Eur Biophys J (2014) 43:477–483
DOI 10.1007/s00249-014-0978-5

ORIGINAL PAPER

EBSA
Biophysics in Europe

Interaction of the antibiotic norfloxacin with ionic micelles: pH-dependent binding

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Received: 22 May 2014 / Revised: 10 July 2014 / Accepted: 18 July 2014 / Published online: 5 August 2014
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Abstract The interaction of the antimicrobial drug norfloxacin (NFX) with anionic sodium dodecyl sulfate (SDS) and cationic cetyltrimethylammonium bromide (CTAB) micelles was studied using the intrinsic spectroscopic properties of NFX to obtain association constants and molecular modifications. Nonionic Tween® 20 micelles were also investigated, but the spectroscopic properties of NFX did not detect interactions with these micelles, and quenching by iodide suggested a weak association constant around 47 M^{-1}. For SDS and CTAB, UV–Vis absorption spectroscopy, steady-state and time-resolved fluorometry were monitored as a function of surfactant concentration ranging from the premicellar to micellar region. It was found that cationic (pH 4.0) and zwitterionic NFX (pH 7.4) associate with SDS micelles, with binding constants equal to 5.4×10^3 and 1.7×10^3 M^{-1}, respectively. Premicellar interaction slightly decreases the critical micelle concentration of SDS. Association of anionic NFX (pH 10.6) is very weak. The fluorescence spectrum and lifetime showed that SDS-associated NFX is cationic and that the heterocycle penetrates the interfacial environment of decreased polarity. Cationic CTAB micelles do not bind cationic NFX, and the association constant with zwitterionic NFX is two orders of magnitude lower than that of SDS micelles. From a pharmacological point of view, it is important that at neutral pH, NFX presented a two orders of magnitude higher affinity for anionic than for cationic sites, and did not interact significantly with nonionic or zwitterionic micelle interfaces.

Keywords Fluoroquinolone · Norfloxacin · Fluorescence · Lifetime · Ionization equilibrium · Membrane binding

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Introduction

Norfloxacin (NFX) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-quinoline-3-carboxylic acid] is a synthetic and potent fluoroquinolone antibacterial agent (Appelbaum et al. 2000; Emami et al. 2005) widely used in clinical treatment of many infections including prostate, skin, pulmonary, digestive, and urinary tract infections. Figure 1 shows the chemical structure of norfloxacin.

Fluoroquinolones target bacterial enzymes, which are essential for DNA replication (Drlica 2009). The cytotoxicity of the drugs is due to a strong binding to the gyrase–DNA complex in the presence of Mg^{2+} (Andriole 2000; King et al. 2000). It is clear that formation of drug-enzyme–DNA complexes is the central event in quinolone action, and that electrostatic interactions are important to the stabilization of the complex. However, the knowledge of these complexes is far from complete (Drlica 2009), and study of interactions with model systems will help to understand the important features for cytotoxicity.

Some physicochemical properties of several quinolone antibiotics have been reviewed (Park et al. 2002). The protonation equilibrium and divalent cation complexation have also been examined. Fluoroquinolone interactions

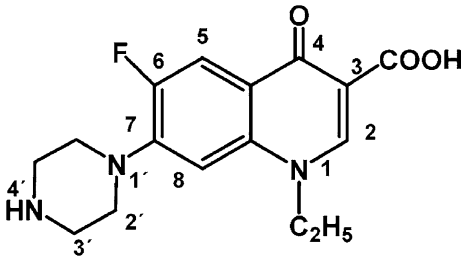

Fig. 1 Structure of norfloxacin

are significantly influenced by their degree of ionization, expressed by pK_a values (Popović et al. 1998; Albini and Monti 2003; Drakopoulos and Ioannou 1997; Luiz et al. 2011). The presence of multiple proton binding sites in fluoroquinolones makes the pattern of acid–base equilibrium rather complex. It has been shown by several techniques that the carboxylate group and the 4′-amine of the piperazine ring are the most significant proton binding sites from a biological point of view [Albini and Monti 2003].

Fluoroquinolones interact with lipids and model membranes (Hernández-Borrell and Montero 2003; Bensikaddour et al. 2008a, b; Sortino et al. 2001). Ciprofloxacin (CPX) was found to interact with neutral liposomes at the surface level and induce a moderate decrease in the bilayer anisotropy (Hernández-Borrell and Montero 2003). The presence of CPX in the core of the bilayer was excluded. A dose-dependent increase of the size of the negatively charged DPPG (1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol) liposomes in the presence of ciprofloxacin was observed, whereas no effect was evidenced for zwitterionic DPPC liposomes (Bensikaddour et al. 2008a). The binding constants were in the order of 10^5 M^{-1} and the affinity is dependent on the negative charge of the liposomes. The results demonstrated that the interactions of fluoroquinolones with lipids depend markedly on the nature of the lipid head groups, and that they interact preferentially with anionic lipid compounds.

Sortino et al. (2001) found a high association constant ($K_{ass} = 5 \times 10^4$ M^{-1}) between the monocationic species of lomefloxacin and sodium dodecyl sulfate (SDS) micelles using iodide quenching of lomefloxacin fluorescence. They also found a 30-fold photochemical stabilization of this fluoroquinolone in SDS micelles. Sortino (2006) also studied the association of norfloxacin with SDS micelles under physiological pH conditions, and found that the dominant form of NFX at physiological pH, zwitterionic, is not the most abundant species in the presence of SDS micelles. These anionic micelles exhibit a high preference for the cationic form of the drug, which becomes the largely dominant species at neutral pH.

There is enough evidence that the interaction of fluoroquinolones with anionic groups in biomolecules is important for the binding properties, stabilization and mechanisms of action. For these reasons, we found it important to investigate more deeply the interaction of the three physiologically relevant NFX species (cationic, NFX^+; zwitterionic, $NFX^{\pm}$; and anionic, NFX^-) with the anionic SDS micelles. Furthermore, we also studied the interaction with cationic cetyltrimethylammonium bromide (CTAB) and non-ionic Tween® 20 (Tw20) micelles. We used the intrinsic fluorescence properties of NFX, e.g., fluorescence emission and lifetimes. A pH titration in the range of 4 to 11 was performed in the presence of SDS micelles. NFX solutions at pH 4.0, 7.5, and 10.6 were titrated with SDS and CTAB in concentrations below and above their critical micelle concentrations (CMC). Premicellar interactions with SDS were observed and association constants were obtained.

Experimental

Materials

Norfloxacin, SDS, CTAB and Tw20 were purchased from Sigma–Aldrich. Buffers were prepared from reagent grade chemicals in ultrapure deionized water. The buffer, consisting of a mixture of borate, phosphate and citrate, was chosen to cover the pH range 4 to 11. The stock solution for the buffer contains 33 mM citric acid, 50 mM phosphoric acid, 50 mM boric acid and 330 mM NaOH. The buffer (20 mM Na) was obtained by a 1:15 dilution, adjusting the pH with a 1 M HCl solution.

Instrumentation

UV–Visible absorption spectra were obtained using a diode array spectrophotometer model 8452A (Agilent). Fluorescence spectra were recorded on a PTI QM1 fluorescence system (Photon Technology International, Birmingham, NJ). Fluorescence lifetimes were measured using an IBH-Horiba-Jobin Yvon TCSPC system. The light source used for excitation was a 330 nm nanoLED N-16, 1.0 ns nominal pulse duration, 1 MHz repetition rate. The pH was measured using a Cole-Parmer Chemcadet 5986-25 pH meter with an Ag/AgCl semimicro combination electrode.

Computer programs supplied by Horiba-Jobin Ivon-IBH, which perform reconvolution fits, were employed for processing the fluorescence decay curves. Fluorescence decay analysis was performed with multi-exponential expressions.

The concentration of norfloxacin was 8 μM in all the experiments. The solutions were prepared from the 1 mM NFX stock solution in ethanol. Titrations of the NFX solutions were carried out using SDS and CTAB stock solutions.

Methods

The fluorescence quantum yields were calculated by the comparative method, using Eq. (1) with the monocationic form NFX^+ as the standard, with fluorescence peak at 442 nm and a fluorescence quantum yield of 0.12, at pH 4.0 (Bilski et al. 1996).

$$\phi_f = \phi_f^{st} \frac{A^{st}}{A} \frac{F_{int}}{F_{int}^{st}} \quad (1)$$

where A is the absorbance at the excitation wavelength, F_{int} is the area under the emission curve, and the superscript st refers to the standard.

The binding constants were quantitatively determined in terms of the two-state model in which micelles and water are considered as separate pseudophases, and the drug (NFX) is free in water or associated with the micelle (Guo et al. 1994). Modifications of the fluorescence spectrum upon binding allowed obtaining the binding constants of the different NFX species to micelles. Fluorescence increments as a function of surfactant concentration were fitted with the Benesi-Hildebrand equation (Benesi and Hildebrand 1949), modified to describe the binding above the CMC.

$$F - F_0 = (F_\infty - F_0)\left(1 + \frac{1}{K_b([S] - \mathrm{CMC})}\right)^{-1} \quad (2)$$

where K_b is the binding constant referred to the surfactant concentration in micellar phase ($[S] - \mathrm{CMC}$), CMC is the critical micelle concentration, and F_0, F and F_∞ are the fluorescence intensity of NFX in the absence of micelles, at a given surfactant concentration, and at saturating surfactant concentrations, respectively. The fitting parameters are K_b, F_∞ and CMC. Equation (2) is valid for micelle concentrations much greater than [NFX].

The pK_2 for the deprotonation of the NFX piperazine distal amine in the presence of SDS micelles was obtained by a least squares fit of Eq. (3) (Luiz et al. 2011), which gives F_λ as a function of the pH, for one titration site:

$$F = \frac{F_1\, 10^{pK} + F_2\, 10^{pH}}{10^{pH} + 10^{pK}} \quad (3)$$

where F_1 and F_2 are the fluorescence intensity at a given wavelength of the protonated and deprotonated amine, respectively.

Results and discussion

The monocationic form NFX^+ is fluorescent, with a fluorescence peak at 442 nm and a fluorescence quantum yield of 0.12, at pH 4.0 (Bilski et al. 1996). The zwitterionic $NFX^\pm$ is the dominant species at pH 7.4. It has a fluorescence peak at 408 nm and a quantum yield of 0.10. The anionic species NFX^-, which strongly predominates at pH around 10.6, is virtually nonfluorescent, with a quantum yield less than 0.005. We choose these three pH values in order to study the association of these three biologically relevant NFX species with anionic SDS, cationic CTAB, and nonionic Tw20 micelles.

Interaction of norfloxacin with Tween® 20

The experiments with Tw20, however, did not produce significant modifications on NFX spectroscopic parameters, suggesting lack of or weak association. An alternative explanation is that NFX binds to Tw20 micelles, but the local environment of NFX molecules at the palisade layer is very similar to the aqueous environment, especially concerning the pH and dielectric constant.

Quenching experiments of NFX fluorescence by iodide were performed to differentiate between these explanations. Titration of NFX solutions (8.10^{-6} M, pH 7.4) with KI in the absence and presence of Tw20 micelles (10 mM) gave Stern–Volmer quenching constants of 16.3 and 13.7 M^{-1}, respectively. A simple geometric argument suggests that if NFX associates at the surface of a Tw20 micelle, the probability of collisions with iodide ions will decrease twice and the bimolecular quenching constant will present a twofold reduction. Assuming this model, the 16 % reduction of the quenching constant suggests that 32 % of NFX molecules bind to Tw20 micelles at the concentrations used. This gives an association constant of 47 M^{-1}.

Interaction of norfloxacin with SDS

Figure 2 presents the evolution of the NFX fluorescence emission spectra with increasing amounts of the anionic surfactant SDS at pH 4.0 (a), 7.4 (b) and 10.6 (c). Fluorescence at pH 4.0 shows an increase in quantum yield from 0.12 to 0.20 and a blue shift from 440 to 432 nm in the presence of SDS micelles. The inset of Fig. 2a shows the fluorescence change at 432 nm. The sharp change in the fluorescence intensity occurs around SDS concentration of 2 mM, and suggests association with micelles. Small changes at concentrations smaller than 2 mM suggest premicellar interactions. Equation (2) fits the data in the inset of Fig. 2a, with best fit parameters in Table 1. The CMC of 1.8 mM is much less than the CMC of SDS in pure water (around 8.3 mM), but only somewhat smaller than that obtained using pyrene fluorescence in the same buffer (2.9 mM). The small decrease in CMC is probably due to the premicellar interactions. In contrast with the sharp fluorescence variation, the NFX absorption spectrum

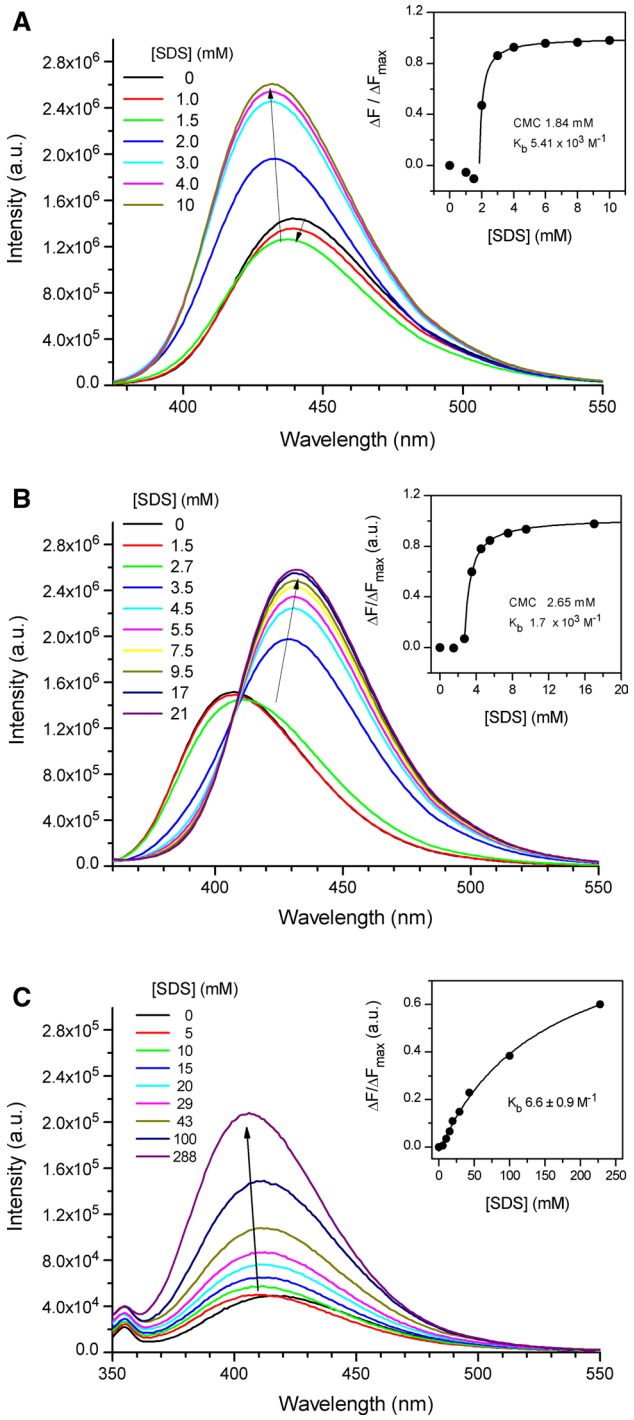

Fig. 2 Fluorescence spectra of NFX in buffer with increasing SDS concentrations. NFX concentration: 8 μM. Excitation at 318 nm. **a** pH 4.0; **b** pH 7.4; **c** pH 10.6. Insets: fractional fluorescence changes at 432 nm (**a, b**) and 410 nm (**c**) as a function of SDS concentration. The *solid lines* are the least squares fits using Eq. 2, with parameters in the legends (Table 1)

(not shown) presents only minor changes, suggesting that NFX^+ keeps its cationic form upon binding to SDS micelles.

Table 1 Association constants of the cationic, zwitterionic and anionic norfloxacin species with SDS micelles; SDS critical micelle concentrations (CMC) in the presence of norfloxacin (buffer $[Na^+] = 20$ mM)[1]; and lifetimes in the absence and presence of SDS

pH	K_b (M^{-1})	CMC (10^{-3} M)	τ (ns)	τ_{SDS} (ns)
4.0	$5.4 (\pm 0.4) \times 10^3$	1.84 (± 0.01)	1.5	2.8
7.5	$1.7 (\pm 0.4) \times 10^3$	2.65 (± 0.01)	1.2	2.8
10.8	6.6 (± 0.9)	2.5 (± 1) 0	(1.2)*	–

[1] Data obtained from least square fits using Eq. (2)

* lifetime associated with the small amount of $NFX^{\pm}$ at this pH

The blue shift indicates a NXF environment of decreasing polarity, and points out that the heterocyclic rings penetrate the micellar core, while the charged amino group remains in the interfacial diffuse layer of electric charges.

The fluorescence spectrum of $NFX^{\pm}$ at pH 7.4 also presents an increase in quantum yield (Fig. 2b), but shows a red shift from 408 to 432 nm in the presence of SDS micelles. It is worth noting that the fluorescence spectra of NFX at saturating SDS concentrations are equal at both pH 4.0 and 7.4. The UV absorption spectrum (not shown) evolves from zwitterionic to cationic type under increasing SDS concentration. We conclude that $NFX^{\pm}$ becomes cationic on binding to SDS micelles, i.e., the carboxylic anion protonates. So, the observed fluorescence red shift is the combined effect of the transition from zwitterionic to cationic species and the change to the environment of decreasing polarity. The inset of Fig. 2b presents the fluorescence change at 432 nm. Equation (2) fits the data with best fit parameters in Table 1. NFX protonation on binding to SDS at pH 7.4 has also been observed by Sortino (2006).

It is worth noting that the negative surface charge density of the SDS micelles increases the local pH relative to the bulk pH according to the Boltzmann equation (Tocanne and Teissié 1990):

$$\left[H^+\right]_{surf} = \left[H^+\right]_{bulk} \exp\left[-e\psi/(kT)\right] \quad (4)$$

$$pH_s = pH_b + 0.434\, e\psi/(kT) \quad (5)$$

where (s) refers to surface and (b) refers to bulk, ψ is the surface potential, e is the elementary charge, k is the Boltzman constant and T (K) is the temperature. Surface potentials from −111 to −137 mV have been reported for SDS micelles (Tocanne and Teissié 1990; Louro et al. 1994). A surface potential of −125 mV lowers the pH by 2 units at ambient temperature. So, for a bulk pH 7.4, the local pH at the micelles surface is about 5.2. It is therefore not surprising that the NFX carboxyl protonates at the SDS micelle surface and NFX molecules become positively charged.

At pH 10.6, NFX^- is virtually non-fluorescent as shown in Fig. 2c. Electrostatic repulsion nearly prevents association

of NFX^- with the anionic SDS micelles. The fluorescence spectrum shows an increase in quantum yield with increasing SDS concentration, but even at concentrations as high as 250 mM, the quantum yield is much less than at pH 7.4 or 4.0 (see the ordinate scale). The inset in Fig. 2(c) also shows that the association constant is very small. Equation (2) fits the data with a K_b three orders of magnitude lower than at pH 4.0 (Table 1). The absorption spectrum (not shown) presents almost no changes, except for a small increase in light scattering at high SDS concentrations.

A pH titration of NFX in the presence of 25 mM SDS was carried out, and the fluorescence results appear in Fig. 3. The fluorescence increase with decreasing pH from 11 to 8 demonstrates the increase in binding of NFX to SDS micelles. Figure 3 also shows that in the pH range from 8 to 4 virtually all the NFX molecules are bound to the micelles. The pK_2 value for the amine protonation shifted from 8.6 in the absence of SDS to 9.1, at 25 mM SDS.

Fluorescence lifetimes were also measured as a function of SDS concentration. Figure 4 shows the NFX fluorescence decay curves at pH 4.0 and 7.4 in the absence and in the presence of saturating amounts of SDS (10 mM for pH 4.0 and 21 mM for pH 7.4). The lifetime is $\tau_1^+ = 1.5$ ns at pH 4.0, for cationic NFX, and $\tau_1^{\pm} = 1.2$ ns at pH 7.4, for zwitterionic NFX. The values are similar to those of Luiz et al. (1.58 ns at pH 4.0 and of 1.15 ns at pH 7.6) (Luiz et al. 2011), and the small discrepancies are due to expected lifetime variations in experiments performed at slightly different experimental conditions. The lifetime increases to $\tau_2 = 2.8$ ns when NFX binds to SDS micelles at both pHs (Table 1). This is in agreement with the steady state measurements, which displays the same emission spectrum at both pHs under saturating SDS concentrations. The inset in Fig. 4 presents the relative contributions of the lifetimes τ_1 and τ_2 as a function of [SDS], obtained from global analysis of the decay curves. The lifetime transition from unbound to bound NFX agrees with the steady state results. Assuming the CMC as the SDS concentration of maximum slope, we obtain 1.6 mM at pH 4.0 and 2.5 mM at pH 7.5, which are similar to the steady state results in Table 1.

Interaction of NFX with CTAB

At pH 4.0, the cationic NFX^+ predominates and CTAB micelles also have a net positive surface charge. So there is an electrostatic repulsion between NFX^+ and the CTAB head groups. As expected, the UV absorption spectrum did not change, indicating that the electrostatic repulsion dominates over hydrophobic interactions, and there is no association. The fluorescence decay was also investigated in the presence of CTAB. Even at high CTAB concentrations (about 60 mM), NFX^+ lifetime remained equal to that in aqueous solution, confirming the initial argument that the electrostatic repulsion dominates and prevents NFX^+ binding to the micelles.

In the case of zwitterionic $NFX^{\pm}$, because of charge symmetry, we expected an association constant with CTAB micelles similar to that with SDS. Figure 5 shows a fluorescence decrease with increasing CTAB concentration. This suggests deprotonation of the NFX piperazine distal amine, which leads to the nonfluorescent NFX^- species. An alternative explanation would be the quenching by the bromide ions of CTAB, but in this case the NFX fluorescence lifetime would decrease (dynamic quenching). This explanation was ruled out, since the lifetime was observed to remain constant.

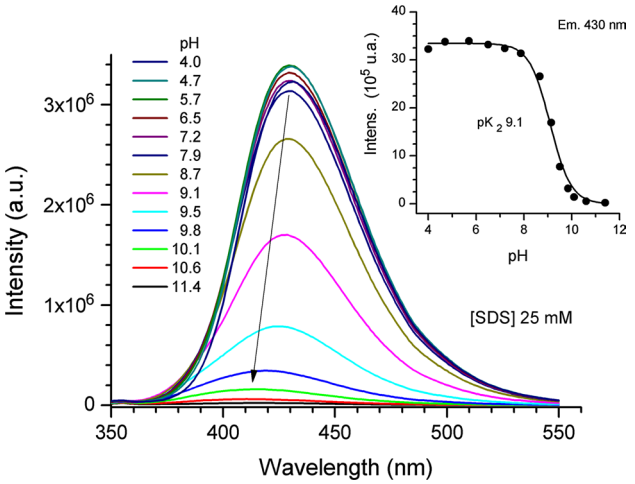

Fig. 3 Fluorescence spectra of NFX in buffer with SDS 25 mM at different pH. NFX concentration: 8 μM. Excitation at 318 nm. The *arrow* indicates increasing pH. Inset: fluorescence emission at 430 nm as a function of pH. The *solid line* is the least squares fit using Eq. 3, with $pK_2 = 9.1$

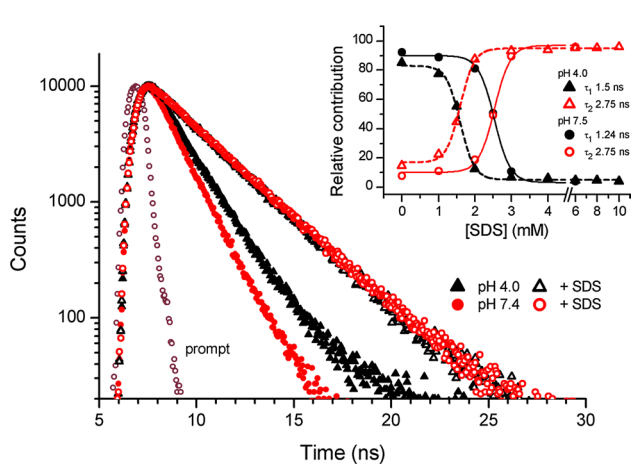

Fig. 4 Fluorescence decay curves of NFX fluorescence at pH 4.0 (*triangles*) and 7.4 (*circles*) in the absence and presence of saturation amounts of SDS (10 mM for pH 4.0 and 21 mM for pH 7.4). Inset: relative contribution (%) of the NFX lifetimes τ_1 (aqueous) and τ_2 (micellar) as a function of SDS concentration

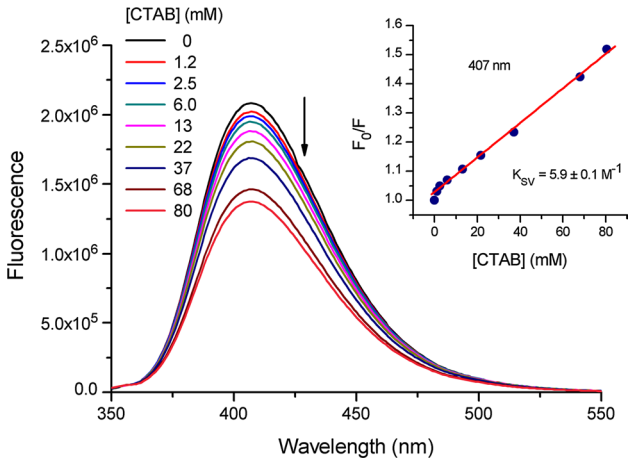

Fig. 5 Fluorescence spectra of NFX at pH 7.4 with increasing CTAB concentrations (see *arrow*). NFX concentration: 8 μM. Excitation at 318 nm. Inset: Stern–Volmer plot of fluorescence quenching. The *solid line* is the least squares fit using Eq. 2, with Stern–Volmer constant $K_{SV} = 5.9 \pm 0.1\ M^{-1}$

Assuming that each bound molecule becomes nonfluorescent, the obtained Stern–Volmer constant will be the association constant, $K_{SV} = 6.0\ M^{-1}$ (see Fig. 5). This is two orders of magnitude lower than with SDS micelles. The zwitterionic species preferentially associate with the anionic sites. This result is quite unexpected and suggests that other interactions unrelated to the charge modify the binding.

Deprotonation of the NFX piperazine distal amine at the micelle surface is probably due to increased local pH at the CTAB micelle surface, because the positive surface charge density repels hydronium cations. As a result, the NFX± molecules change into nonfluorescent anionic species at the micelle surface. In fact, it was observed that CTAB micelles produce an apparent pK shift of −1.7 for the amino group of the local anesthetic dibucaine, a −1.3 shift due to the surface charge density, and −0.4 due to the different dielectric constant (Louro et al. 1994). Assuming this shift, we obtain a local pH of 8.8 at the micelle surface, for a bulk pH of 7.5 (7.5 + 1.3). It is possible to estimate the amount of deprotonated NFX distal amines at the micelle surface using its pK (8.6) (Luiz et al. 2011), and the local pH = 8.8: $NFX^-/NFX^{\pm} = 10(pH - pK) = 1.58 \rightarrow NFX^-/(NFX^{\pm} + NFX^-) = 0.61$. That is, only 39 % of the NFX molecules contribute to the fluorescence. This amount agrees with the fluorescence decrease in Fig. 5 (we obtained an asymptotic value of 42 % of the initial fluorescence intensity). We conclude that the mechanism of NFX fluorescence decrease was in fact the modification of local pH due to the surface electric potential at the CTAB micelle surface.

At pH 10.6, the anionic form of NFX largely prevails, and we would expect an electrostatic attraction between the NFX carboxylate ion and the cationic head groups of the micelles. The polycyclic rings would tend to penetrate the micelle due to hydrophobic interactions. So, the hydrophobic interaction and electrostatic attraction would cooperate to maintain NFX molecules associated with the micelles. However, it was not possible to find the association constant of the anionic NFX^- with CTAB micelles (pH 10.6), because the UV absorption spectrum does not change and the species is not fluorescent.

Conclusion

The association of the three most relevant species of NFX with micelles of three surfactants with different polar head groups was examined using the fluorescence of NFX as a spectroscopic marker for the interaction. It was observed that NFX fluorescence does not change in the presence of the nonionic micelles of Tw20 up to 20 mM. This suggested very weak association of the three species NFX^+, $NFX^{\pm}$ and NFX^- with nonionic micelles. Quenching by iodide suggested an association constant around 47 M^{-1}.

It was found that both NFX^+ and $NFX^{\pm}$ bind to SDS micelles. Association constants are equal to 5.4 x 10^3 and 1.7 x 10^3 M^{-1}, respectively, but the association of NFX^- is very weak. Premicellar interactions were detected, which cause a small decrease in the CMC of SDS. The results showed that NFX molecules associated with SDS micelles are cationic, with fluorescence properties different from those in aqueous environment. The fluorescence peaks at 432 nm, and the quantum yield (0.21) is higher than in solution (maximum of 0.12 at pH 4.0). The fluorescence lifetime (2.8 ns) is also higher than in solution (1.5 ns at pH 4.0). Increasing the pH causes the transition from $NFX^{\pm}$ to NFX^- and dissociation from the SDS micelles with a pK equal to 9.1 (Fig. 3).

The interactions of the various species of norfloxacin with cationic CTAB micelles were not symmetrical with respect to anionic SDS micelles. The electrostatic repulsion between NFX^+ and CTAB prevents association. $NFX^{\pm}$ molecules bind to CTAB micelles with an association constant (6.0 M^{-1}) two orders of magnitude lower than to SDS micelles (1.7 x 10^3 M^{-1}). Although the positive surface potential of the CTAB micelles increases the local pH and tends to stabilize the anionic form of associated NFX, it was not possible to obtain an NFX^- association constant with CTAB because this species is not fluorescent, and a different technique is necessary.

Acknowledgments This work was partly supported by the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and the project inct-INAMI (CNPq).

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