

Characterization of Interaction Between Bergenin and Human Serum Albumin in Membrane Mimetic Environments

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Abstract The interaction between bergenin and human serum albumin (HSA) in AOT/isooctane/water microemulsions was studied by fluorescence quenching technique in combination with UV absorption spectroscopy, circular dichroism (CD) spectroscopy and dynamic light scattering (DLS) technique. Fluorescence data in ω_o 20 microemulsions revealed the presence of a binding site of bergenin on HSA and its binding constants (K) were 1.64×10^4 , 1.44×10^4 , 1.26×10^4 and 1.09×10^4 M^{-1} at 289, 296, 303, and 310 K, respectively. The binding of bergenin with HSA in microemulsions was stronger than that in buffer solution. The alterations of protein secondary structure in the microemulsions in the absence and presence of bergenin compared with the free form of HSA in buffer were qualitatively and quantitatively analyzed by the evidence from CD spectra. Enthalpy and entropy changes for the reaction were calculated to be -14.45 $kJ\ mol^{-1}$ and 30.76 $J\ mol^{-1}\ K^{-1}$. These results indicated that bergenin bound to HSA mainly by a hydrophobic interaction in microemulsions which was in agreement with the result of the molecular modeling study. The DLS data suggested that HSA may locate at the interface of the microemulsion and bergenin could interact with them.

Keywords Microemulsion · Bergenin · Human serum albumin · Spectrum · Binding

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Introduction

The microemulsion systems in question typically consist of discrete, nanometer-sized water domains stabilised by a surfactant, and dispersed in a continuous apolar phase [1]. It has been used extensively as biological membrane models to aid in the understanding of membrane chemistry [2]. Water is readily solubilized in the polar core, forming a so-called “water pool”, which is described by the water-surfactant molar ratio ω_o ($\omega_o = [H_2O]/[S]$). The aggregates containing a large amount of water molecules (above $\omega_o = 15$) are usually called microemulsions whereas reverse micelles correspond to droplets containing a small amount of water (below $\omega_o = 15$) [3]. Reverse micelles have been used as model systems for studying various reactions in confinement, for example, micellar catalysis [4, 5], enzymatic reactions [6, 7], protein purification [8] and so on. By far, the most widely studied without microemulsion systems are the ternary mixture consisting of the anionic surfactant the sodium salt of bis(2-ethylhexyl)sulfosuccinate (AOT)/nonpolar-solvent/water. It is optically transparent and the change in the system could be followed by different spectrophotometric methods.

Human serum albumin is the most abundant protein in blood serum with a concentration of 0.63 mM. It is a globular protein composed of a single polypeptide chain of 585 amino acid residues with a large α -helix. Its amino acid sequence contains a total of 17 disulfide bridges, one free thiol (Cys-34) and a single tryptophan (Trp-214). HSA binds a number of the relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport throughout the circulation. HSA is also capable of binding a wide variety of drugs and xenobiotics [9, 10]. Binding of drugs to plasma proteins is an important pharmacological parameter,

since it frequently affects the distribution and elimination of a drug as well as the duration and intensity of its physiological action [11–13]. Bergenin (structure shown in Fig. 1), is isolated from several medicinal plants and exhibits various biological activities, such as antiarrhythmic [14], hepatoprotective [15], anti-inflammatory [16] and antitumor effects [17] as well as anti-HIV [18], neuro-protective [19], antiulcer [20] and antitussive activities [21]. Given all these, bergenin is very worthy of study.

In previous works, a series of studies concerning the interaction between drugs and proteins in aqueous solution under physiological conditions have been reported [22–25]. Bernard Desfosses et al. [26] compared the behavior of HSA in the presence of three chemically distinct ligands: oxyphenylbutazone, dansylsarcosine, hemin in buffer and reverse micelles. Daniel M. Davis et al. using fluorescence quenching and CD studied the behavior of HSA in AOT reverse micelles [27]. Suzana M. Andrade and Sílvia M. B. Costa investigated the interaction of two water-soluble freebase porphyrins with two drug-carrier proteins (human serum albumin and β -lactoglobulin) in AOT/isooctane/water reverse micelles by steady-state and transient-state fluorescence spectroscopy [28]. Yet the detailed investigation on the binding interaction of bergenin and HSA via diversiform analytical methods in membrane mimetic environments has not been reported.

This study was designed to demonstrate a new array on the interaction of bergenin with HSA in microemulsions. Besides using fluorescence spectroscopy, UV absorption spectroscopy, circular dichroism (CD) and dynamic light scattering (DLS) methods were also applied. Attempts were made to investigate the binding constant (K), the number of binding sites (n) and thermodynamic parameters for the reaction in the membrane mimetic environments. In addition, the molecular modeling was studied with SGI FUEL workstation. These were the first results on bergenin–HSA interactions, which illustrate the nature of the complications in microemulsion.

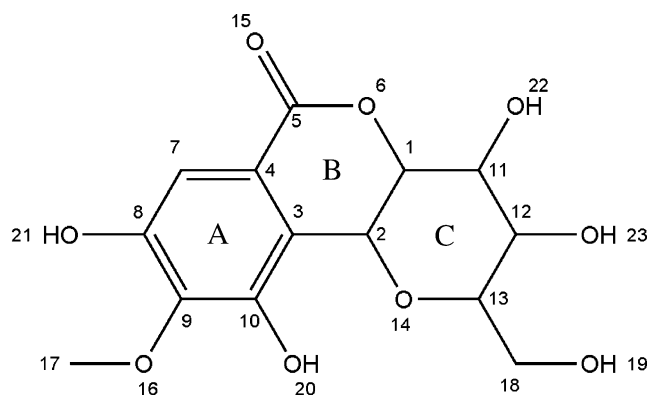


Fig. 1 The chemical structure of bergenin

Materials and methods

Materials

Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) (Fluka, $\geq 96\%$ purity) was used without further purification. Human serum albumin (HSA), purchased from Sigma Chemical Company, was used as received and its molecular weight was assumed to be 66,500 to calculate the molar concentrations. All HSA stock solutions were prepared in the pH 7.40 buffer solution and were kept in the dark at 4°C. Bergenin was of analytical grade, and obtained from the National Institute for Control of Pharmaceutical and Bioproducts (Beijing, China), and the stock solution was prepared in deionized water. Buffer solution (pH 7.40) consisted of Tris (0.2 M) and HCl (0.1 M). Isooctane and other reagents were of analytical grade and deionized water was used throughout all the experiments. All pH were checked with a PHS-10A acidity meter (Xiaoshan Science Instrumentation Factory, Zhejiang, China).

AOT reverse micelle preparation

Microemulsion solutions of desired ω_o ($\omega_o = [\text{H}_2\text{O}]/[\text{AOT}]$) were prepared by adding protein stock solutions, drug solutions or plain buffer (pH=7.40) to a 0.1 M AOT solution in isooctane. Volume of additivity was assumed in calculating AOT concentration and water AOT molar ratios. The samples were gently shaken until complete clarification. The final sample concentration was calculated according to total mole of sample injected and the total volume of the microemulsion.

Apparatus and methods

UV absorption and fluorescence spectroscopic measurements

The absorption and steady state fluorescence measurements were performed using a CARY-100 UV–visible spectrophotometer (Varian, USA) and a RF-5301PC spectrofluorophotometer (Shimadzu), respectively. The fluorescence emission spectra were recorded from 280 to 500 nm (excitation wavelength 280 nm) using 5/5 nm slit widths. Synchronous fluorescence spectra of HSA encapsulated in microemulsion in the absence and presence of increasing amount of bergenin ($0\text{--}4.95 \times 10^{-5}$ M) were recorded λ_{ex} : 280–500 nm.

Fluorometric titration experiments: 2.0 ml microemulsions containing appropriate concentration of HSA were titrated manually by successive addition of a 1.91×10^{-4} M of corresponding microemulsions with bergenin encapsulated (to give a final concentration of 4.65×10^{-6} – $44.0 \times$

10^{-5} M) with trace syringes. The fluorescence intensities were recorded at excitation and emission wavelength of 280 and 311 nm. All fluorescence intensities were measured at four temperatures (289, 296, 303, and 310 K). An electronic thermo regulating water-bath (NTT-2100, EYELA, Japan) was used for controlling the temperature. The fluorescence quantitative data obtained were analyzed by the Scatchard equation [29] to calculate the binding parameters

$$r/D_f = nK - rK \tag{1}$$

where r represents the number of moles of bound drug per mole of protein, D_f represents the molar concentration of free drug, n and K are the number of binding sites and binding constant, respectively. The quantitative analysis of the binding drug–HSA was performed by fluorescence quenching method, which was done by titrating with drug at a given protein concentration. In the drug–HSA system, the concentration of drug was much higher than the concentration of HSA, so the concentration of drug bound to HSA was little, the free concentration of drug can be seen as the total concentration of drug. The total concentration of drug was calculated for the Scatchard plot instead of the free concentration of drug (D_f). On the other hand, drug almost had no fluorescence at 311 nm when the excitation wavelength was kept at 280 nm as shown in the following section, so the fluorescence quenching of HSA can be seen as the decrease of the protein fluorescence (ΔF) owing to the interaction of drug with HSA. Therefore, the value of r can be replaced by the content of the decrease of the fluorescence of protein, that is, $\Delta F/F_0$ (F_0 is the fluorescence of HSA in the absence of drug). To eliminate the inner-filter effect, all the fluorescence intensities were corrected according the relationship [30]:

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2},$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities of HSA, while A_{ex} and A_{em} are the absorbance of bergenin at the excitation (280 nm) and the emission (311 nm) wavelengths of HSA in microemulsion, respectively.

For the anisotropy measurements the excitation and emission band widths were both 5 nm. Steady state anisotropy (r) is defined by

$$r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}),$$

where I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented in vertical and horizontal orientation, respectively. The G factor is defined as: $G = I_{HV} / I_{HH}$, similarly, I_{HV} and I_{HH} are the intensities obtained with the

excitation polarizer oriented horizontally and the emission polarizer oriented in vertical and horizontal orientation.

Thermodynamic data

Thermodynamic parameters were calculated based on the temperature dependence of the binding constant in ω_o 20 microemulsion for bergenin–HSA binding. The temperatures were used 289, 296, 303, and 310 K. The enthalpy change (ΔH^0) was calculated from the slope of the Van’t Hoff relationship:

$$\ln K = -\Delta H^0 / RT + \Delta S^0 / R \tag{2}$$

K is the binding constant at temperature T and R is gas constant. The value of ΔS^0 was obtained from linear Van’t Hoff plot. The value of ΔG^0 was calculated from the equation:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{3}$$

CD measurements

Circular dichroism (CD) spectra were measured with an Olis DSM 1000 Circular Dichroism (American), using a 1 mm cell at 296 K. CD spectra (200–300 nm) were taken in microemulsions with 3.0 μ M HSA encapsulated (ω_o 20) and the results were taken as millidegrees. The α -helical content of HSA was calculated from the molar ellipticity ($[\theta]$) at 208 nm according the equation % helix = $\{(-[\theta]_{208} - 4000) / (33000 - 4000)\} \times 100$ [31]:

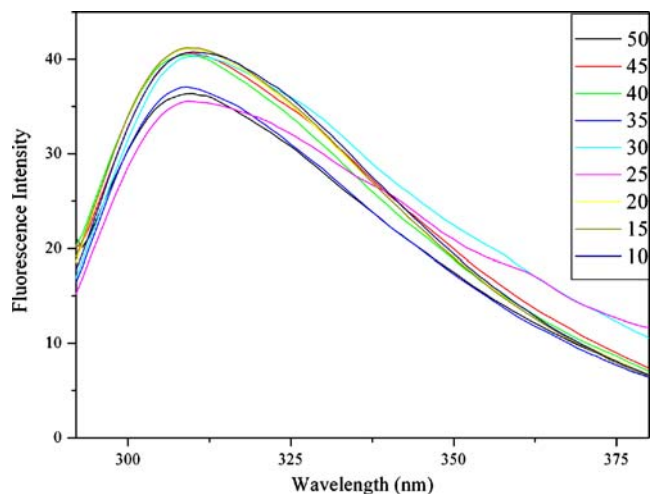


Fig. 2 The fluorescence spectra of HSA (3.0 μ M) in microemulsions with different ω_o

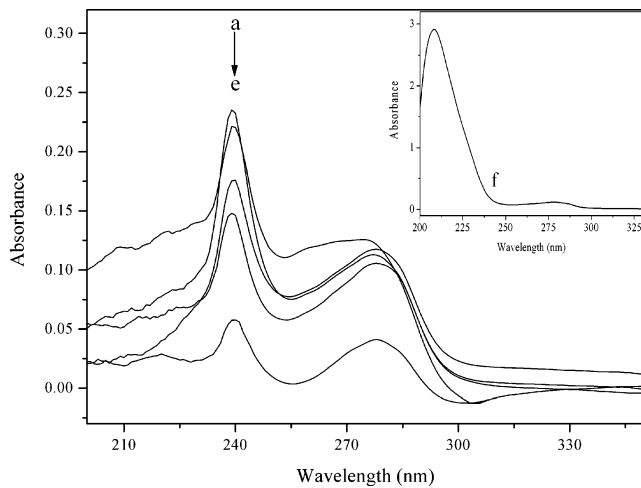


Fig. 3 UV absorption spectra of HSA (3.0 μM). In different ω_0 microemulsions: (a) 15; (b) 30; (c) 20; (d) 10; (e) 25; (f) In Tris buffer, pH=7.40

DLS data

Dynamic light scattering experiments were performed to determine hydrodynamic diameter of AOT microemulsions at room temperature. The measurements were done using a BI-200SM Static and Dynamic laser light scattering system (Brookhaven, America) coupled with a 4 W laser.

Molecular modeling studies

The crystal structure of HSA was taken from the Brookhaven Protein Data Bank (entry codes 1h9z) [32]. The potential of the 3-D structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The

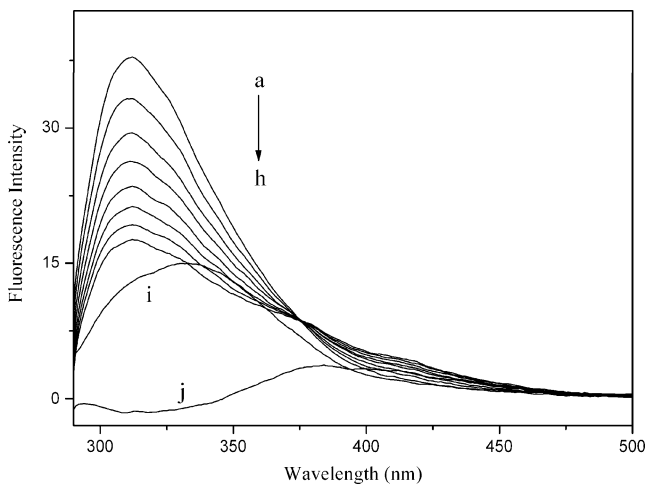


Fig. 4 The fluorescence spectra of bergenin–HSA system in ω_0 20 microemulsion. The concentration of HSA was 3.0 μM while the bergenin concentration corresponding to 0, 9.09, 17.3, 24.9, 31.8, 38.2, 44.0, 49.5 μM from a to h; (j) [bergenin]=49.5 μM in ω_0 20 microemulsion; $T=296$ K; $\lambda_{\text{ex}}=280$ nm, $\lambda_{\text{em}}=311$ nm; (i) 3.0 μM HSA in Tris buffer, pH=7.40; $\lambda_{\text{ex}}=280$ nm, $\lambda_{\text{em}}=332$ nm

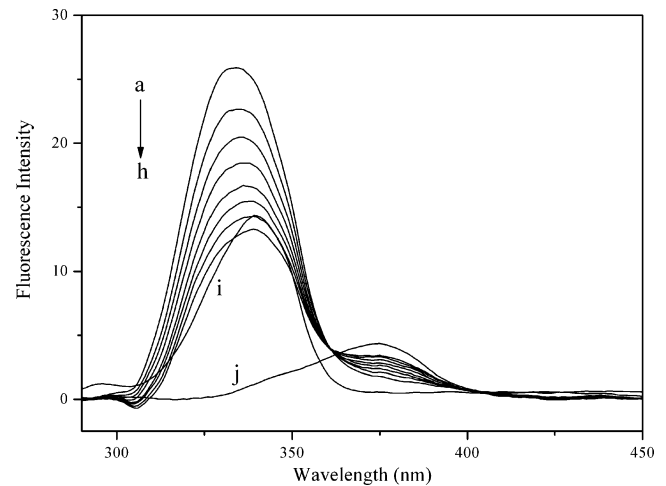


Fig. 5 Synchronous fluorescence spectra of HSA (3.0 μM) in ω_0 20 microemulsion with $\Delta\lambda=60$ nm in the absence and presence of increasing amount of bergenin (μM): (a) 0; (b) 9.09; (c) 17.3; (d) 24.9; (e) 31.8; (f) 38.2; (g) 44.0; (h) 49.5; (j) [bergenin]=49.5 μM in ω_0 20 microemulsion; $T=296$ K; (i) 3.0 μM HSA in Tris buffer, pH=7.40

initial structure of all the molecules was generated by molecular modeling software Sybyl 6.9 [33]. The geometry of the molecule was subsequently optimized to minimal energy using the Tripos force field with Gasteiger–Marsili charges. The FlexX program was used to build the interaction modes between the bergenin and HSA.

Results and discussion

Determination of ω_0 value for microemulsions with HSA encapsulated

To choose a suitable ω_0 value of microemulsions for HSA encapsulated, in which microemulsion HSA's stability is

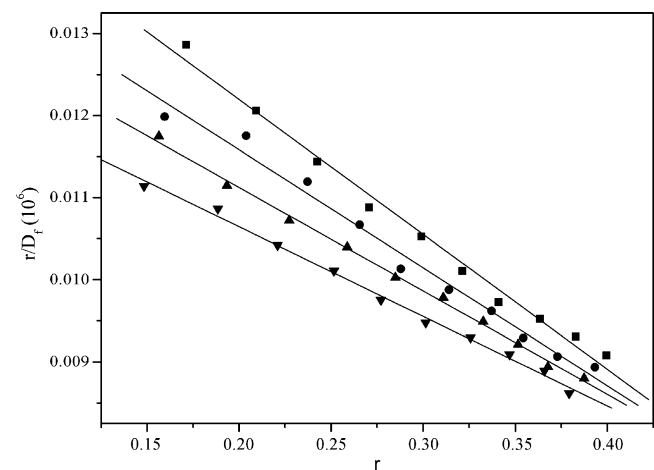


Fig. 6 Scatchard plot for the bergenin–HSA in ω_0 20 microemulsion. HSA concentration: 3.0 μM ; shaded square 289 K; shaded circle 296 K; shaded triangle 303 K; shaded inverted triangle 310 K; $\lambda_{\text{ex}}=280$ nm, $\lambda_{\text{em}}=311$ nm

Table 1 Binding parameters and thermodynamic parameters of bergenin–HSA in ω_o 20 microemulsions

T (K)	K ($\times 10^4 M^{-1}$)	n	Regression coefficient	ΔG^0 (kJ mol $^{-1}$)	ΔH^0 (kJ mol $^{-1}$)	ΔS^0 (J mol $^{-1} K^{-1}$)
289	1.64	0.94	0.99513	–23.33	–14.45	30.76
296	1.44	1.01	0.99240	–23.55		
303	1.26	1.08	0.99856	–23.76		
310	1.09	1.18	0.99757	–23.98		

better, a series of microemulsions with different ω_o from 10 to 50 were prepared. When ω_o equaled to 35 to 50, the clear microemulsions were prone to turbid. When the ω_o changed from 10 to 25, the microemulsions maintained pellucid which were more stable than ω_o 30. According to the research result of Susana Andrade et al. [34], HSA is a spherical protein, the radius of its sphericity r_p (Angstrom)= $0.7 \times Mr^{1/3}$, an empirical equation for radius of AOT micelle inner core is given by r_m (Angstrom)= $4 + 1.5 \times \omega_o$, when $r_p = r_m$, the optimal ω_o for HSA is expected to be around 16. Therefore, 20 was chosen as the optimized ω_o of the microemulsion for the main fluorometric titration experiments and spectroscopic measurements in which microemulsion HSA had the higher fluorescence intensity in Fig. 2. Furthermore, 20 was close to the calculated value 16 and literature values 21 [27], 22.4 [26], respectively.

Fluorescence and UV spectra of HSA in microemulsions of different ω_o

The fluorescence spectra of HSA in microemulsions with various ω_o were measured. In Fig. 2 there was no significant shift in the fluorescence emission maximum (λ_{max}) with the change of ω_o . When ω_o increased from 10 to 50 the λ_{max} were all about 309 nm. There can be two possible reasons for this result: (a) the microenvironment of HSA remains essentially invariant with ω_o , or (b) change in microenvironment does occur, but is not reflected through λ_{max} values. And the effect of ω_o on the relative fluorescence intensity was not large. It is noticed that the properties of reverse micelle solubilized water are different from those of bulk water, even at higher ω_o values, and its apparent microviscosity is six to nine times greater than that of free aqueous solution [35]. Compare 309 nm with λ_{max} 332 nm of HSA in buffer, it may indicate a less polar environment of amino acid residues.

Figure 3 showed the UV absorption spectra of HSA in different ω_o microemulsions. There is an absorbance intensity band of HSA with a maximum at around 210 nm in aqueous solutions. In AOT microemulsions there was a general tendency towards a red shift from 210 to 239 nm for UV absorption spectra when compared with that in water solution. The relative absorption intensities of peaks at 277 nm which character the conjugated double bond of tryptophan, tyrosine and phenylalanine residue to peaks at

239 nm were stronger than that in buffer solution. It means that there is some difference between microenvironment of HSA in microemulsions and in water. Variations of water quantity in microemulsions influenced the absorbance of HSA with a constant peak positions. The absorption of the HSA in ω_o 25 microemulsion was relatively weaker. The possible reasons were that the conformation of HSA was affected most by the microenvironment in ω_o 25 microemulsion or the stability of HSA in this microemulsion was comparatively lower.

Analysis of fluorescence quenching of HSA by bergenin in microemulsion

Fluorescence quenching of the single tryptophan residue in HSA was used to measure drug-binding affinity. The fluorescence spectra of HSA in microemulsion before and after addition of bergenin compared with the native HSA in the pH 7.40 Tris buffer were measured with the excitation wavelength at 280 nm and their representative spectra were shown in Fig. 4. HSA had a strong fluorescence emission with a peak at 311 nm in ω_o 20 microemulsion, a large shift of emission to a shorter wavelength from 332 nm of HSA in aqua. It indicated perturbations of the amino acid residue microenvironment. The intensity of HSA in microemulsion was much higher than in aqueous solutions. Bergenin had a weak fluorescence emission at 380 nm under the presence experiment conditions in microemulsion. With gradual increase in drug concentrations, typical dual fluorescence behavior was observed. It can be seen that the binding of HSA and bergenin in microemulsion quenched the intrinsic fluorescence of the HSA. Moreover, the occurrence of an isoactinic point at 375 nm might also indicate the existence of bound and free bergenin in equilibrium [24]. This behavior was more visible in the succeeding synchronous fluorescence spectra of HSA in the presence of bergenin in ω_o 20 microemulsion.

Table 2 Binding constants of bergenin–HSA in microemulsions of different ω_o and buffer solution at 296 K

ω_o	10	15	20	25	30	Buffer
K ($\times 10^4 M^{-1}$)	1.39	1.42	1.44	1.47	1.46	1.29

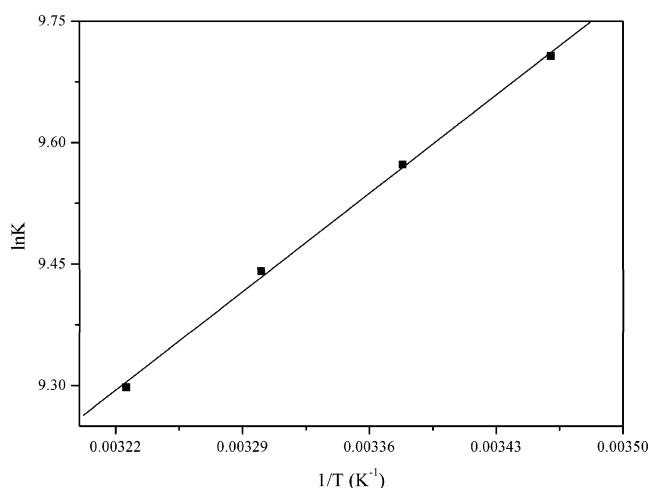


Fig. 7 Van't Hoff plot for the interaction of HSA and bergenin in ω_0 20 microemulsion

The synchronous fluorescence spectra are frequently used to characterize the interaction between fluorescence probe and proteins since it can provide information about the molecular microenvironment in a vicinity of the chromospheres molecules. According to Miller [36], with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of HSA is characteristic of tryptophan residue. Synchronous fluorescence spectral changes of HSA upon addition of bergenin with varied concentration in ω_0 20 microemulsion when $\Delta\lambda=60$ nm were displayed in Fig. 5. Compared with HSA in the Tris buffer, protein encapsula-

tion immediately resulted in a shift of emission to a shorter wavelength from 339 to 333 nm. The addition of the drug led to a dramatic decrease in the fluorescence intensity with a bathochromic shift to 333–339 nm. It is considered that the λ_{\max} of the tryptophan residues is relative to the polarity of microenvironment. λ_{\max} at 330–332 suggests that tryptophan residues are located in the non-polar region, that is, they are buried in a hydrophobic cavity in HSA; λ_{\max} at 350–352 nm indicates that tryptophan residues are exposed to water, that is, the hydrophobic cavity in HSA is disagglomerated and the structure of HSA is looser. Thus Fig. 5 may show that after introducing HSA into microemulsion, there was a less polar environment of tryptophan residues. And bergenin mainly bound to the hydrophobic cavity of HSA, which was in accordance with the results from molecular modeling and the thermodynamics parameters obtained by the experimental data as follows. It was also indicated that the reaction between HSA and bergenin in microemulsion led the polarity around the tryptophan residues increased.

Binding constant and number of binding sites

The quantitative analysis for HSA–bergenin at four different temperature in ω_0 20 microemulsions was carried out using the Scatchard equation Eq. 1. In Fig. 6 the satisfactory linearity of Scatchard plots may indicate that bergenin binds to a class of binding sites on HSA and

Fig. 8 The binding mode between bergenin and HSA, only residues around 10 Å of bergenin are displayed. The residues of HSA are represented using gray ball and stick model and the drug structure is represented by a green one. The hydrogen bond between bergenin and HSA is represented using yellow dashed line

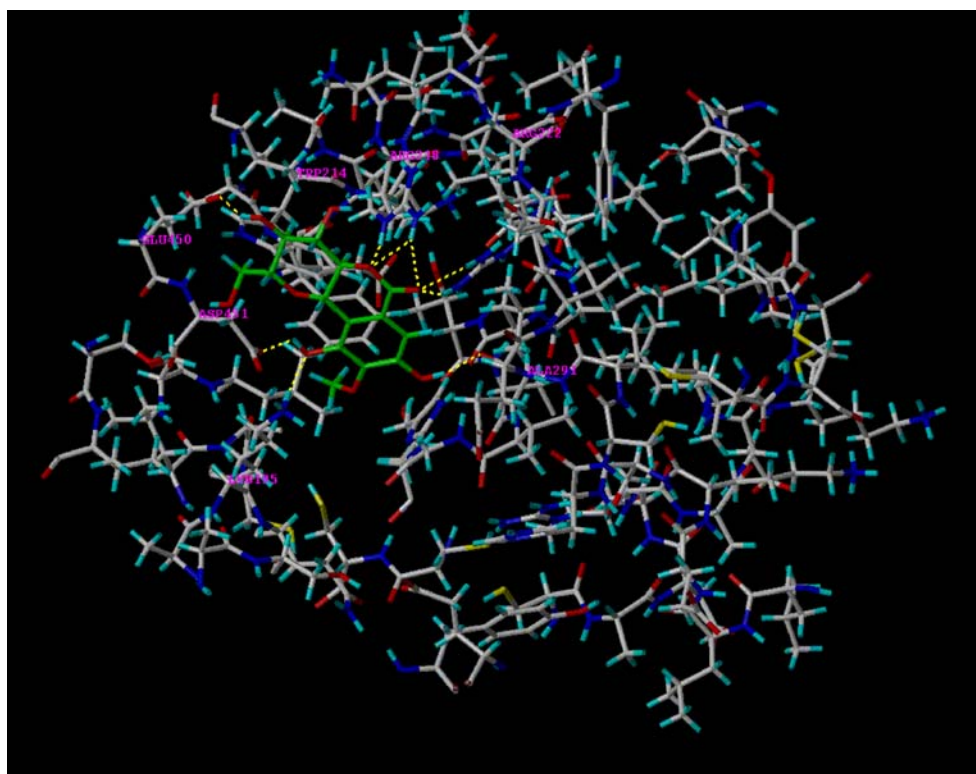


Table 3 Fluorescence anisotropy (r) of HSA in different ω_o microemulsions and buffer solution at 296 K

ω_o	10	15	20	25	30	35	40	45	50	Buffer
r	0.1035	0.1074	0.1025	0.1024	0.0980	0.1054	0.1024	0.0970	0.1034	0.0783

approximate process of the Eq. 1 in “Apparatus and methods” section is logical. The fluorescence quenching data may indicate that the binding site of bergenin on HSA may be the site I in the hydrophobic cavity of sub-domain IIA. It could make the bergenin much closer to the tryptophan residues. The binding constants K decreased with the increasing temperature in microemulsions. Bergenin can be removed by the proteins in the body. The binding constants and the number of binding sites were summarized in Table 1. In Table 2 the binding constants K were listed for bergenin associated with HSA in microemulsions of different ω_o and in buffer solution at 296 K. Binding constants K changed a little in different water pools which have the diverse water capacity. So the change of microenvironment in microemulsions had very small effect on binding of bergenin and HSA. While K of the buffer solution was lower to a small degree than the microemulsions’, this may indicate greater accessibility of bergenin toward HSA when HSA was encapsulated in microemulsion. It may imply that HSA associates with the interface of microemulsion and an interesting conformational change is induced in the protein’s structure.

Binding mode

There are several types of non-covalent interactions modes such as hydrogen bond, van der Waals force, hydrophobic interaction force, and electrostatic force, etc [37]. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS) of reaction are important for confirming binding mode. For this reason, the temperature-dependence of the binding constant of microemulsion (ω_o 20) was studied. The temperatures chosen were 289, 296, 303, and 310 K so that HSA does not undergo any structural degradation. According to the binding constants of bergenin to HSA obtained at the four temperatures in ω_o 20 microemulsions above, the values of ΔH^0 and ΔS^0 were obtained from linear Van’t Hoff plot (Fig. 7) and presented in Table 1. The free energy change ΔG^0 was estimated from the Eq. 3. As shown in Table 1, ΔH^0 and ΔS^0 for the binding reaction between bergenin and HSA were found to be $-14.45 \text{ kJ mol}^{-1}$ and $30.76 \text{ J mol}^{-1} \text{ K}^{-1}$. Thus, the negative sign for ΔG^0 means that the binding process was spontaneous and the formation of bergenin–HSA coordination compound was an exothermic reaction accompanied by positive ΔS^0 value. For typical hydrophobic interactions, both ΔH^0 and ΔS^0 are positive, while negative ΔH^0 and ΔS^0 changes arise from van der Waals force and hydrogen bonding

formation in low dielectric media. However, negative ΔH^0 might play a role in electrostatic interactions [38]. Therefore, it is not possible to account for the thermodynamic parameters of bergenin–HSA coordination compound on the basis of a single intermolecular force model. The binding process of bergenin to HSA in the microemulsions might involve hydrophobic interaction strongly as evidenced by the positive values of ΔS^0 , but the electrostatic interaction could also not be excluded.

Molecular modeling study of the interaction between HSA and bergenin

Descriptions of the 3-D structure of crystalline albumin have revealed that HSA comprises three repeating domains (I–III): I (residues 1–195), II (196–383), III (384–585), and each of which is divided into two sub-domains (A and B). Sudlow et al. [39] have suggested two main distinct binding sites on HSA, site I and site II, which locate in the hydrophobic cavities of sub-domains IIA and IIIA, respectively, and one tryptophan residue (Trp-214) of HSA is in sub-domain IIA [40]. There is a large hydrophobic cavity present in subdomain IIA that many drugs can bind to.

SGI FUEL workstation was used to calculate the partial binding parameters to study the binding site of the HSA–bergenin system, and the best energy ranked result was presented in Fig. 8. It can be seen that bergenin was situated within subdomain IIA in Sudlow’s site I formed by helices. The bergenin molecule was located within the binding

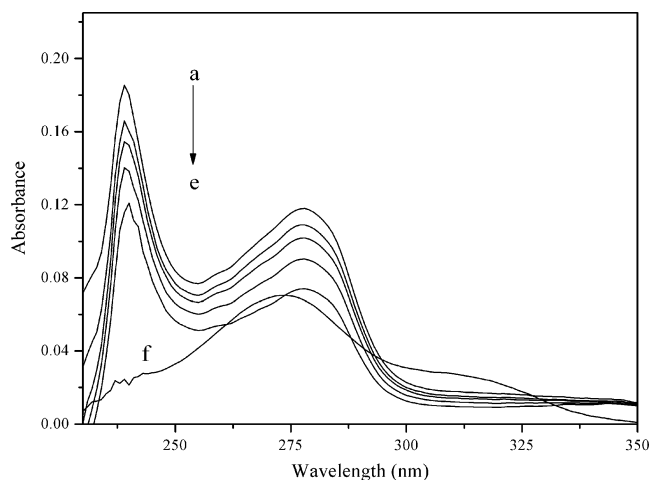


Fig. 9 UV absorption spectra of ω_o 20 microemulsions with HSA (3.0 μM) encapsulated in the presence of different bergenin concentrations (μM): (a) 0; (b) 9.09; (c) 17.3; (d) 31.8; (e) 54.5; (f) [bergenin]=9.09 μM , $T=296 \text{ K}$

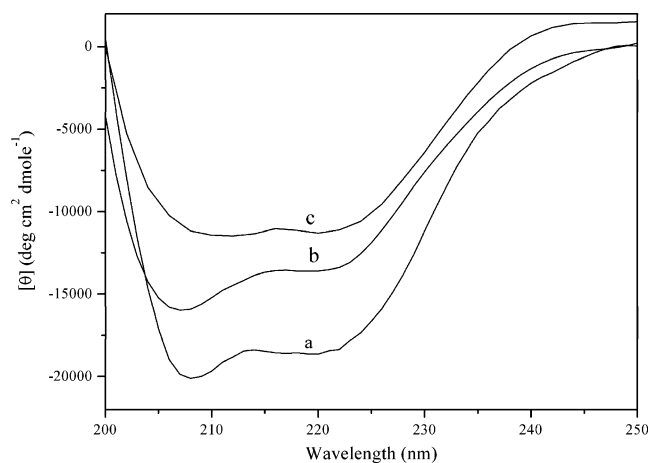


Fig. 10 CD spectra of the HSA–bergenin system. (a) 3.0 μM HSA in Tris buffer; (b) 3.0 μM HSA in ω_0 20 microemulsion; (c) 3.0 μM HSA in the presence of 15.0 μM bergenin in ω_0 20 microemulsion

pocket, and it was important to note that the only tryptophan residue (Trp-214) of HSA was in close proximity to C- and B-rings, suggesting the existence of hydrophobic interaction between them. Further, this finding provided a good structural basis to explain the efficient fluorescence quenching of HSA emission in the presence of bergenin. The interaction between bergenin and HSA was not exclusively hydrophobic in nature since there were many polar and charged amino acid residues on the protein surface, which play a subordinate role in stabilizing the bergenin molecule via electrostatic interaction. It was in agreement with the binding mode study. On the other hand, there were hydrogen bonding interactions between 6-O of bergenin and the residue Arg-218 of HSA; 15-O and Arg-218, Arg-222; 20-OH and Lys-195, Asp-451; 21-OH and Ala-291; 23-OH and Glu-450; The results suggested that the formation of hydrogen bond decreased the hydrophilicity and increased the hydrophobicity to stability in the bergenin–HSA system. The calculated binding Gibbs free energy (ΔG^0) was $-19.72 \text{ kJ mol}^{-1}$, which was not very close to the experimental data ($-23.55 \text{ kJ mol}^{-1}$) to some degree. A possible explanation may be that the X-ray structure of the protein from crystals differs from that of the microemulsions system used in this study. Therefore, the results of modeling indicated that the interaction between bergenin and HSA was dominated by hydrophobic force, which was in accord with the binding mode study.

Fluorescence anisotropy of HSA in various microemulsions

The fluorescence anisotropy measurements of HSA in AOT microemulsions as a function of increasing amounts of water were carried out when λ_{ex} was 280 and the result was shown in Table 3. It is evident that as ω_0 increased, very little change in anisotropy (r) was observed, indicating that there was no appreciable additional change in the average

mobility of HSA molecules. It is noteworthy that all the r were higher than that obtained for HSA in buffer solution, which showed that the mobility of the chromophores inside microemulsion droplets was more limited than in aqueous solution. This effect could be explained by interactions between amino acid side-chains and AOT sulfonate groups or the structured interfacial water layer in which these were encapsulated. It may also be explained simply by the increase of water microviscosity when water acts as a component of the microemulsion.

Binding studies between bergenin and HSA in microemulsions using UV and CD spectrum

Figure 9 was the UV absorption spectra of HSA with increasing amount of bergenin in microemulsion (ω_0 20). As can be seen in Fig. 9, the absorbance of HSA in microemulsion at 239, 279 nm decreased with the addition of bergenin. This result clearly indicated the interaction between bergenin and HSA.

CD spectrum was applied to analyze the changes of HSA secondary structure in the absence and presence of bergenin in microemulsions and compared them with spectrum in buffer; the results were shown in Fig. 10. The CD spectra of HSA exhibit characteristic features of the α -helical structure of protein with negative bands in the ultraviolet region at 208 and 222 nm. In Fig. 10, the CD spectra of free HSA buffer solution had a characteristic of the typical ($\alpha + \beta$)-helix structure with negative bands at 208 and 220 nm. After protein encapsulation in microemulsion, the band intensity of negative Cotton effect of HSA at 208 and 220 nm reduced, indicating the considerable changes in the protein secondary structure with the reduction of the α -helical content in HSA. There may be some co-effect factors contributing to the changes: the unusual properties of water localized in the interior of microemulsions could bring about a different chiral environment for HSA; the contact of HSA molecules with isoctane in the process of solubilization can change the structure of HSA; the interaction of AOT and HSA can also change the structure of protein. The binding of bergenin to HSA in microemulsion kept on decreasing the α -helical content in protein and increased the disorder structure content in the HSA. From the above results, there may be the formation of complex between HSA and bergenin in microemulsion. The quantitative CD data exhibited a reduc-

Table 4 Hydrodynamic diameter (D_H) of AOT microemulsions without HSA and with HSA (8.1 μM), HSA + bergenin ([HSA]/[bergenin]=1:5) at ω_0 20; $T=296 \text{ K}$

	No HSA	HSA	HSA + bergenin
D_H (nm)	10.3	6.3	4.2

tion of α -helices from 55.58% (free HSA in buffer) to 41.10% (free HSA in microemulsion), from 41.10% to 24.74% (bound HSA in microemulsions). This suggested that, in spite of the impact of the environment of microemulsion on the secondary structure of HSA, bergenin could interact with the protein in microemulsion. From CD measurements, Desfosses et al. [26] showed that upon hosted into AOT microemulsions of ω_o 22.4, the α -helical content of HSA decreased by approximately 15%.

DLS data

The dynamic light scattering data was presented in order to have insight on the localization of HSA and its interaction with bergenin which occurred after encapsulation in AOT microemulsions. Hydrodynamic diameters (D_H) of AOT microemulsions with and without HSA, HSA binding drug at ω_o 20 were measured (Table 4). The D_H obtained for ω_o 20 microemulsion was 10.3 nm, which was comparatively close to the literature's value [41]. After HSA immersing in AOT microemulsion, D_H decreased to 6.3 nm. According to Pileni [3], in microemulsion the contribution of the solute to the interface or to the polar volume induces an increase either in the interfacial area, $d\Sigma$, or in the polar volume, dV . The water pool radius expressed as a sphere ($R_w = 3(V + dV) / (\Sigma + d\Sigma)$) changes by increasing either dV or $d\Sigma$. A probe located at the interface induces a decrease in the water pool radius due to an increase in the interface, $d\Sigma$, with a constant volume, V . Therefore HSA may locate at the interface inducing a decrease in the water pool, which may have relation to the change of secondary structure in HSA. Upon adding bergenin, the D_H decreased to 4.2 nm. The reasons may be that when bergenin entered the water pool of microemulsion which contained the HSA, bergenin interacted with HSA at the interface, so the contribution of HSA–bergenin complex to the interface of water pool was different from the HSA's. Another possible reason was that after interacting with bergenin, the interaction between HSA and AOT was changed, which may affect the size of water pool.

Conclusions

In this paper, we chose AOT/isooctane/water microemulsions as membrane mimetic environments for the binding of bergenin and HSA which was studied by fluorescence quenching technique, UV absorption spectroscopy, CD spectroscopy, dynamic light scattering technique and molecular modeling method. The work demonstrated that the secondary structure of HSA was influenced after encapsulating the protein into microemulsions. Addition of bergenin to the microemulsion with HSA encapsulated

kept on changing the conformation of HSA, which was proved by the analysis data of CD spectrum. HSA may locate at the interface of the microemulsion and had the interaction with bergenin according to the DLS data. The main interaction between HSA and bergenin was hydrophobic force but the electrostatic interaction can also not be excluded. The studies presented also clearly indicated that bergenin was a quencher for HSA and bound to HSA with higher affinities in microemulsions than in buffer solution.

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