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Aggregation of lariat ethers attached to a membrane anchoring unit

Natasha K. Djedovič,[a] Riccardo Ferdani,[a] Paul H. Schlesinger[b] and George W. Gokel[a,*]

[a]*Program in Bioorganic Chemistry, Division of Bioorganic Chemistry, Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8103, St. Louis, MO 63110, USA*
[b]*Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA*

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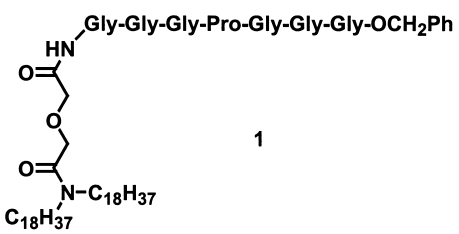
Abstract—The GGGPGGG heptapeptide, when anchored by octadecylamine–diglycolic acid unit forms chloride-selective channels in bilayer. Crown ethers, differently anchored, have been used to form synthetic cation channels. Dialkylamine–diglycolic acid anchored crown ethers failed to form cation-selective channels. This might be due to an inability to insert in the bilayer. Their ability to form stable aggregates, reflective of the latter, was therefore studied. © 2002 Elsevier Science Ltd. All rights reserved.

In more than three decades since the discovery of crown ethers,[1,2] these remarkable compounds[3] have evolved from functioning as alkali metal binding agents[4] to serve as structural elements of general utility in supramolecular chemistry.[5,6] One important role played by crown ethers is as an amphiphile head group. Crown-ether-based amphiphiles have been studied at the air–water interface[7–10] and in aqueous suspension.[11–15] In recent work, crown ethers have served as headgroups or ion portals for synthetic ion channels.[16–21] The synthetic ionophores we have called hydraphiles[17] use crown ethers as both headgroups[22] and as ion relays[23] analogous to the 'water and ion-filled capsule' found in the KcsA channel of *Streptomyces lividans*.[24]

When our efforts to develop synthetic cation channels enlarged to include anions, we considered crown ethers as plausible headgroups or ion portals. Certainly, the complexation of halogens by protonated azacrowns or cryptands has long been known. Admittedly, complexation of halogens has relied to an appreciable extent on H-bond interactions that could profoundly restrict[25] ion flow. Further, the issue of hydration state of transient ions in channels is a difficult[26,27] and currently unresolved question. The hole sizes of 15- and 18-membered crown ethers correspond approximately to the sizes of Na^+ and K^+, respectively. Crystallographic data place the diameters of Na^+ and K^+ at 2.04 Å (6-coordinate) and 3.02 Å (8-coordinate).[28] The same source places chloride's diameter at 3.62 Å with no coordination number specified.[28] Recent computational studies by Zhou and co-workers calculate the hydrated diameters for Na^+, K^+, and Cl^- at 5.98, 5.50, and 6.48 Å.[29] In this work, the authors adopt an effective diameter of 2.76 Å for a water molecule. The range of sizes reported for individual ions underscores the difficulty facing the designer of functional supramolecular assemblies.

We have recently reported a successful chloride-transporting, membrane-anchored heptapeptide, **1**, that appears to function as a dimer.[30] Our current model for chloride transport by **1** requires the diglycolic acid tail and twin hydrocarbon chains to insert in the phospholipid bilayer while the heptapeptide serves as a part of the ion portal.[31] It currently appears that proline, which occurs at the center of the heptapeptide, provides a bend in the chain causing it to adopt a v-shape. As noted, **1** is dimeric in phospholipid bilayers[30] so the two-face-to-face, v-shaped peptides probably form a parallelogram-like opening in the membrane. We reasoned that a similarly anchored system might also function as an ionophore when a crown ether, rather than a pair of peptides, served as the ion portal/headgroup. Crowns have previously been used in cation,[17,18,32] rather than anion, channel models so we anticipated that these novel anchored crowns would be cation selective if they functioned as ionophores at all. We now report the preparation and analysis of three new, anchored macrocycles (**2**–**4**) along with their ion transport and aggregation properties.



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Keywords: crown ethers; amphiphiles; bilayers.

* Corresponding author. Tel.: +1-314-362-9297; fax: +1-314-362-9298/7058; e-mail: ggokel@molecool.wustl.edu

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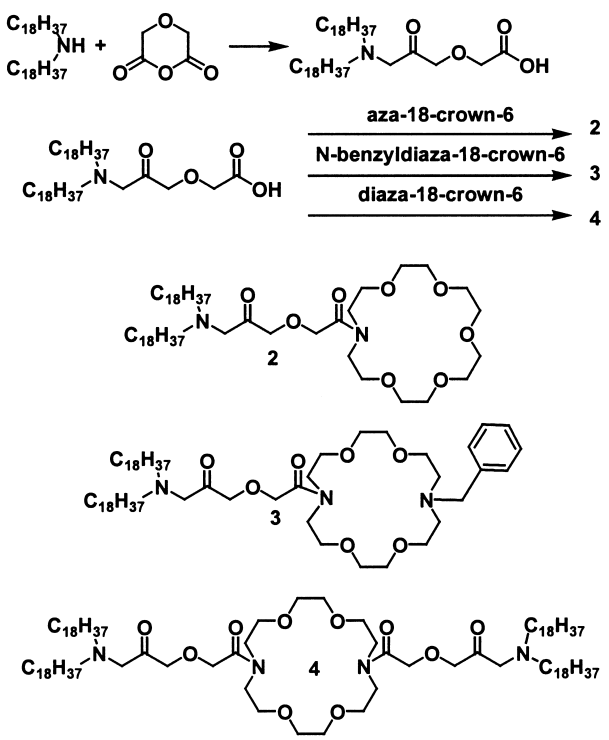
The design of the anchor unit used to develop a chloride channel[30] is apparent from the structure of **1**. Dioctadecylamine provides the hydrocarbon chains that will insert into a phospholipid bilayer. The oxydiacetic (diglycolic) acid residue emulates the midpolar regime of a phospholipid bilayer and serves to connect the hydrocarbon chains to the 'headgroup'. Synthesis of compounds **2**–**4** was accomplished in a straightforward fashion. Diglycolic acid and dioctadecylamine were heated in refluxing toluene for 48 h. The solvent was removed to afford the crude product, $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$, which was crystallized from diethyl ether. We abbreviate this compound as 18_2DGA-OH. This anchor unit was coupled, using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and triethylamine or 1-benzotriazolyloxytris(pyrrolidino)-phosphonium hexafluorophosphate (PyCloP) and diisopropylethylamine (DIEA), on a molar basis with aza-18-crown-6 or *N*-benzyldiaza-18-crown-6 to yield **2** or **3** in 81% (73±8%, 3 runs; 81% yield obtained with PyCloP) and 46% yields, respectively. Carbodiimide mediated coupling of diaza-18-crown-6 with 2 equiv. of the acid afforded **4** in 44% yield (Scheme 1).



Scheme 1.

1. Ion transport studies in phospholipid vesicles

Compounds **2**–**4** could potentially insert in phospholipid bilayers and form ion-conducting channels. Owing to the presence of the crown headgroup, we expected these compounds to be cation transporters if they functioned at all as ionophores. Analysis of either cation or anion transport required the formation of liposomes, which were obtained for these studies as follows. Unilamellar vesicles, 305±72 nm, were prepared containing 2.5 M NaCl (pH=7.0, 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)) and the extravesicular solution was exchanged for 2.5 M choline chloride (pH=7.0, 100 mM HEPES). In anticipation of cation selectivity, expected because of the crown ether headgroup, a sodium-selective electrode was inserted into the buffered medium and its response was continuously recorded. Fig. 1, above, shows the (unfiltered, noisy) baseline response. Addition of compound **2** to the medium had no effect on the electrode response. Addition of the above-described liposomes to the solution in the presence of **2** likewise had no effect on sodium current. If sodium channels formed, we expected to detect an increasing current as **2** gradually inserted into the vesicles. No such current change was observed during nearly an hour of observation.

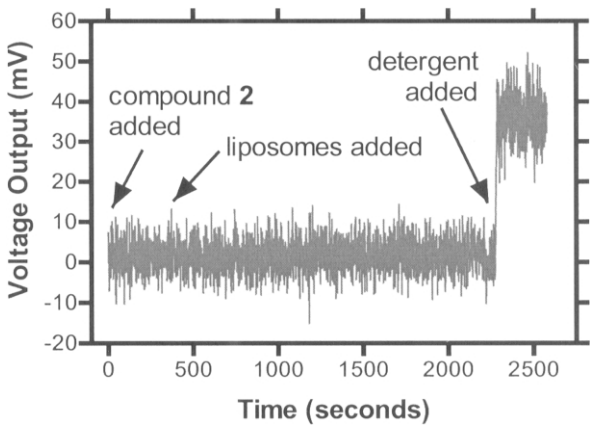


Figure 1. Assay for sodium release by **2** in buffered aqueous solution. No current above noise level was observed until detergent-induced liposome rupture occurred.

Neither the anchored azacrown (**2**) nor the bis(anchored) diazacrown (**4**) showed effective Na^+ release using the experimental procedure described below. The figure above shows the electrode output over ~2000 s when **2** (287 μM) was assayed alone, after addition of liposomes (0.1 mM phospholipid), and after addition of 2% Triton X-100 (detergent) to the system. The only change observed in system response resulted from detergent-induced vesicle rupture. Similar results were obtained for compound **4**.

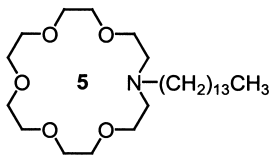
A similar assessment of activity with **3** showed a low level of Na^+ release from liposomes. The activity was reproducibly above the level induced by isopropyl alcohol, the solvent for **3**, but Na^+ release was too low to accurately quantitate.

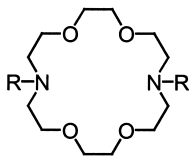
2. Aggregation studies

The failure of **2** and **4** to form sodium-conducting pores in phospholipid liposomes raised the question of their ability to insert in membranes. An excellent indication of this property is whether the monomers themselves readily form aggregates. We therefore attempted to do so in aqueous solution with the expectation that, if formed, the aggregates would be characterized by light scattering. The technique of vesicle formation by reverse phase evaporation failed.[33] We thus formed vesicles by using a solid hydration procedure (see Section 4).

Aggregates formed from the mono-tailed azacrown (**2**) were stable in H_2O (diameter=100–250 nm, 1000–2500 Å) for >24 h, but the presence of Na^+ or K^+ accelerated coalescence of the aggregates as soon as sonication ceased. The twin-tailed analog **4** yielded aggregates in the 200–400 nm range in water. Although it was not possible to obtain stable aggregates when K^+ was present in the medium, the presence of Na^+ was less deleterious. When NaCl was present in the mixture, minimal fluctuations in size were observed over time. Compound **3** contains a benzyl group in place of one of the hydrophobic tails of **4**, and the resulting aggregates were rarely stable for more than 24 h. Dynamic light scattering measurements indicated a bimodal distribution of aggregate diameters immediately following sonication. In water, small aggregates (10–50%) were observed ranging from 5 to 100 nm, while larger particles of about 300 nm were also recorded. No physical significance is necessarily attributed to the latter, and the distribution was often improved by extrusion through a polycarbonate membrane. Results of aggregate sizing experiments (laser light scattering) for compounds **1**–**4** are recorded in Table 1.

In previous studies, we characterized aggregates formed from *N*,*N*-didodecyldiaza-18-crown-6, **6**, by a variety of techniques so the system was well understood.[13] Two other previously studied compounds are relevant to these studies.[13] They are *N*-tetradecylaza-18-crown-6 (**5**), *N*,*N*′-ditetradecyldiaza-18-crown-6 (**7**), and *N*,*N*′-dioctadecyldiaza-18-crown-6 (**8**). The structures are shown.





6, R = $(CH_2)_{11}CH_3$
7, R = $(CH_2)_{13}CH_3$
8, R = $(CH_2)_{17}CH_3$

Table 1. Light scattering data for 18-membered ring crown amphiphiles

Compound[a]	Additive	Size (nm)[b]
18_2DGA-⟨N18⟩ (**2**, 3 mM)	None	171±64
18_2DGA-⟨N18⟩ (**2**, 1 mM)	10 equiv. NaCl	1010±broad
18_2DGA-⟨N18⟩ (**2**, 1 mM)	10 equiv. KCl	5370±broad
18_2DGA-⟨N18N⟩bzl (**3**, 3 mM)	None	172±broad
18_2DGA-⟨N18N⟩bzl (**3**, 1 mM)	10 equiv. NaCl	207±broad
18_2DGA-⟨N18N⟩bzl (**3**, 1 mM)	10 equiv. KCl	391±broad
18_2DGA-⟨N18N⟩-$DGA18_2$ (**4**, 2 mM)	None	244±58
18_2DGA-⟨N18N⟩-$DGA18_2$ (**4**, 1 mM)	10 equiv. NaCl	304±91
18_2DGA-⟨N18N⟩-$DGA18_2$ (**4**, 1 mM)	10 equiv. KCl	1200±broad
C_{12}⟨N18N⟩C_{12} (**6**, 1 mM)	None	200±41
C_{12}⟨N18N⟩C_{12} (**6**, 1 mM)	10 equiv. NaCl	181±60
C_{12}⟨N18N⟩C_{12} (**6**, 1 mM)	10 equiv. KCl	188±58

[a] Aza-18-crown-6 is represented by ⟨N18⟩ and diaza-18-crown-6 by ⟨N18N⟩ according to a previously published shorthand.[34]

[b] The particle size and range are calculated by instrument software and is reported as broad rather than numerically when that is a more apt description.

First, we note that the previously reported studies were conducted in water.[13] Compound **6** formed vesicles in aqueous suspension when sonicated using a tip sonicator. The vesicles were approximately 300 nm (2970±710 Å) in diameter, stable, and insensitive to addition of K^+ (as KCl). Table 1 shows that under the conditions of the present study, vesicles of about 200 nm diameter (2000±410 Å) formed from **6** and these were insensitive to 10 equiv. of either NaCl or KCl. Although the recent studies produced aggregates of **6** that were, on average, larger than those previously reported, their experimentally determined size ranges clearly overlap.

Single-anchored 18_2DGA-⟨N18⟩ (**2**) formed aggregates of 171±64 nm in water. These enlarged considerably (to ~1000 nm) when 10 equiv. of NaCl were added to the suspension. Further enlargement to >5000 nm and loss of stability occurred on addition of 10 equiv. of KCl. These results are interesting when compared to those obtained for **5**.[13] When **5** was sonicated in H_2O, aggregates of only about 10 nm were formed. A comparison of **2** and **5**, the two single-armed aza-18-crown-6 derivatives, is instructive. Whereas **5** forms small aggregates that may be either vesicles or micelles, **2** forms robust aggregates nearly 20× larger. No comparison of salt effects is possible since few of the single-armed crowns form stable aggregates.

Twin-anchored **4** forms aggregates that are about 250 nm in diameter. These are relatively insensitive to addition of NaCl but enlarge substantially when KCl is present in the medium. The latter corresponds to the behavior of **7** and **8** in aqueous suspension.[13] In the absence of salt, **7** and **8** form aggregates reported by light scattering to be 297±71 and 220 nm, respectively. The experimental range in the latter case was reported by the instrument's internal software as 'broad' suggesting that the range of particles was even larger than the typical extent of ±25 to ±33%. There was essentially no change in aggregate size when 1 mM KCl was added to suspensions of **7** and **8**. The corresponding data for **4** are 244±58 nm in the absence of salt and 304±91 nm when 10 equiv. of NaCl were added. Addition of 10 equiv. of KCl gave aggregates that were 1200(±broad) nm or 12000 Å in diameter.

Compound **3** is a diaza-18-crown-6 derivative but it has a single 18_2DGA anchoring unit. A benzyl group protects the 'non-anchored' arm. Amphiphile **3** forms a broad distribution of aggregates having an average value of 172 nm. Addition of 10 equiv. of NaCl enlarges the average size to 207 nm. The latter value is well within the size distribution for the salt-free aggregate suspension. Addition of 10 equiv. of KCl approximately doubles the average aggregate size to 391 nm. This is by far the smallest KCl effect observed for **2**–**4**.

Although rigorous theory concerning the physical chemistry of aggregate formation is not yet available, it is recognized that the relationship between headgroup and anchor size is influential.[35] The hydrocarbon tails of **5**–**8** are far smaller than the dialkyl-DGA anchors of **1**–**4**. Although the headgroups for **2**–**8** are all 18-membered rings, **2**, **3**, and **5** have only one hydrophobic anchor. Compounds **3**, **4**, and **6**–**8** all possess diaza-18-crown-6 headgroups, but **3** has a

single anchor whereas the others have two. We have previously inferred from data obtained for aza- and diazacrown amphiphiles that headgroup association, possibly through a hydrogen-bonding network, influenced aggregate size and stability in alkylazacrown and dialkyldiazacrown derivatives.[13] Steroidal sidechains are much larger than alkyl chains of corresponding length. When the former are present on azacrowns, aggregation behavior different from those possessing alkyl sidearms is observed.[36] Thus, different aggregate sizes and, presumably, stabilities are anticipated when the sidechains differ in number, structure, and length.

Two of the variables that we believe play a role in the systems described here are the presence of two, rather than one, nitrogens in the macroring and the size of the sidechain. A third issue is that when the DGA residue is attached to nitrogen, the latter becomes non-basic. On the other hand, the non-basic nitrogen is adjacent to an amide group that is a good donor for cations. We surmise that the larger anchors prevent a close association of macroring headgroups. This, and the presence of amide donors, makes alkyl-DGA-crown-amphiphiles more susceptible to interactions with cations.

3. Conclusion

Previous studies have shown that tris(crown) hydraphiles insert in bilayer membranes and transport sodium at rates competitive with those of natural channels.[17] Chloride-selective transport in bilayers was previously achieved by using a heptapeptide having the sequence Gly-Gly-Gly-Pro-Gly-Gly-Gly and anchored by octadecylamine–diglycolic acid unit.[30] When the heptapeptide (that presumably functions as a headgroup, entry portal, and selectivity filter in chloride transport) was replaced by a crown ether, little or no sodium transport was observed. The question of membrane insertion was therefore addressed by assessing the ability of anchored macrocycles to form stable liposomes. Novel amphiphiles **2**–**4** did so but showed a surprising sensitivity to the presence of salts.

4. Experimental

4.1. General

All reaction solvents were freshly distilled and reactions were conducted under N_2 unless otherwise specified. Et_3N was distilled from KOH and stored over KOH. CH_2Cl_2 was distilled from CaH_2. Column chromatography was performed on silica gel 60 (230–400 mesh). Thin layer chromatography was performed with silica gel 60 F254 plates with visualization by UV light (254 nm) and/or by phosphomolybdic acid (PMA) spray. Starting materials were purchased from Aldrich Chemical Co, and used as received unless otherwise indicated. ^{1}H NMR spectra were recorded at 300 MHz and are reported in the following manner: chemical shifts are reported in ppm down field from internal tetramethylsilane (integrated intensity, multiplicity (b=broad; s=singlet; d=doublet; t=triplet; m=multiplet, bs=broad singlet, etc., coupling constants in Hertz, assignment). ^{13}C NMR spectra were obtained at 75 MHz and referenced to $CDCl_3$ (δ 77.0). Melting points were determined on a Thomas Hoover apparatus in open capillaries and were uncorrected.

4.1.1. *N,N′*-Didodecyldiaza-18-crown-6. The title compound was prepared according to the previously published procedure.[13]

4.1.2. 18_2DGA-aza-18-crown-6 (2). Aza-18-crown-6 (0.13 g, 0.49 mmol) was dissolved in anhydrous dichloromethane (35 mL). 1-Benzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyCloP, 0.31 g, 0.74 mmol), 18_2DGA-OH (0.47 g, 0.74 mmol), and diisopropylethylamine (DIEA, 0.21 g, 1.62 mmol) were added. The mixture was stirred at room temperature for 4 days. The solvent was evaporated and the residue purified by column chromatography (silica gel, $CHCl_3$–MeOH 95:5) to yield **2** as a deliquescent white solid (0.35 g, 81%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (6H, t, J=6.9 Hz, CH_3), 1.23 (60H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.49 (4H, bs, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.15 (2H, t, J=7.5 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.25 (2H, t, J=7.5 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.63 (24H, m, crown OCH_2 and NCH_2). 4.26 (2H, s, $COCH_2O$), 4.37 (2H, s, $COCH_2O$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.9, 22.5, 26.7, 26.9, 27.5, 28.8, 29.2, 29.3, 29.4, 29.5, 31.8, 45.7, 46.6, 46.8, 48.1, 69.1, 69.2, 69.5, 69.7, 70.3, 70.47, 70.5, 70.56, 70.6, 70.7, 71.0, 168.5, 169.6. Anal. calcd for $C_{52}H_{102}N_2O_8$: C, 70.70; H, 11.64; N, 3.17%. Found: C, 70.46; H, 11.75; N, 3.20%.

4.1.3. 18_2DGA-⟨diaza-18-crown-6⟩-CH_2Ph (3). *N*-Benzyl-4,13-diaza-18-crown-6 (0.30 g, 0.85 mmol) was dissolved in anhydrous CH_2Cl_2 (35 mL) to which were added 1-benzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyCloP, 0.54 g, 1.28 mmol), 18_2DGA-OH (0.82 g, 1.28 mmol) and diisopropylethylamine (DIEA, 0.36 g, 2.78 mmol). The mixture was stirred at room temperature for 48 h, the solvent was removed, and the product purified by column chromatography (silica gel, $CHCl_3$/CH_3OH 95:5→90:10) to give **3** as a light yellow oil (0.35 g, 46%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (6H, t, J=6.9 Hz, CH_3), 1.23 (60H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.50 (4H, bs, $CH_3(CH_2)_{15}CH_2CH_2N$), 2.78 (4H, m, $BzNCH_2CH_2O$), 3.15 (2H, t, J=7.5 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.26 (2H, t, J=7.5 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.57 (16H, m, crown OCH_2), 3.67 (6H, m, CH_2Ph and $C(O)NCH_2CH_2O$), 4.26 (2H, s, $COCH_2O$), 4.34 (2H, s, $COCH_2O$), 7.26 (5H, m, Ph). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3, 22.8, 27.0, 27.2, 27.8, 29.1, 29.5, 29.8, 32.1, 46.0, 47.1, 47.2, 48.3, 53.9, 54.0, 60.2, 69.4, 69.5, 69.9, 70.2, 70.3, 70.4, 70.6, 70.9, 71.0, 127.0, 128.3, 128.9, 139.7, 168.5, 169.5. Anal. calcd for $C_{59}H_{109}N_3O_7$: C, 72.87; H, 11.30; N, 4.32%. Found: C, 72.69; H, 11.41; N, 4.30%.

4.1.4. 18_2DGA-⟨diaza-18-crown-6⟩-DGA18_2 (4). A solution of diaza-18-crown-6 (0.15 g, 0.57 mmol), 18_2DGA-OH (1.10 g, 1.72 mmol) and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.33 g, 1.73 mmol) and triethylamine (0.5 mL) in $CHCl_3$ (25 mL) was stirred at room temperature. After 24 h the solvent was evaporated and the residue purified by column chromatography (silica gel, $CHCl_3$–MeOH 95:5) yielding

the desired product (0.38 g, 44%) as a white solid, mp 56–57°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (12H, t, J=6.9 Hz, CH_3), 1.20 (120H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.49 (8H, bm, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.13 (4H, t, J=8.1 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.25 (4H, t, J=7.8 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.55 and 3.61 (24H, 2bs, crown OCH_2 and NCH_2). 4.25 (4H, s, $COCH_2O$), 4.32 (4H, s, $COCH_2O$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.2, 22.8, 27.0, 27.2, 27.7, 29.1, 29.5, 29.8, 32.1, 46.0, 47.1, 47.3, 48.3, 69.3, 69.6, 69.7, 69.7, 69.8, 70.3, 70.4, 70.6, 70.8, 70.9, 168.4, 169.4. Anal. calcd for $C_{92}H_{180}N_4O_{10}$: C, 73.55; H, 12.08; N, 3.73. Found: C, 73.19; H, 12.20; N, 3.85%.

Compounds **5**–**8** were obtained as previously described.[13]

4.2. Vesicle preparation

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (monosodium salt) (DOP) were obtained from Avanti Polar Lipids as chloroform solutions. Dry lipid films of DOPC–DOP (20 mg, 7:3) were dissolved in diethyl ether (0.5 mL) and 0.5 mL of an aqueous phase (2.5 M NaCl, 100 mM HEPES, pH 7.0) was added. The mixture was sonicated for 60 s to give an opalescent dispersion, after which the organic solvent was removed under reduced pressure. The suspension was filtered through a 200 nm filter membrane (5×) using a mini extruder and passed through a Sephadex G25 column, which had been previously equilibrated by a choline buffer (2.5 M choline chloride, 100 mM HEPES, pH 7.0). The vesicles collected were subsequently characterized using laser light scattering and their diameter was generally found to be 300–400 nm.

4.3. Ion transport experiments

Compounds **2**–**4** were dissolved in 2,2,2-trifluoroethanol to give concentrations between 20 and 50 mM. The sodium-specific electrode was equilibrated in 6 mL of the choline buffer solution. The vesicle solution was added to the stirred buffer solution containing up to 50 μL compound **2**–**4**, and each assay contained 0.1 mM lipid as vesicles. Complete release was obtained by addition of a detergent solution (2% Triton X100). The data were collected at 1 Hz using Axoscope 7.0 and Digidata 1200 digitizer (Axon Instruments) and they were analyzed using Origin 6.1 (OriginLab Corporation).

4.4. Solid hydration procedure

The amphiphile (20–40 mg) was dissolved in CH_2Cl_2 (1 mL), and 1–3 μmol were placed in test-tubes. High vacuum was applied until the amphiphile dried to a thin film (~2 h). Deionized water (1 mL, pH 6.80) was added to obtain an amphiphile concentration of 1–3 mM, and the effect of the presence of 10 equiv. of NaCl or KCl was also investigated. The suspension was then sonicated (20–30°C, 15 min) and the milky solution was filtered (1000 nm nucleopore filter membrane). Aggregates were characterized by light scattering as shown in Table 1. Sonicated aggregate suspensions were diluted to 25–50 nM (H_2O) prior to light-scattering experiments.

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