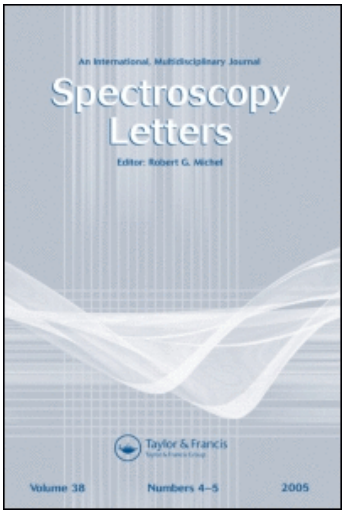

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Effects of Temperature and Ions on Binding Dihydromyricetin to Bovine Serum Albumin by Spectroscopic Method

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ABSTRACT The interaction of dihydromyricetin (DMY) and bovine serum albumin (BSA) in solution was investigated by fluorescence, synchronous fluorescence, and ultraviolet (UV) spectra. These results revealed that DMY quenches the fluorescence of BSA by forming the DMY–BSA complex. The number of binding sites (n), binding constant (K_a), and thermodynamic parameter (ΔG, ΔH, ΔS) were calculated. The primary binding forces between DMY and BSA were found to be the electrostatic force and hydrophobic interaction. The binding distance (r) between the donor (BSA) and acceptor (DMY) was determined as 3.43 nm based on Förster's theory. The effect of metal ions (Ni^{2+}, K^{+}, Cu^{2+}, and Co^{2+}) on the binding constant of DMY–BSA complex was also investigated. The effect of DMY on the conformation of BSA was analyzed using synchronous fluorescence spectroscopy and UV-Vis absorption spectroscopy.

KEYWORDS bovine serum albumin, dihydromyricetin, fluorescence spectroscopy, interaction, ion

INTRODUCTION

The interaction between small molecules and proteins has attracted great interest among researchers.[1–12] The interaction between protein and small molecules results in formation of a stable complex, which may be considered as a model for gaining general fundamental insights into small molecule–protein binding.[8–12] Bovine serum albumin (BSA) has been one of the most extensively studied of proteins particularly because of its structural homology with human serum albumin (HSA).[6–10]

Dihydromyricetin (3,5,7,3′,4′,5′-six hydroxy-2,3-dihydro flavonol, DMY; Fig. 1), is an important dihydro flavonoid from *Hovenia dulcis*[13] and *Salix hulteni*.[14] DMY has anticancer, antilipid-peroxidation, and antioxidative activities. To gain some insights into the medicinal action of puerarin, the interaction between DMY and BSA is investigated. The binding constants were calculated and a binding mechanism was proposed. Meanwhile, the energy transfer between DMY and BSA was reported, and the effect of metal ions on the DMY–BSA complex was also studied.

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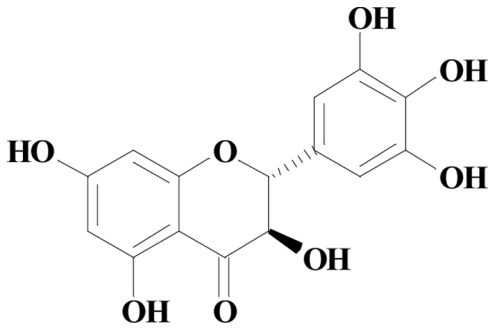

FIGURE 1 Structure of dihydromyricetin.

EXPERIMENTAL

Apparatus

Fluorescence spectra were obtained on a Hitachi FL-4500 spectrofluorometer (Tokyo, Japan). The absorption spectra were measured on a Purkinje TU-1800SPC UV-Vis spectrophotometer (Beijing, China). A quartz cell of 1.00 cm was used for the measurements. The pH measurements were carried out on a Leici PHS-3C Exact Digital pH meter (Shanghai, China), which was calibrated with standard pH buffers.

Reagents

DMY (99.0%) was obtained commercially from the Bioszune Co., Ltd. (Beijing, China). The stock solution of DMY (1.0×10^{-4} M) was prepared by dissolving with methanol–water solution (1:1, v/v). BSA (fraction V) and *Tris*-base were purchased from Beijing Ding Guo Biotechnology (Beijing, China). The working solution of BSA (1.0×10^{-5} mol L^{-1}) was prepared with *Tris*-HCl buffer (0.05 mol L^{-1}, pH 7.4) and stored in a refrigerator prior to use. *Tris*-HCl buffer containing NaCl was selected to keep the pH value and maintain the ionic strength of the solution. Metal ions Cu^{2+}, Co^{2+}, Ni^{2+}, K^{+} of 1×10^{-3} M were prepared in *Tris*-HCl buffer. All other reagents and solvents were of analytical reagent grade and used without further purification. All aqueous solutions were prepared using newly double-distilled water.

Procedure

One milliliter of BSA (1.0×10^{-5} mol/L) was added to a 10-mL volumetric flask including a certain concentration of DMY and then diluted to 10 mL with water, with the final concentration of DMY in the range of 0.0–1.44×10^{-5} mol/L. The resultant mixture was subsequently mixed and incubated at 288, 298, and 310 K for 10 min. The solution was scanned on a fluorometer with the range of 290–550 nm under the excitation at wavelength of 280 nm. The widths of both the excitation slit and emission slit were set at 5.0 nm. In addition, in the presence of 1×10^{-4} M metal ion, other fluorescence spectra could be obtained.

Synchronous fluorescence spectra of BSA with various amounts of DMY were recorded from 260 to 320 nm ($\Delta\lambda = 15$ nm) and from 250 to 310 nm ($\Delta\lambda = 60$ nm), respectively. The UV spectra of DMY (BSA) without and with BSA (DMY) were scanned with the range of 220–400 nm at room temperature. The concentrations of DMY and BSA were kept at 4×10^{-6} and 2×10^{-6} mol L^{-1}, respectively. The dynamic quenching constant (K_{SV}) and the binding constants (K_a) are obtained according to Lakowicz.[15]

RESULTS AND DISCUSSIONS

Characteristics of the Fluorescence Spectra

The interaction of DMY with BSA was evaluated by monitoring the intrinsic fluorescence intensity changes of BSA upon addition of DMY. Fluorescence quenching spectra of BSA in the presence of various concentrations of DMY are shown in Fig. 2.

As shown in Fig. 2, the addition of DMY leads to a concentration-dependent quenching of BSA intrinsic fluorescence intensity along with an obvious red shift

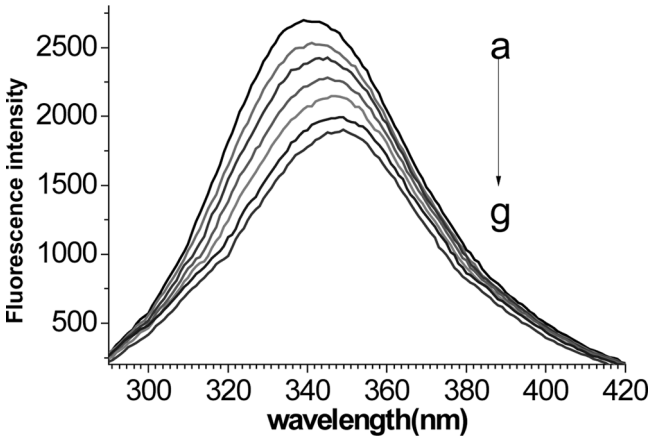

FIGURE 2 The quenching effect of DMY on BSA fluorescence. $\lambda_{ex} = 280$ nm (a)–(g), BSA, 1.00×10^{-6} M; 0.00, 0.24, 0.48, 0.72, 0.96, 1.2, 1.44×10^{-5} M of DMY.

of the maximum emission wavelength, implying that the binding of DMY to BSA occurs and the microenvironment around the chromophore of BSA is changed upon addition of DMY.

Quenching Constants

Fluorescence quenching could proceed via different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature. To elaborate the fluorescence quenching mechanism, the Stern-Volmer equation was utilized for the data analysis:[15]

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

where F_0 and F represent the fluorescence intensities in the absence and in the presence of quencher, K_q is the quenching rate constant of the bimolecular, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher, and [Q] is the concentration of the quencher. Figure 3 displays the Stern-Volmer plots of the quenching of BSA fluorescence by DMY at different temperatures.

The plot shows that within the investigated concentrations, the results agree with the Stern-Volmer Eq. (1) and the plot does not show deviation toward the y-axis obviously, which indicates that either dynamic quenching or static quenching is predominant. The corresponding quenching constant K_{SV} and the quenching rate constant K_q for the interaction between DMY and BSA were calculated and shown in Table 1.

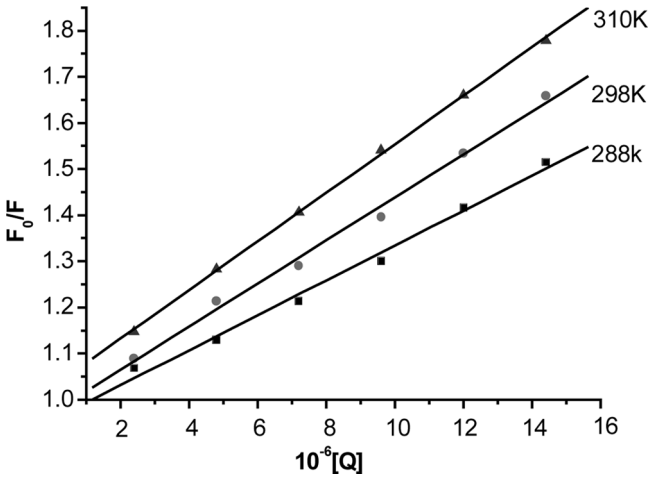

FIGURE 3 Stern-Volmer plots for the quenching of BSA by DMY at different temperatures. c(BSA) = 1.00×10^{-6} M; pH = 7.4; λ_{ex} = 280 nm.

The results show that K_{SV} increases with increasing temperature, indicating that the probable quenching mechanism of fluorescence of BSA by DMY is a dynamic quenching procedure,[19] because higher temperatures will result in faster diffusion and hence larger amounts of collisional quenching. Another explanation is that the quenching is initiated by static quenching, resulting in forming a DMY–BSA complex and the stability of the complex increases with increasing temperature.

According to the literature,[7,8] for dynamic quenching, the maximum collision quenching constant of various quenchers with biopolymers is $2.0 \times 10^{10}\,L\,mol^{-1}\,s^{-1}$. Obviously, the rate constant (K_q) of the protein quenching procedure initiated by DMY is greater than the K_q of the scatter procedure. This means that the quenching is not initiated by dynamic collision but by forming a complex.

Binding Constant and Binding Sites

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the binding constant formula:[15]

$$\log_{10}(F_0 - F)/F = \log_{10} K_a + n\log_{10}[Q] \qquad (2)$$

where K_a is the binding constant, and n is the number of binding sites per BSA. After the fluorescence quenching intensities on BSA at 340 nm were measured, the double-logarithm algorithm was assessed by Eq. (2). Figure 4 shows the double-logarithm curve and Table 2 gives the corresponding calculated results.

As seen in Table 2, the binding constants (K_a) decrease with the increasing temperature. The number of binding sites of the DMY–BSA system also decreases in the higher temperature. The data clearly show that there is one binding site on BSA for DMY.

TABLE 1 Stern-Volmer Quenching Constants for t for the System of DMY–BSA

T (K)	K_{SV} (M^{-1})	K_q ($M^{-1}\,s^{-1}$)	R
288	3.79×10^4	3.79×10^{12}	0.9953
298	4.67×10^4	4.67×10^{12}	0.9969
310	4.70×10^4	4.70×10^{12}	0.9989

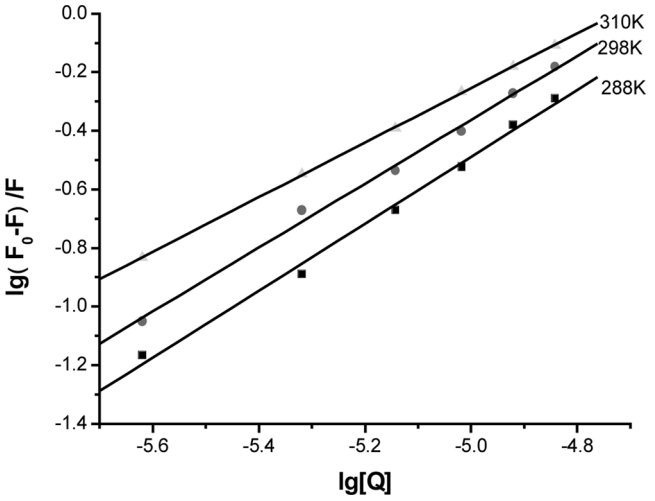

FIGURE 4 **Double-log plots of DMY quenching BSA fluorescence at different temperatures.**

The increasing temperature may result in increasing the diffusion coefficient, and it also leads to lowering the stability of the DMY–BSA system. The competition of the diffusion coefficient and stability of the DMY–BSA system with increased temperature may induce the above results. The temperature also affects the stability of DMY, which was easily oxidized and decomposed under high temperature. The by-products may have lower binding forces with the BSA. Flavonoids can be oxidized to semiquinones and quinones, which may weaken the affinity for BSA.

UV-Vis Absorption Spectroscopy

UV-Vis absorption measurement is a simple but effective method in detecting complex formation. In order to further validate the quenching mechanism, the UV-Vis absorption spectra of BSA and DMY were measured. Figure 5 shows that the absorption spectra of BSA without and with DMY cannot be superposed, and the absorption of DMY changed after addition of BSA. These results reconfirm that the quenching mechanism of fluorescence of BSA by DMY is a static quenching procedure.[16,17] In other words, the fluorescence quenching of BSA resulting in forming complex is predominant, whereas that from dynamic collision could be negligible.

TABLE 2 **Binding Parameters for the System of DMY–BSA**

T (K)	K_a (M^{-1})	n	R
288	1.66×10^5	1.1417	0.9968
298	1.24×10^5	1.0910	0.9975
310	2.50×10^4	0.9315	0.9999

Thermodynamic Parameters and Nature of the Binding Forces

The interaction between drug and biomolecule may involve hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc.[18] In order to elucidate the interaction of DMY with BSA, the thermodynamic parameters were calculated from Eqs. (3)–(5). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta \mathrm{G} = -RT \ln K_a \tag{3}$$

where R is the gas constant, T is the experimental temperature, and K_a is the binding constant at corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from the following equations:

$$\ln \frac{K_2}{K_1} = \left[\frac{1}{T_1} - \frac{1}{T_2}\right] \frac{\Delta H}{R} \tag{4}$$

where K_1 and K_2 are the binding constants at the experiment temperatures T_1 and T_2, respectively.

$$\Delta G = \Delta H - \mathrm{T}\Delta S \tag{5}$$

The thermodynamic parameters for the interaction of DMY with BSA are shown in Table 3. The negative sign for ΔG means that the interaction process is spontaneous. From the point of view of water structure, the positive values of ΔS are evidence of hydrophobic interaction.[19] Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and a negative ΔH. Accordingly, it is not possible to account for the thermodynamic parameters of the DMY–BSA complex on the basis of a single intermolecular force model.[20] It is more likely that hydrophobic, electrostatic interactions are involved in its binding process.

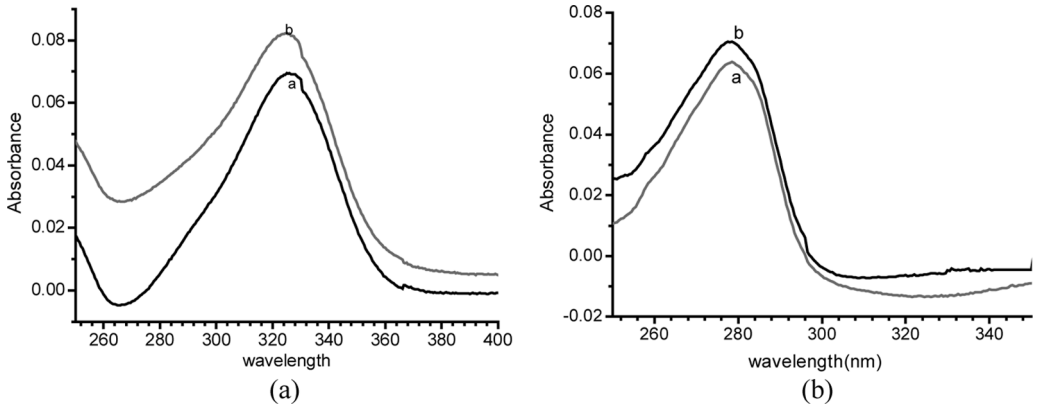

FIGURE 5 Absorption spectra of DMY(A)/BSA(B) without (a) and with (b) BSA/DMY at 298 K. pH = 7.4; c(DMY) = 4.0×10^{-6} mol/L; c(BSA) = 2×10^{-6} mol/L.

Energy Transfer Between DMY and BSA

According to the Förster nonradiative resonance energy transfer theory,[21] the rate of energy transfer depends on (1) the relative orientation of the donor and the acceptor dipoles, (2) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, and (3) the distance between the donor and the acceptor. Here the donor and acceptor were BSA and DMY, respectively. The overlap of the absorption spectrum of DMY and the fluorescence emission spectrum of BSA at 310 K is shown in Fig. 6.

The efficiency of energy transfer between the donor and acceptor, E, could be calculated using the equation

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

where F_0 and F are the fluorescence intensities without and with DMY, respectively. r is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%. R_0^6 can be calculated using the equation

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \tag{7}$$

TABLE 3 Thermodynamic Parameters of DMY–BSA Binding Procedure

T (K)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
288	−20.81	−28.78	27.66
298	−20.81	−29.06	27.66
310	−20.81	−26.10	17.05

where K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the donor; the absorption spectrum of the acceptor given by J is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta\lambda$, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at λ. In the present case, $K^2 = 2/3$, $n = 1.336$, and $\Phi = 0.15$.[26] According to Eqs. (6)–(8), the parameters were found to be $J = 1.73 \times 10^{-14}$ cm^3 L/mol, $R_0 = 2.8$ nm, $E = 0.2256$, and $r = 3.4$ nm. The

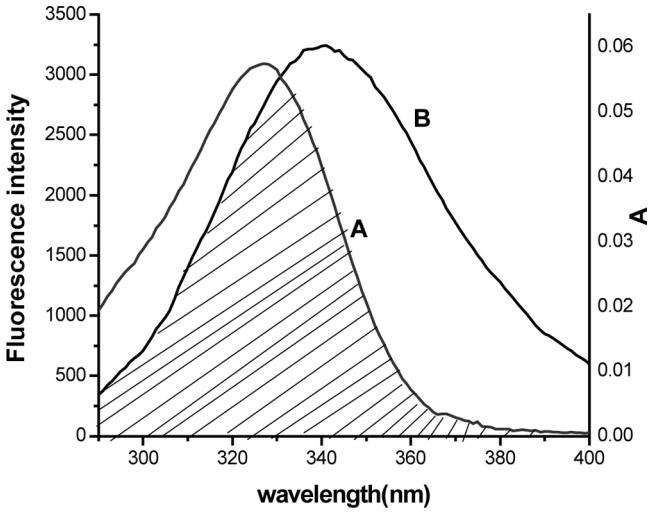

FIGURE 6 The overlap of the absorption spectrum of DMY(A) and the fluorescence emission spectrum of BSA(B). c(BSA) = c(DMY) = 2.0×10^{-6} M.

donor-to-acceptor distance, r, is less than 7 nm,[22] which implies that the energy transfer from BSA to DMY occurs. Larger BSA–DMY distance, r compared to that of R_0 value observed in the present study also reveals the presence of static type quenching mechanism to a larger extent.[23]

Influences of Common Ions on Binding Constant

The metal ions can affect the interaction between drugs and proteins in blood. Effects of four common metal ions (e.g., Ni^{2+}, K^{+}, Co^{2+}, and Cu^{2+}) on the interaction between DMY and BSA were investigated at 310 K. Figure 7 shows the quenching effect of DMY on BSA in the presence of these metal ions. The binding constants (K_a) obtained in the presence of metal ions are listed in Table 4.

It can be seen from Table 4 that the presence of Cu^{2+} and Co^{2+} largely increases the binding constant of DMY–BSA complex. However, the binding constant between DMY and BSA decreases the presence of Ni^{2+} and K^{+}. The higher binding constant possibly results from the formation of ion–DMY complexes via a metal ion bridge.[9] This may prolong the storage period of DMY in blood plasma and enhance its maximum effects. On the contrary, the decrease in binding constant may due to the formation of an ion–BSA complex. From the pharmacokinetics perspective, the formation of an ion–albumin complex is likely to affect conformation of protein, which may decrease the interaction between the drug and serum albumins and prolong the duration in plasma in some way.

Conformation Investigation

To explore the structural change of BSA by addition of DMY, we measured the synchronous fluorescence spectra (Fig. 8) of BSA with various concentrations of DMY. Synchronous fluorescence is a kind of simple and effective means to measure the fluorescence quenching and the possible shift of the maximum emission wavelength λ_{max}, relative to the alteration of the polarity around the chromophore microenvironment. $\Delta\lambda$, representing the value of difference between excitation and emission wavelengths, is an important operating parameter. For $\Delta\lambda = 15$ nm, the synchronous fluorescence offers characteristics of tyrosine residues. When $\Delta\lambda = 60$ nm, it provides the characteristic information of tryptophan residues. When $\Delta\lambda$ is set at 15 or 60 nm, the shift of the λ_{max} and the fluorescence quenching

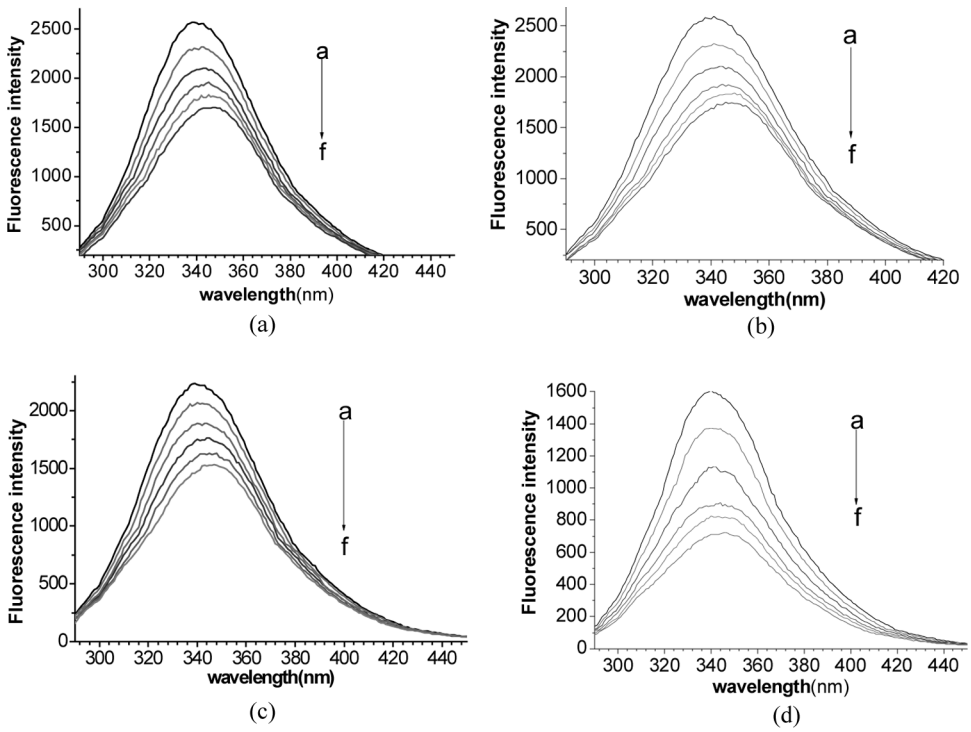

FIGURE 7 The quenching effect of DMY on BSA fluorescence in the presence of Ni^{2+} (a), K^{+} (b), Co^{2+} (c), and Cu^{2+} (d). $\lambda_{ex} = 280$ nm, (a–f) BSA, 1.00×10^{-6} M; 0.00, 0.24, 0.48, 0.72, 0.96, 1.20, 1.44×10^{-5} M of DMY. $c(Ni^{2+}) = c(K^{+}) = c(Co^{2+}) = c(Cu^{2+}) = 1.0 \times 10^{-4}$ M.

TABLE 4 Binding Parameters for the System of DMY–BSA in the Presence of Metal ions at 310 K

System	K_a (L mol^{-1})	n	R
DMY–BSA	2.50×10^4	0.9315	0.9999
DMY–BSA–Ni^{2+}	3.32×10^4	0.9716	0.9999
DMY–BSA–K^+	2.78×10^4	0.9549	0.9958
DMY–BSA–Cu^{2+}	1.52×10^6	1.2350	0.9992
DMY–BSA–Co^{2+}	2.88×10^5	1.1695	0.9996

of BSA imply the alteration of polarity microenvironment around Tyr or Trp residues and the state of drug binding to BSA.[15]

As shown in Figs. 8a and 8b, the fluorescence intensity of BSA weakened regularly along with the adding of DMY, implying that DMY was bound to BSA and located in close proximity to the tyrosine and tryptophan residues. It is apparent from Fig. 8a that the maximum emission wavelength of tyrosine residues does not change. In contrast, an obvious red shift of tryptophan residues was observed (Fig. 8b). The shift effect expresses that the conformation of BSA was changed. It is also indicated that the polarity around the tryptophan residues increased and the hydrophobicity decreased, yet the microenvironment around the tyrosine residues has no discernible change during the binding process.

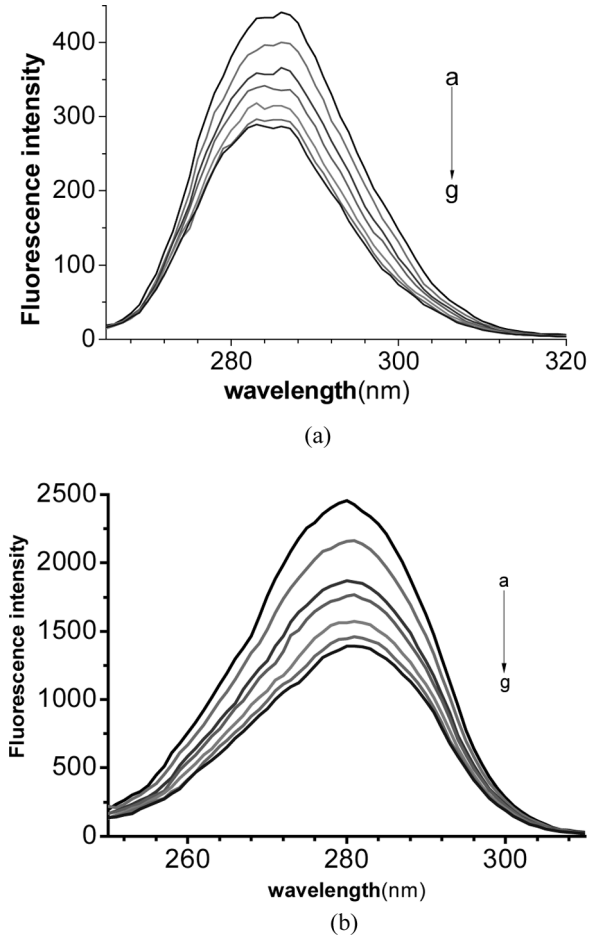

FIGURE 8 Synchronous fluorescence spectra of BSA in the presence of DMY (310 K, pH 7.4). c(BSA) = 10^{-6} M; c(DMY) = 10^{-5} M, (a)–(g): 0.00, 0.24, 0.48, 0.72, 0.96, 1.2, and 1.44 M, respectively; (a) $\Delta\lambda$ = 15 nm, (b) $\Delta\lambda$ = 60 nm.

CONCLUSION

In this article, the interaction between DMY and BSA was studied by fluorescence spectroscopy and UV-Vis absorption spectroscopy. The method is easy to operate and is reliable, practical, and simple. The experimental results indicated that the probable quenching mechanism of fluorescence of BSA by DMY is a static quenching procedure and the binding reaction is spontaneous. The binding force is largely mediated by hydrophobic and electrostatic forces. The binding distance between DMY and BSA was determined to be 3.43 nm. The presence of Co^{2+} and Cu^{2+} increases the binding constant of DMY–BSA complex, which may prolong the storage period of DMY in blood plasma and enhance its maximum effects. The results obtained from synchronous fluorescence spectra show that the structure of BSA molecules is changed dramatically in the presence of DMY.

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REFERENCES

1. Idowu, M.; Lamprecht, E.; Nyokong, T. Interaction of water-soluble thiol capped CdTe quantum dots and bovine serum albumin. *J. Photochem. Photobio. A* **2008**, *198*, 7–12.
2. Lu, Z.; Zhang, Y.; Liu, H.; Yuan, J.; Zheng, Z.; Zou, G. Transport of a cancer chemopreventive polyphenol, resveratrol: Interaction with serum albumin and hemoglobin. *J. Fluoresc.* **2007**, *17*, 580–587.
3. He, Y.; Wang, Y. W.; Tang, L. F.; Liu, H.; Chen, W.; Zheng, Z. L.; Zou, G. L. Binding of puerarin to human serum albumin: A spectroscopic analysis and molecular docking. *J. Fluoresc.* **2008**, *18*, 433–442.
4. Xiao, J. B.; Chen, X. Q.; Jiang, X. Y.; Hilczer, M.; Tachiya, M. Probing the interaction of trans-resveratrol with bovine serum albumin: A fluorescence quenching study with Tachiya model. *Journal of Fluorescence* **2008**, *18*, 671–678.
5. Poncet-Legrand, C.; Gautier, C.; Cheynier, V.; Imberty, A. Interactions between flavan-3-ols and poly(l-proline) studied by isothermal titration calorimetry: Effect of the tannin structure. *J. Agric. Food Chem.* **2007**, *55*, 9235–9240.

6. Xiao, J. B.; Suzuki, M.; Jiang, X. Y.; Chen, X. Q.; Yamamoto, K.; Xu, M. Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. *J. Agric. Food Chem.* **2008**, *56*, 2350–2356.
7. Xiao, J.; Cao, H.; Wang, Y.; Zhao, J.; Wei, X. Glycosylation of dietary flavonoids decreases the affinities for plasma protein. *J. Agric. Food Chem*. **2009**, *57*, 6642–6648.
8. Xiao, J.; Chen, X.; Zhang, L.; Talbot, S. G.; Li, G. C.; Xu, M. Investigation of the mechanism of enhanced effect of EGCG on huperzine A's inhibition of acetylcholinesterase activity in rats by a multispectroscopic method. *J. Agric. Food Chem.* **2008**, *56*, 910–915.
9. Riihimaäki, L. M.; Vainio, M. J.; Heikura, J. M. S.; Valkonen, K. H.; Virtanen, V. T.; Vuorela, P. M. Binding of phenolic compounds and their derivatives to bovine and reindeer *β*-lactoglobulin. *J. Agric. Food Chem.* **2008**, *56*, 7721–7729.
10. Xiao, J. B.; Chen, J. W.; Cao, H.; Ren, F. L.; Yang, C. S.; Chen, Y.; Xu, M. Study of the interaction between baicalin and bovine serum albumin by multi-spectroscopic method. *J. Photochem. Photobio. A* **2007**, *191*, 222–227.
11. Soares, S.; Mateus, N.; Freitas, V. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary r-amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.* **2007**, *55*, 6726–6735.
12. Xiao, J. B.; Shi, J.; Cao, H.; Wu, S. D.; Ren, F. L.; Xu, M. Analysis of binding interaction between puerarin and bovine serum albumin by multi-spectroscopic method. *J. Pharmaceut. Biomed. Anal.* **2007**, *45*, 609–615.
13. Yoo, S. M.; Mun, S.; Kim, J. H. Recovery and pre-purification of (+)-dihydromyricetin from *Hovenia dulcis*. *Process Biochem.* **2006**, *41*, 567–570.
14. Jeon, S. H.; Chun, W.; Choi, Y. J.; Kwon, Y. S. Cytotoxic constituents from the bark of Salix hulteni. *Arch. Pharm. Res.* **2008**, *31*, 978–982.
15. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.
16. Chen, G. Z.; Huang, X. Z.; Xu, J. G.; Zheng, Z. Z.; Wang, Z. B. *The Methods of Fluorescence Analysis*, 2nd ed.; Beijing Science Press: Beijing, 1990.
17. Hu, Y. J.; Yu, H. G.; Dong, J. X.; Yang, X.; Liu, Y. Spectroscopic studies on the interaction between 3,4,5-trimethoxybenzoic acid and bovine serum albumin. *Spectrochim. Acta A* **2006**, *65*, 988–992.
18. Klotz, I. M. Physiochemical aspects of drug-protein interactions: A general perspective. *Ann. New York Acad. Sci.* **1973**, *226*, 18–35.
19. Ross, P. D.; Subramanian, S. Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry* **1981**, *20*, 3096–3102.
20. Murphy, C. B.; Zhang, Y.; Troxler, T.; Ferry, V.; Martin, J. J.; Jones, W. E., Jr. Probing Förster and Dexter energy-transfer mechanisms in fluorescent conjugated polymer chemosensors. *J. Phys. Chem. B* **2004**, *108*, 1537–1545.
21. Förster, T.; Sinanoglu, O. Eds. *Modern Quantum Chemistry*, vol. 3; Academic Press: New York, 1966.
22. Valeur, B.; Brochon, J. C. *New Trends in Fluorescence Spectroscopy,* 6th ed.; Springer Press: Berlin, 1999.
23. Cui, F. L.; Jing, F.; Jian, L.; Hu, Z. Interactions between 1-benzoyl-4-*p*-chlorophenyl thiosemicarbazide and serum albumin: Investigation by fluorescence spectroscopy. *Biorg. Med. Chem.* **2004**, *12*, 151–157.