EVALUATION OF THE INTERACTION BETWEEN AZITHROMYCIN AND HUMAN SERUM ALBUMIN BASED ON $Ru(bpy)_3^{2+}$-DOPED SILICA NANOPARTICLES PROBE

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ABSTRACT

The interaction between azithromycin and human serum albumin (HSA) was investigated by using capillary electrophoresis (CE) coupled with end-column solid-state electrochemiluminescence (ECL). $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuDS) were prepared by an one-pot water-in-oil microemulsion method. The as-prepared RuDS was displayed ECL signal at around 1.18 V. The ECL signal was further increased in the presence of azithromycin, which could be used for end-column detection of azithromycin concentration. Under the optimized condition, the ECL intensity was linear with azithromycin concentration in the range of 0.1 to 140 μmol/L (R^2=0.9983) with a detection limit of 0.05 μmol/L. Based on this, the binding constant between azithromycin and HSA was estimated to be 7.46×10^4 L/mol by nonlinear regression analysis assuming that only one special binding site for drug coupling provided per protein. Warfarin and ketoprofen were used as probes to study their interaction mechanism and the result showed that azithromycin binds at warfarin site (site I) of HSA. The proposed CE-ECL method may be applicable to study a wide range of interactions between drugs and biomacromolecules such as proteins and DNA.

KEYWORDS: Capillary electrophoresis; Electrochemiluminescence; Azithromycin; Human serum albumin; Interaction.

1. INTRODUCTION

Azithromycin, first synthesized in 1980s, [1] is a semi-synthetic 15-membered ring macrolide antibiotic. It has been already widely used as human medicine in treatment of pneumonia, urinary tract infections and tonsillitis and malaria. [2] Its wide antibacterial activity against both gram-positive and gram-negative pathogens [3] is benefits from the interaction with a 50S ribosomal subunit, which effectively inhibits the ribosomal protein synthesis of bacteria and plasmodium. Many spectroscopic techniques (e.g. UV-vis absorption spectroscopy, [4] Fourier transform infrared spectroscopy, [5] fluorescence spectrometry, [6] electrochemical technique [7] and gas chromatography-mass spectrometry[8]) have been used to monitor azithromycin for pharmacokinetic studies.

Drug-protein interactions, especially with the most abundant plasma protein human serum albumin (HSA), play a crucial role in the pharmacology and biological activity of drugs. Binding to HSA alters the free drug concentration, which can cross membrane barriers and be distributed to tissues, and then affects the pharmacokinetics of the drugs including absorption, distribution, metabolism, and excretion. [9] Investigating the binding mechanism of the drug with HSA is not only important for the study of toxicology and pharmacokinetics but also can elucidate the characteristics of the drug–protein complex and can provide useful information on the structural features that determine the therapeutic effectiveness of the drug. Therefore, study of the interaction between drugs and plasma proteins has been an interesting area of research in life sciences, chemical biology and clinical medicine. Several methods have been applied to drug–protein binding studies, including equilibrium dialysis, [10] ultrafiltration, [11] ultracentrifugation, [12] microdialysis, [13] fluorescence spectrometry, [14]surface plasmon resonance assay, [15] and high-performance affinity chromatography. [16] Complementarily, due to the high separation efficiency, capillary electrophoresis (CE) has also been successfully utilized as a technique for characterizing drug-protein interactions in the last decade. [17,18] CE has a prominent advantage with low sample consumption. However, this specialty will have a negative effect on detection sensitivity. Traditionally, most of the CE-based methods adopt ultraviolet detector which has a poor sensitivity. MS and LIF have been also coupled as highly sensitive detectors with CE, however, both the instruments are so expensive and bulky that their extensive applications are restricted. So, more sensitive and convenient detection techniques are needed to explore. Currently, electrochemiluminescence (ECL) has become an attractive detection method which not only has comparative sensitivity to MS and LIF detection but also has other predominant qualities, including low equipment

costs, easy to operate and excellent stability. Recently, $Ru(bpy)_3^{2+}$-based ECL coupled with CE has become a very powerful analytical tool and has been widely used in the areas of clinical analysis,[19] drug metabolism, [20] enzyme activity evaluation [21] and drug-protein interaction. [22-24] However, in most of analytical applications by CE-ECL, the chemiluminescent reagent $Ru(bpy)_3^{2+}$ was directly added in the cathodic cell in liquid form, which resulted in a dilution of $Ru(bpy)_3^{2+}$ near the surface of the working electrode by the CE buffer flowing from the capillary. To solve these problems, a solid-state ECL detector by immobilizing $Ru(bpy)_3^{2+}$ onto the electrode has been developed for coupling with CE. Particularly when immobilized, this recyclable chemiluminescent reagent can be used for reagentless durable detectors. Although so many methods to immobilize $Ru(bpy)_3^{2+}$ have been reported, [25] the solid-state ECL sensor used as detector for CE is very limited. Therefore, there is an urgent need to investigate and expand new areas in applications by using solid-state ECL detector coupling to CE. Predictably, coupling CE with reagentless solid-state ECL detectors is significative and has a good prospect in analytical applications.

In this paper, the interaction between azithromycin and HSA was investigated by using CE coupled with solid-state ECL. It resolved the problems arising from liquid $Ru(bpy)_3^{2+}$ ECL detection that suffer from a dilution of $Ru(bpy)_3^{2+}$ near the surface of the working electrode by the CE buffer flowing from the capillary and the excessive consumption of expensive $Ru(bpy)_3^{2+}$. The microdialysis method was used to simplify chemical analysis by excluding large molecules from complex constituents. In addition, small molecules could transport through the dialysis quickly, and thus it increased the rate of approach to equilibrium by mechanical shaking. Thermodynamic parameters, such as the number of binding sites, binding constant and interaction force mode were calculated and the interaction mechanism was also illustrated by using warfarin and ketoprofen as probes.

2. MATERIALS AND METHODS

2.1. Reagents and apparatus

tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2·6H_2O$), HSA, chitosan (CHIT, low molecular weight, minimum 85% deacetylated), Tween-80, tetraethyl orthosilicate (TEOS), n-hexanol, cyclohexane, ammonium hydroxide (25 wt %), dialysis bag (8000 Da) were purchased from Sigma-Aldrich. Azithromycin standard was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Nanjing China). Azithromycin for injection was from Furentang Pharmaceutical Co., Ltd (Henan China). All

other chemicals were of analytical grade. All solutions were prepared with double-distilled water. Prior to use, samples and buffer solutions were filtered through 0.22-μm cellulose acetate filters (Shanghai Xinya Purification Material Factory). The uncoated fused-silica capillary (25 μm i.d., 375 μm o.d.) from Yongnian Optical Conductive Fiber Plant (Hebei, China) was used for separation.

UV-vis spectra were measured on an UV1102 spectrophotometer (Tianmei Instruments, Shanghai, China). The morphology and size of RuDS were analyzed with a transmission electron microscope (TEM, S-3400N, HITACHI, Japan). ECL measurements were carried out on a MPI-A CE–ECL instrument (Xi'an Remex Analytical Instrument Ltd. Co., China). The end-column ECL cell consists of a three-electrode system with a 1.5 mm diameter graphite disc electrode coated with $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuDS)-CHIT composite film as working electrode, a Pt wire electrode as auxiliary electrode, and Ag/AgCl electrode as reference electrode. The potential of working electrode was supplied using a potentiostat.

2.2 Preparation of $Ru(bpy)_3^{2+}$ modified electrode

The fabrication of $Ru(bpy)_3^{2+}$ modified electrode was according to the previous paper with a minor modification.[27] One-pot synthesis of $Ru(bpy)_3^{2+}$-doped silica nanoparticles by a water-in-oil (W/O) microemulsion method was according to previous reports.[28-29] Briefly, 1.77 mL of Tween-80 were mixed with 7.5 mL of cyclohexane, 1.8 mL of n-hexanol and 80 μL of 100 mmol/L $Ru(bpy)_3^{2+}$ solution in water (340 μL). TEOS (100 μL) was then added as a precursor for silica formation, followed by addition of 60 μL of NH_4OH to initiate the polymerization reaction. The reaction was allowed to continue for 24 h at room temperature by vigorous stirring. After that, RuDS nanoparticles were precipitated from the microemulsion using 4 mL of acetone. To remove physically adsorbed $Ru(bpy)_3^{2+}$ and surfactant on the surface, RuDS nanoparticles were further carefully washed by repetitive ultrasonication and centrifugation with ethanol and water. The diameter of as-prepared $Ru(bpy)_3^{2+}$-doped silica was ca. 100 nm (see TEM image in Fig. 1).

For immobilization of RuDS nanoparticles on the electrode surface, a 1.0 wt % CHIT solution was prepared by dissolving CHIT flakes in a hot acetic acid solution (1.0%) with magnetic stirring for 2 h. Then, 100 μL of 10 mg/mL RuDS nanoparticles suspended in phosphate buffer solution (PBS) was dispersed into 900 μL of 1.0% CHIT acetic acid solution and ultrasonicated for several minutes. After that, a 5-μL aliquot of RuDS-CHIT

composite was placed on the surface of cleaned graphite disc electrode and dried at room temperature. When not in use, the as-prepared RuDS-CHIT modified electrodes were stored at 4 ^{o}C for the following experiments.
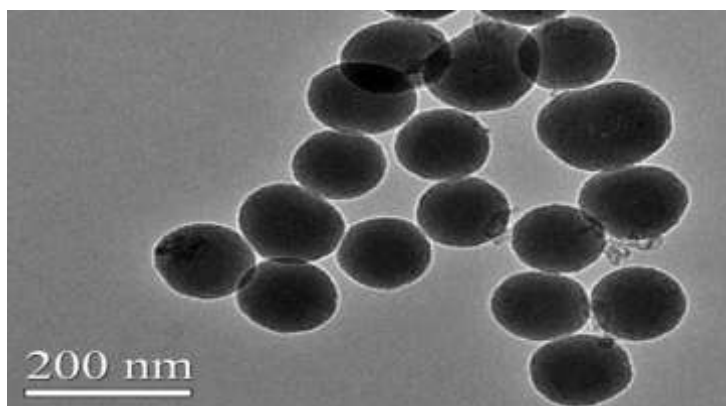

Fig.1 TEM image of Ru(bpy)$_3$$^{2+}$-doped silica nanoparticles.

2.3 Analysis process

The interaction between azithromycin and HSA was investigated by using capillary electrophoresis coupled with end-column solid-state electrochemiluminescence. After incubation in 0.5 mL centrifugal tube, the mixture of HSA and azithromycin were injected into a dialysis bag (21 mm diameter, molecular weight cutoff 8000 Da). The dialysis bag was then kept in a phosphate buffer solution (2 mL) and incubated at 37 ^{o}C for 4 h with shaking at 800 r/min until the equilibrium between the inside and outside of the dialysis bag was obtained. It should be noted that here the mild mechanical shaking accelerated the transportation of small molecules through the dialysis membrane, but did not have any influence on the interaction between proteins and drugs. After the dialysis equilibrium, the solution outside the dialysis bag was injected to a pretreated capillary and the ECL signal was measured with end-column mode.

Prior to each capillary electrophoresis, a new capillary with length of 25 cm was filled with 0.1 mol/L sodium hydroxide over night. It was then washed with 0.1 mol/L sodium hydroxide for 5 min, followed by water for 5 min, and equilibrated with the running buffer (10 mmol/L PBS) for 5 min to maintain an active and reproducible inner surface. In all experiments, the separation voltage for electrophoresis in the capillary was 15 kV and 10 mmol/L PBS at pH 8.0 was used as running buffer. A detection potential of 1.18 V was applied on the working electrode. Sample introduction was accomplished by electrokinetic injection for 10 s at 10 kV. The potential of the photomultiplier tube (PMT) was operated at -800V. The capillary was flushed with the running buffer for 3 min after each separation.

3. RESULTS AND DISCUSSION

3.1 ECL behavior of RuDS modified electrode in azithromycin solution

Azithromycin could enhance ECL of $Ru(bpy)_3^{2+}$ due to its two tertiary amine groups. Fig. 2 shows the ECL-potential curves of RuDS modified electrode in 50 mmol/L pH 8.0 PBS at scan rate of 100 mV/s.
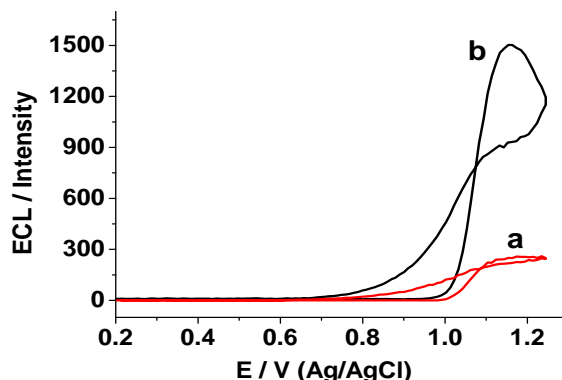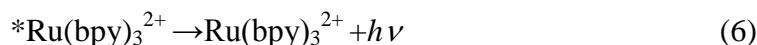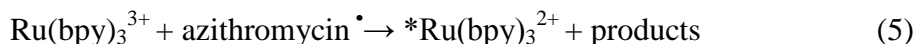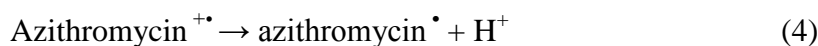

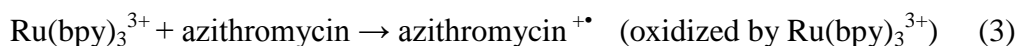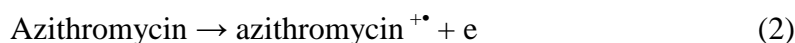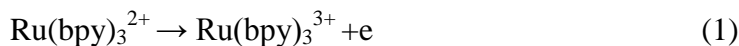**Fig.2 ECL-potential curves in the absence (a) and presence (b) of 60 µmol/L azithromycin. Conditions: 50 mmol/L, buffer with pH 8.0 in the ECL cell; potential scan rate: 100 mV/s; PMT voltage: −800 V.**

The rise of ECL intensity started at about 1.10 V, increased with the increase of potential, and reached the maximum ECL at 1.18 V (Fig. 2a), because of the direct oxidation of $Ru(bpy)_3^{2+}$ in buffer solution. Interestingly, after addition of azithromycin in the detection cell, the ECL intensity of $Ru(bpy)_3^{2+}$ increased remarkably (Fig. 2b). Here, the enhanced emission of $Ru(bpy)_3^{2+}$ was presumably due to the two tertiary amine groups in azithromycin, which could react with the ruthenium species, just similar to that in the traditional tripropylamine/$Ru(bpy)_3^{2+}$ system.[30] Thus, a possible mechanism in ECL procedure was proposed as follows.

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e \qquad\qquad (1)$$

$$\text{Azithromycin} \rightarrow \text{azithromycin}^{+\bullet} + e \qquad\qquad (2)$$

$$Ru(bpy)_3^{3+} + \text{azithromycin} \rightarrow \text{azithromycin}^{+\bullet} \quad (\text{oxidized by } Ru(bpy)_3^{3+}) \quad (3)$$

$$\text{Azithromycin}^{+\bullet} \rightarrow \text{azithromycin}^{\bullet} + H^+ \qquad\qquad (4)$$

$$Ru(bpy)_3^{3+} + \text{azithromycin}^{\bullet} \rightarrow {}^*Ru(bpy)_3^{2+} + \text{products} \qquad\qquad (5)$$

$${}^*Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{2+} + h\nu \qquad\qquad (6)$$

Both $Ru(bpy)_3^{2+}$ and azithromycin at the surface of electrode could be oxidized directly to form $Ru(bpy)_3^{3+}$ (Eq. 1) and azithromycin radical cation (azithromycin $^{+\bullet}$) (Eq. 2). Subsequently, the short-lived azithromycin $^{+\bullet}$ further lost a proton from α-carbon and generate a strongly reducing intermediate (azithromycin $^{\bullet}$, Eq. 4). This reducing intermediate was then reacted with $Ru(bpy)_3^{3+}$ via an electron transit reaction to form emitting species of $*Ru(bpy)_3^{2+}$ (Eq. 5). At the same time, if only $Ru(bpy)_3^{3+}$ produced at the electrode surface, it could homogeneously oxidize azithromycin to generate the radical cation of azithromycin $^{+\bullet}$ (Eq. 3), which could release a proton to form azithromycin $^{\bullet}$ (Eq. 4). Finally, $Ru(bpy)_3^{3+}$ was reduced by the radical azithromycin $^{\bullet}$ to $Ru(bpy)_3^{2+*}$ and emitted light.

3.2 Optimization of pH

As well known that the ECL reaction of $Ru(bpy)_3^{2+}$ with alkylamine was a pH-dependent process. Thus, for a higher emission intensity, pH values of the buffer solution in the detection cell should be optimized.
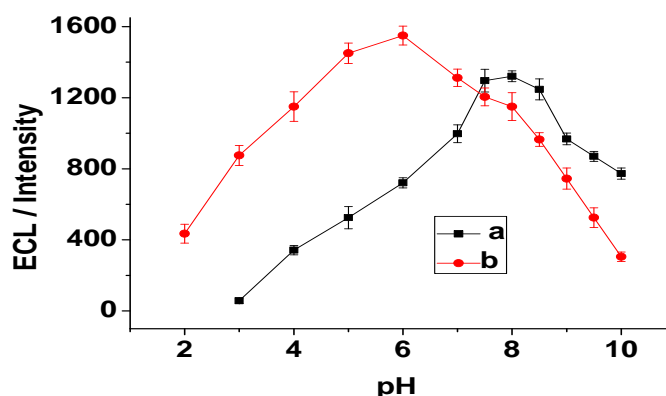

Fig. 3 The influence of the phosphate buffer pH in the ECL cell (a) and running buffer (b) on ECL intensity. Detection conditions: 60 μmol/L azithromycin; electrokinetic injection: 10 kV×10 s; running buffer: 50mmol/L PBS (pH 6.0); separation voltage: 15 kV. ECL cell contents: 50 mmol/L PBS; detection potential: 1.18 V; separation capillary: 25 μm i.d, 30 cm length.

As shown in Fig. 3a, ECL intensity was significantly increased with pH at pH<7.0 because of the protonation of tertiary amine, while it decreased sharply after pH >9.0 due to the reduced availability of $Ru(bpy)_3^{3+}$ by the competitive reaction with the OH$^-$. [31] The suitable pH value was between 7.0-9.0. Here, pH 8.0 was chosen as the optimal condition in the detection cell.

According to the previous study, [24] the pH value of running buffer affected the ECL intensity because the pH value strongly influences the electroosmotic flow (EOF) and the analyte ionization. Therefore it obviously influenced the signal intensity. Fig. 3b illustrated the effect of pH value of running buffer on the detection sensitivity. The ECL intensity increased as running buffer pH value increased from 2.0 to 6.0. When the pH of running buffer exceeded 6.0, the ECL responses decreased. This might be due to the stability of azithromycin in solution. Azithromycin was stable in weak acid conditions and might be hydrolyzed under the strong acid or alkaline conditions. Therefore, the maximum ECL value was obtained when the pH value of running buffer was 6.0.

3.3 Optimization of incubation time of azithromycin with HSA and dialysis time

The interaction between azithromycin and HSA was examined by UV–Vis spectrophotometry, as shown in Fig. 4.
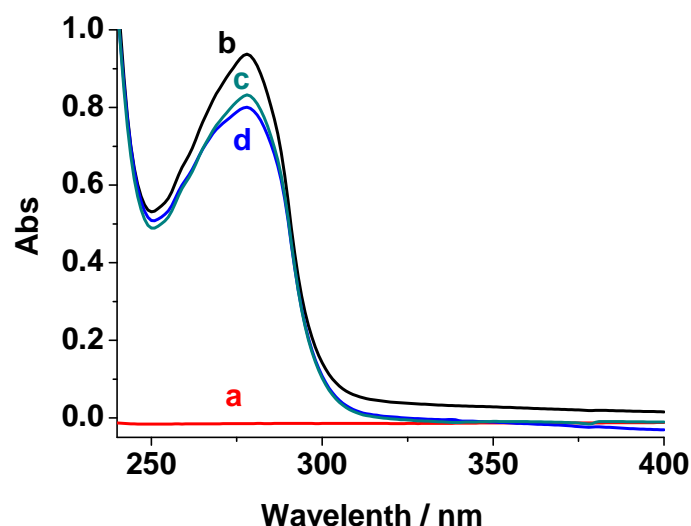

Fig.4 UV–Vis spectra of 10 mmol/L azithromycin (a), 200 µmol/L HSA (b), and their mixture (100 µL 200 µmol/L HSA+10 µL10 mmol/L azithromycin) after incubation for 1 h (c) and 5 h (d), which were diluted into 1 mL with 10 mmol/L pH 7.4 PBS, respectively.

The azithromycin showed no absorbance (Fig. 4a), while the HSA showed strong absorbance at 278 nm (Fig. 4b). Upon addition of azithromycin to the HSA solution, the characteristic peak of HSA at 278 nm decreased (Fig. 4c). In addition, the absorbance of HSA decreased with the incubation time from 1 h (Fig. 4c) to 5 h (Fig. 4d), which confirmed the interaction between HSA and azithromycin. The incubation time was also optimized. Experiments

showed that the absorbance at 278 nm decreased with the incubation time and reached a stable value at 4 h, as shown in Fig.5
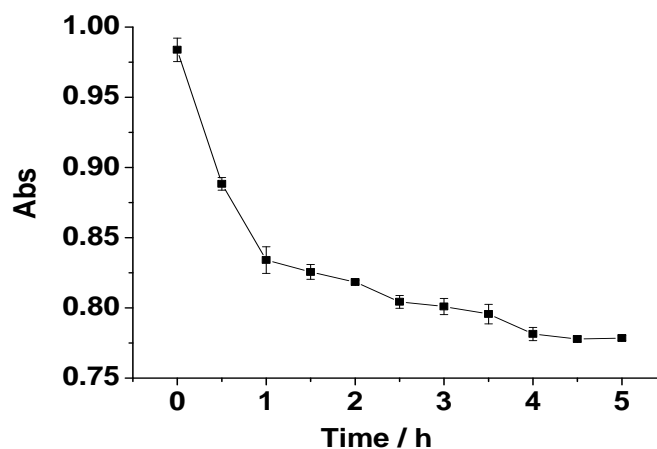

Fig. 5 Effect of incubation time for the mixture of azithromycin and HSA on absorbance of HSA at 278 nm. Incubation temperature: 37 °C; solution:100 μL of 200 μmol/L HSA+10 μL of 10 mmol/L azithromycin which was diluted to 1 mL with 10 mmol/L pH7.4 PBS.

To determine the dialysis time that needed to reach equilibrium, the ECL intensity of free drug outside of the bag was measured every half hour. It was observed that the ECL intensity of free drug outside of the dialysis bag became constant after 3 h. Moreover, the solution outside of the bag showed almost no UV absorbance at 278 nm, which showed that the protein did not leakage from the bag. Therefore, the dialysis time was optimized to 3 hours.

3.4 Linearity, detection limit and reproducibility

ECL responses with end-column detection mode varied with the different concentrations of azithromycin in solutions, thus, it could be used for quantitative analysis of azithromycin. Under optimized conditions, the ECL intensity of azithromycin was linear with its concentrations in the range of 0.1 to 140 μmol/L. A linear equation of *EI* =19.45 $C_{azithromycin}$ (μmol/L) + 54.5 was obtained with the correlation coefficient R^2=0.9983, where *EI* was the ECL intensity and $c_{azithromycin}$ was the azithromycin concentration. The limit of detection for azithromycin was 0.05 μmol/L, calculated as three times signal-to-noise (S/N) ratio. The relative standard deviation (RSD) of the ECL intensity and the migration time for six continuous injections at azithromycin concentration of 10 μmol/L were 1.6% and 1.4% respectively, indicating a good repeatability for the detection of azithromycin by this method.

3.5 Azithromycin–HSA binding parameters under physiological conditions

An important parameter about drug-protein interactions is the number of total drugs bound per protein molecule or the fraction of total binding sites which were occupied. Here, we assumed the binding site and the drug is stoichiometrily as 1:1, i.e., only one specific type of binding site for the drug in the protein. Therefore, the binding parameters of drugs to proteins could be extracted by the following equation:[32-34]

$$r = n\frac{K[D_{free}]}{1+ K[D_{free}]}$$

(Eq. 7)

where n and K are the number of binding sites and the binding constant, respectively; r and [D_{free}] stand for the fraction of bound drug molecules per protein molecule, and the concentrations of free drug.

The experiments were carried out by keeping the concentration of HSA constant and evaluating the percent of azithromycin unbound at different drug concentrations. Thus, 100 μL of 200 μmol/L HSA with a series of different volumes of 10 mmol/L azithromycin were mixed and then be incubated in phosphate buffer solution at 37 ^{o}C for 4 h, respectively. Then, the mixtures were dialyzed in PBS for 3 h at 37 ^{o}C while keeping their total volume constantly at 2 mL. After equilibrium, the free drug concentration [D_{free}] in each mixture was measured by detecting the ECL intensity of the outside solution. The relationship between the fraction of bound drug molecules per protein molecule and the concentration of free azithromycin was evaluated according to Eq. 7, as shown in Fig.6.
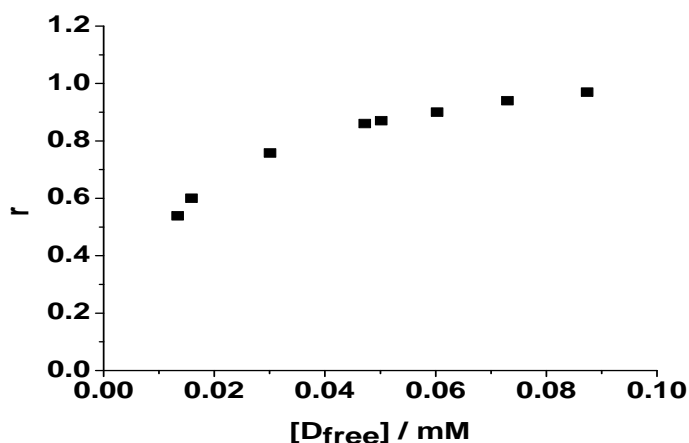

Fig. 6 Binding curve for azithromycin with HSA in phosphate buffer solution (pH 7.4) at 37 ^{o}C; [D_{free}] is the concentration of free azithromycin.

The number of binding sites and the binding constant were estimated to be 0.9797 and 7.46×10^4 L/mol, respectively. The number of binding sites was very close to 0.9706 by fluorescence spectroseopies measurements, [35] while the binding constant was larger than that of 5.44×10^4 L/mol by fluorescence spectroseopies measurements, [35] and 4.67×10^3 L/mol by a surface plasmon resonance (SPR) biosensor based on the immobilization of HSA on SPR chip. [36] These differences were not unexpected because the experiments were carried out in different buffer systems. Furthermore, the different binding parameters may come from different sources of protein and drug batch differences. Thirdly, the biosensor-based assay required the immobilization of the protein on a support, which might alter the conformation and drug binding properties of the protein. Lastly, it is obvious that there was a discrepancy in the affinity constants when different experiment approaches were used with different calculating equations.

According to the previous report, [37] the molecular forces contributing to protein interactions with small molecular substrates may include vander Waals interactions, hydrogen bonds, electrostatic force and hydrophobic interactions and so on. Ross and Subramanian [38] have characterized signs of the thermodynamic parameters associated with the various individual kinds of interactions that may take place in protein association processes. Therefore, the thermodynamic parameters, containing enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) of azithromycin-protein binding reaction were determined as the main evidences for evaluating the binding mode. The values of ΔH, ΔS and ΔG were calculated using the following equations.

$$\text{In } K = -\Delta H/RT + \Delta S/R \qquad\qquad (Eq.8)$$
$$\Delta G = -RT\ln K = \Delta H - T\Delta S \qquad\qquad (Eq.9)$$

where K is the binding constant at the corresponding temperature and R is the gas constant. In order to keep the bioacitivity of HSA, the binding researches were carried out at 20, 25 and 37^oC, respectively. It assumes that ΔH and ΔS did not vary significantly over the temperature range studied. Based on the K values of azithromycin with HSA obtained at different temperatures, the values of ΔH and ΔS were obtained from the slope and intercept of the plot of lnK versus 1/T according to Eq. 8. Then, the values of ΔG were calculated from Eq. 9. The values of thermodynamic parameters were summarized and the corresponding values are presented in Table 1.

Table.1 Thermodynamic parameters of the interaction between azithromycin and HSA

T (K)	K (M^{-1})	n	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS(J mol^{-1} K^{-1})
323	7.88×10^4	0.9690	-4.150	-30.281	80.901
328	7.72×10^4	0.9699	-4.150	-30.685	80.901
335	7.46×10^4	0.9797	-4.150	-31.252	80.901

As shown in table 1, the values of ΔH and ΔS for the binding reaction between azithromycin and HSA are found to be -4.150 kJ mol^{-1} and 80.901 J mol^{-1} K^{-1}. On the basis of observed positive ΔS and negative ΔH values, we could conclud that both enthalpy and entropy combine to drive the binding for azithromycin-HSA interaction. The negative values of ΔG revealed that the interaction process was spontaneous.

At the same time, it also revealed that the main source of ΔG value was derived from a large contribution of ΔH term with a little contribution from factor ΔS. Accordingly, the negative values of ΔH and positive values of ΔS are generally viewed as evidence for predominant electrostatic interaction in the binding processes of azithromycin to HSA. However, negative enthalpy changes demonstrated that the binding reaction was an exothermic, and it usually arised from van der Waals forces and hydrogen formation in low dielectric media, [38] therefore, the hydrophobic interaction could not be ignored.

3.6 The mechanism of the interaction between azithromycin and HSA

Previous studies demonstrated that HSA molecule had two well-defined regions, site I (the warfarin–azapropazone site) and site II (the indole–benzodiazepine site), which would interact with drugs.[39,40] It was known that both warfarin and ketoprofen had a strong binding affinity to the proteins HSA at site I and at site II respectively. [41-42]

In our case, both warfarin and ketoprofen were used as guides to evaluate the binding site by displacement experiments. Briefly, if azithromycin blocks the same site with warfarin or ketoprofen, then warfarin or ketoprofen can displace part of bound azithromycin due to its high specificity and affinity.

As a result, the concentration of free azithromycin outside of the dialysis bag increased. Otherwise, if the azithromycin blocks the different site with warfarin (or ketoprofen), then the concentration of free azithromycin should not change.
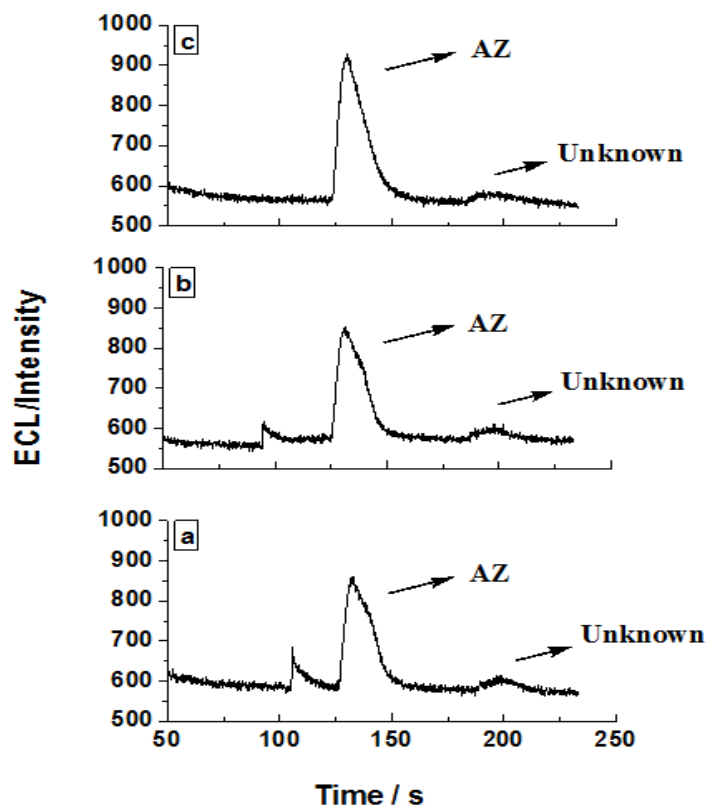
Fig.7 Electropherogram of samples after dialysis of azithromycin + HAS (a) and azithromycin + HSA in presence of ketoprofen (b) or warfarin (c). Electrokinetic injection: 10 kV×10 s; running buffer: 50 mmol/L PBS (pH 6.0); separation voltage: 15 kV. ECL cell contents: 50 mmol/L phosphate (pH 8.0); detection potential: 1.18 V; separation capillary: 25 µm i.d, 30 cm length.

Fig. 7 shows that the ECL signal of free azithromycin outside of the dialysis bag in the mixture of azithromycin + HSA (Fig. 7a) and azithromycin + HSA + ketoprofen (Fig. 7b) were nearly consistent, indicating that ketoprofen could not displace azithromycin from the azithromycin-HSA complex. However, the concentration of free azithromycin increased when warfarin was added into the mixture (Fig.7c). The concentration of free azithromycin for mixtures of azithromycin +HSA and azithromycin + HAS+ ketoprofen was estimated to be 11.6 µmol/L by linear fitting, whereas that for mixtures of azithromycin + HAS+ warfarin was 15.7 µmol/L. It is indicated that the addition of warfarin increased the concentration of free azithromycin, whereas ketoprofen does not produce an apparent effect. This result suggested that warfarin preferentially blocked the site I by displacing the azithromycin from the azithromycin-HSA complexes. Therefore, it is concluded that azithromycin binds at site I of HSA. This was in good agreement with the previous reports for acquisition of information

about the location of drug binding to HSA by using warfarin and ketoprofen as displacement ligands. [43,44]

4. CONCLUSIONS

In this study, the CE coupling with a solid-state ECL detector was used to investigate the interaction between azithromycin and HSA at physiological conditions. ECL responses under end-column detection mode increased with the concentrations of azithromycin in solutions, which could be used for quantitative analysis of azithromycin. The used microdialysis method simplified chemical analysis by excluding large molecules from complex constituents. Moreover, the binding mechanism and binding parameters between azithromycin and HSA were investigated by CE-ECL method with the aid of equilibrium microdialysis. The proposed CE-ECL method may be applicable to study a wide range of interactions between drugs and biomacromolecules such as proteins and DNA.

ACKNOWLEDGMENTS

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