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How Do Amphiphiles Form Ion-Conducting Channels in Membranes? Lessons from Linear Oligoesters

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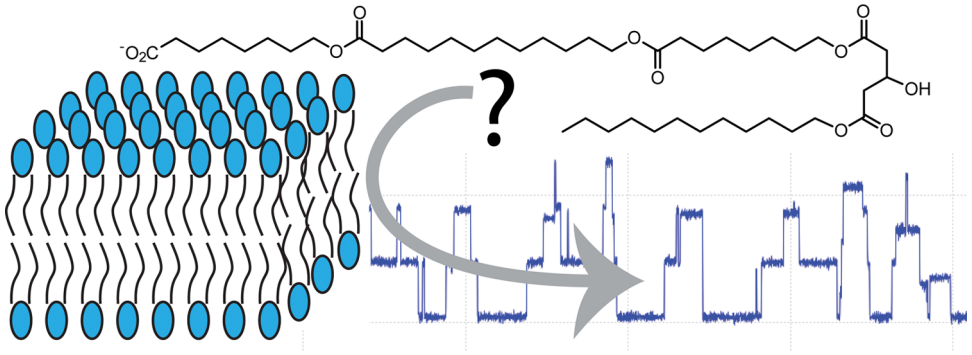
CONSPECTUS



The X-ray crystal structures of biological ion channels are exquisitely complex, but not all natural products capable of forming ion-conducting channels are equally elaborate. Examples such as the peptides gramicidin or alamethicin or the polyene antibiotics amphotericin and nystatin clearly form well-defined channels without requiring a massive protein superstructure. These molecules form the starting point for a supramolecular chemistry challenge: how to create synthetic compounds and systems that catalyze the translocation of ionic species across bilayer membranes mimicking naturally occurring channels. Over the past three decades, supramolecular chemists have developed numerous examples of systems with transport rates and efficiencies that rival natural channels. As the field developed, researchers discovered many compounds that are functional for ion transport but bear very little resemblance to any imagined architectures of ion channels. We and others have followed these lead compounds extensively in a quest to focus on the mechanisms such simple compounds use to achieve their function. These compounds show all the hallmarks of ion channels including high activity, ion specificity, regular time-dependent conductance changes, and in some cases higher-order phenomena such as voltage-dependent activity.

In this Account, we summarize experimental evidence derived from an extensive class of oligoester bolaamphiphiles that illustrates how amphiphilic molecules can form ion-conducting channels in membranes. Examination of increasingly simple compounds over the past two decades has shifted the focus away from biological paradigms towards alternative modes for transmembrane ion transport. We have developed new tools to move beyond simple on–off channel openings to complex bursts of high activity. From the perspective of flux, the highly conducting bursts clearly move ions more efficiently than simple on–off openings. High and sustained conductance, whatever its structural origin, has direct applications in amplification of chemical signals or membrane-disrupting biological activity. These results ensure that simple transporters will continue to fascinate and puzzle for a long time to come.

1. Introduction

We did not start out to make simple compounds.[1] We, and others,[2] were inspired by Tabushi's first report of a β-cyclodextrin derivative, dimers of which were proposed to act as a channel for the transport of transition metal cations into vesicle bilayers.[3] We were also inspired by the proposed active structures of the gramicidin dimer channel[4,5] and of the aggregate channels of amphotericin,[6] both of which exhibit a membrane-spanning tube of roughly 2.5 nm in length with an internal diameter of 0.3 nm or more. But we were also confused by these structures because they appeared to be too short to fully

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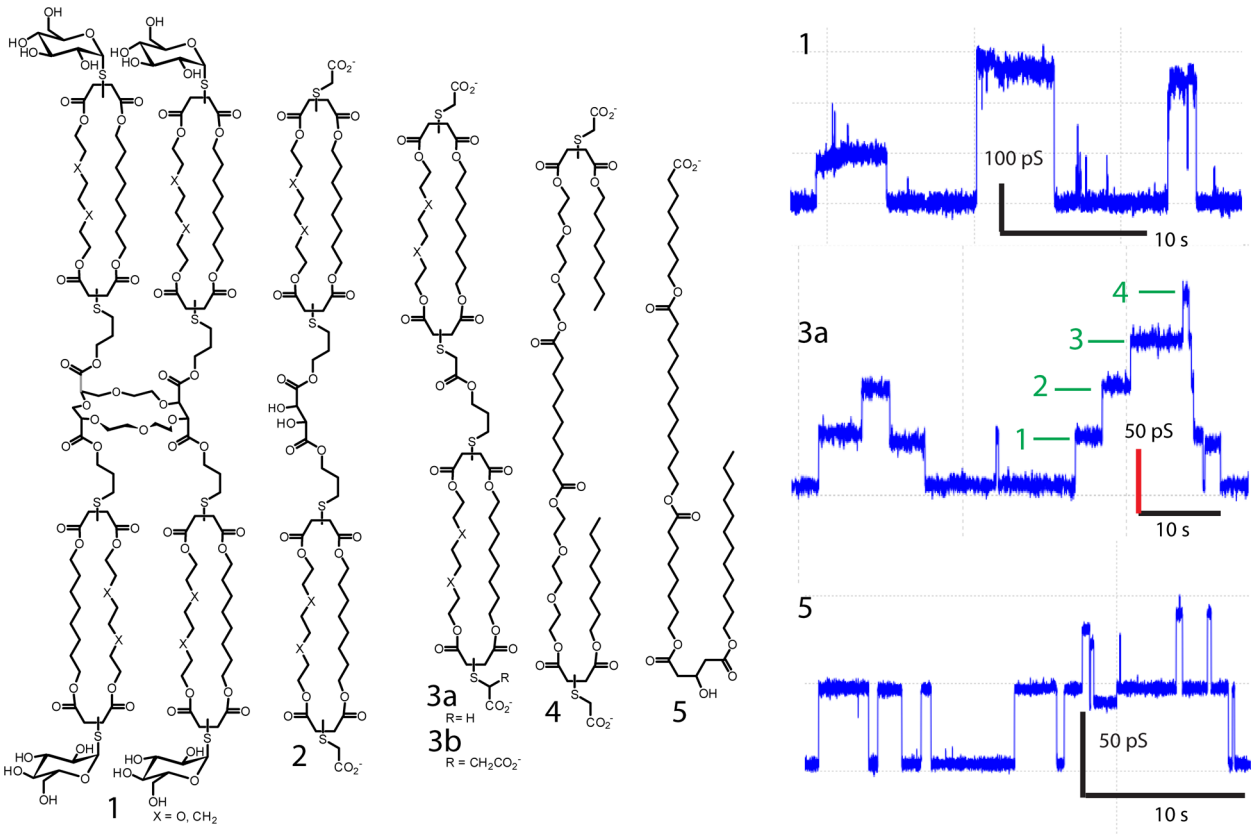

FIGURE 1. Structural evolution of linear oligoesters and their corresponding bilayer conductance–time profiles under comparable conditions (1 M CsCl electrolyte, diphytanoylphosphatidyl choline (diPhyPC) membrane, 100 mV applied potential).

span a bilayer membrane; depending on the lipids and the conditions, bilayer membranes appear to have a thickness of 3.5–4.5 nm.[7] For an ion to traverse a bilayer membrane, it would encounter the headgroup region where charges and residual water could stabilize, followed by passage through the "midpolar" region of the glycerol esters before encountering the nonpolar tail region of the bilayer. At this early stage we did not know which parts of this transit we needed the synthetic transporter to overcome. The required length has immense practical consequences; an additional nanometer would add 750–1000 g/mol to an already large target. In the end, we saw this as solely a synthetic problem, how best to construct an object of 3–4 nm length in a reasonable amount of time and yield, and assumed we could sort out how long was really required in a subsequent refinement. The proposed active structures of gramicidin and amphotericin also suggested other desirable properties: membrane-spanning bola-amphiphilic character, inward facing polar functionality to bind water and ions in transit, and hydrophobic outward facing functionality to stabilize and orient the structure within a bilayer membrane.

Our first completed target (Figure 1, **1**) therefore represented a compromise between structural control and synthetic efficiency. In the final stages, the compound was rapidly assembled from a tetracarboxylate 18-crown-6 and tetraester macrocyclic "wall" units derived from maleic anhydride and mixed diols, which were further modified by the Michael addition of thiols; the terminal thioglucose "head" groups were added at the end.[8] Compound **1** and related compounds proved to be active ion channels as assessed by a variety of vesicle-based methods.[9] Preliminary assessment by the bilayer clamp technique gave the remarkable result that the channels had conductance within the range of natural ion channels and gramicidin. The data shown in Figure 1 were acquired somewhat later but clearly show the on–off behavior expected for single channels. Seen as a catalyst of translocation, these first compounds achieved efficiencies above those of natural channels due in part to the very long duration of the open states.

From a synthetic perspective, this modular approach worked well enough and eventually 15 compounds of this type, having molecular weights between 2000 and 5000 Da, were prepared and characterized.[8] It was gratifying that not

all compounds prepared were active, and our analysis at the time focused on structural influences on a presumed membrane-spanning structure. Related work on amphotericin mimics[10] eventually prompted us to explore "cut-down" versions of **1** and its relatives, of which compounds **2** and **3a** are representative examples.[11,12] We were delighted to discover that these synthetically simpler compounds preserved all the essential features of the activity of the more complex structures as well as producing some novel behaviors.[12] Clearly, the compounds were capable of assembling within the membrane to form transport competent structures, thereby circumventing the need for prior covalent assembly. Although easier to make, these compounds were not easy targets. This was highlighted in the synthesis of compound **3b**, which was achieved in well lower than 1% yield. In this case, the end justified the means because this compound was an early example of a synthetic voltage-gated pore.[13] Voltage-gated channels in Nature underpin the action of nerves.[14] They open only under a defined magnitude and polarity of membrane potential so only fire when a signal is propagating. The current–voltage response is therefore asymmetric; such channels are molecule-scale rectifiers of ionic currents. Asymmetric insertion of our compound was clearly responsible for the rectifying behavior, and we therefore sought other compounds that were inherently dissymmetric dipolar in the same sense as **3b**.

The maleate-derived structures certainly allowed entry into this area, but they have a variety of inherent problems. The Michael additions inevitably produce an inseparable statistical mixture of regio- and stereoisomers, the initial macrocyclization process involves tedious purification, and the subsequent monofunctionalizations required to prepare dissymmetric structures degrade the overall yield considerably. The assumption that "wall units" were an essential design element was by then a decade old and had been asserted in many guises in the introductory sections of our papers. It was simple to test the validity of this assumption, and compound **4** was the first target to establish whether these macrocycles were in fact needed for activity. It was immediately clear that they were not.[15,16] This finding significantly altered the synthetic task, and we turned to rapid syntheses of libraries of compounds that we could assemble via a solid-phase synthesis.[17–19] Compound **5** is typical of the structures this methodology produces, and as is evident, the conductance–time traces[20] appear to be of the same type as earlier and much more complex structures.

In parallel, we were exploring alternative macrocyclic "wall units". The parent compound (**6**, Figure 2) was active but was essentially insoluble;[21] acyclic versions (**7**) were much more tractable and demonstrated sustained regular on–off openings (40 pS under conditions comparable to Figure 1) with good cation selectivity. More recent work in this series with compound **8** uncovered a rare exponential voltage dependence of the mean current carried by these channels.[20] Thus this series of compounds behaves as the earlier series; simplified compounds retain the activity of more complex parents.

We were not working in a vacuum and we were stimulated by reports from others of remarkable activity associated with simple compounds (Figure 2). Notable among these are the ion-pair amphiphiles reported by Kobuke, as exemplified by **9**.[22] Members of this series of compounds show regular step-conductance changes, ionic selectivity,[23] voltage-dependent currents,[24] and, when built on an azobenzenechromophore, photoswitched conductance response.[25] Even simpler are the fatty acid esters of oligo(ethylene glycol) reported by Schäfer, Neumann, and coworkers (**10**), which showed clear structure–activity properties with respect to channel-forming activity and current–time characteristics.[26] All these generally linear amphiphilic compounds potentially could line some type of transmembrane structure, but even that simplistic view does not stretch to an obvious structure for the channels formed by Triton X-100 (**11**).[27,28] There is a fine line between ion channel formation and general membrane disruption; Triton X-100 is apparently capable of straddling this line under some circumstances. The "molecular harpoons", of which **12** is an active example, were reported early on by Regen,[29,30] but at the time, we did not recognize that they were closely similar to the types of simple channels we eventually investigated. More recent examples of clear ion channels from simple compounds are Yang's isophthamide **13**[31,32] and Gokel's picolinamides **14**.[33,34] Not only do **13** and **14** transport chloride in artificial bilayer membranes by mechanisms that include single-channel formation, these compounds show membrane-derived biological activity *in vivo*. To round out this catalogue of simple compounds that form ion-conducting structures are pure membrane lipids themselves; so-called "lipid channels" can form at the phase transition temperatures of lipids[35,36] and perhaps in lipids for which no phase transition is evident.[37]

What are we to make of the activity of these simple channel-forming compounds? How can such simple compounds create structures with defined properties that persist for many seconds? Exploring such questions required a new perspective on the analysis of conductance data and new

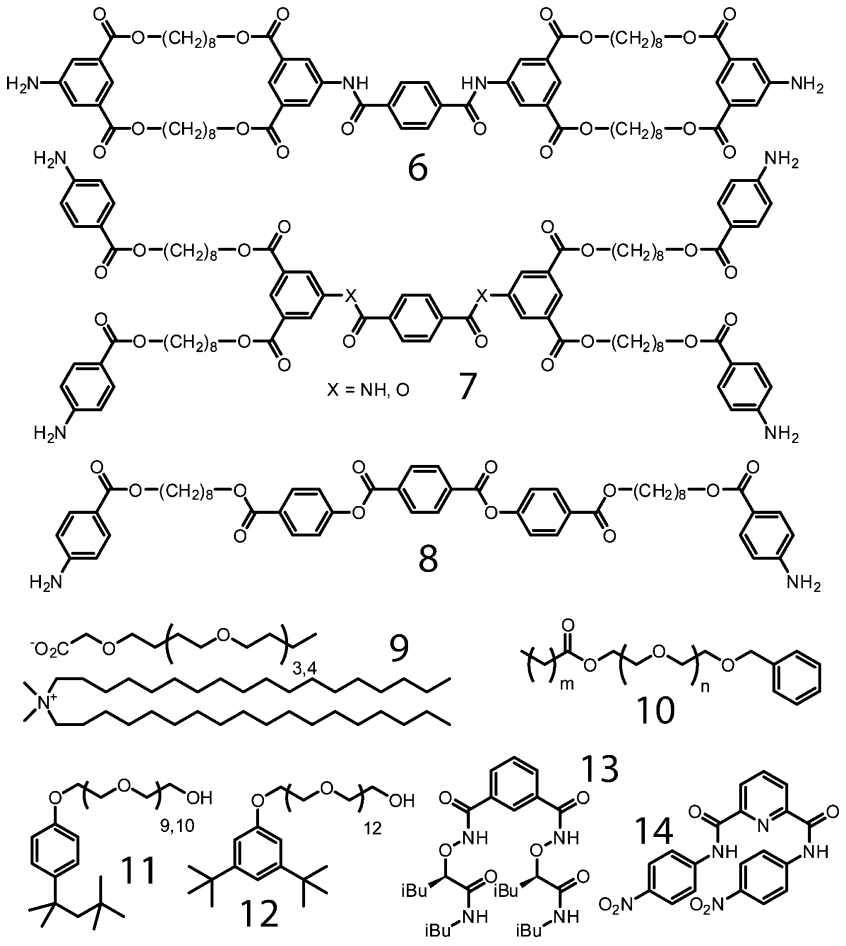

FIGURE 2. Simple compounds that form transporting structures.

compounds that allowed inactive states of the compounds to be observed.

2. Documenting Bilayer Conductance Data

Of the many techniques that can be used to detect and characterize ion channel formation,[38] the bilayer clamp experiment is conceptually the most direct;[39] a planar bilayer is formed across an orifice between two pools of electrolyte, a potential is applied, and the current is monitored as a function of time. In the absence of an ion channel, the insulating bilayer passes no current. When a channel opens, there is an increase in current for the duration of the opening. This ideal on−off behavior, illustrated in Figure 1, is unequivocal evidence of ion channel formation. The magnitude of the current passing relates to the energetic constraints the conducting structure imposes on ionic conduction and can be analyzed as an apparent internal diameter of a channel. The duration of the opening relates to the stability of the conducting structure in the membrane environment.[39] This is a single-molecule technique made possible by the high conductance of ion channels in membranes, which produce sufficient current to fall in the range of direct amplification. Unlike the usual techniques of chemistry, this technique reveals the behavior of molecules and supramolecular species one at a time. It requires sequential observation of individual events in order to infer the collective behavior of a compound, in much the same way that individual observations of marine mammals or birds eventually produce an overall picture of species characteristics. Digital data from bilayer clamp experiments are stochastic: a single molecular/supramolecular state is observed at any instant and time-dependent data represent the random shifts among the accessible states of the system. Simple on−off data are typically analyzed as a Markov process consisting of states separated by static barriers.[40]

The simple two-state model implied by the data in Figure 1, including multiple copies of recurring states, provides an easy link between observation and analysis. Consequently, this sort of observation is what we expect when we think of ion channels. Our initial experimental forays

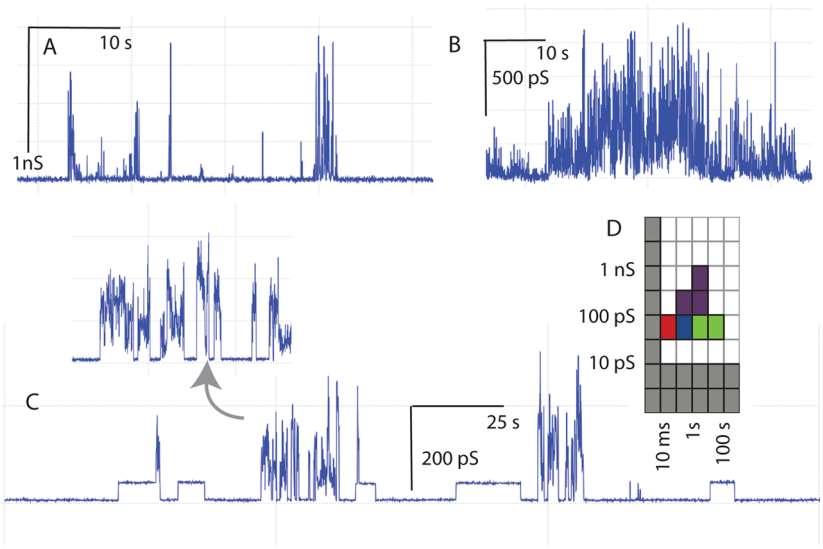

FIGURE 3. Examples of conductance–time data and an example an activity grid: (A) **1** in diPhyPC, 1 M CsCl, 50 mV, 25 min after recording of Figure 1; (B) **1** in diPhyPC, 1 M KCl electrolyte, 100 mV; (C) **8** in diPhyPC, 1 M CsCl, −100 mV; inset shows expansion in the time axis for clarification; (D) activity grid for the trace of panel C.

were directed to finding this type of behavior, and fortunately, it was not rare. Digital storage was relatively expensive, so experimental data sets were relatively limited, and observers chose to record data that could be analyzed. Other data, described in the lab books as "erratic" and other more colorful descriptors, are fairly common but only limited amounts were recorded in the early stages. Some examples are given in Figure 3 for compound **1**; this data occurred 25 min after the Figure 1 (panel A) and on a subsequent day (panel B) under somewhat different conditions. It is easy to ignore such observations. They do not "fit" with expectations, and the technique is inherently prone to artifacts and membrane failures; erratic data can always be dismissed as irrelevant or incorrect. As our confidence in our experimental abilities increased and as digital storage improved, these other behaviors were tolerated and in some cases documented,[12] but we continued to seek "gold standard" data like those illustrated in Figure 1.

This situation of ignoring difficult data persisted until the sheer weight of the problem forced us to confront it. There were two main triggers. One was the observation of apparently fractal characteristics of some of our "erratic" data;[41] the data appeared to be similar no matter what time scale was used to view them. Of more statistical weight, the open time distribution fits to a power-law dependence with high probability.[41] We did not see a clear physical picture of why this was occurring, but it was a revelation that such ugly data did in fact reflect some type of underlying physical process in which we ought to be interested. A more tractable, but still problematic, type of observation is given in Figure 3, panel C, and its inset expansion. Here there are obvious on–off openings of the Figure 1 type ("square tops") along with events of other types. It is clear that these other events are controlled in some sense; they start and stop much like the square tops. Their sole difference is that they do not settle to one level, but occupy numerous conducting states for the duration of the opening. Digital storage by this time had become inexpensive, so long data sets (30–90 min) were acquired from which it was evident that a mixture of behaviors was in fact the characteristic behavior of this and most other compounds we examined. The on–off behavior we could analyze was always present in a mixture with behavior we could not analyze.

In keeping with a natural history approach to these diverse observations, we sought a means to classify the conductance events in a way that would at least allow us to decide whether today's experimental observation of a compound's activity was similar to yesterday's. The result is the activity grid, an example of which is illustrated in Figure 3. The horizontal dimension is the duration of an observed event, the vertical dimension is the (maximum) conductance of the event, and the color coding describes the type of current–time behavior.[39] Given the span of observations, the duration dimension is logarithmic (1 ms to 100 s) as is the vertical dimension (1 pS to 3 nS in steps of factors of log 2). A colored fill describes the existence of an observation of a given conductance/duration. The colors relate to the shape of the conductance–time event: green for "square top"

openings; yellow for openings that "flicker" between two or more defined states; blue for openings with a defined start and stop and an evident set of levels within the opening time of the event; red for "spikes" of short duration or longer duration events that show some type of conductance evolution through the duration; purple for "erratic" events that have no obvious structure during the span of the event. This representation summarizes observations on a very coarse level; only five "species" are recognized, and there is no attempt to describe the frequency of an occurrence.

Our first interest was to work with a significant data set, the published literature on synthetic ion channels, to uncover whether any structural trends control synthetic ion channel behavior. We were initially profoundly disappointed because the data when summed together show that on this coarse scale structure does not matter much.[39] Rather, we showed that the activity of synthetic ion channels fell into well-defined clusters of conductance duration irrespective of the structural details. This is another facet of the similarities evident in Figure 1 but extended over a hundred wildly different structures. The key question is why should summary data be clustered around specific ranges of duration and conductance (e.g., 100 ms to 1 s, 30 pS for green openings)? Observer bias and selectivity, noted above, is probable but is an unlikely explanation; it is as easy to observe and report a 1 nS square top opening as it is to report the smaller prevalent type commonly observed. Nonetheless we selected a small suite of compounds related to **5** and **8** (eight compounds in total) and did a systematic derivation of activity grids for several hours of data collected over several months.[20] The summaries of this experimental data contained a significant proportion of the erratic and the more complex behaviors. Even though more prevalent than in the literature data summaries, the same clustering was evident in both experimental and literature data.[20]

Our conclusion is that the activity grids, crude as they are, point to an underlying energetic landscape in which synthetic ion channels function, and it is the signature of this landscape that we see, modulated by the structures we prepare. This shifts the focus of a mechanistic discussion to a larger system of water, electrolyte, lipid, and compound and to a larger number of observations required to distinguish the compound signal from the inherent system characteristics.[39]

3. Probing Transport-Silent States

As direct as bilayer conductance experiments are, they only provide information on the brief period of activity a compound provokes. This is clearly inadequate if we wish to describe the mechanistic sequence of events as a compound approaches, inserts, and eventually acts within a bilayer. Some of the most persuasive evidence for the location and activity of Gokel's hydraphile channels stems from a clever set of fluorescently labeled hydraphiles that revealed a membrane-spanning orientation.[42] Our interest in probing the mechanism of simpler amphiphiles required something similar, but we were very worried that an appended probe would be too large a perturbation. Our solution incorporates the probing fluorophore into the strand of the linear oligoester. From a dimensional perspective, diphenylacetylene appeared to provide a suitable starting point, and we focused on a group of compounds related to **5** (**15a**,**b**, Figure 4, and related congeners). The solid-phase synthesis route proved to be unworkable because the acetylene degraded under the cleavage conditions. This set-back proved in the end to be an asset because it forced exploration of alternative syntheses. A Sonogashira coupling proved to be a robust and reliable centerpiece of a convergent synthesis, which gave high-purity target compounds in good yields after relatively few steps.[43] Even better, some of the compounds prepared were substantially more active than the lead compound **5**, showing good activity in the range $10-15\ \mu$M.

The origin of the fluorescence of diphenylacteylene is complex and not well-understood,[44] but the first few compounds we made showed it to possess a good mixture of desirable characteristics. In homogeneous solution, monomer emission occurs at 320 nm; upon aggregation in polar solvent, the emission shifts sharply to an excimer emission centered about 380 nm. Both emissions can be efficiently quenched by Cu^{2+} ions with excimer quenching achieved at micromolar concentrations. The monomer/excimer emission therefore allowed the monitoring of the partitioning of the compound from water to vesicles as shown in panel A of Figure 4. This immediately clarified why **15b** was an inactive transporter compared with **15a**, a compound bearing four fewer methylenes. The active compound clearly shifts to the vesicle judging from the sharp increase in monomer emission (solid red line), accompanied by slower changes in the excimer emission (dashed red line) consistent with migration of compound from an aqueous aggregate to the vesicle. The longer homologue **15b** does nothing after vesicles are injected indicating that the aqueous aggregate acts as a sink for the transporter. When examined by bilayer clamp, a mixture of **15b** in lipid certainly shows channels can form, but in the vesicle experiment, aggregation competes and shuts down transport.

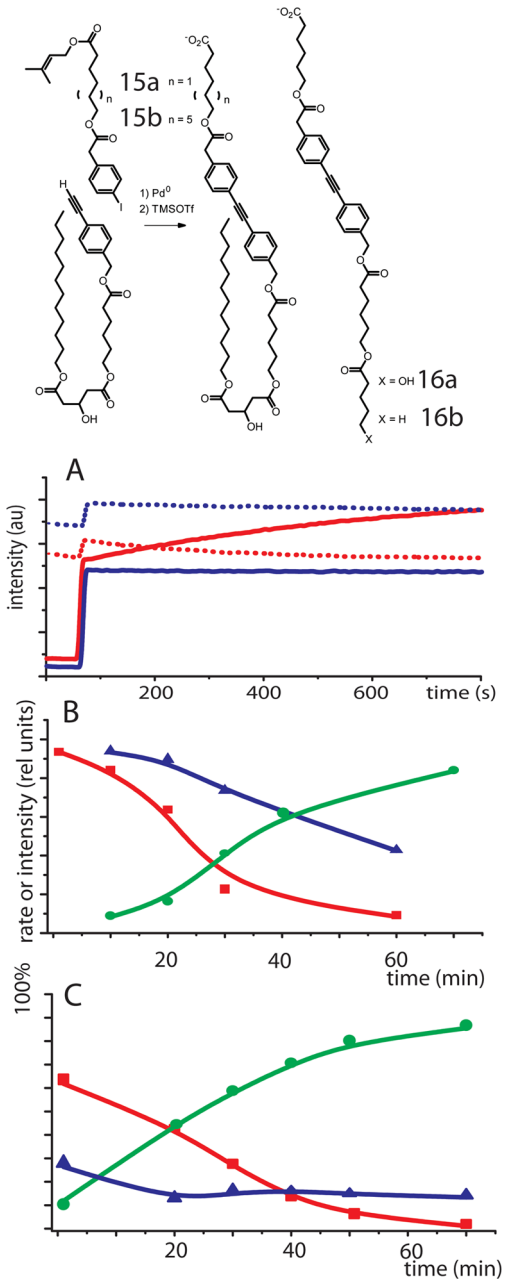

FIGURE 4. Diphenylacetylene-containing oligoesters as probes of partition and transport. (A) Emission at 320 nm (solid) and 380 nm (dashed) following injection of vesicles to a solution a **15a** (red) or **15b** (blue) in aqueous electrolyte (100 mM NaCl, pH 6.4). (B) Normalized time-dependent changes of emission at 320 nm (blue, triangle) and 380 nm (green, circle) and HPTS transport rate (red, square) following injection and incubation of **16b** in vesicles. (C) Proportions of emitting species following injection and incubation of **16b** in vesicles: short-lived component (red, square), medium-lived component (blue, triangle), and long-lived component (green, circles).

Continuing the structural simplification eventually led to compounds **16a** and **16b**.[45] Compared with **15a**, **16a** is about twice as active in an HPTS transport assay, while **16b** is about half as active. Both show time-dependent partitioning from water to added vesicles, similar to Figure 4A, but **16b** shows the unusual property that the transport rate in a vesicle assay falls off with the incubation time following injection of compound (Figure 4B, red). This slow change in transporter efficiency is accompanied by changes in the monomer (Figure 4B, blue) and excimer (Figure 4B, green) emission intensities in which the monomer emission declines over the same slow time frame as the transporter efficiency. Similar slow changes are detected in the fluorescent lifetime of the excimer (Figure 4C). Both **16a**, **b** and related compounds show three types of membrane-bound excimer species differing in lifetime: a short-lived species ($\tau < 1$ ns), a medium species ($\tau \approx 2$ ns), and a long-lived species ($\tau > 4$ ns). The proportions are usually constant with the time following incubation of compound with vesicles. In the case of **16b**, however, there is an evolution of the proportions with the short-lived component (red) declining and the long-lived component (green) increasing over the same period.[45] Emission lifetime relates to the dynamic processes accessible to the excimer; shorter lifetimes suggest a more loosely structured aggregate, while longer emission lifetimes suggest a better structured aggregate species. The changes observed are therefore indicating that initially formed excimers evolve slowly to more structured excimers.

The decrease in transport efficiency, and the decrease in the proportions of monomer and poorly structured excimer illustrated in Figure 4B,C are strikingly similar. We are well aware that correlation does not imply causation, but very few molecular processes fall into such a sluggish time frame. One that does is lipid flip–flop,[7] the process involving the penetration of a lipid headgroup to the opposite leaflet. From this starting point, we reasoned that the photophysical data in Figure 4 were consistent with an initial partitioning to one leaflet of the bilayer, formation of membrane-bound monomer and excimer in that leaflet, slow flip–flop of the compound to the opposite leaflet, and further aggregation/excimer formation.[45] The slowly formed species are less competent transporters.

4. Mechanistic Implications

With a few notable exceptions,[46,47] synthetic ion transporter mechanisms invoke transmembrane structures that provide a continuous pathway from one face of the bilayer barrier to the other.[48,49] To access such structures, the transporter must penetrate the bilayer, a process that has some of the characteristics of lipid flip–flop and is occasionally described as rate-limiting.[48,49] This view is not very consistent with the

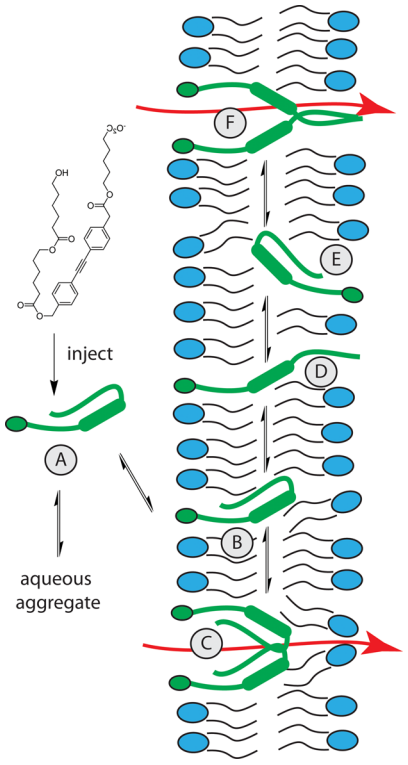

FIGURE 5. A proposed mechanistic framework for transport by oligoester channel-forming compounds.

available data on our simple compounds. First, the bilayer conductance analysis suggests that there are typically a variety of conducting structures formed, so any mechanistic proposal ought to include a number of species, some of which may be membrane-spanning. The fluorescence data show monomeric and several different types of aggregated species and in some cases transport appears to be associated with first-formed and poorly structured species, rather than defined, slower to form structures. A mechanistic framework consistent with these observations is given as Figure 5.

The injection of a compound results immediately in the formation of some monomer state (A) in water. Since channel-forming compounds are hydrophobic, aqueous aggregation is inevitable, and in some cases, the aggregate so-formed can act as a sink that inhibits further progress toward channels. The monomer can potentially associate with the bilayer in a variety of ways, but we assume a flexible amphiphile would rapidly partition to form a U-shaped insert (B). The nature of this type of species would depend on the structure of the compound: where it could bend, what length segments were produced, how these complemented the leaflet thickness, etc. Once inserted, this foreign object would perturb the membrane environment to an extent that aggregation and eventually microphase separation would become a driving force. These loosely structured aggregates (Figure 5C) would produce the kind of short-lifetime excimer emission observed (Figure 4C), and we see these as good candidates for one of the conducting structures formed. A U-shaped insertion of the correct depth would perturb the opposing leaflet of the bilayer, allowing some penetration of water, and thereby opening a conducting pathway. Such a structure would have a relatively low conductance but could be relatively constant over time as the aggregate C would be stabilized by the phase behavior of only one leaflet. The U-inserted species (Figure 5B) could uncoil to a spanning state (Figure 5D) or undergo a complete flip–flop to a U-inserted species in the opposite leaflet (Figure 5E). These new species could undergo homo- or heteroaggregation, for example, to Figure 5F to produce new transporting species. The mechanical forces on spanning structures would be considerable due to the largely independent motions of the two leaflets of the bilayer.[7] As a result, membrane structures like Figure 5F might give shorter-lived or less regular conducting states albeit with potentially much higher conductance. Whatever the true state of the system, a combination of several types of interconverting conducting structures appears to be required in order to rationalize the observations to date.

5. Conclusions

The close examination of increasingly simple compounds over the past two decades has shifted the focus away from biological paradigms toward alternative modes for transmembrane ion transport. As these are coming into somewhat better focus, it is clear that the functions of these ion transporters are just as valuable as more complex and more channel-like compounds. From the perspective of flux, the highly conducting bursts as shown in Figure 3C or the erratic episode of Figure 3B clearly move ions more efficiently than the simple square-tops of Figure 1. High and sustained conductance, whatever its structural origin has direct applications in amplification of chemical signals or membrane-disrupting biological activity. This alone will ensure that simple transporters will continue to fascinate and puzzle for a long time to come.

I am greatly indebted to the many enthusiastic co-workers who generously contributed their hands, hearts, and brains to work sketched in this Account; their names and their much more extensive contributions can be found in the cited references.

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BIOGRAPHICAL INFORMATION

Following doctoral studies in Toronto and postdoctoral studies with Jean-Marie Lehn in Strasbourg, **Tom Fyles** joined the University of Victoria where he is now Professor of Chemistry. His research is focused on supramolecular chemistry with an emphasis on membrane transport processes.

FOOTNOTES

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The authors declare no competing financial interest.

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