Interaction of Bioactive Components Caffeoylquinic Acid Derivatives in Chinese Medicines with Bovine Serum Albumin

Dan TANG, Hui-Jun LI, Ping LI,* Xiao-Dong WEN, and Zheng-Ming QIAN

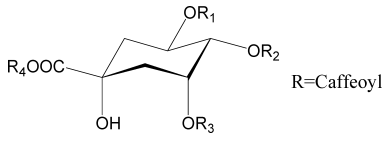
Key Laboratory of Modern Chinese Medicines, Ministry of Education, China Pharmaceutical University; No. 24, Tongjia Lane, Nanjing 210009, P. R. China. Received May 16, 2007; accepted December 1, 2007; published online January 8, 2008

Five caffeoylquinic acid derivatives (CQAs), including methyl 3,4-di-*O*-caffeoylquinate (3,4-diCQM), methyl 3,5-di-*O*-caffeoylquinate (3,5-diCQM), 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) and chlorogenic acid (CA), were isolated from *Lonicera fulvotomentosa* HSU *et* S. C. CHENG to be used as model compounds. The binding of these bioactive components to bovine serum albumin (BSA) was investigated by fluorescence quenching method. The results showed that there were binding affinities for CQAs with BSA, and the binding constants ranked in the following order: 3,4-diCQM>3,5-diCQM≈3,4-diCQA>3,5-diCQA>CA, under the physiological conditions, which suggested that the numbers and the substituted positions of caffeoyl group as well as the esterification of carboxyl group in the molecular structures appeared to contribute moderate effects to the interaction processes. Furthermore, the Stern–Volmer curves demonstrated that CQAs caused the fluorescence quenching through a static quenching procedure. Thermodynamic analysis indicated that both hydrophobic and electrostatic interactions played major roles in stabilizing the complex. The binding distance for each binding reaction was also calculated by the Föster theory.

Key words caffeoylquinic acid derivative; bovine serum albumin; fluorescence quenching

Caffeoylquinic acid derivatives (CQAs, Fig. 1) with diversity and extensive distribution in the plant kingdom are important bioactive components in some Chinese medicines, such as Flos Lonicerae, Herba Erigerontis, Fructus Xanthii. The compounds of this category were well known as potential antioxidants.[1—5] Furthermore, they also possessed significant antitumor,[6] hepatoprotective,[7,8] anti-inflammatory,[9] antimicrobial activities,[10] antimutagenicity[11] and so on. Most important, CQAs have been established as an important class of compounds with their potential effects of inhibiting human immunodeficiency virus (HIV)-1 integrase selectively and preventing HIV-1 replication in tissue culture at nontoxic concentrations.[12—14] Structure–activity relationship studies showed that their antioxidant,[5] antitumor,[6] hepatoprotective[7] and antimutagenicity[11] activities increased in proportion to the number of caffeoyl groups. Recently, it was also concluded that the radical scavenging activity of natural dicaffeoylquinic acids in the biological aqueous system might depend on the positions of caffeoyl ester groups.[15]

Serum albumins are the most abundant proteins in animal including human circulatory system and have many physiological functions.[16] One of the main functions is to regulate plasma osmotic pressure between the blood and tissues, which is chiefly responsible for the maintenance of blood pH.



	R_1	R_2	R_3	R_4
3,4-diCQM	Caffeoyl	Caffeoyl	H	CH_3
3,5-diCQM	Caffeoyl	H	Caffeoyl	CH_3
3,4-diCQA	Caffeoyl	Caffeoyl	H	H
3,5-diCQA	Caffeoyl	H	Caffeoyl	H
CA	Caffeoyl	H	H	H

Fig. 1. Chemical Structure of Caffeoylquinic Acid Derivatives (CQAs)

Another important property is that they serve as depot and transport proteins for a variety of endogenous and exogenous compounds, such as fatty acids, hormones, bilirubin, drugs, and a large diversity of metabolites.[17] Protein–ligand interactions play important roles in pharmacology and pharmacodynamics. It is widely accepted that the distribution, free concentration, and metabolism of various compounds can be affected as a result of binding to serum albumins in the blood stream.[18] From a review of the literatures,[14,19—21] it was proposed that caffeic acid, tissular methylated metabolites (*e.g.* ferulic and isoferulic acids) and hippuric acid were some of the metabolites of CQAs (*e.g.* chlorogenic acid (CA)). Also, the products released from CQAs might also be isomeric *O*-mono/di-methylated, isomeric *O*-mono/di-methyl-glucuronidated and/or glucuronidated metabolites. Therefore, the information on interactions of CQAs with serum albumin can provide a molecular basis for elucidating interaction mechanism and the absorption, metabolism together with distribution of CQAs. In addition, we also have been interested in the relationships between the differences of the molecular structures of CQAs and their binding affinities with serum albumins at the molecular level.

The measurement of the natural fluorescence quenching of serum albumins is an important method to study the interactions of ligands with serum albumins, owing to its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation. This method can reveal the accessibility of quenchers to fluorophore groups in the protein, provide an understanding of binding mechanisms to drugs, and yield clues to the chemistry of the binding phenomenon. The binding of various bioactive components in Chinese medicines to serum albumins have been investigated widely,[22—26] including chlorogenic acid,[27,28] but the interactions between dicaffeoylquinic acid derivatives and serum albumins have not been reported.

In the present paper, five CQAs isolated from *Lonicera fulvotomentosa* HSU *et* S. C. CHENG by authors were used as model compounds and bovine serum albumin (BSA) was se-

* To whom correspondence should be addressed. e-mail: liping2004@126.com

lected as a model protein for its structural homology with human serum albumin (HSA).[29] The characterizations of interactions between CQAs and BSA at different temperatures under physiological conditions utilizing the spectrofluorimetry in combination with UV absorption spectra were investigated. Moreover, the relationships between the binding affinities and the molecular structures were also discussed.

Experimental

Materials and Preparation of Solutions Methyl 3,4-di-*O*-caffeoylquinate (3,4-diCQM), methyl 3,5-di-*O*-caffeoylquinate (3,5-diCQM), 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA), chlorogenic acid (CA) (Fig. 1) were isolated from *L. fulvotomentosa* Hsu *et* S. C. Cheng in our laboratory and their structures were elucidated by UV, IR, MS, ^{1}H- and ^{13}C-NMR in comparison with the data in the references.[8,30,31] The purity of these compounds were determined to be more than 98% by normalization of the peak areas detected by HPLC with DAD, and they showed stable in methanol solution at 4 °C in the refrigerator.

BSA was purchased from Sigma Chemical Company, Tris–HCl (0.05 M) buffer solution containing 0.1 M NaCl was used to keep the pH of the solution at 7.40. BSA stock solution was prepared with the Tris–HCl buffer solution and kept in the dark at 4 °C. The stock solutions (2.0 mM) of CQAs were prepared in anhydrous methanol. All other reagents were of analytical grade and double-distilled water was used throughout the experiments.

Apparatus and Methods Fluorescence spectra were measured by a RF-5301PC spectrofluorophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells and electronic thermostat water bath. The emission spectra were recorded from 300 to 500 nm (excitation wavelength 280 nm) using 3 nm/3 nm slit widths. UV absorption spectra were recorded at 25 °C on a UV-2501PC spectrophotometer (Shimadzu, Japan). The range of wavelength was from 300 to 500 nm. Sample masses were accurately weighted using a microbalance (Sartorius, ME215S) with a resolution of 0.01 mg.

Fluorometric Titration Experiments: 3.0 ml solution containing appropriate concentration of BSA was titrated in the Tris–HCl buffer solution (pH 7.40) by successive addition of CQAs stock solution, respectively. Titrations were done manually by using trace syringes. The CQAs were added from concentrated stock solutions so that volume increment was negligible. All experiments were performed at three different temperatures (298, 310, 318 K).

Displacement Experiments: A 3.0 ml solution of BSA with an appropriate CQAs concentration was titrated by successive addition of warfarin and ibuprofen solutions, respectively. The fluorescence intensity was recorded (excitation at 280 nm using slit widths 3 nm/3 nm) at 298 K. The data were analyzed according to the method of Sudlow.

Results and Discussion

Fluorescence Quenching Mechanism Fluorescence measurement was carried out to investigate whether CQAs bound to BSA. As shown in Fig. 2, BSA had strong fluorescence emission while CQAs almost had none intrinsic fluorescence under the present experiment conditions. And the fluorescence intensities of BSA decreased regularly with CQAs concentrations increasing, accompanied by red shift of the maximum emission wavelengths in the fluorescence spectra. These data elucidated that CQAs could interact with BSA and quench their intrinsic fluorescence. Meanwhile, the red shift signified that the binding of CQAs were associated with changes in the local dielectric environment in BSA,

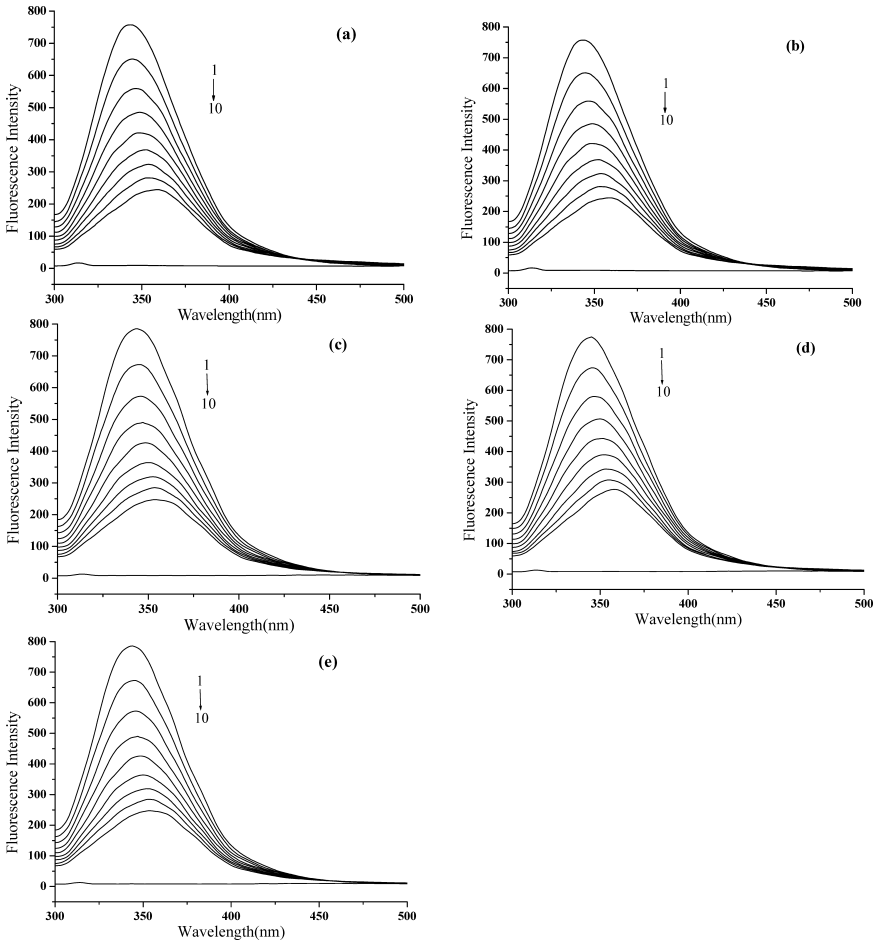


Fig. 2. Fluorescence Emission Spectra of BSA in the Presence of Various Concentrations of 3,4-diCQM (a), 3,5-diCQM (b), 3,4-diCQA (c), 3,5-diCQA (d), CA (e)

T=298 K, λ_{ex}=280 nm; c(BSA)=2.0 μM; c(CQAs)/(μM): (1)—(9): 0; 2.0; 4.0; 6.0; 8.0; 10.0; 12.0; 14.0; 16.0. (10): 10 μM CQAs.

which suggested the chromophore was placed in a decreased hydrophobic environment after the addition of CQAs.[32]

The fluorescence quenching data are usually analyzed by the well-known Stern–Volmer equation (Eq. 1):

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

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, K_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher and its value is 10^{-8} s,[33] [Q] is the concentration of quencher and K_{sv} is the Stern–Volmer quenching constant which can be obtained by the slope (can be deducted from K_q in Table 1). These results indicated that the probable quenching mechanism of fluorescence of BSA by CQAs was a static quenching procedure because the K_{sv} decreased with rising temperatures. Moreover, the values of K_q (listed in Table 1), in the range of 3.60×10^{12} to 2.18×10^{13} l s^{-1} mol^{-1} for all reactions of CQAs–BSA and were far larger than 2.0×10^{10} l s^{-1} mol^{-1}, the maximum diffusion collision quenching rate constant of various quenchers with the biopolymer.[34] Consequently, the CQAs–BSA binding reactions should follow a static quenching process rather than a dynamic one.

Binding Constants and Binding Sites For the static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by Eq. 2[35,36]:

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

where K is the binding constant, and n is the number of binding sites per BSA. Table 2 lists the corresponding calculated results. All correlation coefficients are larger than 0.995, indicating that the interactions between CQAs and BSA agree well with the site-binding model underlied in Eq. 2. As shown in Table 2, binding constants of CQAs–BSA decreased in the following order: 3,4-diCQM>3,5-diCQM≈3,4-diCQA>3,5-diCQA>CA. As regards the structure differences (Fig. 1), all dicaffeoylquinic acid derivatives (3,4-, 3,5-diCQMs and 3,4-, 3,5-diCQAs) possess one more caffeoyl group than the monocaffeoylquinic acid derivative (CA), which demonstrated the caffeoyl group might be the active agent for these interactions. Meanwhile, derivatives with 3,4-substituted structures showed higher binding affinities than ones with 3,5-substituted structures, that is to say, the rate of the binding affinity increased as the intramolecular distance of the two caffeoyl groups decreased.

These results were in agreement with the conclusions of previous structure–activity relationship investigations by other researchers.[5—7,11,15] It was proved that the protein–ligands binding mechanism could be used as a mode to elucidate the biological and pharmacological properties of the ligands. In addition, once the carboxyl groups being esterified, the binding affinities became stronger, which suggested that esterification of carboxyl group also seemed having some effects in the interaction processes.

Similar to HSA, BSA consists of amino acids chains forming a single polypeptide which contain three homologous α-helices domains (I—III). Each domain is divided into two subdomains (A and B).[18] The binding sites of BSA for endogenous and exogenous ligands may be in these domains, and the principal regions of the ligands binding sites of albumin are located in hydrophobic cavities in subdomains IIA

Table 1. Stern–Volmer Quenching Constants for the Interactions of CQAs with BSA at Three Different Temperatures

Compounds	298 K		310 K		318 K	
	K_q (10^{13} l mol^{-1} s^{-1})	r	K_q (10^{13} l mol^{-1} s^{-1})	r	K_q (10^{13} l mol^{-1} s^{-1})	r
3,4-diCQM	2.18	0.9921	1.57	0.9965	1.33	0.9953
3,5-diCQM	1.35	0.9925	1.26	0.9942	1.17	0.9970
3,4-diCQA	1.28	0.9974	1.12	0.9969	1.07	0.9978
3,5-diCQA	1.11	0.9973	1.05	0.9971	1.01	0.9984
CA	0.38	0.9988	0.36	0.9978	0.34	0.9983

r is the correlation coefficient. K_{sv} can be deducted from K_q by equation $K_{sv}=K_q\tau_0$.

Table 2. Binding Parameters and Relative Thermodynamic Parameters of the Systems of CQAs–BSA at Different Temperatures

Compounds	T (K)	K (10^5 M^{-1})	n	r	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	r
3,4-diCQM	298	38.1	1.27	0.9977	−13.76	−37.51	79.68	0.9813
	310	29.1	1.24	0.9998		−38.38		
	318	27.1	1.23	0.9997		−39.10		
3,5-diCQM	298	12.9	1.21	0.9980	−12.43	−34.86	75.26	0.9980
	310	10.8	1.20	0.9989		−35.68		
	318	9.37	1.19	0.9997		−36.36		
3,4-diCQA	298	12.5	1.21	0.9995	−12.99	−34.79	73.14	0.9990
	310	10.3	1.21	0.9995		−35.67		
	318	8.97	1.20	0.9997		−36.25		
3,5-diCQA	298	8.59	1.19	0.9996	−11.94	−33.84	73.49	0.9987
	310	7.04	1.18	0.9988		−34.73		
	318	6.36	1.17	0.9998		−35.31		
CA	298	0.94	1.09	0.9989	−3.56	−28.39	83.31	0.9906
	310	0.90	1.09	0.9990		−29.39		
	318	0.86	1.08	0.9992		−30.15		

r is the correlation coefficient.

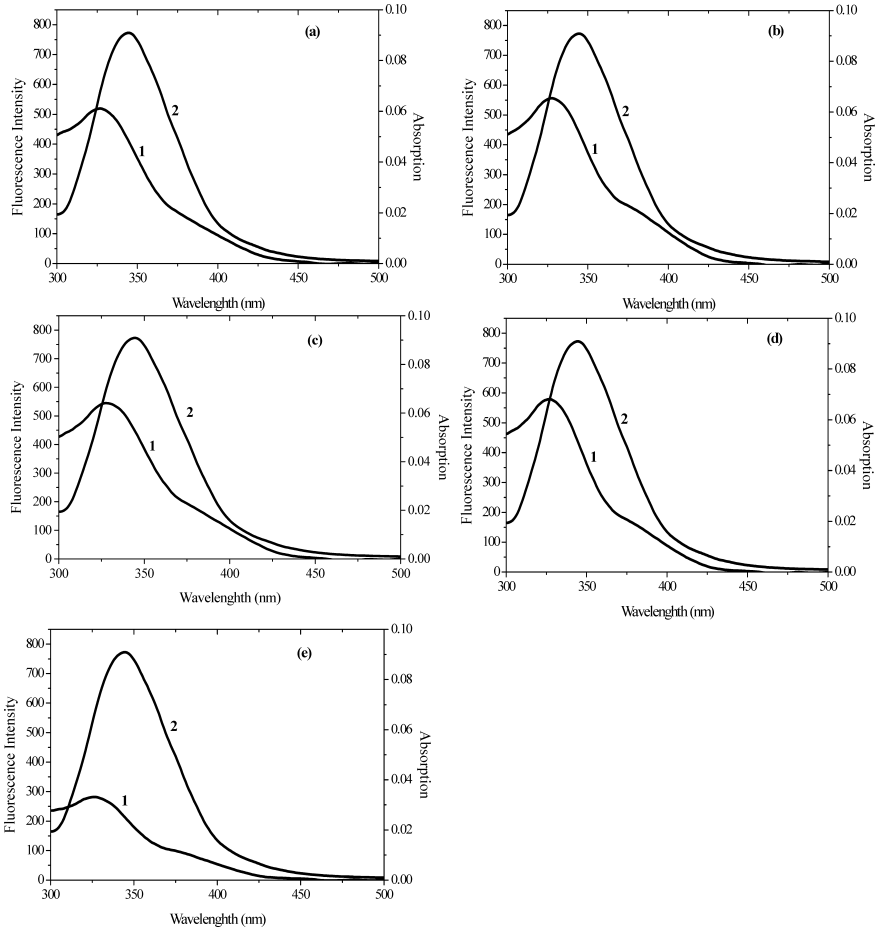


Fig. 3. Overlaps of the Fluorescence Spectra (2) of BSA with the Absorption Spectra (1) of such CQAs as 3,4-diCQM (a), 3,5-diCQM (b), 3,4-diCQA (c), 3,5-diCQA (d) and CA (e)

c(BSA)=c(CS)=2.0 μM (T=298 K).

and IIIA. The binding cavities associated with subdomains IIA and IIIA are also referred to as site I and site II according to the terminology proposed by Sudlow *et al.*[37]

Many ligands bind specifically to serum albumin, for example warfarin for site I, and ibuprofen for site II,[37] but some drugs such as aspirin and iodinated aspirin analogues, show nearly equal distributions between binding sites located in subdomains IIA and IIIA.[38]

In order to determine the specificity of the CQAs binding and the location of the CQAs-binding sites on BSA, the displacement experiments were carried out according to the literatures,[22,39] using some drugs mentioned above. A 3.0 ml solution of BSA with an appropriate CQAs concentration was titrated by successive addition of warfarin and ibuprofen solutions, respectively. The percentage of displacement of the probe was determined according to the method described by Sudlow *et al.*[37]

$$\text{probe displacement (\%)} = F_2/F_1 \times 100$$

where F_1 and F_2 represent the fluorescence of CQAs plus BSA in the absence and presence of probe, respectively.

Figure 4 shows the changes in fluorescence of CQAs bound to BSA with the addition of the probes. The relative fluorescence intensity significantly decreased after the addition of warfarin or ibuprofen, which indicated that both warfarin and ibuprofen had remarkable effect on the binding of

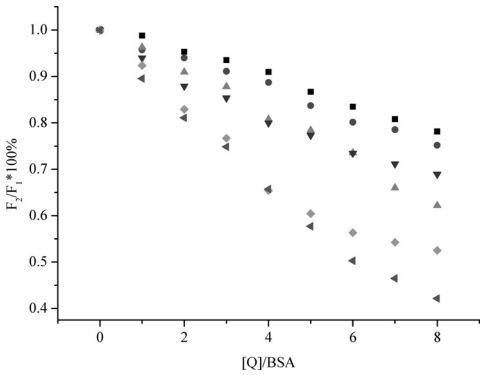


Fig. 4. Effect of Site Maker Probe on the Fluorescence of CQAs–BSA

The concentrations of BSA and CQAs were 2.0 μM and 10 μM, respectively. [Q]: ■ ibuprofen (CA–BSA); ● ibuprofen (3,4-diCQA–BSA); ▼ ibuprofen (3,4-diCQM–BSA); ▲ warfarin (CA–BSA); □ warfarin (3,4-diCQA–BSA); ◀ warfarin (3,4-diCQM–BSA); T=298 K, λ_{ex}=280 nm.

CQAs to BSA. So, we suggest the binding sites of CQAs to BSA are non-specific.

Binding Mode Generally, the acting forces contributing to the macromolecules interactions with small ligands may be hydrogen bond, Van der Waals force, electrostatic interaction and hydrophobic force.[40] The value of the enthalpy change (ΔH^0) and entropy change (ΔS^0) can be evaluated by the Van't Hoff equation if ΔH^0 does not vary significantly in

the temperature range studied. The Van't Hoff equation is:

$$\ln K = \Delta H^0/RT + \Delta S^0/R \quad (3)$$

where K is the binding constant at the corresponding temperature (298 K, 310 K or 318 K) and R is the gas constant. ΔH^0 and ΔS^0 can be calculated from the slopes and ordinates of the Van't Hoff relationship, respectively. The free energy change (ΔG^0) is estimated from the following relationship:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (4)$$

The results of the parameters ΔH^0, ΔG^0 and ΔS^0 are listed in Table 2. The negative signs for ΔG^0 supported the assertion that all binding processes were spontaneous. The values of ΔH^0 are negative and small, while the values of ΔS^0 are reverse. According to the views of Ross and Subramanian,[41] the positive ΔS^0 value was frequently taken as evidence for hydrophobic interaction, and specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS^0 and a negative value of ΔH^0. Therefore, it was not possible to account for the thermodynamic parameters of CQAs–BSA compounds on the basis of a single interaction molecular force model. In Table 2, the main sources of ΔG^0 values are derived from a large contribution of ΔS^0 term with little contribution from the ΔH^0 factor. All results meant that the acting force between CQAs and BSA mainly ascribed to hydrophobic force, but the electrostatic interaction could not be excluded.

Energy Transfer between CQAs and BSA There were good overlaps between the fluorescence emission spectra of free BSA and absorption UV/vis spectra of CQAs (Fig. 3). As the fluorescence emission of protein was affected by the excitation light around 280 nm, the spectra ranging from 300 to 500 nm were chosen to calculate the overlapping integral. According to the Förster theory,[42] the energy transfer efficiency E is given by the equation:

$$E = R_0^6/(R_0^6 + r^6) = (F - F_0)/F \quad (5)$$

where F_0 and F are the fluorescence intensities in the absence and presence of CQAs, respectively, r is the distance between donor and accepter, and R_0 is the distance at 50% transfer efficiency.

$$R_0^6 = 8.8\times10^{-25}(K^2 \cdot \Phi \cdot n^{-4} \cdot J) \quad (6)$$

where K^2 is the spatial orientation factor describing the relative orientation in space of the transition dipoles of the donor and acceptor, n is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor in the absence of the acceptor and J is the overlap integral between the donor fluorescence emission spectra and the acceptor absorption spectra. J is given by

$$J = \sum(F(\lambda) \cdot \varepsilon(\lambda) \cdot \lambda^4 \cdot \Delta\lambda)/\sum(F(\lambda) \cdot \Delta\lambda \quad (7)$$

$F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. So, J could be calculated by integrating the overlap spectra for λ=300—500 nm. Under these experimental conditions, it has been reported for BSA that, K^2=2/3, n=1.36, Φ=0.15.[43] Based on these data, the critical distance R_0 could be calculated by Eq. 6, they were 2.66, 2.69, 2.69, 2.68 and 2.40 nm for 3,4-diCQM, 3,5-diCQM, 3,4-diCQA, 3,5-diCQA and CA interacting with BSA, respectively. Finally, the distances between CQAs and BSA could be obtained from Eq. 5. Consequently, the values of r were 3.32, 3.63, 3.64, 3.68 and 3.74 nm for 3,4-diCQM, 3,5-diCQM, 3,4-diCQA, 3,5-diCQA and CA interacting with BSA, respectively. Obviously, the donor-to-acceptor distances are lower than 7 nm, which indicated that the energy transfer from BSA to CQAs occurred with high probability. Larger BSA–CQAs distance, r compared to that of R_0 values observed in the present study also revealed the presence of static type quenching mechanism.[44]

Conclusions

In this paper, an investigation of the interactions between CQAs and BSA was undertaken using fluorescence spectroscopy, and the interaction characteristics of dicaffeoylquinic acid derivatives with BSA were reported for the first time. The results suggested that there existed binding affinities between CQAs and BSA. At the same time, the binding mechanism, the binding parameters including binding constant (K), the distance (r), and thermodynamic functions were also studied in detail. Such information can provide a molecular basis for elucidating interaction mechanism and the absorption, as well as distribution of these bioactive components.

In the approach to relationships between the binding affinities and the molecular structures characteristics, it was found that their binding affinities increased in proportion to the number of caffeoyl group. Meanwhile, the substituted positions of caffeoyl group as well as the esterification of carboxyl group in the molecular structures also had moderate effects during the interaction processes. Such knowledge of the relationships can provide valuable information for the structural modification of lead compounds or designing of new drugs.

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