ACCOUNTS
of chemical research

Conformationally Controlled Oligocholate Membrane Transporters: Learning through Water Play

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RECEIVED ON DECEMBER 17, 2012

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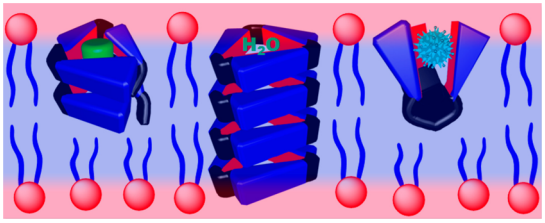


Controlled translocation of molecules and ions across lipid membranes is the basis of numerous biological functions. Because synthetic systems can help researchers understand the more complex biological ones, many chemists have developed synthetic mimics of biological transporters. Both systems need to deal with similar fundamental challenges. In addition to providing mechanistic insights into transport mechanisms, synthetic transporters are useful in a number of applications including separation, sensing, drug delivery, and catalysis.

In this Account, we present several classes of membrane transporters constructed in our laboratory from a facially amphiphilic building block, cholic acid. Our "molecular baskets" can selectively shuttle glucose across lipid membranes without transporting smaller sodium ions. We have also built oligocholate foldamers that transiently fold into helices with internal hydrophilic binding pockets to transport polar guests. Lastly, we describe amphiphilic macrocycles, which form transmembrane nanopores in lipid bilayers through the strong associative interactions of encapsulated water molecules.

In addition to presenting the different transport properties of these oligocholate transporters, we illustrate how fundamental studies of molecular behavior in solution facilitate the creation of new and useful membrane transporters, despite the large difference between the two environments. We highlight the strong conformational effect of transporters. Because the conformation of a molecule often alters its size and shape, and the distribution of functional groups, conformational control can be used rationally to tune the property of a transporter. Finally, we emphasize that, whenever water is the solvent, its unique properties—small size, strong solvation for ionic functionalities, and an extraordinary cohesive energy density (i.e., total intermolecular interactions per unit volume)—tend to become critical factors to be considered. Purposeful exploitation of these solvent properties may be essential to the success of the supramolecular process involved—this is also the reason for the "learning through water play" in the title of this Account.

Introduction

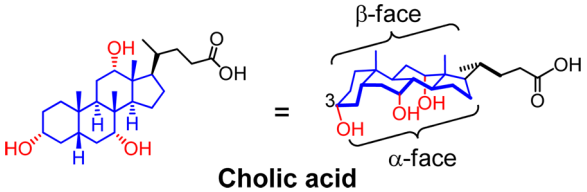
Most membrane transporters work as either a carrier or a channel/pore.[1] A carrier escorts its cargo to diffuse across the membrane. A channel or pore is relatively stationary and provides a transmembrane (TM) conduit for the permeant. A transporter can also operate in a relay.[2] Although the transporter may have significant movement in the last mechanism, the movement mostly involves passing the permeant from one transporter to another before releasing it into the aqueous phase.

A lipid membrane is essentially a supramolecular assembly of lipid molecules driven together by the hydrophobic interactions. In order for a water-soluble molecule or ion to traverse a membrane, some sort of assistance must come into play. This is because, unless the molecule is of sufficient lipophilicity, its desolvation of water and entrance into a hydrophobic environment are thermodynamically unfavorable. Hence, to shield a hydrophilic permeant from lipid hydrocarbon, the transporter needs to have certain degree of amphipathicity, whatever the transport mechanism may

Published on the Web 03/28/2013 www.pubs.acs.org/accounts
10.1021/ar300337f

be. With one side interacting with the hydrophilic permeant and the other side with the lipid tails, the transporter provides a low-energy pathway for the permeant to navigate through the hydrophobic barrier. The amphipathicity usually becomes more important as the permeating species gets larger, as the potential unfavorable hydrophilic−hydrophobic contact with lipid hydrocarbon is directly proportional to the size of the hydrophilic permeant.

One of the most well-known amphipathic molecules is cholic acid, a metabolite of cholesterol in mammals. Its rigidity, facial amphiphilicity, and ease of functionalization make the molecule a favorite building block in supramolecular chemistry,[3−8] including in the construction of ion channels,[9−13] molecule-[14−16] and anion-transporters[17,18] for membrane-related applications. In this Account, we illustrate how different organizations of cholates in combination with rational conformational control can afford membrane transporters with diverse properties. The dramatic correlation between the structure of the oligocholates and their transport properties is the hallmark of this approach. Seemingly unimportant spacers within otherwise similar structures, minute changes in the way how oligocholate blocks are connected, and switching the locations of functional groups in the molecules are shown to impact the transport profoundly, sometimes changing the transport mechanism altogether.



Cholic acid

Cholate-Derived Molecular Baskets

Our interest in cholate-based materials began with the realization that the two opposing faces of cholic acid enable the molecule to interact with a nonpolar and a polar environment simultaneously. Although such is always the case for an amphiphile, the large steroidal backbone of cholic acid greatly enhances the strength of the potential interactions involved. The free energy of a solvophobically driven binding relates directly to the area of solvent-incompatible surface removed from solvent exposure upon binding. In water, each square angstrom of hydrophobic association contributes ca. -0.025 kcal·mol^{-1} of binding free energy.[19] The 100 Å^2 hydrophobic β-face of cholic acid,[20] thus, has the potential to provide -5 kcal/mol of free energy when interacting with an appropriate hydrophobic environment. As a result, a few cholates can afford sufficient driving force for a material that relies on solvophobic interactions to function.

Our first design involves preorganizing multiple cholates on a scaffold such as calixarene or the 1,3,5-2,4,6-hexasubstituted benzene. The resulting molecular baskets, for example, **1**−**3**, were hypothesized to adopt either a micelle-like or reverse-micelle-like conformation, as the corresponding polar or nonpolar faces turn outward to be compatible with the environment.[21−23] The conformational switching was supported by the solubility of **1** in both 20:80 water/methanol and 5:95 methanol/CCl_4. In addition, the aromatic protons *ortho* to the amido groups displayed distinctive splitting at both the high and low end of the polarity scale but appeared as a singlet at intermediate polarity (Figure 1). Since basket **2** had a larger solvophobic difference between the α- and β-face than **1**, its splitting was more sensitive to solvent polarity, consistent with the stronger solvophobic driving force involved. As expected from the two conformers, **1** could bind (polar) phenyl β-D-glucopyranoside in nonpolar solvents and (nonpolar) aromatic hydrocarbons in polar solvents.[24]

The reverse-micelle-like conformer of the cholate baskets should bind polar solvents in its hydrophilic interior.[22] When the bulk solvent was a 10:90 DMSO-d_6/CCl_4 mixture, these baskets were found to contain 50−60% DMSO according to the ^{1}H NMR chemical shifts of the amide and hydroxyl protons. The cooperativity of conformational change was established by dendritic **3**, which used all six cholate arms to form a single polar microenvironment with an encapsulated pool of DMSO.

These amphiphilic baskets seemed particularly suitable for "protecting" hydrophilic molecules in a nonpolar medium. The sugar-binding of **1** made us wonder whether it could carry glucose across lipid membranes. Since the calixarene interfered with the UV-based glucose leakage assay, we turned our attention to **4**−**6**, two of which were reported in our earlier paper.[22] The transport of glucose across lipid membranes was measured by the induced leakage of glucose-filled large unilamellar liposomes (LUVs). The leakage assays revealed that the flexible basket (**5**) was the most efficient transporter for glucose across POPC/POPG membranes. The arrangement of the cholates was critical, as neither methyl cholate nor linear trimer **7** displayed any activity. When the initial leakage rates were plotted against the concentrations of the transporters, two molecules of **5** were found to work cooperatively in the transport while a single molecule was involved in the cases of **4** and **6**,

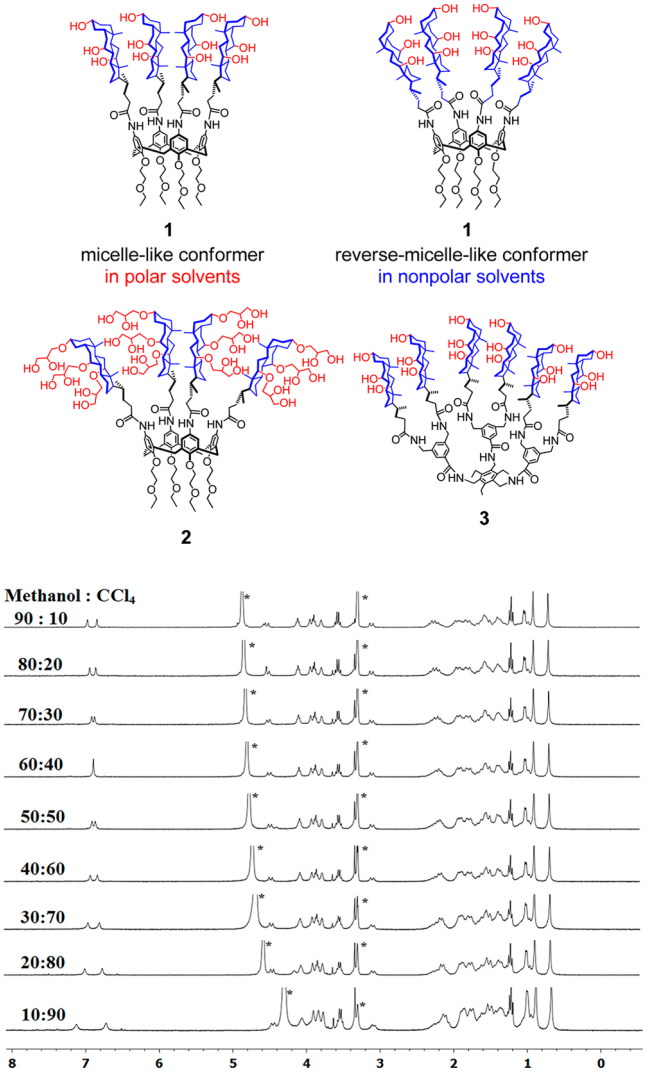
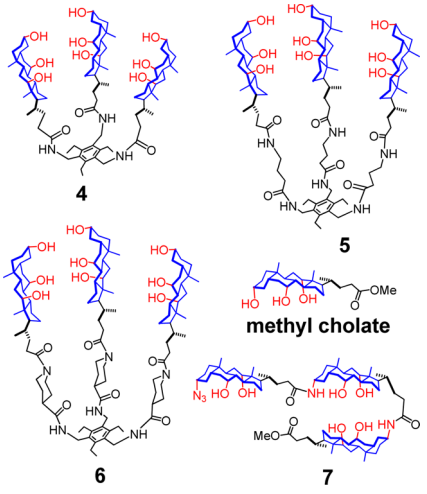


FIGURE 1. ^{1}H NMR spectra of **1** in different solvents. Solvent peaks (CD_3OH and CD_2HOD) are marked with * on the right. (Reprinted with permission from ref 21. Copyright 2004 American Chemical Society.)

supporting a relay mechanism in **5** and carrier-based transport in **4** and **6**.[25]

There could be two main reasons for the relay mechanism in **5**. First, the 4-aminobutyroyl spacers added three secondary amides to the basket. The poor solvation of these hydrophilic groups by the lipid tails is expected to hinder the diffusion of the basket in the membrane. As the carrier mechanism is disfavored, a relay becomes more competitive because it requires far less movement of the transporters within a lipid bilayer. Given the comparable heights of the basket and a single leaflet of a bilayer, a relay involving two baskets is very reasonable. Second, in order for a relay to operate, the basket has to release the entrapped glucose within a hydrophobic membrane. With limited numbers of water molecules in the membrane, the numerous hydroxyl and amide groups of **5** need to "self-solvate" through intramolecular hydrogen-bonds after unloading the guest, possibly with and through some encapsulated or associated water molecules. Without such self-solvation, a huge number of polar groups would face lipid hydrocarbon—a highly unfavorable alternative. Since the flexible tethers in the basket allow the intramolecular cholate–cholate interactions to occur with minimal strain in mixed organic solvents,[22] they should do the same in the membrane and facilitate the binding/release of the glucose in the relay.



Notably, the inclusion of 30 mol % cholesterol in the POPC/POPG membranes switched **4** to the relay mechanism, but the larger, more rigid basket (**6**) remained as a carrier. These results gave additional support for the relay. As the membrane becomes thicker and more hydrophobic upon cholesterol addition, basket **4** would have difficulty diffusing in the membrane, which would disfavor the carrier mechanism but favor the relay. Basket **6** is the most hydrophobic among the three and the least flexible. As mentioned earlier, strong hydrophobicity makes the molecule more compatible with the hydrophobic membrane and rigidity in the structure slows down the binding and release of glucose; since both factors favor the carrier mechanism and/or disfavor the relay, there is little reason for **6** to switch away from the carrier mechanism.

It is interesting to note that very few synthetic sugar carriers have been reported in the literature. Although synthetic nanopores had been employed to permeate carbohydrates across lipid bilayers,[26] organic boronic acids seemed to be the only efficient carriers of sugar in lipid membranes.[27,28] In contrast, most natural glucose transporters (e.g., GluT proteins) operate by the carrier mechanism.[29−32] Carrier-based transporters are considered necessary for the chemical imbalance between the intra- and extracellular media, as a pore (i.e., the alternative mechanism) large enough for

glucose probably would have difficulty preventing the passage of smaller molecules and ions (e.g., Na^+).[30] Amazingly, the selectivity for glucose over sodium ions found in GluT proteins was also observed in our molecular baskets. Under conditions that caused complete leakage of glucose from the LUVs, leakage of sodium ions was essentially absent. Hence, although these baskets could move back and forth in a bilayer or operate in a relay, encapsulation of the smaller sodium ion was unfavorable. It is possible that the limited number of water molecules in the basket simply could not solvate both the polar groups of the cholates and the encapsulated sodium ion(s) simultaneously. Because glucose can hydrogen-bond with the polar groups of the cholates, its desolvation of water and entrance into the basket should be much easier, as supported by the binding of phenyl β-D-glucopyranoside by a similar basket in nonpolar solvents.[24]

Oligocholate Foldamers

The highly preorganized amphiphilic molecular baskets probably do not undergo large-scale conformational changes. Linear oligocholates (e.g., **9**), prepared from monomer **8** by standard amide chemistry, are quite different in this regard.[33] Molecular modeling suggests that the linear oligomer could fold into a helix with a nanometer-sized internal hydrophilic cavity (Figure 2), which seems ideal for membrane transport.

Our solution studies revealed that the folding of the oligocholate is driven by the preferential solvation of its hydrophilic groups by the polar solvent molecules that phase-separate into the central cavity (Figure 2). The folded helix resembles the reverse-micelle-like conformer of the molecular baskets, with a concentrated pool of polar solvent inside the hydrophilic nanocavity formed by the surrounding cholate groups. Due to the comparable size of the solvent and the nanocavity, the preferential solvation and the folding are strongly influenced not only by polarity but also the size and shape of the solvent molecules.[34] The conformation of the oligocholate is extremely sensitive to solvents, with a few percent change in the solvent composition triggering complete unfolding of the helix in some cases.[35−38] As in the amphiphilic baskets, flexible tethers in between the cholates reduce the strain in the folded conformer and are helpful to the folding.[39] The folding/unfolding is highly cooperative, similar to the two-state conformational change of many proteins.[33]

With a cholesterol-like backbone, the oligocholates are great mimics of membrane proteins. The large tunability in their structures seems particularly attractive for molecular transport. A short folded helix potentially can act as a carrier, with the internal, functionalizable cavity binding a suitable guest to shuttle it across a membrane. A sufficiently long folded helix, on the other hand, may span an entire bilayer to form a TM nanopore. Even though we do not have mixed organic solvents in the membranes, water and lipid tails should in principle function as the polar and nonpolar "solvents" to provide similar solvophobic driving force to the folding.

Armed with extensive knowledge of the solvophobic folding in solution and numerous tools to strengthen the folding (e.g., internal salt bridge[40] and metal−ligand complexation[35−37]), we had anticipated a smooth transition when moving into lipid membranes. To our chagrin, the environmental effect turned out far trickier to deal with. Initially, we studied the folding of the oligocholates in surfactant micelles because they were frequently employed as membrane-mimetic environments for membrane-associated peptides and proteins.[41] Even there, surprises turned out quickly. Flexible tethers such as 4-aminobutyroyl that greatly

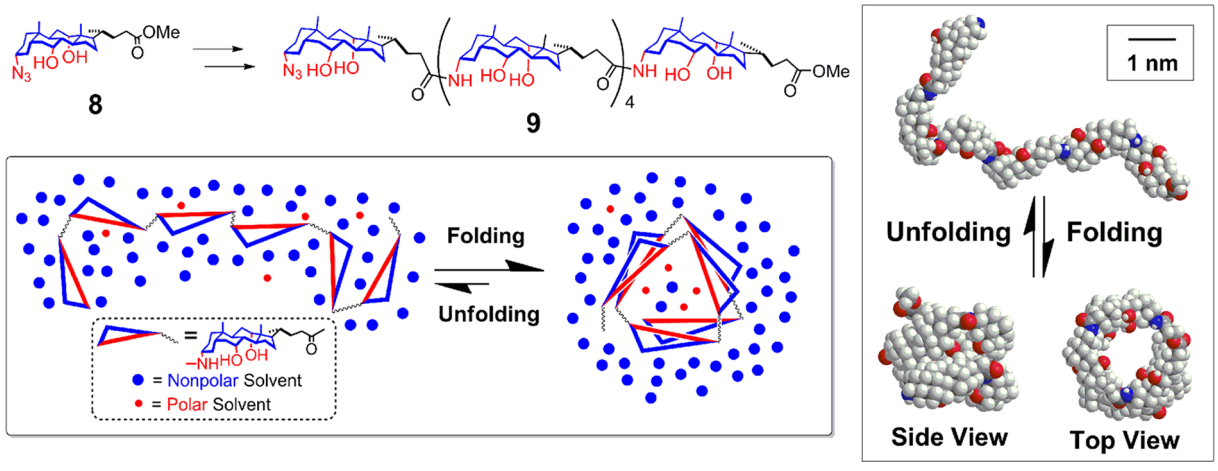
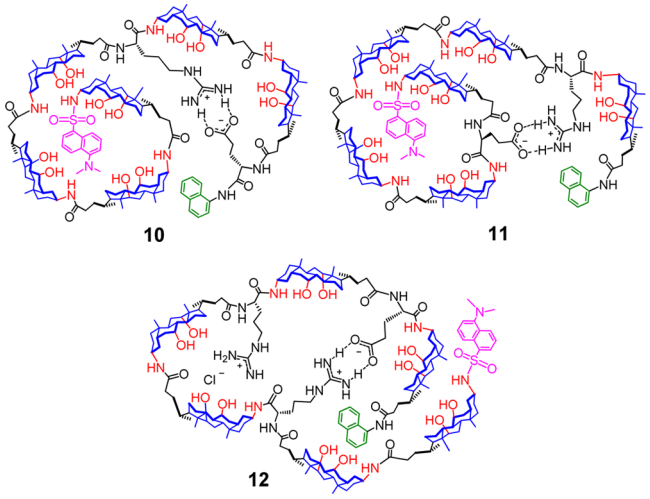


FIGURE 2. Solvophobic folding of the oligocholate and the molecular models of a folded and unfolded hexamer.

assisted the folding of linear oligocholates in mixed organic solvents[42] completely reversed its effect in micelles. The folding of the oligocholates was found to be governed by a completely different mechanism in micelles, mostly related to the rigidity of the oligomer and how it can best meet its solvation needs in a nanosized hydrophobic "cage".[41,43,44]

The strong guanidinium–carboxylate internal salt bridge also turned out as a disappointment in the new environments. Although very helpful to the folding of **10**–**12** in solution, the salt bridge was ineffective and even detrimental once the oligocholate moves into a micelle or membrane environment.[40] Two possible culprits were identified. First, these charged functional groups have a strong need for solvation by water. Instead of forming an internal salt bridge and staying in the hydrophobic core of a micelle or membrane (to stabilize the folded helix), they much prefer to stay at the surface of the micelle or membrane, where an abundance of water is found. Second, the sulfate or phosphate headgroups of the surfactants (e.g., SDS) or phospholipids by sheer abundance competes effectively for the guanidinium group in the oligocholate, making the internal salt bridge even less competitive.

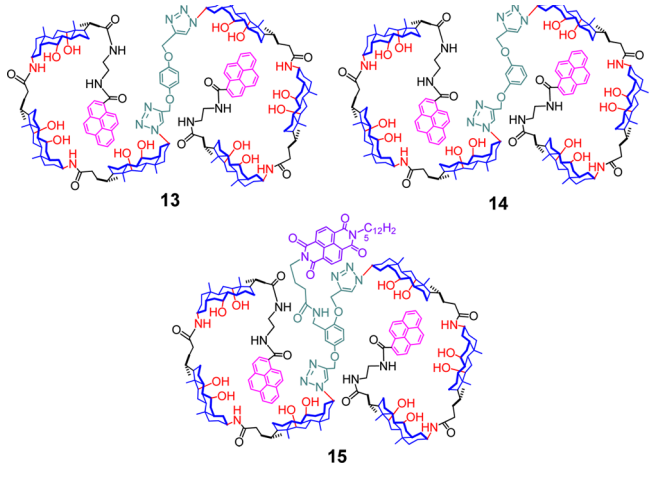


The failure in the above "water play" reminded us again that supramolecular chemistry is a game of competition. In a supramolecular reaction, the environment is never an innocent bystander but an active participant, as similar noncovalent interactions are involved between all parties. In the oligocholates, the environmental effect could completely overwhelm the "inherent foldability" of the molecule, even making the best "folder" in one environment the worst in another and vice versa.[40]

Despite the challenges in folding the oligocholates in lipid membranes, we decided to study their transport of water-soluble guests across lipid membranes. The reasoning was that the molecule did not have to fold permanently to transport a guest. As long as a (transiently) folded oligocholate had a long enough lifetime to carry the guest beyond the central dividing line of the bilayer, unfolding should release the guest to the other side of the membrane.

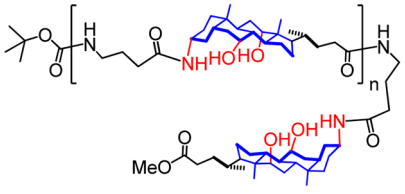
The permeant in our initial study was carboxyfluorescein (CF), whose concentration-dependent self-quenching allowed its efflux from LUVs to be monitored by fluorescence spectroscopy. The results were encouraging. Oligocholate **12**, although unable to fold (permanently) in POPC/POPG membrane, was able to shuttle CF across the membrane efficiently. Its highest activity among **10**–**12** may be understood from its extra arginine that could bind the carboxylate group of CF. The correlation between the foldability and the CF-transport was evident when the "salt-bridge isomers" (**10** and **11**) were compared. Although the two compounds differed only in the location of the salt bridge, **10** had approximately half of the transport activity of **13** but **11** was completely inactive. In mixed organic solvents, **11** could fold somewhat better than **10**, probably because the folding of **10** had to constrain four instead of two cholates and thus bore a higher entropic cost. The difference in foldability was maintained in micelles and apparently was large enough to devoid **11** of its ability to fold even transiently in the membrane.

In a following work, we studied the transport of CF by several nonionic hexacholates (**9**, **13**–**15**).[45] Remarkably, the seemingly trivial change from the 1,3- to 1,4-substitution in the phenylene spacer in **13** and **14** had a pronounced effect on the transporters over 3000 Da in MW. An intriguing trend was observed: poorer folders in solution turned out as more efficient transporters in the membranes. The inverse folding–transport correlation might seem counterintuitive. Nevertheless, for large, cooperatively folded transporters such as the oligocholates, significant changes in the



solvation/desolvation of the amphiphilic backbone occur in the binding and release of the guest. If unfolding of the transporter and its guest-release happen to be rate-limiting in the process, a poor folder understandably would be beneficial, due to its "eagerness" to unload the guest as soon as it migrates over to the opposite side of the membrane.

As mentioned earlier, 4-aminobutyroyl spacers in between the cholates made the flexible oligocholates fold better than the parent, more rigid oligocholates in mixed organic solvents[42] and the opposite effect was observed in micelles.[43] In the lipid membranes, the spacers had yet a still different effect and enabled the rigid, facially amphiphilic, awkwardly shaped cholates to pack tightly in a hydrophobic environment. The result was that, unlike the parent oligocholates that tend to stay near the membrane surface[46] or transiently fold in the hydrophobic core,[40,45] the flexible compounds (**16**–**18**) formed relatively tight aggregates in the membrane, stabilized by intermolecular hydrogen bonds. Glucose could "squeeze through" small "crevices" within these aggregates to cross the membrane, with the leakage rate displaying an unusual zero order dependence on the oligocholate concentration. Larger guests such as CF could not do so and relied on the nonaggregated oligocholates to shuttle them over by the carrier mechanism.[47] Note that, under the same conditions, "spacer-free" trimer **7** was completely inactive, highlighting the importance of the spacers to the intermolecular aggregation of the oligocholates.[48]



16, n = 1; **17**, n = 2; **18**, n = 3

Oligocholate-Based Macrocycles as Nanopore-Forming Agents in Membranes

TM nanopores are extremely useful in the delivery of hydrophilic molecules across cell membranes,[49−54] sensing,[55] and catalysis.[56] Unable to make the linear oligocholates to fold (permanently) in the membrane, we sought another strategy to create a TM nanopore using macrocycles such as **19**. The folded linear oligocholates are helices consisting of three monomer units per turn.[33] Thus, **19** essentially represents the cross-section of a folded helix. Its rigidity, resulting from both the triangular geometry and the fused steroid backbone, should prevent the inner cavity from collapsing—an important prerequisite for a nanopore. We hypothesized that the same driving force to make a linear oligocholate to fold—that is, preferential solvation of the polar groups by the entrapped polar solvent—would drive the macrocycles to stack on top of one another (Figure 3).

Switching from a conformationally mobile chain to a "prefolded" macrocycle was a strategic move in our water play. As discussed earlier, the dominant form of a linear oligocholate in the membrane is the unfolded conformer, which presumably lies at the membrane/water interface with the hydrophilic and hydrophobic faces toward water and the lipids, respectively.[40] At higher concentrations, these oligocholates can form loose, unstable intermolecular aggregates[46] in the membrane through intermolecular hydrogen bonds. Hence, the preferential solvation (with water as the polar solvent and lipid tails as the nonpolar "solvent") is insufficient to make the folded helix the most competitive structure among all possible forms. Solvation of the polar groups of the oligocholates is simply better met in the unfolded and aggregated structures, depending on the oligocholate concentration in the membrane.

For macrocycle **19**, the situation is very different. As the macrocycle enters a membrane, its highly polar interior, with six hydroxyl and three amide groups, cannot be solvated by the lipid tails but prefer to be filled with water molecules. These interior water molecules are "activated" in the membrane, as they are exposed to lipid hydrocarbon through the openings of macrocycles. The unfavorable hydrophilic–hydrophobic contact is lower when the macrocycle stays flat at the membrane/water interface than when it inserts deep into the membrane, but is best avoided if several macrocycles stack to form a TM nanopore (Figure 4). The arrangement allows the water molecules inside the macrocycles to solvate the polar groups of the cholates and still exchange with the bulk water readily. The solvent exchange is entropically favorable to pore formation. Indeed, it is known that, in some cases, the (entropic) cost for trapping a single water molecule can be as high as 2 kcal/mol.[57]

To our delight, **19** was found to transport glucose efficiently across lipid membranes, even much better than molecular baskets **4**–**6**. A Hill coefficient of $n = 4.0 \pm 0.3$ for trimer **19** and 4.4 ± 0.5 for tetramer **20** suggests four macrocycles worked cooperatively to transport glucose. POPC bilayer is about 2.6 nm in the hydrophobic thickness[58,59] and a cholate about 0.6–0.7 nm on the side. Thus, a TM pore consisting of four stacked macrocycles seems to be the active transporter.[60]

The oligocholate nanopores displayed highly unusual behavior as a result of the solvophobically driven pore formation.

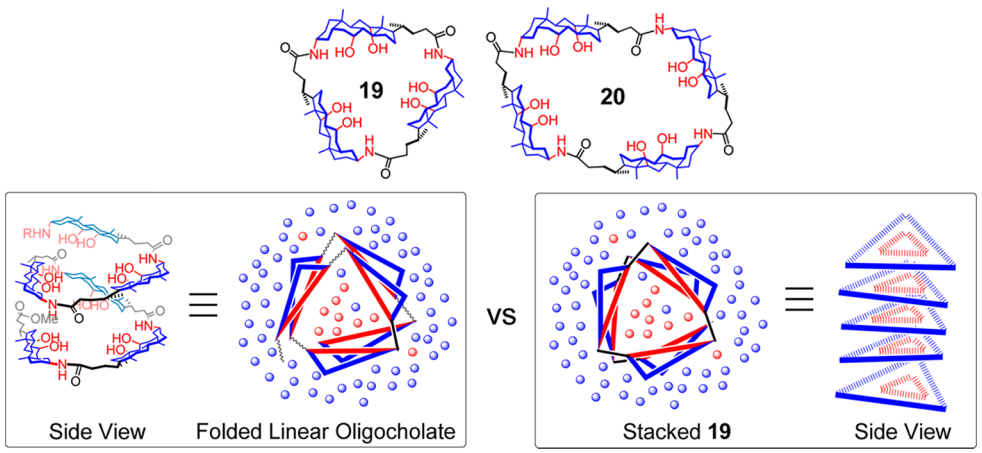


FIGURE 3. Schematic representation of the solvophobically driven folding of a linear oligocholate and the stacking of **19**. (Reprinted with permission from ref 60. Copyright 2011 American Chemical Society.)

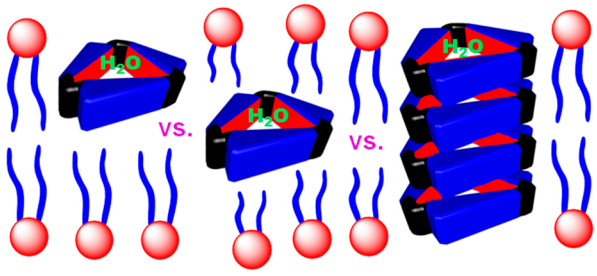
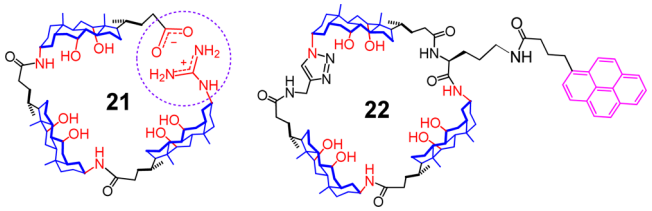


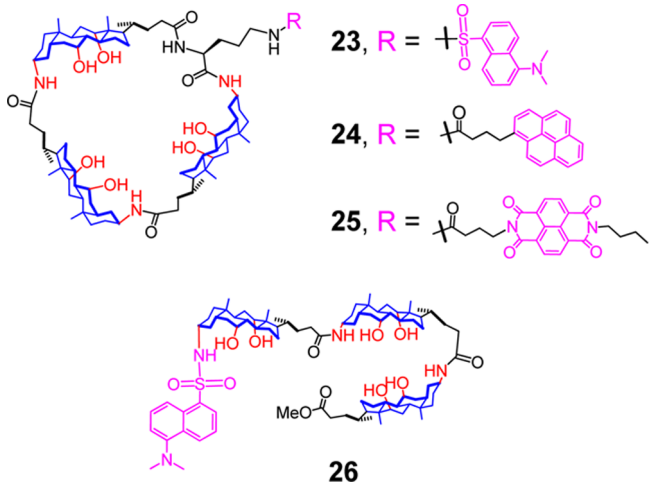
FIGURE 4. Stacking of oligocholate macrocycle **19** in a lipid bilayer membrane to minimize unfavorable water–lipid contact.

Cholesterol is known to increase the hydrophobic thickness of lipid bilayers and decrease their fluidity. Yet, the enhanced hydrophobicity caused by cholesterol facilitated the pore formation of **19**–**20** and increased the permeability of glucose across the cholesterol-containing membranes. Larger hydrophilic molecules normally have difficulty moving across a hydrophobic barrier. The cyclic tetramer (**20**), however, was more effective at permeating maltotriose than glucose, possibly due to the template effect of the trisaccharide, which could thread through multiple macrocycles to induce the pore formation.[60] A similar stacking mechanism was found to operate with "noncovalently linked" macrocycle **21**.[61] Unlike the covalent trimer (**19**), however, **21** could "breathe" and thus allow the passage of molecules larger than that its inner diameter.



The pore formation was confirmed by fluorescence spectroscopy. Macrocycle **22** displayed characteristic, concentration-dependent pyrene excimer emission in lipid membranes. (The triazole-linkage was introduced for synthetic efficiency.)[60] Most importantly, the excimer formation correlated with the hydrophobicity of the membrane, exactly as predicted by the proposed pore-forming mechanism. When the excimer/monomer emission ratio was fitted to the Hill equation, the Hill coefficient was ~1.5 in DLPC membranes, ~3 in POPC/POPG, and ~4 in cholesterol-containing POPC/POPG. These results showed that the number of the macrocycles in the aggregates correlated with the membrane thickness, in full agreement with the pore-forming mechanism and the leakage data.

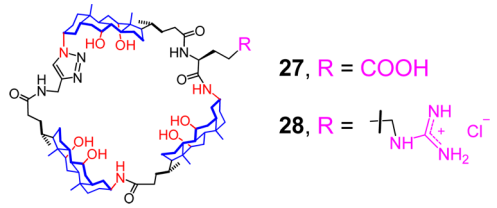
By labeling a cyclic (**23**) and a linear trimer (**26**) with a dansyl fluorophore, we were able to gain additional insights into how the morphology of the oligocholates impacts its aggregation in lipid membranes.[46] Environmentally sensitive emission, red-edge excitation shift (REES), and fluorescence quenching by water- and oil-soluble quenchers consistently supported the better penetration of the cyclic trimer into the hydrophobic core



of the membrane than the linear trimer. The linear trimer prefers to stay at the membrane/water interface; although it can aggregate intermolecularly and move inside the lipid bilayer at high concentrations, the aggregates tend to stay close to the membrane surface and equilibrate rapidly with the dissociated species on the surface. Such behavior is fully consistent with the inability of the linear trimer (**7**) to transport glucose. Similar conclusions were drawn in a solid-state NMR study.[62]

With the pore-forming mechanism firmly established, we became interested in using additional noncovalent interactions to tune the stacking. The idea was that, once we learned to control the stacking, we would be able to open and close the pore on demand. Macrocycles **24** and **25** were labeled with an aromatic donor (pyrene) and an acceptor (naphthalene diimide or NDI), respectively.[63] In the literature, the aromatic donor–acceptor interactions between 1,5-dialkoxynaphthalene and NDI were found to be 1–2 orders of magnitude stronger than the acceptor–acceptor interactions in *polar* solvents.[64] Our leakage data showed that the acceptor–acceptor interactions were far more effective at promoting the stacking of the oligocholate macrocycles in lipid membranes. The results underscore the importance of the environmental effect once again, and were attributed to the poor solvation of NDI groups by the lipid hydrocarbon.

Another surprise was found in the study of **27** and **28**.[65] The guanidinium–carboxylate salt bridge did not help the stacking, a result not surprising given the strong preference of charged groups for membrane surface and the competition from the phospholipid headgroups. What was unexpected, at least to us, was that the hydrogen-bonded carboxylic acid dimer turned out very helpful to the pore-formation. Competition, or the lack thereof, again seemed to be the reason for the oddity. Once a carboxylic acid enters a hydrophobic membrane, it has essentially no other way to lower its polarity except through dimerization. Strong dimerization, indeed, has been proposed to be the responsible for the fast flip–flop of fatty acids in common phospholipid bilayers.[66]



Concluding Remarks

What have we learned through these cholate-derived transporters? First, to translocate a relatively large hydrophilic guest across a lipid bilayer, the transporter has to create a hydrophilic microenvironment within the lipid assemblies. The microenvironment, commonly a binding pocket or a nanopore, is needed simply to shield the permeant from lipid hydrocarbon while opening a low-energy path through the hydrophobic barrier. Second, in addition to having the right size to accommodate the permeant, the hydrophilic microenvironment, whether formed within the covalent framework of the transporter or through the latter's supramolecular assembly, needs to have a fine balance of structural rigidity and flexibility. Rigid construction prevents the binding pocket or pore from collapsing under the lateral pressure imposed by the lipid molecules (which by hydrophobic interactions prefer to stay close together without internal hydrophilic pockets/channels). Flexibility is useful for modulating the transport mechanism, including the kinetics and selectivity. The 4-aminobutyroyl spacers in **5**,[25] the 1,3- versus 1,4-phenylene connector in **13** and **14**,[45] and the covalent versus noncovalent linkages in **19** and **21**[60,61] are good examples for this fine-tuning. Third, the most challenging and interesting part of a supramolecular game, including membrane transport, is probably to "teach" a transporter to bind, release, assemble, or do whatever molecular tasks desired. There is obviously no general solution to this problem but an important lesson was learned in the nanopore formation of the cholate macrocycles.[60] Water molecules are pulled into the membrane by the amphiphilic macrocycles but these water molecules are "unhappy" due to their exposure to lipid hydrocarbon. Stacking of the macrocycles is then "designed" to become the natural way for the entrapped water molecules to ease their "unhappiness". Essentially, in order to guide any molecules along a desired path, one has to *create* reasons for them to do so. These reasons are typically thermodynamic in supramolecular chemistry and encoded within the structure of the transporter and its interactions with the environments. Lastly, water should not be overlooked: although small in size, it can be the biggest player in a game of supramolecular water play.

The membrane transport research was supported by NSF (DMR-1005515). Some fundamental conformational study of the oligocholates was supported by DOE (DE-SC0002142). This account is dedicated to Prof. Steven C. Zimmerman. Y.Z. acknowledges all previous students whose names appear in the papers cited.

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FOOTNOTES

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