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ORIGINAL PAPER

Fluorescence Studies of gold(III)-Norfloxacin Complexes in Aqueous Solutions

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Abstract Formation of gold(III) complexes with the synthetic antibiotic norfloxacin (NF) was investigated in aqueous solution at pH 4.0, 7.5 and 10.6, with the ligand in cationic, zwitterionic and anionic forms, respectively. UV-Visible spectroscopy, steady state and time-resolved fluorometry were used to characterize the complexes. Binding sites, association constants and fluorescence lifetimes of the complexes were obtained. Au^{3+} binding to zwitterionic NF produced a fluorescence decrease and a small red shift. Fluorescence changes as a function of Au^{3+} concentration were fitted using a one-site binding model and the association constant was obtained, $K_b^{zw} = 1.7 \times 10^5\ M^{-1}$. The association of Au^{3+} with cationic NF was much weaker, the obtained binding constant being $K_b^{cat} = 2.4 \times 10^3\ M^{-1}$. The Au^{3+} binding site for these species involves the carboxyl group, in agreement with a much stronger association of the cation with the carboxylate anion than with the neutral acid. Association of Au^{3+} with nonfluorescent anionic NF presented a clear evidence of two binding sites. The highest affinity site is the unprotonated piperazinyl group with $K_b^{pip} \geq 5 \times 10^7\ M^{-1}$, and the low affinity site includes the carboxylate anion. The results point out to important pH dependent differences in complex formation between transition metal ions and fluoroquinolones, leading to different binding sites and association constants that change by several orders of magnitude.

Keywords Fluoroquinolones · Norfloxacin · Gold(III) complexes · Fluorescence · Binding constants · Binding sites

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Introduction

Fluoroquinolones are broad-spectrum widely used synthetic antibiotics, and norfloxacin (NF) is a typical member of this class of antibacterial agents. The behavior of fluoroquinolones is significantly influenced by their physicochemical properties, particularly by their ionization degree expressed by the pK_a values [1]. The presence of multiple proton binding sites in fluoroquinolones makes the pattern of acid–base equilibrium rather complex. It has been shown by several techniques that the carboxylate group and the 4′-amine of the piperazine ring are the most significant proton binding sites from the biological point of view (pK_a in the range of 5–9) [2]. Acid–base equilibrium of several fluoroquinolones has been reported in literature [3]. These data are important for a detailed understanding of absorption, transport and receptor binding of these drugs at the molecular level. The structure of norfloxacin and the interconversion between the three relevant forms in aqueous solution ($4<pH<11$) are presented in Fig. 1. At neutral pH the zwitterionic form bearing protonated N4′ and dissociated 3-carboxyl group is the largely prevailing species. The cationic and the anionic forms predominate in acidic and in basic solutions, respectively. The acid–base equilibrium constants of NF is about 6.3 for the carboxylate at position 3 of the quinolone heterocycle, and about 8.6 for

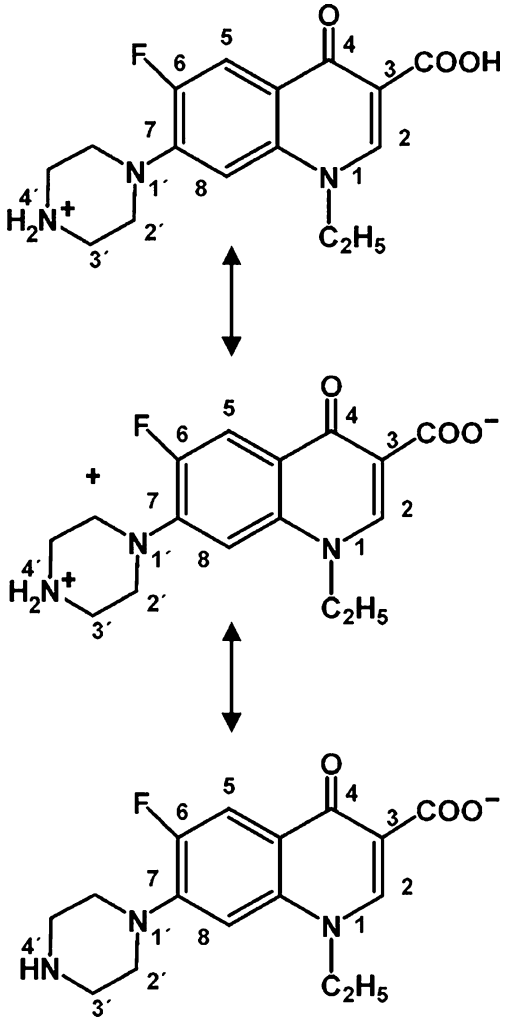

Fig. 1 Molecular structure of norfloxacin and the acid–base equilibrium for 4<pH<11

the 4′ amine of the piperazinyl ring (values are average from several published results) [3, 4]. Hence, cationic NF molecules predominate at pH 4, zwitterionic at pH 7.5, and anionic at pH~10. The structure that probably prevails in apolar solvents is the neutral form with undissociated carboxyl substituent and unprotonated N4′.

Several fluoroquinolones are fluorescent, including norfloxacin, and their fluorescence properties, such as quantum yields, Stokes shifts and lifetimes, reflect the different protonation states of the molecule and the local molecular environment determined by the solvent and biologically relevant ions or molecules [5, 6].

Fluoroquinolones generally form complexes with several metal ions, which greatly influence their antibacterial activity [7]. Usually, the binding sites of metal ions with quinolones clearly involve the 4-oxo and 3-carboxyl oxygen atoms, but it was found that Ag^+ and Au^{3+} ions coordinate with the norfloxacin ligand through the N atom of the piperazinyl ring [8]. Vieira et al. also reported the synthesis and characterization of platinum(II) complexes with ciprofloxacin, levofloxacin, ofloxacin, sparfloxacin, and gatifloxacin, and found complexes coordinated through the nitrogen atoms of the piperazine ring [9].

Recently, research on gold(III) compounds as anticancer drug candidates has been increasing because gold(III) complexes display structural and reactivity features similar to those of platinum(II) complexes, such as a d^8 electronic configuration and strong preference for square-planar geometry [10]. It has been shown that a group of structurally diverse gold compounds are highly cytotoxic toward several tumor cell lines through a variety of biochemical mechanisms [11]. In general, gold(III) compounds possess a considerable potential as cytotoxic and antitumor agents. A comparative analysis of a series of structurally related metal complexes established that the presence of a gold(III) center typically results in the appearance of a more pronounced cytotoxic behavior [12]. Several studies indicated that most of the newly synthesized gold(III) species possess sufficient stability in solution and show pronounced antiproliferative effects in vitro [13].

In this work we studied the interaction of norfloxacin with gold(III) using steady-state and time-resolved fluorescence. At first, Au^{3+}(NF) complex was synthesized as a powder and its fluorescence was studied in aqueous solution as a function of pH. It was observed that Au^{3+} produces pH dependent spectral modifications, attributed to different interactions with the distinct ionic species. Hence, NF solutions at pH 4.0, 7.5 and 10.6 were titrated with $HAuCl_4$ to investigate the association of Au^{3+} with cationic, zwitterionic and anionic NF molecules, respectively. The fluorescence spectral modifications were used to calculate Au^{3+} association constants and to propose association sites.

Experimental

Reagents and Solutions

Norfloxacin (NF) and $HAuCl_4$ were purchased from Sigma-Aldrich (St. Louis, MO). Buffers were prepared from reagent grade chemicals in ultrapure deionized water. The buffer consisting of a mixture of borate, phosphate and citrate was chosen to cover the whole pH range from 4 to 11. The stock solution for the buffer contains 33 mM citric acid, 50 mM phosphoric acid, 50 mM boric acid and 330 mM NaOH. The buffer (20 mM Na) was obtained by a 1:15 dilution, adjusting the pH with a 1 M HCl solution.

Synthesis of the Complex

Complex $[AuCl_2(NF)]Cl$ was prepared as follows: an appropriate quantity of NF (0.25 mmol) was dissolved in

methanol (40 mL), which was then added to a solution of $HAuCl_4$ (0.25 mmol) in methanol (5 mL). Immediately after the addition of the ligand, a precipitate formed. The reaction mixture remained stirring at room temperature for 24 h. The solids obtained were filtered under vacuum, washed with ether, and dried. Elemental analysis was performed on a CE Instruments CHN-O EA 1110. The results (Found: C, 29.02; H, 3.14; N, 6.16%) suggest the presence of one hydration water molecule (Calc. for $[AuCl_2(NF)]Cl$: C, 30.9; H, 2.09; N, 6.70%. Calc. for $[AuCl_2(NF)]Cl\ H_2O$: C, 30.0; H, 3.15; N, 6.56%.). The complex is water soluble at concentrations in the mM range.

Instrumentation and Methods

The pH was measured using a Cole-Parmer Chemcadet 5986–25 pH meter with an Ag/AgCl semimicro combination electrode. UV-Visible absorption spectra were measured using a diode array spectrophotometer model 8452A (Agilent). Fluorescence spectra were recorded on a PTI QM1 spectrofluorometer (Photon Technology International, Birmingham, NJ). Fluorescence lifetimes were measured using an IBH-Horiba-Jobin Yvon TCSPC system. The light source used for excitation was a 330 nm nanoLED N-16, 1.0 ns nominal pulse duration, 1 MHz repetition rate. Computer programs supplied by Horiba Jobin Ivon IBH which perform reconvolution fits were employed for processing the time decay curves. Fluorescence decay analysis of each titration set was performed with multi-exponential expressions using global analysis ($0.9<\chi^2<1.2$).

The concentration of norfloxacin was 10 μM in all the experiments. The solutions were prepared from a 1 mM NF stock solution in ethanol. The fluorescence study of the acid–base equilibrium was performed using different samples for each pH. Titrations of the NF solutions were carried out using freshly prepared 1 mM $HAuCl_4$ solutions. The pH variations were less than 0.1 even for the highest $HAuCl_4$ concentrations used.

Results and Discussion

Absorption and Fluorescence of NF and Au^{3+}(NF) as a Function of pH

Absorption and fluorescence of NF have already been studied as a function of the pH [3, 6]. In this work, the spectra of NF and Au^{3+}(NF) in aqueous solutions were obtained and compared in a pH range from 4 to 11. The absorption spectra of NF and Au^{3+}(NF) were similar over the whole pH range, except for small differences in intensity (not shown). Figure 2a shows the fluorescence

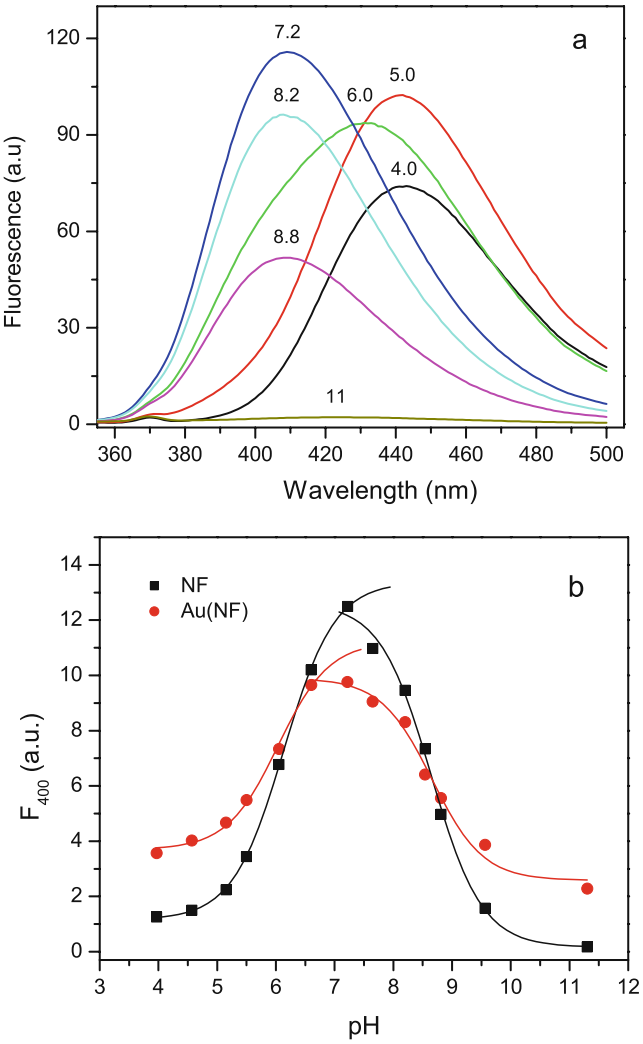

Fig. 2 (**a**) pH dependent fluorescence spectra of norfloxacin. The numbers above each peak correspond to the pH. (**b**) Fluorescence intensity of NF and Au^{3+}(NF) at 400 nm. The lines are least squares fits using Eq. (1) with pK_1=6.1 for NF and 6.0 for Au(NF), and pK_2=8.6 for both

spectra of NF at different pH. It was observed that the spectra of Au^{3+}(NF) and NF presented pH dependent differences. The fluorescence intensity at 400 nm (maximum variation) was plotted as a function of the pH (Fig. 2b). The data were fitted using the Henderson-Hasselbalch Eq. (1) with the fitting parameters being the pK_a and the fluorescence intensities F^A and F^B of the corresponding acidic and basic forms, respectively.

$$F = \frac{F^A 10^{pK_a} + F^B 10^{pH}}{10^{pK_a} + 10^{pH}} \quad (1)$$

The plots show the same general behavior of the ligand and the complex, including the pK_a values for

carboxyl and amine. The intensity, however, changes differently. This result suggests that the complex is partially dissociated at the low concentrations used in fluorescence measurements. It also suggests that the association constant of Au^{3+} with NF is pH dependent. For this reason titrations of NF with Au^{3+} at pH 4.0, 7.5 and 10.6 were carried out and fluorescence changes were used to obtain binding properties with the different NF species.

Titration of Norfloxacin with Au^{3+}

Solutions of cationic, zwitterionic and anionic norfloxacin, at pH 4.0, 7.5 and 10.6, respectively, were titrated with $HAuCl_4$. The UV-Visible absorption spectra, steady-state fluorescence emission and fluorescence decays were measured.

UV–Vis Absorption

Figure 3 presents the UV–vis absorption spectra of NF titrated with different amounts of $HAuCl_4$. In the absence of gold, the spectrum has a band at 285 nm (band I) and a less intense poorly resolved double band at 322–335 nm (band II). The spectrum of $HAuCl_4$ is also presented in Fig. 3. It is observed that the resulting absorption spectra are very similar to a superposition of NF and $HAuCl_4$ spectra, indicating that UV–vis absorption of NF undergoes little modification upon interaction with Au^{3+}. At pH 7.5 and 4.0, there is no absorption in the visible region (Fig. 3a and b). At alkaline pH, however, at Au concentrations greater than ~30 μM the solution becomes slightly blue, and a wide band centered at ~550 nm develops (Fig. 3c). This is due to the surface plasmon resonance band characteristic of aggregated gold nanoparticles (see, for example, [14]). Generally, gold nanoparticles are produced in a liquid by reduction of chloroauric acid ($HAuCl_4$). The diameter of gold nanoparticles and/or aggregates determines the wavelengths of light absorbed, and, therefore, the color of nanoparticle solutions. The color also depends on the pH and ionic strength of the solution. The interaction of gold nanoparticles with NF, however, will not be treated here.

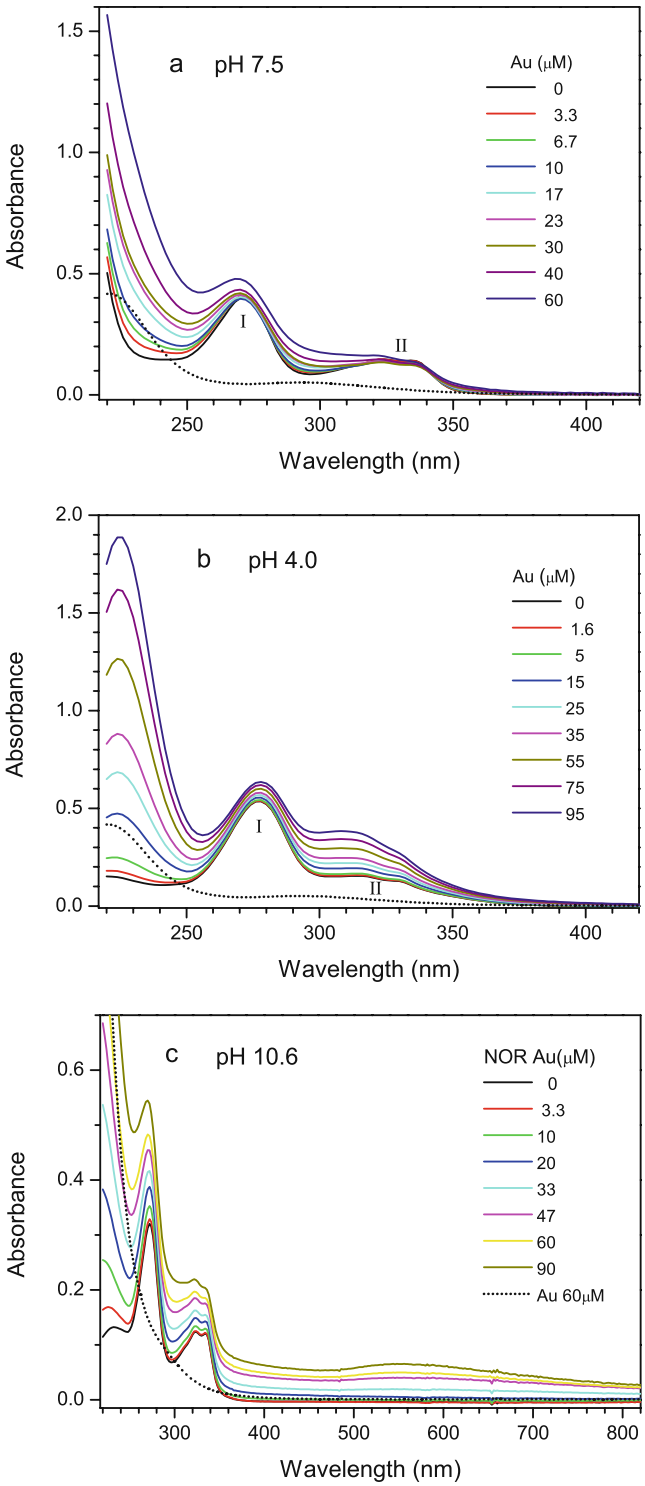

Fig. 3 UV-visible absorption spectra of norfloxacin (10 μM) titrated with $HAuCl_4$ at (**a**) pH 7.5, (**b**) pH 4.0 and (**c**) pH 10.6. The spectrum of $HAuCl_4$ appears in the three figures as dotted lines (20 μM in **a** and **b**; 60 μM in **c**)

Steady-State Fluorescence of Norfloxacin

Zwitterionic norfloxacin (pH 7.5) has a fluorescence emission peaked at 407 nm with a quantum yield of 0.10 [5]. Figure 4 presents the effect of $HAuCl_4$ on the fluorescence emission of zwitterionic NF. It shows that increasing Au concentrations cause a fluorescence decrease combined with a small red shift from 407 to 412 nm at 60 μM Au. The fluorescence change at 407 nm, (F_0–F), was plotted as a function of Au^{3+} concentration (inset of

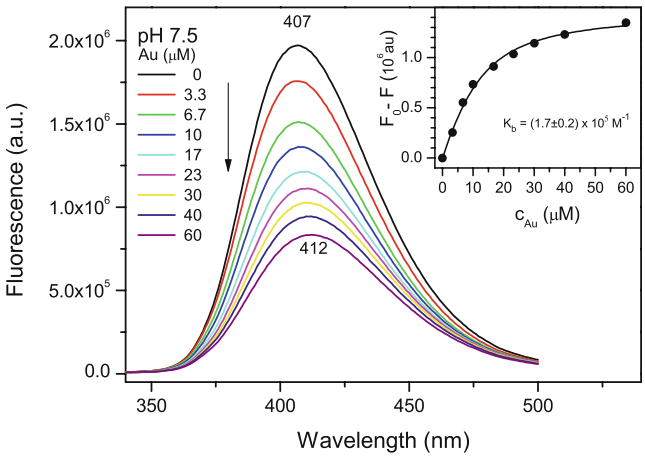

Fig. 4 Fluorescence spectra of norfloxacin (10 μM) at pH 7.5 titrated with $HAuCl_4$. The arrow represents increasing Au concentrations. Inset: fluorescence changes at 407 nm as a function of Au concentration, and the one site binding (Eq. 2) least squares fit with $K_b=1.7\ (\pm 0.2)\times 10^5\ M^1$

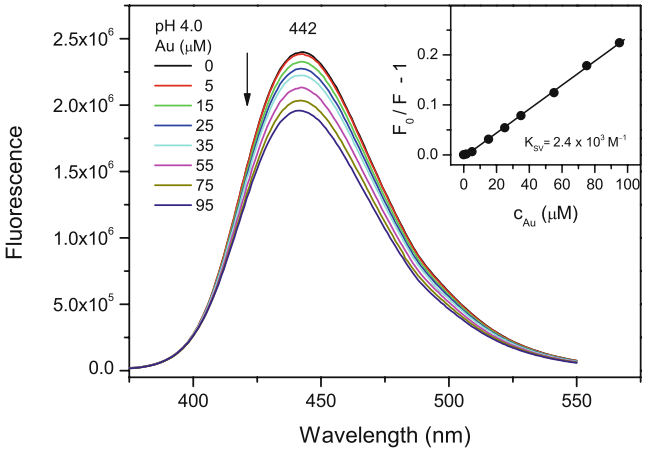

Fig. 5 Fluorescence spectra of norfloxacin (10 μM) at pH 4.0 titrated with $HAuCl_4$. The arrow represents increasing Au concentrations. Inset: Stern-Volmer plot for the fluorescence at 442 nm, and linear fit with the Stern-Volmer constant $K_{SV}=2.4\times 10^3\ M^1$ (Eq. 3)

Fig. 4) and the data were fitted to the exact expression for the one-site binding model [15]:

$$F_0 - F = \frac{F_0 - F_\infty}{2} \left\{ \left(1 + \frac{c_M}{c_L} + \frac{1}{K_b c_L}\right) - \left[\left(1 + \frac{c_M}{c_L} + \frac{1}{K_b c_L}\right)^2 - 4\frac{c_M}{c_L} \right]^{\frac{1}{2}} \right\} \quad (2)$$

where K_b is the binding constant, c_L and c_M are the total ligand (NF) and metal (Au) concentrations, respectively; F_0, F and F_∞ are the fluorescence intensity of the ligand in the absence of metal, at a given metal concentration, and at $c_M >> c_L$, respectively. The nonlinear least squares fit gave $K_b^{zw} = 1.7\times 10^5\ M^{-1}$ (solid line in the inset of Fig. 4). It is interesting to compare the Au^{3+} and Cu^{2+} binding constants with zwitterionic NF. Using fluorescence quenching, the Cu^{2+} binding constant was found as $1.3\times 10^6\ M^{-1}$ at pH 7.4 [16], which is one order of magnitude higher than that of Au^{3+}.

Cationic norfloxacin (pH 4.0) has a fluorescence emission peak at 442 nm with a quantum yield of 0.12 [5]. Titration of NF with $HAuCl_4$ at pH 4.0 produced a much smaller fluorescence decrease than at pH 7.5, and the peak did not shift (Fig. 5). It is impossible to estimate F_∞ from the data in Fig. 5, but it is possible to estimate the binding constant at pH 4.0 using the Stern-Volmer Eq. (3) to fit the quenching data in Stern-Volmer plots.

$$\frac{F_0}{F} = 1 + K_{SV}\,[Q] \quad (3)$$

In static fluorescence quenching, the Stern-Volmer quenching constant K_{SV} is the actual binding constant K_b^{cat} [17]. The inset in Fig. 5 shows the Stern-Volmer plot for NF at pH 4.0, and the linear fit using Eq. (3) gives a K_{SV} of $2.4\times 10^3\ M^{-1}$. It is worth noting that Eq. (2) also fits the data with $K_b^{cat} = 2.4\times 10^3\ M^{-1}$, assuming complete quenching at high Au^{3+} concentrations, i.e., $F_\infty=0$.

The Au^{3+} binding constant with cationic NF (pH 4.0) is, therefore, about 70 times smaller than with zwitterionic NF (pH 7.5). Since the N4′ amine is protonated both at pH 4.0 and 7.5, it cannot be responsible for the different Au^{3+} binding constants. This suggests that Au^{3+} coordinates with the carboxylate ion of zwitterionic NF ligand to form the complex. The association constant with this site is 1.7 $(\pm 0.2)\times 10^5\ M^{-1}$.

Finally, NF was titrated with $HAuCl_4$ at pH 10.6. The fluorescence results are presented in Fig. 6. NF at pH 10.6 is almost non-fluorescent in the absence of Au^{3+} ions. The fluorescence quantum yield of NF is negligible when amine N4′ is unprotonated, and the small fluorescence intensity is probably due to the small fraction of molecules with cationic amine at pH 10.6 (~1%).

Three phases were detected with increasing Au^{3+} concentration (Fig. 6). At concentrations smaller than NF concentration, a fluorescence peak at 420 nm increases almost linearly (a). The second phase is characterized by a fluorescence decrease combined with a blue shift to 407 nm (b). Finally, at Au concentrations greater than 50 μM a small increase is noticed accompanied by an additional blue shift (c). Figure 6d is a plot of the fluorescence intensity versus Au^{3+} concentration, which clearly shows the three phases.

Figure 7 represents the first phase of the plot in Fig. 6d and the curve corresponding to the one-site binding Eq. (2) with $K_b=5\times 10^7\ M^{-1}$. From the increase in fluorescence yield we conclude that the highest affinity binding site for

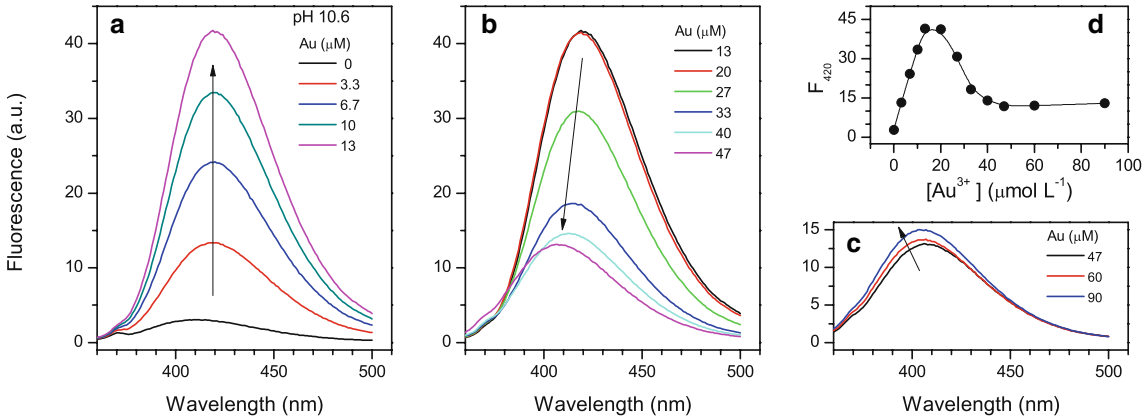

Fig. 6 Fluorescence spectra of NF at pH 10.6 and different $HAuCl_4$ concentrations: **a** from 0 to 13 μM; **b** from 13 to 47 μM; **c** from 47 to 90 μM. The arrows indicate increasing Au^{3+} concentrations. **d** is a plot of the fluorescence intensity at 420 nm versus Au^{3+} concentration

Au^{3+} involves the unprotonated N4′ amine of the piperazine ring. As long as the unprotonated N4′ of NF prevails in apolar solvents, it can be concluded that Au^{3+} binds to the piperazine ring in most organic solvents, in agreement with the site proposed in [8]. NF recovers in part the fluorescence yield upon Au^{3+} binding at this position. The quantum yield of the complex is however about five times smaller than at pH 7.5.

It is important to note that when the affinity is large, i.e. $K_b.c_L>>1$, the K_b determined using Eq. (2) will be inaccurate because the titration curve will consist of two portions of straight lines [15]. This is the case of the data in Fig. 7, then the high value of K_b has to be considered only a lower limit for the binding constant of Au^{3+} with the unprotonated piperazinyl moiety of NF.

The second phase observed in Fig. 6b associated with a fluorescence decrease agrees with the binding of a second Au^{3+} ion to the site containing the carboxylate anion. The fluorescence decrease occurs at an Au^{3+} concentration range that matches the binding to this site observed at pH 7.5.

The last phase occurs after binding of two Au^{3+} ions with one NF molecule. The fluorescence modifications are very small. This phase is out of the scope of this work because it concerns the formation of gold nanoparticles (see Fig. 3c).

Fluorescence Decay of Norfloxacin

Fluorescence intensity decays of norfloxacin were measured using the time domain method. Intensity decays at pH 7.5, 4.0, and 10.6 are presented in Fig. 8. The lifetimes obtained from exponential fits are presented in Table 1. The decay at pH 7.5 was fitted using a single exponential with a time constant $\tau_1=\tau_{zw}=1.15$ ns. The decay at pH 4.0 was fitted with a two-exponential expression. The main lifetime $\tau_1=\tau_{cat}=1.58$ ns (fractional contribution, $f_{cat}=91\%$) is due

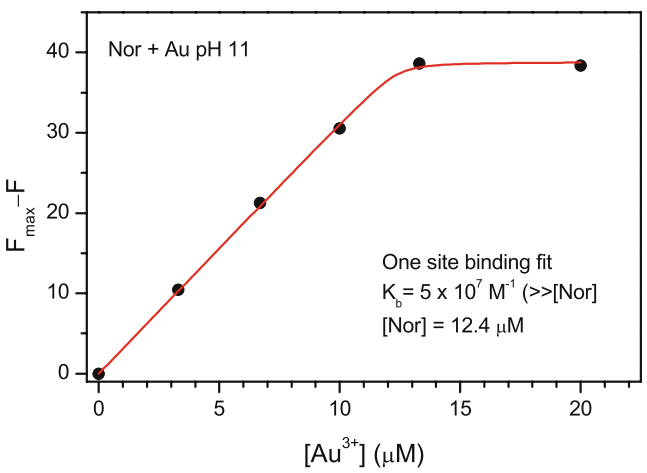

Fig. 7 Fluorescence change of NF at 420 nm, pH 10.6, upon $HAuCl_4$ titration. The line represents the one-site binding least squares fit using Eq. (2) with $K_b=5\times10^7$ M^1 and [NF]=12.4 μM

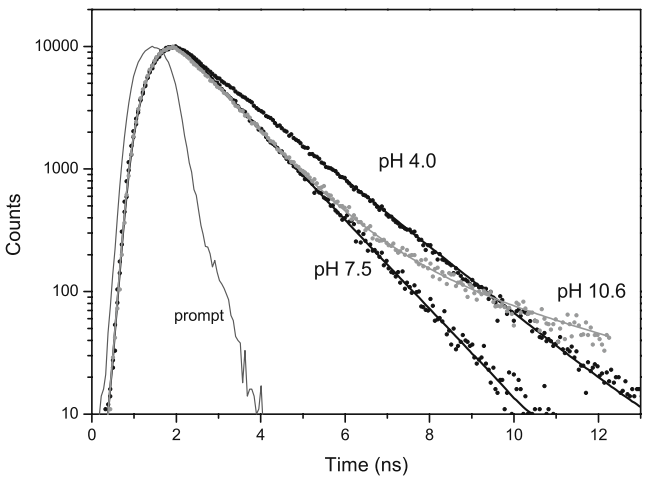

Fig. 8 Intensity decays of norfloxacin at pH 7.5, 4.0, and 10.6 (TCSPC data). Excitation at 330 nm. Emission at 407 nm (pH 7.5 and 10.6), and 440 nm (pH 4.0)

Table 1 Fluorescence lifetimes τ_1 and τ_2 of norfloxacin. Factors f_1 nd f_2 are the fractional contributions at the emission wavelength

pH	τ_1 (ns)	f_1	τ_2 (ns)	f_2
4.0	1.58	91	0.55	9
7.5	1.15	100		
10.6	1.15	90	4.6	10

to cationic NF. It is possible that the shortest lifetime (0.55 ns) is due to a small proportion of another species, since two other nitrogen atoms of NF can protonate at this pH. Finally, at pH 10.6, a two-exponential expression was also necessary to fit the decay. The main lifetime was τ_{zw}=1.15 ns, due to the small amount of zwitterionic NF present at this pH, given that the anionic NF has a negligible fluorescence. The other lifetime (4.6 ns, with fractional contribution of 10%) becomes visible because of the long acquisition time due to the very low fluorescence at this pH.

The effect of Au^{3+} on NF fluorescence decay was also pH dependent. At pH 7.5 a global analysis was performed using a two exponential expression with a fixed lifetime τ_{zw}= 1.15 ns. A second lifetime τ_2=2.42 ns appears, whose contribution increases with increasing Au^{3+} concentration (Table 2). This lifetime is therefore due to the complex formed by Au^{3+} bound at the carboxylate of zwitterionic NF.

The effect of Au^{3+} on NF fluorescence decay at pH 4.0 was also analyzed using global analysis but a three exponential expression was necessary: two lifetimes were fixed, τ_1=1.58 ns and τ_2=0.55 ns (which appeared in the absence of Au^{3+}), and τ_3=6.07 ns fitted the decays (Table 3). The contribution of this third lifetime is very small, but it increases with increasing Au^{3+} concentration.

The fluorescence decays of Au^{3+}-titrated NF at pH 10.6 corresponding to phase one (Fig. 6a, binding to the N4′ amine) are all very similar. A typical decay curve is presented in Fig. 9 together with the curves for pure NF at pH 4.0 and 7.5. A global fit omitting the prompt gave a

Table 2 Fluorescence lifetimes of Au^{3+}- titrated norfloxacin at pH 7.5 obtained by global analysis. τ_1=1.15 ns fixed; τ_2=2.53 ns. α_1 and α_2 are the pré exponential factors; f_1 and f_2 are the fractional contributions at the emission wavelength

[Au]	α_1	α_2	f_1(%)	f_2(%)
0	0.995	0.005	99.0	1.0
5	0.991	0.009	98.0	2.0
10	0.986	0.014	97.0	3.0
20	0.981	0.019	95.8	4.2
30	0.971	0.029	93.9	6.1
50	0.964	0.036	92.5	7.6
100	0.941	0.059	87.8	12.2

Table 3 Fluorescence lifetimes of Au^{3+}- titrated norfloxacin at pH 4.0 obtained by global analysis. τ_1=1.58 ns fixed; τ_2=0.55 ns fixed, τ_3= 6.07 ns. α_1, α_2 and α_3 are the normalized pré exponential factors; f_1, f_2, and f_3 are the fractional contributions at the emission wavelength

[Au]	α_1	α_2	α_3	f_1	f_2	f_3
0	0.885	0.114	0.000	96.0	3.8	0.2
1.7	0.866	0.132	0.001	94.5	5.0	0.5
4.5	0.866	0.132	0.002	94.2	4.8	1.0
15	0.864	0.133	0.003	93.8	5.0	1.2
25	0.864	0.132	0.005	93.2	4.9	1.9
35	0.860	0.133	0.007	92.2	5.0	2.8
45	0.859	0.132	0.009	91.6	4.8	3.6
65	0.857	0.132	0.011	90.5	4.9	4.6
85	0.841	0.141	0.018	87.9	5.0	7.1

single lifetime τ_{AuNF}-=1.52 ns. Thus, this is the lifetime of the complex Au^{3+}(NF) with Au bound at the piperazinyl ring. This lifetime does not change at higher Au^{3+} concentrations (second phase) suggesting static fluorescence quenching upon binding of the second Au^{3+} ion at the site involving the 3-carboxyl and 4-oxo oxygen atoms.

Conclusion

Luminescence properties of norfloxacin were used to study the association of Au^{3+} ions with the cationic, zwitterionic and anionic forms of the drug. Au^{3+} binding to zwitterionic NF produced a fluorescence decrease and a small red shift. The modifications of the fluorescence intensity were fitted with the exact expression for the one-site binding model and the association constant was obtained: $K_b^{zw} = 1.7 \times 10^5\ M^{-1}$. The fluorescence lifetime that was τ_{zw}=1.15 ns for the

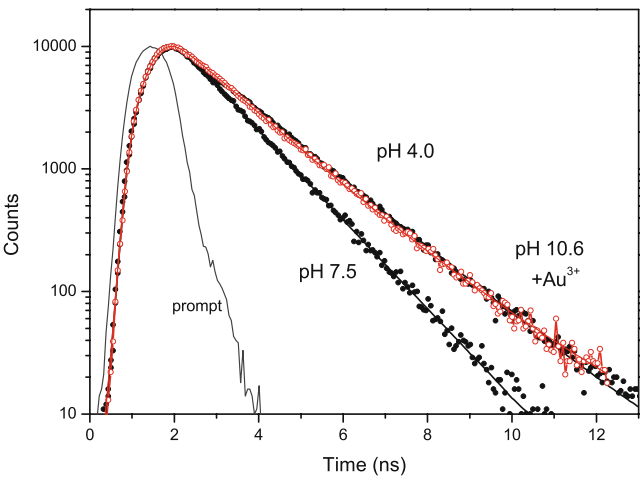

Fig. 9 Decay of Au^{3+}-norfloxacin fluorescence at pH 10.6 (empty dots connected by straight line) compared with the decays of pure NF at pH 7.5, 4.0. [Au^{3+}]:[NF]=1.3. Excitation at 330 nm; emission at 420 nm (pH 10.6), 407 nm (pH 7.5), and 440 nm (pH 4.0)

zwitterionic NF became τ_2=2.42 ns when the complex was formed. The association of Au^{3+} with cationic NF was much weaker. The binding constant obtained from the fluorescence changes was $K_b^{cat} = 2.4 \times 10^3\ M^{-1}$. The results revealed that Au^{3+} binding site to zwitterionic NF involves the carboxyl groups, in agreement with the site proposed by many authors for several metal ions that bind to the carbonyl oxygen and to one oxygen of the 3-carboxylic group [7]. The results also showed that there is a much stronger association of Au^{3+} with the carboxylate anion than with the neutral acid.

Association of Au^{3+} with the nonfluorescent anionic NF, pH 10.6, presented a clear evidence of two binding sites. The fluorescence increase at low Au^{3+} concentrations suggested that the highest affinity site is the unprotonated piperazinyl group. This agrees with authors that showed that Ag^{+} and Au^{3+} [8] and Pt^{2+} ions [9] coordinate with norfloxacin and other fluoroquinolones through the N atom of the piperazinyl ring. Binding of Au^{3+} to this site would restrict the excited state electron transfer that render the quinolone ring nonfluorescent, so that fluorescence is partially recovered upon Au^{3+} binding. The binding constant to this site is very high, and only a lower limit was determined using the fluorescence changes, $K_b^{pip} \geq 5 \times 10^7\ M^{-1}$. The fluorescence decay measurements gave a lifetime of 1.52 ns for the complex. Binding of a second Au^{3+} quenches the Au^{3+}(NF) fluorescence. The second binding site of anionic NF would be the same as the single binding site of zwitterionic NF, e.g., the oxygen atoms of the carbonyl and carboxyl groups.

The results imply that the local pH of an environment is very important to determine the association of metal ions with fluoroquinolones. Binding sites are different at different pH, and binding constants change by several orders of magnitude.

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