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Synthesis and Characterization of Betaine-like Diacyl Lipids: Zwitterionic Lipids with the Cationic Group at the Bilayer Interface

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Abstract

We synthesized and characterized a series of zwitterionic, acetate-terminated, quaternized amine diacyl lipids (AQ). These lipids have an inverted headgroup orientation as compared to naturally occurring phosphatidylcholine (PC) lipids; the cationic group is anchored at the membrane interface, while the anionic group extends into the aqueous phase. AQ lipids preferentially interact with highly polarizable anions (ClO_4^-) over less polarizable ions (Cl^-), in accord with the Hofmeister series, as measured by the change in zeta potential of AQ liposomes. Conversely, AQ lipids have a weaker association with calcium than do PC lipids. The transition temperatures (T_m) of the AQ lipids are similar to the T_m observed with phosphatidylethanolamine (PE) lipids of the same chain length. AQ lipids form large lipid sheets after heating and sonication; however, in the presence of cholesterol, (Chol) these lipids form stable liposomes that encapsulate carboxyfluorescein. The AQ:Chol liposomes retain their contents in the presence of serum at 37 °C, and when injected intravenously into mice, their organ biodistribution is similar to that observed with PC:Chol liposomes. AQ lipids demonstrate that modulating the headgroup charge orientation significantly alters the biophysical properties of liposomes. For the drug carrier field, these new materials provide a non-phosphate containing zwitterlipid for the production of lipid vesicles.

Keywords

charge orientation; drug carriers; lipid vesicle; nanotechnology; permeability

1. Introduction

An increasingly diverse toolbox of lipid headgroups, linkers and hydrophobic domains is being populated by the systematic study of natural lipid components and surfactants. This lipid collection has elucidated the influence of headgroup properties such as hydrogen

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bonding capability, size, and charge on superstructure formation and molecular interactions (Langner and Kubica, 1999; Chemin et al., 2008). For instance, zwitterionic surfactants interact with ions with higher affinity than do surfactants with a nonionic headgroup (Marte et al., 2007). However, there are a number of lipid headgroup architectures that have not been well characterized. In particular, modifications to the phosphocholine headgroup have been investigated less than other classes of lipids, despite their important role in biological membranes. As a result, less is known about the effects of zwitterionic headgroup structure and charge orientation on superstructure formation and lipid interactions.

Naturally occurring zwitterionic headgroups are oriented with an anion proximal to, and a cation distal to the membrane interface. Inverting this charge orientation in single chain surfactants by placing the cation proximal to the interface has yielded insight into the charge interactions of zwitterionic micelles (Tondo et al., 2010). Zwitterionic sulfobetaine micelles and lecithin liposomes can specifically interact with anions; this interaction follows the Hofmeister series and Pearson's hard—soft classification (Kamenka et al., 1995; Kamenka and Chevalier, 1995; Minnes et al., 2008). “Soft” bases with low charge densities and high polarizability, such as ClO_4^- , bind these micelles more effectively than “hard” bases with high charge densities and low polarizability, such as F^- .

Recently, our group has synthesized zwitterionic sulfobetaine diacyllipids and zwitterionic choline phosphate diacyl lipids with interesting salt-dependant thermotropic properties (Perttu and Szoka, 2011; Perttu and Szoka, unpublished data). These diacyllipids have a cationic amine adjacent to the membrane interface and an anionic group (sulfonate, phosphate or ethylphosphate) that extends away from the membrane. The nature of the anionic counterion influences the sulfobetaine lipid thermal transition. In high salt solutions, these lipids exhibit transition temperatures (T_m) similar to that of PC lipids of the same chain length. Conversely, at low salt concentrations, these lipids exhibit elevated T_m similar to those observed in phosphatidylethanolamine (PE) lipids of the same chain length. We propose that the change in T_m is driven by interactions of anions with the cationic amine at the membrane interface (Perttu and Szoka, 2011; Perttu and Szoka, unpublished data).

Here-in, we expand this work by synthesizing a class of inverse zwitterionic lipids that have an anionic acetic acid group extending into the aqueous phase and a cationic quaternized amine adjacent to the bilayer interface (Figure 1A). These Acetate Quaternized amine (AQ) lipid vesicles bind to anions and have interesting phase transition temperatures. To our knowledge, this is the first example of the synthesis of diacyl AQ headgroup lipids and of the characterization of the supramolecular assemblies and biophysical properties of such lipids.

2. Experimental

2.1 Materials

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Trifluoroacetic acid was obtained from AK Scientific (Union City, CA); all other solvents were obtained from VWR Scientific (Radnor, PA). PD-10 sephadex columns were obtained from GE Healthcare (San Francisco, CA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO). Buffers were prepared using Milli-Q deionized water. GraceResolv Silica thin layer chromatography plates were obtained from Grace Davison Discovery Sciences (Deerfield, IL).

2.2 Instruments

NMR measurements were taken on a Bruker (Billerica, MA) 300 MHz Avance system and analyzed using TopSpin software. Chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard. ^{13}C NMR measurements were composed of 10,000 scans while ^1H measurements were composed of 32 scans. ^{13}C experiments were performed in CDCl_3 with 10% MEOD. ^1H experiments were performed in CDCl_3 or deuterated DMSO. MALDI-TOF measurements were taken on PerSeptive Biosystems Voyager-DE from Applied Biosystems (Carlsbad CA). High performance flash chromatography (HPFC) was carried out using a Grace Reveleris Flash System (Columbia, MD) with pre-packed silica gel columns. Zeta potential and size measurements were carried out using a Nano-ZS Dynamic Light Scattering Instrument from Malvern (Westborough, MA). Differential Scanning Calorimetry (DSC) measurements were obtained using a high-temperature MC-DSC 4100 calorimeter from Calorimetry Sciences Corp. (Lindon, UT). Fluorescence measurements were made on a FLUOstar plate reader from BMG Labtech (Durham, NC) with excitation at 485 nm and emission at 518 nm.

2.3 Synthesis

The AAQ lipid library was synthesized from a 3-(dimethylamino)-1,2-propanediol core (**2**) via a three-step synthesis (Scheme 1). The synthesis is straightforward and provides a high yield of the title compounds.

2.3.1 Alkyl acid conjugation (i)—1 molar equivalent (6.3 mmol, 0.75 g) of 3-(dimethylamino)-1,2-propanediol was solubilized in 10 mL dichloromethane (DCM). 2.2 molar equivalents of the alkyl acid chain (myristic acid (**3a**), palmitic acid (**3b**), or stearic acid (**3c**)) were added and the reaction was diluted to a final concentration of 0.1 M with DCM. Next, 0.5 molar equivalents of dimethylaminopyridine (DMAP) and 2.5 equivalents of *N,N'*-Dicyclohexanecarbodiimide (DCC) were added to the reaction. The reaction proceeded overnight at room temperature and was monitored by thin layer chromatography (solvent: 10–20% methanol in DCM, visualized by bromocresol green and iodine vapor) and MALDI-TOF. If necessary, DCC was added to drive the reaction toward completion. In order to purify the product (**3a-c**), the remaining DCC was converted to DCU by addition of 2 mL glacial acetic acid. The produced DCU was removed by vacuum filtration through 55 mm filter paper. To remove residual DCU, the reaction was diluted with 50 mL DCM, washed twice with 10 mL 1 M HCl. The DCM solution was dried over Na_2SO_4 . Solvent was removed by rotary evacuation and then by vacuum. The product was purified using HPFC (0–10% methanol in chloroform); this step had 70–80% yield. The compound structure was confirmed using TLC, MALDI-TOF, and ^1H NMR.

2.3.2 Amine quaternization with tert-butyl bromoacetate (ii)—1 molar equivalent (2 g) of **2a-c** was solubilized in 30 mL DCM by stirring at room temperature. Next, 3 molar equivalents of Hunig's base were added to the reaction. 2 molar equivalents of tert-butyl bromoacetate were gradually dripped into the reaction in order to avoid excessive heating. The reaction proceeded overnight at 40°C in an oil bath and was monitored by TLC (10–20% methanol in DCM) and MALDI-TOF. An additional 0.5 equivalents of tert-butyl bromoacetate and Hunig's base were added to drive the reaction to near completion. The reaction mixture was diluted with 50 mL DCM, washed twice with 10 mL 1 M HCl to remove residual Hunig's base and tert-butyl bromoacetate, and dried over Na_2SO_4 . The quaternized product was purified by precipitation at 0°C in DCM. Solvent was removed by rotary evaporation and vacuum; this step had 90% yield. **4a-c** formation was confirmed by TLC, MALDI-TOF, and ^1H NMR.

2.3.3 Deprotection of tert-butyl ester (iii)—4a-c was solubilized in 20 mL DCM and 10 mL TFA. 1 mL of triisopropylsilane was added as a scavenger of carbocations. The reaction was allowed to proceed for 1 hour at room temperature and was monitored by TLC (10–20% methanol, visualized by bromocresol green and iodine vapor) and MALDI-TOF. The reaction was diluted with 50 mL DCM, washed twice with 10 mL 1 M NaHCO₃ to remove residual TFA, and dried over Na₂SO₄. 2-((2,3-bis(myristoyloxy)propyl)dimethylammonio)acetate (**1a**), 2-((2,3-bis(palmitoyloxy)propyl)dimethylammonio)acetate (**1b**), and 2-((2,3-bis(stearoyloxy)propyl)dimethylammonio)acetate (**1c**) were formed in 50–70% overall yield. Product was confirmed by TLC, MALDI-TOF, ¹H NMR, ¹³C NMR and C, H, N elemental analysis.

2.4 Structural confirmation of reaction products

3a: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (t, 6H); δ 1.26 (m, 40H); δ 1.64 (p, 4H); δ 2.3–2.6 (m, 4H); δ 2.78 (s, 6H); δ 3.1–3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.47 (p, 1H). MALDI-TOF calculated: 539.49, observed: 541.40.

3b: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (m, 6H); δ 1.26 (m, 48H); δ 1.64 (m, 4H); δ 2.3–2.6 (m, 4H); δ 2.78 (m, 6H); δ 3.1–3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.45 (m, 1H). MALDI-TOF calculated: 595.55, observed 598.37.

3c: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (m, 6H); δ 1.26 (m, 56H); δ 1.64 (m, 4H); δ 2.3–2.6 (m, 4H); δ 2.78 (s, 6H); δ 3.1–3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.45 (p, 1H). MALDI-TOF calculated: 651.62, observed 654.11.

4a: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (t, 6H); δ 1.26 (m, 40H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.35 (m, 4H); δ 3.6–3.75 (m, 6H); δ 4.05–4.25 (m, 2H); δ 4.5–4.8 (m, 4H); δ 5.58 (m, 1H). MALDI-TOF calculated: 654.57, observed: 655.59.

4b: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (m, 6H); δ 1.26 (m, 48H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.4 (m, 4H); δ 3.78 (m, 6H); δ 4.05–4.25 (m, 2H); δ 4.5–4.9 (m, 4H); δ 5.6 (m, 1H). MALDI-TOF calculated: 710.63, observed 713.49.

4c: ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (m, 6H); δ 1.26 (m, 56H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.4 (m, 4H); δ 3.78 (m, 6H); δ 4.05–4.25 (m, 2H); δ 4.5–4.9 (m, 4H); δ 5.6 (m, 1H). MALDI-TOF calculated: 766.69, observed 768.86.

1a (DMAQ): ¹H NMR (CDCl₃:MeOD ~10:1): δ .88 (m, 6H); δ 1.26 (m, 40H); δ 1.64 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 4.05–4.25 (m, 2H); δ 4.0–4.4 (m, 6H); δ 5.55 (m, 1H) (Supplementary Figure 1). ¹³C NMR (CDCl₃): δ –0.02, δ 14.10, δ 22.68, δ 24.65, δ 24.74, δ 29.09, δ 29.11, δ 29.25, δ 29.29, δ 29.35, δ 29.56, δ 29.50, δ 29.65, δ 29.68, δ 31.91, δ 33.86, δ 34.20, δ 51.87, δ 52.53, δ 63.26, δ 63.47, δ 65.66, δ 66.61, δ 164.53, δ 172.79, δ 173.09 (Supplementary Figure 2). **MALDI-TOF** calculated: 597.50, observed: 599.72. **Elemental analysis** (% expected, % observed): C (70.3, 70.09), H (11.29, 11.05), N (2.34, 2.36)

1b (DPAQ): ¹H NMR (DMSO): δ .88 (t, 6H); δ 1.26 (m, 48H); δ 1.55 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 3.8–4.1 (m, 4H); δ 4.2–4.4 (m, 4H); δ 5.55 (m, 1H) (Supplementary Figure 3). ¹³C NMR (CDCl₃): δ –0.11, δ 14.02, δ 22.63, δ 24.63, δ 29.29, δ 29.62, δ 31.87, δ 33.78, δ 34.09, δ 51.79, δ 52.26, δ 63.20, δ 63.53, δ 165.80, δ 173.39 (Supplementary Figure 4). **MALDI-TOF** calculated: 653.56, observed: 656.51. **Elemental analysis** (% expected, % observed): C (71.62, 71.28), H (11.56, 12.08), N (2.14, 2.17)

1c (DSAQ): ^1H NMR (DMSO): δ .88 (t, 6H); δ 1.26 (m, 56H); δ 1.55 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 3.8–4.1(m, 4H); δ 4.2–4.4(m, 4H); δ 5.55 (m, 1H). (Supplementary Figure 5). ^{13}C NMR (CDCl_3): δ 3.75, δ 17.89, δ 26.53, δ 28.53, δ 33.20, δ 33.54, δ 35.78, δ 37.70, δ 37.99, δ 55.71, δ 56.07, δ 57.16, δ 67.18, δ 69.49, δ 169.98, δ 177.01, δ 177.44 (Supplementary Figure 6). **MALDI-TOF** calculated: 709.62, observed: 712.66. **Elemental analysis** (% expected, % observed): C (72.73, 72.33), H (11.78, 11.28), N (1.97, 2.00)

2.5 Elemental analysis

5–10 mg of dry lipid was submitted to Microanalytical Laboratory at the University of California Berkeley for elemental analysis determinations.

2.6 Transmission Electron Microscopy (TEM)

TEM images were obtained at the University of California Berkeley Robert D. Ogg Electron Microscope Laboratory. Thin films of DPAQ:Chol formulations were rehydrated in 50 mM HEPES 150 mM NaCl to a final lipid concentration of 20 mM. Preparations were sonicated for 7 minutes at 80 °C. Ten μL of liposome solutions were added to glow discharged copper grids with 400 mesh and Formvar/carbon coatings from Structure Probe, Inc (West Chester, PA). Liposomes were allowed to adsorb on grids for two minutes. Grids were washed three times with distilled water. Liposomes were negatively stained with 1% uranyl acetate and imaged using an FEI Tecnai 12 TEM (FEI, Hillsboro, OR).

2.7 Liposome formation in buffers with various anions

Thin lipid films were hydrated in a range of buffers to a final concentration of 20 mM. Preparations were heated at 80 °C for 10 minutes and then sonicated for 7 minutes at 80 °C. After allowing samples to stabilize at room temperature, zeta potential and diameter were measured on a Malvern Nano-ZS. Zeta potential was fit using the Smoluchowski model, while Mark-Houwink parameters were used to determine diameter. All samples were run in triplicate.

2.8 Effect of $[\text{Ca}^{2+}]$ on vesicle zeta potential and size

Liposomes were prepared as outlined above. Liposome suspensions were spiked into a solution containing between 0 and 10 mM CaCl_2 and made isotonic with NaCl. Zeta potential and size measurements were obtained using the Malvern Nanosizer. All samples were run in triplicate.

2.9 Carboxyfluorescein (CF) release from DMAQ and DMPC vesicles

The carboxyfluorescein encapsulating protocol was adapted from Weinstein *et al* (Weinstein *et al.*, 1977). 10 μmol lipid film was rehydrated in 10 mM Tris, 100 mM carboxyfluorescein (CF), pH 7.4 to a final concentration of 20 mM lipid. Each sample was heated at 80 °C for 10 minutes and subsequently sonicated at 80 °C for 10 minutes. Liposomes were then extruded 13 times through a 100 nm polycarbonate membrane. Free CF was removed by size exclusion chromatography with a PD-10 sephadex column. Two concentrations of liposomes (5 or 20 μL) were incubated in 200 μL of 105 mM NaCl, 10 mM HEPES pH 7.4 with or without 30% (v/v) fetal bovine serum, .02% sodium azide at 37 °C for one week. Leakage was measured with a FLUOstar plate reader with excitation at 485 nm and emission at 518 nm. Percent leakage values were obtained by normalization to the fluorescence of the samples after lysis of liposomes using 0.5% $\text{C}_{12}\text{E}_{10}$. Leakage measurements were run in triplicate.

2.10 Differential scanning calorimetry (DSC)

DSC experiments were based upon a protocol described in (Huang and Szoka, 2008) DSC was performed with three reusable Hastelloy sample ampoules and a reference ampoule. Data were collected over a range of 5–90 °C at 1 °C/min with the relevant buffer as the reference. The CpCalc 2.1 software package was used to convert the raw data into a molar heat capacity. The data was then exported and plotted in Matlab. Liposomes for DSC measurements were prepared as outlined above and loaded into the ampule using a glass syringe (250 µL per sample). Samples were scanned through a heat-cool-heat cycle and data was collected from the second heating cycle.

2.11 Cell viability assay

C26 murine colon carcinoma cells were seeded at 10^4 cells per well in 100 µL media in a cell culture treated 96-well plate (Costar). After 24 hours, liposomes were added in 100 µL at varying concentrations. Two lipid formulations with similar transition temperatures were investigated: DPAQ liposomes: DPAQ:Cholesterol:DPPG (60:40:5 mol ratio) and DSPC liposomes: DSPC:Cholesterol:DSPG (60:40:5: mol ratio). Cells were incubated with liposomes for 72 hours. Cells were washed with Dulbecco's phosphate-buffered saline (D-PBS) without calcium and magnesium and fresh media was added. Ten µL of 12 mM (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and cells were incubated for 4 hours at 37 °C. All but 25 µL of the media was removed and 50 µL of DMSO was added to each well. After a 10-minute incubation at 37 °C, absorbance was read at 540 nm using a OptiMax Microplate Reader from Molecular Devices (Sunnyvale, CA).

2.12 Liposome distribution and pharmacokinetics in vivo

All animal experiments were performed in compliance with the NIH guidelines for animal research under a protocol approved by the Committee on Animal Research at the University of California, San Francisco. Thirty µmol of total lipid was rehydrated in HEPES buffered saline. For liposome stability and liposome fluorescence, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG) and 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine (DiD) were incorporated in liposome formulations, respectively. Two lipid formulations with similar transition temperatures were investigated: DPAQ liposomes: DPAQ:Cholesterol:DPPG:DiD (60:40:5:0.2 mol ratio) and DSPC liposomes: DSPC:Cholesterol:DSPG:DiD (60:40:5:0.2 mol ratio). Lipid formulations with cholesterol showed no transition temperature. To prepare liposomes, the lipids were sonicated at 80 °C for 20 minutes and extruded 9–15 times through 100 nm polycarbonate membranes. Samples were then dialyzed overnight against Hepes buffered saline (10 mM Hepes, 50 mM NaCl) and filtered through a 200-micron membrane. Two hundred µL of each liposome preparation was injected into the tail vein of BALB/C mice tumored with C26 murine carcinoma cells. Relative lipid concentrations were measured using the DiD signal. The mice were divided into two cohorts. Cohort 1 (n=3 mice per formulation) was bled at 10 minutes, 30 minutes, and 300 minutes post injection. Cohort 2 (n=3 mice per formulation) was bled at 90 minutes, 24 hours and 48 hours post injection. Both cohorts were sacrificed after the last bleed. Standard DiD curves were constructed for the relevant organs and the plasma by spiking known quantities of DiD into organ and plasma samples. DiD concentration was then measured according to these curves. In order to calculate pharmacokinetic parameters, data was fit to a two-compartment model using MATLAB.

3. Results

3.1 Synthesis

A straightforward three-step synthetic route was employed to synthesize the AQ lipids (Scheme 1). The starting compound, 3-(dimethylamino)-1,2-propanediol (**2**), was linked to myristic acid (C₁₄), palmitic acid (C₁₆), or stearic acid (C₁₈) by DCC coupling (**i**). After purification via HPFC, the disubstituted product (**3a-c**) was obtained in high yield (70–80%). Next, the amine was quaternized by S_N2 addition of tert-butyl bromoacetate (**4a-c**, **ii**). Finally, the carboxylic acid was deprotected under acidic conditions to give the final product (**1a-c**, **iii**). Column purification was not necessary after the second or third step, as the quaternized compounds readily precipitated out of dichloromethane (DCM). The yields for the last two synthetic steps were effectively quantitative and the overall yield for the synthesis was 50–70% from starting material. The lipids were named using the following acronym: Di-Myristoyl/Palmitoyl/Stearoyl Acetate Quaternized-amine (DMAQ, DPAQ, and DSAQ respectively) (Figure 1A). Lipid products were confirmed by MALD-TOF mass spectrometry, ¹H NMR, ¹³C NMR and C, H, N elemental analysis (**Experimental, 2.4**; Supplementary Figures 1–6).

3.2 Vesicle formation and characterization

The ability of AQ lipids to form superstructures was investigated. Single component AQ lipid formulations formed large aggregated vesicles and planar lipid bilayer sheets after heating and sonication as visualized by negative stain transmission electron microscopy (TEM) (Figure 1B, **left**). Directly after sonication, optically clear suspensions were observed. After 5 minutes at room temperature, these dispersions reorganized into viscous hydrogels or large lipid aggregates. These formulations did not form stable vesicles regardless of saturated chain length or the pH and salt concentration of the hydration buffer. However, addition of 40 mole percent cholesterol to AQ composition allowed for the formation of vesicles. Small diameter uniform vesicular-like structures are observed by TEM (Figure 1B, **right**). The TEM images corroborated the size distribution data obtained by dynamic light scattering (DLS) (Figure 1C). DMAQ formulations including more than 50% molar DPPC formed vesicles at a temperature above the phase transition temperature. However, when the temperature was reduced below the T_m of DMAQ, the vesicles aggregated and their contents released (data not shown).

3.3 Anions bind to DMAQ liposomes according to the Hofmeister series

Zwitterionic sulfobetaine liposomes (Perttu and Szoka, 2011) bind to anions in aqueous solutions to a greater extent than do phosphatidylcholines. To investigate if this binding preference is a general phenomenon associated with the inverted headgroup, we measured the zeta potential of DMAQ:Chol liposomes in the presence of various anions and compared it to the zeta potential of DMPC:Chol liposomes measured under the same conditions. DMAQ:Chol liposomes demonstrate more negative zeta potentials than DMPC:Chol liposomes for each anion investigated (Figure 2A). This result indicates that anions bind DMAQ:Chol liposomes more effectively than DMPC:Chol liposomes. DMAQ:Chol liposomes sequester anions with high polarizability (ClO₄[−], I[−]) more than those with low polarizability (Cl[−]) (Figure 2A), and this preference parallels the Hofmeister series (Zhang and Cremer, 2006). DMPC:Chol liposomes also bind ions according to the Hofmeister series; however, the magnitude of the change in zeta potential is reduced as compared to DMAQ:Chol liposomes (Figure 2A).

3.4 Interaction of calcium with AQ liposomes

We investigated the interaction of calcium with the DMAQ:Chol and DMPC:Chol liposome compositions using the zeta potential as a surrogate for direct calcium binding (Figure 2B). The surface potential of the DMPC:Chol vesicles becomes more positive as the Ca^{2+} concentration increases. While the zeta potential for the DMAQ:Chol vesicles increases along with Ca^{2+} concentration, the rate of change is less than observed in DMPC:Chol vesicles and the surface potential remains negative at 10 mM $[\text{Ca}^{2+}]$ (Figure 2B). Conversely, DMPC:Chol vesicles adopt a positive charge at 10mM $[\text{Ca}^{2+}]$.

In vivo, divalent cations are known to interact with lipid headgroups to induce aggregation and fusion of vesicles (Needham et al., 2000). As such, we measured the impact of increasing Ca^{2+} concentrations on the aggregation of DMAQ:Chol and DMPC:Chol vesicles. In the presence of 10 mM Ca^{2+} neither formulation showed aggregation (data not shown).

3.5 Permeability of AQ liposome to an encapsulated water-soluble anion

The charge at the membrane interface is believed to influence the permeation of charged molecules through the membrane (Khavrutskii et al., 2009). Naturally occurring phospholipids have an anionic phosphate group positioned at the membrane interface, while the AQ lipids have a cationic group adjacent to the interface. To better understand the role of this inverted orientation on the permeability of the membrane to water soluble compounds, we measured the leakage rate of carboxyfluorescein (CF), a water soluble anion, from DMAQ:Chol and DMPC:Chol liposomes (Figure 2C) (Weinstein et al., 1977). In the absence of serum both DMPC:Chol and DMAQ:Chol liposomes showed minimal leakage of CF at 37 °C. In the presence of serum, DMPC:Chol liposomes exhibited slightly more leakage than did the DMAQ:Chol vesicle compositions.

3.6 Phase transition temperatures of lipid dispersions prepared from AQ lipids

The phase transition temperature of a lipid formulation can lend insight into the intermolecular forces governing lipid headgroup and hydrophobic domain interactions. The phase transition temperatures for DMAQ, DPAQ, and DSAQ lipids were investigated by differential scanning calorimetry; all three lipids demonstrated transition temperatures significantly greater than analogous di-substituted saturated chain PC lipids but similar to analogous PE lipids (Figure 3A).

In the presence of anions with low charge density (ClO_4^- , I^-), DMAQ lipids exhibited a downward shift in transition temperature (Figure 3B). A shift in the T_m was not observed in the presence of large cations, such as tert-butyl ammonium (data not shown). DPAQ and DSAQ lipids exhibit no such shift in transition temperatures in the presence of various anions (Figure 3C,D). DPAQ lipids exhibit a pre-transition at low salt concentrations that is eliminated at increased ionic strength (Figure 3C).

3.7 In vitro cytotoxicity of liposomes composed of AQ lipids

To determine if the AQ lipids are tolerated when applied to cells in culture, we compared the viability of C26 colon carcinoma cells exposed to DPAQ:Chol or DSPC:Chol liposomes using an MTT assay. At concentrations that are commonly used in vitro or obtained after in vivo administration, both formulations were well tolerated by cells and demonstrated little toxicity (Figure 4A). At millimolar concentrations, the DPAQ liposomes showed a two fold greater reduction in cell viability in culture than did the DSPC liposomes

3.8 In vivo properties of AQ lipids

After confirming the *in vitro* stability of liposomes prepared from the AQ lipids and the low *in vitro* cytotoxicity, we investigated their biodistribution and pharmacokinetic parameters in BALB/C mice tumored with C26 murine colon carcinoma cells. To measure the *in vivo* distribution of AQ liposomes we incorporated 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine (DiD) into the lipid composition as a fluorescent lipid marker. Two lipid formulations were compared: DSPC:Chol:DSPG:DiD (60:40:5:0.2 mol ratio) and DPAQ:Chol:DPPG:DiD (60:40:5:0.2 mol ratio). Small diameter vesicles were prepared from both lipid compositions (DPAQ: 87 nm, DSPC: 78 nm). It is worth noting that neither formulation contained poly(ethylene glycol)-DSPE. The pharmacokinetic data indicates that the DPAQ liposomes demonstrate an extensive alpha phase and a terminal half-life of 9.5 hours (Figure 4B, D). The DSPC have a shorter distribution phase and a terminal half-life of 12.7 hours. Both formulations accumulate in the liver and spleen, with the AQ composition having a greater uptake into the liver and spleen at 48 hours post-administration than the DSPC liposomes (Figure 4C). Liposome uptake into the tumor was greater for the DSPC liposome composition than for the AQ composition (Figure 4C).

4. Discussion

We describe the synthesis of a new class of inverse-zwitterionic lipids with betaine-like headgroups. These lipids form vesicles in formulations that contain cholesterol and interact with anions with a greater affinity than do liposomes prepared from naturally occurring PC lipids.

In the absence of cholesterol, AQ lipids form a turbid solution of lipid aggregates. TEM reveals that these formulations form structures resembling planar bilayer sheets. AQ:Chol formulations form a clear solution of small lipid vesicles. Cholesterol will act as a spacer between adjacent AQ lipids and may interfere with electrostatic interactions between adjacent AQ lipids. It may also allow for a greater membrane curvature necessary to transition from a planar bilayer to spherical vesicles.

Anion binding to AQ lipid vesicles parallels the Hofmeister series, with low charge density anions binding more favorably than those with a high density. The Hofmeister series is a classification of ions according to their ability to salt in or salt out proteins. Ions with high charge densities (F^- , Cl^-) are strongly hydrated. Conversely, ions with a low charge densities (I^- , ClO_4^-) are weakly hydrated. Large anions with low charge density penetrate further into the bilayer of PC liposomes than do small anions (Sachs and Woolf, 2003). The anion concentration dictates the zeta potential of AQ liposomes according to the Hofmeister series. This result corroborates previous studies that demonstrate that zwitterionic diacyllipids (Perttu and Szoka, 2011; Perttu and Szoka, unpublished data) with the cationic group at the membrane interface and zwitterionic micelles with a cationic group proximal to the micelle interface bind anions according to the Hofmeister series (Priebe et al., 2008). We hypothesize that the location of the quaternary amine at the bilayer interface is responsible for this behavior.

AQ lipids prepared with cholesterol can stably encapsulate the water-soluble dye carboxyfluorescein. The influence of the headgroup charge orientation on the permeability of the bilayer to charged water-soluble molecules and ions has been the subject of considerable investigation. Recent computational studies suggest that the anchoring of the phosphate at the hydrophobic bilayer interface promotes the partitioning of cations into and out of bilayers (Khrutskii et al., 2009). Perttu and coworkers (Perttu and Szoka, 2011) have found that carboxyfluorescein release from sulfobetaine inverse lipids was slow and similar in magnitude to the CF release observed from PC liposomes. Conversely,

carboxyfluorescein leakage was significantly faster from liposomes prepared from inverse choline phosphate lipids than from liposomes prepared from analogous PC lipids (Perttu and Szoka, unpublished data). The AQ lipids behave similarly to the sulfobetaine lipids with regard to CF leakage. However, considerably more permeability studies across a range of charged substances are required to resolve the influence of headgroup charge orientation on charged solute permeability.

The AQ lipids demonstrate transition temperatures between those of PC and PE lipids with similar saturated chain lengths. The transition temperature profiles of the DMAQ and DPAQ lipids are also affected by anion charge density and concentration (Figure 3B,C). DMAQ lipids exhibit a downward shift in transition temperature in the presence of 1 M ClO_4^- or I^- . We hypothesize that these anions intercalate into the interfacial region of bilayer and disrupt lipid packing, which leads to decreased transition temperatures. The bilayer packing disruption may be relatively more pronounced for DMAQ than for DPAQ or DSAQ lipids because of its shorter acyl chains. Alternatively, the anion binding may disrupt hydrogen bonding or electrostatic interactions among adjacent lipids. It is thought that PE lipids show greater transition temperatures than PC lipids with matching hydrophobic tails because of the hydrogen-bonding capabilities of PE headgroups (Mabrey and Sturtevant, 1977). As such, the increased T_m of AQ lipids may be due to an altered headgroup conformation that reduces surface area and increases headgroup packing as compared to PC lipids.

While lipid dispersion prepared from DPAQ lipids do not exhibit a drop in transition temperature in the presence of the polarizable anions, they do exhibit a dampening of the pre-transition with increasing anion polarizability. Generally, PC lipids demonstrate pre-transitions while PE lipids do not. The pre-transition indicates a subtle change in lipid packing conformation. It is not observed in high radius of curvature PC vesicles and can be eliminated by small amounts of lipid impurities such as fatty acids (Knight, 1981). In the presence of large anions, the DPAQ lipids may adopt a packing configuration similar to PE lipids. This change in packing confirmation may be driven by a change in the hydration state of DPAQ lipids in the presence of highly polarizable anions.

The AQ:Chol liposomes demonstrate similar cytotoxicity and biodistribution profiles to PC:Chol liposomes. Both formulations distribute to the liver, spleen and intestine *in vivo*. Although the two compositions have a similar beta phase, the larger alpha phase of the AQ liposomes (Figure 4B) requires further investigation. It may indicate that there is a dose dependent interaction of AQ liposomes with blood components when injected into animals.

The altered biophysical properties of AQ liposomes compared to PC liposomes provide a new tool for exploring the role of charge orientations at membrane interfaces on ion trafficking across the bilayer. AQ lipid membranes may also provide insight into function of charge orientation in regulating interaction soluble protein binding and membrane protein function. Finally, the AQ lipids may have useful properties for preparing liposome-based drug carriers to treat cancer and infectious diseases.

Highlights

- We report novel diacyl acetate quaternized amine (AQ) zwitterionic lipids
- AQ lipids exhibit transition temperatures similar to those of PE lipids
- AQ lipids interact with anions according to the Hofmeister series
- AQ liposomes demonstrate similar *in vivo* properties to DSPC liposomes

- AQ lipids have distinct biophysical properties from PC lipids that may be useful in drug delivery

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Chemin C, Bourgaux C, Péan J-M, Pabst G, Wüthrich P, Couvreur P, Ollivon M. Consequences of ions and pH on the supramolecular organization of sphingomyelin and sphingomyelin/cholesterol bilayers. *Chem Phys Lipids*. 2008; 153:119–129. [PubMed: 18381068]
2. Huang Z, Szoka FC. Sterol-Modified Phospholipids: Cholesterol and Phospholipid Chimeras with Improved Biomembrane Properties. *J Am Chem Soc*. 2008; 130:15702–15712. [PubMed: 18950160]
3. Kamenka N, Chorro M, Chevalier Y, Levy H. Aqueous solutions of zwitterionic surfactants with varying carbon number of the interchange group. 2. Ion binding by the micelles. *Langmuir*. 1995; 11:4234–4240.
4. Kamenka N, Chevalier Y. Aqueous solutions of zwitterionic surfactants with varying carbon number of the interchange group. 1. Micelle aggregation numbers. *Langmuir*. 1995; 11:3351–3355.
5. Khavrutskii IV, Gorfe AA, Lu B, McCammon JA. Free energy for the permeation of Na(+) and Cl(−) ions and their ion-pair through a zwitterionic dimyristoyl phosphatidylcholine lipid bilayer by umbrella integration with harmonic fourier beads. *J Am Chem Soc*. 2009; 131:1706–1716. [PubMed: 19146415]
6. Knight, CG. Liposomes, from physical structure to therapeutic applications. Elsevier-North-Holland Biomedical Press; 1981.
7. Langner M, Kubica K. The electrostatics of lipid surfaces. *Chem Phys Lipids*. 1999; 101:3–35. [PubMed: 10810922]
8. Mabrey S, Sturtevant JM. Incorporation of saturated fatty acids into phosphatidylcholine bilayers. *Biochim Biophys Acta*. 1977; 486:444–450. [PubMed: 856286]
9. Marte L, Beber RC, Farrukh MA, Micke GA, Costa ACO, Gillitt ND, Bunton CA, Di Profio P, Savelli G, Nome F. Specific anion binding to sulfobetaine micelles and kinetics of nucleophilic reactions. *J Phys Chem B*. 2007; 111:9762–9769. [PubMed: 17661510]
10. Minnes R, Ytzhak S, Weitman H, Ehrenberg B. The effect of solution electrolytes on the uptake of photosensitizers by liposomal membranes: a salting-out effect. *Chem Phys Lipids*. 2008; 155:38–42. [PubMed: 18606157]
11. Needham D, Anyarambhatla G, Kong G, Dewhirst MW. A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. *Cancer Res*. 2000; 60:1197–1201. [PubMed: 10728674]
12. Perttu EK, Szoka FC. Zwitterionic sulfobetaine lipids that form vesicles with salt-dependent thermotropic properties. *Chem Commun (Camb)*. 2011
13. Priebe JP, Lal Satnami M, Tondo DW, Souza BS, Priebe JM, Micke GA, Costa ACO, Fiedler HD, Bunton CA, Nome F. The chameleon-like nature of zwitterionic micelles: the intrinsic relationship of anion and cation binding in sulfobetaine micelles. *J Phys Chem B*. 2008; 112:14373–14378. [PubMed: 18582111]

14. Sachs JN, Woolf TB. Understanding the Hofmeister effect in interactions between chaotropic anions and lipid bilayers: molecular dynamics simulations. *J Am Chem Soc.* 2003; 125:8742–8743. [PubMed: 12862466]
15. Tondo DW, Leopoldino EC, Souza BS, Micke GA, Costa ACO, Fiedler HD, Bunton CA, Nome F. Synthesis of a new zwitterionic surfactant containing an imidazolium ring. Evaluating the chameleon-like behavior of zwitterionic micelles. *Langmuir.* 2010; 26:15754–15760. [PubMed: 20849116]
16. Weinstein J, Yoshikami S, Henkart P, Blumenthal R, Hagins W. Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. *Science.* 1977; 195:489–492. [PubMed: 835007]
17. Yatvin MB, Weinstein JN, Dennis WH, Blumenthal R. Design of liposomes for enhanced local release of drugs by hyperthermia. *Science.* 1978; 202:1290–1293. [PubMed: 364652]
18. Zhang Y, Cremer PS. Interactions between macromolecules and ions: The Hofmeister series. *Curr Opin Chem Biol.* 2006; 10:658–663. [PubMed: 17035073]

Figure 1. AQ Lipid Structure and Vesicle Formation

(A) The structures of the synthesized AQ lipids are shown. (B) TEM images of (right) DMAQ lipids after heating and sonication and (left) of DPAQ:Chol liposomes. (C) Size and zeta potential measurements for AQ:Chol liposomes. Formulations composed of 60% AQ lipids and 40% cholesterol form stable vesicles with neutral zeta potential.

Figure 2. Ionic Interactions and Stability of AQ Vesicles

(A) Zeta potential measurements of anionic interactions of DMPC:Chol and DMAQ:Chol vesicles. Anions with low charge density decrease the zeta potential more than those with a high charge density. (B) Calcium interactions of DMAQ:Chol and DMPC:Chol liposomes. Both formulations become increasingly positive in the presence of increasing $[Ca^{2+}]$. DMAQ:Chol vesicles remain negatively charged at 10mM $[Ca^{2+}]$ while DMPC:Chol liposomes adopt a positive charge. (C) DMAQ:Chol vesicles show comparable stability to DMPC:Chol vesicles at 37 °C in 30% serum over one week. For (A)–(C), samples were run in triplicate and error bars represent $\pm$ standard deviation.

Figure 3. AQ Lipids Show Elevated Transition Temperatures

(A) AQ transition temperatures are plotted alongside those for PC and PE lipids. (DMAQ T_m 48.8 °C, DPAQ T_m 58.3 °C, DSAQ T_m 65.5 °C). (B-D) Thermograms of DMAQ, DPAQ, and DSAQ lipid dispersions in various salts.

Figure 4. Cytotoxicity, Pharmacokinetics and Biodistribution of AQ Lipid Vesicles

(A) Viability of C26 colon carcinoma cells in the presence of DPAQ:Chol or DSPC:Chol liposomes. Samples were run in triplicate. Error bar represent $\pm$ standard error of the mean. (B) Pharmacokinetics and (C) biodistribution of DPAQ and DSPC liposomes.

Experiments were run with $n=3$ mice per group. (D) Pharmacokinetic parameters of DPAQ and DSPC liposomes. Error bars represent $\pm$ standard deviation.

Scheme 1.

(i) Dichloromethane, 4-Dimethylaminopyridine, *N,N'*-Dicyclohexylcarbodiimide, RT, 16 hours (ii) Dichloromethane, *N,N*-Diisopropylethylamine, 40 °C, 16 hours (iii) Dichloromethane, Trifluoroacetic acid, RT, 1 hour.