JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Investigation of Flavonoids Bearing Different Substituents on Ring C and Their Cu^{2+} Complex Binding with Bovine Serum Albumin: Structure−Affinity Relationship Aspects

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Supporting Information

ABSTRACT: The effects of 1:1 flavonoid−Cu^{2+} complexes of four flavonoids with different C-ring substituents, quercetin (QU), luteolin (LU), taxifolin (TA), and (+)-catechin (CA), on bovine serum albumin (BSA) were investigated and compared with corresponding free flavonoids by spectroscopic analysis in an attempt to characterize the chemical association taking place. The results indicated that all of the quenching mechanisms were based on static quenching combined with nonradiative energy transfer. Cu^{2+} chelation changed the binding constants for BSA depending on the structures of flavonoids and the detected concentrations. The reduced hydroxyl groups, increased steric hindrance, and hydrophilicity of Cu^{2+} chelation may be the main reasons for the reduced binding constants, whereas the formation of stable flavonoid−Cu^{2+} complexes and synergistic action could increase the binding constants. The changed trends of critical energy transfer distance (R_0) for Cu^{2+} chelation were contrary to those of binding constants.

KEYWORDS: binding affinity, bovine serum albumin, chemical structure, flavonoid, flavonoid−Cu^{2+} complex

INTRODUCTION

Dietary flavonoids are important phytonutrient components widely distributed in plant foods.[1−5] Numerous recent investigations of flavonoids have described their beneficial biological activities, such as antioxidant activity,[4,6] anti-inflammatory activity,[7] antifungal activity,[8] and antitumor activity.[9] Flavonoids have been linked with many health benefits, and foods rich in flavonoids have attracted much attention in recent years. Most flavonoids consist of a benzene ring (A-ring) condensed with a six-membered ring (C-ring) that carries a phenyl group (B-ring) as a substituent in the 2-position. The C-ring is either a γ-pyrone ring (flavonols and flavones) or its dihydro derivative (flavanonols and flavanones). The number and mutual position of hydroxyl groups in the A- and B-rings and the conjugation and resonance effects between the A- and B-rings are related to their biological activities.[10] Flavonoids can chelate with metal ions, forming flavonoid−metal complexes.[11] It has been found that flavonoid−metal complexes are redox active and play an important role in antioxidant, anticancer, anti-inflammatory, and antibacterial properties and that flavonoid−metal complexes are often more active than free flavonoids.[12−15] Moreover, complexes with metal to flavonoid at 1:1 were found to possess stronger antiradical and cytoprotective activities in comparison with those of metal to flavonoid at 2:1.[12] However, the in vivo activities of flavonoids and their metal complexes remain uncertain, and questions concerning their transport, absorption, metabolism, and bioavailability in vivo are still unanswered.[16] Current knowledge suggests that factors such as protein binding may affect drug absorption, bioavailability, and even activities,[17] and the structural differences of drugs may strongly affect the binding process with protein.

Serum albumin is the major soluble protein in circulatory system, which has many physiological functions, such as maintaining the osmotic pressure and pH of blood and functioning as carriers transporting a great number of endogenous and exogenous compounds including fatty acids, amino acids, drugs, and pharmaceuticals.[18] The drug−serum albumin interaction plays a dominant role in drug disposition and efficacy. The study of the interaction between drugs and serum albumin is not only important in providing salient information about the nature of drugs and pharmacokinetics but also helpful in explaining the relationship between the structures and functions of drugs. Up to now, most papers have studied the binding process between flavonoids and serum albumin,[19,20] and a study on structure−affinity relationship indicated that hydroxylation on the A- and B-rings, hydrogenation on the C-ring, and the glycoside substituent affected the binding constants between flavonoids and serum albumin significantly.[21−25] However, little information has been obtained about the binding process of flavonoid−metal complexes with serum albumin in comparison with free flavonoids.[26,27] In this work, bovine serum albumin (BSA) has been selected as the protein model because of its structural homology with human serum albumin (HSA).[24]

Received: July 10, 2011
Revised: August 23, 2011
Accepted: August 25, 2011
Published: August 25, 2011

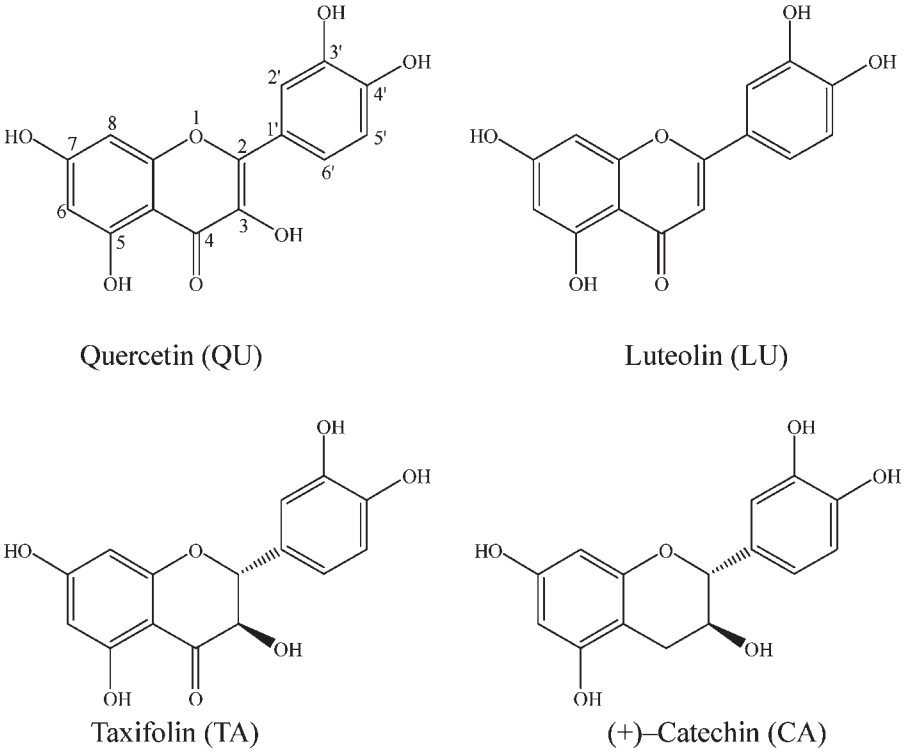

Figure 1. Molecular structures of QU, LU, TA, and CA.

Flavonol quercetin (QU), flavone luteolin (LU), flavanonol taxifolin (TA), and flavanol (+)-catechin (CA) (Figure 1) occur in many common foods.[28−31] There are three possible metal chelating domains in flavonoids that can interact with metal ions: the 3′,4′-dihydroxy group located on the B-ring and the 3- or 5-hydroxy and 4-oxo groups in the C-ring. Although many papers have dealt with the complex formation of QU, LU, TA, or CA with metals, few complexes have been isolated and characterized. The structure and complex formation features are sometimes incomplete and contradictory. Some studies proposed that the chelating properties of flavonoids toward metal ions should be attributed to the presence of the 3- or 5-hydroxy and 4-oxo groups rather than the 3′,4′-dihydroxy group in the B-ring, but there are also some studies that have proposed the 3′,4′-dihydroxy group as the major site for metal chelating.[32,33]

By using quenching fluorescence methods, the aims of this study were to investigate the effects of 1:1 flavonoid−Cu^{2+} complexes of four flavonoids with different C-ring substituents, QU, LU, TA, and CA, on BSA compared with corresponding free flavonoids in an attempt to characterize the chemical association taking place, which would provide additional information about the possibilities of transport, disposition, and efficiency of flavonoids and their corresponding Cu^{2+} complexes in blood plasma.

MATERIALS AND METHODS

Chemicals and Reagents. Four flavonoids, QU, LU, TA, and CA, with purities >99% were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). BSA was purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals, such as Tris buffer with a purity >99.5%, $CuCl_2$, NaCl, HCl, and ethanol, were all of analytical purity and used without further purification; all were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Water used in all experiments was doubly distilled water.

Apparatus. The ultraviolet (UV) spectra were obtained on a Perkin-Elmer Lambda 17 UV spectrophotometer with the wavelength range of 200−450 nm (Perkin-Elmer Corp., Edison, NJ). Electrospray ionization mass spectrometry (ESI-MS) data were measured on a Bruker Esquire 3000+ instrument (Faellanden, Switzerland). All fluorescence spectra were recorded on an F-2000 spectrofluorometer equipped with 1.0 cm quartz cells and a 150 W xenon lamp (Hitachi, Tokyo, Japan). An excitation wavelength of 280 nm was used. The excitation and emission slit widths were both set at 2.5 nm. The weight measurements were performed on an AY-120 electronic analytic weighing scale with a resolution of 0.1 mg (Shimadzu, Japan). The pH value was measured in a pHs-3 digital pH-meter (Shanghai, China).

Preparation of Solutions. Tris-HCl buffer solution (0.1 mol L^{-1} Tris, pH 7.4) containing 0.1 mol L^{-1} NaCl was prepared to keep the pH value and maintain the ionic strength of the solution. The Cu^{2+} stock solution (2×10^{-2} mol L^{-1}) was prepared by dissolving it in Tris-HCl buffer solution. The flavonoid and flavonoid−Cu^{2+} complex stock solutions (2×10^{-4} mol L^{-1}) were prepared by dissolving them in ethanol. The working solution of BSA (1×10^{-4} mol L^{-1}) was prepared by dissolving it in Tris-HCl buffer solution and was stored in a refrigerator at 4 °C prior to use.

Synthesis and Characterization of Flavonoid−Cu^{2+} Complexes. The flavonoid−Cu^{2+} complexes were synthesized according to a modified method in the literature:[34] 0.5 mmol of flavonoid and 25 mL of ethanol were added into a 50 mL two-necked round-bottom flask and then stirred by electromagnetic stirrer until the solid flavonoid was completely dissolved; 25 mL of Cu^{2+} stock solution (2×10^{-2} mol L^{-1}) was then added quickly into the flavonoid solution, and the solution was stirred at room temperature for 4.0 h; the reaction mixture was filtered after stirring, and the filtrate was evaporated slowly at room temperature. The resulting flavonoid−Cu^{2+} complexes were obtained after several washings with ethanol and dried in a vacuum desiccator. The structures of flavonoid−Cu^{2+} complexes were characterized by UV−vis spectra and MS.

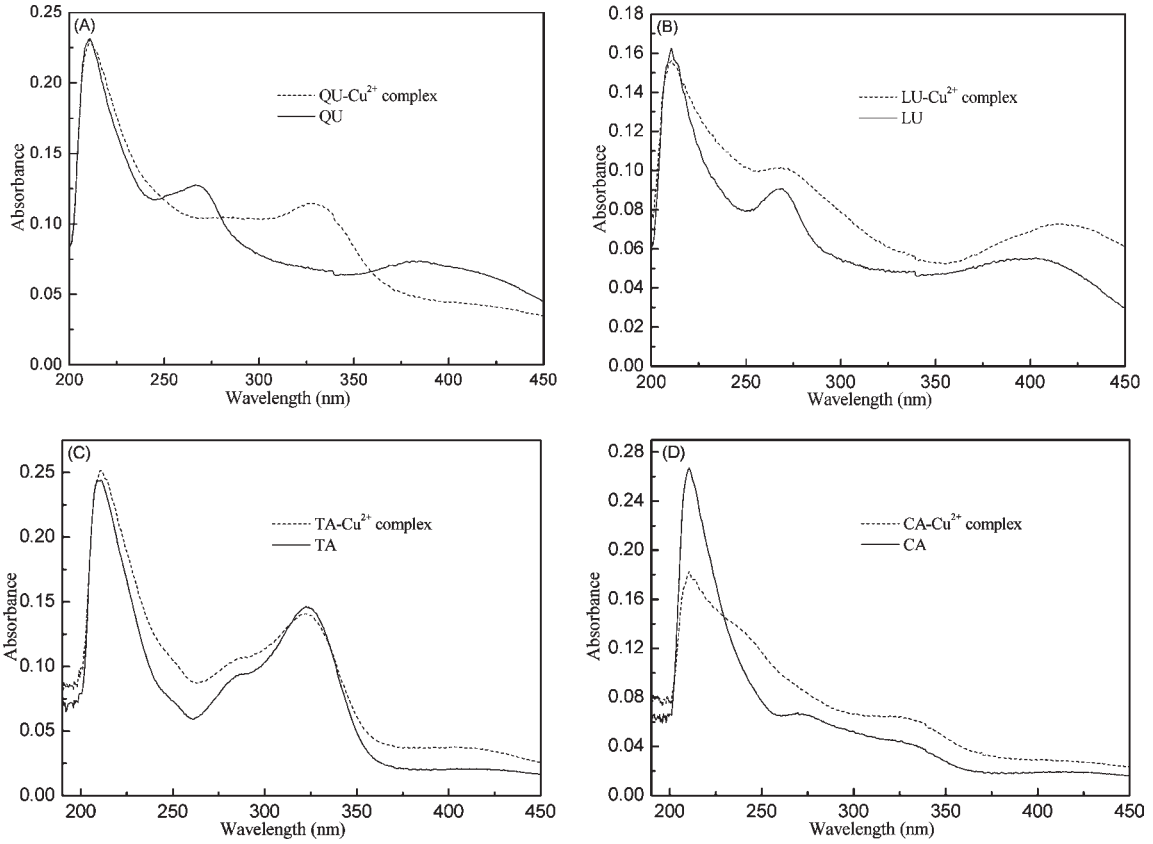

Figure 2. UV−vis absorption spectra of free flavonoids [(A) QU, (B) LU, (C) TA, and (D) CA] and their corresponding Cu^{2+} complexes: (thick solid line) flavonoids (2×10^{-4} mol L^{-1}); (dashed line) flavonoid−Cu^{2+} complexes (2×10^{-4} mol L^{-1}).

Fluorescence Spectra. Three hundred microliters of BSA solution was added to 11 5 mL flasks, respectively, and then appropriate amounts of 2.0×10^{-4} mol L^{-1} flavonoids or their complexes were added; the mixtures were diluted to 5 mL with Tris-HCl buffer. The final concentrations of flavonoids or flavonoid−Cu^{2+} complexes were 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 μmol L^{-1}, and the concentration of BSA was 6.0 μmol L^{-1}. The resultant mixtures were then incubated at 25 °C for 1.0 h. Then the fluorescence emission spectra were scanned in the range of 290−450 nm, and the fluorescence intensity at 340 nm was measured. All experiments were repeated in triplicate and found to be reproducible within experimental error (<1%).

Determination of Binding Parameters. For the dynamic quenching, the fluorescence quenching data are described by the Stern−Volmer equation[35]

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

where F_0 and F denote the steady state fluorescence intensities of serum albumin without and with the existence of quencher, respectively. K_{SV} is the Stern−Volmer quenching constant with units of L mol^{-1}; [Q] is the concentration of the quencher with units of mol L^{-1}. The Stern−Volmer equation was applied to determine K_{SV} by linear regression of a plot of F_0/F against [Q]. k_q is the quenching rate constant with units of L mol^{-1} s^{-1}, and τ_0 is the average lifetime of the serum albumin without any quencher, which is generally equal to 5 ns.[36]

The binding constant (K) and binding sites (n) are calculated by the double-logarithm equation for static quenching:[37]

$$\lg[(F_0 - F)/F] = \lg K + n \lg[Q] \quad (2)$$

The efficiency energy (E) was determined by Förster's energy transfer theory[38]

$$E = 1 - \frac{F}{F_0} = \frac{R_0{}^6}{R_0{}^6 + r^6} \quad (3)$$

where F and F_0 are the fluorescence intensities of BSA with or without the existence of ligand, respectively, r is the distance between acceptor and donor, and R_0 is the critical distance, which is evaluated as follows when the transfer efficiency is 50%:

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

k^2 is the orientation factor, N is the refractive index of the medium, and Φ is the fluorescence quantum yield of the donor. For BSA, $k^2 = 2/3$, $N = 1.36$, and $\Phi = 0.14$.[39] J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, which is approximately given by the equation

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (5)$$

where $F(\lambda)$ is the fluorescence intensity of the donor and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor.

All of the above data points were fit to curves by means of OriginPro 7.5 software (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

UV−Vis Spectroscopic Study of the Flavonoid−Cu^{2+} Complexes. QU, LU, TA, and CA all contain a 3′,4′-dihydroxy group in the B-ring but have different substituents at the C-ring.

LU, TA, and CA share a general chemical structure with QU, except that LU lacks a hydroxyl group at position 3 and TA lacks the C2−C3 double bond, whereas CA has no C2−C3 double bond and a 4-oxo group in C-ring. The appearance of new peaks in UV−vis spectra made it possible to determine whether or not the flavonoid−Cu^{2+} complexes were being formed and the possible metal chelating domains in flavonoids. The formation of flavonoid−Cu^{2+} complexes was described in terms of shifts in band I (300−550 nm) and band II (240−285 nm), which relate to B- and A-ring absorption, respectively. The chelating experiments were conducted at pH 7.4. It is known that the deprotonation on ring B is favorable at pH >5, thus facilitating the formation of a bidentate ligand and, subsequently, the 5-membered chelate ring with the metal ion.[40] Furthermore, the delocalization of the oxygen electrons of the 3′,4′-dihydroxy group was high on ring B, which facilitated the delocalization of the π electrons and then led to the formation of a stable chelate with ring B. The UV−vis spectra of free flavonoids and flavonoid−Cu^{2+} complexes in ethanol are shown in Figure 2. QU exhibited two major absorption bands at 261 and 376 nm, respectively, in the Tris-HCl buffer (pH 7.40).[15] When QU reacted with Cu^{2+}, the intensity of the two bands decreased remarkably, and a new stronger absorption peak appeared at 328 nm, suggesting that the 3′,4′-dihydroxy group in ring B of QU was important for Cu^{2+} chelation.[41] LU exhibited two maxima absorption at 266 and 401 nm. Interaction of Cu^{2+} with LU produced bathochromic shifts in band I of 15 nm, whereas no changes in the position of band II were observed. The presence of the peak at 266 nm demonstrated that ring A remained unchanged. The bathochromic shift could be explained by the extension of the conjugated system with complexation. Therefore, it seemed that Cu^{2+} interacted with LU probably between 3′,4′-dihydroxy groups to form five-membered ring complexes. There were no significant shifts in band I and II maxima for TA and CA. Because CA lacks the 4-oxo group, the chelation involves only the 3′,4′-dihydroxy group in the B-ring. The chelation of Cu^{2+} to TA gave rise to an intensity decrease of band I and an increase of band II, which was attributed to the change of the B-ring system, suggesting that 3′,4′-dihydroxy groups were the most important chelating site. Therefore, all of the UV−vis studies suggested that complexes were formed involving the 3′,4′-dihydroxy B-ring system, whatever the flavonoid structures considered.

MS of the Complexes. ESI-MS is a high-sensitivity technique adequate to characterize the chelation of Cu^{2+} by flavonoids, which provided direct evidence of chelate formation and information about the various stoichiometries of chelates and the chelating site when combined with collision-induced decomposition (CID) studies.[42] The QU−Cu^{2+}, LU−Cu^{2+}, TA−Cu^{2+}, and CA−Cu^{2+} complexes showed MS with molecular ions at m/z 362, 346, 364, and 350 $[M - H]^-$, respectively, which indicated that flavonoid−Cu^{2+} complexes were formed between Cu^{2+} and all of the flavonoids, having the stoichiometries flavonoid/Cu^{2+} 1:1. None of the CID spectra of flavonoid−Cu^{2+} complexes showed the loss of CuCO, which confirmed that flavonoids chelated Cu^{2+} through the 3′,4′-dihydroxy group instead of between the 3- or 5-hydroxyl group and the 4-oxo group.

Fluorescence Quenching of BSA by Free Flavonoids and Their Cu^{2+} Complexes. BSA has three linearly arranged, structurally distinct, homologous domains (I−III), and each domain is composed of two subdomains (A and B). The specific sites binding with BSA are sites I and II, which are located in

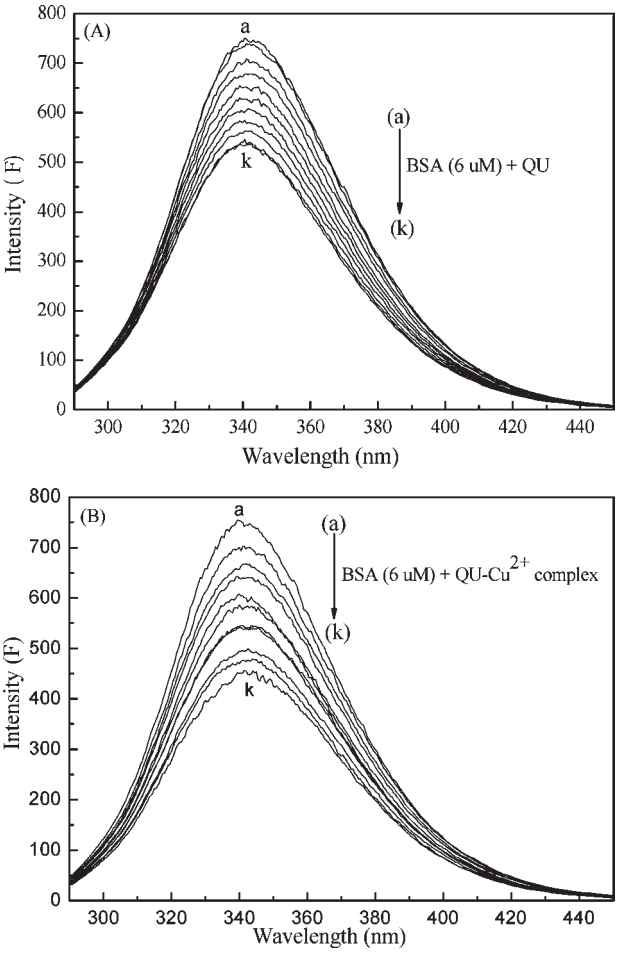

Figure 3. Fluorescence quenching spectra of BSA at various concentrations of QU and QU−Cu^{2+} complex. λ_{ex}, 280 nm; c(BSA), 6.0 μmol L^{-1}; c(QU) = c(QU−Cu^{2+} complex); (a−k) 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 μmol L^{-1} for QU (A) and QU−Cu^{2+} complex (B), respectively; T, 298 K.

hydrophobic cavities in the IIA and IIIA subdomains.[43] When the fluorescence emission spectra of BSA are measured with a series of concentrations of quencher by fixing the excitation wavelength at 280 nm, the fluorescence emission peak of BSA at 340 nm gives the information of tryptophan residues.[44] Therefore, fluorescence quenching can be considered as a technique for measuring binding constants.

Figure 3 shows the representative fluorescence spectra of QU and the Qu−Cu^{2+} complex. The fluorescence intensity of BSA decreased remarkably with the addition of QU or QU−Cu^{2+} complex. About 28.4 and 39.5% of the fluorescence intensities of BSA were quenched by adding 10 μmol L^{-1} of QU and Qu−Cu^{2+} complex, respectively (calculated from Figure 3). QU, LU, TA, CA, and QU−Cu^{2+} complex can quench BSA fluorescence proportionally, whereas LU−Cu^{2+} complex, TA−Cu^{2+} complex, and CA−Cu^{2+} complex quenched BSA fluorescence nonproportionally (see Figure S1 of theSupporting Information). The quenching percentages of BSA at 340 nm induced by LU, TA, CA, and the corresponding Cu^{2+} complexes at 10 μmol L^{-1} were as follows: 35.2, 22.5, 40.2, 31.6, 35.2, and 19.6% (calculated from Figure S1 of the Suppporting Information). The QU−Cu^{2+} complex and the TA−Cu^{2+} complex could quench more fluorescence of BSA than free flavonoids, whereas the LU−Cu^{2+} complex

and the CA−Cu^{2+} complex quenched less fluorescence of BSA than free flavonoids. The extent of the fluorescence attenuation was in the order CA > QU−Cu^{2+} complex > LU ≈ TA−Cu^{2+} complex > LU−Cu^{2+} complex > QU > TA > CA−Cu^{2+} complex. The results indicated that Cu^{2+} chelation of flavonoids could affect their quenching effects on BSA fluorescence, and the quenching effects of flavonoids and their Cu^{2+} complexes on BSA fluorescence depended on their structures.

The effect of flavonoids and their Cu^{2+} complexes on the fluorescence spectra of BSA was seen as a slight blue shift of the maximum emission wavelength (λ_{em}, 2 nm) of BSA fluorescence for QU; however, a slight red shift of λ_{em} (3 nm) was observed for the Qu−Cu^{2+} complex (Figure 3). Larger blue shifts of λ_{em} occurred for LU (4 nm) than for LU−Cu^{2+} complex (1.5 nm), and smaller red shifts of λ_{em} for TA (3 nm) were observed than for the TA−Cu^{2+} complex (4 nm). There was a weak blue shift of λ_{em} for CA (2 nm), but there were no blue or red shifts for the CA−Cu^{2+} complex. The emission of indole may be blue shifts if the group was buried within a native protein, and its emission may shift to longer wavelengths when the protein is unfolded.[45] Therefore, the shift results suggested that the environment of the trypophan and tyrosine residues was changed and that the flavonoids and flavonoid−Cu^{2+} complexes were situated at close proximity to the tryptophan and tyrosine residues for the quenching to occur.

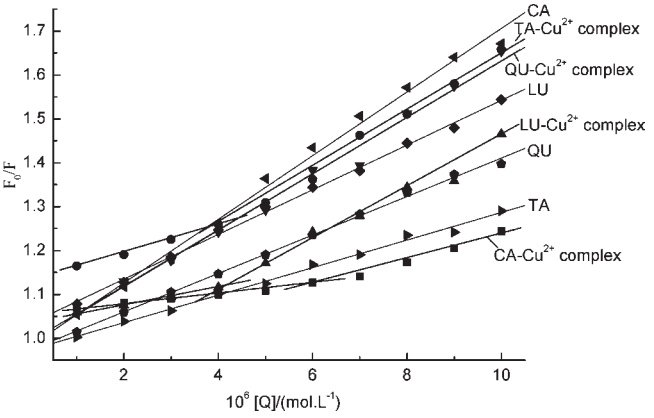

Figure 4. Stern−Volmer plots for BSA fluorescence quenching by QU, LU, TA, CA, and their Cu^{2+} complexes at 298 K.

The Stern−Volmer plots for the fluorescence quenching by QU, LU, TA, CA, and their Cu^{2+} complexes are shown in Figure 4. The Stern−Volmer curves for QU, LU, TA, CA, and QU−Cu^{2+} complex were linear. However, the Stern−Volmer plots for the LU−Cu^{2+}, TA−Cu^{2+}, and CA−Cu^{2+} complexes were nonlinear, and the points could be resolved into two straight lines, which indicated that the interactions between BSA and the LU−Cu^{2+}, TA−Cu^{2+}, or CA−Cu^{2+} complex followed two types of binding sites. In the linear range of Stern−Volmer curves, all of the average k_q values were far greater than 2.0×10^{10} L mol^{-1} s^{-1} (Table 1), which indicated that all of the fluorescence quenching probably originated from the formation of a ligand−BSA complex for static quenching procedure rather than a dynamic quenching process. Therefore, the Cu^{2+} chelation of flavonoids did not change the quenching mechanism.

According to the literature, the quenching mechanism of QU, LU, TA, and CA has been ascribed to a static quenching procedure.[17,24,46−48] To reconfirm that the probable quenching mechanism of fluorescence of BSA by flavonoid−Cu^{2+} complexes was initiated by static quenching, UV−vis absorption spectra were obtained. UV−vis absorption spectroscopy is a very simple but effective method to explore structural change and to recognize complex formation.[49] From Figure 5 it can be seen that the UV−vis absorption spectra of BSA and the difference absorption spectra between BSA−flavonoid−Cu^{2+} and flavonoid−Cu^{2+} at the same concentration could not be superposed within experimental error, which reconfirmed that the probable quenching was mainly a static quenching procedure and at least a BSA−ligand complex with certain new structure formed.[50]

Binding Constants of QU, LU, TA, CA, and Their Cu^{2+} Complexes to BSA and Structure−Affinity Relationship. Determination of the level of drug binding with serum albumin is critical and will directly correlate with the transport, disposition, and in vivo efficacy of the drug. If a drug has low ability to bind with serum albumin, the amount of drug available to diffuse into the target tissue may be significantly reduced, and the efficacy of the drug may then be poor and vice versa.[51] The binding constants (K) and binding sites (n) can be calculated by the double-logarithm equation (eq 2). Plots of $\lg(F_0 - F)/F$ versus $\lg[Q]$ for flavonoids and their Cu^{2+} complexes to BSA are shown in Figure 6, and Table 2 lists the corresponding calculated binding constants and number of binding sites in diverse modes. It is clear that the double-logarithm curves for QU, LU, TA, CA, and the QU−Cu^{2+} complex were linear, but those for the

Table 1. Stern−Volmer Quenching Constants (K_{SV}) and Quenching Rate Constants (k_q) for the Interactions of QU, LU, TA, CA, and Their Cu^{2+} Complexes with BSA at 298 K

	c (μmol L^{-1})	K_{SV} (× 10^4 L mol^{-1})	k_q (× 10^{12} L mol^{-1} s^{-1})	R^a	SD[b]
QU	≤10	4.38	8.76	0.9990	0.006
QU−Cu^{2+} complex	≤10	6.40	12.80	0.9947	0.008
LU	≤10	4.99	9.98	0.9926	0.009
LU−Cu^{2+} complex	≤4	1.92	3.84	0.9978	0.007
	≥4	5.44	10.88	0.9901	0.009
TA	≤10	2.93	5.86	0.9963	0.008
TA−Cu^{2+} complex	≤4	3.19	6.38	0.9979	0.008
	≥4	6.73	13.46	0.9967	0.007
CA	≤10	7.26	14.52	0.9967	0.006
CA−Cu^{2+} complex	≤6	1.16	2.32	0.9914	0.009
	≥6	3.40	6.80	0.9988	0.008

[a] R is the correlation coefficient. [b] SD is the standard deviation.

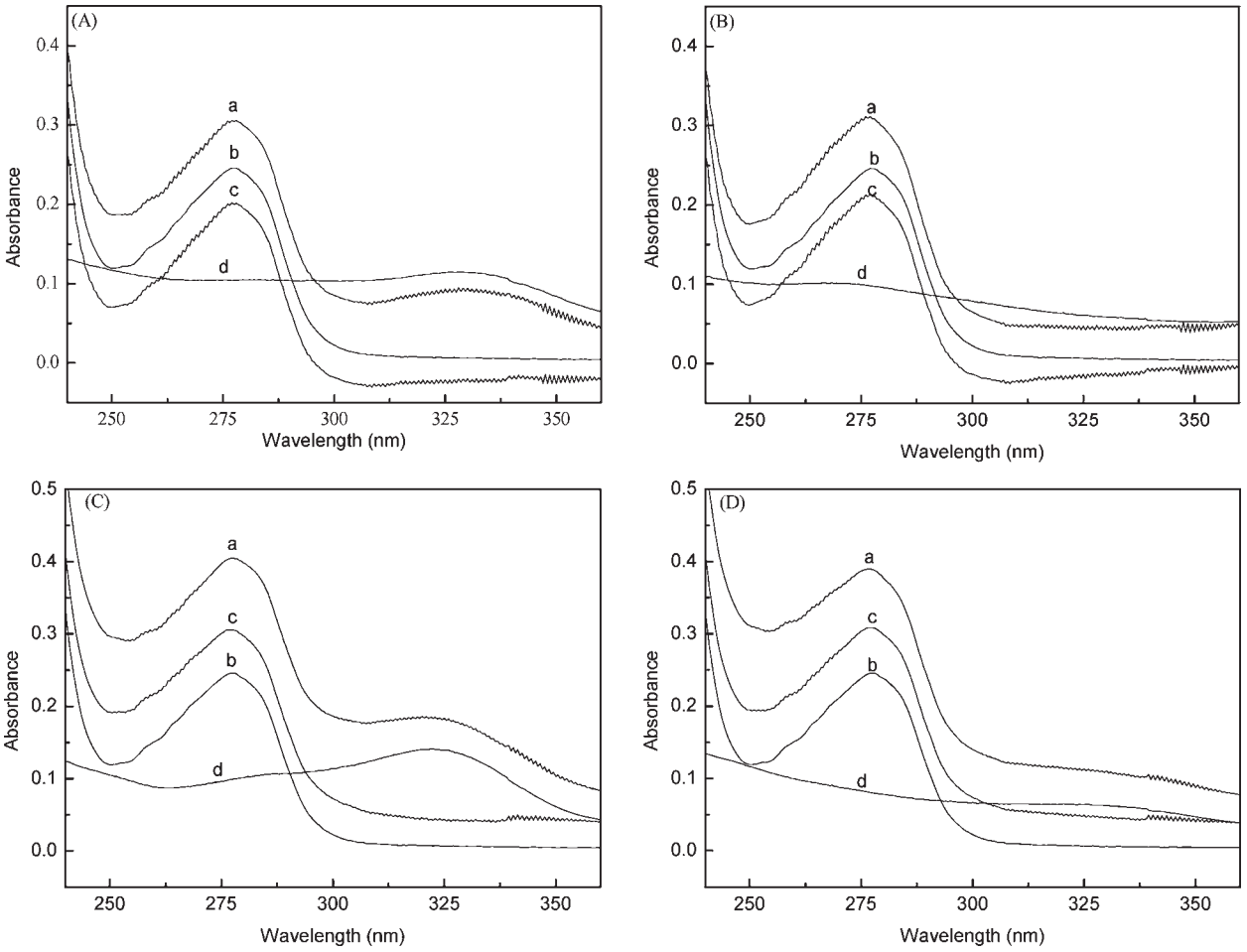

Figure 5. UV−vis spectra of BSA in the presence of QU−Cu^{2+} complex (A), LU−Cu^{2+} complex (B), TA−Cu^{2+} complex (C), and CA−Cu^{2+} complex (D). (a) Absorption spectra of BSA−flavonoid−Cu^{2+} system when the BSA and flavonoid−Cu^{2+} complexes were at the same concentration; (b) absorption spectra of BSA only; (c) difference absorption spectra between BSA−flavonoid−Cu^{2+} and flavonoid−Cu^{2+} complex at the same concentration; (d) absorption spectra of flavonoid−Cu^{2+} complex only. $c_{BSA} = c_{flavonoid-Cu^{2+}complexes} = 6.0\ \mu mol\ L^{-1}$.

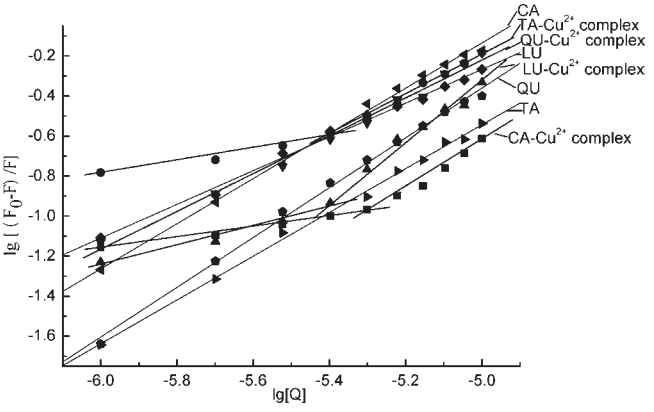

Figure 6. Double-logarithm curves of QU, LU, TA, CA, and their Cu^{2+} complex quenching BSA fluorescence at 298 K.

Table 2. Static Binding Constants and Binding Sites for the Interactions of QU, LU, TA, CA, and Their Cu^{2+} Complexes with BSA at 298 K

	c (μmol L^{-1})	K	n	R^a	SD^b
QU	≤10	7.24×10^5	1.24	0.9984	0.015
QU−Cu^{2+} complex	≤10	3.39×10^4	0.95	0.9953	0.018
LU	≤10	8.91×10^3	0.84	0.9963	0.017
LU−Cu^{2+} complex	≤4	44.67	0.48	0.9856	0.036
	≥4	6.92×10^6	1.44	0.9915	0.020
TA	≤10	8.32×10^4	1.09	0.9985	0.012
TA−Cu^{2+} complex	≤4	7.24	0.28	0.9838	0.031
	≥4	8.13×10^4	1.04	0.9958	0.019
CA	≤10	3.24×10^5	1.13	0.9983	0.016
CA−Cu^{2+} complex	≤6	2.88	0.27	0.9918	0.014
	≥6	1.70×10^5	1.17	0.9907	0.017

[a] R is the correlation coefficient. [b] SD is the standard deviation.

LU−Cu^{2+}, TA−Cu^{2+}, and CA−Cu^{2+} complexes were divided into two straight lines. Both the binding constants and binding sites in lower concentration were smaller compared with those in higher concentration for the LU−Cu^{2+}, TA−Cu^{2+}, and CA−Cu^{2+} complex binding with BSA. These results indicated that flavonoid−Cu^{2+} complexes might facilitate binding sites with lower affinity in lower concentration, whereas at higher concentration, flavonoid−Cu^{2+} complexes might get nearer

tryptophan residues and binding is comparably stronger.[52] The values of binding constants for LU−Cu^{2+}, TA−Cu^{2+}, and CA−Cu^{2+} complex binding with BSA at low concentration were merely 10^1−10^3 mol L^{-1}, which was rather low compared to the common constants of ligands for serum albumin,[20−26] whereas the values of binding constants of QU, LU, TA, CA, and their Cu^{2+} complexes in high concentration to BSA were in the range of 10^4−10^7 mol L^{-1}, which agreed with common BSA−ligand binding.[20−26] Cu^{2+} chelation could lower the constants of QU, TA, or CA binding with BSA by 1−5 orders of magnitude depending on the structures of flavonoids and the detected concentrations. The binding constant of QU for BSA was about 21.38 times higher than that of the QU−Cu^{2+} complex. The binding constants of TA and CA were about 11481.54 and 112201.85 times higher than those of the TA−Cu^{2+} and CA−Cu^{2+} complexes in lower concentrations, respectively, but only 1.02 and 1.91 times higher than those in higher concentrations, respectively. However, the binding constants between LU and BSA reduced after Cu^{2+} chelation by 99.50% in the lower concentration and increased about 776.25 times in higher concentration. All of the values of lg K were proportional to the binding sites (n) with higher correlation coefficient (R = 0.9959) (see Figure S2 of the Supporting Information), which confirmed that the mathematical model used in the experiment was suitable to study the interaction between flavonoids or their Cu^{2+} complexes and BSA.

It is obvious that flavonoids differing in C-ring substitution and conjugation have different binding constants. BSA contains hydrophobic groups in the interior of the tertiary structure and polar groups such as amino, hydroxyl, and sulfhydryl groups at the surface. Hydrogen bonding may take place between hydrogen atoms in flavonoids or flavonoid−Cu^{2+} complexes and polar groups at the BSA surface. The values of the binding constants were in the order QU > CA > TA > LU. The binding constants of QU, CA, and TA with the hydroxyl group at C-3 for BSA are about 81, 36, and 9 times higher than that of LU, respectively. Therefore, hydroxyl groups were important for flavonoid binding with BSA according to the formation of hydrogen bonding between flavonoids and the polar groups at the BSA surface. The existence of a C2−C3 double bond was the next factor, which could increase the binding constants, because the binding affinity of QU for BSA was higher than those of CA and TA, which may be explained by the absence of the double bond in the C-ring not allowing any electronic delocalization; then a strong deviation from ring coplanarity happened, and the noncoplanarity and greater polarity of CA and TA weakened its capacity to penetrate into hydrophobic regions of BSA, which are frequently buried in the interior of the folded protein.[53]

Cu^{2+} chelation changed the binding constants of flavonoids. The values of the binding constants for higher concentration were in the order LU−Cu^{2+} complex > CA−Cu^{2+} complex > TA−Cu^{2+} complex > QU−Cu^{2+} complex. It was obvious that Cu^{2+} chelation reduced two hydroxyl groups in flavonoids, which weakened the possibility of formation of hydrogen bonds between flavonoid−Cu^{2+} complexes and BSA. Another mechanism may be that Cu^{2+} chelation increased the molecular size and hydrophobility, which weakened the capacity of flavonoid−Cu^{2+} complexes to penetrate into the tryptophan-rich hydrophobic regions of BSA.[54] LU had the lowest binding affinity in the free flavonoids; however, the LU−Cu^{2+} complex had the highest binding affinity among the flavonoid−Cu^{2+} complexes. The reason might be that the formation of the more stable LU−Cu^{2+} complex gave different exposure and location in BSA, which was consistent with two straight lines of the Stern−Volmer plot. Another reason may be the existence of synergistic action about LU and Cu^{2+} binding with BSA. Therefore, Cu^{2+} chelation could change the transport, disposition, and pharmacological effects of free flavonoids.

Table 3. J, E, R_0, and r Values of QU, LU, TA, CA, and Their Cu^{2+} Complexes with BSA

	J (cm^3 L mol^{-1})	E (%)	R_0 (nm)	r (nm)
QU	1.52×10^{-14}	19.03	2.67	3.40
QU−Cu^{2+} complex	1.75×10^{-14}	27.71	2.74	3.21
LU	1.36×10^{-14}	26.35	2.62	3.11
LU−Cu^{2+} complex	1.31×10^{-14}	19.32	2.61	3.31
TA	1.16×10^{-14}	14.37	2.56	3.44
TA−Cu^{2+} complex	2.72×10^{-14}	21.13	2.95	3.67
CA	2.96×10^{-14}	30.30	2.99	3.43
CA−Cu^{2+} complex	3.14×10^{-14}	11.23	3.02	4.26

Binding Mode and Binding Distances of QU, LU, TA, CA, and Their Cu^{2+} Complexes to BSA. Förster nonradiation energy transfer theory is an important method to investigate a variety of biological phenomena such as energy transfer processes. According to the Förster nonradiation energy transfer theory,[38] energy transfer could happen under the following conditions: (i) the donor and acceptor dipoles would have relative orientation; (ii) the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor would overlap to some extent; and (iii) the distance between the donor and acceptor was <8 nm. As shown in Table 3, all of the values of r are much smaller than 8 nm, which suggests that the nonradiative energy transfer from BSA to flavonoids or their Cu^{2+} complexes occurred with high possibility. The larger value of r compared to that of R_0 also revealed the presence of a static type of quenching mechanism. Therefore, the quenching mechanisms for QU, LU, TA, CA, and their Cu^{2+} complexes to BSA were static quenching combined with nonradiative energy transfer. Cu^{2+} chelation increased the R_0 of QU, TA, and CA and decreased that of LU. Moreover, the changed trends of R_0 for Cu^{2+} chelation were in contrast with those of binding constants.

■ ASSOCIATED CONTENT

Supporting Information. Figures for the fluorescence quenching spectrum of BSA at various concentrations of LU, TA, CA, and their Cu^{2+} complexes and the relationship between lg K and n. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

This work was supported by the National Scientific Foundation of China (21005089), the Natural Science Foundation of Hunan Province of China (10JJ4006), the Freedom Explore Program of Central South University (201012200015), the Shenghua Yuying project of Central South University, the open fund of the State Key Laboratory of Powder Metallurgy, and the aid program for Science and Technology Innovative Research

Team (Chemicals of Forestry Resources and Development of Forest Products) in Higher Educational Institutions of Hunan Province.

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