Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery [1]

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Abstract

Phase transitions in closed vesicles, i.e., microenvironments defined by the size of the vesicle, its contents, and permeability of its membrane are becoming increasingly important in several scientific disciplines including catalysis, growth of small crystals, cell function studies, and drug delivery. The membrane composed from lipid bilayer is in general impermeable to ions and larger hydrophilic ions. Ion transport can be regulated by ionophores while permeation of neutral and weakly hydrophobic molecules can be controlled by concentration gradients. Some weak acids or bases, however, can be transported through the membrane due to various gradients, such as electrical, ionic (pH) or specific salt (chemical potential) gradients. Upon permeation of appropriate species and reaction with the encapsulated species precipitation may occur in the vesicle interior. Alternatively, these molecules can also associate with the leaflets of the bilayer according to the transmembrane potential. Efficient liposomal therapeutics require high drug to lipid ratios and drug molecules should have, especially when associated with long circulating liposomes, low leakage rates. In this article we present very efficient encapsulation of two drugs via their intraliposomal precipitation, characterize the state of encapsulated drug within the liposome and try to fit the experimental data with a recently developed theoretical model. Nice agreement between a model which is based on chemical potential equilibration of membrane permeable species with experimental data was observed. The high loading efficiencies, however, are only necessary but not sufficient condition for effective therapies. If adequate drug retention within liposomes, especially in the case of long-circulating ones, is not achieved, the therapeutic index decreases substantially. Anticancer drug doxorubicin precipitates in the liposome interior in a form of gel with low solubility product and practically does not leak out in blood circulation in the scale of days. With an antibiotic, ciprofloxacin, the high loading efficacy and test tube stability is not reproduced in in vitro plasma leakage assays and in vivo. We believe that the reasons are higher solubility product of precipitated drug in the liposome, larger fraction of neutral molecules due closer pK values of the drug with the pH conditions in the solutions and high membrane permeability of this molecule. High resolution cryoEM shows that encapsulated anticancer agent doxorubicin is precipitated in the form of bundles of parallel fibers while antibiotic ciprofloxacin shows globular precipitate. Doxorubicin gelation also causes the change of vesicle shape.

Keywords: Vesicle; Gradient loading; Microreactor; Phase transition; Drug encapsulation; Stability; Intraliposomal precipitation; Doxorubicin gel

1. Introduction

Vesicles are becoming, in addition to their medical applications, increasingly important systems to study various physico-chemical, catalytic, and biological phenomena [1]. As a model system in biology they can serve as a two-dimensional solid or fluid matrix to study membrane proteins, biomineralization, cellular transport and inter/intracellular traffic. Recently spontaneous phase transition in the aqueous phase within vesicles were observed due to enhanced accumulation of amphiphilic weak bases into vesicles by the force of transmembrane chemical potential gradient. Precipitation in vesicle interior can be also induced by inclusion of various ionophores in the bilayer and can offer a convenient system to study the mechanism of various crystallizations, biomineralizations, tissue calci-

fication or in the preparation of colloid-sized microcrystals. Of course, it is also extremely important in drug delivery where a stable drug encapsulation is a necessary condition in most applications [1–4].

One of the main obstacles in the development of liposomal therapeuticals is their poor loading capacity, especially in the predominantly used small vesicles (< 100 nm), and stability of the encapsulated drug [1,2]. There are several techniques, such as preparation of liposomes at very high lipid concentrations where they encapsulate most of the space, which enable high loading efficiencies but in most cases they are hampered by poor entrapment stability, i.e., quick release of the encapsulated molecules, and problems with down sizing of vesicles without releasing drug. The problem is still amplified by the use of long circulating liposomes [5,6] which circulate in patients up to a week, in contrast to few (tens of) minutes of most conventional liposome formulations. In addition, upon infusion into human body liposomes can be diluted several 100-fold which makes all the comparisons with most in vitro tests, such as plasma induced leakage, and toxicity/efficacy studies in mice, where dilutions are approx. 5-fold absolutely unrealistic [2,3] because due to simple solution thermodynamics this results in quick reequilibration which can release most of the encapsulated or membrane bound/embedded molecules within microseconds.

In a preliminary report we have presented a concept of drug precipitation in the liposome interior and via an example of drug gelation showed its strongly improved stability of encapsulation [7]. In this article we would like to further characterize the gel formed in liposomes as well as describe another system in which the encapsulated drug is precipitated, as we believe, in a crystalline state. In both cases we used the ammonium sulfate gradient method [8,9] for the loading of vesicles. Concentration driven accumulation of anticancer drug doxorubicin in the liposome interior resulted in the formation of a gel while antibiotic ciprofloxacin precipitated in similar conditions. The stability of encapsulation of this drug was found to be lower than the one exhibited by doxorubicin. Several possible reasons will be given.

A theoretical model which was introduced elsewhere in detail [10] was employed to explain the loading of these two drugs. Very good agreement with experimental data was found.

2. Experimental

2.1. Liposome preparation

In order to be able to follow in vivo stability of the encapsulated drug we have used in most studies sterically stabilized liposomes with the following composition: ^{2000}PEG-DSPE/HSPC/Chol = 1:3:1 (w/w) (PEG, polyethylene glycol; DSPE, distearoyl phosphatidylethanol-

amine; HSPC, hydrogenated soy phosphatidylcholine; Chol, cholesterol). Upon hydration of dry lipid film in 250 mM ammonium sulfate, pH 5.5 (unbuffered), (or ethanol injection followed by diafiltration/dialysis) multilamellar liposomes were extruded/microfluidized to a final size of 80–90 nm [1,5]. External ammonium sulfate was replaced with 10% sucrose, pH 5.5 (unbuffered) by dialysis.

2.2. Loading of liposomes

These liposomes at 50 μmol/ml were mixed with doxorubicin HCl (DOX) solution at 6 mg/ml in isotonic sucrose equivolumetrically. After 1/2 to 1 h incubation at 60°C preparations were cooled to room temperature. If necessary, free drug was removed by ion exchange or gel chromatography. The final preparation was diluted to 20 mM total lipid and doxorubicin concentration of 2 mg/ml.

For active loading of ciprofloxacin (CIP) the above liposomes were mixed with several different concentrations of ciprofloxacin HCl solutions, as indicated in the figure caption, in 10% sucrose. The concentration of liposomes used during the incubation was approx. 50 mM total lipid and the pH of the unbuffered drug solution ranged between 4–4.5. The percent of drug loading was determined spectrophotometrically after separating the free-form from liposome encapsulated-drug by using a Sephadex G-50 column. For large scale preparations, free drug molecules were removed by diafiltration using a hollow fiber cartridge (Amicon). Some samples were prepared in glycine buffer at pH 3.5 without noticeable differences in loading and stability.

2.3. Liposome leakage assays

Test tube stability assay simply consists of measuring the fraction of nonencapsulated drug by separating it from liposomal drug on a desalting column (Pierce 1 × 5 cm) for DOX or G-50 column (1 × 30 cm) for CIP and measuring drug spectrophotometrically in total volume (free drug) and encapsulated drug in the void volume after lysis of liposomes. Dilution induced leakage (DIL) assay consists of diluting liposomes to a specified concentration, (normally a 100-fold dilution), incubation at 37°C for specified time (normally 1 and 24 h) and repeating the encapsulation efficiency assay as described above. Plasma induced leakage (PIL) is equivalent to DIL with exception that 50% plasma is normally used as a dilution medium.

2.4. Small angle X-ray scattering

For SAXS measurements liposomes were pelleted at 240000 rpm ($10^5 \times g$) for 12 h and the pellet was analyzed. In a control experiment, prolonged rocking (1 day) of the pellet resulted in vesicles with equivalent size distribution as before the centrifugation. HSPC/Chol liposomes did not disperse after equivalent treatment.

2.5. Solid phases

Dox-SO$_4$ and Dox-Cl gel: a concentrated sodium sulfate/chloride solution was added to DOX solution in water to a final concentration [Dox] = 10–30 mg/ml, and [SO$_4$,Cl] = 5–250 mM. Heterogeneous gel – a mixture of solid and fluid phases – was heated to 60–70°C. It melted and a homogeneous solution upon cooling solidified into a homogeneous solid. In contrast to sulfate gels which were orange-red and opaque, Cl-gels were dark red and transparent.

CIP-SO$_4$ precipitate: to an aqueous solution of ciprofloxacin HCl concentrated sodium sulfate was added. The mixture was centrifuged at 3000 rpm for 1/2 h and the precipitate was used for SAXS and EM studies. In contrast to doxorubicin gels this precipitate was birefringent and consisted of very long and thin needles, as observed in optical microscope.

Liposomes as well as model gels were studied by several different physico-chemical methods. Cryoelectron microscopy and small angle X-ray scattering measurements were performed as described previously [7,11,12]. Negative stain microscopy was performed by using uranyl acetate.

3. Theory of loading

In contrast to the pH gradient method which can be approximated by Hendersohn-Hasselbalch equation [10,13–16] the loading in the case of ammonium sulfate gradient is a much more complex process.

The rigorous theory of loading of drugs into liposomes, which takes into account all acid/base equilibria in both interacting compartments as well as possible binding of particular species onto inner/outer bilayer surface and the formation of the precipitate inside the liposomes, was described elsewhere [10]. Briefly, two basic assumptions were applied to develop the theoretical treatment of interacting inner and outer solutions in liposome systems. Firstly, concentration gradients of permeable species represent a driving force for permeation of molecules resulting in their concentration equilibration on both sides of the bilayer. Secondly, the permeation of all charged species is assumed to be negligible. This enabled the development of a set of Master Equations, derived from chemical equilibria equations of each species, mass and charge balance, with which concentration of any species inside or outside of the liposomes can be calculated. The equilibrium state in the liposome suspension, i.e., the concentrations of all species, including charged and noncharged forms of drug molecules in either compartment, can be described by three nonlinear x, y and R^p dependant equations, where x and y are concentrations of protons inside and outside of liposomes, respectively, and R^p is the ratio between amounts of immobilized and dissolved drug. These equations take into account all possible initial chemical parameters in both compartments (outer/inner volume ratio, concentration of acids/bases/salts/buffers, their acidity constants (pK_a values) and chemical composition as well as solubility product of the precipitated form of the drug or binding constants of membrane adsorbed drugs). One can solve this system of equations by the Newton-Raphson method and within a particular parameter range calculate values like loading efficiency (percentage of drug molecules inside the liposomes), inner and outer pH, concentration of particular species in either compartment, exchanged amount ratio (influx/efflux), etc. Therefore, the effect of each of the parameters on the behavior of liposome systems can be studied in the form of different diagrams in which loading efficiencies, internal or external pH, etc. are calculated for different values and combinations of all of these parameters. The mathematical formalism is explained in details in a paper by Čeh and Lasic, in which theoretical curves, such as loading efficiency, inner or outer pH as a function of liposome, drug, and buffer concentrations, pK of the drug and similar, for various sets of parameters are shown [10].

Assuming the activity coefficients are equal to 1 (dilute solutions), the particular examples of loading of the CIP and DOX into the liposomes can be defined by the following three equations (for details see Ref. [10]). Master Equation describing the state of the outer solution is in this particular system, as obtained from the general equation [10]:

$$c_1 \frac{1 - K_{11}K_{12}/y^2}{K_v^{-1}\left(\dfrac{x}{y}\right)(1 + R^p)(1 + K_{11}/x + K_{11}K_{12}/x^2) + \left(1 + K_{11}/y + K_{11}K_{12}/y^2\right)}$$

$$+ 2c_3 \frac{1}{\left(\dfrac{x}{y}\right)(1 + K_{31}/x) + K_v(1 + K_{31}/y)} + c_2 \frac{1 - K_{21}K_{22}/y^2}{1 + K_{21}/y + K_{21}K_{22}/y^2} + \left(y - \frac{K_w}{y}\right) - ac_2 - c_1 = 0 \qquad (1)$$

The state of the inner solution is described by an analogous Master Equation:

$$c_1 \frac{1 - K_{11}K_{12}/x^2}{K_v^{-1}\left(1 + K_{11}/y + K_{11}K_{12}/y^2\right)(1 + R^p) + \left(\frac{y}{x}\right)\left(1 + K_{11}/x + K_{11}K_{12}/x^2\right)}$$

$$\times \left(1 - \left(\frac{1}{2}\right)R^p \frac{\left(1 + K_{11}/x + K_{11}K_{12}/x^2\right)(-1 - 2K_{42}/x)}{\left(1 - K_{11}K_{12}/x^2\right)(1 + K_{42}/x)}\right) + c_3 \frac{-1 - 2K_{42}/x}{1 + K_{42}/x}$$

$$+ 2c_3 \frac{1}{(1 + K_{31}/x) + K_v\left(\frac{y}{x}\right)(1 + K_{31}/y)} + \left(x - \frac{K_w}{x}\right) = 0 \qquad (2)$$

and for the solubility product one gets

$$L_p = \left[\frac{c_1}{K_v^{-1}\left(1 + K_{11}/x + K_{21}/x^2\right)(1 + R^p) + \left(\frac{y}{x}\right)\left(1 + K_{11}/y + K_{21}/y^2\right)}\right]^2$$

$$\times \left[\left(c_3 - \left(\frac{1}{2}\right)c_1 R^p \frac{\left(1 + K_{11}/x + K_{21}/x^2\right)}{K_v^{-1}\left(1 + K_{11}/x + K_{11}K_{21}/x^2\right)(1 + R^p) + \left(\frac{y}{x}\right)\left(1 + K_{11}/y + K_{11}K_{21}/y^2\right)}\right)\left(\frac{K_{42}}{K_{42} + x}\right)\right] \qquad (3)$$

Here K_v is the ratio of nonencapsulated versus the encapsulated volume, c_1 is the initial concentration of the drug (CIP, DOX), c_2 the starting concentration of the glycine (only in some cases of CIP loading; in the case of DOX loading $c_2 = 0$), both of them in the outer solution, c_3 is the starting concentration of the ammonium sulfate in the liposomes and a the portion of hydrochloric acid with regards to glycine (for adjustment of the outer pH, in our case 7.5%). The corresponding pK_{ij} values are: for DOX $pK_{11} = 8.2$, $pK_{12} = 10.2$; for CIP, $pK_{11} = 6.0$, $pK_{12} = 8.8$, for glycine, $pK_{21} = 2.34$, $pK_{22} = 9.6$, for ammonia, $pK_{31} = 9.26$, and for sulfuric acid, $pK_{42} = 1.92$. The solubility product for $(CIPH)_2SO_4$ and for $(DOXH)_2SO_4$ were found to be $8.0 \cdot 10^{-6}$ and $1.1 \cdot 10^{-7}$, respectively. Due to the fact that corresponding pH_i/pH_o (inner/outer) pairs, calculated from the Eqs. (1)–(3) in both of the treated systems, are much lower than all the pK_a values, except the first one of glycine and second from the sulfuric acid, the Eqs. (1)–(3) may be reduced to considerably simpler expressions Eqs. (4)–(6). Therefore, for the outer compartment one can obtain the following equation:

$$c_1 \frac{1}{K_v^{-1}\left(\frac{x}{y}\right)(1 + R^p) + 1} + 2c_3 \frac{1}{\left(\frac{x}{y}\right) + K_v} + c_2 \frac{1}{1 + K_{21}/y} + y - ac_2 - c_1 = 0 \qquad (4)$$

where a represents the fraction of buffered glycine (i.e., to lower the pH of solution one has to add HCl, in our case $a = 3/40 = 0.075$). Then, for the inner compartment:

$$c_1 \frac{1}{K_v^{-1}(1 + R^p) + \left(\frac{y}{x}\right)}\left(1 - \left(\frac{1}{2}\right)R^p \frac{-1 - 2K_{42}/x}{1 + K_{42}/x}\right) + c_3 \frac{-1 - 2K_{42}/x}{1 + K_{42}/x} + 2c_3 \frac{1}{1 + K_v\left(\frac{y}{x}\right)} + x = 0 \qquad (5)$$

and finally for the solubility product:

$$L_p = \left[\frac{c_1}{K_v^{-1}(1 + R^p) + \left(\frac{y}{x}\right)}\right]^2\left[\left(c_3 - \left(\frac{1}{2}\right)R^p c_1 \frac{1}{K_v^{-1}(1 + R^p) + \left(\frac{y}{x}\right)}\right)\left(\frac{K_{42}}{x + K_{42}}\right)\right] \qquad (6)$$

Using these equations, as indicated in the discussion and figure captions, we have calculated the loading efficiencies of doxorubin and ciprofloxacin for experimental conditions used in their preparation.

4. Results and discussion

Fig. 1 shows cryoelectron micrographs of doxorubicin and ciprofloxacin loaded in sterically stabilized liposomes, respectively. In the first case one can see the periodic structure of fibrilar gel while in the second one some globular precipitates/crystallites can be observed in the liposome interior.

Although in vitro gel samples could not achieve as high DOX concentrations as in liposome interior (> 0.2 M, which is more than 5-fold aqueous solubility due to the active pumping mechanism), the gel structures look rather similar. Detailed studies (Fig. 2a) of Dox-sulfate gel have shown fibers with diameters of 28 Å interwoven into bundles. Local symmetry appeared to be the same as in liposomes (Fig. 1a). The same periodicity was observed also by negative stain (Fig. 2b). Fibers were also observed in chloride-gel, but in contrast to the sulfate gel the fibers are not aligned. Cl-gel seems to be composed of a random three-dimensional network of thin, separated fibers and this can explain its transparency (Fig. 2c).

Equivalent periodicity of 28 Å in the DOX-sulfate gel in vitro as well as in liposomes was also observed by SAXS [7]. It seems that the periodicity reflects the distance between aligned fibers within a bundle and is proportional to the thickness of the fibers. It is possible that fibers represent stacks of doxorubicin molecules connected by mono/bi dentate sulfate bridges. Such structures are common in liquid crystals. The fibers may be bound together by H-bonds (see below). A weak helical twist with a pitch of approx. 1000 Å can be observed. Chloride gel and dense doxorubicin solution (at 20 mg/ml, self-aggregation starts at < 0.5 mg/ml) did not yield any reflections. In contrast to doxorubicin containing liposomes, the ones loaded with ciprofloxacin do not show any X-ray reflections. This may indicate either amorphous nature of the precipitate or that the size of the crystalline particles is too small to give constructive diffraction. Macroscopic observation of CIP crystallization in optical microscope showed very quick growth of very long (millimeters), thin (micrometers) and rigid fibers. Because doxorubicin, in contrast, gave nonbirefringent (i.e., optically isotropic) gels we believe that precipitate is in crystalline form. From EM observations we can understand optical isotropy by a network of too thin fibers to interact with the wavelength of visible light.

The microscopic similarity between test tube precipitate and intraliposome precipitate was not reproduced in the ciprofloxacin liposomes. Fig. 2d shows test tube precipitate.

In the test tube investigations sulfate and chloride gels exhibited well defined phase transition solid–fluid as could be observed by naked eye in a temperature bath. The temperature of the fluidization/solidification depends on the concentrations of both ions, pH and was in the range from 40–70°C. Higher concentrations of either component

resulted in higher transition temperature. Phase transition exhibited large hysteresis (10–20°C, depending on the cooling rate) upon cooling. DSC measurements with Perkin Elmer model 7 DSC however, did not show any phase transition. This is possibly due to very weak H bonds which hold the gel network together. No phase transition was observed also in the liposome sample. The absence of the bilayer phase transition can be explained by high amount of cholesterol. Visual observations as well as simple qualitative mechanical tests showed that gel was the hardest around pH 5.5–6.

The origin of peculiar vesicle shape change upon encapsulation of doxorubicin is still not clear. It is possible that the new morphology is due to the osmotic imbalance upon precipitation or simply due to mechanical interaction of gel fibers with the membranes. Equivalent lipid composition and efficient loading of ciprofloxacin at unchanged shapes, however, may indicate the second possibility.

We should note that doxorubicin formulations using ammonium sulfate gradient are much more stable with respect to the leakage of drug than the ones with pH gradient in liposomes with similar/equivalent membrane composition [7,8,17,18]. The reasons are the formation of a stable precipitate, pH in the liposomes (not only lipids and therefore their permeability barrier properties and the stability of the entrapment but also gel is the most stable in the pH region between 5 and 6), and the differences in the membrane permeability which can be scaled down to the difference between escape rate of H^+ and SO_4^{2-} (approximately seven orders of magnitude difference [1]). Also, the reservoir of protons is much larger in the case of the ammonium sulfate gradient method.

High membrane permeability of antibiotic ciprofloxacin was inferred from the loading experiments in which the drug could be loaded into liposomes already at 30°C (Fig. 3). In contrast, doxorubicin can be effectively loaded only above 55°C using the same lipid composition. Larger values of solubility product, or other kind of drug association within vesicle, coupled with high drug permeability can therefore result in relatively quick drug release from vesicles. Also, pK values of ciprofloxacin are closer to the values of pH in the system and the fraction of neutral molecules is higher.

Another possible mechanism of the drug leakage may be simple rupture of liposome membrane by the growing crystals. In optical microscope ciprofloxacin crystals showed thin, elongated, needle-like structures. Biomineralization studies of crystal growth inside vesicles have also shown that phosphate and apatite crystals can pierce the vesicle membrane upon controlled growth and induce contents leakage [19]. Cryoelectron microscopy images, however, do not support this picture.

Fig. 4 shows the leakage of ciprofloxacin in in vitro assay. While percent of the drug encapsulation in in vitro stability assays does not change in several months, the dilution with plasma (PIL assay) shows significant leak-

age. Corresponding experiment with doxorubicin causes minimal leakage. While plasma circulation half-lives for doxorubicin encapsulated in long-circulating liposomes in rats and mice are around 22 h [20,21], half of the injected dose of ciprofloxacin encapsulated in sterically stabilized liposomes, as measured by an HPLC assay, is taken up in 1.5 h. At present we do not know if this is caused by leakage or liposome uptake. PIL data and other experiences with sterically stabilized liposomes, such as independence of blood circulation time on the marker, seem to point to the first possibility. Before we continue with the theoretical model we should point out that the model presented below can be in principle also used to study the leakage rate. Detailed composition of the external phase would have to be taken into account.

At present, however, we have developed only a general model for loading of the drugs into preformed liposomes by the influence of various gradients.

Each of the corresponding original Master Eqs. (1)–(2) and (4)–(5) represents a description of particular case of the so-called bidirectional exchange loading [10] or, in our case, ammonium sulfate gradient loading [7–9]. In Figs. 5 and 6 the loading efficiencies ($K_v^{-1}\ c_1^{inside}/c_1^{out,\ start}$) at different K_v values for DOX as well as for CIP liposome systems are given, calculated from the following equation:

$$\alpha = \frac{\left(1 + K_{11}/x + K_{11}K_{12}/x^2\right)\left(1 + R^p\right)}{\left(1 + K_{11}/x + K_{21}/x^2\right)\left(1 + R^p\right) + K_v\left(\dfrac{y}{x}\right)A} \tag{7}$$
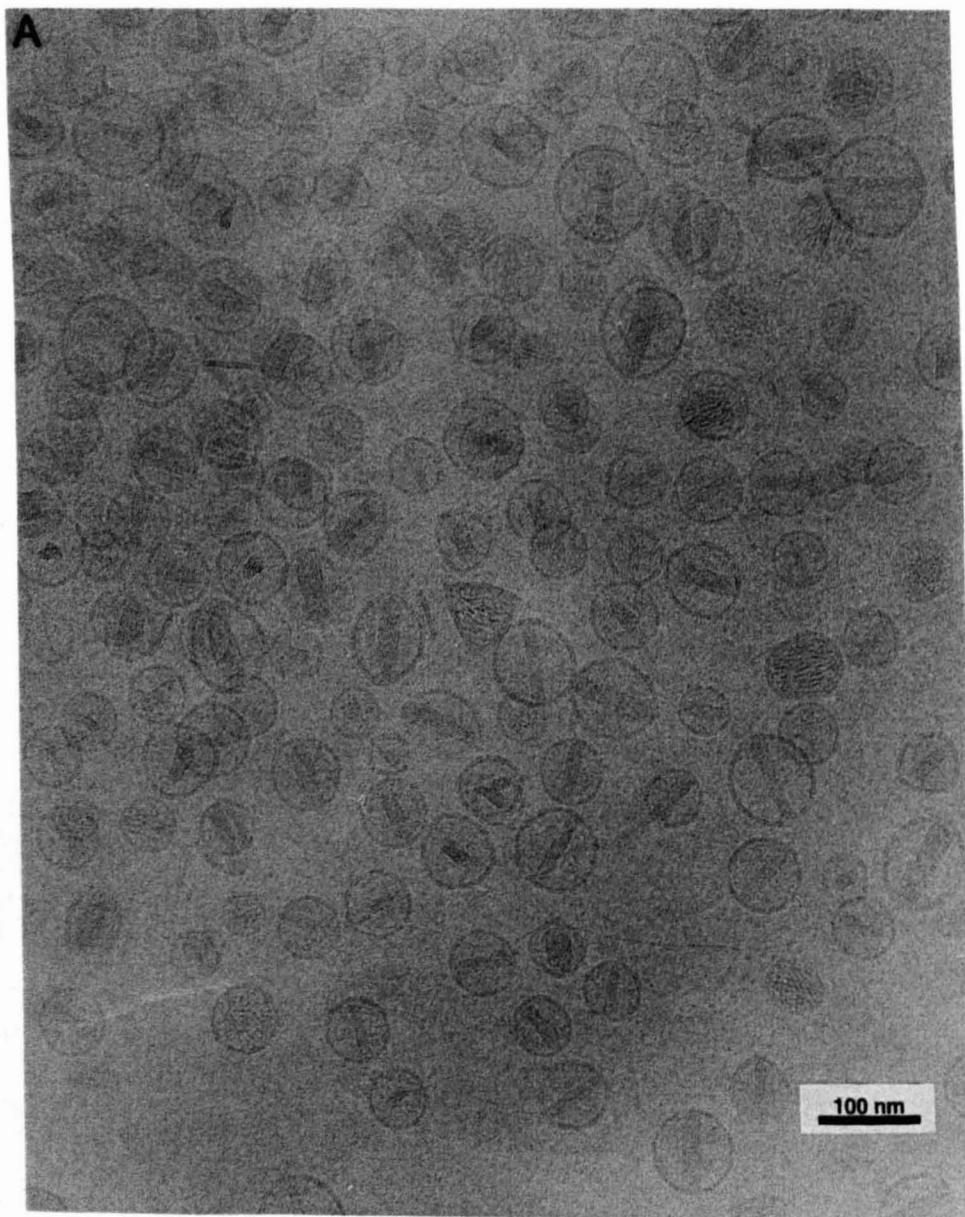

Fig. 1. (A) Cryoelectron micrograph of Stealth doxorubicin liposomes. (B) Cryoelectron micrograph of Stealth ciprofloxacin liposomes. (C) Negative stain micrograph of Stealth doxorubicin liposomes.
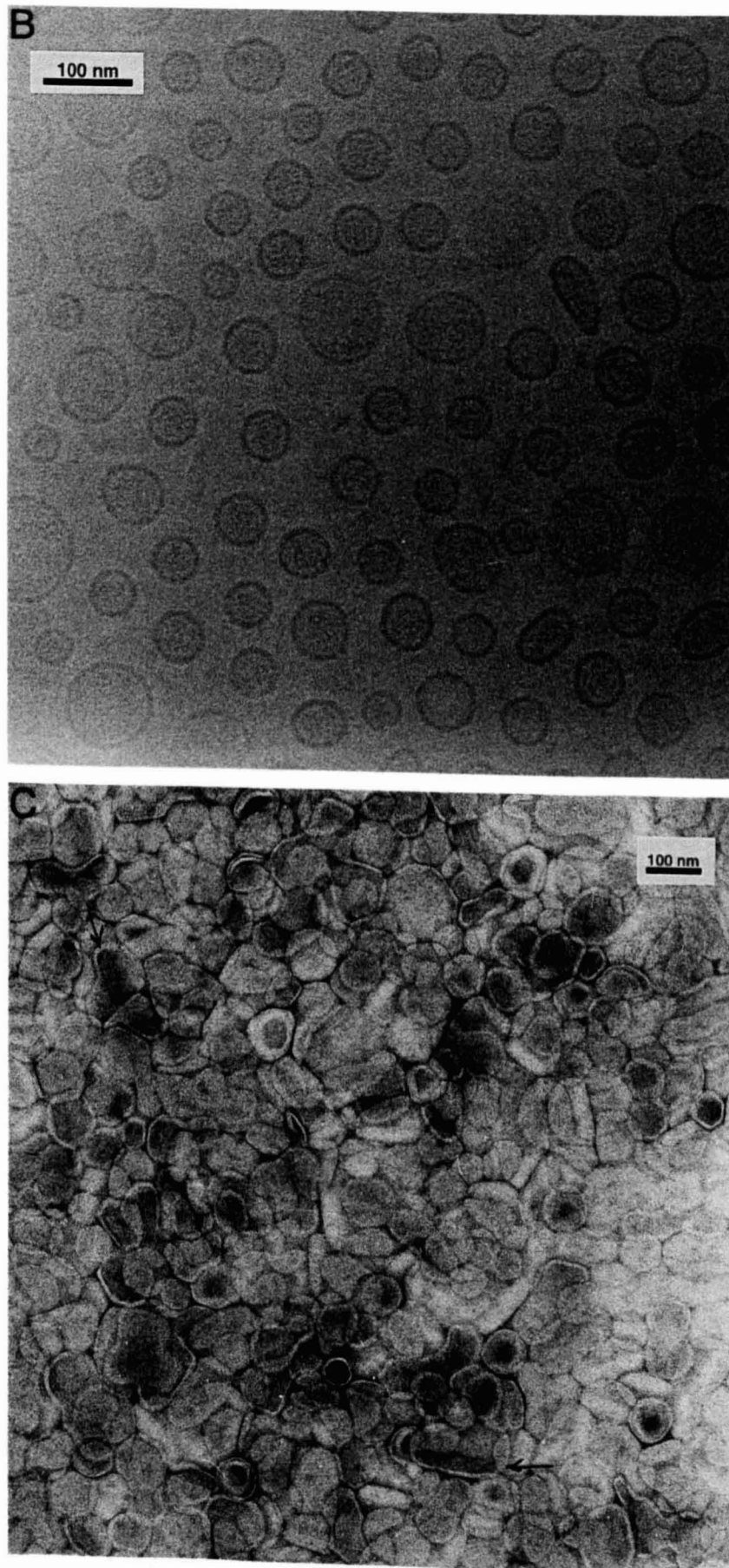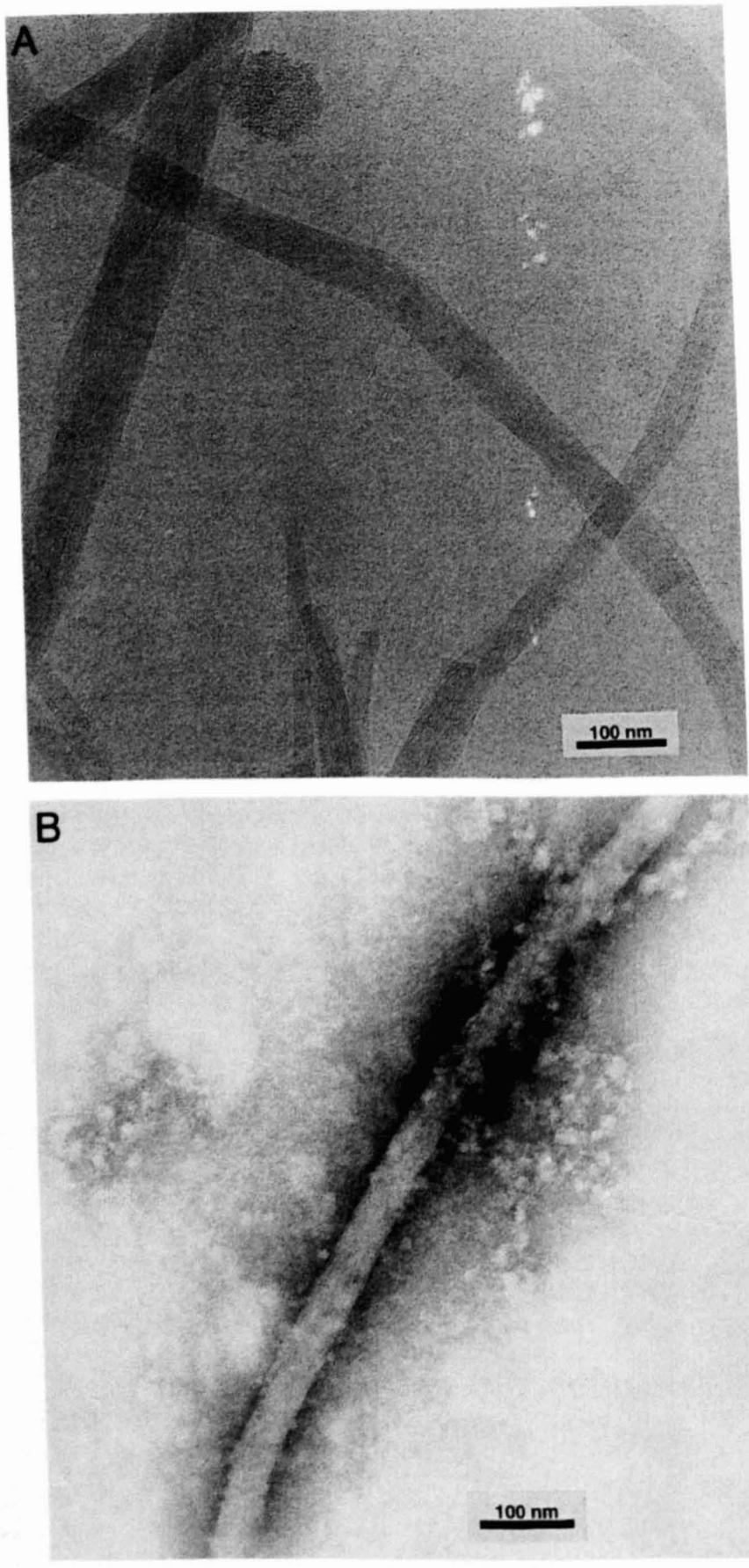
Fig. 1 (continued).

Fig. 2. (A) Cryoelectron micrograph of doxorubicin-sulfate gel. (B) Negative stain micrograph of doxorubicin-sulfate gel. (C) Negative stain micrograph of doxorubicin chloride gel. (D) Negative stain micrograph of ciprofloxacin sulfate gel.
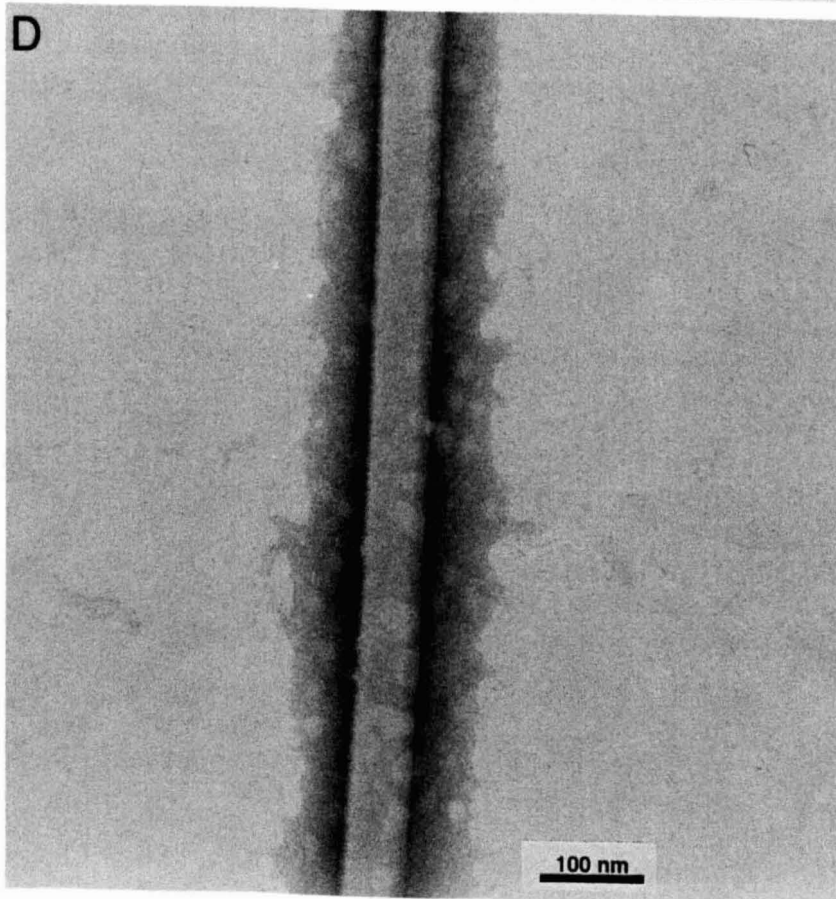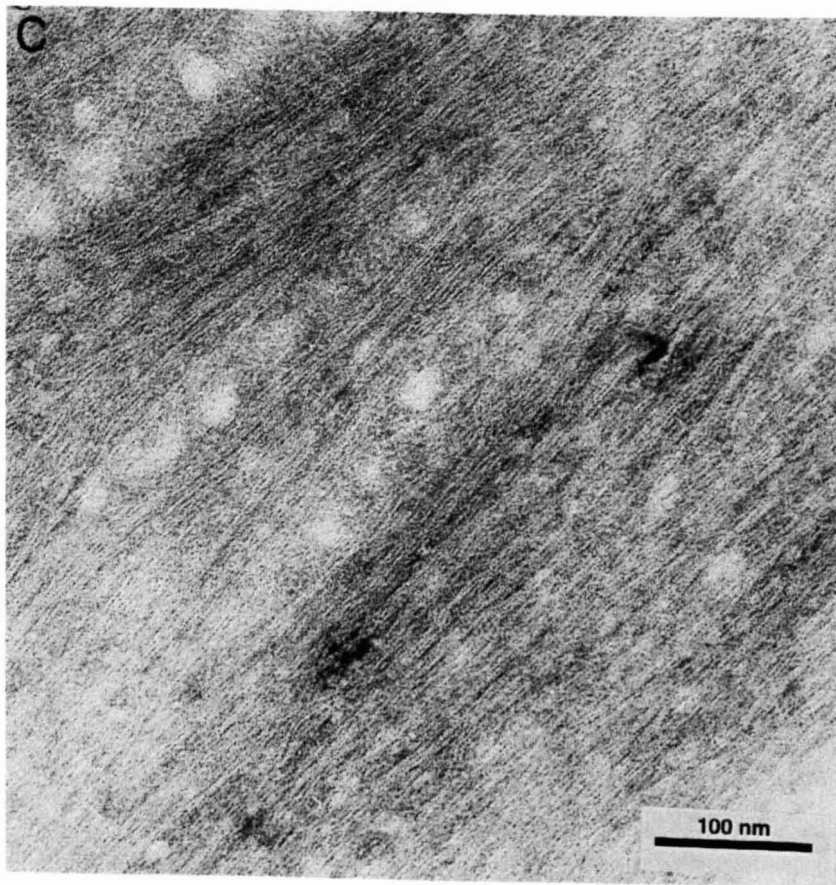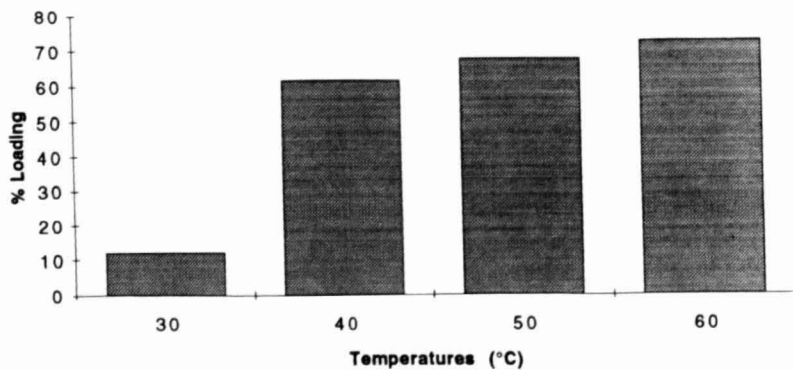
Fig. 2 (continued).

Fig. 3. Loading of ciprofloxacin at different temperatures as described in experimental part.

(where $A = 1 + K_{11}/y + K_{11}K_{12}/y^2$) or, for reasons mentioned above, from its simplified form:

$$\alpha = \frac{(1 + R^p)}{(1 + R^p) + K_v\left(\dfrac{y}{x}\right)} \tag{8}$$

The experimental loading efficiency measurements are also shown. As an illustrative example and to draw comparison with liposome system where no precipitate formation is assumed, in each of the figures one (K_v dependent) curve (B) is drawn for the case of $R^p = 0$ (i.e., excluding the possibility of the precipitate formation), with $K_v = 30$ for DOX loading and $K_v = 20$ for CIP loading (meaning that liposomes encapsulate 3.2 and 4.8% of the aqueous volume, respectively). It is interesting to compare the course of these curves with those where precipitate formation is considered (point of inflection). In both figures it is easy to see a general trend, i.e., diminishing loading efficiency with increasing drug concentration at constant lipid concentration as well as with increasing ratio of outer/inner

volumes, K_v. Also, in both cases the course of the curves which implies the precipitate formation (A, the presence of the point of inflection) drastically differ from that where precipitation is not assumed. The calculated values of loading efficiency are, depending on drug concentration, 10–80% higher in the cases where precipitate is assumed to be formed. Surprisingly, the theoretically calculated loading efficiencies are rather insensitive to L_p-changes (there are hardly changes within a unit or two on a present pL_p scale; calculations not shown).

In the case of DOX the agreement between theoretical and measured values is more than satisfying. Even more, nice agreement between experimental data and theory confirms, in addition to EM and SAXS data, precipitate formation inside the liposomes. At given K_v values (inverse to lipid concentration and size of the vesicles) and drug concentrations the calculations show that most of CIP (over 80%) and DOX (over 98%) is in the precipitated form. The calculated pH values of inner and outer solution after loading moderately and strongly depend on concentration and K_v (Figs. 7 and 8). Because measurements of

**Release of Cipro from S-cipro in Buffer and Rat Plasma
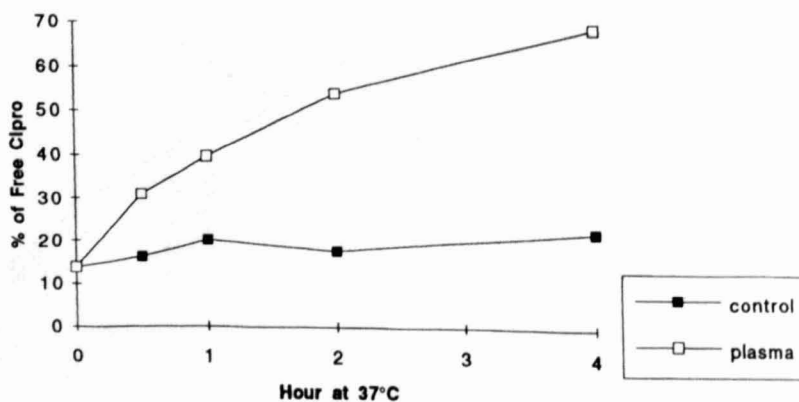(Batch 931-12)**

Fig. 4. Liposomal ciprofloxacin (S-CIPRO) was diluted 100-fold in rat plasma or 5% dextrose solution (control) to a final concentration of approx. 50 μg/ml. The diluted samples were incubated at 37°C for various time. Free and encapsulated ciprofloxacin were separated by a Bio-Gel A-5m column and concentration of the drug was determined by HPLC.
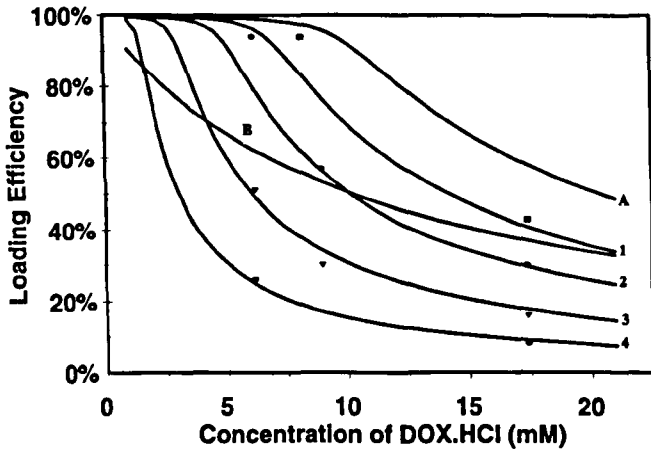
Fig. 5. Percentage of loaded drug as a function of doxorubicin HCl concentration. Experimental points show% of loading for given drug and liposomes concentrations. After loading the encapsulation efficiency was measured spectrophotometrically at 480 nm after separation of liposomal and free drug by Pierce desalting columns. Curve B indicates loading without precipitate formation. Fit parameters: internal concentration of ammonium sulfate, $c = 155$ mM, and internal liposome volume: $K_v = 30$ (A/B), 43 (1; experimental points-rectangles), 60 (2, ellipsoids), 100 (3, triangles), 200 (4, ellipsoids).

pH in such systems are very demanding and rather inaccurate we believe that these results shed additional light on the loading process.

The agreement between theoretical and experimental values in the case of CIP loading is, in comparison to DOX, a little less satisfying, but nevertheless, the formation of precipitate can also be confirmed (Fig. 6). The loading efficiencies at higher concentrations fit in lower K_v values ($K_v \approx 20-30$), those under 15 mM in higher values ($K_v \approx 30-50$). The differences mentioned may be
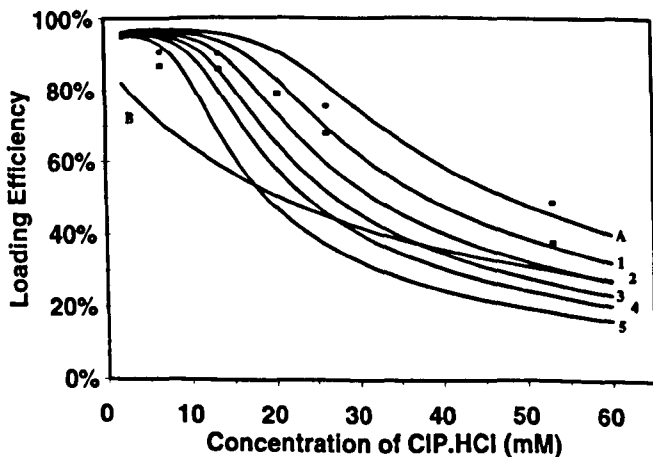

Fig. 6. Percentage of loaded drug as a function of ciprofloxacin HCl concentration. Experiment: liposomes were incubated with drug at different concentrations. After loading the encapsulation efficiency was measured spectrophotometrically at 278 nm after separation of liposomal and free drug on G-25 columns. Curve B indicates loading without precipitate formation. Fit parameters: internal concentration of ammonium sulfate, $c = 250$ mM, $K_v = 20$ (A/B), 25 (1), 30 (2), 35 (3), 40 (4), and 50 (5). Experimental points were determined for $K_v = 20$ (ellipsoids) and 25 (rectangles).
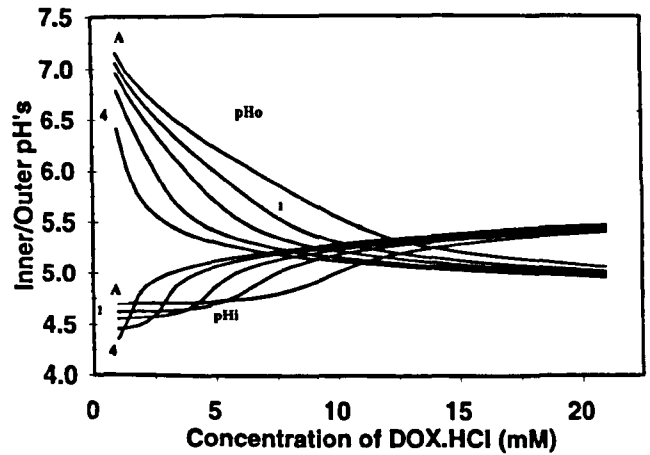

Fig. 7. Internal and external values of pH as a function of doxorubicin HCl concentration for different values of internal volume of liposomes. Internal concentration of ammonium sulfate, $c = 155$ mM, $K_v = 30$ (A/B), 43 (1), 60 (2), 100 (3), 200 (4).

due to relatively lower accuracy in CIP measurements, including its leakage (considerable differences between the measurements at the same conditions are observed here).

Excess of ammonium sulfate in liposomes was often used to explain enhanced stability due to its large reservoir of protons. From the above equations we should add that the influence of co-ion, i.e., large excess of sulfate ions strongly reduces the solubility of doxorubicin. This is similar to a salting out precipitation caused by a co-ion present at large excess.

Very encouraging pre-clinical and clinical trials with doxorubicin encapsulated in Stealth liposomes show that in addition to long circulation times and liposome ability to extravasate at sites of trauma, stable drug encapsulation is necessary for high therapeutic efficacy. Similar liposomes with higher drug leakage rates were found to be therapeutically much less effective [22].

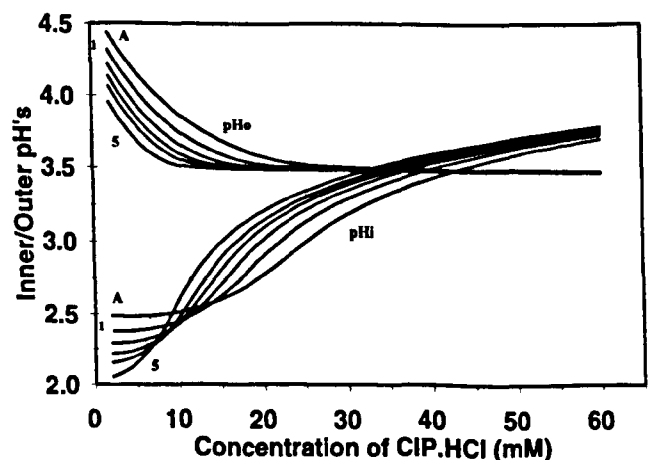The rather quick leakage of ciprofloxacin teaches us

Fig. 8. Internal and external values of pH as a function of ciprofloxacin HCl concentration for different values of internal volume of liposomes. Internal concentration of ammonium sulfate, $c = 250$ mM, $K_v = 20$ (A/B), 25 (2), 30 (3), 35 (4), 40 (5), and 50 (6).

about the complexity of the problem. Excellent test tube stability was not reproduced in in vitro assays and therefore we recommend rigorous in vitro tests, such as stability upon large (up to 1000-fold if sensitivity of analysis allows) dilutions, with buffer and with plasma at several temperatures to assess suitability of particular formulation for in vivo studies.

In order to expect stable encapsulation we believe, solubility product should be low, uncharged drug should have low octanol/buffer partition coefficient and low membrane permeability at physiological conditions and counterion should be present in excess and have very low membrane permeability. Several other means to improve drug encapsulation, such as formation of complex, binding to the membrane or encapsulated (poly)ions and molecular traps, can be envisaged [1,10]. However, our results indicate that all the approaches for drug retention in the liposomes must be carefully considered and evaluated in vitro in order to avoid unpleasant surprises in the efficacy studies. Of course, similar analysis must be done also for hydrophobic and membrane bound drugs. In these cases thermodynamic equilibria establish even faster and upon large dilution all the membrane embedded or associated drug can immediately pop out from the liposome and infusion of liposomal drug actually resembles infusion of empty liposomes and free drug.

Another possibility to improve drug retention are the so-called gelosomes [23,1]. These are liposomes with encapsulated polymerizable agent which can upon polymerization stabilize liposome as well as its cargo, i.e., reduces its leakage. Liposomes are formed at temperature above the solid–fluid phase transition of the medium, preferentially at high lipid concentration (to increase the encapsulated fraction of aqueous medium), and upon dilution and lowering of the temperature gelatine polymerizes in the liposome interior. Experimental conditions, i.e., gelatine concentration, can be tailored to induce the gelation at particular temperature what may offer the development of temperature sensitive release system. Of course, the diffusion constant of the drug in hydrogel should be measured to check if significant reduction is achieved.

These phase transitions were driven by concentration difference of membrane permeable species. Similarly, crystallizations due to selective membrane transfer of particular ions upon incorporation of specific ionophores can be induced. Such systems may offer preparation of minute crystals or amorphous solids and can further shed light on the formation of inorganic colloidal particles as well as explain the growth of bones, calcification of tissues and similar.

In conclusion, we have presented dynamic two compartment system in which chemical potential gradients coupled with asymmetric specific reactions can effectively concentrate particular molecules in the liposome interior to so high concentrations that these substances precipitate inside.

Acknowledgements

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