Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Quantum dots based electrochemiluminescent immunosensor for the detection of pg level phenylethanolamine A using gold nanoparticles as substrate and electron transfer accelerator

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Abstract:

This study reports the development of an electrochemiluminescent (ECL) immunosensor for ultrasensitive detection of phenylethanolamine A (PA) based on CdSe quantum dots (QDs) and gold nanoparticles (GNPs). The GNPs/Ovalbumin-PA/anti-PA-QDs immunosensor was fabricated layer by layer using GNPs as substrate and electron transport accelerator. It greatly enhanced sensitivity for detecting PA due to the excellent electron transportation ability and the large surface area of GNPs carrier allowing several binding events of Ovalbumin-PA on each nanosphere. Transmission electron microscope image (TEM), photoluminescence spectra, ultraviolet-visible absorption spectra and dynamic light scattering (Dls) were used to characterize the QDs and GNPs. The sensor was characterized with electrochemical impedance spectra (EIS), and a strong ECL emission of the modified electrode could be observed during the cathodic process of $S_2O_8^{2-}$ and QDs in air-saturated PBS buffer containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl (pH 7.4). With a competitive immunoassay format, the ECL signal depended linearly on the logarithm of the phenylethanolamine A concentration within a range from 0.02 ng mL^{-1} to 50 ng mL^{-1}, and a detection limit was 0.0047 ng mL^{-1}, much lower than those reported in literature. This ECL immunosensor is rapid, simple and sensitive with acceptable precision, and it will extend the application of QDs ECL in immunoassays of β-agonists and open new avenues for the detection of food additive residue in the future.

Key words*:* Immunosensor; Electrochemiluminescence; CdSe quantum dots; Gold nanoparticles; phenylethanolamine A

Introduction

Phenylethanolamine A [PA, 2-(4-(nitrophenyl) butan-2-ylamino)-1-(4-methoxyphenyl) ethanol, $C_{19}H_{24}N_2O_4$], a new emerging β-adrenergic agonists, is a synthetic substance and the isomer of formoterol (which also belongs to the family of β-agonists), and structurally similar to ractopamine. As we all known, β-adrenergic agonists are often illicitly abused as growth-promoting agents in animal feeds to improve the production of lean meat and as doping drugs to enhance the performance of human athletes [1]. However, the illegal use of β-adrenergic agonists in livestock production has led to toxic effects after human consumption of meat products. Due to the long-term or high dose use, the illicit deleterious physiological side-effects and a large amount of a single dosage may initiate an acute toxic response such as cardiac palpitation, tachycardia, nervousness, muscle tremors and confusion [2]. Poisoning incidents caused by high concentrations of β-agonists residues in edible tissue have occurred in many countries such as Spain, France, Italy, and China [3, 4]. Therefore, β-agonists are used in a restricted manner within a low limit of dosage in many countries, even banned in some countries. Unfortunately, although the governments have made great efforts to strengthen the supervision and monitor the use of β-agonists, new alternatives emerge and bring more harm to livestock production and human health. Recently a new alternative of phenylethanolamine A has been illegally used in livestock in China. Since 2010, PA has been prohibited from being used in feeds and animal drinking water in bulletin No. 1519 issued by the Ministry of Agriculture of China [5]. Based on the above facts, it is very important to develop a rapid, simple, accurate and ultrasensitive method for the detection of PA in order to ensure food safety and public health.

Although many analytical methods have been reported to monitor other

β-agonists (like ractopamine and clenbuterol) in animal feeds, urine, and tissues using instrument methods, including high-performance liquid chromatography (HPLC), electrochemical detection, LC-fluorescence, liquid chromatography-mass spectrometry (LC-MS), chemiluminescence or gas chromatography-mass spectrometry (GC-MS) [6-11] and immunochemical approaches [12, 13]. To the best of our knowledge, few analytical methods have been reported to monitor PA except three studies with LC-MS/MS [14], ELISA [15], immunoaffinity chromatography column [16] and electrochemical methods [17]. In 2010, the Ministry of Agriculture of China issued a standard for the detection of PA in feed using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [5]. Chromatographic tandem mass spectrometry methods are frequently applied in confirmation with preciseness. Nevertheless, the expensive instrumentation and inherently complicated sample pretreatment might limit the extensive application of these methods. Thus, it is not suitable for them to screen large quantities of real samples.

In recent years, the electrochemiluminescent (ECL) assay has drawn more and more attentions due to its good stability against photobleaching, simplified optical setup, low cost, and low background noise [18]. As for ECL assay, the luminescent signal is triggered by the electrochemical reaction, which can be utilized to transduce the recognition substance occurring on the electrode [19]. Semiconductor nanocrystal have been widely used as optical [20] or electric [21] labels in bioanalysis due to their significant advantages of size-control photoluminescence (PL), good stability against photobleaching and subsequent chemical solubilization to release electrochemical detectable ions for sensitive quantitative readout [22, 23]. Among the miscellaneous functional nanomaterials, quantum dots (QDs) are of considerable interest in bioassays and bioimagings owing to the unique size-dependent optical and electronic

properties. The ECL processes from multifarious QDs, e.g. TOPO-capped CdSe or CdTe QDs, and a CdSe/ZnSe core-shell structure, have been extensively investigated in organic solutions. On this account, efficient and stable electrochemiluminescence (ECL) from TGA-CdSe QDs in aqueous solution can be applied as a cathodic potential to the QDs films [24]. Furthermore, in order to improve the ECL intensity, many compounds, such as $S_2O_8^{2-}$ [25], H_2O_2 [26, 27], SO_3^{2-} [28] and amines [29], have been employed as the cathodic or anodic ECL coreactants of QDs. Owing to its favorable spatial and time controllability, ECL assay is deemed to be well suited for the fabrication of immunosensor. Therefore, due to its excellent sensitivity, it is assumed that ECL immunoassay can detect PA residue at very low level and with simple operation.

In this paper, we have successfully designed an ECL immunosensor for the detection of pg level PA using QDs as ECL probe and gold nanoparticles as the basement. Our contribution lies in the use of the GNPs to efficiently accelerate the electron transport in this work. Ultrasensitive PA detection was achieved with a detection limit of 0.0047 ng mL^{-1} and a RSD of 2.12% (n=7). To the best of our knowledge, it is the first time that the ECL immunoassay using CdSe QDs has been used for the detection of PA. This ECL immunosensor is rapid, simple and sensitive with acceptable precision, and it will extend the application of QDs ECL in immunoassays of β-agonists and open new avenues for the detection of food additive residue in the future.

Experimental

Reagents and materials

Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$, 99%), thioglycollic acid (TGA, 99%) isopropyl alcohol (99.7%), Se powder (99.95%), sodium borohydride ($NaBH_4$, 96%),

monopotassium phosphate (KH_2PO_4, 99%), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl, 99.5%), potassium peroxodisulfate ($K_2S_2O_8$, 99.5%), chloroauric acid ($HAuCl_4 \cdot 4H_2O$, 47.8%), trisodium citrate (99%) and potassium carbonate (K_2CO_3, 99%) were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Ethyl-3-(dimethyl aminopropyl) carbodiimide (EDC, 98%), N-hydroxysuccinimide (NHS, 97%) and bovine serum albumin (BSA, 98%) were purchased from Fluka. Ovalbumin (OVA, 99.0%) was bought from Sigma-Aldrich Co. Ltd (USA). Phenylethanolamine A was bought from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Ractopamine, salbutamol, clenbuterol were purchased from National Institutes for Food and Drug Control. (Beijing, China).

Phosphate buffered saline (PBS) containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl was prepared by using Na_2HPO_4, KH_2PO_4 and NaCl, which was used throughout the whole work. 1.0 M HCl and 1.0 M NaOH were used to adjust solution pH to 7.4. All reagents and chemicals were commercially available and of analytical reagent grade or above. All aqueous solutions were prepared with sub-boiling distilled deionized water.

Instruments and measurements

The electrochemical measurement for ECL was carried out on a RST electrochemical working station (Suzhou Risetest Instrument Co., Ltd., China) and chemiluminescent analytical system (Nanjing Henyue Optech Co., Ltd., China) at room temperature with a conventional three-electrode system with a 3-mm-diameter glassy carbon electrode (GCE) as working, a platinum wire as counter, and a Ag/AgCl (saturated KCl solution) as reference electrodes. Cathodic potential within a range from -0.2V to -1.7 V was applied to the GCE by cyclic voltammetric (CV) technique, while the ECL emission was recorded. During measurements, the photomultiplier

tube (detection range from 300 to 800 nm) was biased at -800 V.

Electrochemical impedance spectroscopy (EIS) was carried out with a RST electrochemical working station (Suzhou Risetest Instrument Co., Ltd., China), using the same three-electrode system as that in the ECL detection. UV-vis absorption spectrum was recorded with Agilent 8453 UV-vis photospectrometer (Agilent Co., America). Photoluminescence (PL) spectrum was excited by SDL-405-LM-100T (Shanghai Dream Lasers Technology Co., Ltd., Japan) and performed on Avaspec-2048 fluorospectrometer (Avantes Co., Netherlands). The High Resolution Transmission Electron Microscope (HRTEM) images were obtained from Tecnai G2 F20 S-TWIN 200KV (FEI Co., U.S.A). The dynamic light scattering (Dls) was taken on a Malvern Zetasizer Nano ZS 90 (U.K.).

Preparation of CdSe QDs

The water-soluble CdSe QDs were prepared using TGA as stabilizing agent. 2mL of 50mM NaHSe solution was synthesized by reaction between $NaBH_4$ and Se powder in oxygen-free water for 20 min at 50 ℃. 38 μL TGA was added carefully to 50 mL 0.004 M $CdCl_2$ solution with 1M NaOH to adjust the pH of the solution to 10. Then the solution was bubbled with highly pure N_2 for 30 min. The as-prepared NaHSe solution was injected into this solution to obtain a clear yellow solution of CdSe QDs precursors. The final molar ratio of Cd^{2+}: TGA: Se^{2-} was 1: 2.5: 0.5. The size of QDs was obtained by precisely controlling the refluxing time of 8 h at 100 ℃. The clear orange solution of QDs was then centrifuged to remove a morsel of precipitate and dialyzed against double distilled water for further 10 h. The finally obtained QDs solution was rather stable for more than 3 months when kept in refrigerator at 4 ℃. The as-synthesized QDs were characterized by HRTEM, ultraviolet-visible absorption spectra, dynamic light scattering (Dls) and

photoluminescence (PL) spectra, respectively.

Preparation of the phenylethanolamine A antigen and phenylethanolamine A antibody

The synthesis of the phenylethanolamine A -protein conjugate was performed as described in the literature [30] with a small modification. Briefly, 9.6 mg PA-NH_2 were dissolved in 300 μL of water, and then 9.3 mg $NaNO_2$ dissolved in 300 μL water were added dropwise. The solution was adjusted to pH 1.5 by adding 0.2 mol L^{-1} HCl and the reaction was carried out in the dark for 7 h at 4 ℃. After primary testing with N, N-dimethylaniline, the reaction was stopped by adding a carbamide (11 mg) solution in water (200 μL). The above diazotized PA-NH_2 was added to a solution containing 99 mg of protein (BSA or OVA) dissolved in 1.5 mL of PBS. The pH value of the mixed solution was kept at 7.5 by adding a small amount of 1 mol L^{-1} NaOH. The reaction was continued overnight at 4 ℃ under slightly stirring. The solution was intensively dialyzed in PBS for 4 d with several changes of the dialyzing buffer solution. Finally, the PA-OVA were used as coating-antigen and the PA-BSA were used as immune-antigen.

According to the reported literature [31], four adult New Zealand rabbits were immunized with as-synthesized PA-BSA at 4-week intervals for four subsequent injections. The polyclonal antibody of PA was obtained from the antisera of animals, and stored at -60 ℃ until use. It should be mentioned that all experiments in live animals were performed in compliance with the relevant laws and institutional guidelines of China, and the institutional committees have approved the experiments.

Fabrication of the ECL immunosensor

According to the previous report [32], the conjugation procedures of CdSe QDs with antibody (Ab) were described as follows: 166 μL the as-prepared QDs solution

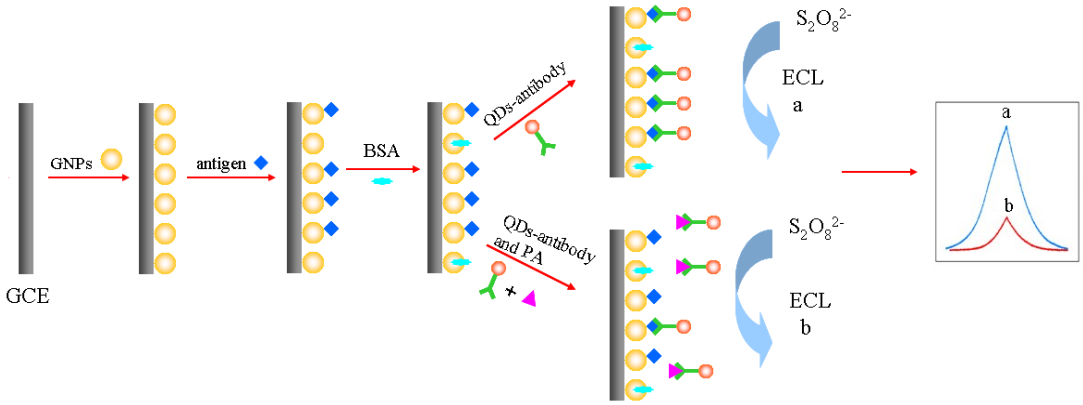
was mixed with 166 μL of isopropyl alcohol and centrifuged at 12000 rpm for 10 min. After removing the supernatant solution, the QDs was washed and dissolved in 10 μL sub-boiling distilled water. Then 20 μL of fresh prepared solution containing of 100 μg mL^{-1} EDC and NHS was added to the QDs solution. After stirring for 10 h under dark, the resulting QDs was collected by centrifugation at 12000 rpm for 20 min. The precipitates were diluted to 20 μL with 0.01 M PBS, followed by adding 20 μL of 10 μg mL^{-1} Ab solution to the mixture and the samples were incubated overnight under shaking. Then 10 μL of 2% BSA solution was used to block the nonspecific binding sites.

The method of preparing GNPs was described in the previous report [33]. 2 mL of 1% sodium citrate solution was added into a boiling solution of 50 mL of 0.01% $HAuCl_4$. The mean size of the GNPs was about 16 nm, which could provide more active binding sites for the immobilization of antigen [34]. The protein could combine with GNPs via electrostatic interaction, so that the PA-OVA conjugates were used as coating antigen to be modified on the electrode. Then 10 μL 10 μg mL^{-1} PA-OVA antigen in 0.01M PBS was mixed with 190 μL as-prepared gold nanoparticles, followed by incubating at 4 ℃ for 2 h and the prepared GNPs-antigen conjugates were collected by centrifugation at 12000 rpm for 45 min. After removing the supernatant liquid, the GNPs-antigen conjugates were decanted and diluted to 10 μL with double distilled water.

A 3 mm diameter glassy carbon electrode was polished with 1.0-, 0.3- and 0.05-μm α-Al_2O_3 powder and electrochemically pretreated in 0.5 M H_2SO_4. 10 μL as prepared GNPs-antigen conjugates were dropped on the surface of the electrode at the room temperature. After being dried in 4 ℃, nonspecific binding sites of the conjugates were blocked by 20 μL of 2w% BSA solution for 1 h at room temperature.

Assay procedure

The fabricated immunosensor was immersed into 20 μL of the solution containing of 15 μL QDs-antibody solutions and 5 μL different concentration PA solutions to establish the competitive immunoassay. During incubating at 37 ℃ for 1 h, the PA in the incubation solution competed with the immobilized coating antigen to react with the limited binding sites of the QDs-antibody to form the immune-complex. Afterwards, 0.01 M PBST (pH 7.4) was used to wash the immune-complex of PA-Ab-QDs and remainder Ab-QDs on the surface of immobilized electrode. Finally, the fabricated electrode was scanned under the cyclic voltammetry from -0.2 to -1.7 V in 0.1 M PBS (pH 7.4) containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl. The ECL intensity of the GCE/GNPs/OVA-PA/anti-PA-QDs immunosensor decreased upon the addition of the PA concentration in the detection solution, which resulted from competitive immunoreaction between coating antigen and target analyte in the solution for limited binding sites on the QDs-antibody conjugate. As shown in scheme 1, The ECL response of immunsensor in the absence of PA (Scheme 1, curve a) was higher than that in the presence of PA (Scheme 1, curve b). The changes of the ECL responses were correlative with the concentration of PA, which could be used as the quantitative criterion of PA.



Scheme 1. Fabrication process and the detection mechanisms of the ECL immunosensor.

Results and Discussion

Characterization of TGA-capped CdSe QDs

The as-prepared QDs solution was diluted twice by using 0.1 M PBS (pH 7.4) buffer as the supporting electrolyte. The processed QDs solution showed a photoluminescence peak (Fig. 1A) at 690 nm, and UV-vis absorption peak occurs at 508nm, indicating the consequence of quantum confinement. The calculated sizes and concentration of the QDs based on the absorption peak in the UV-vis spectrum and the previous empirical equations [35] are 4.6 nm and 0.1205 μg mL^{-1}, respectively. Fig. 1B shows the HRTEM image of the QDs, which indicate that the actual size of QDs was about 4.8 nm. Fig. 1C shows the distribution of the as-prepared QDs by dynamic light scattering, revealing that the sizes of TGA capped CdSe QDs range from 10 nm to 40 nm and the average grain diameter was 21.3 nm. Hydrodynamic diameters included total sizes of CdSe QDs, cysteine and the hydrophilic group around the QDs, so the average diameter was larger than the size obtained from TEM. After kept at 4 ℃ for 3 months, our QDs solution could be still stable, and the polydispersity index (PDI) reached 0.281, suggesting a good dispersion.

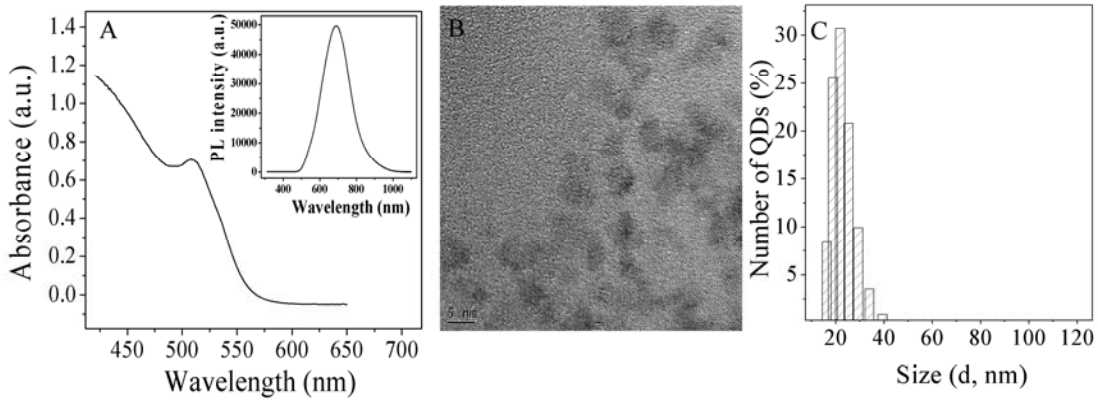


Fig.1. UV-vis , and PL (inset) spectra of the TGA capped CdSe QDs (A). HRTEM image of TGA capped CdSe QDs (B) and the sizes distribution of TGA capped CdSe QDs (C).

Optimization of immunoassay conditions

The decrease of ECL intensity depends upon the amount of coating antigen immobilized on the electrode surface and the concentration of phenylethanolamine A antibody in the incubation solution. Then, the effect of the concentration of OVA-PA from 0 μg mL^{-1} to 20 μg mL^{-1} was examined on the ECL immunosensor performance (Fig. 2A). As shown in Fig. 2A, when the concentration was beyond 10 μg mL^{-1}, the *Δ*ECL (the difference between the ECL responses of immunosensor for phenylethanolamine A detection at 0 and 1000 ng mL^{-1}) response slowly increased, thus 10 μg mL^{-1} of coating antigen was used for immunosensor fabrication. On the other hand, the amount of the anti-PA-QDs conjugates in the incubation solution could also affect the sensitivity of the immunosensor. Thus, the concentration of phenylethanolamine A antibody added in the QDs solution to form the conjugates was tested from 0 μg mL^{-1} to 15 μg mL^{-1} (Fig. 2B). With the increase of the concentration of Ab, the *Δ*ECL intensity increased and reached a plateau at 10 μg mL^{-1}, thus 10 μg mL^{-1} was chosen as the appropriate concentration of phenylethanolamine A antibody to connect the QDs.

The *Δ*ECL intensity reaches a maximum value at pH of 7 with the increasing pH of detection solution from 6.0 to 10.0 (shown in Fig. 2C), indicating that the high pH was unfavorable for bioanalysis applications, on the other hand, the pH 7.4 of the detection solution would be most suitable for the activity of the protein. Then, pH 7.4 PBS buffer in presence of 0.1 mol L^{-1} $K_2S_2O_8$ and 0.1 mol L^{-1} KCl was used as the detection solution.

To the best of our knowledge, both $K_2S_2O_8$ and H_2O_2 are the coreactants for QD cathodic ECL emission, but the effciencies of the two coreactants are quite different. Compared to the previous reports for detecting the same substance using the different system of H_2O_2[23] and $K_2S_2O_8$[24], the detection limit and sensibility were different.

Furthermore, for QDs-based cathodic ECL emission, $S_2O_8^{2-}$ exhibits a higher efficiency than does dissolved oxygen and H_2O_2 [36]. Therefore, the efficiencies of $K_2S_2O_8$ and H_2O_2 were compared in our work. As shown in Fig. 2D, the different ECL emission phenomena have been observed in two different solutions, which was in agreement with the above literatures. The *Δ*ECL signal in $K_2S_2O_8$ solution (curve a) was almost 1.2 times of that in nitrogen-saturated detection solution containing of 0.1 mol L^{-1} H_2O_2 (curve b). Thus, the stronger ECL intensity of the QDs immunosensor with the coreactant $K_2S_2O_8$ was chosen in this experiment.

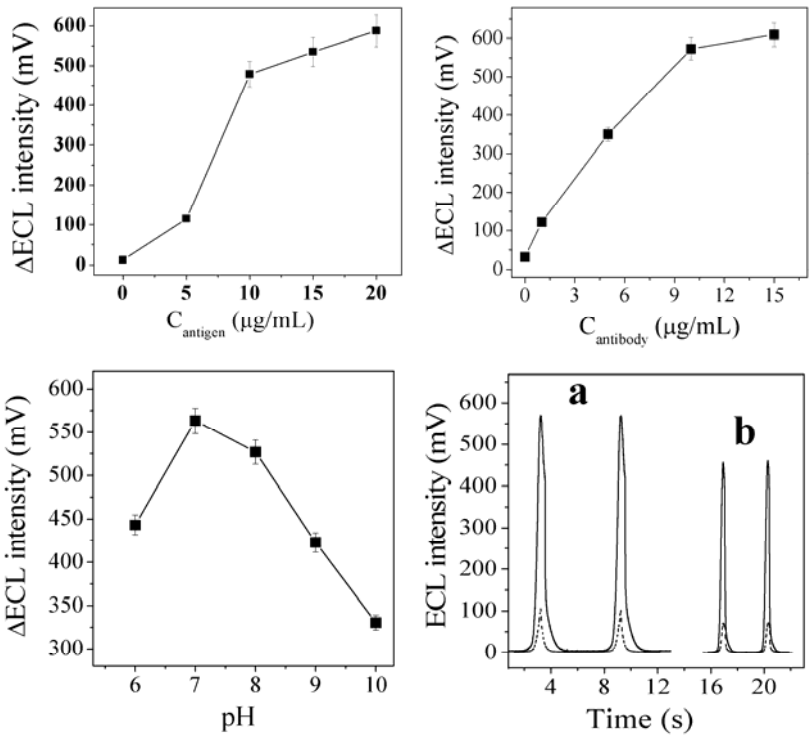


Fig. 2. Optimization of concentrations of PA coating antigen (A) and PA antibody (B) for immunoreaction conditions was operated in pH 7.4 PBS buffer containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl. The *Δ*ECL emission was stands for the difference between the ECL responses of immunosensor for phenylethanolamine A detection at 0 and 1000 ng mL^{-1}. *Δ*ECL-pH plot (C) of the immunosensor was formed in different pH of PBS buffer solution containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl. ECL-time curves (D) of immunosensor was formed at 0 ng mL^{-1} (solid curve) and 1000 ng mL^{-1} PA (dashed curve) stardard solution in air-saturated pH 7.4 PBS containing of 0.1 M $K_2S_2O_8$ (a), and nitrogen-saturated pH 7.4 PBS containing of 0.1 M H_2O_2 (b). Scan rate: 100 mV s^{-1}.

EIS characterization of the immunosensor

The EIS of the resulting electrodes can give further information on the modification process [37, 38]. Fig. 3 shows the EIS of the electrode during the stepwise modification processes in 0.01 M PBS (pH 7.4) containing 2.5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and 0.1 M KCl. In EIS, the diameter of semicircle equals the electrontransfer resistance, R_{et}. The bare GCE showed a relatively small R_{et} (curve a), which was characteristic of a mass diffusion limiting process. After the electrode coated by gold nanoparticles, the electrode (curve b) showed an obviously small R_{et}, implying that the gold nanoparticles had an excellent electrical conductivity and could accelerate the electron transfer [39]. After the GCE was coated by the GNPs-antigen conjugates, the semiconductor film increased the impedance, thus showed larger Ret (curve c). Similarly, BSA could also resist the electron-transfer kinetics of the redox probe at the electrode interface, resulting in the increasing impedance of the electrode (curves d), which testified the immobilization of these substances.

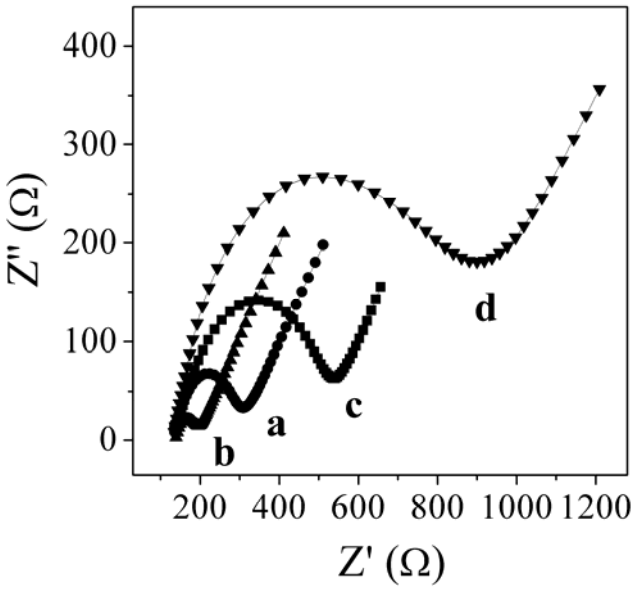


Fig.3. EIS of (a) bare GCE electrode, (b) GCE/GNPs, (c) GCE/GNPs/OVA-PA, (d) GCE/GNPs/OVA-PA/BSA in 0.01 M PBS (pH 7.4) containing of 2.5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and 0.1 M KCl.

Electrochemical and ECL behaviors of the CdSe QDs on the electrode

In PBS buffer containing of 0.1M KCl and 0.1M $K_2S_2O_8$ (pH 7.4), the GCE/GNPs/OVA-PA/anti-PA-QDs modified electrode showed a strong cathodic ECL emission. As shown in inset (curve b) of Fig. 4A, two reduction peaks at about -0.87 V and -1.33 V emerged on the cyclic voltammogram of the immunosensor which showed a strong cathodic ECL emission. While the reduction peak at -0.87 V disappeared and a slight peak at -0.68 V emerged when the electrode immersed in the PBS without $K_2S_2O_8$ (shown in curve a in inset of Fig.4A), thus this peak (-0.87 V) could be attributed to the reduction of $S_2O_8^{2-}$. Followed by immersing the electrode in the PBS without $K_2S_2O_8$ and bubbled with N_2, the peak of -0.68 V disappeared. Thus this peak could be attributed to the reduction of O_2, it could be considered that the O_2 level in the solution has an effect on the ECL, indicating a weak ECL signal (shown in curve a of Fig. 4A). Another slight peak at -1.33 V was attributed to the electrochemical reduction of CdSe QDs on the electrode, which produced negatively charged radicals of $CdSe^{\bullet -}$. At the same time, the large reduction peak at -0.87 V resulted from the reduction of $S_2O_8^{2-}$ to anion sulfate radical $SO_4^{\bullet -}$. The strong oxidant $SO_4^{\bullet -}$ further reacted with $CdSe^{\bullet -}$ to generate the excited state $CdSe^*$ by injecting a hole into the highest occupied molecular orbital of $CdSe^{\bullet -}$. When $CdSe^*$ fell from the excited state to the ground state, light was emitted and detected (shown in curve b of Fig. 4A). Based on the above discussions and the previous report [40], the possible ECL mechanisms could be expressed as follows:

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{\bullet -}$$

$$O_2 + H_2O + 2e^- \rightarrow OOH^- + OH^-$$

$$CdSe + e^- \rightarrow CdSe^{\bullet -}$$

$$SO_4^{\bullet -} + CdSe^{\bullet -} \rightarrow CdSe^* + SO_4^{2-}$$

$2CdSe^{\bullet -} + OOH^- + H_2O \rightarrow 3OH^- + 2CdSe^*$

$CdSe^* \rightarrow CdSe + h\nu$

The better conductivity of GNPs could facilitate the electron transfer and increase the conductive surface area, which improved the electrochemical reaction efficiency of QDs and $K_2S_2O_8$ in the ECL process. Furthermore, the GNPs also played the part of the substrate of coating antigen, as the OVA-PA and OVA-PA/BSA couldn't be modified on the surface of the electrode if GNPs were absent. In order to study the effect of GNPs on ECL, chitosan and glutaraldehyde were chosen to connect PA-OVA with the electrode, and an experiment was made to explain the effect of GNPs on ECL. The experiment results were shown in Fig. 4B. Comparing the ECL responses of the GCE/chitosan/glutaraldehyde/OVA-PA/anti-PA-QDs (curve b in Fig. 4B) with GCE/GNPs/OVA-PA/anti-PA-QDs immunosensors (curve a in Fig. 4B), the effects of ECL intensity vary greatly, experimental results demosntrate that the GNPs can efficiently accelerate the electron transport on ECL process.

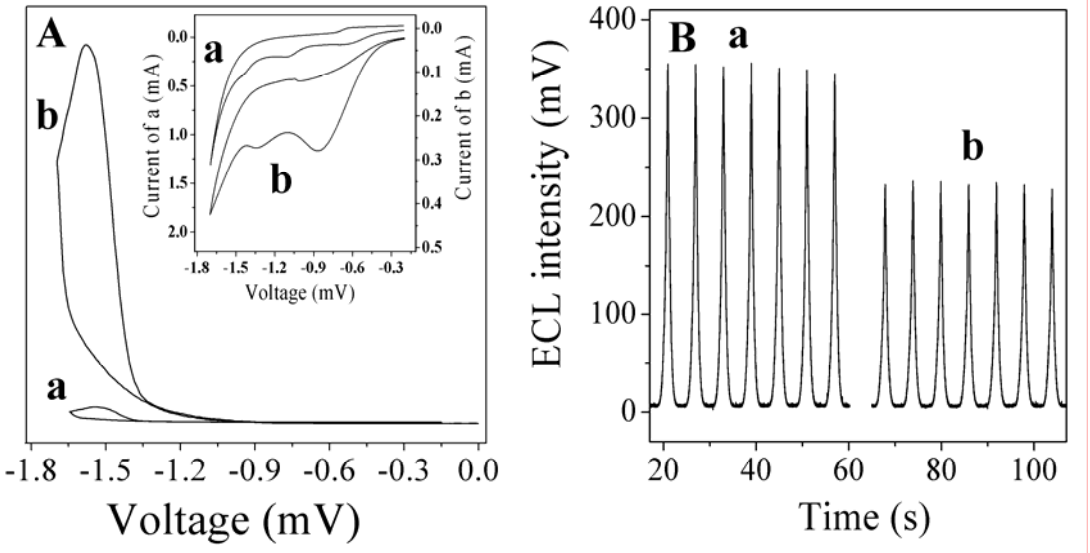


Fig.4. A: I_{ECL}-V curves of GCE/GNPs/OVA-PA/anti-PA-QDs immunosensor for PA detection at 0.5 ng mL^{-1} in PBS (pH 7.4) buffer containing of 0.1 M KCl in presence (b) and absence (a) of 0.1 M $K_2S_2O_8$. Inset: Cyclic voltammograms curves of the GCE/GNPs/OVA-PA/anti-PA-QDs immunosensor in 0.1 M PBS (pH 7.4) containing of 0.1 M KCl in presence (b) and absence (a) of

0.1 M $K_2S_2O_8$ at 100 mV s^{-1}. B: ECL intensities of GCE/GNPs/OVA-PA/anti-PA-QDs (a) and GCE/chitosan/glutaraldehyde /OVA-PA/anti-PA-QDs (b) immunosensors formed at 0.5 ng mL^{-1} phenylethanolamine A in PBS (pH 7.4) buffer containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl.

ECL detection of PA

On the basis of the optimal condition, the competitive immunoassay was applied to the PA detection. The ECL curves of the immunosensor for detecting different concentrations of PA were shown in Fig. 5B. The scatter diagram and standard calibration curve for PA detection is illustrated in Fig. 5A. The linear equation was Y (I_0-I) = 244.9256 +134.5256 log [PA] (ng mL^{-1}) with the correlation coefficient of 0.9943, and the limit of detection (LOD) was 4.7 pg mL^{-1} with a signal-to-noise ratio of 3. In the linear equation, I_0 stands for the ECL intensity for 0 ng mL^{-1} PA, while I stands for the ECL intensity of different concentrations of PA. Compared to the previous literature for detecting PA [14-17], this study has a wider linear range and a lower LOD, suggesting that this immunosensor displays more simple and convenient prospect of application.

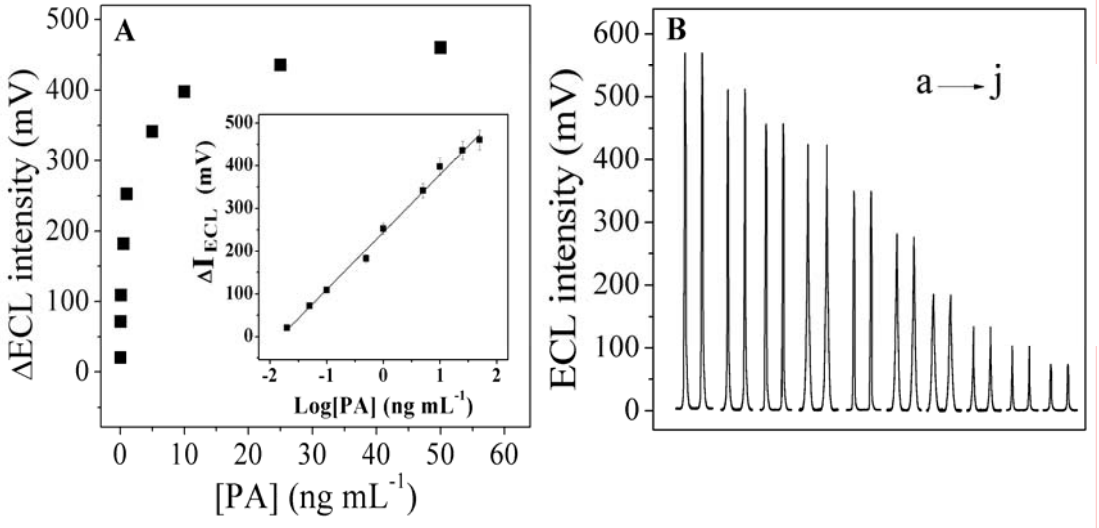


Fig. 5. A: Scatter diagram in different PA concentrations. Inset: Linear calibration curve of the immunosensor in different PA concentrations. B: ECL curves of GCE/GNPs/PA-OVA/Ab-QDs for phenylethanolamine A detection at (a) 0 ng mL^{-1}, (b) 0.02 ng mL^{-1}, (c) 0.05 ng mL^{-1}, (d) 0.1 ng mL^{-1}, (e) 0.5 ng mL^{-1}, (f) 1 ng mL^{-1}, (g) 5 ng mL^{-1}, (h) 10 ng mL^{-1}, (i) 25 ng mL^{-1}, (j) 50 ng mL^{-1}

in pH 7.4 PBS buffer containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl.

Specificity, reproducibility and stability of the immunosensor

The specificity of the immunosensor played an important role in analyzing real samples in situ without separation. Some coexisting β-agonists including salbutamol, clenbuterol and ractopamine were chosen as the interfering species since they might exist in some real samples. 1 ng mL^{-1}, 10 ng mL^{-1} and 50 ng mL^{-1} of interfering species were detected, respectively. Owing to their very analogous chemical structures, ractopamine showed the cross-reactivity about 8.6% with the PA antibody (Fig. 6). Salbutamol and clenbuterol did not cause the interference in the test, which suggests that this immunosensor had an acceptable specificity for the determination of PA.

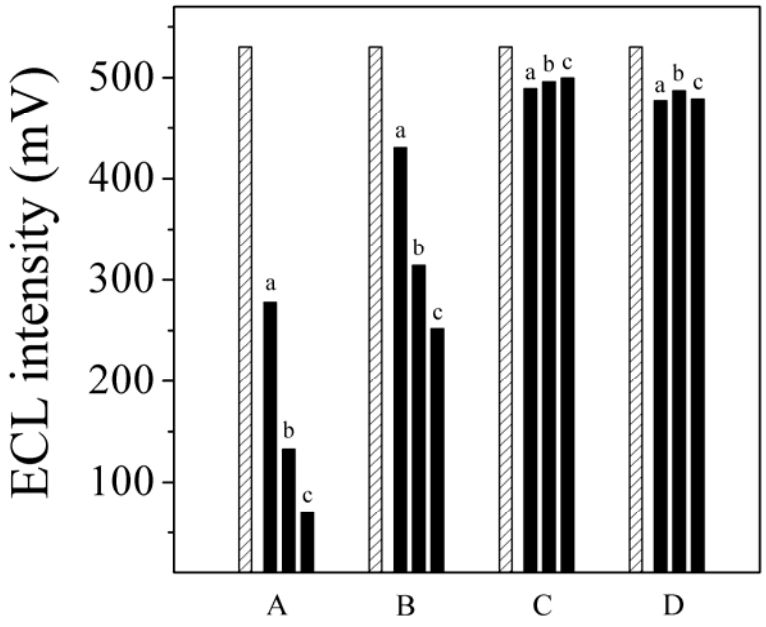


Fig. 6. ECL responses of immunosensor at 1 ng mL^{-1} (a), 10 ng mL^{-1} (b), 50 ng mL^{-1} (c) of phenylethanolamine A (A), ractopamine (B), salbutamol (C) and clenbuterol (D), respectively. Conditions: PBS (pH 7.4) buffer containing of 0.1 M $K_2S_2O_8$ and 0.1 M KCl. Diagonal bars represent the ECL intensity of the immunosensor at 0 ng mL^{-1} of phenylethanolamine A, ractopamine, salbutamol, and clenbuterol, respectively.

Reproducibility and stability of the immunosensor for PA was investigated with

intra- and inter-assay precision. The intra-assay precision was evaluated by continuous cyclic scan of the immunosensor formed at 0.5 ng mL^{-1} PA stardard for 7 cycles (shown in Fig. 7). The inter-assay precision was estimated by testing one PA level with five immunosensors independently. The relative standard deviations of intra- and inter-assay for the detection of 0.5 ng mL^{-1} PA were 2.12% and 5.08%, respectively. The coincident signals indicated the excellent reliability, stability and reproducibility of the immunosensor, which could be applied to the ECL detection.

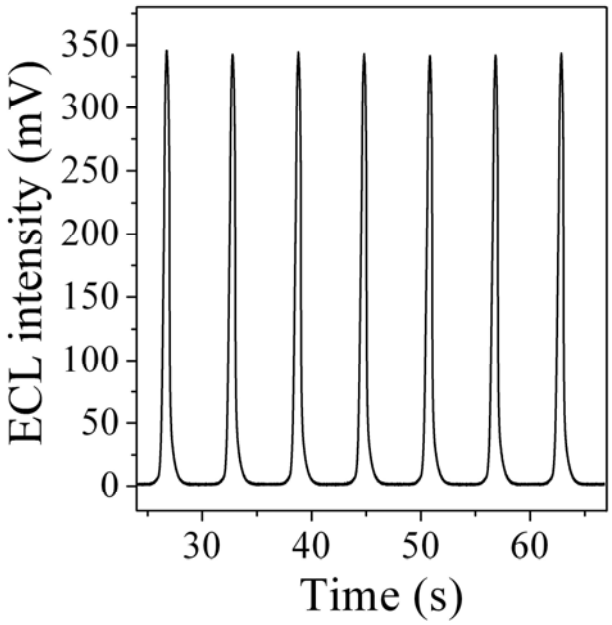


Fig. 7. Continuous cyclic scans of GCE/GNPs/OVA-PA/anti-PA-QDs immunosensor formed at 0.5 ng mL^{-1} PA stardard solution in pH 7.4 PBS buffer containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl. Scan rate: 100 mV s^{-1}.

Real sample analysis

To further investigate the feasibility of the immunosensor for the daily applications, the detection of phenylethanolamine A residue in real samples including pork and liver from swine (collected from the market in Suzhou) and the blank urine samples of healthy people (collected from student volunteers in the laboratory) was carried out. 10 of 1 g of homogenized pork (or liver) obtained from different place were taken for analysis. Extraction was performed with 300 mL 0.01 mol L^{-1} HCl and

kept at room temperature overnight. After the removal of precipitates, the supernatant from pork or liver samples was directly analyzed. The blank urine sample was diluted 1000 times by sub-boiling distilled water in this work. No phenylethanolamine A residue concentration was measured by this immunsensors for all collected samples. Therefore they could be used for blank samples. The samples of pork and liver and blank human urine (n=3) were spiked with PA standard solution at the concentrations of 0.05 ng mL^{-1}, 0.1 ng mL^{-1} and 0.5 ng mL^{-1}. As shown in Table 1, the recoveries were within the range of 82-124%. To evaluate the reliability of the prepared immunosensor, HPLC was established for the detection of PA in the real samples. It turned out that there were no significant differences for the detection of PA between this method and HPLC, which was satisfactory for quantitative assays performed in real samples.

Table 1. Recovery tests of PA spiked in the real samples by the proposed method (n=3)

Samples	Added (ng mL^{-1})	Found (ng mL^{-1})	Recovery (%)
Pork No.1	0.05	0.047 ± 0.005	94
Pork No.2	0.10	0.09 ± 0.02	90
Pork No.3	0.50	0.52 ± 0.04	104
Pork liver No.1	0.05	0.053 ± 0.003	106
Pork liver No.2	0.10	0.08 ± 0.03	82
Pork liver No.3	0.50	0.55 ± 0.07	110
Human urine No.1	0.05	0.062 ± 0.006	124
Human urine No.2	0.10	0.08 ± 0.01	84
Human urine No.3	0.50	0.47 ± 0.03	94

Conclusions

With the Au substrates for signal amplification, a QDs based competitive electrochemiluminesence immunosensor for the detection of pg level PA has been designed. The ECL emission could be generated in the presence of the $S_2O_8^{2-}$ as the coreactant during the cathodic scanning. An ideal substrate of GCE/GNPs has been obtained for antigen immobilization with good stability and bioactivity. Moreover, GNPs were creatively used to accelerate the electron transport between the surface of electrode and QDs, which could efficiently enhance the sensitivity of the immunosensor. The resulting immunosensor possesses the advantages of low-cost, good storage stability and fabrication reproducibility, and exhibits a wider linear detection range with lower detection limit in comparison to the reported methods for PA detection. This proposed method has been used in the detection of real samples with satisfactory results, which shows that it can be a promising method for other small molecular compounds and extend the application of QDs in ECL biosensing.

Acknowledgments

This study was supported by the Science Fund from the National Natural Science Foundation of China (No. 21075087, No. 21175097), the Project of Scientific and Technologic Infrastructure of Suzhou (SZS201207), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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Graphical Abstract