

*Research Paper***Photophysics and Rotational Dynamics of a Fluoroquinolone Drug Norfloxacin in Biomimetic Reverse Micellar Nanocavities**

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(Received on 23 November 2015; Revised on 04 March 2016; Accepted on 16 March 2016)

The present work describes the study of the photophysics and dynamics of a prospective anti-cancer antibacterial drug norfloxacin (NFX) within the micro-heterogeneous environment of Aerosol OT (AOT) reverse micelle (RM). The environment sensitive fluorescence spectroscopic behavior of NFX is found to exhibit a significant red-shift with increasing water pool size ($w_0 = [\text{water}]/[\text{surfactant}]$) of the RM. A prominent decrease of fluorescence anisotropy of NFX with increasing w_0 demonstrates a relaxation of the motional restrictions imposed on the bound drug molecules with enhanced hydration. These results are further substantiated from our time-resolved measurements. Study of the rotational dynamics of NFX within the RM as a function of w_0 exhibits a typical biexponential decay behavior which has been adequately described on the basis of the wobbling-in-cone model. The rotational dynamical parameters are also calculated for wobbling motion of the drug within the RM as a function of w_0 .

Keywords: Norfloxacin; Antibacterial Drug; AOT Reverse Micelle; Fluorescence Spectroscopy; Rotational Dynamics; Wobbling-in-Cone

Introduction

Reverse micelles (RMs; Scheme 1) are spherical aggregates formed by surfactant molecules in non-polar medium and consist of a solvent water pool entrapped in the core of the reverse micellar unit surrounded by the headgroups of surfactants with the non-polar hydrocarbon chains of surfactants projected toward the non-polar bulk medium (Levinger *et al.*, 2002; Bhattacharyya *et al.*, 2003; Baruah *et al.*, 2006; Willard *et al.*, 1998; Silber *et al.*, 1999; Satpati *et al.*, 2009). The water molecules entrapped inside the reverse micelle are characterized to be highly structured and non-homogeneous in nature. The average size of a reverse micelle is dependent on the amount of solubilized water and expressed by water to surfactant molar ratio, $w_0 = [\text{water}]/[\text{surfactant}]$ (Luisi *et al.*, 1984; Kalyansundaram *et al.*, 1987; Luisi *et al.*, 1988; Jain *et al.*, 1989). Several studies have shown that two types of water population exist inside the water pool (Levinger *et al.*, 2002; Bhattacharyya

et al., 2003; Baruah *et al.*, 2006; Willard *et al.*, 1998), namely, water molecules that are strongly bound with the headgroup of the surfactant and hence display distinct characteristics compared to bulk water (Dutt *et al.*, 2004) and the free water molecules that reside deep inside the core of the water pool. Thus the interior of a reverse micelle is truly heterogeneous. Reverse micelles are widely considered as an efficient model for biological systems because of their ability to mimic several essential and important features of biological membranes. Given the structural, compositional and functional complexities associated with biological membranes, researchers have been particularly motivated to find some simple model system to mimic the physicochemical properties of membranes (Fendler *et al.*, 1976; Mitra *et al.*, 2008; Faeder *et al.*, 1989; Venables *et al.*, 2001; Nandi *et al.*, 2000; Bhattacharyya *et al.*, 2000). In our case we have used aerosol OT (AOT) to prepare the reverse micellar system. AOT is a double tailed anionic surfactant which is used for solubilization and

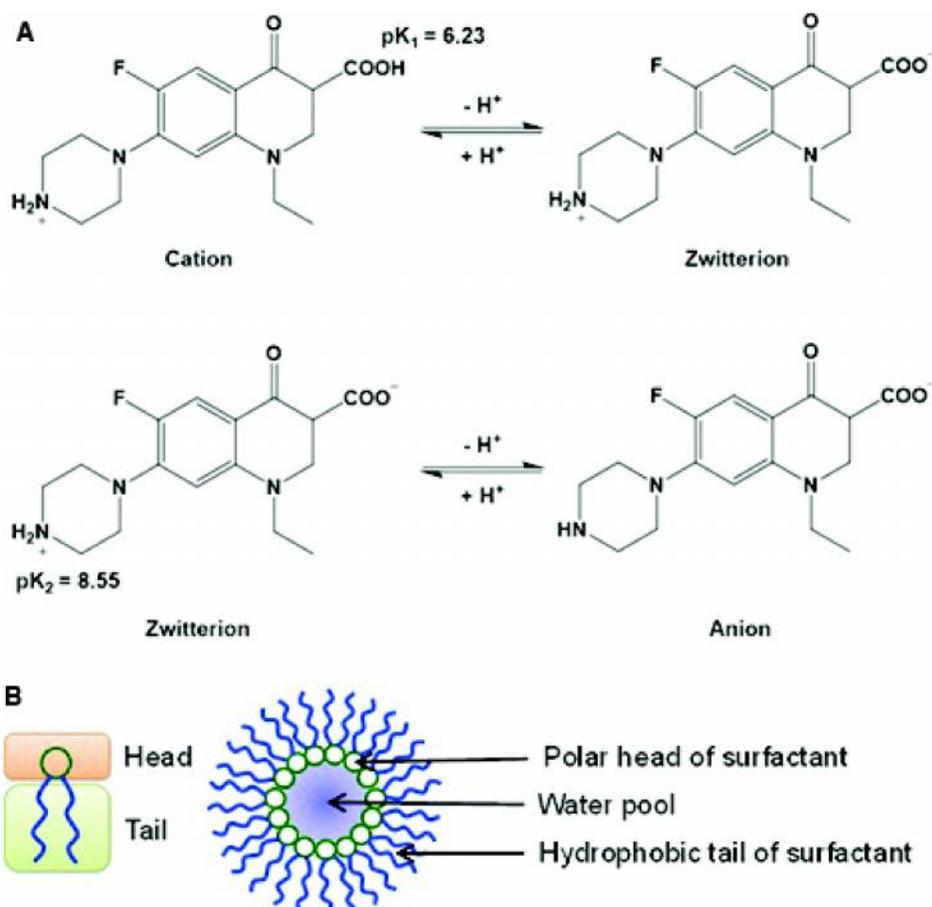
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emulsification. It can form spherical aggregates in wide range of w_0 values (Nave *et al.*, 2000), that is, the AOT reverse micelle can compartmentalize large amount of water into its core.

Fluoroquinolone (FQ) classes of compounds are widely known for their broad spectrum antibacterial activities. FQs are used as antibacterial drugs in the treatment of various infectious diseases (Lipsky *et al.*, 1999; Stahlmann *et al.*, 1999; Anand *et al.*, 2012), sexually transmitted diseases such as prostatitis, skin infection, acute cystitis and selected pneumonias (Ting *et al.*, 2003; Appelbaum *et al.*, 2000; Ball *et al.*, 2000; Blondeau *et al.*, 1999). The mechanism of action of FQs with biological or biomimicking assemblies has been quite unique in the sense that both the hydrophilic as well as hydrophobic pathways have been invoked as the actual mechanistic conduits, and the latter being mainly responsible for the route leading to their entry into the cytoplasm (Nikaido *et al.*, 1993; Berlanga *et al.*, 2000).

Herein, we have carried out a spectroscopic investigation of interaction of a fluoroquinolone drug, norfloxacin (NFX; Scheme 1) with AOT reverse micellar system. The chemical structure of NFX is comprised of a carbonyl group and a piperazinyl moiety at the 7-carbon atom. The 4' 'N' of the piperazinyl ring and the carboxylate group are the most significant proton binding sites from the biological viewpoint which leads to pH-dependent prototropism which in NFX is characterized by two pK_a values, $pK_1 = 6.23$ and $pK_2 = 8.55$ (Albini *et al.*, 2003; Sortino *et al.*, 1998; Monti *et al.*, 2002; Paul *et al.*, 2015).

In this study, we have studied the photophysical properties and rotational dynamics of NFX in AOT reverse micelle with various w_0 values by using steady-state and time-resolved fluorescence spectroscopic techniques. The photophysical and spectroscopic data are very useful in elucidating the interaction of these drug molecules with relevant biological and biomimicking assemblies. Upon



Scheme 1: (A) Prototropic Equilibrium of NFX ($pK_1 = 6.23$ and $pK_2 = 8.55$) and (B) Simplified Cartoon Representation of a Reverse Micellar System

entrapment inside an organized molecular assembly, the drug molecules will experience restriction on its dynamics and mobility. Fluorescence depolarization measurement yields an estimation regarding the dynamical information about the fluorophore in the complex micro-heterogeneous environment such as the reverse micelle. Understanding the rotational behavior of a probe in the microheterogeneous medium is significant for determining the dynamics of drug in biological systems. Hence, in the present study we intend to explore the dynamics of the drug (NFX) within the anionic RMs to understand their rotational behavior in such organized assemblies.

Materials and Methods

Norfloxacin (NFX) (Scheme 1) and sodium bis (2-ethylhexyl) sulfosuccinate (AOT) from Sigma-Aldrich, USA and spectroscopic grade n-heptane from Spectrochem, India were used as procured. Triply distilled deionized Milli pore water was used for all measurements.

Steady-State Spectral Measurements

The absorption and fluorescence emission (and excitation) spectra were acquired on a Cary 100 UV-Vis spectrophotometer and Fluorolog 3-111 fluorometer, respectively. In all measurements the concentration of NFX was maintained at *ca.* 2.0 mM in order to avoid aggregation and reabsorption effects. Only freshly prepared solutions were used for spectroscopic measurements. Appropriate spectral background correction and instrumental correction have been ensured during all spectral measurements.

Steady-State Fluorescence Anisotropy

Steady-state anisotropy (r) was recorded on Fluorolog 3-111 fluorometer. The steady-state anisotropy (r) is defined as (Lakowicz, 1999):

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2.GI_{VH}} \quad (1)$$

$$G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

where, I_{VV} and I_{VH} are the emission intensities when the excitation polarizer is vertically oriented and the emission polarizer is oriented vertically and horizontally,

respectively. G is the correction factor.

Time-Resolved Measurements

Fluorescence lifetimes were obtained by the method of Time Correlated Single Photon Counting (TCSPC) following excitation of the samples at $\lambda_{ex} = 340$ nm (IBH-NanoLED-340, FWHM ~ 750 ps) using picosecond diodes and the signals were collected using a Hamamatsu MCP Photomultiplier (Model R-3809U-50) at the magic angle polarization of 54.7° to eliminate any considerable contribution from fluorescence anisotropy decay (Lakowicz, 1999). The decays were deconvoluted on DAS-6 decay analysis software. The average lifetime $\langle \tau \rangle$ is calculated using the following equation (Lakowicz, 1999):

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i \quad (3)$$

where, α_i is the normalized pre-exponential factor for the i^{th} lifetime component.

For time-resolved fluorescence anisotropy decay measurements, the polarized fluorescence decays for the parallel [$I_{\parallel}(t)$] and the perpendicular [$I_{\perp}(t)$] emission polarizations with respect to the vertical excitation polarization were first collected at the emission maxima of the samples under investigation. The anisotropy decay function, $r(t)$ was then constructed using these $I_{\parallel}(t)$ and $I_{\perp}(t)$ decays on the following equation (Lakowicz, 1999):

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} \quad (4)$$

where, G , the grating factor was determined from long time tail matching technique (Lakowicz, 1999).

Results and Discussion

Steady-State Fluorescence Study

Before discussing the spectral changes of NFX inside AOT reverse micelles, we first focus on the photophysical properties of the drug in bulk solvents which could serve as a comparative avenue for assessing the changes in spectral properties of the drug within the AOT reverse micellar assembly. NFX exhibits highly environment-sensitive spectral properties because of the pH-dependent protropic equilibrium (Scheme 1).

Figure 1a exhibits the absorption spectra of NFX in aqueous solution of different pH(s). The absorption profile of NFX at pH = 7.4 shows a strong absorption at ~ 270 nm due to the presence of the aromatic ring and another absorption band in the 300-350 nm wavelength region with two sub-peaks at ~ 325 nm and 335 nm. This long wavelength absorption is ascribed to the $n \rightarrow \pi^*$ electronic transition (Albini *et al.*, 2003; Paul *et al.*, 2015). With decrease in pH of the solution from pH = 7.4 to an acidic value (pH = 4.0), the short wavelength absorption band is found to undergo a red-shift by ~ 5 nm (from ~ 270 nm to ~ 275 nm), along with the appearance of a tail at the long wavelength region (beyond 350 nm) (Fig. 1A). The absorption spectral changes to this direction have been identified with the formation of the cationic species of NFX in acidic pH (Albini *et al.*, 2003). Figure 1a also shows a considerable decrease of absorption of NFX at alkaline pH (pH = 10.7), in analogy to literature reports (Albini *et al.*, 2003; Paul *et al.*, 2015).

Figure 1b represents the emission spectra of NFX in aqueous medium of different pH(s). The emission profile of NFX at pH = 4.0 (i.e., acidic pH) shows maxima at $\lambda_{em} \sim 445$ nm which is characteristic emission of the cationic species. The emission wavelength at $\lambda_{em} \sim 418$ nm in pH 7.4 (i.e., neutral pH) provides the signature of the zwitterionic form of NFX (Paul *et al.*, 2015). At pH = 10.7 the emission spectra of NFX is centered at ~ 420 nm (Fig. 1B) and the fluorescence yield of NFX at alkaline pH (pH = 10.7) is significantly low. Fluoroquinolone derivatives, e.g., NFX, are also known to exhibit solvent-dependent charge transfer emission from the nitrogen center of the piperazinyl group to the keto oxygen following photo-excitation (Blondeau *et al.*, 1999; Nikaido *et al.*, 1993; Berlanga *et al.*, 2000; Albini *et al.*, 2003).

Figure 1C represents the emission spectra of NFX in various solvents of different polarity. In chloroform (CHCl_3) and 1, 4-dioxane, NOF shows emission maxima at ~ 404 nm attributable to the neutral species (Albini *et al.*, 2003). In aqueous medium, NFX exhibits characteristic emission from the cationic species having emission maxima at ~ 442 nm. Thus, the maximum emission wavelength of NFX at ~ 434 nm in methanol is close to that of the cationic species of NFX.

The fluorescence profile of NFX in AOT reverse micelle with increasing water pool size (designated by w_0 value) is displayed in Fig. 2. A progressive red-shift of the emission wavelength with increasing w_0 value indicates increase of polarity in the vicinity of the drug microenvironment. In this context, it is intriguing to note that the emission wavelength of NFX undergoes a remarkable red-shift to $\lambda_{em} \sim 442$ nm at $w_0 = 22$ from $\lambda_{em} \sim 408$ nm at $w_0 = 1$. On the basis of a direct comparison of the spectral properties of NFX in bulk solvents it can be argued that at low w_0 the probe experiences a predominantly hydrophobic environment in which the neutral form of NFX is preferentially stabilized (as indicated by the characteristic fluorescence maxima, $\lambda_{em} \sim 408$ nm at $w_0 = 1$, which is close to that of the neutral species of NFX, $\lambda_{em} \sim 404$ nm in CHCl_3 (Paul *et al.*, 2015; Blondeau *et al.*, 1999; Nikaido *et al.*, 1993; Berlanga *et al.*, 2000; Albini *et al.*, 2003). Whereas, with increased hydration (high w_0) the microenvironment surrounding the probe becomes considerably hydrophilic in which the cationic form of NFX appears to be preferentially stabilized (as designated by the characteristic fluorescence maxima, $\lambda_{em} \sim 442$ nm at $w_0 = 22$, which closely resemble the characteristic cationic emission of NFX, $\lambda_{em} \sim 445$ nm in acidic pH (Fig. 1) (Paul *et al.*, 2015; Blondeau *et al.*, 1999; Nikaido *et al.*, 1993; Berlanga *et al.*, 2000; Albini *et al.*, 2003). However, it is important to note in this context that besides polarity of the micro-environment, change of pH in the locality of the drug within the AOT reverse micelle could also play a role in modulating the photophysics of the bound drug. A critical discussion on the matter has been attempted based on some specifically designed experimental results in a forthcoming section. Photophysics of the drug Using the anthroloxy probe of varying chain length, Kelkar *et al.* (Kelkar *et al.*, 2004) have shown that the shift of the emission maxima in reverse micellar micro-heterogeneous assemblies is largely governed by its precise location in the RMs. They have reported that the emission maxima of 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-cholesterol is independent of w_0 indicating that the probe is localized away from the core of the RM which is characterized by reduced water penetration (Kelkar *et al.*, 2004). In parity with earlier studies (Kelkar *et al.*, 2004; Sengupta *et al.*, 2013), the possibility of localization of the probe in the bulk solvent (n-heptane) can be

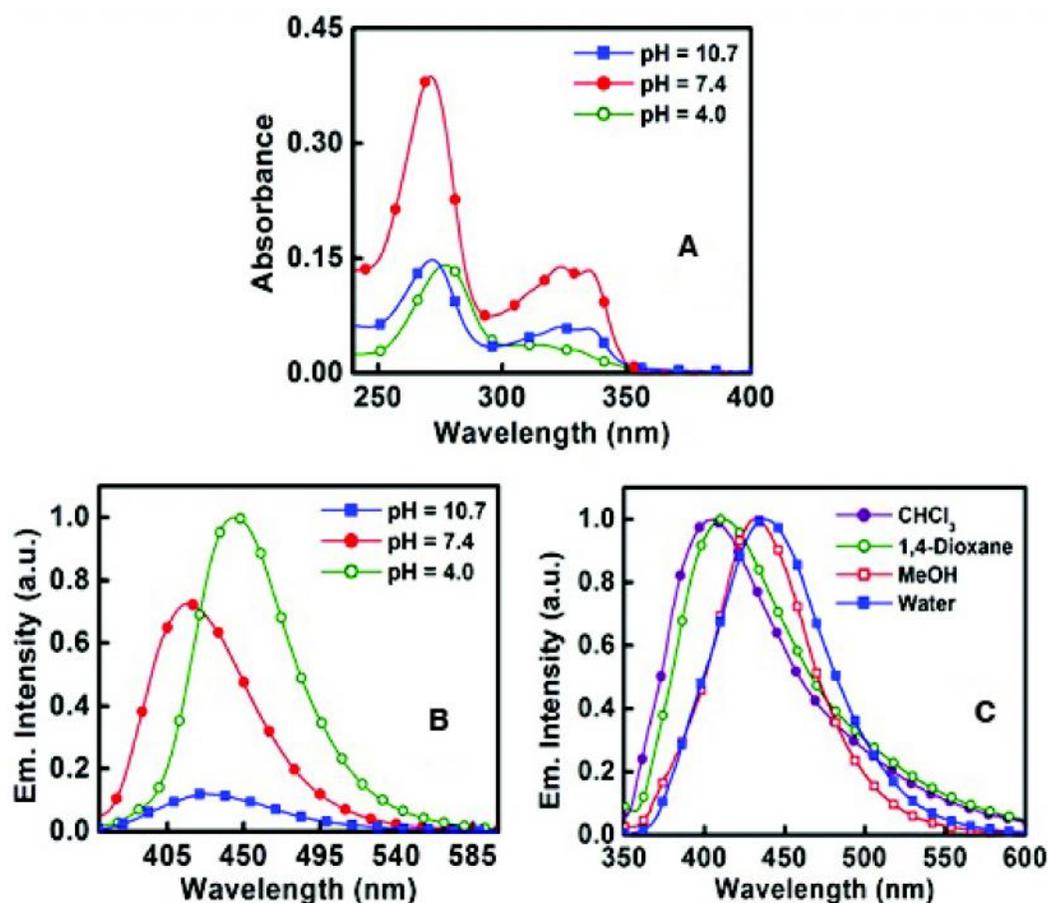


Fig. 1: (A) Absorption spectra of NFX in bulk aqueous solvent of varying pH(s) as indicated in the figure legend (pH = 4.0: -o-, pH = 7.4: -●-, pH = 10.7: -■-), (B) Fluorescence spectra of NFX in bulk aqueous solvent of varying pH(s) as indicated in the figure legend (pH = 4.0: -o-, pH = 7.4: -●-, pH = 10.7: -■-). $\lambda_{\text{ex}} = 335 \text{ nm}$, $[\text{NFX}] = 2.0 \mu\text{M}$ and (C) Fluorescence spectra of NFX in various solvents as indicated in the figure legend (CHCl_3 : -●-, 1,4-dioxane: -o-, MeOH: -□-, Water (pH = 6.0): -■-).

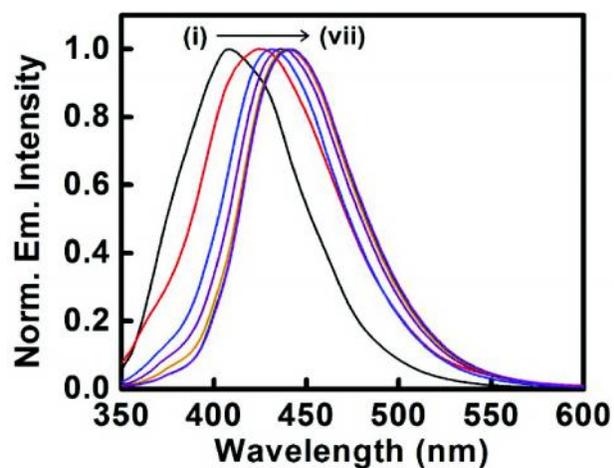


Fig. 2: Normalized fluorescence spectra of NFX ($\lambda_{\text{ex}} = 335 \text{ nm}$, $[\text{NFX}] = 2.0 \mu\text{M}$) in AOT/n-heptane reverse micelles as a function of w_0 . Curves (i) @ (vii) represent $w_0 = 1, 2, 4, 6, 10, 20, 22$

ruled out with a view to the observed marked shift in the emission wavelength as a function of the water pool size (w_0 value). It is also noted that the extent of red-shift of NFX gradually decreases with increasing w_0 and the variation of fluorescence wavelength almost attains saturation beyond $w_0 = 15$ (Fig. 2). This can be rationalized based on the fact that the compartmentalized water in the water pool develops bulk type properties with increase of w_0 value. Calorimetric studies (Goto *et al.*, 1992), determination of dielectric constant (Biswas *et al.*, 2008) and micro-polarity studies inside the reverse micellar water pool (Biswas *et al.*, 2008) have demonstrated that beyond $w_0 \sim 10$, the bulk-like properties become prominent in the water pool.

Fluorescence Anisotropy Study

The photophysical properties of a given drug molecule

in a micro-heterogeneous assembly is largely governed by its precise location. The fluorescence anisotropy can function as sensitive indicator for any modification in rigidity of the surrounding environment of the fluorophore (Paul *et al.*, 2015; Paul *et al.*, 2014). In the present study, the fluorescence anisotropy of NFX is found to be higher inside the reverse micellar microenvironments at all w_0 values compared to that in bulk water ($r \sim 0.013$) (Paul *et al.*, 2015). This result suggests the impartation of motional restriction on the drug molecules inside the AOT RM (Fig. 3).

It is noted that the fluorescence anisotropy of the drug exhibits a specific variation as a function of w_0 value in RM-encapsulated state (Fig. 3). With increase of w_0 value, the anisotropy regularly decreases followed by a gradual saturation. This suggests the impartation of gradual relaxation on motional degrees of freedom of the probe with increasing w_0 inside the RM. This observation probably indicates that the drug molecules move closer to the water pool with increasing w_0 value. These findings are found to be in parity with the results reported (Sengupta *et al.*, 2000).

Time-Resolved Fluorescence Decay

The typical time-resolved fluorescence decay profiles of NFX in RMs as a function of w_0 value are displayed in Fig. 4 and the relevant decay parameters are collected in Table 1. NFX is found to exhibit bi-

exponential decay pattern within the AOT RMs (Table 1), however, the mean (average) fluorescence lifetime is used to explore the behavior of the drug in the micro-heterogeneous reverse micellar environments in various water pool sizes (it is indeed a non-trivial task to assign specific mechanistic models to each decay component in a heterogeneous environment). A gradual decrease of the average fluorescence lifetime of NFX with increasing w_0 (Table 1) points out the fact that with increasing w_0 swelling of the RMs becomes increasingly prominent which in turn accompanies decrease of motional constraint on the bound drug molecules. These results are found to be in keeping with the steady-state results as discussed above. The data summarized in Table 1 show that the

Table 1: Time-resolved fluorescence decay parameters of NFX in AOT reverse micelle with increasing w_0

w_0	τ_1^a (ns)	τ_2^a (ns)	α_1	α_2	$\langle\tau\rangle$ (ns)	χ^2
1	1.31	4.81	0.35	0.65	3.59	1.07
2	1.22	4.38	0.30	0.70	3.43	1.08
4	1.10	4.16	0.24	0.76	3.44	1.02
6	1.02	3.89	0.22	0.78	3.25	1.05
8	0.99	3.64	0.17	0.83	3.18	1.06
10	1.02	3.50	0.35	0.65	2.64	1.01
12	1.04	3.38	0.17	0.83	2.99	1.09
15	1.01	3.27	0.22	0.78	2.77	1.00
22	1.00	3.19	0.34	0.66	2.45	1.01

^a \pm 4%

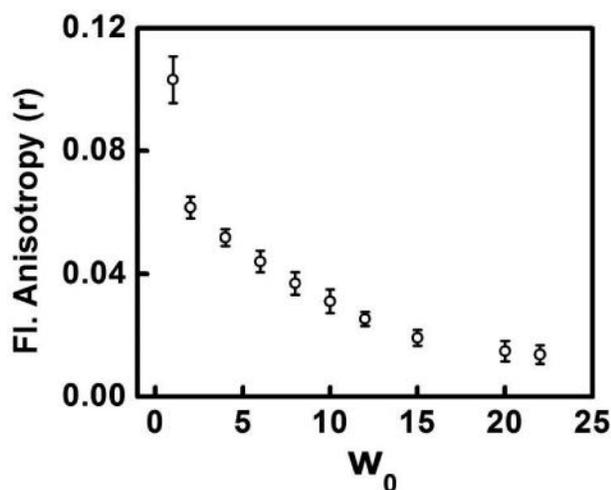


Fig. 3: Variation of steady-state fluorescence anisotropy of NFX ($\lambda_{ex} = 335$ nm, $[NFX] = 2.0$ μ M) in AOT/n-heptane reverse micelles as a function of w_0 . Each data point is an average of 15 individual measurements

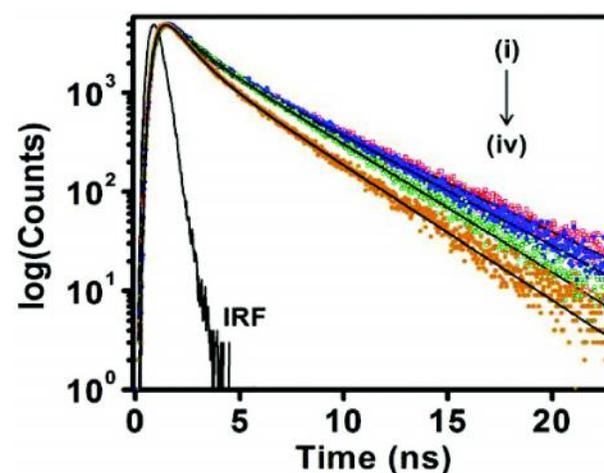


Fig. 4: Time-resolved fluorescence decay transients of NFX ($\lambda_{ex} = 340$ nm, $[NFX] = 2.0$ μ M) in AOT/n-heptane reverse micelle with varying w_0 . Curves (i) \rightarrow (iv) indicate $w_0 = 2, 6, 12, 22$

average lifetime of the probe even at the highest w_0 value studied ($\tau = 2.45$ ns at $w_0 = 22$) is greater than that in aqueous medium ($\tau = 1.91$ ns) and thus suggesting a more restricted environment of NFX within the RMs compared to bulk aqueous phase.

Time-Resolved Fluorescence Anisotropy Decay

To obtain further information about rotational relaxation of the fluorophore and the microenvironment around the fluorophore in reverse micellar environment time-resolved fluorescence anisotropy measurement has been performed as a function of water pool size in RM. The typical anisotropy decay profiles of NFX in RMs at various w_0 values are presented in Fig. 5 and the relevant parameters are summarized in Table 2.

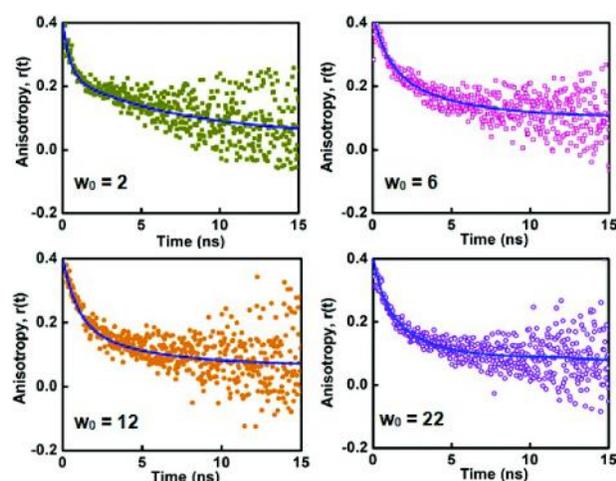
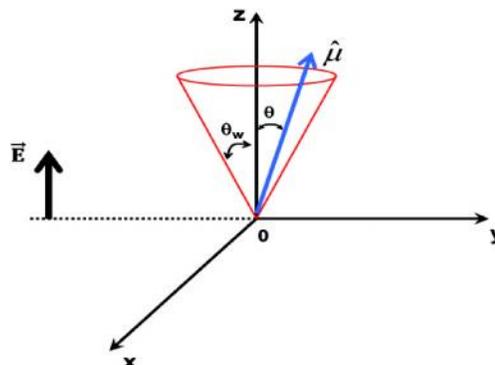


Fig. 5: Time-resolved fluorescence anisotropy decay transients of NFX ($\lambda_{ex} = 340$ nm, $[NFX] = 2.0$ μ M) in AOT/n-heptane reverse micelles at various w_0 values ($w_0 = 2$: \blacksquare , $w_0 = 6$: \blacksquare , $w_0 = 12$: \bullet , $w_0 = 22$: \circ) as specified in the figure legend

Table 2: Time-Resolved Fluorescence Anisotropy Decay Parameters of NFX in AOT Reverse Micelle with Increasing w_0

w_0	τ_{1r}^a (ps)	τ_{2r}^a (ns)	α_{1r}	α_{2r}	$\langle\tau\rangle$ (ns)
2	356	7.25	0.63	0.37	2.91
6	431	6.31	0.76	0.24	1.84
12	527	3.66	0.84	0.16	1.03
22	537	1.89	0.88	0.12	0.61

$\pm 4\%$



Scheme 2: Simplified illustration of the Wobbling-in-Cone Rotation (Here, $\hat{\mu}$ signifies the symmetry axis of the fluorophore, which forms an angle θ with the z-axis which is coincident with the orientation of the E vector of the incident radiation (linearly polarized). There is unrestricted motion around the $\hat{\mu}$ axis which wobbles around the z-axis within a cone of semi-angle, θ_w)

The time-resolved anisotropic decay is found to be biexponential in nature in reverse micellar assemblies of varying water pool sizes. The overall biexponential decay comprises of an ultrafast (τ_{1r}) and a slow (τ_{2r}) component indicating the occurrence of two types of dynamical processes on various time scales. This anisotropy decay is described by the following equation (Lakowicz, 1999):

$$r(t) = r_0 \sum_i \alpha_{ir} \exp(-t/\tau_{ir}) \quad (5)$$

where, α_{ir} is the amplitude of the i^{th} rotational correlation time, τ_{ir} and r_0 is the limiting anisotropy that describes the inherent depolarization of the fluorophore. The average rotational relaxation time is calculated as (Lakowicz, 1999):

$$\langle\tau_r\rangle = \sum_i \alpha_{ir} \tau_{ir} \quad (6)$$

The data compiled in Table 2 reflect a noticeable decrease of the average rotational correlation time ($\langle\tau_r\rangle$) of NFX (that is, enhancement of the rotational motion) with increasing w_0 . This indicates a gradual relaxation of the motional restriction on the AOT-bound drug molecules with increasing w_0 (Kinosita *et al.*, 1977; Paul *et al.*, 2011), in conformity with the steady-state results as discussed earlier (Steady State Fluorescence Study Section).

In tune with numerous literature reports, such a bi-exponential fluorescence depolarization behavior

can be rationalized on the basis of the well-known *wobbling-in-cone* model (Scheme 2) (Kinosita *et al.*, 1977). According to this model, the overall anisotropy decay may arise from three independent motions:

- (i) Wobbling motion of the drug ($r_w(t)$) with a time constant τ_w ,
- (ii) Translational motion of the drug ($r_D(t)$) along the surface of the macromolecule (here reverse micelle) with a time constant τ_D , and
- (iii) Overall rotation of the macromolecule ($r_p(t)$) with a time constant τ_p (Kinosita *et al.*, 1977).

It is often argued that involvement of more than one type of rotational motions leads to deviation of the overall fluorescence depolarization from a mono-exponential pattern. According to this model, the shorter rotational correlation time (τ_{1r}) is interpreted according to a restricted rotor model whose rotational diffusion is restricted in a potential cone of semi angle θ_w about an imaginary axis. The cone itself is considered to rotate on a slower time scale.

Thus, $r(t)$ is decomposed into three independent motions as (Paul *et al.*, 2014; Kinosita *et al.*, 1977; Paul *et al.*, 2011; Tan *et al.*, 2005):

$$r(t) = r_w(t)r_D(t)r_p(t) \quad (7)$$

Further, $r(t)$ in terms of the generalized order parameter S , is given as (Kinosita *et al.*, 1977; Paul *et al.*, 2011; Tan *et al.*, 2005):

$$r(t) = r_0[S^2 + (1 - S^2)\exp[-t(1/\tau_w)]] \exp[-t(1/\tau_D + 1/\tau_p)] \quad (8)$$

Thus, the various rotational dynamical parameters are related to the faster (τ_{1r}) and slower (τ_{2r}) rotational correlation time constants as follows (Paul *et al.*, 2014; Kinosita *et al.*, 1977; Paul *et al.*, 2011; Tan *et al.*, 2005):

$$S = \sqrt{a_{2r}} \quad (9)$$

$$\frac{1}{\tau_w} = \frac{1}{\tau_{1r}} - \frac{1}{\tau_{2r}} \quad (10)$$

Here, S is the generalized order parameter.

Again the order parameter (S) is connected to

the semi angle, θ_w according to (Paul *et al.*, 2014; Kinosita *et al.*, 1977; Paul *et al.*, 2011; Tan *et al.*, 2005):

$$S = \frac{1}{2} \cos \theta_w (1 + \cos \theta_w) \quad (11)$$

The wobbling diffusion coefficient (D_w) of NFX producing information of the cone-restricted motion of the RM-bound drug is given as (Paul *et al.*, 2014; Kinosita *et al.*, 1977; Paul *et al.*, 2011; Tan *et al.*, 2005):

$$D_w = \frac{7\theta_w^2}{24\tau_w}, \quad \text{for } \theta_w \leq 30^\circ \quad (12)$$

For $\theta_w \geq 30^\circ$ D_w is obtained as ($x = \cos \theta_w$):

$$D_w = \{(1 - S^2)\tau_w\}^{-1} \left[\frac{x^2(1+x)^2}{2(x-1)} \left\{ \ln \left(\frac{1+x}{2} \right) + \left(\frac{1-x}{2} \right) \right\} + \left(\frac{1-x}{24} \right) (6 + 8x - x^2 - 12x^3 - 7x^4) \right] \quad (13)$$

In the present case ($\theta_w > 30^\circ$; Table 3), equation 13 is used to elucidate the wobbling diffusion coefficient (D_w) as collected in Table 3.

The calculated rotational parameters (Table 3) show the impartation motional restriction on the drug molecules within the RM-encapsulated state as compared to bulk aqueous phase, whereas the degree of motional restriction imposed on the drug molecules gradually relaxes with increasing water pool size (w_0 value) of the RM. This is distinctly manifested by the decreasing magnitude of the order parameter (S) with w_0 (Table 3). The physical significance of the order

Table 3: Rotational Dynamical Parameters of NFX as Calculated on the Basis of the Wobbling-in-Cone Model

w_0	τ_w (ps)	S	θ_w ($^\circ$)	$D_w \times 10^{-8}$ (s $^{-1}$)
2	374	0.61	44.68	3.91
6	462	0.49	52.48	3.93
12	616	0.40	58.35	3.37
22	750	0.35	61.9	2.96

parameter ($S = \sqrt{a_{2r}}$) can be understood in terms of the degree of spatial restriction on the wobbling-in-cone orientational motion of the drug. The order parameter provides a physical estimate to the orientational motion of the drug within the limit: $S = 0$ for unrestricted motion to $S = 1$ for complete restriction. A decreasing rigidity on the drug molecules within the RMs with increasing w_0 value is reinforced from an enhanced magnitude of the semi angle (θ_w) (Table 3).

Modulated Photophysics of NFX within AOT Reverse Micelle: Effect of pH or Polarity?

In the present context, a natural inquisition may prevail whether the changes in the photophysical properties of NFX with increasing water pool size within the AOT reverse micelle are originating from the modulation of the polarity in the vicinity of the micro-environment of the bound drug or change of pH affecting the protonation/deprotonation equilibrium of NFX.

In order to resolve the issue, we have resorted upon comparison of the photophysical properties of the drug in AOT reverse micelle in which the water pool ($w_0 = 15$) is created with (i) normal water, (ii) acidic water of pH = 4.0 and (iii) alkaline water of pH = 10.0 (Satpati *et al.*, 2009). A detailed pH-dependent study in AOT reverse micelle is, however, not very relevant given the lacuna surrounding the effect on the structure of the reverse micelle of the additives required for changing the pH within the water pool. Figure 6A shows that in all the three cases under investigation, the emission spectra of NFX are strikingly similar to each other (having $\lambda_{em} = 440$ nm). This observation is strongly substantiated from time-resolved fluorescence decay results (Fig. 6B) which

are found to be in close proximity with each other within experimental error limit. Thus, it appears rational to conclude that the observed changes in the photophysical properties of the drug with varying water content within the AOT reverse micelle are more likely to be related to the change in polarity rather than pH in the microenvironment of the bound drug (Satpati *et al.*, 2009).

Conclusions

The present work reports a spectroscopic study of the photophysical properties of NFX within reverse micellar environments as a function the water pool size. Our results reveal that with increasing water pool size inside the micro-heterogeneous reverse micellar core the spectroscopic properties of the bound drug are remarkably modified. The marked red-shift in fluorescence wavelength and decrease of fluorescence anisotropy of NFX with increasing w_0 suggest that the microenvironment in the vicinity of the drug molecules tend to become more bulk like with enhanced hydration (increase in w_0). These results are also found to bear reasonable parity with earlier literature and are further corroborated from time-resolved measurements. The rotational dynamical parameters of NFX within AOT RM as a function of the w_0 value have been calculated using the wobbling-in-cone model which yields meticulous information regarding the degree of motional constraint imposed on the drug molecules in terms of variation of the generalized order parameter (S) and the cone semi angle (θ_w). These results demonstrate the decrease of motional restriction with increase of the water pool size. Furthermore, we have tried to show that our specific experimental results demonstrate the predominating role of change of

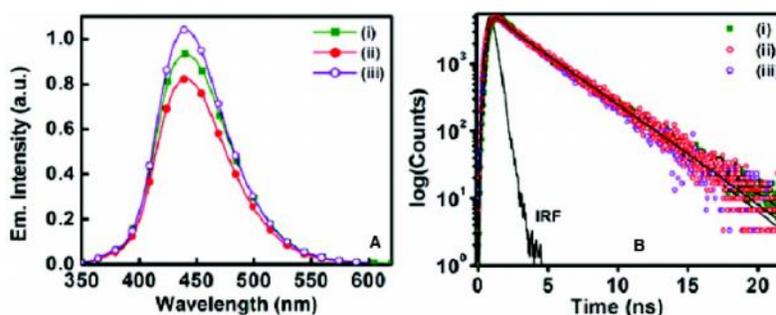
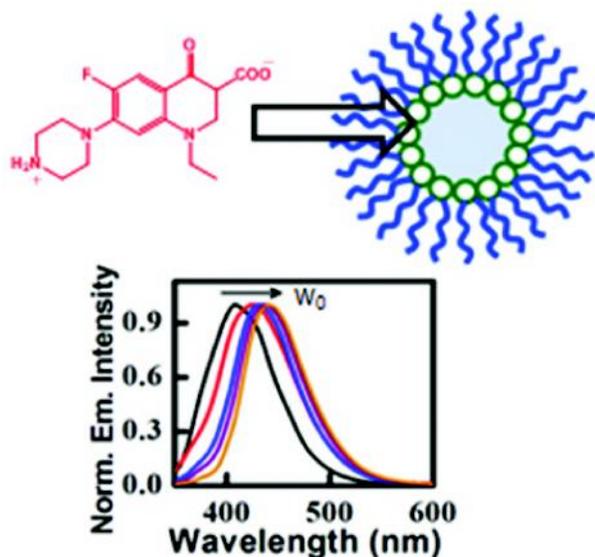


Fig. 6: Comparison of (A) fluorescence spectra and (B) time-resolved fluorescence decay transients (IRF: Instrument Response Function) of NFX in AOT/n-heptane reverse micelles at $w_0 = 15$ with (i) normal water, (ii) water of pH = 10.0, and (iii) water of pH = 4.0

polarity rather than pH in the microenvironment of the bound drug, which is responsible for the observed modulations in the photophysical properties of the drug with water pool size inside AOT reverse micelle.

Graphical Abstract



Acknowledgments

The acknowledge receipt of post-doctoral research fellowship (BKP) from IISER Bhopal, doctoral research fellowships from CSIR (NG & AT) and funding from DST.

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