Transport of chloride ion through phospholipid bilayers mediated by synthetic ionophores

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Chloride has emerged as one of the most vigorously studied ions that occur in nature. A continuing motivation for the study of chloride transport lies in the pathological conditions that relate to or involve chloride transport. The goal of the organic, biological, or supramolecular chemist, thus, is to understand binding strength, binding dynamics, binding selectivity, transport selectivity and dynamics of chloride transporters. Various successful anion transporters or channels are now known, although detailed characterization and elaboration in most cases is ongoing. Several of these synthetic systems will be discussed in this review as we focus our discussion on the efforts to develop artificial transporters for chloride anions.

Introduction

It is probably not surprising that the ion composition of cellular life reflects that of seawater. By far, the most common metal in seawater is sodium, followed by magnesium, potassium, and calcium. These are the most common metals found *in vivo*. Likewise, chloride is overwhelmingly the most abundant anion in the sea. The cations mentioned above are all spherical although sodium and potassium are normally monovalent and calcium and magnesium are normally divalent. Chloride is also spherical but the situation is more complicated for most other anions that occur *in vivo*. These include carbonate, sulfate, and phosphate as well as their protonated forms. Bromide, iodide, nitrate, and various carboxylates are also found *in vivo* but their concentrations are highly variable depending on the organelle or fluid in which they are present. Ions such as fluoride that have been of great interest to the chemical community are rarely encountered in a biological setting.

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Chloride has emerged as one of the most vigorously studied ions that occur in nature. This is expected as it is both common and its transport is critical to maintain cellular osmotic balance. Recent insight[1–3] into the structure[4,5] of the ClC family of protein chloride transporters has intensified the study of chloride transport. A continuing motivation for the study of chloride transport and transporters lies in the pathological conditions[6] that relate to or involve chloride transport. The most common is cystic fibrosis[7,8] which is a genetic disease that occurs in northern Europeans. Other conditions include Dent's disease, which involves mutations in a renal chloride channel.[9]

Supramolecular chemistry and anion recognition

The knowledgeable scholar can trace the origins of supramolecular chemistry to a time far before Lehn's elegant discussion of the concept.[10] The watershed occurred when Pedersen reported crown ethers,[11] Lehn reported cryptands,[12] and Cram *et al.* began their pursuit of cavitands.[13] Taken together these efforts focused almost entirely on cations or molecules as guests and resulted in the joint award of the 1987 Nobel Prize in chemistry. Of course, the hosts were as varied as

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the innovators who designed them. Although the complexation of anions was clearly of interest to a few investigators[14–17] the field languished in the wake of the crown–cryptand[18] juggernaut.

Examples of complexing agents for various anions are now legion. The area has been described in both reviews[19] and monographs.[20–23] Although transport of anions has certainly been documented (see below), the complexation of anions has been the dominant theme for most of the last decade. It is, of course, important to define the interactions that occur between anions and receptors. Such an understanding is likewise important for comprehending transport. In the latter case, however, a certain paradox exists. For transport of a species to occur at all, a combination of binding and dynamics is required.

The goal of the organic, biological, or supramolecular chemist is to understand binding strength, binding dynamics, binding selectivity, transport selectivity and dynamics of chloride transporters. Ideally, one would like to mimic the properties of natural transporters. If this is accomplished, one would have a model that could be varied in structure to probe the mechanism(s) of transport. These are lofty goals but much work has already been done by chemists to address some of these issues. In the following discussion, we focus on the efforts to develop artificial transporters for chloride anions.

Cation *vs.* anion geometries

Alkali metal and alkaline earth metal cations are spherical, notably the biologically relevant cations: Na^+, K^+, Mg^{2+}, and Ca^{2+}. Other cations populate eukaryotic organisms but none is so plentiful as sodium, potassium, or calcium. The common cations are differentiated by size or charge or both. Sodium and potassium are singly-charged ions but they differ in size: 2.0 Å *vs.* 2.7 Å. Divalent calcium and monovalent sodium are almost identical in size but differ in charge. The common halides, F^-, Cl^-, Br^-, and I^- are all singly charged but differ in size. The anions shown in Fig. 1 differ both in charge and in geometry. They were rendered from solid state structures available in the Cambridge Structural Database (CSD).

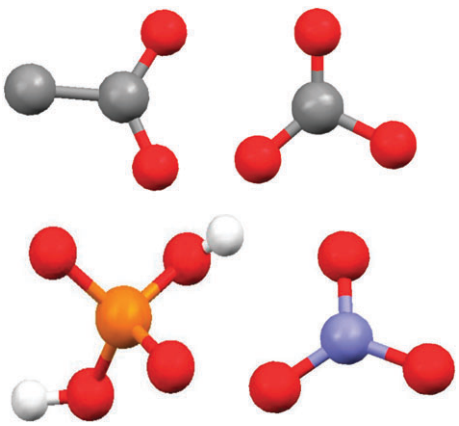


Fig. 1 Crystal structures of non-spherical anions. Top left: acetate; top right: carbonate; bottom left: dihydrogenphosphate; bottom right: nitrate.

The challenge of complexing a spherical cation was met with circular and spherical receptor molecules.[24] These include crown ethers and cryptands.[18,25] The complexation of non-spherical ions such as sulfate[26,27] has been achieved as evidenced by solid state structures of the host–guest ensembles.[28] The discussion below is focused on chloride transport. This requires some discussion on chloride binding as well because recognition cannot occur without at least some binding interaction.

The weak forces that complement anions

Covalent bonds build the primary structure of molecules but supramolecular assemblies all rely on weak force interactions. These can be grouped into three major categories: (a) attraction, (b) repulsion, and (c) exclusion of solvent. Attraction includes opposite charges or opposite polarities. Examples from nature would include salt formation, cation–pi interactions, the formation of $BF_3{\cdot}Et_2O$, hydrogen bonding including C–H interactions, salt bridge formation (Fig. 2), and even the affinity of an amine lone pair of electrons for a carbonyl carbon.

Repulsion arises from the presence of either similar polarities or similar charges that are proximate. Trinitrobenzene is electron poor and it has an affinity for electron rich mesitylene. Trinitrobenzene has no affinity for hexafluorobenzene, which is also electron deficient. The other, and probably more important, type of repulsion is steric hindrance. Two groups cannot occupy the same space so bulky compounds will not interact over short distances.

The third category of supramolecular interaction is exclusion of solvent. Similar entities, such as hydrocarbon chains, interact by dint of London forces. Polarizable systems can interact favorably owing to their local dipoles. The self-affinity of hydrocarbons in water also involves the exclusion of water. The favorable entropy of water (or solvent) exclusion is an important force but it is difficult to assess and predict in the design of supramolecular assemblies.

Two interactions have predominated in the quest to bind anions. These are interactions with positive charges such as quaternary ammonium cations and the formation of anion···H–N hydrogen bonds with amides. Ammonium cations R_4N^+ in which at least one R group is hydrogen have the advantage of both charge and an acidic N–H hydrogen bond. An example of a quaternary ammonium host molecule is the receptor designed by Schmidtchen and reported in 1977.[29] One of the most emulated amide H-bond anion binders was reported by Crabtree and coworkers.[30] Their structures are shown in Fig. 3.

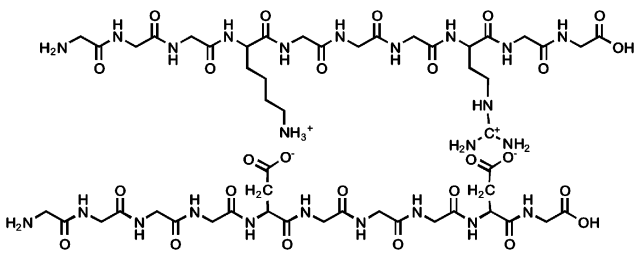


Fig. 2 Interactions between two peptide chains involving a lysine–aspartic acid and an arginine–aspartic acid salt bridge.

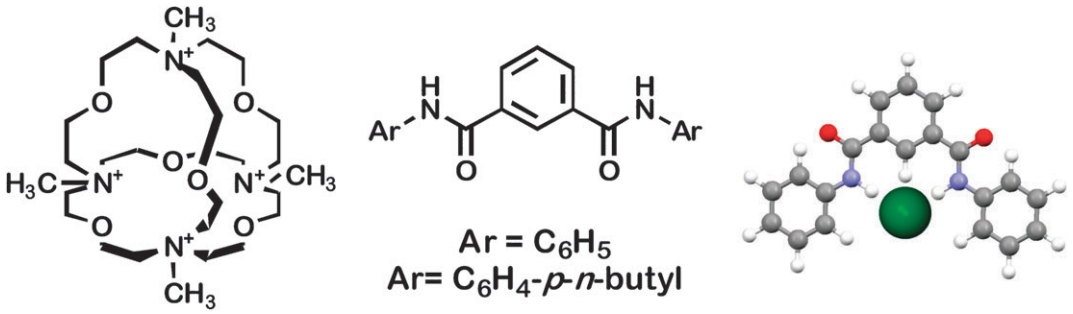


Fig. 3 Anion receptors. Left: Schmidtchen's spheroidal receptor.[31] Center and right, Crabtree's receptor and chloride complex.[32]

The dynamics of complexation

For any host and guest that form a 1 : 1 complex, the reaction is characterized by the equilibrium expression:

$$\text{host} + \text{guest} \underset{k_{\text{decomplex}}}{\overset{k_{\text{complex}}}{\rightleftharpoons}} \text{complex}$$

$$k_{\text{eq}} = k_{\text{complex}}/k_{\text{decomplex}}$$

The equilibrium constant K_{eq} that defines the extent of complexation is further defined by the rates at which the forward and reverse reactions occur. We may write this relationship as $K_{\text{eq}} = k_1/k_{-1}$, $k_{\text{forward}}/k_{\text{reverse}}$, $k_{\text{bind}}/k_{\text{release}}$, *etc*. In a homogeneous solution containing both the host and the guest, the equilibrium constant will vary with solvent, temperature, and possibly concentration. Typically, the more polar the solvent, the lower will be the equilibrium (binding) constant. On those occasions where binding constants are presented without clearly specifying the solvent, it is impossible to assess either the strength of the interactions or to make comparisons with other data.

The issue becomes more complicated when transport, rather than binding alone, is considered. The concept of transport requires something to go from one discrete location to another. Typically, transport of anions involves movement of the ion from one aqueous phase to another through a membrane. In the biological context, this could be from the periplasm to the cytoplasm through a phospholipid bilayer. This process could be modeled by transport through a liposomal bilayer or even by monitoring the passage of an ion through an organic solvent that separates two aqueous phases. In any of these cases, binding between the host and guest must occur or no complex will be present in the membrane. Binding must not be so strong, however, as to prevent dissociation of the complex at the second aqueous-membrane boundary or the transport process will come to naught.

The complexation of anions has been characterized by X-ray crystallography. This gives a structural view of the interactions between host and guest but it does not reveal information on dynamics. It is, of course, very useful to be able to understand the structure of a host–guest complex and it is especially useful to compare it with information about how readily the guest is bound and released by the host. Indeed, the remarkable progress made in the structures of protein chloride transporters,[4] important as they are, begs questions about mechanism and dynamics.

When the equilibrium association constant between host and guest is determined, it informs us of complexation strength in a particular solvent, but not the dynamics. Consider the reaction written above: host + guest = complex. Let us assume that the equilibrium constant for this reaction is 10^5. An equilibrium constant of 10^5 can result from forward and reverse rates of $10^8/10^3$ or $10^5/1$. In the latter case, ion release would likely be too slow for transport efficacy.

Although it involves cations rather than anions as guests, it is interesting to consider the complexation of Na^+ and K^+ by 18-crown-6 in water.[33] Both cations are bound by 18-crown-6 with a rate of 10^8 s^{-1}. The release rates for the Na^+ and K^+ ions are 10^7 and 10^6, respectively. This tells us two important things. First, the selectivity of K^+/Na^+ is approximately 10-fold and it is attributable only to the difference in release rates. The release rates are fast enough for 18-crown-6 to serve as a carrier because it can bind a cation but it releases it rapidly enough that the transport process is practical. In contrast, [2.2.2]-cryptand has binding and release rates of 2×10^5 and 27, respectively, for Na^+ in water.[34] The binding of sodium cation is strong indeed but the release rate is too slow for this macrobicyclic host to be useful as a transporting agent.

Measuring transport by using various dynamic methods is not without its problems either. A very useful approach is to use a glass U-tube[35] as done by Pressman and coworkers in their pioneering studies on valinomycin[36] and gramicidin.[37] The principle is simple. Chloroform is placed at the bottom of the U-tube and aqueous phases are placed in the two upper arms. Ions in one aqueous phase will travel down the concentration gradient to the aqueous phase in the other arm, but only if it can pass through the chloroform membrane (barrier). A chloroform-soluble complexing agent may mediate this transport process, but there are at least three essential requirements. First, the guest must be bound at the salt–chloroform interface. Second, the complex must be stable enough to diffuse to the opposite interface. Third, binding must be sufficiently weak and the decomplexation rate sufficiently high for guest release to occur at the second water–chloroform interface. If these three conditions are met, transport can occur down the concentration gradient until the two aqueous phases are of equal ionic strength.

This process is often likened to the operation of a ferry boat on a river and is referred to as carrier transport. The river is a metaphor for the membrane, while the ferry boat corresponds

to a host or carrier molecule. The host binds a cation on one side of the membrane and ferries it (by diffusion) to the other side. The guest (cation) is released and the host diffuses back to repeat the process. If all of the cations are on one side of the membrane (source phase), the concentration of cations in the receiving phase will gradually increase to 50%. Of course, transport will continue after that, but there will no longer be a concentration gradient and no further changes in concentration will be observed.

A common analytical expedient is to pair the cation with a colored anion such as picrate that can be detected quantitatively by UV-visible spectroscopy.[38] An alternative apparatus is sometimes called the "concentric tube" device. The bottom of a glass tube inserted in a beaker is covered with the dense organic solvent and the aqueous phases are placed above it in the tube or surrounding it.[39] The advantage of concentric, compared to U-tube, devices is that the former is stirred more easily and with less disruptive cavitation.

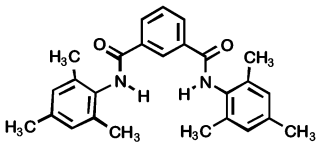
The formation of a channel or a pore in a bilayer membrane is considerably more complicated than the situation encompassed by the ferry boat analogy. A range of processes must occur before the transporter can mediate the passage of ions through the membrane. We may liken transport involving a pore or a channel to a tunnel rather than to a ferry boat. A critical issue is that the tunnel must be built, so to speak, before anything can pass through it. Even a prefabricated tunnel must be inserted into the bilayer in which it will function.

Natural bilayer membranes are extremely complex entities, but they can be modeled by phospholipid liposomes. Both carriers and channels can function in liposomal (or vesicular) membranes. If transport is effective in a U-tube device, it must occur by the carrier mechanism or by diffusion. Channel molecules, including channel proteins,[40] are not usually longer than 60 Å or so. The shortest path one can imagine for a U-tube is tens or hundreds of thousands of times longer than that. On the contrary, cations can be conducted through a bilayer either by a carrier or channel mechanism. It is here that a problem arises as discussed in the following section.

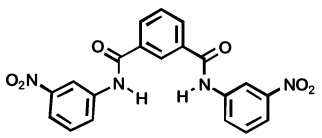
Methods by which chloride complexation and transport can be assayed

The strongest evidence for complexation of an anion by a host molecule is probably the solid state structure. When a solid state structure is obtained that shows an anion surrounded by an obvious host molecule, the inference of complexation is easy to draw. An example may be found in the structures of certain nitrated isophthalamides (Crabtree hosts[41]) that show solid state interactions with F^-.[42] Although the H-bond interactions between the host and F^- are obvious, there is no indication either of the complexation strength or the binding dynamics of this process. Solution binding of Cl^- by closely related molecules has been reported,[19] so it seems reasonable to assume that the nitrated isophthalamides bind F^- in solution as well as in the solid state. If binding inherently implied transport, the solid state structure would suffice. Of course, this is not the case.

Crabtree and coworkers demonstrated *inter alia* F^-, Cl^-, and Br^- complexation by **1** and the non-methylated parent compound. In addition, the X-ray structure of a Br^- complex was reported, as were solution binding studies involving NMR titrations, as well as energies estimated therefrom. The combination of solid state and solution data gives an excellent picture of the interactions that occur between host and guest. Unlike a solid state structure alone, both binding strength and selectivity is reported. Thus, a great deal more is known about **1** and its desmethyl analogs than is known about **2**.



1



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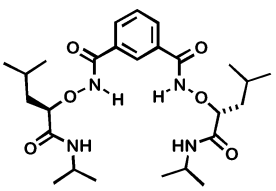


3

Assume that either **1** or **2** or both mediate ion release from liposomes. The efflux of Cl^- can be monitored by inserting a chloride-selective electrode (ion-selective electrode, ISE) in the liposomal suspension.[43] This procedure is described below. A typical ion release curve is shown in Fig. 4. The graph shows that ion release is rapid, although incomplete (<100%). Ion release from liposomes is an excellent way to evaluate various transporters. Although the preparation of liposomes is something of an art, once mastered, a number of ionophores can be evaluated in a reasonable period of time. The difficulty is that ion release can occur either by channel formation or by carrier transport through the bilayer and the release data may look similar.

Chloride transport studies are reported for isophthalamide **3**.[44] A fluorescence assay using the dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ, see below) was used to measure Cl^- transport into egg yolk phosphatidylcholine liposomes. As above, transport could result either from a carrier or channel mechanism. In this case, though, planar bilayer voltage clamp measurements (see below) showed that the membrane exhibited open–close behavior characteristic of channels. How the intriguing oxamide **3** forms channels has not been reported.

The three compounds discussed above demonstrate two issues that pervade the current study of anion transport. The first is the inherent complexity of transport function, even when structurally simple and closely related compounds are involved. Second, owing to the interest or focus of the laboratory reporting the results, incomplete data may be available for comparison. The solid state structures of

complexes involving **1** were reported without either complexation or transport data. Both structural and binding data were reported for **2**, but no information concerning transport. Binding and transport data were reported for **3**, but no structural information was obtained. In the latter case, the critical information concerns how such a small compound can form a channel in a bilayer membrane.

It is worth noting that Smith and coworkers have demonstrated NaCl or KCl transport across phospholipid bilayers by using more than one analytical method. Their transporter is based on the Crabtree isophthalamide receptor joined to a crown ether. Transport by the cation–anion binding hybrid molecule was studied by using ion selective electrodes and both ^{23}Na and ^{35}Cl NMR methods.[45] Likewise, these methods were used in combination to study Cl^- transport mediated by steroid-based receptors.[46]

Ion selective electrode methods to assay transport

Ion-selective electrodes can also be used to measure [Cl^-] in aqueous solution. In this experiment, liposomes are created in a chloride-containing aqueous buffer solution. The suspension is passed over Sephadex and through a filter to obtain uniformly-sized chloride-containing liposomes. The internal buffer is typically KCl and HEPES. The external buffer is normally K_2SO_4 and HEPES. The vesicles are tested for leakage prior to adding the ionophore. After addition, transport is assessed by an increase in Cl^- present in the external solution. After a specified time, the vesicles are lysed by addition of detergent and the final [Cl^-] reading is obtained. The latter value is assumed to be 100% Cl^- release and all values are normalized to it. The curve that is usually obtained is shown in Fig. 4.

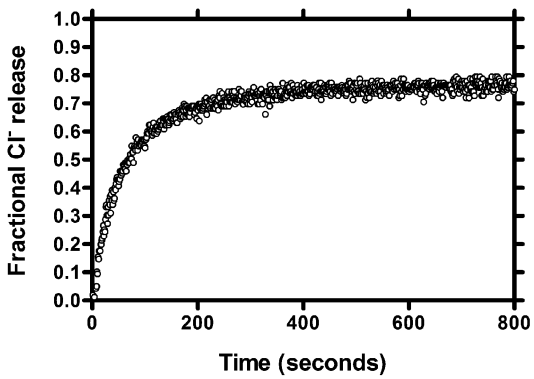


Fig. 4 Typical behavior for ion release from phospholipid vesicles mediated by a transporter.

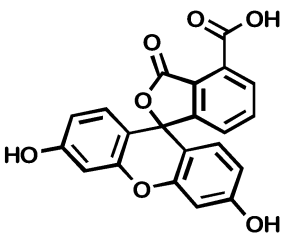
Comparisons can be made among several different ionophores by using identical concentrations of ionophores and comparing release at a fixed time point. Alternately, several different concentrations of ionophore can be tested under otherwise identical conditions to assess the concentration dependence of ion release. The relatively low sensitivity of ion selective electrodes often limits the concentration boundaries of this experiment.

Fluorescence methods to assay transport

Fluorescent dyes can be used in two different ways to monitor transport. These methods usually rely on quenching or dequenching of a fluorescence signal from the dye. Some dyes, such as carboxyfluorescein, self-quench when concentrated. Their release, from liposomes for example, places them in the far less concentrated external phase and a signal is detected owing to dequenching. Lucigenin, on the other hand, is fluorescent but its emission is quenched by the presence of chloride ions.

Carboxyfluorescein

By far, the most common dye assay involves carboxyfluorescein (CF). In this experiment, CF is trapped within vesicles as they are formed and the external buffer is replaced so that no dye persists in the bathing solution. The high concentration of CF within the liposomes causes self-quenching and only a little fluorescence is typically detected. When an ionophore is present that permits CF to pass through the bilayer, the dye passes into the bulk phase surrounding the liposomes and fluorescence is detected. Carboxyfluorescein is highly fluorescent and its release may be quantified over a large concentration range.



carboxyfluorescein

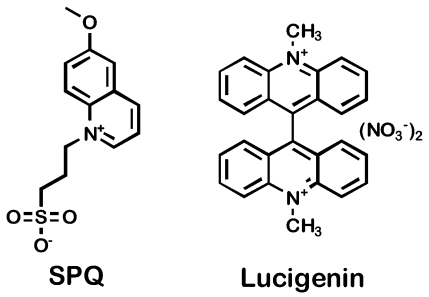
Transport of carboxyfluorescein

Carboxyfluorescein is obviously not Cl^-, but it is an anion. Carboxyfluorescein is larger than Cl^- but not so much larger than hydrated Cl^- as one might think. Although Cl^- alone has an ionic diameter of ~3.5 Å, it is estimated to be about 6.5 Å in diameter when fully hydrated.[47] Carboxyfluorescein is not symmetrical: its dimensions are about 6 Å × 10 Å. If CF is passing through a flexible pore, an oligomeric pore, or being carried through the bilayer, useful information can be obtained. If reproducible transport can be detected over a 10-fold concentration range, a Hill plot can be used to assess cooperativity.[48] If the slope of a plot of $\log_{10}$ ($v/V_{max}-v$) as a function of $\log_{10}$ [ionophore] is greater than 1, it suggests cooperativity. For a pore, the inference is that more than one monomer is required to form the conducting pore. If the slope is 2, two or more ionophores are likely to be involved in the formation of various pores within the structure.

Lucigenin and SPQ

Lucigenin and sulfopropylquinolinium, SPQ, are fluorescent dyes that are quenched by halide ions. As with carboxyfluorescein, the dye is trapped within a liposome. Fluorescence is detected however, because self-quenching does not occur and the interior of the vesicle is chloride-free. The solution

in which the vesicles are suspended contains chloride ion however, and addition of an ionophore that transports chloride passes the ion through the membrane into the dye-containing interior. This leads to quenching of the fluorescent emission that corresponds to the transport of Cl^-.[49]



Correspondence of dye *vs.* ISE methods

In principle, the correspondence between [Cl^-] release measured by the ISE method and the influx of [Cl^-] assayed by fluorescence quenching should be good. The processes differ, however, as do the experimental conditions at least because of the presence of dye in the fluorescence experiment. In addition, transporters may show rectification: transport into the liposome does not occur at the same rate or to the same degree as outward transport. Ideally, one would expect Cl^- release to follow a profile similar to the hypothetical increasing line in the graph of Fig. 5. Chloride influx detected by dye quenching should correspond approximately to the diminishing curve in the hypothetical figure. In fact, experimental differences are observed.[50]

Analytical methods used primarily for demonstrating complexation

As noted above, binding is necessary for recognition, but strong binding may be inimical to transport dynamics. Solid state structures of anion complexes give important insight into bond distances, angles, and molecular arrangement. Nothing about dynamics can be inferred from X-ray data, however. Three other methods that are commonly used to assess binding are calorimetry, NMR titrations, and negative ion mass spectrometry.

Calorimetry is a valuable tool that reveals information about the energetics of solution binding. It has been used extensively in studies on cation and anion binding by a range of macrocycles.[51] As is often the case, those skilled in the use of sophisticated instrumentation and productive synthetic chemists are divergent cohorts. There are, of course, exceptions.[52] Negative ion mass spectrometry is another tool that has been employed relatively little but is certainly useful for obtaining binding information. Of course, mass spectra are obtained in a vacuum. However, electrospray ionization methods are thought to reflect solution interactions and are of particular interest in anion complexation studies.

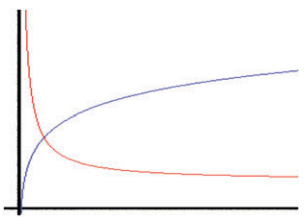
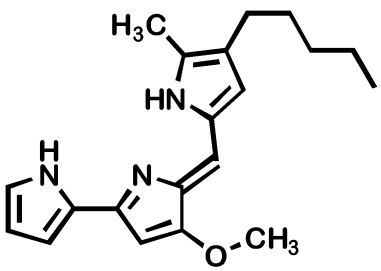


Fig. 5 Hypothetical curves for Cl^- transport detected by CF release (upper line) or lucigenin quenching.

The use of NMR titrations has been a common method to assess complexation strength in solution. This methodology is widely used but it is in any event, beyond the scope of this article. It is mentioned because there is rarely an effort to use more than one method of assay. The combination of mass spectrometry, NMR, and/or negative ion mass spectrometry would increase the credibility of binding strength values obtained by a single method.

Prodigiosin: a natural chloride carrier

Prodigiosin is a deep red colored pigment produced by the Gram negative bacterium *Serratia marcescens*. Colonies of *Serratia*, upon reaching maturity, display a blood-like fluidity and color. The bacterium grows on starch products, particularly bread, where it has the appearance of blood.[53,54] The apparent presence of blood on the Eucharist was seen as a miraculous or "prodigious" event in the middle ages.[50] The correct chemical structure (shown) was established by total synthesis reported in 1962.[55]



Several derivatives of prodigiosin have been discovered that vary in the alkyl substitution on the terminal pyrrole of the dipyrrolemethane subunit. We note that the term "prodigiosin(s)" is typically used to describe a class of molecules, rather than a specific structure.[50] The prodigiosin analogs exhibit similar biological activity but different potencies. Typically, prodigiosins exhibit activity against a wide range of microorganisms; however, their preference for pathogenic microbes over healthy mammalian cells is poor. The prodigiosin family has, however, shown encouraging activity against the human malaria parasite *Plasmodium falciparum*.[56]

Prodigiosins induce apoptosis in a broad range of human cancer cell lines with little or no effect on non-malignant cells. There are three known means of cytotoxicity, all of which appear to be crucial to activity;[50] however, the extent to which each mode plays a role is still unclear. Prodigiosin is known to have potent DNA damaging properties under oxidative conditions and to intercalate with DNA. It is also known to interfere with protein kinase C isozymes. Finally, prodigiosin has the ability to decrease the pH of cellular compartments through the symport of H^+/Cl^-.[53] The chloride binding affinity of prodigiosin arises, most likely, as a

consequence of proton binding followed by interaction with chloride. The transport mechanisms have been discussed elsewhere.[57]

The biomedical importance of chloride channels and synthetic transporters

During the past decade, interest in anion transporters by chemists has intensified and a range of anion complexing and transporting agents has been reported. A significant motivation to develop synthetic chloride transporters lies in the biomedical arena. Cystic fibrosis is the most common genetic disorder among those of northern European descent.[7,8] It affects about 30 000 adults and children in the United States alone.

The transport of chloride is critical to maintaining osmotic balance and the appropriate viscosity of various biological fluids. One of the ion channels that controls this balance is the cystic fibrosis transmembrane conductance regulator or CFTR. When its function is impaired, mucus thickens and lungs become congested, among other problems. A synthetic surrogate for the CFTR would therefore have considerable therapeutic potential.[58–60] The biomedical importance of chloride channels became apparent when studies on the pathogenesis of cystic fibrosis demonstrated that CFTR was a chloride ion channel as well as a regulator of other transport systems. In addition to cystic fibrosis, *myotonia congenita*, Dent's disease, and Bartter's syndrome have all been shown to be serious disorders involving chloride channel dysfunction.

Chloride distribution across membranes is presently an area of intense interest and some controversy. In many cells, E_{Cl} approximates E_M so that chloride distribution appears predominantly passive. Thus, the opening of Cl^- channels generally stabilizes the membrane potential.[61] The importance of excessive epithelial salt secretion in cystic fibrosis has been clear for decades and recently its association with chloride permeability and the CFTR has been demonstrated.[62] In addition, chloride transport is prominent in pH and volume regulation: processes fundamental to all cells.[63] The potential importance of these channels in cellular biology is already broad and they are just beginning to be appreciated in detail.

Synthetic channel model systems

Several channel model systems have been developed over the past two decades. These can generally (but not exclusively) be divided into peptide and non-peptide model systems. Examples of synthetic peptides include the so-called ''synporins'', developed by Montal and coworkers.[64] Mutter[65] and others[66,67] have used ''template-assembled synthetic proteins'' (TASPs) that were expected to form a four-helix bundle in a globular conformation that forms ion channels in lipid bilayers. DeGrado and coworkers[68] developed ''Ser-Leu'' peptides that were shown to exhibit cation transport properties remarkably similar to those found in proteins. In particular, DeGrado and coworkers prepared model peptides containing only leucine and serine residues. A 21-residue peptide H_2N-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)$_3$-$CONH_2$ formed ion channels, with ion permeability and lifetime (opening and closing) characteristics resembling the acetylcholine receptor.

A common shortcoming of studies in the synthetic transporter area is that channels designed to transport cations are often assessed *only* for cations. Early work in the author's lab suffered from this oversight. Of course, the earliest work in the synthetic channel area was focused on demonstrating effective transport of any type of cation[69–71] and selectivity was a secondary consideration. A number of successful transport systems have now been reported. Although touted as cation channels, some may also, or even preferentially, transport anions. It is certainly known that the selectivity of protein channels can be altered at appropriate potentials.

D,L-Cyclic peptide nanotubes

Ghadiri and coworkers[72] reported channel formation from self-assembling peptide ''nanotubes.'' Cyclic peptides incorporating alternating D- and L-amino acids such as *cyclo*[(Trp-D-Leu)$_3$Gln-D-Leu-] were prepared. If planar, the diameter of the internal orifice is 7.5 Å. The stacking of these cyclo-peptides is envisioned as involving H-bond formation similar to that found in a β-pleated sheet. The conductances (M^+) of Na^+ and K^+ chlorides were found to be 60 ± 5 pS. These nanotube channels have been shown to function as antibiotics.[73] No attempt to study anion transport was reported, but the efficacy of these compounds as antibiotics is significant.

Non-peptide channel models

An increasing number of synthetic ion channels has been reported in recent years. Several reviews are available to the interested reader.[74–76] The overwhelming majority of ionophores, transporters, pore-formers, synthetic ion channels, *etc*.have focused on the transport of cations. In many cases, including studies of our own, transport of cations was assayed and the results were reported. No information was reported concerning anion transport. Presumably, the absence of anion transport data meant simply that this was not assayed. One recent review deals with anion transport,[77] which is discussed further in the following sections.

Protein anion transporters

Modern protein channels or pore-forming molecules reside in cellular membranes and regulate the flow of cations, anions, water, and other species through the bilayer. While a great deal is known about what channels do (transport rates, kinetic and open–close behavior, ion selectivity),[78] the chemical mechanisms that underlie these processes remain largely obscure. The extremely active protein channel field has become even more energized in the years since the first channel structures appeared.[79,80] The protein channels known today are remarkably complex and it is certain that structures far simpler met the cellular transport requirement in the earliest organisms, however inadequately. The goal of much of the work in the synthetic anion transporter area has therefore been to develop simple and comprehensible channel models that will throw light on modern protein function. A second goal has been to develop a therapeutic agent that may be of

value to CF patients. An example of a successful approach is found in the studies undertaken in the Tomich laboratory. This work involves a modified peptide rather than a synthetic channel system in the sense described here, but the goal and success are apparent.

A semi-synthetic Cl^--conducting channel

Tomich and coworkers[81] modified the 23-amino acid sequence of the M2 segment of the α-subunit of the glycine-gated Cl^- channel. The sequence is H_2N-PARVG LGITT VLTMT TQSSG SRA-COOH. They have extended this sequence by the addition of four lysines at the carboxy terminus. The resulting sequence is H_2N-PARVG LGITT VLTMT TQSSG SRAKK KK-COOH. They have given this peptide the abbreviated name C-K_4-M2GlyR for C-terminus modified tetralysyl M2 glycine receptor.

It is interesting to note that the scrambled sequence, H_2N-ILAST RSQTG RMALS GTTTP GVVLL LL-COOH, is inactive. Note that the proline has been moved from the N-terminus of M2GlyR to position 20 in the scrambled version. This issue will be discussed below, but for the present it is important to note that Tomich and coworkers have demonstrated (1) Cl^- channel activity, (2) enhanced solubility by incorporation of the tetralysyl terminus, and (3) increased capacity for epithelial anion secretion when studied in Madin–Darby canine kidney and T84 cell monolayers. These are important findings demonstrating the potential of chloride channel models in therapeutic applications.

Abiotic, synthetic models for ion transport

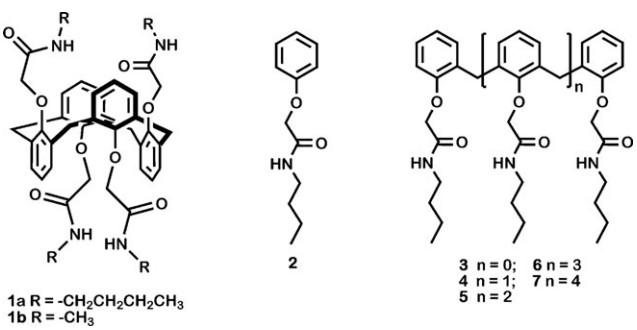
Even today, our understanding of the chemical mechanisms that control "specificity" is poor. The selectivity of a cation-conducting channel for Na^+ over K^+ or *vice versa* is of obvious importance, but the question of why a protein conducts cations rather than anions is equally significant. One hypothesis is that anion-conducting channels possess positive charges at one or more locations in their structure, while cation channels are endowed with negative charges.[82] Such speculation is certainly not unreasonable, but compelling experimental evidence is limited. From the chemical perspective, it seems unreasonable that a highly charged structure could transport an oppositely charged species with reasonable throughput—that is, complexation rather than transport would be the favored process. The cryptands, for example, are excellent alkali metal cation complexing agents that selectively encapsulate a cation of appropriate size. They are, however, poor cation transporters because their cation release rates are extremely low. The same concern presents itself with respect to channels: would a full positive charge permit an ion of negative charge to pass or would the anion be bound?

Many in the biological community have already accepted the value of model channels. Such channel-forming peptides as gramicidin,[83] alamethicin,[84] melittin,[85] and others have been extensively studied in the hope that these "simplified proteins" would yield functional information. Gramicidin is an interesting example. It certainly forms robust, cation conducting pores in bilayer membranes. It is a pentadecapeptide that dimerizes into a channel structure;[86] gramicidin has been a subject in approximately 7000 reports during the last 30 years. If it was not already a part of the landscape, would it be considered a good channel model? It is utterly unlike the KcsA channel. It is *not* formed from α-helices but rather is a π-helix. Its polar residues are *not* localized to a central "capsule" as in the KcsA channel; rather the pore is lined with main chain amide donors. The negatively charged headgroups of the KcsA channel are completely lacking in this accepted channel model. Gramicidin's position in the bilayer is stabilized by a series of tryptophans. Finally, even though gramicidin is so extensively studied and so well accepted as a channel model, it has been the subject of a controversy concerning its functional structure.[87]

In part, because of the extensive studies undertaken on gramicidin, peptide channels have gained greater credibility during the past decade and, accordingly, have been more extensively studied than any other synthetic system.[88] Gramicidin has the great advantage that it is a naturally occurring material and therefore, possesses an inherent legitimacy. It also has the great advantage of function. Like many synthetic organic channel models, its function mimics, but does not duplicate, that of protein channels. Unlike proteins, gramicidin is composed of a mixture of D- and L-amino acids. The KcsA potassium channel organizes a collection of α-helices that form the outer walls of the pore. Cations pass among the α-helices. Gramicidin forms a coil that probably is organized end-to-end. Cations pass through the inside of this coil and may interact directly with main chain carbonyl groups rather than with π-helix sidechains. Notwithstanding these differences, the thousands of studies on gramicidin have advanced our understanding of channel phenomena. The same will likely be true of synthetic anion channel transporters.

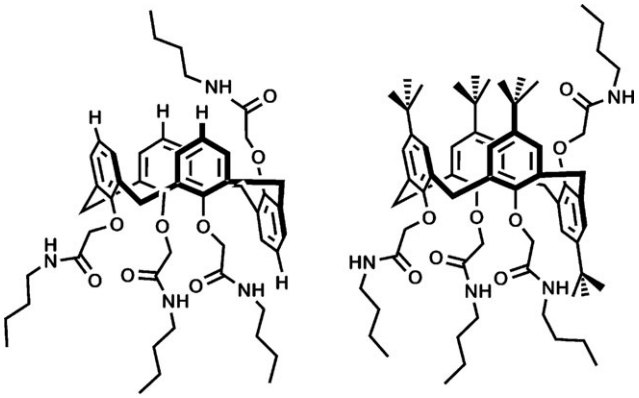
Calixarene-derived chloride transporters

Davis and coworkers demonstrated H^+/Cl^- symport across a lipid bilayer mediated by calixarene **4a**, while the methyl analogue **4b** was inactive.[89] Planar bilayer voltage clamp studies revealed the presence of stable channels, while patch-clamp experiments on HEK cells demonstrated that **4a** mediated voltage-dependent chloride transport.[90] Obviously, calixarene **4a** is not of sufficient size to span the membrane alone. A crystal structure obtained for the methyl analog **4b** revealed that the *1,3-alt* tetraamide motif is capable of organizing, in the presence of Cl^-, into two distinct channel-like structures.[91]



The crystal structure of the **4b**·HCl complex did not show any involvement of the arenes in anion complexation.[88] Six analogs were prepared in which the aromatic rings were retained but the cyclic calixarene structure was not (**5–10**). Trimer **6** was found to be the most efficient chloride transporter, working efficiently even at concentrations of 5 μM (1 mol%).[92] Indeed, **6** was an order of magnitude more active at this concentration than was calixarene **4**. Thus, the initial calixarene backbone is not a crucial component for anion complexation. Additional structural variations showed that amides are crucial to anion complexation, but ester analogs of **6** were completely inactive.[89]

Later studies with calixarene-based transporters showed that **11** was an effective Cl^- transporter but its *tert*-butyl counterpart arene **12** was not.[93] Solid state structures suggested that when the *tert*-butyl groups were present (**11**), steric hindrance prevented the cone organization apparently required for transport. The structures suggested that in the solid state at least, intermolecular interactions involving the amide N–H of **12** take place. To the extent that such interactions occur, chloride transport activity would diminish.



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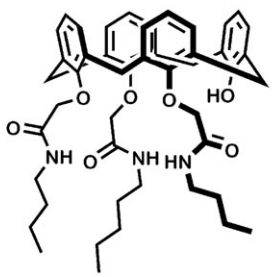

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Further studies on receptor **11** showed that its behavior resembled channel function. In dose-dependent studies, **11** was found to demonstrate pseudo-first-order rate kinetics when increasingly contaminated with inactive **12**. It is also reported that voltage clamp experiments were conducted on these systems, although the data are not presented.[90] Calixarene transporters sensitive to pH have recently been prepared and studied.[94] The activity of **13** was found to decrease as the extravesicular pH was increased, while the activity of a tetraamide control compound did not change. This difference in activity was rationalized by assuming that, when deprotonated, the negative charge on the phenolic oxygen results in unfavorable electrostatic interactions with Cl^- although the mechanism by which transport occurs was not reported.

Cyclodextrin-derived chloride transporters

It is notable that the earliest synthetic channel, prepared by the late Iwao Tabushi and coworkers,[69] was based on cyclodextrin. Gin and coworkers elaborated the β-cyclodextrin module with penta(butylene glycol) chains appended at the primary hydroxyl, which was converted to an amine prior to coupling.[95] The chemical structure is illustrated on the left of Fig. 6 along with a schematic representation of the structure. We refer here to the compound as a hydrobutylated amino-cyclodextrin or hbaCD.

The ^{23}Na-exchange NMR method devised by Riddell and Hayer[96] was used to demonstrate channel function in liposomes. The Na^+ transport activity of hbaCD was found to be 36% that of gramicidin A. Proton transport was also demonstrated by using the pH-sensitive fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HTPS). Little difference in transport was observed when LiOH, NaOH, or KOH were compared. In contrast, when NaCl, NaBr, and NaI were studied, transport efficacy was found to be $I^- > Br^- > Cl^-$.

Further studies were conducted with this compound in an effort to characterize the pH dependence of transport. As above, the ^{23}Na NMR method[97] was used to determine Na^+ cation exchange rates through liposomal bilayers. The vesicles were prepared at pH values of 5.6, 6.3, 7, 8, 9, and 10 and suspended in buffers of the same pH. The observed Na^+ exchange rate (into or out of the vesicles) was somewhat higher at pH values below 7 than at pH = 7. At pH 8, 9, or 10, transport was twice that observed at low pH and about threefold higher than at neutral pH. Halide transport proved more difficult to assess, but both Cl^- and Br^- appeared to be transported more rapidly at higher pH. Iodide has a high membrane permeability and could not be compared in these studies.[98]

Jog and Gin have recently reported the attachment of azobenzene to the larger rim of the cyclodextrin torus.[99] Azobenzene undergoes photochemical *E–Z* isomerization that has been used by Woolley and coworkers[100] as a gate in linked gramicidin monomers and a variety of other peptides.[101] The Jog and Gin design attached azobenzene through one of the 14 secondary hydroxyl groups. When azobenzene was in the ground state *trans* conformation, it inserted into the cyclodextrin's hydrophobic cavity partially blocking transport. When photo-switched to the *cis* configuration, the biarene was less extended, less able to block the hbaCD opening, and ion flux was greater. Photochemical switching is an appealing means of altering transport but it is typically much slower than the open–close transitions observed in proteins and in most synthetic channels.

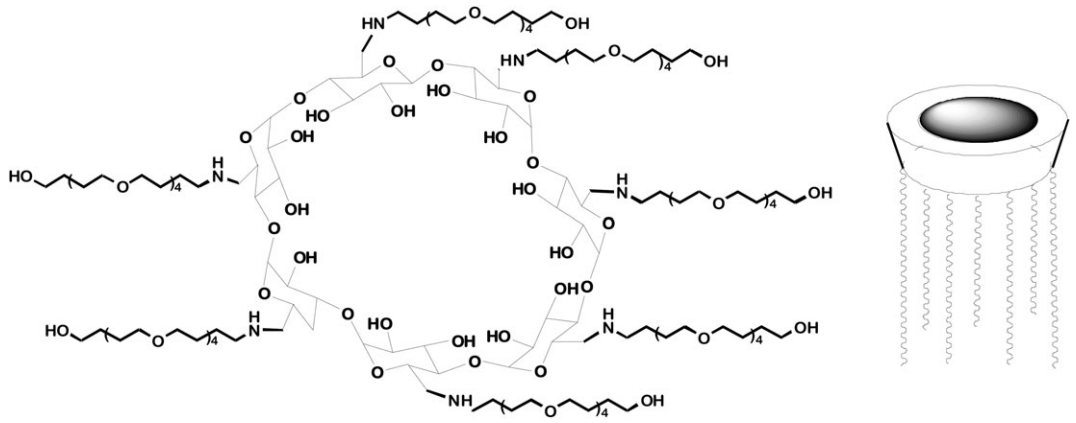


Fig. 6 Structure of a hydrobutylated aminocyclodextrin (hbaCD), left, and a schematic representation.

Oligophenylenes and pi-slides

Matile and coworkers began work in the late 1990's with attempts to mimic the naturally occurring, non-peptide, polyene macrolide antibiotic amphotericin B (AmB).[102] Amphotericin has been referred to as a "rigid-rod" molecule. It is thought to form pores in cellular membranes by organizing into "barrel stave" aggregates.[103,104] Matile and coworkers estimated that synthetic pores would form from rigid rods if they were of appropriate length (~36 Å) and incorporated the three domains bracketed in Fig. 7. These are (A) a terminal anchor at the bilayer–water interface, (B) rigid, hydrophobic, polyene rods which allow favorable interactions with membrane components, and (C) polyols which function as ionic relays.

Matile and coworkers initial rigid-rod channel, reported in 1997, was composed of an octa(*p*-phenylene) backbone having protruding glyceryl residues.[102] The compound was found to function as a unimolecular proton channel. The basic structure is shown in Fig. 8. Three compounds were prepared in an effort to define the length relationship between the transporter and a bilayer membrane. A phospholipid bilayer is typically quoted as having a hydrocarbon or insulator regime that is 30–35 Å thick. Three compounds were prepared that possessed the repeating biphenyl unit. They were estimated to be ~17 Å (tetraphenyl), 26 Å (hexaphenyl), and 34 Å for the octaphenyl rigid rod. The octaphenyl chain was found to be threefold more active than the hexaphenyl rod, and the tetraphenyl rod was inactive. It was speculated that the low activity of the hexaphenyl rod resulted from a combination of being too short to span the insulator regime of the bilayer and a poorer insertion dynamic.

A test that is often used to distinguish carrier from channel function is to alter membrane fluidity. A conductance pore is not affected significantly by a change in rigidity but the diffusive activity of a transporter complex will be altered. Phospholipid bilayers exhibit increased organization in the presence of steroids such as cholesterol (common in mammalian membranes) and ergosterol (common in fungi). The lack of significant variation in the transport rate comports with a

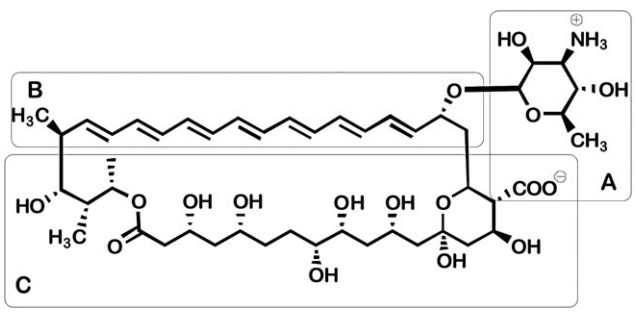


Fig. 7 Amphotericin B (AMP B). Shown are the three domains needed to form barrel staves: (A) an anchor; (B) polyene rods; and (C) polyols.

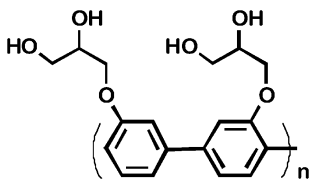


Fig. 8 Proton-conducting, oligophenylene rigid rods. The compounds studied had $n = 2$, 3, and 4.

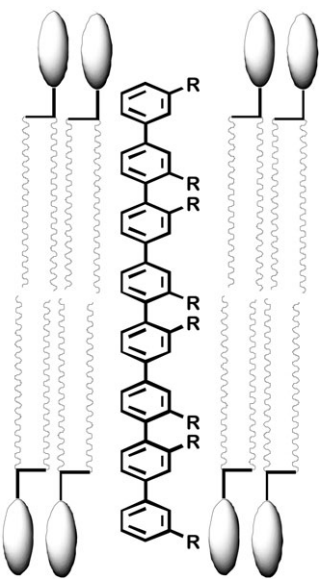


Fig. 9 Schematic representation of an octaphenylene derivative inserted into a phospholipid bilayer.

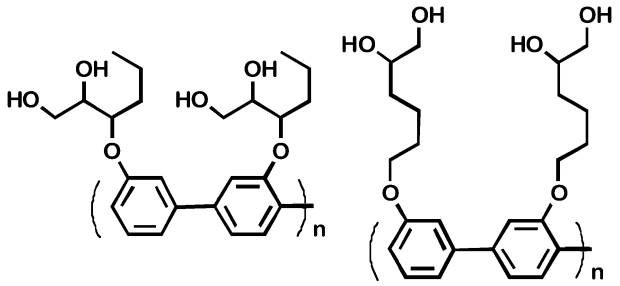


Fig. 10 Hydroxypropylated oligophenylene amphiphiles.

channel or pore mechanism for the oligophenyl compounds. It is notable that the octaphenyl compound was found to conduct chloride through the bilayer.[105]

Despite the success of the oligophenylpolyols as transporters, their insertion into lipid bilayers was problematic. In the development of a second generation of rigid-rod receptors in 1998, Matile recognized a potential difficulty. When the oligophenylene chain is oriented parallel to the fatty acid chain axis, the "R" sidechains shown in Fig. 9 are orthogonal. Whatever the identity of R, the groups must insinuate among the hydrocarbon chains. The compatibility of hydrocarbons and hydroxyl groups is obviously poor. Hydrophobic sidechains were therefore introduced into the rigid-rod molecules in the expectation that solubility would be enhanced.[106]

Two different structures were prepared, both based on the octaphenylene framework. The rigid backbone defines the length of the structure, which is 34 Å. Two variations in R groups were incorporated. In both cases a propylene spacer was included, either substituting or extending the glyceryl terminus as shown in Fig. 10. Both compounds were incorporated more extensively into the bilayer than the parent molecule. Additional studies suggested that the overall orientation within the bilayer was unaltered. Both compounds showed higher proton transport by a factor of about 10^2 compared to the non-propylated parent compound. The greater transport could result simply from a greater extent of insertion. Possible anion transport in these systems was not explored if studied.

Matile and coworkers further elaborated the oligophenylene scaffold to use its π-acid–base properties. The concept of cation–π interactions is well known[107] and has been thoroughly studied by experimental and computational methods.[108] Anion–π interactions are less well studied than are cation–π contacts and remain somewhat controversial.[109] In any event, Matile's hypothesis was that if π-basic *p*-oligophenyl rods function as cation–π scaffolds, then electron-deficient oligo-(*p*-phenylene)-*N,N*-naphthalenediimide (O-NDI) rods should be able to function as anion–π slides (Fig. 11).[110] High-level DFT calculations conducted in advance of experiments confirmed feasibility of the NDI's to participate in anion–π interactions.[111] Even so, the first generation NDI rods were not notably active, due in part to poor transport and in part to "too high" selectivity.

Three second generation π-slide structures are shown in Fig. 12 as **13**, **14**, and **15**. The most effective transport was observed when the oligoarenes were terminated by one charged and one neutral residue. The poorest results were obtained when the termini were both charged. Thus, transport

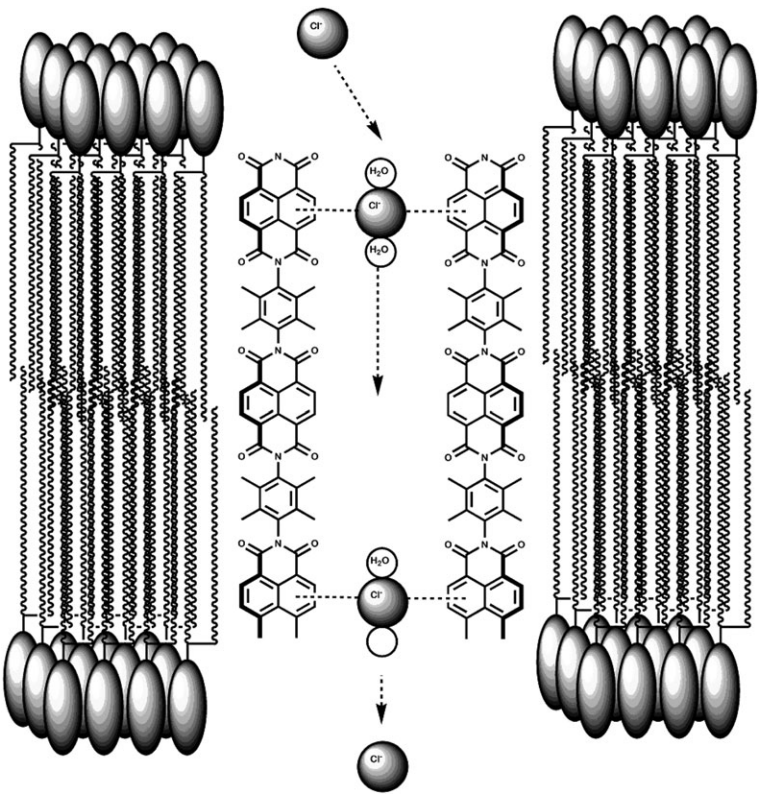


Fig. 11 Concept of anion–π slides in lipid bilayers.

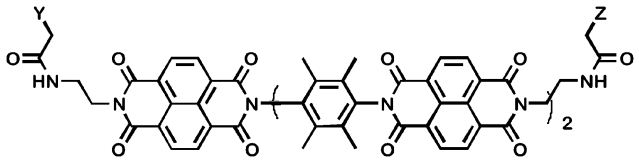
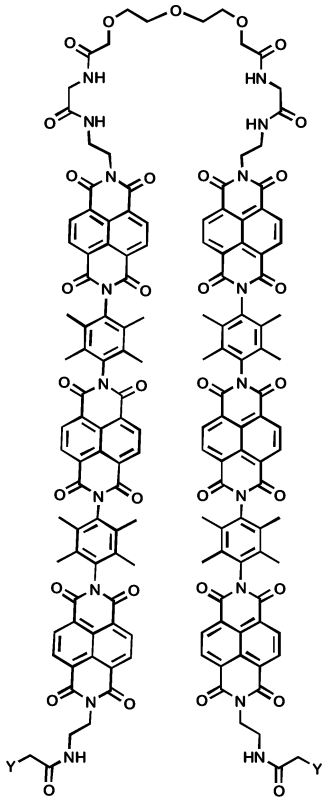


Fig. 12 Second generation anion–π slides. **13**: Y = Z = NHBoc; **14**: Y = NHBoc, Z = $NH_3^+TFA^-$; **15**: Y = Z = $NH_3^+TFA^-$.



16, Y = NH-BOC; 17, Y = NH_2

Fig. 13 Cation–π scaffolds: electron-deficient oligo-(*p*-phenylene)-*N*,*N*-naphthalenediimide (O-NDI) rods.

diminished in the order **14** > **13** > **15**. Transport mediated by **14** was not appreciably affected by changing the extravesicular cations. This observation accords with anion selectivity. The transport selectivity of **13** and **14** among the halides was $Cl^- > F^- > Br^- > I^-$. This order implies strong anion binding, which could account for the low transport activity of the O-NDIs. Although a range of activity was observed, solubility of the oligoarenes remained an issue in the analyses.

In recent work, Matile and coworkers reported a second generation of O-NDIs that incorporate a hairpin turn and create a dimeric bundle of the two most active parent compounds, **13** and **14**. O-NDI **16** (Fig. 13) is a dimer of **13**, while **17** is a dimer of **14**. Interesting as these dimer structures are, their activity was disappointing. This was attributed to solubility problems or possible aggregation into large assemblies.[112]

A third generation of related structures has been reported this year. These O-NDI-type molecules are differently colored and contain photosynthetic qualities.[113] These oligo(*p*-phenylene)-*N*,*N*-perylenediimide (O-PDI) rods **16** and **17**, like their predecessors, act as anion–π slides. Remarkably, they also act as π-acidic semiconductors. Anionic triglutamate tails were appended to one end of each rod to provide the desired water solubility and to ensure delivery to membranes. These new receptors were also found to have a preference for Cl^- anions. The ability of these negatively charged rods to effectively attract anions, rather than cations, comports with an anion–π interaction. The selectivity of Cl^- ($\Delta G_{hydr} \sim 370$ kJ mol^{-1}) over ClO_4^- ($\Delta G_{hydr} \sim 230$ kJ mol^{-1}) suggests that the observed selectivity does not result from the dehydration penalty alone.

Amphiphilic heptapeptides as synthetic anion transporters (SATs)

A family of amphiphilic peptides that function as ion transporters has been designed, prepared, and extensively studied in our laboratory. The transporters were conceptualized as units containing four essential modules, based in part on the elements of modern phospholipids. The four elements of the design are as follows. First, hydrocarbon chains (as a dialkylamine) were incorporated to mimic the fatty ester's hydrocarbon chains. Diglycolic acid, $O(CH_2COOH)_2$, served to link the hydrocarbon chains to the peptide. The peptide's N-terminus was linked as a diglycoylamide and the peptide's C-terminus was capped as an ester. Overall, this gave a general structure as shown in Fig. 14.

A combination of dialkylamine [$(R^1)_2N$] and diglycolic acid ($\sim COCH_2YCH_2CO\sim$, Y = O) were chosen to serve, in combination, as the hydrocarbon anchor and the midpolar regime mimic. The dialkylamines are readily available and the reaction between diglycolic anhydride and a dialkylamine occurs readily without any catalyst or by-product. The anchor unit $\sim COCH_2OCH_2CO\sim$ may be abbreviated as "[DGA]".

The choice of the initial peptide sequence was made on the basis of two observations. First, the so-called *C*-peptide that is cleaved from proinsulin has approximately 30 residues and has been identified as a potential channel-former.[114] Conserved and apparently critical sequences are ^{13}GGGPEA^{19}G (rat) and ^{13}GGGPGA^{19}G (human). Pig *C*-peptide has the sequence ^{13}GGGLGG^{19}L and is inactive. Separately, the putative conductance pore of the ClC chloride transporting proteins has a conserved G-X-X-P sequence. The GGGPGGG sequence further appealed because racemization within the peptide sequence is unlikely to be a problem. It was felt that the C-terminal acid might be too polar for this compound to function as a transporter. Thus, the free carboxyl was esterified. The first compound to be designed, prepared, and characterized was $(C_{18}H_{37})_2N$-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2C_6H_5$.[115] The preparation of the first SAT molecule is shown in Fig. 15.

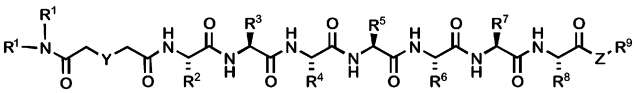


Fig. 14 General structure of amphiphilic heptapeptides that are synthetic anion transports, SATs.

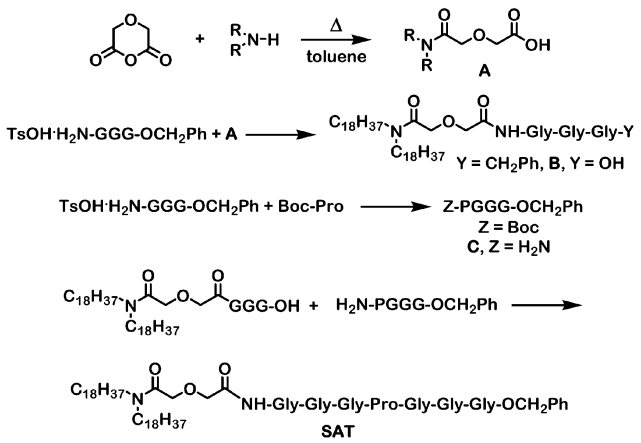


Fig. 15 Preparation of the first amphiphilic heptapeptide synthetic anion transporter.

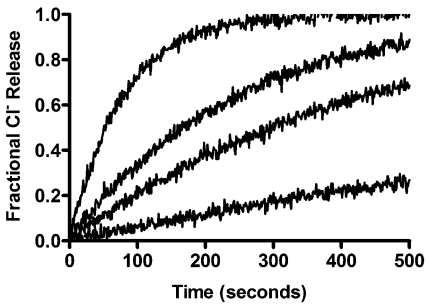


Fig. 16 Fractional chloride release from liposomes mediated by $(C_{18}H_{37})_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OCH_2Ph$ at concentrations of (bottom to top) 24 μM, 44 μM, 73 μM, and 154 μM.

Characterization of ion transport by SATs

Initial evaluation of transport was done by measuring Cl^- release from liposomes. The ISE method described above gave the graph shown in Fig. 16. As the ionophore concentration is increased from 24 μM to 154 μM (~six-fold), anion release increases. It was noted above that porcine *C*-peptide has a GGGLGGL sequence rather than GGGPGGG near its midpoint and that it is inactive. An otherwise identical ionophore in which leucine replaced proline was found to be much less active. Thus, Cl^- release mediated by the SAT having a GGGPGGG sequence at 24 μM was similar to a 154 μM concentration of the GGGLGGG peptide ionophore.

Confirmation of channel behavior

Planar bilayer voltage clamp methods were used to confirm channel behavior. Fig. 17 shows a trace for a SAT molecule in which multiple openings are apparent during an approximately >four second time course. Many different SAT structures exhibited similar, although not identical, behavior. By using the planar bilayer voltage clamp experiment, anion transport was clearly and unequivocally confirmed. In addition, it was found that the gating (open–close) frequency was voltage dependent. Over a range of voltages, the channels were completely open, completely closed, or showing behavior of the type seen in Fig. 17. By using these methods, it was established that $(C_{18}H_{37})_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OCH_2Ph$ showed >10-fold selectivity for Cl^- over K^+.

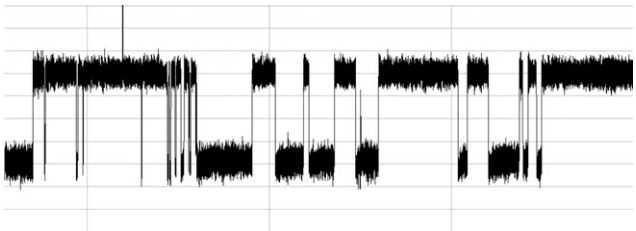


Fig. 17 Open–close behavior of a typical SAT molecule determined by the planar bilayer voltage clamp method.

Structural variations of subgroup modules

By systematically altering the four modules that comprise the SATs, (1) the N-terminal anchors, (2) the connector chain, (3) the peptide, and (4) the C-terminal ("secondary") anchor, a structure–activity relationship can be constructed. The survey began by altering the N-terminal anchors, R^1 $(R^1)_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OCH_2Ph$.[116] The twin N-terminal sidechains (R^1 in Fig. 14) studied were ethyl, propyl, hexyl, octyl, decyl, dodecyl, tetradecyl, hexadecyl, and octadecyl. Although two octadecyl chains were expected to give the best anchoring and thus the best transport results, shorter chains proved to be better. Data were obtained by different assays; Fig. 18 shows data for CF dequenching. The bis(decyl) compound showed very high transport but further study revealed that anion selectivity was essentially lost in the shorter-chained compounds.[117] Indeed, planar bilayer voltage clamp experiments showed that both $Cl^-$and Na^+ were transported by the C_{10} SAT.

In the initial design, the C-terminal ester was intended only to protect the carboxyl group (*i.e.*, prevent ionization) and reduce polarity. Structural variations in this series retained octadecyl as the N-terminal anchors and varied R^2: $(C_{18}H_{37})_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OR^2$. The alkyl ester groups were varied from ethyl to octadecyl and included isopropyl and cyclohexylmethyl. The C-terminal *n*-heptyl ester was the most effective transporter.[118] Studies using a Langmuir trough showed that a third *n*-octadecyl chain

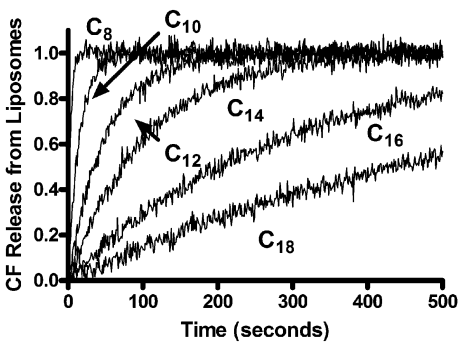


Fig. 18 Release of carboxyfluorescein (CF) from liposomes monitored by fluorescence dequenching. C_{18}–C_8 refer to the alkyl chains R^1 in Fig. 14.

led to a highly organized structure that apparently lacked sufficient flexibility to form pores rather than aggregates.[119,120]

The diglycolic acid unit that connects the dialkylamines with the peptide was also systematically varied, *i.e.*, Y in $(C_{18}H_{37})_2$N-Y-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph. Among the diacids surveyed, succinic, glutaric, diglycolic, 3-thiaglutaric, *N*-methyliminodiglycine, isophthalic, and terephthalic acids, transport was most improved by the presence of 3-thiaglutaric acid.[121] Succinic and glutaric acids also showed improved transport although the latter's Cl^- release curve showed an inflection.

The peptide sequence and chain length were also potentially important variables. The peptide was varied from $(Gly)_2$-Pro-$(Gly)_2$ to $(Gly)_4$-Pro-$(Gly)_4$. The latter was the most effective transporter and the sequence $(Gly)_4$-Pro-$(Gly)_2$ was found to be superior to either isomer $(Gly)_3$-Pro-$(Gly)_3$ or $(Gly)_2$-Pro-$(Gly)_4$. The ion selectivity of these ionophores remains to be established.[122] Altering the peptide sequence also engendered considerable variation in transport efficacy. Replacement of proline in $(Gly)_3$-Pro-$(Gly)_3$ with $(Gly)_3$-Pip-$(Gly)_3$ where Pip is pipecolic acid, the 6-membered ring analog of proline, caused a dramatic loss in transport activity. Several other proline replacements such as leucine, azetidine-2-carboxylic acid, and 3-aminobenzoic acid likewise showed diminished transport.[115,123]

Certain amino acids were deliberately incorporated in the sequence to serve as analytical tools and others were probes of function. Compounds **18** and **19** (Fig. 19), for example, were prepared to probe the influence of charge on transport of ions through the pore.[124,125] Fluorescent residues such as pyrene (**20**) and tryptophan (**21**), were incorporated so that the experiments such as fluorescence resonance energy transfer (FRET) could be studied.[126] The presence of tryptophan also facilitated the study of aggregation and membrane insertion dynamics.[127,128]

Several lines of evidence suggested that **18** functioned as a dimer to transport anions through a phospholipid bilayer. For example, Hill plots have a slope of ~2 corresponding to a dimeric aggregation state. Dextran block experiments suggested that the pore was 7–8 Å in diameter. This is consistent with a dimer structure as determined by molecular modeling. If the pore was dimeric, covalent linkage of two molecules of **18** should produce a compound having activity similar to twice the concentration of **18**. The two compounds shown in Fig. 20 and identified as "C-dimer" and "N-dimer" were thus prepared.[129]

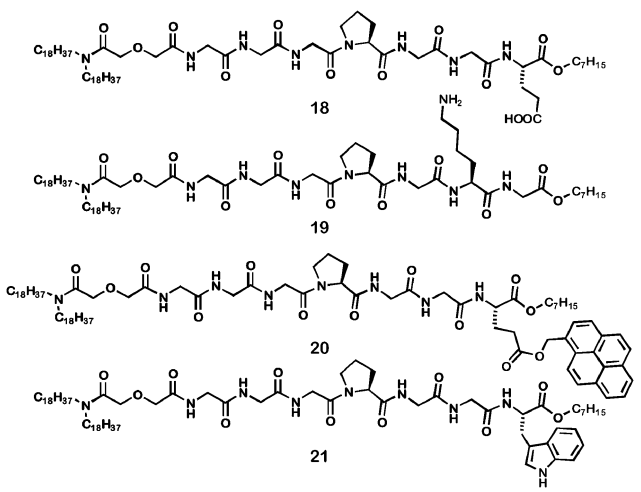


Fig. 19 Variations in the structures of amphiphilic SAT molecules.

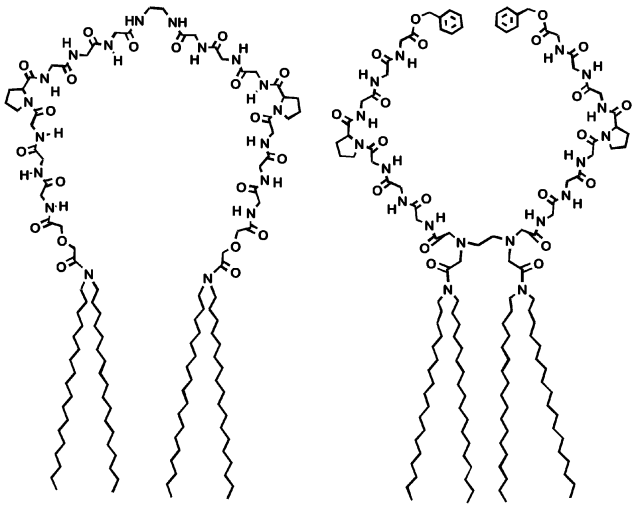


Fig. 20 "C-dimer" (left) and "N-dimer" (right) of the acitive SAT compound **18**.

Chloride binding by the amphiphilic heptapeptides

Although it was clear that the SAT compounds were good transporters, it was of interest to determine how strongly complexation with Cl^- occurred. These studies were conducted by NMR and produced both binding data and a structure for the complex.[130,131] Three key findings emerged from this work. First, peptides of the form R_2N-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OR′ bound Bu_4NCl in $CDCl_3$ with $K_S \approx 1800\ M^{-1}$. This value is nearly the same as that reported for the Crabtree triarene.[30] Second, this study showed that the magnitude of Cl^- complexation depended significantly upon the counter cation.[132] Third, the amide hydrogens of the fifth and seventh amino acid residues (~GGG**P**GGG~, $^5G_{NH}$, $^7G_{NH}$) were specifically identified as the key donors for Cl^- complexation.

Biological activity of amphiphilic peptides

Numerous peptides isolated from a variety of sources have proved to exhibit antibiotic activity. The area has been reviewed.[133] There has been considerable recent interest in developing novel antibiotics by these "hybrid" approaches and there has also been much effort invested in understanding how the combinations of segments cooperate to achieve efficacy.[134,135] Important as the development of novel antibiotics is, other biological applications are possible and promising. For example, it was shown in the authors' laboratory by using an Ussing chamber, that $(C_{18}H_{37})_2$N-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph alters Cl^- transport in vital, mammalian airway epithelial cells. Admittedly, this compound has a molecular weight of 1168 Daltons and it is a peptide. Still, introduction into lungs

could be effected by a simple inhaler such as those used to administer steroids. The critical finding is that these compounds alter chloride transport in mammalian cells.

Conclusions and prospects

Barely two decades ago, synthetic channels that transported biologically relevant cations were just beginning to emerge. Synthetic anion channels were unknown. Today, anion complexation is well established and its continuing study is a vital field. Several successful anion transporters or channels are now known, although detailed characterization and elaboration in most cases is ongoing. The issues of transport mechanism, channel gating, selectivity, and rectification loom large. The prize is equally great because these families of structures constitute abiotic leads for biological activity.

From the chemical aspect of the problem it is important for us to fully characterize each new structure both in terms of its properties and its efficacy. We must challenge our assumptions with control experiments and ask if our designs could lead to functions or behavior different from those that inspired us or that we intended. This is increasingly important as structures become large, syntheses become complex, and analyses become almost too elaborate for others to repeat in any routine way.

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