CyPLOS: a new family of synthetic ionophores†

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The ion transport properties of a new family of synthetic ionophores based on cyclic phosphate-linked oligosaccharide (CyPLOS) macrocycles are described.

Artificial ion transporters, *i.e.* ionophores, are synthetic molecules that mimic at a functional level the activity of naturally occurring ion channels or carriers.[1] The interest in these systems has intensified in recent years because the control of ion-coupled processes occurring in the cell membrane and which regulate most of the physiologically relevant cell processes[2] may lead to new biotechnological and biomedical applications. For example, the group of Matile has shown that artificial ion channels can be used in the development of elegant sensing schemes for biologically relevant analytes.[3] On the other hand, synthetic ionophores have demonstrated antibiotic and antiproliferative activity,[1d] as well as the ability to activate new chloride pathways to overcome the loss of chloride channel function in channelopathies such as cystic fibrosis.[4]

Ion transport is a typical supramolecular function which requires specific intermolecular interactions between the ionophore and the transported ions in order to compensate for the loss of hydration energy during the translocation process across the phospholipid bilayer.[1h] This can be achieved through a variety of different mechanisms, leading to some structural diversity among synthetic ionophores so that they are often functional but not structural mimics of the natural channels. In this context, cyclodextrins decorated on one or both faces with chains of different nature have been shown to form ion channels in phospholipid bilayers.[5] As a matter of fact, one of the first examples of a synthetic ionophore, reported by Tabushi and co-workers in 1982, was based on a β-cyclodextrin with four long hydrophobic chains attached to the lower rim.[5a] This design was re-investigated more recently by the group of Gin, who has described a β-cyclodextrin with seven oligobutylene glycol chains attached to one face.[5d,e] This macrocycle provides a pore at the surface of the membrane, where the chains, inserted in the membrane, represent the polar conduit for the ion transport.

We are interested in the design of new artificial ionophores using macrocyclic scaffolds for the appendage of the active amphiphilic subunits.[6] Recently, some of us have described the synthesis and conformational properties of novel cyclic oligosaccharide analogues, 4,6-linked through phosphodiester bonds, that were named CyPLOS (cyclic phosphate-linked oligosaccharides).[7] These cyclic saccharide surrogates, designed to combine some constitutive elements of both small cyclodextrins and crown ethers, have been further exploited as platforms for preparing different analogues, where the secondary hydroxyls at C-2 and C-3 of the monosaccharide residues have been derivatized with long chains of different lipophilicity.[7c] In this way we obtained the jellyfish-shaped phosphate-linked disaccharides **1–3**, shown in Fig. 1. Our expectation was that these molecules were able to insert in the phospholipid bilayer, with the anionic macrocycle floating on the polar membrane surface and the amphiphilic tentacles dipping in the inner core of the phospholipid bilayer, thus altering its permeability. Herein we report our studies on the ionophoric activity of these novel derivatives together with the investigation of compounds **4** and **5**, used for control experiments.

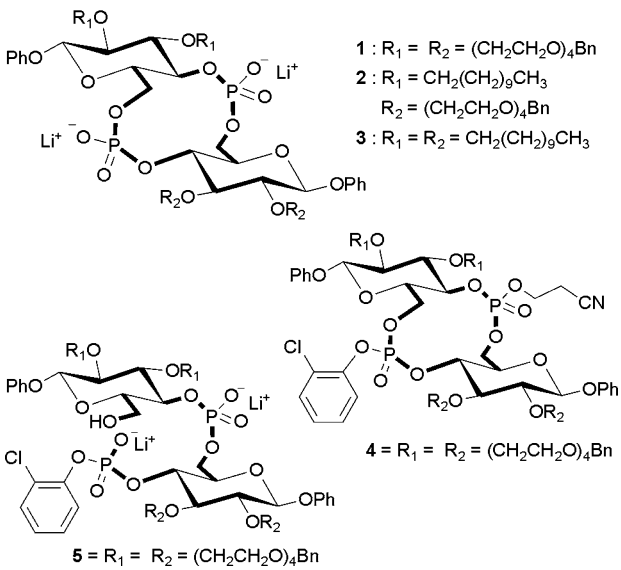

Fig. 1 Chemical structures of CyPLOS derivatives **1–5**.

The synthesis of CyPLOS derivatives **1–3** has been reported in full detail and is based on straightforward and well optimized reactions in oligonucleotide chemistry.[7c] Compounds **1–3** share a common di-anionic macrocyclic structure and differ in the nature of the chains appended at C-2 and C-3 of the two monosaccharide rings. On going from **1** to **3** the number of the tetraethylene glycol (TEG) chains decreases from four to zero, being progressively substituted with C_{11} hydrocarbon tails, thus providing a tetra-polyether (**1**), a mixed (**2**), and a tetra-alkylated (**3**) derivative with increasing hydrophobicity along the series. Compounds **4** and **5** are structurally related to **1** with two main differences: compound

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4 is a fully protected, and therefore neutral, precursor of **1**, while **5** is a linear di-anionic analogue.

The ionophoric activity of the CyPLOS derivatives was investigated with the HPTS assay (HPTS = 8-hydroxypyrene-1,3,6-trisulfonic acid).[8] In brief, HPTS, a pH-sensitive fluorescent dye (pK_a 7.3), is entrapped in the inner water pool of large unilamellar vesicles (LUVs, 100 nm diameter). The liposome suspension is prepared in a pH 7.0 HEPES buffer containing 100 mM NaCl (or MCl and NaX in the experiments for cation and anion selectivity, respectively) and, once the ionophore is added, the external pH is suddenly brought to 7.6 by the addition of NaOH (or MOH in experiments for cation selectivity). All the experiments were carried out at 25 °C, a temperature at which the membrane is in the fluid state. The increase of the HPTS fluorescence in response to the applied transmembrane pH-gradient indicates basification of the inner water pool which may be derived either from H^+ efflux or OH^- influx. This transmembrane charge translocation needs to be counterbalanced and this may be achieved through four possible overall processes: H^+/Na^+ antiport, OH^-/Cl^- antiport, H^+/Cl^- symport and Na^+/OH^- symport. The ionophoric activities of the CyPLOS derivatives are reported in Fig. 2.

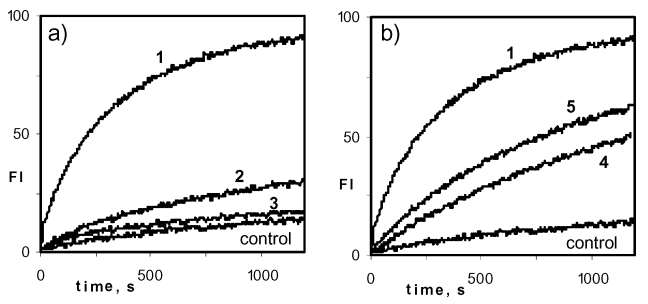

Fig. 2 Normalized fluorescence change in HPTS emission (FI, λ_{ex} 460 nm, λ_{em} 510 nm) as a function of time after addition of the base (50 μL of 0.5 M NaOH), in the presence of 2% concentration of ionophores **1–3** (a) and **1**, **4** and **5** (b), to 95:5 EYPC/EYPG LUVs loaded with HPTS (0.1 mM HPTS, 0.17 mM total lipid concentration, 25 mM HEPES, 100 mM NaCl, pH 7.0, total volume 3 mL). The ionophore concentration is given in percent with respect to the total concentration of lipid. The control trace has been recorded in the absence of ionophore. (HPTS: 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EYPC: egg yolk phosphatidyl choline; EYPG: egg yolk phosphatidyl glycerol.)

Among the CyPLOS derivatives investigated here, **1** shows the highest ionophoric activity, with the pH gradient being completely discharged after about 20 minutes in the presence of 2% ionophore (Fig. 2a). This activity may be strictly correlated to the presence of the TEG chains, as indicated by the lower efficacy of the mixed derivative **2** and by the almost complete inactivity of tetra-alkylated derivative **3**. On the other hand, the comparison with compounds **4** and **5** (Fig. 2b) shows that the anionic character of the candidate ionophore and the presence of the macrocycle are not prerequisites for activity but both guarantee a *ca.* two-fold more active compound, with the first structural motif somehow more important than the second one.[9] On the basis of these results, an in-depth investigation was concentrated on compound **1**.

The ionophoric activity of **1** increases with its concentration and the fitting of the kinetic traces gives the apparent first-order rate constants (k_{obs}, s^{-1}) for the transport process.[10] The values of the rate constant for selected concentrations of ionophore **1** are reported in Table 1 and compared with the rate constant reported by Gin and co-workers for their oligobutylene glycol functionalized β-cyclodextrin[5d] (CD) and with that of amphotericin B (AmB), a naturally occurring ionophore which is often taken as a reference compound.[6c] The activity of **1** is a significant fraction of that of AmB and about 5 times lower than that of CD. This latter comparison suggests that a more rigid macrocyclic scaffold and a higher number of appended hydrophilic chains are beneficial factors for activity.

Table 1 Observed rate constants (k_{obs}, s^{-1}) for the transport process in the presence of the different ionophores[a]

Ionophore	Concentration[b]	10^3 k_{obs}, s^{-1}
none	–	0.32
1	0.5	1.01
1	1.0	1.53
1	2.0	2.98
CD[c]	0.5	4.8
Amphotericin B	1.0	2.33

[a] For conditions see Fig. 2. [b] The ionophore concentration is given in percent with respect to the total concentration of lipid. [c] CD refers to the functionalized β-cyclodextrin reported by Gin and co-workers. The rate constant has been estimated from the original data (ref. 5d).

The dependence of the activity *vs.* the concentration of ionophore **1** is reported in Fig. 3a. The observed rate constant of the transport process increases linearly with the concentration

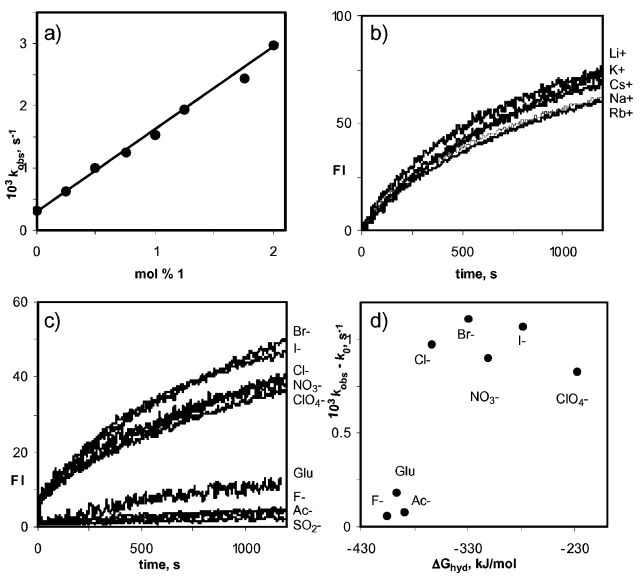

Fig. 3 a) Dependence of k_{obs} on the concentration of ionophore **1**. The original kinetic profiles are reported in Fig. S3.† For conditions see Fig. 2. b) Cation selectivities for ionophore **1** (1% concentration), using the HPTS assay (100 mM MCl, pH 7.0, base pulse by addition of 50 μL of 0.5 M MOH, other conditions as specified in Fig. 2); c) Anion selectivities for ionophore **1** (0.5% concentration), using the HPTS assay (100 mM NaX, pH 7.0, base pulse by addition of 50 μL of 0.5 M NaOH, other conditions as specified in Fig. 2, Glu = glutamate); d) Apparent first-order rate constant corrected for the unmediated electrolyte exchange (k_{obs}–k_0, s^{-1}) as determined from the profiles of Fig. 2c as a function of the anion hydration energy. The data for SO_4^{2-} are not reported due to the very negative hydration energy.

of **1**, suggesting that the active species responsible for the transport process is monomeric.[11]

Selectivity in ion transport was investigated with a protocol proposed by the group of Matile.[8] In this experiment a suspension of LUV loaded with HPTS is diluted in a buffer containing only the cation or anion under investigation. Therefore, the fluorescence time course observed reports on the selectivity in ion transport. The results are illustrated in Fig. 3b and c where the kinetic traces are corrected for the permeation of the anion or cation under investigation in the absence of ionophore. On varying the nature of the cation present in solution among the group I alkali metals, the activity is not sensitively affected (Fig. 3b); on the other hand, the anions (Fig. 3c) show an "off-on" selectivity, with the halogens (except fluoride), nitrate and perchlorate efficiently transported, and fluoride, acetate, glutamate and sulphate little or not transported. The insensitivity of the rate of transport to the nature of the cation, together with the absence of ion flux in the presence of certain anions, where however Na^+ is present as counterion, suggest that anions and not group I cations are involved in the transport process, thus excluding H^+/Na^+ antiport, and Na^+/OH^- symport from the possible pathways for the pH gradient decay.[12] Accordingly, the experiments of Fig. 3b report on the transport of chloride, which is present as the counterion of all the cations. The selectivity observed with anions is more complex. The apparent rate constants for the transport process (k_{obs}, s^{-1}), corrected for anion permeation in the absence of the ionophore (k_0, s^{-1}), show a clear discontinuity for anion hydration energies between those of acetate and chloride ions as shown in Fig. 3d.[13] Apparently, the anions less lipophilic than chloride are not transported; on the contrary, the more lipophilic anions are indeed transported, but with efficiencies scarcely depending on their hydration energy. This behavior seems to exclude the presence of specific interactions between the transported anions and the ionophore, as well as a transport process simply controlled by the dehydration cost of the anions. Taking into account that the pH gradient decay correlates with OH^-/X^- antiport or with H^+/X^- symport, an alternative explanation for the observed "off-on" selectivity is the switching of the transport process rate limiting factor from X^- to H^+ (or OH^-) transport on decreasing the anion hydration energy. Highly hydrophilic anions are not transported but when the dehydration cost decreases below that of acetate, the process is controlled by H^+ (or OH^-) transport and, therefore, all the anions with lower hydration energy are transported at a similar rate. This is also suggested by the observation that the proton carrier FCCP[14] increases the observed rate of transport in the case of chloride by a factor of about 2 [see Fig. S4,† FCCP = carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone]. Finally, the above discussed selectivity data suggest the formation of a poorly structured active species with little or non specific interactions with the transported ions (see the little difference in selectivity among the lipophilic anions).

On the basis of the obtained results, the mode of action of ionophore **1** may be visualized as follows: the polar macrocycle lies on the surface of the membrane with the four amphiphilic chains inserted in the phospholipid bilayer. In the active conformation, the macrocycle anchors the ionophore on the membrane surface while the TEG chains destabilize the phospholipid bilayer underneath, forming a polar conduit which favors ion transit.[15] The overall length of **1** is clearly insufficient to span the entire double layer but activity is often observed in such "short" channel forming ionophores.[16] Probably, the disorder in the phospholipid bilayer induced by the TEG chains generates a defect[17] in the membrane and the ions, when assisted to cross the membrane interface and the first part of the hydrocarbon core, find their way to the opposite side.

In conclusion we described here the ionophoric activity of new macrocycles **1–3**, based on an anionic cyclic disaccharide analogue (CyPLOS) derivatized with long chains of different lipophilicity. Our results indicate that both the presence of the four TEG tentacles and the anionic macrocycle are structural motifs necessary for activity. With respect to other existing preformed macrocycles such as cyclodextrins, CyPLOS are synthetically accessible tools with a higher degree of chemical and conformational diversity and more easily and selectively manipulable moieties. Studies are in progress towards the preparation of a second generation of CyPLOS macrocycles for a better tuning of activity and selectivity and a deeper investigation of their mechanism of action.

Acknowledgements

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9 The same trend was observed with the phosphate-protected or the linear derivatives of **2**, despite the lower general activity. See ESI†.
10 The kinetic traces for the experiments at different concentrations of **1** are reported in Fig. S3.† The data fit well with a first-order kinetic process and the apparent first order rate constants were obtained by non-linear regression analysis of the fluorescence data *vs.* time. The fit error on the rate constant was always less than 1%.
11 Linear activity/concentration profiles are observed also in the presence of a monomer/aggregate equilibrium completely shifted toward the aggregate. However, the critical aggregation concentration of **1** in $CDCl_3$ is between 6 and 8 mM (ref. 7c), a concentration much higher than the one used here in the transport experiments.
12 In the absence of pH gradient, ionophore **1** is however able to transport Na^+ as indicated by ^{23}Na-NMR experiments (see ESI†). Therefore, we cannot exclude that in the HPTS experiment the transport of Na^+ not coupled with H^+ or OH^- is present.
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15 We cannot exclude that **1** acts as a molecular carrier because experiments in gel phase membranes, after incubation of the ionophore above the phase transition temperature, failed due to the low partition of the ionophore in such conditions, see ESI.† However, this appears very improbable due to the molecular structure of **1**.
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