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Ion Transport through Lipid Bilayers by Synthetic Ionophores: Modulation of Activity and Selectivity

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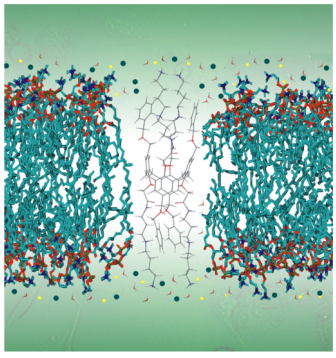
CONSPECTUS



The ion-coupled processes that occur in the plasma membrane regulate the cell machineries in all the living organisms. The details of the chemical events that allow ion transport in biological systems remain elusive. However, investigations of the structure and function of natural and artificial transporters has led to increasing insights about the conductance mechanisms.

Since the publication of the first successful artificial system by Tabushi and co-workers in 1982, synthetic chemists have designed and constructed a variety of chemically diverse and effective low molecular weight ionophores. Despite their relative structural simplicity, ionophores must satisfy several requirements. They must partition in the membrane, interact specifically with ions, shield them from the hydrocarbon core of the phospholipid bilayer, and transport ions from one side of the membrane to the other. All these attributes require amphipathic molecules in which the polar donor set used for ion recognition (usually oxygens for cations and hydrogen bond donors for anions) is arranged on a lipophilic organic scaffold. Playing with these two structural motifs, donor atoms and scaffolds, researchers have constructed a variety of different ionophores, and we describe a subset of interesting examples in this Account.

Despite the ample structural diversity, structure/activity relationships studies reveal common features. Even when they include different hydrophilic moieties (oxyethylene chains, free hydroxyl, etc.) and scaffolds (steroid derivatives, neutral or polar macrocycles, etc.), amphipathic molecules, that cannot span the entire phospholipid bilayer, generate defects in the contact zone between the ionophore and the lipids and increase the permeability in the bulk membrane. Therefore, topologically complex structures that span the entire membrane are needed to elicit channel-like and ion selective behaviors. In particular the alternate-calix[4]arene macrocycle proved to be a versatile platform to obtain 3D-structures that can form unimolecular channels in membranes. In these systems, the selection of proper donor groups allows us to control the ion selectivity of the process. We can switch from cation to anion transport by substituting protonated amines for the oxygen donors.

Large and stable tubular structures with nanometric sized transmembrane nanopores that provide ample internal space represent a different approach for the preparation of synthetic ion channels. We used the metal-mediated self-assembly of porphyrin ligands with Re(I) corners as a new method for producing to robust channel-like structures. Such structures can survive in the complex membrane environment and show interesting ionophoric behavior.

In addition to the development of new design principles, the selective modification of the biological membrane permeability could lead to important developments in medicine and technology.

Introduction

Ion transport across biological membranes is a typical supramolecular function which requires specific interactions between the host (carrier or channel) and the guest (the ion) in order to compensate the loss of hydration energy and to stabilize the dehydrated ion when crossing the phospholipid bilayer.[1] Nature has developed a large number of ionophores, that is, ion transporters, which

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alter membrane permeability through different mechanisms. These compounds are characterized by a large structural diversity, ranging from simple ion carriers or pore-forming molecules to the complex protein architectures of ion channels. However, structural studies and, in particular, the recent definition of the high-resolution X-ray structure of bacterial potassium[2] and chloride[3] channels suggest a unified approach based on hard oxygen donors to stabilize cations and hydrogen bond donor groups to interact with anions. These are the classical noncovalent interactions at the basis of Supramolecular Chemistry, and the recognition of this characteristic has stimulated a large body of studies motivated by mechanistic interests and by the potential applications of synthetic ionophores in technological[4] and biomedical fields.[5]

The main challenge in the design of synthetic ionophores is the correct assembly of donor groups in a well-defined and stable architecture in order to exploit the principles of preorganization and complementarity which may ensure high efficient and selective ion transport, and this is even more complex in the peculiar membrane environment. This has led to the exploration of a huge structural diversity, ranging from simple linear polyethers to complex multiporphyrinic architectures and passing through almost all the typical scaffolds used in Supramolecular Chemistry (macrocycles, peptides, steroids, etc.).[6] Although some remarkable efforts in the rationalization of the structure/activity relationship have been made,[7] the different experimental conditions and inhomogeneity of the data collected with different methodologies make it quite difficult to identify general rules of behavior. Common experiences suggest some useful guidelines: for example, elongated amphiphilic structures with several polar groups (alcohols, amines, etc.) and a length approximately matching the lipid bilayer thickness often show ionophoric activity in a monomeric or aggregate form.[8] Nevertheless, a unifying picture and a clear understanding of the factors influencing the ionophoric activity of synthetic compounds is still far from being achieved, hardly hampering the possibility to efficiently a priori predict their effective ion transport abilities.

Our interest in the control of the activity of synthetic ionophores started several years ago, when we were investigating the use of metal ions as allosteric elements in supramolecular hydrolytic catalysts,[9] and, at the same time, the transport of metal ions across phospholipid membranes exploiting hydrolytic processes as reporter mechanism.[10] In these systems, the complexation of a Zn(II) ion to a peptide-based ionophore switched off the ionophoric activity by inducing a transition from a membrane-spanning extended conformation to a calix-like structure, too short to span the membrane.[11,12] Since then, we have investigated a variety of different systems. In this Account, we describe our contribution to the structure/activity relationship studies on synthetic ionophores, focusing on how, by structurally modifying the molecular scaffolds and/or the donor groups, it is possible to achieve control on the efficiency and selectivity of the transport process. In our studies, we used large unilamellar vesicles (usually 100 nm diameter) made by natural phospholipids as model membrane.[1] In these spherical liposomes, a phospholipid bilayer insulate an inner water pool from outer bulk water and the two compartments may be differentiated by internal trapping of various fluorescent probes, by external addition of NMR shift reagents, of fluorescent quenchers or other specific reactants, and by the application of pH or ion concentration gradients. Depending on the assay chosen, the activity of the ionophore is then studied using commonly available and "easy to use" techniques such as fluorescence spectroscopy, NMR spectroscopy, or ion selective electrodes. In this way, activity data and ion selectivity may be readily collected allowing for a fast and internally consistent study of the structure/activity relationship. Therefore, activity studies in liposomes are a valid alternative to the more complex use of voltage clamp experiments across planar bilayer which is the traditional and, particularly in the biophysics community, preferred method for these studies.[7] The two approaches are complementary, although the second one allows for a more in deep mechanist characterization of the ion transport process and, for example, with vesicles-based experiments is not always easy a clear distinction between channel or carrier behaviors. Nevertheless, the use of liposomes is a fair compromise between complexity of experimental methodologies and quality of the information obtained and is particularly well suited for structure/activity relationship studies.

Steroid-Based Ionophores: Structure/Activity Relationship

Amphipathicity, and in particular facial amphipathicity, in membrane-spanning roughly tubular structures, is a frequent characteristic of natural and synthetic ionophores. We investigated the structure/activity relationships in amphipathic systems starting with polyhydroxylated steroid dimer **2**, synthesized from the precursor **1** in 16 steps (Figure 1).[13] Dimer **2** is 42 Å long in its extended conformation, therefore matching the thickness of the lipid bilayer,

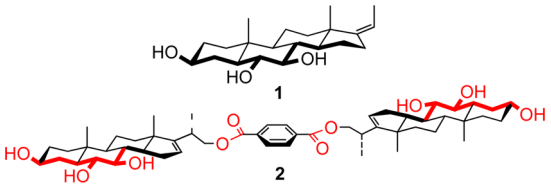


FIGURE 1. Structure of polyhydroxylated steroid **1** and its dimer **2**, with the hydrophilic portion marked in red.

and is characterized by a flat semirigid C_2-symmetric structure, lined on one side by the oxygen atoms, and on the other side by the lipophilic steroid skeletons. Therefore, the facial amphipathicity of the dimer should promote intramembrane self-assembly forming a tubular pore able to modify the membrane permeability. ^{23}Na-NMR experiments showed that **2** is indeed really efficient in transporting cations across liposomes, with a pseudo-first-order rate constant for the transport process $k_{Na} = 0.1\ h^{-1}$ measured at 1% ionophore concentration with respect to the total lipid concentration.[14] Hill analysis of the kinetic data suggested that the active species is a trimer self-assembled in the membrane. In this cluster, the hydrophobic face of the steroid derivative points outward, in contact with the hydrocarbon portion of the bilayer, while the hydrophilic face points inward, defining a cylindrical cavity lined by the oxygen atoms which stabilize the sodium cations crossing the pore. On the contrary, steroid **1** is completely inactive, underlying the requirement of a membrane-spanning structure for the formation of stable, active pores.

As an evolution toward higher synthetic accessibility and structural diversity, we prepared the steroid derivatives **3**–**7** (Figure 2).

Compounds **3a**,**b** structurally resemble dimer **2** but with the lipo- and hydrophilic portions well separated and assembled with a synthetically more accessible modular approach.[15] Steroids **4**–**7** represent further simplified systems in which the two portions are connected in a head-to-tail fashion and differ for the connection between the steroid moiety and the ethylene glycol chain (**4**, **5**), as well as for the number of hydroxyls on the steroid skeleton (**5**–**7**).[16] These molecules are still long enough to completely span the lipid bilayer; however, their amphipathicity is topologically different from that of the dimeric systems and, therefore, their self-assembling mode in membrane should be different. Surprisingly, all these systems, dimers **2** and **3** and steroid derivatives **4** and **5**, show very similar sodium transport activities, although with some differences in the Hill plots. This independence of the activity from the ionophore structure probably indicates that the pores formed, although with

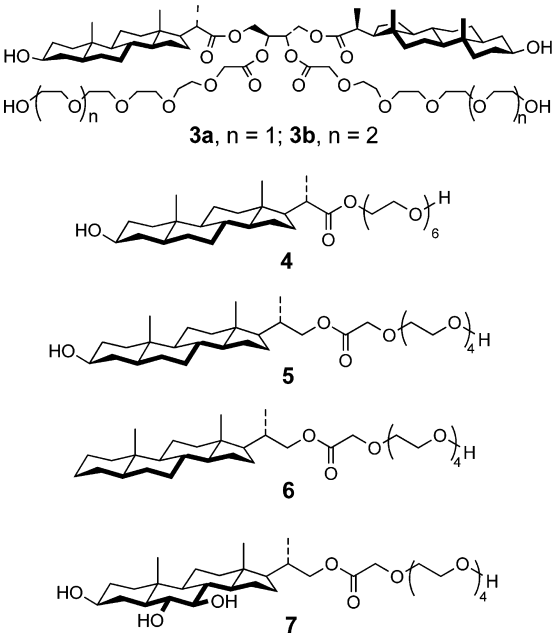


FIGURE 2. Structures of dimeric steroid derivatives **3a**,**b** and of head-to-tail amphipathic steroids **4**–**7**.

different structures, are all poorly defined and unstable; therefore, the observed effects are more related to local perturbations of the membrane structure rather than to the formation of a discrete channel.

A different situation was observed when studying the protonophoric activity of compounds **3**–**7** using a standard assay based on the response of the intravesicular pH-sensitive pyranine (HPTS) fluorophore to a transmembrane pH-gradient established by external addition of a base (usually NaOH). In this case, the dimeric compounds **3a**,**b** are far more efficient than the monosteroid derivatives, with **3b** ($k_H^+ = 0.056\ s^{-1}$ at 0.1% ionophore) about 70 times and **3a** ($k_H^+ = 0.046\ s^{-1}$ at 0.5% ionophore) about 12 times more active than **4** ($k_H^+ = 0.0037\ s^{-1}$ at 0.5% ionophore). This, in turn, results to be slightly more active than **5**–**7** (k_H^+ in the range 0.0016–0.0021 s^{-1} at 0.5% ionophore). Interestingly, Hill analysis of the kinetic profiles indicates that the active species is monomeric in the case of compounds **3** and **7** and dimeric for **4**–**6**. The reason behind such striking difference in the transport of Na^+ and H^+ is probably related to different mechanisms of transport. To cross the membrane, sodium needs to be desolvated and stabilized by interactions with the ionophore which compensate the hydration loss, while H^+ may be transferred by Grotthuss-type mechanisms, in which the proton hops from one molecule of water to the neighbor, along hydrogen-bonded chains of water molecules occasionally formed in the membrane. The role of the ionophore is therefore to stabilize this row of

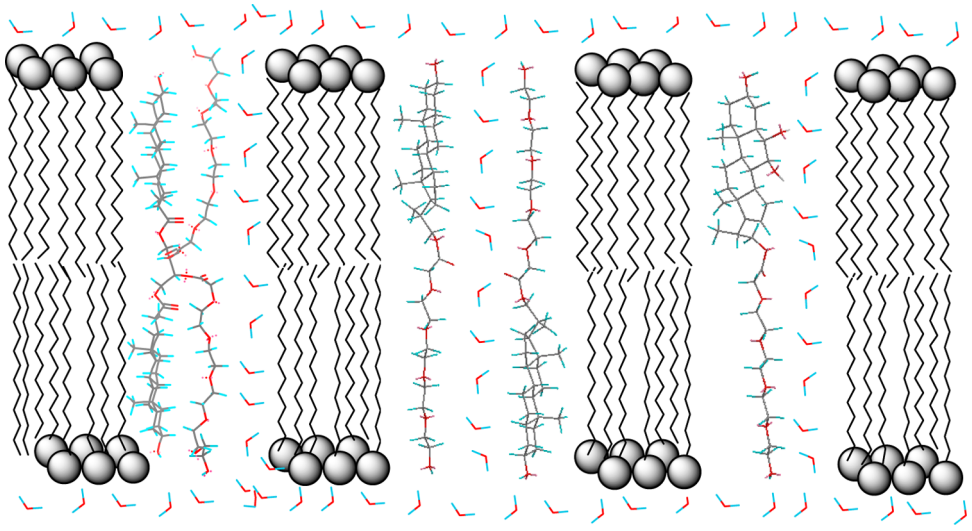


FIGURE 3. Proposed structures for the proton conducting pores formed by **3** (left), **4**–**6** (center), and **7** (right).

water molecules and this can be efficiently ensured by a single molecule of dimer **3** which presents a continuous polar surface spanning the entire membrane thickness (Figure 3, left). On the other hand, in order to form a transmembrane polar conduit, monosteroid derivatives have to self-assemble in dimeric structures, forming less stable clusters and promoting a less efficient proton transport (Figure 3, center). Compound **7** has an intermediate behavior: it is poorly active (for its higher hydrophilicity) and forms unimolecular pores (thanks to the continuous polar surface spanning the membrane; Figure 3, right).

Calix[4]arene-Based Ionophores as Selective Unimolecular Channels

As illustrated above, simple amphipathic molecules form self-assembled pores which are active in the permeabilization of the membrane, though poorly structured and scarcely suitable to promote selective transport of ions. To obtain unimolecular channels with well-defined structures, an evolution from a 2D- (e.g., the terephthaloyl group in **2**) to a 3D-preorganized scaffold was required. We, therefore, chose the versatile calix[4]arene moiety as a starting platform, preparing the steroid conjugates shown in Figure 4.

Compounds **8** and **9** were obtained by conjugating cholic acid derivatives, with different acetylation degree, with the calix[4]arene platform conformationally restricted in the *1,3-alternate* (**8**) and *cone* conformation (**9**).[17] The two conformers differ for the overall length which, from molecular models, was estimated to be ca. 35 and 25 Å, respectively. All the calixarene derivatives are effective in promoting the transport of sodium ions and protons across liposomal

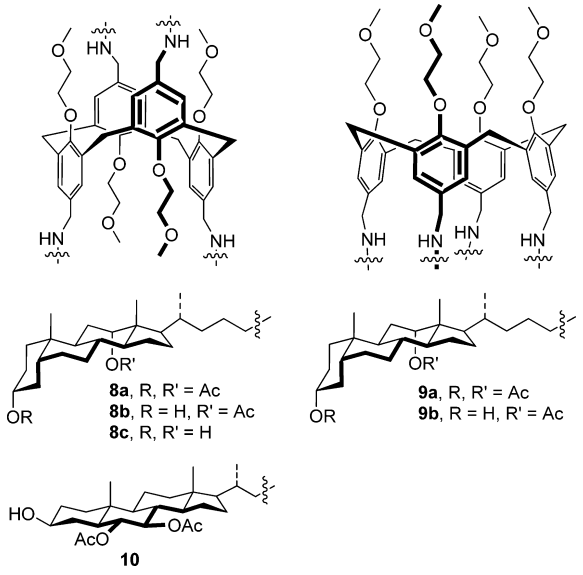


FIGURE 4. Structures of the calix[4]arene-steroid conjugates in the *1,3-alternate* (left) and *cone* (right) conformation.

membranes, with the *1,3-alternate* isomers being more active than the *cone* ones and with little dependence on the number of free hydroxyl groups (**8b** is 3 times more active in the transport of Na^+, $k_{Na}{}^+ = 0.12\ h^{-1}$ at 1.0% ionophore, and 8 times more active in the transport of H^+, $k_H{}^+ = 0.02\ s^{-1}$ at 1.0% ionophore, than **9b**). Although the rate constants for the transport processes are not particularly high, Hill analysis clearly demonstrated that conjugates **8** behave as unimolecular channels also in the case of Na^+ transport, thus confirming the correctness of this approach. Interestingly, the activity strongly depends on the structure of the steroid skeleton: compound **10** (characterized by the flat all-*trans* steroid framework instead of the "folded" AB-*cis*-cholane

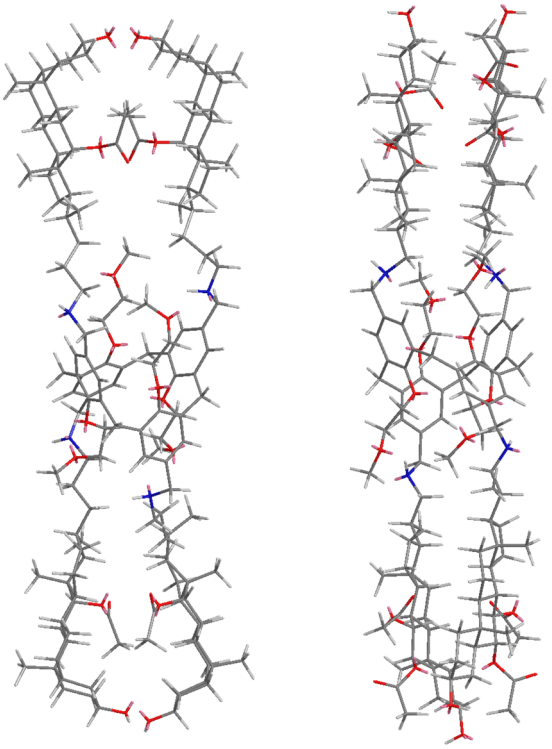


FIGURE 5. Energy minimized structures of compounds **8b** (left) and **10** (right) obtained by molecular modeling.

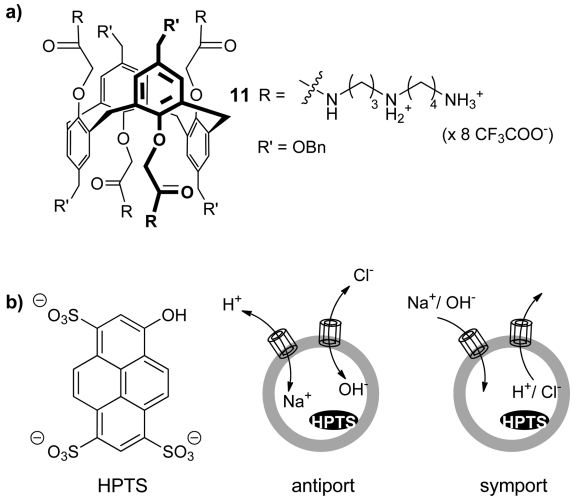


FIGURE 6. (a) Structure of the *1,3-alternate* calix[4]arene-spermidine conjugate **11**. (b) Structure of pyranine (HPTS) and schematic representation of the four possible mechanisms for pH gradient collapse in the HPTS assay in the presence of Na^+ and Cl^- as the transportable ions. Keeping Na^+ constant and changing the anion or keeping Cl^- constant and changing the cation gives information about the influence of the anion/cation nature on the transport rate.

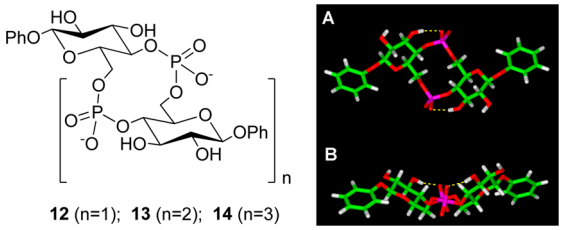
junction of **8**) is completely inactive in the transport of Na^+.[18] Inspection of molecular models of **8b** and **10** (Figure 5) shows that for an AB-*cis* framework (**8b**) the cholic acid residues define a cavity, while in the case of all-*trans* steroid junctions (**10**) the van der Waals forces stabilize the interactions of the facing flat steroid pairs, packing the lipophilic appendage in a more compact morphology. The preorganized inner cavity extending along the bilayer appears, therefore, essential to ensure an efficient Na^+ transport.

Having in hand a suitable platform for the construction of unimolecular channels, we investigated how to achieve cation/anion selective transport. In natural channels as well as in synthetic models, a switch from cation to anion selectivity is obtained by changing from oxygen donors[19] to hydrogen bonding or positively charged groups (often amine moieties). This was obtained by simply replacing the steroid subunits with spermidine chains, partly protonated at neutral pH (Figure 6a).[20]

Ion selectivity was investigated with a modified HPTS assay which addresses the symport or antiport of cations/anions associated with the transport of H^+ (or the kinetically equivalent transport of OH^-) induced by the transmembrane pH-gradient (Figure 6b).[21] Experiments in the presence of different cations and anions gave information on the ionophore selectivity. As expected, due to the positively charged spermidine chains, **11** is not able to transport cations and this was confirmed by ^{23}Na-NMR experiments. On the contrary, it is able to transport anions, showing efficient halides transport (with some selectivity toward iodide and bromide over chloride) and low transmembrane conductance of oxygenated anions (ClO_4^-, glutamate, NO_3^-, SO_4^{2-}). These data correlate well with a halide transport based on the lyotropic sequence (with the less hydrated anion transported more efficiently), but exclude from this trend the little hydrated nitrate ion and other oxygenated anions, suggesting, in these cases, a certain degree of anion binding with the ionophores. This was confirmed by inhibition experiments showing that ClO_4^- and glutamate act as channel blockers, suppressing the transport of chloride.

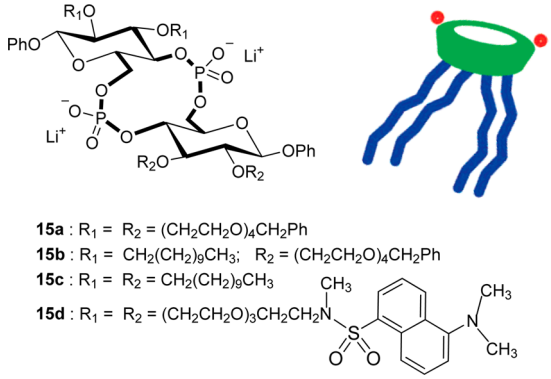
Sugar-Based Synthetic Ionophores

Macrocyclic backbones play an important role in the structural organization and modulation of the functional properties of synthetic ionophores, as demonstrated by the frequent use of these scaffolds in the literature.[6] Among them, cyclodextrins (CDs) occupy a special position because the first synthetic ion channel, reported by Tabushi in 1982, was a cyclodextrin derivative.[22] Since then, several CD-based ionophores have been prepared: however, the use of carbohydrates as structural motifs to build new synthetic ionophores is rare and essentially limited to CDs.[23] This is



12 (n=1); **13** (n=2); **14** (n=3)

FIGURE 7. Chemical structures of CyPLOS **12**–**14** and top (A) and side (B) representations of the best NMR structure of **12**, depicted with carbons in green; oxygens in red; hydrogens in white; phosphorus in magenta. H-bonds are represented with dashed lines.



15a : R_1 = R_2 = $(CH_2CH_2O)_4CH_2Ph$
15b : R_1 = $CH_2(CH_2)_9CH_3$; R_2 = $(CH_2CH_2O)_4CH_2Ph$
15c : R_1 = R_2 = $CH_2(CH_2)_9CH_3$
15d : R_1 = R_2 = $(CH_2CH_2O)_3CH_2CH_2N$

FIGURE 8. Chemical structures of amphiphilic CyPLOS derivatives and cartoon of the jellyfish-like conformation assumed in membrane in which the macrocycle is floating on the surface and the appended chains are dipped in the underneath phospholipid bilayer. The red spots are indicative of the negative charges of the phosphates.

somehow surprising because carbohydrates may result ideal starting materials in the design of novel artificial structures: in fact, they offer various advantages, such as the presence of several, chemically addressable functional groups (OH, NH_2, etc.), well-defined stereochemistry and large accessibility of different building blocks (monomers, dimers, etc.), with the possibility to a priori modulate the flexibility-vs-rigidity ratio in the target compounds. Our contribution in this field was the development of a new family of cyclic glycomimetics, containing phenyl-β-D-glucopyranoside monomers 4,6-linked through stable phosphodiester bonds (**12**-**14**, Figure 7).[24] These compounds, named CyPLOS from the acronym of Cyclic Phosphate-Linked OligoSaccharides, with respect to natural saccharide macrocycles present higher stability to acidic and enzymatic hydrolysis and a modular control of the ring size and functionalization, obtained through standard and well optimized oligonucleotide synthetic protocols.

CyPLOS are characterized by an anionic macrocycle skeleton which, depending on the size, may be arranged in different conformations. NMR studies and theoretical computations have demonstrated that while dimer **12** adopts a quite rigid, 2-fold symmetric, cradlelike conformation in D_2O and in DMSO (stabilized by strong H-bonding interactions between the phosphate groups and the adjacent 3-OH groups, Figure 7), for larger rings, such as trimer **13** and tetramer **14**, several conformations in slow solvent-dependent equilibrium are coexistent. Further synthetic elaboration of **12**, involving the insertion of linear chains of different lipophilicity at the free hydroxyl of the saccharide moieties, led to a set of amphipathic jellyfish-like CyPLOS derivatives **15(a**–**d)**, in which the anionic ring may be anchored to the polar surface of the membrane and the linear chains may be dipped in the phospholipid bilayer (Figure 8).[25–27] In this conformation CyPLOS derivatives topologically resemble the *1,3-cone* calixarene **9** (Figure 4), but with a polar macrocyclic scaffold which should direct the orientation of the molecule in the membrane.

Ion transport experiments with the HPTS assay showed that their activity is strictly correlated to the presence of tetraethylene glycol (TEG) chains: in fact **15a** ($k_H^+ = 0.0015\ s^{-1}$ at 1.0% ionophore) was about 8 times more effective than **15b**, while tetra-alkylated derivative **15c** was almost completely inactive.[28] Comparison of **15a** with a noncyclic and a phosphate-fully protected cyclic parent compound showed that the presence of the macrocycle and its anionic character are not prerequisites for activity, even if both guarantee a ca. 2-fold gain in activity, with the first structural motif more important than the second one. Hill kinetic analysis suggests a monomeric active species while ion selectivity experiments indicate insensitivity from the nature of the cation and a peculiar off-on selectivity for anions. Thus halogens (except fluoride), nitrate and perchlorate are efficiently transported - all at the same rate, i.e. without any apparent selectivity - and fluoride, acetate, glutamate and sulfate are scarcely transported, showing a clear discontinuity for anion hydration energies between those of acetate and chloride ions (Figure 9). These data point to a process rate-controlled by the transport of the anion (based on a OH^-/X^- antiport mechanism or the kinetically equivalent H^+/X^- symport) with a switch in the rate limiting factor from X^- to OH^- (or H^+) transport on decreasing the anion hydration energy. Highly hydrophilic anions are not transported, but when their dehydration cost decreases below that of acetate, the process is controlled by the OH^- (or H^+) transport and, therefore, all the anions with lower hydration energy are transported at a similar rate.

The dansyl groups linked to CyPLOS tentacles in **15d** allowed to obtain information on the insertion mode of the ionophore in membranes. In fact, the emission spectra of

dansyl exhibit large solvent polarity-dependent fluorescence shifts and, therefore, give information on the local environment experienced by the dye. By comparing the fluorescence spectra of **15d** in liposome and in solvents of different polarities, a Dimroth-Reichardt's E_T value intermediate between alcohols and less polar solvents was obtained.[26] This suggests that the dansyl groups of **15d** are located at the midpolar (glyceryl) region of the bilayer and are not deeply inserted in the apolar hydrocarbon portion of the membrane. Therefore, the mode of action of CyPLOS may be visualized as follows: the polar, negatively charged macrocycle lies on the surface of the membrane, with the four amphiphilic chains inserted in the midpolar region of the phospholipid bilayer. In the active conformation, the macrocycle anchors the molecule on the membrane surface, while the TEG chains destabilize the phospholipid bilayer underneath, therefore altering its permeability. The overall length of **15a** is insufficient to span the entire bilayer but activity is often observed in such "short" channel forming ionophores (see the *cone*-calixarene described above). Probably, the disorder in the phospholipid bilayer induced by the chains generates defects in the membrane which allow ion permeation in a moderately efficient though unselective process.

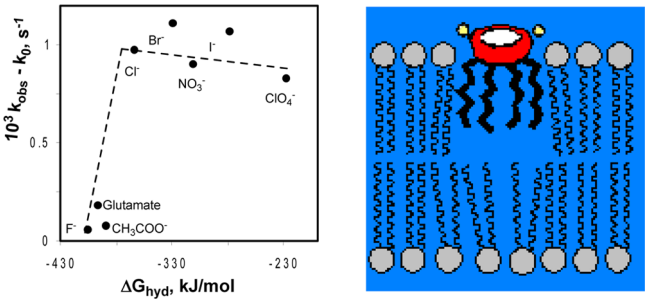


FIGURE 9. Left: Apparent first-order rate constant corrected for the unmediated electrolyte exchange ($k_{obs} - k_0$, s^{-1}) for **15a** (0.5% concentration) as a function of the anion hydration energy. Right: cartoon showing the insertion mode of CyPLOS derivatives within the membrane.

A similar behavior was observed within a small library of sugar-modified guanosine derivatives (Figure 10).[29] Activities in the HPTS assay increase from **16a** to **16c** as the number of the appended oligoethylene glycol chains increases, although remaining moderate on an absolute basis ($k_H^+ = 0.0004$ s^{-1} at 1.0% concentration of **16c**). The active species is monomeric and the rate of transport is independent of the cation. On the contrary, it is strongly influenced by the anions and increases going from fluoride to iodide, following the lyotropic sequence, thus suggesting a transport process governed by the anion translocation and essentially limited by its dehydration cost. Likely, as in the case of CyPLOS derivatives, the amphiphilic guanosines **16a**–**c** partition in the membrane, positioning the guanine moiety and the sugar ring close to the liposome surface, with the appended chains dipped in the membrane. Thus, the membrane is locally destabilized forming a disordered and more permeable zone, which ions are able to cross in an intrinsically poorly selective process, strongly correlated to the lipophilicity of the anions. Somehow disappointingly, while in chloroform these guanosine derivatives form K^+-promoted G-quadruplex structures, we did not observe influence of the potassium ion on their ionophoric activity. This suggests that in phospholipid membranes K^+ is not able to promote the guanosine derivatives self-assembly at the investigated low concentrations as, on the contrary, is observed for the lipophilic guanosine derivatives developed by Davis et al.[30]

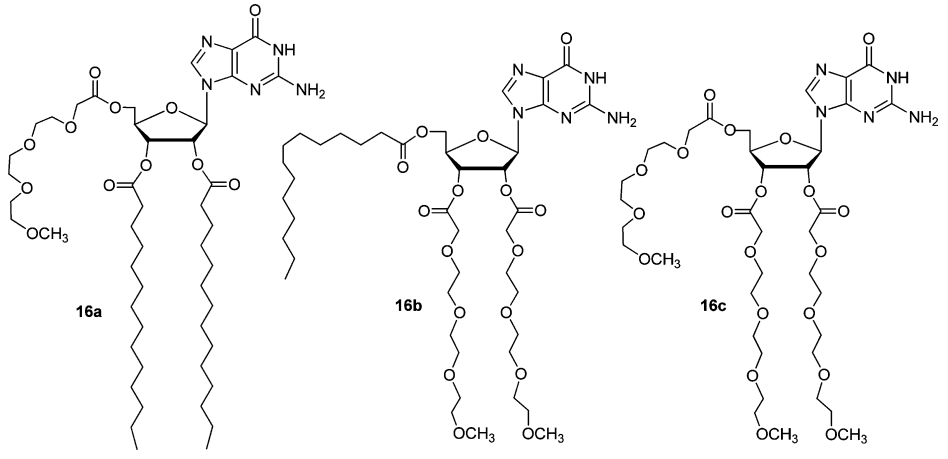


FIGURE 10. Chemical structures of amphiphilic sugar-modified guanosine derivatives **16a**–**c**.

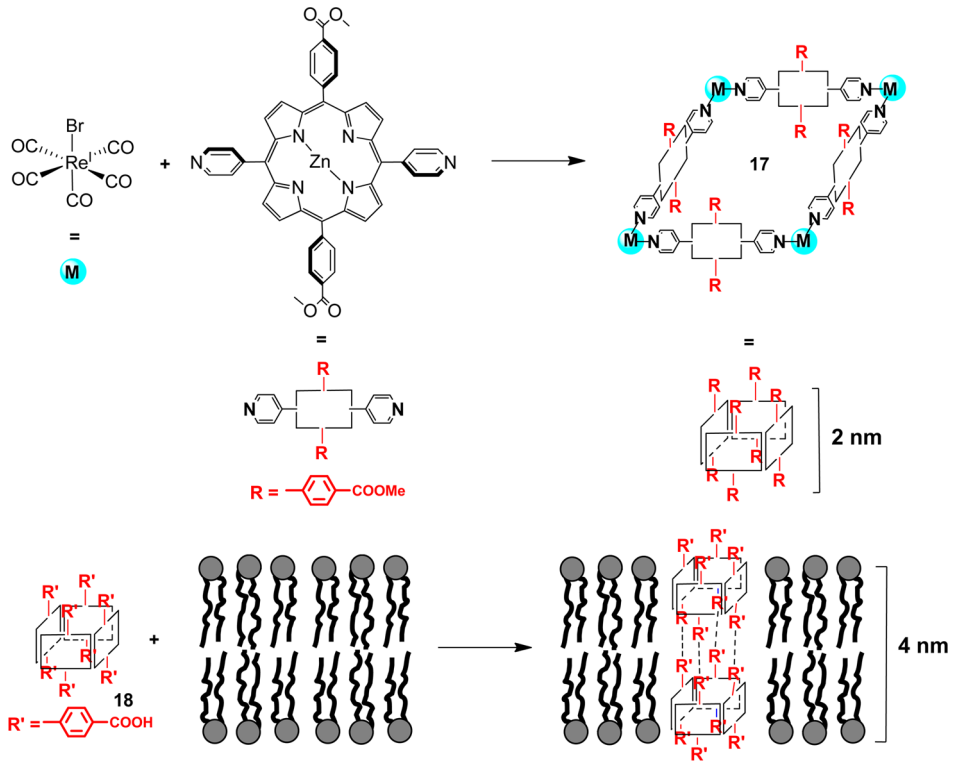


FIGURE 11. Schematic representation of the porphyrin metallacycle **18** and formation of a transmembrane nanopore upon hydrogen-bonding driven dimerization. The carboxy groups are obtained by hydrolysis of the corresponding esters in the preformed metallacycle **17**.

Metal–Organic Transmembrane Nanopores

The above-mentioned ionophores show interesting abilities in perturbing the permeability of a model membrane. However, although with relevant differences, the pores formed are poorly stable and little structurally organized. For several applications, in particular in the field of sensing, thermodynamically and kinetically stable structures forming large pores in the membrane are strongly desired.[4] Inspired by a recent report of Kobuke et al., describing a multiporphyrin covalent system that self-assembles into a dimeric cylindrical transmembrane nanopore with a nanometric sized inner diameter,[31] we decided to exploit the metal-mediated directional-bonding self-assembling approach, the so-called "Fujita-Stang" motif,[32] to develop new, stable structures able to form membrane-active nanopores. This approach has been largely used in the development of complex and functional molecular architectures, but it has found scarce applications in the field of synthetic ionophores.[33] Pioneering work from the groups of Fyles[34] and Webb[35] has demonstrated ionophoric activity in metal-mediated self-assembled systems. However, the use of Pd(II) as metal corner, giving kinetically labile complexes, did not allow to identify the active species among the different linear and cyclic adducts that may form upon self-assembly of the ligands with the palladium center. On this ground, we decided to investigate the formation of transmembrane nanopores using stable metal-mediated macrocycles of porphyrins. In our design, we chose a 10,20-*meso*-dipyridylporphyrin, a linear ligand that upon binding to a *cis*-coordinating metal fragment forms a 4 + 4 metallacycle about 2 nm large. Low spin d^6 *fac*-{$Re^{I}Br(CO)_3$} metal fragments were used as corners, since they are known to form kinetically inert and thermodynamically stable bonds with pyridyl ligands, thus avoiding ligand exchange or scrambling.[36] The resulting adducts roughly define an empty cube that spans ca. half of the thickness of the phospholipid bilayer. Therefore, to promote an efficient dimerization of the metallacycle, and thus the formation of transmembrane nanopores, each porphyrin unit was equipped with two 4-carboxyphenyl groups at the 5,15-*meso* positions. In the resulting tetraporphyrin metallacycle **18** four carboxylic groups point upward and four downward, therefore assisting a hydrogen-bonding driven dimerization of the metallacycle once inserted in the membrane (Figure 11).[37]

HPTS assay showed high ionophoric activity (k_H^+ = 0.011 s^{-1} at 1.0% concentration of **18**) strictly correlated with the preorganization of the metallacycle and with the presence of the peripheral carboxylic acid groups, suggesting

that **18** is indeed capable of forming the proposed transmembrane nanopore via hydrogen bonding dimerization. In this model, each metallacycle has four carboxylic groups pointing inside the membrane involved in the hydrogen bonding network that ensures dimerization of **18**, while the other four point outside, toward the (inner or outer) bulk water, and are likely deprotonated, thus efficiently directing the correct orientation of the metallacycle in the membrane (Figure 11). As expected for a nanopore with an empty internal space, the system does not show selectivity toward first group cations or inorganic anions (Cl^-, Br^-, I^-, NO_3^-, ClO_4^-). However, the large polyanionic calcein dye remains trapped inside the liposome, probably as a consequence of unfavorable electrostatic interactions with the negatively charged portal of the channel (i.e., the deprotonated carboxylic acids). Evidence in support to the proposed model came from the blockage effect of poly(amidoamine) (PAMAM) like dendrimers. This effect is strongly correlated to the size of PAMAM dendrimers and to the number of ionizable amino groups: **G0** is almost ineffective, **G1** reduces only partially the activity, while **G2** completely inactivates the channel. Likely, the cationic dendrimer interacts electrostatically with the carboxylic groups of metallacycle **18** hindering the channel entrance and thus hampering the ion flux. This effect becomes more and more important with the increase of the size and number of ionizable groups of the dendrimer, until the channel is completely blocked. Although further studies are required to better define the mechanism of action of metallacycle **18**, this example demonstrates the suitably of the "Fujita-Stang" motif for the development of stable and organized structures active in biological membranes.

Conclusions and Perspectives

Considering the relatively large structural diversity explored in the cited examples, it is somehow surprising that many compounds with completely different chemical structure show quite similar ionophoric behaviors. CyPLOS derivatives, steroid-based compounds **4**–**6**, *cone*-calixarene derivatives, and guanosine analogues **16** show similar proton conductivity in a moderately effective process, mainly governed by the lipophilicity of the anions. Clustering of effects in structurally unrelated compounds has been recently observed when comparing the ionic conductance of a large body of synthetic channels, and has been interpreted as the effect of additives in a bilayer membrane.[7] The simple insertion of compounds in the bilayer perturbs the membrane and, at the interfacial region between the ionophore and lipids, the formation of defects adjacent to the inserted compound becomes more probable. Compounds not able to span entirely the membrane, as the ones listed above, probably behave in this way independently from their structure. Thus, it is reasonable that, in the absence of specific interaction between the ionophore and the transported ions, the process is limited by the lipophilicity of the anions. More complex and interesting behaviors are observed with membrane-spanning compounds, such as steroid derivative **3** and *alternate*-calixarene **8** and **11**. In particular, the latter ones form unimolecolar channels with ion selectivity that can be switched from cation to anion by selection of the proper donor groups. Clearly, the tridimensional structure of these derivatives and the presence of properly positioned donor groups elicit a channel-like behavior. This observation seems to discourage the minimalist approach to the design of synthetic ionophores, calling for more challenging synthetic efforts. However, it is worth mentioning that for biological applications, for example, in the field of membrane active antibiotics, high activities related to simple chemical structures are by far more desirable than complex behaviors deriving from synthetically struggling compounds.

In recent reviews, Fyles[7] and Matile,[6] the editors of this special issue, encouraged the investigation of new types of transporters in order to expand the landscape on which to base a general discussion on these structure–activity relationships. In particular, Fyles suggests that useful inspiration for chemically innovative and potentially interesting ionophores may be found in structures containing well-defined cavities or enforced openings, such as the Matile's β-barrel structures.[38] In this direction goes our more recent contribution. Although many mechanistic aspects need to be defined, the metal-mediated self-assembling approach seems suitable for the construction of large and robust channel-like structures able to survive in the complex membrane environment. Thanks to the inherent modularity and versatility of this approach, the route to new classes of synthetic ionophores is now open and we believe that this will stimulate further developments in the field of artificial ion transporters.

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Daniela Montesarchio obtained her Ph.D. in Chemistry from Federico II University in Naples in 1993, supervisor Prof. C. Santacroce. After a postdoctoral experience in the lab of Prof. G. Just in McGill University in Montréal, she came back to Federico II University where since 2005 is associate professor of Organic Chemistry. Her research efforts are mainly devoted to the design and synthesis of hybrid systems at the interface between chemistry and biology, including oligonucleotide analogues, peptido- and glycomimetics for innovative therapeutic/diagnostic applications.

Paolo Tecilla obtained his Ph.D. in Chemistry from the University of Padova with Prof. U. Tonellato in 1989. After postdoctoral work with Prof. A. D. Hamilton at the University of Pittsburgh, he got a Lecturer position at the University of Padova. In 1998, he moved to the University of Trieste where he is now full professor of Organic Chemistry. His scientific interests are in the field of supramolecular chemistry and focus on metallo-catalysts of hydrolytic reactions, on fluorescence chemosensors, and synthetic ionophores.

FOOTNOTES

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The authors declare no competing financial interest.
Dedicated to Prof. Maurizio Prato on the occasion of his C60th birthday.

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