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Formation of drug–arylsulfonate complexes inside liposomes: A novel approach to improve drug retention

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Abstract

The development of procedures to enhance drug retention in liposomes is important in order to achieve therapeutically optimized rates of drug release from liposomal carriers. In this study, the ability of lipophilic weak base drugs to complex with arylsulfonates resulting in formation of intravesicular precipitates is investigated as a means to enhance drug retention. It is shown that the arylsulfonates benzenesulfonate and hydroxybenzenesulfonate (HBS) induce precipitation of ciprofloxacin and vinorelbine, two representative weak base drugs that are difficult to retain in liposomal systems. The most complete precipitation was observed at pH values corresponding to charge neutralization of the drug–arylsulfonate complex. HBS is shown to be a much more effective precipitating agent than benzenesulfonate. It is also shown that vinorelbine and ciprofloxacin can be loaded into large unilamellar vesicles (LUV) containing the calcium salt of HBS using an ionophore-based loading method. Following drug loading, the formation of intravesicular drug–arylsulfonate precipitates of vinorelbine and ciprofloxacin was observed by cryo-electron microscopy. In vitro release experiments showed substantial improvements in drug retention for both vinorelbine and ciprofloxacin when HBS was present as compared to standard loading procedures employing $MgSO_4$ as the entrapped solute. In vivo release experiments for vinorelbine in NuNu mice indicated a half-time for release for HBS-containing LUV of ~30 h, compared to 6.4 h for LUV loaded employing $MgSO_4$. It is suggested that encapsulation procedures employing HBS in the internal medium can improve the retention of drugs that are difficult to retain in liposomes, possibly leading to enhanced therapeutic properties.

Keywords: Liposome; Arylsulfonates; Vinorelbine; Ciprofloxacin; Drug release

1. Introduction

The pharmacological properties of many conventional pharmaceuticals can be significantly improved through the use of liposomal drug delivery systems [1–6]. The benefits of liposomal encapsulation, which include reduced toxicity and/or increased efficacy due to enhanced accumulation at disease sites, has resulted in the development of a number of antifungal and anticancer liposomal drug formulations (e.g. Ambisome™, ABELCET™, Doxil™, DaunoXome™) that have received regulatory approval and have demonstrated significant clinical utility. However, the extension of liposome technology to other drugs is often complicated by rapid release of the drug from the liposome following loading. The efficacy of liposomal formulations of certain drugs, such as anticancer agents, can be extremely sensitive to drug release rates, with the slowest releasing systems exhibiting the best efficacy profiles [7–9]. The design of truly optimized liposomal drug delivery systems therefore requires the development of new procedures to further improve drug retention.

While some drugs such as doxorubicin show extremely slow release rates from liposomes in vitro and in vivo [10–12], other drugs such as the antibiotic ciprofloxacin tend to leak out rapidly [10,12,13]. Those differences in release behaviour of different drugs are in part related to the physical state of entrapped drug in the liposomal interior. Thus, whereas doxorubicin encapsulated in liposomes forms precip-

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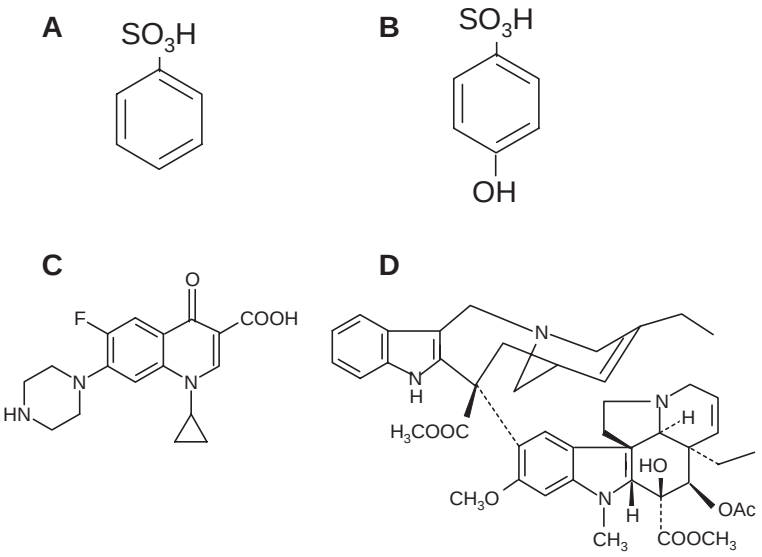

Fig. 1. Chemical structures of benzenesulfonic acid (A), 4-hydroxybenzenesulfonic acid (B), ciprofloxacin (C) and vinorelbine (D).

itated sulfate-based or citrate-based aggregates [11,12,14,15], no intraliposomal precipitation is detected when liposomes are loaded with ciprofloxacin [13]. In the latter case, formation of a solid phase was not observed even for intraliposomal concentrations of ciprofloxacin that exceeded its solubility by orders of magnitude. Another factor that can influence the release rate is the lipophilicity of the drug. Recently, the correlation between lipophilicity and drug retention has been shown for liposomal formulations of vinca alkaloids [16]. Despite the close similarities of their chemical structures, the more lipophilic character of vinorelbine and vinblastine results in much faster release as compared to vincristine.

In the present work, we have investigated the ability of arylsufonates to improve liposomal retention of difficult to retain drugs. Previous work has shown that complexes of arylsulfonates with weakly hydrated organic counter-ions can be soluble in organic solvents and only slightly soluble in water [17–19], leading to the possibility that the presence of the arylsulfonates inside the liposomes may lead to precipitation and enhanced retention of lipophilic, cationic drugs. Results obtained for vinorelbine and ciprofloxacin (for structures, see Fig. 1) are consistent with this proposal as the presence of intravesicular HBS results in the appearance of precipitates as visualized by cryo-electron microscopy following drug loading, and improved retention properties are observed both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Microcon centrifugal filter devices (YM-100) were obtained from Amicon Canada Ltd. (Oakville, ON, Canada). Egg sphingomyelin (ESM) was purchased from Northern Lipids (Vancouver, BC, Canada) and was used without further purification. The reagents 4-hydroxybenzenesulfonic acid (HBSA), 4-hydroxybenzenesulfonic acid sodium salt (NaHBS), benzenesulfonic acid (BSA), benzenesulfonic acid sodium salt (NaBS), calcium hydroxide, magnesium sulfate, ammonium chloride, cholesterol (Chol) and A23187 were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). [^{14}C]Cholesteryl hexadecyl ether (CHE) was custom synthesized by DuPont New England Nuclear (Boston, MA). [^{3}H]CHE was obtained from Perkin Elmer Life Sciences (Boston, MA). Vinorelbine ditartrate was obtained from OmniChem SA (Louvain-la-Neuve, Belgium). [^{3}H]Vinorelbine was synthesized by Moravek Biochemicals Inc. (Breas, CA). Ciprofloxacin hydrochloride, [^{14}C]ciprofloxacin and 1-*O*-(2′-(ω-methoxypolyethylene-glycol)succinoyl)-2-*N*-myristoylsphyngosine (PEG-CerC_{14}) were obtained as a gift from Inex Pharmaceuticals (Burnaby, BC, Canada). All other reagents were reagent grade.

2.2. In vitro precipitation of arylsulfonate complexes of vinorelbine and ciprofloxacin

Aliquots (75 μl) of an aqueous solution of ciprofloxacin (35 mM) containing a trace of [^{14}C] ciprofloxacin and an aqueous solution of vinorelbine (35 mM) containing a trace of tritated vinorelbine were mixed with 300 μl of 0.3 M aqueous solutions of NaHBS or NaBS at pH 4, pH 7 and pH 8.5 (the pH of arylsulfonate solutions was adjusted by the addition of the corresponding free acid (HBSA or BSA) or NaOH). Samples were then allowed to equilibrate for 20 min at room temperature. Separation of solubilized drug from precipitated drug-arylsulfonate complexes was performed using Microcon MY-100 (MWCO of 100,000 Da) ultrafiltration devices. Aliquots of samples (0.25 ml) were removed, placed in Microcon devices and centrifuged at 20,000×*g* for 5 min in a microcentrifuge (Heraeus Biofuge Pico, Kendo Laboratory Products, Germany) equipped with a fixed-angle rotor. Recovery of [^{14}C]ciprofloxacin and [^{3}H]vinorelbine in ultrafiltrates was determined by liquid scintillation counting (Beckman LS 3801).

2.3. Preparation of an aqueous solution of calcium 4-hydroxybenzenesulfonate (Ca^{2+}-HBS)

Solutions of calcium 4-hydroxybenzenesulfonate (~150 mM or ~300 mM) were prepared by mixing calcium hydroxide (60 mg or 120 mg) with 5 ml of distilled water with the subsequent dropwise addition of 65% aqueous hydroxybenzenesulfonic acid to the vortexed suspension until formation of a clear solution ($Ca(OH)_2$/HBSA molar ratio 1:2). As hydroxybenzenesulfonate is monovalent, the most likely stoichiometry of the calcium 4-hydroxybenzenesulfonate is $Ca^{2+}(HBS^-)_2$. The pH of the solution was set at 3.5–4.0.

2.4. Preparation of large unilamellar vesicles (LUVs) exhibiting a transmembrane gradient of calcium hydroxybenzenesulfonate or magnesium sulfate

ESM/Chol (55:45 mol/mol) lipid mixtures, containing trace amounts of either [^{14}C]CHE or [^{3}H]CHE, were prepared by co-dissolving the lipids in chloroform and drying under a nitrogen stream followed by the removal of residual solvent under high vacuum. Lipid mixtures prepared for Ca^{2+}-HBS-containing formulations also included 2 mol% of PEG-CerC_{14}. Dried lipid films were hydrated with an aqueous solution of magnesium sulfate (300 mM) or an aqueous solution of Ca^{2+}-HBS (~150 mM or ~300 mM) and subjected to five freeze–thaw cycles (liquid N_2/60 °C) to give multilamellar vesicle (MLV) suspensions. LUVs were generated by extruding the MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm (10 passes) at 65 °C using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada). The mean diameter of the LUVs was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, California) and found to be 110±25 nm. Phospholipid concentrations were determined by the phosphorus assay of Fiske and Subbarow [20]. The magnesium sulfate and calcium hydroxybenzenesulfonate gradients were formed by dialyzing the LUVs against HEPES-buffered sucrose solutions (pH 6.5). Subsequent addition of the ionophore A23187 to the suspension of LUVs results in the outward movement of one metal cation in exchange for two protons, thus establishing a transmembrane pH gradient, which drives drug uptake [21].

2.5. Drug uptake

Ionophore A23187 (2 μg/mg lipid), EDTA (15 mM final concentration) and the drug solution containing trace amounts of corresponding radiolabeled drug were added to the liposome preparation (5 mM total lipid) to give the desired initial drug-to-lipid ratio. The presence of external chelator (EDTA) was required to bind metal cations released from the vesicles. The samples were then incubated at 60 °C for 45 min. Loaded systems were dialysed for 2 h against sucrose buffer (pH 7.4) to remove untrapped drug and traces of ionophore. The lipid and drug concentrations were determined by liquid scintillation counting (Beckman LS 3801) for [^{3}H] and [^{14}C] labels. Loading efficiencies for molar drug-to-lipid ratios up to 0.3 were approximately 90–95% for all liposomal formulations tested.

2.6. In vitro assay for drug retention

In vitro release experiments were carried out by diluting samples with a HEPES/NaCl buffer containing ammonium chloride (0.5 mM final total lipid, 2 mM final NH_4Cl, pH 7.4 for the release of vinorelbine and 1 mM final total lipid, 2 mM final ammonium chloride, pH 7.4 for the release of ciprofloxacin), followed by incubation at 37 °C. Aliquots at various time points were taken and concentrations of entrapped drug were determined by dual label liquid scintillation counting following removal of released drug by running the aliquots over Sephadex G-50 spin columns. The % retention was defined as the drug-to-lipid ratio at time t divided by the initial drug-to-lipid ratio.

2.7. In vivo experiments

For in vivo studies, LUVs containing 300 mM magnesium sulfate and 150 mM Ca^{2+}-HBS were loaded with vinorelbine to give a drug-to-lipid ratio of 0.15 mol/mol. Both preparations also contained 2 mol% of PEG-CerC_{14} and a trace of [^{3}H]CHE as a lipid label. The NuNu strain of mice was used because of its ability to grow the HT29 tumor. Female HT29 tumor-bearing mice (4 mice per group) were injected with liposomal formulations at a lipid dose of 30 mg/kg. After 18 h, mice were anesthetized and whole blood was collected via cardiac puncture. The blood samples (50 μl) were mixed with 500 μl of Solvable and incubated overnight at room temperature, after which 50 μl of 200 mM EDTA was added followed by 200 μl of H_2O_2 (30% vol.). The samples were then left to decolorize overnight. The next day, 10 μl of 10 N HCl were added followed by 5 ml of Pico Green 40 and the lipid concentrations were determined by scintillation counting. Vinorelbine was measured using a Waters Alliance HPLC system consisting of an Alliance 2695 Separations Module (autosampler, HPLC pump and column heater), a Waters 2996 Photodiode Array detector and Waters Millenium^{32} HPLC software Version 4.0 (Waters Corporation, Milford, MA, USA) [22]. Following dissolution in 100% methanol, samples (10 μl) were injected onto a reverse-phase ACE C8 column with 3 μm packing, 100 mm × 4.6 mm (Advanced Chromatography Technologies, Aberdeen, UK) and eluted with a mixture of water and methanol having 0.1% phosphoric acid. The separation consisted of a gradient method starting at 30% methanol and increasing to 65% methanol with a constant column temperature of 60 °C and a flow rate of 1 ml/min. Vinorelbine was detected at 267 nm.

2.8. Cryo-transmission electron microscopy (cryo-TEM) studies

The cryo-TEM investigations were performed with a Zeiss EM 902A Transmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany). The instrument was

operating at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). An underfocus of 1–2 μm was used to enhance the image contrast.

3. Results

3.1. Ciprofoxacin and vinorelbine form precipitates in the presence of 4-hydroxybenzenesulfonate and benzenesulfonate

Arylsulfonates can form complexes with a variety of amines, resulting in formation of ion pairs with reduced solubility in water compared to that of free ions [17,18]. Two factors play a major role in the formation of these complexes. First, electrostatic interactions between the negatively charged sulfonic group of arylsulfonate and the positive charge on the protonated amino group play a dominant role in formation of the ion pair. Second, hydrophobic effects arising from the interaction between the hydrocarbon regions of complex-forming species can result in a decrease of the "net wetted area". The hydrophobic character of ion pairs can also be affected by the various functional groups (hydroxyl, carboxyl, amino) present in both cationic and anionic components of the complex.

The presence of ionizable functions on the drug renders the solubility of arylsulfonate–amine complexes dependent on the pH of the media. We therefore first investigated the (potential) precipitation of vinorelbine and ciprofloxacin in the presence of an excess of arylsulfonates as a function of pH. The separation of free and precipitated drug was carried out by ultrafiltration, a method that does not affect free drug volume or concentration in the ultrafiltrate and thus allows the direct measurement of the drug remaining in solution. As shown in Fig. 2A–B, the addition of both ciprofloxacin and vinorelbine to NaHBS resulted in precipitation. When ciprofloxacin was added to the NaHBS solution, a precipitate in the form of white needles formed immediately after mixing. The pH of the NaHBS solutions was not affected by the addition of ciprofloxacin. The precipitate formed remained stable over hours, and the extent of precipitation was much higher at lower pH values. Ultrafiltration measurements revealed that about 14% of the drug was in the soluble form at pH 4, whereas almost 80% remained in solution at pH 7 (Fig. 2A). Interestingly, no precipitation was observed after mixing of ciprofloxacin with a NaBS solution at either pH 4 or 7 (Fig. 2A).

The addition of vinorelbine to NaHBS also resulted in immediate precipitation; however, in contrast to the behaviour of ciprofloxacin, the precipitates formed after addition of vinorelbine to NaHBS at pH 4 and pH 7 were not stable and began to re-dissolve after a few minutes; after a 20 min incubation, over 90% of the drug was found in solution at pH 4 (not shown) and about 80% of the drug at pH 7 (Fig. 2B). It was noted that the pH of the NaHBS solution was reduced by approximately two units on addition of vinorelbine due to the low pH of the vinorelbine solution. The initial pH of the

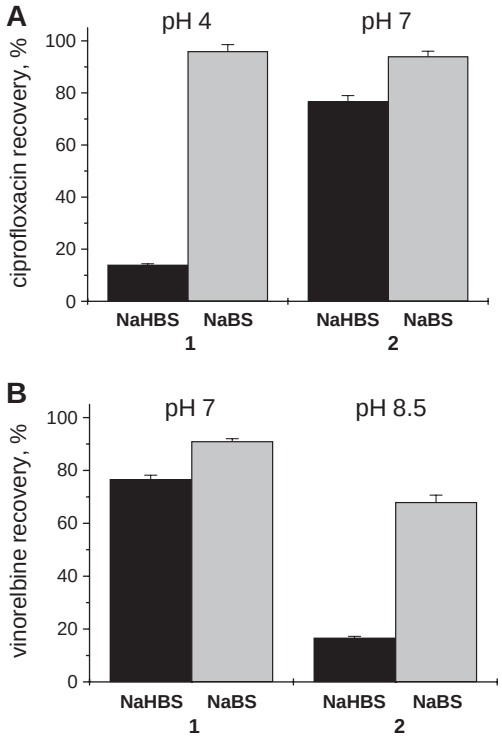

Fig. 2. Recovery of ciprofloxacin (A) and vinorelbine (B) after mixing with NaHBS (■) and NaBS (▩) solutions at different initial pH (A: pH 4 (1), pH 7 (2); B: pH 7 (1), pH 8.5 (2)) and ultrafiltration through Microcon MY-100 devices. Bars represent the means±S.D. of three analyses.

NaHBS solution was therefore raised to 8.5 in order to achieve a final pH of approximately 6. In this case, a stable amorphous precipitate was achieved, with only 16% of the drug remaining in the soluble form after 20 min incubation. As in the case of ciprofloxacin, hydroxybenzenesulfonate was shown to be much more effective precipitate-forming agent compared to benzenesulfonate (Fig. 2B).

3.2. The calcium salt of HBS can be used to drive ionophore-dependent uptake of vinorelbine and ciprofloxacin into LUV

The most efficient way of loading drugs into liposomes is to use transmembrane pH gradients (inside acidic) across the LUV membrane [23] to drive uptake of drugs that are weak bases. Such pH gradients can be generated in a variety of ways, including entrapment of a low pH buffer [23], entrapment of ammonium sulfate [24] and entrapment of salts of divalent cations in combination with the use of an ionophore such as A23187, which exchanges entrapped divalent cations for protons [21]. The ionophore mediated process is the preferred method as it allows the generation of higher drug-to-lipid ratios, which in turn gives rise to superior retention properties [16]. The ionophore A23187 is highly specific for transport of Ca^{2+} [25] and thus we attempted to generate the Ca^{2+} salt of HBS, in order to achieve a system where entrapped Ca^{2+} could be used to drive drug uptake and the entrapped HBS to drive drug precipitation following uptake. These efforts were successful, resulting in Ca^{2+}-HBS solutions with a maximum solubility of 300 mM. Magnesium HBS was also generated;

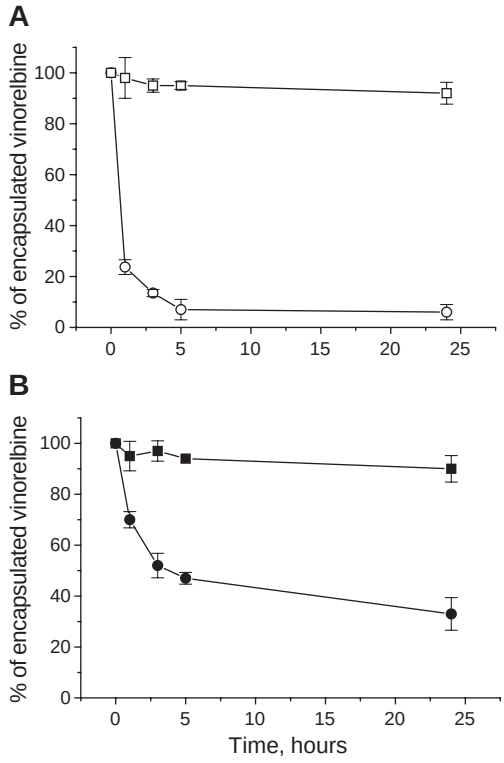

Fig. 3. In vitro release of vinorelbine from 100 nm LUVs loaded using magnesium sulfate (300 mM)/ionophore A23187 procedure (circles) and Ca^{2+}-HBS (150 mM)/ionophore A23187 procedure (squares). The drug-to-lipid ratios were 0.05 mol/mol (A, open symbols) and 0.15 mol/mol (B, closed symbols). Release was measured in the presence of 2 mM ammonium chloride at an incubation temperature of 37 °C. Aliquots were taken at different time points for determination of the lipid and drug concentrations by dual label scintillation counting as described in Materials and methods. The % of retention was defined as the drug-to-lipid ratio at time t divided by the initial drug-to-lipid ratio. The results represent the mean ± S.D.

however, it was much less soluble than Ca^{2+}-HBS and was not used further.

The next step involved generation of LUV containing high levels of Ca^{2+}-HBS, which requires dispersion of lipid in the presence of 150–300 mM Ca^{2+}-HBS with subsequent extrusion through polycarbonate filters with 100 nm pore size. Although it proved possible to disperse ESM/Chol in the Ca^{2+}-HBS solution and extrude the resulting dispersion through 100 nm filters, the LUV formed tended to aggregate. It was found that small amounts of a poly(ethylene glycol) (PEG) coating, provided by the presence of 2 mol% of PEG-CerC_{14} in the lipid mixture, prevented aggregation. Ionophore-mediated drug loading employing LUV containing 150–300 mM Ca^{2+}-HBS in the aqueous interior was then performed as described in Materials and methods. This resulted in trapping efficiencies of 90–95% for ciprofloxacin and 90–99% for vinorelbine for drug-to-lipid ratios as high as 0.15 (mol/mol).

3.3. Loading in the presence of entrapped HBS leads to improved drug retention both in vitro and in vivo

The next set of experiments was aimed at characterizing the benefits of using entrapped HBS in terms of improved drug retention. Comparisons were made to the standard ionophore loading process in which $MgSO_4$ is used as the entrapped agent ($CaSO_4$ cannot be employed as it is insoluble). In order to increase leakage rates to experimentally convenient levels in vitro, low (2 mM) levels of ammonium chloride were included in the HEPES buffered incubation medium. Ammonium chloride dissipates the pH gradient across the liposome membrane, thus raising the interior pH that results in more of the entrapped drug adopting the deprotonated (neutral) form, which is the more membrane permeable form. Aliquots were taken for analysis following incubation at 37 °C for up to 24 h. As shown in Fig. 3, retention of vinorelbine was substantially improved for LUV containing Ca^{2+}-HBS, as compared to $MgSO_4$, with more than 90% of the drug remaining in the liposomes after a 24 h incubation for drug-to-lipid ratios of 0.05 (Fig. 3A, open squares) and 0.15 (Fig. 3B, closed squares). In contrast, less than 10% of entrapped vinorelbine remained in LUV containing $MgSO_4$ at the 0.05 drug-to-lipid ratio (Fig. 3A, open circles) and only 30% for the 0.15 drug-to-lipid ratio (Fig. 3B, closed circles).

The influence of entrapped HBS on the retention properties of ciprofloxacin are illustrated in Fig. 4. As shown in Fig. 4A, when ciprofloxacin was loaded in LUVs containing 150 mM Ca^{2+}-HBS at a drug-to-lipid ratio of 0.05 mol/mol, no significant improvement of drug retention was found over the

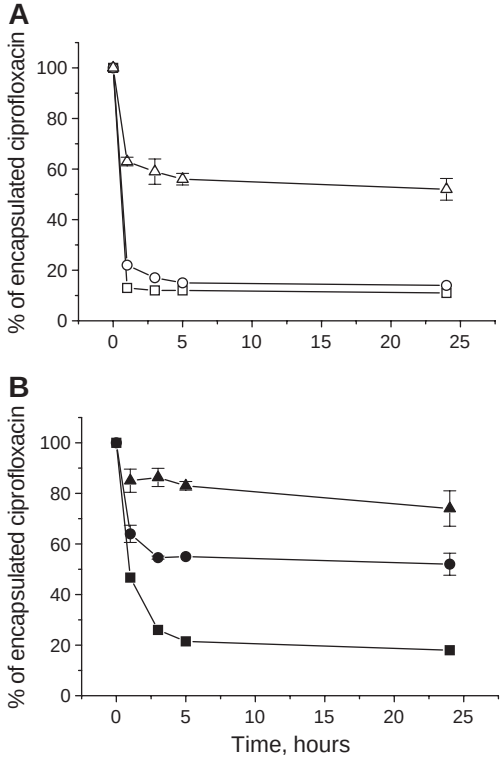

Fig. 4. In vitro release of ciprofloxacin from 100 nm LUVs loaded using magnesium sulfate (300 mM)/ionophore A23187 procedure (circles), Ca^{2+}-HBS (150 mM)/ionophore A23187 procedure (squares) and Ca^{2+}-HBS (300 mM)/ionophore A23187 procedure (triangles). The drug-to-lipid ratios were 0.05 mol/mol (A, open symbols) and 0.15 mol/mol (B, closed symbols). The results represent the mean ± S.D. The drug release was measured under conditions indicated in the legend to Fig. 3.

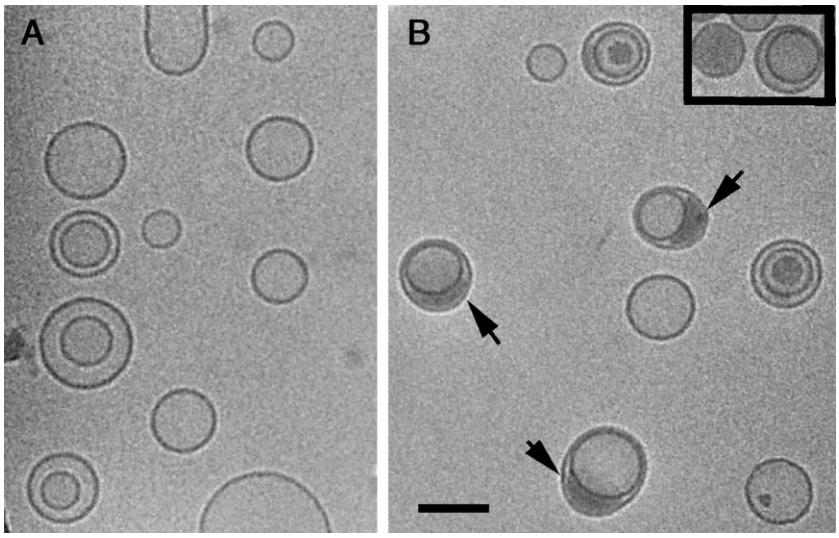

Fig. 5. Cryo-EM micrographs of ESM/Chol/PEG-CerC$_{14}$ (55/45/2) liposomes. (A) Liposomes containing 150 mM Ca^{2+}-HBS in the absence of drug; (B) liposomes loaded with vinorelbine at a drug-to-lipid ratio of 0.15 mol/mol using Ca^{2+}-HBS (150 mM)/ionophore A23187 procedure. Inset shows liposomes loaded with vinorelbine at a drug-to-lipid ratio of 0.15 mol/mol using magnesium sulfate (300 mM)/ionophore A23187 procedure. The size bar represents 100 nm.

magnesium sulfate-containing system (Fig. 4A, open circles). However, there was a measurable improvement in drug retention for the HBS-containing system at the 0.15 drug-to-lipid ratio (Fig. 4B, closed circles). Considerably better retention at both drug-to-lipid ratios was observed when the initial intraliposomal concentration of Ca^{2+}-HBS was increased to 300 mM (Fig. 4A, open triangles; Fig. 4B, closed triangles). It is interesting to note that, for all Ca^{2+}-HBS-containing formulations, the release profiles of ciprofloxacin were biphasic, where rapid drug loss in the first hours of incubation was followed by a slower release phase. This may reflect the co-existence of free and precipitated drug in the liposomal interior, with the rapid phase corresponding to the release of soluble drug (see Discussion). The slower release observed for liposomes 300 mM Ca^{2+}-HBS as compared to 150 mM is consistent with the fact that the presence of higher levels of HBS would shift the equilibrium towards the formation of precipitate.

The in vivo retention properties of Ca^{2+}-HBS containing LUVs loaded with vinorelbine were tested in tumor-bearing NuNu mice using LUV loaded employing entrapped $MgSO_4$ as a control. Both formulations included 2% mol of PEG-CerC$_{14}$. Blood samples were collected at an18 h time point and analyzed for lipid and drug content. It was found that 66±14% of the drug was retained for the HBS-containing LUV, whereas only 14±7% of the vinorelbine remained in the $MgSO_4$ control (data not shown). This corresponds to an increase in the half-time for release in vivo from ~6.4 h to ~30 h.

3.4. The presence of HBS in LUV loaded with vinorelbine and ciprofloxacin leads to intravesicular precipitation as visualized by cryo-transmission electron microscopy (cryo-TEM)

The enhanced retention of vinorelbine and ciprofloxacin in HBS-containing LUV would be consistent with precipitation of

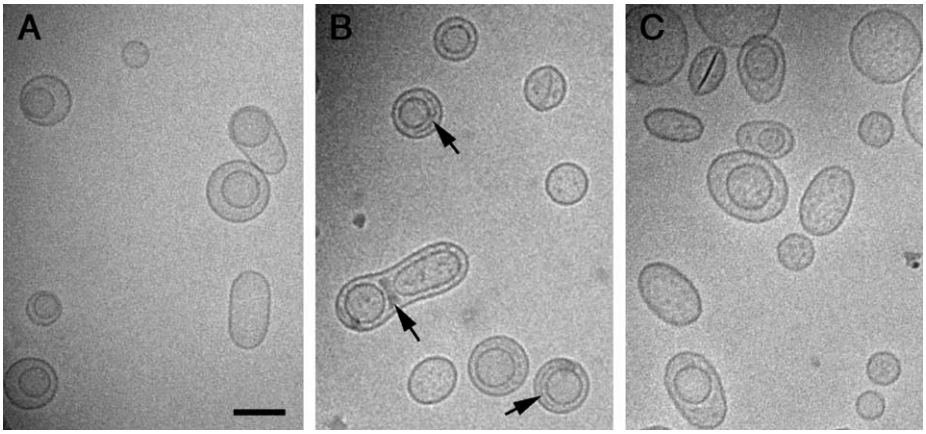

Fig. 6. Cryo-EM micrographs of ESM/Chol/PEG-CerC$_{14}$ (55/45/2) ciprofloxacin-loaded liposomes. (A) Liposomes loaded with ciprofloxacin at a drug-to-lipid ratio of 0.15 mol/mol using magnesium sulfate (300 mM)/ionophore A23187 procedure; (B) liposomes loaded with ciprofloxacin at a drug-to-lipid ratio of 0.15 mol/mol using Ca^{2+}-HBS (150 mM)/ionophore A23187 procedure; (C) liposomes loaded with ciprofloxacin at a drug-to-lipid ratio of 0.15 mol/mol using Ca^{2+}-HBS (300 mM)/ionophore A23187 procedure. The size bar represents 100 nm.

the drug in the vesicle interior, thereby reducing the free concentration of the entrapped drug and thus the net efflux rate. It would also be consistent with the ability of HBS to induce precipitation of vinorelbine and ciprofloxacin as demonstrated in Section 3.1. Cryo-TEM studies of the loaded liposomes were therefore performed to determine whether precipitate formation inside the LUV could be directly visualized. Cryoelectron micrographs of "empty" liposomes and Ca^{2+}-HBS containing vesicles loaded with vinorelbine at drug-to-lipid ratio 0.15 mol/mol are shown in Fig. 5A. Both liposomal populations consist predominantly of mono- and bilamellar vesicles. In the case of the empty liposomes, the LUV show no increased electron density of internal regions in comparison to background. In contrast, the interior of the Ca^{2+}-HBS containing LUV loaded with vinorelbine reveals electron-dense areas consistent with the presence of precipitates. The formation of precipitate between the lamellae of bilamellar vesicles (crescent-shaped structures indicated by the arrows) induces a distortion of the shape of these liposomes. The inset in Fig. 5B shows the magnesium sulfate-containing LUV loaded with vinorelbine at the same drug-to-lipid ratio. Unlike HBS-containing systems, those vesicles demonstrate a homogeneous interior with a slightly increased density.

Finally, as shown in Fig. 6, LUV loaded with ciprofloxacin in response to a $MgSO_4$ gradient shows no indication of intravesicular precipitation, whereas LUV loaded with ciprofloxacin and containing Ca^{2+}-HBS reveals internal structures that are again consistent with precipitation processes. As for vinorelbine-loaded liposomes, the most distinctive electron-dense structures can be seen between the lamellae of bilamellar liposomes (Fig. 6B, indicated by arrows).

4. Discussion

This study demonstrates that drug uptake into LUV-containing calcium salts of HBS can result in improved drug retention that is likely due to precipitation of drug-HBS ion-pair complexes inside the vesicles. There are three major points of interest. The first concerns the mechanism whereby HBS induces precipitation of available drug, whereas the second concerns the mechanism whereby drug precipitation leads to improved retention. Finally, we discuss the utility of methods relying on drug precipitation with arylsulfonates as a general method to improve the retention of weak base drugs in liposomal systems.

With regard to the mechanism whereby HBS induces precipitation of drugs such as vinorelbine and ciprofloxacin, the pH-dependencies of the formation of precipitates give some insight. For example, the results presented here show that ciprofloxacin precipitates much more completely with HBS at pH 4 as compared to pH 7. This behaviour can be related to the ionization state of the carboxyl function ($pK_1 = 6.0$) and amino group ($pK_2 = 8.8$) present on ciprofloxacin [10]. At pH values of 6 and lower, ciprofloxacin would be expected to exist primarily in a positively charged form, and the fact that extensive precipitation is observed at pH 4 indicates that this form readily associates with HBS to give non-soluble ion pairs. Alternatively, at higher pH values, any HBS–ciprofloxacin ion pairs formed would be expected to exhibit a net negative charge, rendering the complex more soluble.

In the case of vinorelbine, the p*K*'s of the two amino functions are not available in the literature; however, they would be expected to be similar to vincristine ($pK_1 = 5.0$, $pK_2 = 7.4$, [10]). At pH values of 5 and below, the molecule would therefore be expected to exist in a protonated form with two positive charges. As indicated by the results of Fig. 2B, the interaction with HBS does not result in stable precipitates in this pH range, which could arise due to steric issues associated with binding of two HBS molecules to a single vinorelbine molecule. However, at pH values above 5, where the molecule exists primarily in a monoprotonated form, stable precipitates with HBS can be formed.

Despite the apparent higher hydrophobicity of benzenesulfonate compared to hydroxybenzenesulfonate, the latter was far superior as a precipitating agent. It may be that the hydroxyl group of hydroxybenzenesulfonate allows hydrogen-bond interactions with the drug molecule, increasing the stability of the complex.

The mechanism whereby precipitation leads to improved drug retention is of particular interest. For a weak base drug obeying the relationship $[DH^+] = [D] + [H^+]$, the release rate is proportional to the concentration gradient of the permeable (neutral) form of the drug across the LUV membrane and the area A of the membrane, giving rise to the relationship $dN_i/dt = -PA([D]_i - [D]_{out})$, where P is the permeability coefficient of the neutral form. In the situation, where no precipitates are formed, then the total concentration of drug inside the LUV is given by $[D]_{tot} = [D]_i + [DH^+]_i$. Using the relation $N_i/V = [D]_{tot}$, where V is the volume of the LUV and, assuming that $[D]_i \gg [D]_o$, we then have that $d[D]^{tot}/dt = -(PA/V)[D]_i$. Under the condition that $[H^+] \gg K_d$, where $K_d = [D][H^+]/[DH^+]$ is the dissociation constant of the drug, we then have $d[D]^{tot}/dt = -(PAK_d/V[H^+])[D]^{tot} = -k[D]^{tot}$ where k is the rate constant, yielding the relationship

$$[D(t)]^{tot} = [D(0)]^{tot} e^{-kt}. \quad (1)$$

Importantly, Eq. (1) predicts that the percentage of the drug remaining in the liposome at time t is not dependent on the initial concentration of the drug. The drug retention expressed as $[D(t)]/[D(0)]$ at time t will be the same for liposomes loaded at different drug-to-lipid ratios (Fig. 7A).

However, this analysis does not hold if a fraction of the encapsulated drug is in a precipitated form. In the presence of counter-ion X^- (in our case, arylsulfonate), the solubility of the drug in the form of DH^+X^- is determined by its solubility product $K_s = [DH^+][X^-]$. Thus, upon achieving the solubility limit at a given concentration of X^-, an equilibrium between the precipitated phase and a saturated solution is established. Further increases of the drug-to-lipid ratio will not affect the concentration of the drug remaining in solution.

The equilibrium between precipitated and soluble drug inside the liposome means that efflux of the drug from the liposome should lead to further dissolution of the precipitate. In

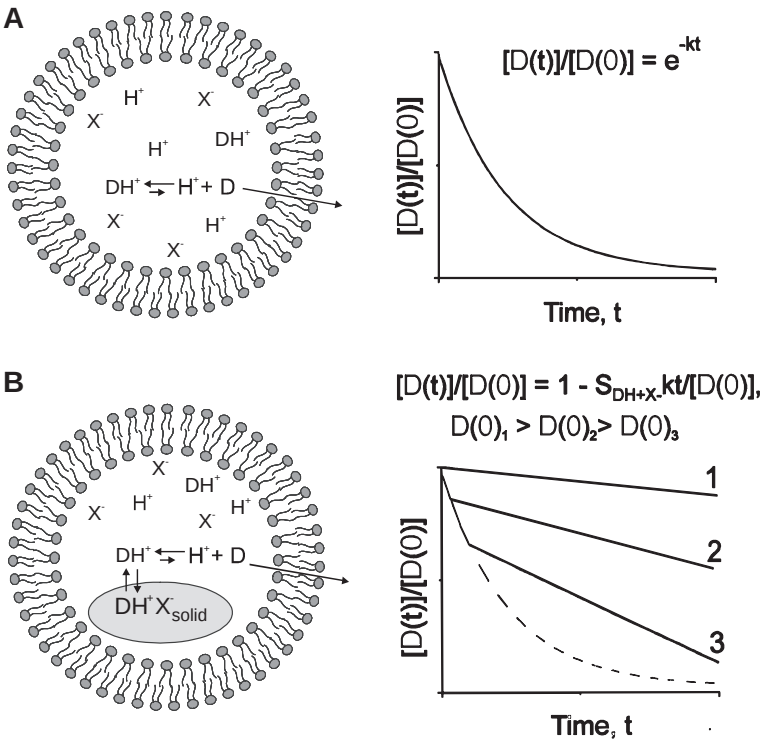

Fig. 7. (A) In the absence of precipitate, the percentage of released drug is not dependent on initial intravesicular drug concentration and obeys first order kinetics. (B) In the presence of a precipitate in equilibrium with the drug in solution, the percentage of released drug is dependent on initial intravesicular drug concentration and exhibits zero order kinetics.

this case, the concentration of drug in solution ($[D(max)]^{tot}$) is kept constant as long as the solid phase is present and is equal to the solubility of the complex $S_{(DH+X-)}$. Under this equilibrium, $d[D]^{tot}/dt = -kS_{(DH+X-)}$ is constant. Integration then gives the result that

$$[D(t)]^{tot} = [D(t)]^{tot} - kS_{(DH^+X^-)}t. \quad (2)$$

Thus, drug release from the liposome containing a saturated suspension in equilibrium with a precipitate is a zero order process, and the percentage of the remaining drug in this case is represented with a straight line with a slope depending on the initial concentration $[D(0)]^{tot}$. However, the actual pattern of release will be also dependent on the dissolution rate of the precipitate. If the latter is slower than the membrane diffusion rate of the neutral species, the release will be biphasic, starting with a rapid loss of the drug and, after establishing an equilibrium, will be followed by a slower linear phase (Fig. 7B, curves 2 and 3). If the efflux rate is slower than the dissolution rate of precipitate, or if the drug exists predominantly in the precipitated form, the percentage of release will be represented with a straight line from the beginning (Fig. 7B, curve 1).

The release profiles obtained for the Ca^{2+}-HBS-containing systems are consistent with the drug precipitation model presented above where ciprofloxacin release at later times is limited by the rate of precipitate dissolution, whereas the release of vinorelbine is limited by the rate of transmembrane diffusion.

The final topic of discussion concerns the utility of precipitation of entrapped drugs with arysulfonates to achieve enhanced retention. Previous work has established that enhanced retention of drugs such as vincristine [7,8] and vinorelbine [22] leads to improvements in efficacy, and thus the ability of HBS to improve retention of these agents would be expected to improve efficacy properties. The major concern would be that the presence of the arylsulfonate makes the formulations more toxic. In this regard, although pure benzenesulfonic acid is a skin and eye irritant, salts of arylsulfonic acids are of low toxicity [26]. For example, the zinc salt of hydroxybenzenesulfonic acid (Phenozin) is widely used as an intestinal antiseptic and locally as an astringent [27] and has a reported (oral) toxicity that is approximately a factor of 10 lower than vinorebine [28].

5. Conclusions

In summary, the use of Ca^{2+}-HBS as an entrapped agent to drive the uptake and precipitation of weak base drugs in LUV is a promising new method for enhancing drug retention in vivo, leading to potential improvements in the therapeutic index of weak base drugs. Work is in progress to find out if the use of liposomal arylsulfonate–drug complexes can lead to enhanced efficacy.

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