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Interaction of bovine serum albumin with synthetic spiropyrimidines

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ABSTRACT

Study of interaction between synthetic spiropyrimidines (SP) with bovine serum albumin (BSA) was investigated by spectroscopy. The fluorescence of BSA was quenched by SP by means of static quenching mechanism. Using Stern–Volmer analysis of the fluorescence quenching data number of binding sites (n) and binding constants (K) at different temperatures were computed. The thermodynamic parameters enthalpy change (ΔH) and entropy change (ΔS) were calculated using Van't Hoff equation (ΔH = -95.16 kJ/mol and ΔS = -251.49 J/mol/ K) and the results clearly indicated binding process is enthalpy-driven but entropically disfavored. The weak force van der Waals interaction and hydrogen bonding is responsible for BSA-SP complexation. Synchronous fluorescence, UV-Vis and circular dichroism (CD) spectra proposed possibility of alteration in conformation of BSA in the presence of SP. Using Forster's non-radiation energy transfer (FRET) theory distance between the donor and the acceptor were found to be <7 nm.

Keywords: Spiropyrimidines; BSA; fluorescence; energy transfer; CD.

Introduction

Proteins are the major targets of many medicines in all organisms of living system [**1-2**] and various studies have revealed that in the circulatory system the distribution, free concentration, and metabolism of a various class of medicines may be strongly affected by medicine–protein interactions and also interaction can influence the stability and toxicity of medicine during the chemotherapeutic process [**3**]. In case of human, serum albumin contains 50–60 % of the total plasma protein and among all abundant protein constituent in the blood stream, albumin is most widely studied protein [**4–6**].

Pyrimidine moiety is the active core of various nucleic acids and shows diverse role in biological, pharmaceutical as well as agrochemical science [**7-8**]. Various pyrimidine and uracil based hetero organic molecules [**9**] have already been synthesized e.g. 3'-azido-3'-deoxy thiamidine (AZT), 2,3-dideoxycytidine (DDC) [**10**]. Introduction of different functional groups at the C-5 and C-6 positions of uracil moiety leads to various biologically important molecules. Spiro-pyrimidine [**11**] derivatives possess suitable pharmacological properties as it has both spiro model and pyrimidine nucleus. Therefore, application of the substituted spiro heterocycles is profound and wide spread from pharmaceutical to electronics.

In case of interaction study due to the structural similarity of BSA with human serum albumin (HSA) it is often used as a model protein [**12-14**]. Fluorescence spectroscopy is one of the potent technical methods to study the interaction between the small molecule compounds and bio-macromolecule [**15**]. The investigation on the interaction mechanism between drugs or small molecule/compounds and serum albumins showed great significance to pharmacokinetics field.

There have been several reports on the fluorescence quenching of BSA/HSA with different synthetic compounds. In recent years few reports have already approved regarding the interaction between serum albumin and organic synthetic compounds [**16-18**]. The interaction between novel Spiro [cyclopropane-pyrrolizin] and BSA has been studied by Yu *et al.* [**19**]. Wang *et al.* recently reported the interaction between clenbuterol hydrochloride with BSA [**20**]. Toprak *et al.* investigated the interaction between orientin and BSA [**21**]. Yao *et al.* studied the interaction of Carteolol Hydrochloride and Urea-induced BSA [**22**]. Yu *et al.* reported the interaction of Phacolysin and BSA [**23**]. Roy *et al.* described the interaction of pyrimidine-annulated spiro-dihydrofuran, spirooxindole-annulated thiopyran derivatives, and pyrano [3, 2-*f*] quinoline derivatives with BSA [**24-26**]. To the best of our knowledge, there is no report on the interactions of SP with BSA. In the featured work the interaction between SP with BSA was studied by UV–vis, fluorescence and CD spectrometry.

Experimental

Materials

The spiropyrimidines required for this study were prepared according to the previously used process [**27**]. Bovine

Serum Albumin (Sigma, USA) was prepared by dissolving BSA in a Tris-HCl (50 mM, pH 7.4) buffer to make the concentration as 0.1 µM and the synthesized SP were dissolved in methanol (1 mM). All other used chemicals were of analytical reagent grade and double distilled water was used throughout.

Apparatus

Fluorescence spectra were recorded on a Cary-Eclipse fluorescence spectrophotometer (Agilent Technologies) well equipped with attach Cary temperature controller. The absorption spectra were obtained by using a Cary UV-Vis spectrophotometer (Agilent Technologies). Circular dichroism spectra were analyzed by a Jasco J-815 CD spectrometer.

Methods

UV–visible spectroscopy: The absorption titration were performed at a fixed concentration of BSA (0.1 µM) while varying the SP concentration (0 to 60 µM) in the wavelength range of 230 to 330 nm at room temperature.

Intrinsic fluorescence: Fluorescence spectra of BSA (0.1 µM) were recorded in the range of 300-450 nm at the excitation wavelength 279 nm by successive additions of (0-60 µM) SP at 293, 303 and 313 K. The range of synchronous scanning were λex = 240 nm, λem = 255 nm where the difference in the wavelengths (Δλ) 15 nm, and λex = 240 nm, λem = 300 nm, where Δλ is 60 nm.

Energy transfer between SP and BSA: The absorption spectrum of SP (1 µM) and the emission spectrum of BSA (0.1 µM) was also recorded and the overlap of the absorption spectrum of SP with the emission spectrum of BSA was used to compute the energy transfer.

Circular dichroism spectra: Spectral changes of BSA (0.01 µM) were monitored in presence of SP (0, 25 and 50 µM) in the range of 190-260nm.

Results and discussion

Absorption characteristics of BSA-SP interaction

Absorption spectra of BSA were monitored in the absence or presence of SP1 for the study of interaction between BSA and SP. **Fig. 1** shows the absorption spectra of BSA in the presence of increasing concentration of SP1 (0, 10, 20, 30, 40, 50 and 60 μM). It is clear from **Fig. 1** that with the increase of SP1concentration, the intensity at the wavelength at 279 nm increases continuously with blue shift of the peak maxima and this increase in intensity reflected the formation of the ground state complex between BSA and SP1.

To study the binding strength of SP to BSA, the apparent association constants (K_{app}) of SP (SP1-4) with BSA were determined. The values of K_{app} were obtained from the BSA absorption according to the published method [**28**]. The K_{app} values were estimated using Benesi and Hildebrand equation (equation 1) [**29**].

$$\frac{1}{A_{obs}-A_0}=\frac{1}{A_C-A_0}+\frac{1}{K_{app}(A_C-A_0)[SP]} \quad (1)$$

where, A_0 is the absorbance of BSA in the absence of SP and A_C is the recorded absorbance at 279 nm for BSA at different SP concentrations. The double reciprocal plot of $1/(A_{obs}-A_0)$ *vs*$1/[SP1]$ is linear and K_{app} has been estimated to be 1.83×10^3 L/mol (R= 0.9990, where R is the correlation coefficient) [**Fig. 1** inset]. In case of SP2, SP3 and SP4; K_{app} values are 5.96×10^2, 1.97×10^2 and 2.49×10^3 L/mol respectively (R= 0.9994, 9993, and 0.9975 for SP2, SP3 and SP4 respectively). The value of K_{app} is considerably small, thereby indicating formation of a weak complex between BSA and SP.

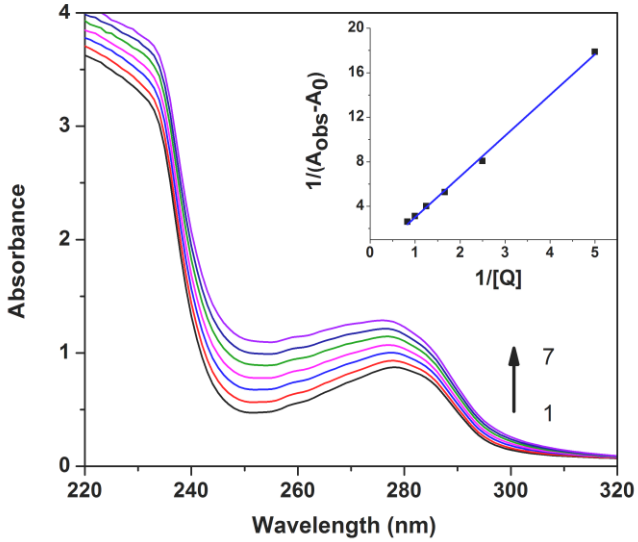

Fig. 1. Absorption spectra of BSA (0.1 μM) in presence of SP1 (1-7): (0, 10, 20, 30, 40 and 50 μM), inset: Calculation of K_{app} of BSA-SP complex.

Characteristics of fluorescence spectra

It is observed in **Fig. 2** that the emission intensity of BSA gradually decreased with the increase addition of SP1 but there seems to be no significant shift of emission wavelength. This observation indicates that SP1 can interact with BSA and quench its intrinsic fluorescence. The quenching properties can be explained using Stern-Volmer equation (equation 2) [**30-33**].

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

where, F_0 and F are the fluorescence intensities in the absence and presence of SP, K_q is the bimolecular quenching constant, K_{SV} is the S-V constant, τ_0 is the average lifetime of the BSA in the absence of a quencher and [Q] is the concentration of the SP. Since the fluorescence lifetime of a biopolymer is 10^{-8} s [**30**], the K_q value can be estimated using the formulae $K_q= K_{SV}/\tau_o$. Quenching data can be measured as plots of F_0/F *vs* [Q] and the values of K_{SV} and K_q at varying temperatures are shown in **Table. 1** (shown in supporting information). F_0/F *vs* [Q] plots for SP1 is shown in **Fig. 2** inset and results showed the quenching constant K_q increases with the rise of

temperature indicating quenching mechanism of BSA by SP is a dynamic quenching process.

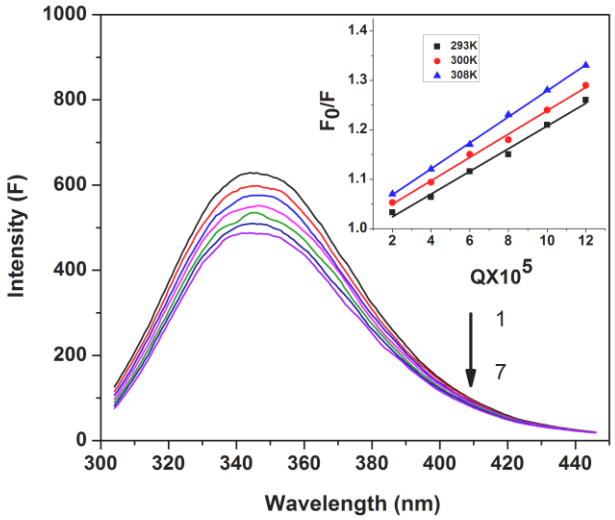

Fig. 2. Fluorescence quenching spectra of BSA in presence of SP (0-60 μM), inset: S-V plot for SP and BSA at 293, 303, and 313K respectively.

Number of binding sites and binding locality

For quenching interaction, if it is assumed that there are similar and independent binding sites in the biomolecules, the relationship between the fluorescence intensity and the quencher is given in following equation [**34-36**]. The number of binding sites (n) and the binding constant (K) between SP and BSA have been calculated using the equation 3.

$$log\frac{F_0 - F}{F} = logK + n\log[Q] \quad (3)$$

The double logarithm plot of log [(F_0 –F)/F] *vs* log [Q] have shown in **Fig. 3** and the values of K and n at 293, 303, and 310 K temperatures for BSA-SP are shown in the **Table 2**. The values for binding constant K and binding sites n of SP1 with BSA were obtained from **Fig. 3**.

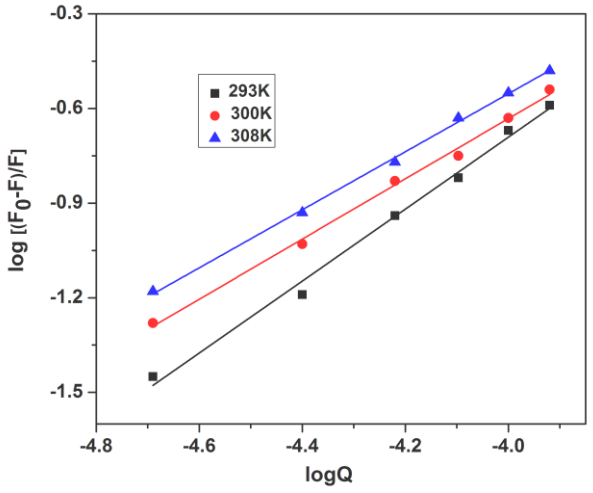

Fig. 3. Plots of the SP quenching effect on BSA fluorescence at 293, 303, and 313K.

Table 2. Binding constant and number of binding site for SP–BSA systems.

Compound	Temperature (K)	K (L/mol)	n	R
SP1	293	7.94×10^8	1.14	0.9967
SP2	293	3.16×10^9	1.19	0.9949
SP3	293	1.0×10^9	1.14	0.9990
SP4	293	1.99×10^8	1.0	0.9979
SP1	303	3.16×10^7	0.95	0.9981
SP2	303	1.99×10^7	0.94	0.9990
SP3	303	1.58×10^7	0.93	0.9979
SP4	303	1.99×10^7	0.90	0.9992
SP1	313	1.0×10^7	0.92	0.9992
SP2	313	1.99×10^5	0.71	0.9910
SP3	313	1.99×10^6	0.77	0.9986
SP4	313	7.94×10^6	0.84	0.9968

The binding constant of the interaction between SP and BSA increased in the following order:

SP4 < SP1 < SP3 < SP2 indicating that SP2 has the highest ability to quench BSA fluorescence and compound SP4 is the weakest to quench BSA.

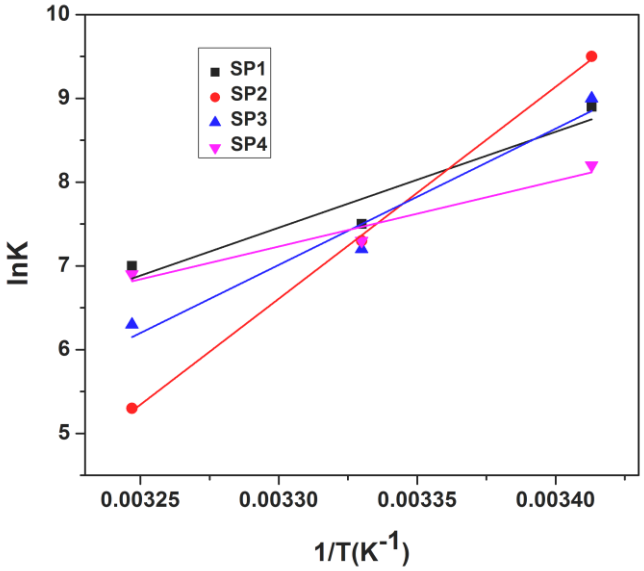

Fig. 4. The Van't Hoff plot for the interaction of BSA and SP.

Thermodynamic parameters and nature of binding forces

We worked out the thermodynamic parameters ΔH and ΔS using the Van't Hoff equation (equation 4). ΔH and ΔS were calculated from Van't Hoff plots shown in **Fig. 4** and the Gibbs free energy (ΔG) is estimated from the equation 5.

$$\ln K = -\Delta H / RT + \Delta S / R \quad (4)$$
$$\Delta G = \Delta H - T\Delta S = -RT\ln K \quad (5)$$

According to the published literature [**37**], the model of interaction process of thermodynamic parameters is as following: 1) ΔH > 0 and ΔS > 0 implies a hydrophobic interaction, 2) ΔH < 0 and ΔS < 0 reflects the van der Waals force or hydrogen bond, 3) ΔH< 0 and ΔS> 0, indicate the presence of electrostatic force of attraction

[**37-39**]. The values of thermodynamic parameters are listed in **Table 3** and results showed that ΔG, ΔH, and ΔS are negative and the negative values of ΔH and ΔS indicate that the binding is mainly enthalpy driven, and the entropy is unfavorable for binding.

Table 3. Thermodynamic parameters for SP–BSA systems.

Compound	Temperature (K)	ΔH (kJ/mol)	ΔS (J/mol/K)	ΔG (kJ/mol)
SP1	293	-95.16	-251.49	-21.48
SP2	293	-210.35	-639.18	-23.07
SP3	293	-145.24	-421.85	-21.65
SP4	293	-70.12	-171.60	-19.84
SP1	303	-95.16	-251.49	-19.71
SP2	303	-210.35	-639.18	-18.60
SP3	303	-145.24	-421.85	-18.70
SP4	303	-70.12	-171.60	-18.64
SP1	313	-95.16	-251.49	-17.70
SP2	313	-210.35	-639.18	-13.48
SP3	313	-145.24	-421.85	-15.35
SP4	313	-70.12	-171.60	-17.27

Characteristics of synchronous fluorescence spectra

Fig. 5 shows the synchronous fluorescence spectra of BSA in presence of SP when Δλ = 15 nm and Δλ =60 nm. It was observed in **Fig. 5a** for Δλ =15 nm that after addition of SP1 fluorescence intensity decreases with small red shift of λ_{max} but in case of Δλ =60 nm the fluorescence intensity decreased regularly without significant shift, as observed in **Fig. 5b** (shown in supporting information). Results indicative of interaction of SP1 with tyrosine residues rather than tryptophan residues and red shift means the reduction of polarity and increase of hydrophobicity around the tyrosine residue [**40**].

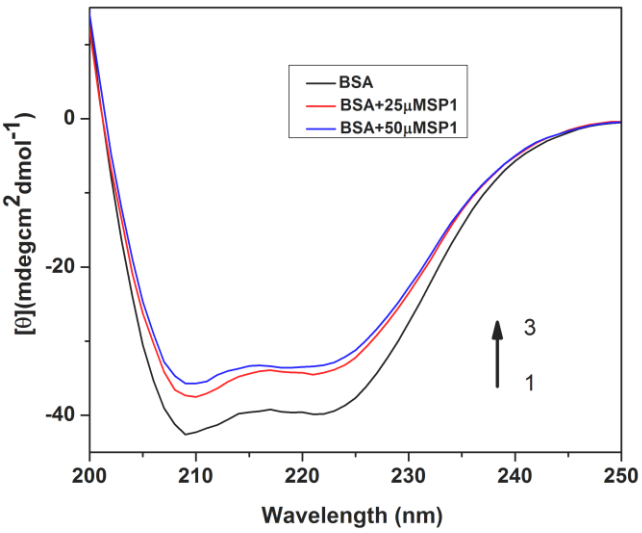

Fig. 6. CD spectra of BSA-SP system, BSA (0.01 μM) and SP (1: 0, 2: 25 μM, 3: 50 μM).

Circular dichroism spectroscopy

CD is well known spectroscopic technique to figure out the secondary structure of protein [**41**]. The CD spectrum of BSA showed two negative minima at 208 nm and 222 nm [**42**]. **Fig. 6** shows the helicity of BSA in the presence of increasing concentration of SP1 (0, 25 and 50 μM).

Results obtained from CD can be stated in terms of mean residue ellipticity (MRE) in mdeg cm^2 $dmol^{-1}$ according to the following equation [**43**].

$$MRE = \frac{obsCD(m\deg)}{Cp \times n \times l \times 10} \quad (6)$$

where, Cp is the molar concentration of the protein, n is the number of amino acid residues and l is the path length. The α-helical contents of free BSA and BSA in presence of SP were calculated from MRE values at 208 nm using the equation 7 [**44**]:

$$\alpha - helix(\%) = \frac{-MRE_{208} - 4000}{33000 - 4000} \times 100 \quad (7)$$

where, MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm and 33000 is the MRE value of a pure α-helix at 208 nm. From the above equation (6-7), the helicity in the secondary structure of BSA can be estimated and results showed that 47.20 % of helicity of BSA in pure BSA and in the presence of SP1, helicity decreases to 40.33 % and 37.50 % in presence of 25 μM and 50 μM SP1, respectively. The result indicated the loss of α-helix due to the interaction of SP1 with BSA. So, the structure of BSA is altered in the presence of SP1, i.e., interaction of SP1with BSA causes some conformational change of the protein [**45**].

Energy transfer between BSA and SP

FRET is a distance-dependent interaction between different electronic excited states of molecules. This process allows the excitation energy to be transferred from one molecule (donor) to another molecule (acceptor) without emission of a photon from the former molecular system. The energy transfer effect, E is also related to the critical energy transfer distance R_0, as described in equation 8.

$$E = 1 - \frac{F}{F_0} = R_0^6 / (R_0^6 + r^6) \quad (8)$$

where, F and F_0 are the fluorescence intensities of BSA in the presence and absence of SP, r is the distance between the acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50 %. This may be determined by the following equation.

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \phi J \quad (9)$$

where, k^2 is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor. The dipole orientation factor, k^2, is the least certain parameter that is used in the calculation of the critical transfer distance. When both the donor and acceptor tumble rapidly and free to assume any orientation, k^2 equals 2/3[**30**]. N is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor and J is the

overlap integral of the fluorescence emission spectrum of the donor. J can be evaluated using equation 10.

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (10)$$

where, F (λ) is the fluorescence intensity of the fluorescent donor at wavelength λ and is dimensionless; ε (λ) is the molar absorption coefficient of the acceptor at wavelength λ. The overlap plot of the UV absorption spectra of SP1 with the fluorescence spectra of BSA is shown in **Fig. 7**.

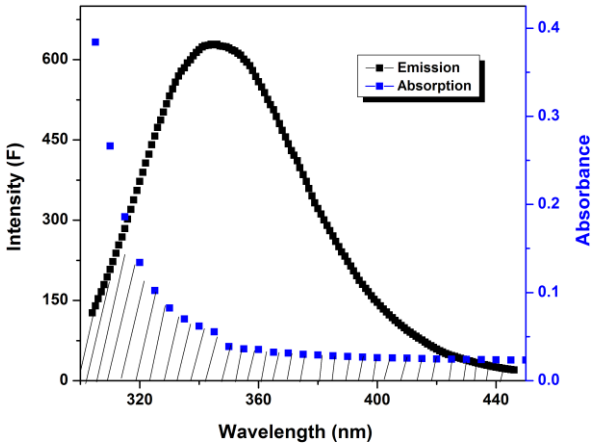

Fig. 7. Overlap plot of the emission spectra of BSA (0.1 μM) and the absorption spectra of SP (1mM).

It has been reported that for BSA that $k^2 = 2/3$, $\phi = 0.15$ and N= 1.336 [**46**]. The experimental results are shown in **Table 4** (shown in supporting information) and the values of r for SP1-4 are less than the predicted value (7 nm) [**47**]. What is more, the distances obtained by this method well agreement with literature values of substrate binding to BSA at site IIA [**48**]. The average distances between BSA and SP are in the 2-3 nm range [**49**] and that indicating high probability with non-radiative energy transfer from BSA to SP.

Conclusion

The study of interaction between SP and BSA showed that probable mechanism of SP interaction with BSA was revealed to be a dynamic quenching process. The thermodynamic parameters as obtained indicated that the binding process was exothermic, entropically disfavored but a spontaneous complexation process and the driving force for this BSA-SP interaction is mainly weak force van der Walls force and hydrogen bonding. The distance between SP and BSA was estimated to be < 7nm using FRET. Moreover, CD, UV-Vis and synchronous fluorescence spectra results revealed the changes of conformation of BSA. This work provides valuable information about the transportation and distribution of SP on serum albumin.

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Supporting information

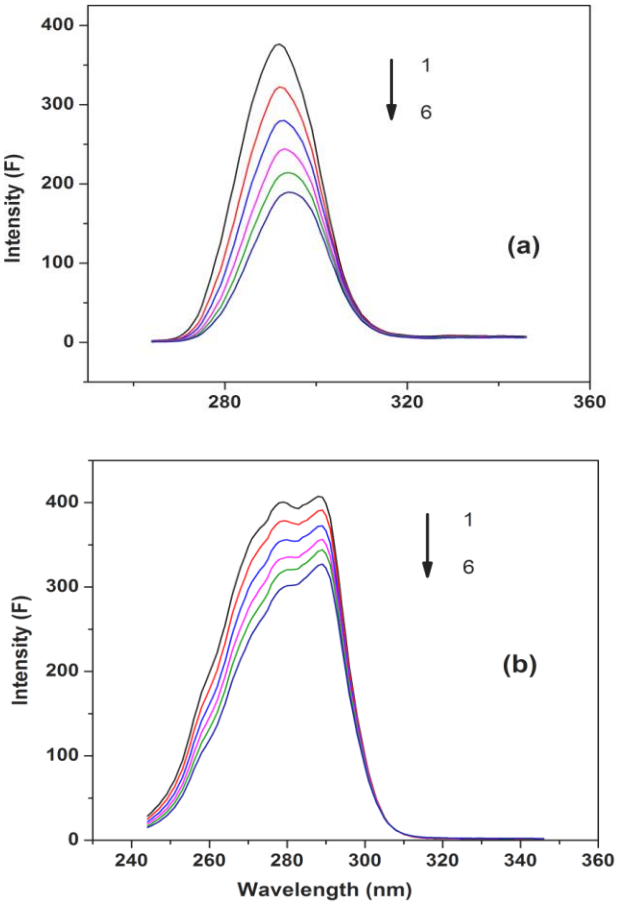

Fig. 5 Synchronous fluorescence spectra of BSA in presence of SP at Δλ = 15 nm (a) and Δλ = 60 nm (b), respectively.

Table 1. Stern–Volmer quenching constants for SP–BSA systems at different temperature.

Compound	Temperature (K)	K_{SV} (L/mol) ×10^2	K_q (L/mol/S)×10^{-6}	R
SP1	293	2.29	2.29	0.9959
SP2	293	2.54	2.54	0.9985
SP3	293	2.49	2.49	0.9980
SP4	293	3.48	3.48	0.9944
SP1	303	2.36	2.36	0.9974
SP2	303	2.38	2.38	0.9986
SP3	303	2.57	2.57	0.9963
SP4	303	3.60	3.60	0.9984
SP1	313	2.63	2.63	0.9996
SP2	313	2.35	2.35	0.9940
SP3	313	3.17	3.17	0.9948
SP4	313	4.10	4.10	0.9990

Table 4. Energy transfer parameter for SP–BSA complex.

System	J (cm^3×L/mol)	E	R_0 (nm)	r (nm)
BSA-SP1	7.41×10^{-16}	0.049	1.65	2.70
BSA-SP2	8.02×10^{-16}	0.054	1.68	2.70
BSA-SP3	7.92×10^{-16}	0.060	1.67	2.64
BSA-SP4	6.86×10^{-16}	0.070	1.63	2.51