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Structural effects on the conformational transition of transferrin induced by binding of flavonoids with different numbers and positions of hydroxyl groups

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Abstract

The effects of conformational changes to transferrin induced by the binding of flavonoids with different numbers and positions of hydroxyl groups were explored using spectroscopic and molecular modeling methods. The flavonoid hydroxyl group is not necessary for conformation changes of transferrin. However, the binding ability was found to increase with increasing numbers of hydroxyl groups and further conformational changes were observed. By molecular modeling calculations, intermolecular energy including van der Waals and electrostatic interactions, together with hydrogen bonding are found to have important roles in binding of flavonoids to transferrin. Additionally, the positions of the hydroxyl groups also affect the binding ability because they can alter the relative acidity of the hydroxyl groups, thereby changing the hydrogen bonding ability. Our results have indicated a mechanism for the interactions between flavonoids and transferrin, and provide information for possible flavonoid modification and design of methods to deliver drug molecules via transferrin to target tissues and cells effectively.

Keywords: Transferrin; Flavonoid; Hydrogen bonding; Conformational transition

1. Introduction

Proteins of the transferrin family – serum transferrin, lactoferrin (Lf), and ovotransferrin – have the characteristic ability to bind, tightly but reversibly, two Fe^{3+} ions together with two associated CO_3^{2-} ions [1]. In humans, transferrin is only 30% saturated with iron, and the vacant sites can bind other metals and act as a natural carrier for anticancer metal ions [2,3] and other chemotherapeutic drugs [4], because transferrin receptors are overexpressed on the surface of tumor cells [5,6]. Transferrin has also been used as a carrier or targeted ligand to deliver anticancer drugs in the form of drug conjugates [7]. However, there have been few reports on the direct binding of transferrin with natural drug molecules through non-covalent bonding, which might be the case for most drug delivery in humans.

The flavonoids are a large group of polyphenolic natural products, widely distributed in higher plants [8]. Such compounds have wide biological activities and important therapeutic applications, including anticancer, antitumor, anti-inflammatory, and anticoagulant drugs [9,10]. Interestingly, many biologically active flavonoids can affect various proteins including enzymes. Thus, understanding the interactions of flavonoids with proteins is important to interpret their biological activity [11]. For example, quercetin, which is abundant in the human diet, is among the group of phytoestrogens suggested to reduce risks of certain cancers [12,13]. Several biochemical and molecular biological investigations have revealed that proteins are often the "target" for therapeutically active flavonoids of natural and synthetic origin [14]. Therefore, investigation on the interaction between flavonoids and proteins is needed to explore the distribution, biotransformation, and ultimately the mechanism of action of flavonoids.

Transferrin (Tf) and human serum albumin (HSA) are two serum proteins involved in the transport of drug molecules. Most research has focused on the interaction between drug molecules

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and HSA [15]. However, few reports have assessed the interaction of flavonoids with transferrin at the molecular level, although the binding of metals to transferrin has intensively investigated in the past [16]. Our previous work has shown that the conformational change of Tf could be induced by a natural compound, quercetin [17]. Owing to the contribution of electrostatic forces to the total binding energy (the sum of van der Waals and electrostatic forces) and strong hydrogen bonding between the hydroxyl groups in quercetin and the polar amino acid residues in transferrin, it may be expected that both electrostatic forces and hydrogen bonding would be important for the binding of quercetin to transferrin. In this paper, we expand on our earlier work and further examine the interaction between Tf and flavonoids with different numbers of hydroxyl groups to understand the effect on the binding of flavonoids to Tf. Our aims are to determine (a) whether the hydroxyl group has an important role in the binding of flavonoids to transferrin; and (b) the structure–function relationship of flavonoid and transferrin binding to improve the targeting delivery ability by transferrin.

2. Materials and methods

2.1. Materials

Human serum transferrin (abbreviated as Tf, Catalog No. T3309, Lot No. 124K0807, 98% purity) was purchased from Sigma–Aldrich Co., and used without further purification. Flavone, 3-hydroxyflavone, galangin, luteolin, and kaempferol were purchased from Sigma–Aldrich Co. and the molecular structures are shown in Fig. 1. Their purities have been confirmed by mass spectrometry (MS) and ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra. The pH 7.4 phosphate buffer saline (PBS) solution containing 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, and 5 mM NaCl was prepared with double-distilled water. All stock solutions of the above flavonoids were first prepared in spectroscopic-grade ethanol and then diluted in PBS to obtain the final solutions (the final ethanol concentration in buffer was <1%, v/v). Stock Tf solution was prepared in phosphate buffer. All reagents and solvents were of analytical grade.

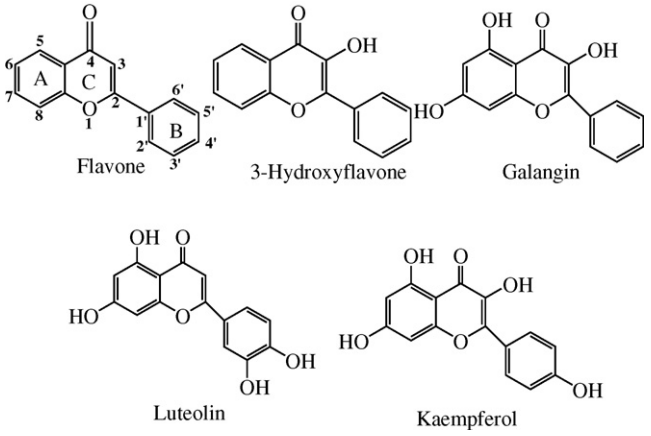

Fig. 1. The structural formulae of flavonoids.

2.2. Spectroscopic measurements

The spectra data were recorded after addition of Tf to flavonoids, and after incubation for 12 h. All the absorption experiments were performed with a 1-cm cuvette on a Shimatzu UV-1601PC spectrometer. The fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrometer with λ_{ex} at 290, 320, 350, 340, and 360 nm for flavone, 3-hydroxyflavone, galangin, luteolin, and kaempferol, respectively. The circular dichroism (CD) spectra were measured using a JASCO J-810 spectropolarimeter, and the spectra were accumulated for six times with a band-width of 1.0 nm, a resolution of 0.025 nm, and a scan speed of 500 nm/min.

2.3. Calculation of association constants

The stereospecific interaction between a ligand (L) and its primary site on the protein (P) can be quantified by the binding constant (K_a):

$$L + P = LP; \quad K_a = \frac{[LP]}{[L][P]} \tag{1}$$

It is evident that

$$[L] = c_L - [LP] \tag{2}$$

and

$$[P] = c_P - [LP] \tag{3}$$

where c_L and c_P represent the total concentrations of the ligand and protein, respectively. Assuming that the quercetin–Tf complex (1:1 stoichiometry) is responsible for the induced CD band, it can be written as [18]

$$CD_{450\,nm}\,(m\mathit{deg}) = k[LP] \tag{4}$$

where $k = 32982.1\Delta\varepsilon l$ ($\Delta\varepsilon$ is the extrinsic molar optical activity at 450 nm of flavonoids bound to Tf in $M^{-1}\,cm^{-1}$ and l is the optical pathlength in cm).

By combination of Eqs. (1)–(4), we obtain

$$CD\,(m\mathit{deg}) = \frac{k}{2}(c_P + c_L + K_a^{-1}) - \sqrt{(c_P + c_L + K_a^{-1})^2 - 4c_P c_L} \tag{5}$$

To calculate K_a, a non-linear regression analysis method was used. The magnitude of negative Cotton Effect at 450 nm was used to calculate the binding constants of all flavonoids.

2.4. Molecular modeling methods

With the aim of characterizing the structure–binding relationship, as well as exploring the mechanism of conformational change, the interaction between flavonoids and human serum transferrin has been investigated. All computations were performed on a SGI silicon graphics fuel workstation using Insight-II 2005 software (Accelrys Inc., San Diego, CA), with

Builder, Biopolymer, Binding Site Analysis and Affinity modules. The X-ray crystal structure of Tf was taken from the Protein Data Bank (PDB entry code 1D3K). The binding sites were located using the Binding Site Analysis module, and the binding of flavonoids to Tf was modeled by Affinity module with pH 7.4 and a CVFF force field. These models were subjected to minimization for 5000 iterations of steepest descents followed by conjugate gradients. Twenty conformations were selected, and of these, 10 with the highest energy rank were selected for the final energy minimization.

3. Results and discussion

3.1. Absorption spectra

Similar to previous reports [19], the absorption spectra of free flavonoid in solution has two main absorption bands commonly referred to as Band I (300–400 nm) and Band II (240–280 nm), shown in Fig. 2. Band I is thought to be associated with the light absorption of the cinnamoyl system (B + C ring) and Band II with the absorption of the benzoyl moiety formed by the A + C

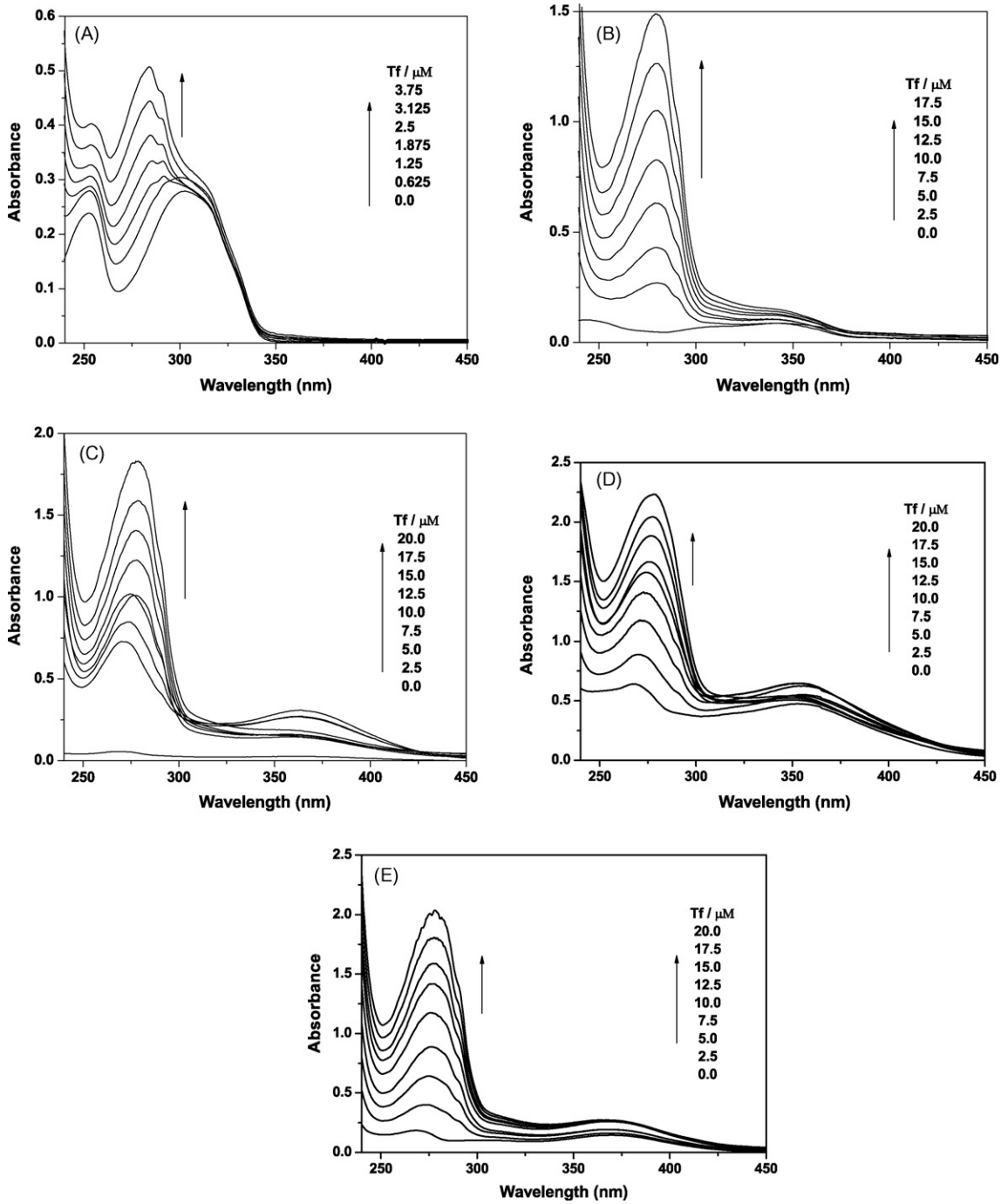

Fig. 2. The absorption spectra of flavonoids in pH 7.4 phosphate buffer after adding Tf of different concentrations. (A) Flavone (10 μM); (B) 3-hydroxyflavone (40 μM); (C) galangin (40 μM); (D) luteolin (40 μM); (E) kaempferol (40 μM).

ring (Fig. 1, flavone). Increasing the number of hydroxyl groups in ring A and ring B shifts the two absorption bands to longer wavelengths; this is particularly true for Band II. For example, flavone (Fig. 1A) shows two absorption bands at 252 and 302 nm in PBS buffer, and these two bands red-shifted to 269 and 370 nm with kaempferol (Fig. 1E) due to the addition of hydroxyl groups. However, ionization of the –OH groups could induce strong bathochromic shifts of all absorption bands [20]. The most acidic phenolic –OH groups of flavonoids are in the 7′, 3′ and 4′ positions [19,20], which would be dissociated at physiological pH. However, this process would be far from complete, resulting in a mixture of neutral and anionic species [21]. Ionized forms of flavonoids have absorption bands at longer wavelengths, but owing to the coexistence of neutral molecules that absorb energy at shorter wavelengths, the two kinds of absorption spectra fuse, resulting in the sum curve shown in Fig. 2. For example, the absorption spectra of kaempferol with 7′, 3′, and 4′ –OH groups shows two main red shifts and significant broadening bands (Fig. 2E) compared with the absorption spectra of flavone.

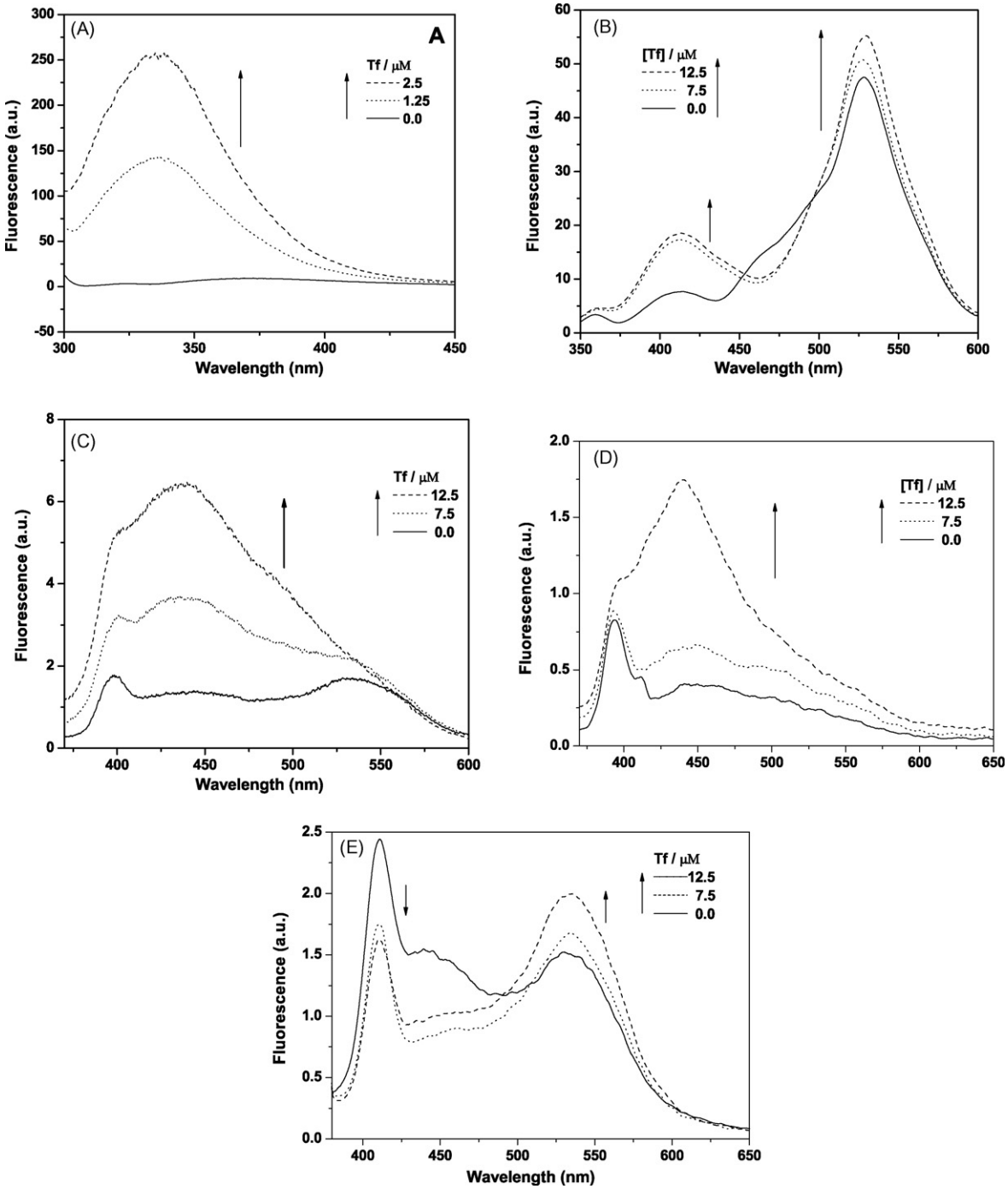

Fig. 3. The fluorescence spectra of flavonoids with different concentration of Tf in pH 7.4 phosphate buffer. (A) Flavone (10 μM), λ_{ex} = 290 nm; (B) 3-hydroxyflavone (40 μM), λ_{ex} = 320 nm; (C) galangin (40 μM), λ_{ex} = 350 nm; (D) luteolin (40 μM), λ_{ex} = 340 nm; (E) kaempferol (40 μM), λ_{ex} = 360 nm.

On addition of Tf the absorption spectra of all flavonoids are generally a little broadened, which could be interpreted as a consequence of their specific non-covalent interactions with the amino acid residues in the binding sites of Tf. A new absorption band at 280 nm also appeared with the addition of Tf, which could be owing to Tf. As mentioned earlier, deprotonation of phenolic –OH substituents leads to a bathochromic shift owing to extension of π-conjugation. However, there was no obvious red-shift in Tf–flavonoid systems, indicating that binding of flavonoids to Tf does not markedly change the deprotonation. In addition, observation of isosbestic points from Fig. 2 is also indicative of the interaction between flavonoids and transferrin in PBS.

3.2. Fluorescence spectra

Intrinsic emission spectra were measured to evaluate the effect of Tf on flavonoids. Fig. 3 shows the fluorescence spectra of flavonoids in the absence and presence of Tf. Owing to the different numbers and positions of hydroxyl groups, the fluorescence spectrum of each flavonoid shows the typical emission pattern: a normal emission band for flavone, well-resolved dual emission bands for 3-hydroxyflavone, galangin, and kaempferol (the shorter wavelength emission and longer wavelength emission could be due to normal state fluorescence and to excited-state intramolecular proton transfer (ESPT) tautomer fluorescence, respectively [22,23], and a normal emission band together with broad emission at longer wavelength for luteolin. On addition of Tf, marked changes in the fluorescence spectra have been observed for all flavonoids. The fluorescence spectra of flavones are dominated by the fluorescence from Tf; the relative ratios of normal state and ESPT fluorescence intensities of 3-hydroxyflavone and kaempferol decrease with Tf, and a new emission band at about 450 nm appears with the lost of the original bands for galangin and luteolin. These results provide evidence of binding of flavonoids to Tf.

The appearance of the normal-state emission requires external H-bond interaction, which would interfere with the ESPT process [24–26]. For 3-hydroxyflavone, galangin, luteolin, and kaempferol, reduction of the normal-state fluorescence with addition of Tf indicate that binding of these molecules to Tf led to the removal of solvent molecules at the protein binding sites. This would reduce the external H-bonding perturbation, increasing the likelihood of a successful intramolecular ESPT process. The less-significant difference in the emission spectra of the 3-hydroxyflavone/Tf system could be due to the relative weak binding of 3-hydroxyflavone to Tf owing to only one hydroxyl group in the molecule. Thus, the external H-bond around the molecule would not markedly affect the normal tautomer at the binding site.

The flavonoids with a 3-OH group, such as 3-hydroxyflavone, galangin, and kaempferol, could induce ESPT tautomer fluorescence, and the intensity of this fluorescence may change with Tf concentration. In chemical and biological systems, the ESPT process is considered one of the simplest of the important photoreactions and has received great attention both experimentally and theoretically [27,28]. The characteristic feature of this process is the large frequency displacement between the normal tautomer lowest $S_0 \rightarrow S_1$ absorption (or $S_1 \rightarrow S_0$ fluorescence) and the proto-transfer (PT) tautomer $S_1 \rightarrow S_0$ fluorescence, which behaves like a normal emission, although strongly wavelength shifted [29]. At the ground state, the hydroxyl group at the 3-position of the benzopyrone ring of flavonol has the main role in the proton transfer of the excited state of hydrogen between the –OH to O=C– groups. The additional hydroxyl groups provide considerable additional electron-donating capacity. The galangin molecule has two extra –OH groups at the 5 and 7 positions, and kaempferol has three additional –OH groups at 3′, 5, and 7 positions. These hydroxyl groups would help to increase the π-electron pool of the flavonol, providing the potential of generating a giant dipole in the proton transfer tautomer *zwitterions* form. Therefore, the observed increased sensitivity in the emission spectra could be due to the binding effect of Tf on the photophysical properties of flavonoids.

3.3. Circular dichroism spectra

To gain a better understanding of the binding mechanism between flavonoids and Tf, CD measurements have been performed. Under physiological condition, flavonoids alone do not show any CD signal owing to their achiral nature. However, addition of Tf to the aqueous solution of achiral flavonoids resulted in three negative–positive–negative CD bands ranging from 290 to 600 nm [17,30]. One example is shown in Fig. 4, and it should be noted that the CD spectra of five flavonoid molecules were parallel in the absence and presence of Tf. The induced signals could not be ascribed to the flavonoids from the peak shapes and band positions because they are quite different from those obtained from the quercetin/human serum albumin system [18]. Additionally, the strong positive and negative peaks, which appeared at 325 and 450 nm respectively, are very similar to those of Tf in the closed state [17,30]. Therefore, these phenomena are likely to be due to the conformational transition of Tf from the open to the closed state that is induced by flavonoids. This result is the same as those obtained in our previous study [17]. The five flavonoids had different numbers of hydroxyl groups; however, the conformational transition of Tf could be observed even with the flavone without a hydroxyl group. Therefore, the

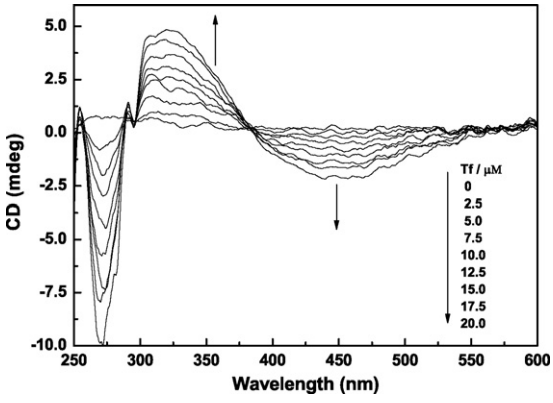

Fig. 4. The CD spectra of 40×10^{-6} M kaempferol with various concentrations of Tf in pH 7.4 phosphate buffer at room temperature.

intermolecular hydrogen bonding is not necessary for induction of the Tf conformational changes induced by flavonoids.

As the CD spectra in the wavelength range of 380–600 nm are mainly due to binding Tf to flavonoids, the magnitude of the negative maximum band at 450 nm can be used to calculate the association constant. The detailed calculation process is outlined in the materials and methods section. By non-linear regression analysis of the CD data, the association constants (K_a) obtained were $(0.69 \pm 0.01) \times 10^5$, $(0.76 \pm 0.05) \times 10^5$, $(0.92 \pm 0.02) \times 10^5$, $(1.95 \pm 0.03) \times 10^5$, and $(1.07 \pm 0.02) \times 10^5\ M^{-1}$ for flavone, 3-hydroxyflavone, galangin, luteolin, and kaempferol, respectively. The results show that the binding ability is generally increased with increasing numbers of hydroxyl groups. However, the association constant of luteolin is greater than that of kaempferol, even though they have the same numbers of hydroxyl groups. Theoretical calculations have shown that the acidities of flavonoids are mainly determined by the positions of the hydroxyl groups [9]. The most acidic positions are the 7-OH, and 4′-OH or 3′-OH, and the least acidic is the 5-OH group. However, the charge stability could be increased by resonances between the B and C rings, resonances between the A and C rings, or intramolecular hydrogen bonding. Therefore, the 3-OH group in kaempferol is not able to bind with amino acids via hydrogen bonds because it is more likely to form anion on physiological condition [9].

3.4. Molecular modeling calculation

With the marked developments in computational chemistry in the past decades, theoretical modeling of ligand–protein interaction chemistry has become increasingly sophisticated. The increasingly important role of computational chemistry in modern chemistry has recently been demonstrated [31]. Many important chemical and physical properties can be predicted from first principles by various computational techniques. Therefore, the molecular modeling method was used to investigate flavonoid–Tf systems. Figs. 5 and 6 show examples of the structural details. As can be seen, the A- and C-rings of flavonoids are almost co-planar, whereas the B rings are rotated around the C_2–C_1 bonds in every flavonoid molecule. The best-energy ranked results are shown in Table 1. It can be seen that the total binding energy decreases with increasing numbers of hydroxyl groups, which is consistent with the CD results. At the same time, the hydrogen bonding ability increases; this could be due to more hydroxyl groups available to form hydrogen bonds with the ionic amino acid residues in the protein. However, the calculated total energy for kaempferol bound to Tf is a littler lower than that for luteolin. Therefore, the obtained binding constant for the former should be larger than that for the latter, which is clearly different from the CD results. As the energy scores in the Affinity module are the sum of the contributions from both van der Waals and electrostatic interaction, the hydrogen bonding is not included. Furthermore, as shown in Table 1, the hydrogen bonding ability of luteolin is much higher than that of kaempferol, which might be why the CD results are not consistent with the energy rank calculated by molecular modeling method. From Table 1, it can be seen that,

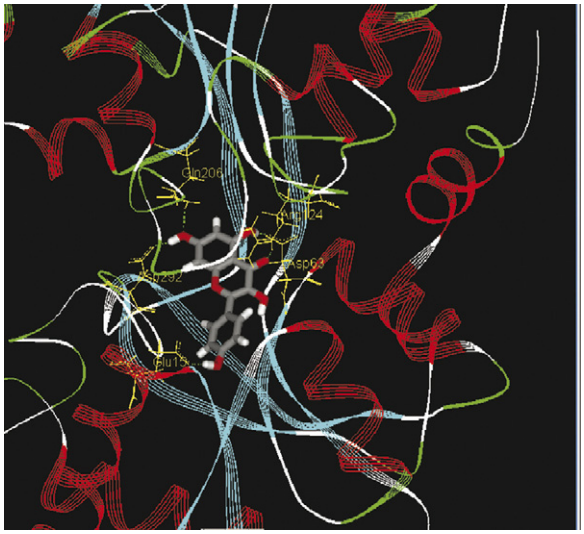

Fig. 5. Structural details of the interaction of kaempferol and Tf obtained by molecular modeling calculations. The kaempferol molecule is shown as a cylindrical model (C: gray; O: red; H: white). Amino acid residues of the binding sites are shown as yellow lines.

except for the hydrophobic and electrostatic interaction, the formation of intermolecular hydrogen bonds between the hydroxyl groups or carboxyl groups of flavonoids and the polar amino residues around the binding packet also have an important role in stabilizing the interactions between flavonoids and Tf.

It has been reported that Fe^{3+} coordinated with distorted octahedral geometry with two oxygens from two tyrosines, one nitrogen from a histidine, one oxygen from an aspartate of Tf, and two oxygens from a bidentate synergistic anion–carbonate through electrovalent bonds. [5] However, binding of flavonoids to Tf is mainly through non-covalent bonding, such as van der Waals forces, electrostatic forces, and hydrogen bonding.

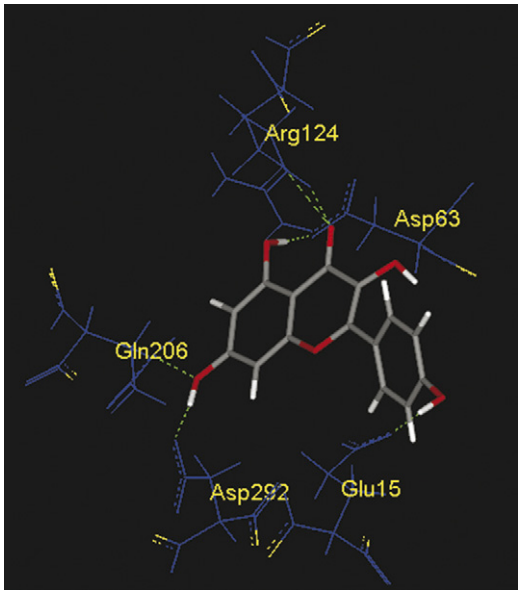

Fig. 6. Stereo drawing of kaempferol (grey) binding to Tf, showing all amino acids within 8 Å of the binding site. Chosen hydrogen bonds are depicted as a green dash lines.

Table 1
Data regarding flavonoid–Tf systems obtained from molecular modeling calculation

Molecules	van der Waals force (kcal/mol)	Electrostatic force (kcal/mol)	Binding energy (kcal/mol)	AA residues bound to flavonoids	Hydrogen bond length (Å)
Flavone	−67.69	−15.57	−83.26	Arg−124	1.95, 2.07
3-Hydroxyflavone	−65.09	−25.12	−90.21	Arg-124	1.96, 1.78
Galangin	−62.94	−37.16	−100.11	Glu-15 Asp-63 Arg-124	1.45, 2.42 1.71 2.07, 2.37
Luteolin	−53.68	−60.97	−114.65	Glu-15 Asp-63 Thr-120 Ala-126 Lys-291	1.36 1.42 1.73 1.74 1.63
Kaempferol	−54.19	−65.39	−119.58	Glu-15 Asp-63 Arg-124 Asp-292	1.47 1.45 1.78 1.39, 2.19

This could explain the large difference between the associated constants of Tf–flavonoid ($0.69–1.95 \times 10^5\ M^{-1}$) and Tf–$Fe^{3+}$ ($10^{22}\ M^{-1}$) complexes. In addition, studies have shown that quercetin, one type of flavonoids, interact with HSA mainly through hydrophobic and electrostatic forces. [15,18] The association constant is about $1.46 \times 10^4\ M^{-1}$ at pH 7.4. This value is markedly lower than those of flavonoids bound to Tf, which may be because the binding site polarities of Tf are much stronger than those of HSA that are known to bind flavonoids, leading to stronger hydrogen bonding and electrostatic force in Tf–flavonoid systems and greater association constants.

It is worth noting that the hydroxyl groups play important roles in the determination of the activity of flavonoids. When flavonoids bind to Tf, the hydrogen bonds form between hydroxyl groups and polar amino residues of Tf, leading to the low activity of flavonoids. It seems that binding to Tf might decrease the beneficial properties of flavonoids. Indeed, transferrin is responsible for the transport and delivery of chemotherapeutic drug molecules. Transferrin binds with flavonoids at pH 7.4 and delivers them to tumor cells via receptor-mediated endocytosis. And then, uptake of flavonoids-Tf complex into an acidic endosome (pH 5.5) could result in exposing flavonoids and releasing them. Thus, free flavonoids could act as effective antioxidants in tumor cells and exhibit high cytotoxicity. However, the precise nature of the targeting delivery and effective release in human remain to be elucidated clearly in the future. Further extension of the present research to encompass comparative studies on biological targeting delivery in tumor cells, should be of considerable interest in relation to studies on biological activities of flavonoids.

4. Conclusions

Absorption, fluorescence, and CD spectra and molecular modeling calculations have been used to investigate the structural effect on the conformational transition of Tf induced by flavonoids. The Tf conformational changes have been detected with flavonoids without hydroxyl groups, indicating that such conformational changes are mainly due to the hydrophobic interactions between the carboxyl and amino groups of the amino acids and the A, B, and C rings of the flavonoid species with the same prototype structure. However, introducing hydroxyl groups increases the electrostatic interactions and hydrogen bonding between the flavonoids and the polar amino acid residues in the protein. The greater the number of hydroxyl groups, the stronger the binding ability. In addition, the relative acidity of the hydroxyl groups in different positions may affect the binding ability. It is worth noting that, although the binding of flavonoids with Tf is much weaker, as noted above, the values of the calculated binding constants of flavonoids and transferrin are greater than those of flavonoids and HSA, indicating that these flavonoids may be transported by Tf to target tissues and cells. Therefore, our results provide a structural model that will allow construction of a delivery system by which Tf can transport flavonoids.

Acknowledgements

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