Preparation and Drug Entrapment Properties of Asymmetric Liposomes Containing Cationic and Anionic Lipids

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Abstract

We developed cyclodextrin-catalyzed lipid exchange method to prepare large unilamellar vesicles (LUVs) with asymmetric charge distributions, i.e. with different net charge on the lipids in the inner and outer leaflets. LUVs contained a mixture of a zwitterionic lipid (phosphatidylcholine), cholesterol and various cationic lipids (O-ethyl phosphatidyl choline or dioleoyl-3-trimethylammonium propane) or anionic lipids (phosphatidylglycerol, phosphatidylserine or phosphatidic acid). Symmetric and asymmetric LUVs with a wide variety of lipid combinations were prepared. The asymmetric LUVs contained cationic or anionic outer leaflets and inner leaflets that had either the opposite charge or were uncharged. The behavior of symmetric LUVs prepared with zwitterionic, anionic or cationic leaflets, was compared to those of asymmetric LUVs. Lipid exchange was confirmed by quantitative thin layer chromatography, and lipid asymmetry by a novel assay measuring binding of a cationic fluorescent probe to the LUVs outer leaflet. For both symmetric and asymmetric LUVs the level of entrapment of the cationic drug doxorubicin was controlled by the charge on the inner leaflet, with the greatest entrapment and slowest leakage in vesicles with an anionic inner leaflet. This shows that it is possible to choose inner leaflet lipids to maximize liposomal loading of charged drugs independently of the identity of outer leaflet lipids. This implies it should also be possible to independently vary outer leaflet lipids to, for example, impart favorable bioavailability and biodistribution properties to lipid vesicles.

Keywords

Asymmetric Vesicles, Drug Delivery, Cationic Lipids, Doxorubicin, and Cyclodextrin.

Introduction

Chemotherapeutic drugs and biomedicines have to be efficiently delivered to their target. This is an especially important issue for charged drugs such as doxorubicin, used for cancer treatment [1], and for RNAs that can be used therapeutically to interfere with gene expression, an approach useful for otherwise undruggable targets [2-3]. As with most drugs, these molecules must be presented at relatively high concentrations at specific targets [4-5]. Direct delivery of a drug can result in poor biodistribution and pharmacokinetics, and so result in unacceptable off-target side effects, short circulation times, drug breakdown and clearance [6].

To avoid such problems, trapping drugs within liposomes has demonstrated many advantages. Liposomes (lipid vesicles) are dispersions of membrane lipids in which a lipid bilayer surrounds an aqueous lumen. The advantages of using liposomes for drug delivery include the ability to trap many types of drugs within their lipid bilayer or aqueous lumen, easy manufacturing procedures, high loading of drug to minimize the dosage needed, the ability to use multi-dosing to maintain an effective drug concentration, the ability to target specific cells, and biocompatibility with long circulation times [7-10].

Much work has been done to study the relationship between liposomal lipid properties and the efficacy of drug delivery in different types of cells [8, 11]. However, the relationship between lipid properties such as charge and the drug-loading ability of vesicles has not been fully optimized. Charge is an important parameter influencing molecular delivery to cells [12]. Cationic lipid vesicles

can be used for delivery of molecules to cultured cells due to their ability to bind to the cell membrane, which facilitates endocytosis, membrane fusion, and endosomal escape [13-15]. In addition, cationic lipids aid the delivery of nucleic acids, which are anionic, because they form complexes [16]. However, if a drug has a positive net charge, use of cationic lipids could decrease the ability of the drug to be loaded within liposomes. Another issue is that cationic lipids on the outside of the vesicle may not be compatible with delivery *in vivo*, as such vesicles will likely stick non-specifically to the many anionic surfaces in a living organism, and can lead to undesirable phagocytosis [17-18].

The limitations of using cationic lipids might be addressed by using asymmetric LUVs. In asymmetric vesicles, the lipids in the inner and outer leaflet are different, and as a result the inner and outer leaflet can have very different properties [19-22]. The use of asymmetric LUVs raises the possibility of independently maximizing the loading efficiency of vesicles and drug delivery, in those cases in which different lipids are needed in the inner and outer leaflet to maximize these parameters. We have developed cyclodextrin exchange methods to prepare asymmetric LUVs [19, 23]. In some cases, studies by our group[19, 24-25] and others[26] prepared vesicles with one charged (anionic) and one neutral or near neutral leaflet. In this report we extend this approach to prepare a series of asymmetric LUVs with one cationic leaflet, and with vesicles in which one lipid leaflet has a charge opposite that of the other leaflet. This includes asymmetric LUVs with cationic outer leaflets and anionic or neutral inner leaflets, as well as asymmetric LUVs with anionic outer leaflets and cationic or neutral inner leaflets. Investigation of asymmetric LUVs properties, and comparison to the corresponding symmetric vesicles, show that the charge of the inner leaflet can maximize doxorubicin entrapment and minimize its leakage out of vesicles independent of the charge on the outer leaflet. This behavior was independent of lipid chemical structure, consistent

with electric charge being the key property controlling drug behavior. These studies open the path to applications of asymmetric LUVs with charge asymmetry in drug delivery and other experimental studies.

Materials and Methods

Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (POePC), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt) (POPA), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were stored in chloroform at -20 °C. Concentrations were determined by dry weight. High performance thin layer chromatography (HP-TLC) plates (Silica Gel 60) were purchased from VWR International (Batavia, IL). Methyl-α-cyclodextrin (MαCD) was purchased from AraChem Cyclodextrin Shop (Tilburg, the Netherlands). It was dissolved in distilled water at close to 300 mM, and then filtered through a Sarstedt (Nümbrecht, Germany) 0.2 μm pore syringe filter. The exact concentration of MαCD was determined by comparing the refractive index of the solutions to a standard curve of refractive index vs. MαCD concentration for a known amount of MαCD dissolved in a known final volume of solution. 1(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMADPH) was purchased from the Molecular Probes (Eugene, OR) division of Invitrogen

(Carlsbad, CA). Ammonium sulfate (AS) was purchased from Fisher Scientific (Boston, MA). Doxorubicin (Dox) was purchased from Cayman Chemical (Ann Arbor, Michigan). 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma-Aldrich (St. Louis, MO). PBS (10X phosphate-buffered saline, diluted to 1X: 10 mM sodium phosphate; and 150 mM sodium chloride, pH ~ 7.4) was purchased from Bio-Rad (Hercules, CA).

Preparation of symmetric LUV

Prior to vesicle preparation, the initial lipid concentrations were measured by gravimetric analysis of the stock solutions. Lipids dissolved in chloroform were mixed in glass tubes, dried under a warm nitrogen stream and subjected to high vacuum for 1 h. The dried lipid mixtures were dispersed to 8 mM lipid concentration with 23 % (w/w) sucrose in 0.83X PBS (sucrose/PBS, ~1009 mOsm, prepared by dissolving sucrose in 1X PBS). For Dox entrapment, lipid mixtures were dispersed in sucrose/PBS with 100 μg/mL of Dox and 50 mM AS. The samples were vortexed briefly and then incubated at 37 °C for 15 min. The lipid mixtures were then cooled to room temperature and subjected to seven cycles of freeze-thaw in a liquid nitrogen bath, alternating with a 27 °C water bath. To form LUVs of uniform vesicle size, the lipid mixtures were then extruded 11 times through 100 nm-pore polycarbonate membranes (Sigma-Aldrich, St. Louis, MO).

If needed to wash away external sucrose (e.g. to prepare acceptor vesicles for lipid exchange or prepare samples for size measurements), 200 μL aliquots of LUV were mixed with 3.8 mL 1X PBS (~325 mOsm) and pelleted by ultracentrifugation at 190,000×g for 30 min at 23 °C using a Beckman L8–80M ultracentrifuge with a SW-60 rotor. Following pelleting, the supernatant was removed, the LUV containing-pellet dispersed in 0.5 mL PBS. When samples had entrapped Dox they were re-centrifuged twice with 4 mL PBS using the same protocol. Finally, the LUV pellet

was dispersed in 500 μL PBS, covered with aluminum foil, and reserved for use. Unless otherwise noted samples were used within 2 h of preparation.

Preparation of donor lipid-loaded MαCD for lipid exchange experiments

Desired ratios of charged lipids (POePC, DOTAP, POPS, POPG or POPA) and zwitterionic POPC dissolved in chloroform were combined in glass tubes, dried under a warm nitrogen stream, and then subjected to high vacuum for 1 h. The dried lipids were placed in a 70 °C water bath and dispersed at 70 °C with an aliquot of pre-warmed PBS, and then an aliquot of pre-warmed MαCD, to give a final concentration of 40 mM MαCD and 16mM lipid. The samples were vortexed briefly, and then vortexed in a multitube vortexer for 2 h at 55°C, cooled to room temperature, covered in foil, and reserved for further use.

Preparation of acceptor LUV for lipid exchange experiments

Desired ratios of charged lipids (POePC, DOTAP, POPS, POPG or POPA), zwitterionic POPC, and cholesterol (40 mol% of total lipid) dissolved in chloroform were combined in glass tubes. LUVs were then prepared as described above for symmetric vesicles.

Outer leaflet lipid exchange

To wash away untrapped sucrose from acceptor LUVs, 500 μL aliquots of acceptor LUVs were diluted with 3.5 ml PBS and subjected to ultracentrifugation at 190,000g for 30 min at 23 °C as above. The supernatant was discarded, the LUV pellets were resuspended to 8 mM lipid concentration with PBS and used immediately. To exchange the outer leaflet of acceptor LUVs, 500 μL of the donor lipid-MαCD mixture and 500 μL of the acceptor LUVs mixtures were combined, covered in foil, and shaken for 45 min at 37 °C. These lipid-exchange mixtures were

layered over 3 mL 7.4 % (w/w) sucrose dissolved in 3.76X PBS (prepared by dissolving sucrose in 4X PBS, ~1448 mOsm) and subjected to ultracentrifugation at 190,000 x g for 45 min at 23 °C. Following centrifugation, most of the supernatant was carefully removed, leaving approximately 750 µL sucrose/4X PBS and loosely pelleted asymmetric LUVs in the bottom of the centrifuge tube. The upper portion of the tube was swabbed with a clean, dry cotton tipped applicator to remove residual adhering donor lipids and MαCD. Approximately 3.25 mL PBS was then added to the tube and thoroughly mixed with asymmetric LUVs and residual supernatant. This mixture was centrifuged a second time as above for 30 min. Following centrifugation, all remaining supernatant was removed, and the pellet was dispersed for immediate use in up to 500 µL PBS or distilled water if samples were for TLC analysis. The asymmetric LUVs lipid concentration was determined by HP-TLC or DPH assay (see below in Methods) and the mean yield was ~10.5% of theoretical maximal yield, see Supplemental Table S1), with a final lipid concentration 0.83 ± 0.22 mM. Entrapped Dox did not appear to reproducibly affect asymmetric LUVs lipid yield.

High-performance TLC (HP-TLC)

Aliquots of samples and lipid standards were dissolved in 1:1 (v/v) chloroform/methanol. Dissolved lipids were applied to HP-TLC (Silica Gel 60) plates (Merck) and chromatographed to within 20% of full plate height in 3:1:1 chloroform:methanol:acetic acid (v/v). After chromatography, the plates were air dried, saturated with 3% (w/v) cupric-acetate-8% (v/v) phosphoric acid by spraying, and then air-dried again. Plates were then charred on a hot place at ~180 °C to develop lipid bands. Lipid band intensity was measured using ImageJ software (National Institutes of Health). Lipids in samples were quantified by comparing background-subtracted band intensity with that of various standard amounts of each lipid chromatographed on the same TLC plate. A sample TLC plate is shown in Supplemental Figure S1. The intensity in the

standard bands was fit to a linear intensity vs. lipid quantity curve using Excel software (Microsoft Corporation, Redmond, WA).

Fluorescence measurements

Fluorescence measurements were carried out using a SPEX FluoroLog 3 spectrofluorometer (Horiba Scientific, Edison, New Jersey) using quartz semimicro cuvettes (excitation pathlength, 10 mm; emission pathlength, 4 mm) or either a BioTek Synergy HTX Multi-Mode microplate reader ot a BioTek Synergy Neo2 HTS Multi-Mode microplate reader (Winooski, VT) using Corning™ 96-Well Solid Black Polystyrene Microplates. TMADPH fluorescence was measured at an excitation wavelength of 364 nm and emission wavelength of 426 nm. Dox fluorescence was measured at an excitation wavelength of 470 nm and emission wavelength of 595 nm. The slit bandwidths were set to 3 mm (about 5 nm bandpass) for both excitation and emission. Fluorescence was measured at room temperature. Background samples, which lacked fluorescent probe, had negligible intensity (<1% of samples with fluorescent probe).

Fluorescence measurement of doxorubicin concentration

We found Dox trapped in LUVs did not have the same fluorescence intensity as when dissolved in solution, likely because it is present within liposomes in an aggregate [1] having a decreased quantum yield. When vesicles with entrapped Dox were dissolved with 1 (v/v) % Triton X-100 Dox fluorescence increased to a level about the same as Dox dissolved in PBS. (Addition of Triton X-100 did not affect the fluorescence of Dox in PBS.) Dissolving the vesicles with 1% Triton increased Dox fluorescence by about 150%, 15%, and 45 % when the Triton X-100 was added to cationic, neutral, and anionic vesicles containing trapped Dox, respectively. This was used as a

correction factor to convert the intensity of fluorescence for vesicle entrapped Dox to that which would be measured for Dox in solution.

Fluorescence measurement of lipid concentration and outer leaflet charge

Lipid concentration in asymmetric LUVs and symmetric LUVs after centrifugation was estimated via TLC or the level of DPH bound as measured by fluorescence [27]. In the latter case, DPH fluorescence was measured at an excitation wavelength of 358 nm and emission wavelength of 430 nm. A standard linear curve of fluorescence vs. lipid concentration was prepared using symmetric LUVs with POPC, 40 mol% cholesterol and with or without 15 mol% of the charged lipids used in the LUVs to be assayed. (However, it should be noted that the standard curves were not affected by the presence or absence of 15 mol% charged phospholipid, and so were averaged to give the final standard curve.) Standard samples were diluted with PBS to the desired concentration.

Experimental samples for which lipid concentration was to be determined were diluted 100 to 400-fold (to ~2-8 μM lipid) by adding aliquots of each sample to quartz semi-micro cuvettes containing enough of PBS to give a total volume of 1 ml and then 20 μL of 18 μM DPH dissolved in ethanol added. To test the lipid concentration of symmetric or asymmetric LUVs with Dox entrapped inside, standard samples were prepared the same way except the lipids were hydrated with 100 μg/mL of Dox and 50 mM AS.

For the TMA-DPH binding assay, samples were diluted to 118 or 257 μM by adding aliquots of LUVs to quartz semi-micro cuvettes containing enough of either PBS or sucrose/PBS to give a total volume of 1 ml, and then 20 μL of 10 μM TMA-DPH dissolved in ethanol was added. The final TMA-DPH concentration was 0.2 μM. A standard curve was prepared from symmetric LUVs

composed of POPC, various amounts up to 15 mol% of the charged lipids of interest and 40 mol% cholesterol. Lipid concentrations for the standard curve were 118 or 257 μM. From the standard curve samples a graph of TMA-DPH fluorescence (F) normalized to that of 0.2 μM TMA-DPH dissolved in ethanol (F_0) vs. net % of charged phospholipid. A fourth order polynomial fit was used for the standard curve. Experimental asymmetric LUVs samples were diluted with PBS to match lipid concentration to that in the standard curve. The value of fluorescence was then normalized to the fluorescence of 0.2 μM TMADPH in ethanol, and the value obtained was compared to the standard curve.

Measurement of dynamic light scattering

LUV size was determined by dynamic light scattering using a ProteinSolution DynaPro instrument (Wyatt Technology, Santa Barbara, CA) at 20°C. AUVs and symmetric LUVs were diluted to ∼50–80 μM using PBS filtered with a 0.2-mm-filter. Vesicle sizes were estimated with the use of the Dynamics V5.25.44 program supplied by Wyatt Technology. Acceptor vesicles before and after exchange of outer-leaflet lipids had similar diameters of 125 ± 20 nm.

Results

Preparation and Final Phospholipid Composition of Asymmetric Vesicles

The MαCD-mediated lipid exchange method developed by our group [28-29] was adapted here to prepare asymmetric LUVs with opposite net charge on their inner and outer leaflets. In the exchange protocol MαCD exchanges phospholipids from donor vesicles with one lipid composition with the phospholipids in the outer leaflet of acceptor LUVs having a different lipid composition, converting the acceptor LUVs into asymmetric LUVs. The acceptor vesicles contain trapped sucrose to aid isolation by centrifugation, and the desired concentration of cholesterol, which is not exchanged by MαCD and so remains in the asymmetric LUVs. When the donor lipid is in excess, the final asymmetric LUVs formed from the acceptor LUVs have an outer leaflet with a phospholipid composition similar to that of the donor vesicles prior to exchange [19, 23] (Figure 1).

Preliminary studies showed yields were highest and most consistent when acceptor vesicles contained 40 mol% cholesterol (relative to vesicles with less or no cholesterol) and when charged lipids were no more than 25 mol % of the total phospholipid (equal to 15 mol% of total lipid). The remainder of the phospholipid used was POPC (45 mol % of total lipid, which is equal to 75 mol% of phospholipid). One change from previous protocols was that to obtain a high yield of vesicles after centrifugation, a higher concentration of PBS was used in the sucrose solution in which centrifugation was carried out. The increase in vesicle yield with cholesterol may arise because it increases vesicle density and so increases vesicle pelleting upon centrifugation. In addition, if lipid exchange between donor and acceptor LUVs is not 1:1 (one lipid added to the acceptor for every lipid removed), a stress between two leaflets would be induced, and this might be alleviated by

redistribution of cholesterol from one leaflet to the other, as cholesterol can flip rapid across the bilayer[30]. Minimizing such stress might minimize vesicle rupture with release of sucrose.

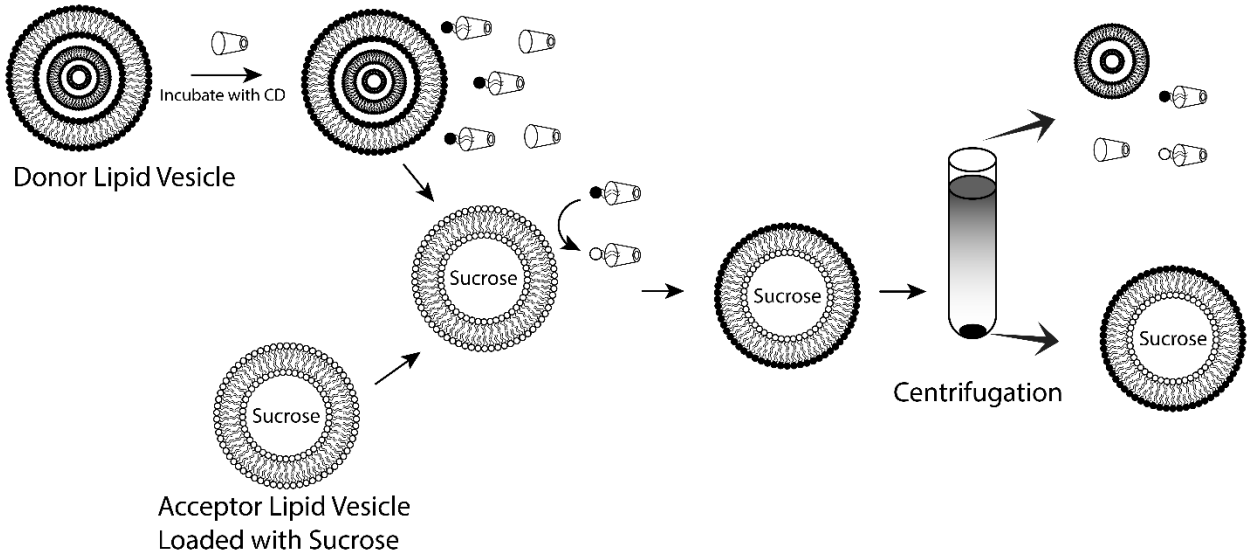

Figure 1. Schematic illustration of asymmetric LUVs preparation.

Using these conditions, asymmetric LUVs with a range of charged lipid compositions were prepared. Asymmetric LUVs were prepared in which the outer leaflet was cationic or anionic, and the inner leaflet had the opposite charge or was uncharged.

Symmetric LUVs were prepared similarly to the asymmetric vesicles but without a lipid exchange step. The symmetric vesicles were composed of POPC, 40 mol% cholesterol and, when desired, 15 mol% of cationic lipid (POePC or DOTAP) or of anionic phospholipid (POPG. POPS or POPA). (Note: although DOTAP is not a phospholipid, for simplicity, when talking about lipids other than cholesterol we will use the term “phospholipid” below instead of the more precise “phospholipid or DOTAP”.)

Donor Phospholipid Composition	Acceptor Phospholipid Composition	Phospholipid Composition in Asymmetric Vesicles	Calculated Outer Leaflet Phospholipid Composition in Asymmetric Vesicles
Charged Outside/Neutral Inside			
POePC:POPC 25:75	POPC	POePC:POPC 8:92	POePC:POPC 17:83
DOTAP:POPC 25:75	POPC	DOTAP:POPC 9:91	DOTAP:POPC 17:83
POPS:POPC 25:75	POPC	POPS:POPC 12:88	POPS:POPC 23:77
POPG:POPC 25:75	POPC	POPG:POPC 9:91	POPG:POPC 18:82
POPA:POPC 25:75	POPC	POPA:POPC 12:88	POPA:POPC 24:76
Anionic Outside/Cationic Inside			
POPS:POPC 25:75	POePC:POPC 25:75	POPS:POePC:POPC 11:16:73	POPS:POePC:POPC 22:7:71
POPG:POPC 25:75	POePC:POPC 25:75	POPG:POePC:POPC 12:18:70	POPG:POePC:POPC 25:10:65
POPA:POPC 25:75	POePC:POPC 25:75	POPA:POePC:POPC 8:13:79	POPA:POePC:POPC 16:1:83
POPS:POPC 25:75	DOTAP:POPC 25:75	POPS:DOTAP:POPC 6:22:72	POPS:DOTAP:POPC* 12:19:69
POPG:POPC 25:75	DOTAP:POPC 25:75	POPG:DOTAP:POPC 11:14:75	POPG:DOTAP:POPC 21:4:75
Cationic Outside/Anionic Inside			
POePC:POPC 25:75	POPS:POPC 25:75	POePC:POPS:POPC 11:14:75	POePC:POPS:POPC 23:2:75
DOTAP:POPC 25:75	POPS:POPC 25:75	DOTAP:POPS:POPC 10:17:73	DOTAP:POPS:POPC 20:10:70
POePC:POPC 25:75	POPG:POPC 25:75	POePC:POPG:POPC 12:21:67	POePC:POPG:POPC 24:17:59
DOTAP:POPC 25:75	POPG:POPC 25:75	DOTAP:POPG:POPC 7:19:74	DOTAP:POPG:POPC 14:12:74
POePC:POPC 25:75	POPA:POPC 25:75	POePC:POPA:POPC 8:13:79	POePC:POPA:POPC 16:1:83

Table 1. Phospholipid composition of asymmetric LUVs. Cholesterol content of acceptor vesicles and asymmetric vesicles is ~40mol%. *Note that due to inefficient exchange using POPS:POPC as the donor and acceptor containing DOTAP and POPC did NOT result in production of asymmetric LUVs with net anionic outer leaflets. The values shown are the average from at least six preperations. The standard deviations are showin Tables S2 and S3.

If exchange was 100% complete, in the asymmetric LUVs the inner leaflet would have the phospholipid composition of the acceptor vesicle and the outer leaflet would have the phospholipid

composition of the donor vesicle. Cholesterol would be in both leaflets because, as noted above, it is not exchanged out of the acceptor vesicles by MαCD. The actual extent of exchange can be influenced by phospholipid structure, which can modulate binding to MαCD, and any differential ability of phospholipids to be extracted from or inserted into lipid vesicles. This is likely to be dependent upon both the structure of the lipids being exchanged and of the lipid composition of the vesicles from which the lipid is being removed or inserted.

Table 1 shows the composition of the donor and acceptor vesicles used for lipid exchange as well as the composition of the asymmetric vesicles prepared from them. To experimentally determine the extent of exchange, the phospholipid composition of the asymmetric vesicles was assayed by quantitative TLC. Average (mean) values for phospholipid composition in vesicles after exchange are summarized in Table 1 (results with both the mean values and standard deviations are shown in Supplemental Tables S2 and S3).

Calculated outer leaflet compositions are shown in rightmost column of Table 1. The composition of the outer leaflet was calculated based on prior observations showing that: 1. exchange is specific to the outer leaflet, and 2. phospholipid flip-flop between leaflets, which would destroy asymmetry, is very slow (days) for the types of lipid compositions studied here [19, 24-25, 31-33]. To calculate the outer leaflet composition, the overall composition determined by TLC was combined with the fact that the exchange only involves lipids in the outer leaflet. The percent of the outer leaflet phospholipid for each lipid = the percent of total phospholipid of that lipid in the asymmetric vesicle after exchange x 2 – the percent of total phospholipid of that lipid in the acceptor for before exchange. Once the % of charged lipid from the donor in the asymmetric vesicles is known, then the efficiency of exchange can be calculated. The % efficiency of exchange = [(% of charged lipid from the donor located in the acceptor outer leaflet after exchange) / (% of

charged donor lipid in the donor)] x 100%. For example, in the first row in Table 1, the efficiency of exchange is (17%/25%) x 100% = 68%.

A significant amount of exchange was achieved when donor lipid contained various combinations of different cationic and anionic lipids with POPC and acceptor vesicles contained POPC and cholesterol. The % of charged donor phospholipid in the asymmetric vesicles prepared from the acceptor vesicles was ~60-95 mol% of the value for complete exchange. There was slightly lower mean exchange efficiency for donor containing cationic phospholipid (~70%) than for donor containing anionic phospholipid (~88%).

The pattern for the efficiency of charged phospholipid exchange asymmetric LUVs when the both the donor and acceptor contained charged phospholipid was somewhat different. There was a relatively high value of efficiency of charged donor phospholipid exchanged into the acceptor vesicles in most cases (~80-100%), but also a few cases with relatively low efficiency of exchange (~50-65%). This had consequences for which types of asymmetric LUVs can be prepared with strongly opposite net charge in each leaflet (see below).

The efficiency of exchange of POPC relative to charged phospholipid is an additional parameter influencing final phospholipid composition. If exchange of POPC and charged phospholipids between donor and acceptor were equally efficient in the samples in which both donor and acceptor contain 25 mol% charged phospholipid, the final fraction of POPC in the phospholipid of the asymmetric LUVs would be the same as before exchange, 75 mol%. The mean experimental values for POPC content as a percent of total phospholipid in experiments in which both donor and acceptor had charged phospholipids was 74 mol%. This indicates that the relative exchange efficiencies of charged lipid and POPC are similar, although some phospholipid compositions

resulted in slightly lower (~60 mol%) or higher (~85 mol%) POPC content in total phospholipid, suggestive of slightly different exchange efficiencies for POPC and phospholipids with net charge. When POPC levels are significantly above 75% it indicates that POPC from the donor must have exchanged into the acceptor more efficiently than the charged lipid from the donor, or that POPC from the acceptor exchanged out less efficiently than did charged lipid originally in the acceptor. When POPC is significantly less than 75% it indicates that POPC from the donor must have exchanged into the acceptor less efficiently than charged lipid from the donor, or that POPC from the acceptor exchanged out more efficiently than did charged lipid originally in the acceptor.

Somewhat unequal (non-random) exchange of different phospholipids is also suggested by the fact that in some experiments in which donor and acceptor both contained 25 mol% charged phospholipid, the final total mol% of phospholipid that was charged in the asymmetric vesicles was somewhat less than or greater than 25 mol%. The former case can occur when charged phospholipid from the donor is not exchanged as efficiently as donor POPC, and/or charged phospholipid from the acceptor is exchanged more efficiently than acceptor POPC. The latter case can occur when charged phospholipid is extracted from acceptor vesicles less efficiently than acceptor POPC, and/or when charged phospholipid is exchanged into acceptor more efficiently than POPC. In such cases, the outer leaflet contains substantial amounts of both anionic and cationic lipid.

Despite these complications, the overall level of exchange in most cases was sufficient to prepare a wide variety of asymmetric LUVs with different signs on the net charges on their inner and outer leaflet (Table 1). One exception was the case in which the donor contained POPS and acceptor DOTAP, in which the level of exchange was so low, that the outer leaflet of the acceptor vesicles remained cationic before and after exchange. Another example of poor exchange was

when the donor contained DOTAP and the acceptor POPG. In that case the level of exchange was only enough to result in a near-neutral outer leaflet rather than the desired highly cationic outer leaflet.

It should be noted that DPH anisotropy, which measures membrane order, was not significantly different for the different preparations of symmetric and asymmetric vesicles (data not shown). This indicates that for the lipids used, charge and asymmetry did not affect membrane order.

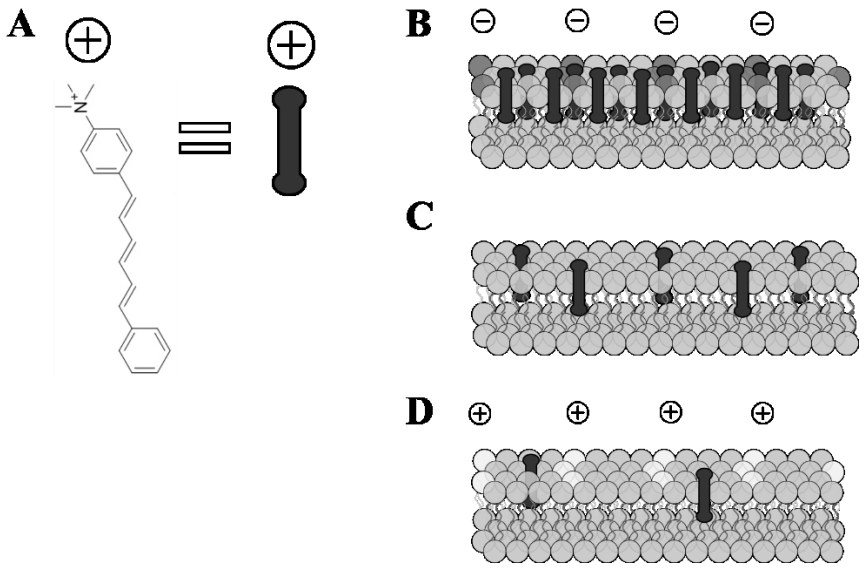

Figure 2. Schematic illustration of TMA-DPH binding assay for measuring outer leaflet charge. A. Structure of TMA-DPH. B., C. and D., binding to net anionic, neutral and cationic outer leaflets, respectively. The signs show relative lipid charge.

TMADPH assay to measure charge in the outer leaflet of asymmetric LUV

Although the outer leaflet phospholipid compositions estimated from the extent of lipid exchange should be valid given the prior demonstration that phospholipid exchange only involves the outer leaflet and lipid flip-flop between leaflets is slow, as noted above, it was desirable to have

a confirmatory method to estimate the charge on the outer leaflet of the asymmetric LUVs. To achieve this a novel TMA-DPH binding assay was developed. The structure of the cationic fluorescent probe TMA-DPH is shown in the Fig 2A. Because TMA-DPH is cationic and does not rapidly cross membranes [19, 34-35], its binding to and insertion into membranes is dependent on outer leaflet charge, with a higher level of binding to anionic membranes, as shown schematically in Fig. 2B. After inserting into the hydrophobic core of the vesicle bilayer, the fluorescence of TMA-DPH greatly increases, which allows facile detection of binding.

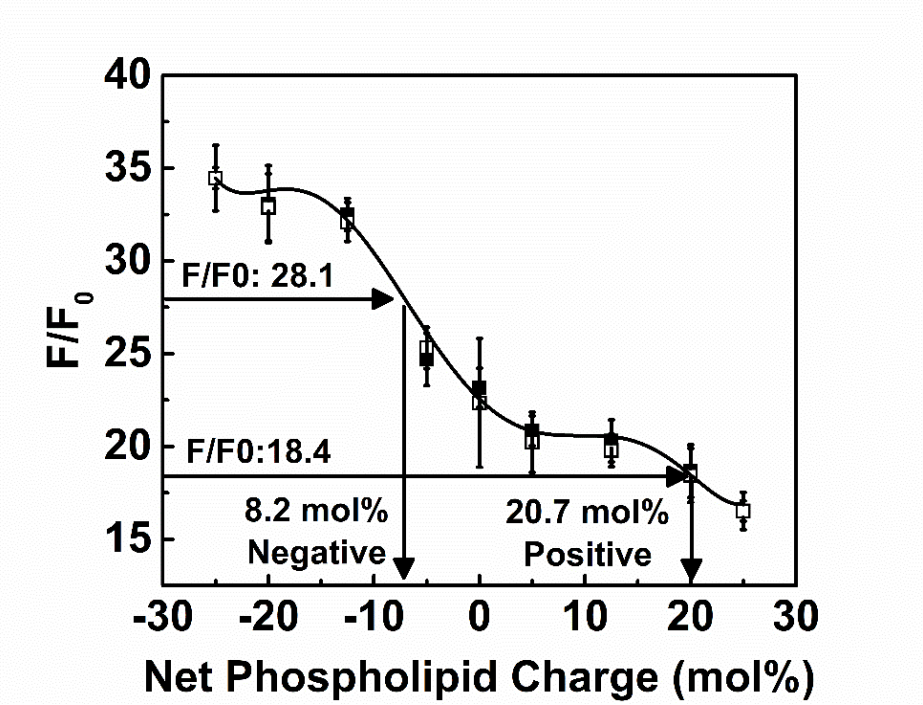

Figure 3. Relationship between normalized fluorescence of TMADPH (F/F_0) and outer leaflet net charge in vesicles containing POePC and/or POPS as charged lipids. F/F_0 equals the fluorescence intensity of TMADPH in vesicle-containing samples/fluorescence of TMADPH in ethanol. Net Phospholipid Charge (mol%) equals the mol% net charge (of phospholipid) in the outer leaflet of LUVs. Solid boxes: Mixed-charges sample: phospholipids were composed of 75 mol% of POPC and 25 mol% of POePC and POPS in various ratios. Open Boxes: Mono-charged sample: phospholipids were composed of various % POePC and POPC or of % POPS and POPC. All samples had 40 mol% cholesterol. Experimental values for specific asymmetric LUVs preparations (arrows) are also shown. These are for the sample compositions in Figure 4 column c. and g. See Results for details. Mean values and standard deviations from three vesicle preparations are shown.

Figure 3 shows an example of a standard curve of TMA-DPH fluorescence vs. charge for estimating charged lipid content in the outer leaflet of LUVs. The standard curve shown is for LUVs composed of 40 mol% cholesterol and mixtures of POPC, POPS, and POePC. Other lipid mixtures gave similar standard curves (Supplemental Figure S2). Two types of standard curves were prepared. In one set the standard curve samples were composed of (in addition to cholesterol) binary phospholipid mixtures containing various ratios of POPC and POPS, to prepare standard samples with 0-25 mol% net negative charged phospholipid. In the second set various ratios of POPC and POePC were used to prepare standard samples with 0-25 mol% net positively charged phospholipid. In the third set of standards cholesterol and a ternary phospholipid mixture of POPC, POPS, and POePC was used, in which total charged phospholipid was fixed at 25 mol% of total phospholipid, but with different ratios of POPS to POePC. To illustrate the difference between these sets of samples, in the first and second set, the samples with zero net charged phospholipid contained only cholesterol and POPC, while in the third set the samples with zero net charged phospholipid contained cholesterol and phospholipid composed of 75 mol% POPC, 12.5 mol % POPS and 12.5 mol% POePC.

As shown Figure 3, the two sets of standard samples gave almost identical similar TMA-DPH fluorescence values for vesicles having equivalent net charge, indicating that TMA-DPH binding was simply sensitive to net phospholipid charge. Similar behavior was observed for the standard curves prepared for other mixtures of cholesterol with binary and ternary phospholipid mixtures (Supplemental Figure S2). We calculated experimental values for TMA-DPH fluorescence for two examples of asymmetric vesicles POPS:POPC out/POePC:POPC in/Chol and for POePC:POPC out/POPS:POPC in/Chol asymmetric LUV. (Asymmetric vesicles are named as follows: “lipid

A:lipid B out/lipid C:lipid D in/Chol") in which "out" refers to the lipids in the outer leaflet which are predominantly from the donor and "in" refers to the lipids in the inner leaflet, which are the same as in the acceptor before exchange. Chol is not designated by in or out because it is in both leaflets). Figure 3 illustrates how these values were used to estimate outer leaflet charge. The values for net outer leaflet charge for these vesicles was 8.2 mol% negative charge and 20.7 mol% positive charge, respectively.

Comparison of TLC and TMA-DPH assay of outer leaflet charge.

Figure 4 compares the results for outer leaflet charge from the TMA-DPH assay to the values estimated from the phospholipid composition of the asymmetric LUVs after exchange. The results show there was good agreement between outer leaflet charge determined by TMA-DPH and that estimated by TLC by assuming that donor phospholipid was transferred only into the outer leaflet. This was true both for vesicles with net negative, net positive, or near neutral outer leaflets. (As noted above, a near neutral outer leaflet was observed in the case of DOTAP:POPC out/POPG:POPC in/Chol vesicles, in which the amount of residual POPG in the outer leaflet was very high (Table 1).) There is some difference between the results from TLC quantification and TMA-DPH binding assay method, especially in Figure 4 columns a, c and g. One possibility is that this just represents experimental error as the precision of TLC assay is limited. In fact, this is one reason we developed the TMA-DPH assay. In the case of column c, there is the additional possibility that the vesicles may have lost some asymmetry.

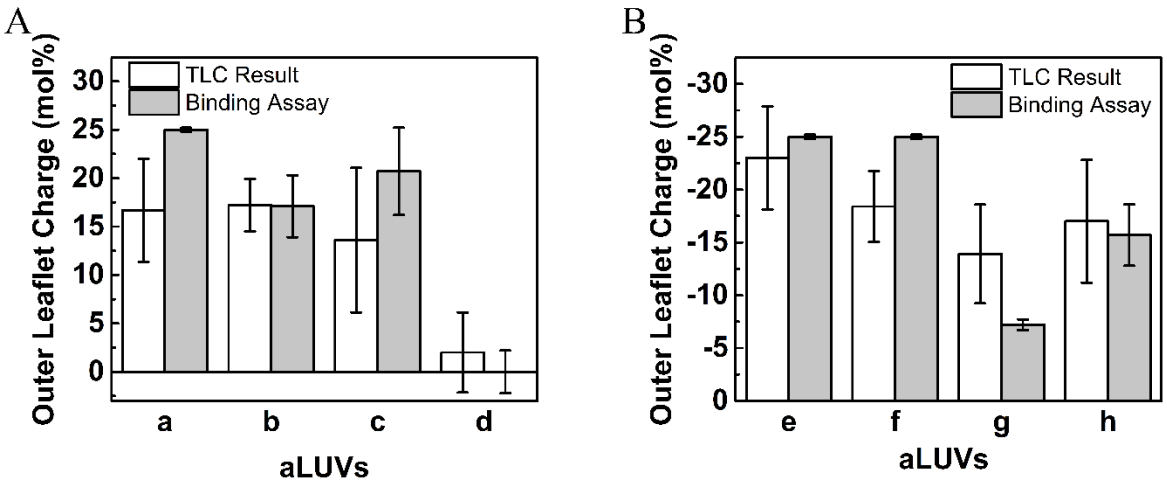

Figure 4. Comparison of TLC and TMADPH binding assay results for outer leaflet charge of asymmetric LUVs A: Cationic/neutral outer leaflet vesicles: (a) POePC:POPC out/POPC in/Chol, (b) DOTAP:POPC out/POPC in/Chol, (c) POePC:POPC out/POPS:POPC in/Chol and (d) DOTAP:POPC out/POPG:POPC in/Chol, and B: Anionic outer leaflet vesicles: (e) POPS:POPC out/POPC in/Chol, (f) POPG:POPC out/POPC in/Chol, (g) POPS:POPC out/POePC:POPC in/Chol and (h) POPG:POPC out/DOTAP:POPC in/Chol. Raw data of a, c, e, g, are analyzed by standard curves in Figure 3 and b, d, f, h from standard curves in Figure S2. TMA-DPH assay results for c and g are the examples shown in Figure 3. Results show mean values and standard deviations from three vesicle preparations.

Stability of Asymmetry

Although prior studies have demonstrated that asymmetry of lipids with palmitoyl and oleoyl acyl chains is stable, often for days [19, 24-25, 31-33], and that cholesterol slows phospholipid flip[36], we used the TMA-DPH assay to confirm the stability of asymmetry. It is noteworthy that TLC would not show any change in overall phospholipid composition as asymmetry is lost, and so cannot be used to detect changes in the level of asymmetry. In contrast, the TMA-DPH assay directly measures outer leaflet charge, and so can be used to assay changes in asymmetry. Asymmetric LUVs were dispersed and incubated at room temperature in PBS/sucrose that was identical, and so osmotically balanced, with the solution inside the vesicles or they were dispersed and incubated

in PBS. The outer leaflet charge of the asymmetric LUVs was measured by the TMA-DPH binding assay after 1 and 2 days. The results in Figure 5 show behavior was similar when vesicles were dispersed in PBS or PBS with sucrose. The net mol% of charged lipids in the outer leaflet of both POePC:POPC out/POPS:POPC in/Chol and POPS:POPC out/POePC:POPC in/Chol asymmetric LUVs was relatively stable, not changing significantly in the first 48 h. This presumably reflects the low flip-flop rate of the lipids used [19, 24-25, 31-33].

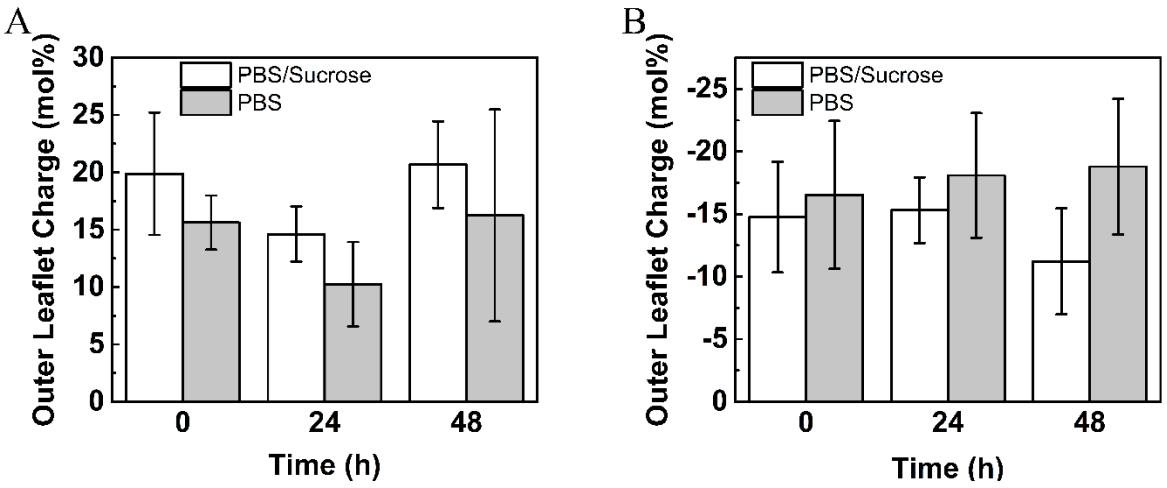

Figure 5. Time dependence of outer leaflet charge assayed with TMA-DPH binding assay for asymmetric LUVs in room temperature: A: POePC:POPC out/POPS:POPC in/Chol and B: POPS:POPC out/POePC:POPC in/Chol in PBS/sucrose or PBS. Results show mean values and standard deviations from three vesicle preparations.

The level and stability of doxorubicin entrapment within asymmetric LUVs

Next, the effect of lipid charge and asymmetry upon liposomal entrapment of the cationic anti-cancer drug doxorubicin (Dox) was measured. Dox intercalates between DNA base pairs, inhibiting topoisomerase II and thus replication [37-39]. It is used encapsulated inside liposomes to lower its toxicity and prolong its circulation time [1, 40-45]. Dox can cross lipid membranes, and be entrapped in their aqueous lumen by a pH-gradient [46-47], or by precipitation induced via a

manganese-gradient [48], a phosphate gradient [49], or a sulfate-gradient [1, 50-51]. In our studies we used liposome-entrapped ammonium sulfate to aid stable entrapment of Dox. We found that with 23% (w/w) sucrose entrapped inside the LUVs, the concentration of ammonium sulfate allowing entrapment of a high amount of Dox could be decreased to 50 mM (data not shown). Using these conditions, symmetric and asymmetric LUVs were prepared, and then the amount of Dox entrapped in the LUVs was assayed by measuring its fluorescence after washing the liposomes (See Methods).

Figure 6 shows the amount of Dox associated with symmetric LUVs in terms of the Dox/lipid ratio. LUVs were composed of either 60mol% POPC and 40 mol% cholesterol, or of 15% POePC, POPS or POPG, 45 mol% POPC and 40 mol% cholesterol. The negatively charged LUVs (containing POPG and POPS) associated with 3-6 times more Dox than neutral (POPC) LUVs, or positively charged LUVs (containing POePC). This indicates that electrostatic interactions between lipids and Dox, has a strong influence on the amount of liposome-associated Dox.

Dox entrapment within symmetric anionic LUVs was highly stable. After pelleting samples and washing in PBS twice, 90 or more % of the initially trapped Dox remained in symmetric LUVs containing anionic phospholipid or lacking charged lipid. In contrast, symmetric LUVs containing cationic lipid only retained 60% of entrapped Dox under these conditions (Supplemental Table S4).

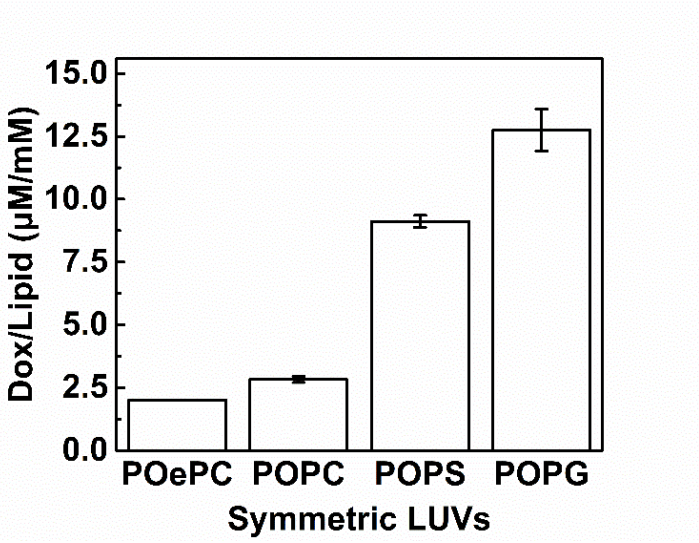

Figure 6. Dox entrapment within symmetric LUVs containing 40 mol% cholesterol and either 60 mol% POPC or 45mol% POPC and 15 mol% POePC, POPS or POPG. These samples were pelleted by centrifugation and washed twice to match the protocol used for asymmetric vesicles (see Methods). Results show mean values and standard deviations from three vesicle preparations.

To determine how asymmetry of lipid charge would affect Dox association with liposomes, these experiments were then repeated with asymmetric LUVs. As shown in Figure 7, asymmetric vesicles with cationic POePC in their outer leaflets and anionic inner leaflets (compositions a and b) trapped the largest amount of Dox, in amounts per lipid similar to those in symmetric vesicles containing anionic lipids in both leaflets. In contrast, asymmetric LUVs with a similar overall lipid composition, but with the opposite asymmetry, in which the inner leaflet was cationic and the outer leaflet was anionic, (compositions d and e) trapped low amounts of Dox, similar to that trapped in symmetric vesicles containing cationic lipids in both leaflets. Compositions with a cationic outer leaflet and neutral inner leaflet (composition c) trapped an intermediate amount of Dox, similar to neutral symmetric vesicles.

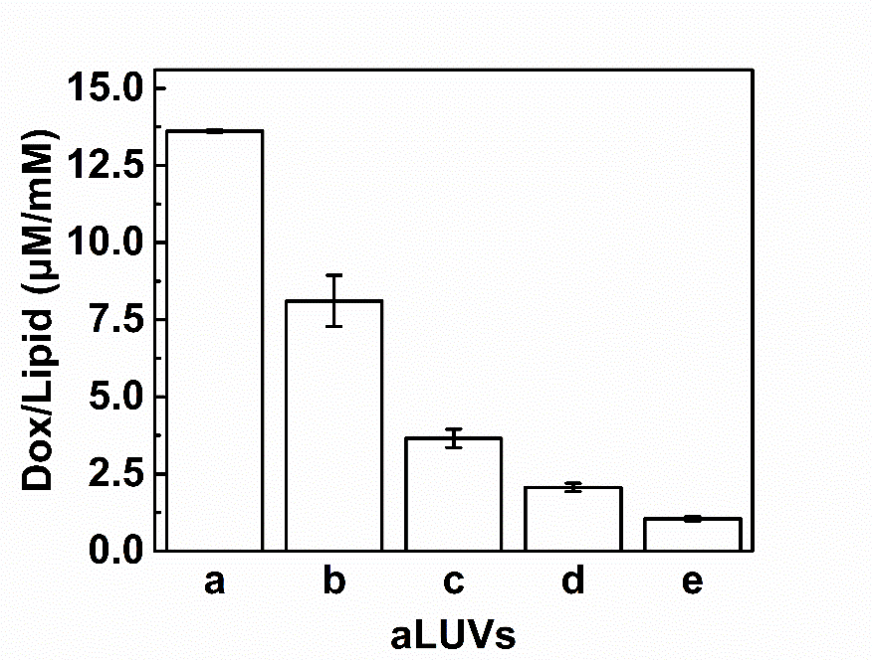

Figure 7. Dox entrapment within asymmetric LUVs. (a) POePC:POPC out/POPG:POPC in/Chol, (b) POePC:POPC out/POPS:POPC in/Chol, (c) POePC:POPC out/POPC in/Chol, (d) POPS:POPC out/POePC:POPC in/Chol, (e) POPG:POPC out/POePC:POPC in/Chol. Results show mean values and standard deviations from three vesicle preparations.

These experiments demonstrate that the charge on the inner leaflet of a lipid vesicle determines how much Dox is trapped within the vesicle, with no appreciable effect of the outer leaflet lipid charge. The observation that vesicle outer leaflet charge has little effect implies it is very unlikely that significant amounts of Dox associate with the outer leaflet of the vesicles. The ability to control Dox entrapment by controlling the inner leaflet independently of the outer leaflet raises the possibility that asymmetric vesicles could have important advantages for drug delivery applications (see below).

Discussion and Conclusion

Liposomal drug delivery is useful because liposomes can improve biodistribution, improve uptake by the target, and protect drugs from degradation, thus reducing side effects [52]. These advantages are affected by the intrinsic characteristics of the liposomes, such as the size of the liposomes, their net charge (or the zeta potential), and the selective binding properties of surface lipids[5, 53].

In this report, we concentrated preparing asymmetrically charged liposomes that might further increase their utility. Asymmetric LUVs have been recently developed as natural membrane models to study the behavior and properties of membranes and membrane domains [54-56]. However, highly asymmetrically charged LUVs have been little explored for drug delivery. In this report, we used cyclodextrin exchange to prepare asymmetric vesicles with various types of lipid charge asymmetry. We found that LUVs with asymmetric charged leaflets could be prepared with a neutral inner leaflet, and positive or negative outer leaflet. It was also possible to prepare vesicles with a cationic inner leaflet and anionic outer leaflet and vice versa. Vesicles with one cationic and one anionic lipid leaflet were of particular interest because to our knowledge they have not been investigated in past studies. It was found that more than one type of anionic or cationic phospholipid could be used. Importantly, lipid asymmetry was stable, at least for 48 hours for the combinations of membrane lipids studied.

Asymmetric LUVs preparations may have several useful properties. One of the most important is increasing the concentration of drug that is trapped in the liposomes. We found that for Dox,

anionic lipid in the inner leaflet can maximize the amount and stability of drug entrapment within the vesicles. This may reflect an attraction of Dox to the anionic lipid surface during vesicle formation. This attraction might also prevent translocation of Dox across the membrane, and so inhibit leakage of Dox from the vesicles. In contrast, the charge on the outer leaflet had no influence upon the amount of Dox that was vesicle-associated. This indicates that it is very unlikely that there is very tight binding to a cationic surface or that significant amounts of Dox are associated with the outer leaflet of the vesicles.

It is possible that asymmetric LUVs with an anionic inner leaflet might be useful for drug Dox delivery. The dose of free Dox that can be delivered without exhibiting cardiotoxicity is 10-50 fold less than with Doxil, which is liposome-encapsulated Dox [1, 57-58]. Doxil does not contain anionic lipid. Thus, using asymmetric LUVs with an anionic inner leaflet, the dose of liposome-encapsulated Dox could potentially be increased several-fold without altering the outer leaflet lipid composition. High intra-liposomal drug-loading is an important parameter for its therapeutic application [59-60]. It will be interesting to determine if similar principles can be used to optimize nucleic acid entrapment.

Asymmetric LUVs had additional properties that may be favorable for drug delivery. The presence of cholesterol significantly improved yield in many cases, and should be useful for reducing uptake of LUVs macrophages, which can clear liposomes from the circulation [61]. It should also be noted that the diameter of the asymmetric LUVs was ~120nm, which is a good size for drug delivery [62]. For delivery to tumor tissues sizes in the range of 100-200nm have been reported to be optimal for prolonging circulation time [63], increasing transfer from vascular to tumor tissue [64-65], accumulating around tumor tissue [66-67], permeating through tumor capillaries [68-69],

retention in tumor interstitial spaces [65, 70-71], reducing side effects relative to free drug [53], reducing degradation by the complement system [72-73], and uptake by mononuclear phagocytes [62].

In the future, it will be important to test the entrapment of drugs or imaging reagents with both different charge and hydrophobicities, as well as the efficiency of drug delivery into cells as a function of outer leaflet composition and charge. Outer leaflet lipids can be optimized for slow clearance from the circulation and vesicle targeting (such as by using monosialoganglioside or polyethyleneglycol containing phospholipids)[9, 74], and to release drug most effectively at certain sites (such as by using pH-sensitive lipid)[75]. Manipulating outer leaflet charge by adjusting the donor lipid composition should itself be important, since the net charge of the outer leaflet of LUVs should alter unfavorable binding to charged surfaces and biomolecules, which can play a role in immunogenicity, screening by spleen or kidney, accumulation in the liver, and cytotoxicity [5, 76]. Outer leaflet charge should also influence drug concentration at the target, which when optimized would reduce drug side effects.

Supporting Information

Additional experimental data including asymmetric LUVs lipid yield, lipid composition of asymmetric LUVs determined by TLC, lipid composition of outer leaflet of asymmetric LUVs calculated from TLC results, concentration of Dox and lipid in symmetric LUVs with entrapped Dox as a function of number of times pelleted, example of TLC plate, and standard curve showing relationship between normalized fluorescence of TMADPH and outer leaflet net charge for asymmetric LUVs containing DOTAP and/or POPG.

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Note

The authors declare no competing financial interest.

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