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ORIGINAL PAPER

Investigation of the Binding Between Pepsin and Nucleoside Analogs by Spectroscopy and Molecular Simulation

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Abstract In this paper, the interactions of pepsin with CYD (cytidine) or nucleoside analogs, including FNC (2′-deoxy-2′-β-fluoro-4′-azidocytidine) and CMP (cytidine monophosphate), were investigated by fluorescence, UV–visible absorption and synchronous fluorescence spectroscopy under mimic physiological conditions. The results indicated that FNC (CYD/CMP) caused the fluorescence quenching by the formation of complex. The binding constants and thermodynamic parameters at three different temperatures were obtained. The hydrophobic and electrostatic interactions were the predominant intermolecular forces to stabilize the complex. The F atom in FNC might weaken the binding of nucleoside analog to pepsin. Results showed that CYD was the strongest quencher and bound to pepsin with higher affinity.

Keywords Pepsin · Nucleoside analogs · Interaction · Fluorescence

Introduction

Pepsin is an enzyme expressed as a prototype of zymogen and pepsinogen and is released by the chief cells in the stomach to degrade food proteins into peptides [1]. According to its physiological role, it has a broad substrate specifcity but preferentially cleaves its substrates at large hydrophobic residues [2]. Pepsin is a member of the large family of aspartic proteases. These enzymes are of great medical and pharmaceutical interest because some of them play important roles in the development of various human diseases (e.g., hypertension), in the formation of gastric ulcers, in HIV viral maturation, and as a prognostic tool for breast tumor invasiveness [3]. Pepsin is fluorescencable because of tyrosine, tryptophan and phenylalanine residues.

Cytidine and its analogues (FNC, CMP) have important pharmacology functions. As shown in Fig. 1, a similar main structure is found in these nucleoside analogs. Cytidine is a nucleoside molecule that is formed when cytosine is attached to a ribose ring (also known as a ribofuranose) via a β-N1-glycosidic bond. Cytidine is a component of RNA [4]. There are a variety of cytidine analogs with potentially useful pharmacology. FNC is a novel cytidine analogue. It is an excellent substrate for deoxycytidine kinase and is phosphorylated with higher efficiency than deoxycytidine [5]. Recent studies have demonstrated that FNC is a highly potent and selective inhibitor of HCV [6]. Cytidine monophosphate, also known as 5′-cytidylic acid or simply cytidylate, is a nucleotide that is used as a monomer in RNA [7]. It is an ester of phosphoric acid with the nucleoside cytidine. CMP consists of the phosphate group, the pentose sugar ribose, and the nucleobase cytosine.

For the first time, this work is designed to demonstrate the binding interactions between FNC (CYD/CMP) and pepsin at different temperatures using spectroscopic methods.

Experimental

Apparatus

All fluorescence spectra were recorded on 970CRT fluorescence spectrophotometer (SANCO, Shanghai, China) equipped with 1.0 cm quartz cell. UV/vis absorption spectra were measured at room temperature with a T6 Xinshiji UV-spectrophotometer (XINKE, Shenyang, China) equipped with 1.0 cm quartz cell. pH values were measured with a pHS-3C acidity meter (Jingke, Shanghai, China). The widths of the

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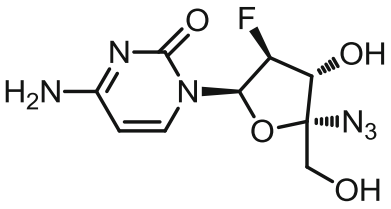
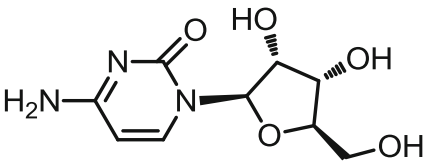
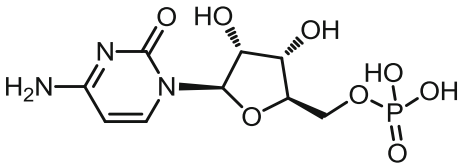


FNC (2'-deoxy-2'-β-fluoro-4'-azidocytidine)



CYD (cytidine)



CMP (cytidine monophosphate)

Fig. 1 Molecular structures of FNC (2′-deoxy-2′-β-fluoro-4′-azidocytidine), CYD (cytidine) and CMP (cytidine monophosphate)

excitation and the emission slits were set to 10.0 nm/10.0 nm for pepsin, respectively.

Reagents

CYD, CMP and pepsin were purchased from Aladdin chemistry Co. Ltd. HCl-CH_3COONa buffer (0.2 mol/L) was used to keep the pH value at 1.6, which is to provide physiological conditions (in stomach) for the chemical reactions. FNC was synthesized in our lab. The deionized water was used throughout whole experiment.

Stock solutions: FNC (2.0×10^{-3} mol/L), CYD (2.0×10^{-3} mol/L), CMP (2.0×10^{-3} mol/L), and pepsin (1.0×10^{-4} mol/L) were prepared in 50 mL volumetric flasks, respectively. All solutions were stored in refrigerator at 4 °C in dark.

Procedures and Methods

Pepsin (0.6 mL 1.0×10^{-4} mol/L) solution, 1.0 mL HCl–CH_3COONa buffer and a certain volume of FNC/CYD/CMP were added into a 4 mL volumetric quartz cell. Deionized water was added to dilute the mixture to 3 mL. The fluorescence intensities of all solutions were measured (excitation at 285 nm and emission wavelengths of 300–450 nm) at three temperatures (298 K, 308 K and 318 K), respectively. Synchronous fluorescence spectra were obtained by setting $\Delta\lambda$=60 nm and $\Delta\lambda$=15 nm.

Results and Discussion

UV/vis Absorption Spectra

UV/vis absorption measurement is a simple method and applicable to explore the structural changes and the complex formation [8]. Figure 2a-c showed the absorption spectra of (a) buffer+pepsin; (b) buffer+pepsin+drugs; (c) buffer; (d) buffer+drugs; (a-c) the absorption spectra of pepsin in the absence of drugs and (b-d) the absorption spectra of pepsin in the presence of drugs.

Spectra (a-c) and (b-d) should be identical in Fig. 2a–c if no interaction occurred between pepsin and drugs. But as we can see there is an obvious different between (a-c) and (b-d). Results indicated that there exists interaction between nucleoside analogs and pepsin.

The UV/vis absorption spectrum of pepsin shows a strong band in the near-UV region with a maximum at 211 nm, which appears due to peptide bond absorption of tryptophan [8].

Quenching Studies of Pepsin

In terms of biological macromolecules, some information of the binding between small molecules and protein, instants of the binding mechanism, binding sites, binding constants and inter-molecular distance, has been given by fluorescence measurements [9].

The intrinsic fluorescence of proteins can provide information about their structure and dynamics, and it is often used to study the folding and associated reactions of protein [10]. Pepsin consists of 327 amino acids residues and is a multi-tryptophan protein that contains five tryptophan (Trp) residues (Trp 39, Trp141, Trp181, Trp190, and Trp300). These Trp residues can be used as intrinsic fluorophores to provide information on the pepsin-ligand interaction and ligand-induced conformational change [11].

Figure 3 shows the fluorescence emission spectra of pepsin in the absence and presence of increasing amount of nucleoside analogs and cytidine. The main fluorescence peak of pepsin was at about 343 nm. Nucleoside analogs and cytidine were non-fluorescent. The fluorescence intensity of pepsin decreased regularly with the increasing nucleoside analogs and cytidine concentration, which indicates that nucleoside analogs and cytidine may interact with pepsin.

Generally speaking, the fluorescence quenching types often include static and dynamic quenching. In order to obtain a clear insight into the quenching mechanism, the fluorescence quenching data were analyzed by the Stern–Volmer equation [12]:

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

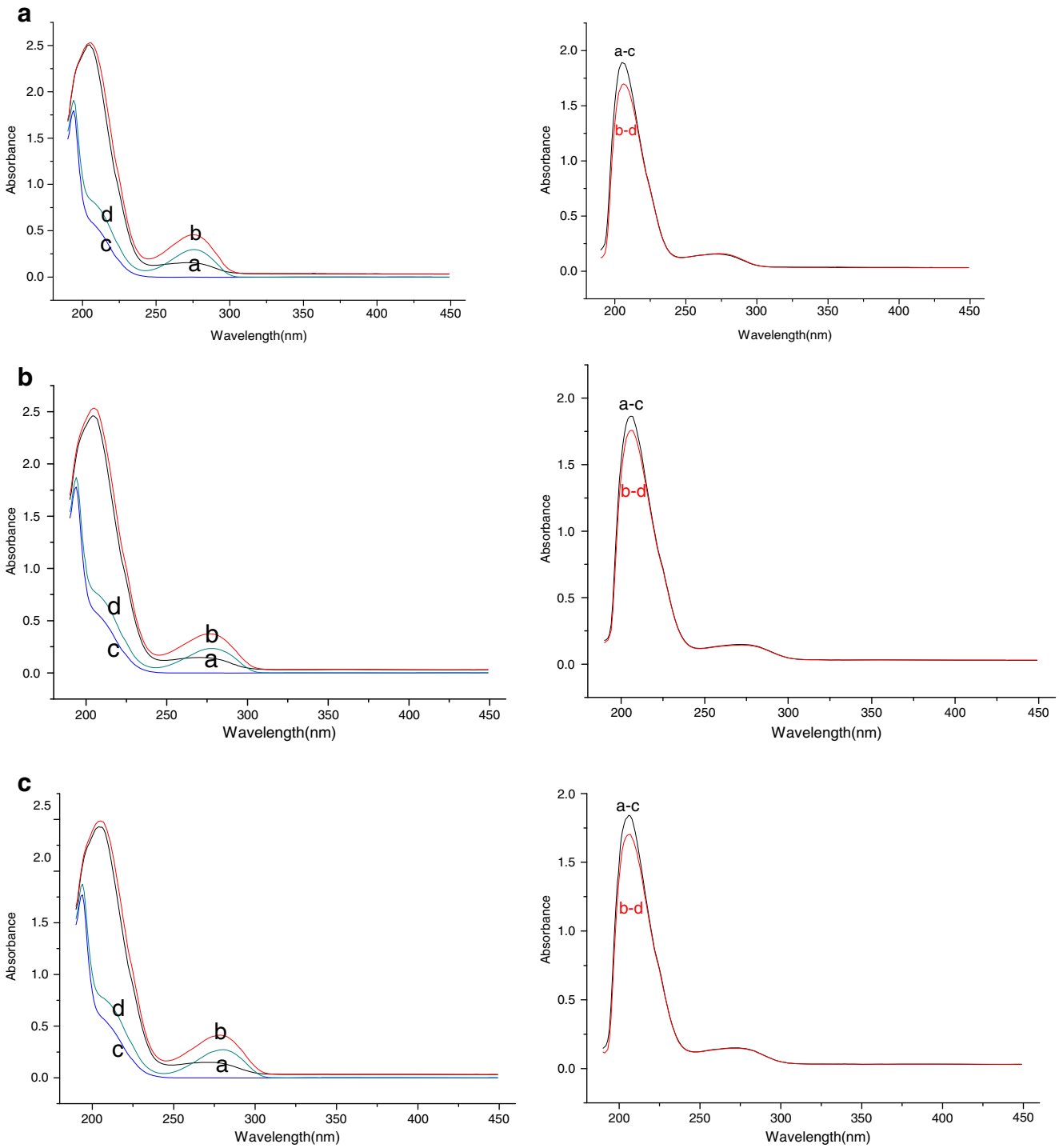


Fig. 2 A. The UV absorption spectra of pepsin-FNC system. Absorption spectra of (a) Buffer+pepsin (2.0×10^{-5} mol/L); (b) Buffer+pepsin (2.0×10^{-5} mol/L)+FNC (2.0×10^{-5} mol/L); (c) Buffer; (d) Buffer+FNC (2.0×10^{-5} mol/L). B. The UV absorption spectra of pepsin-CYD system. Absorption spectra of (a) Buffer+pepsin (2.0×10^{-5} mol/L); (b) Buffer+pepsin (2.0×10^{-5} mol/L)+CYD (2.0×10^{-5} mol/L); (c) Buffer; (d) Buffer+CYD (2.0×10^{-5} mol/L). C. The UV absorption spectra of pepsin-CMP system. Absorption spectra of (a) Buffer+pepsin (2.0×10^{-5} mol/L); (b) Buffer+pepsin (2.0×10^{-5} mol/L)+CMP (2.0×10^{-5} mol/L); (c) Buffer; (d) Buffer+CMP (2.0×10^{-5} mol/L)

F_0 and F are the fluorescence intensities of pepsin in the absence and presence of the quencher, respectively. Kq is the quenching rate constant of protein, and 2.0×10^{10} L mol/L^{-1} s^{-1} is seen as the maximum of dynamic quenching rate constant of protein [13]. Ksv is the Stern–Volmer quenching constant and determined by liner regression of Stern–Volmer equation. τ_0 represents the average lifetime of pepsin without

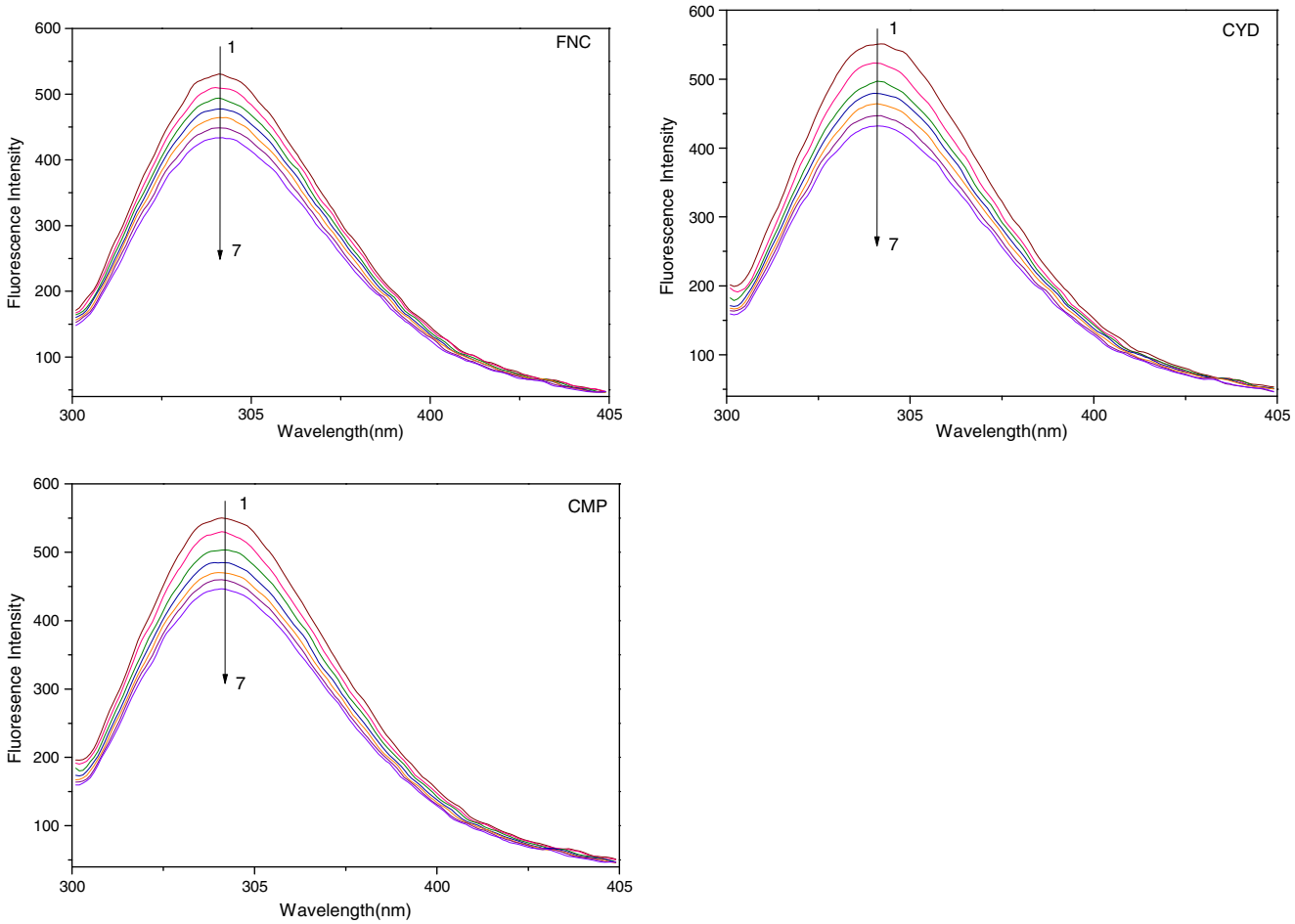


Fig. 3 Effect of FNC (CYD or CMP) on fluorescence spectra of pepsin (T=298 K, pH=1.6, λ_{ex}=285 nm). (1–7) C_{pepsin} =1.67×10^{-5} mol/L, $C_{analogs}$/(μM): 0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 respectively. The concentrations of CYD and analog were same

quencher (usually 10^{-8} s) and [Q] means concentration of quencher.

Within certain concentration, the curve of F_0/F versus [Q] (Stern–Volmer curve) would be linear if the quenching type is single static or dynamic quenching [14]. If the quenching type is combined quenching (both static and dynamic), the Stern–Volmer plot is an upward curvature [15].

Figure 4a–c display the Stern–Volmer plots of the quenching of pepsin fluorescence by FNC, CMP and CYD, respectively. The curves show good linear relationships within the investigated concentrations at different temperatures. Linear Stern–Volmer plots may either reveal the existence of a single type of quenching, or indicate the occurrence of just one binding site for quencher in the proximity of the fluorophore [16]. Ksv and Kq are calculated at corresponding temperature in Table 1. The Ksv values are inversely correlated with temperatures, which indicate that the probable quenching mechanism of fluorescence of pepsin by nucleoside analogs and CYD is not initiated by dynamic collision but complex formation [17]. As a rule, for dynamic quenching, the maximum scatter collision quenching constant of various quenchers is 2.0×10^{10} L mol^{-1} s^{-1}. Kq is approximately 2.0×10^{12} L mol^{-1} s^{-1} for nucleoside analogs and CYD and greater than 2.0×10^{10} L mol^{-1} s^{-1} [18]. Obviously, this indicated that the quenching mechanism of pepsin by nucleoside analogs and CYD is not a dynamic quenching process but a static quenching process. A complex substance was generated by the interaction between pepsin and FNC/CYD/CMP.

The Binding Constants

The Lineweaver–Burk curves were shown in Fig. 4d-f. The curves of $1/(F_0–F)$ versus 1/[Q] were linear. The binding constant (K_A) between pepsin and drugs can be calculated by employing the Lineweaver–Burk equation [19],

$$1/(F_0-F) = 1/F_0 + 1/(K_A F_0[Q]) \quad (2)$$

where F_0 is fluorescence intensities before the addition of FNC (CYD or CMP); F is the fluorescence intensity; [Q] is the concentration of FNC (CYD or CMP). The values of binding constants K_A is determined from the Lineweaver–Burk

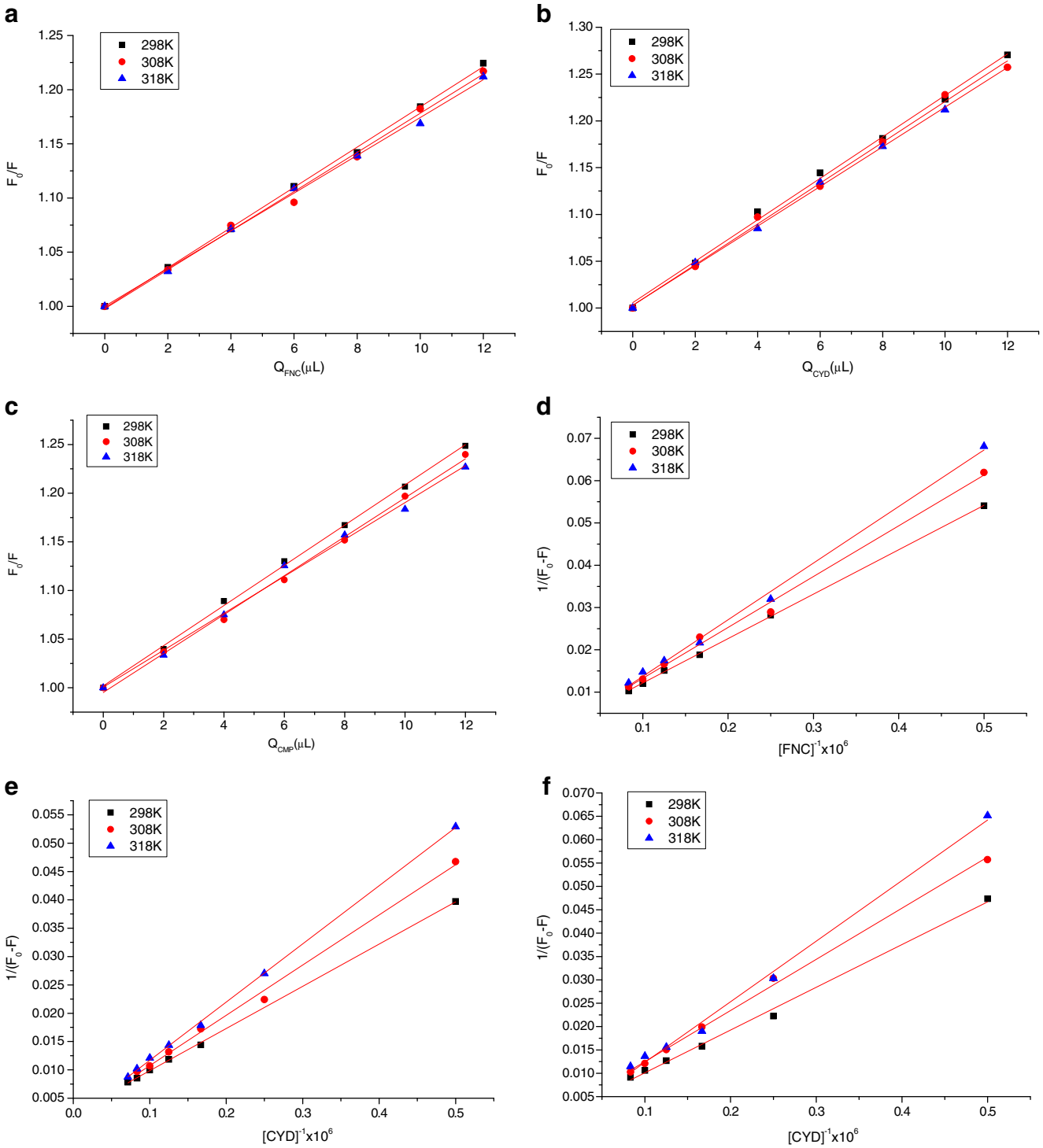


Fig. 4 Stern–Volmer plots (figure A, B and C) and the Lineweaver–Burk curves (figure D, E and F) at different temperature for the quenching of pepsin by FNC, CYD and CMP, respectively. The concentration of pepsin was 1.67×10^{-5} mol/L. pH 1.6, λ_{ex}=285 nm and λ_{em}=344 nm

equation at different temperatures as shown in Table 2. The values of K_A decreased with the increase of temperature. The increasing temperature leads to the lower stability of the complex. Results also showed that the F atom in FNC might weaken the binding of FNC (CYD or CMP) to pepsin.

Thermodynamic Parameters and the Binding Forces

Considering the dependence of binding constant K_A on temperature, a thermo-dynamic process was considered to be responsible for the formation of a complex. Therefore,

Table 1 Stern–Volmer quenching constants of the system at different temperature.

T(K)	EQUATION	R_2	$10^{-4}K_{SV}$(L/MOL)	10^{-12}Kq(L/MOL)
FNC				
298	$F_0/F=1.86\times10^4[Q]+0.9983$	0.9988	1.86	1.86
308	$F_0/F=1.81\times10^4[Q]+0.9976$	0.9954	1.81	1.81
318	$F_0/F=1.74\times10^4[Q]+0.9999$	0.9980	1.74	1.74
CYD				
298	$F_0/F=2.21\times10^4[Q]+1.0057$	0.9970	2.21	2.21
308	$F_0/F=2.18\times10^4[Q]+1.0029$	0.9966	2.18	2.18
318	$F_0/F=2.12\times10^4[Q]+1.0030$	0.9982	2.12	2.12
CMP				
298	$F_0/F=2.07\times10^4[Q]+1.0019$	0.9986	2.07	2.07
308	$F_0/F=2.00\times10^4[Q]+0.9951$	0.9974	2.00	2.00
318	$F_0/F=1.90\times10^4[Q]+1.0006$	0.9946	1.90	1.90

thermodynamic parameters such as enthalpy change (ΔH^0), entropy change (ΔS^0) and free energy change (ΔG^0) were used to characterize the interactions between nucleoside analogs and pepsin.

There are essentially four non-covalent binding types that could play a role in ligand binding to proteins, which embrace hydrogen bond, Van Der Waals forces, hydrophobic interaction force and electrostatic force [20]. On the premise of mild fluctuation of ΔH^0 over the studied temperature range, ΔH^0, ΔS^0 and ΔG^0 can be calculated by the Van't Hoff equation (Eq. (3)) and thermodynamic equation (Eq. (4)) [21],

$$\ln K_A = -\Delta H^0/RT + \Delta S^0/R \tag{3}$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{4}$$

K_A is the binding constant at a certain T. R is the universal gas constant (8.314 J mol^{-1} K^{-1}). The temperatures were chosen at 298, 308 and 318 K at which pepsin did not undergo any structural degradation. According to the binding constants at the three temperatures, the thermodynamic parameters were determined from linear Van't Hoff plot (Fig. 5.) and were presented in Table 2. (The plot of ln K_A versus 1/T gave a straight line according to the Van't Hoff equation).

Masaki Otagiri summed up the thermodynamic law of the interaction that may take place in protein association processes [22]. According the views of Masaki, the positive ΔH^0 and ΔS^0 values are associated with hydrophobic interaction. The negative ΔH^0 and ΔS^0 values are associated with hydrogen bonding and van der Waals interaction in low dielectric medium. Finally very low positive or negative ΔH^0 and positive ΔS^0 values are characterized by electrostatic interactions [23]. Thus it is difficult to interpret the thermodynamic parameters of nucleoside analogs–pepsin interaction with a single intermolecular force. As can be seen from Table 2, the negative signs for ΔG^0 and ΔH^0 indicate the spontaneity and exothermicity of the binding of nucleoside analogs with pepsin. ΔS^0 is a positive value. A positive ΔS^0 value is frequently taken as evidence for hydrophobic interaction, because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic interaction. Negative ΔH^0 value cannot

Table 2 Thermodynamic parameters of pepsin–FNC (CYD or CMP) interactions

T(K)	$10^{-4}K_A$(L/mol)	R^2	ΔH^0(kJ/mol)	ΔG^0(kJ/mol)	ΔS^0(J/mol)
FNC					
298	1.7862	0.9996	−4.631	−24.257	
308	1.6763	0.9950		−24.908	65.85
318	1.5882	0.9970		−25.574	
CYD					
298	2.4410	0.9996	−6.651	−25.030	
308	2.2418	0.9964		−25.652	56.50
318	2.0617	0.9994		−26.264	
CMP					
298	1.9859	0.9962	−7.695	−24.519	
308	1.8167	0.9980		−25.113	65.85
318	1.6332	0.9950		−25.648	

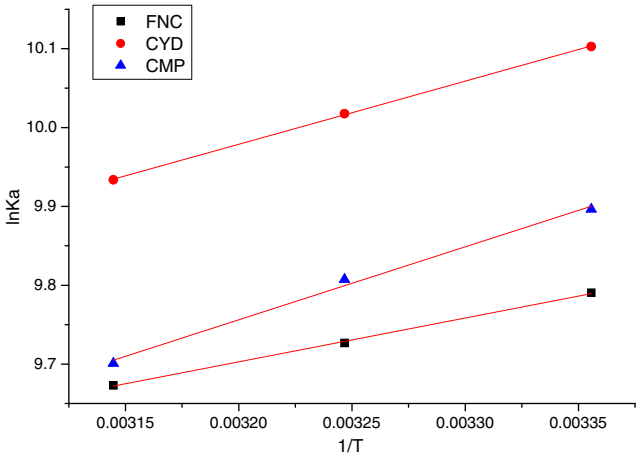


Fig. 5 Van't Hoff plot for the binding of pepsin to FNC (CYD or CMP)

be attributed to electrostatic interactions since for electrostatic interactions, ΔH^0 is very small, almost zero [24]. Accordingly, it is not possible to account for the thermodynamic parameters of pepsin–FNC (CYD or CMP) on the basis of a single intermolecular force model [25]. We inferred that hydrophobic interaction might play the major role in the interactions of FNC (CYD or CMP) with pepsin, and electrostatic interaction was possibly also involved in the binding process.

Energy Transfer

According to Förster's theory [26], energy transfer is likely to happen under the following conditions: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, and (iii) the distance between the donor and the acceptor is less than 7 nm. Here the donor and acceptor were pepsin and nucleoside analogs respectively. There was a spectral overlap between the fluorescence emission spectrum of free pepsin and absorption UV/vis spectra of FNC (CYD or CMP) as shwn in Fig. 6. The spectrum ranging from 300 to 400 nm was chosen to calculate the overlapping integral.

According to Förster's theory, the energy transfer efficiency E is defined as the following equation Eq.(5).

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

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \Phi J \quad (6)$$

$$J = \int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda / \int_0^\infty F(\lambda) d\lambda \quad (7)$$

Here, F and F_0 are the fluorescence intensities in the presence and absence of nucleoside analogs, respectively, r is the binding distance between donor and receptor, and R_0 is the

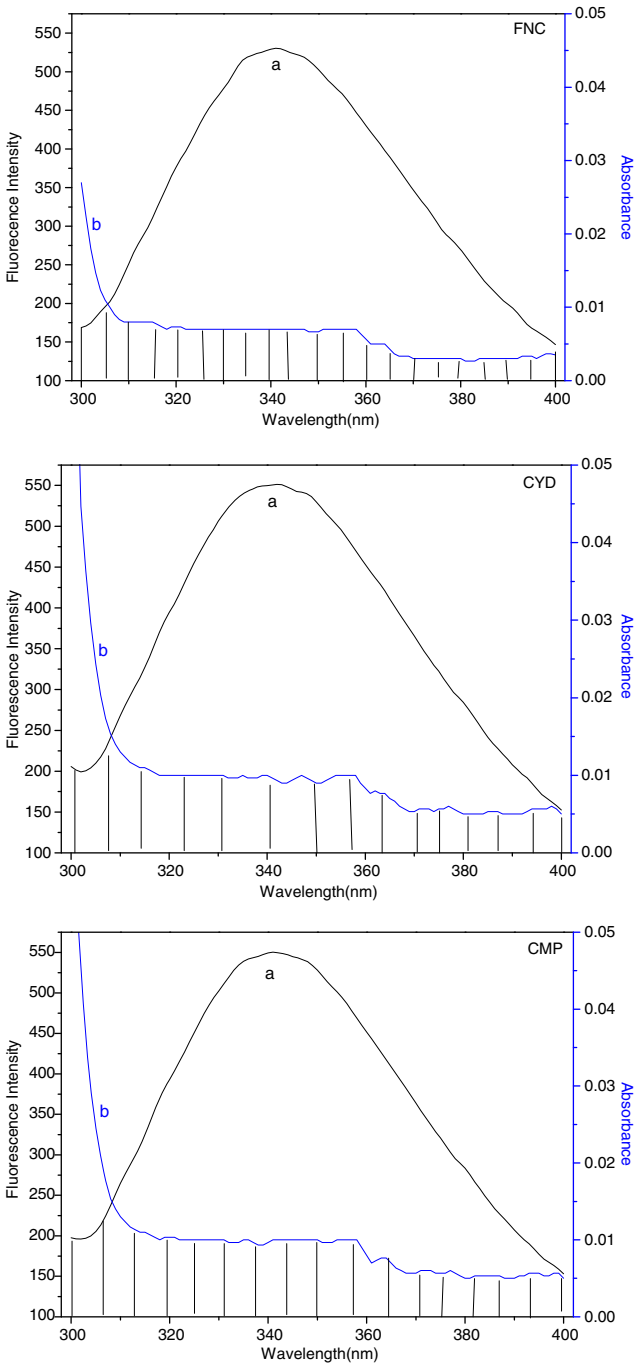


Fig. 6 Overlap of the fluorescence emission of pepsin (a) with the absorption spectrum of FNC (CYD or CMP) (b). $C_{analogs}=C_{pepsin}=1.67\times10^{-5}$ mol/L. The concentrations of CYD and analog were same

critical distance when the efficiency of energy transfer is 50 % [26]. It can be calculated from donor emission and acceptor absorption spectra using the Förster formula Eq. (6). In Eq. (6), 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 Eq. (7), where, F(λ) is the corrected fluorescence intensity of the donor in the wavelength range λ to λ+Δλ; ε(λ) is the extinction coefficient of the acceptor at λ. In the present case, $K^2=2/3$, $N=1.36$, $\Phi=0.146$ [27], according to Eqs. ((5)–(7)), calculated results of J, R_0, E and r were listed in Table 3. The average distance between a donor fluorophore and acceptor fluorophore was less than 7 nm obviously, which indicated that the energy transfer from pepsin to nucleoside analogs occurred with high probability [28], while r was bigger than R_0 in the present study also revealed that FNC (CYD or CMP) could strongly quench the intrinsic fluorescence of pepsin by static quenching [29].

Conformation Investigation

Synchronous fluorescence spectroscopy technique was introduced by Lloyd in 1971 [30]. It involves simultaneous scanning of the excitation and emission mono-chromators while maintaining a constant wavelength interval between them. The synchronous fluorescence spectra can provide information about the molecular environment in a vicinity of the chromospheres molecules and has several advantages, such as sensitivity, spectral simplification, spectral band width reduction and avoiding different perturbing effects [27]. Yuan et al. [31] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum is corresponding to the changes of the polarity around the chromospheres molecule. When the value of Δλ is 60 nm or 15 nm, the synchronous fluorescence gives the characteristic information of tryptophan and tyrosine residues [32]. In order to clearly reveal the quenching mechanism of pepsin with nucleoside analogs, the synchronous fluorescence spectroscopic experiments were carried out.

The synchronous fluorescence spectra of pepsin at various concentrations of nucleoside analogs are shown in Fig. 7a-f. It can be seen that the intensities decrease with the addition of FNC (CYD or CMP) for both tryptophan and tyrosine, but the quenching extents were significantly different. In Fig. 7d-f there is an obvious red-shift on the spectra of tyrosine. It is conveyed that more tyrosine expose to hydrophilic and polar microenvironment [33].

Table 3 The distance parameters of pepsin–FNC (CYD or CMP) interactions

Alkaloid	J(cm^3/mol L^{-1})	R_0(nm)	E	r(nm)
FNC	5.12×10^{-16}	1.53	0.2360	1.86
CYD	7.91×10^{-16}	1.65	0.2726	1.94
CMP	8.00×10^{-16}	1.65	0.2586	1.96

The three-dimensional fluorescence spectra are 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, and the contour spectra can also provide some information [34]. Figure 8. presented the three-dimensional fluorescence contour spectra of pepsin (A), pepsin–nucleoside analogs (B, C, D) and the corresponding projections spectra of pepsin (A'), pepsin–nucleoside analogs (B', C', D'), respectively. The contour map displayed a bird's eye view of the fluorescence spectra. Results showed that three-dimensional fluorescence contour map of pepsin and pepsin–FNC (CYD or CMP) were different obviously. The intensity of one peaks decreased obviously. The phenomena revealed that the interaction of pepsin–FNC (CYD or CMP) induced some microenvironmental and conformational changes in pepsin. A complex between pepsin and FNC (CYD or CMP) was formed [35].

Molecular Simulation

In order for understanding the efficacy of a biologically active drug molecule to function as a therapeutic agent, knowledge of its binding location in the model transport protein environment is very crucial and important. Pepsin is a single chain protein with 327 amino acid residues that consists of three domains. The central domain consists of a six-stranded antiparallel β-sheet that serve as a backbone to the active-site region of the molecular. Molecular modeling is carried out using Autodock 4.2 to investigate whether FNC/CYD/CMP binds to pepsin. The binding mode and binding site are given in Fig. 9. As we can see from Fig. 8 there are MET, PHE, LEU, PRO and ALA five hydrophobic amino acid residues around FNC (CYD or CMP), which means hydrophobic interaction play the major role in the interactions of FNC (CYD or CMP) with pepsin. These results are in agreement with the thermodynamic analysis.

Comparison

The bindings between pepsin and small molecules have been reported, such as Ligupurpuroside A [36], nobiletin [37], silybin [38], chlorogenic acid [39], prulifloxacin [40], fleroxacin [41], and bisphenol A [42].

A static quenching mechanism has been found between pepsin and many small molecules in this study and published work [36–42]. Hydrophobic and electrostatic interactions played major role in the binding process of pepsin with FNC (CYD or CMP), silybin [38], nobiletin [37], and fleroxacin [41]. Van der Waals' forces and hydrogen bonds were

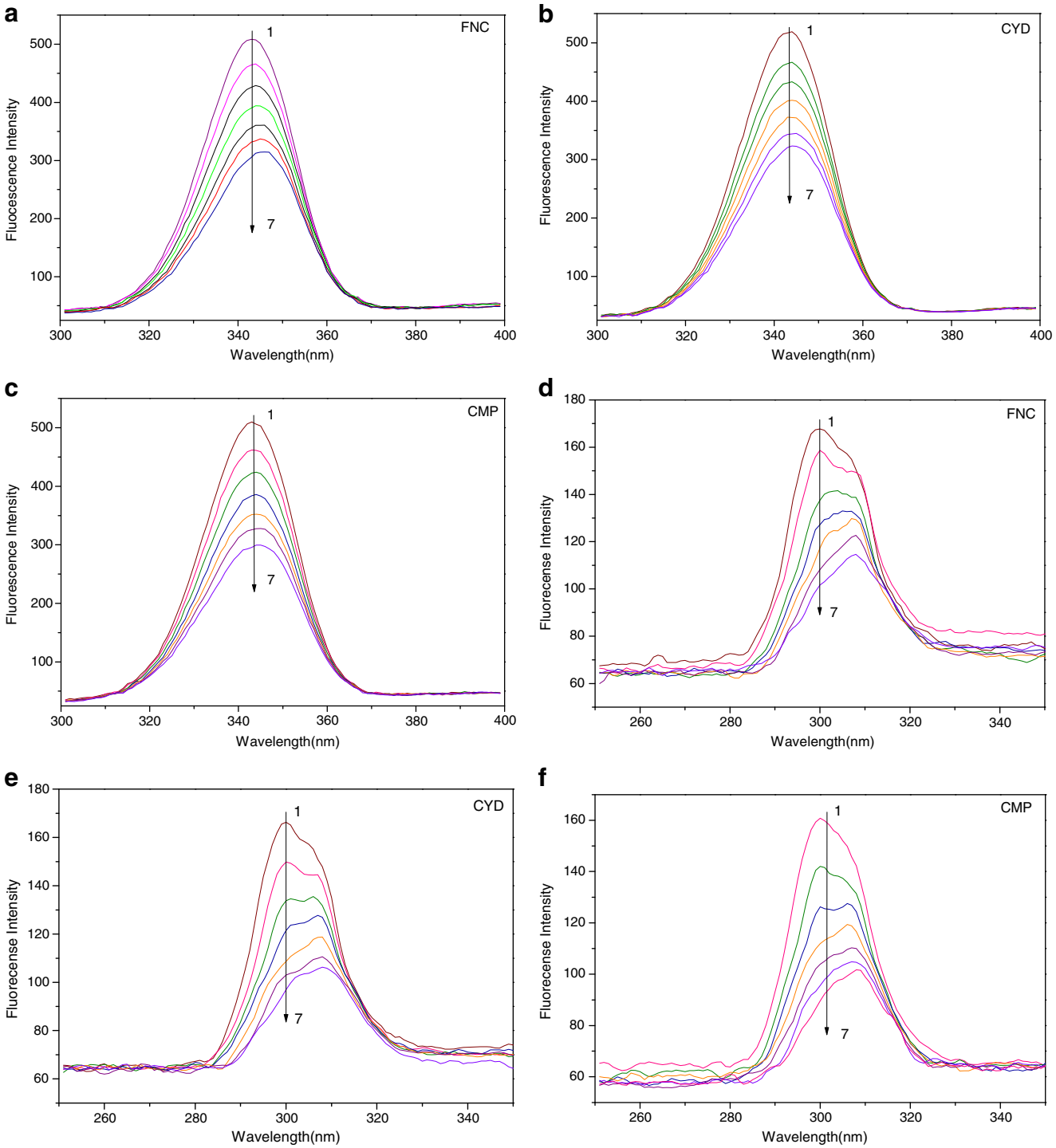
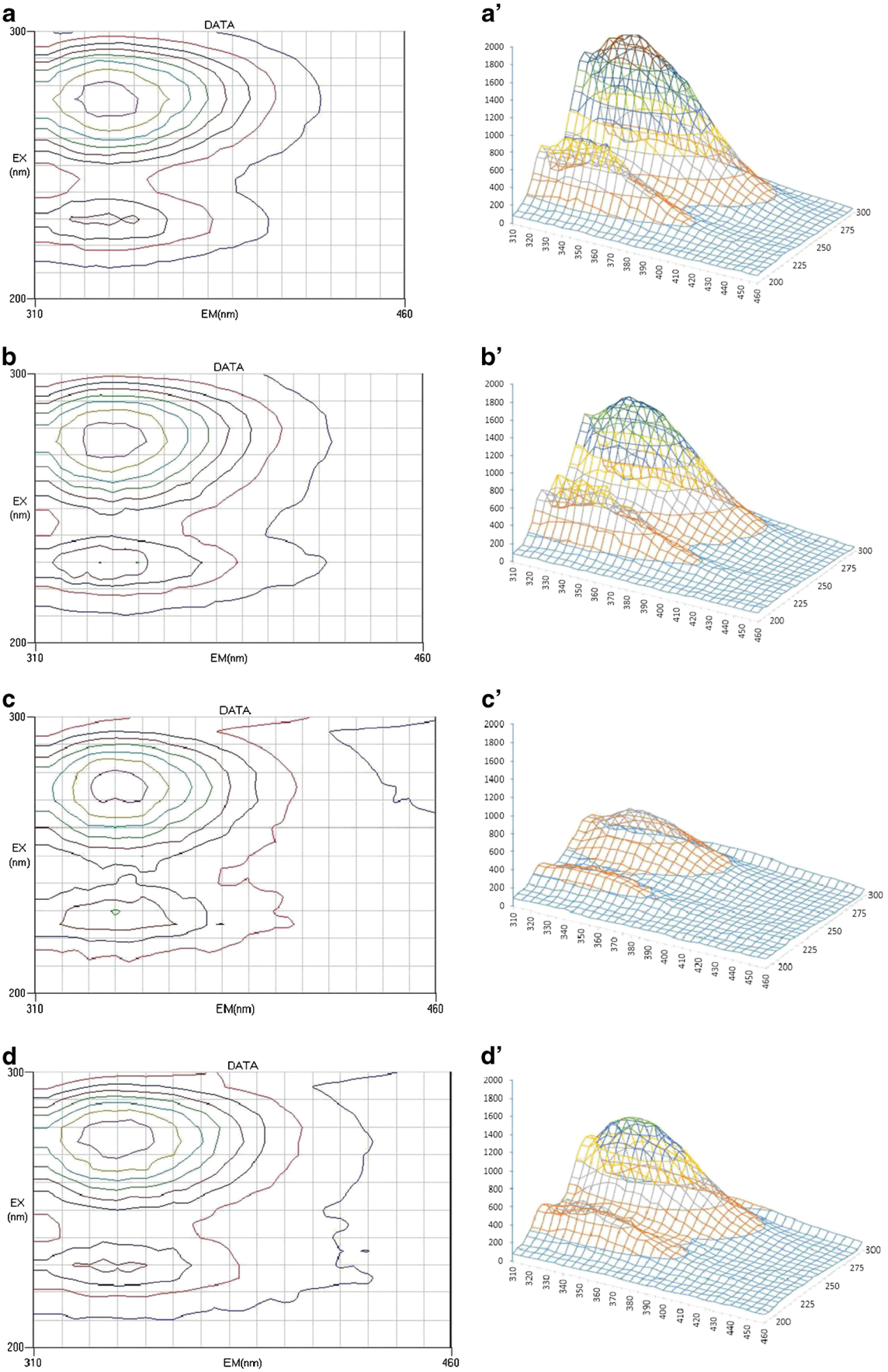


Fig. 7 Influence of FNC (CYD or CMP) on synchronous fluorescence spectra of pepsin. Conditions: $\Delta\lambda$=60 nm (figures A, B and C), pH 1.6, T=298 K. From 1 to 7: C_{pepsin} =1.67×10^{-5} mol/L, $C_{analogs}$=0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0×10^{-6} mol/L. Conditions: $\Delta\lambda$=15 nm (figures D, E and F), pH 1.6, T=298 K. From 1 to 7: C_{pepsin}=5.0×10^{-5} mol/L, $C_{analogs}$=0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0×10^{-5} mol/L. The concentrations of CYD and analog were same

observed for chlorogenic acid [39] and prulifloxacin [40]. Hydrophobic interaction and hydrogen bond were observed for the interaction of pepsin with Ligupurpuroside A [36] or bisphenol A [42].

Different binding constants between pepsin and some small molecules have been reported [36–42]. The binding constants for FNC (CYD or CMP) obtained in this work were at the order of 10^4 L/mol and similar results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◀ **Fig. 8** The three-dimensional fluorescence contour spectra of pepsin (A), FNC–pepsin (B), CYD–pepsin (C), CMP–pepsin (D), and the corresponding projections spectra of pepsin (A'), FNC–pepsin (B'), CYD–pepsin (C'), CMP–pepsin (D'). The concentration of pepsin and FNC/CYD/CMP is 1.67×10^{-5} mol/L

were reported for silybin [38]. The binding constants for bisphenol A was 10^3 L/mol [42]. The binding constants of 10^6 L/mol were observed by Ligupurpuroside A [36] and fleroxacin [41]. The binding constants of 10^5 L/mol were reported for nobiletin [37] and chlorogenic acid [39], respectively. The binding constant for prulifloxacin was 10^8 L/mol [40].

Conclusion

The present study has provided insight into the interactions between pepsin and FNC (CYD or CMP), respectively. The results showed that nucleoside analogs and cytidine could bind to pepsin by a static quenching and form a new complex which becomes unstable with the rising temperature. Hydrophobic and electrostatic interactions played a major role in stabilizing the complex. The influence of molecular structure on the binding aspects was reported. Results showed that CYD was the strongest quencher and bound to pepsin with higher affinity than FNC and CMP. The F atom in FNC might weaken the binding of nucleoside analog to pepsin. The

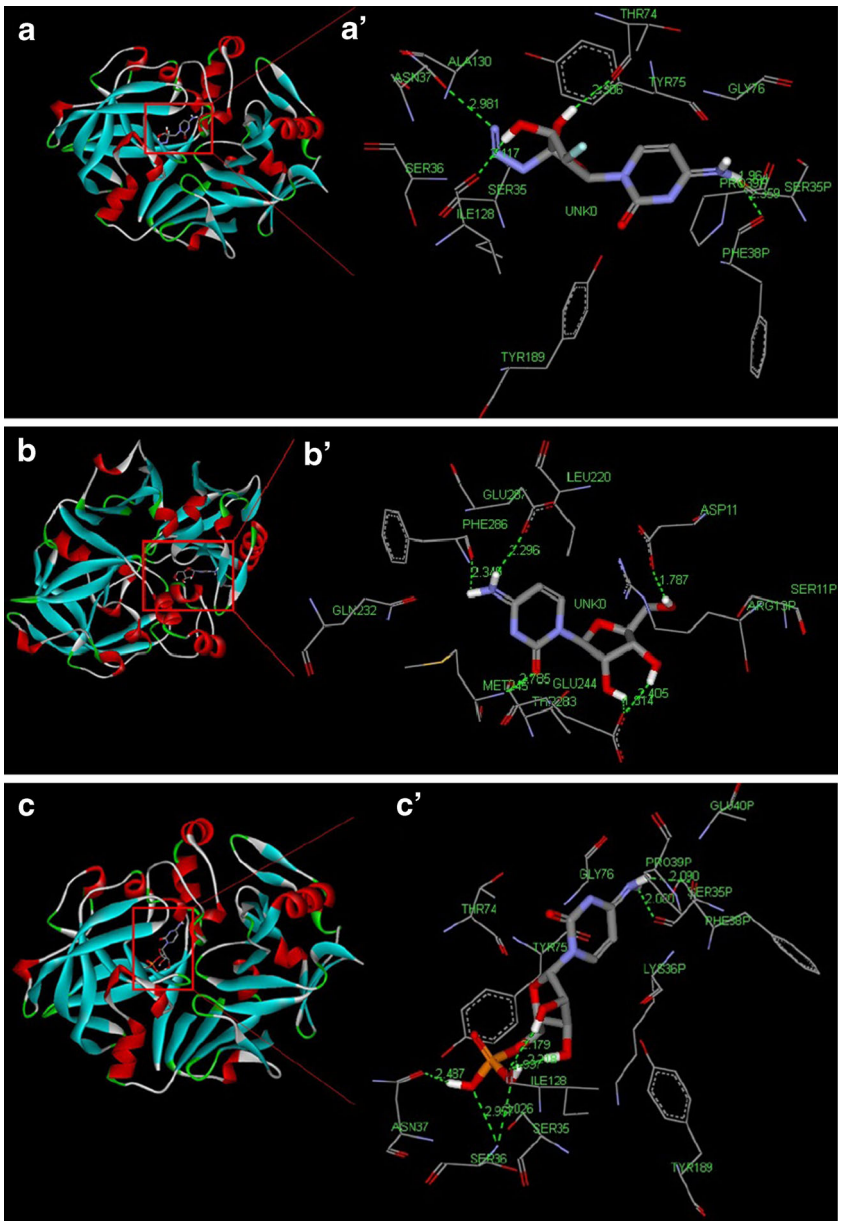

Fig. 9 Stereo view of the docked conformation of the drugs FNC (A), CYD (B) and CMP(C) with pepsin. The lower panels in each case display the protein residues in near vicinity of FNC (A'), CYD (B') and CMP (C') over a molecular surface representation of the protein. The dotted green line denotes hydrogen bond. Color scheme: white for hydrogen atoms, red for oxygen atoms, blue for nitrogen atoms, orange for phosphorus atom and carbon atoms are gray

binding and modeling study could provide useful information on the compatibleness and use of drugs at molecule level. These results will be helpful in understanding the pharmacokinetics and pharmacodynamics of nucleoside analogs and cytidine.

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References

1. Gole A, Dash C, Rao M et al (2000) Encapsulation and biocatalytic activity of the enzyme pepsin in fatty lipid films by selective electrostatic interactions. Chem Commun 4:297–298
2. Fruton JS (2002) A history of pepsin and related enzymes. Q Rev Biol 77:127–147
3. Scorilas A, Diamandis EP, Levesque MA et al (1999) Immunoenzymatically determined pepsinogen C concentration in breast tumor cytosols: an independent favorable prognostic factor in node-positive patients. Clin Cancer Res 5:1778–1785
4. Muramatsu M, Kinoshita K, Fagarasan S et al (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102: 553–563
5. Wang Q, Li Y, Song C et al (2010) Synthesis and anti-HiV acticity of 2′-deoxy-2′-fluoro-4′-C-ethynyl nucleoside analogs. Bioorg Med Chem Lett 20:4053–4056
6. Zheng L, Wang Q, Yang X et al (2012) Antiviral activity of FNC, 2′-deoxy-2′-β-fluoro4′-azidocytidine, against human and duck HBV replication. Antivir Ther 17:679–687
7. Pascal JM (2008) DNA and RNA ligases: structural variations and shared mechanisms. Curr Opin Struct Biol 18:96–105
8. Bi S, Song D, Tian Y et al (2005) Molecular spectroscopic study on the interaction of tetracyclines with serum albumins. Spectrochim Acta A 61:629–636
9. Wang R, Dou H, Yin Y, Xie Y, Sun L, Liu C, Dong J, Huang G, Zhu Y, Song C, Chang J (2014) Investigation of the interaction between isomeric derivatives and human serum albumin by fluorescence spectroscopy and molecular modeling. J Lumin 154:8–14
10. He W, Dou H, Zhang L, Wang L, Wang R, Chang J (2014) Spectroscopic study on the interaction of trypsin with bicyclol and analogs. Spectrochim Acta A 118:510–519
11. Wang R, Xie Y, Zhang Y et al (2013) Comparative study of the binding of pepsin to four alkaloids by spectrofluorimetry. Spectrochim Acta A 108:62–74
12. Lakowicz JR (1999) Principles of Fluorescence Spectroscopy, 2nd edn. Plenum Press, New York
13. Gerbanowski A, Malabat C, Rabiller C et al (1999) Grafting of aliphatic and aromatic probes on rapeseed 2S and 12S proteins: influence on their structural and physicochemical properties. J Agric Food Chem 47:5218–5226
14. Boaz H, Rollefson GK (1950) The quenching of fluorescence. Deviations from the Stern-Volmer law. J Am Chem Soc 72:3435–3443
15. Wagner PJ, Kochevar I (1968) Triplet energy transfer. III. How efficient is diffusion-controlled triplet energy transfer. J Am Chem Soc 90:2232–2238
16. Wang Y, Zhang H, Zhou Q et al (2009) A study of the binding of colloidal Fe_3O_4 with bovine hemoglobin using optical spectroscopy. Colloids Surf A 337:102–108
17. Stedmon CA, Markager S, Bro R (2003) Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. Mar Chem 82:239–254
18. Huang Y, Zhang Z, Zhang D et al (2001) Flow-injection analysis chemiluminescence detection combined with microdialysis sampling for studying protein binding of drug. Talanta 53: 835–841
19. Hu YJ, Liu Y, Shen XS et al (2005) Studies on the interaction between 1-hexylcarbamoyl-5-fluorouracil and bovine serum albumin. J Mol Struct 738:143–147
20. Šoltés L, Mach M (2002) Estimation of drug–protein binding parameters on assuming the validity of thermodynamic equilibrium. J Chromatogr B 768:113–119
21. Becktel WJ, Schellman JA (1987) Protein stability curves. Biopolymers 26:1859–1877
22. Kragh-Hansen U, Chuang VTG, Otagiri M (2002) Practical aspects of the ligand-binding and enzymatic properties of human serum albumin. Biol Pharm Bull 25:695–704
23. Ghuman J, Zunszain PA, Petitpas I et al (2005) Structural basis of the drug-binding specificity of human serum albumin. J Mol Biol 253: 38–52
24. Kandagal PB, Ashoka S, Seetharamappa J et al (2006) Study of the interaction of an anticancer drug with human and bovine serum albumin: spectroscopic approach. J Pharm Biomed Anal 41:393–399
25. Tian J, Liu J, He W et al (2004) Probing the binding of scutellarin to human serum albumin by circular dichroism, fluorescence spectroscopy, FTIR, and molecular modeling method. Biomacromolecules 5: 1956–1961
26. Sklar LA, Hudson BS, Simoni RD (1977) Conjugated polyene fatty acids as fluorescent probes: binding to bovine serum albumin. Biochemistry 16:5100–5108
27. Stratikos E, Gettins PGW (1999) Formation of the covalent serpin-proteinase complex involves translocation of the proteinase by more than 70 Å and full insertion of the reactive center loop into β-sheet A. Proc Natl Acad Sci 96:4808–4813
28. Valeur B, Berberan-Santos MN (2013) Molecular fluorescence: principles and applications. Wiley, New York
29. He W, Li Y, Xue C et al (2005) Effect of Chinese medicine alpinetin on the structure of human serum albumin. Bioorg Med Chem 135: 1837–1845
30. Lloyd JBF (1971) Synchronized excitation of fluorescence emission spectra. Nature 231:64–65
31. Yuan T, Weljie AM, Vogel HJ (1998) Tryptophan fluorescence quenching by methionine and selenomethionine residues of calmodulin: orientation of peptide and protein binding. Biochemistry 37: 3187–3195
32. Tang J, Luan F, Chen X (2006) Binding analysis of glycyrrhetinic acid to human serum albumin: Fluorescence spectroscopy, FTIR, and molecular modeling. Bioorg Med Chem 14:3210–3217
33. Congdon RW, Muth GW, Splittgerber AG (1993) The binding interaction of Coomassie blue with proteins. Anal Biochem 213:407–413
34. Wang R, Chai Y, Wang R et al (2012) Study of the interaction between bovine serum albumin and analogs of Biphenyldicarboxylate by spectrofluorimetry. Spectrochim Acta A 96:324–331
35. Ni Y, Su S, Kokot S (2002) Spectrofluorimetric studies on the binding of salicylic acid to bovine serum albumin using warfarin and ibuprofen as site markers with the aid of parallel factor analysis. Anal Chim Acta 580:206–215
36. Shen L, Xu H, Huang F, Li Y, Xiao H, Yang Z, Hu Z, He Z, Zeng Z, Li Y (2015) Investigation on interaction between Ligupurpuroside A and pepsin by spectroscopic and docking methods. Spectrochim Acta A 135:256–263

37. Zeng HJ, Qi T, Yang R, You J, Qu LB (2014) Spectroscopy and molecular docking study on the interaction behavior between nobiletin and pepsin. J Fluoresc 24:1031–1040
38. Zeng HJ, You J, Liang HL, Qi T, Yang R, Qu LB (2014) Investigation on the binding interaction between silybin and pepsin by spectral and molecular docking. Int J Biol Macromol 67:105–11
39. Zeng HJ, Liang HL, You J, Qu LB (2014) Study on the binding of chlorogenic acid to pepsin by spectral and molecular docking. Luminescence 29:715–721
40. Huang Y, Yan J, Liu B, Yu Z, Gao X, Tang Y, Zi Y (2010) Investigation on interaction of prulifloxacin with pepsin: a spectroscopic analysis. Spectrochim Acta A 75:1024–1029
41. Lian S, Wang G, Zhou L, Yang D (2013) Fluorescence spectroscopic analysis on interaction of fleroxacin with pepsin. Luminescence 28: 967–972
42. Zhang HM, Cao J, Fei ZH, Wang YQ (2012) Investigation on the interaction behavior between bisphenol A and pepsin by spectral and docking studies. J Mol Struct 1021:34–39