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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