A fluorescent assay for chloride transport; identification of a synthetic anionophore with improved activity

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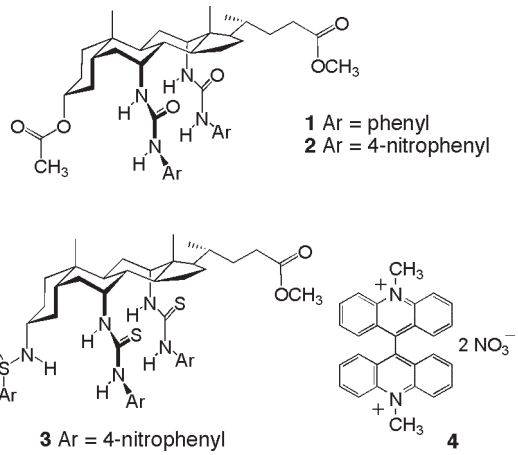
A fluorescent assay based on the chloride-sensitive probe, lucigenin, is developed for monitoring chloride transport into vesicles, and used to compare the effectiveness of three steroid-derived transporters.

A topic of growing interest in supramolecular chemistry is the construction of synthetic membrane transporters for chloride ions with potential applications in biomedical science.[1] Recently, a number of research groups have described prototype designs of transporters that operate in liquid organic and bilayer membranes.[2–4] Our own contribution is the development of a steroid-derived family of "cholapods" and our discovery that they are able to transport chloride ions across vesicle and cell membranes.[3] The lipophilic cholapods partition strongly into bilayer membranes and transport the chloride ions as hydrogen bonded complexes. An important attribute of the cholapod structure is that it can be readily modified to give derivatives with predictable changes in molecular and supramolecular properties. For example, cholapod **2**, with two *N*-(4-nitrophenyl) urea groups is a significantly better chloride binder than cholapod **1** with less acidic *N*-phenyl urea groups. In terms of chloride transport, a preliminary structure/activity study indicated that cholapods with increasing anion affinity induce higher transport fluxes, *i.e.*, cholapod **2** is a significantly better transporter of chloride ions than cholapod **1**.[3] Intuitively, this correlation is not expected to continue indefinitely because at some point the kinetics of anion release from the transporter will become rate-determining. To ascertain how close we are to an end-point, we decided to measure the transport induced by a cholapod with substantially higher anion binding affinity than **2**. An excellent candidate is cholapod **3**,[5] which is known to have a chloride affinity that is two hundred times greater than **2**, and ten thousand times greater than **1** (K_a (Et_4NCl) for **1**, **2**, and **3** in water saturated chloroform is 1.5×10^7, 5.2×10^8, and 1.1×10^{11} M^{-1} respectively).[3,5]†

Cholapods **1**, **2** and **3** were initially tested in a previously reported transport assay that employs a chloride selective electrode.[3,4] This straightforward potentiometric experiment is conducted in the following way. Unilamellar vesicles are prepared with encapsulated sodium chloride. An electrode is inserted into the vesicle dispersion and used to monitor the rate of chloride escape from the vesicles upon the addition of a candidate transporter. When this experiment was conducted with cholapod **3**, essentially no chloride efflux was detected; however, a close inspection of the dispersion indicated that **3** was precipitating as a fine powder which, of course, invalidated the experiment. To avoid this solubility problem we subsequently chose to pre-incorporate each cholapod into the vesicles and test if the vesicles were capable of enhanced chloride influx upon addition of a pulse of sodium chloride. This required an assay that was capable of detecting increases in chloride concentrations inside a vesicle. The literature contains examples of fluorescent proteins,[6] and synthetic dyes[7] that are chloride-sensitive. We were attracted to the commercially available fluorescent probe, lucigenin, **4**,‡ which is commonly used to detect superoxide,[8] but it is also known to be quenched by halide ions and has been employed in assays to detect chloride transport using biological transporters.[9] Chloride influx into vesicles was measured in the following way. Vesicles composed of POPC (1-palmitoyl-2-oleoylphosphatidylcholine)–cholesterol (70 : 30) and an appropriate amount of cholapod were prepared by dispersion of a lipid film in aqueous solution followed by extrusion through 200 nm membranes. Encapsulated inside the vesicles was a solution of lucigenin (1 mM) and sodium nitrate (225 mM). The lucigenin dye that was not encapsulated was removed by filtration through a Sephadex G-50 column loaded with sodium nitrate (225 mM). The transport experiment was initiated by adding an aliquot of sodium chloride in sodium nitrate to give a final concentration in the assay vessel of 0.4 mM lipid, 225 mM sodium nitrate, and 24 mM sodium chloride.



The fluorescent quenching traces in Fig. 1 show the change in lucigenin emission when sodium chloride is added to vesicles containing cholapod **1**, **2**, or **3** (the ratio of transporter to lipid is 1 : 25 000). The data indicate that cholapod **2** is substantially more effective than **1** which is in agreement with our electrode-based results.[3] In addition, it is clear that cholapod **3** is the most effective transporter with an initial rate of influx that is almost two times that for **2**.

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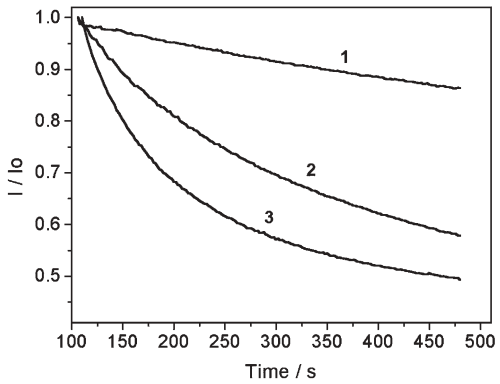

Fig. 1 Chloride transport into vesicles containing **1**, **2**, or **3** at a cholapod to lipid ratio of 1 : 25 000. Inside vesicles: 225 mM $NaNO_3$, 1 mM lucigenin. Outside vesicles: 225 mM $NaNO_3$, 24 mM NaCl. No transport was observed in the absence of cholapod.

The traces in Fig. 2 show that the chloride influx induced by cholapod **3** is concentration dependent and is greatly inhibited if the vesicles encapsulate sodium sulfate instead of sodium nitrate. The same results were observed with compound **2** except that the transport rates were about two times slower (data not shown). The sulfate inhibition of chloride influx, combined with our previous observation that chloride transport flux is independent of counter cation identity,[3] is strong evidence that these cholapods mediate transport by an anion exchange mechanism. That is, to maintain electroneutrality a chloride anion can only enter a vesicle if another anion departs. This is easily achieved if the vesicles contain the relatively lipophilic nitrate anion, whereas, the sulfate dianion is extremely hydrophilic and is not readily transported. Therefore, when the vesicles contain sodium sulfate, the initial influx of chloride quickly induces an electric potential across the membrane (inside negative) which greatly inhibits subsequent transport.

The data clearly demonstrate that compound **3** with an extremely high affinity for chloride is also an excellent chloride transporter. The result extends our empirical observation with the cholapod series, that increased anion binding ability leads to increased chloride transport. Efforts are under way to see if the correlation is maintained with other members of the series. Also ongoing are mechanistic studies, with the preliminary data in favor of a carrier diffusion process.[3] It is worth noting that in the case of sodium cation transport across bilayer membranes, mediated by neutral crown ethers (and known to be a carrier diffusion process), transport rates are observed to increase with cation affinity constant.[10] And from a theoretical perspective, transport flux for a carrier mediated ion exchange process is expected to be maximal when both ions have the same affinity for the carrier.[11]

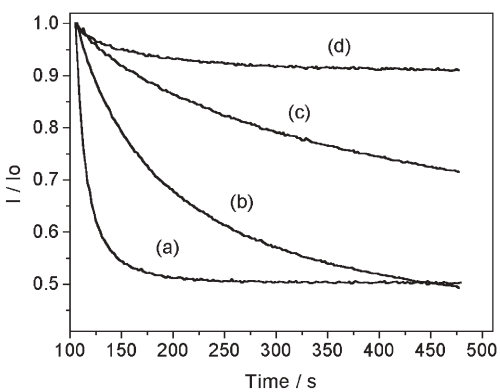

Fig. 2 Chloride transport into vesicles containing cholapod **3** at a cholapod to lipid ratio of: (a) 1 : 2500 (b) 1 : 25 000 (c) 1 : 250 000 (d) 1 : 25 000 and Na_2SO_4 instead of $NaNO_3$.

The fluorescent lucigenin assay will greatly facilitate our future structure/activity studies, especially with highly lipophilic cholapods. In addition, the assay appears to be an excellent way of rapidly testing libraries of synthetic transporter candidates for activity. The method requires small amounts of material and can be readily modified for high throughput screening using fluorescence plate reading technology. Since the lucigenin assay measures chloride influx it is complementary to the electrode method which measures chloride efflux. This research was supported by the National Science Foundation (USA) and the EPSRC (GR/R04584/01). Assistance from the EPSRC National Mass Spectroscopy Service Centre, Swansea, is gratefully acknowledged.

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Notes and references

† The eicosyl ester analogue of **3** is reported in ref. 5. Cholapod **3** was similarly prepared and tested as a receptor for Et_4NCl. In this and other cases, the change in ester side-chain has very little effect on measured affinities.

‡ Lucigenin excitation 455 nm, emission 506 nm. In water the quantum yield is 0.67 and the chloride Stern–Volmer constant is 390 M^{-1}.[7a] Chloride quenching effectiveness is significantly decreased in the presence of POPC/cholesterol vesicles, presumably because the lucigenin weakly associates with the vesicle membranes which inhibits chloride access to the dye. Under the assay conditions (0.4 mM lipid, 225 mM sodium nitrate) the chloride Stern–Volmer constant was determined to be 44 $\pm$ 5 M^{-1}. Thus, the curves in Figs. 1 and 2 plateau at I/I_0 values of around 0.5 because the intravesicle chloride concentration reaches the expected equilibrium value of 23 mM. Lucigenin is reported to be metabolically unstable,[7a] and is weakly quenched by DMSO.

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