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RESEARCH ARTICLE

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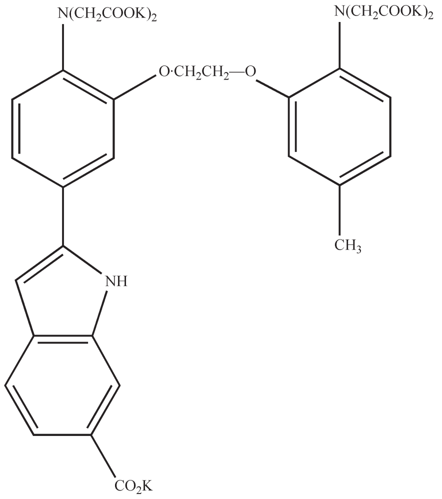
Interaction between bovine serum albumin and Indo-1 using fluorescence spectroscopic method

Abstract This work attempts to calculate the binding-site number using fluorescence spectroscopic method with bovine serum albumin (BSA) and Indo-1 as protein and ligand models, respectively. The method for calculating the binding-site number in BSA for Indo-1 was developed based on the relationships between changes in Indo-1 fluorescence intensity and the analytical concentration of BSA. The interaction between BSA with Indo-1 was investigated comprehensively using fluorescence techniques as well as fluorescence resonance energy transfer, and the thermodynamic parameters were calculated according to the effect of enthalpy on temperature. Three binding sites in BSA for Indo-1 were revealed, and the distances from Trp212 in BSA to the three binding sites were 2.93, 2.57 and 2.40 nm, respectively. It was also proven that Indo-1 embedded into the three hydrophobic cavities of BSA by hydrophobic association. This paper provides a reference on calculating the binding-site number in proteins for ligands and studying their interactions by fluorescence spectroscopic methods. In fluorescent quenching experiments, fluorescence changes were automatically recorded in real time by combining the Microlab 500 Series Dispenser and PTI fluorescence apparatus.

Keywords bovine serum albumin, Indo-1, binding-site number, interaction, fluorescence spectroscopic methods

1 Introduction

Fluorescence techniques are used in environmental monitoring, clinical chemistry, DNA sequencing, genetic analysis, cell identification and cell imaging to reveal the localization and movement of intracellular substances [1]. Measuring fluorescent change is a practical method to study protein interactions with other substances [2,3]. There are many reports on the study of interactions between proteins with ligands using fluorescence spectroscopic methods [4–9]. In studying such interaction, different methods for calculating the binding-site number between proteins and ligands were used [10,11]. Serum albumin is one of the most abundant proteins that plays an important role in the transport and deposition of various ligands in blood [12,13]. The interactions of Indo-1 (Scheme 1) with other proteins have been reported [14–16], but there have been no reports on the interaction of BSA with Indo-1. Thus, bovine serum albumin (BSA) and Indo-1 were selected in this study. In this work, we attempt to provide a fluorescence spectroscopic method for calculating the binding-site number as BSA interacts with Indo-1, and we hope this study can provide some reference in studying the interactions of proteins with ligands.



Scheme 1

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2 Materials and methods

2.1 Materials

BSA (type A-2153) was purchased from Sigma. The stock solution of BSA was prepared by dissolving a certain amount of BSA in 10 ml water and keeping it in a refrigerator. The accurate concentration of BSA was determined by spectrophotometry. The work solution and micromolar concentrations of BSA were prepared by diluting the stock solution with buffer solution. Indo-1, tetrapotassium salt was obtained from Sigma, stored below 0°C and protected from light. The different concentration solutions of Indo-1 were freshly prepared just before the experiment and kept in a dark place during the experimental interval. The buffer solutions were prepared with 1% HEPES. The water used in the experiments was purified by a Millipore Milli-Q system until its resistance reached 18.2 MΩ. All the other reagents were analytical reagent grade.

2.2 Spectral measurements

Emission spectrum, excitation profiles and fluorescence intensity measurements were performed on QuantMaster™ Luminescence Spectrometers from PTI equipped with a sample stirrer and a temperature controller (Heto CBN 8-30 made in Denmark). A fluorescence grade quartz cuvette (1.0 cm path length, 4.0 ml volume) was used with the fluorescence instrument. In quenching experiments, fluorescence was monitored using the TIMEBASED mode of PTI software, and fluorescence intensity was recorded one point each second. At the same time, the sample solution was titrated by the quencher's solution using a Microlab 500 Series manufactured by Hamilton. Thus, the changes of fluorescence intensity were automatically recorded in real time by combining the Microlab 500 Series and PTI fluorescence apparatus. The absorption spectra were measured on a Cary Model 500 spectrophotometer from Varian (USA) with a quartz cuvette (1.0 cm path length, 1.4 ml volume). Except for studying the effect of pH, all the solutions were measured at pH7.4 since it is close to the physiological pH value.

3 Results and discussion

3.1 Interactions between BSA and Indo-1

The measured absorption spectra are shown in Fig. 1. Both concentrations of BSA and Indo-1 are 20 μM whether they existed separately or together. The determined solutions were buffered with 1% HEPES (pH7.4). As BSA and Indo-1 existed separately, they presented an absorption maximum at 280 and 340 nm, respectively. No obvious changes in absorbency around 280 nm were observed, and a new absorption peak emerged at 355 nm as they coexisted. In contrast to the absorption spectrum of Indo-1, the absorption at 300–380 nm decreased significantly and the absorption maximum shifted from 340 nm to 355 nm. These results provided clues on the interaction of BSA with Indo-1.

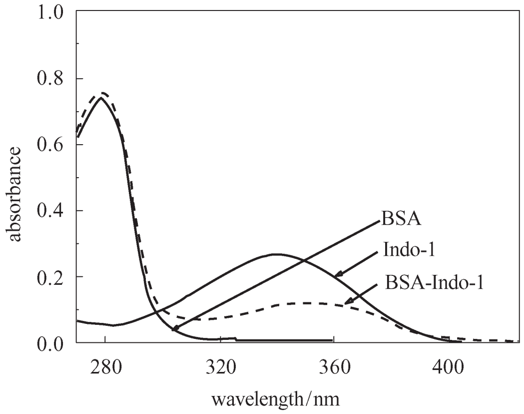

Fig. 1 Absorption spectra of differently determined solutions

Fluorescent measurements also provided clues on the interaction of BSA with Indo-1. Either Indo-1 or BSA quenched the other's fluorescence as they coexisted. Excited at 284 nm, fluorescence intensity at 342 nm decreased with increasing fluorescence intensity at 454 nm when the amount of Indo-1 added was increased (Fig. 2). An isoemissive point indicated that these spectra were linear combinations of two components and reflect equilibrium between these components [17], providing evidence on the interaction of BSA with Indo-1. Furthermore, the emission spectra of Indo-1 in the range of 360–500 nm, excited at

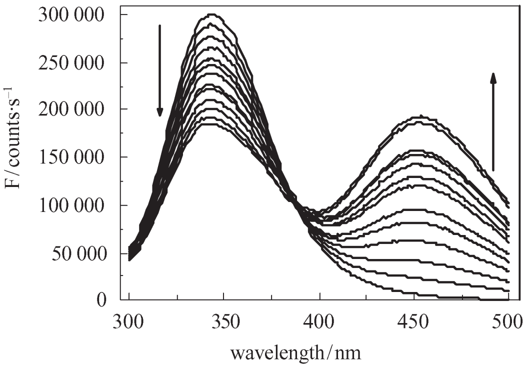

Fig. 2 Emission spectra of BSA-Indo-1 as a function of the concentration of Indo-1. BSA concentration was 2.0 μM. Indo-1 concentration increased from 0 to 6.0 μM, respectively. The measurements were carried out under room temperature with an excitation at 284 nm. The solutions' acidity was pH7.4. The arrows indicate the changes of the signals as a function of Indo-1 concentration

335 nm, were monitored as increasing amounts of BSA and yielded similar results. With the increment in BSA concentration, the fluorescence intensity at 393 nm decreased while the fluorescence intensity at 454 nm increased. All the results indicated that there was fluorescence quenching accompanied by the interactions of BSA with Indo-1, and possibly fluorescence resonance energy transfer (FRET) between BSA and Indo-1.

The new excitation and emission peaks at 362 and 454 nm respectively presumably resulted from the interaction of BSA and Indo-1; moreover, the new spectra that emerged were assumed to belong to BSA-Indo-1 system. Fluorescence spectra in the range of 382–600 nm, excited at 362 nm, were thus used to study the effect of pH on the interaction of BSA with Indo-1.

3.2 Effect of pH on the interaction of BSA with Indo-1

Results showed that the $\lambda_{em.}$ of BSA and BSA-Indo-1 located about 415 and 454 nm respectively at pH4.0–9.6. The emission-peak position of Indo-1 ranges from 393 and 475 nm due to the protoned and nonprotoned forms of Indo-1, which has a pK_a close to 7 [18] and showed different fluorescence forms at different pH [19]. The λ_{em} of Indo-1 changes in the range of 475–393 nm and had a maximum value at 393 nm. At pH7.0–8.0, BSA-Indo-1 has the strongest fluorescence intensity compared to Indo-1 and BSA, and the greatest λ_{em} difference between BSA- Indo-1 and Indo-1. pH7.4, which is close to the physiological pH value, was thus selected for all further studies.

3.3 Fluorescence quenching between BSA and Indo-1

To prove that quenching of BSA fluorescence by Indo-1 is either a static or dynamic process, the quenching measurements were excited at 284 nm and the emission monitored at 342 nm under different temperatures, 275, 290.5 and 310 K. The plots in Fig. 4 were obtained according to the Stern-Volmer equation [1b],

$$F_0/F = 1 + k_q \tau_0 c_q \tag{1}$$

where F_0 and F are the fluorescence intensity in the absence and presence of a quencher respectively, k_q is the biomolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of a quencher, and c_q is the concentration of quencher (in this study, the concentration of Indo-1). The linear regression equations on c_q and F_0/F were obtained by linear fitting. For biological macromolecules, the average value of τ_0 is about 10^{-8}s [15]. From the slopes and τ_0, the values of k_q at temperature 275, 290.5 and 310 K were calculated to be 2.681×10^{13}, 2.892×10^{13} and 3.169×10^{13} $M^{-1}s^{-1}$ respectively. Since the values of k_q are higher than 1.0×10^{10} $M^{-1}s^{-1}$, which is the maximum diffusion constant for all kinds of quenchers to biological macromolecules, the quenching of BSA fluorescence by Indo-1 is not a dynamic but a static one induced by the BSA forming complex with Indo-1 [12].

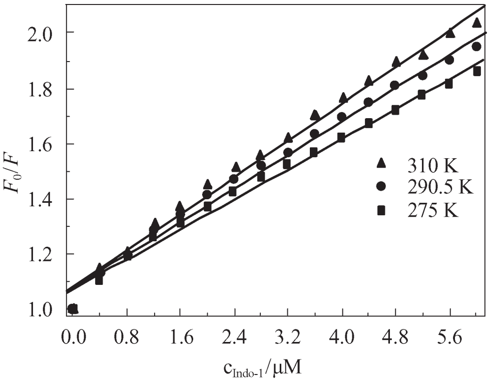

Fig. 3 The Stern-Volmer plots under different temperatures. The BSA concentration is 2.00 μM; the concentrations of Indo-1 were increased from 0 to 6.00 μM. The measurements were carried out under temperature 275, 290.5 and 310 K with an excitation at 284 nm and emission at 342 nm. The solutions pH value is 7.4

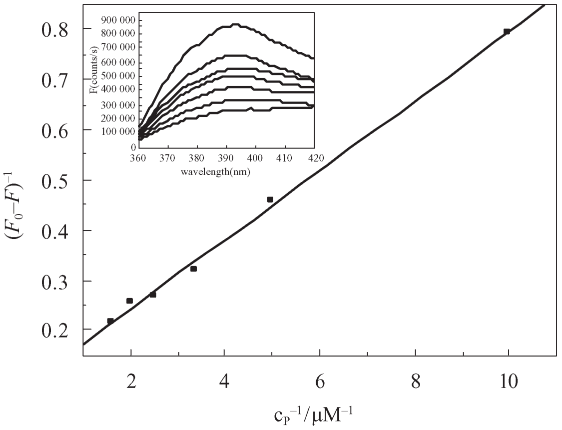

Fig. 4 Plot of $(F_0\text{-}F)^{-1}$ versus c_P^{-1} under room temperature. The concentration of Indo-1 was 2.00 μM and the concentration of BSA increased from 0 to 0.60 μM. The fluorescence intensity at 393 nm was measured with an excitation at 335 nm under pH7.4. As quenched by BSA, the emission spectra of Indo-1 were presented as inserts

3.4 Interaction-force mode of BSA and Indo-1

To understand the interaction mode between BSA and Indo-1, quenching data were analyzed according to the modified Stern-Volmer equation [1c],

$$\frac{F_0}{F_0 - F} = \frac{1}{f_A} + \frac{1}{f_A K_A c_q} \tag{2}$$

where K_A is the Stern-Volmer quenching constant of the accessible fraction and f_A is the fraction of the initial fluorescence accessible to quencher. This modified equation enables f_A and K_A to be determined graphically by the plots of $F_0/(F_0-F)$ versus $1/c_q$ yields $(f_A)^{-1}$ as the intercept and $(f_AK_A)^{-1}$ as the slope. The Stern-Volmer quenching constant K_A was calculated based on the modified Stern-Volmer equation. Table 1 shows that the interaction of BSA with Indo-1 is very strong and the constant K_A increased with increasing temperature.

Table 1 Thermodynamic parameters on the interaction of BSA with Indo-1 at pH7.4

temperature/K	K_A/L·mol^{-1}	ΔG^0/ kJ·mol^{-1}	ΔH^0/ kJ· mol^{-1}	ΔS^0/ J·mol^{-1}·K^{-1}
275	9.89×10^5	−31.6	6.96	140
290.5	1.17×10^6	−33.7		
310	1.40×10^6	−36.5		

To understand the interaction of BSA with Indo-1 in more detail, the thermodynamic parameters were calculated with similar methods to reference [20]. If the enthalpy change (ΔH^0) does not vary significantly over the temperature range in the study, then its value and that of entropy change (ΔS^0) can be calculated from the Van't Hoff equation,

$$\ln K_A = -\Delta H^0/RT + \Delta S^0/R \tag{3}$$

and free energy change can be estimated from the following relationship,

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{4}$$

In Eq.(3) and Eq.(4), K_A refers to the same factor as in Eq.(2) and R is the gas constant.

The values of ΔG^0, ΔH^0 and ΔS^0 calculated from Eq.(3) and Eq. (4) are shown in Table 1. The negative values for ΔG^0 show that BSA binds to Indo-1 spontaneously. Both ΔH^0 and ΔS^0 have positive values, which indicate that Indo-1 binds to BSA by hydrophobic association and possibly including protonation [21]. The development of entropic control of ΔG^0 is a consequence of the hydrophobic interaction dominating the association process [21]. BSA consists of three similar structure domains - Site I, Site II and Site III - and each has a pocket-like structure consisting of two anti-parallel subdomains [22]. Almost all hydrophobic amino acid residues are located in the cavities, which are composed of three hydrophobic cavities. Hydrophobic association dominated the interaction of BSA with Indo-1 and Indo-1 embedded into those three hydrophobic cavities in BSA without excluding protonation.

Furthermore, values of ΔH^0 which were more than zero indicated that the interaction of BSA with Indo-1 was an endothermic reaction, i.e., increasing temperature promoted the interaction of BSA with Indo-1, and also fluorescence quenching between BSA and Indo-1. Those conclusions were consistent with the results in Fig. 3, which indicated that increasing temperature could promote fluorescence quenching and the quenching constants. Based on the relationships of temperature and static fluorescence quenching, increasing temperature should reduce fluorescence quenching, although that conclusion is limited to fluorescent materials and quenchers which have infirm binding force [1d]. Both k_q and K_A had bigger values, which indicated that fluorescent quenching and the interaction of BSA with Indo-1 were intense and the binding force between BSA and Indo-1 was stronger.

3.5 Binding-site number in BSA to Indo-1

Quenching data of Indo-1 fluorescence by BSA were used to calculate the binding-site number. The fluorescence spectroscopic method for calculating the binding-site number was deduced as follows.

Define [L] as the total concentration of Indo-1 unbound to BSA; [L′] as the total concentration of Indo-1 bound to BSA; c_L ($c_{L=}$[L]+[L′]) as the total concentration of Indo-1; [P] as the total concentration of BSA unbound to Indo-1; and c_p as the total concentration of BSA. The experimentally determined fluorescence intensity at 393 nm, excited at 335 nm, can thus be written as,

$$F = k[L] + k'[L'] + k_p[P] \tag{5}$$

where k, k' and k_p are constants corresponding to the fluorescence intensity of each component and its concentration under given conditions. Using Indo-1 concentration as a constant and varying the amount of added BSA, the fluorescence-intensity changes of Indo-1 were monitored under selected conditions. Under these conditions, the added BSA almost binds to Indo-1 completely, indicating that the value of $[P]$ is very small and the value of k_p is significantly less than those of k' and k. Thus, $k_p[P]$ could be omitted and Eq.(5) rewritten as,

$$F = k[L] + k'[L'] \tag{6}$$

Substituting $[L] = c_L - [L']$ into Eq. (6), we derive the following:

$$F = k'[L'] + k\{c_L - [L']\} \tag{7}$$

Letting $\Delta k = k - k'$ and $F_0 = kc_L$, where F_0 is the fluorescence intensity in the absence of BSA, Eq.(7) can be rearranged as,

$$[L'] = (F_0 - F)/\Delta k \tag{8}$$

where Δk is a constant under the given experimental conditions. We then define a new relation as,

$$K_c = [L']/[L]c_P \tag{9}$$

Substituting $[L] = c_L - [L']$ and Eq. (8) into Eq.(9) yields

$$K_c = \frac{(F_0 - F)/\Delta k}{c_L c_P - c_P(F_0 - F)/\Delta k} \tag{10}$$

Where K_c is a constant under given experimental conditions. Our experiments proved this viewpoint (Table 2). Then, rewriting Eq.(10) as

$$(F_0 - F)/\Delta k = K_c c_L c_P - K_c c_P (F_0 - F)/\Delta k \tag{11}$$

Eq. (11) divided by $(F_0 - F)K_c c_L c_P$ is

$$(F_0 - F)^{-1} = (\Delta k c_L)^{-1} + (\Delta k K_c c_L)^{-1} c_P{}^{-1} \tag{12}$$

Table 2 Data used for calculating the value of K_c ($c_L = 2.0$ μM; $\Delta k = 4.57 \times 10^{11}$ counts·s^{-1}·M^{-1})

c_p/M	F/counts·s^{-1}	$(F_0 - F)/\Delta k$	$c_L c_P - c_P(F_0 - F)/\Delta k$	logK_c
0	8.818×10^5	0	0	–
2.0×10^{-7}	6.64×10^5	4.78×10^{-7}	3.04×10^{-13}	6.20
4.0×10^{-7}	5.10×10^5	8.13×10^{-7}	4.75×10^{-13}	6.23
6.0×10^{-7}	4.25×10^5	1.00×10^{-6}	6.00×10^{-13}	6.22
8.0×10^{-7}	3.43×10^5	1.18×10^{-6}	6.57×10^{-13}	6.25
1.0×10^{-6}	2.86×10^5	1.30×10^{-6}	6.96×10^{-13}	6.27
1.2×10^{-6}	2.74×10^5	1.33×10^{-6}	8.04×10^{-13}	6.22
1.4×10^{-6}	2.51×10^5	1.38×10^{-6}	8.67×10^{-13}	6.20
1.6×10^{-6}	2.01×10^5	1.49×10^{-6}	8.16×10^{-13}	6.26
1.8×10^{-6}	1.93×10^5	1.51×10^{-6}	8.85×10^{-13}	6.23
2.0×10^{-6}	1.69×10^5	1.56×10^{-6}	8.81×10^{-13}	6.25

Represent the average binding number of ligand molecules to per protein molecule as

$$\overline{N} = [L']/c_P \tag{13}$$

Substituting Eq. (8) into Eq. (13) yields

$$(F_0 - F) = \overline{N} \Delta k c_P \tag{14}$$

Substituting Eq. (14) into Eq. (11) yields

$$\overline{N} = K_c c_L - K_c \overline{N} c_P \tag{15}$$

Because K_c is a constant and c_L is a fixed value, the maximum number is $N = K_c c_L$, which is the binding-site number per protein for the ligand. Substituting $N = K_c c_L$ into Eq. (12) yields

$$(F_0 - F)^{-1} = (\Delta k c_L)^{-1} + (\Delta k N)^{-1} c_P{}^{-1} \tag{16}$$

Since N, Δk and c_L are fixed values under given conditions, making graph by $(F_0 - F)^{-1}$ versus $c_p{}^{-1}$ can yield a straight line. The results in Fig. 4 suggest that there is a linear relationship between $(F_0 - F)^{-1}$ and $c_p{}^{-1}$ according to Eq. (16). Using 10^5 counts/s and μM as the units of fluorescence intensity and concentration respectively, the regression equation is $(F_0 - F)^{-1} = 0.10943 + 0.06841 c_p{}^{-1}$, with related coefficient $\gamma = 0.9980$. From the slope $(\Delta kN)^{-1}$ and the intercept $(\Delta k c_L)^{-1}$ of the regression equation, the value of N was calculated as 3.07, which indicates there are three binding sites in BSA to Indo-1. It is consistent with the conclusion that there are three similar structure domains in serum albumin [25]. In addition, the relationship between fluorescence intensity of BSA and c_L (concentration of Indo-1) agreed well with a cubic equation, which also indicates that there are three binding sites in BSA to Indo-1.

Furthermore, if c_p is kept constant, the average binding number $N = c_{L,\max}/c_p$ can also be deduced with a method similar to the one above. However, it will produce a relatively bigger error in choosing the datum of $c_{L,\max}$, which is the concentration when Indo-1 binds all sites in BSA. Thus we recommend selecting c_L as a constant to calculate binding-site number.

In studying the interaction of proteins with ligands, different methods for calculating the binding-site number between protein and ligand were used. As a classic method for studying the binding of bio-macromolecules with small ligands, the Scatchard-plot method [10,11] requires the concentration of the free ligand, which is very difficult to realize in fluorescence spectroscopic experiments. Methods based on protein fluorescence and ligand concentration have been used to calculate the binding-site number [12]. Employing these methods require that the binding sites in protein should fit some assumptions, including equality or independence from each other, so that their applications were limited. The method provided in this paper does away with the traces in calculating binding-site number. It can be used to calculate the binding-site number of protein with other ligands whether both of them have fluorescence or not. Although this method has some similarities with literature [23], this method differs from it in principle and has some advantages. It has higher accuracy since fluorescent spectroscopy has more sensitivity than absorption spectrum. In fluorescent analysis, the disturbance factor could be avoided by changing different λ_{ex} or $\lambda_{em.}$ Hence, this method has a larger scope of application and simpler data processing [24,25]. Thus, this method would be more useful than others.

3.6 FRET between BSA and Indo-1

FRET is the transfer of the excited-stated energy from the initially excited donor (D) to the acceptor (A). The donor molecules typically emit at short wavelengths that overlap with the absorption spectrum of the acceptor. The distance between the donor and the acceptor can be calculated according to Förster's theory [26].

$$E = 1 - \frac{F_{DA}}{F_D} = \frac{R_0^6}{R_0^6 + r^6} \tag{17}$$

where F_D and F_{DA} are the corrected fluorescence intensity of the donor in the absence and presence of an acceptor respectively; E is the efficiency of energy transfer; r is the distance between the donor and acceptor; and R_0 is the distance at 50% transfer efficiency.

$$R_0 = 0.211[k^2 n^{-4} \Phi J(\lambda)]^{1/6} \tag{18}$$

where Φ is the quantum yield of the donor in the absence of an acceptor; n is the refractive index of the medium; k^2 is a factor describing the relation orientation in space of the transition dipoles of the donor and acceptor and is usually assumed to be equal to 2/3; and overlap integral J (λ) expresses the degree of spectral overlap between the donor emission and the acceptor absorption,

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \tag{19}$$

F_D (λ) is the fluorescence intensity of the donor in the range from λ to $\lambda + \Delta\lambda$; ε_A (λ) is the extinction coefficient of the acceptor at λ, which is typically in units of $M^{-1}cm^{-1}$. From these relationships, J (λ), R_0 and E can be calculated. Thus, the value of r can also be calculated.

Figure 5 shows the overlap of the emission spectrum of BSA and the absorption spectrum of Indo-1. The overlap integral in Fig. 5 was calculated to be 9.59×10^{13} $M^{-1}cm^{-1}nm^4$. The values of Φ, n and k^2 were taken as 0.118, 1.336 and 2/3 [21], respectively. The value of R_0 calculated based Eq.(18) is thus 2.44 nm. Furthermore, from the experimentally determined data, the energy transfer efficiencies are $E_1 = 0.249$, $E_2 = 0.425$ and $E_3 = 0.523$ when the molar ratios of Indo-1 to BSA are 1:1, 2:1 and 3:1, respectively. Using the same method as literature [12], the distances from Trp212 (donor) to Indo-1 (acceptor) bound at the three binding sites in BSA are $r_1 = 2.93$ nm, $r_2 = 2.57$ nm and $r_3 = 2.40$ nm, respectively. For FRET, the efficient distance between the excited donor and the fluorescent acceptor is typically 2–7 nm [27] and all the calculated values of r are in this range. Hence, there is an energy transfer between BSA and Indo-1.

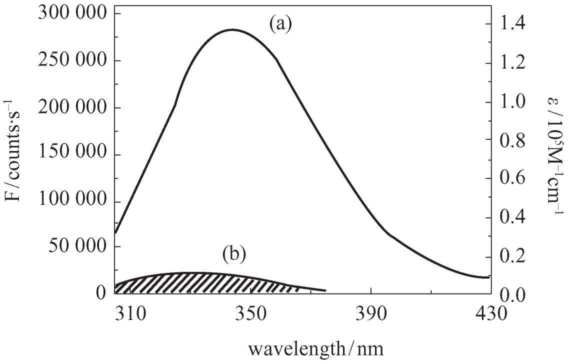

Fig. 5 The fluorescence spectrum (a) of BSA and the absorption spectrum (b) of Indo-1 under room temperature. Both the concentrations of BSA and Indo-1 are 2 μM. All the measurements were carried out with solution acidity of pH7.4. The emission spectrum was excited at 284 nm

4 Conclusions

This study developed an approach for calculating the binding-site number as BSA interacts with Indo-1 using a fluorescence spectroscopic method. First, the interaction of BSA with Indo-1 was proven by the fluorescence quenching experiments. For the interaction mechanism of BSA with Indo-1, the interaction-force mode, binding-site number, quenching form of fluorescence, and binding distance from Trp212 to the binding sties in BSA for Indo-1 were demonstrated. Indo-1, which was shown to have three binding sites in BSA, interacts with BSA by hydrophobic association and forms a new complex with BSA by embedding in the three tub-like hydrophobic cavities of BSA. The results may provide basic knowledge for a better understanding of the properties and structures of the protein involved in the interactions. The method for calculating the binding-site number was developed based on the dependence of Indo-1 fluorescence intensity on the analytical concentration of BSA, which has fewer limitations in contrast to other studies [12,14]. The methods provided in this paper can present some reference in studying the interactions of proteins or enzymes with different ligands such as drugs and probes. This method thus has great importance in terms of methodology, medicine, pharmacology, biology and other fields.

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