Communication

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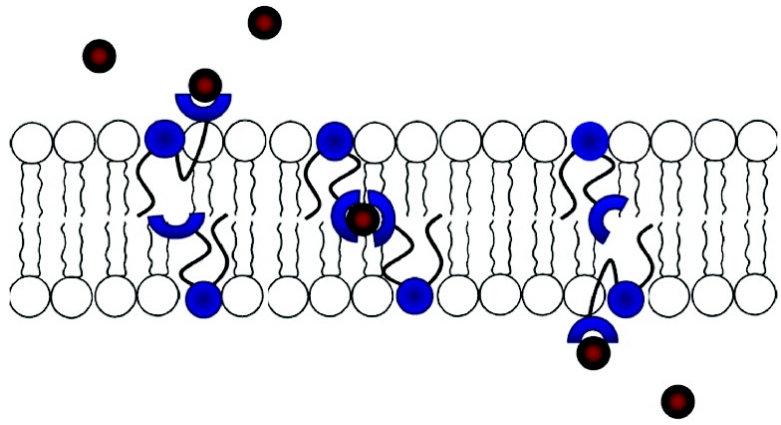

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Membrane Transporters for Anions That Use a Relay Mechanism

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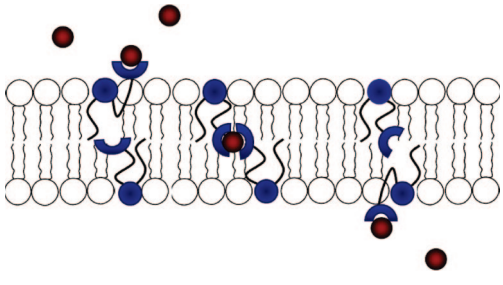
Synthetic bilayer membrane transporters are usually classified mechanistically as mobile carriers or as ion channels.[1] A mobile carrier associates with a target ion to form a discrete supramolecular complex that diffuses across the membrane; whereas, an ion channel is a relatively immobile structure that spans the bilayer and allows a continuous flow of ions.[2] In recent years there has been increased effort to design synthetic membrane transport systems for anions, especially Cl^-.[3] One of the long-term goals of this work is to create transporter replacement therapies that can alleviate the symptoms of diseases caused by diminished levels of endogenous Cl^- transport (e.g., cystic fibrosis).[4] The field of anion transport is still in its early stages with most published studies focusing on fundamental transport studies using model bilayer membranes. In terms of transporter designs, nearly all have been highly lipophilic compounds that partition strongly and nonselectively into any membrane.[5] However, next-generation designs must begin to address the requirements for pharmaceutical success, including the following formulation features: (a) acceptable solubility in physiological solution, (b) appropriate cell targeting and subsequent membrane partitioning, (c) lengthy residence time in the apical plasma membrane of target cells. Suitably designed amphiphilic transporters are likely to exhibit these desirable properties; however, it is quite challenging to design amphiphilic transporters that operate by carrier or ion channel mechanisms. This quandary has prompted us to design a new type of membrane transporter that operates by a relay mechanism.[6]

A generalized picture of the relay transport process is shown in Scheme 1. The transporter structure is a phospholipid derivative with an ionophore appended to the end of its *sn*-2 acyl chain.[7] The ionophore can bind an ion at the membrane surface and then relay it through the bilayer interior to an acceptor molecule located in the opposite leaflet.[8] In this initial study, the ionophore is a simple urea group that can associate with Cl^- via hydrogen bonds.[9] The phosphatidylcholine derivatives **1**−**3** were synthesized in a few steps and high yield using established procedures.[10] Transporter **1** contains a 4-nitrophenylurea group with a relatively high affinity for Cl^-, transporter **2** contains a weaker binding 4-*tert*-butylphenylurea group, and carbamate derivative **3** is a control structure with very weak Cl- affinity.[11]

The ability of compounds **1**−**3** to transport Cl^- into vesicles was measured using a standard fluorescence quenching assay.[12] Briefly, compounds **1**−**3** were preincorporated into separate samples of unilamellar vesicles composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/cholesterol (7:3 molar ratio, diameter 200 nm) and encapsulating the chloride sensitive fluorescent probe lucigenin. Addition of NaCl to the vesicle dispersions induces Cl^- influx and quenching of the lucigenin fluorescence. The traces in Figure 1 clearly show that transporter **1** is superior to **2**, whereas control **3** is essentially inactive. This trend of stronger anionophore producing enhanced transport has been reported before with a separate class of mobile carriers for Cl^- that also utilize urea groups.[13]

Scheme 1. Relay Mechanism for Dimeric Transporter Aggregate



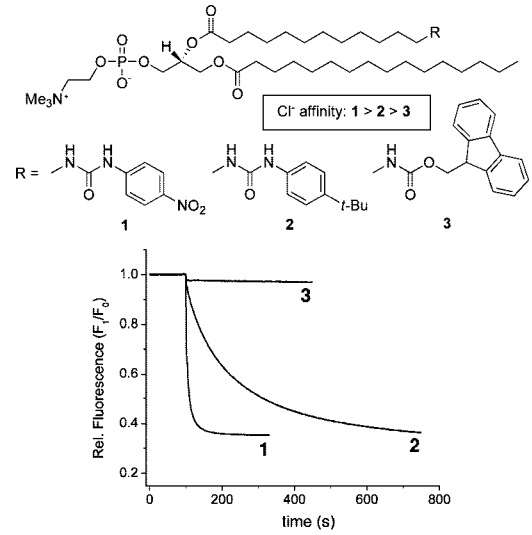


Figure 1. Fluorescence quenching due to Cl^- influx. At 100 s, an aliquot of NaCl (25 mM final concn) was added to vesicles encapsulating the chloride sensitive probe, lucigenin (1 mM) and $NaNO_3$ (225 mM), $T = 25$ °C. The vesicle membranes were composed of POPC/cholesterol (7:3) and either **1**, **2**, or **3** (5 mol %).

To elucidate the transport mechanism, a series of additional experiments were conducted with the most effective transporter, **1**. The first experiment demonstrated that replacing the intravesicle $NaNO_3$ with an isomolar concentration of Na_2SO_4 produced a greatly diminished rate of Cl^- influx (see Supporting Information, Figure S1). This is consistent with an anion exchange process; that is, significant Cl^- influx can only occur if there is a corresponding counteranion efflux, which is greatly diminished with the heavily solvated SO_4^{2-}.[3]

The relay mechanism in Scheme 1 implies that the transporter must reside in both leaflets of the bilayer. This condition was met in the initial experiment which employed vesicles with **1** preincorporated in the membrane. However, no Cl^- transport was observed when the experiment was repeated with one variation, external addition of **1** to preformed vesicles (see Figure S2 for details). In this case, the polar lipid **1** inserts into the outer leaflet of the vesicle membrane (confirmed by UV absorption) and does

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not migrate to the inner leaflet. To be effective, transporter **1** needs to populate both sides of the bilayer membrane.

The dependence of observed Cl^- influx rate constants (k_{obs}) on transporter concentration was determined in two vesicle systems with membranes of different compositions and thickness (i.e., 1,2-dimyristoylphosphatidylcholine (DMPC)/cholesterol (7:3) and the thicker POPC/cholesterol (7:3)). In both cases, the curves were nonlinear (Figure 2) indicating that transport is mediated by kinetically active aggregates of **1**. Furthermore, linear relationships are obtained for the two membrane compositions when k_{obs} is plotted against $[\mathbf{1}]^n$, where $n = 2$ and 4, respectively.[14] Thus, the transporter aggregate number is two for the DMPC/cholesterol membrane which is consistent with the slightly overlapped tail-to-tail dimer shown in Scheme 1. An aggregation of four in the thicker POPC/cholesterol membrane suggests that a pair of transporters are in each leaflet as shown in Scheme 2. An increased transporter aggregation number in thicker membranes has been seen before with self-assembled pore systems.[15]

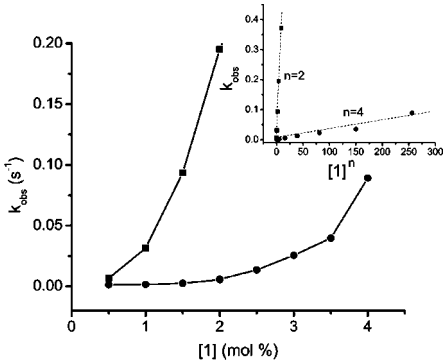
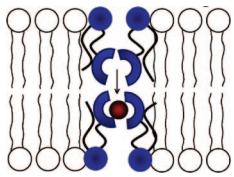


Figure 2. Rate constants (k_{obs}) for Cl^- influx at different concentrations of **1** in vesicles composed of DMPC/cholesterol (7:3) (■) and POPC/cholesterol (7:3) (●), $T = 25$ °C. Inset: linear relationships with $[\mathbf{1}]^n$, where $n = 2$ and 4, respectively.

Scheme 2. Relay Mechanism for Transporter Aggregate of Four



The final mechanistic study with **1** measured Cl^- influx rates as a function of vesicle membrane thickness. Transport was monitored in vesicles composed of phospholipids with increasing acyl chain length, and thus increased membrane thickness.[15,16] Figure S3 shows that increasing the acyl chain carbon number from 14 to 18 produced an incremental decrease in transport rate. Significantly, when the acyl carbon number was increased to 20 and above there was a dramatic drop to essentially zero transport. This membrane thickness threshold effect is consistent with the relay mechanism and not with the two alternatives.[17] When the membrane is relatively thin, the transporters can effectively relay Cl^- across the lipophilic core of the membrane as shown in Schemes 1 and 2. Once the membrane is thicker than the tail-to-tail aggregate in Scheme 2 (whose polar head groups are anchored to their respective membrane interfaces), there is a gap between the urea groups in each leaflet and the energetic barrier for Cl^- relay becomes prohibitively high.

In summary, we report a new class of synthetic membrane transporters whose molecular structures are phospholipids with anionophores appended to the end of the *sn*-2 acyl chain. The current design uses urea groups to bind and transport Cl^-; however, it should be possible to employ other molecular recognition units to produce transporters that are selective for other anions, as well as cations and neutral polar molecules. Mechanistic studies indicate that the transporters operate by a new and distinct membrane relay process. The expected favorable formulation properties of these amphiphilic compounds (e.g., as liposomes, micelles, etc) should facilitate efforts to transform them into tools for biomedical research and perhaps as therapeutic agents.

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Supporting Information Available: Synthetic procedures, transport experiments, and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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