution and purified using solid phase extraction for liquid chromatography–mass spectrometry (LC–MS) analysis.

2.5. ELISA and LC–MS measurements

ELISA measurements of feed samples were similar to that described previously (Shen & He, 2007). In short, 96-well plates were coated with 100 ng/well of clenbuterol-BSA at 37 °C for 1 h, and excess binding sites were blocked by 3% BSA for 1 h. Competing clenbuterol antigen was prepared in PBS containing 0.05% Tween 20 and co-incubated with primary antibody at 2.0 μg/mL for 45 min. After incubation with HRP labelled anti-mouse-IgG (1 h), the colour was developed using *o*-phenylenediamine (OPD) as substrate. A clenbuterol calibration curve was used to determine the clenbuterol level in feed samples.

Chromatographic separation of clenbuterol was achieved on a Waters Symmetry™ C_{18} (2.1 mm × 150 mm; 3.5 μm) column. The mobile phase delivered at a flow rate of 0.2 mL min^{-1}, consisted of A: 0.01 M aqueous ammonium formate (pH 3.8) and B: acetonitrile. A gradient programme was used, starting at an A/B (v/v) composition of 98/2. This composition was changed linearly to reach 70/30 within 5 min, and then to reach 50/50 from 5 to 15 min. A 10-min equilibration time was needed before the next injection. The optimised ionisation conditions for mass spectrometric detection were using a cone voltage of 25 V, a extractor voltage 5 V, and a capillary voltage 3.0 kV. The ion source and desolvation temperatures were 120 °C and 350 °C, respectively. The ionisation was tested in electrospary ionisation positive (ESI^+) mode, and high-purity nitrogen was used as the ESI^+ nebulizing gas. Under moderate ionisation conditions, the selected parent ion *m/z* 277 and the daughter mass ions *m/z* 259 and *m/z* 203 of clenbuterol were monitored with a selected ion mode, and the abundance of parent ion *m/z* 277 was used to quantitative calculation. The clenbuterol spiked in feed samples was quantified by external standard.

2.6. Apparatus and measurement procedures

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed on a CHI 420 electrochemical workstation (CH Instruments). The experiments were carried out in a conventional three-electrode system comprising a platinum wire as auxiliary electrode, an silver wire electrode as reference and a clenbuterol-MWCNT modified GCE as the working electrode. All measurements were carried out in PBS (pH 7.4) containing 2 mM $K_3[Fe(CN)_6]$ redox probe system. Differential pulse voltammetric (DPV) measurements were performed from 500 to –200 mV with the pulse amplitude of 50 mV and the pulse width of 50 ms.

The absorbance values for the ELISA were read in dual-wavelength mode (492–570 nm) using a TECAN SUNRISE Microplate Reader (Salsburg, Austria). LC–MS analysis was performed on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA) coupled to a Macromass ZQ2000 Mass Spectrometer (Manchester, UK). Transmission electron microscopy (TEM) image was taken with an H 600 TEM instrument (Hitachi) operating at 100 kV.

3. Results and discussion

3.1. The principle of the electrochemical immunosensor for clenbuterol

TEM was used to observe the actual dimension of MWCNTs in the aqueous dispersions, showing that the diameter of MWCNT was in the range of 10–20 nm (not shown). In order to immobilize hapten on the surface of carbon nanotube, MWCNTs were firstly acid-oxidized to activate carboxylic acid groups on the surface of the carbon nanotubes. Then carboxylated MWCNTs were activated by EDC, forming a active ester in the presence of NHSS. Finally, the active ester was reacted with the amine group of clenbuterol, forming an amide bond between the MWCNTs and clenbuterol (Fig. 1A). It has been reported that carbon nanotubes could form a stable film on the surface of GCE (Luo, Shi, Li, Gu, & Zhang, 2001). Thus, the clenbuterol-MWCNT conjugates are firstly cast on the GCE. The determination of hapten was based on the the specific interaction between antibody and hapten using a competitive mode. That is, when the electrode was immersed into the solution containing anti-clenbuterol antibody and free clenbuterol, clenbuterol immobilized on the electrode and free clenbuterol in solution can both bind anti-clenbuterol antibody. There is a competition binding between them. The more free clenbuterol molecules in solution can bind antibodies, the less antibodies can bind clenbuterol molecules immobilized on the electrode. On the contrary, the less free clenbuterol molecules in solution, the more antibodies immobilized on the electrode.

$K_3Fe(CN)_6$ probe is a valuable and convenient tool for testing the kinetic barrier of the interface because the charge transfer between solution and the electrode must occur by tunnelling either through the barrier or through the defects in the barrier. When the electrode was immersed into the solution containing specific

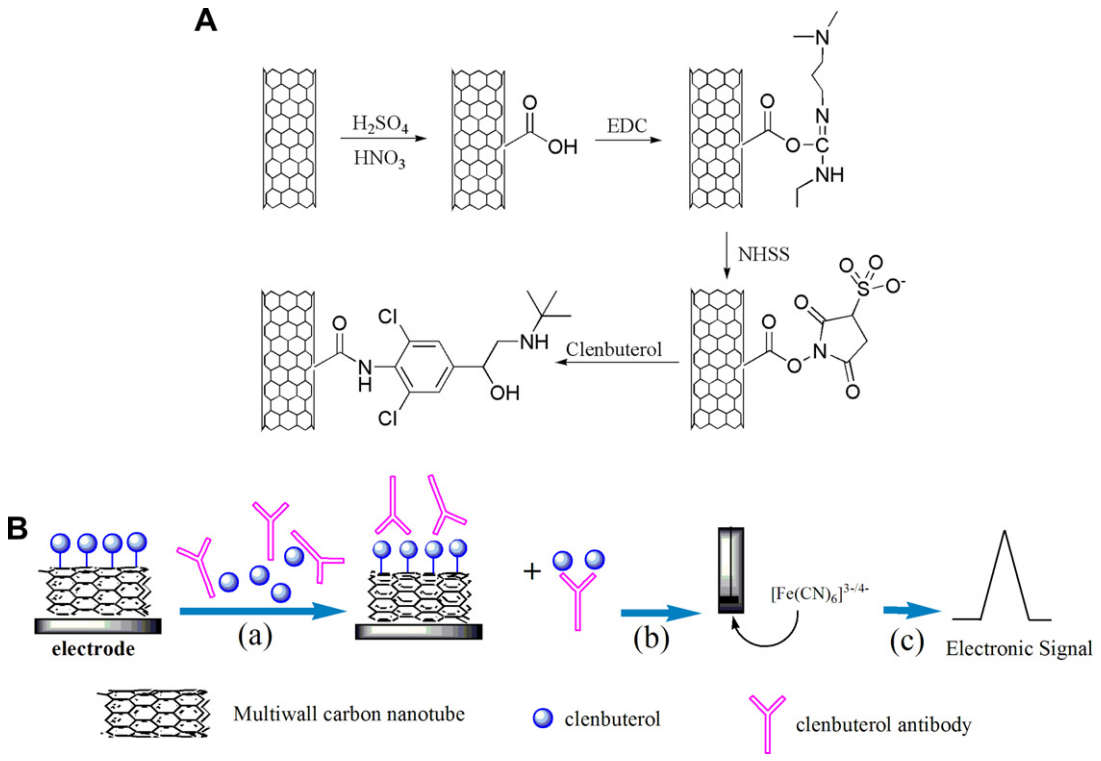

Fig. 1. (A) Schematic representation of the fabrication process of the clenbuterol-MWCNT conjugates. (B) The working principle of the immunosensor with competitive immunoassay: (a) the specific binding of the immobilized hapten to antibody in the competitive setup by free clenbuterol in solution, (b) electrode was washed and transferred into the cell containing 2 mM $K_3[Fe(CN)_6]$, (c) DPV detection.

antibody, the ability of the anti-clenbuterol antibody to specifically bind to the hapten clenbuterol led to the antibody layers to immobilized on the electrode. Formation of antibody-hatpen complex on the surface of the GC electrode led to more steric hindrance on the electrode, which decreased the eletrode active area and hindered probe ions through their pathways into the electrode. Hence, the redox reaction of $K_3Fe(CN)_6$ was suppressed and the peak current decreased. Similar results and corresponding explanation were also reported in the literatures (Gao et al., 2006; Yun et al., 2007; Zhang et al., 2006).

Therefore, Using a competitive reaction, in which clenbuterol contained in solution compete with clenbuterol immobilized on the GC electrode for the antibody in solution, a label-free detection of clenbuterol was possible by monitoring the current response of $K_3[Fe(CN)_6]$ on GC electrode. The principle of the electrochemical immunosensor for clenbuterol was shown in Fig. 1B.

3.2. Optimisation of the immunoassay conditions

Firstly, we studied the temperature effect on the immunoreaction using ELISA. Briefly, the reaction of clenbuterol-BSA and antibody was examined at room temperature, 37 °C, and 50 °C. Substrate colour reaction was found that the absorbance value at 37 °C was the biggest amongst three. The result shows that the antibody exhibits the highest affinity towards clenbuterol at 37 °C. Thus, we employed it as the optimum incubation temperature in this study. The effect of different detection conditions, such as immunoreaction time and antibody concentration, were investigated by DPV method. Using the same antibody concentration, the incubation times were 0, 20, 30, 40 and 60 min in incubation solution, respectively, then the immunosensor was washed carefully with PBS. The DPV peak current of clenbuterol-MWCNTs modified GCE to the redox marker $K_3[Fe(CN)_6]$ in solution decreased with incubation time rapidly up to 40 min and then the peak current decreased slightly at about 60 min (Fig. 2A). Longer incubation time has no significantly effect on the current response. Thus, 40 min was adopted as the optimal incubation time for the immunoassay in the subsequent work.

To investigate the influence of concentration of antibodies in the incubation solutions, the GC electrodes modified with clenbuterol-MWCNTs were immersed in 100 μL of PBS solutions with different concentrations of antibodies at 37 °C for 40 min. As shown in Fig. 2B, the $K_3[Fe(CN)_6]$ peak currents decreased significantly when the concentration of antibody in the solution was changed from 1.4 to 5.6 μg mL^{-1}. With further increase in the antibody concentration to 5.6–22.4 μg mL^{-1}, the decrease of the current response was nearly not observed. The results suggest that an antibody concentration greater than 5.6 μg mL^{-1} in incubation solution would not increase the amount of adsorbed antibody, indicating that the concentration saturation of the binding sites is basically reached. For a competitive immunoreaction, we would choose a concentration that gives a high enough signal but is well below the saturation concentration. Thus, the concentration of 2.8 μg mL^{-1} was chosen as the optimal antibody concentration to construct immunoassay films in the following studies.

3.3. Cyclic voltammetric analysis of clenbuterol-MWCNTs modified electrode

The fabrication of the clenbuterol-modified immunosensor was studied by cyclic voltammetry of $K_3[Fe(CN)_6]$ as the redox marker in pH 7.4 buffer solution. Fig. 3 shows the CVs of different modified electrodes in the redox probe system. As expected, the bare GCE (Fig. 3a) showed a well defined reduction–oxidation peak pair centered at about 0.2 V, characteristic of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple. There was a decrease in overall voltammetric signal after the electrode was modified with clenbuterol-MWCNTs conjugates (Fig. 3b). Further, the redox shapes of the redox probe almost

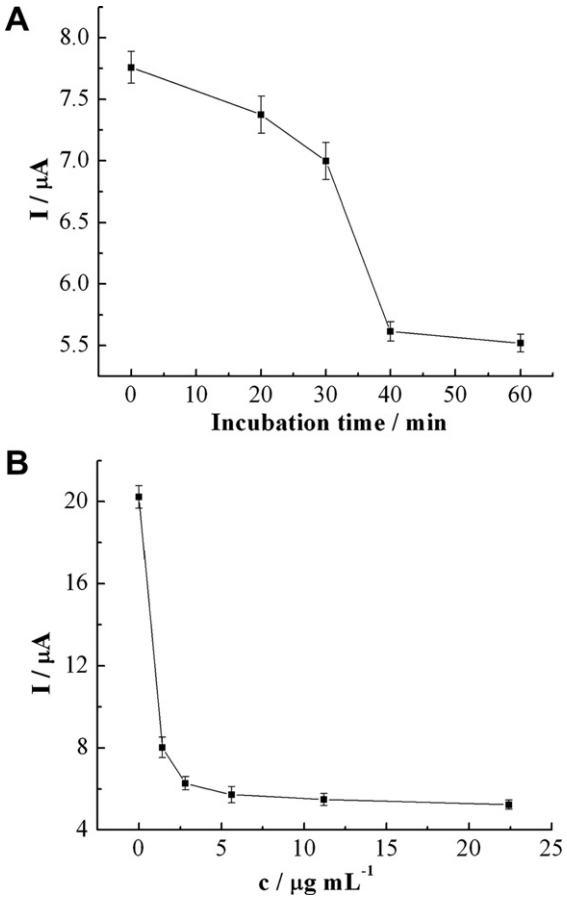

Fig. 2. Effect of incubation time (A) and concentration of antibody (B) in incubation solution on DPV peak current in pH 7.4 PBS solution containing 0.1 M KCl and 2 mM $K_3[Fe(CN)_6]$.

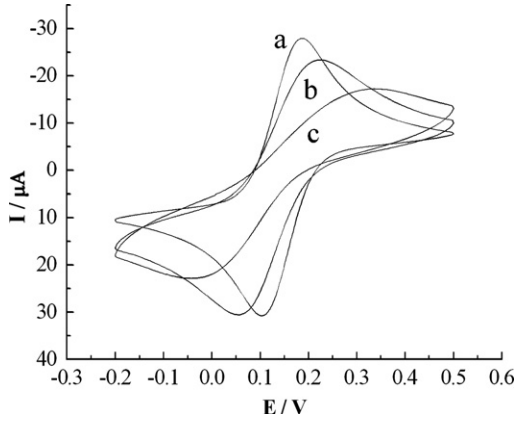

Fig. 3. CV of (a) bare GCE, (b) cleubuterol-carbon nanotube modified GCE, (c) cleubuterol-carbon nanotube modified GCE incubated in PBS containing 2.8 μg mL^{-1} antibody for 40 min. CV conditions: pH 7.4 PBS solution containing 0.1 M KCl and 2 mM $K_3[Fe(CN)_6]$; Scan rate, 50 mV s^{-1}.

disappeared after the modified electrode was reacted with antibody (Fig. 3c). This is similar to that described previously (Yu et al., 2006). The result illustrates that the antigen–antibody complex insulates the electrode and hinders the diffusion of the redox marker toward the electrode surface, and also shows that the clenbuterol-MWCNTs conjugates successfully immobilized on the electrode surface and that direct access by the redox couple was blocked.

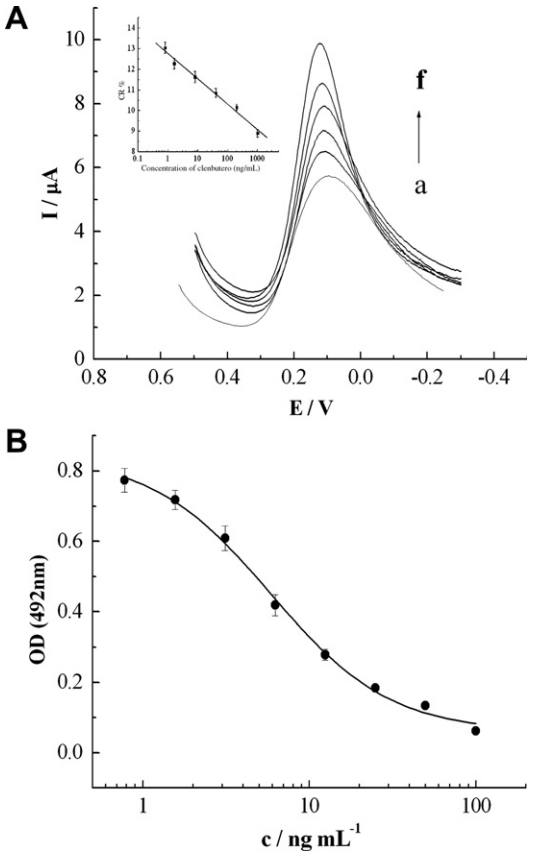

Fig. 4. (A) DPVs of the immunosensor after incubated in PBS buffer containing 2.8 μg mL^{-1} antibody and (a) 0.8 ng mL^{-1}, (b) 1.6 ng mL^{-1}, (c) 8 ng mL^{-1}, (d) 40 ng mL^{-1}, (e) 200 ng mL^{-1}, (f) 1000 ng mL^{-1} cleubuterol. Inset: The calibration curve of free cleubuterol. (B) ELISA calibration curve for clenbuterol.

3.4. Detection of clenbuterol

Since DPV is a very sensitive electrochemical method, the ability of the monoclonal antibody specifically bound to clenbuterol was investigated using DPV. To prepare the incubation solutions, 50 μL of clenbuterol standard solutions with a series of concentrations were mixed with 50 μL of anti-clenbuterol antibody solutions (5.6 μg mL^{-1}) to a total volume of 100 μL. At last, in order to block possible remaining active sites of the electrode, an additional blocking agent BSA was introduced in the system to block uncovered spaces on the electrode surface, since the GCE surface is well known for a large non-specific binding ability. The similar procedures were also done in the fabrication of immunosensors for the detection of specific antigen and hapten in previous reports (Yu et al., 2006; Zhou et al., 2005). Because of the specific interaction between anti-clenbuterol and hapten, the clenbuterol on the electrode would only bind with anti-clenbuterol antibody, not bind with the other antibodies and proteins. When BSA was used to block the uncovered surface of MWCNT and electrode, there is no effect on the determination of clenbuterol.

Then the clenbuterol-MWCNTs modified electrode was immersed in the incubation solution. The clenbuterol in solution competed with the clenbuterol immobilized on the surface of the electrode to bind the clenbuterol antibody in the incubation solution. After incubated for 40 min, the immunosensor was washed carefully with PBS. Fig. 4A showed the DPVs obtained at different concentrations of clenbuterol when the immunosensor was dipped into the PBS buffers containing $K_3[Fe(CN)_6]$. As expected, the DPV peak current increased with an increasing clenbuterol concentration in the incubation solution compared with no clenbuterol. That

is, the higher concentrations of free clenbuterol, the less amounts of antibody to bind the clenbuterol immobilized on the GCE. The percentage of current response reduction (CR%) defined by previous report (Shen & He, 2007) were proportional to the clenbuterol concentrations in the range from 0.8 to 1000 ng mL^{-1}. The detection limit was 0.32 ng mL^{-1}, which was evaluated by the actually DPV response as the lowest concentration of clenbuterol that could be distinguished at a stated level of probability from the PBS buffer only containing 2.8 μg mL^{-1} antibody. The indirect competitive ELISA established above was also applied to detect clenbuterol. A competition calibration curve was prepared using serial dilutions ranging from 1.56 to 100 ng mL^{-1} of free clenbuterol in PBS (Fig. 4B). The limit of detection, defined as detectable concentration equivalent to 10% decline of zero binding, was 0.78 ng mL^{-1}. The results show that it is possible to quantitatively determine clenbuterol using the electrochemical immunosensor, with wider linear response range and higher sensitivity compared to those of ELISA method.

In addition, blank and contrast experiments were performed with negative bovine serum albumin (BSA) and immunoglobulin G (IgG), which could further confirm that the observed DPV responses were indeed based on the specific adsorption between the electrode immobilized clenbuterol and the anti-clenbuterol antibody and were not caused by non-specific adsorption. As expected, when clenbuterol-MWCNTs modified GC electrode was exposed to a high concentration (100 μg mL^{-1}) of non-specific antibody (BSA or IgG), no significant change in the peak current signal was observed (not shown). These results indicate that the DPV changes observed with anti-clenbuterol antibody are virtually due to specific clenbuterol–antibody interaction and not due to non-specific adsorption.

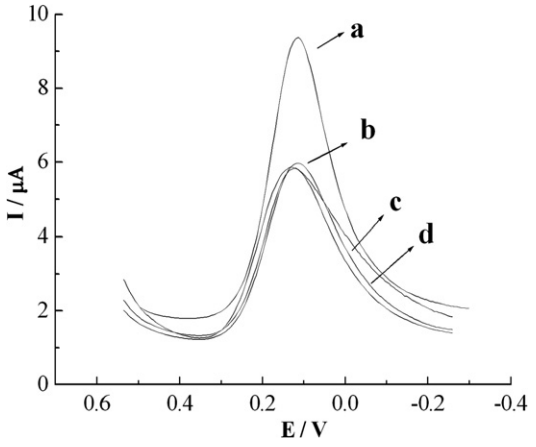

Fig. 5. DPVs of the immunosensor after incubated at 37 °C for 40 min in (a) PBS buffer, (b) PBS buffer containing 2.8 μg mL^{-1} antibody, (c) PBS buffer containing 100 μg mL^{-1} ractopamine and 2.8 μg mL^{-1} antibody, and (d) PBS buffer containing 100 μg mL^{-1} salbutamol and 2.8 μg mL^{-1} antibody.

3.5. Specificity, stability and reproducibility of the immunosensor

To investigate the ability of the immunosensor against interferences arising from the other β-agonists that are expected to exist in real samples, we chose ractopamine, dobutamine, and salbutamol as interferants to evaluate the specificity of the immunosensor. When the incubation solution contained 100 μg mL^{-1} ractopamine, 100 μg mL^{-1} dobutamine, 100 μg mL^{-1} salbutamol, no remarkable difference of peak currents was obtained when compared with the presence of only antibody (Fig. 5). Thus, the immunosensor had a good selectivity to clenbuterol and constituted an appropriate tool for the detection of clenbuterol even existing of a large extent of the interferences from other β-agonists, which have similar chemical structure and physiological character with clenbuterol.

Stability is one of the most important properties required for a biosensor or bioreactor. Stability of clenbuterol-MWCNTs films was tested by DPV under two storage conditions. The electrodes immobilised clenbuterol-MWCNT conjugates were either stored in buffers all the time or stored in air as their dry form, and DPVs were scanned periodically after placing the electrodes into pH 7.4 buffer solution containing redox marker. With both methods, the films showed quite stable responses. The DPV peak potentials and currents maintained essentially unchanged for at least 1 month. That is, the relative standard deviation was less than 10%. Thus, the good stability indicates the fact that the carbon nanotube films may have a promising potential in fabricating the new kind of immunosensors based on the indirect electrochemistry of immunomolecules.

The reproducibility of the immunosensor based on the redox marker in the presence of clenbuterol antibody was also investigated and only a small difference in DPV peak current was observed. The relative standard deviation was less than 10% when 5 repeats with different GC electrodes and different times were measured, indicating that the reproducibility of the immunosensor is acceptable in this experiment.

3.6. Analysis of spiked feed samples

Commercial feed samples were used to assess the accuracy of the immunosensor in real matrixes. The recovery study was performed by spiking blank swine feed samples with clenbuterol at four different levels (1, 2, 10, and 20 mg kg^{-1}), according to the extraction procedure described in the experimental section. For each concentration, five different samples were independently processed. On the basis of the calibration curves prepared in PBS, it was possible to calculate the recovery of the analyte, which ranged from 88% to 92% of clenbuterol with an average of 89.9%. The precision was determined by calculating the standard deviation for the replicate measurements. The statistical results are summarised in Table 1.

Samples were also analysed by ELISA and LC–MS methods. The results are also showed in Table 1. The amounts measured by three methods in Table 1 were analysed using the analysis of variance

Table 1
Recoveries of clenbuterol from the spiked feed samples determined by electrochemical immunosensor, ELISA, and LC–MS

Amount added (mg kg^{-1})	Immunosensor		ELISA		LC–MS		SEM[a]	*P*-value
	Amount measured (mg kg^{-1})	Recovery (%)	Amount measured (mg kg^{-1})	Recovery (%)	Amount measured (mg kg^{-1})	Recovery (%)		
20.0	17.78 ± 0.92	88.9	19.06 ± 1.02	95.3	18.68 ± 0.58	93.4	0.497	0.25
10.0	9.21 ± 0.54	92.1	10.17 ± 0.44	101.7	9.11 ± 0.52	91.1	0.306	0.09
2.0	1.75 ± 0.12	87.5	1.99 ± 0.14	99.5	1.95 ± 0.10	97.5	0.071	0.11
1.0	0.912 ± 0.08	91.2	1.07 ± 0.09	107.0	0.905 ± 0.03	90.5	0.042	0.06

[a] Standard error of the mean.

(ANOVA) procedure of SAS system (version 8.2, SAS Institute, Inc., Cary, NC, USA), and a *P*-value less than 0.05 was considered statistically significant. The statistical results show that the % recoveries for the same concentration level obtained by three different methods are no statistical differences (Table 1), indicating the validity of immunosensor for determination of clenbuterol in animal feeds. For LC–MS analysis of clenbuterol, feed samples require additional solid phase extraction prior to analysis in order to purify sample, making the analysis complex. The immunosensor and ELISA methods offer advantage of ease of sample preparation and high throughout. Moreover, the immunosensor method appears to be less subject to matix effects, or at least these effects are smaller than we have observed in ELISA method. Another advantage of our approach is that clenbuterol-MWCNTs fabrication is simple, and requires only room temperature solution-based fabrication steps.

4. Conclusions

This present work described a novel clenbuterol immunosensor prepared by immobilizing clenbuterol-MWCNTs films on GC electrodes. Using a competitive immunoreaction system, the percentage of reduction current response of the immunosensor was proportional to clenbuterol concentrations in the range of 0.8–1000 ng mL^{-1} with a limit detection of 0.32 ng mL^{-1}. The immunosensor could also be applied to the detection of clenbuterol in feed samples with good precision, high sensitivity, acceptable stability and reproducibility. A satisfactory agreement of analytical results of the proposed method with those of traditional ELISA and LC–MS shows good reliability of the method. Thus, the electrochemical immunoassay can be an alternative method to ELISA or other conventional assays for clenbuterol detection, having the advantages of sensitivity, speed, and simplicity.

Compared with our previous work (Shen & He, 2007), the main difference of the present work is its novel fabrication method of the films modified on the electrode. That is, clenbuterol was covalently linked to multi-wall carbon nanotubes (MWCNTs) through a two-step process using EDC/NHSS as crosslinkers. The clenbuterol-MWCNT conjugates were then cast on the electrode surface. The advantages of this method are that (1) carbon nanotubes (MWCNTs), with excellent conductivity and high surface area, was used as the immobilisation material in fabrication of the biosensor. The electron exchange between redox probe and electrodes are greatly enhanced, and more hapten molecules are immobilized compared with the simple cast methods. Thus, the detection sensitivity of clenbuterol can be significantly enhanced. (2) It replaced the conventional mode for the detection of hapten. Since clenbuterol was covalently immobolized on nanotube surface by this approach, there need not to synthesis coating antigen,which is a complicated synthetic procedure. Thus, the method becomes more simple and rapid than the previous method. In addition, the clenbuterol–MWCNT films showed excellent stability, and the antibodies in the films retained their activity of immunity. Thus, we suggest that the experimental strategy in this paper can easily be applied to investigate other small molecule haptens using appropriate antibodies.

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