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Synthesis, characterization and DNA binding of magnesium–ciprofloxacin (cfH) complex $[Mg(cf)_2] \cdot 2.5H_2O$

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Abstract

Interactions of the tested systems (title compound $[Mg(cf)_2] \cdot 2.5H_2O$ (**1**), ciprofloxacin (cfH) and ciprofloxacin in the mixture with $MgCl_2$), with single and double stranded calf thymus DNA, poly[d(AT)] · poly[d(AT)] and poly[d(GC)] · poly[d(GC)] were studied by UV-spectrophotometric (melting curves) and fluorescence emission measurements. Pronounced quenching of ciprofloxacin's fluorescence intensity has been observed for all the tested compounds after titration with various GC containing DNA molecules. It seems probable that quenching originates in the electron transfer from guanine to the photo-excited fluoroquinolone. The UV-spectrophotometric results obtained for **1** are substantially different from the other solutions and the biggest differences were observed for GC containing DNAs. Solution of **1** provokes a large thermal destabilization of poly[d(GC)] · poly[d(GC)]. This process is irreversible which suggests that the species present in solution of **1** alone inhibit re-annealing by associating irreversibly with the single strands. We have realized that aqueous solutions of **1** are colloidal and we propose that colloidal particles are involved in specific binding to GC containing sequences, most probably in the major groove of DNA.
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Keywords: Magnesium; Ciprofloxacin; DNA; Structure; Spectroscopy

1. Introduction

Quinolones are nowadays clinically the most successful synthetic antibacterial agents [1–3]. Fluoroquinolone member ciprofloxacin (cfH) (Scheme 1) is regularly found among the top 100 most frequently prescribed drugs in North America.

The emergence of drug-resistant bacteria is a growing problem also for quinolones, but maybe a new finding that blocking a protease called LexA could stop bacteria from evolving resistance to some antibiotics (including quinolones) could help to extend their use in the future [4].

Exact mechanism of quinolone action is not yet fully understood and recent review of the most important and sometimes contradictory results was given by Mitscher [1]. However, it is generally accepted that the quinolones target the bacterial enzyme gyrase–DNA complex which is responsible for the supercoiling of bacterial DNA [1].

It is known that nowadays several pharmaceutical companies have left the antibiotic discovery field and are much more interested in the more profitable areas of chronic diseases [5,6]. Therefore it is crucial to understand the molecular mode of action of existing drugs which could help us to exploit them even more efficiently in the unpredictable and never ending battle between bacteria and mankind.

The interactions between metal ions and quinolones have been extensively studied for a long time and a review of metal complexes from this group was given [7]. Generally, quinolones coordinate to the metal as chelates through ring carbonyl and carboxylate oxygen atoms to form discrete molecules. In strongly acidic conditions

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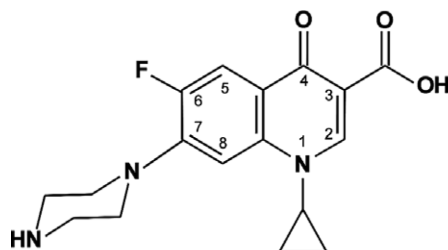

Scheme 1. Formula of ciprofloxacin.

quinolones are protonated and appear as cations in the metal complex compounds. It was also found that in the basic media the quinolones bearing a piperazinyl ring in position 7 (typical representatives ciprofloxacin (cfH), norfloxacin (nfH), pefloxacin), could also form complexes where terminal piperazinyl nitrogen is involved in the coordination to the metal [8–13].

Up to now only few magnesium–quinolone crystal structures have appeared in the literature though it is well known that magnesium ions are important for the activity of these drugs [13–15]. The exact role of magnesium ions in the interaction between quinolone, DNA and DNA–gyrase is also not known yet and several models were proposed to explain the mechanism of action [16–19].

In our previous work we have studied the interaction of cfH with calf thymus DNA, synthetic oligonucleotides and polynucleotides both in the presence and in the absence of metal ions (Cu^{2+}, Mg^{2+}) [20–22]. Our further aim was to prepare new magnesium–quinolone complex(es) and to study their interaction with DNA. Our efforts focused on the complexation in the vicinity of neutral pH that is comparable to the conditions found in the cells. At such pH a 2D molecular square-grid complex $[Mg(cf)_2] \cdot 2.5H_2O$ (**1**) was isolated by a hydrothermal reaction. Just before our paper was submitted we become aware of the work of Xiao et al. [13] who prepared and determined the crystal structures of 14 new metal–ciprofloxacin complexes. Among these also a magnesium complex $[Mg(cf)_2] \cdot 2.5H_2O$ was reported which is analogous to our title complex. In this complex two anionic molecules of ciprofloxacin are coordinated to magnesium ion through ring carbonyl and one of the carboxylate oxygens. The axial positions are occupied by two terminal nitrogen atoms of the piperazinyl residue resulting in the formation of a 2D metal-based molecular square grid. Water molecules are present in the channels that form in the structure. The main difference between crystallographic procedures employed by our group and that of Xiao et al. [13] was the temperature of the measurement. Due to the problem of the water disorder our crystal data were collected at low temperature (150 K), in contrast to Xiao et al. [13] who collected the data at 293 K. Unfortunately free mobility of water molecules in the channels prevents exact determination even at low temperature. Our low temperature data for compound $[Mg(cf)_2] \cdot 2.5H_2O$ have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 297483. Our results were partly reported before [23,24] but there are still some important differences from synthetic and crystallographic view (see Section 3, Supplementary material) which we would like to stress in this paper. From the beginning our main goal was a biological relevance and not a physico-chemical characterization of the magnesium complex. This paper reports the comparison of the interactions between the tested systems (title compound **1**, ciprofloxacin and ciprofloxacin in the mixture with $MgCl_2$), with single and double stranded calf thymus DNA, poly [d(AT)] · poly[d(AT)] and poly[d(GC)] · poly[d(GC)]. Understanding the interactions between studied compounds and DNA may help to elucidate the mechanism of action of this important class of antibacterial agents, and may ultimately lead to the design of better, more potent antibacterial agents.

2. Materials and methods

2.1. General procedures

Infrared spectra (Nujol) were recorded on a Perkin–Elmer FT-1720X spectrometer. Elemental analyzes were performed on a Perkin–Elmer 204C microanalyzer.

2.2. Chemicals

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) (Scheme 1) was purchased from Fluka. The molar extinction coefficient of ciprofloxacin at 275 nm, $\varepsilon_{275} = 35{,}900 \pm 500\ M^{-1}\ cm^{-1}$ in 2% (v/v) dimethyl sulfoxide (DMSO) solution at 25 °C was used [21]. Magnesium chloride hexahydrate was purchased from Sigma Chemical, Ltd. (St. Louis, USA). Both chemicals were of the highest grade commercially available and were used without further purification.

2.3. DNA

Natural genomic DNA (calf thymus DNA) and two synthetic DNA polymers: poly[d(AT)] · poly[d(AT)] and poly[d(GC)] · poly[d(GC)] were purchased from Pharmacia Biotech (Uppsala, Sweden). Before use they were thoroughly dialyzed against corresponding buffer solution. Thermally denatured calf thymus DNA was prepared by heating the sample up to 95 °C and cooling it down to the room temperature. The concentrations of double stranded polynucleotides were determined spectrophotometrically at 25 °C using the following molar extinction coefficients expressed in molar concentration of base pairs: poly[d(AT)] · poly[d(AT)], $\varepsilon_{260} = 13{,}300\ M^{-1}\ cm^{-1}$; poly[d(GC)] · poly[d(GC)], $\varepsilon_{254} = 16{,}800\ M^{-1}\ cm^{-1}$; calf thymus DNA, $\varepsilon_{259} = 12{,}800\ M^{-1}\ cm^{-1}$. These values were either provided by the manufacturer or taken from the literature [25]. For the fluorescence titration experiments the polymeric DNA concentration was ~500 μM (stock) in

base pairs, while for UV-spectroscopic experiments the DNA concentration was 25 μM in base pairs. Unless otherwise stated, the buffer used in our measurements was 2 mM cacodylic acid/sodium cacodylate adjusted to pH 7.4.

2.4. Synthesis

The title compound was synthesized by a hydrothermal reaction from a mixture of cfH (0.50 mmol) and magnesium(II) chloride hexahydrate (0.25 mmol). Both compounds were inserted into the glass tube and 5.0 ml of water was added. The mixture was stirred and the resulting suspension (pH = 7) was then frozen by liquid nitrogen, evacuated and sealed. The ampoule was heated at 150 °C for 3 days to give a small amount of pale violet crystals of $[Mg(cf)_2] \cdot 2.5H_2O$ **1** (yield 10%). A substantial amount of white amorphous precipitate was always additional product of this reaction. The crystals are not stable in mother liquor solution but are stable in air. Anal. Calculated for $C_{34}H_{34}MgF_2N_6O_6 \cdot 2.5H_2O$: C, 55.94%; H, 5.38%; N 11.51%. Found: C, 55.83%; H, 5.42%; N, 11.41%. IR data (cm^{-1}, Nujol) 3380, 3217, 2718, 1627, 1588, 1562, 1537, 1378, 1342, 1307, 1278, 1261, 1220, 1188, 1181, 1119, 1070, 1037, 1006, 899, 831, 790, 750, 721, 668, 633, 554, 542, 501.

The most striking difference in the procedure of isolation between Xiao et al. [13] and this work is pH, which was much higher in their syntheses (pH = 11.5). Our work clearly shows that such complex could form already around neutral conditions. Further differences are different salt used (magnesium(II) nitrate) and longer heating (4 days) of the mixture.

2.5. Fluorescence measurements

Fluorescence emission spectra of **1**, ciprofloxacin in the absence and presence of magnesium ions and after titration by various DNA at 20 °C were measured using 1 cm path length quartz cuvette and Cary Eclipse Fluorescence Spectrophotometer (Varian, Australia). The emission spectra were recorded in the range at 350 to 550 nm at an excitation wavelength of 330 nm. Fluorescence titration profiles were determined by incrementally adding aliquots of various DNAs into cuvette containing a known amount of **1** (0.25 μM) or ciprofloxacin (0.5 μM) in the presence or absence of Mg^{2+}. The emission spectra of **1** and ciprofloxacin, corrected for the solvent blank were multiplied by the dilution factor and corrected for PM-tube response using the instrument's correction spectrum.

2.6. UV-spectrophotometry

UV absorbance was measured in a Hewlett Packard 8453 UV–VIS spectrophotometer (Hewlett Packard GmbH, Waldbronn, Germany) equipped with an electrothermal temperature controller, using 1 cm path length quartz cuvette. pH titration of **1** in the pH range from 1.5 to 12 was followed by measuring the UV-absorption spectra at 25 °C. Absorbance versus temperature profiles (UV-melting curves) of DNAs (25 μM) were measured at 260 nm in the temperature range from 20 to 95 °C. Temperature was raised in 1 °C increments and DNA samples were allowed to equilibrate for 1 min at each temperature. For each optically detected transition, the melting temperature (T_m) of DNA was determined as the transition midpoint. Melting experiments of polymeric DNA at molar ratios of 1:1 (ciprofloxacin:DNA) were performed at the same buffer conditions as described above. To correct for the contribution of **1** or ciprofloxacin to the absorbance spectrum of DNA, the reference cuvette was filled with the buffer solution of **1** or ciprofloxacin at the same concentration as in the sample cuvette.

2.7. Size exclusion chromatography

Size exclusion chromatography (SEC) experiments were performed on GradiFrac liquid chromatograph using a Superdex 75 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were dissolved in distilled water and put on the column which was eluted at a flow rate of 0.5 ml/min and at room temperature with a solution of 5 mM cacodylate buffer, pH 7.4, and 200 mM NaCl as the mobile phase. The wavelength of the UV detector was set at 280 nm. The injection volume was 900 μl.

2.8. pH measurements

The pH values of all solutions were measured using Iskra model MA 5740 pH-meter (Slovenia) and Ag/AgCl combination microelectrode (Mettler Toledo, Switzerland). Absolute error in our pH measurements was ±0.01 pH unit.

2.9. Mass spectrometry (MS)

Mass spectrometry was performed on a VG-Analytical AutospecEQ instrument. Ionization was accelerated with Cs^+ ions with energies of 20–30 keV (Fast Atom Bombardment, FAB) and glycerol was used as a matrix.

3. Results and discussion

3.1. Synthesis

Surprisingly, we were not able to isolate crystallinic complex in the magnesium–ciprofloxacin system in which the typical chelate bonding of ring carbonyl and carboxylate oxygens is present only. Before, several metal (Me) complexes with discrete $[Me(quinolone)_2]$ units were isolated [7]. It was also reported that with norfloxacin (nfH), which is another quinolone family member, a dimeric $[Mg_2(H_2O)_6(nfH)_2]Cl_4 \cdot 4H_2O$ was isolated. This complex was isolated at pH = 6 and the terminal piperazine nitrogen atom is not involved in the bonding to the

metal [15]. However, at pH = 7 we were able to isolate the 2D molecular square-grid complex in which additional involvement of terminal piperazine nitrogen atom in the bonding to the metal was observed. It seems reasonable that the deprotonation of the terminal piperazine nitrogen is not only important for the coordination of quinolone molecules to the metal ions but could be also significant for biological activity. It was namely reported that quinolone derivatives with a piperazine ring at the C7 position are substantially less active at lower pH values [2].

3.2. Solution studies

It was reported that the structurally analogous complexes exert interesting fluorescence properties in solid state but are almost insoluble in most common solvents [8,13]. However, we have realized that magnesium complex **1** is sparingly soluble in water (ca. 2.5×10^{-5} M) which enables us to use some solution techniques (fluorescence, UV spectroscopy) that could help to determine the role of magnesium species in DNA binding and could also be important for elucidating the quinolone activity. Unfortunately, the solubility is still too low to allow the use of common techniques that could give us more structural information in solutions under study.

It was reported before that metal–quinolone complexes dissociate in aqueous solutions [26]. Such solutions therefore contain the mixture of metal–quinolone complexes (1:2, 1:1), free quinolone molecules and hydrated metal ions. Though in the title compound **1** also piperazine nitrogens are involved in bonding to magnesium we assume that similar types of species are present in solution as mentioned above. A proof that the dissociation of **1** gives the mentioned species was also obtained by FAB MS. Peaks at *m*/*z* 685, 686 and 687 were observed in the mass spectrum of **1** corresponding to $[Mg(cf)(cfH)]^+$ ion considering all three isotopes of magnesium. Mass peak at *m*/*z* 332 was also observed which confirms the presence of cfH_2^+ (data not shown).

According to the low solubility of the title compound it is clear that also the concentrations of all species formed by dissociation are very low and it is therefore impossible to determine the species distribution even in a simple aqueous solution. It is certain that the situation in a cell where several other molecules are present is much more complex and we believe intracellular biological conversion of the metal–quinolone complexes is taking place [26]. From all these facts it is obvious that it is nearly impossible to realize which is the active form of the drug in vivo.

It should be born in mind that the intracellular concentration of magnesium ions is much higher than that of quinolone [2,27]. We did not want to use high concentrations of magnesium in our study because this could mask the real effect of metal complexes present in solutions of **1**. It was already reported that the biological activity (antibacterial tests, DNA–gyrase inhibition test) of metal complexes is comparable to that of free quinolone probably due to this fact [7,26]. Our main goal was thus to compare the effect of the solution containing the mixture of magnesium(II) chloride and ciprofloxacin with solution of **1** on various forms of DNA.

3.3. UV-spectrophotometry

No significant changes in the UV absorption of **1** solution above pH 6 indicates a higher stability of **1** in neutral and basic solutions and confirm the applicability of experimental solution conditions (2 mM cacodylate buffer pH 7.4) selected for spectrophotometric measurements (Supplementary material).

UV-melting profiles of polymeric double-stranded DNA in the presence of complex **1**, free cfH, cfH in the mixture with $MgCl_2$, and $MgCl_2$ solution were performed at molar ratio 1:1 of cfH per base pair of DNA at physiological pH 7.4. Natural genomic calf thymus DNA and two synthetic polynucleotides with alternating AT and GC base pairs were chosen with the purpose to determine the possible sequence preferences of the tested compounds. Fig. 1A–C show the effect of various compounds on the melting profiles of poly[d(AT)] · poly[d(AT)], poly[d(GC)] · poly[d(GC)], and calf thymus DNA, respectively.

Complex mixtures containing magnesium:ciprofloxacin complexes (1:1, 1:2), hydrated magnesium ions and ciprofloxacin can interact with different DNAs through several different modes, whose contributions are different. It is therefore very difficult to establish which interaction is the most important but some possibilities are listed and discussed below.

One of the roles of divalent cations in biological systems is to stabilize the structures of macromolecules. The affinity of a cation for a specific site on a nucleic acid structure is a function of its charge, hydration free energy, coordination geometry and complex bond formation capacity. The crystallographic data show that magnesium ion which is

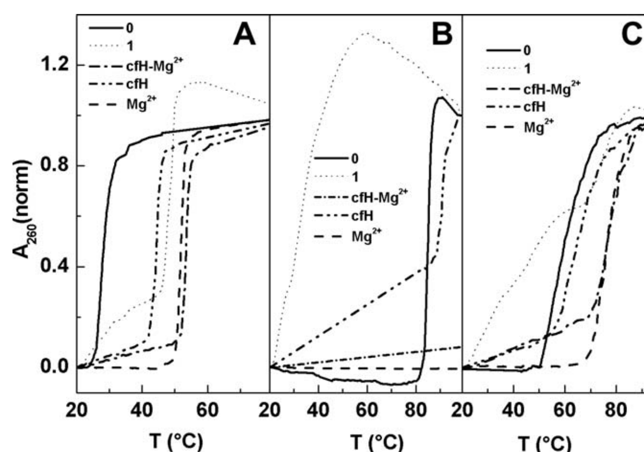

Fig. 1. UV-melting profiles (at 260 nm) of poly[d(AT)] · poly[d(AT)] (A), poly[d(GC)] · poly[d(GC)] (B) and calf thymus DNA (C) in the presence of ciprofloxacin, **1** and cfH–Mg^{2+} mixture; 2 mM cacodylate pH 7.4; $C_{DNA} = 25$ μM; cfH/DNA_{bp} molar ratio 1:1.

hexahydrated in aqueous solutions – $[Mg(H_2O)_6]^{2+}$ most frequently interacts with nucleobase heteroatoms indirectly through hydrogen bonds. Such interactions are sequence specific, because aqua ligands can both donate and accept hydrogen atoms with base atoms. Divalent metal ions are drawn electrostatically to major and minor grooves of DNA, as well as to phosphate groups [28–32]. In contrast, some other atoms (e.g. Ca^{2+}, Mn^{2+}) could coordinate directly to N(7) atom of a guanine, but it was also proposed that at higher concentrations even for magnesium such bonding could be possible [33]. Several possibilities how various magnesium–quinolone complexes could interact with DNA have been proposed in the literature [16–19]. These models include interaction of magnesium with phosphate groups and (or) nucleotide heteroatoms (directly or through hydrogen bonds) but also models where quinolone part of magnesium–quinolone complexes interacts with DNA were proposed. However, none of these models was generally accepted and we indeed believe that in the real system a mixture of more processes is present and it is impossible to realize which interaction is the most important for activity.

Thermal stabilization of poly[d(AT)] · poly[d(AT)] was observed in the presence of all the tested compounds (Fig. 1A). Magnesium chloride solutions thermally stabilize poly[d(AT)] · poly[d(AT)] at applied conditions for 24.7 ± 1.0 °C (Table 1) due to the interactions of magnesium ions with DNA as described above [28–32] and free ciprofloxacin stabilizes poly[d(AT)] · poly[d(AT)] for 17.2 ± 1.0 °C at applied conditions. We have observed previously that free ciprofloxacin itself can bind electrostatically and partially intercalates to various DNA forms, stabilizes them, and shows some sequence preferences for AT rich sequences [21]. Higher thermal stabilization was observed for ciprofloxacin in the presence of magnesium chloride (26.0 ± 1.0 °C) which could be explained as a combination of two stabilization effects: (i) Mg^{2+} interactions with DNA as described above and (ii) cfH intercalation [21]. These two contributions are not simply additive. In addition, magnesium ions are also likely to be involved in formation of the ternary complex between DNA, and quinolone through ciprofloxacin–magnesium 1:1 complex [18,34] which destabilizes double stranded DNA [20].

In the presence of complex **1** the thermal stabilization of poly[d(AT)] · poly[d(AT)] was smaller (22.2 ± 1.0 °C) in comparison to ciprofloxacin in the presence of $MgCl_2$ (26.0 ± 1.0 °C), suggesting that there are some differences in composition of both solutions.

The reversibility measurements of the **1**binding to poly-[d(AT)] · poly[d(AT)] (cooling the sample and reheating it the second time) showed completely super-imposable results onto the first heating scans. This suggests that the species present in solution of **1** alone does not inhibit re-annealing by associating irreversibly with the single strand which is similar to the results obtained for free cfH [21].

The thermal stabilization of poly[d(GC)] · poly[d(GC)] provoked by free ciprofloxacin is much smaller (6.0 ± 1.0 °C) in comparison to poly[d(AT)] · poly[d(AT)] (17.2 ± 1.0 °C) (Table 1). In the presence of magnesium chloride and in the mixture of cfH and magnesium chloride, poly[d(GC)] · poly[d(GC)] was too stable to melt at applied experimental conditions, suggesting the stabilization effect of Mg^{2+}ions and ciprofloxacin in the presence of Mg^{2+}ions. Conversely, the solution of **1** induced a large destabilization of poly[d(GC)] · poly[d(GC)] (−47.0 ± 1.0 °C). These results suggest that the species present in solution of **1**, interact with G-(C) bases and induce the destabilization of DNA double helix. The reversibility measurements of **1** binding to poly[d(GC)] · poly[d(GC)] (cooling the sample and reheating it the second time), which showed that the second heating scan is not superimposable to the first one, suggest that the species present in solution of **1** alone inhibit re-annealing by associating irreversibly with the single strands.

The observed different effect of **1** on the thermal stability of AT and GC alternating sequences is clearly manifested also on biphasic UV-melting profile of calf thymus DNA (Fig. 1C). A part of the UV-melting curve is shifted to lower temperatures and a part to higher temperatures, suggesting two different binding modes which is likely to cause the destabilization of GC and stabilization of AT base pairs rich regions of double stranded calf thymus DNA.

3.4. Size exclusion chromatography

During our experimental work we have realized that solution of **1** is colloidal which was first evidenced by the Tyndall effect. Further support that colloidal particles are present in solution of **1** but not in the solution containing

Table 1
Melting temperatures of different duplexes in 2 mM cacodylate buffer (pH 7.4) in the presence of **1**, ciprofloxacin, the mixture of cfH–Mg^{2+} and Mg^{2+} ions

	poly[d(AT)] poly[d(AT)] T_m = 27.3 ± 0.5 (°C)		dsDNA T_m = 60.9 ± 0.5 (°C)		poly[d(GC)] poly[d(GC)] T_m = 85.0 ± 0.5 (°C)	
	T_m (°C)	ΔT (°C)	T_m (°C)	ΔT (°C)	T_m (°C)	ΔT (°C)
			42.6 ± 0.5	−18.3 ± 1.0		
1	49.5 ± 0.5	22.2 ± 1.0			38.0 ± 0.5	−47.0 ± 1.0
			75.5 ± 0.5	14.6 ± 1.0		
cfH	44.5 ± 0.5	17.2 ± 1.0	65.1 ± 0.5	4.2 ± 1.0	91.0 ± 0.5	6.0 ± 1.0
cfH–Mg^{2+}	53.3 ± 0.5	26.0 ± 1.0	78.4 ± 0.5	17.5 ± 1.0	–	–
Mg^{2+}	52.0 ± 0.5	24.7 ± 1.0	75.8 ± 0.5	14.9 ± 1.0	–	–

the mixture of $MgCl_2$ and ciprofloxacin was obtained by size exclusion chromatography. This is a method of choice for determination of composition and size of colloidal particles and was therefore also used for analysis of solutions in our study. SEC separates different substances on the basis of molecular hydrodynamic volume (or size).

Fig. 2 presents chromatograms of solutions of **1**, ciprofloxacin and mixture of ciprofloxacin with $MgCl_2$. The compound with the smallest hydrodynamic radius retains in the column for the longest time. The void volume was 6.9 ml. The elution volumes of ciprofloxacin and mixture of ciprofloxacin and Mg^{2+} ions were 17.9 and 17.6 ml, respectively. In the solution of **1** first, colloidal, component has elution volume at 13.8 ml and the second peak appears at 19.3 ml. We were able to confirm the presence of Mg^{2+} (8×10^{-7} M) in the second fraction by atomic absorption spectroscopy (AAS). Results of SEC and AAS therefore suggest, that the second fraction contains also magnesium–ciprofloxacin complex. Due to similar elution volumes of complex and ciprofloxacin we can conclude that their sizes are similar and we were not able to separate components with the column used.

We were also not able to determine the exact composition of the colloidal particles but two possibilities could be proposed. First, the colloidal particles could form from stacked $[Mg(cf)_2]$ molecules which are stabilized by the adsorbed charged particles present in solution. Whereas in **1** the charge of Mg^{2+} ions is compensated by ciprofloxacin molecules in the anionic form, in the mixture of $MgCl_2$ and cfH, chloride anions could play this role. In principle, in mixture of ciprofloxacin and $MgCl_2$, a $[Mg(cfH)_2Cl_2]$ complex could form but in solution the chloride ions can easily dissociate to form $[Mg(cfH)_2]^{2+}$ ions which do not have the tendency to form aggregates. In contrast, a 1:2 complex $[Mg(cf)_2]$ formed by decomposition of **1** is neutral and could form larger particles. Low solubility of the neutral magnesium–quinolone complexes suggests that such compounds have a strong tendency to form stacked aggregates even at very low concentrations. The second possibility could be that the colloidal particles contain $Mg(OH)_2$ to which anionic ciprofloxacin and magnesium ions are adsorbed. SEC chromatography of ciprofloxacin in the presence of $Mg(OH)_2$ was namely performed and it was observed that in the mixture of $Mg(OH)_2$ and ciprofloxacin colloidal particles also form (Supplementary material). Elution volumes of colloidal component present in solution of **1** and the mixture of ciprofloxacin and $Mg(OH)_2$ were similar (13.8 and 13.1 ml).

Stabilizing effect of adsorbed ciprofloxacin on the formation of nanoparticles was already reported before [35]. It is known [36,37] that colloidal particles could interact with DNA and change its properties substantially. Different processes could be involved in the binding of the metal containing colloidal particles and biological molecules (adsorption, electrostatic interaction and even covalent linkage) [38]. If we consider all these facts, it could not be excluded that also DNA binding in our study is somehow affected by particle size of the species present in solution and last but not least also the binding of the drug to the enzyme induced pocket in DNA could be affected by this.

3.5. Fluorescence measurements

To get further insight into the nature of interactions between **1** and various DNAs, we have additionally applied the fluorescence measurements. Fig. 3A and B show the fluorescence emission spectra of **1** and ciprofloxacin in the presence of poly[d(AT)] · poly[d(AT)] in 2 mM cacodylate buffer (pH 7.4), respectively. The fluorescence

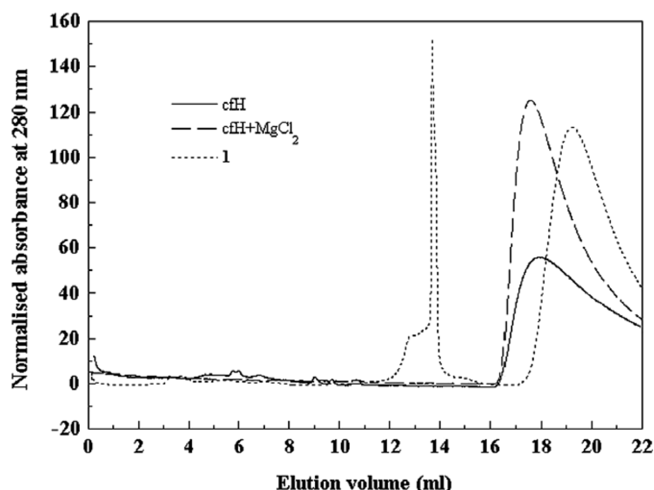

Fig. 2. Separation of 5×10^{-4} M ciprofloxacin (cfH), 2.5×10^{-4} M complex **1** and mixture of 5×10^{-4} M cfH and 2.5×10^{-4} M $MgCl_2$ on Superdex 75 in 5 mM cacodylate buffer (pH 7.4) and 200 mM NaCl. Flow rate was 0.5 ml/min, sample volume was 900 μl and void volume was 6.9 ml. UV detection was performed at 280 nm.

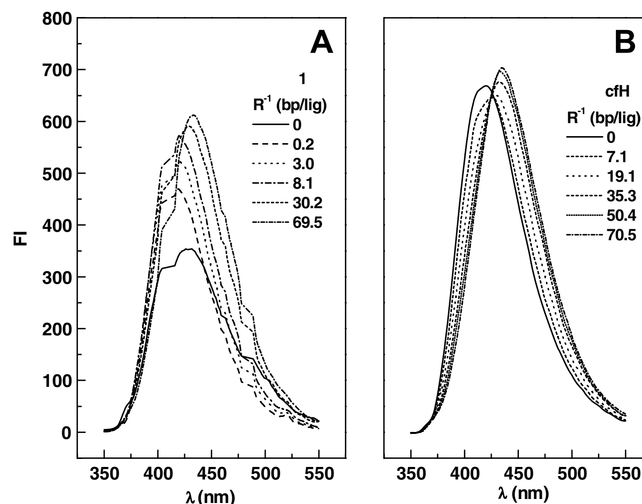

Fig. 3. Fluorescence emission spectra of **1** (A) and cfH (B) in the presence of poly[d(AT)] · poly[d(AT)] at different molar ratios of DNA base pairs and ligands (bp/lig) in 2 mM cacodylate buffer (pH 7.4), $C_{cfH} = 0.5$ μM; $C_1 = 0.25$ μM; $\lambda_{ex} = 330$ nm at 20 °C.

spectrum of **1** is composed of two bands, first at 405 ± 1 nm and the second at 429 ± 1 nm (Fig. 3A), while there is only one band in the fluorescence spectrum of free ciprofloxacin at 420 ± 1 nm (Fig. 3B). The fluorescence emission intensities of **1** and cfH increase after binding to poly[d(AT)] · poly[d(AT)]. A shift in the position of λ_{max} from 420 ± 1 to 435 ± 1 nm at molar ratio 70 DNAbp/ligand is more pronounced after cfH binding to poly[d(AT)] · poly[d(AT)]. In the case of **1** the band at 405 ± 1 nm does not shift at all, while the second band shifts from 429 ± 1 to 433 ± 1 nm. At the beginning of titration of **1** with poly[d(AT)] · poly [d(AT)] a blue shift of ~3 nm in λ_{max} of the peak at 429 ± 1 nm was observed up to the molar ratio of 10 DNAbp per ligand **1**, followed by the red shift at higher molar ratios (Fig. 3A). These results would suggest that at higher drug loading, the hydrophobic moiety of the complex **1** is located in the more hydrophobic environment [39], which could be due to the intercalative mode of binding or stacking interactions.

To compare the effect of binding of different ligands to various DNAs, we have plotted the titration curves of relative fluorescence intensity at 435 nm of **1**, cfH and cfH in the presence of magnesium chloride as a function of molar ratios of DNAbp/drug. The relative increase in the fluorescence intensity of **1** upon the titration by poly[d(GC)] · poly[d(GC)] (Fig. 4B) up to 5 bp/ligand is comparable to that observed for poly[d(AT)] · poly[d(AT)] (Fig. 4A), although in this titration the increase in the fluorescence intensity is not accompanied by significant shift in the position of λ_{max}. At molar ratios higher than 8 base pairs/ligand, the relative fluorescence intensity of **1**starts to decrease after titration with poly[d(GC)] · poly[d(GC)] (Fig. 4B), while it remains constant after titration with poly[d(AT)] · poly[d(AT)] (Fig. 4A). Similar decrease in the fluorescence intensity has been observed after titration of **1** with calf thymus DNA (Fig. 4C).

Additionally, the more pronounced decrease in the fluorescence intensity has been observed for cfH and cfH in the presence of $MgCl_2$ with all DNA containing GC bases including single stranded calf thymus DNA. Therefore it seems appealing to propose that this effect could be due to guanine base quenching the fluorescence. Guanine has namely the lowest oxidation potential of all nucleobases and can be an effective quencher of fluorescence through electron transfer from DNA to photo-excited fluoroquinolone [39,40]. The other possible reason for this effect might also originate in the photochemical activity of fluoroquinolones [41–44]. It was reported [42] that the parent quinolone–nalidixic acid caused photodamage at the 5′-G in consecutive guanines and induced larger amounts of 8-oxodG in double stranded DNA than in single stranded DNA.

3.6. Other possible reasons for different results of 1 in comparison to other studied systems

As realized from the UV-melting profile and fluorescence experiments the results of **1** are substantially different for DNA containing GC base pairs compared to poly[d(AT)] · poly[d(AT)]. This suggests that solution of **1** has different effects on AT and GC base pairs. Apart from the reasons mentioned above, there are also other possible reasons for different actions of the tested compounds. Among these are the steric hindrance due to the presence of different

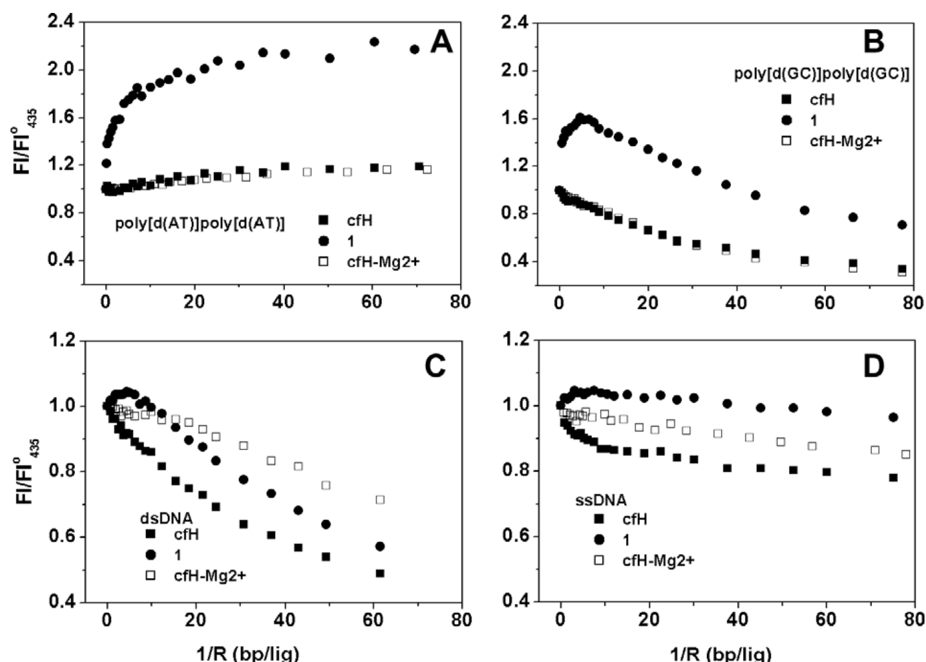

Fig. 4. Relative fluorescence emission intensity at 415 nm (FI/FI^0_{415}), of ciprofloxacin (■), **1** (●) and the mixture of cfH–Mg^{2+} (□) in the presence of different molar ratios of DNA base pairs per ligand ($1/R$) in 2 mM cacodylate buffer (pH 7.4). FI^0 and FI stand for fluorescence emission intensity of ciprofloxacin in the absence and presence of DNA, respectively. C_{cfH} = 0.5 μM; C_1 = 0.25 μM; λ_{ex} = 330 nm at 20 °C.

groups in the minor and major groove, different dimensions of the grooves and different hydration of poly[d(AT)] · poly[d(AT)] in comparison to poly[d(GC)] · poly[d(GC)] [45]. Additionally, it is also well established that several divalent cations, high ionic strength, polycationic species could affect the conformation of duplex and could induce a DNA transition from B- to left-handed Z-form [46]. Interestingly, it was realized in some systems (e.g. nickel–DNA), that in the presence of chloride ions this process was highly disfavored [47]. Obviously the presence of chloride anions is important also for such transitions and could contribute for different results of **1** (containing no chloride) and mixture of ciprofloxacin and $MgCl_2$ (see above). Though in our experiments the conditions were not typical for B- to Z-DNA transition [48], this process could not be totally excluded, especially in the system of poly[d(GC)] · poly[d(GC)] and **1**. Only in this system the melting temperature was substantially lowered. Similar lowering of melting point was found in poly[d(GC)] · poly[d(GC)] and $[Co(NH_3)_6]^{3+}$ system, when B- to Z-DNA transition occurred [49]. It is also interesting to note that in our previous study dealing with the interactions of magnesium, DNA and ciprofloxacin, we have also observed that at very high concentrations of magnesium salt (2.5 M) a B- to Z-DNA transition occurred [20].

4. Conclusions

In our paper we have tried to find the reasons for striking differences between the results obtained for the system **1** in comparison to all other tested systems (free ciprofloxacin, ciprofloxacin and $MgCl_2$, $MgCl_2$).

We assume that in the case of poly[d(AT)] · poly[d(AT)] the prevailing stabilization effects are likely to be the consequence of interactions of magnesium ions with DNA as described above [28–32], intercalation of free ciprofloxacin [21] or magnesium–ciprofloxacin species and the lack of binding site in the major groove of poly[d(AT)] · poly [d(AT)]. In the case of poly[d(GC)] · poly[d(GC)] the stabilization effect of Mg^{2+}, ciprofloxacin and ciprofloxacin in the presence of $MgCl_2$ was observed at applied conditions. The shift in T_m induced by ciprofloxacin binding to poly[d(GC)] · poly[d(GC)] was much smaller than for poly[d(AT)] · poly[d(AT)]. It is therefore possible that ciprofloxacin fits better in the narrow minor groove of AT sequences [21]. The destabilization effect (negative ΔT_m) of **1** on poly [d(GC)] · poly[d(GC)] is likely to be due to the major groove binding of colloidal particles, which is likely to induce a B- to Z-conformational transition. Due to their larger size, such particles could better fit into the wider major groove of DNA. It seems reasonable that this interaction could include a direct binding of magnesium to N(7) atoms of guanine bases. This proposal is supported by the observation that **1** irreversibly associates with poly [d(GC)] · poly[d(GC)]. In the case of poly[d(AT)] · poly [d(AT)] the interaction of complex **1** with the N(7) position of adenine is prevented by the presence of amino group in the proximity of the nitrogen N(7) and the lack of exocyclic O(6).

We are aware that these systems are extremely complex and further work is needed to get a clearer view and to confirm our assumptions. Unfortunately, low solubility of **1** prevents a collection of NMR data and our attempts to prepare appropriate crystals and to determine the crystal structure of the complex with DNA were not successful up to now. Although it is not very probable that polymeric complex **1** forms at physiological conditions, it is possible that colloidal particles present in solution of **1** could somehow form and could be important for the mode of action of quinolones. Our study undoubtedly shows that in these systems very small differences in experimental conditions (in composition of solutions, charge of species, size of particles) affect the results profoundly. This is probably also the main reason why in spite of the big interest there is still no complete understanding of magnesium–DNA–quinolone interactions.

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Appendix A. Supplementary data

Supplementary material for this article is available on request from the authors: Crystallographic data for **1** (Tables: Crystal data and refinement parameters; Selected geometric parameters. Figures: ORTEP view; View of the 2D square grid layer. Description). The pH-dependency of **1** single-wavelength UV absorption at 272 nm. SEC diagram of the mixture of 5×10^{-4} M cfH and 2.5×10^{-4} M $Mg(OH)_2$. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2006.06.003.

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