Effect of Solubilizing Agents on Mupirocin Loading into and Release from PEGylated Nanoliposomes

AHUVA CERN,[1,2] EINAT NATIV-ROTH,[3] AMIRAM GOLDBLUM,[2] YECHEZKEL BARENHOLZ[1]

[1]Laboratory of Membrane and Liposome Research, Department of Biochemistry, IMRIC, The Hebrew University – Hadassah Medical School, Jerusalem, Israel
[2]Molecular Modeling and Drug Design Laboratory, The Institute for Drug Research, The Hebrew University of Jerusalem, Jerusalem, Israel
[3]Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer Sheva, Israel

Received 7 January 2014; revised 7 May 2014; accepted 7 May 2014

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24037

ABSTRACT: Mupirocin was identified by quantitative structure property relationship models as a good candidate for remote liposomal loading. Mupirocin is an antibiotic that is currently restricted to topical administration because of rapid hydrolysis *in vivo* to its inactive metabolite. Formulating mupirocin in PEGylated nanoliposomes may potentially expand its use to parenteral administration by protecting it from degradation in the circulation and target it (by the enhanced permeability effect) to the infected tissue. Mupirocin is slightly soluble in aqueous medium and its solubility can be increased using solubilizing agents. The effect of the solubilizing agents on mupirocin remote loading was studied when the solubilizing agents were added to the drug loading solution. Propylene glycol was found to increase mupirocin loading, whereas polyethylene glycol 400 showed no effect. Hydroxypropyl-β-cyclodextrin (HPCD) showed a concentration-dependent effect on mupirocin loading; using the optimal HPCD concentration increased loading, but higher concentrations inhibited it. The inclusion of HPCD in the liposome aqueous phase while forming the liposomes resulted in increased drug loading and substantially inhibited drug release in serum. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci
Keywords: liposomes; formulation; solubility; nanoparticles; excipients; computer-aided drug design

INTRODUCTION

Liposomes as a drug delivery system have many advantages; they are biocompatible, nontoxic, and in the form of PEGylated nanoliposomes; the prolonged circulation time and nanosize allow them to benefit from the enhanced permeability (EP) effect of blood vessels in inflamed tissues and from the enhanced permeability and retention (EPR) effect in tumors. This explains why liposomes are the drug delivery system of choice for many drugs. So far, more than 10 liposomal drugs are US Food and Drug Administration approved, and many more are at various stages of clinical trials.[1] In order for a liposomal drug to be used clinically, it should be loaded at high concentration per liposome. This is an obligatory requirement to provide a therapeutic dose. In most cases, because of the extremely small volume ($\sim 1 \times 10^{-19}$ L) of nanoliposomes, remote (active) loading of drugs is the only approach to achieve the desired drug concentration. Remote loading uses an ion gradient as the driving force for getting drugs into preformed liposomes, and it is suitable only for amphipathic weak acids or bases.[2–5] The vast amount of data gathered about remote loading has led to the development of models characterizing the effect of both molecular characteristics and experimental conditions on drug remote loading.[4,5] The quantitative structure property relationship (QSPR) models built[5] allowed drug database screening for identifying good candidates for remote liposomal loading.[6] The antibiotic mupirocin was identified, among other molecules, as a good candidate using these models.[6] Mupirocin is a relatively new antibiotic.[7,8] Its structure is different from that of most antibiotics and its mode of action, the inhibition of isoleucyl tRNA synthetase, is not shared by any other therapeutically available antibiotic, so that cross-resistance with other antibiotics is not encountered. It is active *in vitro* against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and has limited activity against gram-negative bacteria. When absorbed into the blood stream or administered parenterally, mupirocin is rapidly degraded to form the inactive monic acid. Therapy with mupirocin is therefore confined to topical application.[7–9] Loading of mupirocin to PEGylated nanoliposomes may protect it from deactivation and passively target it to the infected tissue by the EP effect,[10,11] thus enabling effective administration of mupirocin by the parenteral route. This approach may selectively increase antibacterial drug concentrations at the target location, with the intention to increase the therapeutic efficacy of the antimicrobial treatment.

Many antibiotic drugs when encapsulated in liposomes showed better antibacterial effect then the free drug. For example, amikacin and streptomycin encapsulated in liposomes showed much higher therapeutic efficacy when injected i.v. to mice infected with *Mycobacterium avium–intracellulare* complex compared with the injections of the free drugs.[12] The *in vivo* efficacy of liposomal ciprofloxacin has been evaluated in a murine model of *Salmonella dublin* infection. A single injection of liposomal ciproflaxin was10 times more effective in preventing mortality than a single i.v. injection of free drug.[12] Gentamicin administered in liposomes to a rat model infected with *Klebsiella pneumoniae pneumonia* showed enhanced therapeutic efficacy compared with administration of the free drug.[13]

Correspondence to: Yechezkel Barenholz (Telephone: +972-2-6757615; Fax: +972-2-6757499; E-mail: chezyb@ekmd.huji.ac.il)

Journal of Pharmaceutical Sciences
© 2014 Wiley Periodicals, Inc. and the American Pharmacists Association

Liposomal amikacin (Arikace®) for inhalation has currently completed successfully a phase III study with chronic *Pseudomonas aeruginosa* infections in cystic fibrosis patients.[14] Many more cases of antibiotics encapsulated in liposomes and their *in vivo* activity may be found in the literature.[15–18]

There are many prerequisites to achieve a viable formulation based on nanoliposomes. In this paper, we will focus on two of them: the first one is to achieve a sufficient level of remote loading (see above); the second is that the nanoliposomes retain most of the drug while circulating in the blood and release the drug at the disease site at a rate and level that may be sufficient to result in therapeutic efficacy, similar to what was demonstrated for Doxil®.[19]

Drug remote loading requires sufficient drug solubility, and in this respect, mupirocin is only slightly soluble in aqueous medium and the solubility is pH dependent. In phosphate buffer pH 6.3, mupirocin has a solubility of approximately 28 mM and this concentration is achieved by vigorous stirring and sonication. We found that solubility was increased with the cosolvents polyethylene glycol (PEG) 400, propylene glycol (PG), and the cyclodextrin hydroxypropyl-β-cyclodextrin (HPCD) as solubilizers. The present study investigated the effect of these solubilizers on remote liposomal loading and on release. The solubilizers were added to the incubation solution used for loading. However, the liposomal formulations obtained showed a very fast drug release in the serum, which makes them unsuitable for clinical use. To overcome this major obstacle, we decided to include HPCD in the intraliposome aqueous phase and study its effect on the release from remotely loaded nanoliposomes, with the expectation that it will slow down drug release rate in serum. So far, the use of cyclodextrins for the enhancement of liposome loading and release has been studied mainly for drugs loaded by passive loading.[20] A work was published recently (while we were working on this paper) showing the advantages of HPCD in terms of loading and release by adding it to the intraliposome aqueous phase of liposomes remotely (actively) loaded with Gefitinib.[21] Regarding remote loading of mupirocin, HPCD when added in the extraliposome aqueous medium during remote loading, showed a concentration-dependent effect. At low concentrations, it increased loading, but higher HPCD concentrations inhibited remote loading. When present in the intraliposome aqueous phase, it increased loading and, more importantly, it slowed down significantly the release profile in serum. HPCD was very different from the classical solubilizers: PEG 400 did not alter mupirocin loading; PG increased mupirocin loading, but it did not affect its release.

MATERIALS AND METHODS

Materials

Mupirocin (Teva) was a gift from Foamix Ltd. (Rehovot, Israel). HPCD and Dowex 1×8–200 were obtained from Sigma–Aldrich(St.Louis, MO). Hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(PEG)-2000] (mPEG DSPE), and cholesterol were obtained from Lipoid GmbH (Ludwigshafen, Germany). Sepharose CL-4B was obtained from GE Healthcare (Little Chalfont, UK). Adult bovine serum was obtained from Biological Industries (Beit Haemek, Israel). The solvents used for the analysis were of HPLC grade. All other chemicals were commercial products of reagent grade.

Methods

Preparation of PEGylated Liposomal Mupirocin

Liposomes were prepared using the calcium acetate gradient method.[3] Lipids in a mole ratio of 55:40:5 HSPC–cholesterol–mPEG DSPE were mechanically hydrated by stirring at 65°C with 200 mM calcium acetate pH 5.5, at a weight ratio of 1:9. The liposomal dispersion was downsized by stepwise extrusion by the Northern Lipids (Burnaby, British Columbia, Canada) extruder using polycarbonate filters starting with three times extrusion through a 400-nm pore size membrane, then three times through a 100-nm pore size membrane, and finally 10 times through a 50-nm pore size membrane. Liposomes were then dialyzed, using a Cellu Sep regenerated cellulose membrane (Membrane Filtration Products, Seguin, TX), against a 10% sucrose solution. In the case of HPCD-containing liposomes, lipids were hydrated by 200 mM calcium acetate pH 5.5 containing 15% (w/w) HPCD. Control HPCD liposomes were prepared by hydrating lipids with 15% (w/w) HPCD in phosphate buffer 200 mM pH 6.3. All other preparation steps (downsizing and dialysis) were the same as described above. Remote loading was performed by incubating a solution of the drug at 65°C for 10 min with the liposome dispersion at a volume ratio of 1:1. Liposomes used for loading were freshly prepared and used within 1 week. Drug loading solutions were prepared in 200 mM phosphate buffer pH 6.3. High mupirocin concentrations in phosphate buffer pH 6.3 (28 mM) were achieved by vigorous stirring and 10 min sonication in a bath sonicator. Loading was also tested from 1% to 10% (w/w) HPCD solutions in phosphate buffer pH 6.3. High mupirocin concentration (28 mM) in 1% HPCD solution was achieved by vigorous stirring and sonication as described for phosphate buffer. Mupirocin solutions in higher HPCD concentrations (2.5%–10%) were prepared by stirring only. Loading was also performed from PG and from PEG 400 solutions. In these cases, a stock solution of 100 mM mupirocin was prepared and diluted to the desired concentration with 200 mM phosphate buffer pH 6.3. Phospholipid concentrations in the liposome dispersions used for loading experiments were in the range of 24–60 mM.

Phospholipid Determination

Phospholipid concentration was determined in the blank liposomes as organic phosphorus by a modified Bartlett method.[22] Control HPCD liposomes (containing phosphate buffer) were tested for their phospholipid content by HPLC method that was based on a procedure for assay of lipid blends, received from Lipoid GmbH. It uses a LiChrospher 100 Diol 5 μm, 250 × 4.0 mm^2 column, gradient elution with hexane–2-propanol–water, and evaporative light-scattering detection with an Alltech 3300 ELSD detector (Grace, Deerfield, IL).

Mupirocin Quantification

Drug concentrations were quantified using an HPLC/UV method (HPLC system; Hewlett Packard Series II 1090, Waldbronn, Germany). The column used was a Waters, XBridge C18 column, 5 μm, 4.6 x 150 mm^2. The chromatographic conditions were based on a published method.[23] A resolution solution for the acid hydrolysis products was prepared according to instructions.[23] The resolution between mupirocin hydrolysis products and mupirocin was not less than 2.0. Total (free plus liposomal) drug concentration was determined by a HPLC

 DOI 10.1002/jps.24037

assay of the liposomal dispersion diluted with methanol. Liposomal drug concentration was determined after removing the free drug by mixing the dispersion with Dowex 1 × 8–200 anion exchanger, which binds the free drug.[24–26]

Drug to Lipid Mole Ratio

The initial drug to lipid (D/L) ratio refers to the initial mole ratio used for the remote loading. The initial D/L ratio in the incubation was determined as the mole ratio of total amount of drug used for remote loading to total liposomal phospholipid used for remote loading. The loaded D/L ratio refers to the mole ratio between the liposomal drug and the liposomal phospholipid concentration.

Particle Size Distribution Analysis

Particle size was determined using the well-established dynamic light scattering method, performed with a Zetasizer Nano Series ZEN3600F (Malvern Instruments, Malvern, UK). Mean diameter was based on the volume mean. (For more details, see Barenholz and Amselem[27])

Cryogenic Temperature Transmission Electron Microscopy Images

Transmission electron microscopy at cryogenic temperature was used for direct imaging of solutions and dispersions. Vitrified specimens were prepared on a copper grid coated with a perforated lacy carbon, 300 mesh (Ted Pella, Inc. Redding, CA). A 4-μL drop of the solution was applied to the grid and blotted with filter paper to form a thin liquid film of solution. The blotted samples were immediately plunged into liquid ethane at its freezing point (−183°C). The procedure was performed automatically in the Plunger (Leica, Wetzlar, Germany). The vitrified specimens were transferred into liquid nitrogen for storage. The samples were studied using an FEI Tecnai 12 G2 TEM, at 120 kV with a Gatan cryo-holder maintained at −180°C, and images were recorded on a slow-scan, cooled, charge-coupled device Gatan camera. Images were recorded with the Digital Micrograph software package, at low-dose conditions to minimize electron beam radiation damage.

Release Kinetics of Mupirocin from PEGylated Liposomal Mupirocin

PEGylated liposomal mupirocin was incubated following dilution of 1:20 at 37°C in either 50% adult bovine serum or in saline. Aliquots were taken from these samples at the desired time points and analyzed for level of drug release by gel permeation chromatography, using a Sepharose CL-4B column, which separates liposomal mupirocin from free mupirocin. The column was equilibrated with saline solution and, following sample loading on column, fractions of 0.5 mL were collected and analyzed for mupirocin content. Mupirocin concentration obtained by HPLC at each fraction was plotted against the volume eluted to obtain an elution profile for each sample. The elution profile contained two peaks, the first corresponded to mupirocin in liposomes and the second to free mupirocin. The area under the curve for liposomal mupirocin and free mupirocin was calculated by the trapezoidal method. At each time point, the percent drug retained in liposomes (% retained) was calculated by the following equation:

$$\text{Retained}(\%) = \frac{\text{liposomal mupirocin AUC at } t = x \times 100}{\text{liposomal mupirocin AUC at } t = 0}$$

RESULTS

Effect of Solubilizing Agents on the Remote Loading of Mupirocin into PEGylated Nanoliposomes

Calcium Acetate Liposomes

Mupirocin loading into PEGylated nanoliposomes exhibiting a transmembrane gradient of calcium acetate (calcium acetate liposomes, CA-lip) was evaluated using different loading solution compositions. Mupirocin is not freely soluble in aqueous medium. Being a weak acid, its solubility increases with pH. Mupirocin was soluble in phosphate buffer 200 mM pH 6.3 up to a concentration of 15 mM. Higher concentrations were achieved with vigorous stirring and sonication. Improved solubility (∼100 mM) was achieved with PEG 400 and PG. HPCD solutions of 1%–10% (w/w) in phosphate buffer pH 6.3 also resulted in increased solubility (>34 mM). However, high concentrations (≥28 mM) in 1% HPCD required 10 min sonication to achieve a clear solution. It should be noted that the chromatographic profile of mupirocin in the different solutions tested was similar to that of a standard solution. In an attempt to test the effect of solubility enhancers on mupirocin loading, mupirocin was loaded into the liposomes (exhibiting transmembrane calcium acetate gradient) from incubation solutions containing phosphate buffer pH 6.3 with and without PEG 400, PG, and 1%–10% HPCD. Figure 1 presents loaded D/L ratios as a function of the initial D/L ratios for the different incubation solutions tested. Incubations from phosphate buffer and PEG 400 showed a similar pattern of bell-shaped curves: loaded D/L reached a maximal ratio of 0.23–0.25 D/L and decreased with increase in initial D/L ratios. Loading from PG was high at all initial ratios tested (0.14–0.59) and showed constant increase in loaded D/L, which reached a value of 0.48 for the highest initial ratio tested (0.59). This high loading with PG compared with phosphate buffer and PEG 400 solutions may be a result of PG permeation enhancement characteristics. The loading profile from HPCD solutions was dependent on HPCD concentrations. HPCD (1%) showed slightly higher loaded ratios but a similar bell-shaped loading curve for phosphate buffer. Higher HPCD concentrations (2.5%–10%) showed constant increase in loaded ratio with increase in the initial ratio, and as for PG, loading from these solutions did not result in a bell-shaped pattern. However, the loaded ratios were higher for 2.5% HPCD and decreased with increasing concentrations of HPCD (5% and 10%); high HPCD concentrations seemed to inhibit loading.

CA-lip-Containing HPCD

The effect of HPCD on loading was also determined for CA-lip-containing HPCD (CA-HPCD-lip) in their interior volume. PEGylated liposomes were prepared with calcium acetate buffer containing 15% (w/w) HPCD. The HPCD concentration in the liposome interior volume was above the concentration that inhibited loading (10%; see Fig. 1). This concentration, inside liposomes, was therefore assumed to aid loading by trapping mupirocin in inclusion complexes inside the liposomes and inhibiting its permeation to the outside medium. Figure 2 presents loaded D/L ratio in CA-HPCD liposomes as a function of the initial D/L ratio used and incubation solution composition. In addition, control HPCD liposomes (CTRL-HPCD-lip) containing 15% HPCD without using a calcium acetate gradient were also tested for their mupirocin loading from phosphate buffer solution. As presented in Figure 2, none of the loading

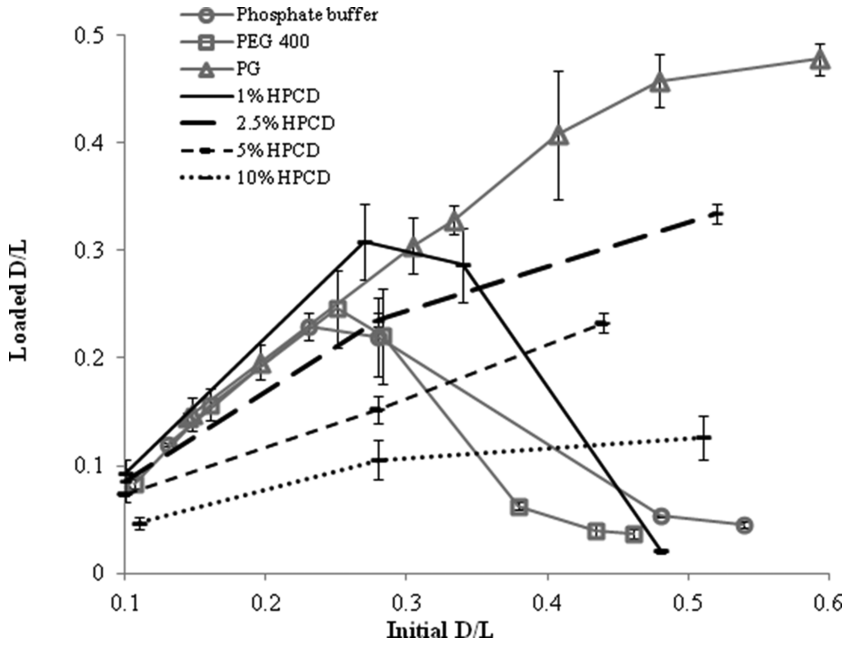

Figure 1. Mupirocin loading into CA-lip as a function of the initial D/L mole ratio and the incubation solution composition. (Mean ± SE, $n = 2$).

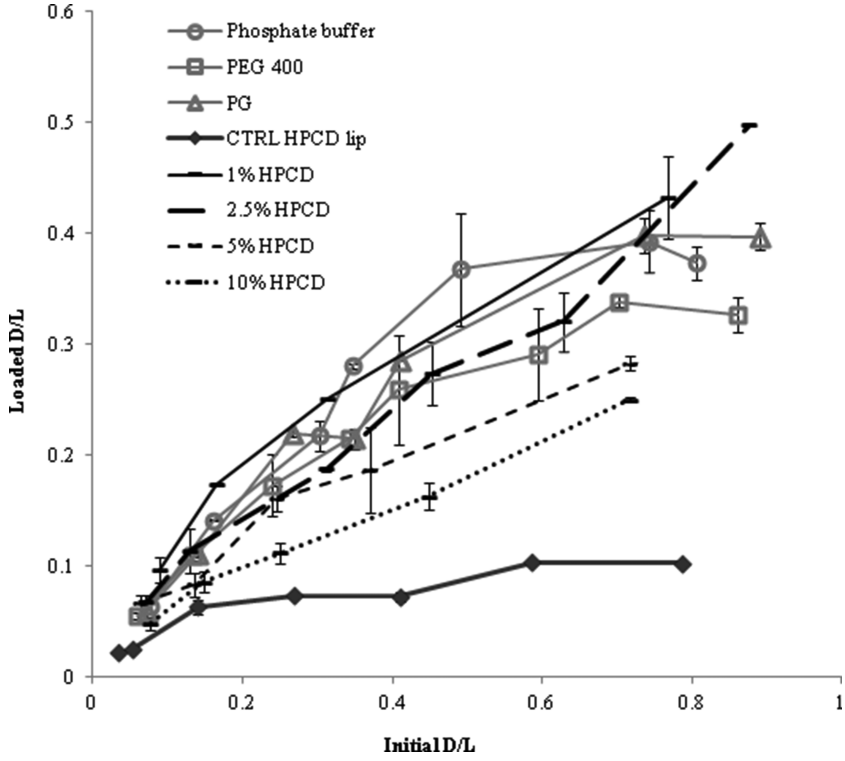

Figure 2. Mupirocin loading into CA-HPCD-lip as a function of the initial D/L mole ratio and incubation solution composition. (Mean ± SE, $n = 2$).

solutions to CA-HPCD-lip showed a bell-shaped curve. Loading to CA-HPCD-lip was similar to loading to CA-lip from incubation solutions that did not show bell-shaped curves for CA-lip (PG and 2.5%–10% HPCD). However, a significant difference was found for loading from phosphate buffer, 1% HPCD, and PEG 400. By loading to these CA-HPCD-lip, the bell-shaped loading obtained for CA-lip disappeared. As shown for CA-lip (Fig. 1), loading to CA-HPCD-lip from HPCD solutions was dependent on HPCD concentration; loading decreased with increase in HPCD concentration in the incubation solution. Figure 2 shows that for loading to CA-HPCD-lip, no solubilizer was required for the incubation solution; phosphate buffer was as good as PG, PEG 400, and 1% HPCD. However, higher HPCD concentrations in the incubation solution (5%–10%) decreased

 DOI 10.1002/jps.24037

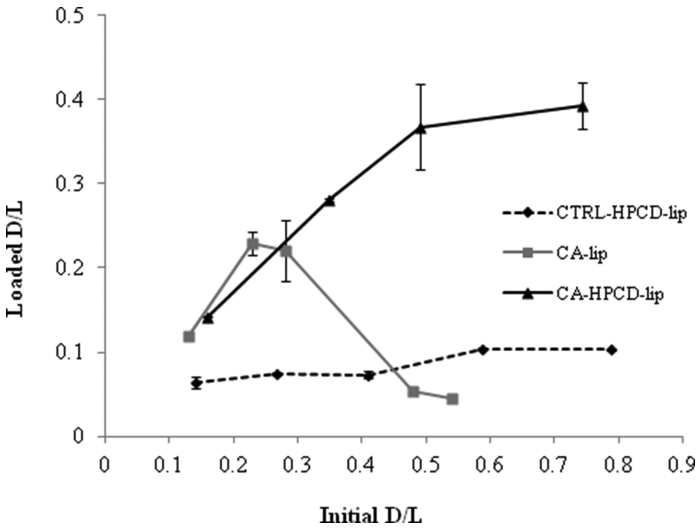

Figure 3. Mupirocin loading from phosphate buffer to liposomes having three different intraliposome aqueous phases, as a function of the initial D/L mole ratio used. (Mean ± SE, $n = 2$).

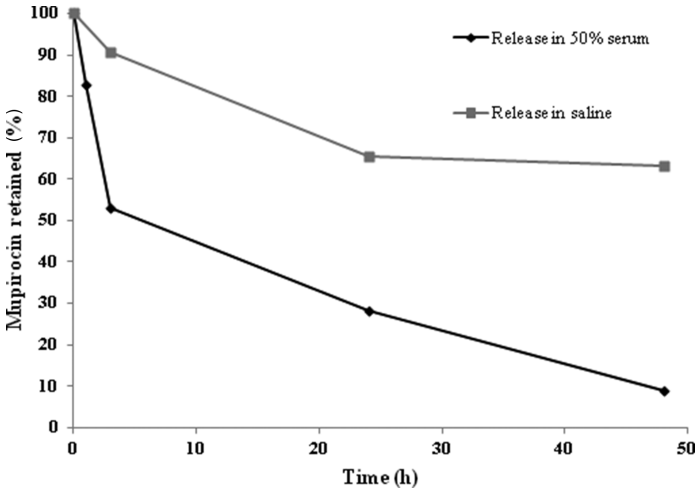

Figure 5. Percent retained liposomal mupirocin in saline and serum over time from CA-HPCD-lip. Each time point represents the liposomal area ratio obtained by the collection of 23 fractions on Sepharose column, as described in the *Methods* section. ($p = 0.04$, calculated by ANOVA: two factor without replication).

loading. Figure 3 shows comparisons of mupirocin loading from phosphate buffer solution to CA-lip, CA-HPCD-lip, and CTRL-HPCD-lip. The comparisons demonstrate the influence of the intraliposome medium on loading pattern. CA-lip showed the bell-shaped loading curve, whereas this pattern was not observed in HPCD-containing liposomes. However, without a CA gradient, the loading was very low.

Mupirocin Release

The release of mupirocin was tested from CA-lip, CA-HPCD liposomes, and control-HPCD liposomes. The release was evaluated in either saline or 50% serum. Figure 4 shows the percentage of mupirocin retained in the liposomes with different intraliposome media after 1 h of incubation at 37°C in either saline or serum. CA-lip containing mupirocin were stable in saline but released drug very fast in the presence of 50% serum

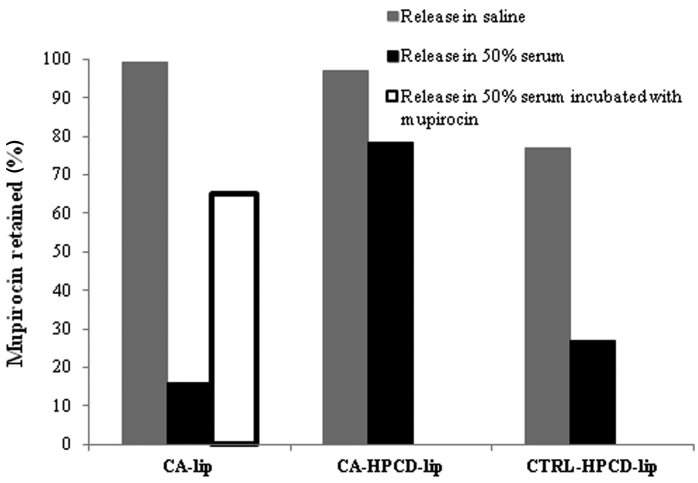

Figure 4. Percent retained liposomal mupirocin following 1 h of incubation at 37°C of different liposomes. Note that the data for CA-HPCD-lip release in saline are an interpolation from the release data at 3 and 24 h (see Fig. 5).

(82% released within 1 h). There was no effect of the loading solution composition on the release rate; CA-lip-loaded from PG solution showed a similar value of release in serum compared with liposomes loaded from phosphate buffer (data not shown). However, the release in serum was significantly reduced in CA-HPCD-lip. Following 1 h of incubation, only 22% was released, and again, no influence was found for loading solution composition (data not shown). Control HPCD liposomes containing mupirocin showed rapid release (73%) after 1 h of incubation. The substantial difference between mupirocin release from CA-lip in saline and serum was postulated to be attributed to the high protein binding affinity of mupirocin (96.5%[9]). To test this assumption, CA-lip containing mupirocin were incubated in serum, which was preincubated with free mupirocin to a concentration of 12.5 μM. The release from CA-lip in this case was reduced substantially to 35%, supporting our working hypothesis on the involvement of serum protein as a released drug sink.

The release profile following 48 h of incubation was tested for CA-HPCD-lip containing mupirocin in the presence of saline and serum (Fig. 5). The release in saline was relatively slow, with 37% release after 48 h of incubation. The release in serum was more rapid, having 47% and 72% release after 3 and 24 h of incubation, respectively. The release in serum from CA-HPCD-lip was substantially lower than the release from CA-lip (82% release after 1 h of incubation; Fig. 4).

Characterization of Liposomal Mupirocin

Cryogenic temperature transmission electron microscopy images of CA-lip and CA-HPCD-lip with and without mupirocin are presented in Figure 6. The images show spherical SUV particles with no observable drug crystals inside them or in the liposome medium. Liposomes were also tested for their size using the Malvern particle size analyzer. The size obtained was small, 77 ± 1.5 nm. The polydispersity index was lower than 0.05 for all samples. No difference in size distribution was found between calcium acetate and calcium acetate–HPCD liposomes.

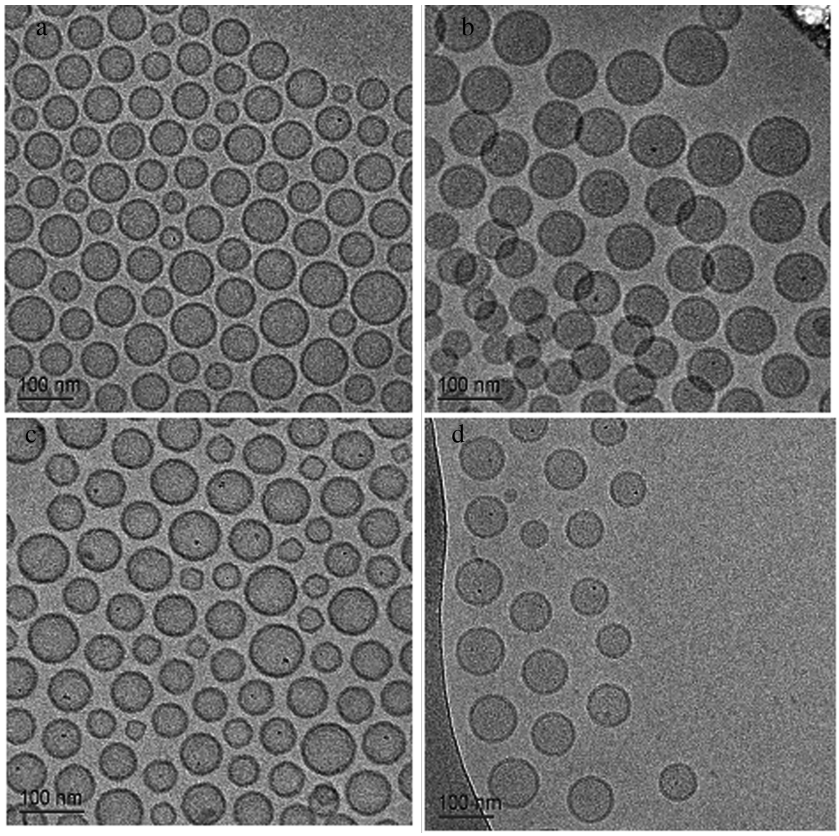

Figure 6. Cryogenic temperature transmission electron microscopy images of calcium acetate and calcium acetate–HPCD liposomes containing mupirocin (a and b, respectively) versus those containing no drug (c and d, respectively).

DISCUSSION

Mupirocin was identified by QSPR screening as a good candidate for remote liposomal loading.[6] Mupirocin administration as a liposomal drug may protect it from degradation to the inactive metabolite monic acid in the circulation, and passively target it to infected tissues, thereby expanding its use from topical to parenteral administration. Mupirocin is slightly soluble in aqueous medium; water-soluble organic solvents (PEG 400 and PG) as well as HPCD were found to increase its solubility. We wanted to investigate whether the solubility enhancements achieved by the solubilizers also affect mupirocin remote loading. Mupirocin was therefore loaded to CA-lip from phosphate buffer pH 6.3, and PEG 400, PG, and 1%–10% HPCD solutions of mupirocin in the same phosphate buffer. Mupirocin loading from phosphate buffer solution showed a bell-shaped curve (Fig. 1); the final D/L mole ratio obtained increased with the initial D/L ratio up to a maximal ratio and decreased thereafter. This behavior was also obtained for PEG 400 solutions and indicated that high mupirocin concentrations outside liposomes inhibited loading. This may be explained by the amphipathic nature of mupirocin, which may form small micelles that above a certain concentration may damage the membrane. We encountered a similar effect with methylprednisolone hemisuccinate.[28] However, the loading pattern from PG solution was different. The final D/L ratio increased with the initial D/L ratio used. It was high at all initial ratios tested and reached a maximal final D/L ratio of 0.48. PG is known to enhance drug permeability through membranes.[29] It is able to penetrate membranes and thereby can transport lipophilic substances via solvent drag.[29] These properties may explain its favorable loading profile. The effect of HPCD on loading was concentration dependent; 1% HPCD in the loading solution resulted in a slight increase compared with phosphate buffer, but the loading pattern of a bell shape was similar. Concentrations of 2.5%–10% did not show the bell-shaped curve, but the maximal loading was obtained for 2.5% HPCD and decreased with increased HPCD concentrations (Fig. 1). Too low (1%) HPCD concentration (a concentration that for complete mupirocin solubilization required sonication and therefore complete solubilization was probably not achieved by forming inclusion complexes) did not alter loading significantly, but too high concentrations (5%–10%), more than was required for solubilizing mupirocin (2.5%), inhibited loading. These findings correlate well with the suggested effect of cyclodextrins on biological membrane permeation of water-insoluble drugs.[30] Cyclodextrins and their complexes cannot permeate biological membranes, and their effect on membrane permeation of water-insoluble drugs is considered to be a result of increasing the availability of dissolved drug molecules in the aqueous layer in proximity to the membrane surface. In addition, excess cyclodextrin (more than is needed to dissolve the drug) will hamper this effect.[30] The liposomes used for mupirocin loading are PEGylated nanoliposomes and, as such, have a large aqueous layer surrounding them.[31] Low HPCD concentrations increased permeation (in our case, loading), whereas higher HPCD concentrations (5%-10%), more than was required for mupirocin solubilization, decreased loading. To further test the effect of HPCD on loading, liposomes

containing HPCD in their interior volume were prepared. These liposomes were prepared with 15% HPCD in calcium acetate. The HPCD concentration used inside the liposomes was chosen to be high so that it will inhibit drug permeation from liposomes (as described above) and thus it aimed to increase drug loading and inhibit drug release. The results showed that loading to CA-HPCD liposomes was indeed higher than loading to CA-lip (Fig. 3). Moreover, when using CA-HPCD liposomes, the bell-shaped pattern obtained for loading from phosphate buffer, PEG 400, and 1% HPCD disappeared; loading from phosphate buffer, 1% HPCD, PEG 400, and PG solutions was similar and increased constantly with increase in initial D/L ratio (Fig. 2). Higher HPCD concentrations (2.5%–10%) decreased loading. To assess that loading was not driven by the HPCD alone, control HPCD liposomes without calcium acetate gradient were prepared. Loading to these liposomes was much lower and reached a maximal loaded D/L value of 0.1.

The release of mupirocin from liposomes was tested in saline and in 50% bovine serum. CA-lip containing mupirocin were relatively stable in saline with respect to drug leakage. However, in 50% serum, mupirocin was rapidly released (84% release after 1 h; Fig. 4). It was postulated that the rapid release in serum compared with saline is a result of the high protein binding affinity of mupirocin (96.5%[9,32]) that enables the serum proteins (or lipoproteins) to act as a highly efficient sink. To test this assumption, mupirocin release from the same liposomes was evaluated in serum that was preincubated with 12.5 μM free mupirocin (which probably saturated serum proteins with the drug). In this case, the release was substantially lower (35% after 1 h), supporting our assumption on the involvement of serum proteins. Although the slow release in this case may also be explained by uptake of the drug by the liposomes that have large residual CA gradient. CA-HPCD liposomes released the drug much more slowly in serum (17%–22% after 1 h). The release probably was inhibited by the protection of the drug in inclusion complexes that was achieved by using a high HPCD concentration inside liposomes. However, the release from control-HPCD liposomes was more rapid than from calcium acetate–HPCD liposomes (73% after 1 h in serum), indicating that the combination of HPCD and calcium acetate gradient is important for this purpose. Liposomes prepared from incubation medium containing PG resulted in a release profile similar to those prepared from phosphate buffer solutions, indicating that in this respect the history of loading has no effect on the release rate.

Cryogenic temperature transmission electron microscopy images of CA-lip and CA-HPCD liposomes showed spherical SUV particles with no observable drug crystals inside them. No observable difference was found between empty liposomes and liposomes containing drug.

Antimicrobial agents formulated in nanoliposomes have already been shown to have improved therapeutic effect and reduced toxicity compared with the free antimicrobial agent, probably because of the EP effect.[10–12] Mupirocin has a specific antimicrobial activity,[7,33] with favorable toxicologic profile.[9] Its administration is currently limited to topical administration because of rapid degradation to its inactive metabolite. Delivery of mupirocin in nanoliposomes may enable its use as a parenteral antibiotic. The liposomal formulation may protect it from degradation in the circulation and passively target it to the infected tissue taking advantage of the EP effect. The CA-HPCD liposomes containing mupirocin presented in this paper are now ready for *in vivo* evaluation in relevant animal models.

ACKNOWLEDGMENTS

We would like to thank Dr. Julia Franz for her help with the release studies. Mr. Alex Lyskin and Mr. Michael Raslin are acknowledged for performing the HPLC phospholipid analysis.

The authors would like to thank Sigmund Geller for editing this paper.

REFERENCES

1. Chang H-I, Yeh M-K. 2012. Clinical development of liposome-based drugs: Formulation, characterization, and therapeutic efficacy. Int J Nanomed 7:49–60.

2. Haran G, Cohen R, Bar LK, Barenholz Y. 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1151:201–215.

3. Clerc S, Barenholz Y. 1995. Loading of amphipathic weak acids into liposomes in response to transmembrane calcium acetate gradients. Biochim Biophys Acta 1240:257–265.

4. Zucker D, Marcus D, Barenholz Y, Goldblum A. 2009. Liposome drugs' loading efficiency: A working model based on loading conditions and drug's physicochemical properties. J Control Release139:73–80.

5. Cern A, Golbraikh A, Sedykh A, Tropsha A, Barenholz Y, Goldblum A. 2012. Quantitative structure–property relationship modeling of remote liposome loading of drugs. J Control Release160:147–157.

6. Cern A, Barenholz Y, Tropsha A, Goldblum A. 2014. Computer-aided design of liposomal drugs: In silico prediction and experimental validation of drug candidates for liposomal remote loading. J Control Release 173:125–131.

7. Pappa K. 1990. The clinical development of mupirocin. J Am Acad Dermatol 22:873–879.

8. Noble WC. 1991. The uses and abuses of mupirocin. J Am Acad Dermatol 3–5.

9. Glaxo Smith Kline Inc. Product monograph Bactroban. Ointment 2% for Dermatologic Use. 2001.07.05.

10. Azzopardi EA, Ferguson EL, Thomas DW. 2013. The enhanced permeability retention effect: A new paradigm for drug targeting in infection. J Antimicrob Chemother68:257–274.

11. Huh AJ, Kwon YJ. 2011. "Nanoantibiotics": A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. J Control Release 156:128–145.

12. Salem II, Flasher DL, Düzgüneş N. 2005. Liposome-encapsulated antibiotics. Methods Enzymol 391:261–291.

13. Schiffelers RM, Storm G, Kate MT, Bakker-woudenberg IAJM, Marian T. 2001. Therapeutic efficacy of liposome-encapsulated gentamicin in rat klebsiella pneumoniae pneumonia in relation to impaired host defense and low bacterial susceptibility to gentamicin. Antimicrob Agents Chemother 45:464–470.

14. Study to evaluate Arikace™ in CF patients with chronic pseudomonas aeruginosa infections. http://clinicaltrials.gov.Last updated March 4, 2014

15. Schiffelers RM, Storm G, ten Kate MT, Stearne-Cullen LE, Hollander JG den, Verbrugh HA, Bakker-Woudenberg IA. 2001. In vivo synergistic interaction of liposome-coencapsulated gentamicin and ceftazidime. J Pharmacol Exp Ther 298:369–375.

16. Labana S, Pandey R, Sharma S, Khuller GK. 2002. Chemotherapeutic activity against murine tuberculosis of once weekly administered drugs (isoniazid and rifampicin) encapsulated in liposomes. Int J Antimicrob Agents 20:301–304.

17. Kadry AA, Al-Suwayeh SA, Abd-Allah AR, Bayomi MA. 2004. Treatment of experimental osteomyelitis by liposomal antibiotics. J Antimicrob Chemother54:1103–1108.

18. Wang D, Kong L, Wang J, He X, Li X, Xiao Y. 2009. Polymyxin E sulfate-loaded liposome for intravenous use: Preparation, lyophilization, and toxicity assessment in vivo. PDA J Pharm Sci Technol63(2):159–167.
19. Barenholz Y. 2012. Doxil®—The first FDA-approved nano-drug: Lessons learned. J Control Release 160:117–134.
20. McCormack B, Gregoriadis G. 1998. Drugs-in-cyclodextrins-in-liposomes: An approach to controlling the fate of water insoluble drugs in vivo. Int J Pharm 162:59–69.
21. Zhou X, Yung B, Huang Y, Li H, Hu X, Xiang GLR. 2012. Novel liposomal gefitinib (L-GEF) formulations. Anticancer Res 32(7):2919–2923.
22. Shmeeda H, Even-Chen S, Honen R, Cohen R, Weintraub C, Barenholz Y. 2003. Enzymatic assays for quality control and pharmacokinetics of liposome formulations: Comparison with nonenzymatic conventional methodologies. Methods Enzymol 367:272–292.
23. USP 35. Mupirocin official monograph.pp 3962–3963.
24. Druckmann S, Gabizon a, Barenholz Y. 1989. Separation of liposome-associated doxorubicin from non-liposome-associated doxorubicin in human plasma: Implications for pharmacokinetic studies. Biochim Biophys Acta 980:381–384.
25. Amselem S, Gabizon A, Barenholz Y. 1990. Optimization and upscaling of doxorubicin-containing liposomes for clinical use. J Pharm Sci 79:1045–1052.
26. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y. 1994. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 54:987–992.
27. Barenholz Y, Amselem S. 1993. Quality control assays in the development and clinical use of liposome-based formulations. In: Liposome technology: Liposome preparation and related techniques; Gregoriadis G, Ed. 2nd ed. CRC Press, Boca Raton, FL. pp 527–616.
28. Avnir Y, Turjeman K, Tulchinsky D, Sigal A, Kizelsztein P, Tzemach D, Gabizon A, Barenholz Y. 2011. Fabrication principles and their contribution to the superior in vivo therapeutic efficacy of nanoliposomes remote loaded with glucocorticoids. PLoS One 6:e25721.
29. Trommer H, Neubert RHH. 2006. Overcoming the stratum corneum: The modulation of skin penetration. A review. Skin Pharmacol Physiol 19:106–121.
30. Loftsson T, Konrádsdóttir F, Másson M. 2006. Influence of aqueous diffusion layer on passive drug diffusion from aqueous cyclodextrin solutions through biological membranes. Pharmazie 61:83–89.
31. Tirosh O, Barenholz Y, Katzhendler J, Priev A. 1998. Hydration of polyethylene glycol-grafted liposomes. Biophys J 74:1371–1379.
32. Sutherland R, Boon RJ, Griffin KE, Masters PJ, Slocombe B, White AR. 1985. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob Agents Chemother 27:495–498.
33. Modi S, Xiang T-X, Anderson BD. 2012. Enhanced active liposomal loading of a poorly soluble ionizable drug using supersaturated drug solutions. J Control Release 162:330–339.