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Fluorescence and electron paramagnetic resonance studies of norfloxacin and N-donor mixed-ligand ternary copper(II) complexes: Stability and interaction with SDS micelles

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ABSTRACT

The stability of ternary copper(II) complexes of a heterocyclic ligand, L (L being 2,2′-bipyridine (bipy) or 1,10-phenanthroline (phen)) and the fluorescent antibacterial agent norfloxacin (NFX) as the second ligand was studied at pH 7.4 and different ionic strengths. Fluorescence quenching upon titration of NFX with the binary complexes allowed to obtain stability constants for NFX binding, K_b, as a function of ionic strength. The K_b values vary by more than two orders of magnitude when buffer concentration varies from 0.5 to 100 mM. It was observed that previously synthesized ternary complexes dissociate in buffer according with the obtained stability constants. This shows that equimolar solutions of NFX and binary complexes are equivalent to solutions of synthesized ternary complexes. The interaction of the ternary copper complexes with anionic SDS (sodium dodecyl sulfate) micelles was studied by fluorescence and electron paramagnetic resonance (EPR). Titration of NFX-loaded SDS micelles with the complexes Cu:L allowed to determine the stability constants inside the micelles. Fluorescence quenching demonstrated that SDS micelles increase the stability constants by factors around 50. EPR spectra gave details of the copper(II) local environment, and demonstrated that the structure of the ternary complexes inside SDS micelles is different from that in buffer. Mononuclear ternary complexes formed inside the micelles, while in buffer most ternary complexes are binuclear. The results show that anionic membrane interfaces increase formation of copper fluoroquinolone complexes, which can influence bioavailability, membrane diffusion, and mechanism of action of the antibiotics.

1. Introduction

Norfloxacin (NFX) is a member of the fluoroquinolone family of antibacterial agents used in the clinical treatment of many bacterial infections [1]. The properties of fluoroquinolones and their interactions with different biologically significant molecules and systems have been studied, and the fact that most fluoroquinolones are fluorescent provides important approaches to investigate molecular mechanisms [2–10].

Interaction with metal ions has important consequences for the solubility, pharmacokinetics and bioavailability of fluoroquinolones, and it is also involved in their mechanism of action [11–14]. Complexation can be a strategy to increase the range of antibiotics action or overcome antibiotics resistance [15]. Several examples of norfloxacin Cu(II) complexes with nitrogen donor co-ligands can be found in the literature [11,16,17]. Studies of their antimicrobial activity and interactions with albumin and DNA have been performed [16,18–21]. Among the metal complexes that have been investigated, those containing N-donor heterocyclic co-ligands like 2,2′-bipyridine (bipy) and 1,10-phenanthroline (phen) have attracted attention [17,22,23].

Fig. 1 shows the chemical structure of the copper(II) complexes of the two nitrogen donor heterocyclic compounds 2,2′-bipyridine, and phenanthroline, and norfloxacin used as co-ligand to the complexes:

Saraiva et al. synthesized the binary copper(II):enrofloxacin and ternary copper(II):enrofloxacin:phenanthroline complexes, and showed that the synthesized complexes and the complex solutions prepared by mixing the individual components in the same stoichiometric proportion exhibited the same behavior in biological studies [24]. They also pointed out that at biological concentrations the copper(II) complexes may dissociate and the antimicrobial activity can be a consequence of the free dissociated fluoroquinolone. On the other hand, the EPR spectra of ternary copper(II) complexes at millimolar concentrations in water (77 K) showed almost

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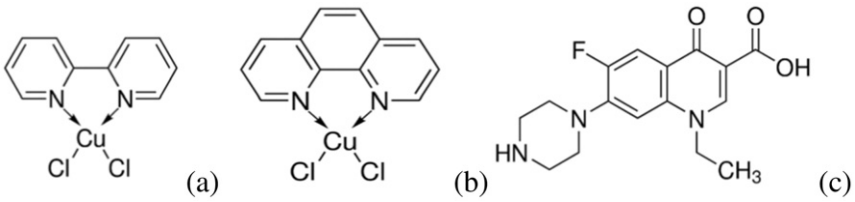

Fig. 1. Structure of (a) copper 2,2′-bipyridine, (b) copper 1,10-phenanthroline, and (c) norfloxacin.

exclusively binuclear species of the type [copper(II):L:NFX]$_2$ where L is bipy or phen [23]. It is therefore important to know the association constants of the complexes under different conditions, and study how the different biological and biomimetic structures influence complex formation.

The interaction of fluoroquinolones with membrane models has been investigated [25–27]. The interaction of ciprofloxacin (Cpx) and its copper(II) ternary complex copper(II):phen:Cpx with unilamellar liposomes of different lipid mixtures was studied using steady-state and time-resolved fluorescence spectroscopy. The results suggested that ciprofloxacin and its metalloantibiotic have different translocation pathways, and the metalloantibiotic may bypass one mechanism of bacterial resistance [26]. In general, it has been noticed that negatively charged groups increase fluoroquinolone affinity for hydrophilic lipophilic interfaces [3,28–29].

The study of drugs association with micelles and liposomes are important for surface engineering of drug delivery systems. NFX molecules associate with micelles of the anionic surfactant SDS, and the fluorescence spectra and lifetimes showed that the heterocycle penetrates the interfacial environment of decreased polarity with the cationic protonated amine localized at the micelle anionic surface [3].

In this work, we first studied the stability of the two mixed ligand copper(II) complexes copper(II):bipy:NFX and copper(II):phen:NFX in phosphate buffer with different ionic strength. Stability constants for binding the second ligand NFX were obtained using the quenching of NFX fluorescence by titration with copper(II):bipy and copper(II):phen. Then, we studied the influence of SDS micelles on formation of the ternary complexes. Fluorescence quenching was used to find association constants, and UV–Visible absorption spectra were obtained to discard trivial causes of quenching. Electron paramagnetic resonance allowed to find details of the complexes inside the SDS micelles and the ligand field around the copper ions.

2. Materials and Methods

2.1. Materials

Norfloxacin, (1-ethyl(-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-quinoline-3-carboxylic acid), 2,2′-bipiridine, and dichloro(1,10-phenanthroline) copper(II) were purchased from Sigma-Aldrich. All other reagents and solvents were from Merck.

The [$CuCl_2$(bipy)] (Fig. 1) precursor was prepared using a method similar to that described in the literature [30]. Briefly, equimolar amounts of the $CuCl_2 \cdot 2H_2O$ and 2,2′-bipyridine (1.74 mmol) were dissolved in about 20 mL of acetone. The mixture was stirred and refluxed for 24 h, and then vacuum filtered.

[CuCl(bipy)(NFX)]Cl and [$CuCl_2$(phen)(NFX)] were prepared according to Martins et al. [23]. Solutions of the binary and ternary Cu(II) complexes (stock 1.0×10^{-3} M) were prepared in 10 mM phosphate buffer at pH 7.4, except otherwise stated.

2.2. Apparatus

X-band electron paramagnetic resonance (EPR) spectra were obtained with a ESP300E spectrometer – Bruker, or a Miniscope MS 400 spectrometer - Magnettech, with a modulation frequency of 100 kHz and a modulation amplitude of 0.2–1.0 mT. Frozen aqueous solutions of the complexes (~1×10^{-3} M) were measured at liquid N_2 temperature (77 K) in Teflon® tubes with a 3 mm internal diameter, which fit a Wilmad LabGlass nitrogen dewar flask.

Steady state fluorescence measurements were performed on a PTI QM1 fluorescence system. UV–Vis absorption spectra were obtained with an Agilent diode array spectrophotometer model 8452A. Fluorescence lifetimes were measured using an IBH-Horiba-Jobin Yvon TCSPC system. A NanoLED (330 nm) with 1.0 ns nominal pulse duration and 1 MHz repetition rate was used for excitation of NFX fluorescence.

2.3. Copper(II):Bipy and Copper(II):Phen Binding to NFX

Quenching measurements of NFX fluorescence were performed using 3 mL solutions of NFX (8×10^{-6} M) in phosphate buffer pH 7.4 at different concentrations (different ionic strengths). The fluorescence emission spectra were obtained at ambient temperature 296 K using an excitation wavelength of 330 nm.

The stability constants K_b for the equilibrium

$$Cu:L + NFX \overset{K_b}{\rightleftarrows} Cu:L:NFX \tag{1}$$

were obtained using the exact expression (2) for the formation of a 1:1 complex [31], under the assumption that the stability constants of Cu:L are much greater than that of Cu:NFX.

$$F_0 - F = \frac{F_0 - F_\infty}{2} \cdot \left\{ \left(1 + \frac{c_{CuL}}{c_{NFX}} + \frac{1}{K_b c_{NFX}}\right) - \left[\left(1 + \frac{c_{CuL}}{c_{NFX}} + \frac{1}{K_b c_{NFX}}\right)^2 - 4\frac{c_{CuL}}{c_{NFX}} \right]^{1/2} \right\} \tag{2}$$

where K_b is the binding constant, c_{NFX} and c_{CuL} are the concentrations of the ligand NFX and the binary metal complex Cu:L, respectively; F_0, F and F_∞ are the fluorescence intensities of NFX in the absence of Cu:L,

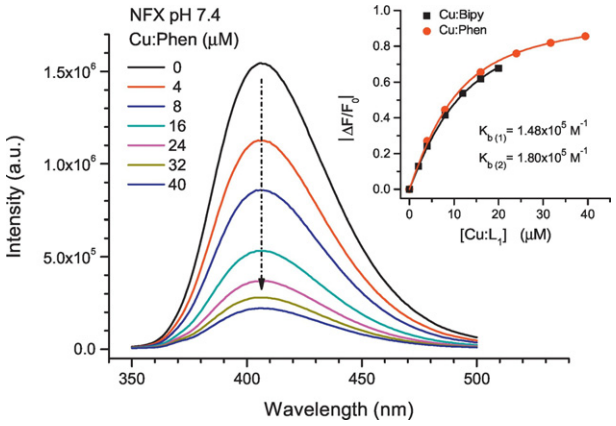

Fig. 2. Quenching of NFX fluorescence (phosphate buffer 20 mM, pH 7,4) by increasing concentrations of Cu:phen. Excitation at 344 nm. Inset: normalized change of NFX fluorescence as a function of the binary complex concentration (▪) Cu:bipy, (●) Cu:phen. Solid lines are the least squares fits using Eq. (2) with $K_b^{(1)} = (1.48 \pm 0{,}02) \times 10^5\ M^{-1}$, and $K_b^{(2)} = (1.80 \pm 0{,}02) \times 10^5\ M^{-1}$, for Cu:bipy and Cu:phen, respectively.

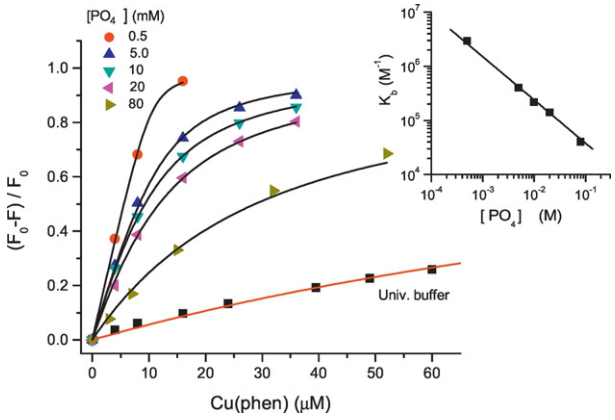

Fig. 3. Normalized change of NFX fluorescence (quenching) as a function of Cu:phen concentration at different buffer concentrations (PB concentrations of 0.5, 5.0, 10, 20 and 80 mM, pH 7.4). Solid lines are the least squares fits using Eq. (2). Results in a buffer containing citrate, phosphate, and borate (Na^+, 20 mM) appear for comparison (squares). Inset: Stability constant K_b as a function of PO_4 concentration (2.9×10^6, 4.0×10^5, 2.2×10^5, 1.4×10^5, 4.0×10^4 M^{-1}, respectively).

at a given Cu:L concentration, and at $c_{CuL} \gg c_{NFX}$, respectively. Here, F_∞ is zero (complete quenching).

For the EPR studies of the complexes in SDS micelles, each binary and ternary copper(II) complex was mixed (final concentration ~ 0.5 mM) with 20 mM SDS solutions in 10 mM phosphate buffer pH 7.4. Also the binary complexes Cu:L were mixed with NFX loaded SDS micelles at equimolar stoichiometry. EPR spectra were obtained at 77 K.

2.4. Effect of SDS Micelles on Copper(II):Bipy and Copper(II):Phen Binding with NFX

To study the association of the binary complexes Cu:L with NFX associated with SDS micelles, 8 μM NXF solutions in 20 mM phosphate buffer, pH 7.4, and 20 mM SDS were titrated with $CuCl_2$, Cu:bipy and Cu:phen. The association was monitored by the quenching of NFX fluorescence upon Cu:L binding.

3. Results and Discussion

3.1. Stability of the Ternary Complexes

The synthesized metal complexes can dissociate in aqueous solutions, and so it is important to evaluate the metal-ligand stability constants. The stability constants of the binary complexes Cu:bipy and Cu:phen are of the order of 10^8 and 10^9 M^{-1}, respectively [32]. Then, ternary complex formation with NFX can be considered a stepwise equilibrium, and the stability constant for binding the second ligand (Eq. (1)) is

$K_b = [Cu:L:NFX]/([Cu:L]\cdot[NFX])$, where L is bipy or phen.

Since copper(II) ions are strong fluorescence quenchers, it can be assumed that NFX fluorescence is quenched in the mixed-ligand copper complexes, so that the fluorescence of the solutions derives only from the dissociated NFX molecules. Thus, fluorescence quenching can be used to obtain the stability constants for binding the second ligand NFX. Indeed, fluorescence quenching was used in a previous work to obtain the stability constant of Cu:phen and NFX in water (neutral pH, but very low ionic strength) [3]. Here, the same method was used to obtain the stepwise stability constants of Cu:L and NFX, for L = bipy or phen. A constant concentration of NFX was titrated with increasing concentrations the binary complexes Cu:L. Fig. 2 shows the quenching results of NFX fluorescence in PB 20 mM, pH 7.4. Fig. S1, in Supplementary material, shows the UV spectra of NFX, Cu:bipy and Cu:phen. It is possible to verify that the copper complexes do not absorb the light at the excitation wavelengths used in the fluorescence quenching experiments, and do not produce trivial quenching effects.

The inset presents the normalized fluorescence changes and the best fits using Eq. (2), with $K_b^{(1)} = 1.48 \times 10^5$ M^{-1}, and $K_b^{(2)} = 1.80 \times 10^5$ M^{-1}, for NFX association with Cu:bipy and Cu:phen, respectively.

These stability constants show that, for concentrations in the mM range, the concentration of the ternary complexes are much greater than that of dissociated NXF. However, in the μM range, dissociated and associated NFX concentrations are of the same order of magnitude.

It is important to mention that the association of Cu:phen with NFX is extremely dependent on the electric charge of NFX. The association constant with the zwitterionic form was about 40 times greater than with the cationic form. It was not possible to obtain the association constant of the binary complexes with anionic NFX using fluorescence quenching, because this species is not fluorescent.

The fluorescence spectra of the synthesized ternary mixed-ligand complexes Cu:L:NFX were obtained in aqueous solution, to test if the fluorescence intensity agree with the obtained stability constants. The fluorescence intensities of NFX and Cu:L:NFX at the same concentrations were compared (8.0 μM, PB pH 7.4, 20 mM). It was observed that the fluorescence intensity of Cu:L:NFX solution was lower than that of pure NFX by factors that agreed with those calculated using the stability constants obtained in Fig. 2. This result shows that the synthesized complexes and the solutions prepared by mixing the components exhibited the same spectroscopic behavior. The equivalence between synthesized complexes and those prepared by mixing was also observed in biological studies using copper(II):enrofloxacin:phen complexes [24].

The influence of ionic strength in the association constant of the binary complexes Cu:phen with NFX was evaluated in phosphate buffer at

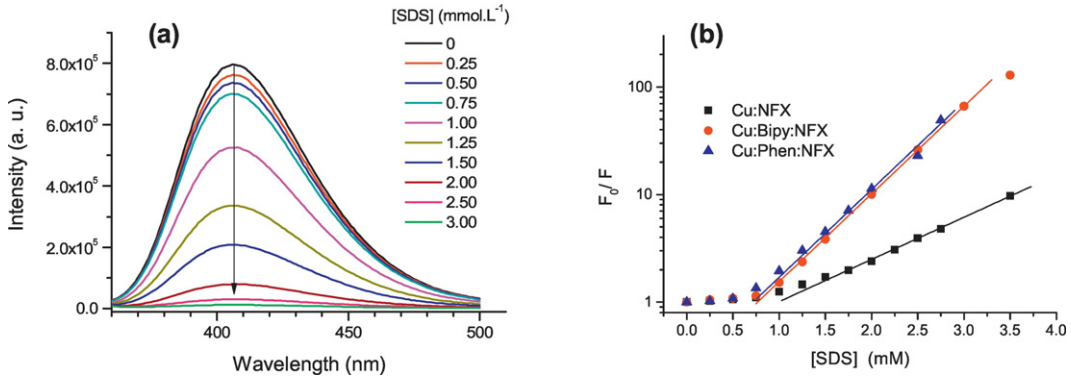

Fig. 4. (a) Fluorescence spectra of Cu:bipy:NFX solution (8 μM) in the absence and presence of increasing SDS concentrations (phosphate buffer 20 mM, pH 7.4). Excitation at 330 nm. (b) Intensity ratio of NFX fluorescence in solutions of the complexes Cu:NFX, Cu:bipy:NFX and Cu:phen:NFX as a function of SDS concentration. Solid lines are the least squares fits using Eq. (3) for [SDS] > [CMC], with parameters in Table 1.

Table 1
Fitting parameters of the data in Fig. 4 (b) using Eq. (3).

	α (10^3 M^{-1})	CMC (10^{-3} M)	R^2
$CuCl_2$	0.90	0.99	0.998
Cu:bipy	1.88	0.77	0.9997
Cu:phen	1.88	0.68	0.997

different concentrations. The different results appear in Fig. 3, which confirm that the stability of these mixed-ligand complexes is highly dependent on the ionic strength.

The inset in Fig. 3 is a logarithmic plot of the stability constant versus phosphate concentration. The straight line is the function $K_b = 6.0 \times 10^3 [PO_4]^{-0.8}$, where $[PO_4]$ is the molar phosphate concentration and K_b is in units of M^{-1}. Fig. 3 also shows the results obtained in universal buffer (borate, citrate, phosphate, 20 mM Na^+). The much weaker stability constant in this buffer ($K_b = 6.3 \times 10^3$ M^{-1}) is due to the presence of citrate ions, which compete with NFX for Cu:L.

3.2. Interaction of the Ternary Complexes With SDS Micelles

It has been observed that the electric charge of the different NFX species in solution is very important in determining their interaction with biomolecules and biomembranes. In particular, it has been found that cationic and zwitterionic NFX species associate with anionic surfactants and lipids in micelles and liposomes. Interaction of NFX with anionic SDS micelles was studied using the intrinsic fluorescence of NFX [3]. It was found that both 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. Both species increase fluorescence quantum yield on binding to SDS micelles, and the emission peak displaces to 432 nm (originally at 407 nm at pH 7.4, and 440 nm at pH 4.0 in aqueous solutions). NFX molecules bound to SDS micelles are cationic (neutral carboxylic group, protonated amine).

In order to determine the influence of negatively charged membrane interfaces on the stability of the copper complexes, we studied the association of the binary Cu:NFX and the ternary Cu:L:NFX complexes (L = bipy or phen) with anionic SDS micelles by titrating the complexes at a fixed concentration with increasing SDS amounts. Fig. 4 (a) shows the fluorescence spectral changes of Cu:NFX with increasing SDS concentration. Similar results were also observed for Cu:L:NFX. At first, a small fluorescence decrease at SDS concentrations up to 0.75 mM suggests premicellar interaction. At higher SDS concentration, the sharp fluorescence quenching indicates that SDS micelles facilitate the association of NFX with Cu or the binary Cu:L complexes.

The fluorescence peak at 407 nm in Fig. 4 (a) indicates that NFX dissociated molecules are in pH 7.4 aqueous environment, because SDS associated NFX molecules has a fluorescence peak at 432 nm [3]. This means that all NFX molecules bound to SDS micelles are associated with copper(II) ions or with the Cu:L complex. This result is an important clue for analyzing molecular mechanisms that drive the interaction of the copper complexes with biological interfaces.

As reported in Section 3.1, the fluorescence of Cu:NFX and the ternary Cu:L:NFX solutions derives only from the dissociated NFX molecules, and a quenching of the fluorescence means association of NFX with copper(II). Fig. 4 shows that SDS addition quenches the fluorescence of Cu:NFX and Cu:L:NFX solutions, meaning that SDS micelles catalyze the association of Cu and Cu:L with NFX.

NFX fluorescence lifetimes were obtained from fluorescence decay measurements for the complexes in the absence and presence of SDS. The lifetimes were all the same, and equal to that of NFX at pH 7.4 [3]. This indicates static quenching, which implies the formation of a ground-state non-fluorescent complex or the existence of a sphere of effective quenching [33]. In the last case, the emission intensity ratio F_0/F is an exponential function of the quencher concentration: $F_0/F = \exp(V_q N_A [Q])$, where V_q is the volume of the sphere around NFX, N_A is the Avogadro's number and [Q] is the molar concentration of quencher.

The quencher concentrations were constant in experiments leading to Fig. 4, but the anionic character of the SDS micelle surface causes an increase of the divalent copper concentration at the diffuse electric double layer surrounding the micelle. In this case, the increasing micelle concentration leads to increased quencher concentration near the micelle surface where NFX molecules bind.

Then, the quenching data was analyzed using a semilog plot of the fluorescence intensity ratio (F_0/F) as a function of SDS concentration (Fig. 4 (b)). The plots suggest a small premicellar interaction, up to about 0.75 mM SDS concentration. Above that, the semilog plot is linear. The following expression fits the data, with the fitting parameters in Table 1:

$$F_0/F = \exp[\alpha([SDS] - CMC)] \quad (3)$$

for [SDS] > [CMC].

The CMC values suggest that the complexes decrease the CMC of SDS, which is about 3 mM at this buffer ionic strength [34].

3.3. Interaction of the Complexes Cu:Bipy and Cu:Phen With SDS Micelle-associated NFX

In the previous section all measurements were performed with the synthesized complexes, and thus the 1:1 molar ratio of Cu(II):NFX was preserved. In this section we studied the association of the Cu:L complexes with NFX already associated with SDS micelles. We used sufficiently high SDS concentration (20 mM) so that virtually all NFX molecules associated with the micelles. NFX fluorescence was monitored in solutions with increasing amounts of $CuCl_2$, Cu:bipy and Cu:phen. The

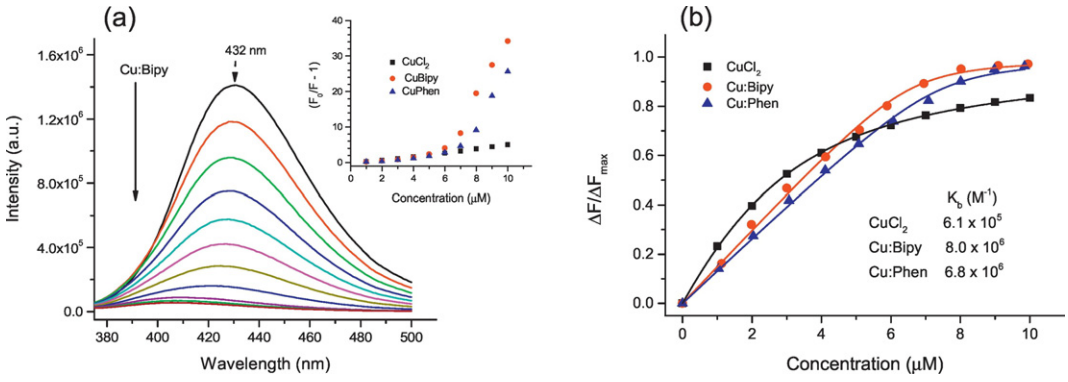

Fig. 5. (a) Fluorescence spectra of NFX (8 μM) in 20 mM SDS micellar solution, and increasing concentrations of Cu:bipy (PB 20 mM, pH 7.4). Excitation at 330 nm. Inset: Stern-Volmer plot of the NFX fluorescence quenching by $CuCl_2$, Cu:bipy and Cu:phen. (b) Normalized change of NFX fluorescence (quenching) as a function of $CuCl_2$, Cu:bipy or Cu:phen concentrations. Solid lines are the least squares fits using Eq. (2).

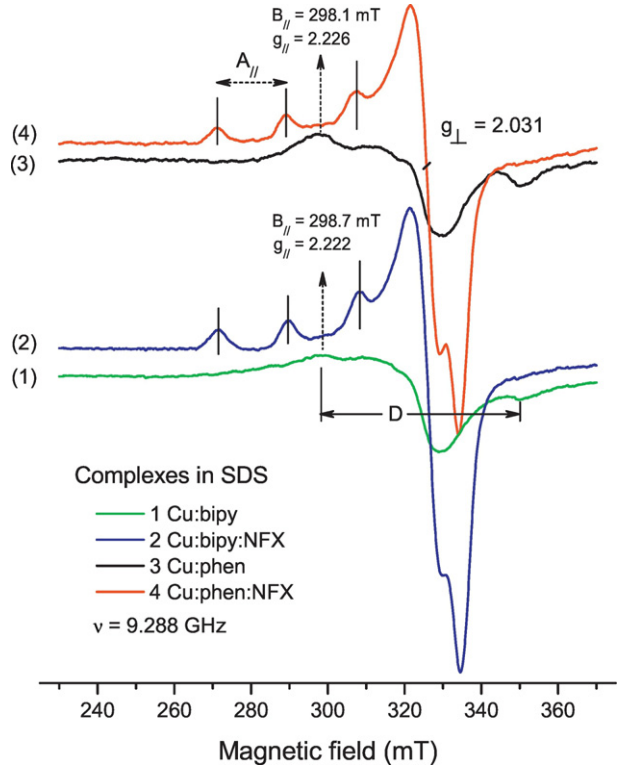

Fig. 6. EPR spectra, X-band, of Cu:bipy (1,2) and Cu:phen (3,4) in SDS micelles (1,3), and in NFX loaded SDS micelles (2,4). [NFX] = [Cu:bipy] = [Cu:phen] = (1×10^{-3} M); [SDS] = 25×10^{-3} M (77 K, PB 10 mM, pH 7.4). EPR parameters in Table 2.

results appear in Fig. 5. UV–Vis absorption was also monitored (see Supplementary material, Figs. S2 and S3). It is possible to notice that the absorption changes at the wavelength of fluorescence excitation are not responsible for the quenching.

The fluorescence peak at 432 nm indicates that NFX molecules are in fact associated with SDS micelles, and Cu:L associates to micelle-bound NFX. From the experimental data (Fig. 5 (b)) it was possible to obtain the stability constants for the formation of the ternary complexes from Cu:L and NFX inside SDS micelles. Least squares fits using Eq. (2) gave binding constants $K_b^{(Cu)}{}_{SDS} = (6.1 \pm 0.1) \times 10^5\ M^{-1}$, for Cu(II), $K_b^{(1)}{}_{SDS} = (8.0 \pm 1.8) \times 10^6\ M^{-1}$, for Cu:bipy, and $K_b^{(2)}{}_{SDS} = (6.8 \pm 1.3) \times 10^6\ M^{-1}$, for Cu:phen. These are much greater than the stability constants in buffer (Section 3.1). For example, for Cu:phen:NFX in SDS, it is about 60 times greater than the one in PB (~$1.0 \times 10^5\ M^{-1}$, see Fig. 3, inset).

3.4. EPR Spectra of the Copper Complexes in SDS Micelles

EPR spectroscopy has shown that the mixed-ligand ternary complexes Cu:bipy:NFX and Cu:phen:NFX in aqueous solutions (concentrations in the mM range) form almost exclusively binuclear complexes with the structure of a dimer $(Cu:L:NFX)_2$ [23].

Table 2
EPR parameters of the Cu(II) complexes.

SDS (77 K)	$g_\perp$	$g_\parallel$	g_{binuc}	D (gauss)
$[CuCl_2(phen)]^*$	2.08	2.22		
(1) Cu:bipy			2.051	530
(3) Cu:phen			2.051	530

SDS (77 K)	$g_\perp$	$g_\parallel$	$A_\parallel$ (gauss)
(2) Cu:bipy:NFX	2.031	2.222	182
(4) Cu:phen:NFX	2.031	2.226	180

* aqueous, unresolved hyperfine splitting, for comparison [19].

According to the results of the previous section, Cu:phen and Cu:bipy associate with SDS micelles, and localize very close to the NFX molecules. However, quenching results can only suggest formation of the ternary complexes Cu:L:NFX bound to the micelles. The EPR technique can answer if the mixed complexes were really formed, since it probes the crystal field around the copper(II) ions.

Stoichiometric amounts of Cu:bipy and Cu:phen were added to solutions containing NFX-loaded SDS micelles. The obtained EPR spectra after equilibration appear in Fig. 6, (2) and (4), together with the spectra of Cu:bipy (1) and Cu:phen (3) in presence of micelles but in absence of NFX, for control (EPR parameters in Table 2).

The EPR spectra show that the crystal field around the copper ions changes completely when the micelles contain NFX molecules, indicating that real mixed-ligand complexes form inside the micelles. The spectrum of the binary complexes Cu:L (Fig. 6, (1) and (3)) is a superposition of binuclear and mononuclear species, but the parameters of the mononuclear species are not well-defined. The ternary complexes formed inside the micelles, however, have very well defined axially symmetric parameters characteristic of mononuclear complexes.

Peisach-Blumberg diagrams are plots of the EPR parameters $A_\parallel$ versus $g_\parallel$ that reflect the nature of the atoms bound to copper(II) [35]. According to these diagrams, values of $g_\parallel \approx 2.22$ and $A_\parallel \approx 180$ G can be expected for 2 nitrogen and 2 oxygen donor atoms in a square planar geometry. This agrees with the structure presumed for the ternary complexes Cu:bipy:NFX and Cu:phen:NFX, since NFX binds to copper ions via the pyridone and carboxylate oxygen atoms, and bipy and phen bind via the pyridine nitrogen atoms.

It follows that the SDS micellar environment with negatively charged surface assists the formation of mononuclear mixed ligand complex.

4. Conclusions

Two mixed ligand ternary copper(II) complexes of norfloxacin and a nitrogen donor heterocyclic ligand, 2,2′-bipyridine or 1,10-phenantroline, were studied in different ionic strength solutions and in anionic SDS micelles. The stability constants for the binding of zwitterionic NFX to Cu:bipy and Cu:phen were obtained by quenching of NFX fluorescence. The influence of ionic strength in the stability constants was also obtained. They varied from 2.9×10^6 to $4.0 \times 10^4\ M^{-1}$ when the ionic strength changed from 0.5 to 80 mM phosphate. It was also observed that the synthesized mixed-ligand ternary complexes in solution are equivalent to stoichiometric mixtures of NFX and the binary complex.

Titration of the complexes with SDS allowed determine the influence of negatively charged interfaces in the stability of the mixed-ligand copper complexes. Above the critical micelle concentration (CMC), SDS catalyzes the formation of the ternary complex. The model based on existence of a sphere of effective quenching described well the decrease of NFX fluorescence at the micellar surface. In SDS micelles the stability constants were about 60 times greater than in buffer.

EPR spectroscopy showed that the mixed ligand ternary complexes formed in SDS micelles are mononuclear, with axially symmetric EPR parameters that agree with the presence of 2 nitrogen and 2 oxygen atoms coordinated to the copper ion. These are very different from the ternary complexes in solution, which mainly form binuclear copper complexes [23]. They are also very different from those of the precursor binary complexes Cu:bipy and Cu:phen in SDS micelles. The results of this work showed that membrane interfaces change the structure and stability constants of copper complexes with pharmacologically important ligands, and that EPR spectroscopy was a valuable tool to provide details of the structural modification of membrane bound copper complexes.

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Appendix A. Supplementary data

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