



Investigation of the interaction between cefoxitin sodium and bovine serum albumin by improved spectroscopy

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ABSTRACT

The reaction mechanism of cefoxitin sodium with bovine serum albumin was investigated using the classical fluorescence spectroscopy with focus on the fluorescence change of protein, as well as the elastic scattering fluorescence spectroscopy with focus on the fluorescence change of drug at different temperatures. The results indicated that cefoxitin sodium could quench the intrinsic fluorescence of bovine serum albumin strongly by a static quenching process. The results of two methods were consistent. In addition, the binding constant obtained from elastic scattering fluorescence spectroscopy was larger than the one obtained from classical fluorescence spectroscopy. At last the correctness of elastic scattering fluorescence spectroscopy method was verified by UV-visible absorption spectroscopy. It is also speculated that "point to surface" interaction between drugs and peptides was existed.

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KEYWORDS

Classical fluorescence spectroscopy;
Elastic scattering fluorescence spectroscopy;
Cefoxitin sodium;
Bovine serum albumin;
Reaction mechanism.

INTRODUCTION

The classical fluorescence spectroscopy studies the reaction mechanism of small molecule drugs and proteins with focus on the fluorescence change of protein^[1-2]. Elastic light scattering is a kind of light scattering, which the radiation light wavelength is the same with incident light wavelength^[3] and is called Rayleigh scattering when the scale of the scattering particles is much smaller than the wavelength of incident light. When Rayleigh scattering is located in the vicinity of the absorption band, it is possible to cause a sharp increase in scattering intensity, and this resonance phenomenon is called Rayleigh scattering,

also known as resonance light scattering (resonance light scattering, RLS). In classical fluorescence spectroscopy, the main source of bovine serum albumin (BSA) fluorescence is Trp-212. Classical fluorescence spectroscopy does not reflect interaction of the other non-fluorescence-emitting residues with drugs and the fluorescence spectrogram only reflects partial information of the interaction of BSA with drug^[4]. As a result, the obtained information is insufficient accuracy. However, fluorescence changes of small molecule drugs can reflect the whole information of interaction between drugs and proteins, so that the fluorescence of small molecule drug reflects the overall fluorescence in the interac-

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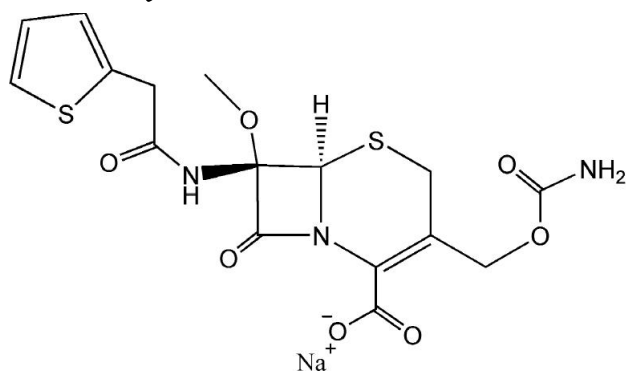


Figure 1 : Chemical structure of cefoxitin sodium

tion. In order to make up for the shortcomings of classical fluorescence spectroscopy, a new method by taking the drug as the object of detection is applied to study the interaction between drugs and proteins.

Cefoxitin sodium (CFXS) is cephalosporin antibiotics, can resist anaerobic bacteria and have stable interaction on enzyme. The structural formula of it is shown in Figure 1. CFXS is widely used due to its strong sterilization, acid-resisting, enzyme-resistant, and relatively small side effects. But along with the increase in clinical application, the adverse reactions reported occasionally. Main show was rash, drug fever, asthma. To make matters worse, severe cases was anaphylactic shock, and even death. Serum albumin is the most abundant protein as important carrier in plasma. It plays an important role in transport, distribution and metabolism of various exogenous ligands like fatty acids, amino acids, drugs and pharmaceuticals due to its unusual ligand binding properties^[5-7]. The binding of hydrophobic drugs with BSA results in solubilization of drugs in cell plasma which then helps drug delivery to cells in vivo and in vitro. Thus BSA plays a dominant role in drug disposition and efficiency^[8]. It is therefore important to study the interaction of drugs of interest with BSA.

EXPERIMENTAL

Apparatus

All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectrofluorophotometer. Absorption was measured with an UV-vis recording spectrophotometer (UV-265, Shimadzu, Japan).

All pH measurements were carried out with a PHS-3C precision acidity meter (Leici, Shanghai, China). All temperatures were controlled by a SYC-15_B superheated water bath (SangLi, Nanjing, China).

Materials

Bovine serum albumin was purchased from Sigma Co. and was of the purity grade inferior 99%. Stock solutions of bovine serum albumin (1.0×10^{-4} mol·L⁻¹) and cefoxitin sodium (1.0×10^{-3} mol·L⁻¹) were prepared. All the stock solutions were further diluted for use as working solutions. Tris-HCl buffer solution containing 0.15 mol·L⁻¹ NaCl was used to maintain the pH of solutions at 7.40 and NaCl solution was used to maintain the ionic strength of the solution. All other reagents were of analytical grade and all aqueous solutions were prepared with newly double-distilled water and stored at 277K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship^[9]:

$$F_{\text{cor}} = F_{\text{obs}} \times e^{(A_{\text{ex}} + A_{\text{em}})/2} \quad (1)$$

Where, F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively. A_{ex} and A_{em} are the absorbance values of cefoxitin sodium at excitation and emission wavelengths, respectively. The fluorescence intensity used in this paper was corrected.

Procedures

Classical fluorescence spectroscopy measurements: In a typical fluorescence measurement, 1.0 mL of Tris-HCl solution, 1.0 mL of 2.0×10^{-6} mol·L⁻¹ BSA solution and different concentrations of CFXS were successively added to a 10-mL colorimetric tube. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 30 min at different temperatures (298K, 310K and 318K). The excitation and emission slits were set at 5 nm. The excitation wavelength for bovine serum albumin was 280 nm and 295 nm, respectively, with a 10 nm path length cell. The intensity of fluorescence was recorded at 340 nm.

Elastic scattering fluorescence spectroscopy measurements: 1.0 mL of Tris-HCl solution, 0.2 mL

of $1.0 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ CFXS solution and different concentrations of BSA were successively added to a 10-mL colorimetric tube. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 20 min at different temperatures (298K, 310K and 318K). The excitation and emission slits were set at 5 nm. The fluorescence spectra were measured ($\Delta\lambda$ at 0 nm and emission wavelengths of 220-450 nm) with a 10 nm path length cell. The fluorescent intensity I was recorded.

UV-visible measurements: 1.0 mL of Tris-HCl solution, 1 mL of $1.0 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ CFXS solution and different concentrations of BSA were successively added to a 10-mL colorimetric tube, and the reference solutions were the corresponding concentration of BSA solutions. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 20 min at different temperatures (298K, 310K and 318K). The UV-vis absorption spectrum of CFXS in the presence and absence of BSA were scanned with 1cm quartz cells over the range from 190 to 350 nm.

RESULTS AND DISCUSSION

Classical fluorescence spectra of BSA-CFXS system

The classical fluorescence spectra of BSA-

CFXS system were shown in Figure 2 with focus on the fluorescence change of protein. As shown in Figure 2, the fluorescence intensity of BSA decreased gradually with the addition of CFXS. The result showed that CFXS could quench the intrinsic fluorescence of BSA significantly and there was an interaction between CFXS and BSA^[10-11].

To evaluate the magnitude and nature of the quenching phenomenon, the fluorescence emission spectra were analyzed using the well known Stern-Volmer Eq. (2)^[12-13]:

$$F_0/F = 1 + K_q \tau_0 [L] = 1 + K_{sv} [L] \quad (2)$$

Where, F_0 and F are the fluorescence intensities of BSA in the absence and presence of the CFXS, respectively. And τ_0 is the average lifetime of fluorescence without quencher, which is about 10^{-8} s ^[14]. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant and $[L]$ is the concentration of the quencher. Based on the linear fit plot of F_0/F versus $[L]$, values of K_{sv} and K_q could be obtained at different temperatures. The calculated results were shown in TABLE 1. From TABLE 1, we could see that the K_{sv} values were inversely correlated with temperatures. That is to say, the extent of fluorescence quenching of CFXS to BSA was reduced with rising temperature, which suggested that the fluorescence quenching of BSA were initiated by the formation of ground-state complex rather than

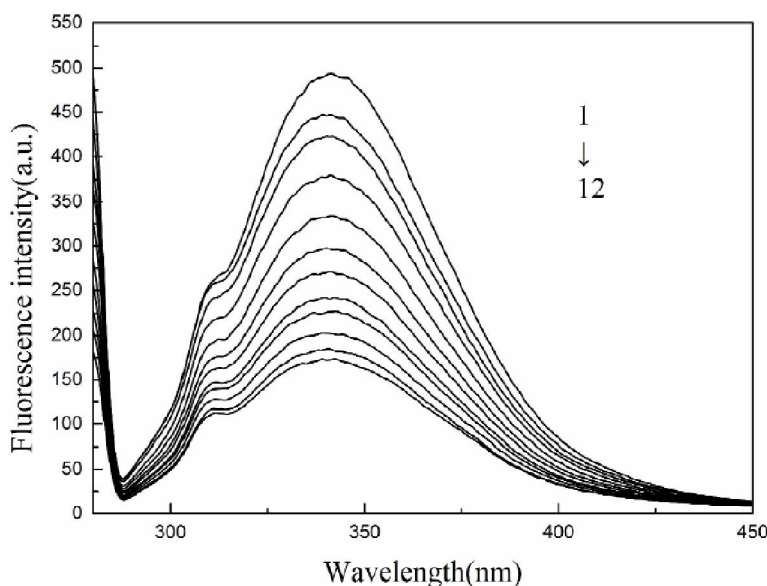


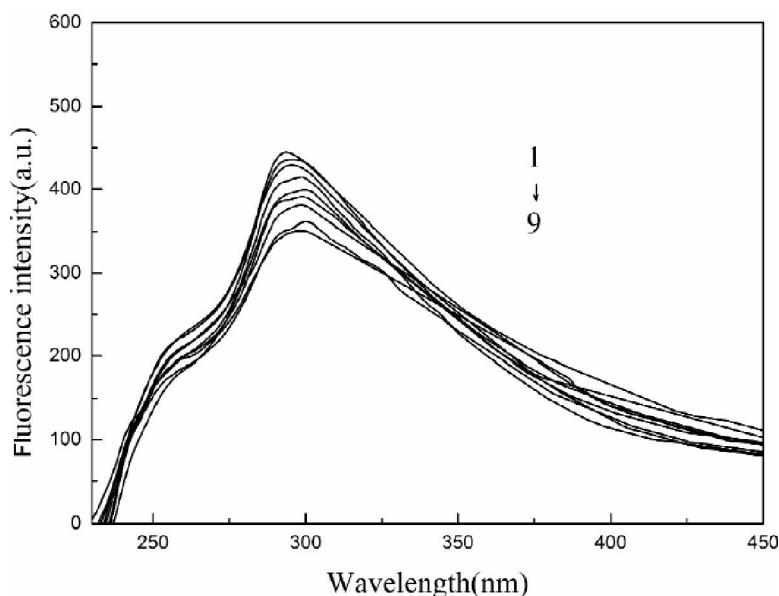
Figure 2 : Fluorescence spectra of BSA-CFXS ($T = 298 \text{ K}$, $\lambda_{ex} = 280 \text{ nm}$) $C_{BSA} = 2.0 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$, 1~12 $C_{CFXS} = (0, 0.1, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$

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TABLE 1 : Quenching reactive parameters of CFXS and BSA at different temperatures

$\lambda_{\text{ex}}(\text{nm})$	$T/(\text{K})$	$K_q/(\text{L}\cdot\text{mol}^{-1}\cdot\text{s}^{-1})$	$K_{sv}/(\text{L}\cdot\text{mol}^{-1})$	r_1	$K_a/(\text{L}\cdot\text{mol}^{-1})$	n	r_2
	298	1.86×10^{12}	1.86×10^4	0.9974	1.68×10^4	0.89	0.9947
280	310	1.66×10^{12}	1.66×10^4	0.9960	1.56×10^4	0.82	0.9953
	318	1.38×10^{12}	1.38×10^4	0.9953	1.43×10^4	0.92	0.9951
	298	6.61×10^{11}	6.61×10^3	0.9948	6.74×10^3	1.01	0.9942
295	310	5.29×10^{11}	5.29×10^3	0.9954	4.83×10^3	0.85	0.9935
	318	4.61×10^{11}	4.61×10^3	0.9941	4.24×10^3	0.72	0.9953

K_q is the quenching rate constant; K_{sv} is the Stern-Volmer quenching constant; K_a is the binding constant; n is the number of binding site; r_1 is the linear relative coefficient of $F_0/F\sim[L]$; r_2 is the linear relative coefficient of $\lg [(F_0-F)/F]\sim\lg [L]$.



$$C_{\text{CFXS}} = 2.0 \times 10^{-5} \text{ mol}\cdot\text{L}^{-1}, 1\sim 9C_{\text{BSA}} = (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0) \times 10^{-7} \text{ mol}\cdot\text{L}^{-1}$$

Figure 3 : Elastic scattering fluorescence spectra of BSA-CFXS system ($T = 298 \text{ K}$)

by dynamic collision^[15]. As a rule, the maximum scatter collision quenching constant, k_q of various quenching with the biopolymer was $2.0 \times 10^{10} \text{ L}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ ^[16]. Considering that in our experiment the rate constants of the protein quenching procedure initiated by CFXS were higher than the maximum value possible for diffusion limited quenching in solution ($\sim 10^{10} \text{ L}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$). It suggested that the static quenching was dominant in the interaction between CFXS and BSA^[17].

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher can be usually described by derived Eq. (3)^[18-19] to obtain the binding constant (K_a) and the number of binding sites (n) in most papers:

$$\lg\{(F_0 - F)/F\} = \lg K_a + n \lg [L] \quad (3)$$

Where, K_a and n are the binding constant and the number of binding sites, respectively. From Eq. (3), the binding parameters can be obtained by the plot of $\lg [(F_0 - F)/F]$ versus $\lg [L]$. The values of K_a and n at 298 K, 310 K, and 318 K are listed in TABLE 1. As shown in TABLE 1, from the value of n , it was found that there was one independent class of binding sites on BSA for CFXS, which indicated that a CFXS molecule was bound to one BSA molecule. Meanwhile, the K_a decreased with the rising temperature, further suggested that the quenching was a static process^[20].

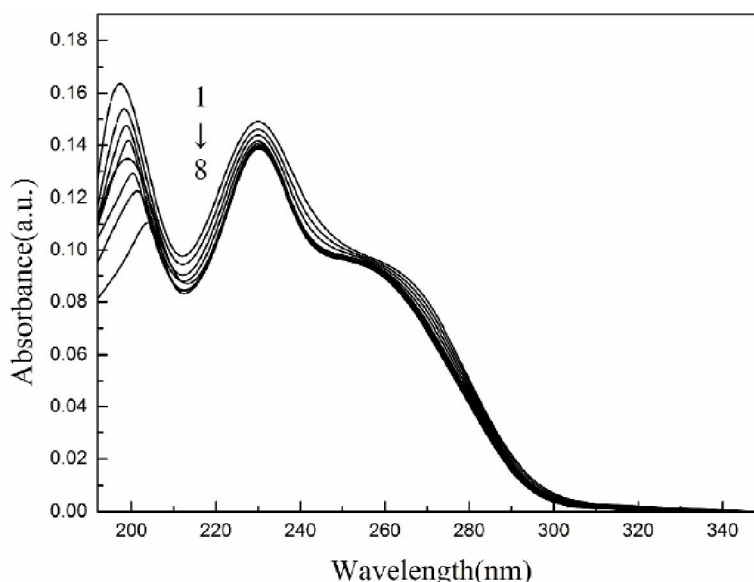
RLS of BSA-CFXS system

According to the experiment as discussed section elastic scattering fluorescence spectroscopy measurements, the interaction between BSA and

TABLE 2 : Elastic scattering fluorescence quenching reactive parameters of CFXS and BSA at different temperatures

$T/(K)$	$K_{qt}/(L \cdot mol^{-1} \cdot s^{-1})$	$K_{svl}/(L \cdot mol^{-1})$	r_3	$K_{al}/(L \cdot mol^{-1})$	n	r_4
298	9.22×10^{13}	9.22×10^5	0.9981	8.15×10^5	0.99	0.9968
310	8.15×10^{13}	8.15×10^5	0.9986	6.71×10^5	0.98	0.9986
318	5.80×10^{13}	5.80×10^5	0.9993	6.12×10^5	1.00	0.9994

K_{qt} is the quenching rate constant; K_{svl} is the Stern-Volmer quenching constant; K_{al} is the binding constant; n is the number of binding site; r_3 is the linear relative coefficient of $F_0/F \sim [L]$; r_4 is the linear relative coefficient of $\lg [(F_0 - F)/F] \sim \lg [L]$



$$C_{\text{CFXS}} = 2.0 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}, 1 \sim 8 \text{ } C_{\text{BSA}} = (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0) \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$$

Figure 4 : Absorption spectra of BSA-CFXS system ($T = 298 \text{ K}$)

CFXS with CFXS as the detection object was investigated. The elastic scattering fluorescence spectroscopy of BSA-CFXS system was shown in Figure 3. As seen in Figure 3, the scattering intensity of CFXS decreased gradually with the addition of BSA to CFXS solution, which suggested that there was an interaction between CFXS and BSA. According to the Eq. (2) and (3), the calculated results were shown in TABLE 2. As seen in TABLE 2, the number of binding site (n) was all approximately to 1. Meanwhile, K_{al} and K_{svl} all were reduced with the rising temperatures, and the values of K_q were much greater than the maximum scatter collision quenching constant of various quenchers ($2 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) under different temperatures, further suggested that the quenching was a static process, which were consistent with the results of classical fluorescence spectroscopy. This indicated that taking protein or drug as the object of detection, although detection methods were different, it could get the same interaction

mechanism between drugs and protein. Moreover, the K_{al} values of elastic scattering fluorescence spectroscopy with drug as detection object were much greater than the K_a values of classical fluorescence spectroscopy with protein as detection object at the same temperature, which showed that the others also interacts with CFXS besides Trp-212 of BSA. In addition to the “point to point” interaction between CFXS and Trp-212, the “point to surface” interaction between CFXS and the other peptides in BSA hydrophobic sub-domain also exists^[21]. This shows that treating drugs as detection object can give more complete and more accurate expression of the interaction information of protein and drugs than classical fluorescence spectroscopy with protein as detection object.

UV-visible spectra studies

The binding constant K_b of BSA with CFXS can be calculated on the following Eq. (4):

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TABLE 3 : The binding constants of BSA-CFXS system by UV absorption spectrometry at different temperatures

T/(K)	K_b /(L·mol ⁻¹)	Linear regression equation	r_s
298	8.90×10^5	$(A_0 - A)^{-1} = 8.280 + 9.295 \times 10^{-6} [L]^{-1}$	0.9942
310	6.81×10^5	$(A_0 - A)^{-1} = 7.523 + 1.105 \times 10^{-5} [L]^{-1}$	0.9981
318	6.53×10^5	$(A_0 - A)^{-1} = 6.689 + 1.025 \times 10^{-5} [L]^{-1}$	0.9998

K_b is the binding constant; r_s is the linear relative coefficient of $(A_0 - A)^{-1} \sim [L]^{-1}$.

$$(A_0 - A)^{-1} = A_0^{-1} + K_b^{-1} A_0^{-1} [L]^{-1} \quad (4)$$

Where, A_0 and A are the absorption values in the absence and presence of quencher, respectively. And $[L]$ is the concentration of the quencher. The UV-vis absorption spectra of CFXS in the absence and presence of BSA were shown in Figure 4. As shown in Figure 4, with gradual addition of BSA to CFXS solution, the intensity of the peak at 195 nm decreased with 10 nm red shifts, indicating that the interaction between BSA and CFXS led to the formation of a complex between drug and protein. Based on the linear regression plot of $(A_0 - A)^{-1}$ versus $[L]^{-1}$, the K_b values can be obtained. The calculated results were shown in TABLE 3. As seen in TABLE 3, the binding constant K_b decreased with rising temperatures, which was consistent with the results of classical fluorescence spectroscopy. The values of K_b were observed to be much larger than K_a of the classical fluorescence spectroscopy and close to K_{el} obtained by the elastic scattering fluorescence spectroscopy. This phenomenon also shows that treating the drug as detection objects can give more complete and more accurate expression for the interaction information of proteins and drugs. In this experiment, because of the calculation formula of reaction between drug and protein was inferred by Scatchard model, the differences of binding constants may be caused by the differences between fluorescence method and UV-visible method.

Thermodynamic parameters and nature of the binding forces

The interaction forces acting between a small molecule and biological macromolecule mainly include hydrogen bond, van der Waals force, electrostatic and hydrophobic interactions, etc. According to the relevant thermodynamic parameters of small molecule drugs with biological macromolecules, the type of interaction force can be simply judged^[22]. If

the temperature does not vary significantly, the ΔH can be regarded as a constant^[23], then its value and that of the entropy change (ΔS) can be determined from Eq. (5)^[24]:

$$R \ln K = \Delta S - \Delta H / T \quad (5)$$

Where, K is the binding constant at corresponding temperature and R is the gas constant. The enthalpy change (ΔH) and the entropy change (ΔS) were obtained from the slope and intercept of Eq. (5) plot based on $R \ln K$ versus T^{-1} .

The Gibbs energy change (ΔG) was estimated from the following relationship:

$$\Delta G = -RT \ln K = \Delta H - T \Delta S \quad (6)$$

The values of thermodynamic parameters K , ΔH , ΔS and ΔG were listed in TABLE 4. As shown in the TABLE 4, the negative value of ΔG clarified a spontaneous reaction between BSA and CFXS. The negative value of ΔH and positive value of ΔS showed that CFXS mainly bound to BSA by the electrostatic attraction^[25]. The thermodynamic parameters obtained by elastic scattering fluorescence spectroscopy, classical fluorescence spectroscopy and UV-visible spectroscopy were consistent, so that we can make a conclusion that the study of reaction mechanism of CFXS with BSA was feasible with treating drugs as detection object by elastic scattering fluorescence spectroscopy and UV-visible spectroscopy.

Hill's coefficient of BSA-CFXS system

According to the Hill's coefficient of BSA-CFXS system, we can make a quantitative analysis for cooperativity between protein and ligands on the basis of the following equation^[26]:

$$\lg \frac{Y}{1 - Y} = \lg K + n_H \lg [L] \quad (7)$$

Where, Y is the fractional binding saturation; fraction of sites occupied with the ligand; n_H is the Hill's coefficient; K is the binding constant. Hill's coeffi-

TABLE 4 : The thermodynamic parameters of BSA-CFXS at different temperatures

Method	T/(K)	K/(L·mol ⁻¹)	ΔH/(KJ·mol ⁻¹)	ΔS/(J·mol ⁻¹ ·K ⁻¹)	ΔG/(KJ·mol ⁻¹)
Classical fluorescence spectroscopy	298	1.68×10 ⁴		60.09	-24.10
	310	1.56×10 ⁴	-6.20	60.28	-24.88
	318	1.43×10 ⁴		60.06	-25.30
Elastic scattering fluorescence spectroscopy	298	8.15×10 ⁵		74.94	-33.72
	310	6.71×10 ⁵	-11.39	74.80	-34.58
	318	6.12×10 ⁵		74.96	-35.23
UV-visible spectroscopy	298	8.90×10 ⁵		71.42	-33.94
	310	6.81×10 ⁵	-12.65	70.84	-34.62
	318	6.53×10 ⁵		71.52	-35.40

K is the binding constant; ΔH is the enthalpy change; ΔS is the entropy change; ΔG is the free energy change.

TABLE 5 : Hill's coefficient of BSA-CFXS systems at different temperatures

T / (K)	Classical fluorescence spectroscopy		Elastic scattering fluorescence spectroscopy		UV-visible spectroscopy	
	n_H	r_6	n_H	r_6	n_H	r_6
298	1.02	0.9995	1.02	0.9966	0.97	0.9957
310	1.00	0.9998	1.01	0.9981	1.04	0.9967
318	0.99	0.9923	0.99	0.9993	1.00	0.9993

r_6 is the linear relative coefficient of $\lg [Y/(1-Y)] - \lg [L]$; n_H is the Hill's coefficient.

coefficient is greater than 1, which exhibits positive cooperativity and its role is enhanced with increasing n_H . Conversely, Hill's coefficient is less than 1, which exhibits negative cooperativity and its role is enhanced by decreasing n_H . A coefficient of 1 indicates non-cooperative reaction^[27].

For fluorescence measurement:

$$\frac{Y}{1-Y} = \frac{Q}{Q_m - Q} \quad (8)$$

$$Q = \frac{F_0 - F}{F_0} \quad (9)$$

Where $1/Q_m$ is intercept of the plot $1/Q$ versus $1/[L]$. According to the formula (7), Hill's coefficient of BSA-CFXS system can be gained from the slope of the plot of $\lg [Y/(1-Y)]$ versus $\lg [L]$. The results were presented in TABLE 5. From TABLE 5, it could be seen that the values of n_H were equal to 1 approximately at different temperatures by classical fluorescence spectroscopy, elastic scattering fluorescence spectroscopy and UV-visible spectroscopy, which indicated that there was non-cooperative reaction between BSA and CFXS. Meanwhile, it illustrated elastic scattering fluorescence spectroscopy

and UV-visible spectroscopy is correct to determine synergy between drug and protein.

CONCLUSION

In this paper, the interaction of cefoxitin sodium with bovine serum albumin was studied at different temperatures by the methods of classical fluorescence spectroscopy and elastic scattering fluorescence spectroscopy and verified by UV-visible spectroscopy. Compared binding constants of the three methods, shows that the K_a values of classical fluorescence spectroscopy was smaller than elastic scattering fluorescence spectroscopy and UV-visible and the later two values are close to each other. That means taking drugs as detection object for elastic scattering fluorescence spectroscopy can be more comprehensive and more accurate when expressing the interaction information between protein and drug in terms of fluorescence. In addition to Trp of bovine serum albumin peptides, the others also interact with cefoxitin sodium. Although the values for the elastic scattering fluorescence spectroscopy and

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UV-visible of binding constant are slightly different, the difference is minimal. It also suggests that the elastic scattering fluorescence spectroscopy method is reasonable. Elastic scattering fluorescence spectroscopy provides a new way to study the interaction more accurately between drugs and proteins, which will further improve the study of the reaction mechanism between drugs and proteins.

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