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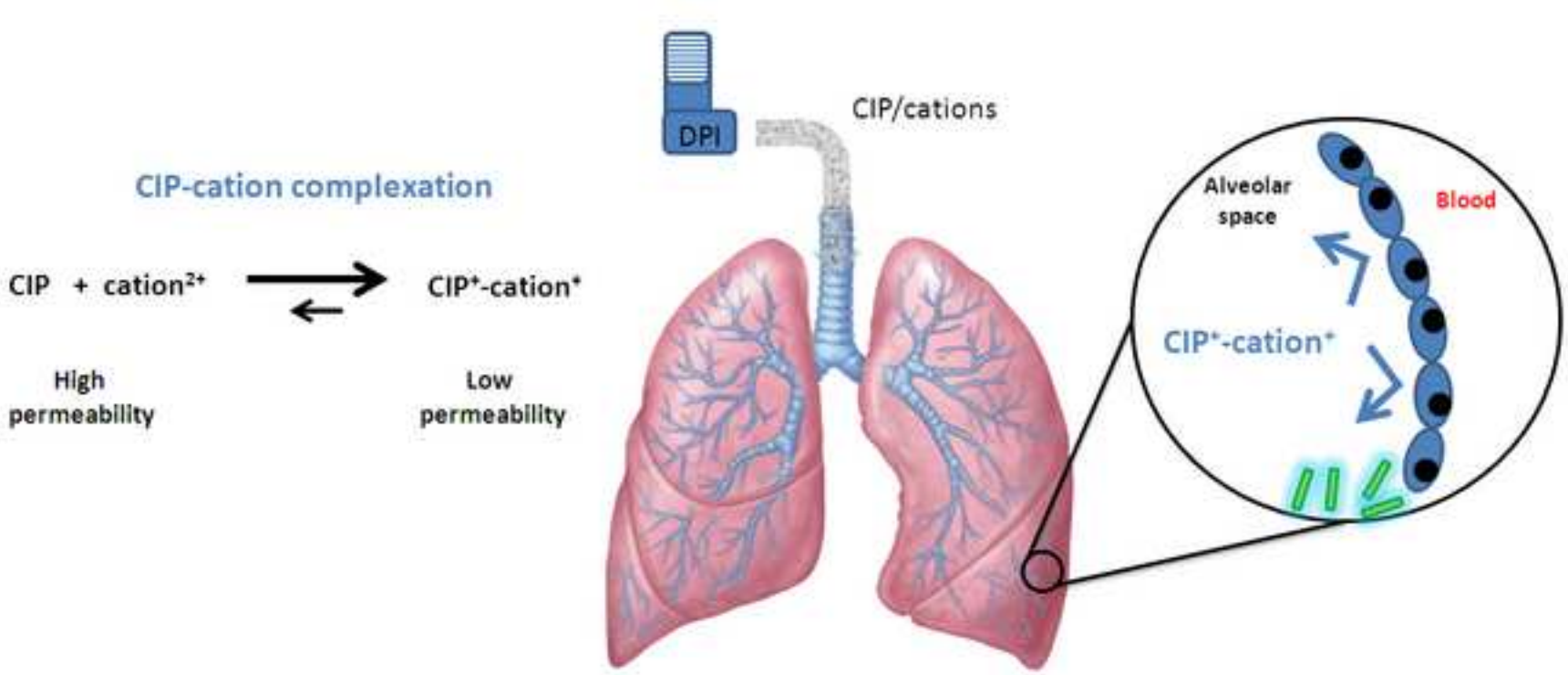
*Graphical Abstract (for review)



Abstract

Pulmonary delivery of fluoroquinolones (FQs) is an interesting approach to treat lung infections as it may lead to high local concentrations while minimizing systemic exposure. However, FQs have a rapid diffusion through the lung epithelium giving the pulmonary route no advantage compared to the oral route. Interactions between FQs and metal cations form complexes which limit the diffusion through the epithelial barrier and would reduce the absorption of FQs and maintain high concentrations in the lung. The effects of this complexation depend on the FQ and the metal cations and optimum partners should be selected through in vitro experiments prior to aerosol drug formulation. In this study, CIP was chosen as a representative FQ and 5 cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+}) were selected to study the complexation and its effects on permeability, antimicrobial efficacy and cell toxicity. The results showed that the apparent association constants between CIP and cations ranked with the descending order: Cu^{2+}>Al^{3+}>Zn^{2+}>Mg^{2+}>Ca^{2+}. When a target of 80% complexation was reached with the adequate concentrations of cations, the CIP permeability through the Calu-3 lung epithelial cells was decreased of 50%. Toxicity of the CIP on the Calu-3 cells, with an EC50 evaluated at 7 µM, was not significantly affected by the presence of the cations. The minimum inhibitory concentration of CIP for *Pseudomonas aeruginosa* was not affected or slightly increased in the range of cation concentrations tested, except for Mg^{2+}. In conclusion, permeability was the main parameter that was affected by the metal cation complexation while cell toxicity and antimicrobial activity were not or slightly modified. Cu^{2+}, with the highest apparent constant of association and with no effect on cell toxicity and antimicrobial activity of the CIP, appeared as a promising cation for the development of a controlled-permeability formulation of CIP for lung treatment.

Keywords: FQ-cation interaction, pulmonary delivery, controlled permeability, ciprofloxacin, Calu-3 cells.

Chemical compounds studied in this article

Ciprofloxacin (PubChem CID: 2764)

1. Introduction

Pulmonary delivery of antibiotics is an interesting approach to treat lung infections as it may lead to high local concentrations with expected high efficiency while minimizing systemic exposure to reduce side effects. This is especially true for drugs that slowly diffuse through the lung epithelium such as colistin or tobramycin (Gontijo et al., 2014b; Marchand et al., 2015). As a matter of facts, the most common antibiotics used in nebulized therapy have also a low lung epithelial permeability, such as tobramycin, colistimethate or aztreonam (Marchand et al., 2010; Marchand et al., 2015, 2016). On the contrary, fluoroquinolones (FQs) such as ciprofloxacin (CIP) have a rapid diffusion through the lung epithelium (Brillault et al., 2010) and following nebulization FQ lung concentrations quickly equilibrate with the plasma concentrations, giving the pulmonary route of administration no advantages compared to the oral route (Gontijo et al., 2014a). However FQs, which are commonly used in patients to treat lung infections by oral administration (Hurley and Smyth, 2012), could benefit from an aerosolized form that would reduce their diffusion through the lung epithelium. Several formulations have been developed to achieve this goal, such as dry powder of CIP but results showed a rapid pulmonary absorption of CIP into the plasma (Stass et al., 2013). Interactions between FQs and metal cations are well-known to decrease the oral absorption and bioavailability (Marchbanks, 1993). FQs form complexes of various

stoichiometries with various metal cations (Uivarosi, 2013). These complexes have a higher molecular weight with an often ionized molecular structure which limits the diffusion through the intestinal barrier. While this interaction is a disadvantage for the oral route, it may be of great interest for the pulmonary route since it would reduce the absorption of FQs and maintain high concentrations of FQs in the lung. Previous work has shown the feasibility of an inhalable calcium-based microparticle formulation of CIP for pulmonary administration (Tewes et al., 2015). In this study calcium (Ca^{2+}) at a high Ca^{2+}/CIP mass ratio of 200 decreased the permeability of CIP to 80% in an in vitro model of the pulmonary epithelium. However, other metal cations and FQs could be used for an optimized controlled-permeability formulation. The optimum partners of FQs and metal cations should be selected through in vitro experiments prior to microparticle formulation, in order to maximize the decrease in FQ permeability, concomitant with a lowest toxicity (*i.e.* lowest amount of cations and FQ that would be administered to the lung) and high efficiency. Considering the numerous available FQs (about 30 compounds) and also the various metal cations that can bind to FQs (Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Cu^{2+}, Ag^{+}, Co^{2+}, Ni^{2+}, Bi^{3+}, etc.), several hundreds of possibilities may be considered. It is then necessary to develop first in vitro assays to screen these combinations and predict their interest for the development of new formulations. In this goal, the first point to consider was the ability of FQs and ions to interact together and how it influences the FQ diffusion through the lung. The second point was to verify if the presence of metal cations and their association with the FQ modified the antimicrobial efficacy and cell toxicity of the antibiotic.

In this study, CIP was chosen as a representative FQ and the 5 five following cations were used to study the complexation with CIP: Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Cu^{2+}. Assays were carried out in medium and conditions that mimic the biological characteristics of the lung:

Interaction between FQs and cations was assayed in a physiological saline buffer, the permeability of CIP in the presence of cations was assayed using the Calu-3 cell line and the antimicrobial efficacy of CIP in the presence of cations was evaluated against *Pseudomonas aeruginosa*, a frequent opportunistic infectious agent in the lung. The main purpose of this study was to better understand the interaction between CIP and metal cations in the lung and to help defining the assays needed for a systematic screening of FQ/metal cation complexes for pulmonary delivery.

2. Materials & Methods

2.1. Chemicals

Sodium fluorescein, CIP, $CaCl_2$, $2H_2O$; $MgCl_2$, $4.5H_2O$; $ZnSO_4$, $7H_2O$; $CuSO_4$, $5H_2O$; $Al(OH)_3$; MOPS, TRIS and HEPES were obtained from Sigma-Aldrich. HPLC-grade acetonitrile was purchased from VWR International (Fontenay sous Bois, France). Mueller Hinton II agar and Mueller Hinton II Broth (cation adjusted) were from Becton Dickinson (Le Pont-de-Claix, France). All other reagents were of analytical grade.

2.2. Apparent constant of association (K)

To study the interaction between FQs and metal cations in medium having ion composition and pH close to biological samples such as the epithelial lining fluid (ELF) of the lung alveoli, CIP was incubated in saline solution (NaCl and KCl) at pH = 7.4. To assess the possible interaction between CIP and buffers, HEPES, MOPS or TRIS buffer was added to a 30 µM CIP solution in water at different concentrations (0, 1, 2.5, 7.5 and 10 mM) and pH was adjusted to 7.4 with 1 M sodium hydroxide. One ml of each solution was placed in 24 well plates and

fluorescence intensity emission spectrum of CIP was recorded at room temperature with a plate reader (λexc = 274 nm and λem ranging from 360 to 500 nm; VarioskanFlash, Thermo Scientific, Villebon sur Yvette, France). MOPS and Tris buffers shifted and lowered the spectrum respectively, HEPES buffer did not affect the CIP fluorescence from 360 to 500 nm with a concentration up to 10 mM (data not shown). Moreover, HEPES is generally considered a non-complexing buffer and is suitable for use in solutions with metal ions (Ferreira et al., 2015). This buffer was then chosen for all the following experiments. Fluorescence emission spectrum of CIP in the presence of increasing concentrations of the different cations did not show a shift of the maximum emission wavelength. The set of 274 and 408 nm for the excitation and emission wavelengths was chosen for all the titration experiments. Fluorescence titration profiles were obtained by adding cation solutions with concentrations ranging from 500 to 10^5 µM for calcium and magnesium ions, from 15 to 500 µM for zinc ions and from 15 to 150 µM for copper and aluminum ions to a 3 µM CIP solution. Solutions contained 5.3 mM KCl and were maintained at constant pH 7.4 with 10 mM HEPES and adjusted with NaCl to a constant ionic strength (155 mM for Mg^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+} and 1205 mM for Ca^{2+}). The final volume of each sample was 1 ml and fluorescence was recorded using the VarioskanFlash plate reader. Binding isotherm equations for 1:1 and 2:1 complexes were built to describe the data according to Hargove et al. (Hargrove et al., 2010). Equations and mathematical development can be found in the Appendix A. The experimental data were fitted with the two models (1:1 and 2:1) and the comparison was evaluated with the Akaike's information criteria (AIC).

2.3. Apparent permeability (Papp) of CIP in the presence of metal cations

2.3.1. Calu-3 cell culture

Calu-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM/Ham's F12 (1/1) supplemented with L-glutamine (2 mM) and 10% foetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany) and incubated at 37°C under 90-95% of relative humidity and 5% v/v of CO_2 in air. The Calu-3 cells at passages 50-60 were seeded at a density of 15 x 10^4 cells/cm^2 onto 12-well plate Transwell inserts (Corning Transwell Clear PET membrane 0.4µm, Thermofischer Scientific). The cells were cultured under air-interface conditions for 15 days. The growth medium in the basolateral compartment (1.5 ml) was replaced by fresh medium every other day.

2.3.2. Transport experiments

Transport experiments were conducted in apical-to-basolateral directions as described elsewhere (Brillault et al., 2010). Briefly, on study day the Calu-3 monolayers were quickly rinsed with the transport medium (TM: NaCl 134 mM, KCl 5.3 mM, glucose 5.5 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1 mM, buffered with 10 mM HEPES and adjusted to pH 7.4) and then incubated for 30 min in TM. This transport medium is a modified formula of the classical HBSS medium without bicarbonate or phosphate known to form complexes with the metal ions. Following the equilibration period, the TM in the donor compartment (apical side) was replaced by fresh TM containing 50 µM of CIP with 0, 5, 10, 50 or 100 mM Mg^{2+}. After 60 min of incubation at 37°C, sample aliquots were taken from the acceptor compartment (basolateral side). Apparent permeability (Papp) was calculated using eqn (1) where Q is the amount of CIP in the acceptor compartment after a time Δt, S is the insert membrane surface (1.12 cm^2), [CIPo] is the initial CIP concentration in the donor compartment.

$$Papp = \frac{Q}{[CIPo].\Delta t.S} \quad (1)$$

156 Papp versus Mg^{2+} concentration data were analysed with the following equation (eqn 2).

157 $$\%\ of\ Papp = 100 - \frac{(100 - Papp\ Min).[X]^{nH}}{EC50^{nH} + [X]^{nH}} \quad (2)$$

158 Where Papp $_{Min}$ is the low plateau of Papp, [X] the Mg^{2+} concentration, EC50 the Mg^{2+}
159 concentration necessary to get 50% of the maximum effect (100- Papp Min) and nH the Hill
160 number. In a second experiment, Papp were determined after cells were incubated in TM
161 with 50 µM of CIP and 40 mM of Ca^{2+}, 5.5 mM of Mg^{2+}, 1.5 mM of Zn^{2+}, 0.1 mM of Al^{3+} or 0.04
162 mM of Cu^{2+} for a 60 min incubation period before sampling in the acceptor compartment.
163 Following the transport studies, routine controls of the monolayer integrity were performed
164 using sodium fluorescein. Briefly, the monolayers were rinsed with TM, fresh TM was added
165 in the basolateral side and a solution of sodium fluorescein in TM (10 µg/ml) was poured in
166 the apical side. The inserts were incubated and samples were taken after 60 min from the
167 acceptor compartment. A threshold P_{app} value of 0.7 x 10^{-6} cm.s^{-1} for fluorescein was
168 retained for the tight junction integrity rejection parameter for all experiments. This
169 corresponds to the transfer of less than 0.5% of the initial amount in the apical compartment
170 (Brillault et al., 2010).

171 **2.3.3. CIP and sodium fluorescein assay**

172 CIP was assayed using a HPLC method with fluorometric detection (λexc =280 nm; λem =460
173 nm) using a Jasco FP-920 fluorescence detector (Jasco France, Lisses, France). The stationary
174 phase was an XTerra MS C18 column, 5 µm, 100 × 2.1 mm (Waters, Milford, MA). The mobile
175 phase (flow rate: 0.25 ml/min using a Hitachi L-2130 pump, Hitachi High technologies Co.,
176 Berkshire, UK) consisted of a 20:80 (v:v) mixture of acetonitrile and water containing 0.1%
177 formic acid and 0.2% heptane sulfonic acid. Samples, standards (7 levels with concentrations

ranging from 1.56 to 50 ng/ml) and quality controls (3.12, 12.5 and 37.5 ng/ml) prepared in the same solvent were injected (75 µl using a Hitachi L-2200 autosampler) and eluted over a run time of 6.5 min. The precision and accuracy were less than 15% for the 3 quality control concentrations. Controls with various concentrations of CIP and cations were assayed to ensure that the presence of cations did not interfere with the analysis of CIP.

Fluorescein concentrations in TM were measured using the VarioskanFlash plate reader with the excitation and emission wavelengths set at 490 nm and 530 nm, respectively.

2.4. Cytotoxicity assay

Calu-3 cells were seeded in 96 well plates at the density of 5000 cells/well. Incubation medium (100 µl) was Gibco MEM (Thermo Fisher Scientific) supplemented with 5% (v/v) foetal calf serum. CIP and ions solutions were prepared at concentrations necessary to achieve 80 % of complex. Namely, 150 µM of CIP were mixed with 40.1 mM of Ca^{2+}, 5.6 mM of Mg^{2+}, 1.6 mM of Zn^{2+}, 0.17 mM of Al^{3+} or 0.125 mM of Cu^{2+}. The cells were then incubated with a serial dilution of these solutions or the CIP alone with concentrations ranging from 0.3 to 150 µM and the plates were returned to the incubator. Control cells were incubated with ions alone with serial dilution from 100 to 0.05 mM for Ca^{2+} and Mg^{2+} and from 5 to 0.0025 mM for Zn^{2+}, Al^{3+} and Cu^{2+}. Following a 24 h incubation time, the incubation medium was removed and replaced with HBSS medium and cell viability was evaluated with a MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, Charbonnières-les-Bains, France) according to the manufacturer protocol. The linearity of the response was verified thanks to preliminary experiments. Data were analysed with the following equation (eqn 3).

$$\% \, of \; viability = Top - \frac{(Top - Bottom).[X]^{nH}}{EC50^{nH} + [X]^{nH}} \quad (3)$$

where [X] is the concentration of CIP or ions, Top and Bottom are plateaus in the units of the Y axis, EC50 is the concentration that gives half of the total effect (Top-Bottom) and nH is the Hill number.

2.5. MIC assay

The effect of cation (Mg^{2+}, Ca^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}) concentrations on the CIP antibacterial activity was assessed by measuring the apparent CIP minimum inhibitory concentration (MIC) against *Pseudomonas aeruginosa* PAO1 (CIP 104116, Institut Pasteur, Paris, France). Bacteria were stored at -80°C. One day before the experiment, they were grown on Mueller-Hinton II agar for 24 h at 37°C. Prior to MIC test, the strains were grown to logarithmic phase in fresh Mueller-Hinton broth II (MHB) for 2-3 hours and then adjusted to 0.5 McFarland standards. The MIC test was performed using the broth microdilution method: The adjusted cultures were diluted 100-times in MHB containing cations, CIP or both and 100 µl of these bacterial suspensions were seeded in 96 well plates. CIP concentrations were 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0 µg/ml. Mg^{2+} concentrations were 20, 10, 5, 2, 1, 0.5, 0 mM. Ca^{2+} concentrations were 100, 50, 20, 10, 5, 2, 1, 0.5, 0 mM. Zn^{2+} concentrations were 1, 0.5, 0.1, 0.05, 0.02, 0.01, 0 mM. Al^{3+} and Cu^{2+} concentrations were 0.1, 0.05, 0.02, 0.01, 0.005, 0 mM. The growth of bacterial cultures at 37°C was determined after 18 hours by monitoring the optical density of the culture at 600 nm using the VarioskanFlash plate reader. Controls without bacteria were made to check the absence of contamination of MHB and to evaluate the effect of the cations on the absorbance reading at 600 nm. Controls without CIP were made to assess the effect of the cations on bacterial growth.

2.6. Statistical analysis

Data analysis and modeling were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results

3.1. Apparent constants of association

Titration curves of CIP with Mg^{2+}, Ca^{2+}, Zn^{2+}, Al^{3+} and Cu^{2+} ions were made in HEPES buffered saline solutions (Figure 1). Increasing the concentration of Ca^{2+}, Mg^{2+} and Zn^{2+} resulted in an increase in the total fluorescence, while with Al^{3+} and Cu^{2+} the total fluorescence was quenched. The equation corresponding to a 1:1 complex model fitted the data well for all the ions with a satisfactory accuracy for the estimated apparent constant of association (Table 1). On the other hand, the equation from the 2:1 complex model resulted in a very broad estimation of the parameters and higher AIC values (data not shown). This suggested a 1:1 (CIP:metal cation) complex stoichiometry. The estimated apparent association constants ranked with the descending order: Cu^{2+}>Al^{3+}>Zn^{2+}>Mg^{2+}>Ca^{2+}, with almost a 9000 fold ratio between the minimum and maximum values (Table 1).

3.2. Influence of complexation on Calu-3 cell layer permeability

In a preliminary experiment, Mg^{2+} was chosen to test the influence of the ion concentration on the apical to basolateral permeability of CIP through the Calu-3 cell model. In the control conditions where no magnesium was added to the transport medium, the Papp was 0.34 ± 0.02 x 10^{-6} cm/s. The results on figure 2A showed a decrease in the CIP Papp dependent on

243 the concentration of Mg^{2+}. As estimated by the modeling, 6.7 mM of added magnesium
244 reduced the Papp to 50% of the control and a plateau was reached at 11.5 % of the control
245 Papp (0.04 x 10^{-6} cm/s). According to the mass balance equations from 1:1 model (Appendix
246 A) and to the apparent constant of association determined in table 1, the percentage of
247 complexation of CIP by Mg^{2+} was calculated and plotted against the Papp (Figure 2B). These
248 data were modeled with eqn 2 and showed that it is necessary to form 86% of complexation
249 to reduce the Papp of 50%. Since the complexation apparently correlated with the decrease
250 in permeability, the transport of CIP was assayed at concentrations of metal cations
251 necessary to reach the same level of complexation. As seen with the Mg^{2+}, a high level of
252 complexation is needed to induce a significant decrease in permeability, but high
253 concentrations of ions, as for Ca^{2+} would lead to hypertonic and deleterious conditions for
254 the cells. For these reasons, a target of 80% complexation was chosen for the next
255 experiment and theoretical ion concentrations were calculated for Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and
256 Al^{3+}. Thus the cells were incubated in the presence of 50 µM of CIP and 40 mM of Ca^{2+}, 5.5
257 mM of Mg^{2+}, 1.5 mM of Zn^{2+}, 0.1 mM of Al^{3+} or 0.04 mM of Cu^{2+}. Results confirmed that such
258 cation concentrations leaded to a decrease in CIP permeability close to 50% (Figure 3).

259 **3.3. Cytotoxicity assay**

260 As shown in figure 4A and table 2, ions may be classified into two groups according to their
261 cytotoxicity: the first group (low toxicity) includes Mg^{2+} and Ca^{2+} with an estimated EC50 at
262 36 x10^3 and 131 x10^3 µM, respectively; the second group (higher toxicity) includes Zn^{2+}, Al^{3+}
263 and Cu^{2+} with EC50 close to 1 x10^3 µM. Cytotoxicity of the CIP was evaluated in the presence
264 of cations. The highest CIP concentration was 150 µM and ion concentrations were chosen
265 to reach 80 % complexation. For Cu^{2+}, Mg^{2+}, Al^{3+} and Ca^{2+}, the concentrations were several

266 times lower than their respective EC50 and for Zn^{2+} equal to its EC50 (Table 2). Thus, the
267 presence of ions at concentrations forming 80% complexes with CIP should not increase the
268 cell toxicity of the CIP. As shown in figure 4B and table 2, the EC50 of the CIP over the Calu-3,
269 which was evaluated to be 7 µM, was not apparently affected by the presence of these ions.

270 **3.4. MIC assay**

271 The influence of the cations was also tested on the antibacterial effect of CIP against *P.*
272 *aeruginosa*. In the presence of the ions alone at all concentrations tested, the bacterial
273 growth was similar to the control (data not shown). The MIC of CIP was 0.25 µg/ml. In the
274 presence of Ca^{2+} or Mg^{2+} the MIC of CIP was increased from an ion concentration of 5 mM
275 (33% of complexation) and 2 mM (50% of complexation) onwards, respectively (Figure 5A).
276 In the presence of Zn^{2+}, the MIC was unchanged up to a concentration of 500 µM (50% of
277 complexation) where it decreased of one level. The MIC remained unchanged for Al^{3+} and
278 Cu^{2+}, except at 50 µM for copper (98% of complexation) (Figure 5B).

279

280 4. Discussion

281 Interactions between FQs and metal cations have been widely studied and it has been
282 shown that the presence of metal cations affects both solubility and membrane permeability
283 of FQs (Stojkovic et al., 2014; Zakelj et al., 2007). The consequence for the oral route is a
284 decrease in their bioavailability. However for the pulmonary route, low permeability with
285 high solubility could be of great advantage for antibacterial lung treatment since it would
286 increase the drug residence time in the lung and lower the systemic side effects.
287 Antimicrobial efficacy is also sometimes affected in a positive or negative way (Uivarosi,

2013). The mechanisms underlying these effects are not clearly understood and may involve FQs electric charge, molecular weight, lipophilicity, membrane permeability and/or binding with their bacterial enzymatic target. These modifications of the bioavailability or efficacy are not predictable and needs to be evaluated. In this work in vitro assays have been developed to study the interaction between CIP and Mg^{2+}, Ca^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+}.

Apparent constants of stability between metal cations and CIP have been evaluated in biological conditions. Since the ionic composition of the ELF is not known, a simple saline buffer (pH 7.4) containing extra-cellular concentrations of sodium, potassium and chloride was chosen. In this medium the apparent association constants K ranked as Cu^{2+}>Al^{3+}>Zn^{2+}>Mg^{2+}>Ca^{2+} (Table 1). These results are in accordance with previous works with an equivalent ranking where the highest interactions were obtained with Al^{3+} and Cu^{2+} (Ma et al., 1997; Seedher and Agarwal, 2010). Interestingly, for Ca^{2+}, Mg^{2+} and Zn^{2+} the interactions increased the fluorescence intensity of the solutions while for Cu^{2+} and Al^{3+} the interactions resulted in a strong decrease of the fluorescence. Similar results had been obtained previously, where Cu^{2+} strongly decreased the CIP fluorescence while Mg^{2+} increased it (Drevensek et al., 2003). This may be due to different sites of interaction on the CIP molecule, which has two main sites of metal chelate formation: one on the carbonyl and carboxyl groups and the second on the piperazin group (Uivarosi, 2013). Quinolone-metal cation chelates may be synthetized as 1:1, 2:1 or 3:1 (FQ:metal cation) complexes depending on the cation, pH and the synthesis process. Here, the titration of CIP in biological conditions showed a spontaneous interaction with metal cations as 1:1 complexes as shown by the modeling of the data (Figure 1). To evaluate if these interactions could affect CIP permeability across the epithelial cells of the lung, CIP apparent permeability was assessed with the Calu-3 cell model in the presence of the different cations (Figure 2 and 3). It should

312 be noted that Mg^{2+} and Ca^{2+} concentrations of about 1 mM are needed for the cell layer
313 integrity since the intercellular junctions that seals the cells together need these two cations
314 to be effective. Thus the transport experiments were made in conditions where Ca^{2+} and
315 Mg^{2+} were already present and would be responsible for 11% and 41% complexation of CIP,
316 respectively. Since the concentration of Mg^{2+} in plasma is close to 1 mM, this also would
317 suggest that in biological fluids CIP is in part present as a Mg^{2+} complex (Jahnen-Dechent and
318 Ketteler, 2012). This might be also the case in the ELF of the alveolar space. For the same
319 reason, in the bacteria cytoplasm where the concentration of free Mg^{2+} is about 1-2 mM
320 (Alatossava et al., 1985), CIP is expected to be present inside the cell mostly as a CIP:Mg^{2+}
321 complex. Nevertheless the results showed a strong correlation between metal cation
322 concentration and decrease in CIP apparent permeability through the cells. With Mg^{2+}, a
323 maximum decrease in permeability of about 90% was observed in conditions were the CIP is
324 almost completely complexed. It is not known whether the 10% remaining apparent flux of
325 CIP is due to the diffusion of the CIP:Mg^{2+} complex or of the free CIP. The permeability
326 experiments with the other cations showed that the apparent association constant is useful
327 to predict the effect of the interaction over the cell permeability. The complexation of 80 %
328 of the CIP affected the apparent CIP permeability with the same extent, whatever the nature
329 of the metal cations. This result suggests that only the electric charges brought by the metal
330 cations are responsible for the decrease in permeability. Indeed, in the case of a 1:1
331 CIP:metal cation interaction, a bicationic complex CIP^{+}-M^{+} appears, while CIP is an overall
332 uncharged zwitterionic molecule at pH 7. It is also unlikely that the extra molecular weight
333 brought by the cations would influence the CIP diffusion since they represent only 7 to 19%
334 of the CIP MW. The apparent association constants varied from 100 to 900000 M^{-1} for Ca^{2+}
335 and Cu^{2+} respectively (Table 1). This difference has huge consequences on the amount of

cations necessary for the complexation of the CIP. For instance, if a pharmaceutical pulmonary drug formulation is designed to deliver in the ELF 1 µg/ml (3 µM) of CIP, which is in the range of MIC for the majority of *P. aeruginosa* from environmental sites and patients (Macdonald et al., 2010), the ion concentration in the ELF needed to reach 80% of complexation would be 1600 µg/ml for Ca^{2+} and only 0.4 µg/ml for Cu^{2+}. Metal cations are known to be potentially toxic to the cells mainly through reactive oxygen species generation and then increased intra-cellular oxidative stress (Stohs and Bagchi, 1995). Cytotoxicity studies showed that for cations with the highest association constant with CIP (Cu^{2+}, Al^{3+} and Zn^{2+}), the relative cell toxicity was about 100 times higher than for Mg^{2+} and Ca^{2+} (Figure 4 and Table 2). However, in the hypothesis of a pulmonary drug formulation that could deliver in the lining fluid of the alveoli a CIP concentration 50 times higher than the MIC for *P. Aeruginosa* (*i.e.* 150 µM), the corresponding concentration of cations necessary to achieve complexation of most of the CIP is several times lower than the EC50 of the cations for the cell toxicity. Thus, the higher toxicity of Cu^{2+}, Al^{3+} or Zn^{2+} may be counterbalanced by the lower concentration needed for complexation compared to that of Ca^{2+} and Mg^{2+}. Several articles have reported that FQ:metal cation complexes had a limited or no effect on the antibacterial activity. For instance, complexes of CIP with Zn^{2+} or Cu^{2+} have been shown to have no influence on the MIC against *S. aureus*, *P. aeruginosa* or *K. pneumoniae* (López-Gresa et al., 2002) while complexes of levofloxacin with Mg^{2+} slightly decreased the antibacterial activity with the same strains (Drevenšek et al., 2006). Here MIC assays have been done to evaluate this influence over *P. aeruginosa* (Figure 5). The growth medium for the bacteria already contains limited amounts of Ca^{2+} and Mg^{2+} (0.5 and 0.4 mM, respectively). However, due to the low concentrations and the weak constants of association of these two ions, their influence can be considered negligible. None or slight increase in

MIC have been shown in the range of cation concentration tested, except for Mg^{2+}at 10 mM where its presence greatly decreased the antimicrobial activity of CIP. This is in agreement with a previous study with sparfloxacin, ciprofloxacin, norfloxacin and pefloxacin where Mg^{2+} was shown to decrease the antibacterial activity of the FQs in correlation with a lower uptake into the bacteria. These results suggested that Mg^{2+} impaired the CIP uptake into the bacteria (Lecomte et al., 1994).

In conclusion, this work showed the influence of metal cations on permeability and antimicrobial activity of the CIP in the goal of pulmonary administration. Permeability was the main parameter that was affected by the metal cation complexation while cell toxicity or antimicrobial activity were not or slightly modified, as shown by the in vitro experiments. While Al^{3+} is suspected of brain toxicity, Cu^{2+} seems to be an interesting cation for the development of a controlled-permeability formulation of CIP for lung treatment and further studies will evaluate the pharmacokinetics of CIP after lung administration of CIP:Cu^{2+} loaded microparticles. More generally, in the goal of selecting the best FQ:metal cation couple for lung delivery formulation, the association constant between FQ and metal cation should be estimated first since this parameter is of great influence on permeability. Lipophilicity of the FQ was not addressed in this study but it should also be taken into account because the effect of complexation over permeability could have a major effect for the most lipophilic drugs such as moxifloxacin, grepafloxacin or pefloxacin. Toxicity and antimicrobial activity should be then investigated with the most interesting couples. Further studies will focus on the effects of metal cation interactions with FQs of different lipophilicities and on in vivo pharmacokinetic evaluations of novel FQ-metal cation formulations.

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Apparent association constant K (M^{-1})	
Ca^{2+}	**1×10^2** [13-188]
Mg^{2+}	**7.2×10^2** [176-1272]
Zn^{2+}	**27×10^2** [1792-3614]
Al^{3+}	**880×10^2** [56540-119406]
Cu^{2+}	**9069×10^2** [849417-964359]

454 Table 1. Apparent association constant between CIP and ions (with the 95% confidence interval) in
455 saline solution at pH 7.4.

456

457

Calu-3 cell toxicity EC50 (µM)			
		CIP	**7** [5-11]
Ca^{2+}	**36×10^3** [22-61 x 10^3]	**CIP:Ca^{2+}**	**5** [4-6]
Mg^{2+}	**131×10^3** [111-152 x 10^3]	**CIP:Mg^{2+}**	**11** [8-14]
Zn^{2+}	**0.9×10^3** [0.7-1.1 x 10^3]	**CIP:Zn^{2+}**	**3** [3-4]
Al^{3+}	**1.0×10^3** [0.7-1.4 x 10^3]	**CIP:Al^{3+}**	**7** [5-9]
Cu^{2+}	**1.1×10^3** [0.7-1.5 x 10^3]	**CIP:Cu^{2+}**	**14** [12-16]

458 Table 2. Cell toxicity EC50 of cations alone or CIP:cations mix in 80% complexation conditions over a
459 24 h incubation period. Values are given with their 95% confidence interval.

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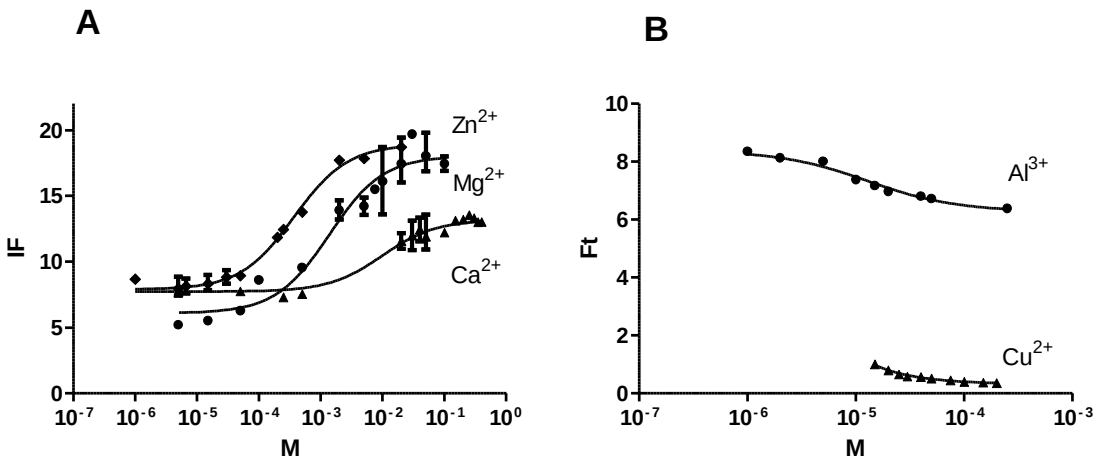


Figure 1. Titration curve of the total fluorescence against the molar concentration of ions. 3µM of CIP were mixed with increasing concentrations of Mg^{2+}, Ca^{2+}, Zn^{2+} (A), Al^{3+} or Cu^{2+} (B). Total fluorescence (Ft) has been recorded at λexc = 274 nm and λem = 408 nm. Data are expressed as means ± range (n=2-3) and were fitted with the 1:1 complex model.

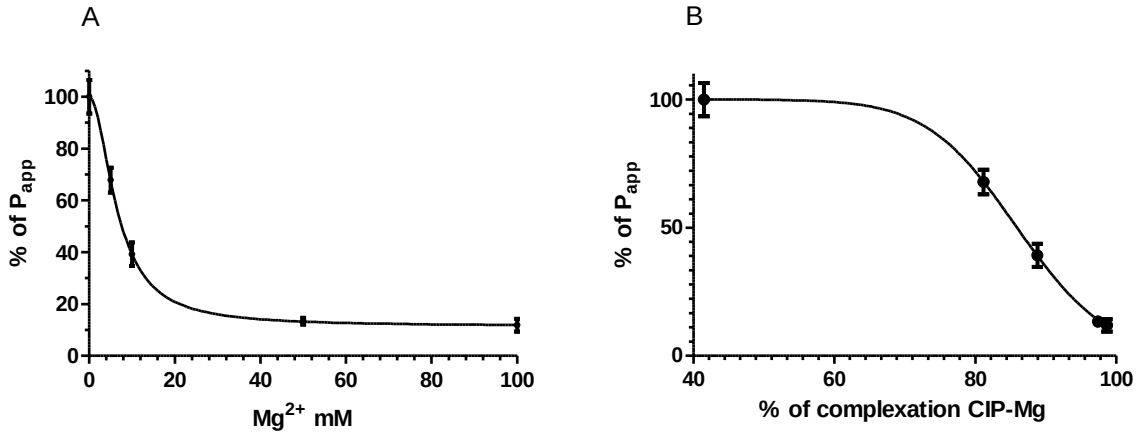


Figure 2. Influence of added Mg^{2+} concentration and Mg-complexation on the apparent permeability for CIP. The apical-to-basolateral permeability of the Calu-3 cell layer for CIP (50 µM) was evaluated in the presence of increasing concentrations of Mg^{2+} (A). Permeability was also plotted against the % of complexation calculated with the constant of association between CIP and Mg^{2+} (B). Results were expressed as a the percentage of the CIP Papp (0.34 ± 0.02 x 10^{-6} cm/s) determined with no Mg^{2+} added to the transport medium (means ± S.E.M., n=3).

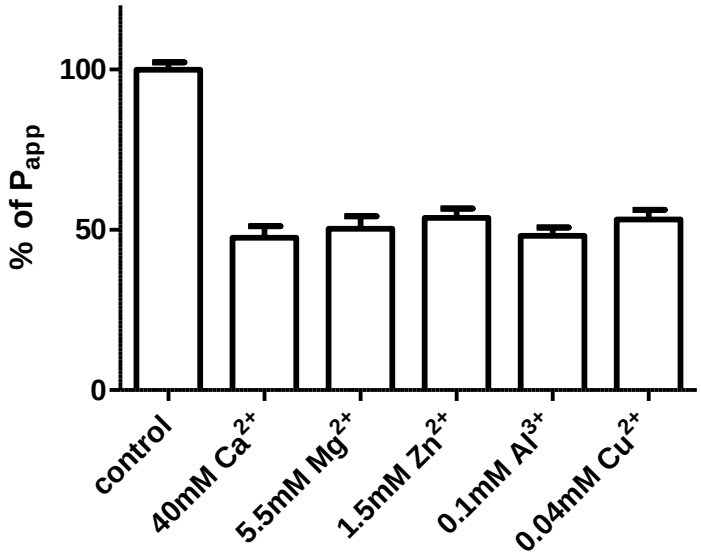
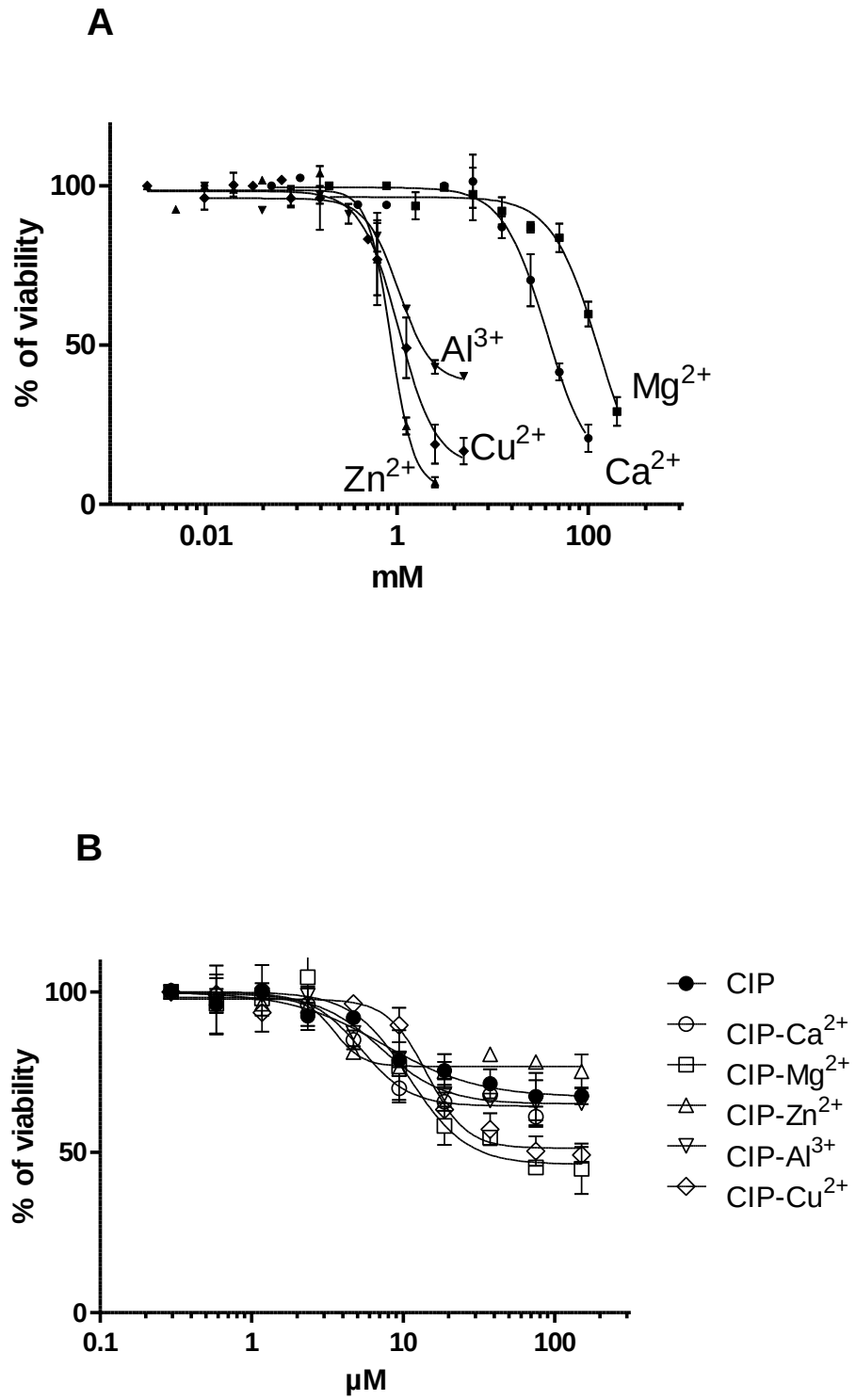


Figure 3. Papp of CIP in conditions of 80% complexation. 50 µM of CIP were incubated with adequate concentrations of Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} or Cu^{2+} ions to achieve a proportion of 80% of CIP:metal cation complexation in solution. Data are expressed as the percentage of the control Papp for CIP (means ± S.E.M., n= 8 to 12). Each condition was significantly different from control using one-way ANOVA and Bonferroni's post hoc test ($P<0.001$).



499

500 **Figure 4. Toxicity of CIP, ions or CIP:ions complexes on Calu-3 cells.** Cells were incubated for 24 h
501 with different concentrations of cations (A) and different concentrations of CIP in the presence of
502 cations (B). Cytotoxicity was evaluated with MTS test kit. 100% correspond to the control condition
503 where cells were cultured for 24 h without CIP and/or cations. Data are expressed as mean ± S.E.M.
504 (n=3).

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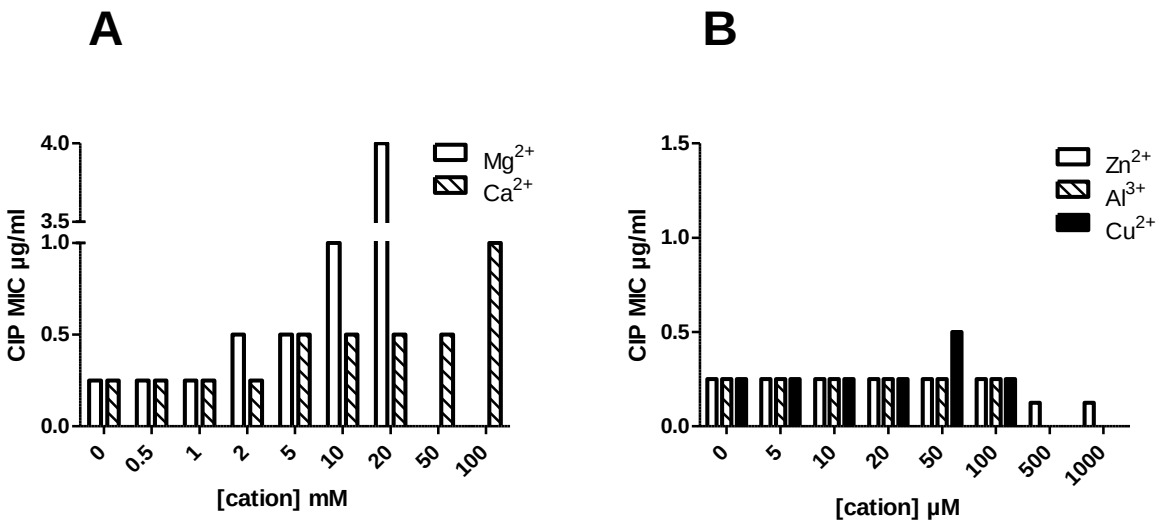

Figure 5. CIP apparent MIC against *P. aeruginosa* in Mueller-Hinton broth II in the presence of increasing concentrations of Ca^{2+}, Mg^{2+} (A), Zn^{2+}, Al^{3+} or Cu^{2+} (B). MICs represent non-continuous data and are accurate to only plus or minus one two-fold dilution.

Appendix A.

For 1:1 complex (CIP:I) between ciprofloxacin (CIP) and a metal ion (I), the equilibrium, apparent binding constant K and mass balance equations are expressed in eqn (1)-(4).

$$CIP + I \leftrightarrows CIP{:}I \quad (1)$$

$$\mathrm{K} = \frac{[CIP{:}I]}{[CIP][I]} \quad (2)$$

$$[CIP]\mathrm{t} = [CIP] + [CIP{:}I] \quad (3)$$

$$[I]\mathrm{t} = [I] + [CIP{:}I] \quad (4)$$

Where [CIP]t and [I]t are the the concentrations of the total CIP and metal ions and [CIP] and [I] are the concentrations of the free CIP and metal ions. [CIP:I] is the concentration of the complexe. Rearranging eqn (2) with eqn (3) and (4) yields to the quadratic equation (5) from which the eqn (6) is a solution.

$$[CIP]^2 + \left([I]\mathrm{t} - [CIP]\mathrm{t} + \frac{1}{\mathrm{K}}\right)[CIP] - \frac{[CIP]\mathrm{t}}{\mathrm{K}} = 0 \quad (5)$$

$$[CIP] = \frac{-\left([I]\mathrm{t} - [CIP]\mathrm{t} + \frac{1}{\mathrm{K}}\right) + \sqrt{\left([I]\mathrm{t} - [CIP]\mathrm{t} + \frac{1}{\mathrm{K}}\right)^2 + \frac{4[CIP]\mathrm{t}}{\mathrm{K}}}}{2} \quad (6)$$

The observed fluorescence (Ft) is the sum of the CIP and CIP:I complex fluorescences (FCIP and FCIP:I), considering the ions are not fluorescent:

$$Ft = FCIP + FCIP{:}I = \delta\mathrm{CIP}[CIP] + \delta\mathrm{CIP{:}I}[CIP{:}I] \quad (7)$$

where δCIP and δCIP:I are the proportionality coefficients between concentration and fluorescence for the CIP and the complex respectively. Rearranging eqn (7) with eqn (3) and into eqn (6) gives the final 1:1 binding isotherm (eqn 8).

$$Ft = (\delta\mathrm{CIP} - \delta\mathrm{CIP{:}\,I)}\left\{\frac{\left([CIP]\mathrm{t}-[I]\mathrm{t}-\frac{1}{\mathrm{K}}\right)+\sqrt{\left([I]\mathrm{t}-[CIP]\mathrm{t}+\frac{1}{\mathrm{K}}\right)^2+\frac{4[CIP]\mathrm{t}}{\mathrm{K}}}}{2}\right\} + \delta\mathrm{CIP{:}\,I}[CIP]\mathrm{t} \quad (8)$$

For 2:1 complex (CIP2:I) between ciprofloxacin (CIP) and a metal ion (I), the equilibriums, binding constants K1 and K2 and mass balance equations are expressed in eqn (9)-(14). For eqn (14), the assumption that the initial concentration of ions ([I]t) is much higher than the initial concentration of CIP ([CIP]t) leads to a simplified equation. Experimentally, ion concentrations were always at least 5 times higher than CIP.

CIP + I ⇆ CIP:I (9)

CIP:I + CIP ⇆ CIP2:I (10)

$$\mathrm{K1} = \frac{[CIP{:}I]}{[CIP][I]} \quad (11)$$

$$\mathrm{K2} = \frac{[CIP2{:}I]}{[CIP{:}I][CIP]} \quad (12)$$

$$[CIP]\mathrm{t} = [CIP] + [CIP{:}\,I] + 2[CIP2{:}\,I] \quad (13)$$

$$[I]\mathrm{t} = [I] + [CIP{:}\,I] + [CIP2{:}\,I] \approx [I] \quad (14)$$

Rearranging eqn (11) and (12) with eqn (13) and (14) yields to the quadratic equation (15) from which the eqn (16) is a solution.

$$[CIP]^2 + \left(\frac{1+\mathrm{K1}[I]\mathrm{t}}{2\mathrm{K1K2}[I]\mathrm{t}}\right)[CIP] - \frac{[CIP]t}{2\mathrm{K1K2}[I]\mathrm{t}} = 0 \quad (15)$$

$$[CIP] = \frac{-\left(\frac{1+\mathrm{K1}[I]\mathrm{t}}{2\mathrm{K1K2}[I]\mathrm{t}}\right)+\sqrt{\left(\frac{1+\mathrm{K1}[I]\mathrm{t}}{2\mathrm{K1K2}[I]\mathrm{t}}\right)^2+\frac{2[CIP]t}{\mathrm{K1K2}[I]\mathrm{t}}}}{2} \quad (16)$$

553 The observed fluorescence (Ft) is the sum of the CIP, CIP:I and CIP2:I complex fluorescences

554 (FCIP, FCIP:I and FCIP2:I), considering that the ions are not fluorescent:

555 $$Ft = FCIP + FCIP{:}I + FCIP2{:}I = \delta\mathrm{CIP}[CIP] + \delta\mathrm{CIP{:}I}[CIP{:}I] + \delta\mathrm{CIP2{:}I}[CIP2{:}I] \quad (17)$$

556 where δCIP, δCIP:I and δCIP2:I are the proportionality coefficients between concentration

557 and fluorescence for the CIP and the complexes. Rearranging eqn (17) with eqn (11) and (12)

558 gives the final 2:1 binding isotherm (eqn 18) together with eqn (16).

559 $$Ft = (\delta\mathrm{CIP} + \delta\mathrm{CIP{:}I\ K1}\ [I]t)[CIP] + \delta\mathrm{CIP2{:}I\ K1\ K2}\ [I]t\ [CIP]^2 \quad (18)$$

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