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Quantitative electrochemiluminescence detection of proteins: Avidin-based sensor and tris(2,2′-bipyridine) ruthenium(II) label

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Abstract

Quantitative electrochemiluminescence (ECL) detection of a model protein, bovine serum albumin (BSA) was achieved via biotin–avidin interaction using an avidin-based sensor and a well-developed ECL system of tris(2,2′-bipyridine) ruthenium(II) derivative as label and tri-*n*-propylamine (TPA) as coreactant. To detect the protein, avidin was linked to the glassy carbon electrode through passive adsorptions and covalent interaction with carboxylate-terminated carbon nanotubes that was used as binder to immobilize avidin onto the electrode. Then, biotinylated BSA tagged with tris(2,2′-bipyridine) ruthenium(II) label was attached to the prepared avidin surface. After binding of BSA labeled with tris(2,2′-bipyridine) ruthenium(II) derivative to the surface-immobilized avidin through biotin, ECL response was generated when the self-assembled modified electrode was immersed in a TPA-containing electrolyte solution. Such double protein labeling protocol with a biotin label for biorecognition and ruthenium label for ECL detection facilitated the detection of protein compared to the classical double antibody sandwich format. The ECL intensity was linearly proportional to the feed concentration of BSA over two orders of magnitude in the range of 15 nM to 7.5 μM. The detection limit was estimated to be 1.5 nM. Further application to the lysozyme analysis was carried out to validate the present approach for an effective and favorable protocol for the quantitative detection of proteins. The dynamic range of lysozyme was from 0.001 g L^{-1} to 0.1 g L^{-1} and the detection limit was 0.1 mg L^{-1}. Electrochemical impedance and cyclic voltammetric measurements along with some necessary control experiments were conducted to characterize the successful formation of self-assembled modified electrodes and to grant the whole detection process.

Keywords: Quantitative electrochemiluminescence; Avidin-based sensor; Tris(2,2′-bipyridine) ruthenium(II) label; Multi-wall carbon nanotube; Protein

1. Introduction

Electrochemiluminescence has been developed as a highly sensitive process in which reactants are oxidized at the surface of an electrode, the resulting strong oxidant and reducing reagent generate an excited state of molecule which decay to the background state to emit light. This technology has many distinct advantages over other detection system (Blackburn et al., 1991; Miao and Bard, 2003) including no light resource and radioisotopes compared to fluorescence and isotope-involved method; an extremely low detection limit (200 fmol L^{-1}) (Blackburn et al., 1991); a very wide dynamic detection range over three to six orders of magnitude (Blackburn et al., 1991). ECL generation method and its applications are regularly reviewed (Richter, 2004). Moreover, the most common tris(2,2′-bipyridyl) ruthenium [$Ru(bpy)_3^{2+}$] ECL as a sensitive detection method was universally applied to detect amine-containing substances (Fähnrich et al., 2001; Fang et al., 2005; Cao et al., 2002) due to its ability to produce ECL response at room temperature in aqueous solutions, especially tertiary amines can result in stronger ECL signal than primary and secondary amines (Knight and Greenway, 1996). We all know amine-containing substances are common constituents in drugs and medicines. If $Ru(bpy)_3^{2+}$ ECL can only detect these small molecules, the development of the ECL technology will be hampered and its superiority cannot be well demonstrated. Bio-macromolecule such as DNA and proteins are lack of tertiary amines and they cannot be detected directly by $Ru(bpy)_3^{2+}$ ECL. So, seeking an available ECL detection protocol to be applied in biological system is of crucial importance.

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A feasible means to achieve the goal is to derivatize $Ru(bpy)_3^{2+}$ to carry some functional groups such as carboxyl, hydroxy and amido group. The functional groups can react with the residues in protein, then the labeled protein can be detected directly while the coreactant of $Ru(bpy)_3^{2+}$ is provided. The $Ru(bpy)_3^{2+}$ derivatives are extremely stable and small enough to label bio-macromolecules, especially, multiple labels can be coupled to proteins or DNA without affecting their biological activities (Blackburn et al., 1991). Bard et al., used commercial $Ru(bpy)_3^{2+}$ phosphoramidite and succinimidyl ester (NHS) to determine immobilized DNA and C-reactive protein on Au(1 1 1) electrode (Miao and Bard, 2003) and further amplified the ECL response by using magnetic beads and microspheres (Miao and Bard, 2004a,b). But the commercial $Ru(bpy)_3^{2+}$ labels, the magnetic beads and microspheres are very expensive and difficult to obtain. Furthermore, the whole sandwich-type procedure for the detection of C-reactive protein and the amplification process of ECL response were very complex, which may cause much more non-specific adsorption problems. Developing a simple and effective approach to be universally applied in the quantitative detection of protein is necessary.

A highly specific biological affinity reaction, biotin/avidin interaction has received diverse and widespread applications in organized assemblies of biomolecules (Anicet et al., 1998), medical diagnostics (Rivera et al., 2003; Guggiero and Sheffield, 1998), immunoassays (Weizmann et al., 2003) and nanotechnology (Caswell et al., 2003). This is essentially due to the strong protein-ligand interaction with a dissociation constant of 10^{-15} M and to the presence of four binding sites for biotin on avidin (Slim and Sleiman, 2004). Their affinity is much greater than that of antigen–antibody ($K_d \approx 10^{-7}$ M) (Zhu et al., 2000).

In our present work, we used biotin/avidin biorecognition pair to quantitatively detect proteins in which an avidin-based sensor and biotinylated protein labeled with $Ru(bpy)_3^{2+}$ derivative were used. The protein was detected by its specifically attaching to the avidin-modified electrode through biotin/avidin interaction after its double labeling with biotin label and ruthenium ECL label. ECL response was generated when the modified electrode was immersed in a TPA-containing electrolyte solution. Comparing with the classical antigen/antibody/antigen sandwich format protocol adopt by Miao et al (Miao and Bard, 2003, 2004a,b), our novel strategy exceeded in facility, convenience and less non-specific adsorption in virtue of its comprising only one step of specific biorecognition. While the obvious disadvantages of the capture/target/detection sandwich methods are the multiple steps including several washings, incubations (Li et al., 2003) and specific interactions which may lead to much non-specific adsorptions. Scheme 1 outlines the schematic assemblies of biomolecules for quantitative detection of model BSA. The present protocol attempted a universally suitable access to quantitative detection of proteins using $Ru(bpy)_3^{2+}$ derivatives. A successful application for lysozyme detection confirmed the feasibility and validity of this approach. Using $Ru(bpy)_3^{2+}$ derivative functionalized with reactive groups opened up new fields of $Ru(bpy)_3^{2+}$ ECL application in bioanalysis and biotechnology.

2. Experimental

2.1. Chemicals and materials

Bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidylester-bis(hexafluorophosphate), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Lysozyme (LYZ), avidin (from egg white), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), biotinamidohexanoic acid *N*-hydroxysuccinimide ester (NHS-LC-biotin), tri-*n*-propylamine (TPA), imidazole, 2-(dibutylamino)ethanol (DBAE), and anhydrous *N*,*N*-dimethylformide (DMF) were purchased from Sigma (St. Louis, MO, USA); Bovine serum albumin (BSA) was bought from Pierce Ins. (P.O. Box 117, Rockford). These reagents were carefully reserved and used without further purification. Other chemicals for synthesis of $Ru(bpy)_3^{2+}$ derivatives were at least reagent grade. Multiwall CNTs were obtained from Shenzhen Nanotech Port Co. Ltd. (China). The preparation of carboxylate MWNTs was described elsewhere (Liu et al., 1998) with slight modification. Double-distilled water was used for all aqueous solution preparation.

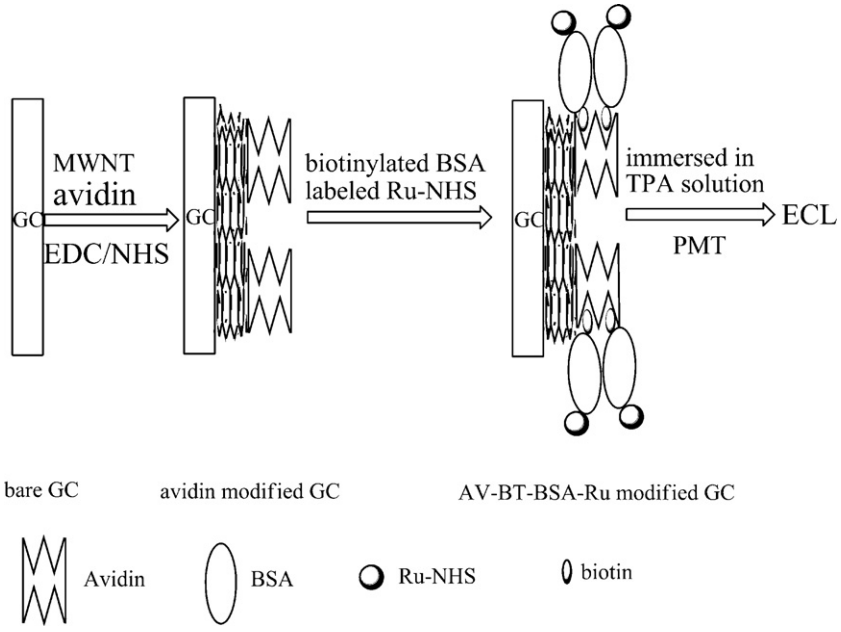
2.2. Apparatus and equipments

Cyclic voltammetric (CV) measurements were performed on a CH Instruments 832 Voltammetric Analyzer (CH Instrument Inc. USA). The ECL response was recorded on a Model BPCL Ultra-weak Luminescent Analyzer (Institute of Biophysics, Chinese Academy of Sciences) in which the photomultiplier tube was biased at −950 V. A conventional three-electrode setup was applied to carry out all CV experiments. Working electrodes were glassy carbon (GC) electrodes modified with different self-assemblies of biomolecules. Ag/AgCl (saturated KCl) reference electrode and platinum counter electrode were used for all the electrochemical and ECL measurements.

The electrochemical impedance experiments of the self-assemblies of biomolecules were performed in the presence of 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) as the redox probe. The spectra were measured by Autolab with PGSTAT 30 (Eco Chemie B.V., Utrecht, Netherlands) and with the aid of a frequency response analysis (FRA) system software under an oscillation potential of 5 mV over a frequency range of 10 kHz to 0.1 Hz.

2.3. Synthesis of bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium-N-succinimidylester bis(hexafluorophosphate) ($Ru(bpy)_3^{2+}$-NHS)

The synthesis of $Ru(bpy)_3^{2+}$-NHS was a modification and improvement of the method cited (Zhou et al., 2005; Sprintschnik et al., 1977; Terpetschinig et al., 1995; Peek et al., 1991). Briefly, 0.019 g NHS and 0.038 g DCC were dissolved in 1 mL cooled DMF. Then



Scheme 1. The schematic diagram of ECL quantification of model BSA.

0.025 g of ruthenium bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) [$Ru(bpy)_3^{2+}$-$(COOH)_2$] dissolved in 0.2 mL cooled DMF was added drop-wise into the mixture. The reaction was taken for 58 h in an ice bath until plenty of white precipitate appeared. The white precipitate was filtered and the filtrate was treated with 40 mL isopropanol. Then, the mixture was kept at 0 °C for 3 h. After filtered and washed with ether, the resulting orange solid was the desired NHS ester.

Characterization of the synthesized $Ru(bpy)_3^{2+}$-NHS by IR and CE-ECL was described in detail in the Supplementary material section.

2.4. Preparation of avidin-based sensor

The GC working electrode was carefully polished with 1- and 0.3-μm Al_2O_3 powder on fine polishing paper and then ultrasonically cleaned in double-distilled water and ethanol. Repeat above mentioned procedures until the peak-to-peak separation of ≤80 mV was obtained in 1 mM ferrocyanide CV measurement, which guaranteed a good reproducibility of GC electrode.

Two milligrams of EDC and 1.2 mg of NHS were freshly dissolved in 40 μL 0.1 M imidazole buffer (pH 7.0). Then, 120 μL carboxylate-terminated MWNT solution of 0.5 g L^{-1} and 40 μL avidin solution of 5 g L^{-1} were mixed together and stirred gently for 3 h at room temperature. Then, a drop of the resulting homogeneous solution (6 μL) was cast on the prepared GC electrode and evaporating the solvent at room temperature in the air, and the avidin-modified electrode was obtained. Before attaching to the biotinylated protein labeled with $Ru(bpy)_3^{2+}$ derivative, the avidin-modified electrode was washed with double-distilled water and 10 mM phosphate buffer (pH 7.5) thoroughly and dried under a high-purity N_2 stream to reduce non-specific adsorptions.

2.5. Labeling and biotinylation of proteins

We chose BSA as a model protein because BSA represents the most abundant of all plasma proteins, which contains 59 lysine residues, and 30–35 of these primary amines capable of being labeled with NHS activated esters (Zhou et al., 2003). Then, we applied this protocol to quantitatively detect another protein, LYZ. Both BSA and LYZ followed the same biotinylation and labeling procedures except that ECL labeling reagent for model BSA was commercial standard product from Sigma and that for LYZ were synthesized by our laboratory.

BSA was labeled with $Ru(bpy)_3^{2+}$-NHS and biotinylated with NHS-LC-biotin by the following procedures with some necessary modifications (Dong et al., 2004; Yin et al., 2005).

1.2 mg commercial $Ru(bpy)_3^{2+}$-NHS was dissolved in 30 μL anhydrous DMF and aliquoted to 6 silanized tube loaded with BSA. Every tube had a total volume of 300 μL, which allowed the concentration of BSA to be 0, 1.5 nM, 15 nM, 150 nM, 1.5 μM and 7.5 μM by adding appropriate amount of 0.1 M sodium phosphate buffer (pH 7.6). All the different concentrations of BSA were labeled with a large molar excess of $Ru(bpy)_3^{2+}$-NHS and the labeling reactions were almost completely processed. After 3 h incubation in the dark, 1.2 mg NHS-LC-biotin was dissolved in 30 μL anhydrous DMF and aliquoted to above six $Ru(bpy)_3^{2+}$-NHS labeled BSA tubes, and incubated with slightly stirring for 2 h more. The resulting mixtures were transferred to dialysis tubes with cutoff molecule weight of 8000. As a result, unreacted biotin and ruthenium esters were removed by 3-day dialysis against 10 mM phosphate buffer (pH 7.6) at room temperature. The remaining solutions were carefully kept at −4 °C for attaching to avidin-modified electrode. In the control experiment, BSA was only labeled with $Ru(bpy)_3^{2+}$-NHS, but not biotinylated. We named it non-biotinylated BSA-Ru.

In the application part, LYZ was labeled with self-synthesized $Ru(bpy)_3^{2+}$-NHS and biotinylated following similar procedures of that of BSA. Self-synthesized $Ru(bpy)_3^{2+}$-NHS was not very pure and there was some unreacted $Ru(bpy)_3^{2+}$-$(COOH)_2$ remained in the product (see Supplementary material). The impurity did not affect the subsequent application of NHS esters because $Ru(bpy)_3^{2+}$-$(COOH)_2$ did not participate in the reactions and can be removed from dialysis. In the biotinylation and labeling of LYZ, various concentrations of LYZ were 0.1, 0.03, 0.01, 0.003, and 0.001 g L^{-1} to be labeled with totally 2 mg self-synthesized $Ru(bpy)_3^{2+}$-NHS and 1 mg NHS-LC-biotin which were aliquoted to 5. The subsequent process was the same of that of BSA.

The biotin and ruthenium labeling ratio to protein can be determined by HABA/avidin reagent (Sigma) and UV–vis spectroscopy, respectively (Dong et al., 2004). The dye/protein ratio for direct labeling is generally in the range of 2–4 while biotin/antibody ratio is often in the range of 8–14 (Li et al., 2003). Our labeling procedure was similar to Dong et al. (2004). They had reported the ratio of biotin/ruthenium/protein was 3.2:3.4:1 (Dong et al., 2004).

2.6. Quantitative detection of proteins

To quantitatively detect the proteins, the prepared avidin-modified electrode was immersed in various concentrations of biotinylated proteins labeled with ECL derivative (BT-protein-Ru). The biotin–avidin interactions were completed by letting them stand for 40 min at room temperature. After rinsed with double-distilled water and PB buffer thoroughly and dried under a N_2 stream, the avidin–biotin–protein–$Ru(bpy)_3^{2+}$ (AV-BT-protein-Ru)-modified electrode was immersed in 50 mM TPA/0.1M PB solution. The electrode was swept to an applied potential over the range of 0–1.3 V and the ECL signal was generated and recorded by the PMT.

3. Results and discussion

3.1. Avidin-based sensor

Carbon nanotubes are ideal material for biosensor fabrication due to its high electrical conductivity, high stability, and extremely high electrocatalytic ability (Wang et al., 2002; Sheeney-Haj-Ichia et al., 2005). They have been extensively used to covalently attach biomolecules such as proteins and DNA through amide formation between carboxyl acid groups functionalized on CNT and amines in the biomolecules with the aid of condensation reagents (Jung et al., 2004; He and Dai, 2004; Li et al., 2005). In our work, we used MWNT as a binder to immobilize avidin onto the GC electrode for two reasons: strong adsorptive ability to proteins via hydrophobic interactions (Balavoine et al., 1999; Chen et al., 2004; Shim et al., 2002) and availability to covalently combine avidin onto the working electrode in the presence of EDC and NHS through amide bond formation (Jung et al., 2004; He and Dai, 2004).

Electrochemical impedance spectra (EIS) as an efficient tool to characterize the formation of self-assemblies of biomolecule

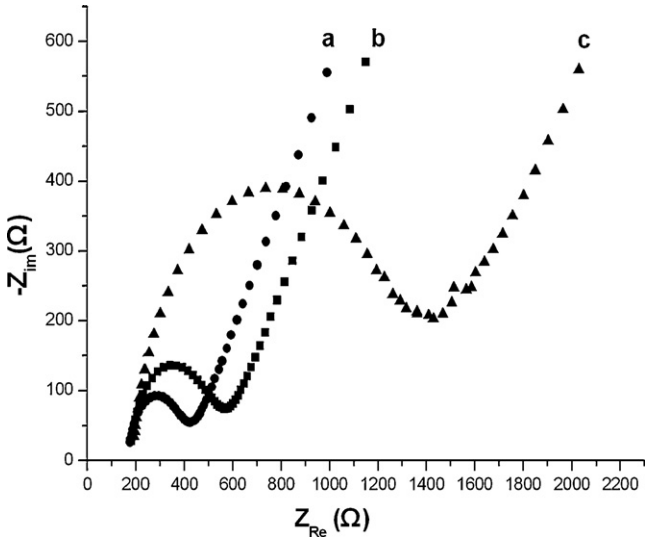


Fig. 1. Nyquist plot for the Faradaic impedance measurements in the buffer containing 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) as redox probe and 0.1 M KCl as supporting electrolyte. (a) A bare GC electrode, (b) an avidin-modified GC electrode, and (c) the avidin-modified electrode after incubation with 0.1 mM biotinylated BSA-Ru.

film were well addressed in the previous work (Xu et al., 2005; Wang et al., 2006; Zayats et al., 2006). The generous Randles model was used as the equivalent circuit for the EIS experiments. The electronic elements in the equivalent circuit include Rs, the ohmic resistance of the electrolyte solution; Zw, the Warburg impedance, resulting from the diffusion of the ions from the bulk electrolyte solution to the electrode surface; Cd, the double layer capacitance, and, R_{et}, the electron-transfer resistance (Patolsky et al., 1999). Nyquist plot of EIS measurement shows a semicircle in the high-frequency region associated with resistance and capacitance elements in parallel while shows a straight line in the low-frequency region associated with mass transfer (Barreira et al., 2004). R_{et} represents electron-transfer resistance and can be accurately readout in the parameters obtained from the fit of the equivalent circuit. It is the most decisive and directive parameter to reflect the changes of insulating features of electrode/electrolyte interface (Xu et al., 2005). Fig. 1 demonstrates the EIS results of bare electrode, avidin-modified electrode and avidin-modified electrode after immersed in BT-BSA-Ru solution. Though we can see in Fig. 1, the EIS dots did not start from zero, it was due to the high frequency we scanned was not high enough. In addition, we carried out the EIS experiments in the frequency range of 100 kHz to 0.1 Hz, the EIS dots were precisely started from zero. And our EIS measurements agree quite well with the fit of the Randles equivalent circuit model. We can see the value of R_{et} of the avidin-modified electrode increased from 255 Ω to 384 Ω compared to the bare electrode. This attributes to the prevention of charge transfer when avidin was immobilized onto the electrode (Garza et al., 2006). The attachment of avidin to the electrode may play a blocking effect and inhibited the rate of charge transfer. Considering the fact avidin was immobilized through MWNT, we also carried out relative EIS experiments of bare and MWNT-modified electrode. As a result, the value of R_{et} of the MWNT-modified elec-

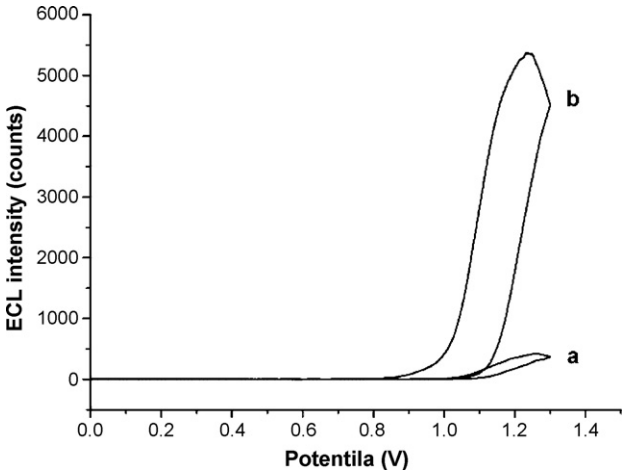


Fig. 2. ECL intensity vs. potential profile for the comparison of biotinylated BSA-Ru with non-biotinylated BSA-Ru attached to the avidin-modified electrodes under certain conditions. (a) Non-biotinylated BSA-Ru and (b) biotinylated BSA-Ru. The BSA concentration was 0.5 mM. ECL experiments were carried out in 50 mM TPA/0.1 M PBS electrolyte solutions at a scan rate of 50 mV/s. The sweep potential range is from 0 V to 1.3 V.

trode decreased 200 Ω compared to the bare electrode (data not shown). This is ascribed to the dual metal and semi-conductor properties of CNT which can accelerate the electron transfer of the probe ion from the bulk solution to the surface of the electrode. CNT and avidin attached onto the electrode may play opposite role on the rate of electron transfer so that the avidin-based sensor bridged with MWNTs inhibits the electron transfer to a lesser extend. After all, the above experimental facts confirmed avidin was successfully bound to the surface of the electrode.

3.2. Confirmation of interaction of avidin and biotin

In Fig. 1, the values of R_{et} of avidin-modified electrode before and after immersed in BT-BSA-Ru solutions were 384 Ω and 1210 Ω. Similar to the effect of avidin attached to the electrode, the attachment of BT-BSA-Ru onto the avidin-modified electrode could further increase the barrier of electron transfer because of BSA so that R_{et} values of AV-BT-BSA-Ru-modified electrode increased much more. The EIS results ensure BT-BSA-Ru was successfully bound to the avidin surface via biotin–avidin interaction.

Further evidence for avidin/biotin interaction was conducted by control experiment of comparing the ECL responses of avidin-modified electrodes after immersed in biotinylated and non-biotinylated ruthenium labeled BSA (BSA-Ru) solutions. The plot of ECL intensity versus sweep potential was well illustrated in Fig. 2 in which curve a corresponds to non-biotinylated BSA-Ru and curve b corresponds to biotinylated BSA-Ru, respectively. As shown in the figure, the biotinylated BSA-Ru attached avidin-modified electrode gave much higher ECL response than non-biotinylated one after immersed in a TPA-containing electrolyte solution. It is indicated that the ECL label was successfully assembled onto the electrode through biotin–avidin interaction. Besides, non-biotinylated BSA-Ru can also result in a weak ECL response. It is due to the non-specific adsorption of non-biotinylated BSA-Ru onto the electrode. The ECL response resulting from this kind of non-specific adsorption was 7.8% of the signal from the specific avidin/biotin interaction. The small non-specific adsorption has a negligible effect on the quantification of the proteins.

3.3. Method validations

Typical ECL responses of avidin-modified electrodes after exposure to different concentrations of biotinylated BSA-Ru were recorded as shown in Fig. 3. From curve c to f, the ECL intensities of the AV-BT-BSA-Ru-modified electrodes increased with the concentrations of BSA increasing. This indicated that the avidin-based sensor can respond to different concentrations of analyte and can be used to quantify the concentration of protein. We seriously analyzed the ECL peak intensities versus the concentrations of BSA and got a satisfying linear relationship. The linear range was from 15 nM to 7.5 μM as shown in the inset with a correlation coefficient of 0.994 and a linear regression equation of $y = 804.2 + 0.7x$ (the unit of x is nM). This dynamic range further validates the proposed approach a reasonable, effective and available protocol for protein quantification. Meanwhile, the detection limit was estimated to be 1.5 nM by measuring the minimum concentration of target BSA reliably being distinguished from the blank. It was reflected by curve a and b in Fig. 3 which is the ECL response of the blank and the minimum amount of detected analyte, respectively. The precision and accuracy was reflected by the error bar in the inlet of Fig. 3, and the R.S.D.s of the ECL response values were calculated to be less than 8.7%.

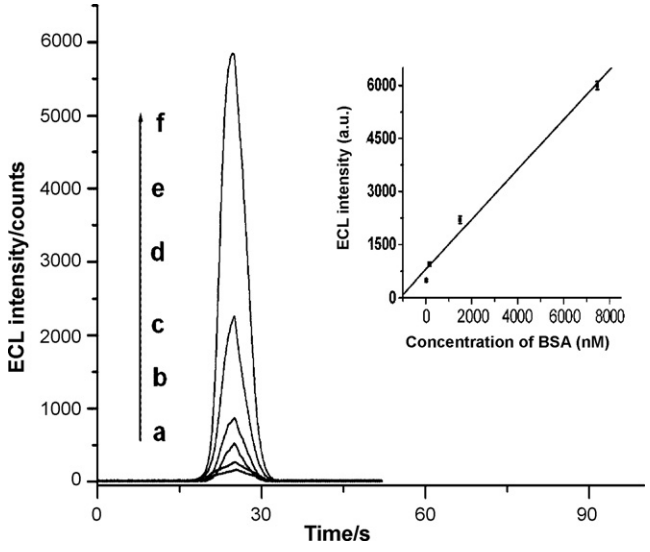


Fig. 3. ECL profiles of AV-BT-BSA-Ru-modified electrodes of different concentrations of BSA after immersed in a TPA-containing electrolyte solution. The concentrations of BSA are (a) 0, (b) 1.5 nM, (c) 15 nM, (d) 150 nM, (e) 1.5 μM, and (f) 7.5 μM. The inlet is the linear relationship between the ECL intensity and the concentration of BSA. The conditions of ECL measurements are the same as in Fig. 2.

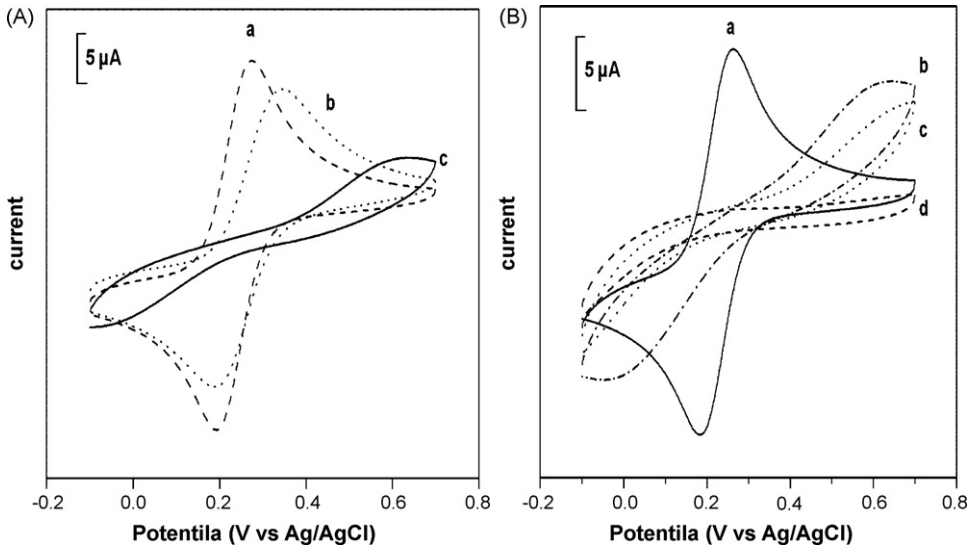


Fig. 4. Cyclic voltammograms of 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) redox probe in 0.1 M KCl (A) obtained at (a) a bare GC electrode, (b) an avidin-modified electrode, and (c) the avidin-modified electrode after incubation with biotinylated LYZ-SRu; (B) obtained at avidin-modified electrodes after incubation with various concentrations of biotinylated LYZ-SRu. The feed concentrations of LYZ are (a) 0, (b) 0.003 g L^{-1}, (c) 0.01 g L^{-1}, and (d) 0.1 g L^{-1}. The scan rate is 50 mV/s.

3.4. Application to quantitative detection of LYZ

From the point of view of protein quantification, we applied this approach for the assay of another protein, LYZ. LYZ is a naturally antimicrobial enzyme which has received attractive applications as food preservative, in cosmetics and medical applications, and as a consequence of its enzymatic stability in wines at various concentrations (Marchal et al., 2000; Delfini et al., 2004; Touch et al., 2003). The detection and quantification of LYZ are of great importance during wine processing or aging, grape crushing and alcoholic fermentation (Delfini et al., 2004).

Cyclic voltammetric (CV) measurements and comparative experiments were used to characterize and confirm the validity of the application part of the method.

Firstly, we performed CV measurements to characterize the avidin-based sensor and to confirm the immobilization of LYZ. Cyclic voltammograms of 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) redox probe with the bare electrode, avidin-modified electrode, and avidin-modified electrode after interacting with biotinylated LYZ labeled with self-synthesized Ru-NHS (LYZ-SRu) were shown in Fig. 4A. It can be seen that the peak currents decreased and the peak-to-peak separation increased with the avidin-modified electrode compared to the bare electrode. It is a typical reflection of the interface alteration of electrode/solution. The reduction in peak current probably originated from the blocking effect of avidin on the electrode. And the increase of peak-to-peak separation was likely due to some blocking of the effective electrode area by avidin. The facts suggest avidin was bound to the electrode. Although the increasing and decreasing ratios are not so announced, it may be due to the influence of MWNT which was also attached to the surface of the electrode as a binder to bind avidin to the electrode. MWNT is a well-known charge transfer accelerator and has electrocatalytic property when binding to the electrode (Wang et al., 2002; Sheeney-Haj-Ichia et al., 2005). To see if LYZ was successfully bound to the electrode, cyclic voltammogram of 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) redox probe with avidin-modified electrode after reacting with biotinylated LYZ-SRu was shown as curve c. As expected, the peak current decreased and the peak-to-peak separation increased a lot compared to that of avidin-modified electrode. This is due to the binding of biotinylated LYZ-SRu onto the electrode which increased the barrier of electron transfer of the probe ions. All these facts suggested LYZ was successfully immobilized on the electrode.

Secondly, we used five different feed concentrations of LYZ to be labeled and biotinylated for the establishment of the dynamic range of LYZ. In Fig. 4B, we present CV profiles of 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) redox couple with bare electrode and avidin-modified electrodes after exposure to various concentrations of labeled and biotinylated LYZ. It is evident to see the CV profile of the bare electrode shows a well-defined redox wave and the AV-BT-LYZ-SRu electrodes exhibit less reversible redox peaks with reduced peak currents and large peak-to-peak separations. With the feed concentration of LYZ increasing, the peak currents of the corresponding assembly modified electrode decreased on one hand; on the other hand, an irreversible behavior of the redox probe emerged as the concentration of biotinylated LYZ-SRu became 0.1 g L^{-1}. These all indicate that the larger the feed concentration of LYZ to be labeled and biotinylated, the more LYZ to be attached to the electrode and the much more inhibition effect on the electron transfer.

Because ECL responses were more sensitive than electrochemical currents, we recorded typical ECL responses of the AV-BT-LYZ-SRu electrodes of five different concentrations of LYZ after immersed in a TPA-containing electrolyte solution. Based on the ECL data, we got a dynamic range of LYZ from

0.001 g L^{-1} to 0.1 g L^{-1} with a coefficient of 0.997 and the linear regression equation of $y = 86.6 + 1507.5x$ (the unit of x is g L^{-1}). The detection limit of LYZ was estimated to be 0.1 mg L^{-1} and it is sensitive enough for the practical application of LYZ in wine lactic bacterial in which the concentration of LYZ is at 0.25–0.50 mg L^{-1} (Delfini et al., 2004).

4. Conclusions

$Ru(bpy)_3^{2+}$ ECL detection method dominates in the drug analysis and several clinic applications based on its intrinsic high sensitivity and selectivity. But, high selectivity brings limitations that $Ru(bpy)_3^{2+}$ ECL can only detect small molecules, especially molecules containing tertiary amine moiety. To enlarge the application scope of $Ru(bpy)_3^{2+}$ ECL detection to the biological fields such as protein and DNA detection is undoubtedly an obligatory task We demonstrated here an effective universal ECL protocol for the quantitative detection of proteins after $Ru(bpy)_3^{2+}$ was functionalized with carboxyl acid group and activated as succinimide ester. So, biotinylated proteins labeled with $Ru(bpy)_3^{2+}$ succinimide ester attached to the avidin-modified electrode via biotin/avidin interaction can generate ECL response in the presence of its coreactant, TPA. In this way, a model BSA was quantitatively detected by an organic combination of double protein labeling protocol with the avidin-based sensor under certain conditions. Obviously, the facile and strong avidin/biotin strategy can reduce the complexity and hardness involved in the classical sandwich protocol. Moreover, served as an extremely sensitive detection means, $Ru(bpy)_3^{2+}$ ECL attracts much attention. But the commercial reagents used in the ECL detection were very expensive, especially the ECL NHS ester labels. Our method was not only addressed by the novelty and feasibility of application of $Ru(bpy)_3^{2+}$ ECL detection in protein detection but also provided a valuable ECL label by self-synthesis. Furthermore, an application to LYZ analysis using self-synthesized ECL label was performed and successfully validated the proposed approach useful and reliable. From this perspective, ECL quantification of protein was realized and the goal of extending $Ru(bpy)_3^{2+}$ ECL application to bioanalysis was fulfilled to some extent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.01.023.

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