Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 5077

www.rsc.org/obc

PAPER

Hydrogen bond-assisted macrocyclic oligocholate transporters in lipid membranes†

Lakmini Widanapathirana, Xueshu Li and Yan Zhao*

Received 10th February 2012, Accepted 30th April 2012
DOI: 10.1039/c2ob25301a

Three macrocyclic oligocholates containing a carboxyl group, a guanidinium ion, and a Cbz-protected amine, respectively, were studied as membrane transporters for hydrophilic molecules. To permeate glucose across lipid bilayers, the macrocycles stacked over one another to form a transmembrane nanopore, driven by a strong tendency of the water molecules in the internal cavities of the amphiphilic macrocycles to aggregate in a nonpolar environment. To transport larger guests such as carboxyfluorescein (CF), the macrocycles acted as carriers to shuttle the guest across the membrane. Hydrogen-bonds between the side chains of the macrocycles strongly affected the transport properties. Surprisingly, the carboxyl group turned out to be far more effective at assisting the aggregation of the oligocholate macrocycles in the membrane than the much stronger carboxylate–guanidinium salt bridge, likely due to competition from the phosphate groups of the lipids for the guanidinium.

Introduction

Controlled passage of molecules and ions through protein-based pores and channels is one of the main methods for cells to regulate the traffic across their membranes. The process, taking place both on the plasma membrane that separates the cell from its environment and on the membranes of many organelles within the cell, is vital to many biofunctions.[1] Although developing a detailed understanding of membrane transport is essential to biology, structural characterization of transport proteins is difficult. The challenge comes not only from the difficulty in crystallizing membrane proteins; many pore-formation mechanisms operate with certain lipid compositions and/or in the presence of other membrane proteins. Static characterization techniques under idealized experimental conditions could easily miss the working structures that exist under the biological settings.

Chemists can contribute to this effort by synthesizing simpler and yet functional transmembrane channels and pores.[2] Synthetic transmembrane pores with an inner diameter of 1 nm or larger, in particular, have attracted much attention in recent years.[3] Knowledge gained through such studies can help us understand biological pore formation, as similar covalent and noncovalent forces are often involved in both types of nanopores. More importantly, synthetic pore-forming materials may have a number of practical applications including sensing,[4] drug delivery,[2] DNA sequencing,[5] and catalysis.[6]

Unlike ion channels constructed frequently from crown ethers and other open chain, flexible structures,[2,7] pore-forming materials need to have significant rigidity to withstand the external membrane pressure to keep the internal pore from collapsing.[8] Despite the significant effort devoted to synthetic nanopores, limited designs are available currently. One of the earliest such examples was reported by Ghadiri, who assembled cyclic D/L-peptides into peptide nanotubes.[9] Matile and coworkers developed an extremely versatile class of β-barrel pores from oligo(phenylene) derivatives[6,10] and applied them to artificial photosynthesis[11] and catalysis.[6] Other reported examples include the porphyrin-based nanopores by Satake and Kobuke,[12] the π-stacked aromatic heterocycles by Gong *et al.*,[13] Fyles's metal-coordinated nanopores,[14] and the guanosine quartet-based giant ion channels by Davis *et al.*[15]

Two of the most prevalent interactions in synthetic nanopores are hydrogen bonds[3] and metal–ligand complexation.[12,14] We recently reported amphiphilic macrocyclic oligocholates (**1–3**) that formed nanopores through *hydrophobic interactions*, a noncovalent force normally expected to operate in water instead of in a hydrophobic environment.[16] Being overall hydrophobic, these macrocycles prefer lipid membranes instead of water. Once entering the membrane, however, they need to solvate their introverted hydrophilic groups by water instead of the lipid hydrocarbon. The dilemma is solved when the macrocycles stack over one another to form a transmembrane pore, enabling the water molecules in the interior to interact with one another, solvate the polar groups of the cholates, and still be able to

Department of Chemistry, Iowa State University, Ames, Iowa 50011-3111, USA. E-mail: zhaoy@iastate.edu; Fax: +1-515-294-0105; Tel: +1-515-294-5845

†Electronic supplementary information (ESI) available: Experimental procedures for the liposome assays and ^{1}H NMR spectra of key compounds. See DOI: 10.1039/c2ob25301a

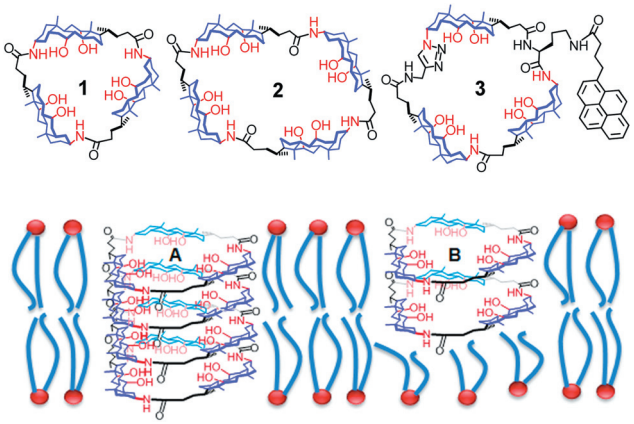


Fig. 1 Schematic representation of two ways of aggregation for oligocholate **1** in a lipid bilayer membrane.

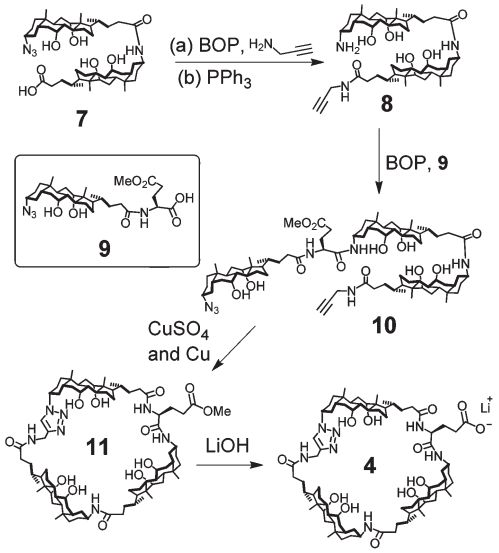
exchange with the bulk water (Fig. 1). The driving force for the stacking is essentially the hydrophobic interactions among the internal, "activated" water molecules that prefer to aggregate instead of facing the lipid hydrocarbon. The exchange of these water molecules with the bulk water could also be important, as the entropic cost for trapping a single water molecule can be as high as 2 kcal mol^{-1} under certain conditions.[17]

To regulate the traffic across the membrane, the nanopore should be tunable, preferably through noncovalent interactions. This paper reports our initial effort toward this goal and aims to control the pore formation through hydrogen-bonds introduced on the side chains of several functionalized cholate macrocycles. A surprising discovery was that the carboxyl group was far more effective than the carboxylate–guanidinium salt bridge in the lipid membrane—the latter is known to have exceptional strength in typical nonpolar environments.[18] The trend was also maintained whether the functionalized oligocholate macrocycles operated through the pore-forming or carrier-based mechanism.

Results and discussion

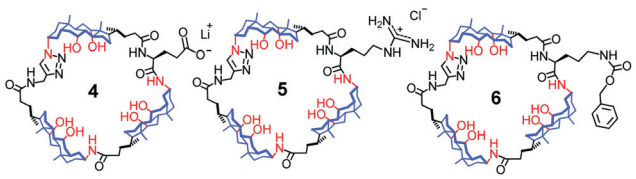
Our previous study revealed that it took four molecules of the cholate macrocycles to form the transmembrane pore in POPC/POPG bilayers (Fig. 1, **A**).[16a] The molecularity results from the matching between the hydrophobic thickness of the membrane and the stacked macrocycle. Other evidence for the hydrophobically driven pore formation includes the correlation between the rigidity of the macrocycle and the transport of glucose, the inactivity of the linear tricholate, the unusual increase in the transport rate of glucose with an *increase* of membrane hydrophobicity, and the counterintuitive faster translocation of maltotriose over glucose. Moreover, when an analogous "clicked" tricholate (**3**) was incorporated into lipid bilayers, the formation of pyrene excimer scaled with the thickness and hydrophobicity of the membrane, providing spectroscopic evidence for the pore formation.

In this paper, we synthesized oligocholates **4**–**6**, which are identical to **3** in the macrocyclic structure but different in the side chain. Two considerations went into the design of the molecules. First, the iterative synthesis of our oligocholates always



Scheme 1 Synthesis of macrocycle **4**.

leaves behind an azide and an ester at the two chain ends, respectively.[19] The most efficient way to synthesize an oligocholate macrocycle, therefore, is to hydrolyze the ester, couple it to an alkyne-terminated amine (*e.g.*, propargyl amine), and cyclize through the highly efficient click reaction.[20] Second, since the pore formation of the macrocycles occurs in a nonpolar environment, other noncovalent forces such as hydrogen-bonds should have sufficient strength to be useful. If the pore formation can be tuned by noncovalent interactions introduced through the side chain of the macrocycle, we should have a rational way to control the traffic across the lipid membrane.



The carboxylate–guanidinium salt bridge is strong in most nonpolar environments[18] and has been reported to work well at the lipid–water interface.[21] Fortunately, the amide linkage in the oligocholates makes it easy to introduce the acid and guanidinium groups by L-ornithine and L-arginine, respectively. Compound **5** was synthesized by deprotecting the Cbz group of **6** and guanidinating the resulting amine derivative with 1-*H*-pyrazole-1-carboxamidine hydrochloride. As shown in Scheme 1, the carboxylated macrocycle (**4**) was synthesized from dimer acid **7**, which was converted to the azide–alkyne-terminated dimer **8** according to a previously reported procedure.[22] The azido group of **8** was reduced by triphenylphosphine. The resulting amine was coupled to glutamic acid-functionalized cholate **9**[23] to afford trimer **10**, followed by the click cyclization and basic hydrolysis to afford **4**.

Tricholate **1** has a triangularly shaped internal cavity approximately 1 nm on the side, large enough for glucose to pass through.[16a] To understand the transport abilities of the functionalized macrocycles, we employed the glucose leakage assay.

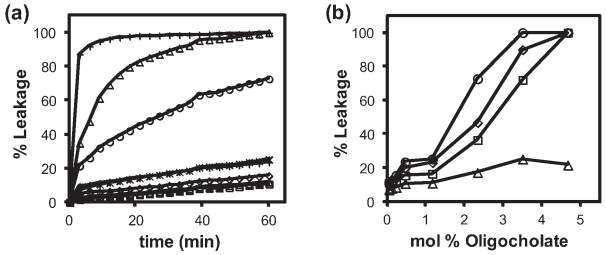


Fig. 2 (a) Percent leakage of glucose from POPC/POPG LUVs upon the addition of different concentrations of **4**. The concentrations of the oligocholate added were 0, 0.125, 0.25, 0.5, 1.25, 2.5, 3.75, and 5 μM from bottom to top. The concentration of glucose was 300 mM within the LUVs. (b) Percent leakage of glucose at 60 min from POPC/POPG LUVs as a function of oligocholate concentration for **4** (○), **5** (□), **6** (△), and a 1 : 1 mixture of **4** and **5** (◊) at ambient temperature. [phospholipids] = 107 μM. These leakage experiments were typically done in duplicate, with the error of the two <10%.

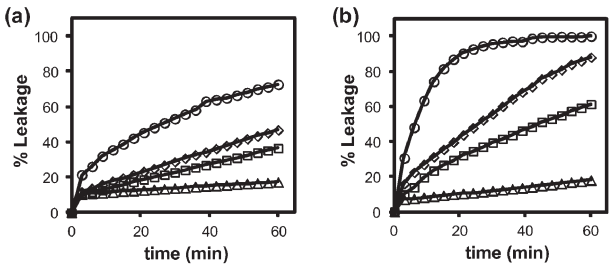


Fig. 3 Percent leakage of glucose from (a) POPC/POPG LUVs and (b) POPC/POPG LUVs with 30% cholesterol upon the addition of **4** (○), **5** (□), **6** (△), and a 1 : 1 mixture of **4** and **5** (◊). [Oligocholate] = 2.5 μM. [phospholipids] = 104 μM. The liposomes were lysed at 60 min upon addition of 1% Triton X-100.

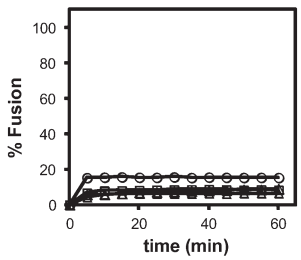


Fig. 4 Percent fusion of LUVs as a function of time for **4** (○), **5** (□), and **6** (△). The data points are connected to guide the eye. [Oligocholate] = 2.5 μM. [phospholipids] = 54 μM.

Typically, a high concentration (300 mM) of glucose was first trapped inside large unilamellar vesicles (LUVs) prepared by the extrusion method.[24] The liposomes were formulated with a neutral lipid (POPC) and an anionic one (POPG)—the latter was added mainly to enhance the colloidal stability of liposomes. After the external glucose was removed by gel filtration, hexokinase, glucose-6-phosphate dehydrogenase, NADP, and ATP were added to the liposomal solution. In the absence of transporting agents, glucose stays inside the LUVs and remains intact. If an added reagent causes leakage of the liposomes, the escaped glucose will be converted by the enzymes to glucose-6-phosphate while NADP reduced to NADPH. Because of the fast enzymatic kinetics, the formation of NADPH at 340 nm normally correlates directly with the rate of glucose efflux.[9a] At the end of the experiments, a nonionic surfactant, Triton X-100, is added to destroy the liposomes and the amount of NADPH formed is used as the reference for 100% leakage.

Fig. 2a shows the percent leakage of glucose triggered by the carboxylated tricholate (**4**) from the LUVs over a period of 60 min. The leakage increased with an increasing concentration of the macrocycle. Fig. 2b compares the induced glucose leakage of the three newly synthesized macrocycles as a function of their concentrations in the membrane.[25]

Cholesterol is known to increase the hydrophobic thickness[26] of POPC bilayer and decrease its fluidity.[27] Cholesterol-containing bilayers have been shown to be much less permeable to hydrophilic molecules, including glucose.[28] A highly unusual observation in the oligocholate-induced glucose leakage was the *faster* leakage in *more hydrophobic* membranes. Although contrary to conventional thinking, the result is fully expected from the hydrophobically driven pore-forming mechanism. As the membrane becomes more hydrophobic, the (hydrophobic) driving force for the pore formation increases, making guests pass through the membrane more easily.[16a] Fig. 3 compares the glucose leakage induced by **4**–**6** from POPC/POPG LUVs with and without 30 mol% cholesterol in the membrane. A very notable *increase* of glucose leakage was observed for all the active transporters (*i.e.*, **4**, **5**, and the **4**/**5** mixture). The results were opposite to what is expected from other transport mechanisms such as carrier-based transport. In a recent work of ours, the same level of cholesterol *decreased* the activity of carrier-based oligocholate foldamer transporters.[29]

We also performed a lipid-mixing assay to confirm the integrity of the lipid bilayers. In this assay, a batch of unlabelled LUVs is mixed with another batch labeled with 1 mol% NBD- and rhodamine-functionalized lipids. Any processes that destroys the membranes (*e.g.*, disintegration of the lipid bilayer) or causes the liposomes to fuse or aggregate will change the fluorescence resonance energy transfer (FRET) between the fluorescent labels.[30] As shown in Fig. 4, even at 5 mol%, a concentration that caused complete leakage of glucose, all the macrocycles displayed <15% mixing of the lipids, indicating that none of the above-mentioned membrane-disrupting processes were significant under our experimental conditions.

Although all the leakage data obtained so far were fully consistent with the hydrophobically driven pore-forming mechanism, several trends were quite unexpected. The Cbz-protected macrocycle (**6**), for example, was almost completely incompetent (Fig. 2b and 3), at least within the 5 mol% tested concentrations.[31] The result was somewhat surprising to us because **6** was very similar to the pyrene-labeled macrocycle **3**, which aggregated in lipid membranes by the hydrophobic mechanism.[16a,32]

Because the parent tricholate macrocycle (**1**) was very potent,[16a] the low activity of **6** should *not* be caused by the lack of a charged functionality. There are two possible reasons for the incompetency of this transporter. First, the macrocycle is considerably more flexible in comparison to the parent macrocycle **1**. Both the ornithine and the triazole moiety in the clicked

structure introduced rotatable bonds. Since the pore formation relies on the "reverse micelle-like", amphiphilic configuration of the cholate macrocycle, it is important that the hydrophilic groups of the macrocycle point inward to create the hydrophilic microenvironment in the center of the molecule. For the parent macrocycle (**1**), the hydrophilic groups are forced to turn inward by the curvature of the cholate backbone, caused by the *cis*-fused AB rings. As more rotatable bonds are present in the macrocycle and its size gets larger, it is easier for the polar groups of **6** to rotate outward. The less preorganized the macrocycle is for the "reverse micelle-like" conformation, the more difficult it is for the hydrophobically driven pore formation to operate.[16a] Second, the Cbz-side chain introduces a carbamate group. If the hydrogen-bonding needs of the carbamate are not properly satisfied in the stacked nanopore, the group may prefer to stay near the surface of the membrane and thus hinder the formation of the transmembrane pore.

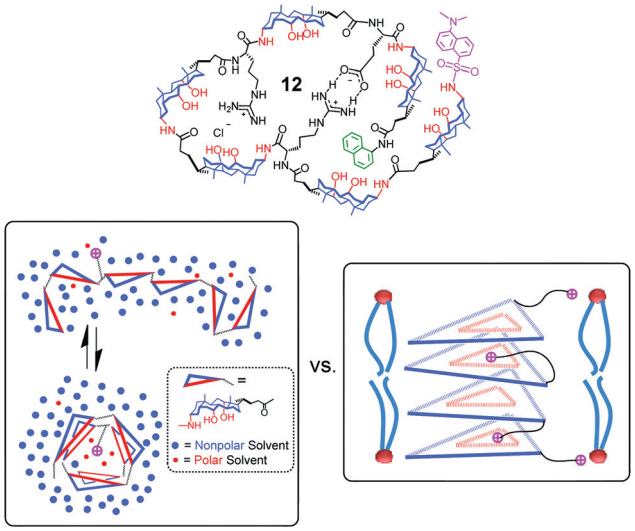
(It should be pointed out that the low activity of **6** was not a problem in the current study. The compound simply provided a reference point for the clicked macrocyclic structure in this study.)

All the leakage data indicated that the guanidinated compound (**5**) was much more active than the nonionic **6**. The result might be perplexing, as placing a charged group in a nonpolar environment is unfavorable.[33] For the pore-forming mechanism, the result is particularly disconcerting because it seems very unreasonable to stack multiple charged macrocycles in a nonpolar environment.

The guanidinium group is an unusual functionality. Although highly polar, it can form strong hydrogen-bonded salt bridges with anions such as carboxylate and phosphate.[18] Once hydrogen-bonded with a lipophilic anion, the guanidinium groups is known to migrate easily into nonpolar solvents and lipid membranes.[34] Our liposomes are made of phospholipids and have net negative charges due to the anionic POPG lipid. The guanidinated macrocycle should be electrostatically attracted to the liposomes. It is possible that, once neutralized by the phosphate headgroup of POPG, the guanidinated macrocycle could stack fairly easily inside the membrane.

In a previous work of ours, a carboxylate–guanidinium-bridged oligocholate foldamer (**12**) with an extra, non-engaging guanidinium side chain was found to fold particularly well in nonpolar solvents containing a small amount of a polar solvent.[35] The effect was attributed to the solvation of the guanidinium group. Essentially, the guanidinium group needs to be solvated by the polar solvent (the minor ingredient in the solvent mixture), regardless of the conformational state of the molecule. The folded helix has an internal hydrophilic cavity filled disproportionately with the polar solvent. Because the folded conformer can satisfy the solvation need of the guanidinium group better than the unfolded conformer, the solvation of the guanidinium indirectly favors the former (Scheme 2, left panel).

Our oligocholate macrocycles were inspired by the folded oligocholate conformers. In fact, the folding of the oligocholate foldamers and the stacking of the cholate macrocycles are driven by exactly the same solvophobic force.[16a] Thus, when the guanidinium group is placed inside the polar solvent-filled hydrophilic cavity, the same "self-solvation" that helped the folding of



Scheme 2 Enhanced folding of **12** due to the solvation of the guanidinium group and possible stacking of guanidinium-containing macrocycle **5** in a lipid bilayer.

12 should facilitate the stacking of **5** in the lipid membrane. It should be mentioned that, in this model, not all four molecules of **5** need to place their guanidinium groups in the stacked nanopore. Instead, only the middle two macrocycles have to do so and the two macrocycles near the membrane surface could form salt bridges with the phosphate headgroups (Scheme 2, right panel). Such an arrangement not only avoids lining up four positive charges inside the nanopore but also anchors the two peripheral macrocycles at the membrane–water interface.

Both of the above proposed models for the higher activity of **5** over **6** deal with the "solvation" needs of the guanidinium group in the nonpolar membrane. The difference between the two is how the guanidinium is stabilized in a nonpolar membrane, whether by salt-bridging with the phosphate group or by insertion into the hydrophilic cavity of the macrocycle. It is possible that both mechanisms could be operating simultaneously, depending on where the macrocycle is located in the hydrophobic core of the membrane or near the surface where phosphate groups exist in abundance.

The overall transport activity follows the order of **4** > 1 : 1 **4**/**5** mixture > **5** over a broad range of concentrations (Fig. 2b). Hence, there was no benefit in having the carboxylated and guanidinated macrocycles in the same membrane.[25] The intermediate activity of the **4**/**5** mixture suggests that the two macrocycles were probably operating independently—a very surprising result given the strength of the salt guanidinium–carboxylate salt bridge in nonpolar media including at the membrane–water interface.[18]

The most likely reason for the noninvolvement of the carboxylate–guanidinium salt bridge is the competition from the phosphate. Both carboxylate and phosphate can form strong salt bridges with guanidinium.[18] In order for **5** to engage in the salt bridge with **4**, it has to do so selectively in the presence of a large excess of phosphate groups on the membrane surface. Unless there are special reasons for **4** and **5** to interact with each other, such selectivity would be difficult.

Another unexpected result—most surprising to us—was the consistently high activity of the carboxylated macrocycle **4**. Over a broad range of concentrations (Fig. 2b) and in the presence or absence of cholesterol (Fig. 3), this compound outperformed other clicked macrocycles in the glucose transport. Provided that the glucose leakage mainly occurs through the nanopore formation of the oligocholates, the carboxyl side chain must have provided special benefit to the stacked nanopore.

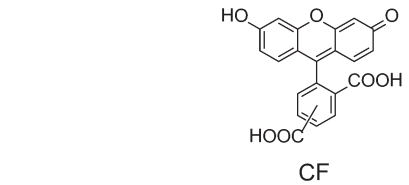
Our rationale for the effectiveness of **4** involves the hydrogen-bonded dimer formed between the carboxyl side chains. Lipophilic acids such as fatty acids have a pK_a of *ca.* 4 in solution and 7.5 when bound to lipid membrane.[36] Under physiological conditions, therefore, a significant amount of the acid is in the protonated, uncharged form in the membrane. Uncharged fatty acids are known to readily partition into lipid membranes and diffuse across the bilayer. In fact, the flip-flop of fatty acids in common phospholipid bilayers has a half life of less than 10 ms without any protein transporters.[37] Macrocycle **4** is essentially a lipophilic acid with an internal hydrophilic cavity. Since bile acids permeate lipid bilayers in a similar fashion as fatty acids,[38] macrocycle **4** should be able to readily partition into the membrane in the protonated form.

Once getting into the hydrophobic membrane, **4** has two potential interactions to assist its stacking: the activated water molecules in its interior promote the pore formation by the aforementioned hydrophobic interactions, and the hydrogen-bonded carboxyl dimer between the side chains should also be effective. The dimerization constant of carboxylic acids is reported to be 10^3–10^4 M^{-1} in nonpolar solvents such as CCl_4 and heptane, translating to 4–5 kcal mol^{-1} in binding free energy.[39] Once the dimer is formed through the side-chain interactions, it is much easier for two dimers to stack and form the transmembrane pore. Of course, the carboxyl dimerization is not limited to specific pairs of macrocycles, any neighboring pairs could engage in such interactions, helping the transmembrane pore formation.

Changing the size of the permeant is a useful way to probe the transport mechanism. Guests too large to pass through the pore would have to move across the membrane by alternative mechanisms. We thus studied the permeation of carboxyfluorescein (CF) through the POPC/POPG membrane. The fluorescent probe is commonly used in liposome research to study transmembrane movement due to its self-quenching at relatively high concentrations (*e.g.*, 50 mM).[40] Our previous work indicated that the probe was too large to pass through the pore formed by stacked tricholate **1**. Instead, the molecule seemed to move across a membrane as being sandwiched between two cholate macrocycles.[16b]

The functionalized macrocycles (**4**–**6**) were able to permeate CF through POPC/POPG membranes as well.[41] The leakage profile for guanidinated compound (**5**) is shown in Fig. 5a as an example. The other compounds displayed similar profiles.

Because 30% cholesterol increases the hydrophobic thickness[26] of the POPC bilayer from 2.58 to 2.99 nm and decreases its fluidity,[27] carrier-based transport generally slows down upon cholesterol incorporation.[2e] Consistent with the changed transport mechanism, all three oligocholates displayed *lower* CF transport across the cholesterol-containing membranes (Fig. 5b, c). The result was exactly the opposite to what was observed with glucose as the permeant (Fig. 3a,b).



CF

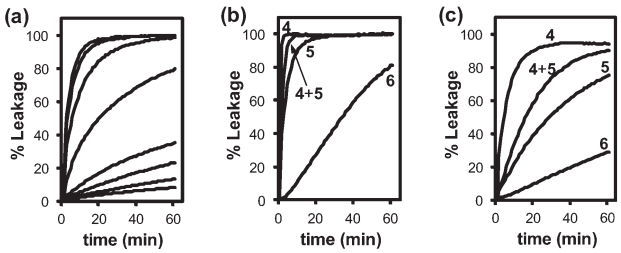


Fig. 5 (a) Percent leakage of CF from POPC/POPG LUVs upon the addition of different concentrations of **5**. The concentrations of the oligocholate added were 0, 0.005, 0.0125, 0.025, 0.05, 0.125, 0.25, 0.375, and 0.5 μM from bottom to top. The concentration of CF was 50 mM within the LUVs. [phospholipids] = 2.9 μM. The liposomes were lysed at 60 min upon addition of 1% Triton X-100. (b,c) Percent leakage of CF from POPC/POPG LUVs (b) and POPC/POPG LUVs with 30% cholesterol (c) upon the addition of **4**, **5**, **6**, and a 1 : 1 mixture of **4** and **5**. [Oligocholate] = 0.25 μM. [phospholipids] = 2.9 μM. The liposomes were lysed at 60 min upon addition of 1% Triton X-100.

Most interestingly, the activity of the functionalized macrocycles followed the same order in the CF transport, *i.e.*, **4** > 1 : 1 **4**/**5** mixture > **5** (Fig. 5b,c). Unlike glucose, CF has two carboxyl groups and should be able to bind to the guanidinated macrocycle (**5**). It is, therefore, quite significant that the carboxylated macrocycle remained as the most active transporter. Quite likely, the carboxyl dimer interaction assisted the dimerization of **5**, making it better able to sandwich CF and shield it while passing through the membrane. It is also possible that the strong guanidinium–phosphate salt bridge formed between **4** and the phosphate groups on the membrane hindered the transmembrane movement of the macrocycle, interfering with the carrier-based transport.

Conclusions

The lipid membrane is a unique environment due to its amphiphilicity, nanodimension, liquid crystallinity, and possible microphase separation of lipids when multiple components exist. Chemists clearly need to recalibrate their thinking when moving their molecules from homogenous solution to the lipid membrane.[29,35] The guanidinium–carboxylate salt bridge finds numerous applications in supramolecular chemistry.[18] In the phospholipid membranes, however, it fails to help the aggregation of the oligocholate macrocycles, whether when the macrocycles engage in transmembrane pore formation or as carriers to shuttle the guest across. The results once again remind us that supramolecular chemistry is a game of competition. Although the carboxylate–guanidinium salt bridge is strong in typical nonpolar environments,[18] the carboxylated macrocycle (**4**) needs to compete with the abundant phosphate groups to interact with the guanidinated compound (**5**). The carboxyl dimer interaction, on the other hand, operates easily in the membrane without major

competitors. The cooperativity between the hydrophobic interactions of the entrapped water molecules in the carboxylated macrocycles and the hydrogen-bonds among side chains makes **4** a particularly effective transporter, whether as a pore-forming molecule or a transmembrane carrier. These results should be useful for the design of additional functionalized transporters in the future.

Experimental

General

The syntheses of **6**,[16a] **7**,[22] **8**,[22] and **9**[23] were reported previously. All reagents and solvents were of ACS-certified grade or higher, and were used as received from commercial suppliers. Millipore water was used to prepare buffers and the liposomes. UV-vis spectra were recorded on a Cary 50 Bio UV-visible spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse Fluorescence spectrophotometer.

Synthesis

Compound 10. Compound **9** (118.6 mg, 0.2 mmol), compound **8** (183.5 mg, 0.22 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 48.6 mg, 0.36 mmol), and benzotrazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 159 mg, 0.36 mmol) were dissolved in anhydrous DMF (5 mL). Diisopropylethylamine (DIPEA, 174 μL, 1 mmol) was added. The reaction mixture was stirred at 40 °C under N_2 overnight and was poured into 1 N HCl (100 mL). The precipitate was collected by suction filtration, washed with water (3 × 10 mL), dried in air, and purified by column chromatography over silica gel using 9 : 1 CH_2Cl_2–MeOH (9 : 1) as the eluent to give an off-white powder (205 mg, 73%). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 4.32 (br, 1H), 4.30–3.92 (m, 5H), 3.83 (s, 3H), 3.69 (s, 3H), 3.53 (br, 2H), 3.17 (br, 1H), 2.40–1.05 (series of m, 80H), 1.02 (d, 9H), 0.94 (s, 9H), 0.71 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 174.6, 174.0, 173.3, 170.6, 79.1, 72.4, 72.4, 72.4, 70.50, 67.51, 67.4, 67.4, 61.0, 52.1, 51.1, 49.6, 49.3, 49.1, 48.8, 46.4, 46.3, 45.9, 41.6, 41.6, 41.3, 41.2, 39.1, 39.0, 35.7, 35.7, 35.4, 35.2, 35.1, 35.0, 34.4, 34.3, 34.3, 34.1, 34.0, 33.0, 32.9, 32.5, 32.5, 32.4, 32.4, 31.7, 31.3, 31.3, 29.8, 29.7, 29.1, 28.1, 27.8, 27.7, 27.3, 27.1, 26.8, 26.2, 26.1, 22.7, 22.9, 21.9, 16.5, 16.5, 16.4, 11.8. HRMS (ESI) (*m*/*z*): $[M + H]^+$ calcd for $C_{81}H_{130}N_7O_{12}$, 1392.9777; found 1392.9772.

Compound 11. A solution of **10** (129 mg, 91.6 μmol) in 2 : 1 THF–MeOH (9 mL) was added *via* a syringe pump to a vigorously stirred solution of $CuSO_4{\cdot}5H_2O$ (0.1 M, 1.83 mL, 183 μmol) and sodium ascorbate (72.5 mg, 366 μmol) in 2 : 1 : 1 THF–MeOH–H_2O (31 mL) at 50 °C under N_2 for 4 h. After the addition was complete, the reaction mixture was stirred for another 4 h at 50 °C. The solvents were removed by rotary evaporation and the residue was purified by column chromatography over silica gel using 8 : 1 CH_2Cl_2–MeOH as the eluent to give an off-white powder (108 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 4.39–4.22 (m, 4H), 4.10 (m, 1H), 3.94–3.90 (m, 3H), 3.80–3.75 (m, 3H), 3.64–3.59 (m, 4H), 3.47 (m, 1H), 2.50–0.73 (series of m, 98H), 0.68–0.65 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 176.2, 174.6, 172.2, 145.3, 121.5, 74.0, 68.7, 68.6, 68.4, 62.3, 53.9, 52.2, 43.0, 42.9, 42.78, 42.7, 40.4, 40.3, 37.6, 37.0, 36.8, 36.7, 36.6, 36.5, 35.6, 35.5, 35.4, 35.3, 35.2, 32.7, 32.6, 31.1, 30.4, 29.2, 29.1, 28.6, 28.4, 27.9, 27.8, 27.6, 27.5, 27.4, 23.9, 23.1, 22.9, 17.7, 13.0. HRMS (ESI) (*m*/*z*): $[M + H]^+$ calcd for $C_{81}H_{130}N_7O_{12}$, 1392.9777; found 1392.9740.

Compound 4. Compound **11** (60 mg, 43 μmol) was dissolved in 1 : 1 THF–MeOH (5 mL). A solution of LiOH (2 M, 0.2 mL, 0.4 mmol) was added and the reaction mixture was stirred until the starting material was consumed. After the solvent was removed under reduced pressure, the residue was purified by column chromatography on silica gel using 4 : 1 CH_2Cl_2–MeOH as the eluent to give a white powder (55 mg, 93%). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 4.53–4.41 (m, 3H), 4.30 (m, 1H), 4.00–3.95 (m, 3H), 3.83 (bs, 3H), 3.52–3.49 (m, 2H), 2.39–0.93 (series m, 95H), 0.74–0.71 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 175.4, 175.3, 174.7, 171.4, 144.4, 120.6, 72.7, 67.8, 67.7, 67.5, 61.4, 53.2, 49.9, 49.4, 46.6, 46.4, 46.3, 46.2, 46.1, 46.0, 45.3, 42.1, 41.8, 41.7, 39.4, 39.3, 36.0, 35.8, 35.7, 35.6, 34.8, 34.7, 34.6, 34.5, 34.4, 34.3, 34.2, 28.1, 27.4, 26.6, 26.5, 23.0, 22.27, 2.2, 22.1, 16.7, 12.0. HRMS (ESI) (*m*/*z*): $[M + H]^+$ calcd for $C_{80}H_{128}N_7O_{12}$, 1378.9621; found 1378.9633.

Compound 5. A mixture of the amino derivative of compound **3**[16a] (15 mg, 0.01 mmol) and 1*H*-pyrazole-1-carboximidamide (18 mg, 0.12 mmol) was dissolved in anhydrous DMF (0.25 mL). DIPEA (80 μL, 0.5 mmol) was added to the above mixture, which was stirred at 50 °C overnight. The reaction was monitored by ^{1}H NMR spectroscopy. When the reaction was complete, the mixture was poured in brine and the precipitate was collected by suction filtration, dissolved in methanol, and precipitated again in acetonitrile to give an off-white powder (11 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ): 7.82 (br, 1H), 4.37 (m, 3H), 3.92 (br, 3H), 3.79 (br, 1H), 3.50 (br, 2H), 3.17 (br, 2H), 2.74 (br, 1H), 2.39–0.76 (series of m), 0.67 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD = 1 : 1, δ) 175.4, 174.9, 172.0, 157.6, 136.8, 128.5, 128.0, 127.5, 72.8, 66.5, 62.7, 53.7, 53.6, 46.8, 46.8, 46.0, 40.2, 35.2, 33.0, 29.0, 26.9, 22.5,17.1, 12.5, 12.4, 12.3. ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{81}H_{134}N_{10}O_{10}$, 1407.0279; found, 1407.0247.

Acknowledgements

We thank NSF (DMR-1005515) for supporting the research.

Notes and references

1 (*a*) A. G. Lee, *Biomembranes: A Multi-Volume Treatise*, JAI Press, Greenwich, Conn., 1995; (*b*) G. Menestrina, M. Dalla Serra and P. Lazarovici, *Pore-Forming Peptides and Protein Toxins*, Taylor & Francis, London, New York, 2003; (*c*) F. G. v. d. Goot, *Pore-Forming Toxins*, Springer, Berlin; New York, NY, 2001; (*d*) L. K. Tamm, *Protein-Lipid Interactions: From Membrane Domains to Cellular Networks*, Wiley-VCH, Weinheim, 2005; (*e*) R. J. C. Gilbert, *Cell. Mol. Life Sci.*, 2002, **59**, 832.
2 (*a*) W. D. Stein, *Carriers and Pumps: An Introduction to Membrane Transport*, Academic Press, San Diego, CA, 1990; (*b*) G. W. Gokel and

I. A. Carasel, *Chem. Soc. Rev.*, 2007, **36**, 378; (*c*) T. M. Fyles, *Chem. Soc. Rev.*, 2007, **36**, 335; (*d*) B. A. McNally, W. M. Leevy and B. D. Smith, *Supramol. Chem.*, 2007, **19**, 29; (*e*) J. T. Davis, O. Okunola and R. Quesada, *Chem. Soc. Rev.*, 2010, **39**, 3843.
3 (*a*) S. Matile, A. Som and N. Sorde, *Tetrahedron*, 2004, **60**, 6405; (*b*) A. L. Sisson, M. R. Shah, S. Bhosale and S. Matile, *Chem. Soc. Rev.*, 2006, **35**, 1269.
4 S. Litvinchuk, N. Sorde and S. Matile, *J. Am. Chem. Soc.*, 2005, **127**, 9316.
5 (*a*) J. J. Kasianowicz, E. Brandin, D. Branton and D. W. Deamer, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 13770; (*b*) M. Akeson, D. Branton, J. J. Kasianowicz, E. Brandin and D. W. Deamer, *Biophys. J.*, 1999, **77**, 3227; (*c*) A. Meller, L. Nivon, E. Brandin, J. Golovchenko and D. Branton, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 1079; (*d*) W. Vercoutere, S. Winters-Hilt, H. Olsen, D. Deamer, D. Haussler and M. Akeson, *Nat. Biotechnol.*, 2001, **19**, 248; (*e*) S. Howorka, S. Cheley and H. Bayley, *Nat. Biotechnol.*, 2001, **19**, 636; (*f*) J. Clarke, H. C. Wu, L. Jayasinghe, A. Patel, S. Reid and H. Bayley, *Nat. Biotechnol.*, 2009, **4**, 265.
6 N. Sakai, N. Sorde and S. Matile, *J. Am. Chem. Soc.*, 2003, **125**, 7776.
7 (*a*) G. W. Gokel and A. Mukhopadhyay, *Chem. Soc. Rev.*, 2001, **30**, 274; (*b*) U. Koert, L. Al-Momani and J. R. Pfeifer, *Synthesis*, 2004, 1129; (*c*) G. W. Gokel and O. Murillo, *Acc. Chem. Res.*, 1996, **29**, 425; (*d*) M. Jung, H. Kim, K. Baek and K. Kim, *Angew. Chem., Int. Ed.*, 2008, **47**, 5755; (*e*) X. Li, B. Shen, X. Q. Yao and D. Yang, *J. Am. Chem. Soc.*, 2009, **131**, 13676.
8 A. Som and S. Matile, *Chem. Biodiversity*, 2005, **2**, 717.
9 (*a*) J. R. Granja and M. R. Ghadiri, *J. Am. Chem. Soc.*, 1994, **116**, 10785; (*b*) J. Sanchez-Quesada, H. S. Kim and M. R. Ghadiri, *Angew. Chem., Int. Ed.*, 2001, **40**, 2503.
10 (*a*) N. Sakai, J. Mareda and S. Matile, *Acc. Chem. Res.*, 2005, **38**, 79; (*b*) G. Das, P. Talukdar and S. Matile, *Science*, 2002, **298**, 1600.
11 S. Bhosale, A. L. Sisson, P. Talukdar, A. Furstenberg, N. Banerji, E. Vauthey, G. Bollot, J. Mareda, C. Roger, F. Wurthner, N. Sakai and S. Matile, *Science*, 2006, **313**, 84.
12 A. Satake, M. Yamamura, M. Oda and Y. Kobuke, *J. Am. Chem. Soc.*, 2008, **130**, 6314.
13 A. J. Helsel, A. L. Brown, K. Yamato, W. Feng, L. H. Yuan, A. J. Clements, S. V. Harding, G. Szabo, Z. F. Shao and B. Gong, *J. Am. Chem. Soc.*, 2008, **130**, 15784.
14 T. M. Fyles and C. C. Tong, *New J. Chem.*, 2007, **31**, 655.
15 L. Ma, M. Melegari, M. Colombini and J. T. Davis, *J. Am. Chem. Soc.*, 2008, **130**, 2938.
16 (*a*) H. Cho, L. Widanapathirana and Y. Zhao, *J. Am. Chem. Soc.*, 2011, **133**, 141; (*b*) H. Cho and Y. Zhao, *Langmuir*, 2011, **27**, 4936.
17 J. D. Dunitz, *Science*, 1994, **264**, 670.
18 (*a*) F. P. Schmidtchen and M. Berger, *Chem. Rev.*, 1997, **97**, 1609; (*b*) M. D. Best, S. L. Tobey and E. V. Anslyn, *Coord. Chem. Rev.*, 2003, **240**, 3.
19 Y. Zhao and Z. Zhong, *J. Am. Chem. Soc.*, 2005, **127**, 17894.
20 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596.
21 K. Ariga and T. Kunitake, *Acc. Chem. Res.*, 1998, **31**, 371.
22 Z. Zhong, X. Li and Y. Zhao, *J. Am. Chem. Soc.*, 2011, **133**, 8862.
23 Z. Zhong and Y. Zhao, *Org. Lett.*, 2007, **9**, 2891.
24 S. C. Kinsky, J. A. Haxby, D. A. Zopf, C. R. Alving and C. B. Kinsky, *Biochemistry*, 1969, **8**, 4149.
25 None of these compounds have any significant water-solubility due to their dominant hydrophobicity. We expect all the compounds to readily enter the lipid membrane instead of staying in water.
26 F. A. Nezil and M. Bloom, *Biophys. J.*, 1992, **61**, 1176.
27 J. C. M. Holthuis, G. van Meer and K. Huitema, *Mol. Membr. Biol.*, 2003, **20**, 231.
28 (*a*) R. A. Demel, K. R. Bruckdor and L. L. van Deene, *Biochim. Biophys. Acta*, 1972, **255**, 321; (*b*) D. Papahadjopoulos, S. Nir and S. Ohki, *Biochim. Biophys. Acta*, 1972, **266**, 561.
29 S. Zhang and Y. Zhao, *Chem.–Eur. J.*, 2011, **17**, 12444.
30 D. K. Struck, D. Hoekstra and R. E. Pagano, *Biochemistry*, 1981, **20**, 4093.
31 The background leakage in these experiments was typically 6–10% over 60 min.
32 Although **6** and **3** both could aggregate in the membranes by the same hydrophobic mechanism, the aggregational states required for the formation of the pyrene excimer and for the glucose leakage could be quite different. Because the two processes operate on a very different time scale and have different sensitivity, an aggregational state sufficient for the pyrene excimer may not allow glucose to permeate the membrane.
33 I. Gitlin, J. D. Carbeck and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 2006, **45**, 3022.
34 N. Sakai and S. Matile, *J. Am. Chem. Soc.*, 2003, **125**, 14348.
35 H. Cho and Y. Zhao, *J. Am. Chem. Soc.*, 2010, **132**, 9890.
36 J. A. Hamilton,in *Carbon-13 NMR Spectroscopy of Biological Systems*, ed. N. Beckmann, Academic Press, San Diego, 1995, p. 117.
37 (*a*) F. Kamp and J. A. Hamilton, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 11367; (*b*) F. Kamp, D. Zakim, F. Zhang, N. Noy and J. A. Hamilton, *Biochemistry*, 1995, **34**, 11928; (*c*) J. A. Hamilton, W. Guo and F. Kamp, *Mol. Cell. Biochem.*, 2002, **239**, 17.
38 F. Kamp and J. A. Hamilton, *Biochemistry*, 1993, **32**, 11074.
39 (*a*) J. Wenograd and R. A. Spurr, *J. Am. Chem. Soc.*, 1957, **79**, 5844; (*b*) D. S. Goodman, *J. Am. Chem. Soc.*, 1958, **80**, 3887.
40 R. R. C. New, *Liposomes: A Practical Approach*, IRL Press, Oxford, 1990.
41 Although the concentrations of the transporters in the CF leakage (Fig. 5) were much lower than those in the glucose leakage assays (Fig. 1), the data do not mean that these compounds were better transporters for the larger CF. Because the UV-based glucose assay had lower sensitivity than the fluorescence-based CF assay, a much higher concentration of LUVs (107 *vs.* 2.9 μM) had to be used in the former. Since the oligocholates were essentially insoluble in water, the effective concentration of the transporter in the membrane would increase with a decrease in the phospholipid concentration.