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Biochimica et Biophysica Acta 1374 (1998) 9–20

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a ^{1}H-NMR study

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Received 2 March 1998; accepted 11 May 1998

Abstract

Many drugs are weak bases and can be accumulated into liposomes in response to a pH gradient to achieve high internal drug concentrations. This study is aimed at gaining an understanding of the relationship between the retention of the fluoroquinolone antibiotic ciprofloxacin in liposomes and the intraliposomal form and location of this drug. ^{1}H-NMR spectroscopy was used to probe the interactions experienced by ciprofloxacin following uptake into large unilamellar liposomes (LUV). It is shown that ciprofloxacin is located in the aqueous interior of the liposomes and is self-associated in the form of small stacks. It does not precipitate out of solution even though the intraliposomal ciprofloxacin concentration can exceed its solubility in aqueous solutions by almost two orders of magnitude. The results also indicate that little entrapped ciprofloxacin partitions into the inner monolayer of the LUV. As a result of the lack of precipitation and rapid exchange properties, ciprofloxacin can respond quickly to changes in electrochemical equilibria such as depletion of the pH gradient. This provides a rationale for the rapid leakage of this drug in response to serum destabilization or depletion of the pH gradient. 0005-2736/98/$ – see front matter

Keywords: Nuclear magnetic resonance; Drug–membrane interaction; Fluoroquinolone; Transmembrane pH gradient; Drug loading; Solubility

1. Introduction

Liposomes are widely used as carriers for anticancer, antibiotic and antifungal drugs [1,2]. Encapsulation of drugs in liposomes can reduce their acute toxicity and may result in increased efficacy due to the ability of small, long-circulating liposomes to preferentially accumulate at the site of disease. Increased potency of antibiotics can occur as a consequence of the natural clearance of liposomes from the circulation by phagocytic cells, which are fre-

Abbreviations: LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; ΔpH, transmembrane proton gradient; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; chol, cholesterol; CHE, cholesteryl hexadecyl ether; CHES, 2-*N*-cyclohexylaminoethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; RCF, relative centrifugal force; d/l ratio, molar drug-to-lipid ratio (mol/mol)

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00125-4

quently the site where intracellular pathogens localize [3]. Ciprofloxacin, a synthetic quinolone derivative with activity against a broad spectrum of Gram-positive and Gram-negative bacteria, is one of the most widely used antibiotics for the treatment of respiratory, urinary and enteric infections [4,5]. Its bacterial action results from the inhibition of the enzyme DNA gyrase which is needed for the synthesis of bacterial DNA [6,7]. However, the use of liposomal carriers for ciprofloxacin is severely impaired by rapid leakage of this drug from liposomes.

Ciprofloxacin is one of a large number of drugs with weak base character which can be actively accumulated in liposomes in response to a pH gradient [8–10]. However, while some drugs such as the anticancer drug, doxorubicin, are only slowly released from the liposomal carrier in vitro or in vivo, other drugs including ciprofloxacin leak out rapidly. In order to improve these systems knowledge about the relationship between retention and the form and location of these drugs in liposomes is important. Encapsulated drugs could be partitioned in the membrane, reside in the aqueous interior as a precipitate or remain in solution. The nature and location of the drug in the liposome interior together with factors such as membrane permeability determine how quickly the encapsulated drug can respond to disturbances of thermodynamic equilibria such as depletion of the pH gradient.

Ciprofloxacin loaded into liposomes in response to transmembrane pH gradients has been suggested to be precipitated out in the liposome interior [11]. Reasons proposed for its rapid release in vitro and in vivo were larger solubility product than other drugs which are precipitated, other kind of drug association within the vesicles, pK values close to neutrality which results in higher fractions of the neutral membrane-permeable form or rupture of the liposomal membrane by growing crystals.

This study was aimed at achieving a better understanding of the factors which determine the retention of ciprofloxacin after accumulation into large unilamellar vesicles driven by a transmembrane proton gradient (ΔpH), where the ΔpH was generated in response to entrapped ammonium sulfate. Because of the sensitivity of NMR signals to changes in molecular environment and dynamics, the interaction of ciprofloxacin with itself and with phospholipid vesicles was investigated using ^{1}H-NMR spectroscopy. It is shown that ciprofloxacin is located in the aqueous interior of the liposomes in the form of stacks consisting of a small number of ciprofloxacin molecules. The drug is not precipitated although the intraliposomal ciprofloxacin concentration can exceed its solubility by orders of magnitude. This is the first time such a phenomenon has been observed in a liposomal system. Previous work has shown that other drugs such as doxorubicin can be entrapped at concentrations substantially higher than their aqueous solubility; however, this can be explained by precipitation or partitioning into the liposome membrane [9,12]. The high solubility of entrapped ciprofloxacin together with the rapid exchange into and out of the lipid membrane allow it to respond very quickly to changes in thermodynamic equilibria such as depletion of the pH gradient.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and palmitoyl-oleoyl PC (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL), and were $>99\%$ pure. Cholesterol (chol) and ammonium sulfate (AS) were obtained from Sigma (St. Louis, MO), and deuterium oxide (D_2O) and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) from Cambridge Isotope Laboratories (Andover, MA). [^{14}C]Cholesteryl hexadecyl ether (CHE) was custom synthesized by DuPont New England Nuclear (Boston, MA), [^{3}H]CHE, [^{3}H]methylamine, [^{14}C]sucrose and [^{3}H]glucose were from DuPont as well. Normal mouse serum was purchased from Cedar Lane Laboratories (Hornby, ON, Canada). Ciproflaxacin hydrochloride as well as [^{14}C]ciprofloxacin were a gift from INEX Pharmaceuticals (Vancouver, B.C., Canada).

2.2. Preparation of liposomes

DPPC/chol (55:45 mol/mol) liposomes were prepared by dissolving the lipids in t-butanol and lyophilizing for 6 h. POPC was directly used for the preparation of POPC liposomes. Multilamellar

vesicles (MLVs) were formed by hydrating the lipid with a 300 mM ammonium sulfate solution followed by five freeze–thaw cycles [13]. The lipid concentration was approximately 100 mM. The resulting MLVs were directly extruded 10 times through two stacked Nuclepore polycarbonate filters with pore sizes of 80, 100 and 200 nm to obtain LUVs of corresponding sizes. The size of the vesicles was measured by dynamic light scattering (Nicomp Particle Sizing Systems, Santa Barbara, CA). Average hydrodynamic diameters were found to be 115 ± 20 nm and 190 ± 30 nm for the DPPC/chol LUVs extruded through 100 and 200 nm filters, respectively, and 75 ± 20 nm and 155 ± 30 nm for POPC LUVs extruded through 80 and 200 nm filters. Lipid concentrations were determined using the phosphorus assay of Fiske and Subbarow [14].

2.3. Generation of the pH gradient

The ammonium sulfate gradient was generated by exchanging the extravesicular solution with 150 mM sodium chloride (NaCl) on G-50 Sephadex spin columns [15]. This concentration gradient drives the formation of a pH gradient of approximately 3 units. For the NMR experiments the spin columns had to be repeatedly washed with a 150 mM $NaCl/D_2O$ solution in order to remove residual ethanol in the Sephadex gel. The pH gradient was determined using [^{14}C]methylamine and was performed as previously described [16].

2.4. Uptake of ciprofloxacin into LUVs

Ciprofloxacin was added to these liposomes and the samples were incubated for 15 min at 60°C. Encapsulation efficiencies were determined as follows. Residual unentrapped drug was removed by size exclusion chromatography on Sephadex G50 spin columns and ciprofloxacin concentrations determined by scintillation counting using [^{14}C]ciprofloxacin or UV-absorption at 274 nm after a Bligh–Dyer extraction into 200 mM NaOH as aqueous phase [17]. Lipid concentrations were determined either by scintillation counting using the radiolabeled lipid [^{3}H]CHE or by the Fiske–Subbarow phosphorus assay. Typical encapsulation efficiencies for molar drug-to-lipid (d/l; mol/mol) ratios up to 0.6 were between 90% and 95%. The average size of the liposomes was not affected by the uptake of the drug as was shown by dynamic light scattering measurements.

2.5. Drug release experiments

In vitro release experiments were carried out in test tubes by diluting samples 1:1 with normal mouse serum and incubation at 37°C. The pH gradient was depleted by incubation in 150 mM ammonium acetate solution at 25°C. Samples at various time points were taken and concentrations of entrapped drugs were determined following removal of unentrapped drug by size exclusion chromatography as described above for drug uptake experiments.

2.6. Trapped volume determination

Trapped volumes of the liposomes were determined under the same conditions as for the drug uptake using radiolabeled sucrose or glucose as aqueous trap markers. 100 μmol DPPC/chol (55:45 mol%) were hydrated in 1 ml of a 300 mM ammonium sulfate, 10 mM sucrose or glucose solution spiked with 0.5 μCi [^{14}C]sucrose or [^{3}H]glucose, respectively, freeze–thawed and extruded as described above. The external solution containing unentrapped marker was exchanged against a 150 mM sodium chloride solution on Sephadex G50 spin columns. The lipid concentrations were determined by the phosphorus assay and the amount of trapped radioactive marker as well as total marker in the initial lipid dispersion by liquid scintillation counting. Trapped volumes were expressed as μl of trapped aqueous volume per μmol of total lipid. The internal volume of 200 nm DPPC/chol LUVs was 2.0 ± 0.1 μl/μmol lipid.

2.7. Solubility

Solubilities were determined at 25°C as a function of ammonium sulfate concentration and pH and in the presence of liposomes. For the concentration dependence, excess drug was added to 5 mM sodium acetate solutions containing the appropriate amounts of ammonium sulfate. If necessary the pH was adjusted to pH 5. DPPC/chol liposomes in 25 mM ace-

tate buffer at pH 5 were added to 10 mM ciprofloxacin in water. Lipid concentrations ranged from 5 to 20 mM. Subsequently ammonium sulfate was added to a concentration of 300 mM. It was not necessary to readjust the pH. The pH dependence was determined in 300 mM ammonium sulfate solutions which were titrated with NaOH to the appropriate pHs. The solutions contained 3 mM CHES, Hepes and citric acid to allow better pH adjustment. No attempt was made to keep the ionic strength constant since its increase by NaOH addition has no significant effect on the solubility at 300 mM ammonium sulfate. Subsequently the samples were equilibrated for 24 h at 25°C and the concentration of ciprofloxacin determined after centrifugation for 15 min at $3000 \times g$ in the supernatant by UV absorption at 274 nm, as described previously.

2.8. Binding studies

Binding studies were performed using a Beckmann TL-100 ultracentrifuge with a TLA 100.2 fixed-angle rotor. DPPC/chol 200 nm LUVs (2.5 mM lipid) were incubated in the presence of increasing concentrations of ciprofloxacin (0.25–10 mM) at 25°C for 2 h in 20 mM sodium acetate/100 mM NaCl (pH 5) and pelleted by ultracentrifugation for 90 min at a RCF_{max} of $436\,000 \times g$ (100 000 rpm). The supernatants as well as the lipid pellets were assayed for drug and lipid using the methods described above. The pellets were quickly rinsed with cold buffer and resuspended in H_2O. The amount of drug associated with the lipid pellets makes up a few percent ($\leq$3%) of the total drug. This, however, does not take the aqueous volume which is included in the pellets into account. Therefore, the actual amount of partitioned drug is even lower. The lipid in the supernatant was less than 5%. The level of association cannot be determined by assaying the drug in the supernatant using fluorescence or UV spectroscopy, as it corresponds to the accuracy of the measurements.

2.9. ^{1}H-NMR experiments

All salts used in the ^{1}H-NMR experiments were dissolved in D_2O and subsequently lyophilized to exchange protons against deuterons. This procedure was repeated once. Whenever D_2O was used as the solvent, pH-meter readings are reported rather than pD values (pD values can be derived by adding 0.4 units to the pH-meter reading [18]). The ^{1}H-NMR experiments were performed on a Bruker MSL 200 FT-NMR spectrometer operating at 200 MHz (47 T). If not otherwise mentioned, the temperature was maintained at 20°C. Typically, the pulse width was 2.5 μs (60° pulse) and the interpulse delay 3 s (the longest T_1 was 0.9 s). The digital resolution was 0.244 Hz/point with a spectral width of 2000 Hz. An exponential multiplication corresponding to 0.5 Hz line broadening was applied to the free induction decay prior to Fourier transformation. The chemical shifts are referenced to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The spin-lattice relaxation times, T_1, were measured by the inversion-recovery method (180°–τ–90° pulse sequence). In all T_1 experiments the incrementation of the recovery interval was nonmonotonic in order to avoid systematic errors. Peak heights were used for the calculation of T_1s. Assignments were made in accordance with ^{1}H-NMR spectra published elsewhere for ciprofloxacin and related fluoroquinolones [19–21]. The H_5 and H_8 protons were distinguished by the relative splitting of the signal. Due to its close proximity to the fluorine in position 6, H_5 was assigned to the signal with the larger coupling constant. The structure of ciprofloxacin is shown in Fig. 1. The central structural unit is a quinolone ring which has a fluorine atom at the 7-position, a piperazine moiety at the 6-position, a cyclopropyl ring at position 3 and a carboxyl group at position 1. The pK values are 6.0 for the carboxyl group and 8.8 for the 4′ nitrogen [22].

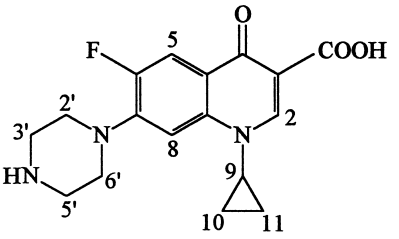

Fig. 1. Structure of ciprofloxacin. The central structural unit is a quinolone ring with a fluorine atom at the 7-position, a piperazine moiety at the 6-position, a cyclopropyl ring at position 1 and a carboxyl group at position 3. There are two pK values, $pK_{a,COOH} = 6.0$ and $pK_{a,N4'} = 8.8$.

2.10. Line-broadening calculations

In the following it is demonstrated how exchange of ciprofloxacin between the membrane and the aqueous phase can lead to significant broadening of the NMR signals, even though the membrane-associated fraction is very small. The exchange process can be described by equations which were derived for nuclear relaxation in solutions containing paramagnetic ions where exchange occurs between the coordination shell of the paramagnetic ion and the bulk solvent [23,24]. They were specifically applied to study the diffusion of water across lipid bilayers, where the internal and external water were distinguished by the presence of paramagnetic ions in either compartment [25,26]. The spin–spin relaxation is responsible for the line broadening ($\Delta\nu = 1/\pi T_2$). The observed relaxation times, T_2, depend on the population fractions and exchange rate ($1/\tau_{ex}$) according to:

$$T_2 = \frac{p_{aq}}{p_{mem}}(T_{2,mem} + \tau_{ex}) \quad (1)$$

where $T_{2,mem}$ is the relaxation time in the membrane in the absence of exchange, p_{aq} and p_{mem} the population fractions of ciprofloxacin in the aqueous phase and the membrane, and τ_{ex} the exchange time of ciprofloxacin between aqueous phase and membrane (or the average residence time of ciprofloxacin in the membrane) [26].

Although insufficient information is available concerning the parameters in Eq. 1 to calculate T_2, a lower limit can be estimated from a theoretical lower bound for $T_{2,mem}$. The lower limit for $T_{2,mem}$ is attained if the molecules enter the membrane in a certain state (orientation, conformational state) and exit the membrane without undergoing a change of state. In this case the NMR line shape can be approximated by a Gaussian function and the free induction signal is given by $FID(t) \sim \exp(-M_2^2 t^2/2) \sim \exp(-t^2/2T_{2,eff}^2)$ with an effective $T_{2,eff} \cong (1/M_2)^{1/2}$ [27]. Abragam [28] gives the following formula for the second moment M_{2j} of a spin j coupled by dipolar interactions to N spins k ($k = 1,2,...N$) having the same gyromagnetic ratio, γ, as spin j: $M_{2j} = 3/5\gamma^4\nu^2 I(I+1)\sum 1/r_{jk}^6$, where I is the spin of each nucleus and r_{jk} the distance from spin j to spin k [28]. $T_{2,eff}$ can be taken as a lower limit for T_2 in the membrane: $T_{2,mem} \geq T_{2,eff} = (1/M_2)^{1/2} = 2.3 \times 10^{-6} r$ (Å)^3 (for $r = 4$ Å, $T_{2,mem} \geq 1.5 \times 10^{-4}$ s and for $r = 2$ Å, $T_{2,mem} \geq 1.8 \times 10^{-5}$ s). Eq. 1 leads to the inequality $T_2 \geq (p_{aq}/p_{mem})T_{2,mem}$, thus yielding an upper limit to the predicted line broadening ($\Delta\nu = 1/\pi T_2$) due to the exchange process. Representative upper limits to the line width for $p_{mem} = 0.03$ are $\Delta\nu \leq 67$ Hz for $r = 4$ Å and $\Delta\nu \leq 549$ Hz for $r = 2$ Å, respectively. The majority of distances of neighboring protons in ciprofloxacin are within 3 Å (for a modeled three-dimensional structure of ciprofloxacin see NIH Three Dimensional Drug Structure Bank at http://cmm.info.nih.gov/modeling/drugbank.html).

3. Results

3.1. Ciprofloxacin is released rapidly from LUVs in the presence of serum

It has been demonstrated that ciprofloxacin can be accumulated to high concentrations inside liposomes in response to a transmembrane pH gradient [11,29,30]. This is illustrated in Fig. 2. Ciprofloxacin is rapidly accumulated into DPPC/chol LUVs at 60°C, with the uptake going to completion within 5 min. However, it is also rapidly released from the LUVs on incubation in 50% mouse serum at 37°C or depletion of the pH gradient at 25°C in 150 mM ammonium acetate (Fig. 2, right side). Serum proteins permeabilize the membrane, whereas ammonium acetate dissipates the pH gradient by equilibration of ammonia and acetic acid across the membrane. Ciprofloxacin is also released upon external addition of ammonium sulfate. From this it is evident that ciprofloxacin is able to respond rapidly to changes of thermodynamic equilibria.

3.2. Ciprofloxacin does not precipitate in the liposome interior

The concentration of ciprofloxacin inside liposomes can be very high (up to 300 mM). The solubility of ciprofloxacin was determined under the conditions prevailing inside the liposomes. The internal pH after uptake was found to be 4.9 ± 0.1 utilizing radiolabeled methylamine. Before uptake the internal ammonium sulfate concentration is 300 mM; how-

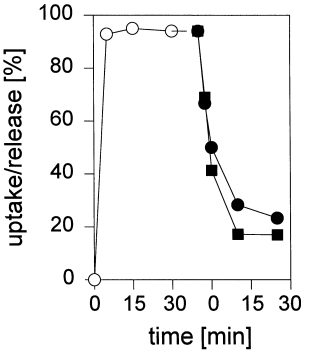

Fig. 2. Time course of a typical uptake of ciprofloxacin into 200 nm DPPC/chol LUVs at a d/l ratio of 0.3 and incubation at 60°C for 30 min (open circles). Ciprofloxacin was loaded using the ammonium sulfate gradient technique with 300 mM ammonium sulfate. The full symbols represent the release of ciprofloxacin from the LUVs in 50% mouse serum (full circles) and in 150 mM ammonium acetate (depletion of the pH gradient, full squares).

ever, the ammonium concentration is reduced by the amount of ciprofloxacin loaded into the LUVs during uptake. Fig. 3 shows the solubility of ciprofloxacin as a function of ammonium sulfate concentration at 25°C and pH 5, the inset describes the pH dependence in 300 mM ammonium sulfate solutions. The ciprofloxacin precipitate had the form of white needles. The presence of liposomes (5–20 mM DPPC/chol) had no significant effect on the solubility of ciprofloxacin in 300 mM ammonium sulfate solution at pH 5 and 3. The solubility at pH 5 and 300 mM ammonium sulfate is 5 mM and increases to about

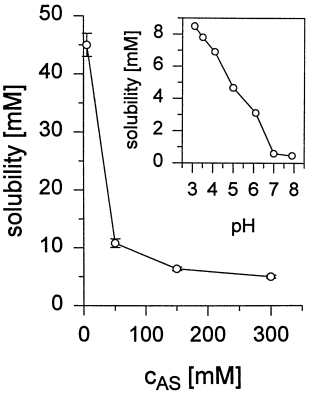

Fig. 3. Solubility of ciprofloxacin as a function of ammonium sulfate concentration at pH 5. The pH corresponds to the internal pH after uptake of ciprofloxacin into 200 nm DPPC/chol LUVs at a d/l ratio of 0.5. The inset gives the solubility in 300 mM ammonium sulfate solutions as a function of pH. Experiments were repeated three times.

8.5 mM at pH 3. Solubilities were slightly lower in D_2O than in H_2O (20–30%). The intraliposomal concentration of ciprofloxacin at a molar drug-to-lipid ratio of 0.5, 90–95% uptake and an internal volume of 2 μl/μmol lipid for 200 nm DPPC/chol LUVs is between 225–240 mM. This exceeds the solubility of free ciprofloxacin by almost 50-fold.

The ^{1}H-NMR spectrum of ciprofloxacin loaded into 200 nm DPPC/chol LUVs at a drug-to-lipid ratio of 0.5 is depicted in Fig. 4a. For comparison the spectra of the free drug at the same concentration as used for the uptake and that of empty DPPC/chol liposomes are included (Fig. 4b,c). Two features of the spectrum of encapsulated ciprofloxacin are prominent. First, all resonances are clearly visible though significantly broadened. The integrated intensities are within 10% of those of the same amount of free ciprofloxacin. Second, large upfield shifts of the aromatic proton resonances (H_2, H_5, H_8) as compared to free ciprofloxacin are observed. The small satellite peaks correspond to non-encapsulated ciprofloxacin in the external medium. The encapsulation efficiency was 90% with 10% external ciprofloxacin as determined using the procedures described under Section

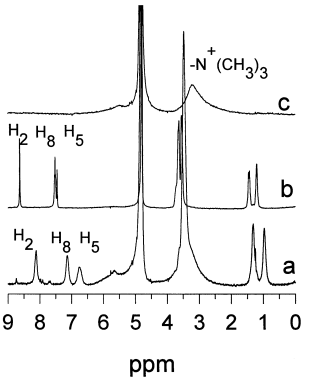

Fig. 4. (a) ^{1}H-NMR spectrum of ciprofloxacin loaded into 200 nm DPPC/chol LUVs (d/l = 0.5). Approximately 90% of drug is inside the liposomes. The rest is in the external medium represented by the small satellite peaks in the low field region. (b) ^{1}H-NMR spectrum of ciprofloxacin in 150 mM NaCl solution in the absence of liposomes at the same concentration as was used for the uptake above (c_{cipro} = 9 mM). The assignments of the aromatic resonances are given in the figure. For a full assignment of all proton resonances see Fig. 5A. The signals of protons H_5 and H_8 overlap. They can be distinguished by their relative splittings. H_5 has the larger coupling constant. (c) ^{1}H-NMR spectrum of 200 nm DPPC/chol LUVs (c = 40 mM). The trimethylammonium resonance of the lipid phosphocholine head group is indicated.

2. The solubility data predict that ciprofloxacin should precipitate inside the liposomes. Formation of crystals, however, would lead to disappearance of NMR resonances due to the line broadening associated with the immobilization of the molecules. Precipitation of free ciprofloxacin induced by increasing the pH of the solution results in a proportional loss of signal with no broadening of the lines.

3.3. Ciprofloxacin molecules self-associate into small stacks

Experiments were performed to demonstrate that the tendency of ciprofloxacin molecules to self-associate in small stacks in aqueous solution also occurs in the LUV interior. Before describing the experiments, the interactions affecting the NMR spectra are described briefly. The interactions of aromatic molecules with themselves in solution and their packing in crystals are governed by strong attractive interactions between the aromatic π-systems [31,32]. As a consequence of these π–π interactions, ciprofloxacin molecules form stacks in aqueous solutions as well as in crystals where the molecules lie on top of each other in an head-to-tail arrangement [19,33]. The self-association process in aqueous solution depends on concentration and is highly dynamic with molecules constantly associating and dissociating. Monomers exist only at concentrations below 1 mM. In NMR experiments, the ring currents associated with the circulation of the π electrons in the plane of the aromatic rings lead to a shielding of protons above and below this plane [34]. The signals of these protons, which in our case are located on neighboring molecules, will appear upfield shifted.

The extent of stacking increases with increasing concentration. This is illustrated in Fig. 5 for ciprofloxacin in D_2O by the increasing upfield shifts of the aromatic proton resonances. Spectra of ciprofloxacin in D_2O at 5 mM (bottom) and 115 mM (top) are depicted in Fig. 5A, whereas the relative changes of the chemical shift values as a function of concentration are plotted in Fig. 5B. The effect of ciprofloxacin concentration on the shifts of the non-aromatic proton resonances are comparatively small. The chemical shift values are listed in Table 1. The line widths are not significantly affected at any concentration. In these experiments the pH was not adjusted. It was shown elsewhere that below pH 5 there is practically no effect of pH on chemical shifts [21]. The highest pH in these experiments was 4.8.

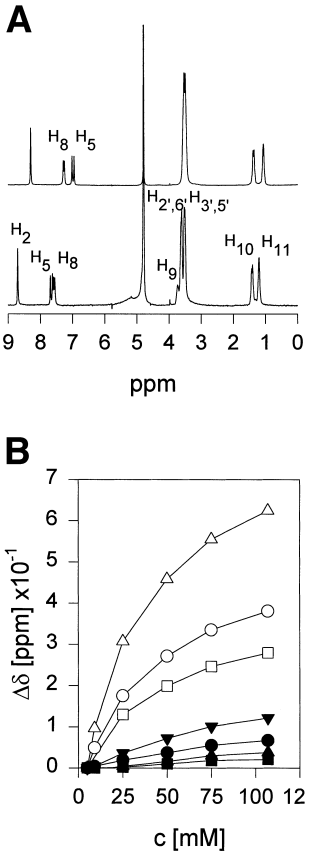

Fig. 5. Stacking of ciprofloxacin molecules with increasing concentrations demonstrated by the increased upfield shifts of the aromatic proton signals. The other resonances are much less affected. (A) ^{1}H-NMR spectra of ciprofloxacin in D_2O at 5 mM (bottom) and 115 mM (top). Spectra were normalized to the same height. (B) Changes of chemical shift values with concentration relative to 5 mM ciprofloxacin ($\Delta\delta = \delta_{5\text{ mM cipro}} - \delta_{x\text{ mM cipro}}$). Chemical shift values are listed in Table 1. The aromatic protons are represented by the open symbols, the closed symbols indicate the non-aromatic protons. H_2 (○), H_5 (△), H_8 (□), $H_{2'}$, $H_{6'}$ (●), $H_{3'}$, $H_{5'}$ (■), H_{10} (▲), H_{11} (▼).

Inside liposomes ciprofloxacin shows the same behavior as far as stacking is concerned. Increasing the drug-to-lipid ratio from 0.05 to 0.7 leads to increasing upfield shifts, the ring protons are affected most (Fig. 6, Table 2). The small satellite peaks again correspond to non-encapsulated ciprofloxacin. The shifts are much larger for encapsulated ciprofloxacin than for ciprofloxacin free in solution. This can be attributed to the much higher ciprofloxacin concen-

trations inside the liposomes (up to 300 mM). In addition, the different counterions, chloride for the concentration dependence of the drug alone and sulfate inside the liposomes, may affect the shifts slightly differently. A concentration series in ammonium sulfate solutions is not possible because of the low solubility of ciprofloxacin (5 mM in 300 mM ammonium sulfate at pH 5, see Fig. 4).

3.4. Interaction of ciprofloxacin with the lipid bilayer is responsible for the line broadening

The line widths of entrapped ciprofloxacin decrease with increasing concentration (see Fig. 6, Table 2). The broadest lines were obtained at the lowest drug-to-lipid ratio (d/l = 0.05) where the internal ciprofloxacin concentration is in the range of 25 mM. This is in contrast to the behavior of ciprofloxacin in solution where the line width did not change significantly with increasing concentration up to 115 mM which is just slightly less than its solubility in D_2O. The broadening also appears larger in POPC liposomes than in DPPC/chol LUVs (Table 2). External addition of ciprofloxacin to 200 nm POPC liposomes with no transmembrane pH gradient leads to significant line broadening (Fig. 7 and Table 2, d/l = 0.5 with 150 mM NaCl in- and outside). The line width

Table 1
Chemical shifts of ciprofloxacin alone in D_2O and ciprofloxacin taken up into 200 nm DPPC/chol and POPC LUVs

	H_2	H_8	H_5	$H_{2'}$,$H_{6'}$	$H_{3'}$,$H_{5'}$	H_{10}	H_{11}
A. Ciprofloxacin in D_2O[a]							
5 mM	8.721	7.580	7.662	3.618	3.528	1.422	1.209
9 mM	8.672	–	7.566	3.614	3.528	1.426	1.205
25 mM	8.545	7.450	7.355	3.599	3.526	1.418	1.173
50 mM	8.449	7.381	7.204	3.581	3.518	1.406	1.137
75 mM	8.386	7.334	7.107	3.563	3.510	1.392	1.108
107 mM	8.340	7.301	7.038	3.551	3.507	1.384	1.087
B. Uptake into 200 nm DPPC/chol LUVs							
d/l 0.05	8.447	7.350	–			1.405	1.124
d/l 0.25	8.165	7.162	6.810			1.332	0.998
d/l 0.5	8.112	7.116	6.718			1.308	0.960
d/l 0.7	8.085	7.106	6.690			1.300	0.942
C. Uptake into 200 nm POPC LUVs							
d/l 0.5	8.119	7.144	6.746				

[a]The coupling constants are 7.1 Hz for H_8, 13 Hz for H_5 and 6 Hz for H_{10}.

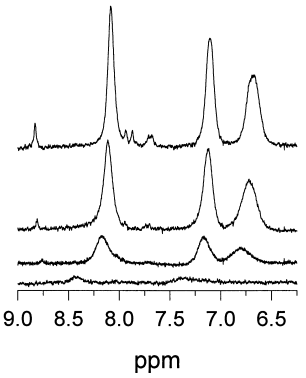

Fig. 6. ^{1}H-NMR spectra of ciprofloxacin loaded into 200 nm DPPC/chol LUVs at different d/l ratios. Only the downfield regions with the aromatic proton resonances are shown. The d/l ratios increase from the bottom to the top in the order 0.05, 0.25, 0.5 and 0.7. The small satellite peaks are drug in the external medium. The line widths are listed in Table 2.

of the H_2 proton increases with decreasing concentration much more than the others (not shown). The width of the water peak does not change in the presence of liposomes. Fig. 7a,c are the spectra of ciprofloxacin alone in 150 mM NaCl and ciprofloxacin loaded into POPC liposomes at the same d/l ratio. Therefore, the main contribution to the line broadening must arise from an interaction of ciprofloxacin with the liposomes.

3.5. Only a small proportion of ciprofloxacin partitions into the lipid bilayer

Binding studies performed by ultracentrifugation demonstrated that the amount of ciprofloxacin associated with DPPC/chol liposomes is less than 3% of the total amount of drug initially added (for the conditions see Section 2). This percentage was determined by assaying the lipid pellet and does not account for the drug in the aqueous volume included in the pellet. A determination in the supernatant was not possible since its accuracy corresponds to the level of association. Therefore, this 3% value represents an upper limit for the amount of drug associated with the lipid. This corresponds to a maximum partition coefficient, K_p, of 0.03, where $K_p = c_{mem}/c_{aq}$ with c_{aq} and c_{mem} being the concentrations of drug in the membrane and aqueous phase, respectively. These data clearly indicate that almost all ($\geq 97\%$) of the drug is located in the aqueous interior of the liposomes. This is also corroborated by integration

of the NMR spectra of free and entrapped drug (see above). A further quantitation was not attempted.

In addition, if significant amounts of drug were associated with the lipid effects on the lipid headgroup, proton resonances would be expected. The trimethyl ammonium group should be particularly affected by the ring currents associated with the aromatic rings of ciprofloxacin if significant amounts of ciprofloxacin reside in close proximity (<8 Å). Shifts between 10 Hz and more than 100 Hz have been found for benzylalcohol, indole-3-acetic acid, aromatic lipophilic drugs such as dibucaine as well as the surface potential probe ANS (1-anilino-8-naphthalenesulfonate) [35–38]. However, uptake of ciprofloxacin into 80 nm diameter POPC liposomes does not result in an upfield shift of the trimethyl lipid headgroup protons, even at a d/l ratio of 0.5. Addition of Pr^{3+} to the external medium separates the signal of the trimethyl protons of the inner monolayer from that of the outer by shifting the latter downfield [39]. The signal from the inner monolayer remained essentially unchanged (shifted upfield by 1–2 Hz) in the presence of entrapped drug.

The above data indicate that self-association and lipophilicity can be excluded as the source of line broadening for encapsulated ciprofloxacin. The most likely explanation involves rapid exchange of drug into and out of the membrane, where only a small fraction of drug actually resides in the membrane.

Table 2
Line widths (full width at half maximum), $\Delta\nu_{1/2}$ (Hz), of ciprofloxacin in solution, after uptake into 200 nm DPPC/chol and POPC LUVs and upon external addition to 200 nm POPC LUVs exhibiting no transmembrane proton gradient

	H_2	H_8	H_5	H_{10}	H_{11}
A. Ciprofloxacin free in solution					
5 mM	3.8	–	–	13	10.4
115 mM	3.8	12	16.6	13.6	10.9
B. Uptake into 200 nm DPPC/chol LUVs					
d/l 0.25	29.6	28.9	44.4	22.7	24.9
d/l 0.5	21.8	22.2	38.0	20.8	20.7
d/l 0.7	14.8	18.6	29.4	–	18.5
C. Uptake into 200 nm POPC LUVs					
d/l 0.5	24.4	32.6	35.9		
D. External addition of ciprofloxacin to 200 nm POPC LUVs					
d/l 0.1	37.8	28.8[a]			
d/l 0.25	23.8	26.6[a]			
d/l 0.5	15.2	23[a]			

[a] H_5 and H_8 overlap, $\Delta\nu_{1/2}$ is their combined width.

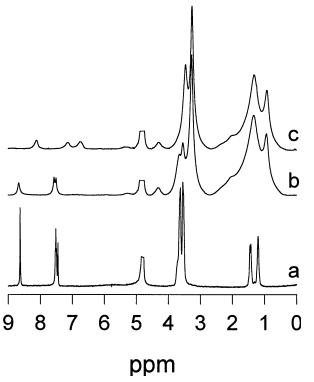

Fig. 7. ^{1}H-NMR spectra. (b) External addition of ciprofloxacin to 200 nm POPC LUVs in the absence of a transmembrane proton gradient (d/l = 0.5, c_{cipro} = 9 mM). (a) 9 mM ciprofloxacin in 150 mM NaCl solution. (c) uptake of ciprofloxacin into 200 nm POPC LUVs (d/l = 0.5, c_{cipro} = 9 mM).

4. Discussion

Many drugs, which are weak bases, can be accumulated to high concentrations in liposomes in response to a transmembrane proton gradient. However, uptake and retention properties are strongly drug dependent. For example, accumulation of drugs such as doxorubicin leads to high levels of drug uptake and excellent retention [8,9,12,40,41]. Alternatively, drugs such as ciprofloxacin exhibit poor retention properties. The interrelationship between the retention properties of ciprofloxacin loaded into LUVs and its nature and location inside liposomes is the subject of this study. The following discussion will focus on the anomalous solubility of ciprofloxacin and the lack of precipitation in the liposome interior, and second, the exchange of ciprofloxacin into and out of the lipid membrane with regard to NMR line-broadening effects.

The ciprofloxacin concentrations inside liposomes can exceed 250 mM. However, the drug does not precipitate inside the liposomes even though the solubility of ciprofloxacin in bulk aqueous solution under the same pH and ionic strength conditions as in the liposome interior is only 5 mM. Self-associa-

tion of ciprofloxacin molecules into small stacks is observed. The extent of stacking as indicated by the upfield shifts of the NMR lines is larger in the liposome interior due to the higher internal drug concentrations. The reason for the anomalously high solubility of ciprofloxacin in the LUV interior remains obscure. One possibility concerns the nature of the water in the LUV interior. It is known that the properties of water at interfaces and confined to small spaces are very different from that of bulk water. Water is highly structured at interfaces, with lower density and higher viscosity than bulk water [42,43]. The distances over which the water structure is affected is a matter of dispute, estimates range from 10–30 Å up to 1000 Å [44,45]. Anomalous properties of water were also reported in Sephadex gels where the internal water could dissolve significant amounts of water-insoluble dyes [46,47].

An alternative possibility is that the phospholipid surface may act as an inhibitor of crystallization in the same way as macromolecules exert specific control of mineralization processes [48–50]. Biomimetic studies of mineral formation in synthetic phospholipid vesicles have shown that the lipid composition has a dramatic effect on the precipitation kinetics and the size and location of the intravesicular precipitate [51]. For example, the rate and extent of precipitation of calcium phosphate in the interior of liposomes was extremely low in the presence of 10 mol% phosphatidic acid (PA), but not in the presence of other anionic phospholipids [52,53].

The results presented here indicate that almost all ($\geq 97\%$) of the encapsulated drug is located in the aqueous interior of the liposomes. Nevertheless, all ^{1}H-NMR resonances are considerably broadened with line widths up to 45 Hz as compared to 4–15 Hz for free ciprofloxacin (see Table 2). The decrease in line widths at high intraliposomal ciprofloxacin concentrations and the negligible effect of stacking on line widths rules out self-association and precipitation of ciprofloxacin as sources of broadening. However, exchange of ciprofloxacin between the membrane and the aqueous phase is a reasonable mechanism for the observed line broadening (see Section 2.10). According to this model, the NMR resonances are broader the higher the fraction of ciprofloxacin in the membrane and/or the 'faster' the exchange (see Section 2, Eq. 1). For water exchange between the bulk solution and the aqueous interior of DPPC SUVs it has been shown that τ_{ex} dominates the relaxation times at low temperatures [26]. The partition coefficient of small molecules for POPC membranes is higher than for DPPC/chol membranes [54–56]. This could explain why ciprofloxacin resonances are broader in POPC than in DPPC/chol liposomes. The fact that the line widths decrease with increasing internal ciprofloxacin concentrations can be explained by considering the change in relative population fractions. Reduced p_{mem} or higher p_{aq} lead to a decrease in line width. Increasing the total concentration of ciprofloxacin causes an increase in the fraction of drug in the aqueous phase when the membrane is saturated. Concomitantly, a second competing exchange process, the increased self-association of ciprofloxacin with increasing concentration, may contribute to reduce the amount of the exchangeable species, the monomer.

The high solubility of ciprofloxacin in the intraliposomal space and its ability to rapidly exchange into and out of a lipid membrane correlate well with the high tissue penetration of this drug and its accumulation within mammalian cells, factors which are responsible for the broad spectrum of activity of the fluoroquinolone antibiotics against extracellular as well as intracellular pathogens [57–59].

Finally, strategies to improve the retention of ciprofloxacin are discussed. While depletion of the pH gradient results in rapid leakage of ciprofloxacin from liposomes other drugs such as doxorubicin are retained much longer. Thus the effect of the pH gradient can be modulated by other parameters. Precipitation of drugs in the liposome interior as well as drug partitioning into the lipid bilayer have been identified as leakage retarding factors [9,12,41]. In a subsequent study we have shown that the retention of ciprofloxacin can be improved upon inclusion of negatively charged lipids into the LUV formulations consistent with the partitioning model [29,41].

In summary, ciprofloxacin does not precipitate inside liposomes following accumulation in response to a transmembrane pH gradient even though the internal drug concentration can be much higher than its solubility in the bulk aqueous phase. In addition, only a very small proportion of the entrapped drug can partition into the inner monolayer of the LUV. We suggest that the soluble nature of internalized

ciprofloxacin is directly related to the rapid leakage of the drug following changes in electrochemical equilibria.

Acknowledgements

This work was supported by the Medical Research Council of Canada. We would like to thank Myer Bloom for his help with the line-broadening calculations.

References

[1] P.N. Shek, Liposomes in Biomedical Applications, Harwood Academic Publishers, New York, 1995.
[2] G. Gregoriadis, Engineering liposomes for drug delivery: progress and problems, TIBTECH 13 (1995) 527–537.
[3] M.J. Hope, K.F. Wong, Liposomal formulation of ciprofloxacin, in: P.N. Shek, (Ed.), Liposomes in Biomedical Applications, Harwood Academic Publishers, New York, 1995, pp. 121–134.
[4] S.R. Norrby, Treatment of urinary tract infections with quinolone antimicrobial agents, in: D.C. Hooper, J.S. Wolfson (Eds.), Quinolone Antimicrobial Agents, American Society of Microbiology, Washington, 1993, pp. 273–283.
[5] B.E. Scully, Therapy of respiratory tract infections with quinolone antimicrobial agents, in: D.C. Hooper, J.S. Wolfson (Eds.), Quinolone Antimicrobial Agents, American Society of Microbiology, Washington, 1993, pp. 339–362.
[6] D.C. Hooper, J.S. Wolfson, Mechanism of quinolone action and bacterial killing, in: D.C. Hooper, J.S. Wolfson (Eds.), Quinolone Antimicrobial Agents, American Society of Microbiology, Washington, 1993, pp. 53–75.
[7] L.L. Shen, Quinolone–DNA interaction, in: D.C. Hooper, J.S. Wolfson (Eds.), Quinolone Antimicrobial Agents, American Society of Microbiology, Washington, 1993, pp. 77–95.
[8] T.D. Madden, P.R. Harrigan, L.C.L. Tai, M.B. Bally, L.D. Mayer, T.E. Redelmeier, H.C. Loughrey, C.P.S. Tilcock, L.W. Reinish, P.R. Cullis, The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey, Chem. Phys. Lipids 53 (1990) 37–46.
[9] P.R. Harrigan, K.F. Wong, T.E. Redelmeier, J.J. Wheeler, P.R. Cullis, Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients, Biochim. Biophys. Acta 1149 (1993) 329–338.
[10] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim. Biophys. Acta 1151 (1993) 201–215.
[11] D.D. Lasic, B. Ceh, M.C.A. Stuart, L. Guo, P.M. Frederik, Y. Barenholz, Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery, Biochim. Biophys. Acta 1239 (1995) 145–156.
[12] D.D. Lasic, P.M. Frederik, M.C.A. Stuart, Y. Barenholz, T.J. McIntosh, Gelation of liposome interior. A novel method for drug encapsulation, FEBS Lett. 312 (1992) 255–258.
[13] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.
[14] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[15] U. Pick, Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures, Arch. Biochem. Biophys. 212 (1981) 186–194.
[16] T.E. Redelmeier, L.D. Mayer, K.F. Wong, M.B. Bally, P.R. Cullis, Proton flux in large unilamellar vesicles in response to membrane potentials and pH gradients, Biophys. J. 56 (1989) 385–393.
[17] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[18] P.K. Glasoe, F.A. Long, Use of glass electrodes to measure acidities in deuterium oxide, J. Phys. Chem. 64 (1960) 188–190.
[19] C.M. Riley, D.L. Ross, D. van der Velder, F. Takusagawa, Characterization of the complexation of fluoroquinolone antimicrobials with metal ions by nuclear magnetic resonance spectroscopy, J. Pharm. Biomed. Anal. 11 (1993) 49–59.
[20] M. Helena, S.F. Teixeira, L.F. Vilas-Boas, V.M.S. Gil, F. Teixeira, Complexes of ciprofloxacin with metal ions contained in antacid drugs, J. Chemother. 7 (1995) 126–132.
[21] S. Lecomte, X. Tabary, N.J. Moreau, M.-T. Chenon, NMR investigation of pefloxacin–cation–DNA interactions 1. Influence of pH and concentration,, Magn. Reson. Chem. 34 (1996) 458–466.
[22] D.L. Ross, C.M. Riley, Dissociation and complexation of the fluoroquinolone antimicrobials – an update, J. Pharm. Biomed. Anal. 12 (1994) 1325–1331.
[23] T.J. Swift, R.E. Connick, NMR relaxation mechanisms of O^{17} in aqueous solutions of paramagnetic cations and the lifetime of water molecules in the first coordination sphere, J. Chem. Phys. 37 (1962) 307–320.
[24] Z. Luz, S. Meiboom, Proton relaxation in dilute solutions of cobalt(II) and Nickel (II) ions in methanol and the rate of methanol exchange of the solvation sphere, J. Chem. Phys. 40 (1964) 2686–2692.
[25] T. Conlon, R. Outhred, Water diffusion permeability of erythrocytes using an NMR technique, Biochim. Biophys. Acta 288 (1972) 354–361.
[26] J. Andrasko, S. Forsen, NMR study of rapid water diffusion across lipid bilayers in dipalmitoyl lecithin vesicles, Biochem. Biophys. Res. Commun. 60 (1974) 813–819.

[27] M. Bloom, H.S. Sandhu, NMR line-shape studies in methane using pulse techniques, Can. J. Phys. 40 (1962) 292–295.
[28] A. Abragam, The Principles of Nuclear Magnetism, Clarendon Press, Oxford, 1961.
[29] E. Maurer-Spurej, K.F. Wong, N. Maurer, D. Fenske, P.R. Cullis, Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles, Biochim. Biophys. Acta (1997) submitted.
[30] M.S. Webb, N.L. Boman, D.J. Wiseman, D. Saxon, K. Sutton, K.F. Wong, P. Logan, M.J. Hope, Antibacterial efficacy against an In Vivo Salmonella typhimurium infection model and pharmacokinetics of a liposomal ciprofloxacin formulation, Antimicrob. Agents Chemother. 42 (1998) 45–52.
[31] H. Porumb, The solution spectroscopy of drugs and drug–nucleic acid interactions, Prog. Biophys. Mol. Biol. 34 (1978) 175–195.
[32] C.A. Hunter, J.K.M. Sanders, The nature of π–π interactions, J. Am. Chem. Soc. 112 (1990) 5525–5534.
[33] I. Turel, K. Gruber, I. Leban, N. Bukovec, Synthesis, crystal structures, and characterization of three novel compounds of the quinolone family member norfloxacin, J. Inorg. Biochem. 61 (1996) 197–212.
[34] J.A. Pople, W.C. Schneider, H.J. Bernstein, High-resolution Nuclear Magnetic Resonance, McGraw-Hill, New York, 1959, pp. 180–183, 424–428.
[35] C.M. Colley, J.C. Metcalfe, The localisation of small molecules in lipid bilayers, FEBS Lett. 24 (1972) 341–346.
[36] G.L. Jendrasiak, T.N. Estep, The interaction of 1-anilino-8-naphthalene-sulfonate with lipids: a nuclear magnetic resonance study, Chem. Phys. Lipids 18 (1977) 181–198.
[37] G.P. Jones, A. Marker, L.G. Paleg, Complex formation between indole-3-acetic acid and phospholipid membrane components in aqueous media. 1. Parameters of the system, Biochemistry 23 (1984) 1514–1520.
[38] G.L.E. Erikson, Interaction of charged amphiphilic drugs with phosphatidyl-choline vesicles studied by NMR, Biophys. Chem. 26 (1987) 9–18.
[39] M.S. Fernandez, J. Cerbon, The importance of the hydrophobic interactions of local anesthetics in the displacement of polyvalent cations from artificial lipid membranes, Biochim. Biophys. Acta 298 (1973) 8–14.
[40] B. Ceh, D.D. Lasic, A rigorous theory of remote loading of drugs into liposomes, Langmuir 11 (1995) 3356–3368.
[41] P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, D.B. Fenske, Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles, Biochim. Biophys. Acta 1331 (1997) 187–211.
[42] D.E. Woessner, Relaxation theory with applications to biological studies, in: J.W. Pettegrew (Ed.), NMR: Principles and Applications to Biomedical Research, Springer, New York, 1990, pp. 37–67.
[43] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning, Marcel Dekker, New York, 1995, pp. 50–74.
[44] F.M. Etzler, W. Drost-Hansen, Bioelectrochemistry: Ions, Surfaces, Membranes, Advances Chem. Series 180, Am. Chem. Soc., Washington, 1980, pp. 485–497.
[45] R.P. Rand, V.A. Parsegian, Hydration forces between phospholipid bilayers, Biochim. Biophys. Acta 988 (1989) 351.
[46] M. Janado, K. Takanaka, H. Nakamori, Y. Yano, Solubilities of water-insoluble dyes in internal water swollen Sephadex gels, J. Biochem. 87 (1980) 57–62.
[47] J. van Steveninck, M. Paardekooper, T. Dubbelman, E. Ben-Hur, Anomalous properties of water in macromolecular gels, Biochim. Biophys. Acta 1115 (1991) 96–100.
[48] L. Addadi, S. Weiner, Stereochemical and structural relations between macromolecules and crystals in biomineralization, in: S. Mann (Ed.), Biomineralization. Chemical and Biochemical Perspectives, VCH, Weinheim, Germany, 1989, pp. 133–156.
[49] S. Mann, Crystallochemical strategies in biomineralization, in: S. Mann (Ed.), Biomineralization. Chemical and Biochemical Perspectives, VCH, Weinheim, Germany, 1989, pp. 35–62.
[50] H. Furedi-Milhofer, S. Sarig, Interactions between polyelectrolytes and sparingly soluble salts, Prog. Cryst. Growth Char. 32 (1996) 45–74.
[51] B.R. Heywood, Biomineralization: New directions in crystal science, Microsc. Res. Technique 27 (1994) 376–388.
[52] B.R. Heywood, E.D. Eanes, An ultrastructural study of the effects of acidic phospholipid substitutions on calcium phosphate precipitation in anionic liposomes, Calcif. Tissue Int. 50 (1992) 149–156.
[53] E.D. Eanes, Mixed phospholipid liposome calcification, Bone Mineral 17 (1992) 269–272.
[54] J. de Gier, J.G. Mandersloot, J.V. Hupkes, R.N. McElhaney, W.P. van Beek, On the mechanism of non-electrolyte permeation through lipid bilayers and through biomembranes, Biochim. Biophys. Acta 233 (1971) 610–618.
[55] M. Luxnat, H.J. Galla, Partition of chlorpromazine into lipid bilayer membranes: the effect of membrane structure and composition, Biochim. Biophys. Acta 856 (1986) 274–282.
[56] S.E. Wright, J.C. White, L. Huang, Partitioning of teniposide into membranes and the role of lipid composition, Biochim. Biophys. Acta 1021 (1990) 105–113.
[57] P.M. Tulkens, Intracellular distribution and activity of antibiotics, Eur. J. Clin. Microbiol. Infect. Dis. 10 (1991) 100–106.
[58] D.J. Winston, Use of quinolone antimicrobial agents in immunocompromised patients, in: D.C. Hooper, J.S. Wolfson (Eds.), Quinolone Antimicrobial Agents, American Society of Microbiology, Washington, 1993, pp. 435–471.
[59] D. Greenwood, Synthetic antibacterial agents and miscellaneous antibiotics, in: D. Greenwood (Ed.), Antimicrobial Chemotherapy, Oxford University Press, Oxford, 1995, pp. 54–55.