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Original article

Fluorescence spectroscopic investigation of the interaction between chloramphenicol and lysozyme

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ABSTRACT

The interactions between chloramphenicol and lysozyme were studied using fluorescence, UV/vis and circular dichroism spectra. The results proved the mechanism of fluorescence quenching of lysozyme by chloramphenicol is due to the formation of lysozyme–chloramphenicol complex. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) for the reaction, were calculated to be -12.41 kJ mol^{-1} and 37.99 J mol^{-1} K^{-1}, which indicated that hydrophobic force and hydrogen bond were the dominant intermolecular forces in stabilizing the complex. The distance $r = 3.99$ nm between donor and acceptor was obtained according to Förster's theory. In addition, the alterations of lysozyme secondary structure in the presence of chloramphenicol were confirmed by the evidences from circular dichroism, synchronous and three-dimensional fluorescence spectroscopy.

1. Introduction

Lysozyme, also called muramidase, a protein discovered by Alexander Fleming in 1922 [1] is a bacteriolytic agent having an ability to lyse the cell walls of bacteria by hydrolyzing the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan. Lysozyme is antimicrobial protein widely distributed in various biological fluids and tissues including avian egg and animal secretions, human milk, tears, saliva, airway secretions, and secreted by polymorphonuclear leukocytes [2]. It has many physiological and pharmaceutical functions, such as anti-inflammatory, anti-viral, immune modulatory, anti-histaminic and anti-tumor activities [3–7]. So it is extensively used in the pharmaceutical and food fields. Intriguingly, lysozyme also has the capacity to carry drugs. It can cure some illness via the binding with these drugs. In view of the multifarious functions of lysozyme and its important practical role from a medicinal point of view, studies on the interactions between drugs and lysozyme have important meaning on realizing the transport and metabolism process of the drugs, the relationship of structure and function of lysozyme, and the chemical essence of the interaction between biomacromolecule and small molecule.

Chloramphenicol (2,2-dichloro-*N*-[(a*R*,b*R*)-*b*-hydroxy-*a*-hydroxymethyl-4-nitrophenethyl] acetamide, CAS 56-75-7, structure shown in Fig. 1) is a bacteriostatic antimicrobial originally derived from the bacterium *Streptomyces venezuelae*, isolated by David Gottlieb, and introduced into clinical use in 1947 [8,9]. It is a cheap and effective broad-spectrum antibiotic that has been widely used in the treatment of pneumococcal and meningococcal disease and also in patients with meningitis caused by *Haemophilus influenzae* [10]. Chloramphenicol exerts its antimicrobial effect by inhibiting bacterial protein synthesis through binding at the 50S ribosomal subunit, thereby interfering with the requisite peptidyl transferase [11]. Transportation, distribution, physiological and pharmacological actions of chloramphenicol is closely related to their binding with proteins (including enzymes), so the investigation on binding of chloramphenicol with proteins is very significant. The binding parameters are helpful in the study of pharmacokinetics and the design of dosage forms. In previous work, Panov et al. [12] reported ^{1}H and ^{13}C high resolution NMR spectroscopic studies to characterize the interaction of chloramphenicol with human serum albumin (HSA), however, HSA and lysozyme are different proteins with different binding features to chloramphenicol, and the investigation on binding interaction of chloramphenicol to lysozyme has not been reported. In this paper, we will report our studies on the interaction of chloramphenicol with lysozyme using fluorescence, UV/vis, circular dichroism (CD) and three-dimensional fluorescence spectroscopy at different

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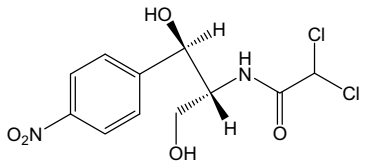

Fig. 1. Chemical structure of chloramphenicol.

temperatures. Attempts were made to investigate the binding mechanism, binding constants, binding sites, the thermodynamic functions and the effect of chloramphenicol on the secondary structure of lysozyme.

2. Results and discussion

2.1. Fluorescence quenching

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching [13]. Such decrease in fluorescence intensity is called quenching. In order to investigate the binding of chloramphenicol to lysozyme, the fluorescence emission spectra were recorded in the range of 300–500 nm upon excitation at 280 nm. Fig. 2 shows the fluorescence emission spectra of lysozyme in the presence of various concentrations of chloramphenicol at 298 K. Under the same condition, no fluorescence of chloramphenicol itself was observed. When different amounts of chloramphenicol were titrated into a fixed concentration of lysozyme, the fluorescence intensity of lysozyme decreased regularly with no shift of the emission wavelength, suggesting that chloramphenicol could interact with lysozyme and quench its intrinsic fluorescence [14].

The fluorescence quenching data were analyzed by the well-known Stern–Volmer equation [15]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_{SV} is the Stern–Volmer quenching constant and [Q] is the concentration of quencher. k_q is the quenching rate constant of the biomolecule macromolecule and $k_q = K_{SV}/\tau_0$. τ_0 is the average lifetime of the biomolecule without any quencher and the fluorescence lifetime of the biopolymer is 10^{-8} s [16]. For dynamic quenching, the maximum scattering collision quenching constant of various quenchers is 2.0×10^{10} L mol^{-1} s^{-1} [17]. Fig. 3 displays the Stern–Volmer plots of the quenching of lysozyme fluorescence by chloramphenicol. The plot shows that within the investigated concentrations, the results agree with the Stern–Volmer equation (1). Table 1 shows the calculated K_{SV} and k_q at each temperature studied. The results showed that the values of Stern–Volmer quenching constants K_{SV} decreased with increasing temperature and the values of k_q were much greater than 2.0×10^{10} L mol^{-1} s^{-1}, which indicated that the probable quenching mechanism of fluorescence of lysozyme by chloramphenicol is not initiated by dynamic collision but compound formation [18]. In other words, the fluorescence quenching of lysozyme results from complex formation is predominant, while from dynamic collision could be negligible.

The fluorescence data was further examined using modified Stern–Volmer equation [19]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a f_a[Q]} \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_a is the effective quenching constant for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. Fig. 4 displays the modified Stern–Volmer plots and the corresponding results of K_a values at different temperatures are shown in Table 2. From Table 2, it can be seen that the decreasing trend of K_a with increasing temperatures was in accordance with K_{SV}'s dependence on temperature as mentioned above. Most ligands are bound reversibly and display moderate affinities for proteins (binding constants in the range $1–15 \times 10^4$ L mol^{-1}), therefore, the K_a values show that the binding between lysozyme and chloramphenicol was moderate [20], which indicated that a reversible lysozyme–chloramphenicol complex formation and chloramphenicol can be stored and carried by lysozyme in the body.

2.2. Binding constant

When ligand molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the

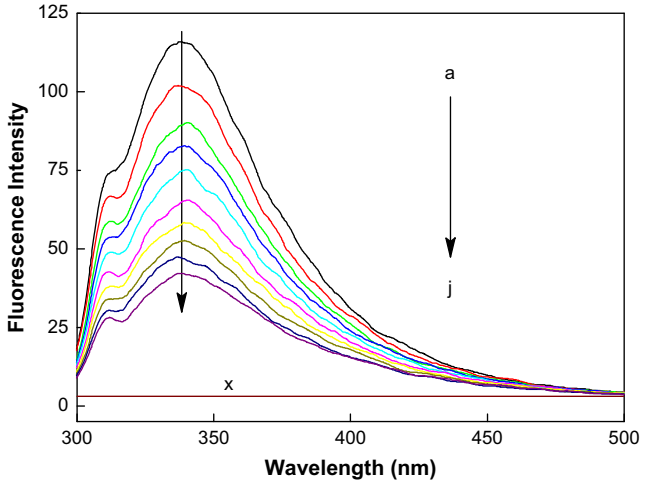

Fig. 2. The fluorescence emission spectra of the lysozyme–chloramphenicol system. (a) 2.0×10^{-6} mol L^{-1} lysozyme; (b→j) 2.0×10^{-6} mol L^{-1} lysozyme in the presence of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5×10^{-5} mol L^{-1} chloramphenicol; (x) 0.5×10^{-5} mol L^{-1} chloramphenicol. $\lambda_{ex} = 280$ nm, pH = 7.40, $T = 298$ K.

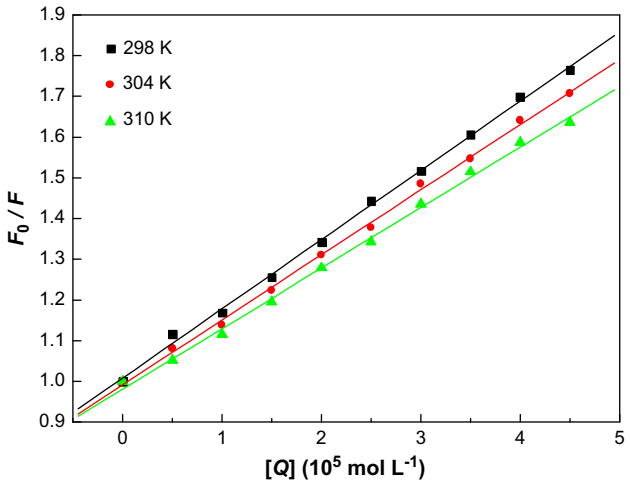

Fig. 3. Stern–Volmer plots for the quenching of lysozyme by chloramphenicol at different temperatures. c (lysozyme) = 2.0×10^{-6} mol L^{-1}; pH = 7.40.

Table 1
Stern–Volmer quenching constants for the interaction of chloramphenicol with lysozyme at three different temperatures.

T (K)	K_{SV} ($\times 10^4$ L mol^{-1})	k_q ($\times 10^{12}$ L mol^{-1} s^{-1})	R[a]	S.D.[b]
298	1.699	1.699	0.9991	0.012
304	1.597	1.597	0.9992	0.011
310	1.483	1.483	0.9986	0.013

[a] R is the correlation coefficient.
[b] S.D. is the standard deviation.

Table 2
Modified Stern–Volmer association constants K_a and relative thermodynamic parameters of the lysozyme–chloramphenicol system.

T (K)	K_a ($\times 10^4$ L mol^{-1})	R[a]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
298	1.442	0.9994	−12.41	−23.73	37.99
304	1.331	0.9993		−23.98	
310	1.187	0.9984		−24.18	

[a] R is the correlation coefficient for the K_a values.

numbers of binding sites (n) can be found from the following equation [21]:

$$\log\frac{F_0 - F}{F} = n\log K_b + n\log\left(\frac{1}{[Q_t] - \frac{F_0 - F}{F_0}[P_t]}\right) \quad (3)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, $[Q_t]$ and $[P_t]$ are the total quencher concentration and the total protein concentration, respectively. Fig. 5 is the plots of $\log(F_0 - F)/F$ versus $\log(1/([Q_t] - (F_0 - F)[P_t]/F_0))$ for the lysozyme–chloramphenicol system at different temperatures, the calculated results are presented in Table 3. As can be seen from Table 3, K_b decreased with the increasing temperature, which indicated the forming of an unstable compound in the association reaction. The unstable compound would be partly decomposed with the rising temperature, therefore, the values of K_b decreased. The values of n at the experimental temperatures were approximately equal to 1, which indicated that there was one class of binding sites in lysozyme for chloramphenicol. The intrinsic fluorescence of lysozyme primarily originates from tryptophan (Trp) 62 and 108, and Trp 62 is more exposed to the polarity environment [22], so, from the value of n, it may be speculated that chloramphenicol most likely binds to the Trp 62 and quench its intrinsic fluorescence.

2.3. Binding mode

Generally, the acting force between a small molecule and macromolecule mainly includes hydrogen bond, van der Waals force, electrostatic and hydrophobic interactions [23]. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS) of reaction, are important for confirming the acting force. For this reason, the temperature dependence of the binding constant was studied. The temperatures chosen were 298, 304 and 310 K so that lysozyme does not undergo any structural degradation. The thermodynamic parameters can be determined from the van't Hoff equation:

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

K is analogous to the effective quenching constants K_a because lysozyme has two primarily fluorophores, one of which (Trp 108) is not accessible to the chloramphenicol and R is gas constant. The value of ΔH and ΔS were obtained from linear van't Hoff plot (Fig. 6) and are presented in Table 2. The value of ΔG is calculated from the relation:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

As shown in Table 2, the signs for ΔH and ΔS of the binding reaction between lysozyme and chloramphenicol were found to be negative and positive, respectively. Thus, the formation of the complex was an exothermic reaction accompanied by positive ΔS value. Ross and Subramanian [24] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes, as described below. From the point of view of the solvent structure around lysozyme and chloramphenicol, a positive ΔS value is frequently taken as typical evidence for hydrophobic interaction. Furthermore, the negative ΔH value (−12.41 kJ mol^{-1}) observed can't be mainly attributed to electrostatic interactions since for electrostatic interactions ΔH is very small, almost zero [24,25]. Negative ΔH value indicates there is hydrogen bond in the

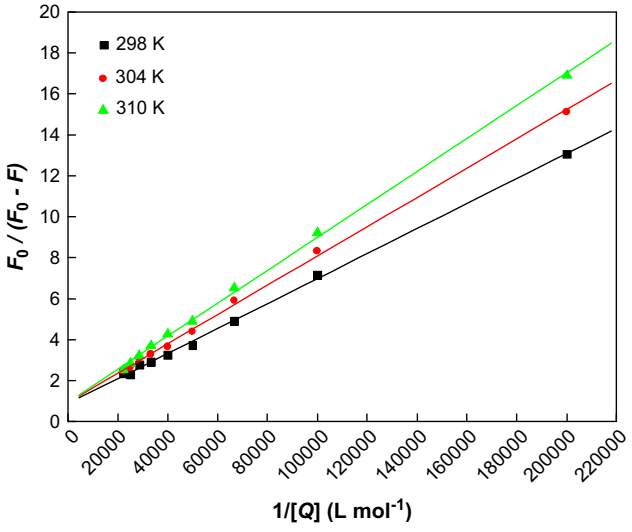

Fig. 4. Modified Stern–Volmer plots for the quenching of lysozyme by chloramphenicol at different temperatures. c (lysozyme) = 2.0×10^{-6} mol L^{-1}; pH = 7.40.

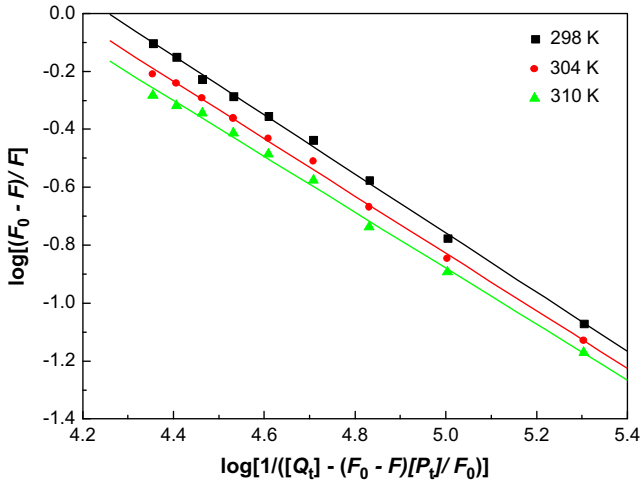

Fig. 5. The plots of $\log(F_0 - F)/F$ versus $\log(1/([Q_t] - (F_0 - F)[P_t]/F_0))$. c (lysozyme) = 2.0×10^{-6} mol L^{-1}; pH = 7.40.

binding. Therefore, it is not possible to account for the thermodynamic parameters of lysozyme–chloramphenicol binding reaction on the basis of a single intermolecular force model. So, in the binding of chloramphenicol to lysozyme process, hydrophobic forces most likely played a major role, but hydrogen bond also could not be excluded.

2.4. Energy transfer

Fluorescence resonance energy transfer (FRET) is a distance dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecule (donor) to another molecule (acceptor) without emission of a photon from the former molecular system. According to Förster's theory [26], the efficiency of FRET depends mainly on the following factors: (i) the extent of overlap between the donor emission and the acceptor absorption; (ii) the orientation of the transition dipole of donor and acceptor, and (iii) the distance between the donor and the acceptor. Here the donor and acceptor are lysozyme and chloramphenicol, respectively. There was a spectral overlap between the fluorescence emission spectrum of lysozyme and UV/vis absorption spectrum of chloramphenicol (Fig. 7). The efficiency (E) of energy transfer is related to the distance (R) between lysozyme and chloramphenicol by

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{6}$$

where F_0 and F are the fluorescence intensities without and with chloramphenicol, respectively. r is the binding distance between donor and acceptor, and R_0 is the critical distance between donor and acceptor, at which 50% of the excitation energy is transferred to acceptor and can be obtained from donor emission and acceptor absorption spectrum using the equation:

$$R_0^6 = 8.79 \times 10^{-25} K^2 \cdot n^{-4} \cdot \varphi \cdot J \tag{7}$$

where K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles; n is the average refractive index of medium in the wavelength range where spectral overlap is significant; φ is the fluorescence quantum yield of the donor; J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which could be calculated by the following equation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta\lambda$ and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. In the present case, $K^2 = 2/3$, $n = 1.36$, $\varphi = 0.14$ [27]. According to equations (6)–(8), the values of the parameters were found to be $J = 1.975 \times 10^{-14}\ \mathrm{cm^3\,L\,mol^{-1}}$, $R_0 = 2.79$ nm, $E = 0.105$, $r = 3.99$ nm. The donor-to-acceptor distance was less than 8 nm, which indicated that the energy transfer from lysozyme to chloramphenicol occurred with high probability [28]. This accorded with conditions of Förster's theory and indicated again a static quenching between lysozyme and chloramphenicol.

2.5. Conformation investigations

2.5.1. Synchronous fluorescence spectra

Synchronous fluorescence spectroscopy introduced by Lloyd [29] has been used to characterize complex mixtures providing fingerprints of complex samples [30]. It involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval between them. In the synchronous spectra, the sensitivity associated with fluorescence is maintained while offering several advantages, such as spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. According to the theory of Miller [31], when $\Delta\lambda$ between excitation and emission wavelength is stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine (Tyr) or Trp residues, respectively. Fig. 8 showed the effect of the addition of chloramphenicol on the synchronous fluorescence spectrum of lysozyme when $\Delta\lambda$

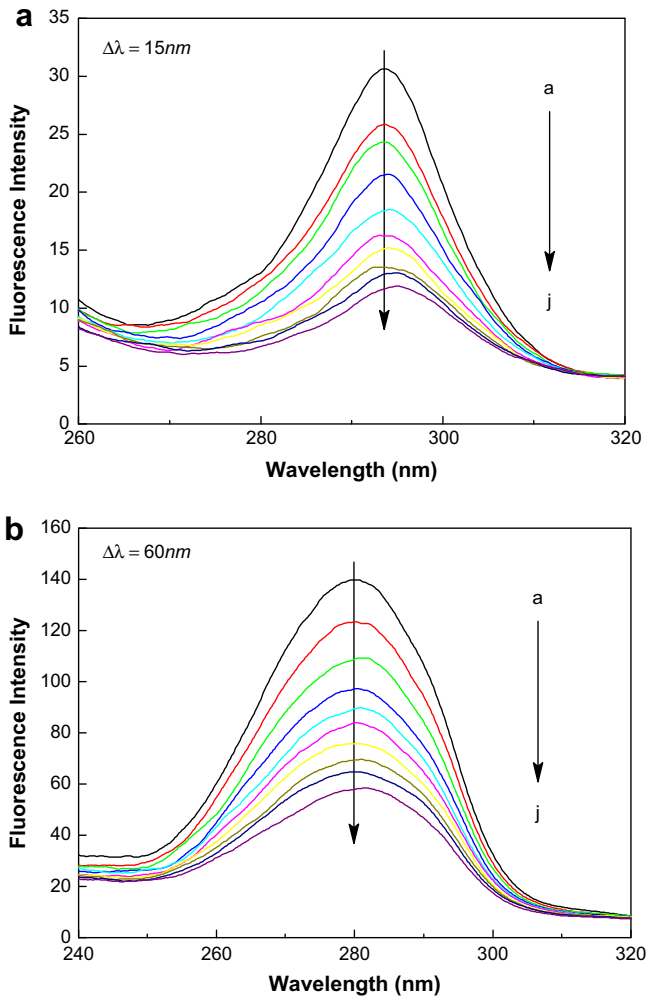

Fig. 8. Synchronous fluorescence spectrum of lysozyme in the absence and presence of chloramphenicol (pH = 7.40, $T = 298$ K). (a) 2.0×10^{-6} mol L^{-1} lysozyme; (b→j) 2.0×10^{-6} mol L^{-1} lysozyme in the presence of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5×10^{-5} mol L^{-1} chloramphenicol, respectively.

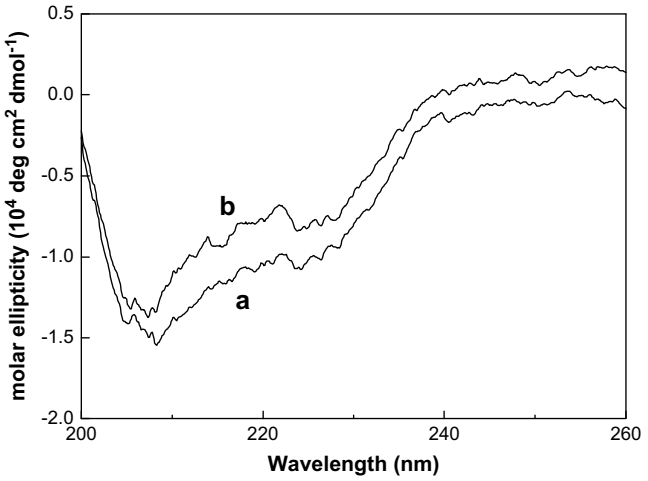

Fig. 9. CD spectra of the lysozyme–chloramphenicol system. (a) 2.0×10^{-6} mol L^{-1} lysozyme; (b) 2.0×10^{-6} mol L^{-1} lysozyme + 8.0×10^{-6} mol L^{-1} chloramphenicol. pH = 7.40, $T = 298$ K.

conformation of lysozyme was changed. Moreover, the fluorescence intensity decreased regularly with the addition of chloramphenicol, which further demonstrated the occurrence of fluorescence quenching in the binding process.

2.5.2. Circular dichroism

Further evidence of conformational changes of lysozyme upon addition of chloramphenicol was provided by CD spectra. CD is a sensitive technique to monitor conformational changes in protein upon interaction with ligands. The raw CD spectra of lysozyme in the absence and presence of chloramphenicol are shown in Fig. 9. The CD spectra of lysozyme exhibited two negative bands in the far-UV region at 208 and 222 nm, characteristic of α-helical structure of protein. The reasonable explanation is that the negative peaks between 208 and 209 nm and 222 and 223 nm are both contributed to $n \rightarrow \pi^*$ transition for the peptide bond of α-helix [32]. The binding of chloramphenicol to lysozyme caused only a decrease in band intensity without any significant shift of the peaks, clearly indicating that chloramphenicol induced a slight decrease in the α-helical structure content of lysozyme. The α-helical content of lysozyme was calculated from equation (10). The calculated results displayed a reduction of α-helical structure from 39.40% to 32.51% at a molar ratio of lysozyme to chloramphenicol of 1:4. From the above results, it was apparent that the effects of chloramphenicol on lysozyme caused a secondary structure change of the protein, with the loss of α-helix stability [33].

2.5.3. Three-dimensional fluorescence spectra

The three-dimensional fluorescence spectra have become a popular fluorescence analysis technique in recent years [34,35]. It can comprehensively exhibit the fluorescence information of the sample, which makes the investigation of the characteristic conformational change of protein to be more scientific and credible. If there is a shift at the excitation or emission wavelength around the fluorescence peak, or appearance of a new peak or disappearance of existing peak, it could be an important hint to suggest conformational changes to the protein. Fig. 10 presented the three-dimensional fluorescence spectrum and their characteristics of lysozyme (A) and lysozyme–chloramphenicol (B), with the corresponding parameters presented in Table 4. In Fig. 10, peak *a* is the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$), peak *b* is the second-ordered scattering peak ($\lambda_{em} = 2\lambda_{ex}$). Peak 1 mainly reveals the spectral behavior of Trp and Tyr residues. The reason is that when lysozyme is excited at 280 nm, it mainly reveals the intrinsic fluorescence of Trp and Tyr residues. Beside peak 1, there is another strong fluorescence peak 2, which mainly exhibit the fluorescence characteristic of polypeptide backbone structures [36]. The fluorescence intensity of peak 2 decreased a lot after interacting with chloramphenicol, which implied that the peptide strand structure of lysozyme was changed and this was in accordance with the decreased of α-helical content in the CD spectra. Analyzing from the intensity changes of peaks 1 and 2, they both decreased obviously but to different degrees: the fluorescence intensity of peak 1 has been quenched of 31.15% while peak 2 of 21.99%. The decrease of the fluorescence intensity of the two peaks in combination with the synchronous fluorescence and CD results indicated that there was specific interaction occurring between lysozyme and chloramphenicol, and the binding of lysozyme–chloramphenicol induced the slight unfolding of the polypeptides of lysozyme, which resulted in a conformational changes of lysozyme to increase the exposure of some hydrophobic regions that had been buried [37]. All these phenomena and analyses show that the binding of chloramphenicol to lysozyme induced secondary structure changes in lysozyme.

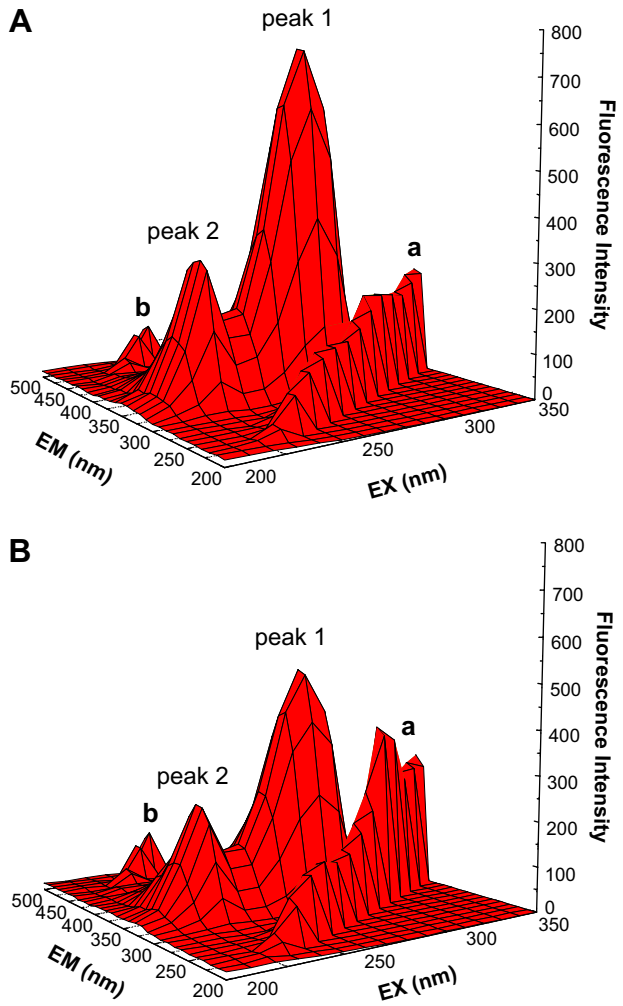

Fig. 10. Three-dimensional fluorescence spectra of lysozyme (A) and the lysozyme–chloramphenicol system (B). (A): c (lysozyme) = 2.0×10^{-6} mol L^{-1}, c (chloramphenicol) = 0; (B): c (lysozyme) = 2.0×10^{-6} mol L^{-1}, c (chloramphenicol) = 2.0×10^{-6} mol L^{-1}. pH = 7.40, T = 298 K.

2.6. Further considerations

Proteins containing two polypeptide chains are termed dimers, and the individual polypeptide chains of which they are composed are termed monomers or subunits. The forces that stabilize these aggregates are hydrogen bonds and electrostatic bonds formed between residues on the surfaces of the polypeptide chains [38]. Lyophilized lysozyme usually has a covalent dimer. There is evidence in the literature [39] that lysozyme can form non-covalent dimers as a function of concentrations. In this study, both hydrophobic forces and hydrogen bonds played major role in lysozyme–chloramphenicol reaction. Therefore, it could be that the state of association changes as the lysozyme concentration is changed. All these aspects have to be taken into account because they affect binding results.

Table 4
Three-dimensional fluorescence spectral characteristics of lysozyme and lysozyme–chloramphenicol system.

Peaks	Lysozyme			Lysozyme–chloramphenicol		
	Peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	Stokes $\Delta\lambda$ (nm)	Intensity F	Peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	Stokes $\Delta\lambda$ (nm)	Intensity F
Fluorescence peak 1	280.0/336.0	56.0	759.6	280.0/338.0	58.0	523.0
Fluorescence peak 2	230.0/333.0	103.0	315.6	230.0/335.0	105.0	246.2

3. Conclusions

In summary, the interaction of chloramphenicol with lysozyme has been studied by fluorescence emission spectra in combination with UV/vis, CD and three-dimensional fluorescence spectroscopy. It was shown that the fluorescence of lysozyme has been quenched for reacting with chloramphenicol and formed a certain kind of new compound. The quenching belongs to static fluorescence quenching type, with non-radiative energy transfer happening within single molecule. The enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be -12.41 kJ mol^{-1} and 37.99 J mol^{-1} K^{-1}, which indicated that hydrophobic force and hydrogen bond were the dominant intermolecular forces in stabilizing the complex. Synchronous fluorescence, CD and three-dimensional fluorescence spectra revealed the chloramphenicol binding to lysozyme under the studied conditions induced small changes in the secondary structure of lysozyme. The binding study of drugs with proteins is of great importance in pharmacy, pharmacology and biochemistry. This study is expected to provide important insight into the interactions of lysozyme with drugs.

4. Materials and methods

4.1. Materials

Lysozyme and chloramphenicol were purchased from Sigma–Aldrich Chemical Company. All other reagents were of analytical reagent grade. The Milli-Q ultrapure water was used throughout the experiments. NaCl (1.0 mol L^{-1}) solution was used to maintain the ionic strength at 0.1. Tris (0.2 mol L^{-1})–HCl (0.1 mol L^{-1}) buffer solution containing NaCl (0.1 mol L^{-1}) was used to keep the pH of the solution at 7.40. Dilutions of the lysozyme stock solution (2.0×10^{-5} mol L^{-1}) in Tris–HCl buffer solution were prepared immediately before use, and the concentration of lysozyme was determined spectrophotometrically using ε_{280} (lysozyme) = 36,500 L mol^{-1} cm^{-1} [40]. The stock solution (2.5×10^{-4} mol L^{-1}) of chloramphenicol was prepared in Tris–HCl buffer solution.

4.2. Apparatus and methods

The UV/vis absorption spectrum was recorded at room temperature on a Lambda-25 double-beam spectrophotometer (Perkin–Elmer, USA) equipped with 1.0 cm quartz cells. All fluorescence spectra were recorded on an F-4500 fluorophotometer (Hitachi, Japan) equipped with 1.0 cm quartz cuvette and a thermostat bath. The excitation and emission slits with a band pass of 5.0 nm were used for all the measurements.

Fluorometric titration experiments: 2.5 mL solution containing appropriate concentration of lysozyme was titrated by successive additions of a 2.5×10^{-4} mol L^{-1} stock solution of chloramphenicol (to give a final concentration of 4.0×10^{-5} mol L^{-1}). Titrations were done manually by using trace syringes, and the fluorescence intensity was measured. All experiments were measured at three temperatures (298, 304 and 310 K). The fluorescence intensity was corrected for absorption of exciting light and re-absorption of the emitted light to decrease the inner filter effect using the relationship [13]:

$$F_{cor} = F_{obs} \times e^{\frac{A_{ex} + A_{em}}{2}} \quad (9)$$

where F_{cor} and F_{obs} are the fluorescence intensities corrected and observed, respectively; and A_{ex} and A_{em} are the absorption of the

systems at the excitation and the emission wavelength, respectively. The intensity of fluorescence used in this paper is the corrected fluorescence intensity.

The three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength was recorded between 200 and 500 nm, the initial excitation wavelength was set to 200 nm with increment of 10 nm, the number of scanning curves was 16, and other scanning parameters were just the same as those of the fluorescence quenching spectra.

Circular dichroism spectra were recorded with a Jasco-810 spectropolarimeter (Jasco, Japan) using a 0.1 cm path length quartz cell. Measurements were taken at wavelengths between 200 and 260 nm with 0.1 nm step resolution and averaged over three scans recorded as a speed of 50 nm min^{-1}, the slit width was set at 5.0 nm. All observed spectra were baseline subtracted for buffer and the α-helical content of lysozyme was calculated on the basis of change of molar ellipticity ($[\theta]$) value at 208 nm using the following equation [41]:

$$\%\ \alpha\text{-helix} = \frac{-[\theta]_{208} - 4,000}{33,000 - 4,000} \tag{10}$$

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