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Ciprofloxacin Metalloantibiotic: An Effective Antibiotic with an Influx Route Strongly Dependent on Lipid Interaction?

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Abstract Fluoroquinolones are antibiotics that have a large spectrum of action against bacteria, especially Gram-negative. A strategy to enhance their pharmacological behavior, and try to counteract bacterial resistance, is their coordination to divalent metal ions and 1,10-phenanthroline. These stable complexes modify fluoroquinolones potency and specificity, possibly due to their alternative translocation through the bacterial membranes. In this work, we determined the interaction of ciprofloxacin and its copper(II) ternary complex with unilamellar liposomes of DMPC, POPE/POPG (0.75:0.25), POPE/POPG/cardiolipin (0.67:0.23:0.10), and *E. coli* total extract, using time-resolved and steady-state fluorescence spectroscopy. The association constants determined show that the interaction of both compounds depends on membrane lipids composition and is always higher for the metalloantibiotic, a trend already observed for other fluoroquinolone metalloantibiotics. Nevertheless, the interaction of ciprofloxacin metalloantibiotic is, normally, higher, which reflects the fluoroquinolone species that are present in solution at physiological pH. In overall, the results obtained suggest that ciprofloxacin and its metalloantibiotic have different translocation pathways, proposing that the diffusion of the metalloantibiotic is a hydrophobic mechanism and suggesting that this new metalloantibiotic may be a good choice to replace the pure ciprofloxacin and bypass, at least, one of the mechanisms of the bacterial resistance to fluoroquinolones.

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Introduction

Fluoroquinolones are a family of antibiotics widely used against Gram-negative and some Gram-positive bacteria. Apart from their large spectrum of action, these antibacterial agents are used in clinical practice due to its improved bioavailability and pharmacokinetics properties (Andersson and MacGowan 2003; Scholar 2002; Van Bambeke et al. 2005). The major permeability cellular barrier of Gram-negative bacteria for quinolones is the outer membrane. To access their target site and exercise their antibacterial activity, quinolones have to overcome this barrier by penetrating this structure and three different routes are known: the hydrophobic pathway, the porin pathway, and the self-promoted route, used by cationic compounds (Bryan and Bedard 1991; Vázquez et al. 2001b). Some fluoroquinolones are capable of diffusing through lipid bilayers, due to their lipophilic character and their uncharged form at physiological conditions. Thus, the interaction between the drug and the lipid bilayer is, probably, the first process involved in the passage through the membrane (Bensikaddour et al. 2008; Fresta et al. 2002; Neves et al. 2005; Vázquez et al. 2001b).

The increasing use and misuse of the antibiotics promoted bacterial adaptations and prompted the emergence of different bacterial resistance mechanisms. The two main bacterial resistance mechanisms developed against the action of the quinolones are the change of the target molecules (topoisomerases) and the reduction of the intracellular concentration of the antibiotic through the decreasing

of the membrane permeability or the existence of efflux pumps (Scholar 2002; Van Bambeke et al. 2005). Due to this, the knowledge of the molecular mechanism and kinetics of quinolone entry into the bacterial cell is essential.

Biological membranes are complex systems due to its lipids and membrane proteins composition. Some fluoroquinolones need porins to arrive at their target, mainly OmpF and/or OmpC (Bryan and Bedard 1991; Nikaido 2003; Nikaido and Vaara 1985). However, porin mutations are one of the major causes of bacterial resistance mechanisms. To try to overcome this issue, the use of the novel transition metal complexes derivatives of fluoroquinolones, ternary complexes of metal, fluoroquinolone, and phenanthroline (Phen) seems a promising route to pursue. Thus, the synthesis of fluoroquinolone ternary metal complexes (known as metalloantibiotics) has been carried out to determine their biophysical properties in model membrane lipidic systems with different compositions. These studies were performed in an attempt to clarify their antibacterial activity in *E. coli* strains, based on antimicrobial susceptibility tests (Feio et al. 2014; Saraiva et al. 2010; Sousa et al. 2012). The biological activity of these metalloantibiotics seems to combine the antibacterial activity of fluoroquinolones and the DNAase activity of copper(II)/Phen complexes, which makes them an even more promising path to try to counteract bacterial resistance (Hernández-Gil et al. 2009; Macías et al. 2003; Saha et al. 2004). Furthermore, their drug–lipid interaction is high, which could favor an alternative influx pathway that may overcome the porin mutation resistance mechanism (Lopes et al. 2013; Ribeiro et al. 2011; Saraiva et al. 2010).

The interaction of antimicrobial agents with bacterial model membranes (biophysical studies) is generally assessed through the determination of the drug/liposomes association (Gaber 2004; Lorin et al. 2004), that is normally quantified in drug/liposomes suspensions using spectroscopic techniques (Chatterjee and Agarwal 1988; Rodrigues et al. 2002; Santos et al. 2003).

In this work, we studied the interaction of Ciprofloxacin (Cpx), a second generation fluoroquinolone (Oliphant and Green 2002; Scholar 2002; Van Bambeke et al. 2005), and its ternary complex of copper(II) and Phen (CuCpxPhen) with three different lipidic mimetic systems of *Escherichia coli* membrane: a binary system composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) in the proportion POPE/POPG (0.75:0.25), a ternary system comprising POPE, POPG, and cardiolipin (CL) in the proportion POPE/POPG/CL (0.67:0.23:0.10), and a third natural system of *E. coli* total lipid extract. These binary (Lopes et al. 2013; Picas et al. 2010a; Ribeiro et al. 2011) and ternary (Lopes et al. 2010, 2013; Ribeiro et al. 2011) mixtures are widely used to mimic bacterial membranes as they comprise the main phospholipid components of the *E. coli* membrane, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and CL (Cheng et al. 2011; Dowhan 1997; Lopes et al. 2010; Picas et al. 2010b). The interaction of the drug and its metalloantibiotic with the mimetic systems of the bacterial membranes was performed through the determination of partition coefficients/association constants using steady-state and time-resolved fluorescence spectroscopy. The study of the CuCpxPhen metalloantibiotic was also performed in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes, as it is a membrane mimetic system of eukaryotic cells.

The results obtained show that the interaction of metalloantibiotic/liposomes is much stronger than that observed for Cpx/liposomes. These results may suggest a different translocation route for the metalloantibiotic, largely governed by metalloantibiotic–membrane interaction. Moreover, our results propose that CuCpxPhen may be more active than pure Cpx and even than similar ternary copper(II)/Phen complexes of other fluoroquinolones as enrofloxacin and moxifloxacin (Lopes et al. 2013; Ribeiro et al. 2011).

Materials and Methods

All compounds were used as received. Cpx (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) was from Fluka. 1,10-phenanthroline (Phen) and *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (HEPES) were from Sigma (grade *pro analysis*). DMPC, POPE, POPG, CL (natural extract from *E. coli*), and *E. coli* total lipid extract were from Avanti Polar Lipids. All other chemicals were from Merck. All solutions were prepared with 10 mmol dm^{-3} HEPES buffer (0.1 mol dm^{-3} NaCl; pH 7.4) using double deionised water. The $Cu(NO_3)_2{\cdot}3H_2O$ solution used in the metalloantibiotic preparation was previously titrated with EDTA.

Liposome Preparation

Chloroform solutions containing the appropriate amount of the several used lipids (DMPC, *E. coli* total lipid extract, POPE:POPG and POPE:POPG:CL mixtures) were dried under a stream of argon. The obtained films were evaporated under vacuum for 3 h, at least, to ensure the total removal of the organic solvent. Multilamellar vesicles (MLVs) were obtained by redispersion of the lipidic film in 10 mmol dm^{-3} HEPES buffer (0.1 mol dm^{-3} NaCl; pH 7.4). The samples were vortexed, and the vesicles were submitted to a ten times cycle of frozen/thawed. The freezing was done in liquid nitrogen and thawing in a water

bath with a temperature above the phase transition temperature of each system (DMPC: ~24 °C; binary system: ~21 °C; ternary system and *E. coli* total lipid extract: ~63 °C) (Lopes et al. 2010; Neves et al. 2009). Large unilamellar vesicles (LUVs) were obtained by 10 times MLVs extrusion through 100 nm polycarbonate filters on a Lipex Biomembrane extruder attached to a water bath. The extrusions were performed above the phase transition temperature of each mixture (DMPC and binary system: above 37 °C; ternary system and *E. coli* total lipid extract: above 65 °C).

Spectroscopic Measurements

Absorption spectra were carried out on a UV–Vis-NIR (UV-3600) Shimadzu spectrophotometer equipped with a temperature controller (Shimadzu TCC-CONTROLLER). Spectra were recorded at 37.0 ± 0.1 °C, in 1 cm quartz cuvettes, with a slit width of 5 nm, in a wavelength range from 225 to 450 nm. Steady-state fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorometer equipped with a "single cell peltier accessory" temperature controller, in 1 cm quartz cuvettes, with a slit width of 5 nm, scan rate of 120 nm/min, and data range of 1 nm. Excitation wavelength of 270 nm and emission wavelength range from 280 to 525 nm were used for Cpx solutions (2.0 μmol dm^{-3}), while excitation wavelength of 290 nm and emission wavelength range from 300 to 525 nm were used for Cpx and metalloantibiotic solutions (5.0 μmol dm^{-3}).

Small aliquots (μL) of LUV's of each system under study were successively added to an aqueous solution of Cpx (2.0 and 5.0 μmol dm^{-3}), or of its metalloantibiotic (5.0 μmol dm^{-3}), to achieve final lipid concentrations in the range of 0–1.0 mmol dm^{-3}. The mixtures were left to incubate, at 37.0 ± 0.1 °C, for 5 min after which the emission spectra were obtained.

The metalloantibiotic used was prepared by mixing the three components (Cpx, Cu(II), and phen) in stoichiometric proportions (1:1:1), as previously reported for other fluoroquinolones metalloantibiotics (Lopes et al. 2013; Ribeiro et al. 2011; Saraiva et al. 2010; Sousa et al. 2012).

The antibiotic and metalloantibiotic concentrations used were chosen according to their Lambert–Beer law (absorbance value <0.1 at the excitation wavelength) in order to avoid the inner filter effect (Cox et al. 2000; Ohno 2002).

Dynamic Light Scattering

The size distribution of the prepared liposomes was determined by dynamic light scattering on a Zeta Sizer Nano Zs of Malvern Instruments. All measurements were performed at 37.0 ± 0.1 °C.

Time-Resolved Fluorescence Spectra

The fluorescence lifetimes were measured on a Fluoromax-4 spectrophotometer, attached to a single photon counting controller (FluoroHub), both from Horiba Jobin–Yvon, at 37 ± 0.1 °C. A set of samples with a final volume of 1.5 mL was prepared by adding a known volume of Cpx solution, or its metalloantibiotic, and small volumes of LUVs suspension in HEPES. The experiments were performed with a lipid concentration range from 0 to 1.0 mmol dm^{-3}. The fluorescence excitation was performed with a Horiba Nano LED source of 290 nm, and fluorescence emission was recorded at the maximum wavelength for each solution (415 nm for both solutions). The lamp profile was recorded by placing a scatter (dilute solution of LUDOX in water) in place of the sample (Bhattacharya and Samanta 2008).

Data Analysis

Steady-State Fluorescence

Cpx and its metalloantibiotic have fluorescent properties which enable the partition/association constants determination by fluorescence spectroscopy without using external probes.

Partition coefficient (K_p) of any compound between vesicle suspensions and an aqueous solution is defined as

$$K_p = \frac{(C_L / C_T) / [L]}{(C_W / C_T) / [W]}, \quad (1)$$

in which C_T, C_L, and C_W are the total drug molar concentration and the drug molar concentration in lipid and in aqueous media, respectively; [*L*] and [*W*] represent lipid and water molar concentrations (Chong et al. 2010). Furthermore, this equation can be rearranged as

$$K_{ass} = \frac{(C_L / C_T) / [L]}{(C_W / C_T)}. \quad (1a)$$

The partition coefficients/association constants were determined, without phase separation of drug/liposome suspension, by analysis of the experimental emission fluorescence spectra obtained. Steady-state fluorescence experimental data of Cpx or its metalloantibiotic were always corrected for the dilution effect (Coutinho and Prieto 1993). Spectral changes resulting from antibiotic–lipid interactions can be used to obtain the association constants since the background signals from liposome scattering, at the maximum emission wavelength, do not interfere under the used experimental conditions (Fig. 1a).

All the experimental data were first treated by linear and nonlinear graphical methods, at the wavelength of maximum intensity (using the computer program Origin 7®), to

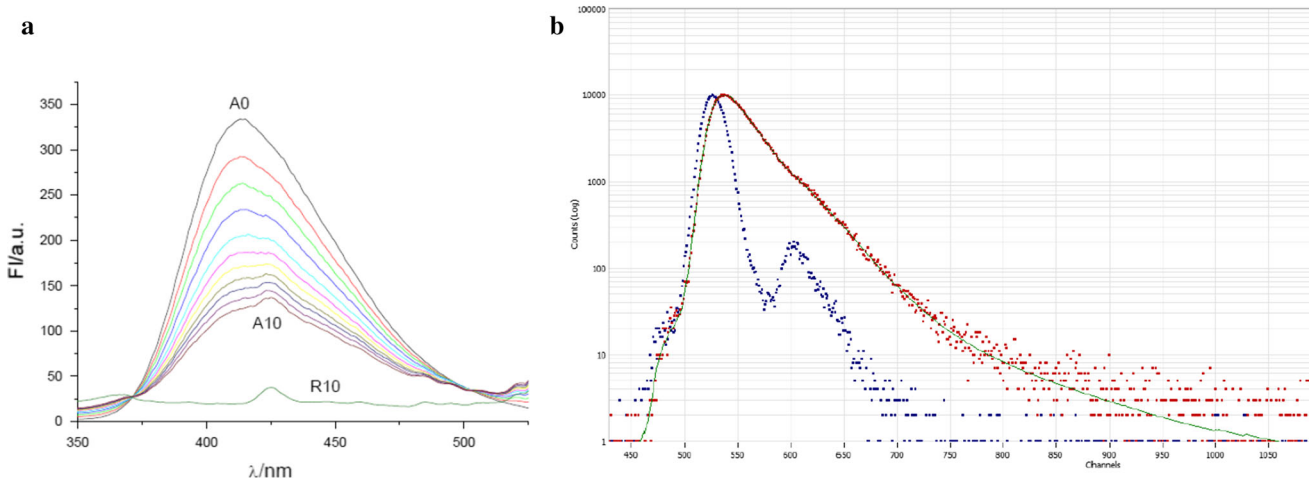

Fig. 1 Experimental data obtained from steady-state (**a**) and time-resolved (**b**) fluorescence spectroscopy, at the maximum emission wavelength of Cpx. **a** Emission fluorescence spectra of Cpx solution, in the absence (*A0*) and presence (*A1–10*) of LUVs of *E. coli* total lipid extract, and background signal from liposome scattering of the higher concentration ($\sim$1 mmol dm^{-3}) of the respective lipidic system (R10), with excitation wavelength of 270 nm. Each spectrum is a mean of five measurements. **b** Time-resolved fluorescence intensity decay of Cpx in the presence of LUVs of *E. coli* total lipid extract ($\sim$1 mmol dm^{-3}). The *blue peak* on the *left side* is the LUDOX and the *red peak* on the *right side* is the decay profile, fitted with a two-exponential function (*green line*)

validate the model and to determine a first approximation of the drug/liposome interaction values.

As antibiotic–lipid interaction resulted in a quenching of the fluorescence, the experimental data were first analyzed by a linear method using the Stern–Volmer equation (Callaghan et al. 1997; Rodrigues et al. 2002):

$$\frac{FI_0}{FI} = 1 + K_{SV}\,[Q], \tag{2}$$

where FI and FI_0 are the fluorescence intensities of the fluoroquinolone/metalloantibiotic measured in the presence and absence of the quencher Q (lipid vesicles), respectively; K_{SV} is the Stern–Volmer constant.

The nonlinear method was performed using the following equation (Lopes et al. 2013; Ribeiro et al. 2011; Rodrigues et al. 2002; Santos et al. 2003):

$$\Delta I = \frac{K_p\,\Delta I_{max}\,[L]}{1 + K_p\,[L]}, \tag{3}$$

where ΔI is the difference between the fluorescence intensity of the fluoroquinolone/metalloantibiotic measured in the presence (FI) of lipid vesicles (L) and in its absence (FI_0); [L] represents lipid molar concentration; K_p is the association constant. $\Delta I_{max} = FI_\infty - FI_0$, where FI_∞ is the limiting value of FI. This equation can be used due to the definition of partition coefficient (Eq. 1a) used and the K_p values obtained are identical, within the experimental error, with the association constants, K_{ass}.

All the data obtained from the experimental spectra were also analyzed with the computer program HypSpec (Gans et al. 1996, 1999), in order to obtain more precise K_{ass} values for drug/liposome interaction. The analysis was performed in the range from 370 to 500 nm for Cpx and its metalloantibiotic.

Time-Resolved Fluorescence

Data analysis of the fluorescence intensity decays was carried out using a nonlinear least squares iterative convolution method with computer program DAS6 v6.5 (Fig. 1b). Two exponentials were required to the fit, and its goodness was assessed from the global Chi-square value ($\chi^2 \leq 1.3$ was considered acceptable) (Teixeira et al. 2010), weighted residuals, and autocorrelation plots (Santos et al. 1998).

For a two-exponential decay, the average fluorescence lifetime of a fluorophore, $\overline{\tau}$, is (Lakowicz 2006):

$$\overline{\tau} = \frac{\sum \alpha_i\,\tau_i^2}{\sum \alpha_i\,\tau_i}, \tag{4}$$

where τ_i represents each component of the fluorescence lifetime and α_i the respective pre-exponential factor, which is proportional to the concentration of the component.

Changes in the average fluorescence lifetime, in the presence of lipid vesicles, can be correlated with the amount of drug in the different phases. Considering drug(D)/liposome(L) interaction as (Connors 1987)

$$D + L \rightleftharpoons DL\;K_{ass} = \frac{[DL]}{[D]\,[L]},$$

where [D], [L], and [DL] are the concentrations of the drug, of the lipid, and of the drug interacting with the liposome, respectively; K_{ass} is the drug/liposome association constant.

Considering that $\overline{\tau}_0$ and $\overline{\tau}$ are the average fluorescence lifetimes of the drug in the aqueous media and in the presence of a lipidic suspension, respectively, and that the fluorescence lifetime changes due to the drug/liposome interaction, Eq. 5 can be obtained:

$$\frac{\overline{\tau}_0}{\overline{\tau}} = 1 - K_{ass}\,[L]\,. \tag{5}$$

All the experimental data were treated by this linear graphical method, at the wavelength of maximum intensity, using the computer program Origin 7®.

Results and Discussion

Determination of Drug/Liposome Association Constants by Steady-State Fluorescence Spectroscopy

Like most fluoroquinolones (Lopes et al. 2013; Ribeiro et al. 2011; Saraiva et al. 2010), Cpx has two ionizable functional groups, a carboxylic group at position 3 of the quinolone ring and a basic piperazinyl group at position 7 with $pK_{a1} = 6.18 \pm 0.05$ and $pK_{a2} = 8.76 \pm 0.03$ (Vázquez et al. 2001a), respectively. These values are in the range of those observed for other fluoroquinolones (Langlois et al. 2005; Martinez et al. 2006; Neves et al. 2007; Saraiva et al. 2010), which accounts for the 90 % of zwitterionic form (HCpx) and 10 % of anionic form of Cpx at physiological pH (Neves et al. 2007; Rodrigues et al. 2002).

Synthesis and speciation studies of several metalloantibiotics (metal/fluoroquinolone/phenanthroline complexes) are currently under study, and the speciation and biological studies show that only copper/fluoroquinolone/phenanthroline complexes are stable under physiological conditions (Feio et al. 2014; Fernandes et al. 2014; Saraiva et al. 2010; Sousa et al. 2012). The speciation studies performed for CuCpxPhen with the HySS computer program (Alderighi et al. 1999) show that, at physiological pH 7.4, the predominant species, CuHLA (~95 % at 2–5 μmol dm^{-3}), is cationic and has a stability constant of 17.96 ($\log K_{CuHLA}$ value) (Buchanan et al. 2012).

The variation of the emission fluorescence spectra of Cpx with the increase of lipid concentration of each bacterial mimetic studied system is shown in Fig. 2. All spectra show a decrease (quenching) of the fluorescence intensity (FI) with the increase of lipid concentration, as observed for other fluoroquinolones (Lopes et al. 2013; Ribeiro et al. 2011).

For Cpx in DMPC, it was not possible to determine a value for their interaction. This result was expected as it has been elsewhere demonstrated (Bedard and Bryan 1989; Hernández-Borrell and Montero 2003; Lasic et al. 1995; Maurer et al. 1998; Wąsik et al. 2013) that Cpx at neutral pH shows little tendency to bind zwitterionic liposomes. For the other lipidic systems used, the fluorescence experimental data were first analyzed with the linear Stern–Volmer equation (Eq. 2), followed by the fitting of (Eq. 3) to the experimental data (Fig. 3). The values obtained for the association constants with these equations and with the program HypSpec (K_{Hyp}), for the two concentrations described in methodology, are summarized in Table 1.

Despite the diversity of denomination and the differences in their mathematical equations, all determined constants represent the interaction between the drug and the liposome bilayer and can be designated, generally, as association constants.

The values obtained for the two used concentrations (2.0 and 5.0 μmol dm^{-3}) are, generally, identical, within the experimental error, for the three membrane model systems. Nevertheless, the values obtained for the *E. coli* total lipid extract at 270 nm are higher than those obtained at 290 nm. These results can be explained as *E. coli* total lipid extract is a natural heterogeneous mixture, and the lipid scattering is normally higher for shorter wavelengths (270 nm) (Cohen et al. 2012; Sommer 1989).

Analyzing the results obtained for the three different lipidic studied systems (POPE/POPG, POPE/POPG/CL, and *E. coli* total lipid extract) is possible to conclude that the values obtained for the drug/liposome interaction are similar, within the experimental error, although the value in the binary system is always slightly higher than that in *E. coli* total extract. This behavior was already observed for moxifloxacin (Lopes et al. 2013) and for enrofloxacin (Ribeiro et al. 2011) for the same lipidic systems. Nevertheless, the values of enrofloxacin are slightly smaller, which can be explained by the fluoroquinolone species that are present in solution at physiological pH (Lopes et al. 2013; Ribeiro et al. 2011): ~90 % zwitterionic form for moxifloxacin and ciprofloxacin and ~70 % for enrofloxacin.

The emission fluorescence spectra of the metalloantibiotic in the absence and presence of increasing lipid concentration, for the different lipidic systems, are depicted in Fig. 4. The fluorescence spectrum of the metalloantibiotic is similar to that observed for Cpx alone ($\lambda_{exc} = 290$ nm and $\lambda_{em} = 415$ nm).

Nevertheless, the FI of the metalloantibiotic increases with the increase of lipid concentration in the presence of POPE/POPG and *E. coli* total lipid extract LUVs. This result was already observed for similar studies with enrofloxacin and moxifloxacin (Lopes et al. 2013; Ribeiro et al. 2011) and was ascribed to a different change of the dipole moment of the molecule upon electronic transitions, being a consequence of specific interactions between solvent and solute that will influence both the non-radiative and radiative coupling between ground and excited state (Bilski et al. 1998; Cuquerella et al. 2006; Park et al. 2004; Pávez

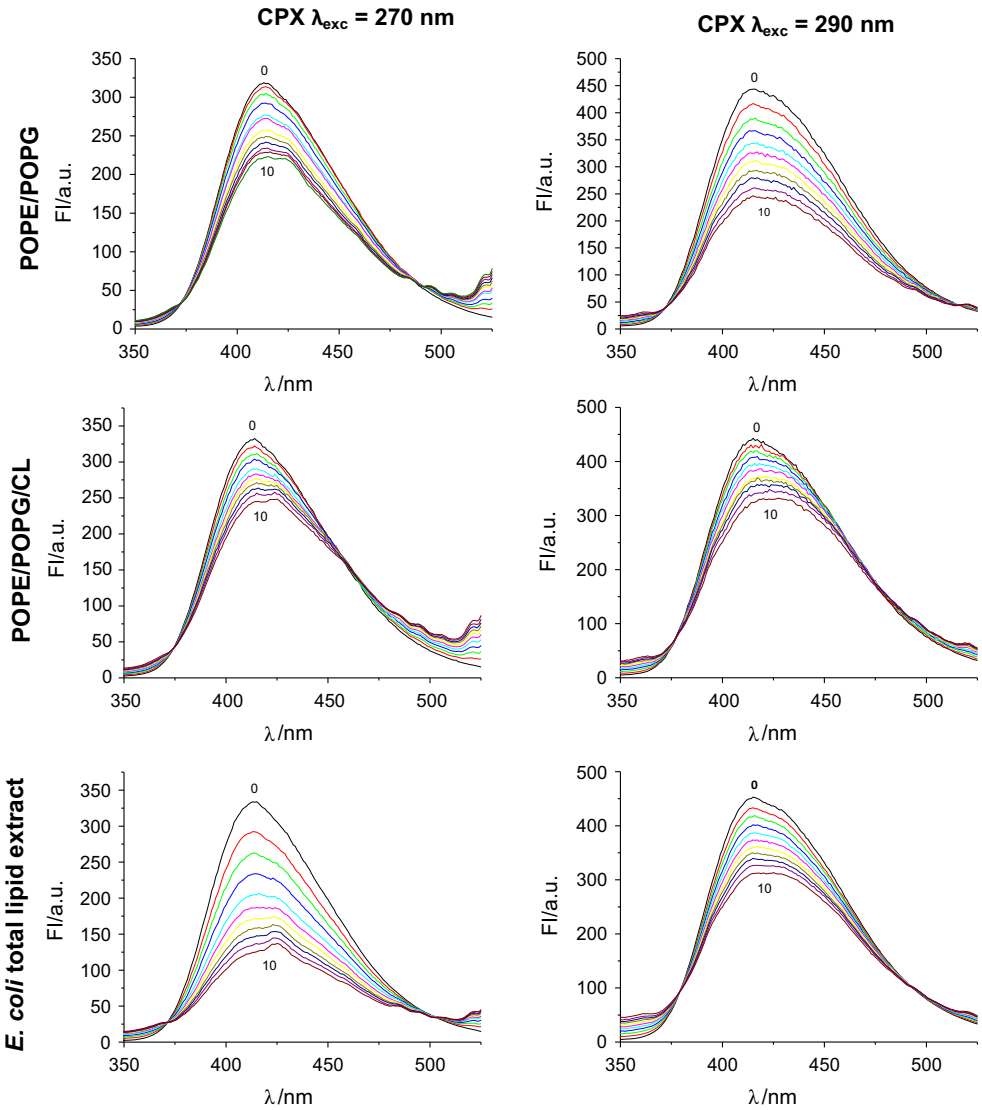

Fig. 2 Emission spectra of Cpx solution in the absence (*0*) and presence (*1–10*) of LUVs of POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract. Each spectrum is a mean of five measurements

et al. 2007). This different behavior points out for the fact that different interactions govern fluoroquinolones and its metalloantibiotics membrane's association.

The experimental data were first analyzed with Eq. (3), and the values obtained were further refined with the program HypSpec (K_{Hyp}) (Table 2).

The values obtained for the association constants obtained in DMPC and POPE/POPG/CL model systems are identical among them and comparable to those found for Cpx alone. The values obtained for POPE/POPG and *E. coli* total lipid systems are higher than those found for the previous systems (and for Cpx) but similar among themselves.

These results show that Cpx metalloantibiotic interacts with the ternary membrane model system similarly to Cpx and more strongly with the binary and the *E. coli* total lipid extract systems, suggesting that the use of the metalloantibiotic may be more efficient than the use of the fluoroquinolone alone. Furthermore, this metalloantibiotic has a higher interaction with *E. coli* liposomes than others already described in the literature (Lopes et al. 2013; Ribeiro et al. 2011).

Determination of the Drug/Liposome Association Constants by Time-Resolved Fluorescence Spectroscopy

In addition to the steady-state fluorescence methodology used above for the study of the interaction of Cpx and its metalloantibiotic with the different lipidic systems, drug/liposome interaction was analyzed by time-resolved fluorescence spectroscopy, where any artifact caused by the

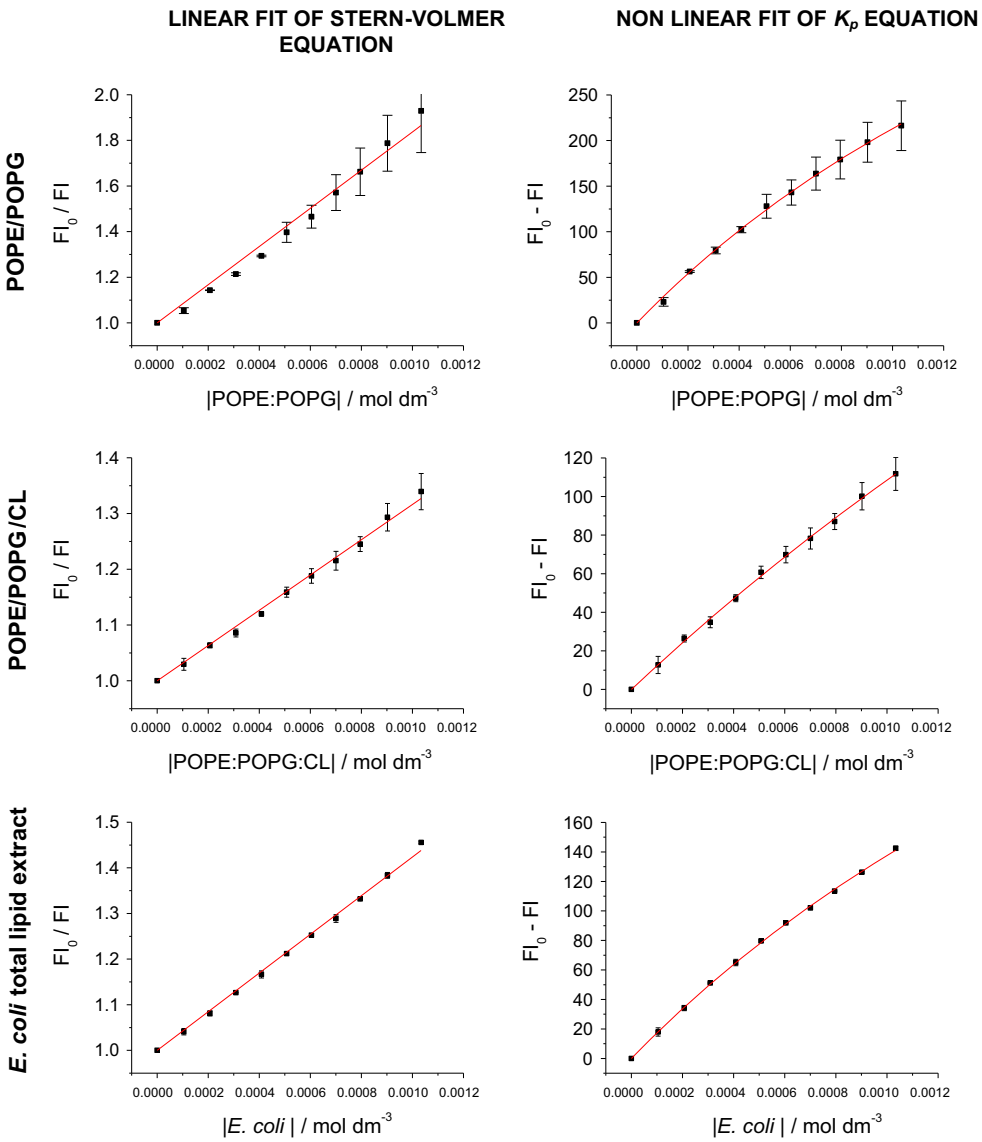

Fig. 3 Graphical treatment of the fluorescence data of Cpx (5.0 μmol dm^{-3}) in LUVs of POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, at 415 nm. The presented data are a mean of, at least, three independent measures

Table 1 Association constants of Cpx with LUVs of POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, using steady-state fluorescence spectroscopy, determined at $\lambda_{em} = 415$ nm by Eq. 2 (K_{SV}), Eq. 3 (K_p), and by HypSpec computer program (K_{Hyp})

Lipid model system	$\lambda_{exc} = 270$ nm |Cpx| = 2.0 μmol dm^{-3}			$\lambda_{exc} = 290$ nm |Cpx| = 5.0 μmol dm^{-3}		
	log K_p	log K_{SV}	log K_{Hyp}	log K_p	log K_{SV}	log K_{Hyp}
POPE/POPG	2.49 ± 0.14	2.58 ± 0.01	2.61 ± 0.37	2.56 ± 0.05	2.92 ± 0.01	2.65 ± 0.01
POPE/POPG/CL	2.34 ± 0.09	2.49 ± 0.01	2.49 ± 0.14	2.17 ± 0.10	2.50 ± 0.01	2.34 ± 0.21
E. coli total lipid extract	3.12 ± 0.01	3.19 ± 0.01	3.12 ± 0.04	2.47 ± 0.03	2.63 ± 0.01	2.45 ± 0.01

The presented values are the mean of, at least, three independent measures

presence of significant light scattering from the vesicle suspension is completely avoided (de Almeida et al. 2009; Santos et al. 1998).

The average fluorescence lifetimes of Cpx and its metalloantibiotic in the absence of liposomes ($\overline{\tau}_0$) calculated by Eq. 4 have a value of 1.5 ± 0.1 and 1.7 ± 0.1 ns,

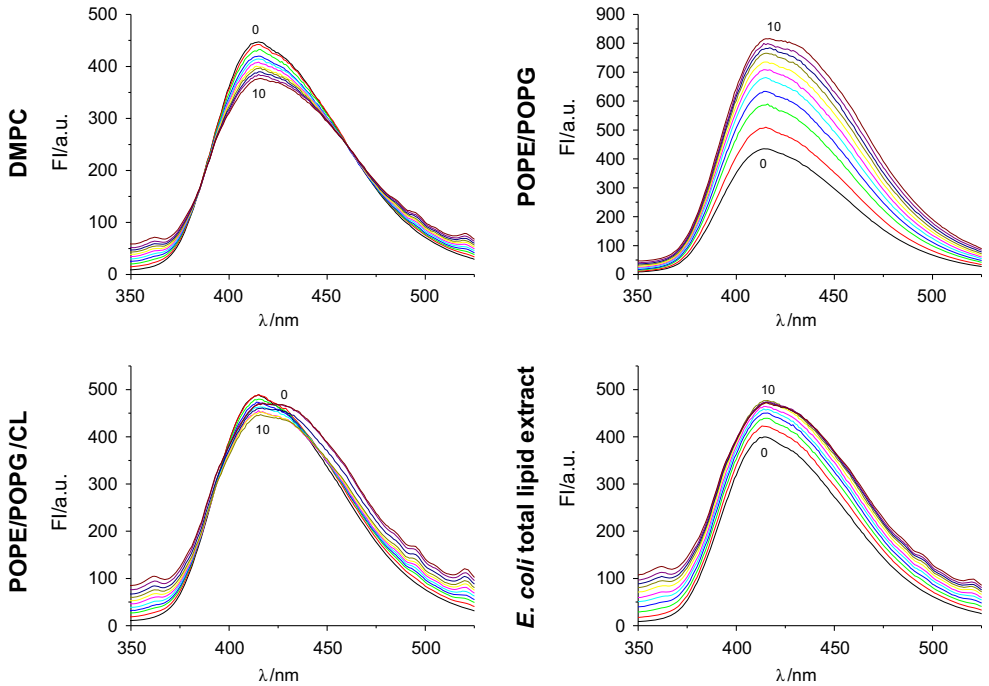

Fig. 4 Emission spectra of metalloantibiotic solution in the absence (*0*) and presence (*1–10*) of LUVs of DMPC, POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract. Each spectrum is a mean of five measurements

Table 2 Association constants of Cpx metalloantibiotic with LUVs of DMPC, POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, obtained by steady-state fluorescence spectroscopy, determined at $\lambda_{em} = 415$ nm by Eq. 3 (K_p) and by HypSpec computer program (K_{Hyp})

Lipid model system	$\lambda_{exc} = 290$ nm |metalloantibiotic| $= 5.0$ μmol dm^{-3}	
	log K_p	log K_{HYP}
DMPC	2.40 ± 0.11	2.64 ± 0.17
POPE/POPG	3.20 ± 0.02	3.09 ± 0.02
POPE/POPG/CL	–	2.76 ± 0.03
E. coli total lipid extract	3.58 ± 0.09	3.30 ± 0.10

The presented values are the mean of, at least, three independent measures

respectively. The value obtained for Cpx is in the range of those find in the literature (1.5 ns) (Polishchuk et al. 2012).

The average fluorescence lifetimes values, obtained for Cpx and its metalloantibiotic, in the absence ($\overline{\tau}_0$) and in the presence ($\overline{\tau}_1$ to $\overline{\tau}_{10}$) of increasing lipid concentrations, for all lipidic systems used, were analyzed with Eq. 5. The results show that the average fluorescence lifetime of Cpx remains constant in the presence of DMPC and POPE/POPG LUVs, as $\overline{\tau}_0 \approx \overline{\tau}_{10}$. However, an increase of the average fluorescence lifetime of Cpx was observed in the presence of POPE/POPG/CL and *E. coli* total lipid extract liposomes, which was more significant in the presence of LUVs of *E. coli* (Table 3).

Table 3 Fluorescence lifetime parameters obtained for Cpx (5.0 μmol dm^{-3}) in the absence and presence of LUVs of DMPC, POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, determined with $\lambda_{ex} = 290$ nm and $\lambda_{em} = 415$ nm by Eq. 4

Lipid model system	$\overline{\tau}_0$ (ns)	$\tau_{1\ (ns)}$	α_1	$\tau_{2\ (ns)}$	α_2
|Lipid| $= 0$ μmol dm^{-3}					
DMPC	1.6	1.3	95.2	3.8	4.8
POPE/POPG	1.5	1.3	91.8	2.6	8.2
POPE/POPG/CL	1.6	1.3	94.6	3.5	5.4
E. coli total lipid extract	1.5	1.3	96.4	3.5	3.6
Lipid model system	$\overline{\tau}_{10}$ (ns)	$\tau_{1\ (ns)}$	α_1	$\tau_{2\ (ns)}$	α_2
|Lipid| $= 1000$ μmol dm^{-3}					
DMPC	1.7	1.3	92.2	3.3	7.8
POPE/POPG	1.6	1.3	92.5	3.1	7.5
POPE/POPG/CL	1.8	1.3	94.3	4.2	5.7
E. coli total lipid extract	3.6	1.3	94.7	9.3	5.3

The presented values are the mean of, at least, three independent measures

For the metalloantibiotic, the average fluorescent lifetime increases in the presence of increasing concentrations of POPE/POPG, POPE/POPG/CL, and *E. coli* total lipid extract but remained constant in the presence of DMPC. Once again, the most significant increase of the average fluorescence lifetime was observed in the presence of LUVs of *E. coli* (Table 4).

Table 4 Fluorescence lifetime parameters obtained for Cpx metalloantibiotic (5.0 μmol dm^{-3}) in the absence and presence of LUVs of DMPC, POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, determined with $\lambda_{ex} = 290$ nm and $\lambda_{em} = 415$ nm by Eq. 4

Lipid model system	$\bar{\tau}_0$ (ns)	$\tau_{1\ (ns)}$	α_1	$\tau_{2\ (ns)}$	α_2
|Lipid| = 0 μmol dm^{-3}					
DMPC	1.6	1.3	95.6	3.8	4.4
POPE/POPG	1.5	1.3	94.8	3.1	5.2
POPE/POPG/CL	1.7	1.3	96.0	4.5	4.0
E. coli total lipid extract	1.7	1.3	96.8	4.8	3.2
Lipid model system	$\bar{\tau}_{10}$ (ns)	$\tau_{1\ (ns)}$	α_1	$\tau_{2\ (ns)}$	α_2
|Lipid| = 1,000 μmol dm^{-3}					
DMPC	1.8	1.3	93.6	4.1	6.4
POPE/POPG	2.4	1.3	91.5	5.4	8.5
POPE/POPG/CL	2.3	1.3	92.9	5.3	7.1
E. coli total lipid extract	4.2	1.3	92.5	9.3	7.5

The presented values are the mean of, at least, three independent measures

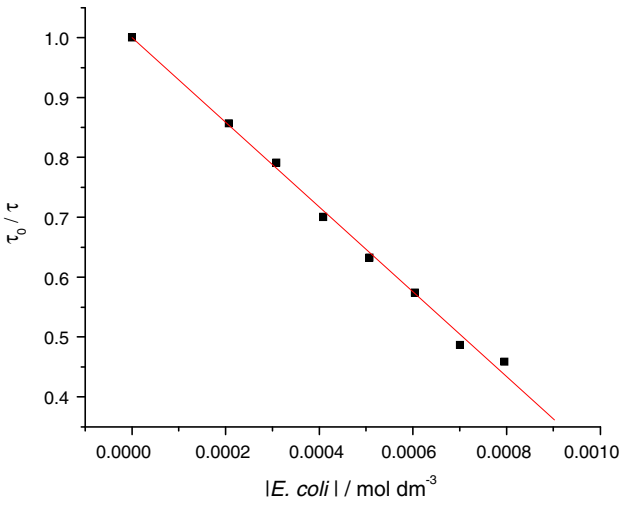

Fig. 5 Graphical treatment of the average fluorescence lifetime data of Cpx (5.0 μmol dm^{-3}) in LUVs of *E. coli* total lipid extract, at 415 nm. The presented data are a mean of, at least, three independent measures

The increase of the average fluorescence lifetime of a molecule in the presence of lipidic suspensions reveals its interaction with the lipid bilayer (Bolean et al. 2010; Goddard et al. 2013; Santos et al. 2003). The increase observed in the lifetime values suggests a partition between the aqueous and the lipidic media, even if there is a decrease in the steady-state fluorescence intensity. The lifetime is an absolute measurement, an intrinsic molecular property independent of concentration, unlike the steady-state intensity which is relative since it is sensitive to changes in concentration caused by photobleaching or diluting.

When carrying out a time-resolved fluorescence spectroscopic study of the interaction of a fluorescent partitioning molecule with a membrane system, ideally two exponentials would describe the experimental fluorescence intensity decay, one corresponding to the molecule in aqueous media and the other to the molecules in the aqueous/lipidic mixture environment. However, in most cases, the decays in both environments are complex and the total decay mixes up all contributions (Santos et al. 2003). Therefore, in this work, the average fluorescence lifetimes of the fluorophores, calculated with Eq. 4, were used to determine drug/liposomes interaction (de Almeida et al. 2009; Neves et al. 2009). The association constant values, obtained using the Eq. 5 (Fig. 5), are summarized in Table 5.

For Cpx, time-resolved fluorescence spectroscopy did not allow the determination of association constants in DMPC or POPE/POPG lipidic systems. The average fluorescence lifetime of this compound in these two lipidic environments is identical, within experimental error, to that obtained in aqueous media and shows no variation in the presence of the respective lipidic suspensions. Nevertheless, for POPE/POPG/CL and *E. coli* total systems, there is a significant change of the lifetimes of Cpx. When compared to the steady-state results, the values obtained for the association constants of Cpx are similar in the ternary system and show an increase in *E. coli* total lipid extract.

For the metalloantibiotic, only the average fluorescence lifetime obtained in the DMPC media was identical, within experimental error, to that obtained in aqueous media. For the other three lipidic systems, a significant change of the metalloantibiotic lifetime value occurred in the presence of the lipidic suspensions. The values of the association constants of the metalloantibiotic in POPE/POPG, POPE/POPG/CL, and *E. coli* total extract showed the same trend (low interaction with the ternary system and higher with *E. coli* extract) observed in the steady-state results. However, all lipidic systems have association constants values lower than those obtained by the steady-state fluorescent.

These results are in agreement with those obtained by steady-state, confirming that Cpx metalloantibiotic interacts more strongly with the bacterial mimetic systems than Cpx. Likewise, these results reinforce the suggestion that the use of the metalloantibiotic may be more efficient than the use of the fluoroquinolone alone.

The fact that no changes were observed in the average fluorescence lifetimes of Cpx and its metalloantibiotic in DMPC liposomes indicates that probably these drugs interact almost negligibly with the phosphatidylcholine (PC), the main lipid component of mammalian cells. This

Table 5 Association constants (K_{ass}) of Cpx and its metalloantibiotic with LUVs of POPE/POPG (0.75:0.25), POPE/POPG/CL (0.67:0.23:0.10), and *E. coli* total lipid extract, using time-resolved fluorescence spectroscopy (at $\lambda_{em} = 415$ nm), determined with Eq. 5

Lipid model system	$\lambda_{exc} = 290$ nm	
	|Cpx| = 5.0 μmol dm^{-3}	|Metalloantibiotic| = 5.0 μmol dm^{-3}
	log K_{ass}	log K_{ass}
POPE/POPG	–	2.71 ± 0.02
POPE/POPG/CL	2.15 ± 0.01	2.41 ± 0.01
E. coli total lipid extract	2.85 ± 0.01	2.91 ± 0.03

The presented values are the mean of, at least, three independent measures

result suggests that, when used to target bacterial membranes, Cpx and its metalloantibiotic leave the mammalian cells unaffected, showing that the metalloantibiotic can be a promising drug to counteract the resistance already developed for Cpx.

Conclusions

An important strategy to counteract bacterial resistance is the design of new drugs. Due to the known bacteria resistance mechanisms, the development of antibiotics that attack directly on bacterial membranes seems to be a promising route. Recent biological studies with fluoroquinolones and transition metal ions clearly show that Cu^{2+} can form very stable ternary complexes with fluoroquinolones and phenanthroline and that these complexes are as efficient antimicrobials as the free antibiotic (Feio et al. 2014; Saraiva et al. 2010; Sousa et al. 2012). Furthermore, biophysical studies strongly suggest that the cell intake route of both species may be different supporting, therefore, the complexes suitability as candidates for further biological testing in fluoroquinolone-resistant microorganisms.

The results obtained in this work, corroborating the previous biophysical studies, show that CuCpxPhen has a much higher interaction with *E. coli* liposomes than the free antibiotic, which may result in a different mechanism of entry in bacterial cell, strongly dependent on drug–lipid interactions. The complex species that exists at physiological pH, Cu-HLA, is cationic and promotes an electrostatic interaction with the negatively charged head groups of the lipids, considerably increasing its interaction with the membrane. The results obtained by steady-state and time-resolved fluorescence spectroscopy have the same trend, although the time-resolved values show always a slightly smaller drug/lipid interaction. These results could be expected as changes in fluorescence lifetimes are independent of concentration, reflecting only hydrophobicity changes. The values of the association constants determined in the studied lipidic systems show that the composition of the mimetic bacterial membranes is a crucial factor for the interaction of Cpx metalloantibiotic, contrary to what is observed for Cpx. These results are in agreement with fluoroquinolones speciation studies that show that Cpx exists in a zwitterionic/neutral form and the complex in a cationic form. Furthermore, the interaction of the metalloantibiotic in the binary lipidic system (POPE/POPG) is similar to that found for *E. coli* total extract. These results suggest that, contrary to enrofloxacin and moxifloxacin, the interaction of the metalloantibiotic with the *E. coli* membrane does not depend on CL composition (Lopes et al. 2013; Ribeiro et al. 2011).

As a final conclusion, the results obtained in this work point to a possible mechanism of entry of Cpx metalloantibiotic based on the hydrophobic pathway, while the pure Cpx requires the lipid/porin interface or the porin to be internalized. Moreover, the high values of the association constants obtained for Cpx metalloantibiotic, compared to those described in literature for enrofloxacin and moxifloxacin metalloantibiotics (Lopes et al. 2013; Ribeiro et al. 2011), indicate that this new metalloantibiotic may be a good choice to replace the pure Cpx and bypass at least one of the mechanisms of the bacterial resistance to fluoroquinolones.

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