http://dx.doi.org/10.5012/bkcs.2011.32.9.3261

Interaction of Bilobalide and Ginkgolides B with Bovine Serum Albumin: A Fluorescence Quenching Study

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Received January 21, 2011, Accepted July 18, 2011

The interaction of bilobalide (BB) and ginkgolides B (GB) with bovine serum albumin (BSA) was investigated by fluorescent technique and UV/vis absorption spectroscopy. The results showed that BB and GB could intensively quench the fluorescence of BSA through a static quenching procedure. The binding constants (*Ka*) and the average binding distance between the donor (BSA) and the acceptor (ginkgolides) were obtained (r_{BB} = 5.33 nm and r_{GB} = 4.20 nm) by the theory of non-radiation energy transfer, and then the thermodynamic parameters such as ΔS^0 (0.17-0.32 kJ/mol), ΔG^0 (-20.76 ~ -17.79 kJ/mol) and ΔH^0 (32.47-76.52 kJ/mol) could be calculated, respectively. All these results revealed that the interaction of BB and GB with BSA were driven mainly by hydrophobie force. The synchronous fluorescence spectroscopy was applied to examine the effect of two ginkgolides on the configuration of BSA. The configuration alteration of BSA could be induced by the hydrophobicitv environment of tyrosine with the increase of the drug concentration.

Key Words : Bilobalide, Ginkgolides B, Bovine serum albumin, Fluorescence spectroscopy, Interaction

Introduction

Bovine serum albumin (BSA) is the most abundant carrier protein of bovine plasma. As endogenous and exogenous protein, it is capable of binding with many substances reversibly and therefore the amount of BSA could be used to assume the transport and vehicle functions in the body. The affinity of albumin for ligands depends on the hydrophobic character of the molecules and their charge. Molecules with long alkyl chains and negatively charged groups are bound very firmly, while molecules with short chains and positively charged groups are bound less firmly,[1] the rapid metabolism of drugs and the release of drug to the receptor were prevented. The most important function of albumin in the body is transporting the lipids and free fatty acids.[2,3] Distribution and metabolism of many bioactivities compounds (drugs, natural products, etc.) of the body are correlated with their affinities toward serum albumin.[4] Therefore, study of the interaction between protein and drug molecules is an important content in pharmacokinetics and clinical pharmacology.

Ginkgolides (GK) is the main efficacy components of *Ginkgo biloba* L. leaf extract, which is current research and development of herbal compounds. Ginkgolides is widely used to prevent memory decline, Alzheimer's, cardiovascular and cerebrovascular diseases.[5,6] Ginkgolides A, B, C and J, together with BB, unique terpenoid components of the *Ginkgo biloba* tree, are specific and efficient strong inhibitors of platelet activating factor receptor and the only natural substances that have tert-butyl.[7] Figure 1 shows chemical structure of BB and GB. Currently GK have been applied to cure cardiovascular disease, but only few studies were conducted on the role of GK in serum albumin.

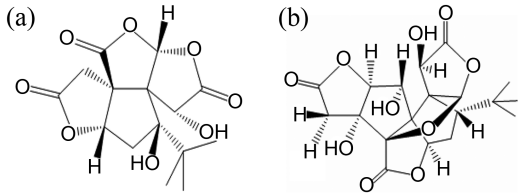

Figure 1. The chemical structure of the compounds. bilobalide (a) and ginkgolides B (b).

Several experimental and theoretical approaches have been developed to study the recognition process between ligands and receptors. While conventional approaches such as affinity and size exclusion chromatography,[8,9] equilibrium dialysis,[10] ultrafiltration,[11] predictive model[12] and ultracentrifugation,[13] compared with spectroscopic techniques,[14-17] suffered from lack of sensitivity, long time and high protein concentrations requirements exceeding dissociation constant for the drug-protein complex and drug-protein interaction studies.[18]

In this paper, the interaction of BB and GB with BSA was investigated by fluorescence spectrometry, and the mechanism of ginkgolides-BSA could provide experimental data and theoretical basis.

Experimental Section

Materials. GB and BB were supplied by Hefei University of Technology Laboratory, Anhui, China (the content is greater than 97.2%). Bovine serum albumin was obtained from Beijing Solarbio Science & Technology Co., Ltd (China). The stock solution of BB and GB (1.0×10^{-3} mol·L^{-1}) were prepared with methyl alcohol. BSA dissolved in Tris-

HCl buffer (pH=7.4) with 0.1 mol·L^{-1} NaCl to maintain the ionic strength at 0.05 mol·L^{-1} to prepare stock solution (1.0 × 10^{-5} mol·L^{-1}, based on its molecular weight of 65,000). All other chemicals were analytical reagent grade and double distilled water was used throughout. All stock solutions were stored at 0-4 °C.

Apparatus. Fluorescence measurements were performed with a spectrofluorimeter Model F-4500 (Hitachi Corp., Japan). The widths of both the excitation slitand emission slit were set at 5.0 nm. DK-S26-type electric water bath pot was obtained from Shanghai Precision Experiment Equipment Co., Ltd (China). The absorption spectra were measured on a U-4100 Ultraviolet-Visible-Near-Infrared Spectrophotometer (Hitachi Corp., Japan). A quartz cell of 1.00 cm was used for the measurements.

Procedure. The absorption spectra of BSA, GB, BB and their complexes were performed at room temperature and the fluorescence measurements were performed at different temperatures (300 and 310 K) in the range of 290 to 500 nm. BSA concentration was kept fixed at1.0 × 10^{-5} mol·L^{-1} and drugs concentrations were varied from 0 to 16.0 × 10^{-5} mol·L^{-1}. The widths of both the excitation slit and the emission slit were set to 5.0 nm. Thus, a series of solutions containing different amounts of drugs and a definite amount of BSA were obtained and they were mixed up and held in the thermostat water-bath for 3 min before the fluorescence measurement was performed under the above selected conditions. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence. The determination conditions and methods of the synchronous fluorescence spectra of BSA with drugs was the same as the above-mentioned two-dimensional fluorescence spectroscopy.

Results and Discussion

Fluorescence Quenching. When BSA was exicted at 280 nm, it showed a maximum fluorescence peak at 349 nm due to tryptophan in BSA.[19] The fluorescence of BSA at different temperatures with the addition of GB and BB as quencher was obtained (shown in Figure 2) and the results showed that a gradual decrease in the fluorescence intensity of BSA was induced but there was no significant emission wavelength shift with the addition of GB and BB, which indicated that the microenvironment around tryptophan in BSA had not changed after interacting with BB and GB. However, under the same conditions the BB had an obvious quenching effect on the protein system. It was concluded that the reason for this result was structural differences. BB is sesquiterpene and GB is diterpenes, the stereospecific blockade of GB is stronger than that of BB, so BB is more likely to contact with BSA, which was revealed in Figure 2.

Fluorescence Quenching Type. There are two quenching types in characterizing the mechanism of the binding of quencher and macromolecules: static and dynamic (or collision) quenching.[20] If it is assumed that the fluorescence quenching of BSA induced by the drugs are dynamic quenching process, fluorescence quenching is described by

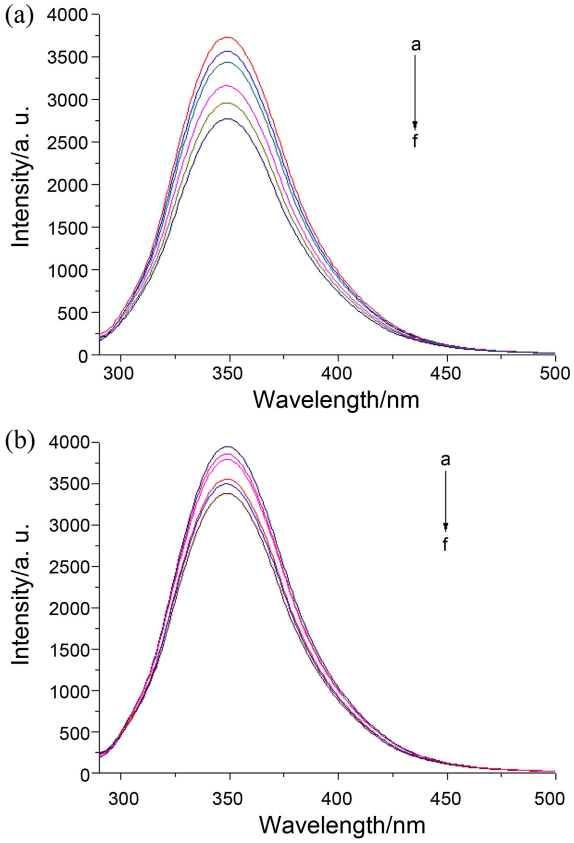

Figure 2. Fluorescence quenching spectra. (a) BB-BSA, (b) GB-BSA. 37 °C, C_{BSA} (mol·L^{-1}): 1 × 10^{-5}; $C_{compound}$ (1.0 × 10^{-5} mol·L^{-1}) from a to f: 0.0, 0.5, 1.0, 2.0, 8.0, 16.0.

the Stern-Volmer equation:[21]

$$F_0/F = 1 + Kq\tau o\,[Q] = 1 + K_{SV}\,[Q]$$

where F_0 and F are the fluorescence intensities of BSA before and after the addition of the GB and BB, respectively, Kq the bimolecular quenching constant, τo the lifetime of the fluorophore in the absence of quencher (for BSA, the lifetime of the fluorophore is approximately 1 × 10^{-8} s), [*Q*] the concentration of drugs and K_{SV} is the Stern-Volmer quenching constant.[22] For the drug-BSA system, the Stern-Volmer graphs were presented in Figure 3 and the values of K_{SV} obtained from the plots at different temperatures were listed in Table 1. The possible quenching mechanisms could be distinguished with the Stern-Volmer plots shown in Figure 3. The results showed that the Stern-Volmer plots were linear and the slopes decrease with increasing temperature. The static quenching between drugs and BSA was indicated by these plots. The values of K_{SV} obtained from the plots at different temperatures were listed in Table 1. It was found that all Kq's were larger than 2.0 × 10^{10} L·S^{-1}·mol^{-1} the maximum diffusion collision quenching rate constant of various quenchers with the biopolymer.[15] It could be concluded that the nature of quenching was not dynamic but probably static, resulting from the formation of ginkgolides-BSA complex.

The Binding Constant and the Number of Binding

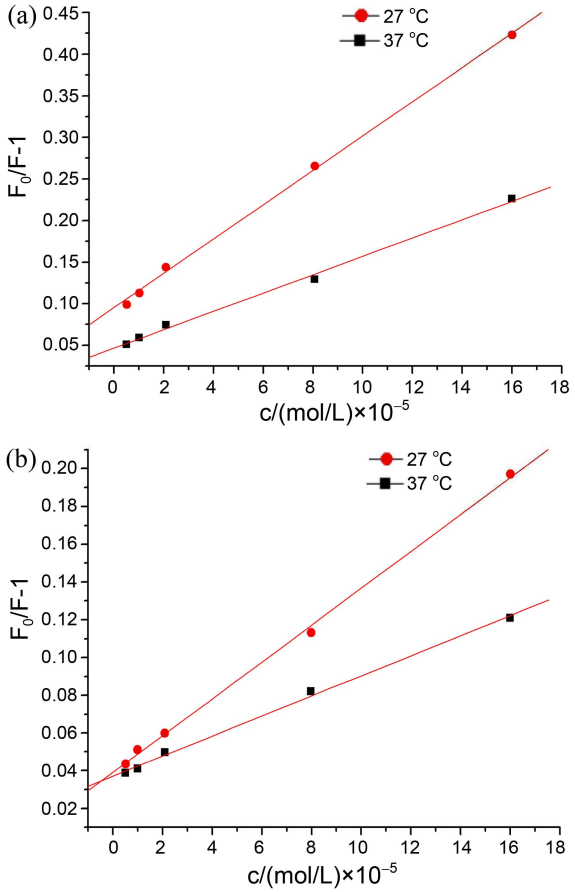

Figure 3. Stern-Volmer curves. (a) BB *vs* BSA, (b) GB *vs* BSA.

Table 1. Quenching constant of GB/BB with BSA at different temperatures

Compound	T (k)	K_{SV} ($L \cdot mol^{-1}$)	Kq ($L \cdot mol^{-1} \cdot s^{-1}$)	R
GB	290	0.998×10^3	9.980×10^{10}	0.998
	310	0.640×10^3	6.400×10^{10}	0.995
BB	290	2.092×10^3	2.092×10^{11}	0.996
	310	1.125×10^3	1.125×10^{11}	0.995

Sites. That the Kq value of BSA quenching by ginkgolide was greater than the Kq of the biopolymer. It showed that ginkgolide binding to BSA was quite strong and the quenching process involves a static quenching mechanism. The relationship between the fluorescence intensity and the quencher can be deduced from the following formula:[23]

$$P + nQ = QnP$$

Where P is the biomolecule with fluorophore, Q is the quencher molecule, QnP is the quenched biomolecules and the resultant constant Ka is given by

$$Ka = [QnP]/[P]\,[Q]^n$$

If the overall amount of biomolecules (bound or unbound with the quencher) is P_0, then $[P_0] = [QnP] + [P]$, here [P] is the concentration of unbound biomolecules, then the relationship between fluorescence intensity and the unbound biomolecule as $[P]/[P_0] = F/F_0$ that is:

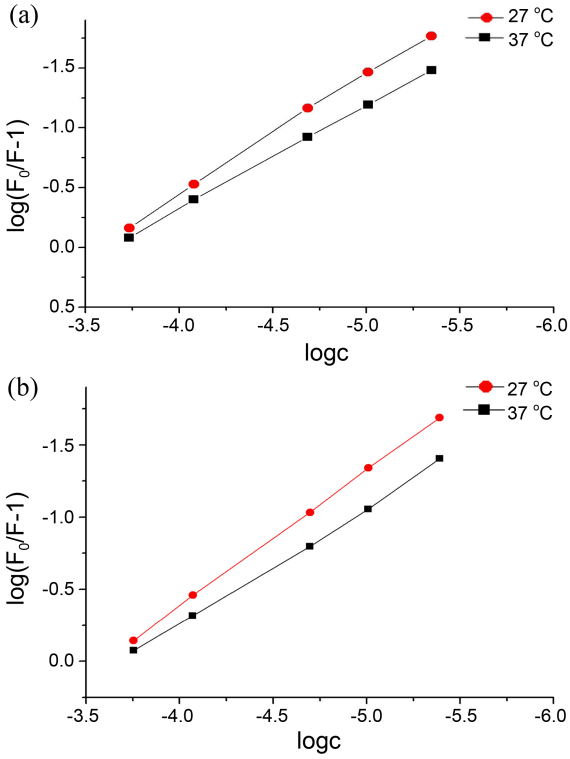

Figure 4. A plot of log (F_0/F-1) *vs* $\log c_{compound}$. (a) log (F_0/F-1) *vs* $\log_{GB}$, (b) log (F_0/F-1) *vs* $\log_{BB}$.

$$\log Ka + n\log [Q] = \log (F_0/F-1)$$

Where Ka is the binding constant of drugs with BSA,[24] which can be determined by the slope of the lg[(F_0–F)/F] versus log[Q] curves as shown in Figure 4. Thus we can obtain binding constant "Ka" and binding sites "n" of ginkgolide with BSA from the intercept and slope of Figure 4.

The decreasing trend of Ka with the increasing temperature was in accordance with K_{SV}'s dependence on the temperature as mentioned above, which indicated that ginkgolide-BSA would be partly decomposed as the temperature changed. The values of *n* at the experimental temperatures were approximately 1, which showed that the interaction of ginkgolide with BSA seemed to be in the presence of one high affinity binding site (obtained Table 2). The polarity of BB was greater than that of GB, so the hydroxyl of BB contact with BSA was easier than GB's, which was revealed from n value.

Determination of Binding Force. The interaction forces between a drug and biomolecule may include hydrophobic force, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc.[25] The thermodynamic parameters such as ΔS^0, ΔG^0 and ΔH^0 could be calculated by the following thermodynamic equations:

$$\Delta G = -RT\ln K = \Delta H - T\Delta S$$
$$\ln K_2/K_1 = [1/T_1 - 1/T_2]\,\Delta H/R$$

The results were listed in Table 3. For the BB-BSA and GB-BSA binding, the fact that ΔH and ΔS are both positive

Table 2. Binding constants of GB/BB with BSA at different temperatures

Compound	K (T)	n	Ka/L·mol^{-1}	R
GB	300	1.01	2181	0.9998
	310	0.94	1479	0.9993
BB	300	1.13	3170	0.9991
	310	0.97	1258	0.9995

Table 3. Thermodynamic constants for the binding of GB/BB with BSA

Compound	T (K)	ΔS (kJ/mol)	ΔH (kJ/mol)	ΔG (kJ/mol)
GB	300	0.17	32.47	-17.79
	310	0.17	32.47	-20.76
BB	300	0.32	76.52	-18.20
	310	0.31	76.52	-19.81

suggests a strong contribution of the hydrophobic effect.[26] The negative sign for ΔG indicates that the binding process is spontaneous.[27] Studies have shown that the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes.[28] For drug-protein interaction, positive entropy is frequently taken as the evidence for hydrophobic interaction, but it has been pointed out that positive entropy may also be a manifestation of electrostatic interaction. Furthermore, the main source of ΔG value is derived from a large contribution of ΔS term with little contribution from the ΔH factor, so the main interaction is by hydrophobic contact, but the electrostatic interaction cannot be excluded.

Effects of Ginkgolides on the Conformation of BSA. 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.[29] To explore the structural change of BSA by addition of drugs, synchronous fluorescence spectra (Figure 5) of BSA with various amounts of drugs were measured. The synchronous fluorescence spectra give information about the molecular environment in a vicinity of the chromosphere molecules. When the D-value (Dl) between excitation wavelength and emission wavelength was stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues.[30] The effect of ginkgolide on BSA synchronous fluorescence spectroscopy is shown in

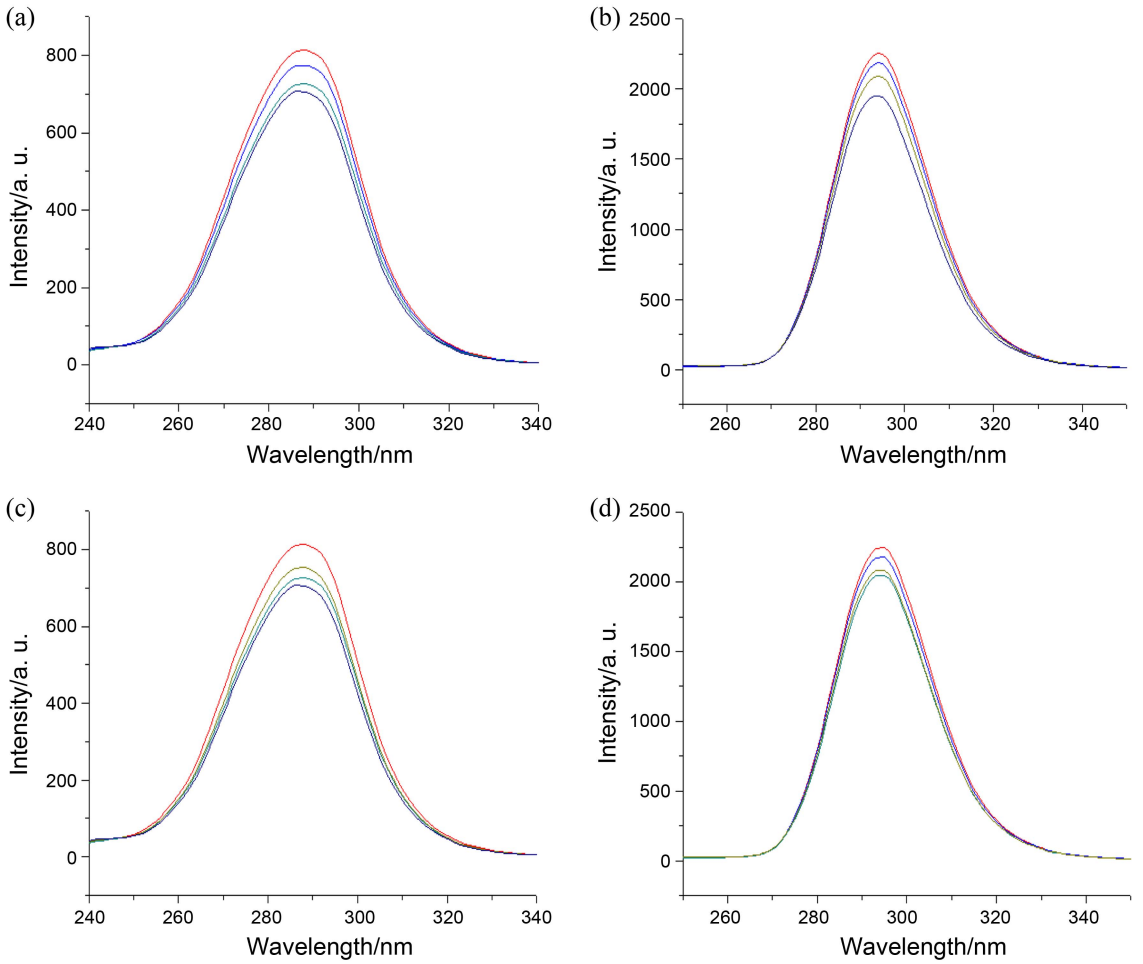

Figure 5. (a) Δλ = 15 nm, BB *vs* BSA (b) Δλ = 60 nm, BB *vs* BSA (c) Δλ = 15 nm, GB *vs* BSA (d) Δλ = 60 nm. GB *vs* BSA Synchronous fluorescence spectra of BSA.

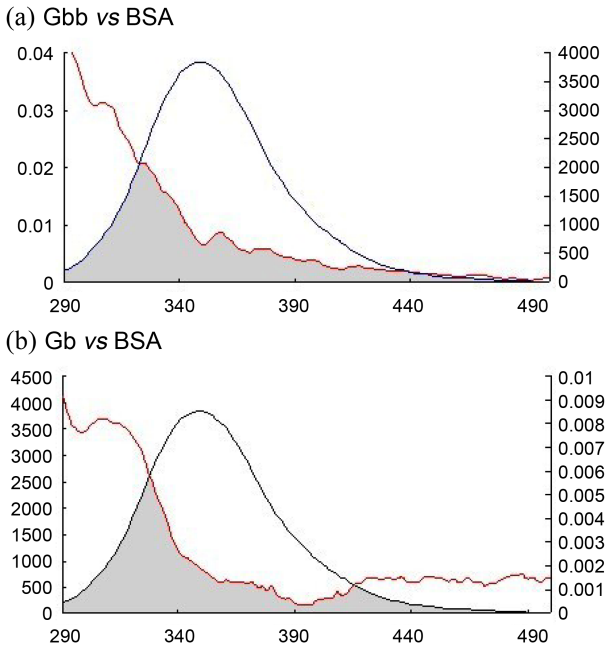

Figure 6. Spectral overlap of Gk absorption with BSA fluorescence at 310 K, c (BSA) = c (Gk) = 1.0×10^{-5} mol L^{-1}.

Figure 5. It was apparent that the emission maximum of tryptophan residues had no significant shift and the little red shift (from 288 to 290 nm) of tyrosine residues fluorescence, which indicated that the polarity around the tyrosine residues was increased and the hydrophobicity was decreased, but the microenvironment around the tryptophan residues had no obvious changes during the binding process.

The Binding Distance between BSA and GK. Föster's theory of dipole-Cdipole energy transfer was used to determine the distances between the protein residue (donor) and the bound drug (acceptor) in BSA. According to Förster theory, the efficiency of energy transfer between the donor and acceptor, *E*, could be calculated by the following equation: [31,32]

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

Where *r* is the distance between the donor and acceptor, and R_0 is the critical distance at 50% transfer efficiency, and can be obtained by the following equation:

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

Where *N* is the refractive index of the medium, K^2 is the orientation factor, and φ is the quantum yield of the donor. The spectral overlap integral (*J*) between the donor emission spectrum and the acceptor absorbance spectrum is approximated by the following summation:

$$J = \frac{\Sigma F(\lambda) \cdot \varepsilon(\lambda) \cdot \lambda^4 \cdot \Delta\lambda}{\Sigma F(\lambda) \cdot \Delta\lambda} \quad (3)$$

Where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to λ+Δλ; ε(λ) is the extinction coefficient of the acceptor at λ. In the present case, $K^2 = 2/3$, n = 1.36, φ = 0.118.[33] According to Eqs. (1)-(3) it was then calculated that: $J_{BB} = 1.4 \times 10^{-13}$ cm^3·mol, $J_{GB} = 3.7 \times 10^{-13}$ cm^3·mol, R_{0BB} = 3.8 nm, R_{0GB} = 2.9 nm, E_{BB} = 0.12 and r_{BB} = 5.33 nm, E_{GB} = 0.10 and r_{GB} = 4.20 nm. The donor-to-acceptor distance $r < 8$ nm, and $0.5R_0 < r < 1.5R_0$, indicate that the energy transfer from BSA to Gk occurs with high possibility.

Table 4. Characteristic parameters of the three-way excitation-emission fluorescence spectra

System	Concentration ($mol \cdot L^{-1}$)	$\lambda_{ex}/\lambda_{em}$	Intensity	Stokes shift (Δλ (nm))
BSA	1×10^{-5}	285.0/345.0	82.97	60
BSA-BB (1:1)	1×10^{-5}	285.0/355.0	76.39	70

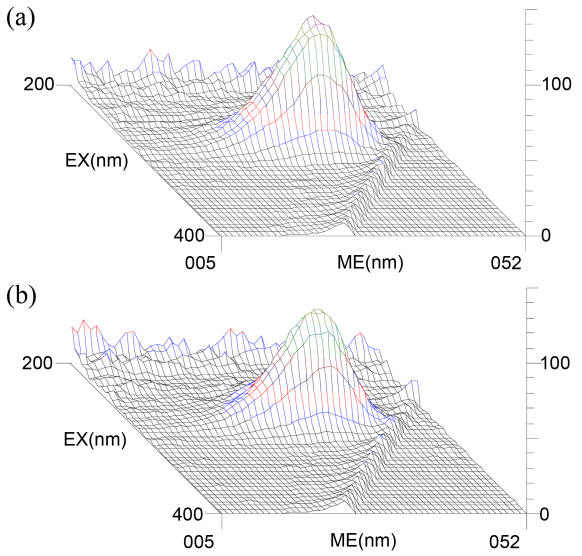

Figure 7. Three-dimensional fluorescence spectra of BSA (a) and BB-BSA system (b) 310 K, C_{BSA} ($mol \cdot L^{-1}$): 1×10^{-5}, C_{BB} ($mol \cdot L^{-1}$): 1×10^{-5}.

Three-dimensional Fluorescence Spectrometry. Figure 7 as well as the information in Table 4, were derived from the three-way excitation-emission spectral array. They demonstrated that the stokes shift and fluorescence intensity related to Peak 1 decreases and the peak maximum showed a red shift. This red shift effect indicated that conformational changes have occurred in the BSA structure as the polarity around the tryptophan and tyrosine residues decreases and the hydrophobicity increases.[34,35] Clearly, an experiment which investigates the ligands with BSA should be supplied for further study.

Conclusion

In this paper, we have studied the interaction of BSA with GB and BB using many spectroscopic techniques. The results of synchronous fluorescence spectra and UV/vis spectra indicated that the secondary structure of the protein was changed in the presence of GB and BB. The experi-

mental results also indicated that a complex was formed between ginkgolides and BSA through the static quenching procedure. The thermodynamic parameters, enthalpy change (ΔH^0) and entropy change (ΔS^0) indicated that both hydrogen bonds and van der Waals forces played a role in the binding of ginkgolides to BSA.

The binding study of 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 drugs. Information is also obtained from the effect of environment on BSA structure, which may be correlated to its physiological activity.

Acknowledgments. The authors gratefully acknowledge the financial support of this study by Foundation for Key Research Program of Education Department of Anhui Province, China (KJ2011A017) and National Natural Science Foundation of China (30800194).

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