RESEARCH ARTICLE

Structure–affinity relationship of flavones on binding to serum albumins: Effect of hydroxyl groups on ring A

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Four flavones (flavone, 7-hydroxyflavone, chrysin, and baicalein) sharing the same B- and C-ring structure but a different numbers of hydroxyl groups on the A-ring were studied for their affinities for BSA and HSA. The hydroxylation on ring A of flavones increased the binding constants (K_a) and the number of binding sites (n) between flavones and serum albumins. The affinities of 7-hydroxyflavone for BSA and HSA were about 800 times and 40 times higher than that of flavone, respectively. It appears that the optimal number of hydroxyl groups introduced to the ring A of flavones is one. As more hydroxyl groups were introduced to positions at C-5, C-6, and/or C-7 of flavones, the affinities for serum albumins decrease. The critical energy transfer distances (R_0) between the hydroxylated flavones (1–3 OH on the ring A) and serum albumins decreased with the increasing affinities for serum albumins.

Received: September 12, 2009
Revised: October 7, 2009
Accepted: October 24, 2009

Keywords:
7-Hydroxyflavone / Baicalein / Chrysin / Flavone / Serum albumins

1 Introduction

Flavonoids are important dietary polyphenols present in many plant foods [1–4]. Investigations of flavonoids from dietary sources have attracted great interest for their nutritional and medical effects. Most of the bioactivities of flavonoids are likely related to their antioxidant abilities [5–8]. The presence of 2,3-double bond in conjugation with a 4-oxo group and the catechol unit (1,2-dihydroxybenzene) are usually required for the antioxidant activities of flavonoids [9]. The structural difference of flavonoids also strongly affects the binding process with plasma proteins. The serum albumins are the major transport protein in blood. In addition to being the most abundant protein in the circulatory system and buffering properties of body fluids, one of the most outstanding properties of serum albumins is the ability to bind small molecules reversibly, such as fatty acids, amino acids, drugs, and inorganic ions. Flavonoid–albumin interaction is expected to modulate the bioavailability of flavonoids. The interactions between flavonoids and proteins have attracted great interest among researchers [10–17]. The interactions between proteins and flavonoids result in forming a stable protein–flavonoid complex, which may be considered as a model for gaining general fundamental insights into flavonoid–protein binding [18–19].

Fluorescence quenching spectroscopy is an appropriate method to determine the binding parameters between drugs and proteins [14–20]. Tryptophan is the dominant amino acid contributing to the fluorescence spectrum of proteins. The quantum yield and emission maximum wavelength (max) associated with intrinsic fluorescence are very sensitive to the polarity of the environment and the structural changes in biomacromolecules [21].

Most reports focused only on the binding process between flavonoids and serum albumins, such as the binding constant, binding site, forces involved in binding (such as hydrogen bonding, hydrophobic forces, and electrostatic forces), binding distance, and energy transfer. However, few reports have focused on the structure–affinity relationship of flavonoids binding to proteins. Dufour and Dangles [18–19] determined the binding constants between the flavonol quercetin and its

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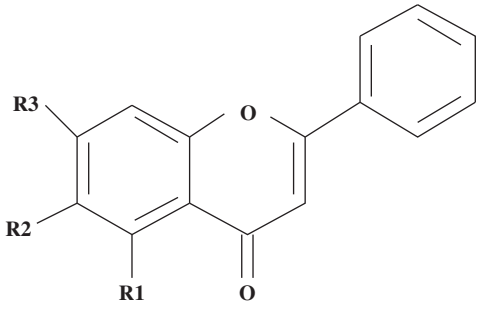
3-glycosides with serum albumins and concluded that glycosylation of flavonoids could lower the affinity to albumins by one order of magnitude. We have reported the influence of B-ring hydroxylation on binding of flavonols to BSA [22]. This study mainly concerns about the effect of hydroxylation on ring A of flavones on binding to BSA and HSA. Four flavones (flavone, 7-hydroxyflavone, chrysin, and baicalein) sharing the same structure on the rings B and C but with different numbers of hydroxyl groups on the ring A (Fig. 1) were studied for their affinity with BSA and HSA.

2 Materials and methods

2.1 Apparatus and reagents

Fluorescence spectra were recorded on a *JASCO FP-6500* fluorometer (Tokyo, Japan). UV–vis spectra were performed on a Shimadzu *UV-2450* spectrophotometer (Kyoto, Japan). The pH measurements were carried out on a *PHS-3C* Exact Digital pH meter (Cole-Parmer Instrument, IL, USA). BSA (fraction V), HSA (essentially fatty acid free, ~99% (agarose gel electrophoresis), lyophilized powder), and 7-hydroxyflavone (99.5%) were purchased from Sigma (MO, USA) and were used without further purification. Flavone, chrysin, and baicalein (99.5%) were obtained commercially from Wako Pure Chemical Industries (Osaka, Japan) and were used without further purification. The working solution of flavones (1.0×10^{-4}–1.0×10^{-5} M) was prepared by dissolving flavone with methanol-water solution (2:8, v/v) (This solution does not affect the fluorescence of BSA.). Tris-HCl buffer (0.20 M, pH 7.4) containing 0.10 M NaCl was selected to keep the pH value and maintain the ionic strength of the solution. The working solutions of BSA and HSA (1.0×10^{-5} M) were prepared with the Tris-HCl buffer and stored in refrigerator prior to use. All other reagents and solvents were of analytical grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.



flavone, R1=R2=R3=H; 7-hydroxyflavone, R1=R2= H and R3=OH; chrysin, R1=R3=OH and R2=H; baicalein, R1=R2=R3=OH.

Figure 1. Structures of flavones in this study.

2.2 Fluorescence spectra

Different concentrations of flavone solutions were transferred to a 10 mL flask, and then 1.0 mL of BSA solution was added and diluted to 10 mL with Tris-HCl buffer. The resultant mixture was subsequently incubated at 300.15 K for 0.5 h (The equilibrium in the interaction requires about 0.5 h. The time course experiment for the equilibration was not given here.). The fluorescent intensity at 340 nm was determined under the excitation wavelength of 280 nm. The fluorescence emissions of these four flavones within the range of 300–450 nm were not observed under this excitation wavelength. Each fluorescence intensity determination was repeated three times. The bandwidths for excitation and emission were 3 and 5 nm, respectively. The dynamic quenching constant (K_{SV}) and the binding constants (K_a) were calculated according to [23–24].

2.3 UV–vis measurements

UV–vis spectra were performed on a Shimadzu *UV-2450* spectrophotometer at 25°C in the range 220–500 nm using a quartz cell with 1 cm path length. Each UV spectra determination was repeated three times.

3 Results and discussion

3.1 Effect of flavones on BSA/HSA fluorescence spectra

The fluorescence spectra of BSA with addition of these four flavones are shown in Fig. 2 (The fluorescence spectra for HSA were not shown here.). In all cases, the fluorescence intensities of BSA/HSA decreased remarkably with the addition of these four flavones. The red shifts of the maximum λ_{em} of fluorescence of BSA and HSA were observed for flavone (A/B) (data not shown). For 7-hydroxyflavone, chrysin, and baicalein, however, the blue shifts of the maximum λ_{em} of fluorescence of BSA and HSA were observed. This means that the molecular conformation of the protein was effected, which was agree to recent studies that have shown that the tertiary structures of proteins were changed upon binding with polyphenols [10–22]. HSA has two primary hydrophobic binding sites commonly referred to as site I and site II, located at domains IIA and IIIA, respectively [25]. 7-Hydroxyflavone was found to bind to the inter-domain IIA-IIIA in close proximity to Trp214 [26] and chrysin was found to bind to sub-domain IIA of HSA [27], which resulted in the dramatic shifts (about 5 and 4 nm) of the maximum λ_{em} of HSA fluorescence. Compared with BSA molecule (residue located in the first sub-domain IB and Trp-212 in sub-domain IIA), the residue in HSA is more sensitive to the flavones, which was illustrated by the lager shifts of the maximum λ_{em}.

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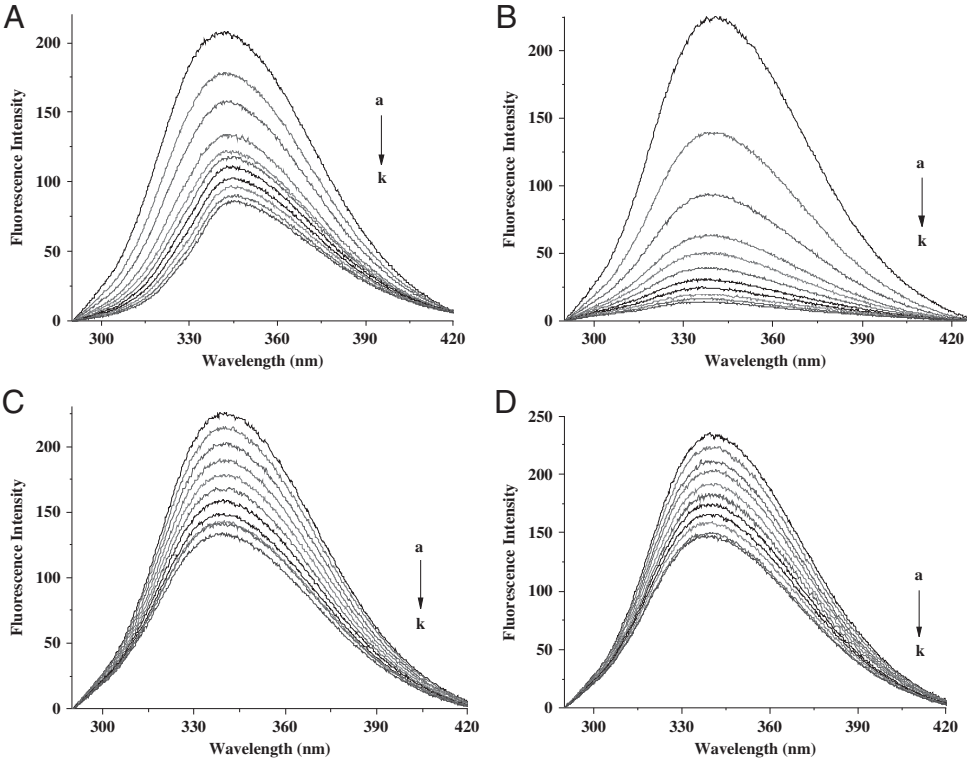

Figure 2. The quenching effect of flavones on BSA fluorescence intensity at 300.15 K. $\lambda_{ex} = 280$ nm; BSA, 1.00 μM; a–k: 0.50, 1.00, 1.50…5.00 ($\times 10^{-5}$ mol/L) of flavone (A) and 7-hydroxyflavone (B), 0.50, 1.00, 1.50…5.00 ($\times 10^{-6}$ mol/L) of chrysin (C), and baicalein (D).

3.2 Quenching of BSA/HSA fluorescence by flavones

The quenching ratio ($(F_0-F)/F_0$) of BSA and HSA fluorescence with addition of these four flavones is shown in Fig. 3. As shown in Fig. 3A, 1.0×10^{-5} M of 7-hydroxyflavone was found to lead to 90% fluorescence quenching, whereas flavone can quench only 60–70% fluorescence. The intensities of BSA and HSA fluorescence decreased rapidly with the addition of 7-hydroxyflavone, but decreased slower by increasing flavone concentration. As shown in Fig. 3B, chrysin can quench more fluorescence of BSA and HSA than that of baicalein under the same condition. These results indicated that the quenching effect of flavones on BSA and HSA fluorescence depended on the structure of flavones.

Figure 4 shows the Stern–Volmer plots for the BSA/HSA fluorescence quenching by flavone, 7-hydroxyflavone, chrysin, and baicalein. A linear Stern–Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to the quencher. In many cases, the fluorophore can be quenched both by collision and by complex formation with the same quencher. When this is the case, the Stern–Volmer plot exhibits an upward curvature, concave toward the *y*-axis at high [*Q*], and F/F_0 is related to [*Q*] by the following modified form of the Stern–Volmer equation [28]:

$$F_0/F = (1+K_D[Q])(1+K_S[Q]) \quad (1)$$

where K_D and K_S are the dynamic and static quenching constants, respectively.

This modified form of the Stern–Volmer equation is second order with respect to [*Q*], which accounts for the upward curvature observed at high [*Q*] when both static and dynamic quenching occur. As shown in Figs. 4A and B, it was found that both dynamic and static quenching were involved for these four flavones on BSA and HSA fluorescence, which demonstrated by the fact that the Stern–Volmer plots largely deviated from linearity toward the *y*-axis at higher flavone concentrations.

In the linear range of Stern–Volmer regression curve the average quenching constants (K_{SV}) for flavone, 7-hydroxyflavone, chrysin, and baicalein (having the lowest quenching effect, inset in Fig. 4) at 300.15 K is listed in Table 1. As summarized in Table 1, the hydroxylation of flavones on ring A affected the quenching effects on BSA and HSA fluorescence. The K_{SV} values for BSA and HSA were determined as follows: 7-hydroxyflavone > chrysin > baicalein > flavone.

3.3 Binding constants and structure–affinity relationship

Figure 5 shows the double-logarithm curves of flavones quenching BSA and HSA fluorescence at 300.15 K and Table 2 summarizes the corresponding calculated results. As summarized in Table 2, the hydroxylation on the ring A of flavones increased the binding constants (K_a) and the number of binding sites (*n*) between flavones and BSA/HSA. Flavone, with no hydroxyl group, showed lowest binding constants. The affinities of 7-hydroxyflavone for BSA and

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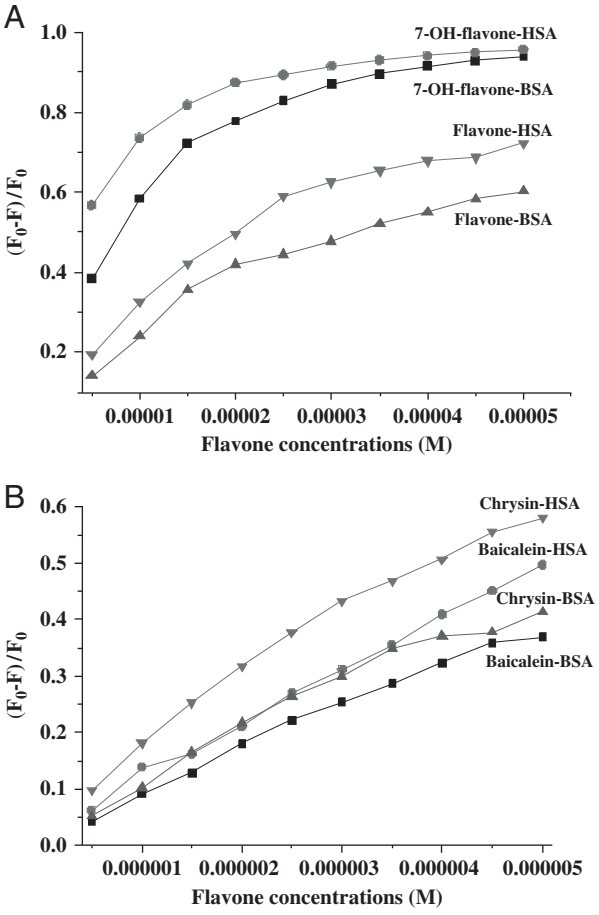

Figure 3. The quenching ratio ($(F_0-F)/F_0$) of BSA and HSA fluorescence with addition of flavone, 7-hydroxyflavone, chrysin, and baicalein.

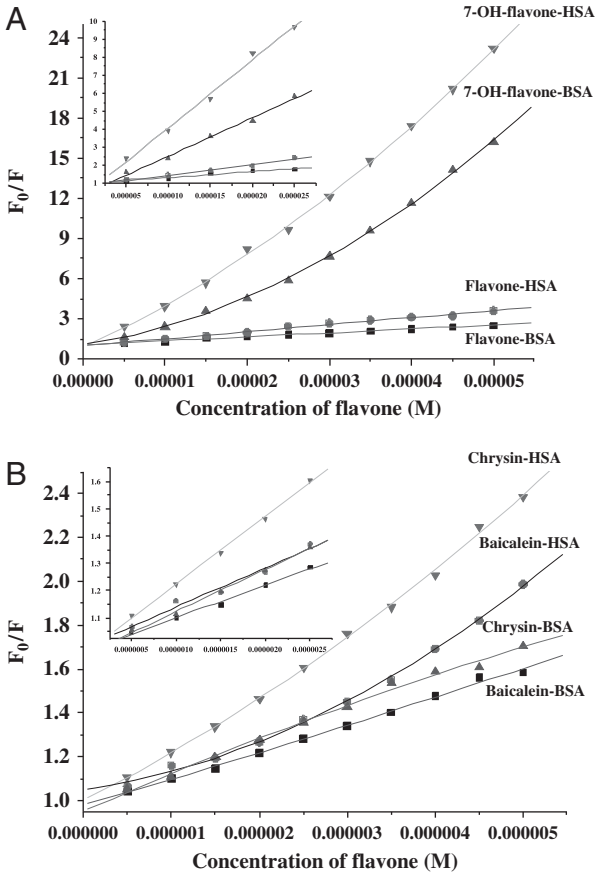

Figure 4. The Stern–Volmer plots for the BSA/HSA fluorescence quenching by flavone, 7-hydroxyflavone, chrysin, and baicalein at 300.15 K. Inset shows the Stern–Volmer curves with low concentration of flavones.

HSA are about 800 times and 40 times higher than that of flavone, respectively. Therefore, it appears that the optimal number of hydroxyl groups introduced to the ring A of flavones is one, since the highest binding was observed with 7-hydroxyflavone containing only one hydroxyl group. As more hydroxyl groups are introduced to positions at C-5, C-6, and/or C-7 of flavones, the affinities for serum albumins decreased. The binding constants (K_a) values for BSA and HSA were determined as: 7-hydroxyflavone (1 OH) > chrysin (2 OH) > baicalein (3 OH) > flavone (0 OH).

The hydrogen bond force may take place between the hydroxyl groups of flavones and the polar groups of BSA/HSA. Compared with 7-hydroxyflavone, the hydroxyl group in C5 position of chrysin can form the intra-molecular hydrogen bond with carbonyl group in C4 position (Fig. 6A), which weakens the formation of hydrogen bonds between chrysin with BSA/HSA. The hydroxyl groups in C5, C6, and C7 positions of baicalein also easily form the intra-molecular hydrogen bonds (Fig. 6B), which weakens the hydrogen bond force between baicalein and BSA/HSA. The decreasing hydrogen bond force between flavones with BSA/HSA may explain the decreasing binding constants with the increasing hydroxyl groups on ring A of flavones. Another mechanism may be that the hydroxylation of the flavone increased the molecular size and polarity and weakened the capacity to penetrate into the tryptophan-rich hydrophobic regions of albumins, which are frequently buried in the interior of the folded albumins. The different exposure and location of all these fluorophores in albumins may result in a deviation from linearity of the Stern–Volmer plots [22].

3.4 Critical energy transfer distance (R_0) between flavones and serum albumins

According to the Förster non-radioactive energy transfer theory [20], the energy transfer effect is related not only to the distance between acceptor and donor, but also to the critical energy transfer distance (R_0) [23]:

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

where K^2 is the spatial orientation factor of the dipole, N the refractive index of the medium, Φ the fluorescence quantum

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Table 1. The quenching constants of flavones on BSA and HSA fluorescence

Flavone	BSA		HSA	
	K_{SV}(L/mol)	*R*	K_{SV}(L/mol)	*R*
Flavone	3.86×10^4	0.9963	4.96×10^4	1
7-Hydroxyflavone	1.96×10^5	0.9970	3.82×10^5	0.9943
Chrysin	1.51×10^5	0.9973	2.37×10^5	0.9997
Baicalein	1.15×10^5	0.9960	1.26×10^5	0.9938

Every fluorescence intensity determination was repeated three times.

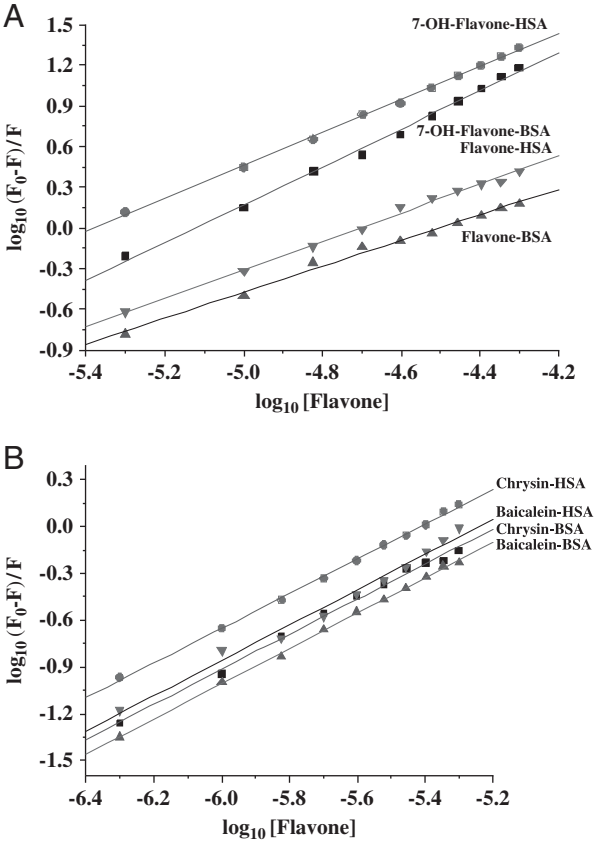

Figure 5. Double-logarithm curves of flavones quenching BSA and HSA fluorescence at 300.15 K.

yield of the donor, and *J* the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor was given by

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \quad (3)$$

where $F(\lambda)$ and $\varepsilon(\lambda)$ represent the fluorescence intensity of the donor and the molar absorption coefficient of the acceptor, respectively, at the wavelength λ. The overlap area was obtained by integrating the overlap region with the OriginPro 7.5 software.

The overlap of the absorption spectrum of flavones and the fluorescence emission spectrum of BSA is shown in Fig. 7. The spectra for HSA are not shown here. The overlap integrals and the critical distances (R_0) are listed in Table 3. The critical energy transfer distances (R_0) between hydroxyflavones (1–3 OH on the ring A) and serum albumins decreased with the increasing affinities for serum albumins (Fig. 8). Flavone, no hydroxyl group on the ring A, showed different manners with others. Furthermore, the critical energy transfer distances (R_0) remained constant when the measuring parameters of fluorescence intensity such as excitation slits, emission slits, and volt were changed (data not shown).

3.5 Relationship between the binding constants (K_a) and the number of binding sites (*n*)

The obtained values *n* (0.9529–1.4030) correspond to the binding sites with high affinity; however, the existence of

Table 2. The binding constants of flavones on BSA and HSA fluorescence

Flavone	BSA			HSA		
	$\log_{10}K_a$	*n*	*R*	$\log_{10}K_a$	*n*	*R*
Flavone	4.29	0.95	0.9959	4.92z	1.05	0.9975
7-Hydroxyflavone	7.17	1.40	0.9974	6.55	1.22	0.9991
Chrysin	6.08	1.17	0.9992	6.03	1.11	0.9992
Baicalein	5.67	1.08	0.9995	5.96	1.14	0.9926

Every fluorescence intensity determination was repeated three times.

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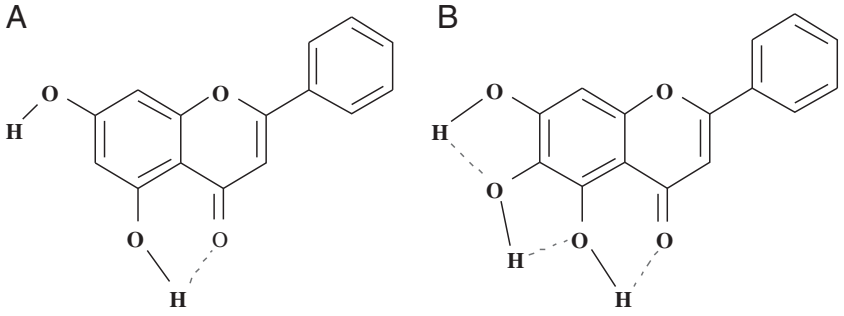

Figure 6. The intramolecular hydrogen bonds in chrysin (A) and baicalein (B).

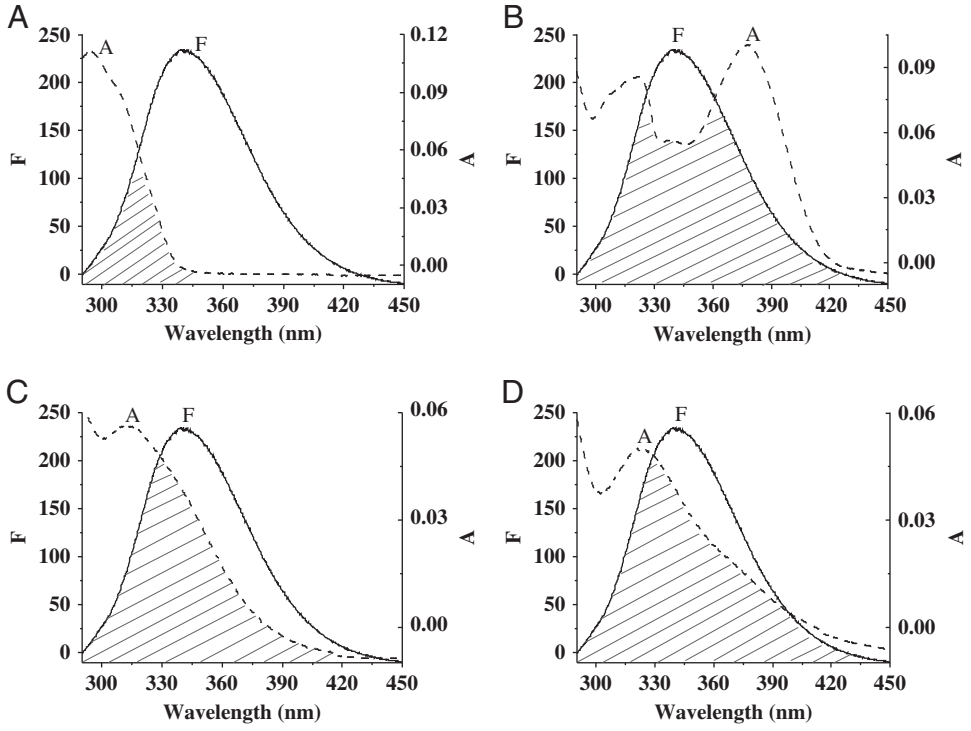

Figure 7. The overlap of the absorption spectrum (A) of flavone (A), 7-hydroxyflavone (B), chrysin (C), and baicalein (D), and the fluorescence emission spectrum (F, λ_{ex} = 280 nm) of BSA.

Table 3. The overlap integrals and the critical distance

Flavones	BSA		HSA	
	J ($cm^3\,L\,mol^{-1}$)	R_0/nm	J ($cm^3\,L\,mol^{-1}$)	R_0/nm
Flavone	0.65×10^{-14}	3.36	0.70×10^{-14}	3.40
7-Hydroxyflavone	0.59×10^{-14}	3.18	0.58×10^{-14}	3.29
Chrysin	1.26×10^{-14}	3.75	0.98×10^{-14}	3.59
Baicalein	1.46×10^{-14}	3.84	$1.05-10^{-14}$	3.64

J was calculated from Eq. (3); R_0 was calculated from Eq. (2) where $K^2 = 2/3$, $N = 1.336$, and $\Phi = 0.118$ [23].

the low affinity sites was not studied in this work. Recently, Berezhkovskiy illustrated that the calculated number of binding sites increased with the increase of compound concentration using the measured values of unbound drug fraction [29]. The number of binding sites (n) is different from the number of molecules actually bound to the sites [29, 30]. The number of molecules bound to the binding sites of a biomacromolecule follows a binomial distribution, if the number of binding sites is fixed [30]. When binding to the receptor with n sites of the same reaction is considered, and $K_d = 1/K_a$ is the dissociation binding constant (affinity of the binding site), it is necessary to have the ligand

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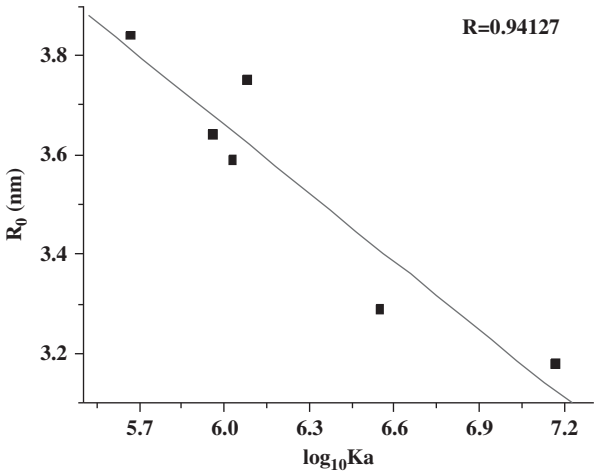

Figure 8. The relationship between the critical energy transfer distances (R_0) and affinities ($\log_{10}K_a$) of hydroxylated flavones (1–3 OH on the ring A) for serum albumins.

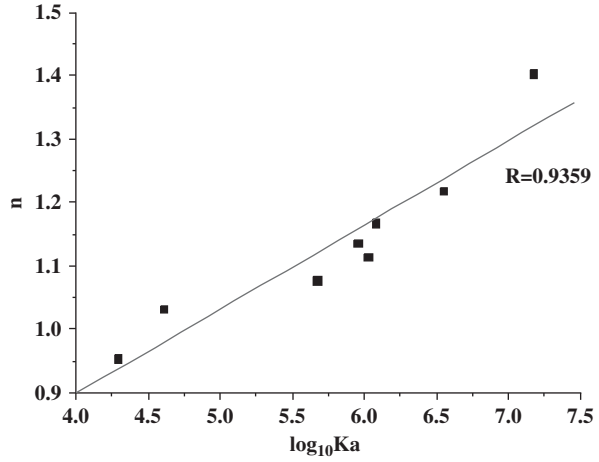

Figure 9. The relationship between the affinities ($\log_{10}K_a$) and the number of binding sites (n) between flavones and serum albumins.

concentration roughly equal to $10 \times K_d$ to occupy 90% of the binding sites. The low affinity site (K_d about or greater than $1000\,\mu M^{-1}$) was not practically occupied (compared to the binding sites with high affinity) and thus was not detected at low concentration of flavones used in the experiments. Though if the number of low affinity sites is significant (for instance 10 with $K_d = 1000\,\mu M^{-1}$), binding to them will be comparable to binding to a single high affinity site. This is because the increase of the quantity of sites leads to the increase of ligand bound to them. Hence, the number of binding sites increasing with increasing binding constant can be considered as one theory to evaluate these models. The relationship between the $\log_{10}(K_a)$ and the number of binding sites (n) between flavones and serum albumins is shown in Fig. 9. The values of $\log_{10}(K_a)$ are proportional to the number of binding sites (n). This result confirms the method used here is suitable to study the interaction between flavones and BSA. For example, Diniz *et al.* determined the binding constant between flavone and HSA as $\log_{10}(K_a) = 4.52$ by capillary electrophoresis-frontal analysis [31], which is near to present data ($\log_{10}(K_a) = 4.92$).

4 Concluding remarks

The structure–affinity relationship of flavones on binding to serum albumins was investigated by studying the effect of hydroxyl groups on ring A on the binding constants. The hydroxylation on ring A of flavones increased the binding constants (K_a) and the number of binding sites (n) between flavones and serum albumins. It appears that the optimal number of hydroxyl groups introduced to the ring A of flavones is one. As more hydroxyl groups were introduced to positions at C-5, C-6, and/or C-7 of flavones, the affinities for serum albumins decreased. The further work will focus on studying the relationships between the interaction data for flavonoids (flavone, falvonol, isoflavone, *etc.*) and the biological data.

The authors are grateful for financial sponsored by Natural Science Foundation of Shanghai (10ZR1421700), "Chen Guang" project supported by Shanghai Municipal Education Commission and Shanghai Education Development Foundation (09CG46), Innovation Program of Shanghai Municipal Education Commission (10YZ68), National Natural Science Foundation of China (Grant No. 20775092), and National High Technology Research and Development (863) Program of China (2008AA10Z322).

The authors have declared no conflict of interest.

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