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Short communication

Spectroscopic studies on the interaction between silicotungstic acid and bovine serum albumin

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Abstract

The interaction between silicotungstic acid and bovine serum albumin (BSA) was investigated using fluorescence and UV/vis. The experimental results showed that the fluorescence quenching of BSA by silicotungstic acid is a result of the formation of SiW–BSA complex; static quenching and non-radiative energy transferring were confirmed to result in the fluorescence quenching. The binding site number n, apparent binding constant K_A and corresponding thermodynamic parameters were measured at different temperatures. The process of binding SiW molecule on BSA was a spontaneous molecular interaction procedure in which entropy increased and Gibbs free energy decreased. Hydrophobic interaction force plays a major role in stabilizing the complex. The effect of silicotungstic acid on the conformation of BSA was analyzed using synchronous fluorescence spectroscopy.

Keywords: Silicotungstic acid; Bovine serum albumin; UV/vis spectroscopy; Fluorescence spectroscopy; Thermodynamic parameters

1. Introduction

Serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions [1]. The most important property of this group of proteins is that they serve as a depot protein and as a transport protein for many drugs and other bioactivity small molecules [2]. Bovine serum albumin (BSA) has been one of the most extensively studied of this group of proteins, especially of its structural homology with human serum albumin (HSA) [1]. BSA is made up of three homologous domains (I, II, III), which are divided into nine loops (L1–L9) by 17 disulfide bonds. The loops in each domain are made up of a sequence of large–small–large loops forming a triplet. Each domain in turn is the product of two sub-domains [3]. BSA has two tryptophans, Trp–134 and Trp–212, embedded in the first sub-domain IB and sub-domain IIA, respectively. HSA is a globular protein composed of 585 amino acid residues in three homologous α-helices domains (I–III). Each domain contains 10 helices and is divided into antiparallel 6 helix and four sub-domains (A and B) [4]. There is only one tryptophan located at position 214 along the chain, in sub-domain IIA of HSA.

The molecular interactions between proteins and many compounds have been investigated successfully including dyes and other some organic small molecules [3,5–10]. However, the binding of some inorganic ions to proteins has seldom been investigated [11,12]. Heteropoly acids, belonging to the heteropoly compounds, are sorts of inorganic acid radical anions [11]. Because of their unique combination of physical and chemical properties, heteropoly compounds have been widely used in analytical and clinical chemistry, catalysis (including photocatalysis), medicine (antitumonal, antiviral and even anti-HIV activity), biochemistry (electron transport inhibition) and solid-state devices [13]. The use of heteropoly acids for protein determination is well established [11,14]. However, other parameters such as mode of interaction, association constant and number of binding sites are important, when heteropoly acids are used as drugs [12]. Investigating the interaction of drugs

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to serum albumins can elucidate the properties of drug–protein complex, as it may provide useful information of the structural features that determine the therapeutic effectiveness of drugs [15].

Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, various possibilities of structural rearrangements in the environment of the fluorophore may lead to a similar fluorescence signal; they can complicate interpretation of the experimental result and be exploited to obtain unique structural and dynamic information [16–18]. Silicotungstic acid (SiW) with the Keggin structure inhibits the replication of rubella [19], rubeola [20] and Moloney virus [21] as well as HIV [22,23]. In the present work, we used SiW as model drug and reporting the mechanism of binding of heteropoly acid with BSA. In order to attain these objectives, we planned to carry out detailed investigation of SiW–BSA association using fluorescence spectroscopy and UV/vis absorption spectroscopy. Through fluorescence resonance energy transfer (FRET), we planned to further investigate the energy transfer parameters of BSA for transfer to SiW. In addition, the conformational change of BSA is discussed on the basis of synchronous fluorescence spectra.

2. Materials and methods

2.1. Materials

Bovine serum albumin (Fraction V, approximately 99%) was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Silicotungstic acid was obtained from ino-Biotechnology Company (Shanghai, China). The Tris buffer was purchased from Acros (Geel, Belgium), and NaCl, HCl, etc. were all of analytical purity. BSA solution (5.0 μmol L^{-1}) was prepared in pH 7.40 Tris–HCl buffer solution (0.05 mol L^{-1} Tris, 0.1 mol L^{-1}NaCl). The SiW solution (2.5×10^{-4} mol L^{-1}) was prepared in pH 7.40 Tris–HCl buffer.

2.2. Equipments and spectral measurements

The UV/vis spectrum was recorded at room temperature on a GBC UV/vis916 spectrophotometer (Australia) equipped with 1.0 cm quartz cells and a slit width of 5 nm with a nominal resolution of 0.5 nm. All fluorescence spectra were recorded on LS–50B Spectrofluorimeter (Perkin-Elmer USA) equipped with 1.0 cm quartz cells and a thermostat bath, the widths of both the excitation slit and the emission slit were set to 5.0 nm with a nominal resolution of 0.5 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence.

2.3. Procedures

A 2.5 mL solution, containing appropriate concentration of BSA, was titrated by successive additions of a 2.5×10^{-4} mol L^{-1} stock solution of SiW (to give a final concentration of 8.0 μmol L^{-1}). Titrations were done manually by using micro-injector. The fluorescence spectra were then measured (excitation at 290 nm and emission wavelengths of 280–500 nm) at two temperatures (299, 309 K). The UV/vis absorbance spectra of SiW with concentration of 5.0 μmol L^{-1} were recorded at room temperature.

2.4. Principles of fluorescence quenching

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions viz., excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching [24]. Such decrease in intensity is called fluorescence quenching. Fluorescence quenching is described by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_q, K_{SV}, τ_0 and $[Q]$ are the quenching rate constant of the bimolecular, the Stern–Volmer dynamic quenching constant, the average lifetime of the bimolecular without quencher ($\tau_0 = 10^{-8}$ s) and the concentration of the quencher, respectively. Obviously,

$$K_q = \frac{K_{SV}}{\tau_0} \quad (2)$$

Hence, Eq. (1) was applied to determine K_{SV} by linear regression of a plot of F_0/F versus $[Q]$.

2.5. Calculation of binding parameters

When small molecules bind independently to a set of equivalent sites on a macromolecule, the apparent binding constant K_A and binding sites n can be obtained from Eq. [25]:

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

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, $[Q]$ is the total quencher concentration. By the plot of $\log(F_0 - F)/F$ versus $\log[Q]$, the number of binding sites n and binding constant K_A can be abstain.

If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then the thermodynamic parameters ΔH, ΔS, ΔG can be determined from the following Eq. [26]:

$$\ln\frac{(K_A)_2}{(K_A)_1} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (4)$$

$$\Delta G = -RT\ \ln K_A \quad (5)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (6)$$

3. Results and discussion

3.1. UV/vis absorption spectroscopy

UV/vis absorption measurement is a very simple method and applicable to explore the structural change and to know the

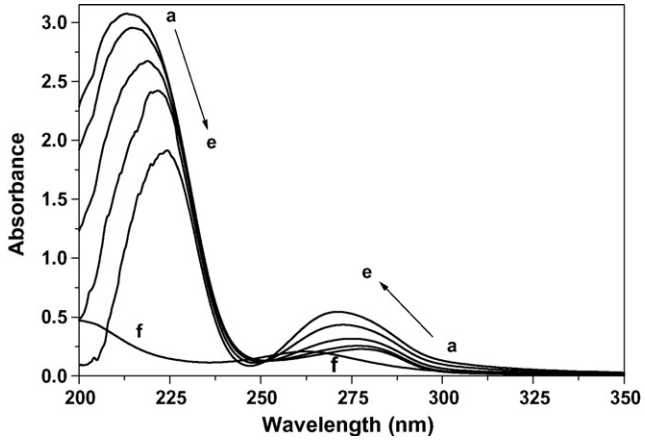

Fig. 1. Absorption spectra of BSA, SiW and SiW–BSA system. BSA concentration was at 5.0 μmol L^{-1} (a); SiW concentrations for SiW–BSA system were 5.0 (b), 10.0 (c), 20.0 (d) and 30.0 μmol L^{-1} (e); a concentration of 5.0 μmol L^{-1} (f) was used for SiW only.

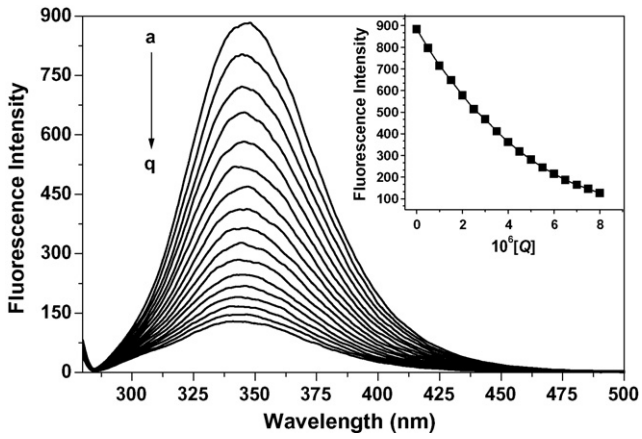

Fig. 2. Effect of fluorescence spectrum of BSA quenched by SiW (T = 299 K, pH 7.4 and λ_{ex} = 290 nm). From curve a–q, BSA concentration was at 5.0 μmol L^{-1}, SiW concentrations: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 μmol L^{-1}, respectively. The inset corresponds to the fluorescence quenching plots of BSA titrated with SiW.

complex formation [7]. Hence, absorption spectra of BSA in presence and absence of SiW were recorded (Fig. 1).

Fig. 1 showed that the absorption wavelengths of BSA, SiW and SiW–BSA were different. The absorption band of 210 nm of BSA is the characteristic of α–helix structure of BSA. Clearly, BSA has strong absorbance with a peak of 212 nm. The absorbance (212 nm) of SiW–BSA system decreased with increasing concentration of SiW and the peak has a red shift (from 212 to 224 nm). In addition, the absorption peaks in the UV/vis spectra at approximately 277 nm rise gradually (from curve a to curve e) and shift to the blue about 7 nm with increasing concentration of SiW. The results indicated that there exists interaction between SiW and BSA and ground state complex formed. Moreover, the occurrence of an isosbestic point at 252 nm might also indicate the existence of bound and free SiW in equilibrium [27].

3.2. Fluorescence quenching

BSA molecule has two tryptophan residues that possess intrinsic fluorescence: Trp–134 in the first sub-domain IB of the albumin molecule and Trp–212 in sub-domain IIA. Trp–212 is located within a hydrophobic binding pocket of the protein and Trp–134 is located on the surface of the albumin molecule [28,29]. A valuable feature of intrinsic fluorescence of proteins is the high sensitivity of tryptophan to its local environment. Changes in emission spectra of tryptophan are common in response protein conformational transitions, subunit association, substrate binding or denaturation [17]. So, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and if often considered on the study of protein folding and association reactions. BSA solutions excited at 290 nm emit fluorescence attributable mainly to tryptophan residues [30].

The effect of SiW on tryptophan residues fluorescence intensity is shown in Fig. 2. As the data shows, the fluorescence intensity of BSA decreased regularly and a slight blue shift with the increasing concentration of SiW, which indicates that SiW can bind to BSA and the chromophore of BSA, is changed. In order to discuss the results within the linear concentration range, we selected carrying out the experiment within the linear part of Stern–Volmer dependence (F_0/F versus [Q]). Fig. 3 displays the Stern–Volmer plots of the quenching of BSA tryptophan residues fluorescence by SiW at different temperatures. It shows that the curves have linear relationships at low concentrations of SiW, and the slopes increasing temperature. It indicates the occurrence of dynamic quenching interaction between SiW and BSA. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In Table 1, the binding constants obtained for the Stern–Volmer method are listed for SiW with BSA. Generally, the collisional quenching constant of various kinds of quenchers with biopolymer is 2.0×10^{10} L mol^{-1} s^{-1}. However, the rate constants of the protein quenching procedure initiated by SiW are greater than that one; it means that the quenching process is static. In

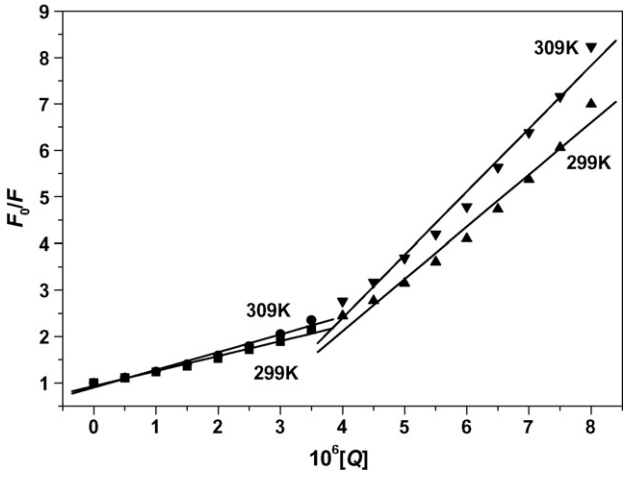

Fig. 3. Stern–Volmer plots for the quenching of BSA by SiW at different temperature. BSA concentration was at 5.0 μmol L^{-1}, pH 7.40, λ_{ex} = 290 nm and λ_{em} = 350 nm.

Table 1
Stern–Volmer quenching constants of the system of SiW–BSA at different temperatures

T (K)	c (SiW) $\leq 3.5\ \mu mol\ L^{-1}$			c (SiW) $\geq 4.0\ \mu mol\ L^{-1}$		
	$10^{-5}K_{sv}$ (L mol^{-1})	$10^{-13}K_q$ (L mol^{-1} s^{-1})	R	$10^{-6}K_{sv}$ (L mol^{-1})	$10^{-14}K_q$ (L mol^{-1} s^{-1})	R
299	3.2	3.2	0.9918	1.1	1.1	0.9889
309	3.8	3.8	0.9872	1.4	1.4	0.9910

R is the correlation coefficient.

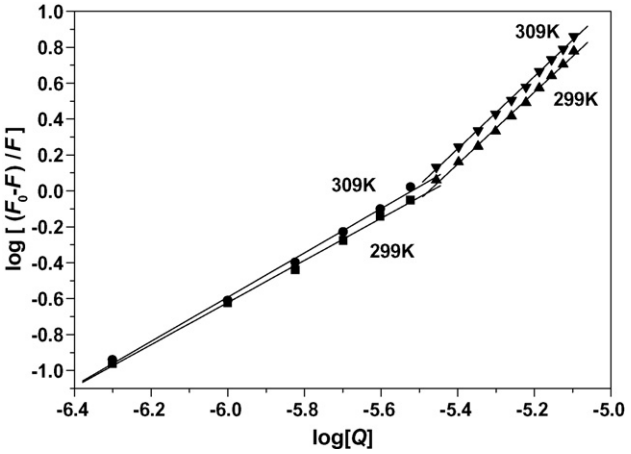

Fig. 4. The plots of log $(F_0 - F)/F$ vs. log [Q] at pH 7.4, BSA concentration was at 5.0 μmol L^{-1}, λ_{ex} = 290 nm, λ_{em} = 350 nm.

addition, ground state complex obtained by absorption spectra (Fig. 1) has also indicated that the static quenching is involved. Because dynamic quenching only affects the excited state of quenching molecule while it has no function on the absorption spectrum of quenching substances [31]. Linear Stern–Volmer plots may either reveal the occurrence of just a binding site for quencher in the proximity of the fluorophore, or indicate the existence of a single type of quenching. BSA titrated by SiW gives obviously concave upward for SiW concentration higher than 3.5 μmol L^{-1}, which indicated that the interaction mechanism becomes more complex and there maybe exist more one biding site in the neighborhood of the tryptophan residues and/or exist the combined quenching [31].

3.3. Binding constant and binding sites

Fig. 4 is the plots of log $(F_0 - F)/F$ versus log [Q] for the SiW–BSA system at different temperatures obtained from the fluorometric titration. In Table 2, the binding constants K_A and binding sites n are listed for SiW associated with BSA.

Table 2
Binding parameters of the system of SiW–BSA at different temperatures

T (K)	c (SiW) $\leq 3.5\ \mu mol\ L^{-1}$			c (SiW) $\geq 4.0\ \mu mol\ L^{-1}$		
	$10^{-6}K_A$ (L mol^{-1})	n	R	$10^{-10}K_A$ (L mol^{-1})	n	R
299	2.6	1.2	0.9990	8.6	2.0	0.9983
309	6.3	1.2	0.9984	13.1	2.0	0.9992

R is the correlation coefficient.

It can be seen from Fig. 4 that the plots of log $(F_0 - F)/F$ versus log [Q] has two regression curves intersecting at c (SiW) = 3.5 μmol L^{-1}. In Table 2, it clearly showed that when c (SiW) $\leq$ 3.5 μmol L^{-1}, the numbers of binding site were near 1.2; when c (SiW) $\geq$ 4.0 μmol L^{-1}, the numbers of binding site were near 2.0, the binding constants were higher than that of c (SiW) $\leq$ 3.5 μmol L^{-1} at corresponding temperatures. These results indicated that at low concentration, there maybe a single class-biding site in the neighborhood of the tryptophan residues, while at high concentration SiW involved other sites with higher binding affinity and selectivity.

3.4. Thermodynamic parameters and nature of the binding forces

In the past decade, a complete thermodynamic description of the self-association of many proteins and their interactions with small molecular substrates has become available [32]. There are essentially four types of non-covalent interactions that could play a key role in ligand binding to proteins. These are hydrogen bonds, van der Waals forces, electrostatic and hydrophobic bonds interactions [33]. The thermodynamic parameters, free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of interaction are important to interpret the binding mode [32]. To obtain such information, the temperature dependence of the binding constant was studied. Experiments were carried out at 299 and 309 K, since BSA does not undergo any gross structural change in this temperature range. From the temperature dependence of the binding constant, it is possible to calculate the thermodynamic functions involved in the binding process. The binding parameters of BSA–SiW complex were calculated from the Van't Hoff equation. From Table 3, it can be seen that the negative sign for ΔG indicates the spontaneity of the binding of SiW with BSA. ΔH and ΔS are positive value.

According the views of Timasheff [34], and Ross and Subramanian [32], the positive ΔH and ΔS value is associated with hydrophobic interaction. The negative ΔH and ΔS values are associated with hydrogen bonding and van der Waals interaction in low dielectric medium. Finally very low positive or negative ΔH and positive ΔS values are characterized by electrostatic interactions. Thus, it is difficult to interpret the thermodynamic parameters of BSA–SiW interaction with a single intermolecular force. Therefore, the binding of SiW to BSA might involve hydrophobic interaction strongly as evidenced by the positive values of ΔS and the electrostatic interaction can also not be excluded. In addition, Long et al. [14] reported that some heteropoly anions coexist with protein taking positive charge by the virtue of hydrophobic and electrostatic force interactions. At pH

Table 3
The relative thermodynamic parmeters of the system of SiW–BSA

T (K)	c (SiW) $\leq 3.5\ \mu mol\ L^{-1}$			c (SiW) $\geq 4.0\ \mu mol\ L^{-1}$		
	$\Delta H(1)$ (KJ mol^{-1})	$\Delta G(1)$ (KJ mol^{-1})	$\Delta S(1)$ (J mol^{-1} K^{-1})	$\Delta H(2)$ (KJ mol^{-1})	$\Delta G(2)$ (KJ mol^{-1})	$\Delta S(2)$ (J mol^{-1} K^{-1})
299	68.2	−36.7	350.7	32.1	−62.6	316.6
309		−40.2	350.7		−65.8	316.6

7.4 solutions, the serum albumin (isoelectric point pI = 4.7) bear negative charge because of the ionization of amino acid residues. So the binding of SiW and BSA by electrostatic force became very difficult. That is to say, the hydrophobic interaction might play a major role in the binding SiW to BSA and the increase of entropy might be based on the destruction of the iceberg structure induced by the hydrophobic interaction [35]. In addition, when c (SiW) $\leq 3.5\ \mu mol\ L^{-1}$, ΔH and ΔS were all higher than that of c (SiW) $\geq 4.0\ \mu mol\ L^{-1}$ at corresponding temperatures. These results indicated that at low concentration, SiW reached the high affinity hydrophobic cavities in sub-domain IIA with a single class-binding site.

3.5. Energy transfer from BSA to SiW

Fluorescence resonance energy transfer is a distance dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecular (donor) to another molecular (acceptor) without emission of a photon from the former molecular system. According to Förster's theory [36], the efficiency of FRET depends mainly on the following factors: (i) the extent of overlap between the donor emission and the acceptor absorption, (ii) the orientation of the transition dipole of donor and acceptor and (iii) the distance between the donor and the acceptor. FRET is an important technique for investigating a variety of biological phenomena including energy transfer processes [37]. Here the donor and acceptor are BSA and SiW, respectively. There is a spectral overlap between absorption UV/vis spectra of SiW (Fig. 1(f)) and the fluorescence emission spectrum of free BSA (Fig. 2(a)). As the fluorescence emission of protein was affected by the excitation light around 290 nm, the spectrum ranging from 280 to 500 nm was chosen to calculate the overlapping integral.

According to Förster's theory the energy transfer efficiency E is defined as the following Eq. (7). Where r is the distance from the ligand to the tryptophan residue of the protein, and R_0 is the Förster critical distance, at which 50% of the excitation energy is transferred to the acceptor [36]. It can be calculated from donor emission and acceptor absorption spectra using the Förster formula Eq. (8).

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6} \tag{7}$$

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \Phi J \tag{8}$$

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4\,\mathrm{d}\lambda}{\int_0^\infty F(\lambda)\,\mathrm{d}\lambda} \tag{9}$$

In Eq. (8), 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 the average refractive index of medium in the wavelength range where spectral overlap is significant; Φ the fluorescence quantum yield of the donor; J the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which could be calculated by Eq. (9), where, $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$; ε (λ) is the extinction coefficient of the acceptor at λ. In the present case, $N = 1.36$, $\Phi = 0.15$[24], according to Eqs. (7)–(9), we could calculate that $J = 2.08 \times 10^{-15}\ cm^3\ L\ mol^{-1}$, $E = 0.68$, $R_0 = 3.68$ nm, $r = 3.12$ nm. The average distances between a donor fluorophore and acceptor fluorophore on the 2–8 nm scale, and $0.5R_0 < r < 1.5R_0$ [38] indicate that the energy transfer from BSA to SiW occurs with high probability [24], while R_0 is bigger than r in the present study also reveals that SiW could strongly quench the intrinsic fluorescence of BSA by non-radiative energy transferring and static quenching.

3.6. Conformation investigation

It is proverbial that the fluorescence of BSA comes from the tyrosine, tryptophan and phenylalanine residues. The spectrum of BSA was sensitive to the micro-environment of these chromophores and it allows non-intrusive measurements of protein in low concentration under physiological conditions. The synchronous fluorescence spectra give information about the molecular environment in a vicinity of the chromosphere molecules and have several advantages, such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects [30]. Yuan et al. [39] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum corresponding to the changes of the polarity around the chromophore molecule [40]. When the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [41]. The effect of SiW on BSA synchronous fluorescence spectroscopy is shown in Fig. 5.

It is apparent from Fig. 5 that the emission maximum of tryptophan residues does not significant shift and the little blue shift of tyrosine residues fluorescence, which indicated that the polarity around the tyrosine residues was decreased and the hydrophobicity was increased [42], but the interaction of SiW with BSA does not obviously affect the conformation of tryp-

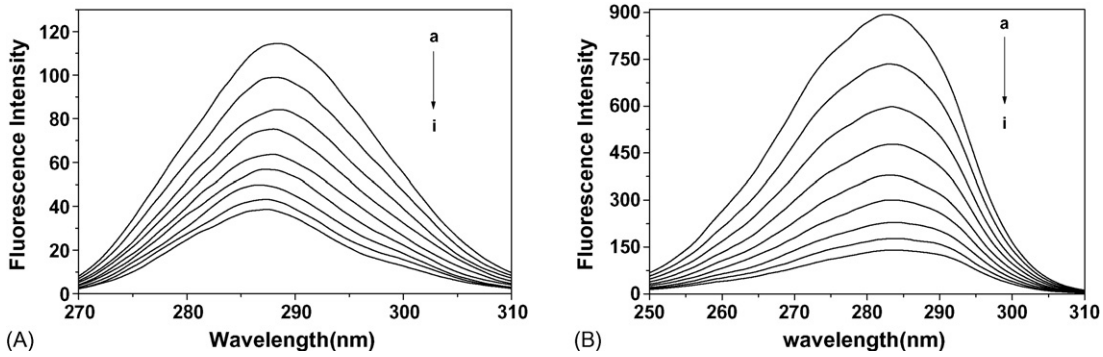

Fig. 5. Synchronous fluorescence spectrum of BSA ($T = 299$ K, pH 7.4), from curve a to i, BSA concentration was at 5.0 μmol L^{-1}, SiW concentrations: 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 μmol L^{-1}, respectively. (A) $\Delta\lambda = 15$ nm and (B) $\Delta\lambda = 60$ nm.

tophan micro-region [43]. In addition, Tyrosine residues are located mainly in sub-domain IB (Tyr 140, Tyr 148, Tyr 150, Tyr 156, Tyr 157) [1]. SiW could involve the second site (sub-domain IB) with higher binding affinity and the formation of complex led to the observation of the little blue shift of tyrosine residues fluorescence when c (SiW) ≥ 4.0 μmol L^{-1}.

4. Conclusions

A fluorescence method for the rapid and simple determination of the interaction between SiW and BSA was provided. The method is easy to operate and is reliable, practical and simple. Analysis was made in the present work using the date for protein fluorescence changes induced by inorganic ions. The results obtained give preliminary information on the binding of SiW to BSA. The experimental results showed that the fluorescence quenching of BSA by silicotungstic acid is a result of the formation of SiW–BSA complex; static quenching and non-radiative energy transferring were confirmed to result in the fluorescence quenching. Hydrophobic interaction force plays a major role in stabilizing the complex. At low concentration (c (SiW) ≤ 3.5 μmol L^{-1}), SiW reached the high affinity hydrophobic cavities in sub-domain IIA with a single class-binding site and when c (SiW) ≥ 4.0 μmol L^{-1}, SiW involved sub-domain IB with two binding sites. The distance $r = 3.12$ nm between BSA and SiW was obtained according to fluorescence resonance energy transfer. The results of synchronous fluorescence spectroscopy and UV/vis absorption spectroscopy indicate that the conformation of BSA was changed in the presence of SiW.

The binding study of inorganic drugs with proteins is of great importance in pharmacy, pharmacology and biochemistry. This study is expected to provide important insight into the interactions of the physiologically important protein BSA with inorganic ions. Information is also obtained about the effect of environment on BSA structure which may be correlated to it's physiologically activity.

Acknowledgements

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