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Interaction between felodipine and bovine serum albumin: fluorescence quenching study

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ABSTRACT: The fluorescence quenching spectrum of bovine serum albumin (BSA) was investigated in the presence of felodipine (FLD) by spectroscopic methods including fluorescence spectroscopy and UV–Vis absorption spectroscopy. Stern–Volmer quenching was successfully applied and the corresponding thermodynamic parameters, namely enthalpy change (ΔH), free energy change (ΔG) and entropy change (ΔS) at different temperatures (304, 314 and 324 K) were calculated according to the Van't Hoff relation. This revealed that the hydrophobic interaction plays a major role in stabilizing the complex. The fluorescence spectrum of BSA was studied in presence of various concentrations of SDS surfactant. The distance (r) between donor (BSA) and acceptor (FLD) was obtained according to fluorescence resonance energy transfer (FRET). The synchronous fluorescence spectroscopy was used to investigate the effect of FLD on BSA molecule. The result shows that the conformation of BSA was changed in the presence of felodipine. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: bovine serum albumin; felodipine; fluorescence resonance energy transfer; fluorescence quenching; thermodynamic parameter

Introduction

Felodipine (FLD) is a dihydropyridine drug and a popular calcium chanel blocker. It is known to inhibit the entry of excess calcium into vascular and cardiac muscles, which results in lowering of blood pressure, thereby reducing oxygen demand in the heart and relieving anginal pain. It is a yellowish crystalline powder and has molecular structure as shown in Fig. 1. The melting point and molecular weight are 142–145°C and 383.07 g respectively. Earlier studies[1–3] have shown that the felodipine has been used in the treatment of angina, arrhythmias and hypertension.

Serum albumin such as bovine serum albumin (BSA) is widely studied because of its many physiological functions and being a major soluble protein constituent of the circulatory system. 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.[4–6] In addition to this it is cheap and readily available. BSA is known as 'Fraction V', a serum albumin protein that has numerous biochemical applications in blots, enzyme linked immunisorbent assay (ELIA) and immunohistochemistry. It is used to stabilize certain enzymes and also to prevent adhesion of the enzymes to reaction tubes and other vessel in the process of restriction digestion of DNA. 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.[7] It is therefore important to study the interaction of drugs of interest with BSA.

Although various researchers have studied the structure and properties of serum albumin and its interaction with small molecules such as dyes, drugs and toxic chemicals using the FRET process,[8–14] there has been no report on the drug–protein interaction, especially FLD and BSA. In this regards, in the present work, we made a detailed and insightful study on the interaction between FLD and BSA by using nondestructive FRET spectroscopy. The binding mechanism between FLD and BSA with respect to binding constant, binding site number and thermodynamic parameters was investigated. The interaction of FLD may bring conformational changes in BSA; such conformational changes were evaluated by measuring synchronous fluorescence intensity during protein–drug interaction.

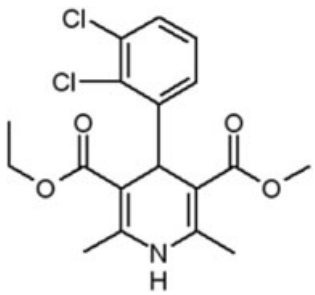

Figure 1. Molecular structure of felodipine.

Experimental

Apparatus and reagents

The fluorescence and fluorescence excitation spectra were recorded on a PC-based spectrofluorophotometer (Jasco model FP-750, Japan) equipped with a xenon lamp source and 1.0 cm quartz cell. The absorption spectra were measured on UV–vis–NIR

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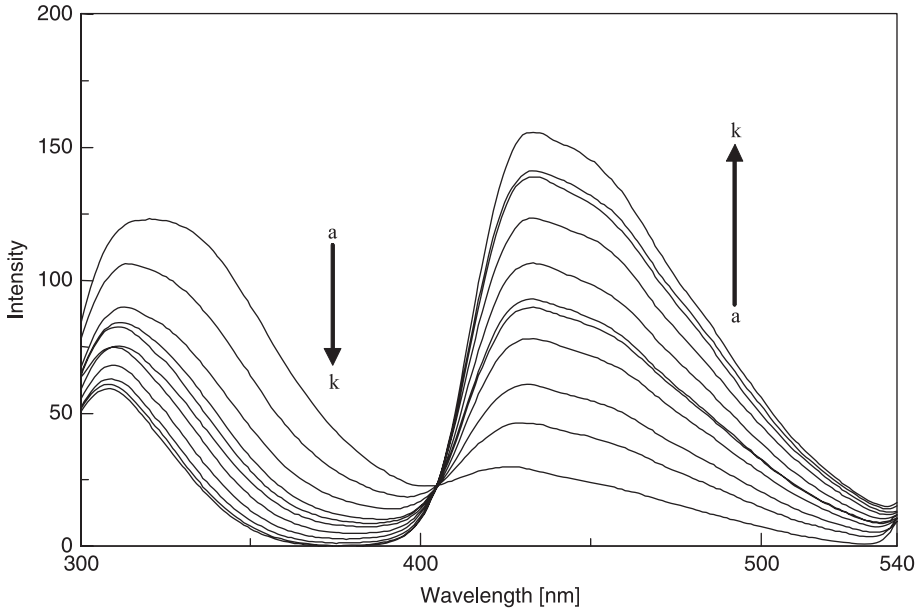

Figure 2. Effect of FLD on fluorescence spectrum of BSA. [BSA] = 2×10^{-6} mol L^{-1}, [FLD] from a to k = 0 – 3×10^{-4} mol L^{-1}.

spectrophotometer (Shimadzu UV-3600). The sample masses were accurately weighed using a microbalance (K-Roy Analytical Balance).

FLD was obtained as gift sample from Cipla Limited Mumbai (batch no. FW 70359). BSA was purchased from Sigma-Aldrich chemicals. The stock solutions of FLD (1×10^{-3} mol L^{-1}) and BSA (1×10^{-5} mol L^{-1}) were prepared by dissolving their appropriate amounts in 4 M acetic acid. The acetic acid procured from Merck was diluted by double distilled water to prepare 4 M acetic acid.

Procedure

The drug–protein solutions were prepared by mixing solution of FLD and BSA in the 4 M acetic acid. BSA concentration was fixed at 2.0×10^{-6} mol L^{-1} while the FLD concentration was varied from 0 to 3.0×10^{-4} mol L^{-1}. Fluorescence quenching spectra were recorded at temperature 304 K in the range of 300–540 nm at excitation wavelength 280 nm. The experiments were repeated to study fluorescence quenching at temperature 314 and 324 K. The range of synchronous scanning was 280–320 and 310–370 nm where the difference in the wavelength $\Delta\lambda$ is 15 and 60 nm, respectively.

Result and discussion

Fluorescence quenching mechanism

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in fluorescence quenching of excited state fluorophores. These include molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. Figure 2 shows the fluorescence spectra of BSA in absence and the presence of FLD. The fluorescence spectrum of BSA shows a sharp broad band with maximum at about 320 nm. It is observed that the fluorescence intensity of BSA decreases with increasing concentration of FLD, having a maximum fluorescence emission wavelength that underwent an obvious blue shift from 320 to 308 nm. The appearance of a peak at 430 nm can also be seen with a clear isoemmesive point at 405 nm. Therefore the FLD-BSA interaction proves the efficient energy transfer between them.

Table 1. Stern–Volmer quenching constants and quenching rate constants for interaction between FLD and BSA at various temperatures

T (K)	K_{SV} ($\times 10^3$ $L mol^{-1}$)	k_q ($\times 10^{11}$ $L mol^{-1}$ S^{-1})	R
304	3.924	3.924	0.9974
314	3.513	3.513	0.9978
324	3.285	3.285	0.9958

R is the correlation coefficient.

The quenching of fluorescence may be static or dynamic and can be recognized by temperature dependence studies. The quenching rate constants are expected to decrease with increase in temperature for static quenching while for dynamic quenching reverse effect is observed.[15] The fluorescence quenching data are analyzed by the Stern–Volmer equation

$$F_0 / F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, and K_{SV} and $[Q]$ are the Stern–Volmer quenching constant and concentration of quencher respectively. The Stern–Volmer plots at various temperatures are shown in Fig. 3. The plots are linear and slope (K_{SV}) decreases with increasing temperature, as given in Table 1. It can be concluded that the quenching is not initiated by dynamic quenching but probably by static quenching, resulting in the formation of BSA-FLD complex. The shift in emission wavelength from 320 to 308 nm further indicates the formation of complex by binds of FLD with BSA sites. The probable mechanism of static quenching is given in Scheme 1.

The variation of F_0 / F against Q (FLD concentration) fits in the equation of $y = mx + c$ with correlation coefficient greater than

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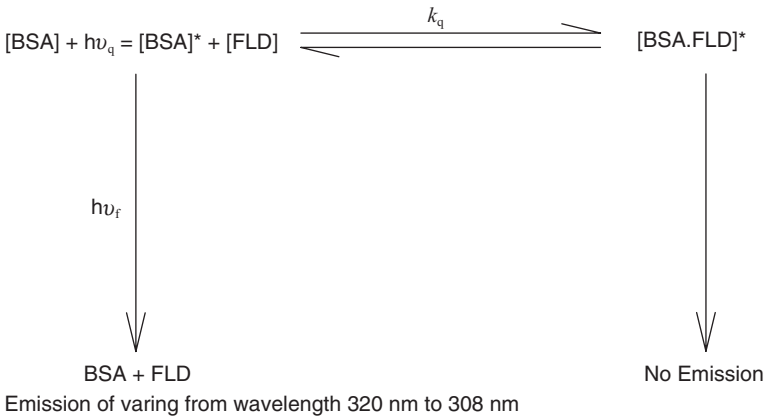

Scheme 1.

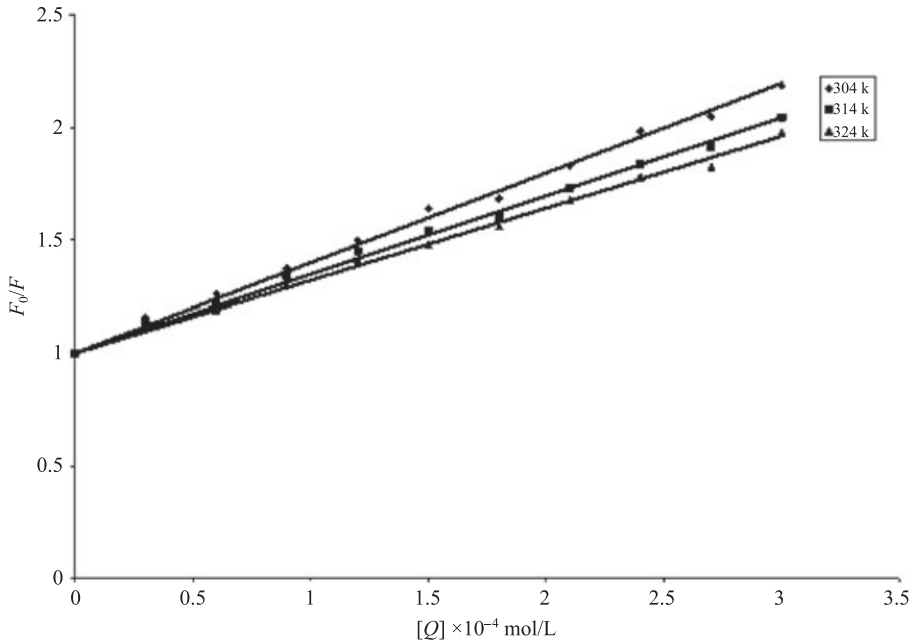

Figure 3. Stern–Volmer plots describing BSA quenching caused by FLD at three different temperatures.

0.9978. The estimated values of kinetic data along with correlation coefficient are given in Table 1. The value of the correlation coefficient indicates that the static quenching fits in the Stern–Volmer relation as considered. The quenching rate constant (k_q) at different temperatures was estimated from the values of K_{SV} and the knowledge of fluorescence life time of biopolymer BSA ($\tau_0 = 10^{-8}$ s)[16] without FLD using the equation:

$$k_q = K_{SV} / \tau_0 \quad (2)$$

Binding constant and binding sites

The binding of FLD with BSA to form complex in the ground state is further understood on the basis of available binding site number and binding constant of the complex formation process. For static quenching, the following equation was used to calculate the binding constant and binding sites:[17,18]

$$\log\left[F_0 - F/F\right] = \log K + n\log[Q] \quad (3)$$

where K and n are the binding constant and binding site number respectively. The plots of log $F_0 - F/F$ vs log [Q] presented in Fig. 4 at different temperatures are linear. The values of binding constant (K) and the binding site number (n) could be calculated from the intercept and slope as shown in Table 2 along with regression coefficient R. The values of regression coefficient nearly equal to one indicate validity of eqn (3). From the table it is also seen

Table 2. The binding parameters for the system of FLD and BSA

T (K)	K (× 10^2 M^{-1})	n[a]	R
304	3.31	0.727	0.9859
314	3.63	0.736	0.9848
324	4.17	0.757	0.9880

[a]The binding site number (n) approximated to 1.
R is the correlation coefficient.

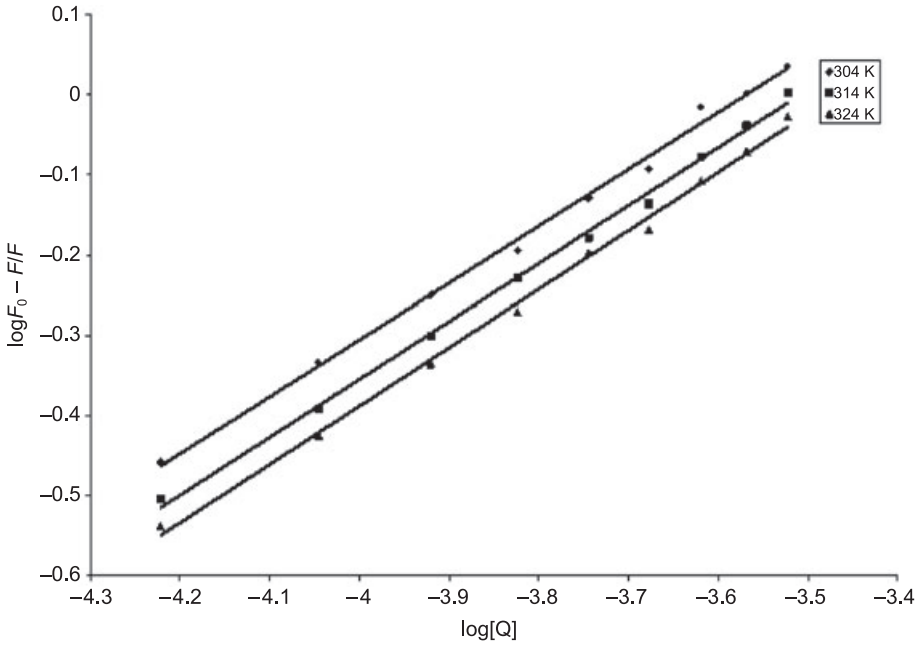

Figure 4. Plots of log $F_0 - F/F$ vs log [Q].

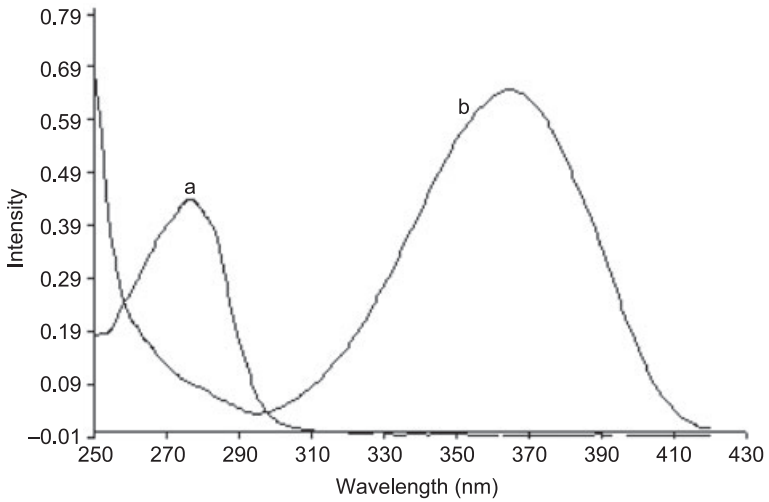

Figure 5. UV–vi. absorption spectra of BSA (a) and FLD (b); [BSA] = 2×10^{-6} mol L^{-1} and [FLD] = 2×10^{-4} mol L^{-1}.

that the binding constant changes very slightly with temperature. This observation suggests that the binding site does not change with temperature. Therefore the present studies involve binding of single molecule of FLD with one molecule of BSA by hydrophobic interaction.

Thermodynamic parameters

The interaction forces between drug and biomolecule include hydrogen bonds, van der Waals forces, electrostatic and hydrophobic interactions.[19] In order to elucidate the interaction of FLD with BSA the temperature dependence binding studies were carried out at 304, 314 and 324 K. The thermodynamic parameters can be evaluated from the Van't Hoff equation:

$$\ln K = -\Delta H / RT + \Delta S / R \quad (4)$$

where K is the binding constant at corresponding temperature T and R is the gas constant. The enthalpy change (ΔH) and entropy change (ΔS) can be obtained from the slope and the ordinates at the origin of the Van't Hoff plot respectively (Fig. 7). The free energy change, ΔG is determined from the following relationship:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

The values of ΔG, ΔS and ΔH are calculated and summarized in Table 3. From the table it can be seen that the negative sign for ΔG indicates that the interaction process is spontaneous.[20] Positive ΔH and ΔS value indicate the less dominant hydrogen bond formation and predominant hydrophobic force between FLD and BSA[21] respectively. Thus the nonpolar hydrophobic group pyrrole in tryptophan residue of BSA may be involved in

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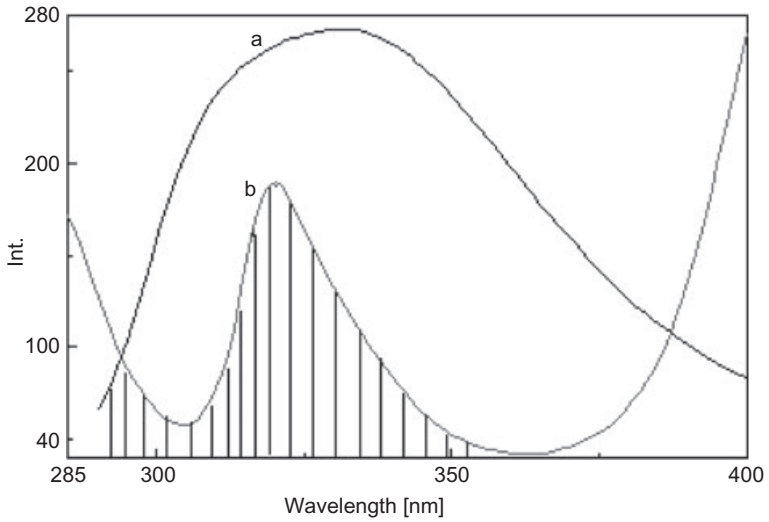

Figure 6. Overlap spectra of fluorescence emission spectra (a) of BSA; [BSA] = 2×10^{-6} mol L^{-1} and excitation spectra (b) of FLD; [FLD] = 2×10^{-4} mol L^{-1}.

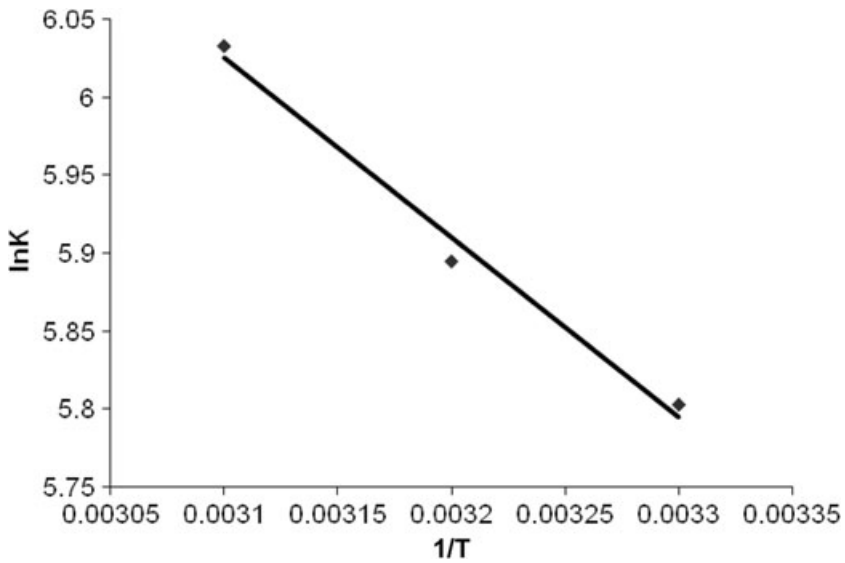

Figure 7. Van't Hoff plot for binding of FLD with BSA.

Table 3. Thermodynamic parameters for FLD-BSA interaction

T (K)	ΔH (kJmol^{-1})	ΔG (kJmol^{-1})	ΔS (J mol^{-1}K^{-1})	R
304	9.587	−14.665	79.77	0.9933
314		−15.388	79.53	
324		−16.25	79.74	

R is the correlation coefficient.

the binding of FLD and BSA. Its basic structure is 1,4 dihydropyridine and it has a hydrophobic cavity at the pyridine site.[3] Similarly the BSA has a hydrophobic pyrrole in tryptophan 212 residue. In solution when both molecules are brought closer at distance r equal to 4.9 nm, the hydrophobic interaction becomes more probable. This is also supported by the fact that the hydrophobic forces are inversely proportional to the seventh power the distance r. The estimated smaller value r indicates that two hydrophobic forces are appreciably large to favor the hydrophobic interaction rather than electrostatic interaction.[22]

Energy transfer from BSA to FLD

FRET is an important technique for investigating a variety of biological phenomena including energy transfer processes.[23] Here the donor and acceptor are BSA and FLD respectively. The absorption spectra of FLD and BSA shown in Fig. 5 are widely separated with the λ_{ab} at 364 and 280 nm respectively. The emission peak of BSA (320 nm) is close to the absorption peak of FLD (364 nm) and the excitation of solution of mixture of BSA–FLD by 280 nm wavelength from source excites BSA only. However, at this wavelength FLD has weak absorption and fluoresce negligibly. Therefore fluorescence energy of BSA is absorbed by

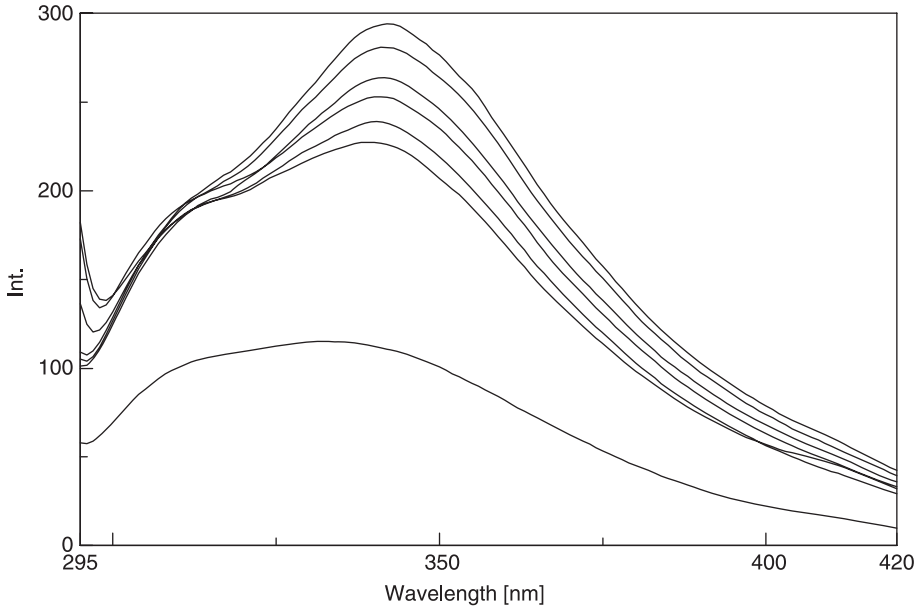

Figure 8. Emission spectra of BSA in the presence of various concentrations of surfactant; [SDS] = 0.0–3 × 10^{-2}mol L^{-1} and [BSA] = 1 × 10^{-5}.

FLD. The large region of overlap seen in Fig. 6 between emission of BSA and excitation spectra of FLD further supports the possibility of fluorescence resonance energy transfer from BSA to FLD molecules in solution. The region of integral overlap is used to calculate the critical energy transfer distance (R_0) between BSA (donor) and FLD (acceptor) using Förster's equation:[15,24]

$$E = R_0^6 / R_0^6 + r^6 \quad (6)$$

where R_0 is the critical distance when the transfer efficiency is 50%

$$R_0^6 = 9000\Phi \ln 10 k^2 / 128\Pi^5 n^4 N_A \int^{f(V)E(V)dv} / v^4 \quad (7)$$

In eqn (7) K^2 is the special orientation factor of the dipole, Φ the fluorescence quantum efficiency of donor, n the refractive index of the materials, N_A Avogadro's number, J the integral overlap area and E the energy transfer efficiency obtained by following equations.

$$J = \int^{F(V)E(V)dv} / v^4 \quad (8)$$

$$E = 1 - F / F_0 \quad (9)$$

From the overlapping of the absorption spectrum of acceptor and fluorescence spectrum of donor, $J = 5.2509 \times 10^{-14}\,dm^3\,cm^3\,mol^{-1}$ can be evaluated by eqn (8) and the energy transfer efficiency, $E = 0.14$, from eqn (9). Under these experimental calculations we found $R_0 = 3.6$ nm from eqn (7) using $K^2 = 2/3$, $n = 1.338$ and $\Phi = 0.15$ for the aqueous solution of donor. The distance r between FLD and amino acid residue in BSA is 4.9 nm. Obviously, it is lower than 7 nm after interaction between FLD and BSA. This result indicates that the nonradiative energy transfer from BSA to FLD occurs with high possibility.[25] Furthermore the value of r is higher than R_0 in the present study, indicating the presence of static quenching interaction between BSA and FLD.[26]

Effect of concentration of surfactant

Figure 8 shows the emission spectra of BSA in the presence of various concentrations of surfactant. From these spectra, the maximum of emission wavelength shows a red shift from 332 to 342 nm in the presence of increasing concentration of SDS surfactant. This indicates that different species are formed in solution. The shift of the maximum of emission wavelength from 332 to 342 nm is consistent with the fact that the change in the environment of the tryptophan residue is occurring and an increase in hydrophobicity in the vicinity of this residue takes place.[27]

Synchronous fluorescence spectroscopy

The conformational changes of BSA was evaluated by the measurement of the synchronous fluorescence intensity of protein amino acid residues before and after the addition of FLD. Fluorescence measurements give information on the molecular environment in the vicinity of the fluorophore functional groups and have several advantages such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects.

This is a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum. The shift in position of emission maximum corresponds to the changes of the polarity around the chromophore molecule. When the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristics information of tyrosine or tryptophan residues respectively.

It is shown in Fig. 9 that the emission maximum of tryptophan residue has a slight blue shift (from 338 to 336 nm) at the investigated concentration range when $\Delta\lambda = 60$ nm. It is also indicated that the polarity around the tryptophan residue was decreased and the hydrophobicity was increased but the microenvironment around the tyrosine residue did not obviously change during the binding process.[28]

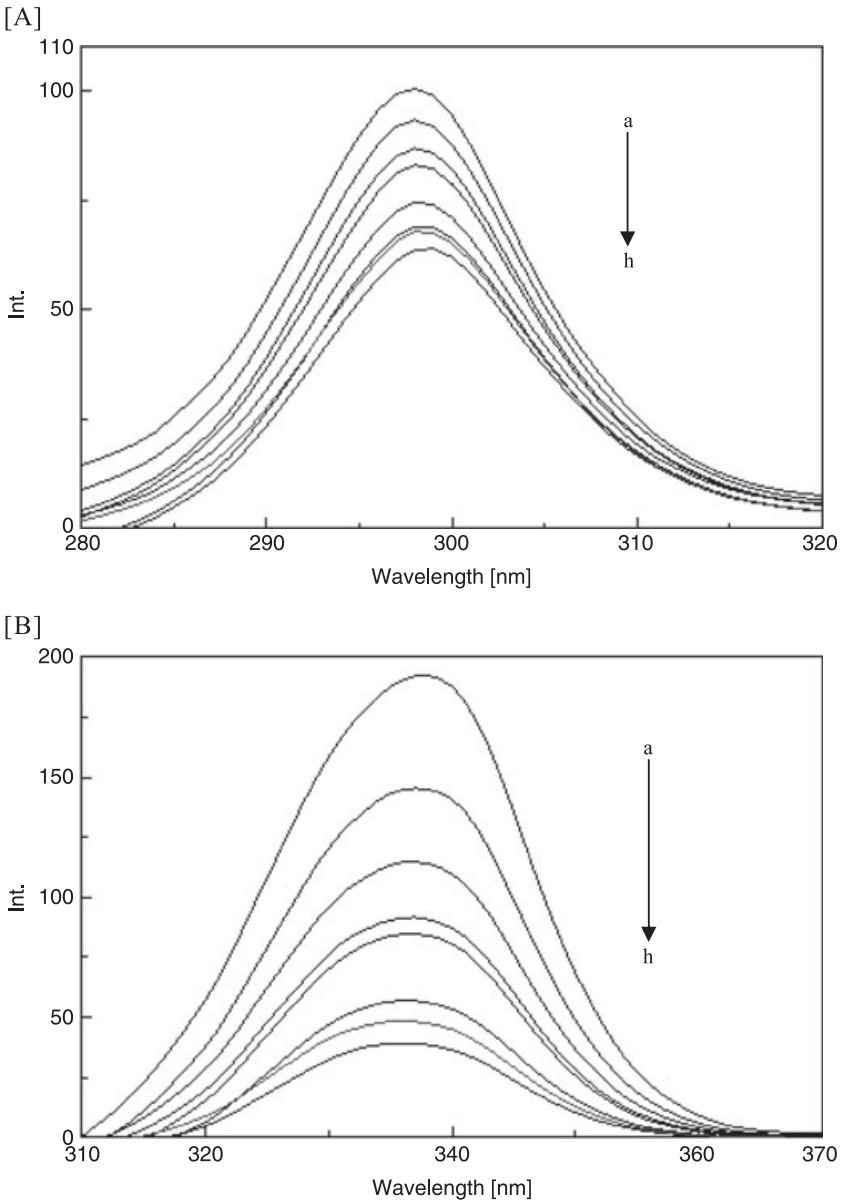

Figure 9. Effect of the FLD on the synchronous fluorescence spectra of BSA. (A) $\Delta\lambda = 15$ nm; (B) $\Delta\lambda = 60$ nm; [FLD] from a to h 0.0 to 2.1×10^{-4}.

Conclusion

A fluorescence method of binding interaction between BSA and FLD in 4 M acetic acid solution was studied using fluorescence and absorption spectrum. The fluorescence spectrum of BSA was also investigated in presence of SDS surfactant. The results of k_q, K_{SV}, binding constant $K = 3.31 \times 10^2$, 3.63×10^2, 4.17×10^2 M^{-1} and number of binding sites, $n = 0.727$, 0.736, 0.757 were calculated at different temperatures based on fluorescence quenching methods. The thermodynamic parameters like enthalpy change (ΔH) and entropy change (ΔS) were estimated to be 9.587 KJ mol^{-1} and 79.68 J mol^{-1} K^{-1} according to the Van't Hoff equation, and the results indicate that the hydrophobic interactions play a major role in FLD–BSA association. The distance r between donor (BSA) and acceptor (FLD) was found to be 4.9 nm according to Förster's energy transfer theory. The result of synchronous fluorescence spectrum revealed that the conformation of BSA molecule changed significantly in the presence of FLD.

Acknowledgements

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