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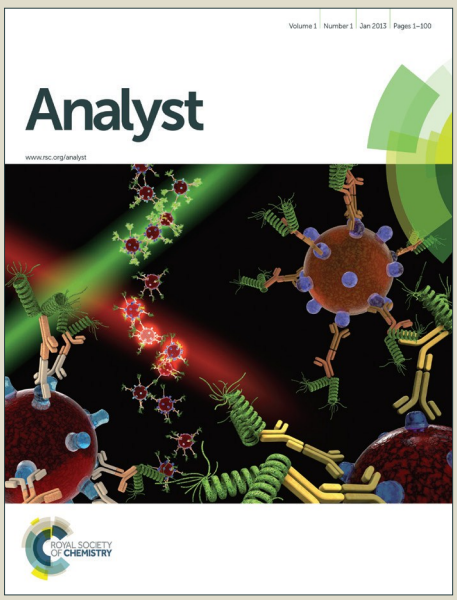

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Dual-Signal Amplified Electrochemiluminescent Immunoassay for Salbutamol Based on Quantum Dots and Gold Nanoparticles-Labeled Horseradish Peroxidase

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ABSTRACT:

This study described a novel electrochemical immunosensor to amplify electrochemiluminescence （ECL） signal for ultrasensitive detection of salbutamol (SAL) using quantum dots (QDs) and gold nanoparticles (AuNPs) conjugated horseradish peroxidase (HRP). The electrochemical detection was based on the HRP catalyzed o-phenylenediamine (OPD) to consume the self-produced H_2O_2, which has been extensively used as a co-reactant of QDs. The enzymatic reaction rate was proportional to the amount of HRP bound to the electrode. In the presence of SAL standard solution, the immobilized SAL coating antigen competed with SAL solution for the Ab-AuNPs-HRP complexes. With the increase of the SAL concentration, the amount of immobilized HRP decreases, which led to the increase of ECL intensity. Under optimized conditions, ECL intensity changed linearly with the logarithm of SAL concentration in the range of 0.05–500 ng mL^{-1} with the detection limit of 0.017 ng mL^{-1} (S/N=3). The ECL immunosensor possesses high sensitivity, satisfied reproducibility and selectivity, and may provide a feasible route for practical application.

Keywords: Electrochemiluminescence; Competitive immunosensor; CdSe quantum dots; Dual-signal amplification; Salbutamol; Gold nanoparticles.

1. Introduction

β-agonists such as ractopamine, salbutamol, terbutaline, and formoterol are usually used in the treatment of respiratory diseases and asthma.[1,2] In addition, β-agonists play an important role in promoting the animal body protein deposition and reducing animal fat levels,[3] many criminals abuse of β-agonists in animal feed in dosage of 4~9 times higher than clinical levels. Residues of β-agonists in illegally treated animal could be a serious threaten to human health.[1] Therefore, many countries have strictly banned the usage of β-agonists in stockbreeding.[4] Salbutamol (SAL) is one of the most often abused β-agonists following the clenbuterol, thus it is meaningful for the detection of SAL.

Many analytical methods of β-agonists have been established including high performance liquid chromatography (HPLC),[5] liquid chromatography-mass spectrometry (LC-MS),[6] gas chromatography-mass spectrometry (GC-MS),[7,8] enzyme-linked immuno sorbent assay (ELISA) and capillary electrophoresis (CE).[9] These chromatographic methods are with high sensitivity and precision, however HPLC, LC-MS and GC-MS need sophisticated and expensive instrumentations and time-consuming sample pretreatments, which were not suitable for on-site analysis and rapid screening. CE provides many advantages over them for the analysis of β-agonists,[9] but its reproducibility is poor. Although ELISA was highly sensitive and selective, the shortages of this method were time consuming, labor-intensive and complexity.[10] Therefore, it is crucial to develop a low-cost, sensitive and specific analytical method to meet the current situation need. Electrochemiluminescence (ECL), an attractive method for the analysis of β-agonists, possesses typical advantages of simplicity, low cost, high sensitivity and simple preparation of sample. Although the preparation of the SAL antibody and HRP-AuNPs-antibody composites

is relatively complex, the high specificity and high loading of enzyme are the key important. Owing to the high specificity and acceptable reproducibility of ECL analytical technique, electrochemical immunosensors have been quickly developed for sandwich-type,[11,12] competitive[13,14] and separation-free[15] immunoassay of proteins.

Recently, gold nanoparticles (AuNPs), a common noble metal nanoparticles, are always used in electrochemiluminescent immunoassay. They served as excellent carriers for preparing probes by loading numerous signal tags such as enzymes,[16] quantum dots,[17] oligonucleotides[18] and dyes.[19] Horseradish peroxidase (HRP) labeled AuNPs were used in this study and the high loading of enzyme on AuNPs for each immuno-recognition greatly magnifies the signal. O-phenylenediamine (OPD), an aromatic amine, reacts with the self-generated H_2O_2 under the catalysis of HRP.[20,21]

As finding new luminophores with an intensive ECL emission for biosensor is an inexhaustible motive force, and the members of luminophores have been expanded from conventional molecular emitters to other virous nano-scale semiconductor materials in recent years.[22] Compared with conventional molecular luminophores, quantum dots (QDs) possess several unique merits like unique optical, electronic, and electrochemical characteristics.[23] QDs based ECL has been widely applied in biosensing and immunoassay.

In this study, we designed a new QDs-based ECL quenching method for sensitive detection of SAL by using thioglycolic acid (TGA)-capped CdSe and HRP-AuNPs-antibody composites. Salbutamol coating antigen and salbutamol competed to the HRP-AuNPs-antibody composites, which made the HRP immobilize on the surface of GCE. The ECL signal was dual-amplified by the employment of HRP and AuNPs. The HRP played a role in enzyme cycle amplification and the AuNPs provided a matrix to anchor a large number of HRP. In the absence of OPD,

the self-produced H_2O_2, a coreactant in the ECL reaction, was consumed inconsiderably. With the concentration of standard salbutamol decreasing, the amount of HRP immobilized on the electrode was increased, but the ECL intensity was hardly changed. Contrarily, in the presence of OPD, the coreactant H_2O_2 was consumed in varying degrees by the catalysis of HRP.[20,24] The amount of HRP immobilized on the electrode changed with the concentration of salbutamol standard solution, therefore the ECL intensity was respond to the concentration of salbutamol standard solution. This ECL immunosensor is sensitive and rapid for bioassays, thus it would be an alternative method for the analysis of food additive residues.

2. Experimental section

2.1. Chemicals and materials

Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$, 99%), Se powder (99.95%), thioglycollic acid (TGA, AR), sodium borohydride ($NaBH_4$, 96%), isopropyl alcohol (99.7%), tris (hydroxymethyl) aminomethane, potassium nitrate (KNO_3, AR), glutaraldehyde solution (GLD, 25%), chloroauric acid ($HAuCl_4 \cdot 4H_2O$, 47.8%), trisodium citrate, o-phenylenediamine (OPD, 99.5%) and horseradish peroxidase (HRP, BR) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China, www. Sinoreagent. com). Aluminum oxide polishing powder (Al_2O_3, 0.3 and 0.05 μm, 99%) was obtained from Tianjin Aidahengsheng Technology Co., Ltd. (Tianjin, China). Bovine serum albumin (BSA, 98%) and ovalbumin (OVA, 99%) were purchased from Sigma-Aldrich Co., Ltd. (USA, www. sigmaaldrich. com/united-states. html).

0.1 M pH 7.4 Phosphate buffer saline (PBS) containing NaCl, Na_2HPO_4 and KH_2PO_4 was used as an incubation buffer. ECL detection buffer was prepared by 0.1 M Tris-HCl buffer containing 0.1 M KNO_3 and 1.5 mM OPD. All reagents were used as received with the analytical grade or above. All aqueous solutions were prepared

with sub-boiling doubly distilled water.

2.2. Apparatus

The ECL emissions were recorded using a MPI-A multifunctional electrochemical analytical system (Xi'An Remax Electronic Science & Technology Co. Ltd., Xi An, China) in the ECL detection buffer, and the working potential was 0 to -1.3 V with the negative high voltage of photomuitplier tube (PMT) set at 650 V. The experiment applied a conventional three-electrode system composed of a modified glassy carbon working electrode (φ = 3 mm), a Ag/AgCl reference electrode (KCl-saturated), and a Pt wire counter electrode.

Electrochemical impedance spectroscopy (EIS) was carried out on a RST electrochemical working station (Suzhou Risetest Instrument Co., Ltd., Suzhou China; www.rst9999.com). High resolution transmission electron microscopy (HRTEM) images were obtained from a Tecnai G2 F20 S-TWIN 200KV transmission electron microscope（FEI Co., Hillsboro, OR, USA).

2.3. Preparation of the Salbutamol Antigen and Salbutamol Antibody

To prepare SAL coating antigen and salbutamol antibody, we firstly synthesized salbutamol derivative (SAL-HS) by succinic anhydride to decorate the active sites on salbutamol.[25] Briefly, 120 mg salbutamol was dispersed in ethanol, and 61 mg succinic anhydride was added gradually under stirring in room temperature for 5 hours, during which the white suspension generated gradually. The reaction process was monitored by silica gel thin-layer chromatography (TLC). The resulting suspension centrifuged for 15 min to separate the precipitate, the precipitate were washed with ethanol and dried at 50 ℃ for 5 hours. Then the obtain SAL-HS was dispersed in 100 mL water and mixed with 100 mL of 0.15 M EDC/NHS under continuous stirring overnight in room temperature. After that, the mixture was

coupled to carry proteins (BSA or OVA) by covalent interactions under slightly stirring for 24 hours. Next the solution was dialyzed at 4 °C for 4 days by using $(NH_4)_2CO_3$ solution with several changes. Finally, the salbutamol-protein conjugates were lyophilized and stored at -40 °C before use. The prepared salbutamol-OVA and salbutamol-BSA were respectively served as coating-antigen and immunogen to establish indirect competitive immunoassay.

The preparation of the polyclonal antibodies (pAb) was described in our previous literature.[25] Polyclonal antibodies were derived from the antisera of adult New Zealand rabbits immunized with immunogen, and stored at -60 °C before use. The above animal experiments have been approved by the institutional committees, and were performed in compliance with the relevant laws and institutional guidelines.

2.4. Preparation of CdSe QDs, AuNPs and Ab-AuNPs-HRP composites

Thioglycolic acid (TGA) modified CdSe QDs and AuNPs were synthesized according to the previously reported methods.[26,27] The prepared AuNPs acted as the nanocarriers to load more antibody and HRP, which could remain the activity of the protein and amplify the efficiency of the electrochemical quenching. To generate pAb-AuNPs-HRP composites, 100 μL HRP and 10 μL of 5 μg mL^{-1} pAb were simultaneously added to AuNPs solution, and the mixture was then shaken overnight at 4 °C. During this process, HRP and pAb were attached to AuNPs through electrostatic interactions and interaction between AuNPs and NH_2 groups of pAb and HRP. Afterward, the mixture centrifuged at 10000 rpm for 10 min, and the supernatant was discarded. The obtained compound was dispersed in doubly distilled water and stored at 4 °C for further use.

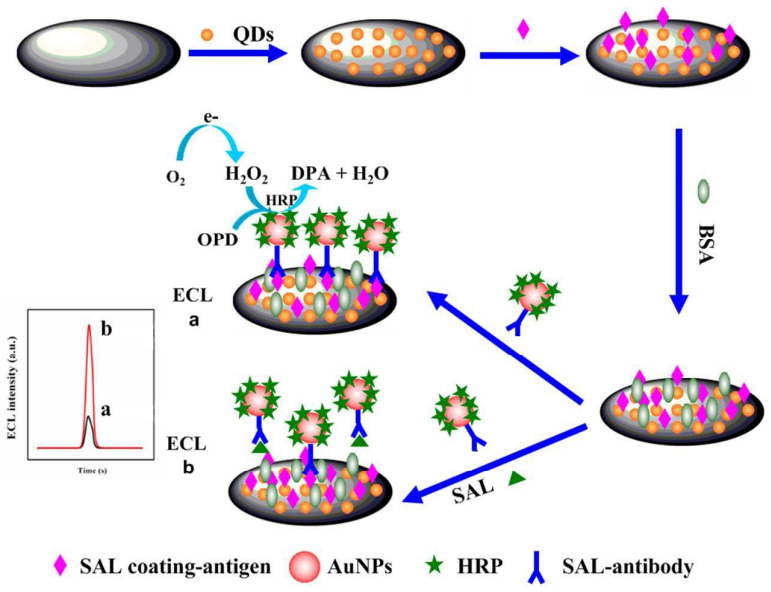
2.5. Construction the ECL Immunosensor

Scheme 1 shows the fabrication steps of the ECL biosensor. A glassy carbon

electrode (GCE) with 3-mm diameter was polished with 0.3 and 0.05 μm α-Al_2O_3 powder followed by rinsing thoroughly with distilled water. Then, 200 μL of QDs solution was concentrated by mixed with the same volume of isopropyl alcohol and centrifuged at 10000 rpm for 5 min. After discarding the supernatant solution, the precipitation was dissolved in distilled water and dropped on the pretreated GCE. Until the electrode dried, ten microliters of 0.025% chitosan was then dropped on the CdSe QDs film and dried in air again. 10 μL of 2% glutaraldehyde activated the chitosan film for 1 h at room temperature and then 10 μL of 10 μg mL^{-1} SAL coating antigen were coated on the electrode by an overnight incubation at 4 °C. Subsequently, 10 μL of 5% BSA was applied to block the nonspecific binding sites for 1 hour, and the ECL immunosensor was formed after washing with PBST dropwise.

2.6. ECL detection

To obtain the incubation solution, 5 μL of SAL standard solution with specified concentration was mixed with 5 μL of pAb-AuNPs-HRP bioconjugates. The as-prepared ECL immunosensor was immersed in the incubation solution and incubated at 37 °C for 1 h to establish the competitive immunoassay. During this process, the SAL standard solution competed with the immobilized SAL coating antigen to react with the limited binding sites of the SAL antibody to form the immunocomplex. After being rinsed with PBST, the fabricated GCE was scanned in ECL detection buffer and the ECL signals related to the SAL concentration was measured.



Scheme 1. Illustrative ECL detection mechanism for SAL based on GCE/CdSe/chitosan/SAL coating-antigen/ Ab-AuNPs-HRP.

3. Results and discussion

3.1. Characterization of CdSe and AuNPs

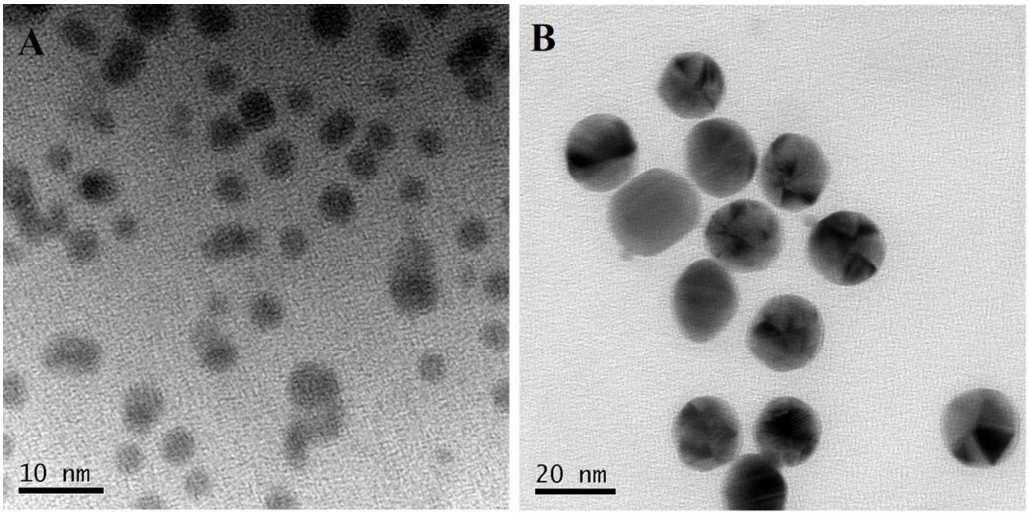


Figure 1. (A) HRTEM images of CdSe QDs and (B) AuNPs.

Fig. 1A showed the HRTEM characterization of the CdSe quantum dots, most of the particles are uniform and the average size of CdSe is about 4 nm. The HRTEM image of AuNPs was shown in Fig. 1B, from which we could see AuNPs dispersed

uniformly without aggregation. The average size of the as-prepared AuNPs was about 16 nm (shown in Fig. 1B), which would provide more active binding sites for the immobilization of antibody.

3.2. EIS Characterization of the Immunosensor

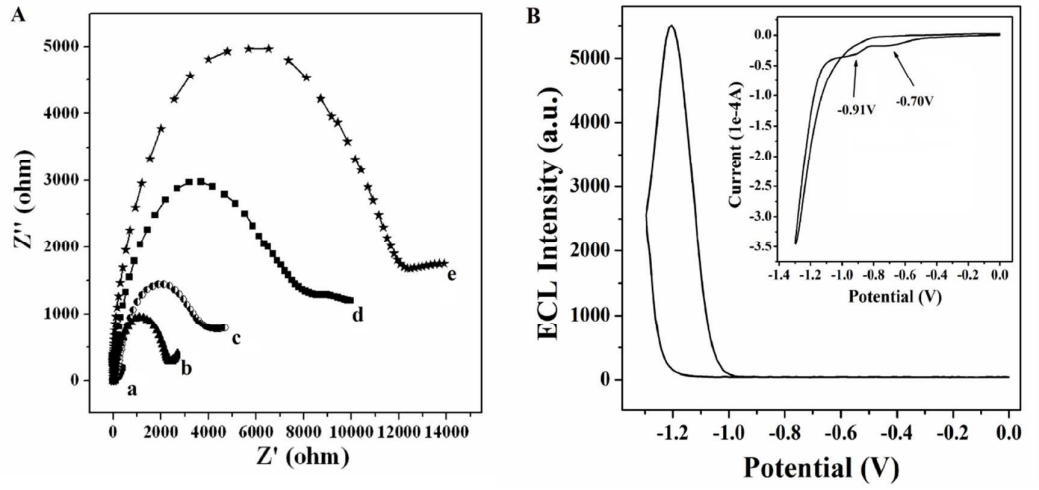


Figure 2. (A) EIS spectra of (a) bare GCE, (b) GCE/CdSe, (c) GCE/CdSe/chitosan, (d) GCE/CdSe/chitosan/Ag and (e) GCE/CdSe/chitosan/Ag/BSA in 0.1 M KCl solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$; (B) ECL curve and cyclic voltammogram (inset) of the detection SAL at 50 ng mL^{-1} in oxygen-saturated pH 9.0 Tris–HCl buffer containing 0.1 mM KNO_3 and 1.5 mM OPD at 100 mV s^{-1}.

Electrochemical impedance spectroscopy (EIS) at each modification process were recorded to monitor the changes in the surface features of ECL immunosensor. The EIS spectrum includes a semicircular part and a linear part, and the diameter of semicircle corresponds to the electron-transfer resistance, R_{et}. The stepwise modification process of the immunosensor was characterized in 0.1 M KCl containing 2.5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. As shown in Fig. 2A, the bare GCE displayed a relatively low R_{et} value (curve a). After the CdSe QDs was coated onto the electrode, a larger R_{et} (curve b) was shown because of the increasing of the impedance. Subsequently the modified electrode was coated with the chitosan and a larger R_{et} was showed (curve c), which suggested that the chitosan formed an additional barrier.

Additionally, coating antigen and BSA could also prevent the electron from being transfered to the surface of the electrode (curves d and e), these results confirmed that substances immobilized successfully in turn on the surface of GCE.

3.3. Electrochemical and ECL Behavior of the Immunosensor

In oxygen-saturated pH 9.0 Tris-HCl buffer, the cyclic voltammogram of GCE/CdSe/chitosan/SAL coating antigen/Ab-AuNPs-HRP showed two reduction peaks at the voltage of -0.70 and -0.91 V (inset in Fig. 2B), similar to that of CdTe QDs.[24] After bubbling with N_2 in the Tris-HCl buffer, the peak at -0.70 V disappeared, which could be ascribed to the reduction of saturated oxygen. The dissolved O_2 could be reduced to H_2O_2, a common coreactant for QD ECL emission, leading to an intensive ECL emission peaked at the voltage of -1.20 V. During cathodic potential scan, the electron-injected $QD^{-\bullet}$ formed at -0.91 V reacted with the H_2O_2 to produce excited QDs (CdSe*). When the excited QDs returned to the ground state, light was emitted and detected by photomuitplier tube. The ECL mechanisms could be expressed as follows:

$$CdSe + e^- \rightarrow CdSe^{-\bullet} \quad (1)$$

$$O_2 + 2e^- + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (2)$$

$$H_2O_2 + 2CdSe^{-\bullet} \rightarrow 2CdSe^* + 2OH^- \quad (3)$$

$$CdSe^* \rightarrow CdSe + h\nu \quad (4)$$

$$OPD + H_2O_2 \xrightarrow{HRP} DAP + H_2O \quad (5)$$

H_2O_2, as coreactant of the excited QDs, generated in situ from the oxygen-saturated Tris-HCl buffer and was consumed by OPD in the presence of the HRP (eq 5), thus resulting in a quenching effect. However, the decrease could be ignored when HRP or OPD is absent (not shown). Previous studies have shown that HRP and OPD can consume the H_2O_2.[20]

3.4. Optimization of Experimental Conditions

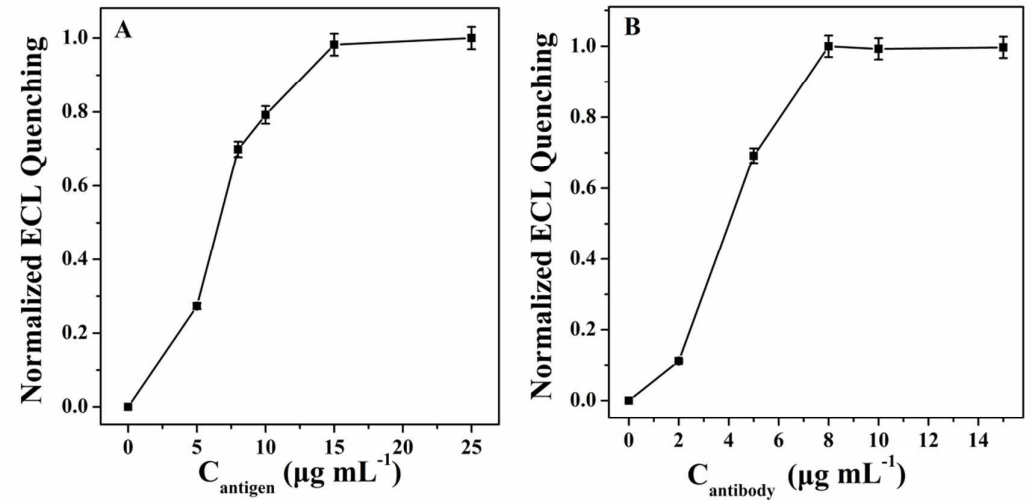


Figure 3. (A) Optimizations of the coating antigen and (B) the antibody concentrations.

To obtain high sensitivity, several experimental parameters were optimized. The amount of coating antigen highly influenced the quenching efficiency of ECL. The ECL intensity increased with the concentration of coating antigen from 0 to 15 μg mL^{-1}, and reached a platform after 15 μg mL^{-1}. Thus, 15 μg mL^{-1} SAL coating antigen was selected as the optimal amount dropped on the electrode surface.

The concentrations of antibody affect not only the quenching efficiency of ECL, but also the quantification of SAL. If the concentration of antibody is low, the introduced HRP is fewer, which affected the efficiency of ECL quenching. However, it could not be too high, because the SAL would conjugate with the excess antibody. As shown in Fig. 3B, when the concentration of antibody was beyond 8 μg mL^{-1}, the ECL tended a plateau revealing the optimal concentration of antibody. Thus, the 15 μg mL^{-1} coating-antigen and 8 μg mL^{-1} antibody were selected as the optimal concentration.

3.5. ECL Detection of SAL with the Immunosensor

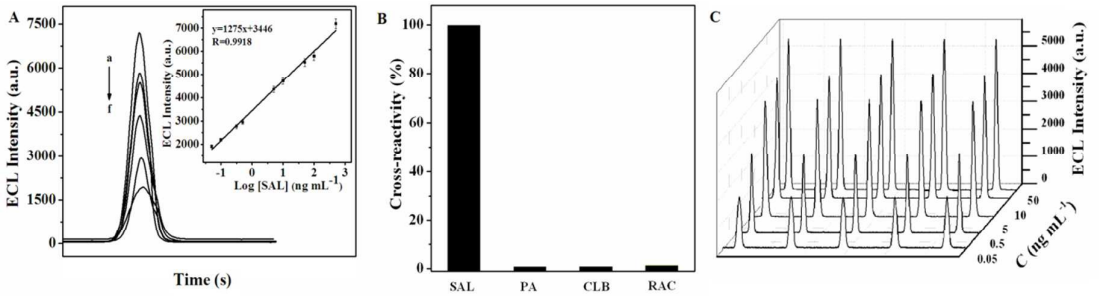


Figure 4. (A) ECL intensity changes with SAL concentrations (ng mL^{-1}) (a) 500, (b) 100, (c) 50, (d) 5, (e) 0.5 and (f) 0.05 ng mL^{-1}; Inset: linear calibration curve for SAL detection (n = 3); (B) ECL responses of the as developed immunosensor to SAL, PA, CLB and RAC; (C) Continuous cyclic scans of immunosensor formed at 0.05, 0.5, 5, 10 and 50 ng mL^{-1} salbutamol standard, respectively. The other conditions are the same as in Figure 2.

Under optimized conditions, the sensitivity immunosensor was evaluated toward SAL standards. As shown in Fig. 4A, the ECL intensity of the immunosensor increased with the increase of SAL concentration in range from 0.05 to 500 ng mL^{-1} with a low detection limit of 0.017 ng mL^{-1} at S/N of 3. The linear equation was y = 1275 log[C_{SAL}/(ng mL^{-1})] + 3446 (y was the ECL intensity) with the correlation coefficient R = 0.9918. Compared to the comparable methods for detecting SAL (Table 1), this immunosensor had a wider detecting range and a lower LOD, indicating that the proposed immunosensor could be applied to detect the concentration of SAL quantitatively.

Table 1. Properties of comparable methods for determination of salbutamol

Methods	LODs	detection ranges	References
HPLC	0.2 ng g^{-1}	1.2–600 ng g^{-1}	[5]
LC-MS	0.029 ng g^{-1}	0.05–5 ng g^{-1}	[6]
GC-MS	0.5 ng mL^{-1}	1.25–100 μg mL^{-1}	[8]
CE	1.03 μg mL^{-1}	2.0–100 μg mL^{-1}	[9]
ECL	0.017 ng mL^{-1}	0.05–500 ng mL^{-1}	This work

3.6. Reproducibility, Stability and Specificity of the Immunosensor

To investigate the specificity of the fabricated immunosensor, we selected

phenylethanolamine A (PA), clenbuterol (CLB) and ractopamine (RAC) as interfering substances into the incubation solution with 10 ng mL^{-1}. As shown in Fig. 4B, no significant signal variation was observed after PA, CLB and RAC reacted with the immunosensor, suggesting that this immunosensor displayed excellent specificity for the determination of SAL.

The stability and reproducibility of the immunosensor were evaluated by intra-assay and inter-assay precision. The inter-assay precision of five immunosensors fabricated independently was evaluated for detecting 50 ng mL^{-1} SAL with a relative standard deviation (RSD) of 3.55%. The stability of the immunosensor was investigated by employing five sensors with different concentration of SAL under consecutive potential scanning for five times. As shown in Fig. 4C, the ECL intensity did not show any obvious decline and the relative standard deviation (RSD) were in the range of 0.18%~1.02%. All the above investigations indicated the acceptable reproducibility, stability and specificity of the immunosensor.

3.7. Application of Real Samples Analysis

Table 2. Recovery tests of SAL in spiked real samples (n = 3)

sample	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%)	Recovery (%)
Pork sample 1	1	0.89±0.02	2.25	88.8
Pork sample 2	5	4.04±0.13	3.22	80.8
Pork sample 3	10	9.48±0.23	2.40	94.8
Liver sample 1	1	0.95±0.03	3.08	95.5
Liver sample 2	5	5.30±0.19	3.45	106.0

Liver sample 3	10	9.23±0.11	1.14	92.3

The proposed immunosensor was applied to real samples including pork and swine liver randomly collected from the market in Suzhou. The real samples preparation processes have been introduced in previous literature.[25] To be brief, 1 g of real samples were pounded in a mortar respectively and were extracted with 30 mL 0.01 mol L^{-1} HCl overnight. Then, the extracts were filtered by 0.45 μm membrane to get rid of some insolubles. The salbutamol residues measured by the immunosensor for all extracts was not detectable, therefore the real samples could be used as blank samples. Recovery experiments were performed by standard addition methods in the pretreated real samples. And the recoveries, as listed in Table 2, were in the range of 80.8%~106.0%, which revealed the developed immunoassay may provide an efficient method for detecting trace amounts of SAL in different real samples.

4. Conclusions

With the AuNPs labeled-HRP and enzymatic cycle for dual-signal amplification, a high sensitivity and low background of QDs-based ECL immunosensor for the determination of SAL has been developed by immobilizing the CdSe QDs on the electrode surface via chitosan. Compared with previous immunoassay, the presence of AuNPs increased the amount of antibody and HRP, which greatly enhanced the electrochemical quenching. The CdSe QDs could react with H_2O_2 to obtain a high ECL emission during the cathodic scan. In a relatively low concentration of SAL, the dual-signal amplification greatly increased the ECL relative intensity and enlarged the detecting range in presence of enzymatic substrate (OPD). The proposed ECL immunoassay exhibited satisfied selectivity, acceptable reproducibility and wide linear range. This method has been applied to detect SAL in real samples successfully.

It will be a promising approach for the detection of SAL and other small molecular compounds.

Acknowledgment

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CE	1.03 μg mL^{-1}	2.0–100 μg mL^{-1}	[9]
ECL	0.017 ng mL^{-1}	0.05–500 ng mL^{-1}	This work

Table 2. Recovery tests of SAL in spiked real samples (n = 3)

Sample	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%)	Recovery (%)
Pork sample 1	1	0.89±0.02	2.25	88.8
Pork sample 2	5	4.04±0.13	3.22	80.8
Pork sample 3	10	9.48±0.23	2.40	94.8
Liver sample 1	1	0.95±0.03	3.08	95.5
Liver sample 2	5	5.30±0.19	3.45	106.0
Liver sample 3	10	9.23±0.11	1.14	92.3

Figure captions

Scheme 1. Illustrative ECL detection mechanism for SAL based on GCE/CdSe/chitosan/SAL coating-antigen/ Ab-AuNPs-HRP.

Figure 1. (A) HRTEM images of CdSe QDs and (B) AuNPs.

Figure 2. (A) EIS spectra of (a) bare GCE, (b) GCE/CdSe, (c) GCE/CdSe/chitosan, (d) GCE/ CdSe/chitosan/Ag and (e) GCE/CdSe/chitosan/Ag/BSA in 0.1 M KCl solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$; (B) ECL curve and cyclic voltammogram (inset) of the detection SAL at 50 ng mL^{-1} in oxygen-saturated pH 9.0 Tris–HCl buffer containing 0.1 mM KNO_3 and 1.5 mM OPD at 100 mV s^{-1}.

Figure 3. (A) Optimizations of the coating antigen and (B) the antibody concentrations.

Figure 4. (A) ECL intensity changes with SAL concentrations (ng mL^{-1}) (a) 500, (b) 100, (c) 50, (d) 5, (e) 0.5 and (f) 0.05 ng mL^{-1}; Inset: linear calibration curve for SAL detection (n = 3); (B) ECL responses of the as developed immunosensor to SAL, PA, CLB and RAC; (C) Continuous cyclic scans of immunosensor formed at 0.05, 0.5, 5, 10 and 50 ng mL^{-1} salbutamol standard, respectively. The other conditions are the same as in Figure 2.