



Mode of encapsulation of Linezolid by β -Cyclodextrin and its role in bovine serum albumin binding



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ABSTRACT

We describe, in this article, the associative interaction between Linezolid and β -Cyclodextrin, and the influence of β -Cyclodextrin on Linezolid's binding to Bovine serum albumin. β -Cyclodextrin forms a 1:1 inclusion complex with Linezolid, with a binding constant value of $3.51 \times 10^2 \text{ M}^{-1}$. The binding is studied using ultraviolet–visible absorption, fluorescence, nuclear magnetic resonance, and rotating-frame overhauser effect spectroscopic techniques. The amide substituent on the oxazolidinone ring of Linezolid is involved in its binding to β -Cyclodextrin. The binding of the Linezolid to bovine serum albumin, in the absence and the presence of β -Cyclodextrin, is studied by analyzing the fluorescence quenching and Förster resonance energy transfer. The Stern–Volmer quenching constant, the binding constant, and energy transfer occurring on the interaction of the Linezolid with BSA are found to be smaller in the presence of β -Cyclodextrin than in water.

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1. Introduction

β -Cyclodextrin (β -CD, Cyclomaltoheptaose) is a truncated cone-shaped macrocyclic oligosaccharide formed by seven glucopyranose units. It has two hydrophilic rims where the hydroxyl groups are located, and a hydrophobic cavity capable of accommodating guest molecules (Enoch & Swaminathan, 2007, 2006a, 2006b; Enoch, Rajamohan, & Swaminathan, 2010). The formation of inclusion complexes between a guest and Cyclodextrin (CD) is driven by the enthalpic contribution, primarily resulting from the hydrophobic interactions between the guest and the host. The hydrophobic internal cavity of Cyclodextrin provides a suitable microenvironment for an apolar guest, if the guest molecule has a suitable size to fit within the cavity (Wenz, Han, & Muller, 2006). The guest molecules carrying polar substituents may interact with the hydroxyl rims of Cyclodextrin forming hydrogen bonds (Enoch & Swaminathan, 2007; Liu, Han, & Zhang, 2004). Moreover, there are about 6.5 water molecules per β -CD ring and the release of these water molecules from the Cyclodextrin cavity into the bulk phase

plays a favorable entropic role in the formation of the inclusion complex (Lindner & Saenger, 1978).

Bovine serum albumin (BSA) is the most abundant plasma protein. It is globular, water-soluble, and has 583 amino acids in a single polypeptide chain. With its ability to bind various types of small molecules, BSA plays an inevitable role in the determination of physiological function (Gani, Mukherjee, & Chatteraj, 1999; Tobitani & Ross-Murphy, 1997). Over the years, various works on the interactions between BSA and small molecules have been reported (Chen, Wu, & Johnson, 1995; Gelamo, Silva, Imasato, & Tabak, 2002; Li, Wang, Chen, & Lu, 2014; Mishra, Barik, Priyadarshini, & Mohan, 2005; Nielsen, Borch, & Westh, 2000; Santos, Zanette, Fischer, & Itri, 2003; Shoba Narayan, Rajagopalan, Reddy, & Chadha, 2014; Turro, Lei, Ananthapadmanabhan, & Aronson, 1995; Vasilescu, Angelescu, Almgren, & Valstar, 1995). Research, on the binding of drugs to serum albumin, aids the comprehension of the drug metabolism and its clinical effectiveness in the human body (Rosso, Gonzalez, Bagatolli, Duffard, & Fidelio, 1998; Romanini, Avale, Farruggia, Nerli, & Pico, 1998).

Although the studies on protein binding of drugs are commonly found in the literature, research work on the influence of β -CD on drug-BSA binding remains rare (Sideris, Koupparis, & Macheras, 1994; Sudha & Enoch, 2011). β -Cyclodextrin improves the stability of drugs, solubilizes water-insoluble drugs, and cover up the smell of medicine. β -Cyclodextrin is used to encapsulate orally

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administered drugs, for instances, Benexate HCl, Piroxicam, and Tiaprofenic acid, although parenteral formulation is difficult with it (Loftsson, Jarho, Måsson, & Järvinen, 2005). Further, the study of the interaction of drug-bound Cyclodextrin to protein is facilitated on using native Cyclodextrins (Sudha, Chandrasekaran, Premnath, & Enoch, 2014), and hence they are preferred over modified Cyclodextrins, because side chains of modified Cyclodextrins can interact with serum albumins. The above points prompted us to choose β -Cyclodextrin for our study.

Moreover, this kind of study could help understanding the mechanism of medicines on protein level to cure diseases. As β -CD is a carrier molecule which transports drugs and aids in their slow and sustained release, it is quite rational to study the stoichiometry of the complexes it forms, their binding strength, and the binding mode, and correlate it to the serum albumin binding ability, in order to understand the pharmacokinetics of the drugs in β -CD encapsulated form.

Linezolid (LZ) is a member of the oxazolidinone class of drugs. It is active against vancomycin-resistant enterococci, and methicillin-resistant *Staphylococcus aureus* (Brickner, 1996). LZ was approved for use in 2000 and it is the first commercially available 1,3-oxazolidinone antibiotic. It inhibits protein synthesis in bacteria, stops their growth and acts as a bacteriostatic agent. Although being a useful drug, it has a few adverse effects, including allergic reactions, pancreatitis, and elevated transaminases (French, 2003). One usually applies CD to effect better solubility and/or less irritation or damage (Stella & He, 2008). Intrigued by the above points, herein we report the β -CD-binding properties of the LZ, and the role of β -CD on the binding of the LZ to BSA.

2. Materials and methods

2.1. Chemicals and solvents

Linezolid was purchased from Sigma-Aldrich, Bangalore, India. Crystalline bovine serum albumin, β -Cyclodextrin, and HEPES were purchased from Hi Media, India. All reagents and solvents (obtained from Merck) were of spectral grade, which were used without further purification. Doubly distilled water was used throughout the experiments.

2.2. Preparation of test solutions

A stock solution (5×10^{-4} mol dm $^{-3}$) of LZ was made with methanol due to LZ's less water solubility. The working solutions were prepared by appropriate dilutions of the stock solutions of LZ, β -CD, and BSA. The test solutions had the concentration of methanol as 1%. HEPES buffer (0.1 M) was used to prepare the stock solution of the BSA (3.0×10^{-5} mol dm $^{-3}$). The binding titration of BSA against LZ was carried out by having the solution of BSA (3.0×10^{-5} mol dm $^{-3}$) and successive addition of different concentrations of LZ viz., 0 , 2.5×10^{-6} , 5.0×10^{-6} , 7.5×10^{-6} , 1.0×10^{-5} , 1.5×10^{-5} , 2.0×10^{-5} , 2.5×10^{-5} , and 3.0×10^{-5} mol dm $^{-3}$. Similarly, the titration of BSA with LZ- β -CD was carried out by keeping the concentration of BSA fixed (3.0×10^{-5} mol dm $^{-3}$) and adding successively different concentrations of LZ viz., 0 , 2.5×10^{-6} , 5.0×10^{-6} , 7.5×10^{-6} , 1.0×10^{-5} , 1.5×10^{-5} , 2.0×10^{-5} , 2.5×10^{-5} , and 3.0×10^{-5} mol dm $^{-3}$ in the presence of β -CD (1.0×10^{-2} mol dm $^{-3}$). Titrations were done by the addition of solutions using a micro-injector. All the experiments were carried out at an ambient temperature of $25 \pm 2^\circ$ C. The test solutions were homogeneous after the addition of all the additives. The absorption and the fluorescence spectra were recorded against appropriate blank solutions.

2.3. Preparation of LZ/ β -CD solid complex

LZ (0.035 g, 2×10^{-3} mol dm $^{-3}$) was dissolved in 5 ml of methanol. Thirty-five milliliter of aqueous β -CD (0.09 g, 2×10^{-3} mol dm $^{-3}$) was prepared separately. A solution of LZ was added slowly to the β -CD solution at room temperature and sonicated in an Ultra-sonicator for 30 min, in order to get a homogenous solution. The mixture was warmed at 50° C for 10 min and then kept at room temperature for two days. The solid obtained (Yield: 94%) was collected and analyzed.

2.4. Instrumentation

A Jasco V-630 double beam UV-Visible spectrophotometer was used to record absorption spectra, using 1 cm path length cells. A Jasco FP-750 spectrofluorimeter, equipped with a 120 W Xenon lamp for excitation, served the measurement of fluorescence. Both the excitation and the emission bandwidths were set up at 5 nm. Ultra-sonicator PCI 9L 250H, India was used for sonication. A two dimensional Rotating-frame Overhauser Effect Spectrum (2D ROESY) of the complex of LZ- β -CD was recorded on a Bruker AV III instrument, operating at 500 MHz, using DMSO- d_6 as the solvent. The mixing time was 200 ms under the spin lock condition. The internal standard used was tetramethylsilane (TMS) and the chemical shift values were obtained downfield from TMS in part per million (ppm). The molecular docking of the guest molecule, LZ with the host (β -CD) and the BSA, was studied using the Schrödinger suite 2013, update 2, Glide 5.5.

3. Results and discussion

3.1. Host-guest association of LZ with β -CD

The absorption spectra of the LZ with various added amounts of β -CD are shown in Fig. 1A. In aqueous solution, LZ shows an absorption maximum at 251 nm (corresponding to the $n-\pi^*$ transition) and, at the addition of β -CD in aliquots, shows a hyperchromic shift. At 1.0×10^{-2} mol dm $^{-3}$ of β -CD, the absorbance of the LZ is enhanced by about 75% from the absorbance of LZ in water. The absorption maximum also shows a red shift of 4 nm at the addition of β -CD. The red shift observed at growing concentrations of β -CD suggests the formation of a complex between LZ and β -CD (Enoch et al., 2010).

The fluorescence spectra of the LZ with various concentrations of β -CD are shown in Fig. 1B. LZ shows a dual fluorescence in water. The lower energy emission (412 nm) is more distinct and intense compared to the high energy emission (314 nm). With the addition of β -CD, LZ shows an enhancement of fluorescence along with a significant blue shift of about 6 nm. The enhancement of fluorescence occurs due to the shielding of LZ from the water environment and suppression of the non-radiative process in the restricted micro-environment provided by Cyclodextrin (Pahari et al., 2013). The blue shift is a consequence of the dislodging of LZ from a polar protic environment to a non-polar, hydrophobic environment offered by the interior of β -CD (Chandrasekaran & Enoch, 2013). The changes in absorption and fluorescence spectra on the addition of β -CD suggests that the formation of the inclusion complex of LZ- β -CD occurs. The equilibrium of the LZ- β -CD binding can be expressed as,



$$K_1 = \frac{[\text{LZ} - \beta\text{-CD}]}{[\text{LZ}][\beta\text{-CD}]} \quad (2)$$

where $[\text{LZ} - \beta\text{-CD}]$ stands for the concentration of the LZ- β -CD complex and K_1 is the equilibrium constant.

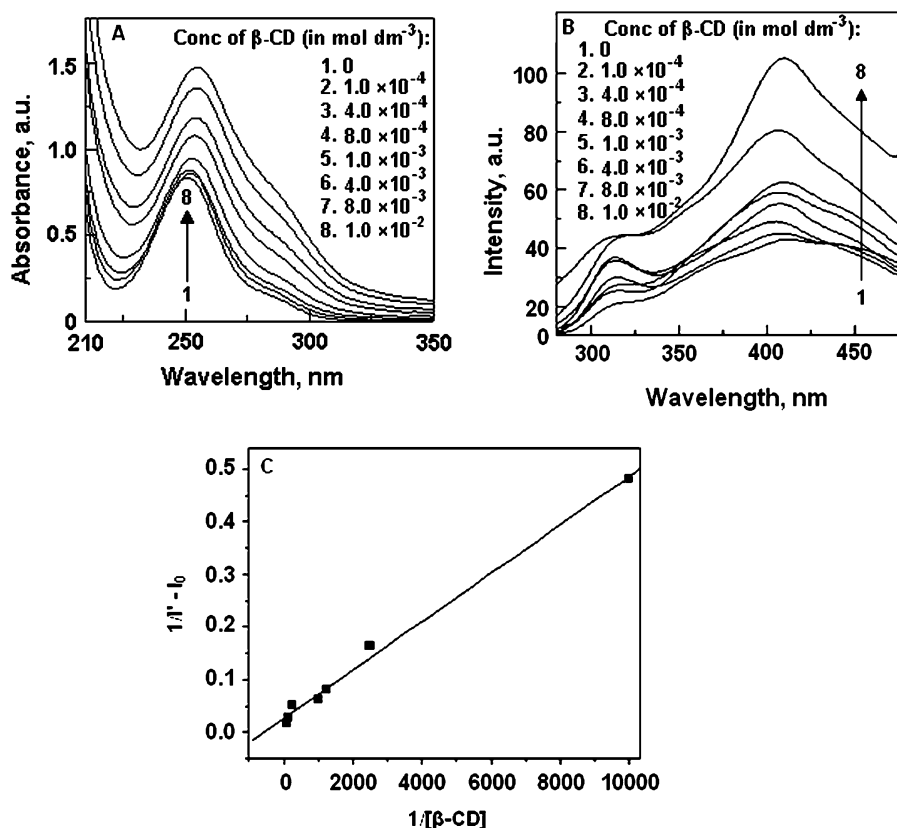


Fig. 1. Absorption spectra of LZ in the presence of various concentrations of β -CD (A). Fluorescence spectra of LZ in the presence of various concentrations of β -CD (B). The plot of $1/I' - I_0$ against $1/[\beta\text{-CD}]$ for the interaction of LZ in the presence of various concentrations of β -CD (C).

The Benesi–Hildebrand relation (Benesi & Hildebrand, 1949) used for plotting the change of intensity on formation of the host–guest complex is,

$$I = \frac{I_0 + I_1 K_1 [\beta\text{-CD}]}{1 + K_1 [\beta\text{-CD}]} \quad (3)$$

where I_0 , I and I' are the fluorescence intensity of the LZ in water and that at a given amount of β -CD, and $[LZ]$ and $[\beta\text{-CD}]$ represents the concentrations of LZ and β -CD, respectively. Rearranging Eq. (3), we get

$$\frac{1}{I - I_0} = \frac{1}{I' + I_0} + \frac{1}{(I' - I_0)K[\beta\text{-CD}]} \quad (4)$$

The Benesi–Hildebrand double reciprocal plot yields a straight line in the event of a 1:1 host: guest complex. Fig. 1C shows that the double reciprocal plot, i.e., $1/(I - I_0)$ vs. $1/[\beta\text{-CD}]$ corresponding to the fluorescence of LZ at various concentrations of β -CD is linear. The variation of $1/(I - I_0)$ vs. $1/[\beta\text{-CD}]^2$, which is a plot made considering a 1:2 complex, does not show a straight line (figure not shown). Hence, it is inferred that only a 1:1 complex is formed between LZ and β -CD in aqueous solution, and the calculated binding constant of the 1:1 complex is $3.51 \times 10^2 \text{ M}^{-1}$ ($R = 0.9967$).

In order to optimize the structure of the LZ– β -CD complex, a 2D ROESY spectrum of the complex was recorded (Chandrasekaran, Sameena, & Enoch, 2014). The ^1H NMR chemical shifts of the LZ, LZ– β -CD complex, and the complexation-induced chemical shift (CIS) values are given in Table 1. The ^1H NMR signals corresponding to the protons of LZ are found shifted in position in the spectrum of the LZ– β -CD complex. The phenyl protons of LZ are deshielded and the other protons indicate the occurrence of more shielding in β -CD complexed form than in its uncomplexed form. The deshielded and shielded proton signals of LZ– β -CD complex NMR resulted in the

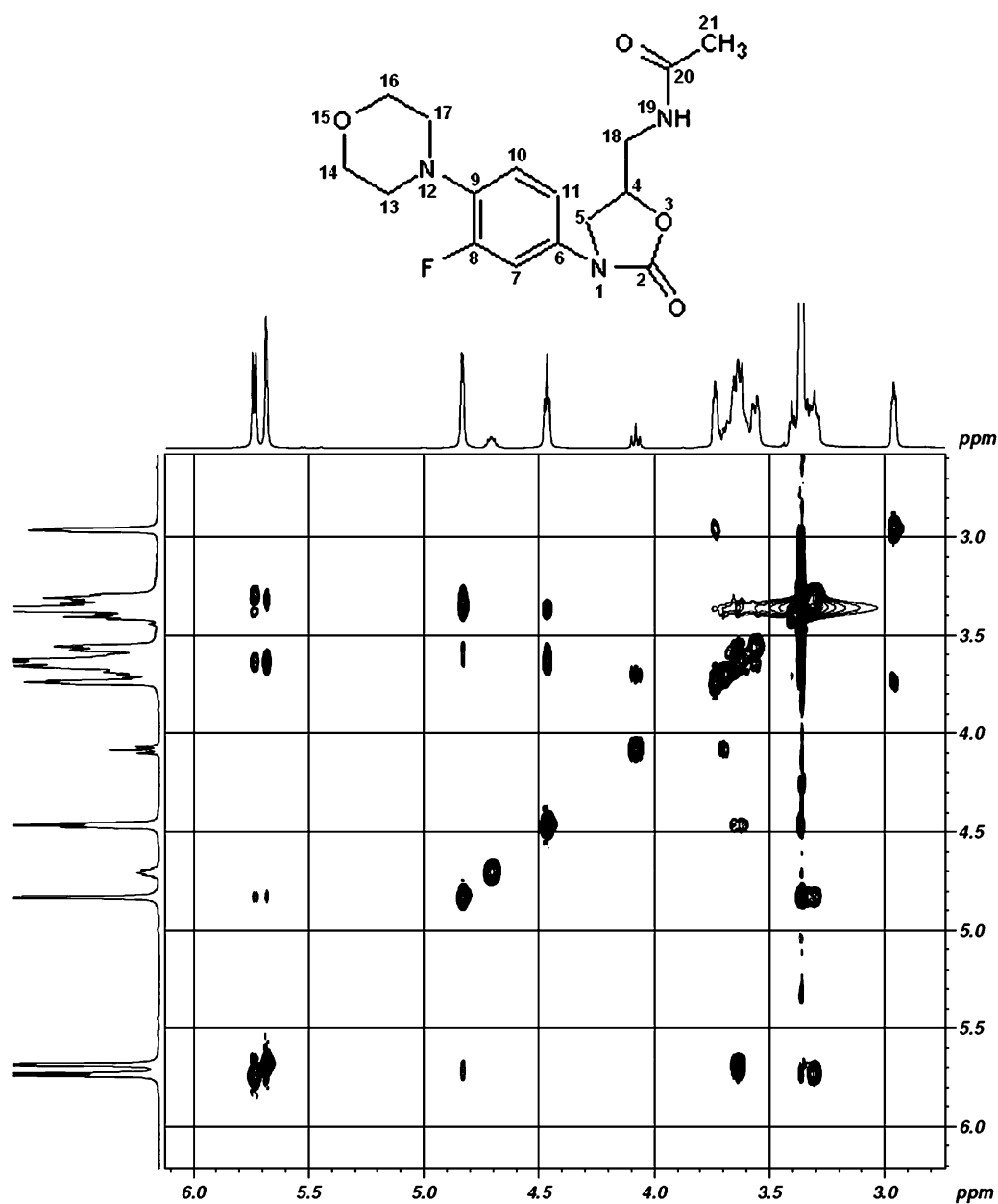
respective negative and positive complexation-induced chemical shift values. The change of the chemical shift values is due to the binding of the LZ to β -CD.

The 2D ROESY NMR spectrum of LZ– β -CD complex is shown in Fig. 2. There are diagonal and off-diagonal peaks observed for the proton signals of LZ– β -CD complex. The cross correlation peaks between the protons of LZ and β -CD suggest that the LZ molecule is accommodated inside the β -CD cavity. A cross peak is observed corresponding to the cross correlation of the methylene protons of the LZ at position 5 and the secondary hydroxyl protons of β -CD. This reveals the inclusion of LZ in the β -CD cavity involving the amide chain of LZ, and the close proximity between the methylene protons (5) and the secondary hydroxyl protons of β -CD located at its outer rim. The methylene protons at the position 18 result in cross peaks due to interaction with the H-1 and the primary hydroxyl protons of β -CD. These cross peaks are obtained because of the possible engulfing of the methylene protons in the amide chain by the β -CD cavity. These H-1 and primary hydroxyl protons exist in the mid and lower rim of the β -CD cup respectively, and the signals correlate with the methylene protons (18) of LZ due to the formation of a host:guest complex.

The mode of LZ– β -CD binding is theoretically studied by molecular docking. The possible mode of interactions between LZ and β -CD are (A) hydrogen bonding, (B) hydrophobic, and (C) electrostatic interactions shown in Fig. 5A, B, and C respectively. The carbonyl group of oxazolidinone ring and the amide group were involved in hydrogen bonding with the hydroxyl protons of β -CD, with a bond length of 1.997 Å and 2.326 Å respectively. The N–H proton of the amide group acts as a hydrogen bond acceptor with the hydroxyl groups of β -CD (1.933 and 2.407 Å). Clearly, the possible electrostatic and hydrophobic interaction could be through the aliphatic amide chain substituted in the oxazolidinone moiety.

Table 1¹HNMR chemical shifts of LZ and LZ-β-CD complex and its Complexation Induced Shift (CIS).

Protons	Position	LZ chemical shift (δ), ppm	LZ-β-CD complex chemical shift (δ), ppm	Complexation Induced Shift (CIS)
CH ₃	21	2.01 (s)	1.83 (s)	0.18
NH	19	4.02 (t)	4.08 (t)	−0.06
CH ₂	5	3.65 (t)	3.56 (t)	0.09
CH ₂	18	3.77 (q)	3.64 (q)	0.13
CH ₂	13 and 17	3.04 (t)	2.96 (t)	0.08
CH ₂	14 and 16	3.85 (t)	3.73 (t)	0.12
CH	4	4.78 (m)	4.70 (m)	0.08
Aromatic protons				
CH	7	6.90 (t)	7.06 (t)	−0.16
CH	10	7.05 and 7.07 (dd)	7.19 and 7.17 (dd)	−0.14 and −0.10
CH	11	7.40 and 7.43 (dd)	7.47 and 7.50 (dd)	−0.07 and −0.07

**Fig. 2.** 2D ROESY spectrum of LZ-β-CD complex.

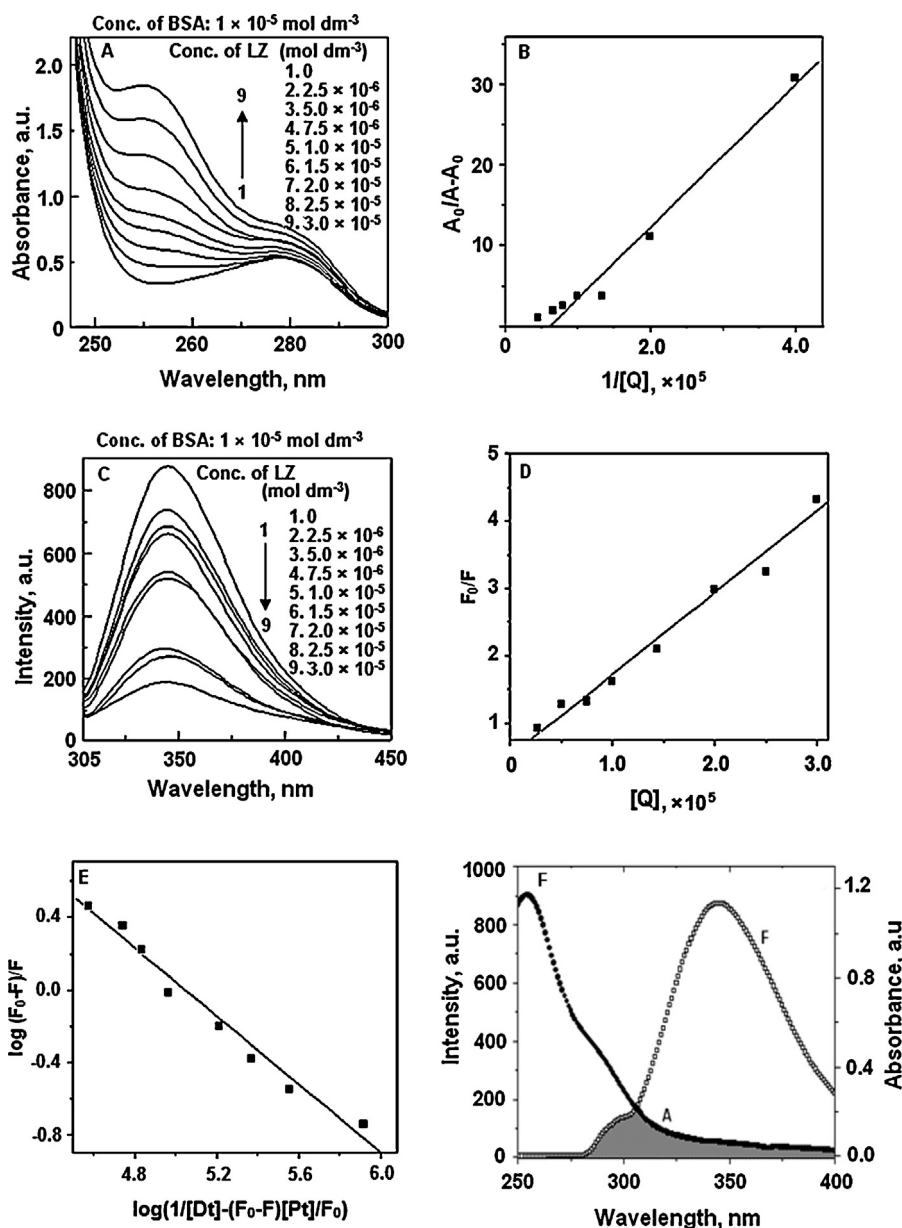


Fig. 3. Absorption spectra of BSA in the presence of various concentrations of LZ (A). The plot of $A_0/(A - A_0)$ against $[LZ]^{-1}$ for the interaction of BSA in the presence of various concentrations of LZ (B). Fluorescence spectra of BSA in the presence of various concentrations of LZ (C). The plot of F_0/F against $[LZ]$ for the interaction of BSA in the presence of various concentrations of LZ (D). The plot of $\log [F_0 - F]/F$ against $\log (1/[D] - (F_0 - F)/[P]F_0)$ for the interaction of BSA with various concentrations of LZ (E). Spectral overlapping between the UV absorption spectrum of LZ and the fluorescence spectrum of BSA (F).

Hence, we conclude that the formation of LZ- β -CD inclusion complex occurs through the inclusion of the amide chain, substituted in the oxazolidinone moiety, in the β -CD cavity.

3.2. Binding interaction of LZ with BSA

The absorption spectra of the binding titration of LZ against BSA are shown in Fig. 3A. The absorption spectrum of BSA shows a single band at 278 nm. Upon the addition of the LZ in increasing concentrations, the absorbance increases, i.e., shows a hyperchromic shift along with the formation of a new band at 250 nm. This occurs at the addition of the LZ. The change of absorbance is used to plot $A_0/(A - A_0)$ vs. $[LZ]^{-1}$ (Fig. 3B), which follows from the equation,

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_G}{\varepsilon_{P-D} - \varepsilon_D} + \frac{\varepsilon_G}{\varepsilon_{P-D} - \varepsilon_D} \frac{1}{k[LZ]} \quad (5)$$

where A and A_0 represent the absorbances of the free LZ and A is the absorbance at varying concentrations of the LZ. ε_D and ε_{P-D} represent the absorption coefficients of the drug and the BSA-LZ complex, respectively. The binding constant (K) of the binding of LZ to BSA is $4.84 \times 10^4 \text{ M}^{-1}$ (correlation coefficient = 0.9904). The change of absorbance and the derived binding constant value indicate that there is a ground state complex formed between LZ and BSA.

Fluorescence quenching is a fundamental phenomenon and it is a source of information of the molecular contact between the fluorophore and the quencher. In static quenching, a complex is formed between the fluorophore and the quencher, and this complex is non-fluorescent. In order to determine the accessibility of the fluorophore in BSA to the LZ molecule, the fluorescence quenching of BSA by LZ is analyzed. The fluorescence spectra of BSA at the various added amounts of LZ are shown in Fig. 3C. The quenching

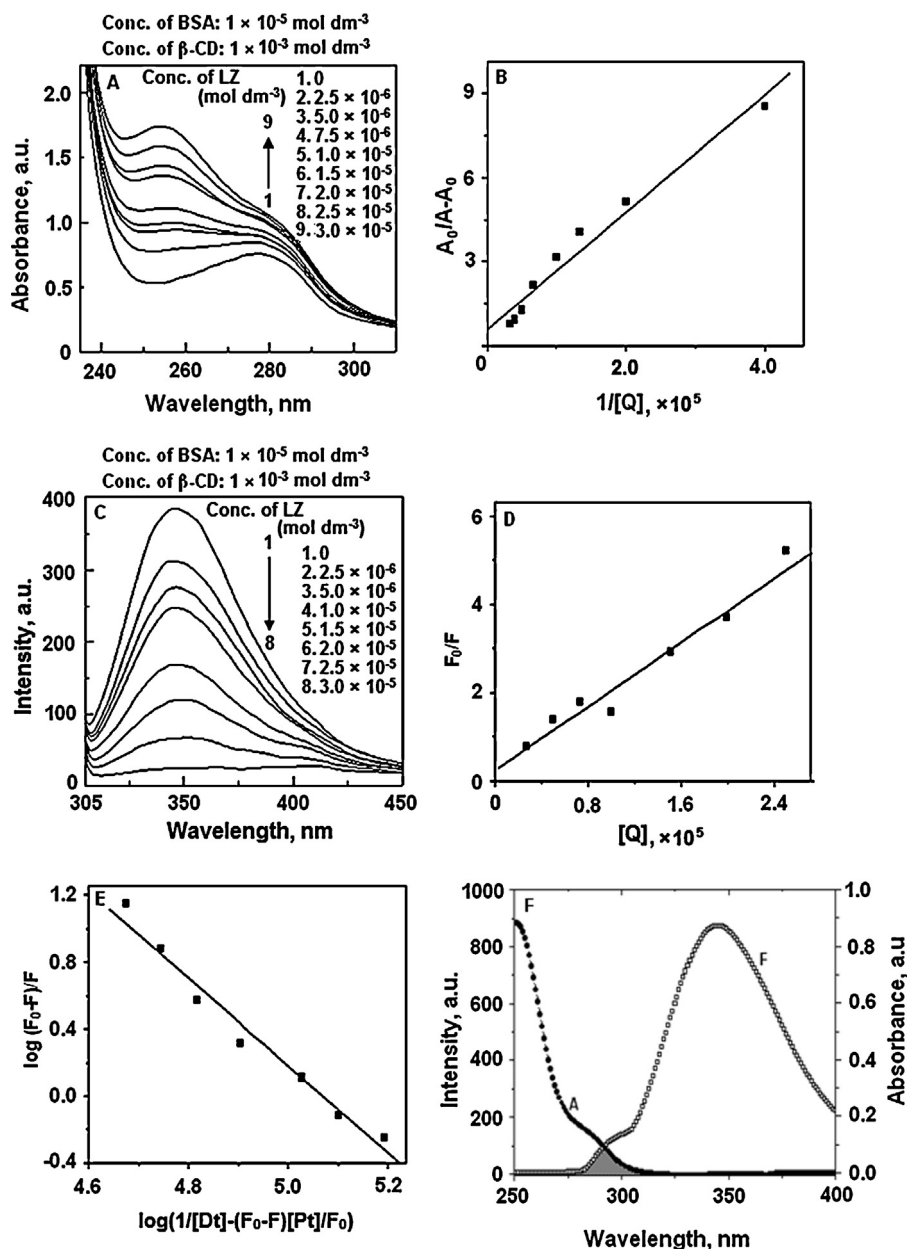


Fig. 4. Absorption spectra of BSA in the presence of various concentrations of LZ-β-CD (A). The plot of $A_0/(A - A_0)$ against $[LZ]^{-1}$ for the interaction of BSA in the presence of various concentrations of LZ-β-CD (B). Fluorescence spectra of BSA in the presence of various concentrations of LZ-β-CD (C). The plot of F_0/F against $[LZ]$ for the interaction of BSA in the presence of various concentrations of LZ-β-CD (D). The plot of $\log \{F_0 - F\}/F$ against $\log \{1/[D_t] - (F_0 - F)[P_t]/F_0\}$ for the interaction of BSA with various concentrations of LZ-β-CD (E). Spectral overlapping between the UV absorption spectrum of LZ-β-CD and the fluorescence spectrum of BSA (F).

of fluorescence is described by the Stern–Volmer equation (Bi et al., 2005).

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (6)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of the quencher, and $[Q]$ is its concentration. The Stern–Volmer quenching constant is given by $K_{SV} = k_q \tau_0$. The Stern–Volmer plot (F_0/F vs. $[Q]$) of the quenching of fluorescence of BSA by LZ is shown in Fig. 3D. The linearity of the Stern–Volmer plot is indicative of the fluorophores being equally accessible to the quencher. The calculated K_{SV} is $1.81 \times 10^5 \text{ M}^{-1}$ (correlation coefficient, 0.9921). Fig. 3E

shows the plot of $\log(F_0 - F)/F$ vs. $\log\{1/[D_t] - (F_0 - F)[P_t]/F_0\}$, corresponding to the binding of the LZ to BSA, according to Eq. (7).

$$\log_{10} \left(\frac{F_0 - F}{F} \right) = n \log_{10} K_A - n \log_{10} \left(\frac{1}{[D_t] - (F_0 - F)[P_t]/F_0} \right) \quad (7)$$

In the above equation, F_0 and F are the intensities of BSA before and after the addition of the LZ respectively. $[D_t]$ and $[P_t]$ represent the total concentration of the LZ and BSA respectively. The plot shown in Fig. 3E is linear and the calculated binding constant of the LZ–BSA binding is $2.24 \times 10^5 \text{ M}^{-1}$ (correlation coefficient, 0.9945). The number of binding sites is 1. Hence, it is obvious that there exists one binding site.

The distance between the tryptophan (donor) in the BSA and the drug (LZ) can be calculated using the Förster resonance energy transfer theory (FRET). BSA acts as the donor and LZ as the acceptor. This is indicated by the overlap of the absorption spectrum of the

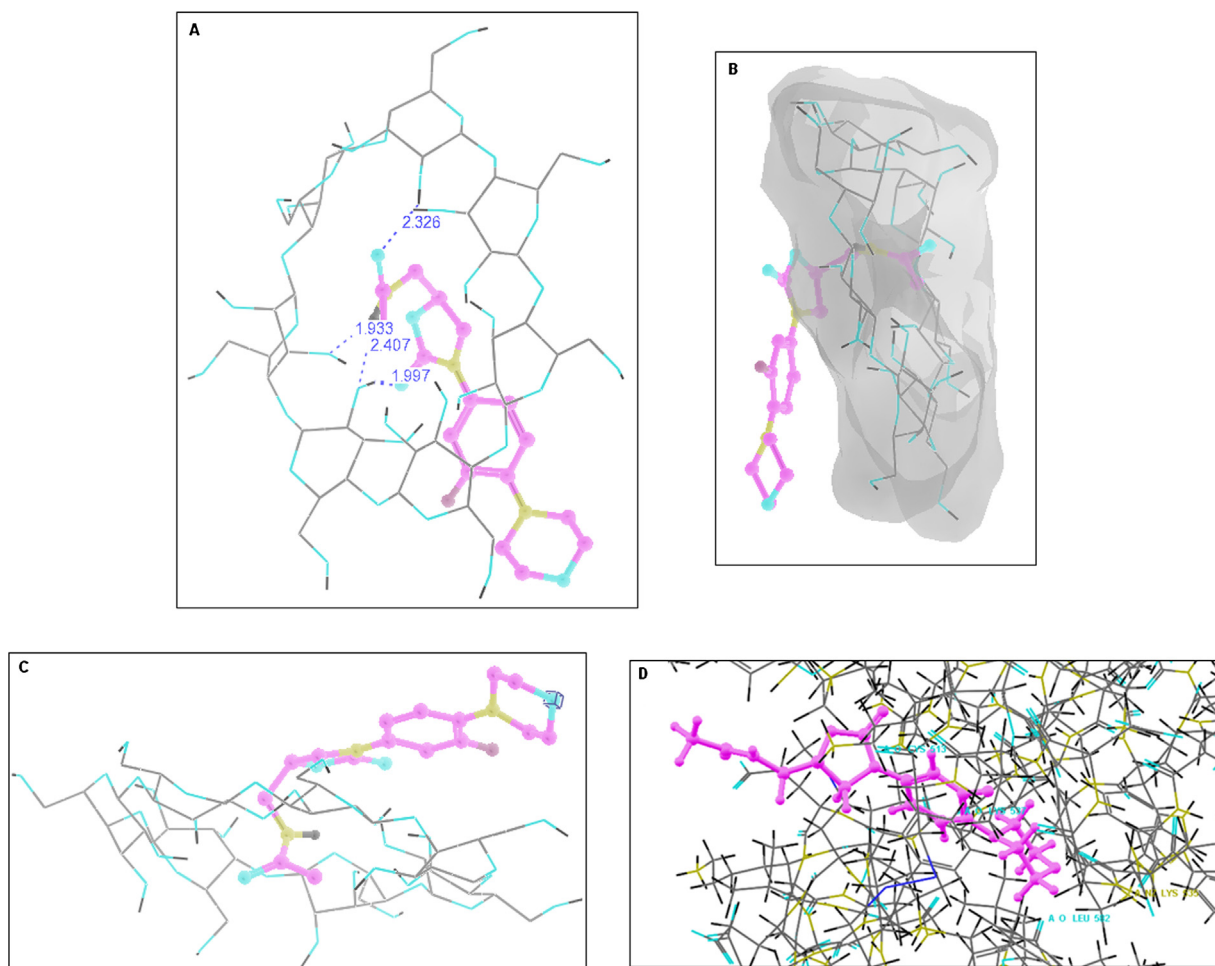


Fig. 5. Molecular docking poses of hydrogen bonding interactions of LZ with β -CD (A). Docking poses of hydrophobic interactions of LZ with β -CD (B). Molecular docking poses of electrostatic interactions of LZ with β -CD (C). Docking poses of LZ-BSA binding (D).

acceptor molecule and the fluorescence emission spectrum of the donor molecule. The efficiency of energy transfer, E , can be given by the following equation

$$E = 1 - F/F_0 = R^6/(R^6 + r^6) \quad (8)$$

where r is the binding distance between the acceptor and the donor, R_0 is the critical distance when the transfer efficiency is 50%, which can be determined as

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \Phi J \quad (9)$$

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

$$J = \frac{\sum F(\lambda) \varepsilon(\lambda) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda} \quad (10)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelengths λ , and $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at the wavelength λ . Fig. 3F shows the spectral overlap of the fluorescence emission spectrum of BSA and the absorption spectrum of the LZ. The calculated overlap integral J is $1.03 \times 10^{-19} \text{ cm}^3 \text{ mol}^{-1} \text{ dm}^3$. The K^2 value here is 2/3 and then $\Phi = 0.15$, $n = 1.33$, and $E = 0.1897$. The calculated R_0 is 3.7781 nm and r is 4.8119 nm. We observe that the value of r is smaller than 7 nm, which suggests that the energy transfer from BSA to LZ occurs with a good probability.

3.3. Binding interaction of LZ/ β -CD complex with BSA

As described in the previous sections, β -CD forms a 1:1 inclusion complex with LZ. This encapsulation can modulate the BSA-LZ binding interaction as the β -CD molecule acts like a partial protective sheath (Sivasubramanian, Thamby & Park, 2013; Yang et al., 2011) around the LZ. The influence of the β -CD on the binding of the LZ to BSA is discussed in this section. Fig. 4A shows the UV-Visible absorption spectra of the LZ- β -CD complex in the presence of various added amounts of BSA. The absorption maximum of BSA shifts slightly to the blue (from 277 to 273 nm), at the addition of the LZ. Moreover, there is a new band formed at higher concentration range of the LZ. This corresponds to the absorption spectral band of the LZ, which contains only one six membered aromatic ring. There is a hyperchromic shift, i.e., an increase of absorbance at each addition of the LZ. These observations lead to the inference that there is a ground state complex formed between LZ and BSA. The lower wavelength band also shows a blue shift of 4 nm at the maximal concentration of LZ, which suggests that a hydrophobic effect operates during the binding of the LZ to BSA. The plot of $A_0/(A - A_0)$ vs. $[LZ^{-1}]$ is shown in Fig. 4B. The calculated binding constant is $2.11 \times 10^4 \text{ M}^{-1}$ (correlation coefficient, 0.9832).

The quenching of fluorescence of BSA by LZ- β -CD is shown in Fig. 4C. The intensity of fluorescence of BSA is decreased along with an observed red shift of fluorescence by about 5 nm. This suggests that the quenching occurs by the formation of a bound complex of BSA and LZ- β -CD. The Stern-Volmer plot for the

above quenching of fluorescence is shown in Fig. 4D. The calculated K_{SV} is $1.21 \times 10^5 \text{ M}^{-1}$ (correlation coefficient = 0.9893). The Stern–Volmer plot is linear and indicative of one type of quenching mechanism. Here, the K_{SV} is smaller than that in the case of quenching of BSA fluorescence by the free LZ. Hence, β -CD hinders the strong collision of the LZ with BSA, by complex formation. The plot of $\log(F_0 - F)/F$ vs. $\log\{1/[D_L] - (F_0 - F)/[P_L]/F_0\}$ of the BSA–LZ/ β -CD binding is shown in Fig. 4E. The calculated binding constant of the binding of β -CD–encapsulated LZ to BSA is $1.11 \times 10^5 \text{ M}^{-1}$ (correlation coefficient, 0.9907), which is half the magnitude of the binding constant of the free LZ–BSA binding.

Fig. 4F shows the overlap of the absorption spectrum of LZ– β -CD with the fluorescence emission spectrum of BSA. There is a considerable area of overlap between both the spectra. There is an energy transfer occurring between BSA and LZ– β -CD and it is analyzed similarly to the FRET described in the previous section. The calculated E is 0.1589, the R_0 = 2.2846 nm and the r = 3.0159 nm. The energy transfer efficiency and the donor (BSA)–acceptor (LZ) distance are less than those calculated for the free–LZ binding to BSA.

3.4. Molecular docking of LZ–BSA binding

Molecular docking is used to extend further insight into the mode of LZ–BSA binding. The structures of the molecules were refined by assigning the bonds, bond orders, charge and hybridization, creating explicit hydrogen and the process was repeated. BSA consists of three homologous domains (I, II, and III): I (residues 1–183), II (184–376), III (377–583), each containing two sub-domains (A and B) that assemble to make it a heart shaped molecule. There is a large hydrophobic cavity in sub-domain IIA to accommodate the drug molecule, which plays an important role in the transportation of drugs in BSA. The best energy ranked results in Fig. 5B reveal that LZ is located within the sub-domain III hydrophobic cavity in close proximity to the residues, such as Lys-535 and 537, Leu-582, and Cys-513 suggesting the existence of hydrophobic interaction between them. Moreover, clear hydrogen bonding interactions are revealed at these sites. Hence, this finding provides a good structural basis to explain the efficient quenching of fluorescence of BSA by LZ. Furthermore, there are also a number of hydrophobic interactions, because several apolar residues in the proximity of the ligand play an important role in stabilizing the molecule via phobic interactions. As shown in the figure the hydrogen bonding occurs, due to the presence of the carbonyl of the LZ molecule, with the Lys-535 and 537, Leu-582, and Cys-513 residues of BSA. These hydrogen bonds increase the stability of the LZ–BSA bound system. Therefore, it can be concluded that the interaction between the LZ and BSA is dominated by hydrophobic forces as well as hydrogen bonds, and correlate well with the binding mode observed from the fluorescence quenching of BSA.

4. Conclusions

We studied the interaction of the LZ in free and β -CD-bound form to BSA mainly using UV–Visible absorption and Fluorescence spectroscopy. The stoichiometry of the inclusion complex of the LZ with β -CD is 1:1, and the binding constant is $3.51 \times 10^2 \text{ M}^{-1}$. The structure of the inclusion complex is proposed from the observed correlation peaks of 2D ROESY NMR spectrum. The complex formation with β -CD occurs due to the encapsulation of the amide chain substituted in the oxazolidinone moiety. The fluorescence quenching of BSA by LZ occurred as a result of the formation of LZ–BSA complex. The calculated K_{SV} was $1.81 \times 10^5 \text{ M}^{-1}$ and the binding constant was $2.24 \times 10^5 \text{ M}^{-1}$. In the presence of β -CD, the binding of the LZ with BSA showed a diminished K_{SV} ($1.21 \times 10^5 \text{ M}^{-1}$) and

a smaller binding constant value ($1.11 \times 10^5 \text{ M}^{-1}$). The encapsulation of LZ by β -CD decreases the strength of binding of the LZ to BSA by blocking the hydrogen bonding and phobic interaction of the LZ. The donor-to-acceptor distance (4.812 nm) confirms that static quenching occurs, with the energy transfer from BSA to LZ. This distance is smaller in the case of β -CD–complexed LZ binding with BSA. β -Cyclodextrin alters this distance by engulfing a part of the LZ. Even though β -Cyclodextrin covers up the LZ molecule, a partial inclusion in the host cavity renders the molecule available for binding to the protein molecule. The modulation of the binding of the LZ to BSA has indeed occurred as evidenced by the alteration of the binding strength.

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