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Affinity of Two Novel Five-Coordinated Anticancer Pt(II) Complexes to Human and Bovine Serum Albumins: A Spectroscopic Approach

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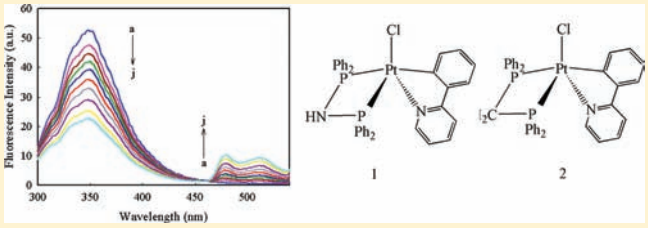
Supporting Information

ABSTRACT: The interactions of two organoplatinum complexes, [Pt(C^N)Cl(dppa)], **1**, and [Pt(C^N)Cl(dppm)], **2** (C^N = N(1), C(2′)-chelated, deprotonated 2-phenylpyridine, dppa = bis(diphenylphosphino)amine, dppm = bis(diphenylphosphino)methane), as antitumor agents, with bovine serum albumin (BSA) and human serum albumin (HSA) have been studied by fluorescence and UV−vis absorption spectroscopic techniques at pH 7.40. The quenching constants and binding parameters (binding constants and number of binding sites) were determined by fluorescence quenching method. The obtained results revealed that there is a strong binding interaction between the ligands and proteins. The calculated thermodynamic parameters (ΔG, ΔH, and ΔS) confirmed that the binding reaction is mainly entropy-driven, and hydrophobic forces played a major role in the reaction. The displacement experiment shows that these Pt complexes can bind to the subdomain IIA (site I) of albumin. Moreover, synchronous fluorescence spectroscopy studies revealed some changes in the local polarity around the tryptophan residues. Finally, the distance, *r*, between donor (serum albumin) and acceptor (Pt complexes) was obtained according to Förster theory of nonradiation energy transfer.



1. INTRODUCTION

Cisplatin is a widely used anticancer drug that is highly effective against testicular and ovarian cancers but has a number of side effects such as nephrotoxicity, ototoxicity, and allergy and a limited spectrum of activity due to inherent and/or acquired resistance.[1] Therefore, much attention has been focused on designing new platinum compounds with improved pharmacological properties, broader range of antitumor activity, and wider spectrum of activity.[2] Although most of the platinum anticancer compounds are classical platinum complexes, study on organometallic platinum complexes is not ample. Traditional belief of incompatibility of organometallic compounds with biological systems may be the main reason for lack of study in this area. Recently, a number of promising cyclometalated platinum(II) complexes have been investigated as anticancer drugs.[3−8]

Several reports have demonstrated that the binding of platinum complexes to the N7-nitrogens of two adjacent guanosine bases may disrupt DNA duplication leading to cell death, but exact mechanism remained unknown. Platinum complexes are also known to react with many other cell components including glutathione and other S-containing biomolecules present in relatively high doses inside the cell.[9] This binding eventually would lead to altered protein conformation and changes in biological activity, especially when enzymatic reactions are affected.[10] Thus, reactions of cisplatin with bionucleophiles other than DNA are of no lesser biological importance because these interactions play central roles in modulating the activity of the platinum-based antitumor drugs. It has also been suggested that more than 90% of platinum in the blood, following intravenous administration of cisplatin, is covalently bound to the plasma proteins.[11,12] Therefore, the study of cisplatin−protein is of a major biological interest that can provide useful information regarding the cisplatin−DNA protein recognition.[13] Among the possible non-DNA targets, albumin is the most used.

Serum albumins, as the most abundant proteins in the circulatory system, act as transporter and disposer of many endogenous and exogenous compounds.[14,15] The crystal structure analyses of human serum albumin (HSA) have revealed that the drug binding sites are located in subdomains IIA and IIIA.[16] A large hydrophobic cavity is present in IIA subdomain. The geometry of the pocket in IIA is quite different from that found for IIIA. Bovine serum albumin (BSA) is structurally homologous to human serum albumin (HSA).[17] HSA has one tryptophan (Trp-214) in subdomain IIA, whereas BSA has two tryptophan moieties (Trp-134 and Trp-213) located in subdomains IB and IIA, respectively.[18,19] The affinities of drugs to protein would directly influence the concentration of drugs in the blood and in the binding sites and the duration of the effectual drugs and consequently contribute

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to their magnitude of biological actions in vivo. Generally, the weak binding leads to a shorter lifetime or poor distribution, while strong binding decreases the concentration of free drug in plasma. Because of these, studies on this aspect can provide information on the structural feature that determines the therapeutic effectiveness of drugs and standardized screens for protein binding in new drug design and for fixing dose limits.[20,21] Therefore, the binding of drugs to serum albumin in vitro, considered as a model in protein chemistry to study the binding behavior of proteins, has been an interesting research field in chemistry, life sciences, and clinical medicine.[22]

Anticancer drugs are used for human treatments as well as veterinary applications. Therefore, finding pharmacokinetics of drugs in both human and animals would be interesting. BSA presents 76% sequence identity with HSA. Although they have a similar folding and a well-known primary structure, and they have been associated with binding of many different categories of small molecules,[23,24] they represent a slight difference in drug binding.[23,25] Thus, in biophysical and biochemical research BSA and HSA are studied simultaneously to figure out the differences and similarities in function of these albumins.[23−26]

Very recently, we synthesized two new five coordinated cyclometalated platinum(II) complexes, [Pt(C^N)Cl(dppa)], **1**, and [Pt(C^N)Cl(dppm)], **2**, where C^N = N(1), C(2′)-chelated, deprotonated 2-phenylpyridine, dppa = bis(diphenylphosphino)amine, dppm = bis(diphenylphosphino)methane, with interesting high antitumor activities (Figure 1).[8]

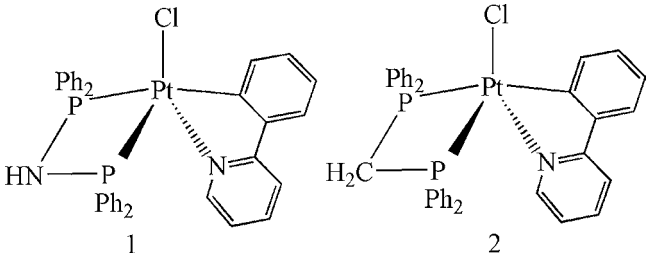

Figure 1. Chemical structure of Pt complexes.

Both in vivo and in vitro studies showed that both platinum complexes exhibit potent anticancer activity superior to than cisplatin. Interestingly, both complexes showed protesaome inhibitory activity toward purified 20S proteasome and intact 26S proteasome in human breast cancer cells. Moreover, spectrofluorimetric analysis of the DNA binding of the complexes with DNA (using ethidium bromide as displacement ligand) indicated that the ligand intercalated into the base pairs of DNA.[8]

In this work, we investigated the binding of these two new Pt(II) complexes to the blood carrier proteins HSA and BSA at physiological pH and temperatures of 27 °C (room temperature) and 37 °C (physiologic temperature). Using different spectroscopic methods, the binding information, including quenching mechanisms, binding parameters, thermodynamic parameters, binding modes, high-affinity binding site, intermolecular distances, and conformation changes, was investigated. Also, this study investigates whether there is any significant difference between BSA and HSA with regard to binding as they have a different number of intrinsic fluorophores (tryptophan residues).

2. EXPERIMENTAL SECTION

2.1. Materials. HSA, BSA, warfarin, and ibuprofen were purchased from Sigma and used without further purification. The stock solutions of proteins (1.00×10^{-4} mol L^{-1}) were prepared by dissolving the solid HSA and BSA in 0.05 M phosphate buffer at pH 7.4 and stored at 0−4 °C in the dark for about a week and then diluted to 1.30×10^{-6} mol L^{-1} using phosphate buffer (pH 7.4, 0.05 M) when used. The concentrations of BSA and HSA were determined from optical density measurements, using the values of molar absorptivity of ε_{280} = 44 720 and 35 700 M^{-1} cm^{-1} for BSA and HSA, respectively.[18,27] Warfarin and ibuprofen stock solutions (2.00×10^{-3} mol L^{-1}) were prepared by directly dissolving their corresponding crystals in dimethyl sulfoxide (DMSO), and then diluting to the required volume with distilled water. The Pt(II) complexes **1** and **2** were synthesized on the basis of a literature method.[8] Stock solutions of complexes **1** and **2** (3.00×10^{-3} mol L^{-1}) were prepared in DMSO because of their lower solubility in water. All other chemicals were of analytical reagent grade, and doubly distilled water was used throughout. All experiments were done in 0.05 M phosphate buffer (pH 7.4).

2.2. Apparatus. All fluorescence measurements were performed on an LS-55 spectrofluorimeter (Perkin-Elmer) equipped with a thermostatic bath and a 10 mm quartz cuvette. Fluorescence emission spectra were recorded at two different temperatures (27 and 37 °C).

UV−vis absorption spectra were recorded on a Shimadzu UV-1650PC UV−vis spectrophotometer (Japan) in a 10-mm cuvette. The UV Probe (version 1.10) software of the instrument was used to collect the absorbance data in a spreadsheet (in 1.0 nm intervals).

The pH was potentiometrically measured using a Metrohm 654 pH-meter equipped with a combined glass electrode (pH Electrode Blue Line 23 pH, Schott).

2.3.1. Procedures. *Fluorescence Measurements.* Quantitative analyses of the interaction between Pt(II) complexes **1** and **2** and biomacromolecules were performed by fluorimetric titration. A 3.0 mL portion of aqueous solution of protein (1.3×10^{-6} mol L^{-1}) was titrated by successive additions of Pt(II) complex solution (to give a final concentration of 5.0×10^{-6} mol L^{-1}). Titrations were done manually by using a trace syringe. For every addition, the mixture solution was shaken and allowed to stand for 20 min at the corresponding temperature (300 and 310 K), and then the fluorescence intensities were measured with an excitation wavelength of 280 nm and emission wavelengths in the interval 300−540 nm. No correction for inner filter effect was applied since the Pt(II) complexes represented very low absorbance (less than 0.09) at the excitation and emission wavelengths. The width of the excitation and emission slit was set to 10.0 and 4.0 nm, respectively, and the scanning speed was set at 1000 nm per min. In the meantime, the synchronous fluorescence intensity of the mixture solution was measured at $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm, respectively.

For fluorescence enhancement experiments, the solution of Pt(II) complexes in 0.05 M phosphate buffer of pH 7.4 (5.0×10^{-6} mol L^{-1}) was titrated with proteins. Each resultant solution through the titration was allowed to stand for 10 min, and then its fluorescence emission spectrum was recorded in the wavelength range 380−600 nm (using an excitation wavelength of 320 nm). Both the excitation and emission slits were set to 10 nm. The dilution effect in the corresponding spectra was corrected in the different experiments.

2.3.3. Site Marker Competitive Experiments. Binding location studies between Pt(II) complexes and HSA or BSA in the presence of two site markers (warfarin and ibuprofen) were measured using the fluorescence titration methods.

2.3.3.1. Warfarin as Marker of Site I. The displacement experiments were performed using the site probe warfarin by preparing equimolar mixtures of biomacromolecule and warfarin (each 1.3×10^{-6} mol L^{-1}), which were then thoroughly mixed and equilibrated at room temperature for 1 h. A 3.0 mL portion of the solution was transferred to a cell, and then it was titrated by successive additions of Pt(II) solution. After thorough mixing of the resultant solution at each

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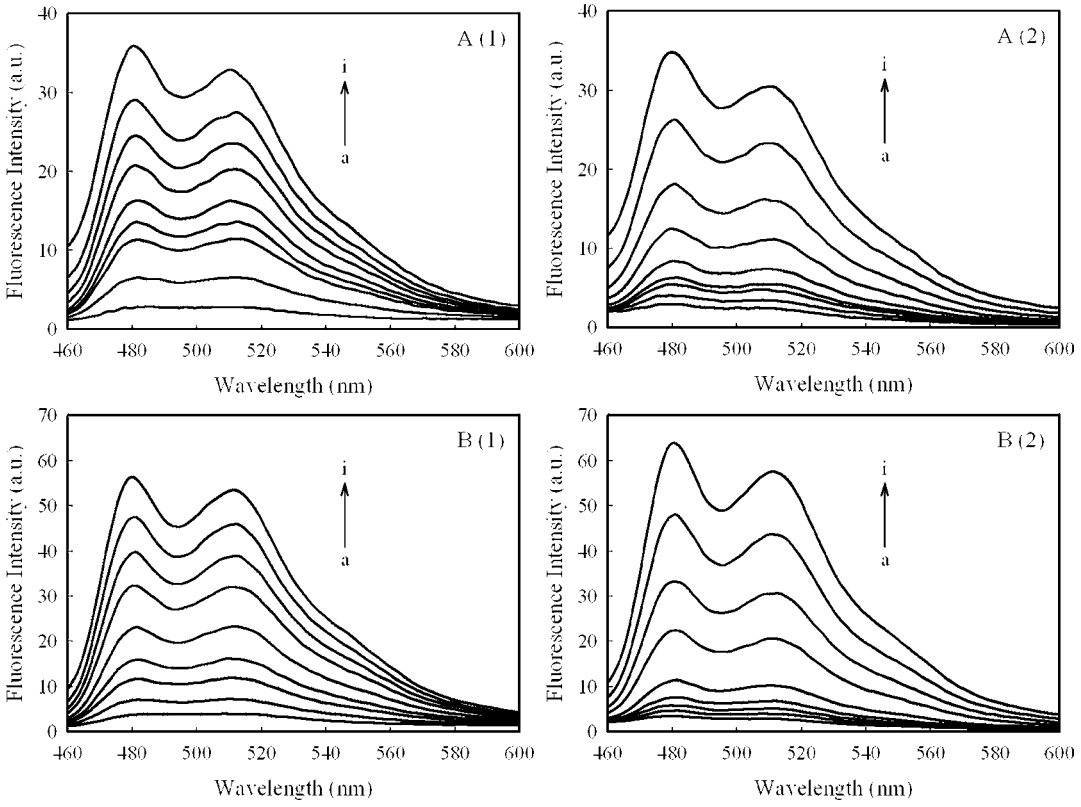

Figure 2. Fluorescence emission spectra of **1** (left, 1) and **2** (right, 2) with HSA (top, A) and BSA (bottom, B). The concentration of Pt complexes was 5.0×10^{-6} mol L^{-1}, and SA concentration was varied from (a) 0.0 to (i) 5.0×10^{-6} mol L^{-1}; pH 7.4 and λ_{ex} 320 nm.

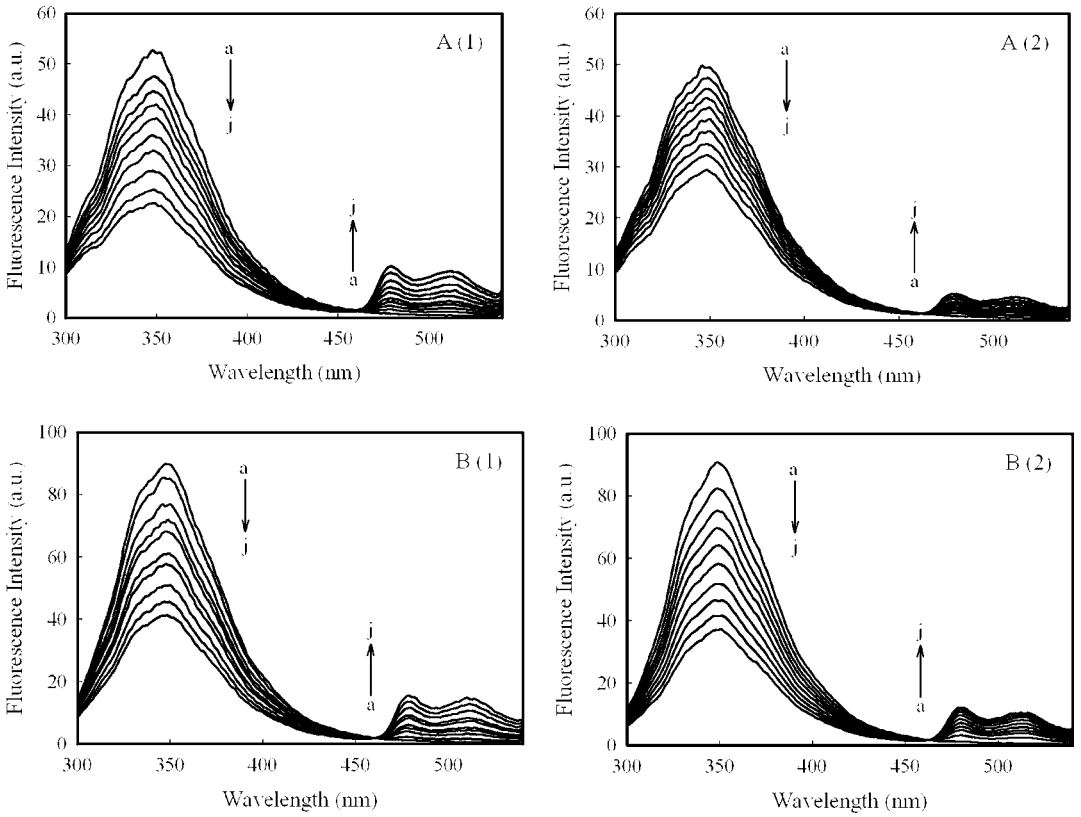

Figure 3. Changes in the fluorescence spectra of HSA (top, A) and BSA (bottom, B) through their titration with **1** (left, 1) and **2** (right, 2) at 300 K. The concentration of both proteins is 1.3×10^{-6} mol L^{-1}, and Pt(II) complex concentration was varied from (a) 0.0 to (j) 5.0×10^{-6} mol L^{-1}; pH 7.4 and λ_{ex} 280 nm.

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titration step, the solutions were allowed to stand for 20 min. An excitation wavelength of 320 nm was selected, where both warfarin and Pt complexes are fluorescent whereas HSA and BSA are not. The emission fluorescence spectra were recorded in the wavelength range 340−600 nm.

2.3.3.2. Ibuprofen as Marker of Site II. Equimolar mixtures of protein and Pt(II) complex (1.3×10^{-6} mol $L^{-}1$) were thoroughly mixed and allowed to equilibrate at room temperature for 1 h. A 3.0 mL portion of the solution was transferred to the spectrofluorimetric cell, and then ibuprofen was gradually added to this solution. The other procedure was the same as that in Section 2.3.3.1.

3. RESULTS AND DISCUSSION

3.1. Effect of HSA/BSA on the Fluorescence Emission of Pt Complexes. Two five coordinated Pt(II) complexes **1** and **2** (Figure 1) were studied in this work. These complexes are weakly fluorescent molecules; however, their fluorescence turns on by the addition of biomacromolecules. The spectrofluorimetric titrations of the Pt(II) complexes with HSA and BSA were thus carried out in phosphate buffer of pH 7.4, and representative spectra are presented in Figure 2. As seen, an increase in the concentration of HSA or BSA results in an enhancement of the fluorescence intensity of the Pt(II) complexes and thus the fluorescence quantum yield (Φ_f) of the complexes. Both compounds exhibit a twin-peak emission spectrum at about 480 and 515 nm (λ_{ex} = 320 nm). The effects of BSA and HSA in the emission spectra are similar. However, one can observe a slight difference between the effects of addition of macromolecules on the emission spectra of **1** and **2**. The changes in the emission intensity of **1** at all protein concentrations seem similar whereas those of **2** are larger at higher concentrations of proteins. The observed changes in the fluorescence spectra of the Pt complexes can be rationalized in terms of binding of the probe compounds with the proteins leading to a less polar microenvironment around the fluorophore. Binding of the complexes to the binding pocket of the proteins decreases the freedom of these Pt(II) complexes to rotate or vibrate. This increase in rigidity can be considered as another reason for increased emission intensity of the Pt(II) complexes. A blue shift in the fluorescence maximum also suggests a reduction in the polarity of the microenvironment.[28]

3.2. Fluorescence Quenching Studies. The interaction of Pt(II) complexes with HSA and BSA was also monitored by studying the quenching the fluorescence of HSA and BSA with increasing concentration of Pt(II) complexes. Thus, the emission spectra of HSA and BSA in the presence of different concentrations of Pt(II) complexes were recorded in the wavelength range 300−540 nm by exciting the protein at 280 nm. The representative fluorescence emission spectra are given in Figure 3. As seen, in both cases, the fluorescence intensities of the proteins are decreased regularly with increasing concentration of the probe compounds, indicating the binding of probes to the proteins. A decrease in the fluorescence intensity of both HSA and BSA at 348 nm accompanies the appearance of two peaks at about 480 and 512 nm, which can be attributed to the fluorescence spectra of the Pt(II) complexes bound to the proteins.

Commonly, fluorescence quenching can be described by the following Stern−Volmer equation (eq 1)[29]

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

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the Stern−Volmer quenching constant, $[Q]$ is the total concentration of quencher, k_q is the bimolecular quenching constant, and τ_0 is the average lifetime of protein in the absence of quencher, and its value is 10^{-8} s. The corresponding Stern−Volmer quenching constants K_{SV} and quenching rate constants k_q are given in Table 1. All the plots represent a good linear relationship (Supporting Information, Figure S1). As it is known, linear Stern−Volmer plots represent a single quenching mechanism, either static (the formation of a complex between quencher and fluorophore) or dynamic (a collisional process).[30]

Table 1. Quenching Parameters of the Interaction of Pt Complexes with HSA/BSA at Different Temperatures[a]

	T (K)	10^{-5} K_{SV} (M^{-1})	10^{-13} k_q (M^{-1} s^{-1})	R
HSA-**1**	300	2.585	2.585	0.9965
	310	3.028	3.028	0.9954
HSA-**2**	300	1.274	1.274	0.9977
	310	1.702	1.702	0.9992
BSA-**1**	300	2.328	2.328	0.997
	310	3.575	3.575	0.9961
BSA-**2**	300	2.828	2.828	0.9975
	310	3.491	3.491	0.9984

[a] R is the linear correlated coefficient.

The dynamic and static quenchings can be distinguished by differing in their dependence on temperature. In the case of dynamic quenching, higher temperature results in faster diffusion, and consequently, the quenching rate constant increases with increasing temperature. In contrast, in the case of static quenching, increasing temperature is likely to result in decreasing complex stability and hence in lowering the value of the static quenching constant.[30] Table 1 shows that K_{SV} increases with rising temperature, indicating that the fluorescence quenching of HSA and BSA by the Pt(II) complexes is likely to occur via a dynamic quenching mechanism. The obtained bimolecular quenching constants for these compounds are on the order of 10^{13} L mol^{-1} s^{-1}, which is 1000-fold higher than the maximum value possible for diffusion controlled quenching (i.e., 2.0×10^{10} L mol^{-1} s^{-1}).[31] This observation suggests that there is a specific interaction between the proteins and Pt(II) complexes and the probable quenching mechanism was not initiated by dynamic quenching but by a static one.

A static process for the observed quenching can also be supported by the UV−vis spectra of the fluorophore. For a dynamic quenching mechanism, the absorption spectra of the fluorescent substance is not changed, and only the excited state fluorescence molecule is influenced by quenchers, while, for static quenching, a new compound is formed between the ground state of the fluorescent substance and quencher and, therefore, the absorption spectra of fluorescence substance would be considerably influenced.[30] Figure 4 shows the UV absorption of the proteins in the presence and absence of the Pt(II) complexes. The influences of the absorbance of Pt(II) complexes were eliminated by adding in the reference cells the solutions of Pt complexes of the same concentrations as in the sample solution. As can be seen from Figure 4, both proteins possess two absorption peaks at around 210 and 280 nm, where that at 210 nm represents the content of α-helix in the protein.[32] A dramatic decrease in the 210 nm absorbance peak of the proteins is observed upon addition of Pt(II) complexes

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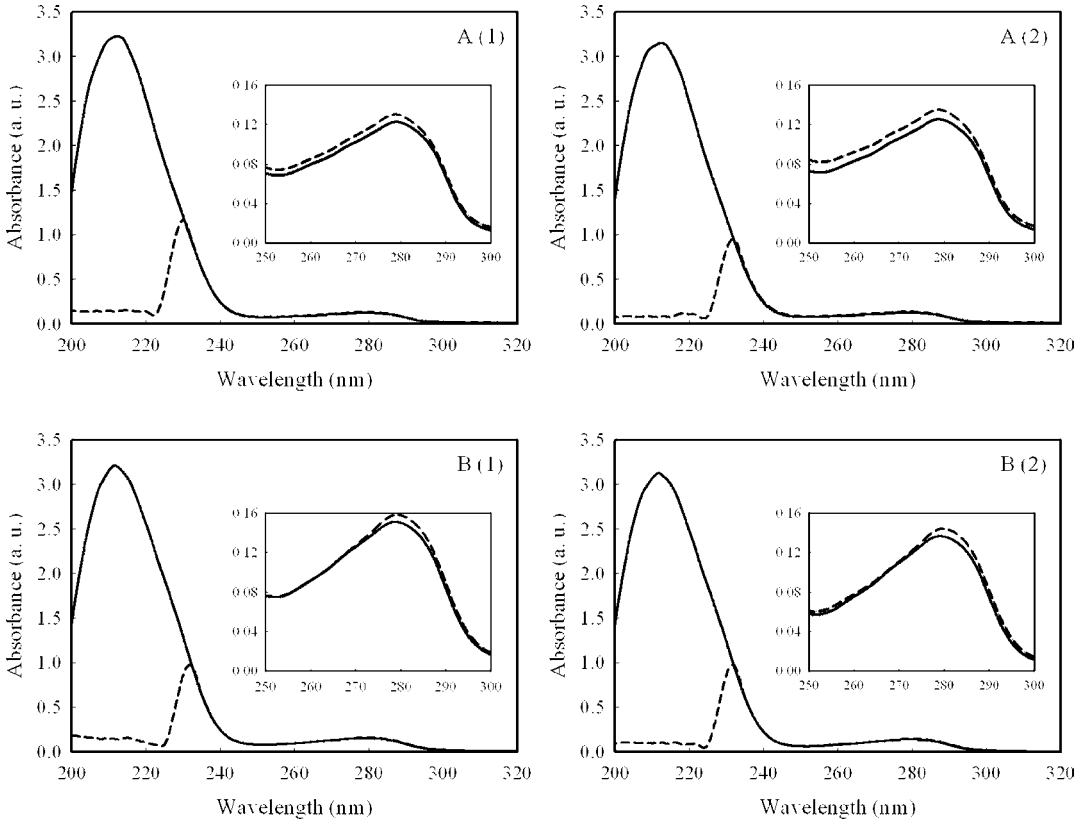

Figure 4. UV−vis absorption spectra of HSA (top, A) and BSA (bottom, B) in the absence and presence of **1** (left,1) and **2** (right, 2). Solid line: the absorption spectrum of proteins. Dashed line: the absorption spectrum of proteins in the presence of Pt complexes at the same concentration, c(HSA) = c(Pt complexes) = 3.5×10^{-6} mol L^{-1}. The inset shows the enlarged spectra in the wavelength range 250−300 nm.

Table 2. Modified Stern−Volmer Association Constant K_a and Relative Thermodynamic Darameters of SA-Pt(II) Complex Systems[a]

	T (K)	10^{-5} K_a (M^{-1})	R	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
HSA-**1**	300	2.975	0.9985	78.384	−31.435	366.064
	310	3.539	0.9933		−32.929	359.078
HSA-**2**	300	1.282	0.9944	78.481	−29.335	359.386
	310	1.530	0.9982		−30.768	352.414
BSA-**1**	300	3.236	0.9963	78.979	−31.644	368.744
	310	4.248	0.9948		−33.400	362.515
BSA-**2**	300	2.759	0.9958	78.506	−31.247	365.846
	310	3.344	0.9983		−32.784	359.002

[a] R is the linear correlated coefficient.

to the proteins. This can be attributed to the induced perturbation of α-helix of proteins by a specific interaction with the ligands.[33] Furthermore, an obvious red shift in the position of the absorbance peak (i.e., from 210 to 231 nm) could also be observed with the addition of Pt(II) complexes. Meanwhile, the absorption intensity of the 280 nm peaks is increased (Figure 4) by addition of Pt(II) complexes, indicating that more aromatic acid residues were extended into the aqueous environment. Trp-214 in HSA and Trp-213 in BSA, which are originally buried in a hydrophobic pocket, were exposed to an aqueous milieu to a certain degree.[32] This result indicated that the microenvironment of the three aromatic acid residues was altered and the tertiary structure of HSA and BSA was destroyed. These results show that the interaction between Pt(II) complexes and HSA and BSA was mainly a static quenching process.

To ensure that the whole complexes interact with the albumins instead of the ligands by themselves, we investigated the binding of dppa and dppm ligands to HSA. It was observed that the fluorescence quenching behavior of ligands was different as compared with those of five-coordinated complexes **1** and **2** (Supporting Information, Figure S2). In addition, the fluorescence spectrum of complexes at around 480−600 nm is not observed. Moreover, the quenching constant (K_{SV}) of the ligands calculated as 8.702×10^4 and 5.875×10^4 M^{-1} for dppa and dppm at 300 K, respectively, were lower than corresponding K_{SV} of **1** and **2**, which were 2.285×10^5 and 1.274×10^5 M^{-1} for complexes **1** and **2**, respectively. Two points are concluded from these observations: (i) The ligands have high affinity toward albumins so that the interactions between Pt complexes and albumins can be mainly attributed to the interaction of hydrophobic ligands with albumins, and (ii) the higher binding constant of complexes confirms the

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significant role of the configuration of ligands around Pt. In addition, we previously showed that the complexes remain pentacoordinated in aqueous solution.[8] So, the observed interactions between complexes and albumins can be attributed to both ligands and whole complexes.

According to the aforementioned explanation, the quenching data were analyzed according to the modified Stern–Volmer equation:[34]

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

In the present case, K_a is the effective quenching constant for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. The plots show a good linear relationship (Figure S3), and corresponding K_a values at different temperatures are given in Table 2. The results show that the binding constants between Pt(II) complexes and HSA and BSA are quite high. Thus, the Pt(II) complexes **1** and **2** can be stored and carried by proteins in the body.

3.3. Binding Parameters. When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the numbers of binding sites (n) can be determined using the following equation[9]

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

where K_b is the binding constant, reflecting the degree of interaction HSA and BSA and the Pt(II) complexes, and n is the number of binding sites. According to the experimental results (Figure 3), the linear fitting plots of $\log((F_0 - F)/F)$ versus $\log[Q]$ can be observed (Figure S4). The corresponding K_b and n values, evaluated from the slopes and intercepts of the linear plots, respectively, are summarized in Table 3. As seen,

Table 3. Binding Parameters of the System of the Interaction of Pt Complexes with HSA/BSA at Different Temperatures[a]

	T (K)	10^{-5} K_b (M^{-1})	n	R
HSA-**1**	300	1.051	0.93	0.9943
	310	1.849	0.96	0.9964
HSA-**2**	300	1.053	0.98	0.9974
	310	1.395	0.98	0.9983
BSA-**1**	300	1.032	0.94	0.9963
	310	1.907	0.95	0.9958
BSA-**2**	300	2.094	0.97	0.9983
	310	2.987	0.99	0.9978

[a] R is the linear correlated coefficient.

the value of n is nearly 1 for binding of both complexes to the proteins used, which indicates that, in the binding reactions the molar ratio of protein to drug is 1:1. The binding constant data suggest that there is no preference for HSA to bind to each one of the Pt(II) complexes, whereas BSA binds to complex **2** about 2 times stronger than to complex **1**.

3.4. Thermodynamic Parameters and Binding Mode. Generally, small molecules are bound to macromolecules through four binding modes, namely hydrogen bonding, van der Waals, electrostatic, and hydrophobic interactions. The sign and magnitude of the thermodynamic parameters such as ΔG (free energy change), ΔH (enthalpy change), and ΔS (entropy change) of the reaction are important for the study of the interaction forces. Considering the constancy of the reaction enthalpy change for small temperature changes, the thermodynamic parameters were calculated from the following equations

$$\ln\frac{K_2}{K_1} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R} \quad (4)$$

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

where K is the equilibrium binding constant, which is analogous to the effective quenching constants K_a at the corresponding temperature, K_1 and K_2 are the binding constants at temperatures T_1 and T_2, respectively, and R is the gas constant. The calculated thermodynamic parameters are given in Table 2.

Ross and Subramanian[35] have characterized the sign and magnitude of the thermodynamic parameters associated with various kinds of interactions that may take place in protein association processes. From the point of view of water structure, a positive ΔS and ΔH value is frequently taken as evidence for hydrophobic interactions, while hydrogen bond and van der Waals power may decrease them. Furthermore, specific electrostatic interactions between ionic species in aqueous solutions are characterized by a positive value of ΔS while $\Delta H \approx 0$. According to the data given in Table 2, a negative value of ΔG indicates the spontaneity of the interaction. The positive values obtained for both ΔH and ΔS indicate that a hydrophobic association is the major binding force and that the interaction is an entropy driven process. In addition to hydrophobic interaction, a possible covalent bonding may be also considered. However, the value of ΔH obtained here (about 78 kJ mol^{-1}) is considerably below what would be expected for a covalent bond formation, which should be ≥120 kJ mol^{-1}.[36] In addition, we found that these complexes remain neutral pentacoordinated in the assay condition[8] and there is no empty position for coordination on Pt(II) to form covalent bond with albumin. Moreover, as it was explained previously, the aromatic ligands play a central role in the interaction of the complexes with albumins. As the ligands possess more hydrophobic character, hydrophobic interaction between complexes and albumins is supported.

3.5. Site-Selective Binding of Pt Complexes on HSA/BSA. The crystal structure analyses[37] revealed that SA contains three homologous domains (I–III), each of which is composed of two subdomains A and B. While Sudlow et al.[38] have suggested two distinct binding sites on HSA, namely site I and site II. The former showed affinity for warfarin, phenylbutazone, etc., whereas the binding pocket of the latter is well suited for ibuprofen, diazepam, fluofenamic acid, etc. To identify the binding site location of Pt(II) complexes on the region of SA, competitive binding experiments were carried out, using warfarin, a characteristic marker for site I, and ibuprofen as one for site II. Then information about the Pt(II) complexes binding site can be obtained by monitoring the changes in the fluorescence spectra of Pt(II) complexes bound to SA, brought about by site I and site II markers.

Warfarin is a well-known marker of site I (hydrophobic subdomain IIA) of SA.[39] The weak fluorescence intensity of warfarin is enhanced upon binding with SA due to its interaction with Trp 214 in HSA or Trp 213 in BSA,[40] when excited at 320 nm. The fluorescence intensity of warfarin, in its bound state to SA, decreases if a second ligand competes for the site occupied by it. Thus, the accessibility of Trp 214 and Trp 213 in HSA and BSA, respectively, by ligands can be confirmed by monitoring the displacement of warfarin by Pt(II) complexes in the SA–warfarin complex. As demonstrated in

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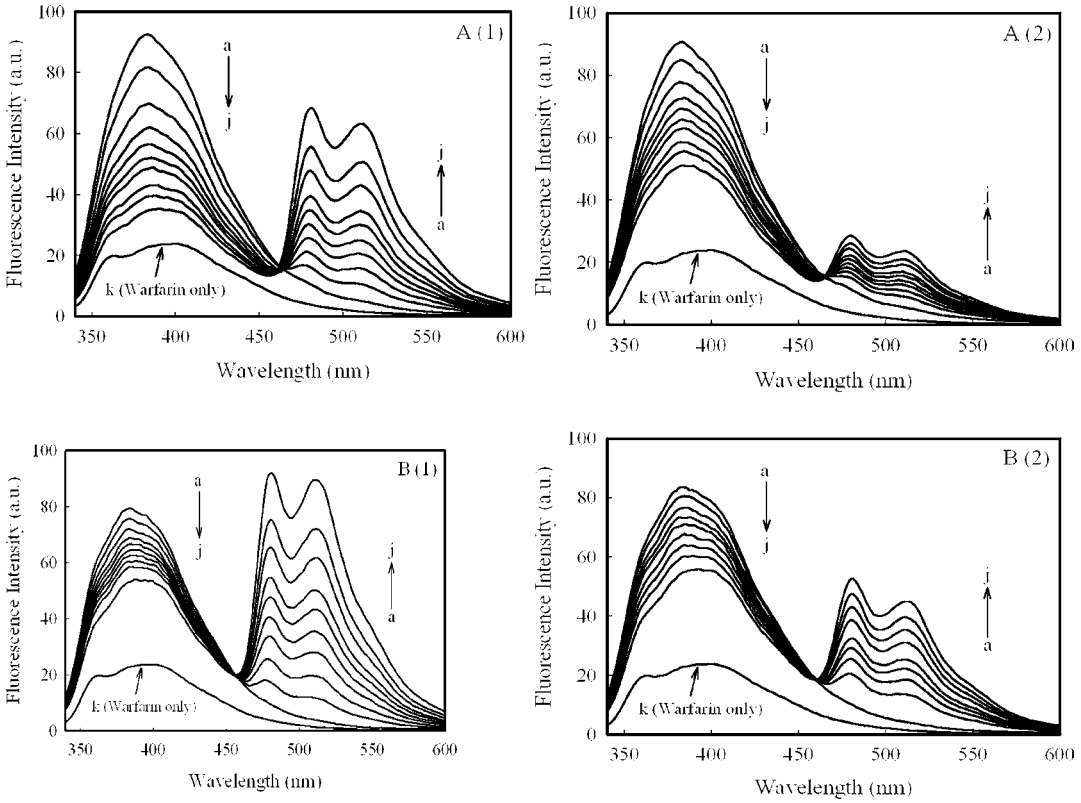

Figure 5. Effect of **1** (1) and **2** (2) complexes to warfarin–HSA (A) and warfarin–BSA (B) system (λ_{ex} = 320 nm). (a–g) c(warfarin) = c(SA) =1.3 × 10^{-6} mol L^{-1}; **1** and **2** concentration was from (a) 0.0 to (j) 11.7 × 10^{-6} mol L^{-1}. Curve k shows the emission spectrum of warfarin only.

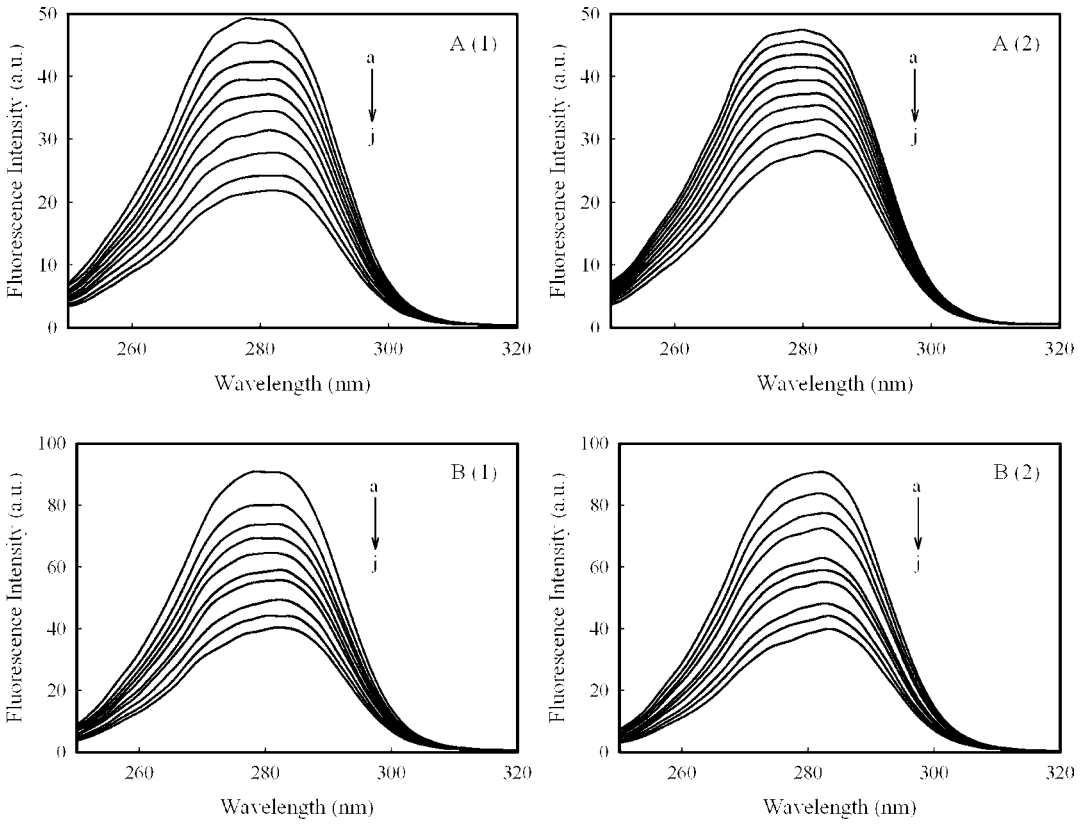

Figure 6. Synchronous fluorescence spectra of HSA (A) and BSA (B) (1.3 × 10^{-6} mol L^{-1}) upon addition of **1** (1) and **2** (2); $\Delta\lambda$ = 60 nm. The concentration of Pt complexes from a to j was 0.0 − 5.0 × 10^{-6} mol L^{-1}.

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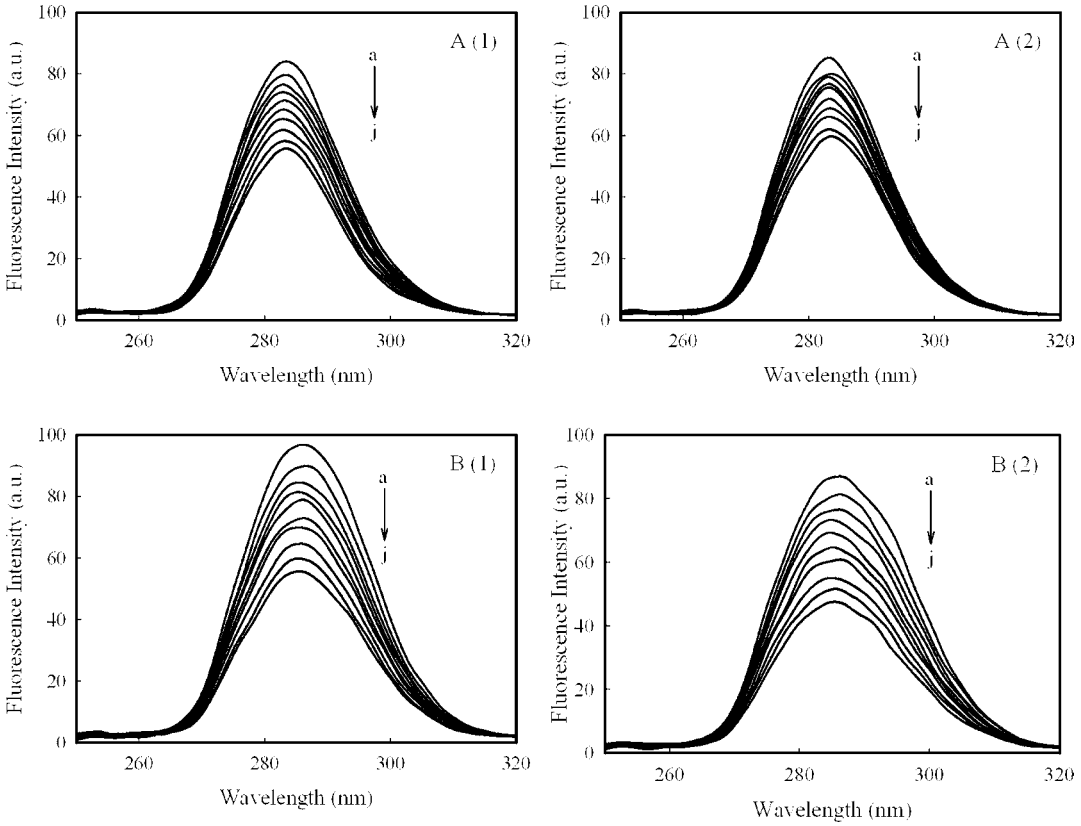

Figure 7. Synchronous fluorescence spectra of HSA (A) and BSA (B) (1.3×10^{-6} mol L^{-1}) upon addition of **1** (1) and **2** (2); $\Delta\lambda$ = 15 nm. The concentration of Pt complexes from a to j was 0.0 − 5.0×10^{-6} mol L^{-1}.

Figure 5, a decrease in the emission intensity of SA−warfarin (in the wavelength region of 340−440 nm, excited at 320 nm) is observed when the Pt(II) complexes are added. The strong quenching of warfarin bound to protein upon addition of Pt(II) complexes reflects a reduction in the warfarin binding capacity at the primary binding site of SA and preferential accessibility of Trp 214 in HSA and Trp 213 in BSA by both Pt(II) complexes. It is very likely that the Pt(II) complex binding occurs at the warfarin site I, located in subdomain IIA.

To investigate whether the Pt(II) complexes are also tending to bind to the binding site II of albumin, an ibuprofen competitive experiment was conducted. Ibuprofen was gradually added to the solution containing equimolar concentrations of SA and each of Pt(II) complexes, and the change in fluorescence intensity of Pt(II) complexes (λ_{ex} = 320 nm, λ_{em} = 520 nm) was monitored. The binding constant of ibuprofen to SA is in the range 3.0×10^5−3.6×10^6 M^{-1}, which is higher than those of the studied Pt complexes. So, if the Pt complexes bind to site II of albumin, they would be substituted by ibuprofen. As it was explained in Section 3.1, the unbound Pt complexes have weaker fluorescence emission with respect to the albumin-bound complexes. Thus a decrease in the flourscence intensity of Pt complexes upon addition of ibuprofen is expected if the complexes bind to site II of albumin. However, it was found that the fluorescence intensities of albumin-bound Pt complexes did not change upon addition of ibuprofen, and it was almost the same as that in the absence of ibuprofen (the data not shown). These data do not support the binding of Pt(II) complexes to the site II of albumin.

3.6. Synchronous Fluorescence Spectroscopy Studies.

Since the introduction of synchronous fluorescence spectroscopy in the 1970s by Lloyd and Evett,[41] this technique can give information about the molecular environment in the vicinity of the fluorophore molecules at low concentrations under physiological conditions.[42] They have several advantages like spectral simplification, spectral bandwidth reduction, and avoidance of different perturbing effects.[43] Therefore, synchronous fluorescence spectra are frequently used to characterize the interaction between fluorescence probe and proteins.[44] When $\Delta\lambda$ of 15 nm is used, the obtained synchronous fluorescence spectrum indicated the spectral property of tyrosine residues, whereas $\Delta\lambda$ of 60 nm indicates that of tryptophan residues.[45] The maximum emission wavelengths of tryptophan and tyrosine residue in the protein molecule are related to the polarity of their surroundings; changes of the maximum emission wavelengths can reflect changes of protein conformation. The effect of Pt(II) complexes on HSA and BSA synchronous fluorescence spectroscopy with $\Delta\lambda$ = 60 nm and $\Delta\lambda$ = 15 nm is shown in Figures 6 and 7, respectively. It can be seen from Figure 6 that the maximum emission wavelength represents a slight red shift (from 280 to 284 nm) at the investigated concentration range when $\Delta\lambda$ was equal to 60 nm. However, as seen in Figure 7, the maximum emission wavelength kept the position at the investigated concentrations range when $\Delta\lambda$ was equal to 15 nm. The red shift of the maximum emission wavelength indicates that the conformation of HSA and BSA was changed and the polarity around the tryptophan residues was increased, whereas the hydrophobicity was decreased.[46] However, the microenvironment around the

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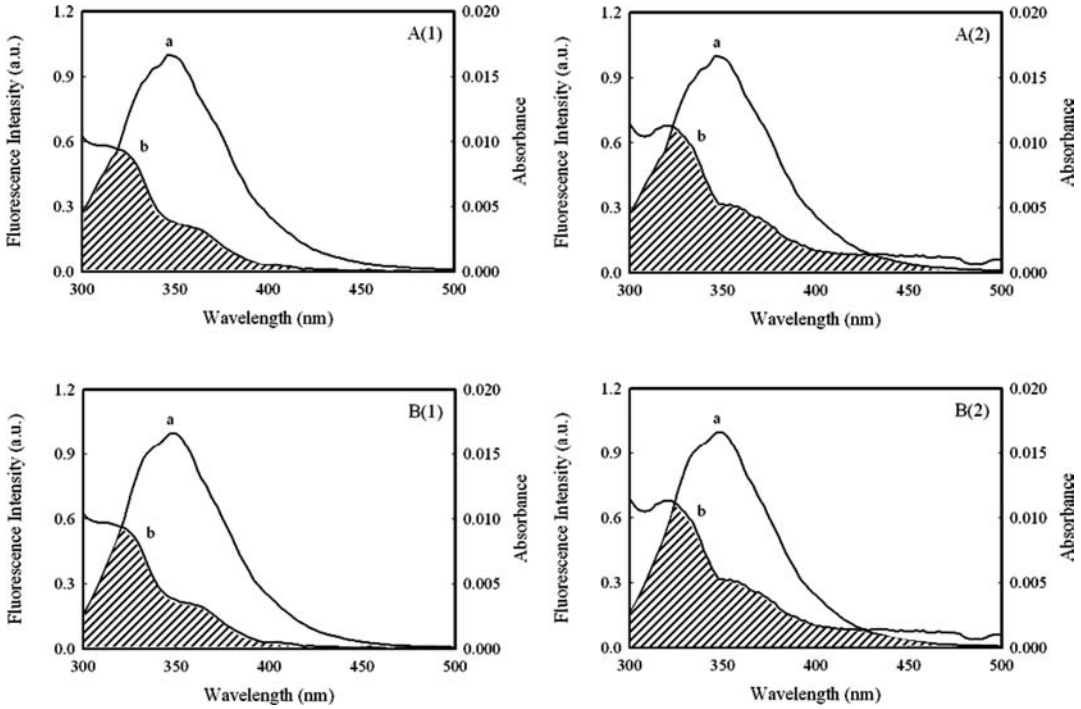

Figure 8. Overlap of the fluorescence spectra (a) of HSA (A) and BSA (B) and the absorbance spectra (b) of **1** (1) and **2** (2) complexes, c(SA) = c(Pt complex) = 1.3 μM (300 K).

tyrosine residues did not undergo obvious changes during the binding process.

3.7. Energy Transfer between Pt Complexes and SA.

The spectral studies suggested that the Pt(II) complexes form complexes with HSA and BSA. To further confirm the vicinity of the Pt(II) complexes to the fluorophore SAs (the drug binding site distance between the site and the fluorophore), fluorescence resonance energy transfer (FRET) from protein to ligands has been verified. According to the Förster theory of nonradioactive energy transfer,[47] the transfer of energy can take place through a direct electrodynamic interaction between the primarily excited molecule and its neighbors.[48] Using the fluorescence resonance energy transfer (FRET), the distance r of binding between Pt complexes and BSA could be calculated by the equation

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

where E denotes the efficiency of energy transfer between the donor and acceptor, and r is the distance between the donor and acceptor. R_0, the critical distance at which the transfer efficiency equals to 50%, is given by the following equation:[49]

$$R_0^6 = 979(K^2 n^{-4} \phi J)^{1/6} nm \tag{7}$$

In eq 7, K^2 is the orientation factor to the donor and acceptor of dipoles and is equal to $^2/_3$ for random orientation as in fluid solution, n is the refraction index of medium, Φ is the fluorescence quantum yield of the donor, and J expresses the degree of spectral overlap between the donor emission and the acceptor absorption (Figure 8), which could be calculated by the equation

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \tag{8}$$

where $F(\lambda)$ is the normalized donor emission spectrum in the range from λ to $\lambda + \Delta\lambda$, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, we took n = 1.36 and Φ = 0.15.[50] According to the above equations and experimental data, $J(\lambda)$, R_0, r, and E were evaluated (Table 4). It has been reported that the energy transfer will most probably take place when the average distance between a donor fluorescence and acceptor fluorescence is on the 2−8 nm scale[51] and $0.5R_0 < r < 1.5R_0$.[52] This is in accord with conditions of Föster's nonradioactive energy transfer theory which indicated again a static quenching interaction between Pt(II) complexes and SA.[53]

Table 4. Förster Energy Transfer Parameters of the Interaction of Pt Complexes with HSA/BSA

	E	$J \times 10^{13}$ (M^{-1} cm^3)	R_0 (nm)	R (nm)
HSA-**1**	0.26	4.37	2.20	2.63
HSA-**2**	0.17	6.42	2.34	3.06
BSA-**1**	0.26	4.38	2.20	2.63
BSA-**2**	0.29	6.45	2.35	3.02

4. CONCLUSIONS

The binding mechanisms of two newly designed antitumor Pt(II) complexes interacting with HSA and BSA, as two important carrier proteins, were investigated by spectroscopic methods. Experimental results suggested that these Pt(II)

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complexes could bind to the serum albumins by high affinity and quench the fluorescence of serum albumins through a static quenching mechanism. On the basis of the Stern−Volmer equation, the quenching rate constants were calculated, and their values suggested that the fluorescence quenching proceeds through a static process. The binding constants of Pt(II) complexes interacting with HSA and BSA were obtained at two temperatures. Hydrophobic interactions were found to play the most important contributions to the binding process. On the basis of the nonradiation energy transfer theory, the energy transfer efficiency and the binding acting distance between studied drugs and serum albumins were obtained. The synchronous fluorescence spectroscopy revealed that the microenvironmental changes that take place around HSA or BSA molecules were also changed in the presence of Pt(II) complexes. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple ligands. The obtained binding constants for the studied complexes with serum albumins are in the intermediate range so that they are not too low to prevent efficient distribution and are not so high to lead to decreased plasma concentration.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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