Biochimica et Biophysica Acta 1778 (2008) 2535–2543

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Interactions of ciprofloxacin with DPPC and DPPG: Fluorescence anisotropy, ATR-FTIR and ^{31}P NMR spectroscopies and conformational analysis

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ARTICLE INFO

Article history:
Received 5 March 2008
Received in revised form 11 August 2008
Accepted 12 August 2008
Available online 6 September 2008

Keywords:
Ciprofloxacin
DPPC
DPPG
Steady-state fluorescence anisotropy
Melting temperature
ATR-FTIR
^{31}P NMR
Conformational analysis

ABSTRACT

The interactions between a drug and lipids may be critical for the pharmacological activity. We previously showed that the ability of a fluoroquinolone antibiotic, ciprofloxacin, to induce disorder and modify the orientation of the acyl chains is related to its propensity to be expelled from a monolayer upon compression [1]. Here, we compared the binding of ciprofloxacin on DPPC and DPPG liposomes (or mixtures of phospholipids [DOPC:DPPC], and [DOPC:DPPG]) using quasi-elastic light scattering and steady-state fluorescence anisotropy. We also investigated ciprofloxacin effects on the transition temperature (T_m) of lipids and on the mobility of phosphate head groups using Attenuated Total Reflection Fourier Transform Infrared-Red Spectroscopy (ATR-FTIR) and ^{31}P Nuclear Magnetic Resonance (NMR) respectively. In the presence of ciprofloxacin we observed a dose-dependent increase of the size of the DPPG liposomes whereas no effect was evidenced for DPPC liposomes. The binding constants K_{app} were in the order of 10^5 M^{-1} and the affinity appeared dependent on the negative charge of liposomes: DPPG>DOPC:DPPG (1:1; M:M)> DPPC>DOPC:DPPC (1:1; M:M). As compared to the control samples, the chemical shift anisotropy ($\Delta\sigma$) values determined by ^{31}P NMR showed an increase of 5 and 9 ppm for DPPC:CIP (1:1; M:M) and DPPG:CIP (1:1; M:M) respectively. ATR-FTIR experiments showed that ciprofloxacin had no effect on the T_m of DPPC but increased the order of the acyl chains both below and above this temperature. In contrast, with DPPG, ciprofloxacin induced a marked broadening effect on the transition with a decrease of the acyl chain order below its T_m and an increase above this temperature. Altogether with the results from the conformational analysis, these data demonstrated that the interactions of ciprofloxacin with lipids depend markedly on the nature of their phosphate head groups and that ciprofloxacin interacts preferentially with anionic lipid compounds, like phosphatidylglycerol, present at a high content in these membranes.

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1. Introduction

The introduction of fluoroquinolones, such as ciprofloxacin, more than 20 years ago offered clinicians a range of antimicrobial agents that have a broad spectrum of activity [2] together with an activity against several intracellular bacteria [3]. The central structural unit of ciprofloxacin (Fig. 1) is a quinolone ring with a fluorine atom at the 6-position, a piperazine moiety at the 7-position, a cyclopropyl ring at position 1 and a carboxyl group at position 3. The primary mechanism of action of these compounds involves inhibition of the intracellular DNA gyrase and topoisomerase IV [4]. Before they can exert their antibacterial effect, fluoroquinolones must enter bacterial cells. While the outer membrane protein F (OmpF) plays an important role in the uptake of fluoroquinolones for Gram-negative bacteria together with a direct uptake by a lipid mediated pathway [5], the passage through the phospholipid bilayer is the major process involved for inner membrane of Gram-negative bacteria and membrane of Gram-positive organisms [6]. For their intracellular activity, fluoroquinolones have also to penetrate into cells and to accumulate within. Diffusion and efflux processes modulate this cellular accumulation [7–9].

With respect to the interactions of ciprofloxacin with lipids, it has been established that ciprofloxacin had a small but definite and measurable interaction with neutral and charged membranes at the headgroup region [10]. The binding appeared as the result of (i) hydrophobic forces between the lipid bilayer and ciprofloxacin [11–13] and (ii) an ionic interaction between negatively charged phosphate groups of the phospholipid and the positively charged piperazine ring at the C-7 position of the quinolone [12–14].

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doi:10.1016/j.bbamem.2008.08.015

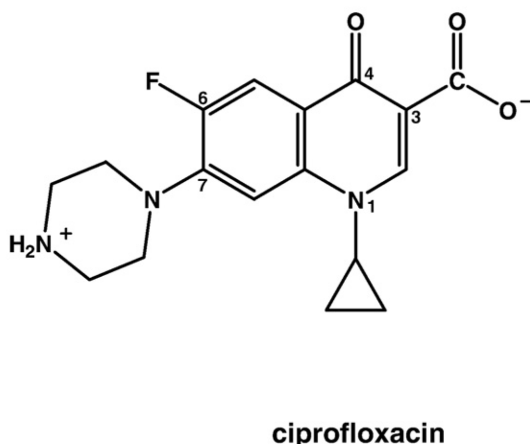

Fig. 1. Structural formula of ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid).

To gain further information at the molecular level on the interaction between ciprofloxacin and lipids, we previously showed [1] in the presence of this antibiotic (i) the erosion of DPPC domains in the DOPC fluid phase as demonstrated by AFM, (ii) the shift of the surface pressure area—isotherms of DOPC:DPPC:ciprofloxacin monolayer towards the lower area per molecule as evidenced by Langmuir studies, and (iii) the decrease of the all-*trans* conformation of acyl lipid chains of DPPC together with a change of their orientation measured using ATR-FTIR.

Unfortunately, only few data [6,15] are available to compare the interaction of fluoroquinolones with both zwitterionic (DPPC) and negatively charged (DPPG) lipids in terms of their binding parameters together with the effect of the drugs on the dynamics of these lipids. Moreover, consequences of this interaction on temperature-dependent parameters such as the disorder of the hydrophobic tails of the lipids representing the major components of mammalians and bacterial membranes are still largely unexplored. Such information is however critical since the lipid composition of both bacterial and eukaryotic membranes markedly differs [16]. The bacterial plasmatic membrane mainly consists of a high amount of anionic lipids like phosphatidylglycerol whereas the membrane of eukaryotic cells appear uncharged and consisting almost exclusively of zwitterionic lipids, like phosphatidylcholine [17].

In this work, using DPPC, a zwitterionic lipid, and DPPG, an anionic lipid, mimicking the bulk lipid of eukaryotic membranes and the major component of bacterial membranes respectively, we therefore turned our efforts (i) to determine the binding parameters of ciprofloxacin to lipids using steady-state fluorescence anisotropy, (ii) to characterize the effect of the drug on the structure and dynamics of multilamellar vesicles using ^{31}P NMR and (iii) to follow, the influence of ciprofloxacin on the acyl chain order as a function of temperature, using ATR-FTIR. Results were related to the location of ciprofloxacin at the hydrophilic/hydrophobic interface, to the mean surface occupied by each DPPC and DPPG molecules in the presence and in the absence of ciprofloxacin, and to the energy of interaction between ciprofloxacin and DPPC or DPPG, as determined by molecular modeling.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidyl glycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and stored at −20 °C. Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) was supplied as hydrochloride salt (Mw = 367.85 g/mol; microbiological standard potency, 85.5%) by Bayer Healthcare AG (Leverkusen, Germany). Stock solutions were prepared in 10 mM Tris (pH 7.4).

2.2. Methods

2.2.1. Preparation of liposomes (MLVs, LUVs)

The large unilamellar vesicles (LUVs) consisting of DPPG, DOPC: DPPG (1:1; M:M), DOPC:DPPC (1:1; M:M), or DPPC were prepared as follows. Lipids were mixed in chloroform:methanol (2:1; V:V), evaporated under nitrogen flow and dessicated under vacuum overnight to remove any residual solvent. The dried films were then resuspended at 40 °C from the walls of the glass tube by vigorous vortexing either in Tris:NaCl buffer 10:100 mM (pH 7.4) for binding experiments, or 10 mM Tris (pH 7.4) for binding stoechiometry, ATR-FTIR and ^{31}P NMR studies. Multilamellar vesicles (MLVs) were obtained by five heat-cooled cycles. Large unilamellar vesicles (LUVs) were then prepared by extrusion (ten times) of the MLV suspension through 100 nm of diameter polycarbonate filters (Nucleopore Costar Corporation, Badhoevedorp, Netherlands), using a thermostated (40 °C) extruder (Lipex Biomembranes, Vancouver, Canada). The actual phospholipid content of each preparation was determined by phosphorus assay [18] and the concentration of liposomes was adjusted for each experiment.

2.2.2. Quasi-elastic light scattering measurements

The apparent average diameter of the LUVs and MLVs and the zeta potential of LUVs were determined using a Zetasizer (Zen 3600, Malvern Instruments, U.K.) with the following specifications: 60 s sampling time; 0.8872 cP medium viscosity; 1.33 refractive index; 90° scattering angle; 25 °C temperature. Data were analyzed using the multimodal number distribution software included in the instrument for size determinations. The zeta potential values were calculated using Helmholtz–Smoluchowski's equation. All the measurements were performed using liposomes at a concentration of 0.5 mg/ml.

2.2.3. Steady-state fluorescence anisotropy

Fluorescence anisotropy is a powerful technique for investigating macromolecular dynamics, in which the sample is excited by linearly polarized light. The anisotropy was evaluated by observing the fluorescence at polarizations parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation according to:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}). \tag{1}$$

The anisotropy titrations of LUV liposomes by ciprofloxacin were performed with a fluorescence spectrophotometer (Perkin Elmer LS-55, Beaconsfield, U.K.) in the T-format at 25 °C, by adding increasing concentrations of liposomes to a fixed amount of antibiotic (5 μM). Anisotropy measurements were performed every 20 s during 4 min. The emitted light was monitored through 390 nm interference filters. Excitation and emission bandwidths were both 9 nm. The excitation and emission wavelengths were set at 280 nm and 430 nm, respectively. The Scatchard equation was rewritten (Eq. 2) to directly fit the anisotropy r, as described previously [19]

$$r = r_0 - \frac{(r_0 - r_t)}{L_t} \frac{(1 + (L_t + nN_t)K_{app})^2 - \sqrt{(1 + (L_t + nN_t)K_{app})^2 - 4L_t nN_t K_{app}^2}}{2K_{app}} \tag{2}$$

where, r_0 and r correspond to the anisotropy of the ciprofloxacin in the absence and in the presence of a given concentration of the LUV liposomes. L_t and N_t designate the concentration of ciprofloxacin fixed at 5 μM and the concentration of lipids in LUV liposomes added, respectively. r_t is the anisotropy at the plateau value. K_{app} and n correspond to the apparent binding constant and the number of

binding sites, respectively. All fitting procedures were carried out with Origin™ 7.5 software.

2.2.4. ^{31}P nuclear magnetic resonance (NMR)

The effect of ciprofloxacin on the mobility of phosphate headgroups of DPPC and DPPG prepared as MLV was investigated by static ^{31}P NMR experiments. For a nonspherical charge distribution about the phosphorous nucleus, the shielding constant largely results from a local diamagnetic shielding contribution and a local paramagnetic shielding contribution. Because the charge distribution in a phosphorus molecule will be far from spherically symmetrical, the major contribution to the ^{31}P chemical shift comes from the paramagnetic term. Asymmetry in the charge distribution implies that the ^{31}P chemical shifts (or shielding constants) vary as a function of the orientation of the molecule relative to the external magnetic field. This gives rise to a chemical shift anisotropy ($\Delta\sigma$) which can be defined by three principal components σ_{11}, σ_{22} and σ_{33} of the shielding tensor and which is a frequently used parameter to characterize the ^{31}P NMR spectra. For molecules which are axially symmetrical, with σ_{11} along the principal axis of symmetry, $\sigma_{11}=\sigma_{||}$ (parallel component), and $\sigma_{22}=\sigma_{33}=\sigma_{\perp}$ (perpendicular component). The ^{31}P chemical shift anisotropy is sensitive to both headgroup geometry and local dynamics. Rapid motion with limited amplitude along the molecular axis of the phospholipid normal to the membrane produces a supplementary averaging of the anisotropy tensor. The effective tensor still has axial symmetry but the chemical shift anisotropy is reduced. When ^{1}H decoupling is applied, ^{31}P spectra are obtained that result solely from the chemical shift anisotropy. The chemical shift anisotropy can be measured by taking the difference of chemical shift between the low field shoulder ($\sigma_{||}$) and the high-field peak ($\sigma_{\perp}$): $\Delta\sigma=\sigma_{||}-\sigma_{\perp}$ [20].

Control samples of 450 µl were prepared from MLV suspension (50 mg/ml) in 5 mm outer diameter tubes, by adding 50 µl of D_2O for locking on the deuterium signal. To investigate the effect of the ciprofloxacin on the mobility of lipid (DPPC and DPPG) headgroups, a defined concentration of drug was added to MLV liposomes to reach a molar ratio of one.

Broadband proton-decoupled ^{31}P NMR spectra were acquired by 1D NMR methods on a Bruker AVANCE 500 spectrometer at 202.5 MHz (11.7 Tesla) equipped with a Bruker 5 mm BBI (broad band inverse) probe. Typical Fourier transform spectral parameters were: 45° (6 µs) flip angle, 50 kHz spectral width, 8K data points, 0.6 s repetition time. Sixty thousands transients were accumulated. An exponential multiplication corresponding to 70 Hz line broadening was applied to the free induction decay prior to Fourier transformation. All chemical shift values were quoted in parts per million (ppm) and were referenced to the isotropic chemical shift of H_3PO_4 (0 ppm). All spectra were recorded at a constant temperature of 45 °C.

2.2.5. Infrared spectroscopy

Attenuated Total Reflection Fourier Transform Infra-Red (ATR-FTIR) is particularly well suited for the study of membranes and to characterize the effect of drugs on melting temperature [21]. This technique is based on internal reflection of the infrared light within an internal reflection germanium plate, which creates an evanescent field at the surface of the plate where the lipid bilayer (and eventually the bound proteins or drugs) resides [22].

The internal reflection element was a 52×20×2 mm trapezoidal germanium ATR plate (ACM, Villiers St Frédéric, France) with an aperture angle of 45° yielding 25 internal reflections. 10 µl of the sample containing DPPC or DPPG liposomes (50 mg/ml in Tris buffer 10 mM, pH 7.4) was dried under a stream of nitrogen on one side of the germanium internal reflection element at 20 °C. Under these conditions a well ordered multilayer stack is formed [23] and the stack is stable under a buffer flow [24]. The ciprofloxacin was added to the lipids at a ratio 1:1 as suggested by the stoechiometry binding experiments.

The germanium crystal was then placed in an ATR holder for liquid samples with an in- and out-let (Harrick, Ossining, NY, USA). The liquid cell was placed at 45 incidence on a Specac vertical ATR setup. The temperature was controlled with temperature-regulated water flowing in a cavity of the steal cell. The temperature was raised degree by degree.

An elevator under computer control made it possible to move the whole setup along a vertical axis (built for us by WOW Company SA, Nannine, Belgium). This allowed the crystal to be separated in different lanes. Here, one such lane contained the membrane film, the other was used for the background.

IR Spectra were obtained on a Bruker IFS55 FTIR spectrometer (Ettlingen, Germany) purged with N_2 as described previously [25]. Briefly, typically interferograms were recorded with 2 cm^{-1} spectral resolution with broadband MCT detector between 4000–800 cm^{-1}, 128 scans were averaged for one spectrum.

The spectra were corrected from the water vapor and CO_2 contributions. They were then apodized in the Fourier domain in order to yield a final resolution of 4 cm^{-1}. Peak positions were determined according to a classical peak picking method. All the software used for data processing was written under MatLab 6.5 (Mathworks Inc., Natick, MA).

2.2.6. Assembly of ciprofloxacin with phospholipids by molecular modeling using the Hypermatrix procedure

The Hypermatrix method described elsewhere [26] was successfully used to study the interaction between different molecules and lipids [27–29]. In the Hypermatrix procedure, the lipid/water interface was taken into account by varying linearly the dielectric constant ε between 3 and 30.

The initial position and orientation of ciprofloxacin and of phospholipids (DPPC and DPPG) were those defined when using the TAMMO procedure [26]. To obtain a multimolecular assembly of ciprofloxacin and DPPC or DPPG with a 1:1 molar ratio, we first carried out the matching between one molecule of ciprofloxacin and one molecule of lipid. For that, the position of the ciprofloxacin was set constant while the lipid molecule was translated towards the ciprofloxacin molecule along the x axis by steps of 0.05 nm. The lipid rotated by steps of 30 around its z' axis and around the x axis. l was the number of positions tested along the x axis, m was the number of rotations around ciprofloxacin and n was the number of rotations around the lipid itself. For each set of l, m and n values, the energy of interaction between the ciprofloxacin and the lipid was calculated as the sum of van der Waals, electrostatic and hydrophobic terms [30]. Then, the lipid molecule was moved by 0.01 nm step along the z' axis perpendicular to the interface and the angle between the z' axis was bent by +1° with respect to the z axis. The most stable ciprofloxacin:lipid complex was used in the next step for the calculation of the multimolecular assembly. Following the same procedure, one ciprofloxacin:lipid complex was set constant and another 1:1 complex was moved towards it with the same combination of rotations and translations than that described above. The energy values together with the coordinates of all assemblies were stored in a matrix and classified according to a decreasing order. The position of the second complex corresponded to the most stable assembly in the matrix. The position of the next 1:1 complex was then defined as the next most energetically favorable orientation stored in the matrix, taking into account the steric and energy constraints due to the presence of the other molecules. The process ended when the central 1:1 complex was completely surrounded.

An assembly of lipids alone was made following the same process, with the central molecule being the lipid of interest. The mean area occupied by one lipid or one ciprofloxacin molecule in the multimolecular complex or in the lipid assembly was estimated by projection on the x–y plane using a grid of 1 Å^2.

Pex2dstats files [31] were generated during the simulations and allowed an easy and detailed analysis of each complex. All calculations were performed on an Intel® Pentium® 4, CPU 3.80 GHz, 4.00 Go of RAM.

3. Results

3.1. Size and zeta potential of LUV liposomes

In order to characterize the homogeneity of the LUV liposomes together with their surface charge, we investigated the size and zeta potential of the liposomes used (DPPG, DOPC:DPPG, DOPC:DPPC and DPPC). The results showed minor differences in the apparent mean diameters of liposomes which were centered on 100 nm (DPPG [124 nm], DOPC:DPPG [104 nm], DOPC:DPPC [92 nm], and DPPC [101 nm]). As expected for negatively charged (DPPG) liposomes, the zeta potential was more negative (−66 mV) than for DOPC:DPPG (−34 mV), DOPC:DPPC (−6 mV) or DPPC (−5 mV) vesicles.

3.2. Binding of ciprofloxacin to LUV liposomes

As a first step, to investigate the effect of ciprofloxacin on the lipid membrane models, we checked the effect of increasing amounts of ciprofloxacin on the size of the liposomes, by quasi-elastic light scattering (Table 1). The fluoroquinolone had no effect on the average diameter of DPPC LUVs whatever the molar ratio investigated (Lipid: drug ratios from 1:0.2 to 1:1). In contrast, for DPPG LUVs, the average diameter increased with the increase of the ciprofloxacin concentration, reaching a value of 256 nm for a lipid:drug ratio of 1. The average diameter was of 155 nm and 195 nm for a molar ratio of 1:0.2 and 1:0.5, respectively.

As a second step, we determined the binding constant, K_{app}, by adding increasing liposome concentrations to 5 μM ciprofloxacin in 10 mM Tris, 100 mM NaCl (pH 7.4) and following the steady-state fluorescence anisotropy. Fitting the experimental values according to Scatchard equation (Fig. 2), K_{app} values were close to 10^5 M^{-1} (Table 2). By comparing the affinity of ciprofloxacin to DPPG liposomes and DOPC:DPPG (1:1; M:M), it appeared that a decrease in the concentration of negatively charged lipids (DPPG) in the liposome mixture, led to a 2.5 fold K_{app} value decrease. The critical effect of DPPG on the interaction of ciprofloxacin was confirmed by comparing the K_{app} values of DPPC *versus* those of DPPG (3.5 times lower) or those of DOPC:DPPC *versus* those of DOPC:DPPG (3 times lower). Taken together, our data showed that the ciprofloxacin bound to lipid vesicles with the following preference: DPPG>DOPC:DPPG (1:1; M:M)>DPPC>DOPC:DPPC (1:1; M:M). To get further data on the binding between ciprofloxacin and lipids and to evaluate the stoechiometry of this binding, we reproduced these experiments in conditions in which NaCl was omitted. Saturation points for fitted binding curve approximately occur for lipids concentrations equimolar to respective ligand concentrations suggesting a stoechiometry of (1:1) (Fig. 2-insert). This was observed whichever the nature of the lipids investigated (DPPG or DPPC).

3.3. Effect of ciprofloxacin on the size and the mobility of phosphate heads of DPPC and DPPG MLV's

From quasi-elastic light scattering studies and binding parameters, the association of ciprofloxacin to lipids appears as strongly dependent on the presence of the negatively charged phospholipid, DPPG. To further explore this interaction, we measured by ^{31}P NMR spectroscopy the effective chemical shift anisotropy ($\Delta\sigma$) of the MLV liposomes of DPPC and DPPG in the presence and in the absence of ciprofloxacin at 45 °C. As shown in Fig. 3, typical spectra obtained with multilamellar vesicles for both DPPC (top panel) and DPPG (bottom panel) were characteristic of a bilayer organization with a high-field maximum and a low field shoulder. In addition, the spectrum of ^{31}P NMR for DPPG revealed two peaks respectively at −10 ppm and 0 ppm. The latter was probably due to the presence of a liposome population (10%) of 150 nm of size as revealed by Nanosizer measurements. Moreover, we observed a marked difference between the chemical shift anisotropy ($\Delta\sigma$) value of DPPC as compared to that of DPPG, 41.5 and 25.5 ppm respectively. This can be related to the mean diameter of the major population of the MLVs which were 2100 nm for DPPC (100%) and 1375 nm for DPPG (90%) vesicles.

In the presence of ciprofloxacin, the $\Delta\sigma$ value was higher than that of the control, for both types of liposomes. We obtained an increase of the chemical shift anisotropy ($\Delta\sigma$) values of 5 and 9 ppm for DPPC and DPPG, respectively. The presence of a population of larger size, estimated around 5000 nm for DPPG vesicles and >10 μm for DPPC vesicles could also be related to the difference observed in terms of chemical shift anisotropy.

3.4. Effect of ciprofloxacin on the melting temperature of the lipids

As a sequel of the binding experiments, our further objective was to determine the effect of the interaction between ciprofloxacin on DPPC or DPPG on the melting behavior of the lipids.

To this end, we used ATR-FTIR spectroscopy. This method allowed us the detection of the transition of the lipid hydrocarbon chains from *trans* to *gauche* conformation which corresponds to the transition of the lipid bilayer from gel (L_β) to liquid-crystalline phase (L_α). It is known that the absorbance of the peaks corresponding to the

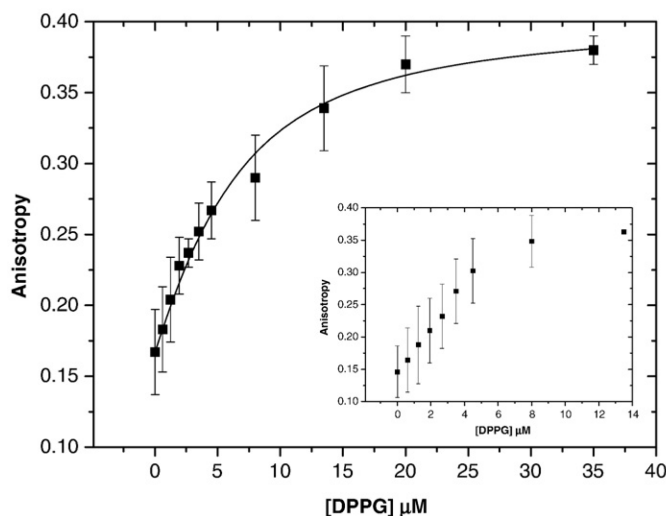

Fig. 2. Binding of ciprofloxacin to DPPG vesicles. Binding of ciprofloxacin to lipid vesicles was followed by steady-state anisotropy in the presence of increasing amounts of DPPG. The antibiotic concentration was 5 μM in Tris:NaCl buffer 10:100 mM (pH 7.4). The continuous line corresponds to the fit of the experimental points with Eq. (2) in order to determine the binding constant. The stoechiometry of the interaction between ciprofloxacin and lipids (inset) was performed in Tris buffer 10 mM (pH 7.4).

Table 1
Size of large unilamellar vesicles in the presence of increasing amounts of ciprofloxacin as determined by quasi-elastic light scattering

Lipid:drug molar ratio	Average size (nm)	
	DPPC	DPPG
1:0	101±1	124±1
1:0.2	100±1	155±2
1:0.5	98±1	195±2
1:1	105±1	256±5

Table 2
Binding parameters obtained from steady-state anisotropy experiments

LUV liposomes composition	K_{app} (10^5 M^{-1})
DPPG	8.6±0.5
DPPC	2.5±0.1
DOPC:DPPG (1:1; M:M)	3.2±0.9
DOPC:DPPC (1:1; M:M)	1.1±0.2

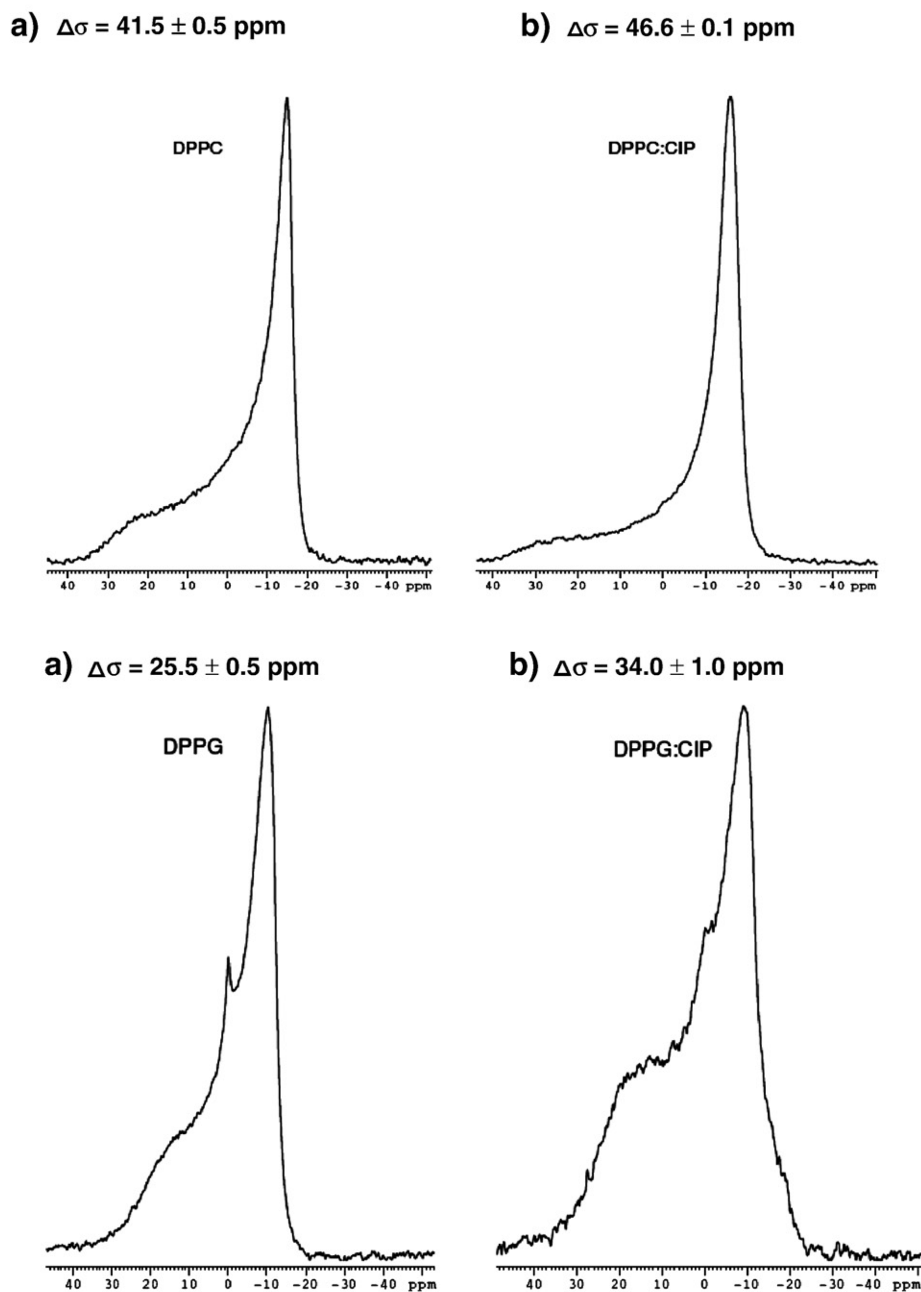

Fig. 3. Effect of ciprofloxacin on ^{31}P NMR spectra of multilamellar vesicles of DPPC (upper panel) and DPPG (lower panel). Liposomes (50 mg/ml) were prepared at pH 7.4 in 10 mM Tris buffer. The experiments were performed at 45 °C. The chemical shift anisotropy ($\Delta\sigma$) values are indicated at the top of the spectra in the absence (a) and in the presence (b) of ciprofloxacin added at a molar ratio of one.

asymmetric $\nu_{as}(CH_2)$ (2920 cm^{-1}), and symmetric $\nu_s CH_2$ (2850 cm^{-1}) stretching bands are sensitive to the intramolecular vibrational coupling and thus to the lateral packing of the hydrocarbon chains.

In order to determine accurately the peak maxima, a Gaussian line was fitted on the upper half of the position of the maximum for the asymmetric CH_2 stretching vibration ($\nu_{as}(CH_2)$) of DPPC (Fig. 4a) and DPPG (Fig. 4b) vesicles and the result was plotted as a function of temperature. Pure DPPC and DPPG vesicles (in Tris 10 mM, pH 7.4 buffer) exhibited a clear phase transition between the gel phase (L_β) and a liquid-crystalline phase (L_α). As expected, the DPPG melting temperature is slightly lower than for DPPC (42 °C) and is near (40 °C).

The melting curve obtained for DPPC in the presence of ciprofloxacin (Fig. 4a), showed a minor effect of the drug before and after the main transition temperatures of DPPC since the frequencies of the two dominant bands observed at 2917 ($\nu_{as}(CH_2)$) and 2849 cm^{-1} ($\nu_s(CH_2)$; not shown) were slightly below those observed for pure DPPC. The melting temperature remained unchanged (42 °C).

In contrast, addition of ciprofloxacin to anionic lipid DPPG vesicles induced a significant change in the melting curve (Fig. 4b). Indeed, a net positive shift of $\nu_{as}(CH_2)$ stretching bands was observed at the pre-transition temperature (between 20 and 37.5 °C). At a temperature between 38 and 40 °C, the two bands centered at 2918 cm^{-1} ($\nu_{as}(CH_2)$) and 2850 cm^{-1} ($\nu_s(CH_2)$; not shown) were significantly changed. With further increase in temperature (>40 °C), the peak positions of stretching band remained significantly below those of the pure DPPG. These data suggest that the ciprofloxacin increased the membrane fluidity in the gel phase, as shown by the increase of the frequency of $\nu_{as}(CH_2)$ asymmetric vibration below T_m and decreased the membrane fluidity, notably in the liquid-crystalline phase (L_α), as shown by the decrease the frequency of $\nu_{as}(CH_2)$ asymmetric vibration above T_m.

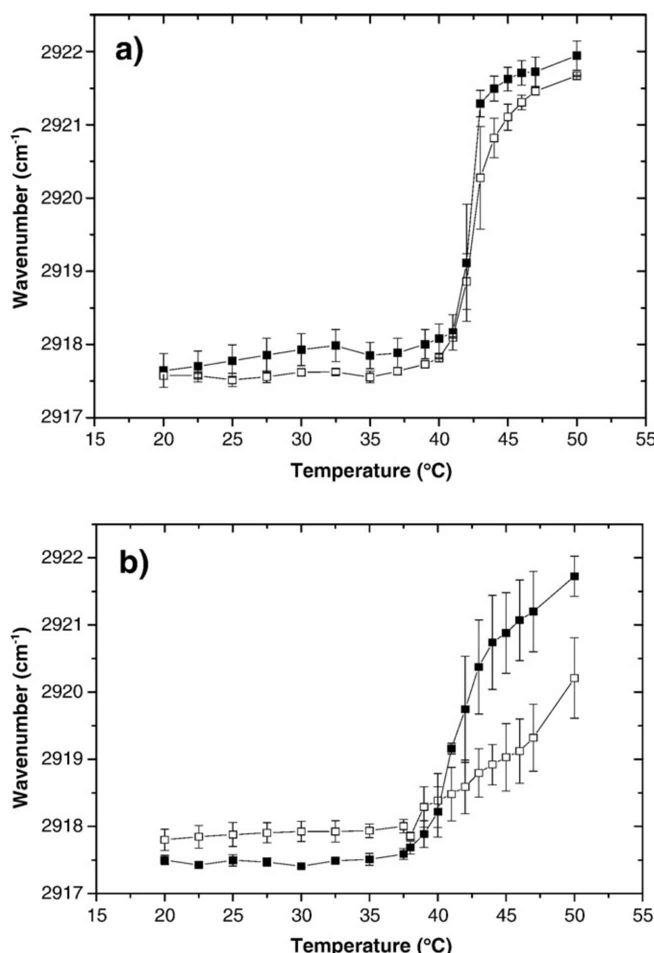

Fig. 4. Evolution of the maximum frequency of the $\nu_{as}(CH_2)$ as a function of temperature for DPPC (a) or DPPG (b) in the absence (closed square ■) or in the presence (open square □) of ciprofloxacin. The concentration of phospholipids was 50 mg/ml. The lipid: ciprofloxacin molar ratio was 1:1.

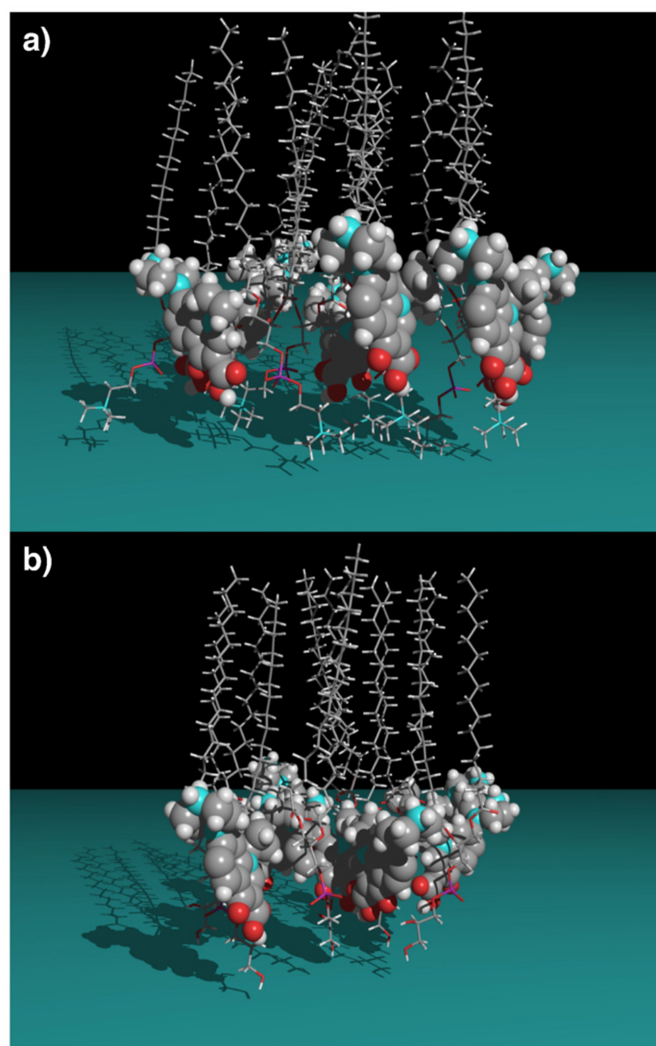

Fig. 5. Assembly of ciprofloxacin with DPPC (a) or DPPG (b) in a 1:1 molar ratio. Ciprofloxacin is represented in Corey–Pauling–Koltum (CPK) mode, whereas the phospholipids are in skeleton mode.

3.5. Molecular simulation of the interaction between ciprofloxacin and DPPC or DPPG

The interaction between ciprofloxacin and DPPC or DPPG at a (1:1) molar ratio was calculated using the Hypermatrix procedure [26] as described in the Materials and methods section. As shown in Fig. 5, ciprofloxacin is inserted at the level of the phospholipid headgroup/acyl chain interface for both lipids, as already suggested [1]. The calculation of the interaction energy between DPPC or DPPG and ciprofloxacin (Table 3) indicates that the association is more stable with DPPG, the energy going from −44.4 kcal/mol for DPPC: ciprofloxacin to −53.4 kcal/mol for DPPG:ciprofloxacin. Furthermore, the presence of ciprofloxacin in DPPG significantly increases the lipid interfacial area, the DPPG value going from 66 Å^2 in a monolayer to 76 Å^2 in the calculated multimolecular assembly. The increase is less important for DPPC, going from 63 Å^2 for the lipid alone to 66 Å^2 in the assembly (Table 3).

4. Discussion

While numerous studies have shown a critical role of the porin hydrophilic pathway for penetration of fluoroquinolones through the outer membrane of Gram-negative bacteria, the present work may shed new light on their passage through the inner membrane of these organisms (and through the membrane of Gram-positive bacteria) as well as through the pericellular membrane of eukaryotic cells. In the absence of demonstrated inward transporter, a direct interaction of fluoroquinolones with lipids allowing their diffusion through the so-called hydrophobic pathway and/or self-promoted pathway must indeed be considered critical for both the antibacterial activity and the tissue distribution and cell accumulation properties of fluoroquinolones.

In order to gain further insight in the interactions of fluoroquinolones with lipids, especially those found in bacterial membranes like the negatively charged phospholipid phosphatidylglycerol, as compared to the bulk lipids of eukaryotic membranes, phosphatidylcholine, we therefore determined (i) the binding parameters of ciprofloxacin in interaction with these lipids using steady-state fluorescence anisotropy, (ii) the effect of ciprofloxacin on the mobility

Table 3
Energy of interaction energy between lipid molecule and ciprofloxacin (CIP) and molecular area of the lipid molecule at the interface

	Energy of interaction (kcal/mol)	Area (Å^2)
DPPC/DPPC	−30.5	63
DPPC/CIP	−44.4	66
DPPG/DPPG	−30.8	66
DPPG/CIP	−53.4	76

of phosphate headgroups using ^{31}P NMR spectroscopy and (iii) the effect of ciprofloxacin on the acyl chain order as a function of temperature using ATR-FTIR.

Steady-state anisotropy experiments showed that ciprofloxacin binds to the lipid vesicles tested with a stoechiometry of 1 and a moderate affinity (10^5 M^{-1}). This result is in agreement with the fair hydrophobicity of ciprofloxacin and derivatives at neutral pH [6]. A partial selectivity (DPPG>DOPC:DPPG (1:1)>DPPC>DOPC:DPPC (1:1)) was however evidenced. So, the greatest affinity observed with the negatively charged liposomes of DPPG or DOPC:DPPG reflected the potential role of electrostatic interactions between negatively charged phospholipid heads together with the H-bonding [12,32–34]. This is in agreement with the results from quasi-elastic light scattering which showed an effect of ciprofloxacin on the size of DPPG liposomes whereas no effect was observed with DPPC. This is also in accordance with the values of the energy of interaction as calculated by molecular modeling. These calculations are based on a semi-empirical force field [30] that can be compared to experimental values in terms of relative value (allowing a "ranking" of the different molecules in terms of preferential interaction) but not in terms of absolute value. The experimental interaction energy does not exactly correspond to calculated values of binding energy since (i) the calculated values depend on the energy force field and (ii) solvation, ionic strength, e.g. that contribute to the experimental measurements, are not taken into account in the calculations. Interestingly, even though the difference in the corresponding binding energy might be less than 1 kcal/mol, it might result from a compensating compromise between the enthalpy and the entropy of association as is often the case for biological systems and regulations. The preferential role of DPPG as compared to DPPC for the interaction between ciprofloxacin and lipids has been also showed with other fluoroquinolones like grepafloxacin [15] and ofloxacin [14] or with cyclic antimicrobial peptides [35,36]. However, in accordance with literature [11–13] and with calculations of the hydrophobic/hydrophilic environments of ciprofloxacin/lipid complex using the Molecular Hydrophobicity Potential (MHP), hydrophobic interactions could also play a critical role in the interaction between ciprofloxacin and lipids. Indeed, such computational analysis clearly revealed that most interactions are hydrophobic whatever the nature of the lipid selected (unpublished result). Because the main objective of the study is to shed light about the peculiar interactions between ciprofloxacin and DPPG, a lipid representative of bacterial membranes, on one hand and DPPC, a lipid mimicking the mammalian membranes, on the other hand, the relative contribution of electrostatic, hydrogen bonding, van der Waals interactions… has not been further investigated in this work.

The potential role played by the nature of the phosphate headgroup lead us to follow by ^{31}P NMR, the chemical shift anisotropy ($\Delta\sigma$) which depends on the local motions of the phosphodiester moiety and its orientation. Below their L_α-liquid-crystalline phase transition temperature, DPPG and DPPC in L_β-gel phase produce both an axially symmetric component and an asymmetric component required to fit their ^{31}P spectra. When the temperature is raised above the L_α-liquid-crystalline state phase transition value, the two static ^{31}P NMR components of the lipids collapse into a single axially symmetric contribution. This dynamically averaged ^{31}P NMR powder pattern results from axial rotation of the phosphodiester moiety about the bilayer normal, bond librations and overall lipid fluctuations and rotations [37]. Accordingly, the substantially smaller $\Delta\sigma$ of the L_α state compared to the L_β state is attributed to the considerable decrease in the correlation times of the ^{31}P headgroup motions by more than one to two orders of magnitude.

The $\Delta\sigma$ value obtained for DPPC is similar to that reported in the literature [38]. The lower value of the chemical shift anisotropy for DPPG as compared to DPPC could be due to the size of the main populations of DPPG liposomes (1375 nm) as compared to DPPC liposomes (2100 nm) together with the presence of a population smaller liposomes of DPPG (centered on 150 nm). Unfortunately, values obtained by quasi-light scattering showed a large variability from one measurement to another which may be accounted for by the fact that this type of measurement is strongly influenced by the presence of small number of large vesicles [39].

Because rapid motions might exist for high curvature regions or isotropic phases, therefore giving rise to a motionally averaged isotropic chemical shift [40], the increase of $\Delta\sigma$ values in the presence of ciprofloxacin in interaction with DPPG, and in a lesser extent with DPPC, suggested the appearance of such structures. Our results also reflect the location of the fluoroquinolone at the polar heads and near the lipid–water interface [6,13]. The results that we obtained for the interaction of ciprofloxacin with DPPC differ from those of Grancelli et al. [13]. The latter reported a remarkable decrease of the second spectral moment in solid state ^{31}P NMR above T_m without modifications below T_m, suggesting an enhancement of the mobility of the headgroups of DPPC above T_m. At 45 °C, we observed an increase of $\Delta\sigma$ reflecting a decrease in the local mobility of the phosphate groups or a reorientation of the phosphate moiety in the presence of the drug, in accord with the binding of the drug to lipids. This discrepancy has not yet received satisfactory explanations. A possibility could be found in the preparation of the MLVs, and in the way the ciprofloxacin is added to interact with lipids. This appeared as critical since when the DPPG MLVs were prepared with the ciprofloxacin, we observed a precipitation of the sample, which could be interpreted as revealing a very strong electrostatic interaction between the negative charge of the phosphate groups and the electric dipole moment of ciprofloxacin. When ciprofloxacin was added into the DPPG MLVs, the mixture didn't flocculate, suggesting the probable existence of a metastable state between both partners. This phenomenon wasn't observed in the case of DPPC MLVs which are electrically neutral and possess an electric dipole moment implying a softer electrostatic interaction with ciprofloxacin.

At first glance, the ^{31}P chemical shift anisotropy variation upon addition of ciprofloxacin into the DPPC and DPPG samples indicates strongly that the drugs interact with the headgroups of the phospholipids. Nonetheless, ^{31}P magnetic relaxation dispersion measurements would be necessary to describe more precisely the microscopic details of the interactions and to know whether or not all the lipid layers of the vesicles are involved and contact the ciprofloxacin molecules.

To investigate further the effect of ciprofloxacin in interaction with lipids on the melting temperature of either DPPC or DPPG, ATR-FTIR experiments were performed. Lipids can exist in several lamellar phases depending on the temperature. For saturated phosphatidylcholines, such as DPPC, there are four recognized lamellar phases; namely, a liquid-crystalline phase (L_α), and phases with ordered hydrocarbon chain arrangements: ripple phase (P_β); gel phase (L_β); and subgel or crystal phase (L_c) [41]. The phase transition in lipid bilayers involves a cooperative structural change from a state in which the lipids are closely packed and their chains fully extended, to a state in which a large fraction of the molecules exhibits as many *gauche* rotations per molecule.

The frequencies of the CH_2 stretching vibrations reflect the order of the acyl chains. Highly ordered acyl chains with an all-*trans* conformation as observed in the gel phase lead to lower vibrational frequencies. With increasing fractions of *gauche* isomers and decreasing van der Waals attractions in the liquid-crystalline phase the absorption maxima of the stretching bands will be shifted to higher frequency [42]. The results indicated that ciprofloxacin did not affect dramatically the DPPC melting curve. However, we noted that ciprofloxacin increased the order of the acyl chains both below and above the transition temperature. This is in agreement with the ability of ciprofloxacin to alter the tilt angle of the acyl chain of DPPC as demonstrated previously by ATR-FTIR [1]. The slight effect of ciprofloxacin on the order of the acyl chains of DPPC is in agreement

with the fact that neither iodobenzene nor iododecanoic acid, both known as hydrophobic quenchers, were able to quench the fluorescence of ciprofloxacin in the presence of DPPC liposomes [34]. In contrast, the effect of ciprofloxacin on DPPG is major. It was reasonable to suggest that ciprofloxacin decreased the ordering of the acyl chain of DPPG below the transition together with its ability to increase the order above this temperature. These observations were similar to those reported for cholesterol [43], a lipid well-known for broadening the gel to fluid transition temperature [44]. Moreover, our observations to compare the effect of ciprofloxacin on the frequencies of the CH_2 stretching vibrations of DPPC and DPPG are consistent with the higher quenching of ciprofloxacin by iodide, in DPPC:DPPG as compared to DPPC vesicles [45]. Surprisingly, however, no effect in steady-state polarization of TMA-DPH as a function of temperature was observed when ciprofloxacin was added to DPPC:DPPG as compared to the effect on DPPC [45]. Further investigations have to be done in this respect to explain this intriguing observation.

Globally speaking the major effect of ciprofloxacin on DPPG as compared to the one on DPPC confirmed the importance of the nature of the polar heads of phospholipids. The role played by the electrostatic interactions between ciprofloxacin and lipids has however to be confirmed using other negatively charged lipids than DPPG, like phosphatidylserine, cardiolipin, sulfatides, gangliosides [46]. The ability of ciprofloxacin to interact with lipids through hydrogen bonding and van der Waals interactions must also be investigated. This would explain the mode of insertion of ciprofloxacin within the lipids.

The binding parameters as determined by steady-state fluorescence anisotropy were correlated with the mobility of phosphate heads as determined by ^{31}P NMR and the acyl chain order as monitored by ATR-FTIR. Moreover, the increase of the area of DPPG at the lipid–water interface as evidenced by conformational analysis can be related to the local perturbations of the lipid bilayer observed by ^{31}P NMR which in turn may upset physiological properties of membranes.

All our results support the existence of a primary step in a binding of ciprofloxacin to the phospholipid bilayer surface and a possible accumulation in enriched domains formed by negatively charged lipids. This could allow the establishment of a concentration gradient that would promote the diffusion of the drug through the bilayer.

This work thus provides new molecular insights in the interaction between a fluoroquinolone antibiotic, as a drug model, and lipids mimicking those found in bacterial and eukaryotic membranes. Finally, these results suggest that biophysical studies combined with conformational analyses might be a powerful additional tool for the characterization of the interactions between antibiotics and lipids.

Acknowledgements

H.B. is a doctoral assistantship recipient of the Catholic University of Louvain (UCL), E.G and R.B. are Research Directors, F.V B. is Senior Research Associate and L.L. is Research Associate of the National Foundation for the Scientific Research (F.N.R.S.). The support of the *Région wallonne*, of the F.N.R.S., of the *Université catholique de Louvain* (*Fonds Spéciaux de Recherche, Actions de Recherche Concertées*), of the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme) is gratefully acknowledged. We also thank Bayer Healthcare AG for providing us with ciprofloxacin.

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