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Interaction of cromolyn sodium with human serum albumin: A fluorescence quenching study

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Abstract—The interaction between cromolyn sodium (CS) and human serum albumin (HSA) was investigated using tryptophan fluorescence quenching. In the discussion of the mechanism, it was proved that the fluorescence quenching of HSA by CS is a result of the formation of a CS–HSA complex. Quenching constants were determined using the Sterns–Volmer equation to provide a measure of the binding affinity between CS and HSA. The thermodynamic parameters ΔG, ΔH, and ΔS at different temperatures were calculated. The distance r between donor (Trp^{214}) and acceptor (CS) was obtained according to fluorescence resonance energy transfer (FRET). Furthermore, synchronous fluorescence spectroscopy data and UV–vis absorbance spectra have suggested that the association between CS and HSA changed the molecular conformation of HSA and the electrostatic interactions play a major role in CS–HSA association.

1. Introduction

Protein–drug binding greatly influences absorption, distribution, metabolism, and excretion properties of typical drugs.[1] Human serum albumin (HSA) is the most abundant protein in the systemic circulation, with HSA comprising 60% in plasma.[2] Its principal function is to transport fatty acids, it is also capable of binding an extraordinarily broad range of drugs,[3] and much of the clinical and pharmaceutical interest in the protein derives from its effects on drug pharmacokinetics.[4] The crystallographic analyses of HSA have revealed that the protein, a 585 amino acid residue monomer, contains three homologous α-helical domains (I –III) and a single tryptophan (Trp^{214}).[5] Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cells in vivo and in vitro; they can play a dominant role in drug disposition and efficacy.[6]

This paper investigates the association of HSA with cromolyn sodium (CS); CS (molecular structure: Fig. 1; formula: $C_{23}H_{14}Na_2O_{11}$; CAS Registry Number: 15826-37-6; molecular weight: 512.33), which is a mast cell stabilizer and inhibits chemotaxis, activation, degranulation, and cytotoxicity of neutrophils, eosinophils, and monocytes, has been shown to prevent the release of mediators that are involved in the process of inflammation.[7,8] Furthermore, as an anti-inflammatory agent, CS has been used in the prophylactic treatment of bronchial asthma[9–12] and allergic rhinitis,[11–13] and its activity both in vitro and ex vivo is associated with a very low toxicity.[14] It is widely accepted in the pharmaceutical industry that the overall distribution, metabolism, and efficacy of many drugs can be altered based on their affinity to serum albumin. In addition, many promising new drugs have been rendered ineffective on

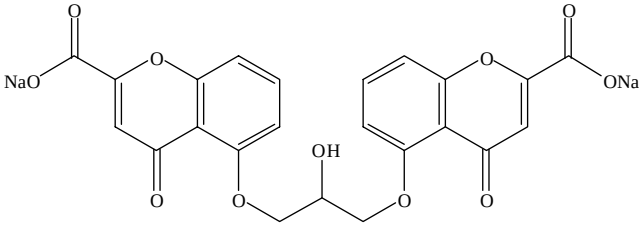

Figure 1. Molecular structure of cromolyn sodium.

Keywords: Cromolyn sodium; Human serum albumin; Fluorescence quenching; Thermodynamic parameters; Fluorescence resonance energy transfer.
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account of their unusually high affinity for this abundant protein.[15] Obviously, an understanding of the chemistry of the various classes of pharmaceutical interactions with albumin can suggest new approaches to drug therapy and design. Hence, it is important to understand and predict ligand/drug displacement interactions for a variety of endogenous and exogenous ligands/drugs. However, detailed investigations of the interaction of HSA with CS are scanty. Because of its medically important nature, our work should be valuable.

Fluorescence quenching is the decrease of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with the quencher molecule. Application of the fluorescence quenching technique can reveal the reactivity of chemical and biological systems in low concentration under physiological conditions, and there have been several studies on fluorescence quenching of albumin induced by drugs or other bioactive small molecules.[16–20] In this paper, quenching of the intrinsic tryptophan fluorescence of HSA has been used as a tool to study the interaction of CS with this transport protein in an attempt to characterize the chemical association taking place.

2. Materials and methods

2.1. Materials

HSA and CS were obtained from Sigma. The Tris buffer had a purity of no less than 99.5% and NaCl, HCl, etc., were all of analytical purity. The samples were dissolved in Tris–HCl buffer solution (0.05 mol L^{-1} Tris, 0.15 mol L^{-1} NaCl, pH 7.4 ± 0.1). All solutions were used with doubly distilled water.

2.2. Equipment

All fluorescence spectra were recorded on a F-2500 Spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells and a thermostat bath. The UV spectrum was recorded at room temperature on a TU-1901 spectrophotometer (Puxi Analytic Instrument Ltd. of Beijing, China) equipped with 1.0 cm quartz cells. Sample masses were accurately weighted using a microbalance (Sartorius, ME215S) with a resolution of 0.1 mg.

2.3. Spectroscopic measurements

The absorption spectroscopy of CS was performed at room temperature. The fluorescence measurements were performed at different temperatures (298, 304, and 310 K). The widths of both the excitation slit and the emission slit were set to 2.5 nm. An excitation wavelength of 295 nm was chosen since it provides no excitation of tyrosine residues and therefore neither emission nor energy transfer to the lone indole side chain would be non-negligible. Appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence.

2.4. Principles of fluorescence quenching

Fluorescence quenching is described by the well-known Stern–Volmer equation[21]

$$\frac{F_0}{F} = 1 + K_{SV}[Q], \tag{1}$$

where F_0 and F denote the steady-state fluorescence intensities in the absence and in the presence of quencher (CS), respectively, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher. Hence, Eq. 1 was applied to determine K_{SV} by linear regression of a plot of F_0/F against [Q].

3. Results and discussions

3.1. Effect of cromolyn sodium on human serum albumin spectra

A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity.[21] Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants.

To discuss the results within the linear concentration range, we selected to carry out the experiment within the linear part of Stern–Volmer dependence (F_0/F against [Q]). In this experiment, the concentrations of HSA solution were stabilized at 1.0×10^{-5} mol L^{-1}, and the concentration of CS varied from 0 to 6.4×10^{-5} mol L^{-1} at increments of 0.8×10^{-5} mol L^{-1}. The effect of CS on HSA fluorescence intensity is shown in Figure 2. As can be seen from Figure 2, addition of increasing concentrations of CS caused a progressive reduction in fluorescence intensity, accompanied by an increase of wavelength emission maximum λ_{max} in the albumin spectrum. Thus, the fluorescence was strongly quenched, whereas λ_{max} was increased from 337.5 to 349.5 nm by addition of 6.4×10^{-5} mol L^{-1} CS, a shift that can be reasonably attributed to an increased polarity (or a decreased hydrophobicity) of the region surrounding the Trp^{214} site.[22] The inset in Figure 2 shows that within the investigated concentration range, the results agree with the Stern–Volmer equation.

The calculation of K_{SV} from Stern–Volmer plots (Fig. 3) demonstrated that varying temperatures have a moderate effect on fluorescence quenching by CS. Table 1 gives the calculated K_{SV} at each temperature studied, while the results show that the Stern–Volmer quenching constant K_{SV} is inversely correlated with temperature, which

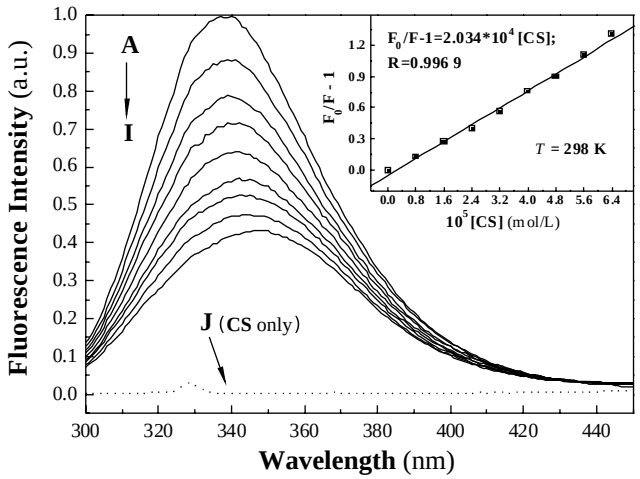

Figure 2. Emission spectra of HSA in the presence of various concentrations of CS (T = 298 K, λ_{ex} = 295 nm); c(HSA) = 1.0×10^{-5} mol L^{-1}; c(CS)/(10^{-5} mol L^{-1}); A–I: 0; 0.8; 1.6; 2.4; 3.2; 4.0; 4.8; 5.6; 6.4; curve J shows the emission spectrum of CS only. The inset corresponds to the Stern–Volmer plot.

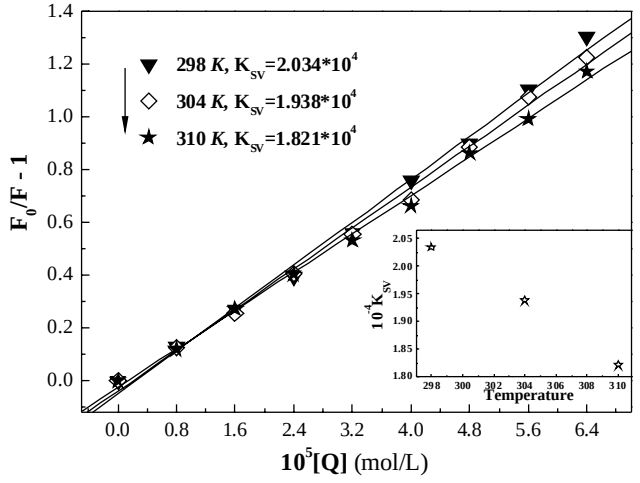

Figure 3. Stern–Volmer plot at three different temperatures. The inset shows the relationship of the Stern–Volmer quenching constants K_{SV} and temperature.

Table 1. Stern–Volmer quenching constants for the interaction of CS with HSA at different temperatures

pH	T (K)	$10^{-4}K_{SV}$ (L mol^{-1})	R[a]	SD[b]
7.4	298	2.034	0.9969	0.0379
	304	1.938	0.9976	0.0317
	310	1.821	0.9984	0.0242

[a] R is the correlation coefficient.
[b] SD is the standard deviation for the K_{SV} values.

indicates that the probable quenching mechanism of a CS–HSA binding reaction is initiated by compound formation rather than by dynamic collision. Therefore, the quenching data were analyzed according to the modified Stern–Volmer equation[23]

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a}. \quad (2)$$

In the present case, ΔF is the difference in fluorescence in the absence and presence of the quencher at concentration [Q], f_a is the fraction of accessible fluorescence, and K_a is the effective quenching constant for the accessible fluorophores, which are analogous to associative binding constants for the quencher–acceptor system.[24]

The dependence of $F_0/\Delta F$ on the reciprocal value of the quencher concentration $[Q]^{-1}$ is linear with the slope equaling the value of $(f_a K_a)^{-1}$. The value f_a^{-1} is fixed on the ordinate. The constant K_a is a quotient of the ordinate f_a^{-1} and the slope $(f_a K_a)^{-1}$. The corresponding results at different temperatures are shown in Table 2. The decreasing trend of K_a with increasing temperature is in accordance with K_{SV}'s dependence on temperature as mentioned above. It shows that the binding constant between CS and HSA is moderate and the effect of temperature is small. Thus, CS can be stored and carried by this protein in the body.

3.2. The determination of the force acting between cromolyn sodium and HSA

The interaction forces between drugs and biomolecules may include electrostatic interactions, multiple hydrogen bonds, van der Waals interactions, hydrophobic, and steric contacts within the antibody-binding site, etc.[25] To elucidate the interaction between CS and HSA, the thermodynamic parameters were calculated from the van't Hoff plots.

If the enthalpy change (ΔH) does not vary significantly in the temperature range studied, both the enthalpy change (ΔH) and entropy change (ΔS) can be evaluated from the van't Hoff equation

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}, \quad (3)$$

where K_a is analogous to the effective quenching constants at the corresponding temperature and R is the gas constant. The temperatures used were 298, 304, and 310 K. The enthalpy change (ΔH) is calculated from the slope of the van't Hoff relationship. The free energy change (ΔG) is then estimated from the following relationship

$$\Delta G = \Delta H - T\Delta S. \quad (4)$$

Figure 4, by plotting the data in Table 2, shows that assumption of near constant ΔH is justified. Table 2 shows the values of ΔH and ΔS obtained for the binding site from the slopes and ordinates at the origin of the fitted lines.

The negative values of free energy (ΔG), seen in Table 2, support the assertion that the binding process is spontaneous. The negative enthalpy (ΔH) and positive entropy (ΔS) values of the interaction of CS and HSA indicate that the electrostatic interactions played a major role in the binding reaction.[26]

Table 2. Modified Stern–Volmer association constants K_a and relative thermodynamic parameters of the system of CS–HSA

T (K)	$10^{-4}K_a$ (L mol^{-1})	SD[a]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	R[b]
298	1.190	0.0521		−23.249		
304	1.154	0.0335	−3.932	−23.638	64.818	0.9998
310	1.119	0.1326		−24.027		

[a] SD is the standard deviation for the K_a values.
[b] R is the correlation coefficient for the van't Hoff plot.

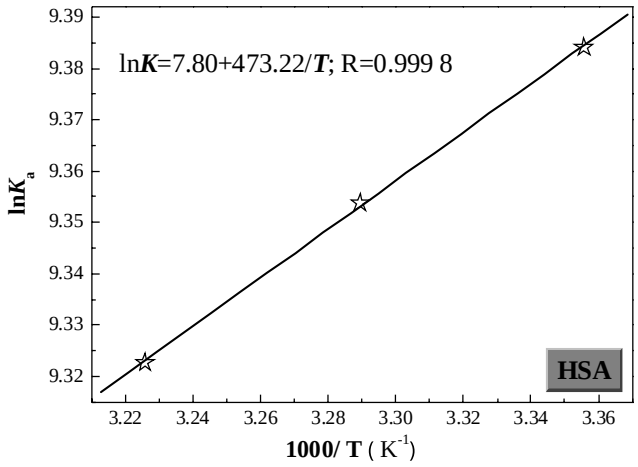

Figure 4. van't Hoff plot, pH 7.40, c(HSA) = 1.0×10^{-5} mol L^{-1}.

3.3. Energy transfer from HSA to cromolyn sodium

FRET is a non-destructive spectroscopic method that can monitor the proximity and relative angular orientation of fluorophores, and the donor and acceptor fluorophores can be entirely separate or attached to the same macromolecule. A transfer of energy could take place through a direct electrodynamic interaction between the primarily excited molecule and its neighbors,[27] which will take place under conditions: (i) the donor can produce fluorescence light; (ii) fluorescence emission spectrum of the donor and UV–vis absorbance spectrum of the acceptor have more overlap; and (iii) the distance between the donor and the acceptor is approached and is lower than 8 nm.[28] Using FRET, the distance r between CS and HSA (Trp^{214}) could be calculated by the equation[20]

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6}, \tag{5}$$

where E denotes the efficiency of transfer between the donor and the acceptor, and R_0 is the critical distance when the efficiency of transfer is 50%

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J. \tag{6}$$

In Eq. 6, K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles, and $K^2 = 2/3$ for random orientation as in fluid solution; n is the average refracted index of the medium in the wavelength range where spectral overlap is significant; ϕ is the fluorescence quantum yield of the donor; and J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 5), which could be calculated by the equation

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \mathrm{d}\lambda}{\int_0^\infty F(\lambda)\mathrm{d}\lambda}, \tag{7}$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range from λ to $\lambda + \Delta\lambda$; $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ.

In the present case, $n = 1.36$, $\phi = 0.074$.[29] According to Eqs. 5–7, we calculate that $J = 1.351 \times 10^{-14}$ cm^3 L mol^{-1}; $E = 0.134$; $R_0 = 2.356$ nm; and $r = 3.216$ nm. The average distances between a donor fluorophore and acceptor on the 2- to 8-nm scale[30] and $0.5\ R_0 < r < 1.5R_0$, [31] indicate that the energy transfer from HSA to CS occurs with high probability. In addition, other bioactive small molecules with structures similar to that of CS may exhibit a similar UV–vis absorbance spectrum, indicating the binding reactions between these molecules (acceptor) and HSA (donor) to be in accord with the conditions of energy transfer theory mentioned above. Hence, molecules with structures similar to that of CS are likely to have a fluorescence quenching effect on HSA.

3.4. Conformation investigation

Trp^{214}, conserved in mammalian albumins, plays an important structural role in the formation of HSA.[5] Spectroscopy is an ideal tool to observe conformational changes in proteins since it allows non-intrusive measurements of substances in low concentration

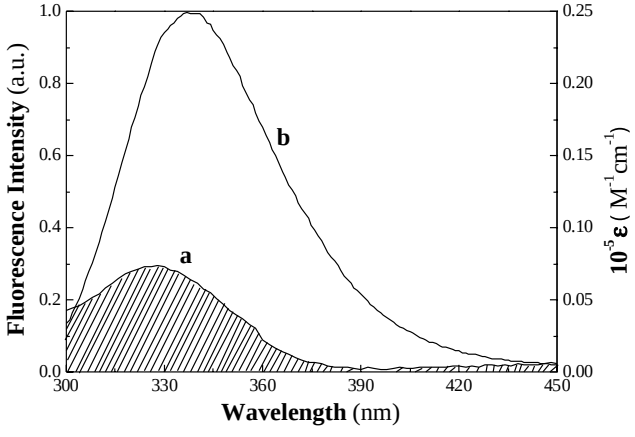

Figure 5. Spectral overlap of CS absorption (a) with HSA fluorescence (b). c(HSA) = c(CS) = 1.0×10^{-5} mol L^{-1}. (T = 298 K).

under physiological conditions. It is advantageous to use intrinsic fluorophores for these investigations to avoid complicated labeling with an extrinsic dye.[16] Synchronous fluorescence spectroscopy introduced by Llody,[32,33] involves the simultaneous scanning of excitation and the fluorescence monochromators of a fluorimeter, while maintaining a fixed wavelength difference ($\Delta\lambda$) between them. The synchronous fluorescence spectra give information about the molecular environment in the vicinity of the chromosphere molecules. In the synchronous spectra, the sensitivity associated with fluorescence is maintained, while offering several advantages: spectral simplification, spectral bandwidth reduction, and avoiding different perturbing effects. The authors[34] have suggested that a useful method to study the environment of amino acid residues was to measure the possible shift in wavelength emission maximumλ_{max}, which corresponds to changes in polarity around the chromophore molecule. When the D value ($\Delta\lambda$) between excitation wavelength and emission wavelength is stabilized at 60 nm, synchronous fluorescence spectroscopy gives the characteristic information of tryptophan residues.[35] The effect of CS on HSA synchronous fluorescence spectroscopy is shown in Figure 6.

It is apparent from Figure 6 that the maximum emission wavelength redshifts (from 341 to 344 nm) at the investigated concentration range when $\Delta\lambda = 60$ nm. The redshift of the emission maximum suggests a more polar (or less hydrophobic) environment of the Trp^{214} residue, the conclusion agreeing with the analytical result given in Figure 2.

For reconfirming the structural change of HSA by addition of CS, we measured the UV–vis absorbance spectra of HSA with various amounts of CS. Figure 7 displays the UV–vis absorbance spectra of HSA at different contents of CS. It is clear from the figure that the baselines of the UV–vis absorbance spectra at 300–250 nm are raised and the absorption spectra maximum blueshift

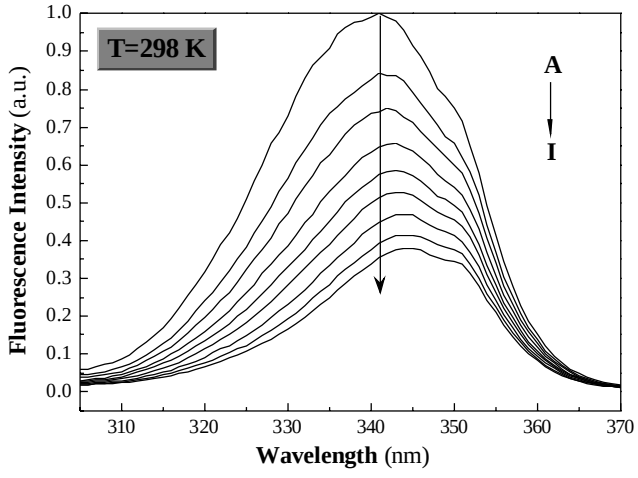

Figure 6. Synchronous fluorescence spectrum of HSA ($T = 298$ K, $\Delta\lambda = 60$ nm). c(HSA) = 1.0×10^{-5} mol L^{-1}; c(CS)/(10^{-5} mol L^{-1}); A–I: 0; 0.8; 1.6; 2.4; 3.2; 4.0; 4.8; 5.6; 6.4.

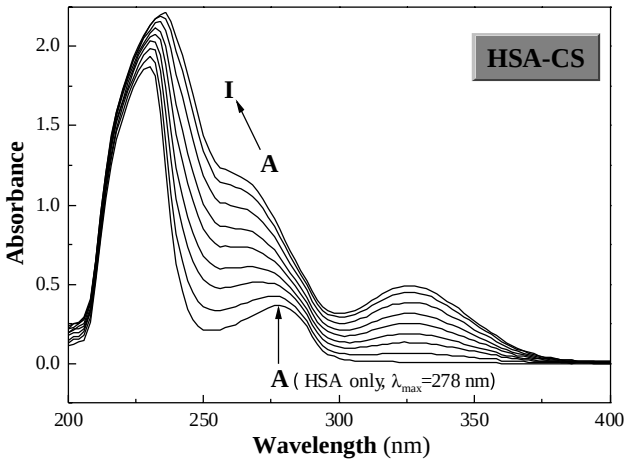

Figure 7. UV–vis absorbance spectra of HSA in the presence of CS. A–I, c(HSA) = 1.0×10^{-5} mol L^{-1}; c(CS)/(10^{-5} mol L^{-1}): 0; 0.8; 1.6; 2.4; 3.2; 4.0; 4.8; 5.6; 6.4. The arrow indicates the sequence of spectral changes upon increase in CS concentration, corresponding to an increase in intensity, blueshift.

(from 278 to 258 nm), indicating that the HSA molecules associate with CS to form a HSA–CS complex and the peptide strand extended even more, while the hydrophobicity was decreased (or became more polar). The conclusion agrees with the result of conformational changes by synchronous fluorescence spectra, which indicates that the approach of synchronous fluorescence spectroscopy is scientific.

4. Conclusions

In this paper, we investigated the nature and magnitude of the interaction of CS with HSA by spectroscopic methods including fluorescence spectroscopy and UV–vis absorption spectroscopy. The results show that the Stern–Volmer quenching constant K_{SV} is inversely correlated with temperature, which indicates that the probable quenching mechanism of the CS–HSA binding reaction is initiated by compound formation. The maximum emission wavelength of synchronous fluorescence spectra redshifts progressively at the investigated concentration range when $\Delta\lambda = 60$ nm, which suggests a more polar (or less hydrophobic) environment of the Trp^{214} residue. The thermodynamic parameters $\Delta G < 0$, $\Delta H < 0$, and $\Delta S > 0$ at different temperatures indicate that the binding process is spontaneous and the electrostatic nature is a major factor in the interaction.

Acknowledgments

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