Translocation of Hydrophilic Molecules across Lipid Bilayers by Salt-Bridged Oligocholates

Hongkwan Cho  
Iowa State University

Yan Zhao  
Iowa State University, zhaoy@iastate.edu

Follow this and additional works at: http://lib.dr.iastate.edu/chem_pubs
Part of the Chemistry Commons

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/chem_pubs/176. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Chemistry at Iowa State University Digital Repository. It has been accepted for inclusion in Chemistry Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Translocation of Hydrophilic Molecules across Lipid Bilayers by Salt-Bridged Oligocholates

Abstract
Macrocyclic oligocholates were found in a previous work (Cho, H.; Widanapathirana, L.; Zhao, Y. J. Am. Chem. Soc. 2011, 133, 141–147) to stack on top of one another in lipid membranes to form nanopores. Pore formation was driven by a strong tendency of the water molecules in the interior of the amphiphilic macrocycles to aggregate in a nonpolar environment. In this work, cholate oligomers terminated with guanidinium and carboxylate groups were found to cause efflux of hydrophilic molecules such as glucose, maltotriose, and carboxyfluorescein (CF) from POPC/POPG liposomes. The cholate trimer outperformed other oligomers in the transport. Lipid-mixing assays and dynamic light scattering ruled out fusion as the cause of leakage. The strong dependence on chain length argues against random intermolecular aggregates as the active transporters. The efflux of glucose triggered by these compounds increased significantly when the bilayers contained 30 mol % cholesterol. Hill analysis suggested that the active transporter consisted of four molecules. The oligocholates were proposed to fold into “noncovalent macrocycles” by the guanidinium–carboxylate salt bridge and stack on top of one another to form similar transmembrane pores as their covalent counterparts.

Disciplines
Chemistry

Comments
Translocation of Hydrophilic Molecules across Lipid Bilayers 
by Salt-Bridged Oligocholates

Hongkwan Cho and Yan Zhao*

Department of Chemistry, Iowa State University, Ames, Iowa 50011-3111, United States

ABSTRACT: Macrocyclic oligocholates were found in a previous work (Cho, H.; Widanapathirana, L.; Zhao, Y. J. Am. Chem. Soc. 2011, 133, 141–147) to stack on top of one another in lipid membranes to form nanopores. Pore formation was driven by a strong tendency of the water molecules in the interior of the amphiphilic macrocycles to aggregate in a nonpolar environment. In this work, cholate oligomers terminated with guanidinium and carboxylate groups were found to cause efflux of hydrophilic molecules such as glucose, maltotriose, and carboxyfluorescein (CF) from POPC/POPG liposomes. The cholate trimer outperformed other oligomers in the transport. Lipid-mixing assays and dynamic light scattering ruled out fusion as the cause of leakage. The strong dependence on chain length argues against random intermolecular aggregates as the active transporters. The efflux of glucose triggered by these compounds increased significantly when the bilayers contained 30 mol % cholesterol. Hill analysis suggested that the active transporter consisted of four molecules. The oligocholates were proposed to fold into “noncovalent macrocycles” by the guanidinium—carboxylate salt bridge and stack on top of one another to form similar transmembrane pores as their covalent counterparts.

INTRODUCTION

Synthetic transmembrane pores with an inner diameter of 1 nm or larger have attracted a great deal of attention in recent years.1−3 Part of the motivation comes from the fact that their biological congeners, membrane-associated pore-forming proteins, are involved in critical functions such as signaling, metabolism, and bacterial or viral infection.3−5 Since it is notoriously difficult to obtain detailed structural information for complex membrane proteins, chemists have sought to construct simpler biomimetic nanopores and study their behavior under more easily controlled conditions. Knowledge generated from such studies not only is useful to the understanding of biological pore formation but also helps create materials with potential applications ranging from single-molecule detection of DNAs and RNAs6−13 to drug delivery.14

Crown ethers and open chain compounds, which worked extremely well for ion channels,16−21 normally are too flexible for nanopore formation. To keep the pore open, the structure must be able to withstand the external membrane pressure when incorporated into a bilayer.22 Despite the significant efforts devoted to synthetic nanopores, limited designs exist currently. An early example was Ghadiri’s cyclic D/L-peptides, which self-assembled into transmembrane pores large enough for glucose and glutamic acid to pass through.23,24 In recent years, the β-barrel pores constructed from oligo(phenylene) derivatives by Matile and co-workers25−27 proved particularly versatile and useful in many applications including sensing26 and catalysis.27 Other examples include the porphyrin-based nanopores by Satake and Kobuke,28 Gong’s T-stacked aromatic heterocycles,29 the metal-coordinated nanopores by Fyles,30 and the guanosine quartet-based giant ion channels by Davis.31 Whereas the majority of the synthetic nanopores in the literature relied on hydrogen bonds and metal−ligand coordination for pore formation, we recently reported amphiphilic macrocyclic oligocholates (1−3) that formed nanopores through hydrophobic interactions, a noncovalent force normally expected to work in water instead of a hydrophobic environment (Figure 1).32

The amphiphilicity and rigidity of the cholate macrocycles are the main reasons for the hydrophobic interactions to operate in bilayer membranes. When included in the lipid bilayer, the oligocholate macrocycles pull water molecules from the aqueous phase into the membrane by their hydrophilic interior. These water molecules are “activated” in the sense that they have a strong tendency to aggregate in a hydrophobic environment. For a nonaggregated macrocycle, the entrapped water molecules have to face hydrocarbon at one or both openings of the macrocycle, depending on whether the macrocycle is located near the surface of the bilayer or deep inside. Aggregation in the z direction eliminates the unfavorable hydrophobic−hydrophilic contact and creates a transmembrane nanopore. Upon pore formation, the water molecules inside the pore are able to solvate the introverted polar groups of the oligocholates and still exchange with the bulk water readily. The latter could be an important reason for the pore formation, as the entropic cost for trapping a single water molecule can be as high as 2 kcal/mol in some cases.33

In this paper, we report the finding that the guanidinium/carboxylate-functionalized oligocholates such as 4 are also able to

Received: February 8, 2011
Revised: March 8, 2011
Published: March 29, 2011
transport hydrophilic molecules across lipid membranes. Transport seems to derive from a similar pore-forming mechanism in which the salt-bridged oligocholates stack on top of one another to allow the hydrophilic guests to pass through. A noted benefit of the “noncovalent macrocycles” is their ability to expand and translocate guests too large to go through the pores formed by the covalent macrocycles.

**EXPERIMENTAL SECTION**

**General.** All reagents and solvents were of ACS-certified grade or higher and used as received from commercial suppliers. Millipore water was used to prepare buffers and liposomes. Routine ¹H and ¹³C NMR spectra were recorded on a Bruker DRX-400 or on a Varian VXR-400 spectrometer. MALDI-TOF mass spectra were recorded on a Thermo-bioanalysis Dynamo mass spectrometer. UV–vis spectra were recorded at ambient temperature on a Cary 100 Bio UV–vis spectrophotometer. Fluorescence spectra were recorded at ambient temperature on a Varian Cary Eclipse fluorescence spectrophotometer. Dynamic light scattering (DLS) was performed on a PD2000DLSPLUS dynamic light scattering detector (see Scheme 1).

The syntheses of compounds 1, 2, 3, 7, 8, 9, 10 were previously reported.

**General Procedure I (Hydrolysis of Methyl Ester-Terminated Oligocholates).** The methyl ester of an oligocholate (0.10 mmol) was dissolved in a mixture of THF (1 mL) and MeOH (1 mL). A solution of 2 M LiOH (0.5 mL, 1 mmol) was added. The reaction was monitored by TLC and complete in 10–24 h. The organic solvents were removed by rotary evaporation. After addition of a dilute HCl solution (0.05 M, 30 mL), the precipitate formed was collected by suction filtration or centrifugation, washed with water, and dried in vacuo. The product was generally used in the next step without further purification.

**General Procedure II (Guanidination of Amine-Terminated Oligocholates).** The hydrolyzed oligocholate (0.10 mmol, prepared according to General Procedure I) and 1-H-pyrazole-1-carboxamidine hydrochloride (0.50 mmol) were dissolved in anhydrous DMF (1.0 mL). N,N-Diisopropylethylamine (DIPEA, 1.0 mmol) was added. After being stirred overnight at room temperature, the reaction mixture was poured into acetonitrile. The precipitate was collected and purified by column chromatography over silica gel.

**Synthesis of Compound 4.** Compound 4 was obtained from 8 by hydrolysis (General Procedure I) and guanidination (General Procedure II). The product was purified by column chromatography over silica gel using CH₂Cl₂/EtOAc/MeOH (1:1:2) as the eluent (86% yield). ¹H NMR (400 MHz in CDCl₃/CD₃OD = 1:1): δ = 3.97 (br, 3H), 3.80 (br, 3H), 3.49 (br, 2H), 3.19 (m, 1H), 2.38–0.82 (series of m, 91H), 0.71 (s, 9H). ¹³C NMR (100 MHz in CDCl₃/CD₃OD = 1:1): δ = 175.6, 157.2, 73.8, 73.7, 68.9, 68.7, 52.8, 50.7, 50.6, 48.0, 47.7, 47.6, 47.3, 43.1, 42.8, 42.7, 40.6, 40.5, 37.1, 36.9, 36.6, 36.4, 35.7, 35.6, 35.4, 34.1, 34.0, 33.4, 33.1, 33.0, 29.2, 28.5, 28.2, 27.5, 24.0, 23.3, 23.2, 17.8, 13.1. MALDI-TOF MS Calcd for C₇₃H₁₂₂N₅O₁₀ [M + Na]⁺: 1251.8. Found: 1252.1. Calcd for C₇₃H₁₂₁N₅NaO₁₀ [M + H]⁺: 1229.8. Found: 1230.4.

**Synthesis of Compound 5.** Compound 5 was obtained from 9 by hydrolysis (General Procedure I) and guanidination (General Procedure II). The product was purified by column chromatography over silica gel using CH₂Cl₂/MeOH (1:2) as the eluent (53% yield). ¹H NMR (400 MHz in CDCl₃/CD₃OD = 1:3): δ = 3.95 (br, 4H), 3.79 (br, 4H), 3.49 (br, 3H), 3.17 (m, 1H), 2.38–0.82 (series of m, 120H), 0.69 (s, 12H). ¹³C NMR (100 MHz in CDCl₃/CD₃OD = 1:3): δ = 183.7, 175.8, 175.7, 175.5, 79.2, 73.9, 73.8, 69.0, 68.8, 53.1, 50.9, 50.9, 50.0, 48.2, 47.9, 47.5, 43.4, 43.1, 43.0, 42.9, 40.8, 37.3, 37.0, 36.8, 36.5, 36.3, 35.8, 35.7, 35.6, 34.3, 34.2, 33.9, 33.4, 33.3, 30.7, 29.5, 29.4, 28.4.
27.8, 24.2, 23.4, 23.3, 17.9, 17.8, 13.2, 13.1. MALDI-TOF MS: m/z calcd for C_{49}H_{80}Ni_{4}O_{13} [M + Na]$: 1641.3. Found: 1642.0. Calcd for C_{50}H_{82}Ni_{4}O_{13} [M + H]: 1619.4. Found: 1620.9.

Synthesis of Compound 6. Compound 6 was obtained from 10 by hydrolysis (General Procedure 1) and guanidination (General Procedure II). The product was purified by column chromatography over silica gel using CH_{2}Cl_{2}/EtOAc/MefOH (1:1:2) as the eluent (86% yield). ^1H NMR (400 MHz in CDCl_{3}/CD_{3}OD = 1:3): $\delta = 3.96$ (br, 2H), 3.79 (br, 2H), 3.49 (br, 1H), 3.16 (m, 1H), 2.38–0.82 (series of m, 60H), 0.69 (s, 6H). ^13C NMR (100 MHz in CDCl_{3}/CD_{3}OD = 1:3): $\delta = 183.6, 175.4, 157.1, 73.8, 68.7, 52.8, 50.5, 48.0, 47.5, 47.3, 43.0, 42.7, 42.6, 40.5, 40.4, 37.0, 36.9, 36.8, 36.5, 36.3, 36.1, 35.6, 35.5, 35.3, 33.6, 32.8, 30.4, 28.1, 27.5, 24.0, 23.9, 23.3, 17.8, 13.1. MALDI-TOF MS: m/z calcd for C_{49}H_{80}Ni_{4}O_{13} [M + H]: 840.2. Found: 839.4.

Liposome Preparation. Glucose-loaded LUVs were prepared according to a slightly modified literature procedure. ^36 A chloroform solution of POPC (25 mg/mL, 198 $\mu$L) and POPG (50 mg/mL, 10.0 $\mu$L) was placed in a 10 mL test tube and dried under a stream of nitrogen. The residue was dried further under high vacuum overnight. A solution of p-(-)-glucose (300 mM) in 50 mM Tris buffer (0.5 mL, pH = 7.5) was added. Reduction of the lipids was allowed to continue for 30 min with occasional vortexing. The opaque dispersion was subjected to 10 freeze–thaw cycles. The resulting mixture was extruded 29 times through a polycarbonate filter (diameter = 19 mm, pore size = 100 nm) at room temperature using an Avanti Mini-Extruder. A portion (0.3 $\mu$L) of the liposome solution was passed through a column of Sephadex G-50 using Tris buffer (50 mM Tris, 150 mM NaCl, pH = 7.5) as the eluent to remove the extravesicular glucose. The liposome fractions were combined and diluted to 5.0 mL with the Tris buffer, with the concentration of phospholipids in the final solution being 0.86 mM.

Lipid-Mixing Assay. Unlabeled POPC/POPG LUVs were prepared with a mixture of POPC (25 mg/mL, 198 $\mu$L) and POPG (50 mg/mL, 10.0 $\mu$L) using HEPES buffer (10 mM HEPES, 107 mM NaCl, pH = 7.0, 95 mL NaCl, 3.5 mM MgCl_{2} and 0.15 mM CaCl_{2}), the enzyme solution (400 $\mu$L, 100 units/mL of $\alpha$-glucosidase in the above HEPES buffer), the enzyme II solution (500 $\mu$L, 10 units/mL of hexokinase/glucose-6-phosphate dehydrogenase and 2 mM ATP dissolved in the above HEPES buffer), and NADP solution (500 $\mu$L, 1 mM dissolved in the above HEPES buffer) were placed in a series of cuvettes. The concentration of phospholipids in each cuvette was 107 $\mu$M. Aliquots of the oligocholate solution (20 $\mu$L) in DMSO were added to different cuvettes via a microsyringe. The amount of DMSO introduced to each sample was $\pm 20$ $\mu$L. The absorbance of NADPH at 340 nm was measured by the same procedure as the glucose leakage assay. After 1 h, the liposomes were lysed by addition of 10 $\mu$L of Triton X-100 (1% v/v) and the absorbance at 340 nm ($A_{max}$) was used to calculate the percent leakage [% leakage = ($A_{0} - A_{e}$)/($A_{max} - A_{e}$) × 100]. $A_{0}$ and $A_{e}$ are the initial and intermediate absorbance, respectively.

Maltotriose Leakage Assay. Maltotriose-loaded LUVs were prepared in the same method mentioned above except 200 mM maltotriose in Milipore water for lipid rehydration and HEPES buffer (50 mM HEPES, 100 mM NaCl, pH = 7.0) for gel filtration were used. Maltotriose released from the liposomes was measured enzymatically by a modified literature procedure. ^36 Aliquots of the above LUV solution (250 $\mu$L), HEPES buffer (350 $\mu$L, 50 mM HEPES, pH = 7.0, 95 mM NaCl, 3.5 mM MgCl_{2} and 0.15 mM CaCl_{2}), the enzyme I solution (400 $\mu$L, 100 units/mL of $\alpha$-glucosidase in the above HEPES buffer), the enzyme II solution (500 $\mu$L, 10 units/mL of hexokinase/glucose-6-phosphate dehydrogenase and 2 mM ATP dissolved in the above HEPES buffer), and NADP solution (500 $\mu$L, 1 mM dissolved in the above HEPES buffer) were washed in a series of cuvettes. The concentration of phospholipids in each cuvette was 107 $\mu$M. Aliquots of the oligocholate solution (20 $\mu$L) in DMSO were added to different cuvettes via a microsyringe. The amount of DMSO introduced to each sample was $\pm 20$ $\mu$L. The absorbance of NADPH at 340 nm was measured by the same procedure as the glucose leakage assay. After 1 h, the liposomes were lysed by addition of 10 $\mu$L of Triton X-100 (1% v/v) and the absorbance at 340 nm ($A_{max}$) was used to calculate the percent leakage as in the glucose leakage assay.

**CF Leakage Measurement.** Preparation of CF-containing LUVs and CF leakage experiment were carried out according to a previously reported procedure. ^38 A solution of CF (0.5 $\mu$L, 50 mM CF, 10 mM HEPES, 10 mM NaCl, pH = 7.4) was used for rehydration of lipid. The liposome solution was subjected to 10 freeze–thaw cycles, followed by extrusion (29 times) through a polycarbonate filter (diameter = 19 mm, pore size = 100 nm). A portion (0.1 $\mu$L) of the liposome solution was passed through a column of Sephadex G-50 using HEPES buffer (10 mM HEPES, 107 mM NaCl, pH = 7.4) as the eluent to remove the extravesicular CF. The liposome fractions were combined and diluted to 10.0 mL with the HEPES buffer. The concentration of phospholipids in the stock solution was 0.14 mM. For fluorescence measurements, aliquots of the above LUV solution (40 $\mu$L) were diluted with the HEPES buffer (1.98 mL, 10 mM HEPES, 107 mM NaCl, pH = 7.4) in a series of cuvettes, resulting in a lipid concentration of 2.9 $\mu$M in each cuvette. Aliquots of appropriate oligocholate in DMSO were added to different cuvettes via a microsyringe. The amount of DMSO introduced to each sample was $\pm 20$ $\mu$L. The change of emission intensity at 520 nm ($A_{max}$) was monitored over time. After 1 h, 40 $\mu$L of Triton X-100 (1% v/v) was added, disrupting the vesicles and releasing the remaining CF (100% release). The percent leakage was defined as [% leakage = ($F_{i} - F_{0}$)/($F_{max} - F_{0}$) × 100], in which $F_{i}$ and $F_{0}$ are the initial and intermediate emission intensity, respectively, and $F_{max}$ is the maximum fluorescence intensity measured after lysis of the LUVs by Triton X-100.

**RESULTS AND DISCUSSION**

Design and Synthesis. Cholic acid derivatives are popular building blocks for membrane-related applications because of their rigid, amphiphilic backbone. ^35–39 Macroycles 1–3 were inspired by other reported cholate-based receptors/transpor ters ^48–57 and our own oligocholate foldamers. ^4,38,58–60 After
they turned out to be effective pore-forming agents, we became interested in the guanidinium/carboxylate-functionalized 4 and 5 as potential transporters. These molecules were synthesized by standard procedures from the corresponding azide/carboxylic acid ester-terminated oligocholates.\textsuperscript{34} Molecules 6 and 7 were prepared as control compounds to probe the structure–activity relationship and the transport mechanism.

The cholate backbone prefers the trimeric periodicity, as shown by Sanders’ work with cyclic oligocholate esters\textsuperscript{52} and our study of the amide-linked oligocholate foldamers.\textsuperscript{35} Although 7 is too short to fold,\textsuperscript{34} 4 should fold readily, benefiting from both the trimeric structure and the guanidinium–carboxylate salt bridge. The hydrogen bond-reinforced salt bridge is weak in water but has binding constants as high as 10\textsuperscript{4} M\textsuperscript{–1} in nonpolar environments. \textsuperscript{62} The cholate backbone for the trimeric folded structure and the guanidinium–carboxylate salt bridge. The hydrogen bond-reinforced salt bridge is weak in water but has binding constants as high as 10\textsuperscript{4} M\textsuperscript{–1} in nonpolar environments. \textsuperscript{62}

We expect compound 4 to easily migrate into a nonpolar lipid bilayer if the internal salt bridge is formed. Even with theionic groups, 4 is insoluble in water, due to the dominance of hydrophobic groups. Guanidinium derivatives are known to migrate into lipid bilayers when complexed with lipophilic anions (carboxylate, phosphate, or others).\textsuperscript{63} With the natural preference of the cholate backbone for the trimeric folded structure and the internal guanidinium/carboxylate groups, 4 is poised to adopt a similar conformation as the covalent macrocycle (1).

Glucose Leakage from POPC/POPG LUVs. Our first experiments were based on the well-established glucose leakage assay in liposomal technology (Scheme 2).\textsuperscript{22} In general, a high concentration (300 mM) of glucose is first trapped inside large unilamellar vesicles (LUVs). After the external glucose is removed by gel filtration, hexokinase, glucose-6-phosphate dehydrogenase, NADP, and ATP are added to liposomal solution. In the absence of transporting agents, the glucose stays inside the LUVs and remains intact. If an added reagent causes leakage of the liposomes, the escaped glucose will be phosphorylated and oxidized by the enzymes while NADP reduced to NADPH. Because of the fast enzymatic kinetics, formation of NADPH at 340 nm normally correlates directly with the rate of glucose efflux.\textsuperscript{23} 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.

Figure 2a shows the percent leakage of glucose over a period of 60 min when different amounts of 4 were added to POPC/POPG LUVs. The anionic POPG was incorporated to render the liposomes anionic and less prone to aggregation or fusion. Leakage became apparent with about 1 μM of 4 and quickly increased as more macrocycles were added. At 5 μM or at 5 mol % of the total lipids, the cholate trimer caused complete leakage of all the glucose within 6 min.

The liposomes were prepared by the extrusion method using 100 nm polycarbonate filters.\textsuperscript{64} As shown in Figure 2b ( ), the size of the liposomes averaged 110–120 nm and stayed quite constant after addition of 5 mol % of 4 (the highest oligocholate/lipid ratio in the leakage assays). The invariant liposome size excluded any mechanisms that cause vesicle aggregation or membrane fusion as well as those that destroy the lipid bilayers.

Further support for the intact membranes came from the lipid-mixing assay. In this experiment, the fluorescence resonance energy transfer (FRET) from NBD- to rhodamine-functionalized lipids was monitored after labeled and unlabeled LUVs were incubated together.\textsuperscript{65} The 100% end point can be measured either after a surfactant such as Triton X-100 is added to destroy the liposomes or through a “mock” fusion product (i.e., liposomes whose probe density corresponds to that of completely fused liposomes). Although the Triton method gave similar results, we mainly used the latter in our experiments because Triton X-100 impacted the quantum yield of NBD.\textsuperscript{66} Once again, even at 5 mol %, a concentration that caused complete leakage of glucose, 4 afforded <10% mixing of the lipids, indicating the absence of membrane fusion under the experimental conditions (Figure 2b, open squares).

In Figure 3a, we compare the leakage profiles of different oligocholates. We included the leakage data of the covalent macrocycles (1 and 2) and linear trimer 7. The covalent macrocycles were shown to transport glucose via hydrophobically driven pore formation.\textsuperscript{32} Interestingly, among the three salt-bridged...
behavior and can be analyzed by the Hill equation (eq 1). The sigmoidal leakage profile is characteristic of cooperative behavior and can be analyzed by the Hill equation (eq 1). The sigmoidal leakage profile is characteristic of cooperative behavior. The Hill equation relates the fractional activity (Y) of a supramolecule to the monomer concentration (c). EC₅₀ is the concentration of the monomer that produces 50% activity. The Hill coefficient (n) in an indicator of the number of monomers involved in the self-assembly.

\[ Y = Y_{\text{low}} + \left( Y_{\text{high}} - Y_{\text{low}} \right) / \left[ 1 + \left( \text{EC}_{50} / c \right)^n \right] \]  

(1)

Figure 3 shows the fitting of the leakage data to the Hill equation for the active transporters. The percent leakage at 30 min as the fractional activity since more data points will be in the transition region of the curve, giving more reliable fitting. The Hill coefficient was \( n = 4.0 \pm 0.3 \) for cyclic trimer 1 and \( n = 4.4 \pm 0.5 \) for tetramer 2. The salt-bridged trimer 4 gave a very similar value (\( n = 4.6 \pm 0.3 \)). Thus, all three compounds operate through an aggregated form consisting of approximately four molecules of oligocholates.

It is difficult to imagine that four oligocholates are needed to transport a single glucose in a carrier-based mechanism. Although the amphiphilic oligocholates conceivably can aggregate randomly to form ill-defined “puddles” or “pores” to allow the sugar to pass through, it is unlikely that such structures can only be formed by the trimer or through the cooperative interactions of four molecules. Instead, given the hydrophobic thickness of the POPC bilayer (ca. 2.6 nm = 4 × 0.65 nm), and the width of a cholate (ca. 0.6–0.7 nm), the most likely transporter is a transmembrane pore consisting of four stacked macrocycles. For the covalent cholate macrocycles, pore formation was confirmed by pyrene excimer formation in 3, which scales with the bilayer hydrophobicity and thickness. Additional evidence came from the effects of macrocycle topology, the lipid composition, and the guest size on the transport (vide infra).

It is possible that salt-bridged oligocholate 4 could operate through a similar mechanism as the covalent macrocycles. After all, nearly all the reasons that favor the pore formation of 1 still apply to 4. The rigidity resulting from the triangular geometry, for example, is true for both noncovalent and covalent macrocycles. The preference of the cholate backbone for the cyclic trimer and the strong guanidinium-carboxylate salt bridge suggest that 4 has a strong propensity to adopt the ring-closed conformation resembling 1. Strong “internal hydrophilicity”, which provides the hydrophobic driving force for stacking, is found in both compounds. In fact, assuming that the intramolecular salt bridge is formed, 4 is even more hydrophilic in the interior than its covalent analogue, due to the presence of the ionic groups on the side.

The chain length effect in 4–6 is also consistent with pore formation. A triangle cannot change its shape as long as the sides are rigid, but a quadrilateral can bend and twist even if the sides are rigid. Because pore formation requires reasonable contact of the macrocycles, stacking of a shape-persistent macrocycle is a lot easier than that of a conformationally mobile one. The pore-forming mechanism thus predicts that the cyclic trimer would be a better pore-forming agent than the tetramer. The prediction was confirmed earlier in the covalent macrocycles and is also true for the salt-bridged compounds (Figure 3a). For the guanidinium-carboxylate-functionalized dimer 6, its incompetency was probably a result of the intramolecularly salt-bridged macrocyclic structure being either too small to allow the guest to pass through or too strained to form. Moreover, since the driving force for pore formation is the hydrophobic interactions of the water molecules within the macrocycles (i.e., their strong tendency to aggregate in a nonpolar membrane), even if the cyclic conformer can form in 6, its interior would contain far fewer water molecules than 4, making pore formation difficult.

Effects of Lipid Composition. An important consequence of the hydrophobically driven pore formation is that the permeability of the membrane for hydrophilic molecules can increase as the membrane becomes more hydrophobic. This is a very counterintuitive result, as hydrophobicity is the reason why a lipid bilayer acts as a barrier for hydrophilic molecules.

Figure 4a compares the glucose leakage for LUVs with and without 30 mol % cholesterol in the membrane. Cholesterol is known to increase the hydrophobic thickness of POPC bilayer and decrease its fluidity. Cholesterol-containing bilayers have been shown to be much less permeable to hydrophilic molecules, including glucose.
Nevertheless, addition of cholesterol afforded a very notable increase in the glucose leakage triggered by the salt-bridged oligocholates. The EC_{50} for 4 went from 1.6 to 0.6 \mu M (compare the data points (\square) connected by the solid and dashed lines). Remarkably, tetramer 5 benefited even more from cholesterol; its ability to transport glucose was almost as good as that of trimer 4. Dimer 6 also benefited, although to a much smaller extent.

The higher transport in the cholesterol-containing membranes is difficult to be explained with the carrier- or relay-based transport. The data is also against random aggregates of the oligocholates or poorly defined “puddles” or “pores”, as such water-filled structures are expected to be less stable and likely will collapse in a more hydrophobic membrane. The proposed pore formation, on the other hand, remains consistent with the experimental observations. As the membrane gets more hydrophobic, the water molecules within the cyclic oligocholates have a stronger driving force to aggregate, directly helping the stacking of the oligocholates. Cholesterol potentially can induce lateral heterogeneity in lipid membranes.71–78 Cholic acid is a metabolite of cholesterol. Its cholesterol-like backbone suggests that the oligocholates would want to associate with cholesterol-rich domains if indeed phase separation of lipids occurs, akin to the “like-dissolves-like” principle in solubility.

For the salt-bridged oligocholates, a more hydrophobic membrane may stabilize the macrocyclic conformer needed for pore formation. Normally, because charged groups are not solvated well in a nonpolar environment, it is quite unfavorable to bury them within lipid bilayers or in the interior of a protein.79 For the proposed pore formation, however, the guanidinium and carboxylate groups are exposed to the water molecules inside the transmembrane pore, and thus, their needs for solvation may be met reasonably well. On the other hand, both hydrogen-bonding and electrostatic interactions become stronger in a less polar environment. Hence, the intramolecular guanidinium—carboxylate salt bridge should be more stable in the cholesterol-containing membranes.

The leakage data for the cholesterol-containing membranes also fit well to the Hill equation (Figure 4b). The Hill coefficient for the trimer remained about 4 (n = 3.9 \pm 0.5); that for the tetramer was smaller (n = 2.6 \pm 0.2). It is not clear why the tetramer had a lower Hill coefficient. The Hill coefficient can change significantly with minor structural modification of even well-behaved systems.69,80–84 Similar observations were made in biology, e.g., in hemoglobin—oxygen binding. The Hill equation is known to work best when extreme positive cooperativity exists between the binding of the first and second molecule.85 Such a condition may not be met when cholesterol changes the driving force of the pore formation.

It should be mentioned that we cannot rule out the formation of intermolecular salt bridges between the neighboring molecules of 4 along the pore axis. In such a case, the transporting supermolecule resembles the folded helix of a linear oligocholate.34,38,58–60 It is even possible that both inter- and intramolecular salt bridges are formed for a given transmembrane pore, and they interconvert rapidly. These scenarios, although structurally different, affect neither the transport nor the conclusions drawn in this paper.

**Effect of Guest Size.** Changing the size of the permeating guest is a useful tool to probe the transport mechanism. As the guest gets larger, its diffusion (as the carrier—guest complex) in the membrane slows down. Because it is more difficult for a larger hydrophilic guest to hop from one station to another in a hydrophobic membrane, relay-based transport will become less efficient as well. Transport by a nanopore, on the other hand, should not be affected significantly as long as the cross section of the guest is smaller than the pore diameter.

We, therefore, investigated the permeation of maltotriose across POPC/POPG membranes. This trisaccharide is too large to fit within a single cholate macrocycle.82 If two oligocholates are needed to shield the sugar from the lipid hydrocarbon tails, a carrier-based mechanism would be severely impacted by the much larger carrier—guest complex. As shown by Figure 5, however, leakage of the longer sugar did not slow down at all. In fact, because the hydrolysis of maltotriose by \( \alpha \)-glucosidase is not as efficient as the phosphorylation/oxidation of glucose by hexokinase/glucose-6-phosphate dehydrogenase, the data shown for the maltotriose only represent the lower limits for the leakage.

The covalent tetramer (2) displayed a significant “template effect” in the transport of maltotriose.82 The effect, caused by the long sugar threading through more than one macrocycle to facilitate pore formation, makes maltotriose leak faster than glucose, another counterintuitive result of the pore-forming mechanism. The template effect seemed to be operating as well for the salt-bridged oligocholates. Although the difference was not very large, tetramer 5 indeed was consistently more effective at transporting maltotriose than glucose, especially considering that...
the leakage rates for the maltotriose were only the lower limits. The template effect was small/absent in both the covalent trimer (1) and the salt-bridged trimer (4). For the covalent trimer, molecular modeling previously suggested that multiple hydrogen bonds formed between maltotriose and the inner pore of the tricholate. These hydrogen bonds probably hampered diffusion of the sugar through the pore and offset the template effect. The same could be true for the noncovalent macrocyclic trimer (4).

As for the tetramers, their larger internal cavity probably geometrically prohibited some of the hydrogen bonds, making the passage of the sugar less hindered.

Although an increase in the length of the guest did not slow down the transport, an increase in the cross section was a completely different story. Carboxyfluorescein (CF) is water soluble when deprotonated. It displays strong self-quenching above 50 mM and is frequently employed to probe transmembrane activity in liposomes, as its leakage from a liposome would dilute the dye and enhance its emission.

The molecular models (Figure 6) suggest that CF would have difficulty going through the inner cavity of 1 even in the fully dehydrated form. Indeed, transport by the covalent trimer displayed saturation (Figure 7a). The Hill coefficient decreased to \( n = 2.0 \pm 0.1 \) (Figure 7b) in the meantime, suggesting that a dimer was possibly involved in shuttling CF across the membrane. The covalent tetramer 2 was more effective in the CF transport than the trimer as expected from its larger cavity but also displayed saturation at high concentrations.

The saturation behavior of the covalent macrocycles was reasonable from the viewpoint of their concentration-dependent pore formation. Akin to the micellization of surfactants, the macrocycles are expected to aggregate above a critical aggregation concentration (CAC). Because glucose is small enough to go through the inner pore, its leakage increases as more macrocycles begin to stack and goes to completion once the membranes contain enough nanopores. CF, on the other hand, cannot go through the nanopores even when more of them are formed in the bilayers. If this larger guest indeed relies on the nonaggregated macrocycles to move across the membrane, its leakage will plateau once the concentration of the macrocycle reaches the CAC, as the concentration of the nonaggregated macrocycles will stay constant above the CAC.

The salt-bridged trimer 4 showed no such behavior. It transports CF more efficiently across the membranes than the covalent trimer 1, and complete leakage occurred with [4] = 0.25 \( \mu \)M (Figure 7a). Significantly, the leakage data fit well to the Hill equation, with \( n = 3.7 \pm 0.5 \) (Figure 7b), suggesting that the same transmembrane pores probably were involved, despite the larger guest size. Presumably, unlike the rigid covalent trimer, the noncovalent macrocycle can open and close reversibly, allowing a larger guest to pass through. It is also possible that the hydrogen-bonding interactions between CF and the guest contributed to the transport.

As for the tetramers, their larger internal cavity probably geometrically prohibited some of the hydrogen bonds, making the passage of the sugar less hindered.

Although an increase in the length of the guest did not slow down the transport, an increase in the cross section was a completely different story. Carboxyfluorescein (CF) is water soluble when deprotonated. It displays strong self-quenching above 50 mM and is frequently employed to probe transmembrane activity in liposomes, as its leakage from a liposome would dilute the dye and enhance its emission.

The molecular models (Figure 6) suggest that CF would have difficulty going through the inner cavity of 1 even in the fully dehydrated form. Indeed, transport by the covalent trimer displayed saturation (Figure 7a). The Hill coefficient decreased to \( n = 2.0 \pm 0.1 \) (Figure 7b) in the meantime, suggesting that a dimer was possibly involved in shuttling CF across the membrane. The covalent tetramer 2 was more effective in the CF transport than the trimer as expected from its larger cavity but also displayed saturation at high concentrations.

The saturation behavior of the covalent macrocycles was reasonable from the viewpoint of their concentration-dependent pore formation. Akin to the micellization of surfactants, the macrocycles are expected to aggregate above a critical aggregation concentration (CAC). Because glucose is small enough to go through the inner pore, its leakage increases as more macrocycles begin to stack and goes to completion once the membranes contain enough nanopores. CF, on the other hand, cannot go through the nanopores even when more of them are formed in the bilayers. If this larger guest indeed relies on the nonaggregated macrocycles to move across the membrane, its leakage will plateau once the concentration of the macrocycle reaches the CAC, as the concentration of the nonaggregated macrocycles will stay constant above the CAC.

The salt-bridged trimer 4 showed no such behavior. It transports CF more efficiently across the membranes than the covalent trimer 1, and complete leakage occurred with [4] = 0.25 \( \mu \)M (Figure 7a). Significantly, the leakage data fit well to the Hill equation, with \( n = 3.7 \pm 0.5 \) (Figure 7b), suggesting that the same transmembrane pores probably were involved, despite the larger guest size. Presumably, unlike the rigid covalent trimer, the noncovalent macrocycle can open and close reversibly, allowing a larger guest to pass through. It is also possible that the hydrogen-bonding interactions between CF and the guest contributed to the transport.

As for the tetramers, their larger internal cavity probably geometrically prohibited some of the hydrogen bonds, making the passage of the sugar less hindered.

Although an increase in the length of the guest did not slow down the transport, an increase in the cross section was a completely different story. Carboxyfluorescein (CF) is water soluble when deprotonated. It displays strong self-quenching above 50 mM and is frequently employed to probe transmembrane activity in liposomes, as its leakage from a liposome would dilute the dye and enhance its emission.

The molecular models (Figure 6) suggest that CF would have difficulty going through the inner cavity of 1 even in the fully dehydrated form. Indeed, transport by the covalent trimer displayed saturation (Figure 7a). The Hill coefficient decreased to \( n = 2.0 \pm 0.1 \) (Figure 7b) in the meantime, suggesting that a dimer was possibly involved in shuttling CF across the membrane. The covalent tetramer 2 was more effective in the CF transport than the trimer as expected from its larger cavity but also displayed saturation at high concentrations.

The saturation behavior of the covalent macrocycles was reasonable from the viewpoint of their concentration-dependent pore formation. Akin to the micellization of surfactants, the macrocycles are expected to aggregate above a critical aggregation concentration (CAC). Because glucose is small enough to go through the inner pore, its leakage increases as more macrocycles begin to stack and goes to completion once the membranes contain enough nanopores. CF, on the other hand, cannot go through the nanopores even when more of them are formed in the bilayers. If this larger guest indeed relies on the nonaggregated macrocycles to move across the membrane, its leakage will plateau once the concentration of the macrocycle reaches the CAC, as the concentration of the nonaggregated macrocycles will stay constant above the CAC.

The salt-bridged trimer 4 showed no such behavior. It transports CF more efficiently across the membranes than the covalent trimer 1, and complete leakage occurred with [4] = 0.25 \( \mu \)M (Figure 7a). Significantly, the leakage data fit well to the Hill equation, with \( n = 3.7 \pm 0.5 \) (Figure 7b), suggesting that the same transmembrane pores probably were involved, despite the larger guest size. Presumably, unlike the rigid covalent trimer, the noncovalent macrocycle can open and close reversibly, allowing a larger guest to pass through. It is also possible that the hydrogen-bonding interactions between CF and the guest contributed to the transport.

As for the tetramers, their larger internal cavity probably geometrically prohibited some of the hydrogen bonds, making the passage of the sugar less hindered.

Although an increase in the length of the guest did not slow down the transport, an increase in the cross section was a completely different story. Carboxyfluorescein (CF) is water soluble when deprotonated. It displays strong self-quenching above 50 mM and is frequently employed to probe transmembrane activity in liposomes, as its leakage from a liposome would dilute the dye and enhance its emission.

The molecular models (Figure 6) suggest that CF would have difficulty going through the inner cavity of 1 even in the fully dehydrated form. Indeed, transport by the covalent trimer displayed saturation (Figure 7a). The Hill coefficient decreased to \( n = 2.0 \pm 0.1 \) (Figure 7b) in the meantime, suggesting that a dimer was possibly involved in shuttling CF across the membrane. The covalent tetramer 2 was more effective in the CF transport than the trimer as expected from its larger cavity but also displayed saturation at high concentrations.

The saturation behavior of the covalent macrocycles was reasonable from the viewpoint of their concentration-dependent pore formation. Akin to the micellization of surfactants, the macrocycles are expected to aggregate above a critical aggregation concentration (CAC). Because glucose is small enough to go through the inner pore, its leakage increases as more macrocycles begin to stack and goes to completion once the membranes contain enough nanopores. CF, on the other hand, cannot go through the nanopores even when more of them are formed in the bilayers. If this larger guest indeed relies on the nonaggregated macrocycles to move across the membrane, its leakage will plateau once the concentration of the macrocycle reaches the CAC, as the concentration of the nonaggregated macrocycles will stay constant above the CAC.

The salt-bridged trimer 4 showed no such behavior. It transports CF more efficiently across the membranes than the covalent trimer 1, and complete leakage occurred with [4] = 0.25 \( \mu \)M (Figure 7a). Significantly, the leakage data fit well to the Hill equation, with \( n = 3.7 \pm 0.5 \) (Figure 7b), suggesting that the same transmembrane pores probably were involved, despite the larger guest size. Presumably, unlike the rigid covalent trimer, the noncovalent macrocycle can open and close reversibly, allowing a larger guest to pass through. It is also possible that the hydrogen-bonding interactions between CF and the guest contributed to the transport.
It should be noted that although the concentrations of the transporters in the CF leakage (Figure 7a) were much lower than those in the glucose leakage assays (Figure 3a), the data do not mean that these compounds were better transporters for the larger CF. Because the UV-based glucose assay has 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 are essentially insoluble in water, the effective concentration of the transporter in the membrane would increase with a decrease in the phospholipid concentration. When expressed as percentages of the total phospholipids, the EC50 of 4 was 1.5% and 3.8% for the leakage of glucose and CF, respectively, from POPC/POPG LUVs.

### CONCLUSIONS

The salt-bridged trimeric oligocholate (4) seems to have inherited most of the attributes from its covalent parent (1). The much higher activity of 4 in comparison to other similarly structured compounds (e.g., 5–7) argues against random intermolecular aggregation of the oligocholates as the reason for the transport. The Hill analysis indicates that four molecules were involved in the transport. Collectively, the data give quite strong support for the transmembrane nanopores formed by the stack-ming of the salt-bridged oligocholate. The high activity of 4 probably derives from the preference of the cholate backbone for a trimeric folded structure and the strong guanidinium–carboxylate salt bridge. Consistent with the hydrophobically driven pore formation, the permeability of glucose becomes higher as the membranes become thicker and more hydrophobic. Compared with the covalent structure, the biggest benefi of the noncovalent macrocycle is its ability to expand and permeate guests larger than that its pore size. Such a design may be useful in the construction of “forgiving” pores that do not have strict size selectivity.

### ASSOCIATED CONTENT

 Supporting Information. Figure 1S and NMR data for the key compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

### AUTHOR INFORMATION

**Corresponding Author**

*Phone: 515-294-5845. Fax: 515-294-0105. E-mail: zhaoy@iastate.edu.*

### ACKNOWLEDGMENT

We thank the NSF (DMR-1005515) for financial support of the research.

### REFERENCES


(81) Bhosale, S.; Matile, S. Chirality 2006, 18, 849–856.


(87) Different from covalent bonds, noncovalent linkages result from an equilibrium favoring the closed form. From the molecular models in Figure 6, CF is slightly larger than the inner cavity of the covalent trimer. The salt-bridged trimer should not need to open too much for the guest to pass through.

(88) According to eq 1, the Hill coefficient n is independent of the unit of concentration used for the compounds.