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Characterizing the Interaction between Tartrazine and Two Serum Albumins by a Hybrid Spectroscopic Approach

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ABSTRACT: Tartrazine is an artificial azo dye commonly used in food products. The present study evaluated the interaction of tartrazine with two serum albumins (SAs), human serum albumin (HSA) and bovine serum albumin (BSA), under physiological conditions by means of fluorescence, three-dimensional fluorescence, UV−vis absorption, and circular dichroism (CD) techniques. The fluorescence data showed that tartrazine could bind to the two SAs to form a complex. The binding process was a spontaneous molecular interaction procedure, in which van der Waals and hydrogen bond interactions played a major role. Additionally, as shown by the UV−vis absorption, three-dimensional fluorescence, and CD results, tartrazine could lead to conformational and some microenvironmental changes of both SAs, which may affect the physiological functions of SAs. The work provides important insight into the mechanism of toxicity of tartrazine in vivo.

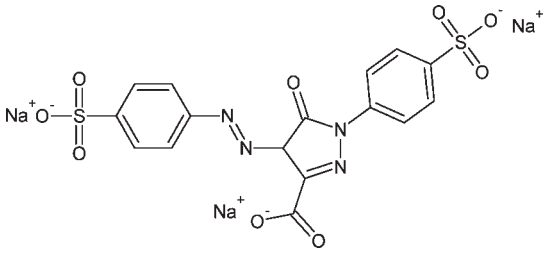
KEYWORDS: tartrazine, serum albumin, fluorescence spectroscopy, toxicological evaluation

INTRODUCTION

Tartrazine (Scheme 1) is an orange-colored, water-soluble powder with wide applications in food products, drugs, cosmetics, textiles, and pharmaceuticals. Since its first safety assessment conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1964, more than 300 studies on laboratory animals and clinical trials on human beings have been conducted in the toxicological fields.[1−3] It has been reported that tartrazine has no carcinogenic potential and produces little adverse effect on reproductive and developmental toxicological studies.[4,5] Thus, it would appear that the levels of actual dietary intake of tartrazine are unlikely to produce any adverse effects in humans.[6] Nevertheless, it may be incorporated, directly or indirectly, during the growth, storage, or processing of foods to increase their acceptability and attractiveness.[7] During its transport and metabolism process in body, it could pose potential biological toxicity risk to humans. Hence, it is very important to study the toxicity of tartrazine at the functional macromolecular level, especially at lower concentrations.

Serum albumins (SAs), the most abundant proteins in blood plasma, play important roles in the transport and deposition of a variety of endogenous and exogenous substances including fatty acids, amino acids, metals, and pharmaceuticals in blood.[8] The binding of toxins to serum albumins may have physiological importance, because they controls their free, active concentrations and affect the duration and intensity of their effects. The free concentration available for toxic action can be effectively reduced by high binding to proteins.[9] In recent years, human serum albumin (HSA) and bovine serum albumin (BSA) have been the two most widely used model proteins in evaluating protein−ligand interactions,[10−12] because they have a similar folding and a well-known primary structure and they have been associated with the binding of many different categories of small molecules. From the spectroscopic point of view, one of the main differences between the two proteins is that BSA has two tryptophan residues (Trp131 and Trp214), whereas HSA has only one (Trp214).

Scheme 1. Molecular Structure of Tartrazine



Herein, BSA can serve as a model protein for characterizing tartrazine−protein interactions using fluorescence spectroscopy, three-dimensional fluorescence, UV−vis absorption, and circular dichroism (CD) techniques. Additional tests are performed with HSA over a range of tartrazine, for comparison to the results of tartrazine−BSA interactions. This paper could provide quantitative binding data for studies on the biological toxicity of tartrazine.

MATERIALS AND METHODS

Reagents. BSA (electrophoretic grade regent, 90% purity) was purchased from Beijing Chemical Reagent Corp., whereas HSA (96% purity) was acquired from Sigma. They were both dissolved in ultrapure water to form a solution of 5.0×10^{-5} mol L^{-1} and then stored at 0−4 °C until use. Tartrazine (95% purity) from TCI (Japan) was prepared at a concentration of 1.0×10^{-4} mol L^{-1} and diluted before

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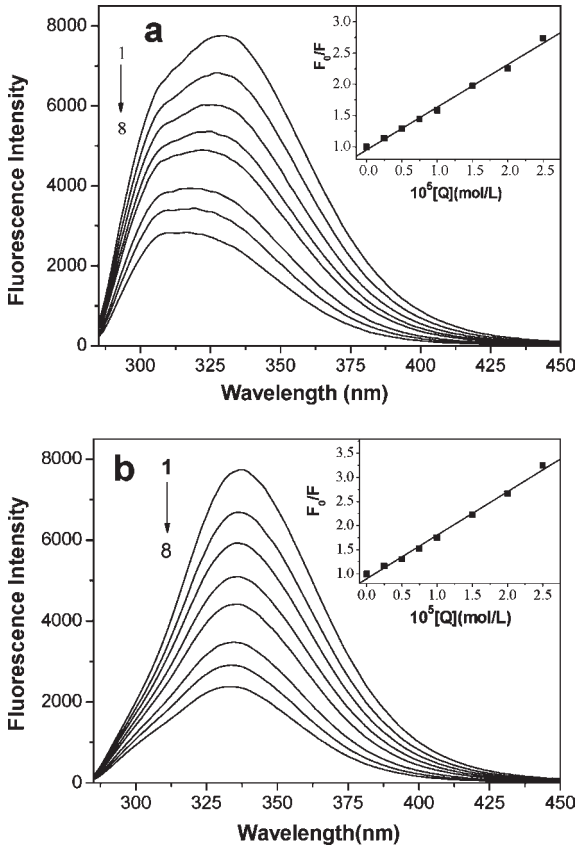

Figure 1. Effect of tartrazine on the fluorescence of HSA (a) and BSA (b). Conditions: T = 300 K, λ_{ex} = 278 nm; buffer, NaH_2PO_4−Na_2HPO_4; pH 7.4; c(HSA) = 5×10^{-6} mol^{-1}; c(BSA) = 5×10^{-6} mol^{-1}; c(tartrazine) 1−8, 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 2.5×10^{-5} mol^{-1}, respectively.

use. The 0.2 mol L^{-1} NaH_2PO_4−Na_2HPO_4 solution was used as the buffer. Ultrapure water was used throughout the experiments.

Fluorescence Quenching Measurements. All fluorescence spectra were recorded on an F-4600 spectrofluorometer (Hitachi, Japan) equipped with a xenon lamp light source and 1.0 cm quartz cells. One milliliter of phosphate buffer, 1 mL of BSA (HSA), and various amounts of tartrazine solution were added to a 10 mL colorimetric tube in sequence, then made up to the mark with ultrapure water, and incubated for 20 min to equilibrate the system. The equilibrated solution was transferred into the quartz cells for analysis, and the corresponding fluorescence emission spectra were recorded over the range of 290−450 nm using an excitation wavelength of 278 nm at a scan rate of 1200 nm/min during 300 and 320 K. The excitation and emission slit widths were both set at 5 nm.

Three-dimensional fluorescence spectra was measured under the following conditions: the initial excitation wavelength was set at 200 nm with increment of 2 nm, and the emission wavelength was recorded between 200 and 400 nm at a scanning rate of 30000 nm/min.

UV and Circular Dichroism (CD) Studies. The UV−vis absorption spectra were collected at room temperature on a UV-2450 spectrophotometer (Hitachi, Japan) in 1.0 cm quartz cells. Circular dichroism (CD) spectra in the presence and absence of tartrazine were measured by a J-810 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature under constant flushing with nitrogen at a scan rate of 200 nm/min.

RESULTS AND DISCUSSION

Fluorescence Quenching of SAs upon Addition of Tartrazine. Protein is considered to have intrinsic fluorescence mainly originating from the tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues.[13] When it interacts with other compounds, its intrinsic fluorescence often changes with the ligand's concentration. Consequently, fluorescence can be regarded as a technique for measuring the mechanism of interactions between the ligands and proteins.[14] The fluorescence spectra of both HSA and BSA upon the addition of tartrazine are illustrated in Figure 1. The fluorescence intensities of both HSA and BSA reduce gradually with increasing tartrazine concentrations, and a blue shift is also observed for the emission wavelengths of both SAs, which suggests that the fluorescence chromophore of SAs is placed in a more hydrophobic environment after the addition of tartrazine.

Fluorescence quenching is the decrease of the fluorescence quantum yield from a fluorophore induced by a variety of molecular interactions, such as excited-state reactions, energy transfer, ground-state complex formation, and collisional quenching. The quenching mechanisms are usually classified into dynamic quenching and static quenching, which can be distinguished by their different dependence on temperature and viscosity or, preferably, by lifetime measurements.[15] For dynamic quenching, when the temperature of the system rises, the effective collision times between molecules, the energy transfer efficiency, and the fluorescence quenching constants will all increase. In contrast, the increase of temperature is likely to result in decreased stability of complexes; thus, the values of the static quenching constants are expected to be smaller.

If the quenching mechanism belongs to the dynamic quenching, it should follow the well-known Stern−Volmer equation[16]

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

where F_0 and F denote the steady-state fluorescence intensities in the absence and presence of quencher, respectively. K_q is the quenching rate constant of the biological macromolecule; K_{SV} is the Stern−Volmer quenching constant, [Q] is the concentration of quencher, τ_0 is the average lifetime of the molecule without any quencher, and the fluorescence lifetime of the biopolymer is 10^{-8} s. The fluorescence intensity spectra of two SAs analyzed by plotting F_0/F verus [Q] at 300 and 320 K are shown in Figure 2, and the calculated K_{SV} and K_q values are presented in Table 1. The Stern−Volmer quenching constant K_{SV} is inversely correlated with temperature. Besides, the quenching constant K_q (Table 1) is far greater than the maximum scatter collision quenching constant (2.0×10^{10} L mol^{-1} s^{-1}), so we conclude that this quenching process is static quenching.

The fluorescence quenching of BSA and HSA by tartrazine should be analyzed using the modified Stern−Volmer equation[16,17]

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

In that case, ΔF is the difference in fluorescence intensity between the absence and presence of the quenching compound at concentration [Q], K_a is the effective quenching constant for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. The dependence of $F_0/\Delta F$ on the reciprocal value of the quenching compound concentration $[Q]^{-1}$ is linear, with slope equal to the value of $(f_aK_a)^{-1}$ (Figure 3). The decreasing trend of K_a with increasing temperature (Table 2) was in accordance with the dependence K_{SV} on temperature as mentioned above. Therefore, this result again confirms that the

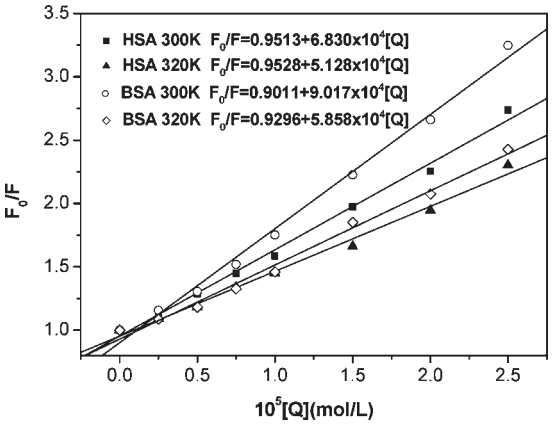

Figure 2. Stern−Volmer plots for the tartrazine−BSA and tartrazine−HSA systems at two temperatures. Conditions: c(HSA) = 5 × 10^{-6} mol L^{-1}; c(BSA) = 5 × 10^{-6} mol L^{-1}; buffer, NaH_2PO_4−Na_2HPO_4, pH 7.40.

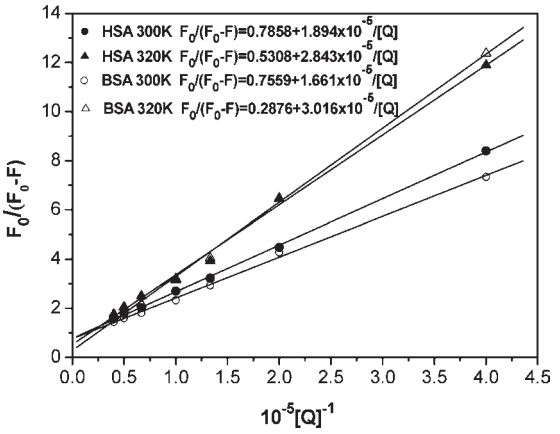

Figure 3. Modified Stern−Volmer plots for the tartrazine−BSA and tartrazine−HSA systems at two temperatures. Conditions: c(HSA) = 5 × 10^{-6} mol L^{-1}; c(BSA) = 5 × 10^{-6} mol L^{-1}; buffer, NaH_2PO_4−Na_2HPO_4, pH 7.40.

Table 1. Stern−Volmer Quenching Constants for the Interaction of Tartrazine with BSA and HSA at Different Temperatures

	pH	T (K)	K_{SV}	k_q	R	SD
BSA	7.4	300	9.017×10^4	9.017×10^{12}	0.99656	0.07075
	7.4	320	5.859×10^4	5.859×10^{12}	0.99597	0.04977
HSA	7.4	300	6.830×10^4	6.830×10^{12}	0.99687	0.05116
	7.4	320	5.128×10^4	5.128×10^{12}	0.99554	0.04585

quenching mechanism between tartrazine and SA belongs to the static quenching. Furthermore, the K_a values in Table 2 reveal that the binding between tartrazine and SA is moderate, which indicated the presence of a reversible tartrazine−SA complex.

Thermodynamic Parameters and Binding Forces. The interaction forces between a small organic molecule and a biological macromolecule mainly consist of four types: hydrophobic interactions, hydrogen bonding, van der Waals force, and electrostatic interactions. Ross and Subramanian have characterized the signs and magnitudes of the thermodynamic parameters (ΔH and ΔS) associated with various individual kinds of interaction that may take place in protein association process.[18] That is, if $\Delta H > 0$ and $\Delta S > 0$, the main force is hydrophobic interaction. If $\Delta H < 0$ and $\Delta S < 0$, van der Waals and hydrogen-bonding interactions play major roles in the reaction. Electrostatic forces are more important when $\Delta H < 0$ and $\Delta S > 0$. If the temperature changes only a little, the enthalpy change (ΔH) can be regarded as a constant in the formula[19]

$$\ln\left(\frac{K_2}{K_1}\right) = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (3)$$

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

where K_1 and K_2 are the binding constants (analogous to K_a in eq 2) at T_1 and T_2, respectively, and R is the universal gas constant.

The enthalpy change (ΔH), free energy change (ΔG), and entropy change (ΔS) for the interaction between tartrazine and HSA (BSA) were calculated according to the van't Hoff equation (eq 3) and thermodynamic equation (eq 4; Table 2). The free energy change (ΔG) is negative, indicating that the binding process is spontaneous. The negative ΔH and ΔS values indicate that van der Waals interactions and hydrogen bonds play a major role in the formation of the tartrazine−SA complex.

Identification of the Binding Parameters. For a static quenching interaction, the binding constant (K_b) and the number of binding sites (n) can be determined using the formula[20]

$$\log\frac{F_0 - F}{F} = \log K_b + n \log[Q] \quad (5)$$

where F_0, F, and [Q] are the same as in eq 2, K_b is the binding constant, and n is the number of binding sites per BSA/HSA molecule. According to eq 5, values of n and K_b at physiological pH 7.4 were calculated. According to a plot of $\log[(F_0 - F)/F]$ versus log[Q], the binding constant for BSA at 300 K is 5.327 × 10^5 and the number of binding sites is 1.171. In addition, the values of K_b and n for HSA are 1.845 × 10^5 and 1.096, respectively. The values of n for both SAs are approximately equal to 1, indicating that there is one binding site in both BSA and HSA for tartrazine during their interaction.

Energy Transfer between Tartrazine and SAs. Energy transfer phenomena have wide applications in energy conversion process. According to Förster's nonradioactive energy transfer theory,[21] the energy transfer will happen under the following conditions: (a) the donor could produce fluorescence light, (b) the fluorescence emission spectrum of the donor and the UV absorption spectrum of the acceptor have more overlap, and (c) the distance between the donor (BSA or HSA) and the acceptor (tartrazine) is <8 nm. The efficiency (E) of energy transfer between the donor and the acceptor could be calculated by the equations[22,23]

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

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J \quad (7)$$

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

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Table 2. Modified Stern−Volmer Association Constants K_a and Relative Thermodynamic Parameters of the Tartrazine−SA Systems at Different Temperatures

	T (K)	pH	K_a	R	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1})
BSA	300	7.4	4.551×10^4	0.9989		−26.75	−118.77
					−62.38		
	320	7.4	0.9533×10^4	0.9990		−24.38	−118.75
HSA	300	7.4	4.150×10^4	0.9997		−26.52	−17.87
					−31.88		
	320	7.4	1.867×10^4	0.9982		−26.16	−16.75

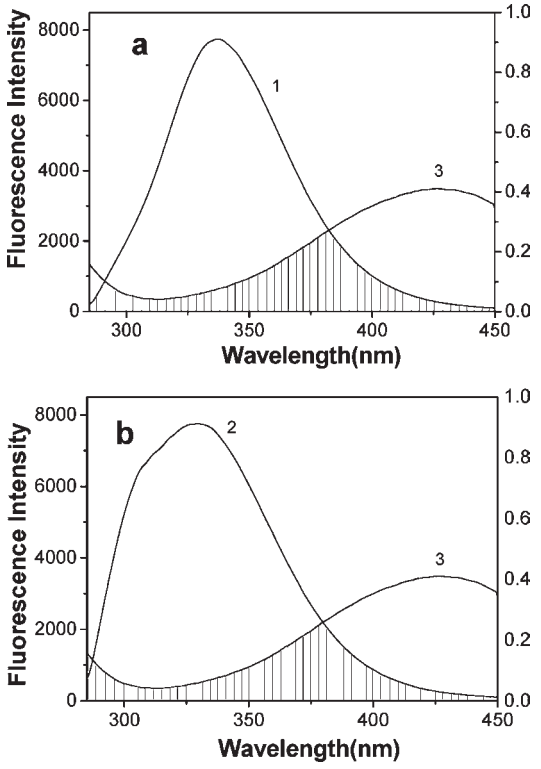

Figure 4. Spectral overlap of UV−vis absorption spectra of tartrazine with the fluorescence emission spectra of BSA (a) and HSA (b). Conditions: 1, fluorescence emission spectrum of BSA; 2, fluorescence emission spectrum of HSA; 3, absorption spectrum of tartrazine; c(BSA) = c(HSA) = c(tartrazine) = 5×10^{-6} mol L^{-1}, T = 300 K.

where r represents the distance between a donor and an acceptor; R_0 represents the critical distance at which transfer efficiency equals 50% is given by the equation;[7] K^2 is the orientation factor related to the geometry of the donor−acceptor dipole; n is the refractive index of medium; ϕ is the fluorescence quantum yield of the donor; J expresses the degree of spectral overlap between the donor emission and the acceptor absorption; $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ; $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ.

The overlap of the absorption spectrum of tartrazine and the fluorescence emission spectrum of BSA and HSA are shown in Figure 4. In the present case, $K^2 = 2/3$, $n = 1.36$, and $\phi = 0.15$. Therefore, we could calculate the parameters based on eqs 6−8. The distance r between tartrazine and the Trp residue

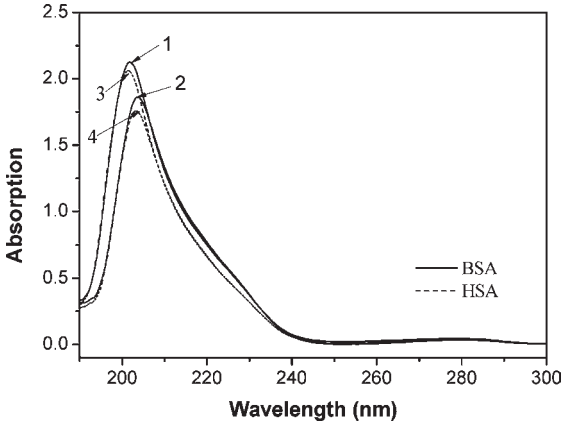

Figure 5. UV−vis absorption spectra of BSA and HSA in the presence of tartrazine. Conditions: 1, BSA; 2, BSA + tartrazine; 3, HSA; 4, HSA + tartrazine; c(BSA) = 1×10^{-6} mol L^{-1}; c(HSA) = 1×10^{-6} mol L^{-1}; c(tartrazine) = 2.5×10^{-5} mol L^{-1}; T = 300 K; buffer, NaH_2PO_4−Na_2HPO_4, pH 7.4.

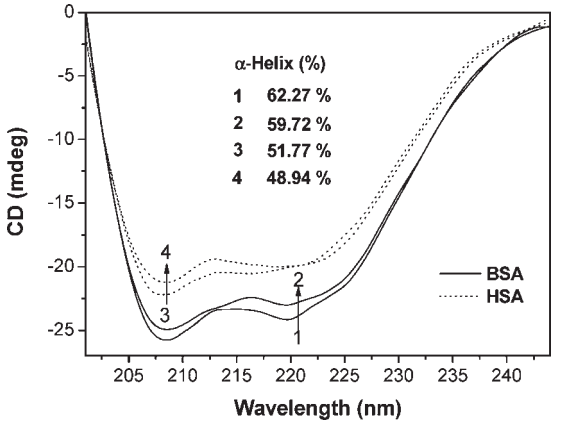

Figure 6. CD spectra of BSA and HSA by tartrazine. Conditions: c(BSA) = 2×10^{-7} mol L^{-1}; c(HSA) = 2×10^{-7} mol L^{-1}; c(tartrazine) 1−4, 0, 1, 0, 1 $\times 10^{-6}$ mol L^{-1}; buffer, NaH_2PO_4−Na_2HPO_4, pH 7.4.

in HSA after interaction is 3.541 nm, whereas that in the tartrazine−BSA system is 3.679 nm. Hence, the donor to acceptor distance in either system is <8 nm, which accords with conditions of Förster's nonradiative energy transfer theory, indicating again the static quenching interaction between tartrazine and SAs.

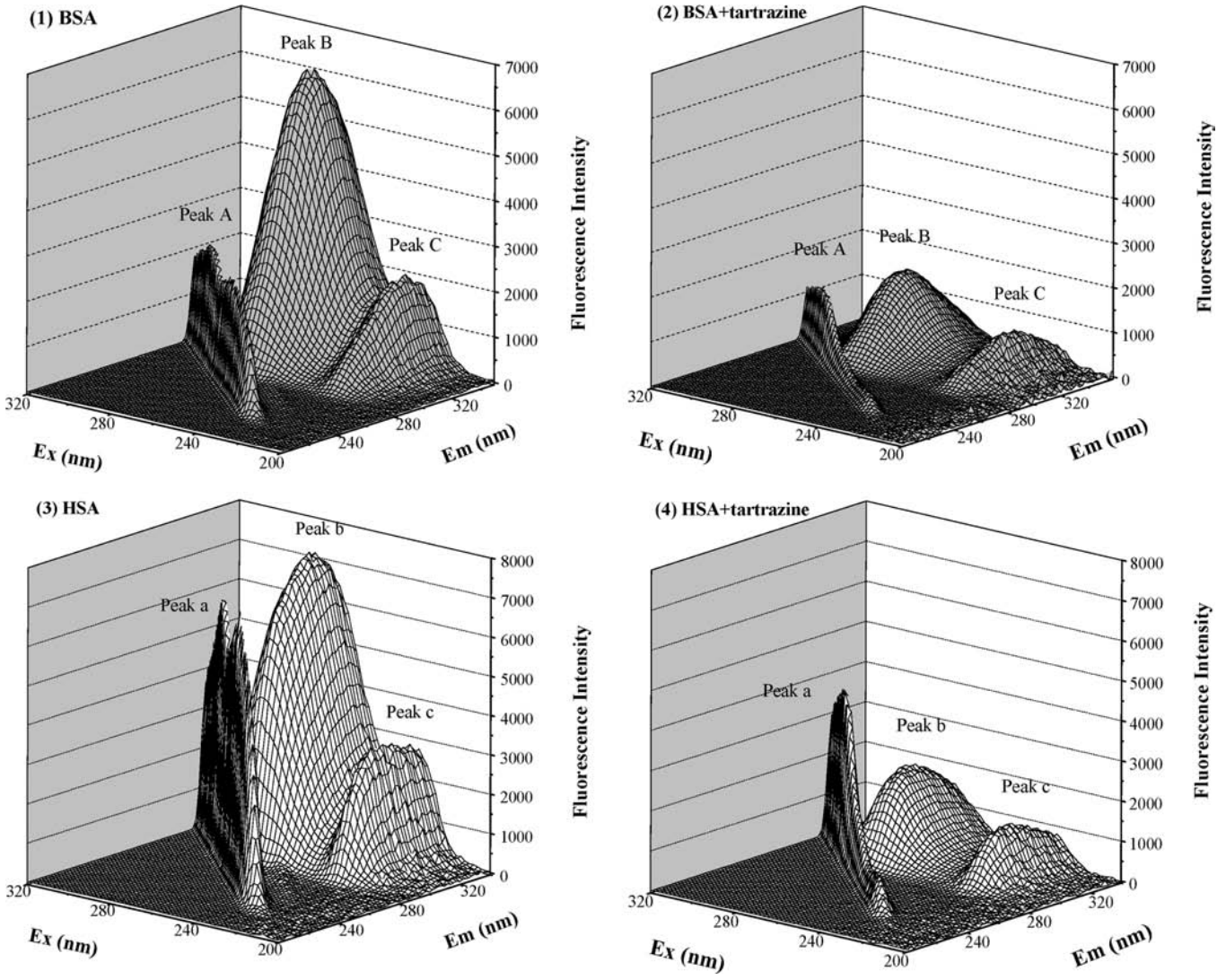

Figure 7. Three-dimensional fluorescence spectra of BSA (1), tartrazine−BSA system (2), HSA (3), and tartrazine−HSA system (4). Conditions: c(BSA) = 5 × 10^{-6} mol L^{-1}; c(HSA) = 5 × 10^{-6} mol L^{-1}; c(tartrazine) = 2.5 × 10^{-5} mol L^{-1}; buffer, NaH_2PO_4−Na_2HPO_4, pH 7.4.

Conformational Analysis. Although it has been confirmed that the binding of tartrazine to SA results in the fluorescence quenching of SA, it is still a puzzle about whether the binding affects the structure and the microenvironment of SA. Therefore, we utilized the methods of UV−vis absorption, CD, and three-dimensional fluorescence spectroscopy to further investigate the conformational changes of SAs.

UV−Vis Absorption Spectra of the Systems. UV−vis absorption spectroscopy technique can be used to explore the structural changes of protein and to investigate protein−ligand complex formation.[24] The UV−vis absorption spectra of SAs in the absence and presence of tartrazine obtained by utilizing the mixture of tartrazine and phosphate buffer at the same concentration as the reference solution are shown in Figure 5.

SA has two main absorption peaks. The strong absorption peak around 208 nm reflects the absorption of the backbone of the protein. The weak absorption peak at around 279 nm is due to the aromatic amino acids (Trp, Tyr, and Phe).[20] With gradual addition of tartrazine, the intensity peak of BSA or HSA at 208 nm decreases with a red shift and the intensity of the peak at 279 nm has minimal changes, which indicate that the interaction between tartrazine and SAs leads to the loosening and unfolding of the protein backbone and decreases the hydrophobicity of the microenvironment of SAs.

CD Response of SAs to Tartrazine. To gain a better understanding on the conformational behavior of the SA−tartrazine system, the CD spectra of both HSA and BSA in the absence and presence of tartrazine were obtained. The CD results were expressed in terms of mean residue ellipticity (MRE) in degree cm^2 $dmol^{-1}$ according to eq 9

$$\text{MRE} = \frac{\text{observed CD (mdeg)}}{C_p \times n \times l \times 10} \quad (9)$$

$$\alpha\text{-helix (\%)} = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000} \times 100 \quad (10)$$

where C_p is the molar concentration of the protein, n is the number of amino acid residues, and l is the path length.

The CD spectra of SA exhibit two negative peaks in the ultraviolet region at 208 and 222 nm, which are characteristic of the α-helical structure in proteins. In Figure 6, the proportion of

Table 3. Three-Dimensional Fluorescence Spectral Characteristic Parameters of SA and Tartrazine−SA Systems

		SA		tartrazine−SA		
	peak	peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	intensity F_0	peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	intensity F	F_0/F
BSA	A	230/230 → 320/320	49.63 → 1709	235/235 → 320/320	109.2 → 1047	
	B	278/335	6184	283/335	1724	3.59
	C	234/337	2120	230/331	948	2.24
HSA	a	227/227 → 320/320	53.38 → 3455	235/235 → 320/320	317.6 → 2766	
	b	278/329	7270	282/320	2154	3.38
	c	234/316	3138	234/315	1327	2.36

α-helical content in either BSA or HSA exhibits a decrease upon the addition of tartrazine. The data suggest that the tartrazine makes SA adopt a more open conformation and an increased exposure of hydrophobic cavities that were seen by the UV−vis absorption experiments. Secondary structure is related closely to the biological activity of proteins, and a decrease in α-helical content could result in a loss of the biological activity of SA upon interaction with a low concentration of tartrazine.

Three-Dimensional Fluorescence Spectra Analysis. Three-dimensional fluorescence spectroscopy has drawn much attention oin the analysis of proteins in recent years, because it has the advantage of making the investigation of the characteristic conformational change of protein more scientific and credible.[25,26] If there is a shift at the excitation or emission wavelength around the fluorescence peak, or the appearance of a new peak or disappearance of existing peak, it could be an important hint to suggest conformational changes to the protein. The conformational and microenvironmental changes of two SAs were investigated by comparing the spectral changes in the absence and presence of tartrazine, as shown in Figure 7, with the corresponding parameters shown in Table 3. Peak A or a is the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$), whereas the strong peak (B or b) mainly reveals the spectral characteristic of Trp and Tyr residues. Besides peak B or b, there is another strong fluorescence peak (C or c), which mainly exhibits the fluorescence spectral behavior of polypeptide backbone structures. Analysis of the intensity changes of peak B or b and peak C or c) of both SAs showed different degrees of differences after the addition of tartrazine. This implies that the interactions of tartrazine with SAs induce a slight unfolding of the polypeptide backbone of the protein, resulting in conformational changes that increase the exposure of some hydrophobic regions that had been buried.[27] These results are in accordance with the UV and CD spectral data. All of these phenomena indicate that the binding of tartrazine to SAs induces conformational and microenvironmental changes in both BSA and HSA.

In conclusion, interaction of tartrazine with two SAs was elucidated at the molecular level using fluorescence, combined with UV−vis absorption, CD, and three-dimensional fluorescence spectroscopy under physiological conditions. The experimental results showed that tartrazine could bind with SAs to form a tartrazine−SA complex with one binding site. The binding process was spontaneous, and hydrogen bonding and van der Waals force played major roles in stabilizing the complex. The microenvironment and the conformation of two SAs were changed in the presence of tartrazine as was demonstrated by the UV−vis absorption, CD, and three-dimensional fluorescence spectra. This study provides important information about the molecular interactions between tartrazine and SAs and the resulting conformational changes in the proteins. This may provide useful information in clarifying the dynamics of toxicity of tartrazine in vivo.

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ABBREVIATIONS USED

SA, serum albumin; BSA, bovine serum albumin; HSA, human serum albumin; CD, circular dichroism; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine.

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