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Study on the binding behavior of bovine serum albumin with cephalosporin analogues by chemiluminescence method

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ABSTRACT

The luminol–bovine serum albumin chemiluminescence system was proposed for the first time. It was found that the hydrophilic luminol bound to the hydrophilic domain at Trp^{134} of BSA with accelerating the electrons transferring rate of excited 3-aminophthalate, which led to the enhancement CL intensity of luminol at 425 nm. The increment of chemiluminescence intensity was proportional to the concentrations of bovine serum albumin from 5.0×10^{-11} to 1.0×10^{-8} mol L^{-1} with the linear equation of $\Delta I = 7.47 C_{BSA} + 4.89$ ($R^2 = 0.9950$). Based on the remarkable quenching effect of cephalosporin on the luminol–bovine serum albumin chemiluminescence system, the interaction of bovine serum albumin–cephalosporin was studied by flow injection-chemiluminescence method. A valuable model for studying the interaction of bovine serum albumin–cephalosporin was constructed and the formula $\lg[(I_0 - I)/I] = \lg K_D + n \lg[D]$ was obtained. The binding parameters calculated by the model did agree very well with the results obtained by fluorescence quenching method. The major binding force of bovine serum albumin with cephalosporins was the hydrophobic effect. The binding ability of cephalosporin analogues to bovine serum albumin followed the pattern: cefoperazone, ceftriaxone and cefotaxime > cefuroxime and cefaclor > cefadroxil, cefradine and cefazolin, which was close to the order of their antibacterial ability. Using flow injection chemiluminescence method also obtained the stoichiometric ratio, the average of association constant K_P and dissociation degree α of luminol–bovine serum albumin were 1:1, 1.12×10^7 L mol^{-1} and 0.086, respectively.

1. Introduction

The study of protein–drug interaction has become the hot spot in the fields of chemistry, medicine and biology because most drugs undergo a greater or lesser extent of reversible binding to plasma proteins and blood cells [1–3]. There are various methods for evaluating protein–drug interaction [4–6]. Spectrometry is one kind of the most commonly tools among them, such as fluorescence spectroscopy [7,8], circular dichroism [9], resonance light scattering [10], nuclear magnetic resonance spectroscopy [11] and Fourier transform infrared [12]. The relationship of bovine serum albumin (BSA)–drug has also been indirectly investigated by flow injection (FI)-chemiluminescence (CL) with some CL systems [13–15].

BSA is the most abundant protein in plasma contributing to osmotic blood pressure. It plays important roles in the transport, distribution and metabolism of many exogenous ligands. Being the major binding protein for drugs and other physiological substances, it is considered as a model protein for studying protein–drug interaction in vitro. Thus, BSA was studied extensively in the past years, partly because of its structural homology with human serum albumin (HSA) [16–18]. Cefradine, cefadroxil, cefazolin, cefaclor, cefuroxime, cefotaxime, ceftriaxone and cefoperazone (illustrated in Scheme 1), the 1st-, 2nd- and 3rd-generation agents of cephalosporin antibiotics, are semisynthetic antibiotic substances obtained from fungi, characterized by a broad antibacterial spectrum for a wide range of both Gram-positive and Gram-negative bacteria [19,20]. Cephalosporin was grouped into "generations" based on the timing of introduction and antibacterial properties, and each newer generation of cephalosporin has significantly greater Gram-negative antimicrobial properties than the preceding generation in general [21].

Since FI analysis was conceived by Ruzicka and Hansen [22], it has revolutionized the way of performing analytical and bioanalytical chemistry [23–25]. Combining with CL, FI-CL has become a very useful analytical tool owing to its advantages of simple apparatus, high sensitivity, wide dynamic ranges, reproducibility and automatability as well as less reagent consumption [26–30]. In this work, the interaction of BSA with cephalosporin analogues was studied using FI-CL method in detail. The luminol–BSA

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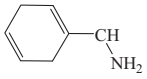
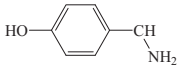
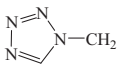
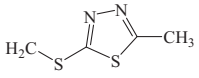
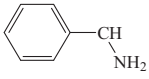
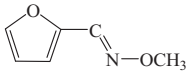
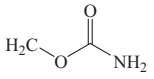
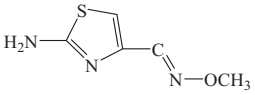
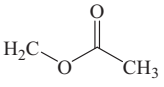
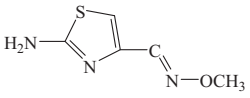
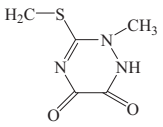
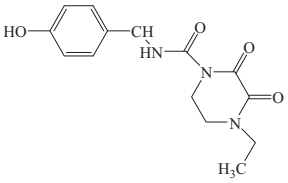
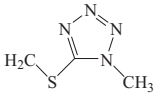
nucleus cephalosporanic			
drugs	R_1	R_2	R_3
first generation			
cefradine		CH_3	H
cefadroxil		CH_3	H
cefazolin			H
second generation			
cefaclor		Cl	H
cefuroxime			H
third generation			
cefotaxime			H
ceftriaxone			H
cefoperazone			H

Scheme 1. The chemical structure of the studied cephalosporins.

FI-CL system was described and the photochemical mechanism of luminol–BSA–cephalosporin was investigated. It was hypothesized that Trp^{134} of BSA could accelerate the rate of excited 3-aminophthalate's electrons transferring and enhance the chemiluminescence signal of luminol, and then produce steady-state chemiluminescence in the flow injection system with the relative standard deviations less than 3.0%. In the presence of cephalosporin, Trp^{212} of BSA might be occupied by cephalosporin formed complex, led to a conformational change of BSA, then the chemiluminescence from luminol–BSA was quenched. Based on the relationship between luminol/BSA and cephalosporin, a model of BSA–cephalosporin interaction was established, and interaction parameters were obtained by the model.

2. Materials and methods

2.1. Apparatus

The FI-CL system used in this work was shown schematically in Fig. 1. A peristaltic pump of the IFFM-E Luminescence Analyzer (Xi'an Remax Electronic Science-Tech. Co. Ltd., Xi'an, China) was applied to deliver all streams. PTFE tubing (1.0 mm i.d.) was used throughout the manifold for carrying the CL reagents. A six-way valve with loop of 100.0 μL was used for sampling. The CL signal produced in flow cell was detected without wavelength discrimination, and the photomultiplier tube (PMT) output was recorded by PC with a IFFE-E client system. A Hitachi F-4500 fluorophotome-

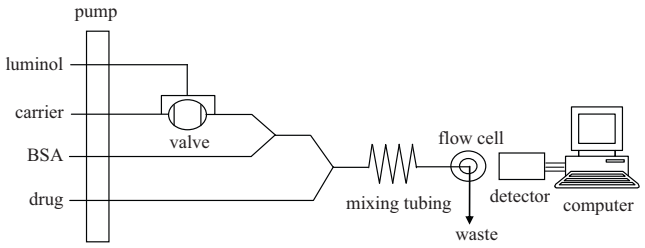


Fig. 1. Schematic diagram of the present FI-CL system.

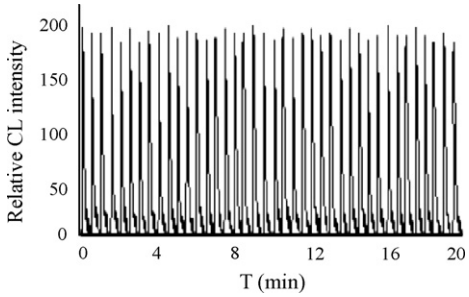


Fig. 2. CL signals with 10 cm mixing tube length. The concentrations of luminol and BSA were 2.5×10^{-5} and 5.0×10^{-9} mol L^{-1}, respectively.

ter (Tokyo, Japan) was applied for the fluorescence measurements. A Lambda-40 UV–Vis spectrophotometer (Perkin Elmer, USA) was employed for acquiring the absorption spectra.

2.2. Reagents

All reagents used in this work were of analytical reagent grade unless specified and doubly deionized water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) used for the preparation of solutions in the whole procedure. Luminol (Fluka, Biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. BSA (Sigma) was used as received without further purification. Cephalosporin analogues (Scheme 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. All stock solutions of cephalosporin were prepared in doubly deionized water at the concentration of 1.0×10^{-3} mol L^{-1}. Working standard solutions of cephalosporin analogues were prepared daily from the above stock solution by appropriate dilution as required. The stock solution of BSA with the concentration of 5.0×10^{-5} mol L^{-1} was prepared with doubly deionized water. Luminol of 2.5×10^{-2} mol L^{-1} was prepared by dissolving 0.44 g luminol in 100 mL of 0.01 mol L^{-1} NaOH solution in a brown calibrated flask. The stock solutions were stored at 4 °C, and all working solutions were prepared freshly.

2.3. General procedure

As shown in Fig. 1, flow lines were inserted into luminol, carrier (pure water), BSA and the drug solutions, respectively. The pump was started at a constant speed of 2.0 mL min^{-1} to wash the whole system until a stable baseline was recorded. Then 100.0 μL of luminol solution was injected into the carrier stream by injection valve and merged with BSA, which was then mixed with the cephalosporin stream. The mixed solution was delivered into the CL cell, producing CL emission, detected by the PMT and luminometer. The PMT negative voltage was set as 700 V. The concentration of cephalosporin drugs could be quantified on the basis of the decrement of CL intensity, $\Delta I = I_0 - I_s$, where I_s and I_0 were CL signals in the presence and in the absence of cephalosporin analogues, respectively. The related solution was scanned on the fluorophotometer with the range of 290–450 nm and the fluorescence intensity was measured at 340 nm with the excitation wavelength at 280 nm.

3. Results and discussion

3.1. Design of the FI-CL system and optimization of the experimental conditions

Two different manifolds were tested in FI-CL system. It was found that when the streams of luminol and drug (cephalosporin) exchanged, viz. 100.0 μL of luminol or drug solution was injected into the carrier stream, then two significantly different results were observed. The whole analysis process, including sampling and washing, could be accomplished in 0.5 min, offering the sampling throughput of 120 h^{-1} accordingly and obtained better precision and signal-to-noise when injection of luminol, as in the manifold described in Fig. 1. Whereas it took more than 1.0 min and more sample consumption when the injection of drug. Therefore, the manifold depicted in Fig. 1 was chosen for this work.

The flow rate and mixing tube length had great effect on the CL intensity. The influence of flow rate on determination was examined by investigating the signal-to-noise (S/N) under different flow rate. Flow rate of 2.0 ml min^{-1} offering highest S/N ratio was then chosen as suitable condition considering analytical precision. The effect of the mixing tube length on CL intensity was tested from 5.0 to 20.0 cm. It was observed that the CL intensity was much stronger and the repeatability of the analytical signals was better using 10.0 cm mixing tube than that of other mixing tube in the presence of 2.5×10^{-5} mol L^{-1} luminol and 5.0×10^{-9} mol L^{-1} BSA (Fig. 2).

The effect of luminol or BSA concentration on the CL intensity was tested over the ranges of 5.0×10^{-7} to 5.0×10^{-4} mol L^{-1} and 5.0×10^{-11} to 1.0×10^{-7} mol L^{-1}, respectively. As shown in Fig. 3, the stable and strong CL intensity could be obtained when using a concentration of 2.5×10^{-5} mol L^{-1} luminol and 5.0×10^{-9} mol L^{-1} BSA.

Due to the nature of the luminol reaction, which was more favored under alkaline conditions, NaOH was added into the luminol solution to increase the sensitivity of the system. A series of NaOH solutions with different concentration ranging from 1.0×10^{-3} to 1.0×10^{-1} mol L^{-1} were tested. At 0.025 mol L^{-1} NaOH, the CL signal could reach a maximum value. Hence, 0.025 mol L^{-1} NaOH was the optimum concentration of this system and used in the subsequent experiments. The introducing of carbonates buffer solution into carrier and luminol solution could maintain the alkaline pH, but the CL intensity evidently depressed in the presence of the carbonates buffer solution. Therefore, the carbonates buffer solution was not been added to the FI-CL system.

3.2. The relative CL intensity-time profiles

The relative CL intensity-time profile of luminol–BSA reaction was examined firstly in the flowing system. As shown in Fig. 4, the maximum CL intensity time (T_{max}) of luminol changed from 4.5 s to 3.8 s and the CL intensity increased from 125 to 210 in the presence of BSA. It was found that the increment of CL intensity was proportional to the concentrations of BSA from 5.0×10^{-11} to 1.0×10^{-8} mol L^{-1} with the linear equation of $\Delta I = 7.47 C_{BSA} + 4.89$ ($R^2 = 0.9950$). Fig. 5 showed the CL intensity of luminol–BSA was quenched remarkably in the presence of cefotaxime with the same T_{max}. The decrement of CL intensity was proportional to the logarithm of cefotaxime concentration over the range from 0.1 to 100 nmol L^{-1}, giving the regression equation $\Delta I = 5.59 \ln C_{cefotaxime} + 18.3$ ($R^2 = 0.9950$,

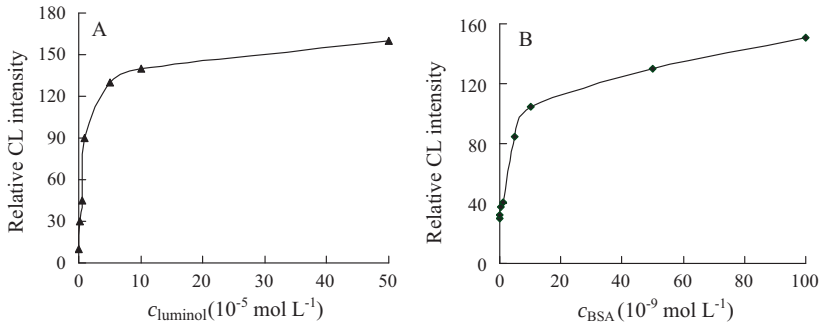


Fig. 3. Effect of concentrations of luminol (A) and BSA (B) on the CL intensity. (A) The concentration of BSA was 5.0×10^{-9} mol L^{-1}. (B) The concentration of luminol was 2.5×10^{-5} mol L^{-1}.

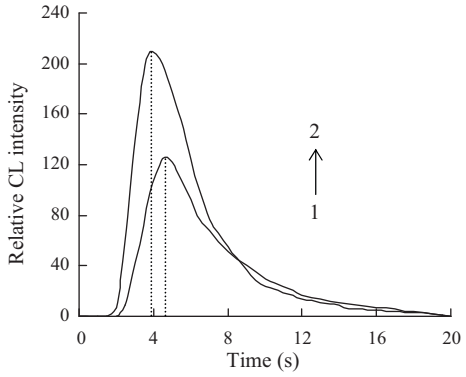


Fig. 4. Relative CL intensity-time profile of luminol–BSA CL system. 1: CL intensity in the absence of BSA; 2: CL intensity in the presence of BSA. The concentrations of luminol and BSA were 2.5×10^{-5} and 5.0×10^{-9} mol L^{-1}, respectively.

Table 1
Calibration curves and detection limits (LOD) of cephalosporins.

Drug	Linear equation	Linear range, nmol L^{-1}	LOD, nmol L^{-1}	R^2
Cefradine	$\Delta I_{CL} = 2.48 \ln C_{cefradine} + 13.5$	1.0–300	0.3	0.9971
Cefadroxil	$\Delta I_{CL} = 10.2 \ln C_{cefadroxil} + 30.1$	0.1–500	0.03	0.9959
Cefazolin	$\Delta I_{CL} = 6.59 \ln C_{cefazolin} + 19.1$	0.5–700	0.2	0.9961
Cefaclor	$\Delta I_{CL} = 3.31 \ln C_{cefaclor} + 8.05$	0.1–100	0.03	0.9934
Cefuroxime	$\Delta I_{CL} = 3.10 \ln C_{cefuroxine} + 11.8$	0.1–100	0.03	0.9940
Cefotaxime	$\Delta I_{CL} = 5.59 \ln C_{cefotaxime} + 18.3$	0.1–100	0.03	0.9950
Ceftriaxone	$\Delta I_{CL} = 5.47 \ln C_{ceftriaxone} + 8.36$	0.3–300	0.1	0.9936
Cefoperazone	$\Delta I_{CL} = 7.85 \ln C_{cefoperazone} + 19.1$	0.1–300	0.03	0.9965

$n = 7$), with a detection limit of 0.03 nmol L^{-1} (3σ). The results of luminol–BSA–cephalosporin CL system were listed in Table 1.

3.3. Possible luminescence mechanism of luminol/BSA/cephalosporin reaction

BSA contains 607 tactic amino acid residues and forms three cylindrical hydrophobic cavity at the molecular interior. There is reported that the drug bound to BSA mainly via hydrophobic interaction entered into the hydrophobic cavity [31,32]. BSA have two tryptophan moieties, Trp^{134} and Trp^{212}. Trp^{134} located on the surface of the molecular, Trp^{212} located in the hydrophobic cavity of the protein which specified for drug binding [33]. The possible luminescence mechanism of luminol–BSA–cephalosporin reaction was studied by FI-CL and UV method. As shown in Fig. 4 that in the presence of BSA, CL intensity of luminol enhanced remarkably and the T_{max} changed from 4.5 to 3.8 s. Fig. 6 showed the UV absorption spectra of luminol–BSA differed from with UV absorption spectra of luminol or BSA. It is hypothesized that the hydrophilic luminol bound to the hydrophilic domain at Trp^{134} of BSA with accelerating the electrons transferring rate of excited 3-aminophthalate, which led to the enhancement CL intensity of luminol and producing the effect of complexation enhancement of CL (CEC).

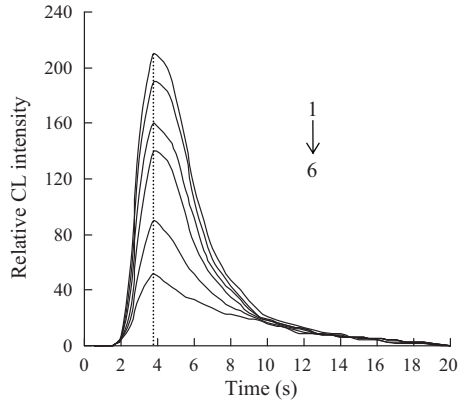


Fig. 5. Relative CL intensity-time profile of luminol–BSA–cefotaxime CL system. The concentrations of cefotaxime ranging from 1 to 6 were 0, 1.0×10^{-9}, 5.0×10^{-9}, 1.0×10^{-8}, 5.0×10^{-8}, 1.0×10^{-7} mol L^{-1}. The concentrations of luminol and BSA were 2.5×10^{-5} and 5.0×10^{-9} mol L^{-1}, respectively.

Fig. 6 also showed the UV absorption spectra of different reaction systems. It was clear that spectra of luminol with drugs did not change, but the spectra of BSA with drugs (cefradine, cefuroxime and cefotaxime) had a little blue shift. It might be due to cephalosporin entered into the hydrophobic cavity and bound with Trp^{212} led to a conformational change of BSA, resulting in the inhibition of CL intensity from luminol–BSA system and causing the effect of complexation enhancement of quenching (CEQ). The luminescence mechanism of luminol–BSA–cephalosporin reaction was illustrated in Fig. 7, which described the CL process as the following two steps:

1. Luminol entered into the sites of Trp^{134} in BSA producing CEC effect and CL signal enhanced.
2. Cephalosporin entered into the site of Trp^{212} in BSA leading CEQ effect and CL signal disappeared. Then, based on the decrement of luminol CL intensity in the present of cephalosporin, the interaction of BSA with cephalosporin was investigated.

3.4. Constructing the FI-CL model

The interaction of BSA with cephalosporin was studied using luminol as luminescence probe and a hypothetic binding model

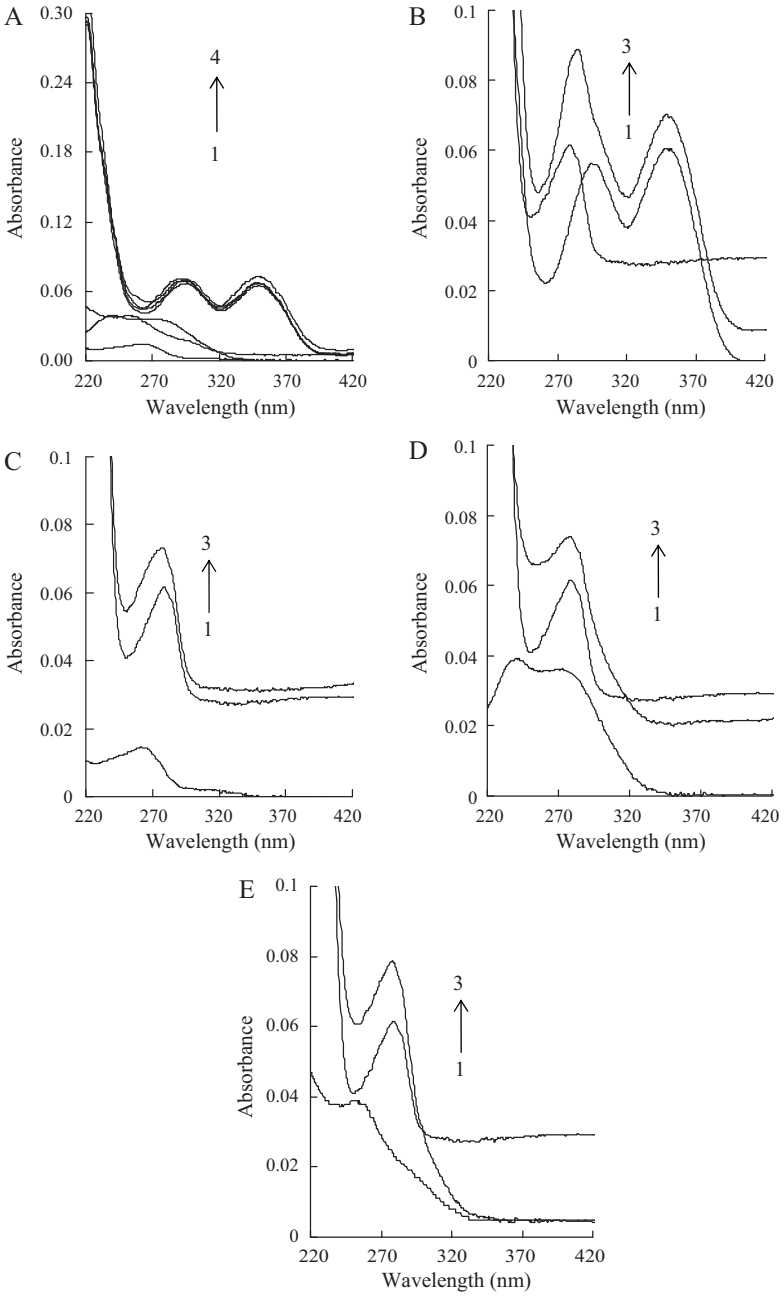


Fig. 6. UV absorption spectra profile of luminol–drugs (A), luminol–BSA (B) and BSA–drugs (C–E). (A) 1, cefradine; 2, cefotaxime; 3, cefuroxime; 4, luminol and luminol–drugs. (B) 1, BSA; 2, luminol; 3, luminol–BSA. (C) 1, cefradine; 2, BSA; 3, BSA–cefradine. (D) 1, cefuroxime; 2, BSA; 3, BSA–cefuroxime. (E) 1, cefotaxime; 2, BSA; 3, BSA-cefotaxime. The concentrations of luminol, BSA and drugs were 1.0×10^{-5}, 1.0×10^{-6} and 1.0×10^{-6} mol L^{-1}, respectively.

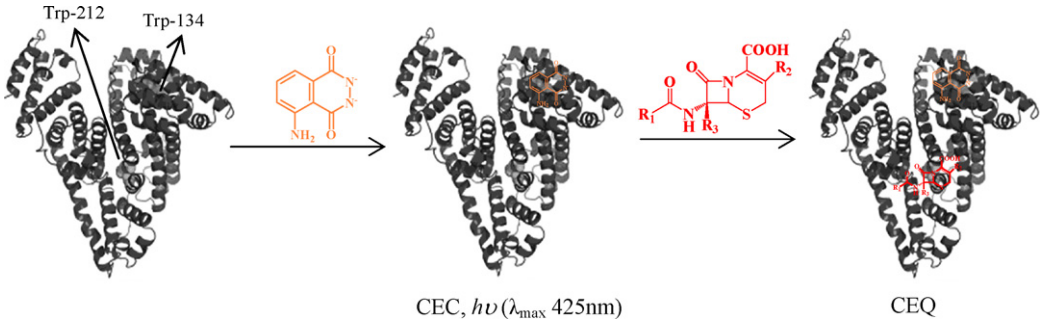


Fig. 7. Schematic diagram of luminol–BSA–cephalosporin interaction process. CEC: complexation enhancement of chemiluminescence; CEQ: complexation enhancement of quenching.

was constructed. As shown in Fig. 1, when luminol was injected into the carrier stream, mixed with BSA in the FI-CL system, BSA mainly existed in the form of $P\cdots L^*$ complex due to the excess of luminol in the mixed local (the concentrations of BSA and luminol were 5.0×10^{-9} and 2.5×10^{-5} mol L^{-1}, respectively), which produced steady-state CL signal with the relative standard deviations (RSD) less than 3.0%. The steady-state FI-CL intensity can be measured by CL. The process was expressed as

$$P + L \overset{K_P}{\rightleftharpoons} P\cdots L^* \rightarrow P\cdots L + h\upsilon \quad (\lambda_{max} \quad 425 \quad \text{nm}) \tag{1}$$

where P and L refer to BSA and luminol, K_P is the association constant of luminol/BSA complex.

After the stream of drug solution mixed with $P\cdots L^*$ stream, the CL was quenched, and formed $D_n\cdots P\cdots L$ ternary complex online. The interaction of luminol–BSA–cephalosporin can be described as follows:

$$nD + P\cdots L^* \overset{K_D}{\rightleftharpoons} D_n\cdots P\cdots L \tag{2}$$

where D refer to cephalosporin drug. The binding constant K_D can be expressed as

$$K_D = \frac{[D_n\cdots P\cdots L]}{[D]^n[P\cdots L^*]} \tag{3}$$

where $[P\cdots L^*]$, $[D]$ and $[D_n\cdots P\cdots L]$ are the concentration of the luminol/BSA complex, cephalosporin and luminol/BSA/cephalosporin complex; $[P\cdots L^*]_0$ is the total concentration of the luminol/BSA complex and equals $[P\cdots L^*]+[D_n\cdots P\cdots L]$, whose substitution into Eq. (3) can lead to

$$K_D = \frac{[P\cdots L^*]_0 - [P\cdots L^*]}{[D]^n[P\cdots L^*]} \tag{4}$$

Eq. (4) can change to the form of common logarithm

$$lgK_D = lg\frac{[P\cdots L^*]_0 - [P\cdots L^*]}{[P\cdots L^*]} - n\,lg[D] \tag{5}$$

$D_n\cdots P\cdots L$ does not emit photon, the mole fraction of unassociated luminol/BSA can be expressed by the corresponding CL intensity

$$\frac{I}{I_0} = \frac{[P\cdots L^*]}{[P\cdots L^*]_0} \tag{6}$$

substituting Eq. (6) into Eq. (5) can be obtained

$$lgK_D = lg\frac{I_0 - I}{I} - n\,lg[D] \tag{7}$$

and

$$lg\frac{I_0 - I}{I} = lg\,K_D + n\,lg[D] \tag{8}$$

where K_D is the binding constant and n is the number of binding potential point. According to the proposed model, the interaction parameters (K_D and n) and the nature of the binding force for BSA/cephalosporin can be obtained.

4. Applications of the proposed FI-CL model

4.1. Calculation of the interaction parameters of BSA/cephalosporin

Utilizing Eq. (8), the interaction parameters could be obtained from the plot of lg[$(I_0-I)/I$] against lg[D]. The calculated results of binding constant K_D and the number of binding potential point n were listed in Table 2. Meanwhile, at the same temperature (298 K), the binding parameters were also studied by fluorescence quenching (FQ). The results obtained by the proposed method agreed well with the results obtained by fluorescence quenching. The binding constant K_D values of the drugs mostly were at 10^3–10^5 level suggesting that there existed a high binding affinity of cephalosporin to BSA. The binding ability of the studied cephalosporin drugs followed the pattern: cefoperazone, ceftriaxone and cefotaxime > cefuroxime and cefaclor > cefadroxil, cefradine and cefazolin, which consisted with that of their antibacterial ability [34]. The n value was ranging from 0.76 to 1.16 approximately equal to 1.0 indicating that there was one class of binding site to cephalosporin analogues in BSA. The interaction parameters calculated by proposed model agreed well with the results obtained by classical FQ model illuminated that the proposed model exerted very good stability and reliability. For FI-CL has many merits, such as high sensitivity, wide linear range, rapid measurement process and simple instrumentation, the proposed model would be extensively used in the study of protein–drug interaction.

Table 2
Binding parameters of BSA/cephalosporin determinated by CL and FQ method.

Drug	Method	K, L mol^{-1}	n	R^2
Cefradine	CL	5.56×10^3	0.87	0.9951
	FQ	7.30×10^3	1.01	0.9924
Cefadroxin	CL	2.63×10^3	0.76	0.9924
	FQ	4.36×10^3	0.82	0.9943
Cefazolin	CL	4.85×10^2	0.86	0.9934
	FQ	7.88×10^2	0.96	0.9952
Cefaclor	CL	3.33×10^3	0.84	0.9902
	FQ	6.72×10^3	1.02	0.9931
Cefuroxime	CL	3.07×10^4	0.97	0.9951
	FQ	1.08×10^4	0.99	0.9988
Cefotaxime	CL	1.30×10^5	1.02	0.9936
	FQ	7.25×10^4	0.95	0.9992
Ceftriaxone	CL	3.04×10^5	1.16	0.9927
	FQ	5.09×10^5	1.03	0.9991
Cefoperazone	CL	1.04×10^5	1.08	0.9909
	FQ	2.25×10^5	1.22	0.9949

4.2. Calculation of the thermodynamic parameters of BSA/cephalosporin

The acting forces between a drug and a biomolecule include hydrogen bond, van der Waals forces, hydrophobic interaction and electrostatic attraction etc. The thermodynamic parameters of binding reaction are the main evidence for confirming the binding force. In order to estimate the interaction force of BSA with cephalosporin, the thermodynamic parameters of cefradine, cefadroxil, cefazolin, cefaclor, cefuroxime, cefotaxime, ceftriaxone and cefoperazone binding with BSA were calculated using the von't Hoff equation [35], respectively. The values of ΔH°, ΔS° and ΔG° at different temperatures obtained were summarized in Table 3. It can be seen that $\Delta G^\circ < 0$, $\Delta H^\circ > 0$ and $\Delta S^\circ > 0$. The negative sign for ΔG° meant that the binding process was spontaneous and the formation of BSA/cephalosporin was an endothermic reaction with positive enthalpy change. According to the sign and the magnitude of the thermodynamic parameters in protein-ligand association process characterized in the literature [36], it was deduced that the binding force was mainly on the hydrophobic interaction.

4.3. Determination of stoichiometry for luminol/BSA

The molar ratio of luminol/BSA complex was obtained by Continuous-Variations method and Molar-Ratio method. The results in Fig. 8 showed that both curves for the association indicated the formation of a 1:1 complex. The measured CL intensity

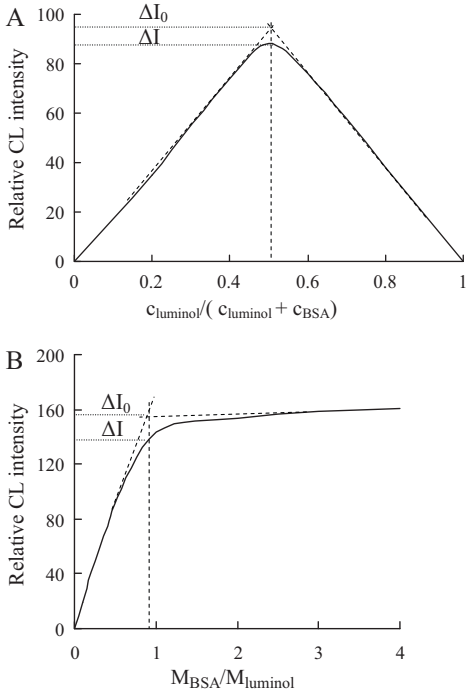


Fig. 8. Measuring the stoichiometric ratio of luminol/BSA complex. (A) Continuous-Variation method, the total concentration of both luminol and BSA were 2.5×10^{-5} mol L^{-1}. (B) Molar-Ratio method, the concentration of luminol was 1.0×10^{-5} mol L^{-1}, the concentration of BSA ranged from 1.0×10^{-6} to 4.0×10^{-4} mol L^{-1}. Every measuring compared with the same concentration of luminol as blank.

(ΔI) at inflexion was compared to theoretical ΔI_0, derived by extrapolation of linear parts of the curve to their intersection, and the values of ΔI and ΔI_0 in curve A and B were 88, 96 and 143, 157, respectively. The quotient of these values represented the degree of association $(1-\alpha)$

$$\frac{\Delta I}{\Delta I_0} = 1 - \alpha \tag{9}$$

where α was the degree of dissociation. According to Eq. (1), association constant for luminol/BSA complex could be written as

$$K_P = \frac{[P\cdots L^*]}{[P][L]} = \frac{1-\alpha}{c\alpha^2} \tag{10}$$

where c were 1.25×10^{-5} mol L^{-1} in Continuous-Variations method and 1.0×10^{-5} mol L^{-1} in Molar-Ratio method, combined with ΔI and ΔI_0 mentioned above, the average of K_P and α were 1.12×10^7 L mol^{-1} and 0.086. The dissociation constant $K_{P'}$ with 0.089 μmol L^{-1} obtained by $1/K_P$ was $\ll$5 μmol L^{-1}, which indicated stronger binding affinity of luminol to BSA [37].

Table 3
Thermodynamic parameters of BSA/cephalosporin.

Drug	T, K	ΔH°, kJ mol^{-1}	ΔS°, J mol^{-1} K^{-1}	ΔG°, kJ mol^{-1}
Cefradine	288	131.52	513.65	−16.23
	298			−21.36
Cefadroxil	288	49.89	232.21	−16.92
	298			−19.24
Cefazolin	288	69.66	285.17	−12.47
	298			−15.32
Cefaclor	288	19.60	133.19	−18.76
	298			−20.09
Cefuroxime	288	74.48	335.83	−22.24
	298			−25.60
Cefotaxime	288	26.88	188.11	−27.29
	298			−29.17
Ceftriaxone	288	21.79	178.08	−29.49
	298			−31.28
Cefoperazone	288	102.29	451.22	−27.66
	298			−32.17

5. Conclusions

The interaction of BSA–cephalosporin reaction was studied by FI-CL, fluorescence quenching and UV method. Based on the relationship between luminol/BSA and cephalosporin, a valuable model for studying the binding of BSA with cephalosporin by FI-CL method was deduced for the first time and the calculated results of binding constant K_D indicated that there existed a high binding affinity of cephalosporin drugs to BSA. The calculated thermodynamic parameters testified that the hydrophobic effect was the major binding force in the interaction of BSA with cephalosporins. Association parameters of luminol/BSA was also obtained by FI-CL method. With stoichiometric ratio of 1:1, the average of association constant K_P and dissociation degree α were 1.12×10^7 L mol^{-1} and 0.086. The study indicates that the FI-CL is a feasible method for studying the interactions between protein and drug.

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