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Cationic triple-chain amphiphiles facilitate vesicle fusion compared to double-chain or single-chain analogues

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Abstract

Cationic, triple-chain amphiphiles promote vesicle fusion more than structurally related double-chain or single-chain analogues. Two types of vesicle fusion experiments were conducted, mixing of oppositely charged vesicles and acid-triggered self-fusion of vesicles composed of cationic amphiphile and anionic cholesteryl hemisuccinate (CHEMS). Vesicle fusion was monitored by standard fluorescence assays for intermembrane lipid mixing, aqueous contents mixing and leakage. Differential scanning calorimetry was used to show that triple-chain amphiphiles lower the lamellar–inverse hexagonal ($L_\alpha - H_{II}$) phase transition temperature for dipalmitoleoylphosphatidylethanolamine. The triple-chain amphiphiles may enhance vesicle fusion because they can stabilize the inversely curved membrane surfaces of the fusion intermediates, however, other factors such as extended conformation, packing defects, chain motion, or surface dehydration may also contribute. From the perspective of drug delivery, the results suggest that vesicles containing cationic, triple-chain amphiphiles (and cationic, cone-shaped amphiphiles in general) may be effective as fusogenic delivery capsules.

Keywords: Membrane fusion; Cationic liposome; Molecular shape; Drug delivery; Membrane curvature; Calorimetry

1. Introduction

The mechanism of bilayer membrane fusion [1–4], a critical cellular process, is the subject of ongoing experimental [5–11] and theoretical studies [12–14]. Although major progress has been made over the past decade, there is still only a moderate mechanistic understanding at the molecular level. Much of the experimental work uses vesicle systems as biomimetic models, and a significant effort has been made to identify natural and abiotic compounds that promote or inhibit vesicle fusion. As a result of this work, it is known empirically that cone-shaped amphiphiles (relatively small head cross-section and large tail cross-section) often promote membrane fusion, and also increase the propensity of lamellar membranes to undergo phase changes to non-lamellar structures such as inverse hexagonal or cubic phases [15–19]. The most popular mechanistic rationalization of this observation uses the pore-stalk fusion model and proposes that cone-shaped amphiphiles stabilize common intermediate membrane structures with inversely curved surfaces [20,21]; however, other explanations have been put forth (see below).

From an applied perspective, much attention has been directed towards vesicles containing cationic lipids because of the potential uses in transfection and drug delivery [22–24]. Although vesicle-mediated delivery is a multistep event, there is evidence that the rate-determining step for certain cell types is endosomal escape, which can occur either by a fusion or disruption process [25,26]. Thus, there is a need for cationic lipids that can effectively promote the fusion or disruption of bilayer membranes [27]. Recently, Hafez et al. [28] reported that binary mixtures of cationic and anionic polar lipids adopt inverted nonbilayer structures, and they used this effect to trigger vesicle fusion by acidification. In addition, Smisterová et al. [29] found that

Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonate; CHEMS, cholesteryl hemisuccinate; DSC, differential scanning calorimetry; DiPoPE, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPX, *N*,*N*′-*p*-xylenebis(pyridinium bromide); egg PC, phosphatidylcholine derived from egg yolk; egg PE, phosphatidylethanolamine made by transphosphatidylation of lecithin; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; Rh-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid; TREN, tris(2-aminoethyl)amine

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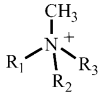
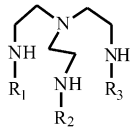
the shape of a series of related cationic amphiphiles controls the structure of cationic amphiphile/DOPE–DNA complexes and the efficiency of gene delivery. These results suggest that cationic, cone-shaped amphiphiles may promote vesicle fusion better than cylinder-shaped or inverse-cone-shaped cationic amphiphiles [30–33]. This hypothesis has lead us to compare the fusogenicity of cationic amphiphiles with one, two or three hydrocarbon chains. In this study, we evaluate the abilities of two groups of structurally related amphiphiles (**1**–**3** and **4**–**7**) to induce membrane fusion and find, for each group, that vesicles containing the triple-chain versions are the most fusogenic. Although triple-chain amphiphiles have been studied before [34–41], they have never been previously directly attributed with strong fusogenic activity.



1 $R_1 = R_2 = CH_3$, $R_3 = C_{12}H_{25}$
2 $R_1 = CH_3$, $R_2 = R_3 = C_{12}H_{25}$
3 $R_1 = R_2 = R_3 = C_{12}H_{25}$



4 $R_1 = R_2 = C_2H_5$, $R_3 = C_{18}H_{37}$
5 $R_1 = C_2H_5$, $R_2 = R_3 = C_{18}H_{37}$
6 $R_1 = R_2 = R_3 = C_{14}H_{29}$
7 $R_1 = R_2 = R_3 = C_{16}H_{31}$ (*cis*-C9)

2. Materials and methods

2.1. Materials

All polar lipids, including the fluorescent labeled ones were obtained from Avanti Polar Lipids Inc. The fluorescent markers 1-aminonaphthalene-3,6,8-trisulfonate (ANTS) and *N*,*N*′-*p*-xylenebis(pyridinium bromide) (DPX) were obtained from Molecular Probes and carboxyfluorescein from Eastman Kodak Co. Dodecyltrimethylammonium bromide, **1**, didodecyldimethylmmonium bromide, **2**, and tridodecylmethylammonium chloride, **3**, were purchased from Aldrich Chemical Co. Compounds **4**–**7** were prepared using the method of Thomas et al. [42]. First tris(2-aminoethyl)amine (TREN) was acylated with the appropriate acid chloride(s). The resulting triamides were then reduced their corresponding amines with excess $LiAlH_4$. *Spectroscopic data*: **4**: ^{1}H NMR (500 MHz) δ 2.74–2.57 (m, 18H), 1.50 (pentet, J=7 Hz, 2H), 1.32–1.22 (m, 28H), 1.14 (br. t, J=6.5 Hz, 6H), 0.87 (t, J=7 Hz, 3H); ^{13}C NMR (75 MHz, some signals in aliphatic region overlap) δ 54.2, 50.0, 47.7, 47.5, 44.1, 31.9, 30.1, 29.7, 29.64, 29.62, 29.59, 29.3, 27.4, 22.7, 15.2, 14.1. FAB-HRMS (m/z) $[M+1]^+$ calculated for $C_{28}H_{62}N_4$ 455.5057, found 455.5080. **5**: ^{1}H NMR (300 MHz) δ 2.72–2.56 (m, 18H), 1.49 (pentet, J=7 Hz, 4H), 1.34–1.18 (m, 56H), 1.12 (br. t, J=7 Hz, 3H), 0.87 (t, J=7 Hz, 6H); ^{13}C NMR (75 MHz, some signals in aliphatic region overlap) δ 54.2, 50.0, 47.7, 47.5, 44.0, 31.9, 30.1, 29.7, 29.6, 29.4, 27.4, 22.7, 15.1, 14.1. HRMS-FAB (m/z): $[M+H]^+$ calculated for $C_{44}H_{94}N_4$, 679.7562; found, 679.7564. **6**: ^{1}H NMR (300 MHz) δ 2.64 (br. q, J=5.4 Hz, 6H), 2.62–2.54 (m, 12H), 1.47 (br. t, J=6 Hz, 6H), 1.26 (br. s, 66H), 0.88 (t, J=6.6 Hz, 9H); ^{13}C NMR (75 MHz) δ 54.8, 50.3, 47.9, 31.9, 30.4, 29.7(21 C), 29.4, 27.5, 22.7, 14.1. HRMS-FAB (m/z): $[M+H]^+$ calculated for $C_{48}H_{102}N_4$, 735.8189; found, 735.8204. **7**: ^{1}H NMR (300 MHz) δ 5.40–5.26 (m, 6H), 2.68–2.52 (m, 18H), 1.99 (br. d, J=5.4 Hz, 12H), 1.46 (br. t, J=5.7 Hz, 6H), 1.28 (br. s, 54H), 0.87 (t, J=5.4 Hz, 9H); ^{13}C NMR (75 MHz, two signals in aliphatic region overlap) δ 129.9, 129.8, 54.5, 50.2, 47.8, 31.7, 30.3, 29.8, 29.7, 29.6, 29.5, 29.3, 28.9, 27.5, 27.2, 22.6, 14.0. HRMS-FAB (m/z): $[M+H]^+$ calculated for $C_{54}H_{109}N_4$, 813.8659; found, 813.8676.

2.2. Unilamellar vesicle preparation

Measured aliquots of the appropriate phospholipids in chloroform were placed in a 10 ml round-bottomed flask. The chloroform was evaporated under reduced pressure on a rotary evaporator and dried under vacuum for at least 1 h. The dried lipids were hydrated with 100 mM NaCl/5 mM TES buffer (pH 7.4) while vortexing using glass bead to promote removal of the lipids from the flask walls. The vesicle solution underwent a rapid freeze/thaw procedure 10 times unless they were to be used for a lipid mixing assay or differential scanning calorimetry (DSC) where this procedure was omitted. The vesicle solution was extruded, at room temperature, 21 times through a polycarbonate filter with 100 nm diameter pores using a hand-held Basic LiposoFast extruder from Avestin, Inc. In the case of contents mixing or leakage assays, the unencapsulated marker compound was removed by overnight dialysis. ^{1}H and ^{13}C NMR was used to determine vesicle composition before and after dialysis. The vesicles were dried under high vacuum and then redissolved in $CDCl_3$. The ratio of cationic amphiphile to phosphocholine was determined from NMR peak integrations and did not change significantly after dialysis.

Dynamic light scattering (Beckman Coulter N4 Plus) showed a vesicle size of about 100 ± 20 nm before fusion. After fusion, the size distribution was very broad and did not provide useful information.

2.3. Lipid mixing assay

All fluorescence experiments were performed using Perkin-Elmer LS50B fluorimeter. The probe dilution method was used which measures the fluorescence resonance energy transfer between an emitter, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD-PE), and a quencher 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl (Rh-PE). Initially, both probes are in the same population of vesicles. Upon fusion of the labeled vesicles with unlabeled vesicles, the probes are diluted and quenching by resonance energy transfer is decreased resulting in increased fluorescence. The excitation wavelength for the NBD-PE probe was set at 470 nm with emission measured

at 530 nm using a 515 nm cutoff filter to reduce stray light noise.

Two sets of lipid mixing experiments were conducted. The first set involved mixing of oppositely charged vesicles. Cationic unlabeled donor vesicles were added to the anionic labeled acceptor vesicles in a 1:1 ratio in 3 ml of 100 mM NaCl/5 mM TES buffer at pH 7.4. The second set of fusion experiments involved acid-triggered self-fusion of vesicles composed of cationic amphiphile and CHEMS. An aliquot of 0.5 M HCl was added to a 3.0 ml sample of vesicles in 100 mM NaCl/5 mM TES buffer, pH 7.4. The ratio of labeled to unlabeled vesicles was 1:5. At the end of each run, 2.5 mM octaethylene glycol monododecyl ether was added to determine 100% lipid mixing, and the final pH determined with an electrode.

2.4. Contents mixing assay

Vesicles were prepared as above encapsulating either 25 mM ANTS/40 mM NaCl (anionic acceptor vesicles) or 90 mM DPX in 5 mM pH 7.4, TES buffer (cationic donor vesicles). After vortexing, the lipid mixture was subjected to 10 rounds of freezing and thawing in an ethanol/dry ice bath and 45 °C water bath, respectively. The dispersions were then extruded as above and dialyzed overnight in 150 mM NaCl using 15,000 MW cutoff dialysis tubing.

The excitation wavelength for ANTS was set at 354 nm with emission measured at 530 nm using a 515-nm cutoff filter to reduce stray light noise. Assays were performed by mixing the donor/acceptor vesicle populations (50 μM final phospholipid concentration) in 3 ml of 100 mM NaCl/5 mM TES buffer (pH 7.4) and the fluorescence monitored over time. Detergent (2.5 mM) was added to the samples after 300 s. The intensity of an initial sample and a reference population of 12.5 mM ANTS/45 mM DPX/20 mM NaCl vesicles were set to 100% and 0% contents mixing, respectively.

2.5. Leakage assay

Acceptor vesicles were prepared as above, but encapsulating 50 mM carboxyfluorescein/100 mM NaCl in 5 TES buffer (pH 7.4). After vortexing, the lipid mixture was subjected to 10 rounds of freezing and thawing in an ethanol/dry ice bath and 45 °C water bath, respectively. The lipids were then extruded as above and dialyzed overnight in 150 mM NaCl using 15,000 MW cutoff dialysis tubing. The excitation wavelength was set at 495 nm with emission measured at 520 nm. The vesicles were added to 3 ml of 100 mM NaCl/5 mM TES buffer (pH 7.4). At 100 s cationic donor vesicles, encapsulating100 mM NaCl/5 mM TES buffer (pH 7.4) were added. Leakage were determined from the initial fluorescence of the sample and after addition of 2.5 mM octaethylene glycol monododecyl ether (0% and 100%), respectively.

2.6. Differential scanning calorimetry

Vesicles were prepared as above, using 99 mol% dipalmitoleoylphosphatidylethanolamine (DiPoPE) and 1% of the compounds **1**–**4**, or **7**, to a final lipid concentration of 14.5 mM in 100 mM NaCl/5 mM TES buffer (pH 7.4). All the samples and the buffers were degassed prior to use. A VP-DSC (Microcal, Amherst, MA) high-sensitivity scanning microcalorimeter was used with a cell volume of 0.5 ml. Continuous scans were recorded at a rate of 60 °C/h from 10 to 60 °C.

3. Results and discussion

Unilamellar vesicle fusion was induced by mixing cationic donor vesicles with an equal population of anionic acceptor vesicles [43,44]. In the case of quaternary ammonium cations, **1**–**3**, the donor vesicles were composed of 5:3.5:1.5, egg phosphatidylethanolamine: egg phosphatidylcholine:X (eggPE:eggPC:X) where X is **1**, **2**, or **3**, and the

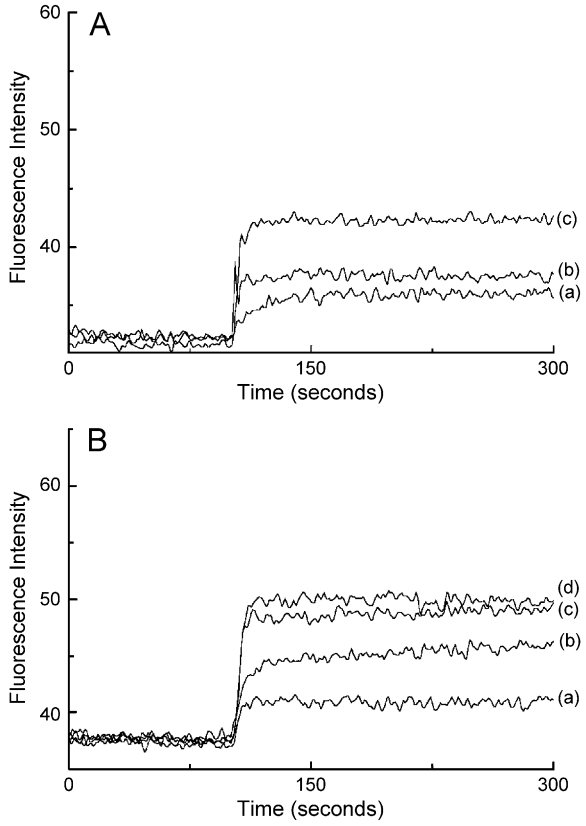


Fig. 1. Lipid mixing. A population of unlabeled cationic donor vesicles (25 μM total lipid) was added at $t = 100$ s to an equal population of anionic acceptor vesicles (containing 0.6% of the probes NBD-PE and Rh-PE) in 100 mM NaCl/5 mM TES buffer, $T = 25$ °C, pH 7.4. (A) Donor vesicles are eggPE:eggPC:X (5:3.5:1.5) and acceptor vesicles are eggPE:eggPC:POPA (5:3.5:1.5). (a) X = **1**, (b) X = **2**, (c) X = **3**. (B) Donor vesicles are eggPE:egg PC:X (5.5:3.5:1) and acceptor vesicles are eggPE:egg PC:POPA (5:3.5:1.5). (a) X = **4**, (b) X = **5**, (c) X = **6**, (d) X = **7**.

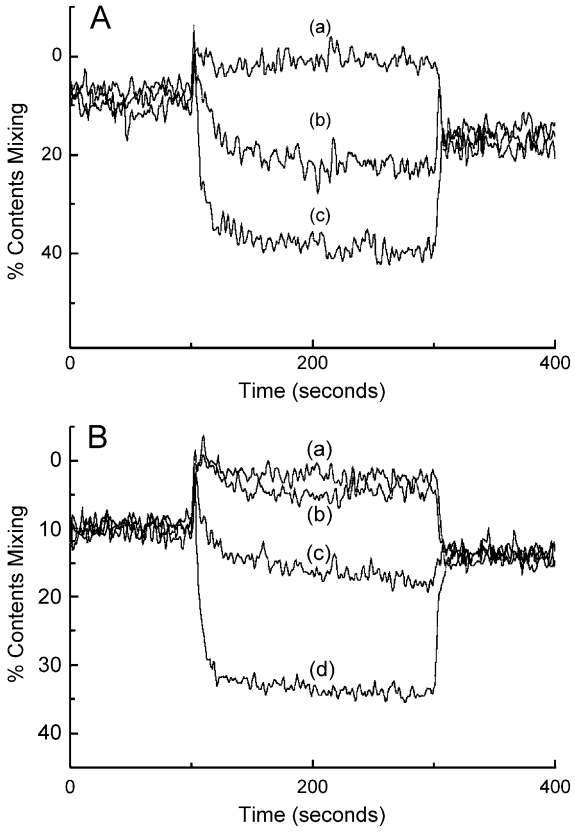


Fig. 2. Contents mixing. A population of cationic donor vesicles (25 μM total lipid) containing DPX (90 mM) are added at $t=100$ s to an equal population of anionic acceptor eggPE:eggPC:POPA vesicles containing ANTS (25 mM) in 100 mM NaCl/5 mM TES buffer, $T=25$ °C, pH 7.4. At $t=300$ s, octaethylene glycol monododecyl ether is added to lyse the vesicles. (A) Donor vesicles are eggPE:eggPC:X (5:3.5:1.5) and acceptor vesicles are eggPE:eggPC:POPA (5:3.5:1.5), (a) X=**1**, (b) X=**2**, (c) X=**3**. (B) Donor vesicles are eggPE:eggPC:X (5.5:3.5:1) and acceptor vesicles are eggPE:eggPC:POPA (5:3.5:1.5). (a) X=**4**, (b) X=**5**, (c) X=**6**, (d) X=**7**.

anionic acceptor vesicles were composed of 5:3.5:1.5, eggPE:eggPC:POPA (POPA is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid). Vesicle fusion was monitored at 25 °C and pH 7.4 by standard fluorescence assays for intermembrane lipid mixing, aqueous contents mixing and leakage [45]. Lipid mixing was monitored by the probe dilution method which uses the fluorescently labeled phospholipid, NBD-PE, and its resonance energy transfer quencher, Rh-PE. One vesicle population containing 0.6% of each of the probes, is added to another population that is unlabeled. Lipid mixing is indicated by an increase in NBD-PE fluorescence intensity due to diminished quenching as the two probes are diluted. As shown in Fig. 1A, rapid lipid mixing occurred when unlabeled cationic vesicles were added to labeled anionic vesicles. The extent of lipid mixing depended on the identity of the cationic amphiphile and increased in the order, **1**<**2**<**3**. Control experiments with the probes in both vesicle populations showed that the fluorescence increases were not due to scattering effects. More compelling evidence for vesicle fusion was gained from contents mixing assays which started with two populations of vesicles, anionic acceptors encapsulating the fluorophore ANTS, and cationic donors containing the quencher DPX. Only complete vesicle fusion and mixing of aqueous contents can produce a decrease in fluorescence intensity. The amounts of aqueous contents mixing after 2 min was 0%, 22% and 42% for **1**, **2**, and **3**, respectively (Fig. 2A). Leakage studies using acceptor vesicles containing carboxyfluoroscein revealed that fusion occurred without any leakage of aqueous contents (Fig. 3A).

Essentially identical fusion experiments were conducted with donor vesicles containing the amine amphiphiles **4**–**7** (Figs. 1B and 2B), and the same trend was observed, i.e., the order of fusogenic activity is **4**<**5**<**6**<**7**. In addition to this trend, we found that vesicles with unsaturated analogue **7** (containing three *cis*-hexadec-9-enyl chains) induced more fusion and leakage (Figs. 2B and 3B) than vesicles with saturated analogue **6** (containing three tetradecyl chains), a structure/activity difference that has been noted before and

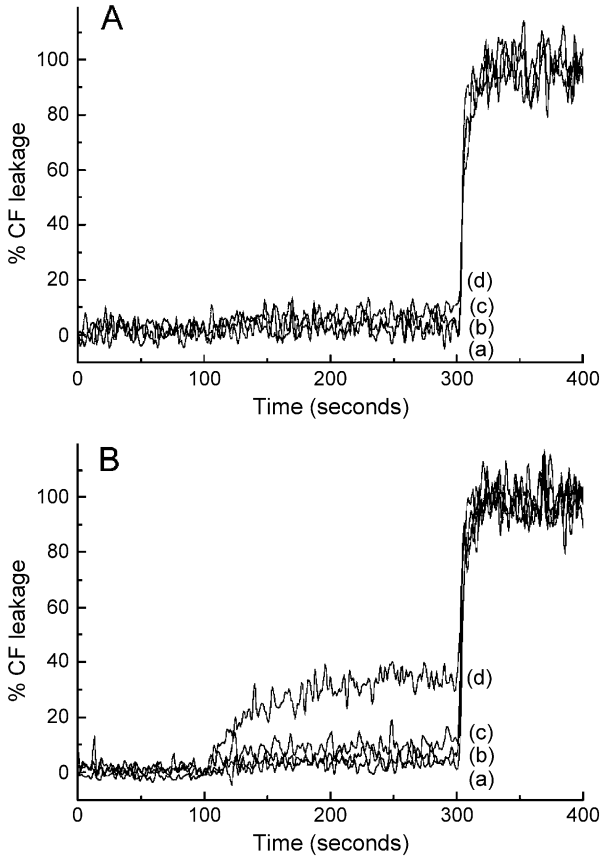


Fig. 3. Leakage. A population of cationic donor vesicles (25 μM total lipid) was added at $t=100$ s to an equal population of anionic acceptor vesicles containing carboxyfluoroscein in 100 mM NaCl/5 mM TES buffer, $T=25$ °C pH 7.4. (a) X=**1**, (b) X=**2**, (c) X=**3**. Octaethylene glycol monododecyl ether, was added at 300 s. (A) Donor vesicles are eggPE:eggPC:X (5:3.5:1.5) (25 μM) and acceptor vesicles are eggPE:eggPC:POPA (5:3.5:1.5), (a) X=**1**, (b) X=**2**, (c) X=**3**. (B) Donor vesicles are eggPE:eggPC:X (5.5:3.5:1) and acceptor vesicles are eggPE:eggPC:POPA (14:3:3). (a) X=**4**, (b) X=**5**, (c) X=**6**, (d) X=**7**.

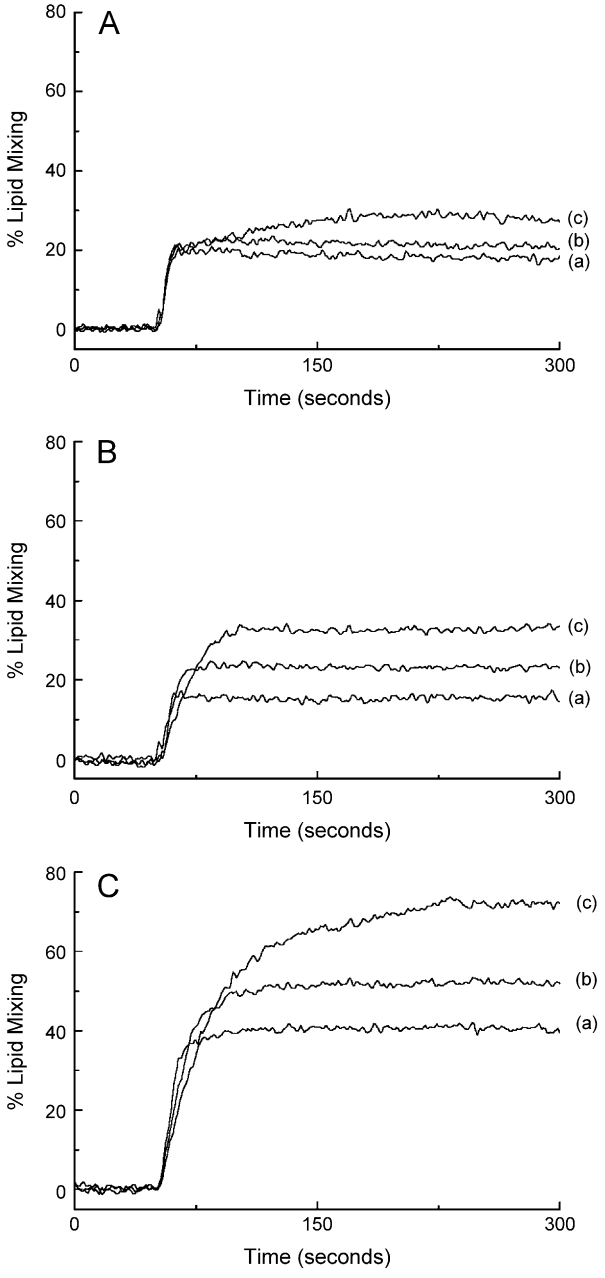


Fig. 4. Acid-triggered lipid mixing. An aliquot of 0.5 M HCl was added at $t = 100$ s to a 1:5 mixture of labeled (containing 0.6% of the probes NBD-PE and Rh-PE) and unlabeled vesicles composed of CHEMS:X (85:15) (150 μM total lipid concentration) in 100 mM NaCl/5 mM TES buffer, pH 7.4. A, X = **1**; B, X = **2**; C, X = **3**. The pH after HCl addition was (A) pH = 6.2, (B) pH = 5, (C) pH = 4.

attributed to differences in amphiphile shape and chain motion [46].

A fusogenicity order of **1** < **2** < **3** was also observed in pH-dependent fusion experiments using vesicles composed of CHEMS:X (85:15) where CHEMS is cholesteryl hemisuccinate and X is either **1**, **2** or **3**. Recently, Hafez et al. [28] showed that mixtures of CHEMS and cationic lipids can form stable vesicles at neutral pH but these liposomes undergo fusion as the pH is lowered. The critical point at which fusion occurs is when the vesicle surface charge is zero. Shown in Fig. 4 are acid-triggered lipid mixing profiles for the CHEMS:X (85:15) vesicles. As the pH is lowered, the initial fusion rates decrease although the final extent of lipid mixing increases (leakage studies showed that fusion occurred without any leakage of aqueous contents, data not shown). Our trend of decreasing initial fusion rate with increasing acidity is opposite of that observed by Hafez et al., and most likely reflects an artifact due to different acidification procedures. Hafez et al. triggered fusion by injecting the vesicles into a buffered acid solution, whereas we added an aliquot of acid to the vesicles. It is likely that our method produced pH gradients resulting in an initial fraction of the vesicles becoming highly cationic and thus undergoing a slower rate of membrane fusion. In any case, this artifact is tangential to our observation that the extent of lipid mixing is **1** < **2** < **3**. Overall, we find for both amphiphilic structural groups, and for both methods of vesicle fusion, that the order of fusogenic activity is single-chain < double-chain < triple-chain.

The intuitive idea that the more branched, triple-chain amphiphiles stabilize inversely curved membrane surfaces compared to the double-chain or single-chain analogues

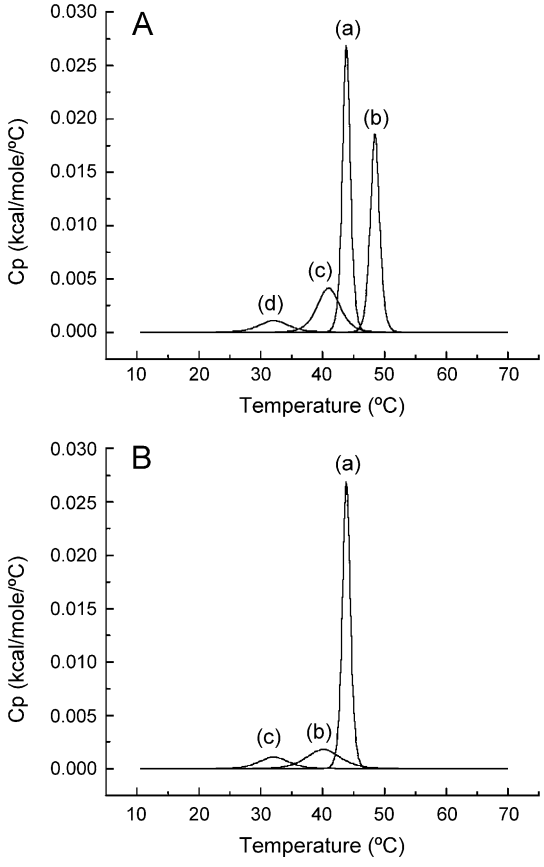


Fig. 5. Differential scanning calorimetry. (A) Lamellar to inverse hexagonal phase transitions in pure DiPoPE (a) and mixtures of 99 mol% DiPoPE and 1 mol% of compounds **1** (b), **2** (c) and **3** (d). (B) mixtures of 99 mol% DiPoPE and 1 mol% of compounds **4** (b) and **7** (c).

[36,47], was confirmed by using DSC to measure the effects of the cationic lipids on the lamellar–inverse hexagonal phase transition temperature (T_H) for DiPoPE. Compounds that lower the T_H for DiPoPE are considered to stabilize inversely curved membrane surfaces [20]. As shown in Fig. 5, the presence of 1 mol% of single-chain **1** raises T_H from 44 to 49 °C, whereas 1% of triple-chain **3** lowers it to 32 °C. The triple-chain DSC results were initially surprising since it was recently reported that charged lipids raise the bilayer–nonbilayer phase transition temperature for zwitterionic lipids [33]. It appears that the inverse curvature stabilizing effect due to the cone-shape of the triple-chain lipids overwhelms the destabilizing effect of the charged head group. Similarly, the presence of 1% of triple-chain analogue **7** lowers T_H for DiPoPE to 32 °C, whereas it is 40 °C in the presence of single-chain analogue **4**.

In addition to inverse membrane curvature stabilization, other factors may contribute to the enhanced fusogenic activity of the triple-chain amphiphiles, such as domain formation, increased membrane packing defects, increased chain motion, or increased surface dehydration [48]. While domain formation is a possibility in the case of the egg-PE:eggPC:X vesicles, it is much less likely in the case of CHEMS:X vesicles because membrane mixing is favored by electrostatic attraction of the oppositely charged membrane components. In addition, domain formation of CHEMS:X requires counter-ion immobilization which is entropically costly [49]. A particularly intriguing mechanistic proposal, which may be relevant in this case, is the extended conformation hypothesis. To use the nomenclature of Kinnunen and Holopainen [50], a cone-shaped, triple-chain amphiphile is ‘‘sterically frustrated’’ in a bilayer membrane which prefers to accommodate cylinder-shaped lipids. During the fusion process, the triple-chain lipid can momentarily relieve this steric frustration by adopting an extended conformation that inserts one hydrocarbon chain into the outer leaflet of an adhered, dehydrated bilayer. Recent molecular dynamics calculations provide supporting evidence for this type of physical picture [51].

In summary, two examples are described where cationic, triple-chain amphiphiles promote vesicle fusion more than structurally related double-chain or single-chain analogues. The same trend is found for the transition of lamellar to inverse hexagonal phase. The fusion enhancement is likely due to differences in lipid dynamic shape and their effects on membrane curvature (the cone-shaped triple-chain amphiphiles best stabilize the inversely curved membrane surfaces of the fusion intermediates). The structural simplicity of these cationic amphiphiles, especially compounds **1**–**3** which are commercially available, makes them highly amenable for detailed mechanistic study. From the perspective of drug delivery, our results suggest that vesicles containing cationic, triple-chain amphiphiles (and cationic, cone-shaped amphiphiles in general) may be effective as fusogenic delivery capsules.

Acknowledgements

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