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Review Commentary

The determination of the ion selectivity of synthetic ion channels and pores in vesicles†

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ABSTRACT: Organic chemists entering the field of synthetic ion channels and pores are often restricted to the characterization of their creation by fluorescence kinetics in vesicles and do not have access to conductance experiments in planar bilayer membranes. This limitation excludes the application of methods to evaluate key characteristics such as ion selectivity or voltage gating that are routine in the membrane biophysics community. The objective of this account is to offer to the non-expert an introduction to the assessment of ion selectivity in vesicles with examples from years of research on functional rigid-rod molecules in biomembranes (For a complementary introduction to the determination of voltage dependence in vesicles, see Sakai N, Matile S. *Chem. Biodiv.* 2004, 1: 28).

KEYWORDS: fluorescence; conductance; anion/cation selectivity; selectivity sequence; selectivity topology; Eisenmen; Hofmeister; halides; proton selectivity; antiport; symport

INTRODUCTION

Herein, we review six representative synthetic ion channels/pores with rigid-rod scaffolds we have made and studied over the past decade with emphasis on their ion selectivity and the methods used for their determination, focusing particularly on vesicles (Fig. 1). Rigid-rod polyol **1** was designed to selectively transport protons across lipid bilayer membranes.[1,2] Experimental evidence supported the expectation that Onsanger's classical 'hop-and-turn' mechanism (Fig. 1, arrows) does not only apply in bioenergetics[3] but also for selective proton movement through the hydrogen-bonded chain created along the membrane-spanning *p*-octiphenyl scaffold of **1**.

Ligand-induced assembly of *p*-septiphenyls **2** was designed to selectively transport potassium across lipid bilayer membranes.[1,4] Conceived at a time when cation-π interactions were believed to account for the selectivity of biological potassium channels,[5,6] the found Eisenman IV topology provided, ironically, excellent experimental support for a mechanism that ultimately turned out not to occur in nature (except for the blockage with the classical cation-π probe TEA^+).

The electron-deficient rigid-rod π-stack architecture of ion channel **3** was designed to open in response to charge-transfer complex formation with intercalating π-donors.[7,8] In this case, experimental evidence for ligand gating was of highest importance to verify the functional innovation of interest. The determination of the ion selectivity was nothing more (and nothing less) than part of a clean characterization of the obtained ligand-gated ion channel.

Voltage gating was the topic of interest with ion channel **4**.[1,9,10] Voltage sensitivity was introduced with the permanent axial macrodipole of rigid push-pull rods, that is, *p*-octiphenyls with a π-donor at one end and a π-acceptor at the other, and amplified by parallel self-assembly in polarized membranes. As with ligand gating, the determination of the parameter describing voltage gating, that is the gating charge, was of highest importance in this case, whereas the determination of the ion selectivity helped to reveal the characteristics of the obtained voltage-gated ion channel.

Synthetic multifunctional pores **5** and **6** were created to combine molecular translocation with molecular recognition and transformation, in other words, to recognize and, in some cases, transform molecules on their way through the pore across the bilayer membranes.[1,11–16]

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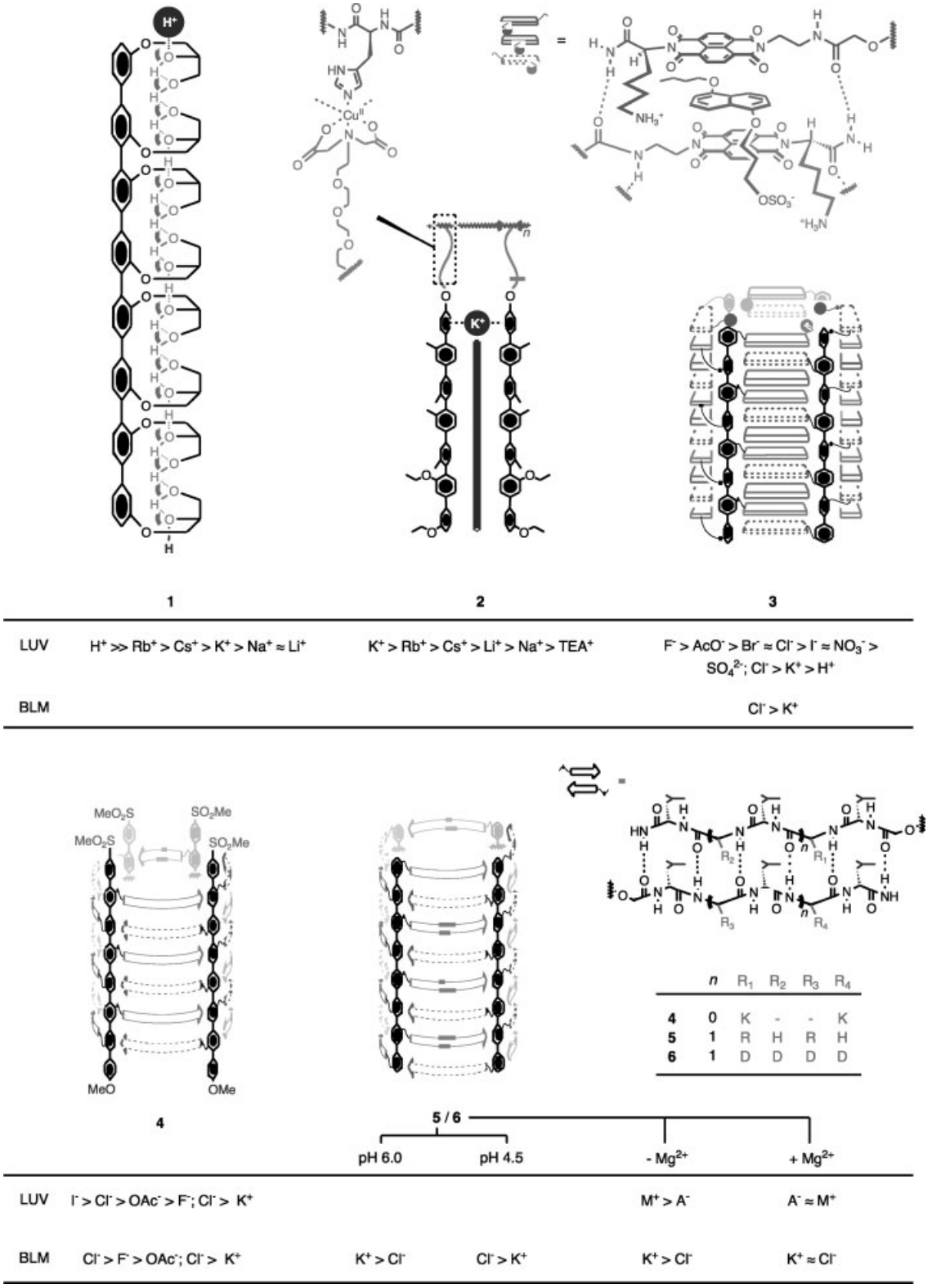

Figure 1. Ion selectivity in vesicles (LUVs) and planar bilayers (BLMs) of representative synthetic ion channels and pores that are constructed from rigid-rod *p*-oligophenyl scaffolds and contain hydrogen-bonded chains for proton transport (**1**), ligand-assembled π-slides for potassium transport (**2**), π-stacks for ligand gating and electron transport (**3**), push-pull rods for voltage gating (**4**) and β-sheets for multifunctionality (molecular recognition and transformation of guests and substrates, **4**–**6**). See recent accounts[1,11] or original literature for details beyond ion selectivity

With the discovery of synthetic access to the rigid-rod β-barrel motif, internal and external pore design became available.[1,11] Simple variations of the amino-acid residues positioned at inner and outer pore surfaces allow to design chemical and physical properties in a rational manner. With synthetic multifunctional pores, the characterization of molecular recognition as well as applications to sensing and catalysis are of highest importance. However, the determination of ion selectivity turned out to be crucial and interesting because of significant contributions to molecular recognition. Selected examples given in the following will illustrate this relationship.

These six examples were selected from years of research on synthetic ion channels and pores with rigid-rod scaffolds because they illustrate the most important lessons learned concerning the determination of ion selectivity in large unilamellar vesicles (LUVs). The objective of this review is to provide a brief and practical introduction on this topic for organic chemists entering the field of synthetic ion channels and pores[17–35] without access to conductance experiments in bilayer lipid membranes (BLMs). Comparison of results from BLMs and LUVs suggest that not only the determination of ion selectivity (Fig. 1) but also the determination of voltage dependence[10,36] is possible and relatively unproblematic in vesicles. Ironically, it is the experimental evidence for the existence of ion channels as such (rather than the presence of alternative modes of transport) that remains most difficult to obtain in vesicles.[18] In the following, we first summarize classical methods to determine the ion selectivity of synthetic channels and pores in BLMs. Shifting attention from BLMs to LUVs, we then describe our favorite methods to determine anion/cation selectivity and selectivity sequences, provide examples how ion selectivity can be expressed in different assays and introduce the use of additives to identify proton selectivity, potassium selectivity, and weak anion/cation selectivity.

PLANAR BILAYER CONDUCTANCE EXPERIMENTS

The traditional and particularly in the biophysics community preferred method to determine the selectivity of ion channels analyzes single- or multichannel conductances in various salt gradients.[37]

A conventional set-up for BLM conductance measurements uses two chambers that are filled with buffer and arbitrarily named *cis* and *trans* (Fig. 2). Typically, the *cis* chamber is connected to an input electrode and the *trans* chamber to a reference electrode (virtual ground). Samples are usually added to the *cis* chamber, and depending on the charges of the synthetic channels or pores, positive or negative membrane potentials are applied to drive the molecule into the BLM between the chambers. The current flowing through the ion channel or pore in the BLM in response to an applied voltage is then recorded and used to obtain insights on the electric properties of the interior of the functional supramolecule. The dependence of this current on varied voltage follows Ohm's law for ohmic channels (Fig. 2a), whereas non-ohmic channels and pores exhibit an exponential *IV* profile determined by their gating charge.

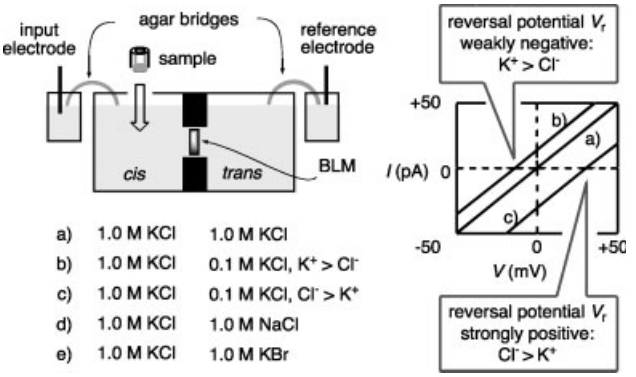

Figure 2. Determination of ion selectivity in planar bilayers (BLMs). The reversal potential V_r at zero current *I* in the *IV* profile shifts from 0 mV for identical content in *cis* and *trans* chamber (a) to negative or positive values in the presence of ion gradients from *cis* to *trans* depending on the anion/cation selectivity (b, c), the cation selectivity sequence (d) or the anion selectivity sequence (e) of the synthetic ion channel or pore in the BLM. Note that this graph is highly schematic: *IV* relations, for example, follow the GHK *current* equation and are therefore often curved when a salt concentration gradient is applied[37]

The anion/cation selectivity of an ion channel describes the overall preference for either anions or cations. To determine the anion/cation selectivity in BLMs, typically a concentration gradient of KCl is applied between *cis* and *trans* chamber (Fig. 2b and c). Because of this gradient, a current will flow without applied voltage, and the direction of this zero current will depend on the preferred movement of K^+ or Cl^- through the channel. The reversal potential V_r is the applied voltage required to cancel this gradient driven current. The reversal potential is used to quantify anion/cation selectivity as permeability ratios with the Goldman–Hodgkin–Katz (GHK) *voltage* equation:

$$P_{Cl^-}/P_{K^+} = \frac{[a_K cis - a_K trans\ \exp(-V_r F/RT)]}{[a_{Cl} cis\ \exp(-V_r F/RT) - a_{Cl} trans]}$$

With this method, the following permeability ratios were measured for rigid-rod ion channels and pores **3–6**: The weak anion selectivity of ligand-gated ion channel **3** with permeability ratio $P_{Cl^-}/P_{K^+} = 1.4$ was as expected for a cationic interior.[8] The cationic interior of ion channel **4** was as well reflected in $P_{Cl^-}/P_{K^+} = 3.0$ as the anionic interior of pore **6** in $P_{K^+}/P_{Cl^-} = 5.6$.[10,15] The puzzling ability of the polycationic pore **5** to attract

cations rather than anions, that is, $P_{K^+}/P_{Cl^-} = 2.0$ at pH 6, was found to originate from internal charge inversion by counteranion scavenging.[15] This surprising result of a routine determination of the ion selectivity of a synthetic ion channel marked the beginning of a new and fruitful research program on the general contributions of counteranions to what is commonly referred to 'arginine magic' in biomembranes.[38,39]

The selectivity sequence describes the preference of an anion channel between different anions or that of a cation channel between different cations.[40–45] In BLMs, selectivity sequences are determined like the anion/cation selectivity, except that the gradient between *cis* and *trans* chamber is not established with different concentrations of the same salt but between usually identical concentrations of different salts. To determine cation selectivity sequences, the anion is kept constant and the cation is varied (Fig. 2d), and vice versa for anion selectivity sequences (Fig. 2e). In the latter case, the zero current will now depend on the preferred movement of Br^- or Cl^- through the anion channel, and permeability ratios calculated from the reversal potential will quantify the discrimination between these two anions. For example, $P_{Cl^-}/P_{OAc^-} = 1.8$ was found for the voltage-gated rigid-rod anion channel **4**.[10]

Because there is no current at the reversal potential, permeability ratios describe the ability of ions to enter a channel, that is, the change in energy from ion stabilization by hydration to ion stabilization by its interaction with the channel. The ability of an ion to move through the channel and be released at the other side is described by the conductance, that is, the slope of the *IV* profile for given ion. Selectivity sequences derived from permeability ratios and conductance can differ significantly.[40–45]

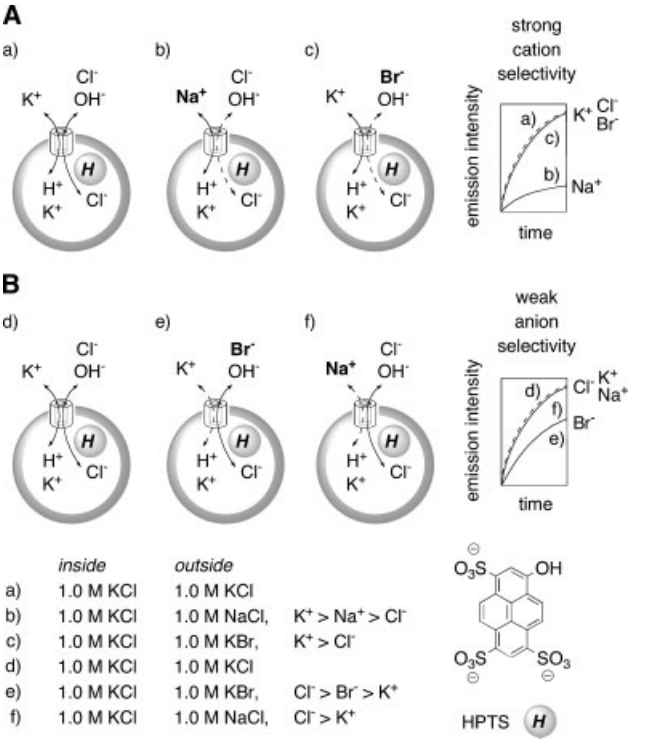

Figure 3. Determination of (A) cation and (B) anion selectivity in vesicles (LUVs). Synthetic ion channels added to LUVs loaded with the pH-sensitive fluorophore HPTS and exposed to a pH gradient mediate the collapse of the latter by either H^+/K^+ or OH^-/Cl^- antiport. Sensitivity of the measured rates to external (b) cation but not (c) anion exchange identifies cation selectivity, sensitivity to external (e) anion but not (f) cation exchange identifies anion selectivity

DETERMINATION OF ANION/CATION SELECTIVITY IN VESICLES FOR SYNTHETIC ION CHANNELS

For an organic chemist entering the field without BLMs,[17–35] LUVs provide a user friendly and, according to the comparative studies discussed below, valid alternative. Among several possible methods to determine anion/cation selectivity of synthetic ion channels in vesicles, the HPTS assay worked best in our hands (Fig. 3). HPTS is a pH-sensitive fluorescent dye with a convenient $pK_a = 7.3$ and one excitation maximum at 405 nm decreasing and another one at 450 nm increasing with increasing pH.[46,47] Entrapped in vesicles, HPTS can serve as intravesicular pH meter that ratiometrically detects the collapse of an applied transmembrane pH gradient as well as the ability of synthetic ion channels to accelerate this process.

A synthetic ion channel can mediate the increase of intravesicular pH in response to an extravesicular base pulse by facilitating either proton efflux or OH^- influx (Fig. 3Aa). These transmembrane charge translocations require compensation by cation influx in response to proton efflux or anion efflux in response to OH^- influx. Below results suggest that focus on antiport without consideration of the osmotically disfavored symport should be reasonable in most cases. The detected increase in HPTS emission with time after channel addition reports on the preferred ion exchange process, that is, either OH^-/Cl^- or H^+/K^+ antiport. The observed rate is more influenced by less favored ion and less influenced by the more favored ion involved in the operational antiport (besides contributions from ion channel partitioning, open probability, lifetime, and so on). In the present configuration, this is H^+ efflux or K^+ influx for cation selective channels and OH^- influx or Cl^- efflux for anion selective channels. All that has to be done to determine the anion/cation selectivity of the channel is, therefore, extravesicular cation and anion exchange.

A large difference in the rate of HPTS fluorescence intensity change upon exchange of external K^+ with Na^+ demonstrates that the channel transports cations with high selectivity (Fig. 3Aa vs b). Insensitivity to external

exchange of Cl^- with Br^- confirms that anion antiport does not occur (Fig. 3Aa vs c). Because of the multiply coupled processes involved, cation selectivity is still detectable with proton selective transporters like **1**,[2] although the observed changes upon external cation exchange are necessarily weaker compared to potassium selective channels like **2** (compare below discussion of proton selectivity).[4]

Anion selectivity can be found analogously. A small change of the detected increase in HPTS emission with time upon exchange of external Cl^- with Br^- demonstrates that the channel transports anions with weak selectivity (Fig. 3Bd vs e). Insensitivity to external K^+/Na^+ exchange confirms that cation antiport does not occur (Fig. 3Bd vs f).

DETERMINATION OF SELECTIVITY SEQUENCES IN VESICLES

By using the same assay, selectivity sequences can also be determined. The selectivity sequences of cation channels are best described as Eisenman sequences or topologies.[40] These 11 sequences cover all reasonable possibilities between cation selectivity completely determined by dehydration penalty (Eisenman I, $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$) or by cation binding in the channel (Eisenman XI, $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$). In Eisenman topologies, ion selectivities are plotted as a function of the reciprocal radius of the cation (Fig. 4c). Application of the Eisenman theory to anion selectivity gives seven meaningful halide sequences reaching from the most frequent 'Wright-Diamond' sequence I determined only by dehydration penalty ($I^- > Br^- > Cl^- > F^- > Li^+$) to the very rare sequence VII dominated by anion binding in the channel ($F^- > Cl^- > Br^- > I^-$).[41] Anion selectivity topologies can be described using the reciprocal radii of the anions (Fig. 4f).[42] However, as the mismatches of size (e.g., $AcO^- > F^-$) and dehydration energy (e.g., $AcO^- \approx F^-$, Fig. 4e) are often found with anions other than halides, direct use of the latter in anion selectivity topologies is often more meaningful.[41–43] Complete dependence on dehydration energy (i.e., sequence I for halides) is also referred to as Hofmeister or lyotropic sequence, with hydrophobic anions named chaotropes (structure breakers) and hydrophilic anions named kosmotropes (structure makers).[44] Many variations and refinements on the theory of ion selectivity exist.[40–45] Recommended references for organic chemists entering the field are the classical 40 (for cations) and 41 (for anions).

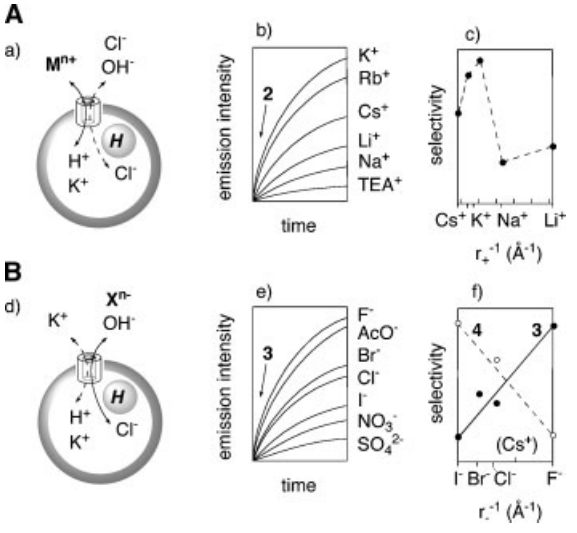

Figure 4. Determination of (A) cation and (B) anion selectivity sequences in vesicles using the HPTS assay (a,d, compare Fig. 3). For cation channel **2**, an Eisenman topology IV with Li^+ anomaly (c) and TEA^+ blockage is obtained from the observed rates for varied external cations M^{n+} (a, b). For anion channel **4**, a halide topology I (or Hofmeister sequence) is obtained from the observed rates for varied external anions A^{n+} (d, f), whereas the very unusual halide topology VII is found for anion channel **3** (d–f)

The determination of cation selectivity sequences with the HPTS assay in vesicles follows directly from the determination of anion/cation selectivity described in the preceding section (Fig. 4A). Namely, the changes of the increase in HPTS emission with time in the presence of different external cations M^{n+} are measured (Fig. 4a and b). For potassium channel **2**, the Eisenman topology IV of biological ion channels with the usual Li^+ anomaly (i.e., the influx of at least partially hydrated but still small enough Li^+) were found (Fig. 4b and c). Blockage of **2** by TEA^+ completed the similarity with biological potassium channels and supported cation-π interactions as origin of potassium selectivity (Fig. 4b).[4] Without the programmed *p*-septiphenyl assembly with the multivalent polyhistidine ligand, the selectivity sequence of **2** degenerated to a rubidium-selective Eisenman III topology. The proton transporter **1** exhibited a less interesting, almost exclusively dehydration dominated Eisenman II sequence.[2]

The determination of anion selectivity sequences in vesicles using the HPTS assay with high external pH is less straightforward. The evaluated external anions X^{n-} primarily compete with OH^- influx and qualify for antiport only indirectly after exchange with internal Cl^- (Fig. 4d). Anion channel **4** with internal ammonium cations exhibited the most frequently observed Hofmeister-like or lyotropic halide sequence I determined by anion dehydration only (Fig. 4f, empty circles).[10] Comparison with permeability ratios from BLMs demonstrated that sequences determined under these conditions directly reflect anion selectivity (Fig. 1).[10] This comparison with permeability ratios corroborating the validity of the halide sequence I for **4** from BLMs was particularly important with regard to the very unusual halide sequence VII determined under identical conditions for the smaller, ligand-gated ion channel **3** (Fig. 4e and f, filled circles).[7] Internal ammonium cations as in

the lyotropic channel **4** were unlikely to account for the implied anion binding, and similar preference of the equally kosmotropic acetate and F^- excluded contributions from simple size exclusion. Multiple anion binding and possibly contributions from anion-π interactions within the electron-deficient naphthalenediimide stacks of channel **3** were therefore likely to contribute to this remarkable halide sequence VII.

Although permeability ratios from BLMs confirm that the determination of anion selectivity under these conditions gives empirically correct sequences (Fig. 1), an inversion of the transmembrane proton gradient would ideally be preferable to simplify the assay. Like with the above determination of cation selectivity sequences by H^+/M^{n+} antiport, external anion exchange would then directly report on OH^-/A^{n-} antiport.

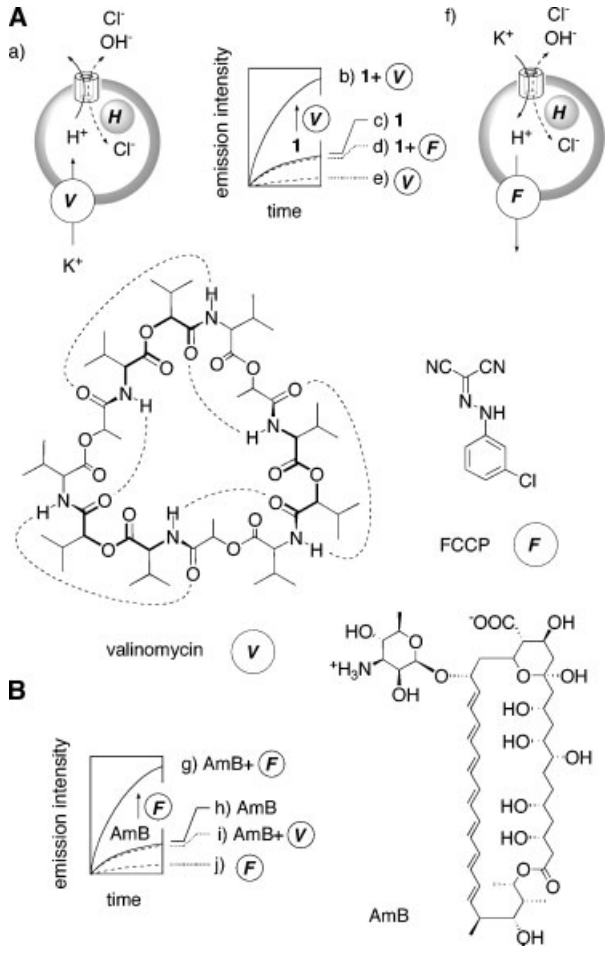

Figure 5. The potassium carrier valinomycin and the proton carrier FCCP as additives for the determination of (A) proton and (B) $M^+ > H^+$ selectivity in vesicles using the HPTS assay (a, f compare Fig. 3). A: Proton 'wire' **1** was identified by sensitivity of the measured rates to the presence of valinomycin (b vs c) but not FCCP (d vs c) at concentrations where valinomycin alone is inactive (e). B: The complementary $M^+ > H^+$ selectivity of the biological cation channel AmB was confirmed by sensitivity of the measured rates to the presence of FCCP (g vs h) but not valinomycin (i vs h) at concentrations where FCCP alone is inactive (j)

DETERMINATION OF PROTON SELECTIVITY WITH VALINOMYCIN

Proton selectivity can be readily determined with the HPTS assay in vesicles (Fig. 5A).[2] In this assay, the apparent activity of proton channels *decreases* with increasing proton selectivity because the rate of the disfavored potassium influx influences the detected velocity more than the favored proton efflux. The rate of this disfavored cation influx can, however, be 'artificially' increased by addition of the potassium carrier valinomycin (Fig. 5a). The increase in apparent activity of the proton channel in response to the addition of valinomycin is therefore a direct measure for proton selectivity, with the absolute selectivity being at least as high as maximal measurable increase. For the proton 'wire' **1**, $H^+/K^+ \geq 16$ was found (Fig. 5b vs c). This value further increased with the application of a K^+ gradient (replacing internal K^+ with Na^+; **1**: $H^+/K^+ \geq 33$). Important controls concerning the determination of proton selectivity include evidence that the valinomycin concentrations are low enough to exhibit no activity without the proton channel (due to disfavored H^+ efflux, Fig. 5e), insensitivity to the proton carrier FCCP (Fig. 5d, see below) and insensitivity to external anion exchange (i.e., evidence for cation > anion selectivity, Fig. 3, see above).

DETERMINATION OF $M^+ > H^+$ SELECTIVITY WITH FCCP

External cation/anion exchange can inform on cation/anion selectivity (Fig. 3) and both anion and cation selectivity sequences (Fig. 4) but not on cation/proton selectivity. The introduction of valinomycin as additive is sufficient for the determination of proton selectivity (Fig. 5A). Insensitivity to valinomycin does, however, not necessarily imply $M^+ > H^+$ selectivity. This complementary information can be obtained using the proton carrier FCCP as additive (Fig. 5B). In strict analogy to above proton selectivity, it is very important to realize that the apparent activity of cation channels *decreases* in the HPTS assay with increasing $M^+ > H^+$ selectivity because of the influence of the rate of the disfavored proton efflux on the overall rate observed. The classical potassium channel amphotericin B (AmB), for example, can exhibit surprisingly poor activity in the HPTS assay because of high $M^+ > H^+$ selectivity (Fig. 5h).[48] As with valinomycin for proton channels, the rate of the disfavored proton efflux with cation channels can be 'artificially' increased, in this case with the proton carrier FCCP (Fig. 5f). The increase in apparent activity of AmB in response to the addition of FCCP is therefore a direct measure for $M^+ > H^+$ selectivity (Fig. 5g vs h). The necessary controls are as above, including the confirmation that the observed increase in activity does not come from FCCP alone (Fig. 5j).

SENSITIVITY OF ANION CHANNELS TO FCCP AND VALINOMYCIN

An anion channel identified with the HPTS assays from insensitivity to external cation exchange and sensitivity to external anion exchange (Fig. 3) should be insensitive to cation-specific additives like valinomycin or FCCP (Fig. 5). The confirmed anion channel **3** (Fig. 6a), however, was sensitive to FCCP but not to valinomycin (Fig. 6d–g).[49] How to interpret such a result? The acceleration of proton transport with FCCP may either enhance Cl^-/H^+ symport (not shown) or K^+/H^+ antiport (Fig. 6b). The first explanation would indicate $Cl^- > OH^-$ selectivity that is not supported by the high sensitivity toward external anion exchange that occurs only with $OH^- > A^-$ (compare above discussion of $H^+ > M^+$ for rod **1**). The weak anion/cation selectivity with sequence $Cl^- > K^+ > H^+$ implied by the second explanation, however, was in excellent agreement with $P_{Cl^-}/P_{K^+} = 1.4$ obtained in BLMs. FCCP sensitivity of anion channels may therefore indicate $A^- > M^+ > H^+$ (Fig. 6b), whereas valinomycin sensitivity of anion channels may indicate $A^- > H^+ > M^+$ (Fig. 6c). However, at this stage, this interpretation should not be considered as established method to determine the magnitude of anion selectivity. Rather, the FCCP-sensitivity of anion channel **3** should be considered as excellent illustration for the constant adventures experienced during studies on ion selectivity in vesicles: It is important to make meaningful controls, and the thoughtful interpretation of results, particularly unexpected results, requires a thorough understanding of the system and can often lead to new insights or even new assays. This general remarks apply particularly to the more complex situation with synthetic pores discussed in the following.

DETERMINATION OF THE ANION/CATION SELECTIVITY OF SYNTHETIC PORES

Whereas synthetic ion channels are compounds made from abiotic scaffolds to transport inorganic ions across lipid bilayer membranes, synthetic pores are made to transport molecules. A pore can also serve as ion channel, whereas an ion channel is too small to act as pore. The determination of anion/cation selectivity of synthetic pores in vesicles is more complex, less explored and often less interesting because of a natural shift of attention from inorganic ions to molecules. The usefulness of the HPTS assay for the determination of the ion selectivity and for the characterization of synthetic pores in general is questionable. The possibilities of HPTS efflux through a large enough pore or pore blockage by HPTS complicate the situation and are not trivial to differentiate from above detection of pH gradient collapse with intravesicular HPTS. Although less useful to study molecular translocation through synthetic pores, we point out that modified HPTS assays have been extremely useful, in our hands, to study molecular transformations by synthetic catalytic pores in vesicles[50,51] and to determine the endovesiculation of vesicles.[52,53]

We have preferred to use the CF and the ANTS/DPX assay to characterize synthetic pores in vesicles (Fig. 7). In the former assay, LUVs are loaded with CF at

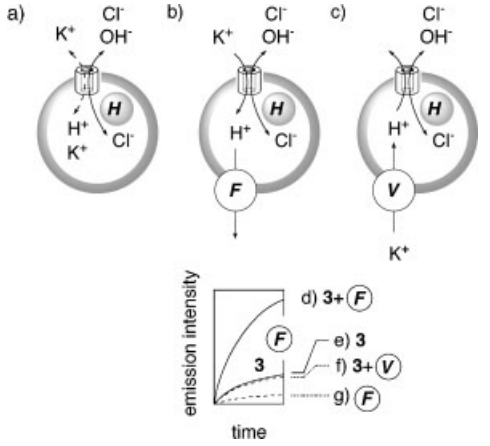

Figure 6. Sensitivity of the confirmed anion channel **3** (a, Fig. B) to FCCP (d vs e plus g) but not valinomycin (f vs e) implies weak anion/cation selectivity with selectivity sequence $Cl^- > M^+ > H^+$ (b), the complementary sensitivity of an anion channel to valinomycin but not FCCP would suggest weak anion/cation selectivity with selectivity sequence $Cl^- > H^+ > M^+$ (c, no example available)

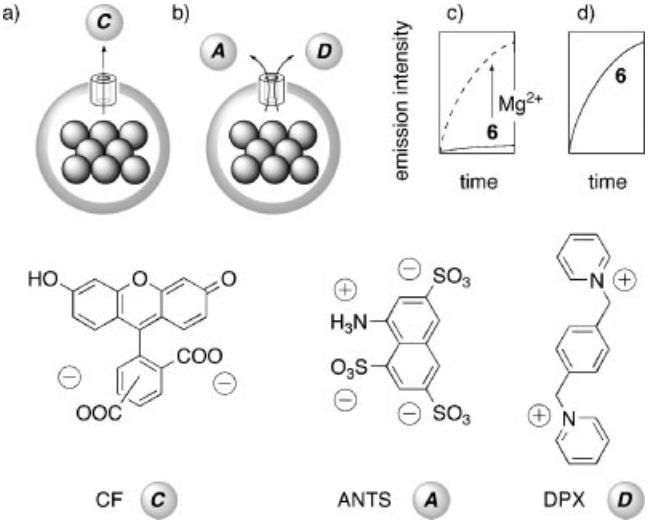

Figure 7. Determination of cation/anion selectivity of synthetic pores in vesicles exemplified with inactivity of cation selective pore **6** in the CF assay (a, c) but activity in the ANTS/DPX assay (b, d). Activation of pore **6** in the CF assay with Mg^{2+} demonstrates deletion or inversion of cation selectivity

concentrations high enough for self-quenching (Fig. 7a). CF efflux through large enough pores results in fluorophore dilution, and the disappearance of self-quenching is detected as an increase in CF emission. In the ANTS/DPX assay, the fluorophore ANTS is entrapped in LUVs together with its quencher DPX (Fig. 7b). Efflux of either the anionic ANTS or the cationic DPX through a pore can then be monitored as an increase in ANTS emission.

Whereas the determination of the ion selectivity of synthetic pores as such may be less important than that of synthetic ion channels, it is crucial to understand how it influences the observed pore activity in vesicles. The synthetic pore **6** provided a particularly instructive example. Although designed to be large enough to let the probe pass through, this synthetic pore was completely inactive under the typical conditions for the CF assay (Fig. 7c, solid).[13] Without careful consideration of the ion selectivity of both pore and assay, this could have been the end of a project, leaving much excitement with the marvelous multifunctionality of this pore undiscovered because of a careless interpretation.[1,11–14] Conductance measurements in BLMs confirmed that the cation selectivity of pore **6** was as high as expected for an inner surface covered with carboxylates ($P_{K^+}/P_{Cl^-} = 5.6$).[15] This high permeability ratio may also serve as instructive example to correct the frequent misconception that the large inner diameter of pores is incompatible with anion/cation selectivity: It is the chemical nature rather than the size of the pore that matters, absence of anion/cation selectivity does not identify pores (and vice versa). The inactivity of pore **6** observed in the CF assay, therefore, is a direct consequence of the incompatibility of the ion selectivity of pore and assay. In other words, the anion CF does not move through pore **6** because the pore is cation selective and not because the pore is too small or because the pore does not exist.

This conclusion could be readily confirmed by reduction of the anion/cation selectivity of either assay or pore. The anion/cation selectivity of the ANTS/DPX assay is much lower than that of the CF assay because the efflux of either the cation DPX through a cation selective pore or the anion ANTS through an anion selective pore results in the same increase in fluorescence emission. Not surprisingly, pore **6** was active in the ANTS/DPX assay (Fig. 7b and d, solid).[12,13,16] In other words, inactivity in the CF assay and activity in the ANTS/DPX assay determines cation > anion anion selectivity of synthetic pores in vesicles. The cationic probe complementary to CF to determine anion > cation selectivity of synthetic pores in vesicles remains to be described, although activity in the CF assay as such already implies at least anion permeability.

Reduction of the cation selectivity of pore **6** by binding of Mg^{2+} to the internal carboxylate arrays resulted in the appearance of activity in the CF assay (Fig. 7c, dotted vs solid).[13] BLM experiments confirmed that Mg^{2+}-binding reduced the permeability ratio from $P_{K^+}/P_{Cl^-} = 5.6$ to $P_{K^+}/P_{Cl^-} = 1.4$[15] and, together with circular dichroism spectroscopy measurements, that this functional change is not caused by a change in pore structure.

The CF assay is also sensitive to pH and can only be used above pH $\sim$ 6.5. The poor sensitivity of ANTS/DPX assay not only to the ion selectivity of the pore but also to pH is important for the determination of pH profiles, that is, the dependence of pore activity on pH. For example, the synthetic pore **5** was found to undergo a pH-gated inversion of ion selectivity from $P_{Cl^-}/P_{K^+} = 0.5$ at pH 6.0 to $P_{Cl^-}/P_{K^+} = 3.8$ at pH 4.0.[15] Several lines of evidence demonstrated that the puzzling cation selectivity of the cationic pore **5** near physiological pH originates from internal charge inversion by scavenged and immobilized phosphate counteranions. As mentioned previously, the study of the contributions of counteranions to the biological functions of oligoarginines in biomembranes in general corroborated the importance of this key finding.[38,39] Confirmed by extensive control experiments, the insensitivity of the ANTS/DPX assay allowed to record a meaningful pH profile of pore **5** despite the pH-gated inversion of anion/cation selectivity.[11,16]

CORRELATION OF RESULTS ON ION SELECTIVITY FROM BLMs AND LUVs

To confirm validity of the ion selectivities determined in vesicles, comparison to the results obtained in BLM was crucial (Fig. 1). So far, all comparisons made are in good agreement, most are mentioned throughout above discussion. To summarize, the anion/cation selectivity of ion channels **3** and **4** obtained from the HPTS assay was identical with that obtained in BLMs.[7–10] The selectivity sequence of anion channel **4** obtained from the HPTS assay was partially identical with that obtained in BLMs ($Cl^- > F^-$, $Cl^- > AcO^-$). The remaining weak discrepancy between the two kosmotrops F^- and AcO^- may be understood considering above mentioned differences between permeabilitiy ratios and channel conductance in BLMs (LUVs: $AcO^- > F^-$, BLMs: $F^- > AcO^-$).[10,40–45]

The anion/cation selectivity of pore **6** obtained by comparison of CF and ANTS/DPX assay was identical with that obtained in BLMs.[13,15] The same was true for the deletion of the cation selectivity of pore **6** with Mg^{2+}.[13,15] Similarly satisfactory agreement has been found for the determination of voltage dependence of ion channel formation (i.e., gating charges)[10,36] and blockage (i.e., electric distances)[54] in BLMs and LUVs.

SUMMARY AND PERSPECTIVES

In general, the more selective synthetic ion channels and pores, the more difficult to detect their activity. For example, proton selectivity of proton channel **1** is only detectable with valinomycin as additive, or the activity of the established, biological ion channel AmB will

appear rather weak without the addition of FCCP. The same rule-of-thumb applies to methods of detection: The more selective the assay, the more difficult to use (ease of use: HPTS > ANTS/DPX > CF). New synthetic ion channels and pores of unknown activity and selectivity are therefore best studied first with the least selective HPTS assay followed by an assessment of the inner diameter (i.e., discrimination between ion channels and pores) with the ANTS/DPX assay under nearly identical conditions. With regard to the specific topic of this article, the bottom line is that with careful use of the HPTS assay, it is possible to reliably determine at least (a) the anion/cation selectivity (Fig. 3), (b) anion and cation selectivity sequences (Fig. 4) as well as (c) proton selectivity (Fig. 5) of synthetic ion channels in vesicles. Moreover, the combined use of the CF and the ANTS/DPX assay allows also to determine at least the anion/cation selectivity of synthetic pores in vesicles (Fig. 7).

The objective of this review was to summarize insights on the determination of ion selectivity in vesicles as a simplified introduction for organic chemists who are entering the field with their main interest in creating new and exciting molecules (For a complementary introduction to the determination of voltage gating in vesicles, please see Ref. 36). Arguably, ion selectivities determined in vesicles are sufficient to contribute to a well-rounded picture of a newly created ion channel or pore. Moreover, vesicle experiments have the advantage to be not only easier to do for an organic chemist but also to be directly comparable with structural studies. However, insights from LUVs will never have the precision and depth of information that is available from BLMs. BLM conductance experiments will always be necessary for a detailed mechanistic understanding. For organic chemist entering the field, these insights can be obtained in collaboration with a BLM expert.

We reiterate that the methods to determine ion selectivity in vesicles summarized above have been continuously developed or refined with the objective to characterize new ion channels and pores such as **1**–**6** rather than to invent new assays. The lessons learned from these adventures are necessarily fragmentary and call for more systematic research on an exciting topic with much room for new and important discoveries.

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