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Impact of host-guest complexation between norfloxacin and β-cyclodextrin on fluorescence quenching: Steady-state and time resolved fluorescence study

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The behaviour of host-guest interaction of the drug i.e. norfloxacin (NFX) with β-cyclodextrin (β-CD) has been investigated by the fluorescence quenching method in acidic and neutral pH using copper as a quencher at various temperatures. The stoichiometry of the host-guest complication has been estimated using the Benesi−Hildebrand relationship. The incorporation of NFX inside the cavity of β-CD is revealed by fluorescence anisotropy and fluorescence lifetime measurements. The effect of temperature on the Stern-Volmer quenching constant (K_{SV}) and binding constant (K) has been analyzed, and the type of quenching was confirmed from the fluorescence lifetime measurement. Using van't-Hoff equation and Gibbs-Helmholtz equation, thermodynamic parameters like change of enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) have been determined. The negative value of ΔH and the positive value of ΔS validate the hydrophobic interaction between drug and quencher, and the negative value of ΔG suggests that the complex is spontaneous in nature.

Keywords: Norfloxacin, β-Cyclodextrin, Host-guest complexation, Quenching, Fluorescence anisotropy

The host-guest complex formation depends on molecular recognition and non-covalent bonding interactions. The binding is very specific in the host-guest complex, and there is always an equilibrium between the unbound state and the bound state. The thermodynamics of binding between host and guest is very vital. Quantitative analysis of binding constant values provides useful thermodynamic information about the complex[1]. The interaction between host and guest can either be hydrophobic or covalent. Hydrophobic interactions between host and guest are considered "encapsulated," while covalent interactions are considered to be conjugated[2].

Solubility and hydrophobicity of drug molecules play an important role in the delivery of a drug to the target area. Most drugs lack these properties, so in the last few decades, scientists have focused on drug delivery by nanosized vehicle like cyclodextrin, micelle, reverse micelle, lipid bilayer etc. The formation of inclusion complexes is emerging as a promising means of enhancing the solubility and bioavailability of poorly water soluble drugs. The use of dendrimers with a high proportion of cyclodextrin in medicine improves the efficiency of the drug delivery process by increasing the solubility and bioavailability of the drug. These inclusion complexes are the result of the ability of cyclodextrin to form host-guest complexes with hydrophobic molecules[3].

Cyclodextrin is a cyclic oligosaccharide consisting of (α-1,4)-linked α-D-glucopyranose units that contain a lipophilic central cavity and a hydrophilic outer surface. Cyclodextrin is mostly available in the form of α-, β- and γ-cyclodextrins, which consist of six, seven, and eight glucopyranose units, respectively. β-cyclodextrin (β-CD) and its derivatives, such as hydroxypropyl-β-CD (HP-β-CD) and methyl-β-CD (M-β-CD), are the main excipients commonly used in pharmaceutical formulations[4]. From a structural aspect, cyclodextrin appears to be a truncated cone with a hydrophilic exterior surface and a non-polar interior cavity. The central cavity is lined with skeletal carbons and ethereal oxygen from the glucose residues. Certain drug molecules with compatible size and shape may be fitted within the CD's created lipophilic microenvironment to form an inclusion complex[4]. The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution, and it has the ability to form inclusion complexes with different sized guest molecules[5]. The van der Waals attractions, hydrogen bonding and hydrophobic attractions play an important role in host-guest chemistry[6].

Norfloxacin (NFX) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-quinoline-3-carboxylic acid] (Fig. 1) is a quinolone antibiotic of the third generation with low solubility and permeability[7]. The poor solubility of NFX reduces the bioavailability to around 30 to 40%[8]. The presence of a fluorine atom at the C-6 position and a piperazinyl group at the C-7 position improves NFX's antimicrobial activity, as fluorine increases activity against gram-positive pathogens, including those resistant to lactam antibiotics and sulfonamides[9], and the piperazinyl group improves activity against gram-negative microorganisms. The nitrogen in the piperazine ring and the carboxylate group are the most significant proton-binding sites[10].

The enhancement in the water solubility and dissolution rate of NFX was investigated by many scientists to ensure effective drug delivery. According to Dua et al.[11], complexation improved the solubility and dissolution rate of NFX in α-cyclodextrin. Guyot and coworkers[12] and Loh and coworkers[13-14] have reported that complexation of NFX with CD plays an important role in the solubility and dissolution rate of norfloxacin. Zhang and co-workers[15] have reported that NFX shows a different band in the UV-visible range with a variation in pH due to the existence of different species. Muniz et al.[16] have investigated the interactions of cationic (NFX^+), zwitterionic ($NFX^\pm$) and anionic (NFX^-) with the anionic sodium dodecyl sulphate (SDS) cationic cetyltrimethylammonium bromide (CTAB) and non-ionic Tween-20 (TW20) micelles. Park et al.[17] have also reported the effect of pH on the lifetime of NFX in surfactant medium. Our group has also reported the behaviour of host-guest interaction of ofloxacin (OFX) with β-Cyclodextrin (β-CD) using the fluorescence quenching method in acidic and neutral medium using copper as quencher[18]. Using NMR data, Li et al.[19] have proposed a spatial configuration of complexes between norfloxacin with 2-methyl-β-cyclodextrin (Me-β-CD) where the piperazine ring of NFX situated outside of the Me-β-CD cavity.

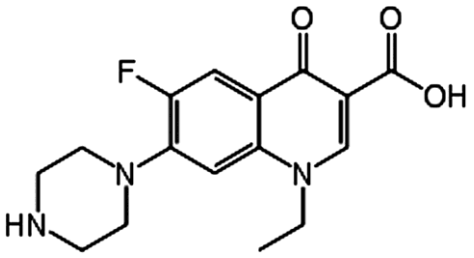


Fig. 1 — Structure of norfloxacin (NFX)

In this work, an attempt has been made to investigate the localization of NFX in β-CD from anisotropy and rotational correlation time experiments in neutral pH and to ascertain the nature of complexation between NFX and β-CD. The effect of localization and orientation of NFX in β-CD is also studied through fluorescence quenching techniques in acidic and neutral pH to know the nature of quenching through steady-state and time-resolved fluorescence data. In order to understand the stability of the complex formation, the thermodynamic parameters are calculated by varying the temperature.

Experimental Details

Chemicals and reagents

Analytical grade β-CD, NFX from Sigma Aldrich, Germany were taken without further purification. Other chemicals used were from Merck, Germany and were of analytical grade. Milipore water was used throughout the experiments. The stock solution of 1.0 mM NFX was prepared in an aqueous medium using 1-2 drops of 1.0 mM HCl in order to make it soluble. The experimental concentration of NFX was maintained at 0.01 mM and the concentration of β-CD was varied between 0.5-4 mM. In order to study the fluorescence quenching between NFX and Cu^{2+} in β-CD, the concentration of β-CD was fixed at 1 mM and the concentration of quencher was varied between 0.002–0.012 mM in neutral pH and 0.2–1.0 mM in acidic pH.

Spectroscopic measurements and techniques

The absorption spectra of the fluorophore were recorded by a UV-visible Shimadzu-2450 spectrophotometer using the same media as references. The fluorescence spectra were recorded by a Hitachi F-7000 spectrofluorimeter. The excitation wavelength was chosen at around the absorption maximum of the fluorophore. Narrow excitation and emission slit widths (half band with = 2.5 nm, 2.5 nm) were chosen. NFX shows a fluorescence band at 440 nm in acidic pH and 430 nm in neutral pH. The fluorescence lifetimes of NFX were measured using the time correlated single photon counting technique (TCSPC) in the Horiba JobinYvon time-resolved fluorescence spectrophotometer. A Nano LED of 280 nm was used as the light source. The fluorescence decays were deconvoluted using the Data station software for acquisition. The decay times were determined using the non-linear least square method by the TCSPC technique. The goodness of the fits

was evaluated by the χ^2 value. The average fluorescence lifetimes (τ) for biexponential decays were calculated using the Eq. 1;

$$\tau_{avg} = \alpha_1\tau_1^2 + \alpha_2\tau_2^2/\alpha_1\tau_1 + \alpha_2\tau_2 \quad \ldots (1)$$

where α_i is a pre-exponential factor representing the fractional contribution to the time resolved decay of the component with a lifetime τ_i.

Results and Discussion

Absorption and fluorescence spectra

In neutral pH, NFX shows a sharp absorption band at 278 nm due to the aromatic ring and a broad band around 318-335 nm due to the n–π* (HOMO-LUMO) transition. As discussed earlier, this drug exists as NFX^+, $NFX^{\pm}$ and NFX^- in acidic, neutral, and alkaline pH respectively (Scheme 1).

In acidic pH, a slight red shift of 2 nm is observed in the spectrum[5] due to NFX^+. The drug shows an emission band at 430 nm in neutral pH and 440 nm in acidic pH (Fig. 2). Bilski and coworkers[20] have reported that in acidic pH, the emission maxima at 442 nm is due to the presence of a cationic form of drug (NFX^+) where as in neutral pH the emission maxima at 408 nm is due to the presence of its zwitterionic form ($NFX^{\pm}$) in the CTAB surfactant system. The anionic form of the drug (NFX^-) found to be non-fluorescent with a quantum yield of less than 0.005. This is due to the ICT, pro-ICT, and non-ICT phenomena of NFX.

Host-guest interaction of NFX in β-cyclodextrin

In order to determine the nature of the inclusion complex (stoichiometric ratio) and the binding constant of the inclusion complex between NFX and β-CD, fluorescence spectra of a fixed concentration of NFX were recorded in various concentrations of β-CD (Fig. 3). NFX initially shows emission maxima (λ_{em}) at 430 nm in water and red shifted to 450 nm with the addition of β-CD. It suggests that there must be some interaction between NFX and β-CD. As the concentration of β-CD increases, the fluorescence intensity increases up to 2 mM of β-CD and after that it remains constant. It implies that NFX is incorporated inside the cavity of β-CD due to which fluorescence intensity increases and remains constant after 100% incorporation of the drug.

Fluorescence anisotropy and rotational correlation time always give useful information to verify the rigidity of the probe inside the cyclodextrin cavity. The mobility of the probe (NFX) can cause a change in the rotational time of the molecules, which is

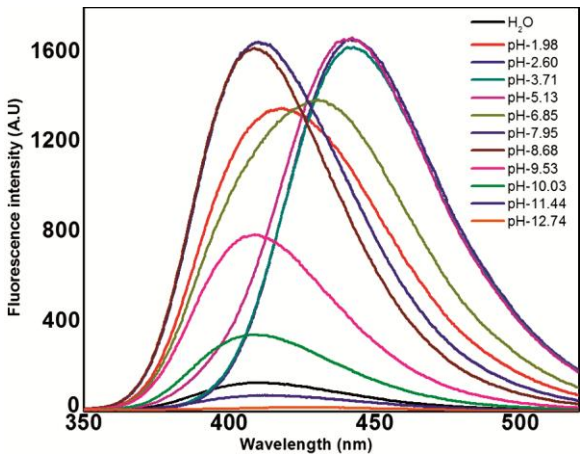


Fig. 2 — Fluorescence spectra of NFX in various pH in aqueous medium

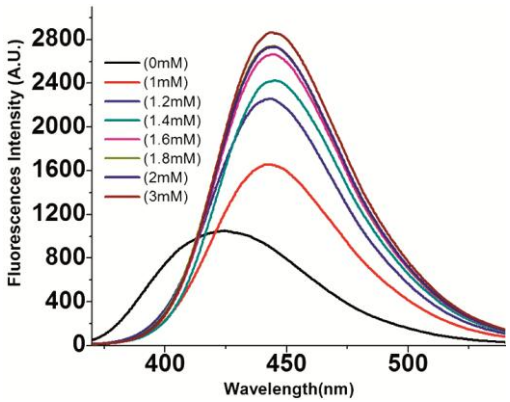
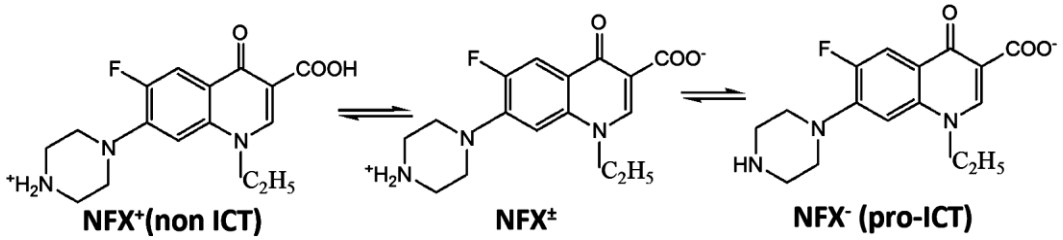


Fig. 3 — Fluorescence spectra of NFX in various concentration of β-CD



Scheme 1 — Protonation and deprotanation of NFX in different pH[20]

reflected in the change in anisotropy value. These intensity values are used to calculate the anisotropy using Eq. 2.

$$r = \frac{(I_{VV} - G.I_{VH})}{(I_{VV} + 2G.I_{VH})} \quad \ldots (2)$$

where I_{VH} and I_{VV} are the intensities obtained from the excitation polarizer oriented vertically and the emission polarizer oriented in horizontal and vertical directions, respectively. The factor G is defined as $G = I_{HV}/I_{HH}$. The rotational correlation time (Θ) can be determined using the Perrin equation (Eq. 3);

$$\frac{r_0}{r} = 1 + {}^{\tau}/_{\theta} \quad \ldots (3)$$

where τ is the fluorescence lifetime, Θ is the rotational correlation time. r is the measured anisotropy, and r_0 is the fundamental anisotropy, which is 0.3 in this case[21].

Anisotropy values (r) and rotational correlation time (Θ) of norfloxacin in various concentrations of β-CD are determined. A plot of the fluorescence anisotropy (r) of NFX in various β-CD concentrations is reported in Fig. 4. From this figure, it can be observed that the anisotropy value increases as the β-CD concentration increases, and it remains constant after a certain concentration of β-CD. A similar trend is also observed in the case of rotational correlation time. An increase in anisotropy values is due to restrictions in rotation of the molecules. Hence, as NFX is incorporated inside the β-CD cavity, the restriction increases, and the anisotropy value as well as rotational correlation time increase. So it suggests the incorporation of NFX inside the cavity of β-CD. After incorporation of all the NFX molecules inside the cavity (beyond 2 mM of β-CD), the anisotropy value and rotational correlation time remain almost constant.

In our earlier work[18], while investigating the host-guest interaction between ofloxacin (OFX) and β-CD, the anisotropy value (r) of OFX found to be 0.005 at 2 mM of β-CD where as in this case it is 0.018. This implies better incorporation of NFX compared to OFX. This may be due to the presence of an extra methyl group in the piperazine moity of OFX which restricts the incorporation of guest molecules inside the cavity of β-CD.

Assuming a 1:1 stoichiometric ratio for the nature of the inclusion complex between NFX and β-CD, the binding constant was determined using the Benesi-Hildebrand equation (Eq. 4)[22];

$$\frac{1}{I - I_0} = \frac{1}{(I_\infty - I_0)\, K_b\, [\beta\text{-CD}]} + \frac{1}{I_\infty - I_0} \quad \ldots (4)$$

where I_0 and I is the intensities of fluorescence in the absence and presence of β-CD concentration, rspectively, I_∞ is the maximum fluorescence intensity due to the incorporation of NFX in β-CD and K_b is the binding constant. The binding constant (K_b) of NFX with β-CD was determined from the Benesi-Hildebrand plot of ($1/I-I_0$) against 1/[β-CD]. The plot is given in Fig. 5. The linearity of the plot implies 1:1 complexation of NFX with β-CD and the binding constant determined from the slope, which is found to be 1.33×10^4 M.

In order to establish the stability of the complex, free energy was calculated using the Gibbs-Helmholtz equation (Eq. 5), which is found to be -0.27 kJ/mol. The negative ΔG value suggests that the complex is stable in nature.

$$\Delta G = -RT \ln K_b \quad \ldots (5)$$

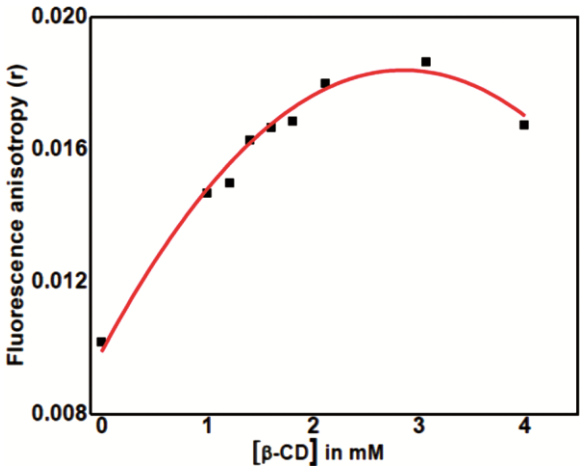


Fig. 4 — Plot of fluorescence anisotropy (r) of NFX in β-CD

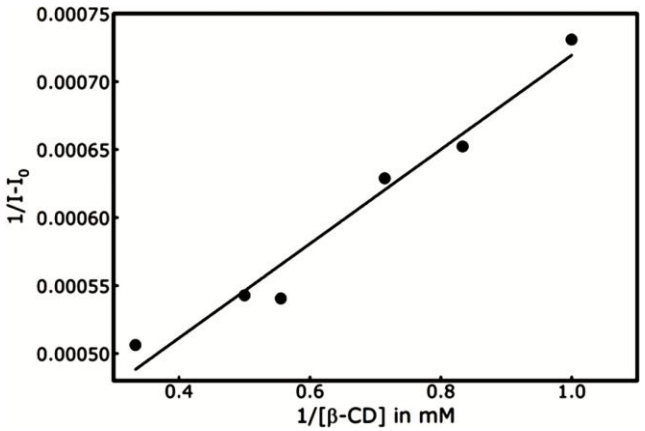


Fig. 5 — Benesi-Hildebrand plot for NFX in β-CD

Fluorescence quenching of NFX by Cu^{2+} in aqueous medium

Fluorescence quenching of NFX by Cu^{2+} was investigated in an aqueous medium at acidic and neutral pH. In order to establish the nature of the quenching mechanism, the fluorescence data in the presence and absence of quencher were analysed using the Stern-Volmer equation (Eq. 6)[23],

$$[(I_0/I)-1]=K_{SV}[Q] \quad \ldots (6)$$

where I_0 and I are the intensities of the fluorophore in the absence and presence of the quencher, respectively, K_{SV} is the Stern-Volmer quenching constant, and [Q] is the concentration of the quencher. The fluorescence spectrum of NFX in various concentrations of quencher, i.e. Cu^{2+} is reported in Fig. 6. It can be observed from Fig. 6, as the concentration of quencher (Cu^{2+}) increases, the fluorescence intensity of NFX decreases without any changes in λ_{em} value.

The Stern-Volmer plots obtained from the fluorescence intensity data are found to be linear in both neutral and acidic pH (Figs. 7 & 8). The Stern-Volmer quenching constant (K_{SV}) calculated from the plot, found to be 7.31×10^4 M^{-1} and 8.69×10^2 M^{-1} for neutral and acidic pH, respectively. The K_{SV} value of acidic pH is less than that of neutral pH. This may be due to the electrostatic force of repulsion between cationic species (NFX^+) of the probe and quencher (Cu^{2+}) in acidic pH, whereas the electrostatic force of attraction between probe and quencher in the case of neutral pH. The linearity of the SV plot suggests that the quenching may be dynamic (collisional) in nature or may be due to static (ground state complex formation). In order to understand the nature of quenching, the fluorescence lifetime of the probe in the absence (τ_0) and presence (τ) of quencher is determined experimentally. The plot of τ_0/τ vs quencher concentration is found to obey the SV equation in the case of acidic pH, whereas approximate τ_0/τ values are found to be unity in neutral pH (Figs. 7 & 8). This suggests that quenching is dynamic in the case of acidic pH and static in the case of neutral pH.

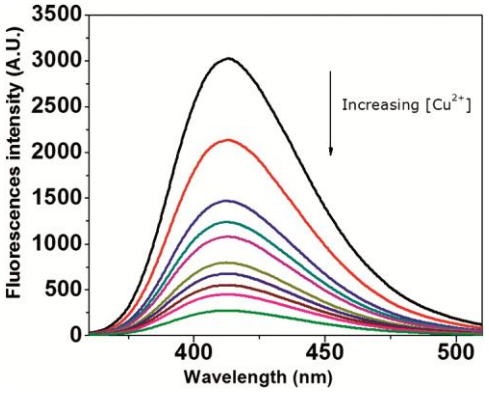


Fig. 6 — Fluorescence spectrum of NFX in presence of various concentration of Cu^{2+} in aqueous medium

Fluorescence quenching of NFX by Cu^{2+} in β-cyclodextrin

The fluorescence quenching of NFX by Cu^{2+} in neutral and acidic pH was investigated in -CD at three different temperatures: 293, 303, and 313 K. The Stern-Volmer plots are linear for acidic pH, whereas the SV plots are linear up to a limiting concentration (0.004 mM) of Cu^{2+} at neutral pH (Fig. 9). This implies that either dynamic (collisional) or static quenching processes operate at an acidic pH in the entire concentration of quencher, whereas at neutral pH at a low concentration of quencher. Beyond the

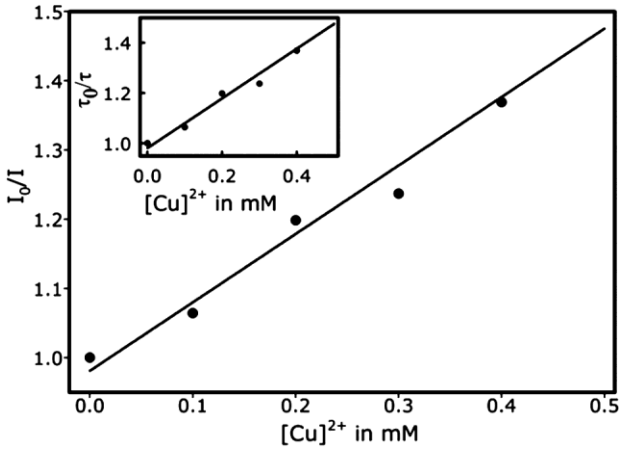


Fig. 7 — Plot of (I_0/I) vs. $[Cu]^{+2}$ and inset shows the plot (τ_0/τ) vs. $[Cu]^{+2}$in aqueous medium at acidic pH (pH 3.2)

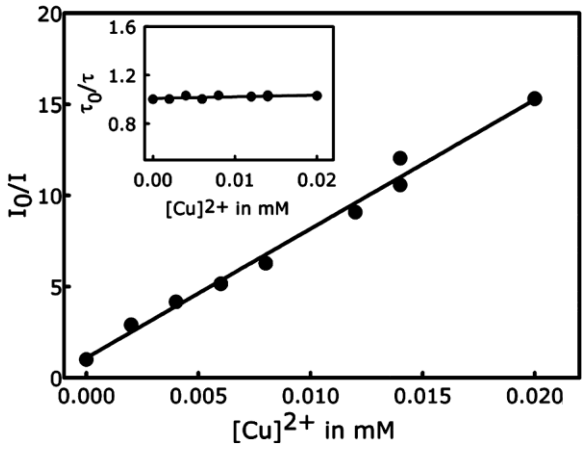


Fig. 8 — Plot of (I_0/I) vs. $[Cu]^{+2}$ and inset shows the plot (τ_0/τ) vs. $[Cu]^{+2}$ in aqueous medium at neutral pH

Table 1 — The Stern-Volmer quenching constant (K_{SV}), binding constant (K) and binding site (n) of NFX in β-CD medium in acidic and neutral pH

	Acidic pH			Neutral pH		
	293 K	303 K	313 K	293 K	303 K	313 K
K_{SV} (M^{-1})	$2.60x10^2$	$2.84x10^2$	$3.12x10^2$	$2.56x10^5$	$2.16x10^5$	$1.92x10^5$
K (M^{-1})	$2.07x10^2$	$2.61x10^2$	$3.09x10^2$	$1.30x10^3$	$1.11x10^3$	$0.79x10^3$
n	0.97	0.97	0.99	1.26	1.31	1.32

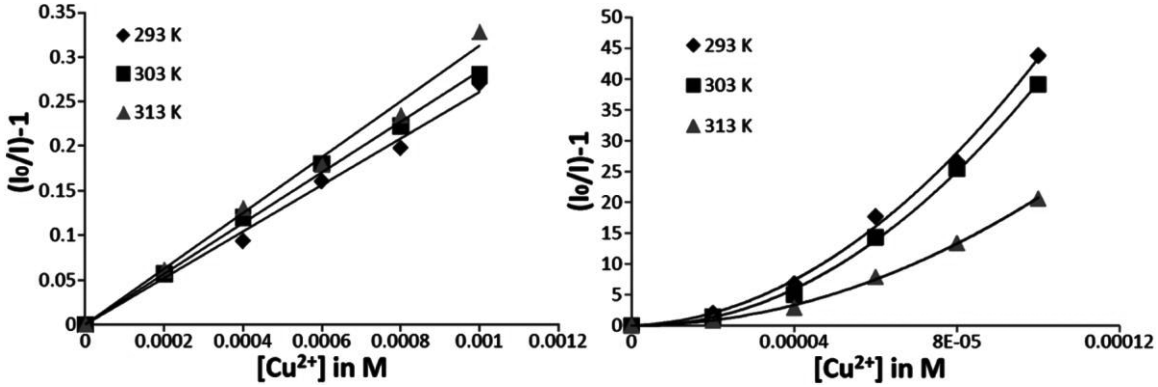


Fig. 9 — Stern-Volmer plot of NFX in β-CD in (a) acidic pH and (b) neutral pH

limiting concentration of Cu^{2+}, the SV plots show a positive deviation in neutral pH. Similar types of results have been reported by Thulborn et al.[24] while investigating the quenching behaviour of anthroyloxy derivatives by dimethyl aniline in a lipid–water system. The authors have taken a series of fluorescent fatty acids in which an anthracene group is attached by an ester linkage at various positions along the alkyl chain. From the fluorescence quenching study, they found that the probes are located at a graded series of depths in the bilayer and observed a positive deviation in the SV plots when both the anthroyloxy derivative and dimethyl aniline were in the same environment. Similarly, in this case, both NFX and Cu^{2+} are in a similar environment. The Stern-Volmer quenching constant was determined using Eq. 6 and is reported in Table 1.

In order to establish the nature of quenching (dynamic or static), the effect of temperature on Stern-Volmer quenching constant (K_{SV}) is investigated. With the increase in temperature, the K_{SV} value increases in acidic pH and decreases in neutral pH (Table 1). The frequency of collision increases with the increase in temperature, which in turn increases the probability of dynamic quenching. As a result, K_{SV} value increases in the case of acidic pH. This suggests that the quenching is dynamic in nature. But in the case of neutral pH, the K_{SV} value decreases with an increase in temperature. In the case of static quenching, an increase in temperature results

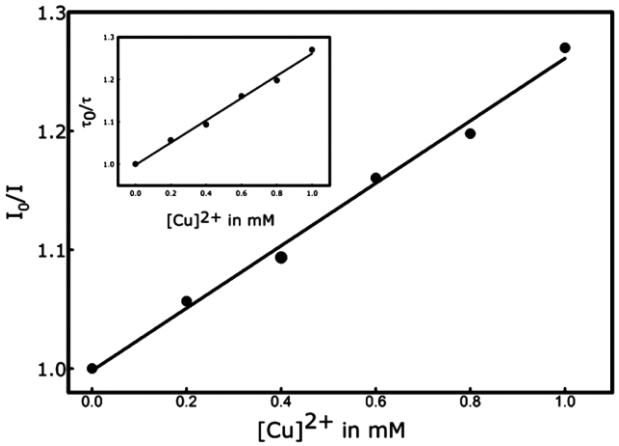


Fig 10 — Plot of (I_0/I) vs. $[Cu]^{+2}$ and inset shows the plot (τ_0/τ) vs. $[Cu]^{+2}$ in β-CD at pH 3.2

in an increase in the diffusion coefficient due to which the stability of the ground state complex decreases, so quenching efficiency decreases. This suggests that quenching is static in nature in the case of neutral pH. The fluorescence lifetime of NFX in the absence (τ_0) and presence (τ) of quencher (Cu^{2+}) was determined experimentally for both neutral and acidic pH. The plot τ_0/τ vs [Q] plot is found to be linear with, intercept similar to that of SV plots at acidic pH (Fig. 10). This suggests that fluorescence quenching between NFX^+ and Cu^{2+} is dynamic (collisional) in nature. In case of neutral pH, the plot τ_0/τ vs [Q] plot is found to be linear with an intercept of one (Fig. 11). This suggests that fluorescence quenching

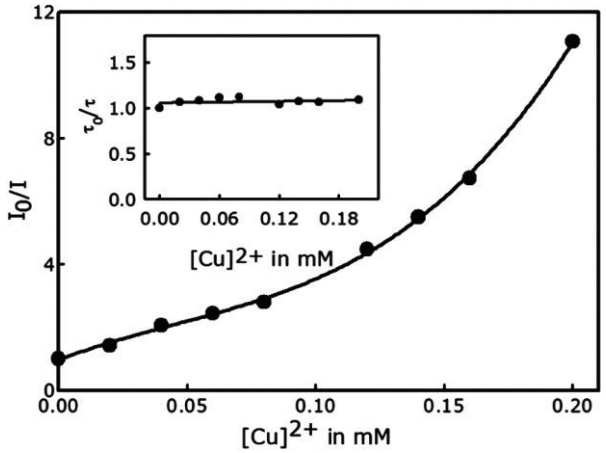


Fig. 11 — Plot of (I_0/I) vs. $[Cu]^{+2}$ and inset shows the plot (τ_0/τ) vs. $[Cu]^{+2}$ in β-CD at neutral pH

Table 2 — Analysis of thermodynamic parameter change in enthalpy, change in entropy and change in free energy in neutral, acidic and alkaline pH

pH	T (K)	ΔG (kJ $mole^{-1}$)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
Neutral	293	2.43	2.273	-0.0005
	303	2.61		
	313	2.44		
Acidic	293	-5.26	-18.39	11.61
	303	-5.38		
	313	-5.49		

between $NFX^{\pm}$ and Cu^{2+} is due to ground-state complex formation (static quenching). Non-linearity in the Stern-Volmer (SV) plot suggests that a dynamic (collisional) quenching process starts playing a role in higher quencher concentration. The quenching mechanism can be explained by modifying the Stern-Volmer equation (Eq. 7) which is second order with respect to quencher concentration[21].

$$I_0/I = (1 + K_S [Q]) (1 + K_D [Q]) \quad \ldots (7)$$

where K_S is the static quenching constant and K_D is the dynamic (collisional) quenching constant.

Analysis of binding constant and binding sites

In order to establish the equilibrium between drugs and quencher in β-CD the binding constant (K) and binding sites (n) of the drug were analysed using (Eq. 8) and the data determined from the plot of log [(I_0/I) -1] against log Q are reported in Table 1.

$$\log \frac{I_0 - I}{I} = \log K + n \log[Q] \quad \ldots (8)$$

It can be seen from Table 1 that the binding constant (K) increases with an increase in temperature in acidic pH, whereas the reverse effect is seen in the case of neutral pH. While investigating the binding between NFX and BSA, Naseri and coworkers[25] have observed that the quenching constant and binding constant decrease with an increase in temperature. The authors have concluded that the quenching is static in nature. Similarly, in the case of acidic pH, the quenching is dynamic in nature, whereas in neutral pH the quenching is static. The binding sites are found to be less than one in acidic pH and it is found to be greater than one in neutral pH, which suggests that there must be one binding site in the case of acidic pH and more than one binding site in neutral pH. It may be due to the nature of species, i.e., NFX^{+} and $NFX^{\pm}$ in acidic and neutral pH, respectively.

Analysis of thermodynamic parameter

In order to understand the binding behaviour between NFX-β-CD-Cu^{2+}complex in neutral and acidic pH, the change in enthalpy (ΔH) and entropy (ΔS) due to the formation of the inclusion complex is determined using the van't Hoff equation (Eq. 9).

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \ldots (9)$$

where K is the binding constant, R is the gas constant, and T is the absolute temperature. The enthalpy change (ΔH) and entropy change (ΔS) calculated from the slope and intercept of the van't Hoff plot, i.e. ln K against 1/T are reported in Table 2. The free energy change (ΔG) is determined from the Gibbs-Helmholtz equation (Eq. 5) and the results are reported in Table 2.

From Table 2, it can be observed that ΔG and ΔH are negative, whereas ΔS is positive in the case of acidic pH. A positive ΔS value indicates a hydrophobic interaction between NFX-β-CD, whereas a negative ΔH value indicates the possibility of a hydrogen bond between NFX-β-CD in both neutral pH[26]. The negative ΔG values indicate that the binding is spontaneous, and the formation of the NFX-β-CD-Cu^{2+} complex is an exothermic reaction[27] accompanied by negative ΔH. Thus, both hydrogen bonding and hydrophobic interactions are present in the NFX-CD complex.

Conclusion

This paper focuses on host-guest interaction of norfloxacin (NFX) with β-Cyclodextrin (β-CD). Linearity in Benesi-Hildebrand plot suggests 1:1 relationship between host-guest molecules. Increase

in Stern-Volmer quenching constant (Ksv) and binding constant (K) with increase of temperature suggest that quenching is purely dynamic in acidic pH, whereas decrease of K_{SV} with increase of temperature suggest that quenching is static in nature in neutral pH. The electrostatic force of attraction between probe and quencher plays an important role in nature of quenching and binding. The ΔH and ΔS determined using van't Hoff's equation reveals that acting forces are mainly due to hydrophobic interaction. The negative value of ΔG suggests that the binding process is spontaneous in nature.

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