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Study on the binding of puerarin to bovine serum albumin by isothermal titration calorimetry and spectroscopic approaches

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Abstract The interaction of a flavonoid molecule (puerarin) with bovine serum albumin (BSA) was characterized by isothermal titration calorimetry (ITC), optical spectroscopic technique, and molecular modeling method under physiological conditions. The binding parameters for the reaction were calculated according to ITC experiments at different temperatures. The thermodynamic parameters, negative enthalpy changes (ΔH), and positive entropy (ΔS) indicated that the binding processes were entropically driven. The alterations of protein secondary structure in the presence of puerarin in aqueous solution were estimated by the evidences from FT-IR and CD spectroscopy with reductions of α-helices. On the basis of fluorescence resonance energy transfer (FRET) between excited tryptophan in BSA and BSA bound puerarin, the critical transfer distance and mean distance between tryptophan in BSA and puerarin were estimated.

Keywords Bovine serum albumin · Puerarin · Binding · ITC · FRET · Thermodynamic parameters

Introduction

Puerarin, a naturally occurring isoflavone C-glycoside, is isolated from pueraria lobota [1], one of the most popular Chinese herbal medicines that is traditionally used to reduce febrile symptoms and is also used as an antiinebriation agent [2]. The chemical name of this compound is 8-β-D-glucopyransyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one. Scheme 1 shows the chemical structure of puerarin. The biomedical effects of puerarin, which have been experimentally or clinically demonstrated [3, 4], include the improvement of blood circulation, prevention of cardiovascular diseases, control of alcoholism, and treatment for arrhythmia [5, 6]. Being one of the precious Chinese herbs with its potential pharmaceutical benefit led us to investigate the interaction of puerarin with protein, so that it can provide a molecular basis for elucidating the mechanism of the drug acting and predicting unfavorable drug interaction.

A number of biochemical and molecular biological investigations have revealed that proteins are frequently the 'targets' for the rapeutically active flavonoids of both natural and synthetic origin. Here, we have explored the interaction of puerarin with bovine serum albumin (BSA). Serum albumin as one of most abundant carrier proteins plays an important role in the transport and disposition of endogenous and exogenous ligands present in blood [7]. Almost every pharmaceutical compound injected in the blood finds itself in the presence of a high concentration of serum albumin, known to have a strong affinity for a variety of chemical species. Distribution and metabolism of many biologically active compounds (drugs, natural products, etc.) in the body are correlated with their affinities toward serum albumin. Thus, the investigation of such molecules with respect to albumin's binding mechanism is of imperative and fundamental importance. The molecular interactions between serum albumin and many compounds have been investigated successfully including many drugs [8–11]. In this article, attempts were made to investigate the binding mechanism of puerarin to BSA with respect to the binding constants (K), the number of binding sites (N), the thermodynamic functions, and the effect of puerarin on

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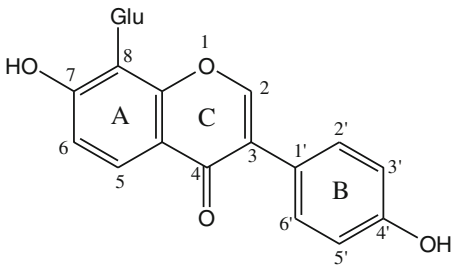

Scheme 1 Structure of puerarin

the protein secondary structure. These are the first results on puerarin–BSA interactions determined by the ITC experiments, and they can illustrate the nature of puerarin–protein complications in vitro.

Materials and methods

Materials

Albumin bovine fraction V (+99%, Acros Chemical Corporation) was purchased and used as received. The stock solution of 5.0×10^{-4} mol L^{-1} was prepared by dissolving the solid BSA in double-distilled deionized water and stored at 0–4 °C in 1.0×10^{-4} mol L^{-1} phosphate buffer (pH 7.4). Puerarin of pharmaceutical purity grade was kindly provided by Nanjing Chemical Reagent Plant (China). All the samples of puerarin were dried in vacuum at (105–110 °C) for 2 h, and used without further purification. All other reagents were of analytical purity without further purification and prepared in water from a Millipore Mili-Q system.

Isothermal titration calorimetry

Heats of dilution were measured using a VP–ITC titration microcalorimeter (MicroCal, Inc., Northampton, MA). In isothermal titration calorimetry (ITC) experiments, one measures directly the energy (enthalpy changes) associated with processes occurring at constant temperature, in our case at a temperature range of 20–35 °C. The titrant and sample solutions were made from the same stock buffer solution, and both experimental solutions were degassed before each titration. The solution in the cell was stirred by the syringe at 307 rpm, which ensured rapid mixing but did not cause foaming on the protein solution. Typically, small aliquots of a stock solution of puerarin were injected under automatic control from the system computer into a known volume (1.436 mL) of an aqueous BSA buffered solution held in the calorimeter cell. Repeated additions of the stock solution gave the heat evolved (Q) as a function of puerarin concentration. The volume of each injection was 5 μL, and the intervals between injections were 240 s to allow correct equilibration. In order to correct for the dilution effect by the injection of penicillin solution, two controls were obtained: titrating the BSA solution by the buffer for BSA dilution, and titrating the buffer solution by the puerarin solution for dilution of the corresponding puerarin. The heat of dilution was, therefore, subtracted from experimental titrations, although resulting in very small heats. The overflow of the reaction mixture by injection of the ligand solution was corrected by the computer. The direct analysis of ITC data curves for puerarin binding to BSA allowed the determination of the binding enthalpy (ΔH), and entropy change (ΔS).

Data analysis

ITC data are analyzed using Origin 7.0 software provided by MicroCal [12]. The ITC profiles are best-fitted to a single set of identical binding sites model. The total heat content, Q, of the solution contained in the active cell volume, V_0 (determined relative to zero for the unligated species) at fractional saturation, Θ, is given by

$$Q = n\Theta M_t \Delta H V_0 \tag{1}$$

where ΔH is the molar heat of ligand binding, M_t the total concentration of the macromolecule, and N the number of binding sites in the macromolecule. The heat released from the ith injection for an injection volume dVi ($\Delta Q(i)$) is then given by the following equation:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \tag{2}$$

The plot of change in enthalpy against temperature is used to calculate the change in heat capacity upon binding, according to

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p \tag{3}$$

The value of the van't Hoff enthalpy at each temperature is calculated, including ΔC_p values, based on the following equation:

$$\Delta_{vH} H(T_1) = \frac{\left\{\ln\left(K(T_2)/K(T_1) - \left(\Delta C_p/R\right)\right)\ln(T_2/T_1) + \left(\Delta C_p T_1/R\right)\left[(1/T_1) - (1/T_2)\right]\right\} \times R}{\left[(1/T_1) - (1/T_2)\right]} \tag{4}$$

CD and FT-IR spectra

Circular dichroism (CD) measurements were made on a Jasco-810 automatic recording spectropolarimeter (Japan), using a 1-mm cell at 298 K. The spectra were recorded in the range of 190–300 nm, and the scan rate is 100 nm min^{-1} with a response time of 4 s. CD determinations of pure BSA and BSA–puerarin mixtures were carried out using the buffer solutions of puerarin at a corresponding concentration as the reference. The results were expressed as ellipticity (mdeg), which was obtained in mdeg directly from the instrument. FT-IR measurements were carried out at room temperature on a Brucker Equinox 55 FT-IR spectrometer. All the spectra were taken via the attenuated total reflection (ATR) method with resolution of 4 cm^{-1} and 32 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition. Then, the absorbance of buffer solution was subtracted from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 1800 and 1300 cm^{-1} was a smooth straight curve.

Fluorescence and UV–vis spectra

Fluorescence spectra were measured with a RF-5301PC spectrofluorophotometer (Shimadzu), using 5/5-nm slit widths. The excitation wavelength of BSA was 280 nm, and the emission was read at 300–500 nm. The UV–vis absorbance spectra of puerarin with concentration of 5.0×10^{-5} mol L^{-1} was recorded at room temperature.

Results and discussions

Thermodynamics of puerarin–BSA interactions

With recent advancement in the sensitivity and reliability of the calorimeter, ITC has become an important tool for the direct measurement of thermodynamic parameters in various biological interactions [13, 14]. Moreover, ITC represents a universal detector that is dependent only on the exchange of heat during a reaction. Therefore, it provides an invaluable tool to monitor a variety of reactions independent of spectroscopic changes that occur during the reactions. ITC yields thermodynamic parameters such as Gibbs free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS), along with the number of binding sites (N) and affinity constant (K) in a single experiment. Also, determination of binding enthalpy as a function of temperature yields changes in heat capacity associated with an interaction that provides a valuable insight into the types and magnitude of forces involved therein.

A representative calorimetric titration profile of 8.0×10^{-3} mol L^{-1} puerarin with 5.0×10^{-4} mol L^{-1} BSA in aqueous solutions and at 298.15 K is shown in Fig. 1. Each peak in the binding isotherm (see Fig. 1a) represents a single injection of the drug into the protein solution. Figure 1b shows the plot of the amount of heat liberated per injection as a function of the molar ratio of the drug to the protein. A standard nonlinear least-squares regression binding model, involving a single class of non-interacting sites fitted well to the data. The smooth solid line shown in Fig. 1b is the best fit to the experimental data. The observed enthalpy does not have significant contribution from the buffer ionization, because phosphate has a small value for the enthalpy of ionization [15]. Therefore, the observed enthalpy is practically the binding enthalpy of the drug to the protein.

As seen in Fig. 1, the ITC titrations of puerarin with BSA yielded negative heat deflection, which indicate that the binding is an exothermic process at all of the studied temperatures, and the temperature dependency of the thermodynamic parameters accompanying the binding of puerarin to

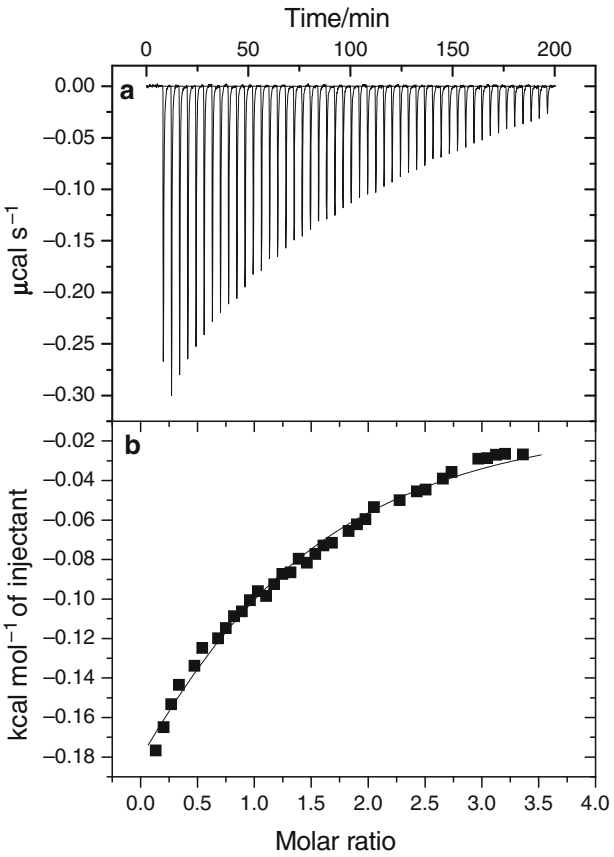

Fig. 1 **a** Raw data for the titration of 8.0×10^{-3} mol L^{-1} puerarin with 5.0×10^{-4} mol L^{-1} BSA at pH 7.4 and 25 °C, showing the calorimetric responses as successive injections of the ligand are added to the sample cell. **b** Integrated heat profile of the calorimetric titration shown in **a**. The *solid line* represents the best nonlinear least-squares fit to a single binding-site model

Table 1 Binding parameters accompanying the titration of 8.0×10^{-3} mol L^{-1} puerarin with 5.0×10^{-4} mol L^{-1} BSA at pH 7.4 and at various temperatures

Temperature, T/K	N	K/L mol^{-1}	ΔH/J mol^{-1}	ΔS/J mol^{-1}
293	1.12	$4.78 \times 10^3 \pm 64$	$-5.39 \times 10^2 \pm 12.1$	6.86×10^2
298	1.02	$1.44 \times 10^3 \pm 205$	$-1.75 \times 10^3 \pm 279.8$	5.43×10^2
303	0.96	$7.20 \times 10^2 \pm 12$	$-3.66 \times 10^3 \pm 101.6$	4.26×10^2
308	0.85	$3.26 \times 10^2 \pm 13$	$-5.27 \times 10^3 \pm 237.5$	3.09×10^2

BSA is summarized in Table 1. Each value in Table 1 is an average of three independent experiments. From Table 1, it can be seen that ΔH is a small negative value, whereas ΔS is a positive value. In these experiments, puerarin–BSA complexes are accompanied by negative enthalpy (ΔH) and positive entropy (ΔS) changes, which indicate that the binding processes are entropically driven. The negative sign for ΔG ($\Delta G = \Delta H - T\Delta S$) indicates the spontaneity of the binding of puearin with BSA. For typical hydrophobic interactions, both ΔH and ΔS are positive, while negative enthalpy and entropy changes arise from van der Waals force and hydrogen bonding formation in low dielectric media [16, 17]. Moreover, specific electrostatic interaction between ionic species in an aqueous solution is characterized by positive ΔS value and negative of small ΔH value. Puerarin is a negatively charged molecule at pH 7.4 ($pK_1 = 6.91$, $pK_2 = 9.93$) [18], and it is expected to bind at the sites that are composed of the positively charged amino acid residues and hydrophobic side chains on the protein. The exothermicity observed in the binding suggests the involvement of electrostatic interactions in the binding process. For puerarin–BSA complex system, the main source of ΔG value is derived from a large contribution of ΔS term with little contribution from the ΔH factor, and so the main interaction is of hydrophobic contact, but the electrostatic interaction can not be excluded.

In general, the value of the stoichiometry of the binding (N) is about 1. With the temperature increasing, the binding site is decreased, which affects the unfavorable interaction of the protein with puerarin. This could possibly be assigned to the interaction of the negatively charged oxygen group of puerarin with one protein molecule and via hydrophobic interactions of the aromatic puerarin group with the other protein molecule and thus decreasing the stoichiometry from 1.12 at 293 K to 0.89 at 308 K. This explanation is consistent with a decrease in the entropy of binding with an increase in temperature, indicating more restriction of the protein molecule, as a result of binding.

A plot of ΔH against temperature (Fig. 2) yields a straight line with a correlation coefficient $R = 0.997$ and yielding a value of $\Delta C_p = -321.0$ J K^{-1} mol^{-1} from the slope, which implies that there is a burial of hydrophobic groups due to the binding process. The value of the van't Hoff enthalpy is calculated from the temperature dependence of the binding

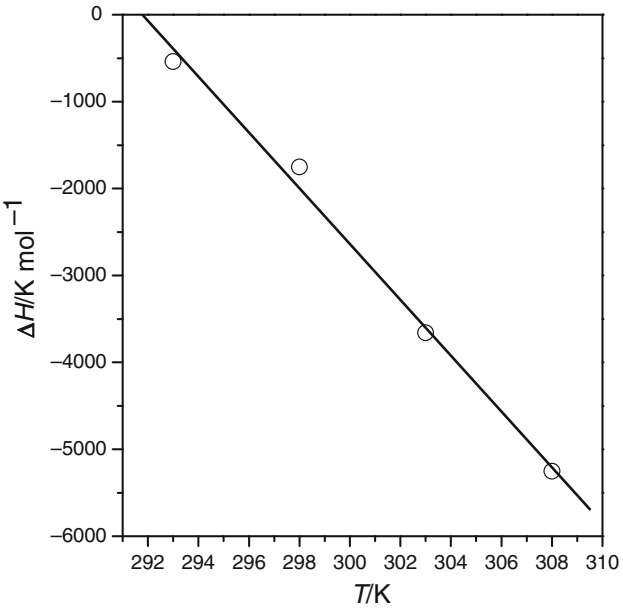

Fig. 2 Plot of enthalpy against temperature for the binding of puerarin to BSA at pH 7.4

constant K and ΔC_p, using Eq. 4 (data not shown). If the van't Hoff enthalpy agrees well with the calorimetric enthalpy at each temperature, then this indicates that adherence to two-state binding process and the binding of drug to the protein do not alter its conformation appreciably. In our experiments, this calculated van't Hoff enthalpy does not match with the calorimetric enthalpy. Possible reasons for this mismatch could be assigned to alteration in the conformation of the protein upon binding, as well as with the increase in temperature [12].

Effect of puerarin binding on the conformation of BSA

In order to gain a better understanding on the effect of puerarin binding on the conformation of BSA, the additional CD and FT-IR spectroscopic measurements are performed on puerarin and the puerarin-BSA complex. Figure 3 shows the CD spectra of the BSA and BSA-puerarin complex obtained at pH 7.4. The CD spectra of BSA exhibit two negative minima at 208 and 217 nm, which are the typical characterizations of the α-helix

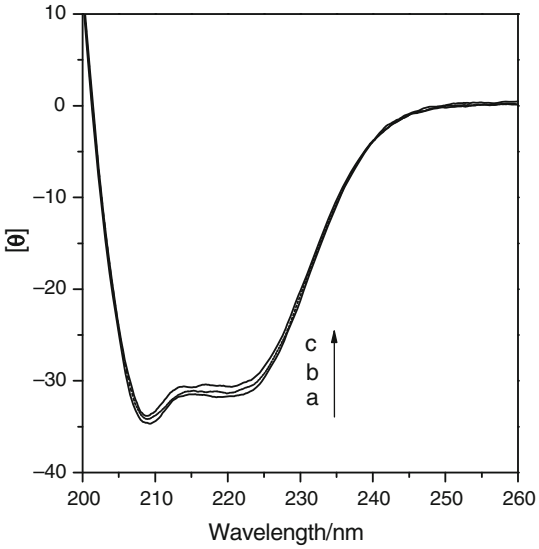

Fig. 3 CD spectra of the BSA–puerarin system obtained in 1.0×10^{-5} mol L^{-1} phosphate buffer of pH 7.4 at room temperature. BSA concentration was kept fixed at 3.0×10^{-6} mol L^{-1} (*a*). In BSA–puerarin, the puerarin concentration was at 1.0×10^{-5} mol L^{-1} (*b*) and 6.0×10^{-5} mol L^{-1} (*c*)

structure of class proteins [19]. The binding of puerarin to BSA causes a decrease in band intensity at all the wavelength of the far-UV–CD spectra, indicating the decrease of the α-helical content in protein. However, the CD spectra of BSA in the presence and the absence of puerarin are similar in shape, indicating that the structure of BSA is also predominantly α-helical. From the above results, it is apparent that the effect of puerarin on BSA causes a conformational change of the protein, with the loss of α-helical stability. The calculated results exhibit a reduction of α-helix structures from 47.8 to 44.2%, and 37.4% at the concentrations of puerarin 0, 1.0×10^{-5} and 6.0×10^{-5} mol L^{-1}, respectively.

Infrared spectroscopy has long been used as a powerful method for investigating the secondary structures of proteins and their dynamics. In the IR region, the frequencies of bands due to the amide I, II, and III vibrations are sensitive to the secondary structure of proteins [20]. Particularly, the amide I band is useful for the secondary structure studies. Both the protein amide I band 1600–1700 cm^{-1} (mainly C=O stretch) and amide II band 1568 cm^{-1} (C–N stretch coupled with N–H bending mode) have a relationship with the secondary structure of protein. However, the amide I band is more sensitive to the change of protein secondary structure than amide II band. Figure 4a shows the FT-IR spectrum of free BSA in PBS buffer, and the difference spectra of BSA–puerarin are also displayed in Fig. 4b. The peak position of amide I moves from 1636.1 to 1634.6 cm^{-1}, and amide II moves from 1568.1 to 1572.3 cm^{-1} in BSA IR spectrum after interaction with puerarin, which indicates that the secondary structure of BSA has been changed because of the

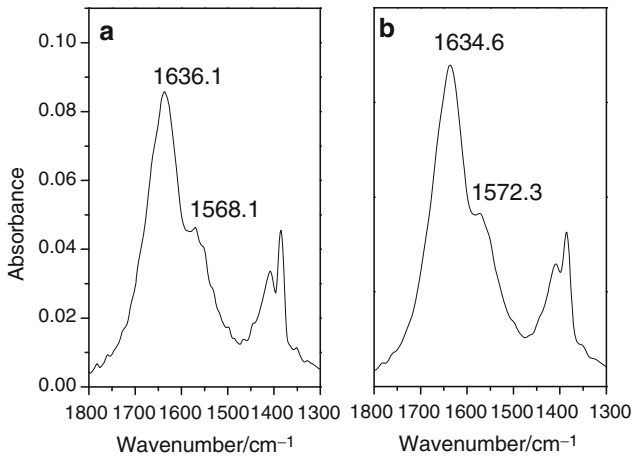

Fig. 4 FT-IR spectra and difference spectra of BSA in aqueous solution. **a** FT-IR spectrum of free BSA; **b** FT-IR difference spectrum [(BSA solution + puerarin solution) − (puerarin solution)] in buffer solution in the region of 1300–1800 cm^{-1} (5.0×10^{-5} mol L^{-1} BSA; 8.0×10^{-5} mol L^{-1} puerarin); pH = 7.4

interaction of puerarin with BSA. These results are also in agreement with the results of CD experiments.

Energy transfer between puerarin and BSA

According to Föster's non-radiative energy transfer theory [21], the energy transfer effect is related not only to the distance between the acceptor and donor (r_0), but also to the critical energy transfer distance (R_0), that is

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \tag{5}$$

where E denotes the efficiency of transfer between the donor and the acceptor, and R_0 is the critical distance when the efficiency of transfer is 50%.

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

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

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \mathrm{d}\lambda}{\int_0^\infty F(\lambda)\mathrm{d}\lambda} \tag{7}$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range from λ to $\Delta\lambda$; $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. Then, the energy transfer efficiency E is given by

$$E = 1 - \frac{F}{F_0} \tag{8}$$

The overlap of the absorption spectrum of puerarin and the fluorescence emission spectrum of BSA is shown in

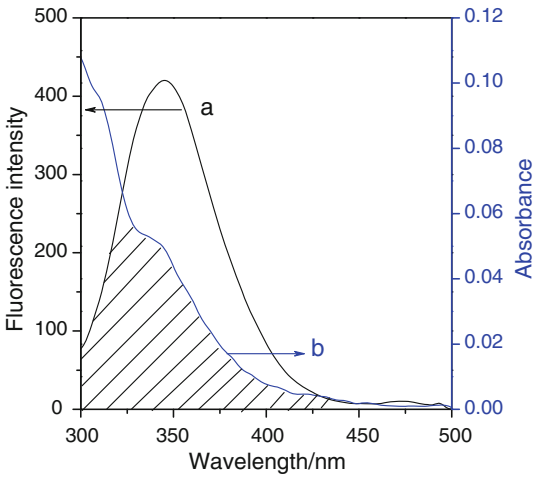

Fig. 5 The overlap of the fluorescence spectrum of BSA and the absorbance spectrum of puerarin (λex = 280 nm, λem = 355 nm, c(BSA)/c(puerarin) = 1:1). The fluorescence spectrum of BSA (*A*) and the absorption spectrum of puerarin (*B*)

Fig. 5. J can be evaluated by integrating the spectra in Eq. 7. Under these experimental conditions, we found that $R_0 = 2.27$ nm from Eq. 6 using $K^2 = 2/3$, $\Phi = 0.15$, $N = 1.36$ [22], the energy transfer effect $E = 0.142$ from Eq. 8, and the distance between puerarin and tryptophan residue in BSA is 3.11 nm. The average distances between a donor fluorophore and acceptor fluorophore on the 2–8 nm scale and $0.5R_0 < r < 1.5R_0$ [23] indicate that the energy transfer from BSA to puerarin hydrochloride occurs with high probability.

Conclusions

The interactions between puerarin and BSA were investigated by ITC combined with CD, FT-IR spectrophotometric techniques, and fluorescence resonance energy transfer under simulative physiological conditions. ITC was an attractive approach for the study of biomolecular with protein such as the interaction of polyphenols with proteins. The binding parameters for the interactions between puerarin and BSA were calculated according to ITC experiments at different temperatures. Experimental results also showed that the binding of puerarin to BSA induced a conformational change of BSA, which was further proved by the analysis data of CD and FT-IR spectra. It is noteworthy that the research described herein signifies a promising approach—exploiting the new use of Chinese medicine puerarin for probing their interactions with relevant target proteins. The binding study of drugs to proteins is greatly important in pharmacy, pharmacology, and biochemistry, and so on. Therefore, this is also expected to open the door to new avenues in the screening and design of appropriate flavonoid-based drugs that will be of important in modern medical research.

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