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2,4,7-triphenylbenzimidazole : the monomeric unit of supramolecular helical rod-like transmembrane transporters

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We describe the ability of 2,4,7-triphenylbenzimidazole to form a robust self-assembly inside phospholipid bilayers. The 2,4,7-triphenylbenzimidazole monomers possess a planar structure and a lipophilicity that allow them to self-assemble into helicoidal rod-like aggregates promoting anionic diffusion via helical pathways on their aromatic exterior. Their potent anionophoric activity is demonstrated by very low EC50 values (70 µmol/l).

The cell membrane is a highly lipophilic barrier that regulates all exchanges between the intracellular and extracellular environment. This phospholipid bilayer exhibits a certain permeability and fluidity, allowing the passage of small polar molecules like water or carbon dioxide[1, 2] and repelling charged molecules, such as ions. Ions are essentials in many biological and cellular regulation processes[1] and chloride is one of the most important anions in the organism, being involved in the regulation of the cells volume.[3, 4] Dent's diseases, Bartter syndrome and cystic fibrosis are only a few examples of diseases resulting from a deficiency in the chloride transport process across the cell membrane.[5] Cystic fibrosis, a common lethal genetic disease in Caucasians, with approximately 70 000 cases in the entire world in 2013,[6] is the consequence of a mutation of the transmembrane conductance regulator protein (CFTR),[7, 8] leading to an interruption of the chloride efflux across the cell membrane. A major effect of this ion concentration perturbation is the formation of sticky mucus in the lungs,[9] causing bacterial infections that often result in an elevated morbidity of the patients.

Over the past ten years, many research groups have focused on the development of different strategies to compensate chloride transport failure using synthetic anion transporters. Different classes of supramolecular assemblies using weak interactions such as hydrogen bond[10-12], π-anion[13-16] and π-cation[17] interactions have been developed as anion transporters.

Our group has recently reported the anionophoric properties of benzimidazolium salts,[18-20] forming dimeric channels, where anions can circulate through a succession of anion-π interactions with the aromatic rings of the (4-phenylethynyl)benzyl units. The 2,4,7-triphenylbenzimidazole motif was previously reported by Loeb *et al*. as template for the assembly of 2-pseudorotaxanes and was also incorporated into metal organic frameworks (MOF's).[21, 22] The 2,4,7-triphenylbenzimidazole scaffold can be seen as a rigid analog of our previously reported benzimidazolium salts, possessing self-association properties and multiple π-anion interaction sites. We report here the anionophoric properties of 2,4,7-triphenylbenzimidazole and its analogous chloride salt. We describe the benefit of the aromaticity and planarity of the 2,4,7-triphenylbenzimidazole unit for chloride transport across lipid membranes, demonstrating that the anionophoric properties of 2,4,7-triphenylbenzimidazole result from the formation of a new type of aromatic rigid rod and discussing the kinetics and the mechanism of the chloride transport process.

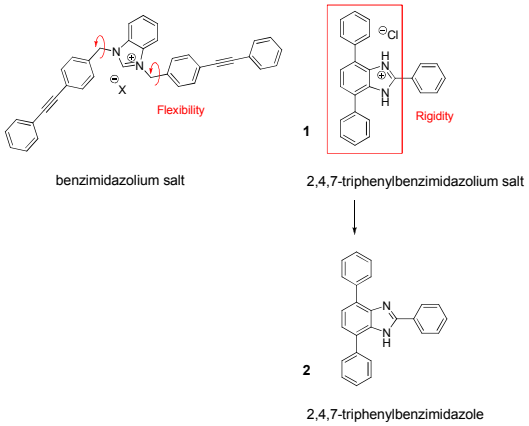

Fig 1 : Structure of the benzimidazolium salts previous studied in our group, the 2,4,7-triphenylbenzimidazolium salt **1** and its unprotonated analog **2**.

Results and discussion

The 2,4,7-triphenylbenzimidazolium chloride salt **1** was synthesized as previously described in the literature.[21] Crystals of **1** were grown by slow diffusion of hexane in chloroform and their self-assembly in the solid state is shown in Fig. 2. The maximization of the π-π interactions between all the aromatic rings results in the formation of interpenetrated aromatic sheets formed by 4 monomers. The positive charges of the benzimidazolium cations are located inside the tetramer.

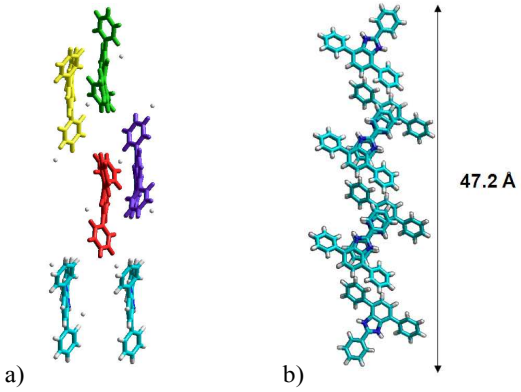

Fig 2 : Packing motif of **1** illustrating the self-assembly in the solid state. Chloride anions are shown in grey in a) and are not shown for clarity in b). The 4 monomers forming the unit cell are represented in 4 different colors in a).

Due to its high aromaticity, compound **1** has a pKa = 4.48 ± 0.10, indicating that 98% of the molecules are deprotonated in the conditions generally used for chloride transport assays (pH=6.4). Crystals of the unprotonated 2,4,7-triphenylbenzimidazole **2** were obtained by slow diffusion of hexane in a chloroform solution and their self-assembly in the solid state is shown in Fig. 3.

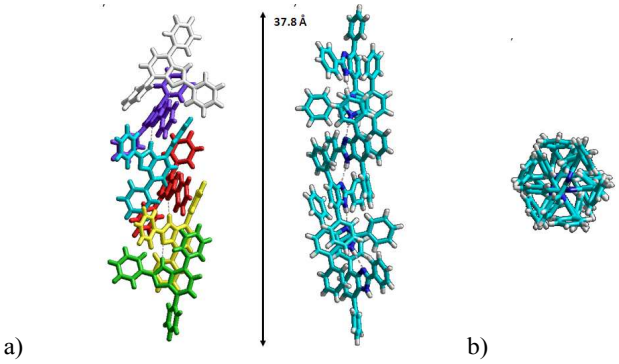

Fig 3 : Packing motif of **2** illustrating the self-assembly of **2** in the solid state : side views in a), and top view of the rod in b). The formation of the helical can be observed when different colors are used.

The crystal organization of **2** shows the formation of helicals rods promoted by the formation N-H···N hydrogen bonds between the benzimidazole moieties. This supramolecular self-assembly is also stabilized by π-stacking interactions between the triphenyl aromatic rings forming a hydrophobic aromatic exterior of the rod (Fig. 3 a and b).

The possibility to preserve this kind of aromatic rod-like self-assembled structure in the presence of phospholipids, was evaluated by a molecular modeling study of four monomers of **2** in an Egg Yolk phosphatidylcholine (EYPC) bilayer. Fig. 4 shows the assembly of the four monomers in a rod-like helical supramolecular structure that matches the thickness of the hydrophobic part of the bilayer (around 30 Å).[23, 24] This rod-like structure is less compact, compared to the organization in the crystal, but the same H-bonds and π-stacking interactions were observed in the self-assembled structure in the presence of phospholipids. This compact helical rod does not form an open channel across the phospholipid membrane, but could allow anions to slide along the aromatic hollows observed at the surface of the rod, by a succession of anion-π interactions on the exterior edges of the supramolecular structure (Fig. 4c)

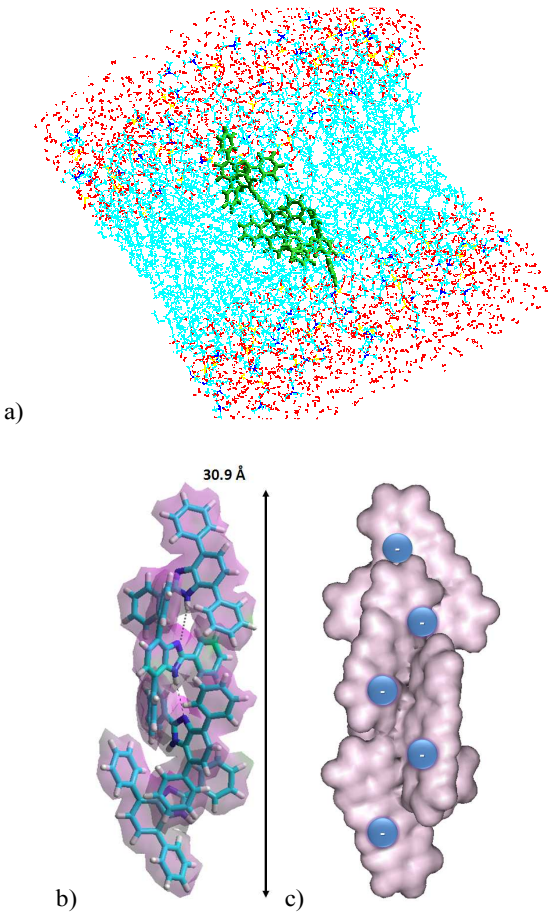

Fig 4 : Molecular modeling results obtained by semi-empirical PM3 geometry optimization of **2** in an EYPC bilayer 50 x 50 Å. a) Self-assembly of the tetramer shown in green in the hydrophobic interior of the bilayer. b) Details of the H bonds formed in the tetramer. c) Exterior surface of the aromatic rod and possible anion-π interactions

The self-association of **2** in a hydrophobic media was experimentally followed by fluorescence. The fluorescence of **2** was measured in chloroform in order to mimic the lipophilic environment inside a phospholipid bilayer. The self-association of **2** at a concentration of 2.0 mmol/L in chloroform was confirmed by the decrease of the fluorescence intensity observed after a few minutes (Fig. 5a). The decrease of the fluorescence intensity may be the result of the Dexter exchange transfer, where the photon absorbed by one monomer is transfered to another one spatially close, via a non radiative path.[25, 26]

A different behavior was observed when **2** was added to an EYPC liposomes solution in sodium nitrate/phosphate buffer (Fig. 5b). In this case, the maximum fluorescence band of **2** was shifted to 423 nm, due to the stabilization of the excited state in a polar environment. Contrary to our previous results obtained in chloroform, an increase of fluorescence was observed after a few minutes. This result suggests the formation of a less compact aggregate, where electron transfer between different monomers cannot occur. This is in accordance to the molecular modeling results, where a less compact aggregate was formed in the bilayer.

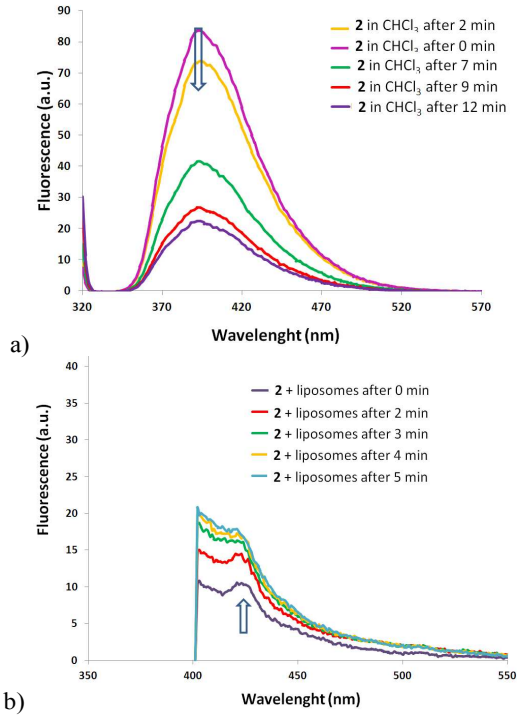

Fig 5 : a) Fluorescence of **2** (0.2 mmol/l) in $CHCl_3$ at 37 °C. b) Fluorescence of **2** (10 mol %, 1.0 mmol/l) in the presence of liposomes at 37 °C. Intravesicular: 100 mM $NaNO_3$, 10 mM phosphate buffer. Extravesicular: 100 mM $NaNO_3$, 100 mM NaCl, 10 mM phosphate buffer (pH 6.4).

The chloride transport properties of **2** were evaluated using EYPC liposomes containing lucigenin, a fluorescent probe, which fluorescence is quenched in the presence of chloride anions.[27] For these studies, lucigenin was encapsulated in the liposomes and chloride anions were present in the extravesicular solution.[28] The concentration of **2** was varied from 0.1 to 30 mol % relative to the concentration of EYPC (10 mM) and the decrease of fluorescence as a function of time was observed. A decrease of the fluorescence intensity of lucigenin when the transporter was added was specific to the capacity of the transporter to translocate chlorides inside the liposomes and quench lucigenin's fluorescence (Fig. 6).

Based on kinetic results shown in Fig. 6, and using a dose response analysis, the transport efficiency (EC_{50}) of **2** was determined. The EC_{50} value in a NO_3^-/Cl^- antiport system was 0.67 ± 0.1 mol % (relative to EYPC concentration), almost five times lower than the EC_{50} value of our previously reported benzimidazoliums salts (2.99 mol %),[20] and comparable to the efficiency of other classes of synthetic transporters reported in the literature.[12, 29] The Hill coefficient *n* obtained from the dose response analysis is 0.92 ± 0.19. The Hill coefficient generally reports the stoichiometry of the stable supramolecular aggregate required to perform the chloride transport process across the membrane. In this case, the Hill parameter close to 1 suggests the formation of a stable assembly in the phospholipid bilayer, responsible of the chloride transport process.[30]

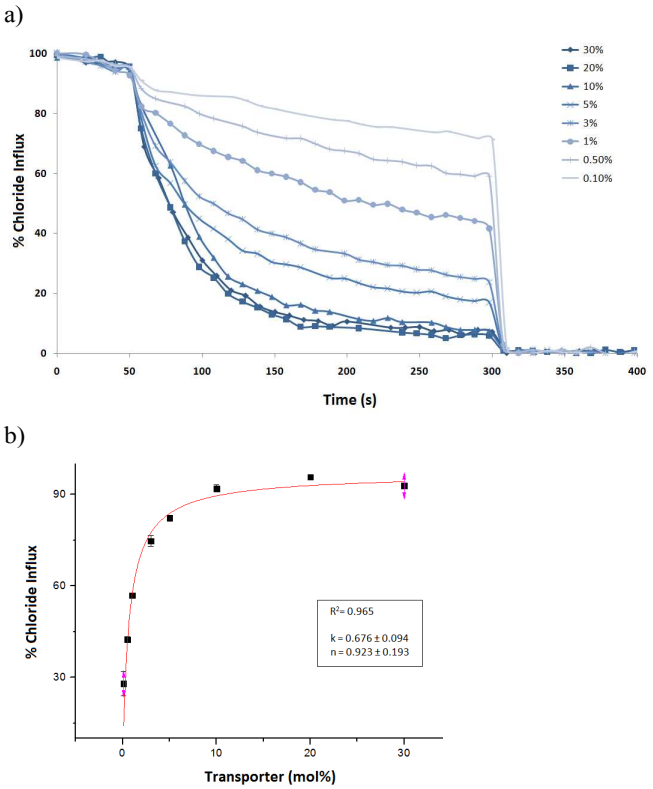

Fig 6 : a) Efflux of Cl^- in EYPC liposomes (10 mM) containing 0.1, 0.5, 1, 3, 5, 10, 20 and 30 mol % (relative to phospholipid) of **2** at 37 °C. Intravesicular: 100 mM $NaNO_3$, 10 mM phosphate buffer, lucigenin 2 mM. Extravesicular: 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 6.4). 100 mM NaCl was added before experiments. **2** was added at 50 s and Triton X was added at 300s. Each curve is the average of three independent measurements. (b) Hill plot analysis obtained from data shown in a).

In order to gain a better understanding of the mechanism of transport of **2**, similar chloride transport studies were performed in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposomes. These phospholipids possess a phase transition temperature at 41°C, below and above this temperature the liposomes are respectively in the gel and the fluid state.[1, 31] In the gel state, a reduction of chloride efflux may be observed in the case of a mobile carrier, and no or slight variation of chloride transport is usually noted for transmembrane channels. Fig. 7 shows the maximum of chloride efflux after 250 seconds of transport obtained with 10 mol% of **2** at different temperatures. These results support the formation of a transmembrane channel, as the fluidity of the membrane did not affect the chloride efflux rate.

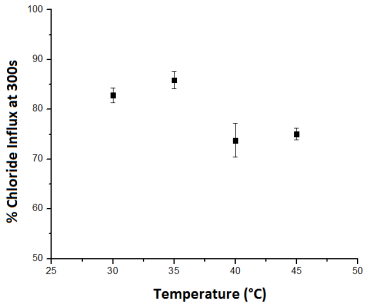

Fig 7 : Chloride efflux at 250 s in DPPC liposomes (10 mM) at 30°C, 35°C, 40°C and 45°C obtained using 10 mol% of **2** (relative to DPPC concentrations). The data at each temperature are the average of triplicates.

As increasing the temperature may also influences the diffusion process, chloride transport studies were also performed in EYPC:cholesterol (ratio 7:3) liposomes. Cholesterol rigidifies liposomes membrane by increasing the energy barrier of phospholipids rotations, lateral diffusion or phospholipid flip-flop inside the membrane.[32, 33] In this case, a mobile transporter may encounter some difficulties to pass through the phospholipids/cholesterol membrane, while a transmembrane assembly may not be influenced by the presence of cholesterol.[34] Obtaining the same chloride transport in the presence of **2** in EYPC and EYPC/cholesterol liposomes confirms the DPPC results and the formation of a transmembrane assembly responsible for chloride transport (see ESI).

Different anion permeations and electron transfer processes in hybrid membranes were previously reported by Creager et al., when studying electroactive species.[35, 36] They postulated that the anion exchange in the membrane is dependent on the hydrophobic nature of the anion and the ionic content of the external buffer.[37, 38]

We thus decided to investigate the ability of **2** to exchange other ions for the transported chloride anions in liposomes containing 8-hydroxy-1,3,6-pyrenetrisulfonate (HTPS) (Fig. 8). This pH-sensitive probe possesses a protonated and a deprotonated form with excitation wavelentghts at 403 nm and 460 nm respectively, and the ratio between the protonated (I_0) and the deprotonated (I_1) form is directly related to the transport of protons across the phospholipid bilayer.[39] For these studies intracellular media was composed by a solution of NaCl (100 mM, 10 mM phosphate buffer pH=6.4) and the variation of the intravesicular pH is indicative of proton transport across the phospholipid membrane.

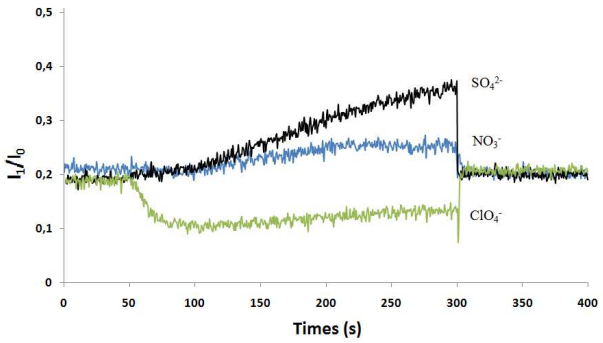

Fig 8 : HPTS-based transport assay. Intravesicular: 100 mM NaCl, 10 mM phosphate buffer (pH 6.4), HPTS 0.1 mM. Extravesicular: 100 mM NaX (X^- = NO_3^-, ClO_4^-) ou Na_2SO_4, 10 mM phosphate buffer (pH 6.4). 10 mol% of **2** was injected at 50s, triton X was injected at 300 s. The Y-axis ''I_1/I_0'' refers to the ratio of HPTS fluorescence emission at 510 nm, where I_0 is the excitation at 403 nm (acidic form of HPTS) and I_1 the excitation at 460 nm (basic form of HPTS). Each curve represents the average of three independent measurements.

The anion selectivity for **2** follows the Hofmeister series ClO_4^- > NO_3^- > Cl^- > SO_4^{2-}. Indeed, alkalinization of the intracellular media by release of protons under the effect of the transporter was observed only when the extravesicular anion was more hydrophilic than the internal chloride anion.[39] For nitrate which possess an hydration enthapy close to that of chloride (ΔH = -320 kJ/mol and ΔH = -390 kJ/mol respectively), a very low alkalinization of the intracellular media was observed (Fig. 8). This result suggests a low transport of protons and confirms the antiport process NO_3^-/Cl^- previously observed in the lucigenin assays when sodium nitrate was used as intracellular buffer. In the case of the more hydrophilic SO_4^{2-} anion (ΔH = -1080 kJ/mol), an increase with time of the ratio I_1/I_0 revealed a proton transport across the bilayer. In the presence of this anion, the antiport process is unfavoured due to the anion hydratation layer which makes it too hydrophilic to cross the phospholipid membrane. The symport mechanism H^+/Cl^- was favoured and probably occured in parallel to the low antiport mechanism SO_4^{2-}/Cl^-. An opposite behaviour was observed for ClO_4^- which is more lipophilic (ΔH = -260 kJ/mol)[40] than the internal chloride. Protons were transported inside the liposomes, suggesting a faster symport H^+/ClO_4^- process. The rapid acidification of the liposome internal media was followed by a second, slower process, where protons were transported outside the liposomes and observed as an increase of the I_1/I_0 ratio.

Based on these results, investigations of the pH influence on chloride transport in EYPC liposomes containing lucigenin were conducted. Several chloride transport assays with different external phosphate buffers at pH 6.00, 6.23 and 6.83 were

performed but no influence of the pH was observed on the maximum of chloride influx (data not shown).

The results obtained in the HPTS assays suggest a faster transport of ClO_4^- compared to Cl^- in the presence of **2**. Binding affinity studies of compound **2** for ClO_4^- and Cl^- were performed by NMR titrations in $CDCl_3$ with tetrabutylammonium chloride (TBACl) and tetrabutylammonium perchlorate ($TBAClO_4$). A low association constant of $K_a \approx 10\ M^{-1}$ and a 1:1 binding stoechiometry were obtained for both Cl^- and ClO_4^- anions. This lack of difference in the association constant values obtained by NMR titrations suggests that the most important parameter in the transport process is the lipophilicity of the transported anion. The lipophilic character of **2** (log P = 7.29, calculated with HyperChem) allows it to penetrate the lipophilic interior of the bilayer, where the monomers can self-assemble into the helical rod described earlier. When the helicoidal rod is formed the transport of different anions depends only on the hydration of these anions.

Having studied the role of the extravesicular anion on the transport, several extravesicular solutions with different cations were used in order to determine if a symport M^+/Cl^- mechanism can simultaneously occur[41]. The size and the lipophilicity of the cations were modified and chloride transport studies in lucigenin-loaded EYPC liposomes were performed. All the transport assays performed with Na^+, NH_4^+ and NMe_4^+ showed no variations in the chloride transport rate (see ESI), suggesting that no symport M^+/Cl^- processes occurred.

Chloride transport can occur by a transport mechanism or by the destabilization of the membrane in the presence of **2.** The integrity of the liposomes during the transport assays was confirmed by dynamic light scattering (DLS) measurements.[42] A blank was realized with liposomes in presence of 50 µl of MeOH and an average diameter of 120 nm was obtained. When 30 mol% of **2** (relative to the concentration of EYPC) in MeOH were added to the liposomes, the diameter of the liposomes increased from 120 nm to 135 nm. This 10% increase in the liposomes size is usually the result of the insertion of the transporter into the phospholipid membrane.[43] All the liposomes were monodisperse after addition of the transporters showing that the liposomes were intact and eliminating the hypothesis of liposomes destruction. Considering that such a high loading of **2** in the liposomes membrane does not affect the integrity of the bilayer, the use of a smaller amounts of transporter **2** should not influence the stability of the liposomes either.

Conclusions

In conclusion, we described here the ability of 2,4,7-triphenylbenzimidazole to form a robust self-assembly in the phospholipid bilayer. Its planar structure and its lipophilicity are making of this compound an attractive scaffold for the development of active chloride transporters, the one described here possessing an interesting EC_{50} value of 70 µmol/l. The 2,4,7-triphenylbenzimidazole monomers self-assemble into a helicoidal rod-like aggregate promoting anionic diffusion via an helical pathway on its aromatic exterior. To the best of our knowledge, this is the first example of a self-assembled aromatic rod-like supramolecular structure containing benzimidazole units, able to efficiently transport anions in phosphoplipid bilayers. These results are encouraging us to optimize the structure of the 2,4,7-triphenylbenzimidazole unit, by increasing the conjugation and the planarity of the molecule that might result in improved chloride transport properties.

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Notes and references

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† Electronic Supplementary Information (ESI) available: experimental details for measurement of chloride transport using lucigenin and HPTS assays, DLS mesurments of liposomes, ^1H NMR titrations, molecular modeling procedures and details of the crystal structures. See DOI: 10.1039/b000000x/

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