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Comparative study of the interactions between ovalbumin and three alkaloids by spectrofluorimetry

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Abstract The interaction between ovalbumin (OVA) and three purine alkaloids (caffeine, theophylline and diprophylline) was investigated by the aid of intrinsic and synchronous fluorescence, ultraviolet-vis absorbance, resonance light-scattering spectra and three-dimensional fluorescence spectra techniques. Results showed that the formation of complexes gave rise to the fluorescence quenching of OVA by caffeine, theophylline, and diprophylline. Static quenching was confirmed to results in the fluorescence quenching. The binding site number n, apparent binding constant K_A and corresponding thermodynamic parameters were measured at different temperatures. The binding process was spontaneous molecular interaction procedures in which both enthalpy and Gibbs free energy decreased. Van der Waals forces and hydrogen bond played a major role in stabilizing the complex. The comparison between caffeine, theophylline, and diprophylline was made, and thermodynamic results showed that diprophylline was the strongest quencher and bound to OVA with the highest affinity among three compounds. The influence of molecular structure on the binding aspects was reported.

Keywords Fluorescence · Ovalbumin · Caffeine · Theophylline · Diprophylline

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Introduction

Caffeine, theophylline, and diprophylline, which have important pharmacology function, are purine alkaloids with similar structure as shown in Fig. 1. Caffeine has been generally believed to abolish chemical or radiation induced delays in cell cycle progression and enhance the toxicity of radiation and anticancer agents [1]. Theophylline is widely used as a bronchodilator for the treatment of bronchial asthma, coronary insufficiency and neonatal apnea [2, 3]. Low doses of theophylline has anti-inflammatory and immune adjustment function. Theophylline can activate the enzyme activity of histone deacetylase, and strengthen the anti-inflammatory effects of glucocorticoids. Diprophylline has the same pharmacology function as theophylline. It was reported that diprophylline compared with theophylline for the treatment of bronchial asthma and coronary insufficiency [3].

OVA is the major egg-white protein. It is a phosphorylated and glycosylated globular protein of 385 amino acids having molecular weight 45 kDa, soluble in water [4]. OVA contains three tryptophan residues: Trp_{148} in helix F, Trp_{267} in helix H, and Trp_{184} as the nearest neighbor residue of the carboxyl terminus of strand 3A [5–7]. OVA has surface active property and can adsorb at the air water interface by exposing its hydrophobic moieties to the interface [8, 9]. Owing to molecular properties of OVA, the poor foaming behavior is responsible for its slow adsorption kinetics at air water interface. However, when the concentration of the protein was between 0.01 and 3 mg/mL, OVA did not form foam [8]. It was reported that OVA was applied to scientific research which can remedy an etiology of infertility in vitro fertilization [10].

In this paper, for the first time the interaction of OVA with caffeine, theophylline, and diprophylline was

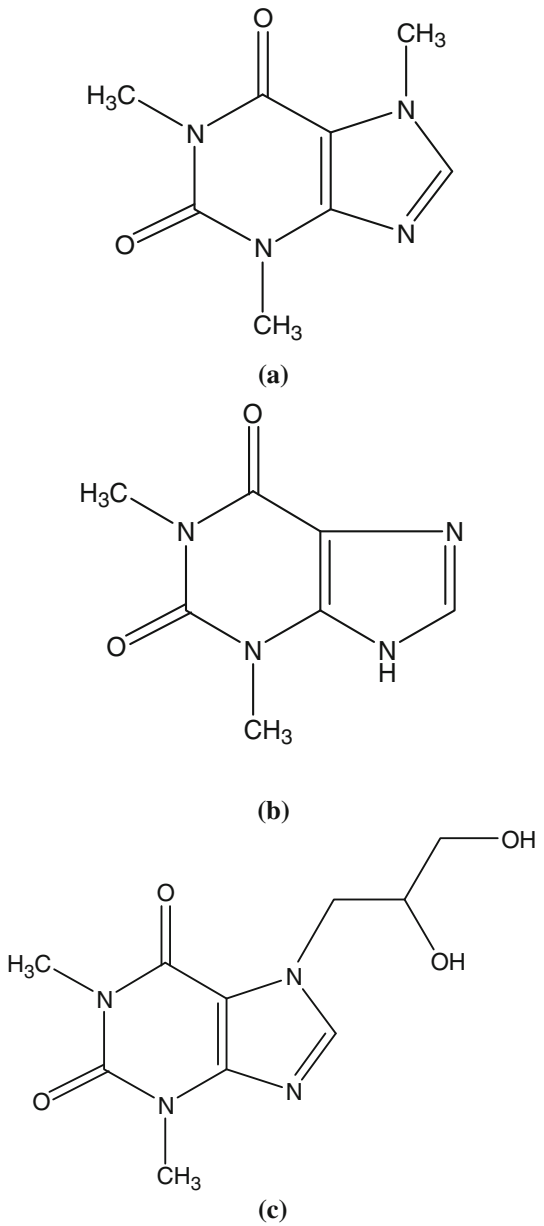

Fig. 1 The molecular structures of caffeine (**a**), theophylline (**b**) and diprophylline (**c**)

investigated by fluorescence, UV/vis absorption, resonance light scattering (RLS), synchronous fluorescence, and three-dimensional fluorescence spectra techniques under physiological pH 7.40. Based on quenching fluorescence of OVA and Stern–Volmer equation, the dynamic quenching constants for caffeine, theophylline, and diprophylline were calculated respectively. The thermodynamic parameters (ΔH, ΔS and ΔG) and the acting forces were studied. The binding site number n and apparent binding constant K_A at different temperatures were calculated. The Van der Waals forces and hydrogen bond played a major role in stabilizing the complex. According to Förster's theory, the distance r between donor and acceptors were obtained.

Materials and methods

Materials

OVA (Sigma) was directly dissolved in 0.10 mol L^{-1} Tris–HCl buffer solution (pH 7.40) with 0.08 mol/L NaCl. The stock solution (9.0×10^{-5} mol L^{-1} OVA) was kept at 0–4 °C; 1.0×10^{-2} mol L^{-1} caffeine, theophylline, and diprophylline (Aladdin) solution were obtained by dissolving them in water. Double distilled water was used throughout and all other chemicals were of analytical grade.

Apparatus

Fluorescence measurements were carried out on a 970–CRT spectrofluorimeter (San Ke, Shanghai) equipped with 1.0 cm quartz cells, using 5/5 nm slit widths. All pH values were measured by a pH-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass electrode. The UV–Vis absorption spectra were obtained using a UV/V-16/18 spectrophotometer (Shanghai). The temperature was regulated by using D2KW-4 thermostat water bath (Beijing). The 3D fluorescence spectra and resonance light-scattering spectra were obtained by F-4500 Spectrofluorophotometer (Japan). Usually, all the experiments were carried out at room temperature.

Procedures

A 3.0 mL solution, containing appropriate concentration of OVA, was titrated by successive additions of a 0.5 μL stock solution of one of three alkaloids (to give a final volume of 45 μL). Titrations were done manually by using microinjector. The fluorescence spectra were obtained (excitation at 287 nm and emission wavelengths of 295–500 nm) at different temperatures (287, 297 K). Synchronous fluorescence spectra of OVA in the presence of one of three alkaloids (caffeine, theophylline, and diprophylline) were recorded with the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength stabilized at 15 or 60 nm respectively. The three-dimensional fluorescence spectrum was performed with the emission wavelengths at 290–470 nm and the excitation wavelength at 200–305 nm. The UV–Vis absorbance spectra of caffeine, theophylline, diprophylline were obtained at room temperature respectively. Resonance light-scattering spectra were measured by synchronous scanning with the wavelength range of 200–800 nm on the spectrofluorophotometer.

Results and discussion

Fluorescence quenching

For macromolecules, the fluorescence measurements can give information of the binding of small molecule substances to protein at the molecular level, such as the binding mechanism, binding mode, binding constants, intermolecular distances, etc. [11]. OVA is a monomer phosphorus glycoprotein which contains three tryptophan residues: Trp_{148} in helix F, Trp_{267} in helix H, and Trp_{184}. The Trp_{148}, Trp_{267}, Trp_{184} residues are the most dominant fluorophores and play an important role in the quaternary state change upon ligand binding. Like most proteins, the characteristic of the intrinsic fluorescence of OVA is very sensitive to its microenvironment. Some factors such as protein conformational transitions, subunit association, substrate binding and denaturation, can result in the intrinsic fluorescence changes of protein [12]. Therefore, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics. Figure 2 illustrates emission fluorescence spectra of OVA in the absence and presence of caffeine, theophylline and diprophylline, respectively. The fluorescence intensity of OVA decreased regularly. Results also indicated diprophylline showed the strongest quenching ability at the same concentration as caffeine or theophylline.

The quenching mechanism

In order to reveal the quenching mechanism (static quenching or dynamic quenching), the Stern–Volmer graph at various temperatures (14, 24 °C) was plotted as shown in Fig. 3. F_0 and F are the fluorescence intensities of OVA in the absence and presence of the quencher, respectively. It was shown that the ratio of F_0/F was linearly proportional to the concentration of caffeine, theophylline and diprophylline, respectively. The slope of the quenching curve at 14 °C was larger than that at 24 °C. If it is static quenching, the stability of the compound formed and the quenching constant would decrease with increasing the temperature. If it is dynamic quenching, the interaction would increase the effective collision number, enhance the energy transfer and increase the quenching constant of the fluorescence substance with increasing the temperature [13]. It indicated that the fluorescence quenching between OVA and drugs (caffeine, theophylline and diprophylline) is static quenching.

To confirm this view, the fluorescence quenching data are analyzed by the Stern–Volmer equation [14]:

$$F_0/F = 1 + Kq\tau_0[Q] = 1 + Ksv\,[Q] \quad (1)$$

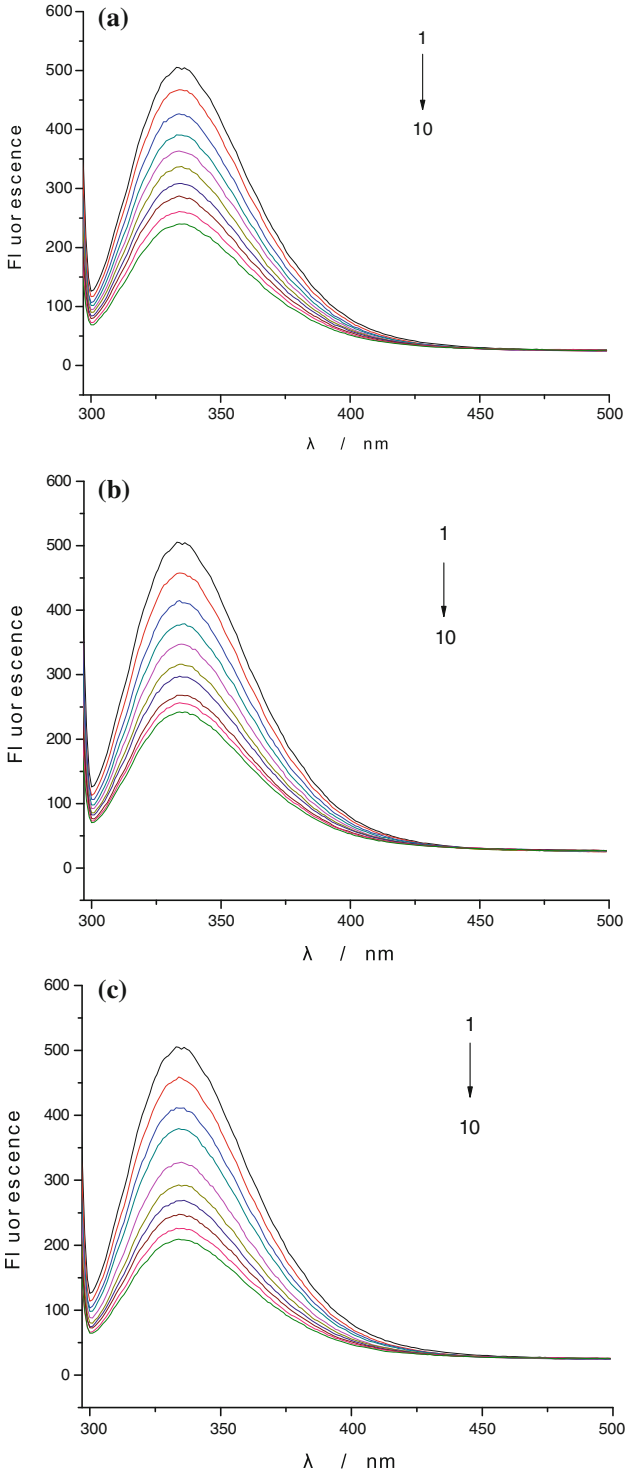

Fig. 2 The fluorescence spectra of OVA in the presence of caffeine (**a**), theophylline (**b**) and diprophylline (**c**). Tris–HCl pH 7.40; NaCl 0.08 mol L^{-1}; OVA 3.0×10^{-5} molSuperscript>/Superscript>L^{-1}. The concentration of caffeine (**a**), theophylline (**b**) and diprophylline (**c**) were 0, 1.60×10^{-5}, 3.33×10^{-5}, 5.00×10^{-5}, 6.67×10^{-5}, 8.33×10^{-5}, 1.00×10^{-4}, 1.17×10^{-4}, 1.33×10^{-4}, 1.50×10^{-4} mol L^{-1} (from *top to bottom*)

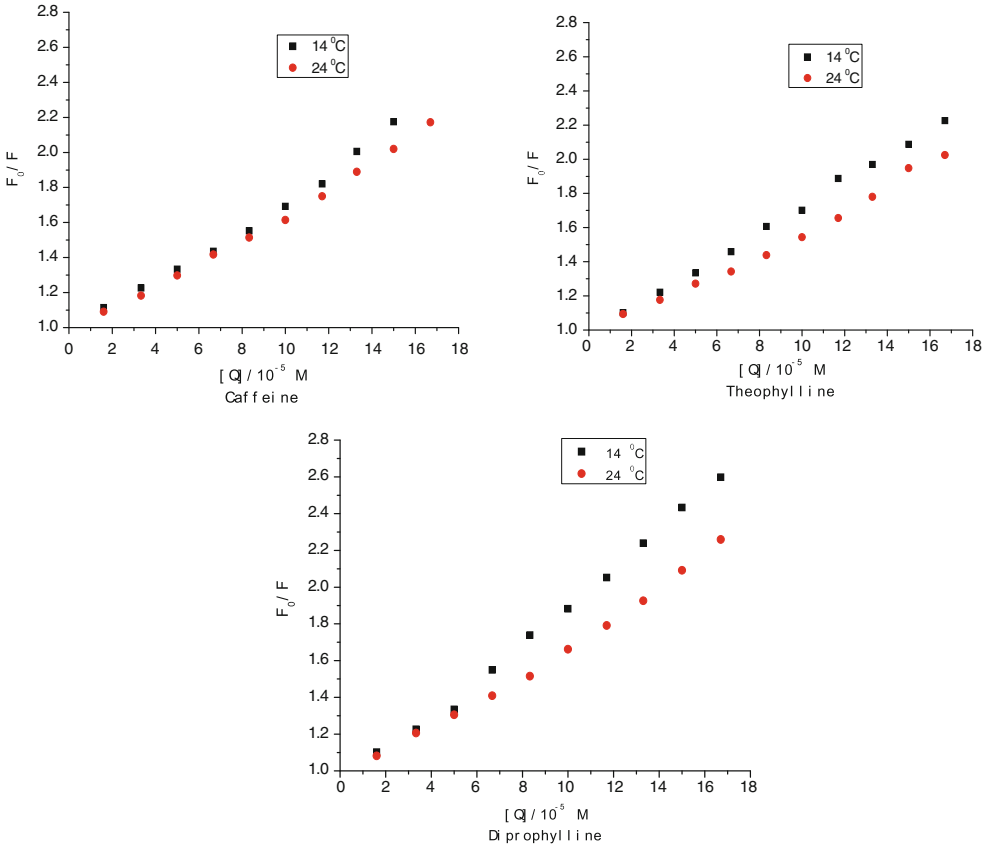

Fig. 3 The Stern–Volmer curves at different temperature (14, 24 °C from *top to bottom*) for caffeine, theophylline and diprophylline quenching the fluorescence of OVA. Other experimental conditions were the same as described in Fig. 2

where Kq, τ_0, Ksv, and [Q] are the quenching rate constant of the bimolecular, the average lifetime of the OVA without quencher, the Stern–Volmer dynamic quenching constant, and the concentration of the quencher respectively. F_0 and F are the initial fluorescence intensity and the fluorescence intensity after the addition of the quencher, respectively. The quenching constants can be calculated by using τ_0 of biopolymers, 10^{-8} s [15]. Results were summarized in Table 1 and indicated that Kq decreased with increasing the temperature. Thus, this quenching was initiated with the formation of compound. In addition, the maximum scatter collision quenching constant of various quenchers with the biopolymer is 2.0×10^{10} L/mol s [16]. From Table 1, the rate constants of protein quenching procedure initiated by three alkaloids (caffeine, theophylline, and diprophylline) are greater than the Kq of the scatter procedure. It also shows that the quenching is initiated by compound formation. What's more, results showed Kq for diprophylline was the largest one among the three alkaloids at the same concentration.

The binding constants and the number of binding sites

For the static quenching process, when small molecules bind independently to a set of equivalent sites on a biomacromolecule, the equilibrium between free and bound molecules is given by the following equation [17]:

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

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. K_A is the binding constant, [Q] is the concentration of quencher and n is the number of binding sites per albumin molecule. Figure 4 showed the plots of log $(F_0-F)/F$ versus log[Q] for the caffeine (theophylline, diprophylline)—OVA system at different temperatures. The calculated binding constants (K_A) and the number of binding sites (n) were presented in Table 2. These results illustrate that there is a strong binding force between alkaloids and OVA, and almost one molecular of alkaloids binds to one molecular of OVA with high affinity. The correlation coefficients are larger than 0.9960, indicating that the interaction between caffeine (theophylline, diprophylline) and OVA agrees fairly well with the site-binding model underlying Eq. (2). The values of K_A illustrate that there is a strong binding force between these alkaloids and OVA [13].

Thermodynamic parameters and binding forces of interaction

There are essentially four types of non-covalent interactions that could play a role in drugs binding to proteins.

Table 1 The quenching constants (L mol^{-1} S^{-1}) between alkaloids and OVA by Stern–Volmer equation

Complex	*T* (°C)	Stern–Volmer equation	Kq (L mol^{-1} S^{-1})	*R*
Diprophylline	14	$Y = 0.8829 + 1.0179 \times 10^4[Q]$	1.0179×10^{12}	0.9983
Caffeine	14	$Y = 0.9465 + 0.7793 \times 10^4[Q]$	7.7930×10^{11}	0.9943
Theophylline	14	$Y = 0.9304 + 0.7520 \times 10^4[Q]$	7.5200×10^{11}	0.9996
Diprophylline	24	$Y = 0.9210 + 0.7681 \times 10^4[Q]$	7.6810×10^{11}	0.9963
Caffeine	24	$Y = 0.9465 + 0.7119 \times 10^4[Q]$	7.1190×10^{11}	0.9976
Theophylline	24	$Y = 0.9704 + 0.6296 \times 10^4[Q]$	6.2960×10^{11}	0.9958

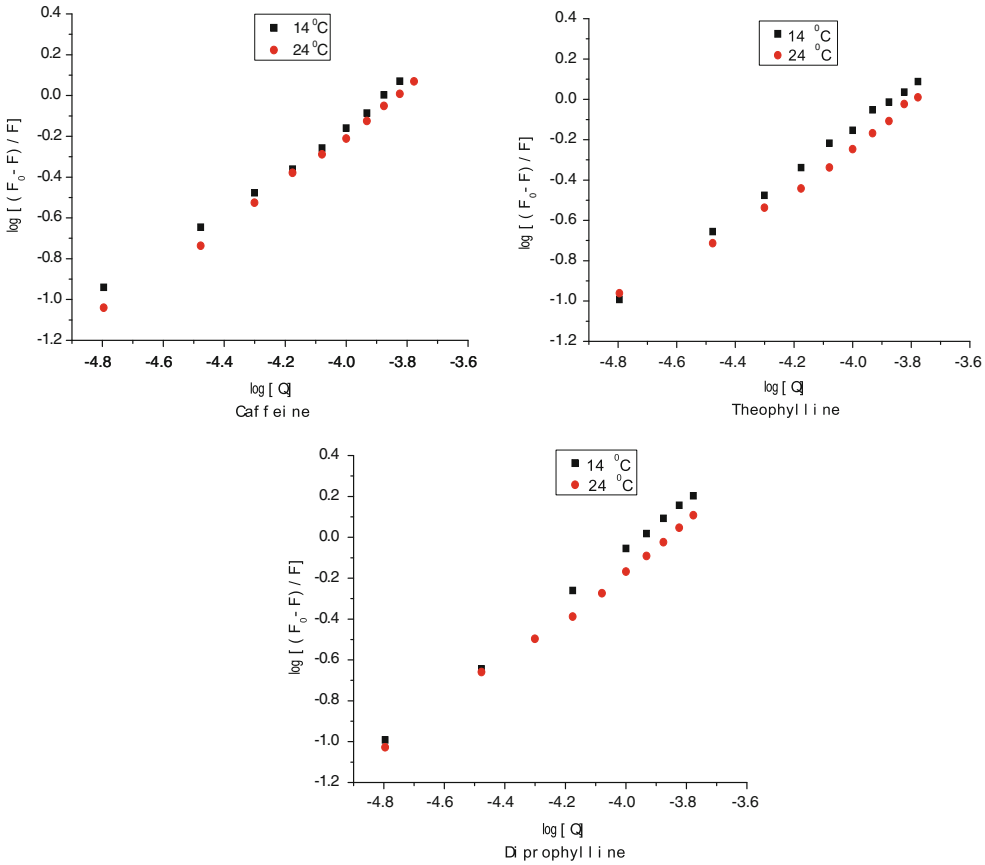

Fig. 4 The double logarithmic curves at different temperature (14, 24 °C from *top to bottom*) for caffeine, theophylline and diprophylline quenching the fluorescence of OVA. Other experimental conditions were the same as described in Fig. 2

Table 2 The binding constants (L mol^{-1}) between alkaloids and OVA by double logarithmic equation

Complex	*T* (°C)	Double logarithmic equation	K_A (L mol^{-1})	*n*	*R*
Diprophylline	14	$Y = 4.6905 + 1.1869\log[Q]$	4.9034×10^4	1.1869	0.9998
Caffeine	14	$Y = 4.2134 + 1.0904\log[Q]$	1.6345×10^4	1.0904	0.9971
Theophylline	14	$Y = 4.1284 + 1.0684\log[Q]$	1.3440×10^4	1.0683	0.9996
Diprophylline	24	$Y = 4.2244 + 1.0965\log[Q]$	1.6765×10^4	1.0964	0.9985
Caffeine	24	$Y = 4.0347 + 1.0603\log[Q]$	1.0831×10^4	1.0603	0.9991
Theophylline	24	$Y = 3.6476 + 0.9700\log[Q]$	0.4442×10^4	0.9700	0.9960

These are hydrogen bonds, van der Waals forces, electrostatic, and hydrophobic interactions [14]. To obtain such information, the temperature changed little (14, 24 °C), the reaction enthalpy change is regarded as a constant. The values of the thermodynamic parameters can be calculated according to Eqs. (3) and (4)

$$\ln K_A = -\Delta H / RT + \Delta S / R \quad (3)$$

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_A \quad (4)$$

K_A is the binding constant at corresponding temperature and R is the gas constant. ΔH is enthalpy change. ΔS is entropy change on binding. ΔG is free energy change. The thermodynamic parameters (ΔH, ΔS) were calculated from the linear relationship between $\ln K_A$ and the reciprocal absolute temperature. According to Eq. (4), ΔG was calculated. The results were listed in Table 3. From the view of thermodynamic theory [18, 19], the interaction can occur between OVA and caffeine (theophylline, diprophylline). In addition, it can be deduced that the acting force of this interaction are mainly van der Waals forces and hydrogen bond [18].

Energy transfer from OVA to alkaloids

The efficiency of energy transfer was studied according to the Förster energy transfer theory [20]. Figure 5 shows the overlap of the fluorescence spectrum of OVA and the absorption spectrum of alkaloids (caffeine, theophylline, and diprophylline). According to Förster non-radiative energy transfer theory, the efficiency of energy transfer (E) is described by the following Eqs. (5)–(7):

$$E = 1 - F/F_0 = R_0^6 / \left(R_0^6 + r^6\right) \quad (5)$$

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

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

where F(λ) is the fluorescence intensity of the fluorescent donor at wavelength λ, ε(λ) is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, $N = 1.36$, $\varphi = 0.062$ [21]. Based on Eqs. (5)–(7), J, R_0, E and r were calculated and shown in Table 4. Obviously, the binding distance is shorter than 7 nm, which according with conditions of Förster energy transfer theory. The results indicated that the energy transfer from OVA to drugs may occur with high probability [22]. The r was bigger than R_0 in this paper, which indicated that alkaloids

Table 3 Thermodynamic parameters of OVA–alkaloids interaction at pH 7.40

Complex	T (°C)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
Diprophylline	14	−25.85	−78.95	−184.99
Caffeine	14	−23.19	−30.25	−24.61
Theophylline	14	−22.81	−81.46	−204.36
Diprophylline	24	−24.01		
Caffeine	24	−22.94		
Theophylline	24	−20.77		

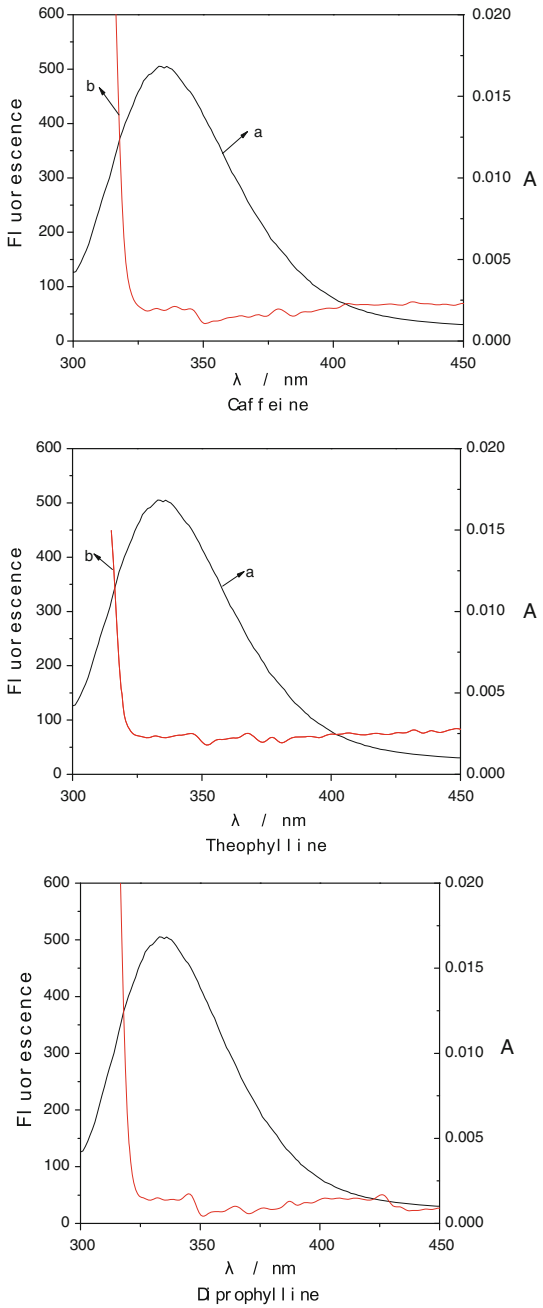

Fig. 5 The overlap of the fluorescence emission spectra of OVA and the UV absorption spectra of caffeine, theophylline, diprophylline. Curve *a* is the fluorescence emission spectrum of OVA (3.0×10^{-5} mol L^{-1}). Curve *b* is the UV absorption spectrum of caffeine, theophylline and diprophylline (3.33×10^{-5} mol L^{-1}). Other experimental conditions were the same as described in Fig. 2

Table 4 Energy transfer parameters in the OVA–alkaloids system

System	J (cm^3 L mol^{-1})	E	R_0 (nm)	r (nm)
OVA–diprophylline	1.6877×10^{-15}	0.7252	1.8971	2.23
OVA–caffeine	0.7554×10^{-15}	0.8599	1.6592	2.25
OVA–theophylline	2.1757×10^{-15}	0.6548	1.9792	2.20

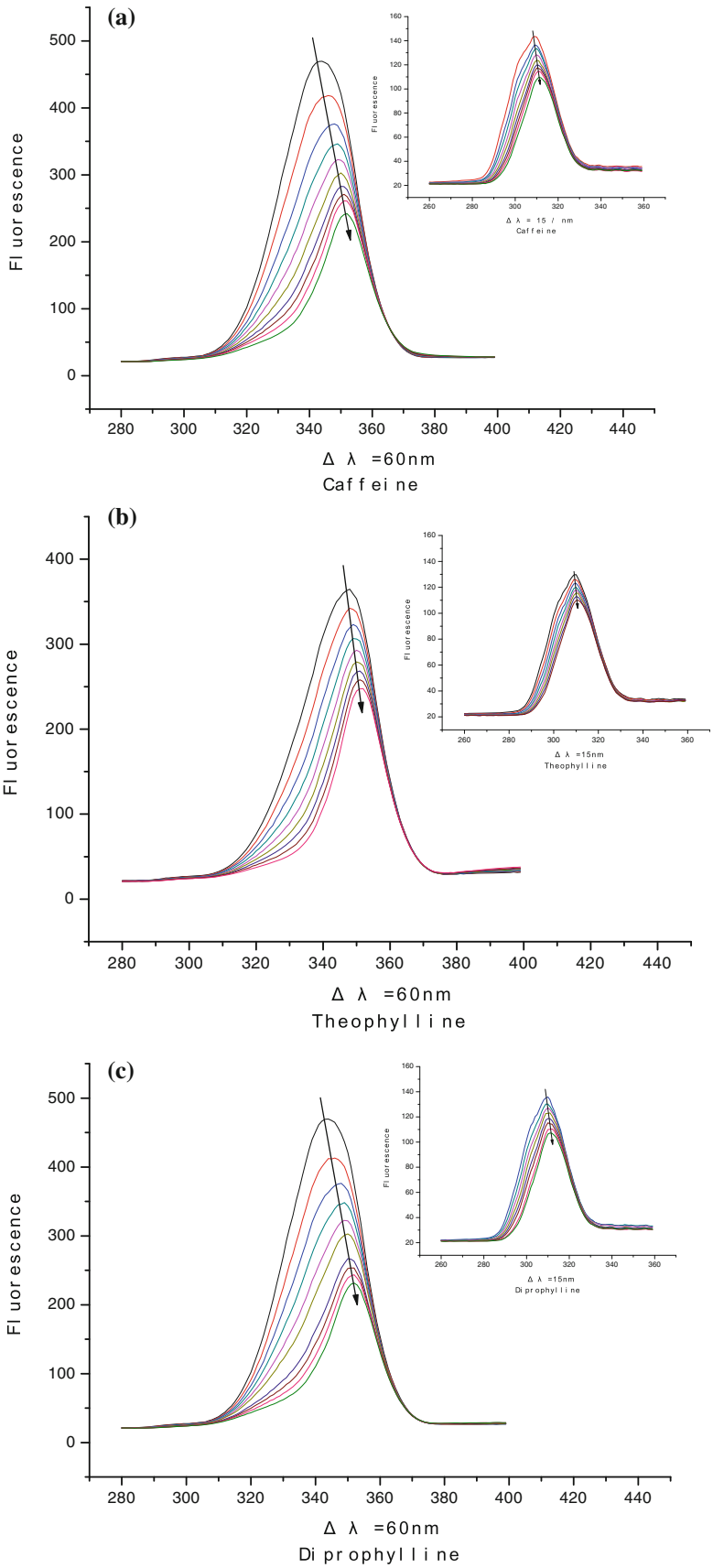

Fig. 6 The synchronous fluorescence spectra of OVA ($\Delta\lambda = 15, 60$) in the presence of different concentration of caffeine (**a**), theophylline (**b**) and diprophylline (**c**). Tris–HCl pH 7.40; NaCl 0.08 mol L^{-1}; OVA 3.0×10^{-6} mol L^{-1}. The concentration of caffeine (**a**), theophylline (**b**) and diprophylline (**c**) were 1.60×10^{-5}, 3.33×10^{-5}, 5.00×10^{-5}, 6.67×10^{-5}, 8.33×10^{-5}, 1.00×10^{-4}, 1.17×10^{-4}, 1.33×10^{-4}, 1.50×10^{-4}, 1.67×10^{-4} mol L^{-1} (from *top to bottom*)

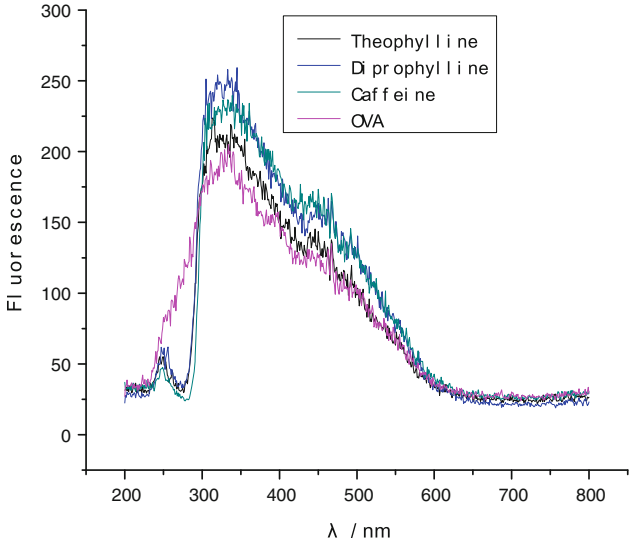

Fig. 7 The RLS spectra of OVA and OVA–caffeine (or theophylline, diprophylline). Tris–HCl pH 7.40; NaCl 0.08 mol L^{-1}; OVA 3.0 × 10^{-6} mol L^{-1}; caffeine (or theophylline, diprophylline) 1.0 × 10^{-4} mol L^{-1}

could strongly quench the intrinsic fluorescence of OVA by static quenching [23].

Conformation investigation

To investigate the conformation change of OVA in the presence of alkaloids, we obtained the synchronous fluorescence, the RLS and three-dimensional fluorescence spectra of OVA.

The synchronous fluorescence spectra give information about the molecular environment in a vicinity of the chromosphere molecules. It involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval between them. When the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [24]. The effect of alkaloids on OVA synchronous fluorescence spectroscopy was shown in Fig. 6. It was apparent from Fig. 6 that the emission maximums of tryptophan and tyrosine residues showed significant red shift which indicated that the polarity around the tryptophan and tyrosine residues was increased [25]. The conformations of OVA were changed while binding alkaloids.

The RLS spectra of alkaloids on OVA were recorded synchronously scanning from 200 to 800 nm with $\Delta\lambda = 0$ nm. The results were shown in Fig. 7. Upon addition of trace amount of alkaloids to OVA solution, we can observe a remarkably increased RLS by caffeine, theophylline and diprophylline, respectively. The RLS intensity is dominated primarily by the particle dimension of the formed aggregate in solution [26]. Bearing these points in mind, it is inferred from the results that the added alkaloids may interact with OVA in solution, forming a new complex that could be expected to be an aggregate. The size of alkaloids-OVA particles may be larger than that of OVA, and thus the increased light scattering signal occurred under the given conditions [27].

The three-dimensional fluorescence is a rising fluorescence analysis technique. The excitation wavelength, the emission wavelength and the fluorescence intensity can be used as the axes in order to investigate the synthetically information of the samples. The three-dimensional fluorescence spectra of OVA and OVA–caffeine were presented in Fig. 8 (spectra for OVA–theophylline and OVA–diprophylline were not shown). The result showed that the three-dimensional fluorescence spectra of OVA were obviously quenched by caffeine. Therefore it can be concluded that there was specific interaction occurring between OVA and alkaloids. Caffeine, theophylline and diprophylline complexed with OVA and changed its conformation [28].

Comparison between caffeine, theophylline and diprophylline

The same main structure was found in the molecular of caffeine, theophylline and diprophylline. Diprophylline has two hydroxide radical groups. Caffeine has three methyls, and theophylline has two methyls.

In this experiments caffeine showed similar quenching ability for OVA as theophylline. Diprophylline showed different characters. For example, the solubility of diprophylline in water was maximum and the hydrogen bond was also the strongest among these compounds. The binding constant of diprophylline was maximum at tested temperature, which showed that the interaction between diprophylline and OVA was the strongest. The phenomena may be explained by the activities of hydroxide radical groups.

Conclusion

At present, the study of the interaction between small molecule and protein is active, but there is few report about the interaction of protein with a series of analogs. No comparison was reported. In this paper, the fluorescence properties of three alkaloids had been investigated.

This paper presents spectroscopic studies on the interaction of three alkaloids (caffeine, theophylline and diprophylline) with OVA by fluorescence, UV/vis absorption, RLS, synchronous fluorescence, and three-dimensional fluorescence spectral techniques for the first time. In

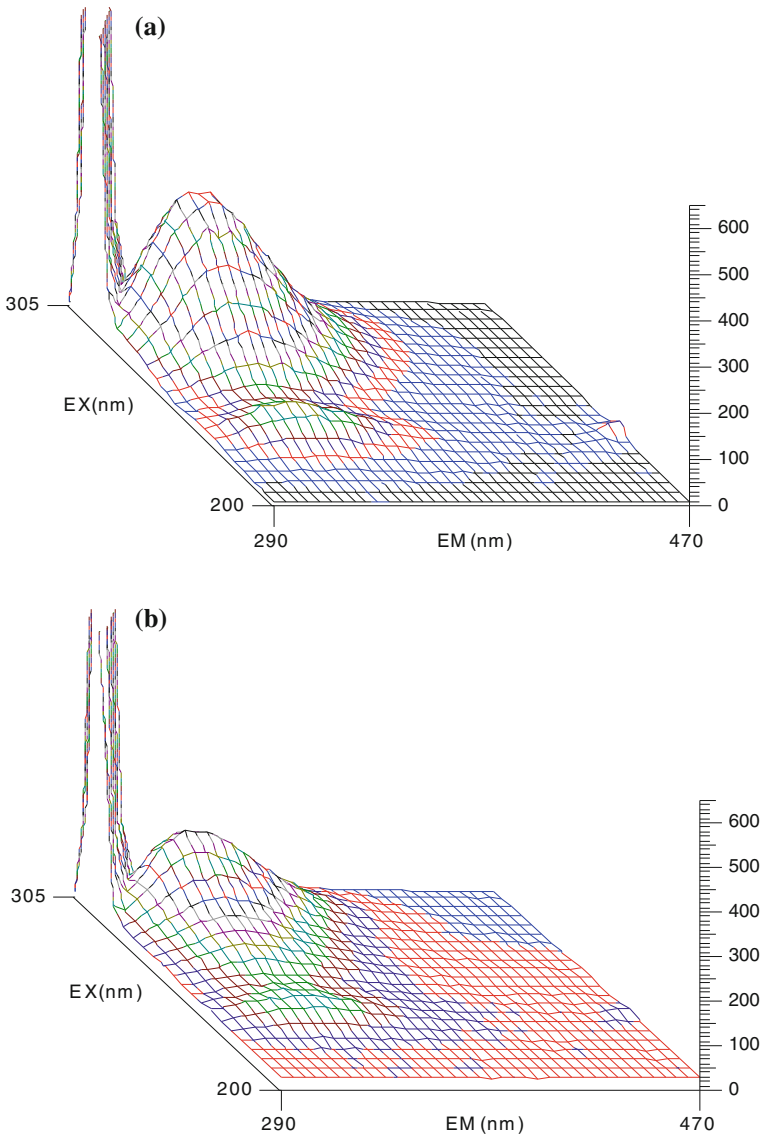

Fig. 8 **a** The three-dimensional fluorescence contour map of OVA. **b** The three-dimensional fluorescence contour map of OVA–caffeine. OVA 3.0×10^{-5} molSuperscript>/Superscript>L^{-1}; caffeine 1.0×10^{-4} molSuperscript>/Superscript>L^{-1}. Other experimental conditions were the same as described in Fig. 2

summary, the fluorescence quenching of OVA by three alkaloids are results of the formation of caffeine (theophylline, diprophylline)-OVA complex. Static quenching was confirmed to result in the fluorescence quenching. OVA molecules have a relatively high affinity with caffeine, theophylline and diprophylline through van der Waals and hydrogen bond. The microenvironments of tyrosine and tryptophan residues of OVA were disturbed by caffeine, theophylline and diprophylline. The influence of molecular structure on the binding aspects was reported. Thermodynamic results also showed that diprophylline was the strongest quencher and binded to OVA with the highest affinity among three compounds. The binding study of caffeine, theophylline and diprophylline with OVA has toxicological and medical importance. This study is expected to provide important insight into the interactions of the physiologically proteins with caffeine, theophylline and diprophylline.

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