Boronic acid mediated ion transport through bilayer membranes

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ABSTRACT

All cells of cellular organisms need to communicate with each other and their surroundings. Generally cellular communication occurs by moving substances into or out of the cell through the cell membrane via ion channels or ionophores. We wished to mimic this ion transport behaviour using some boronate synthetic ionophores containing cholate. These were new compounds and it was unclear if they transported ions through a carrier or channel mechanism. To investigate the mechanism both hydroxypyrene trisulfonate (HPTS) and U-tube assays were carried out; the latter suggested a carrier mechanism. Furthermore we wished to observe if ion transport by the boronic acid was affected by the presence of sugar, and found a strong decrease in ion transport caused by fructose. Finally an unsuccessful resynthesis of these boronate ionophores containing cholate was attempted.
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Chapter 1:

Introduction
1.1 Cell membranes

The smallest unit of life is the cell and cells are the basic structural and functional unit of multicellular organisms.\(^1\)

Every cell has a very thin outer layer which is called the membrane or plasma membrane. The cell membrane separates the cell from its environment.\(^2\) However, in order to function; cells need to communicate with each other and the surrounding environment. A group of biological cells that perform a similar function is called tissue (e.g. nerve, connective tissue), and a collection of tissues joined in a structural unit to present a common function is called an organ (e.g. brain, kidney, heart). All these structures require correct functioning of cells together, which also requires communication through the cell membrane, for example by controlled transport of ions through biological membranes. This shows the study of cell membrane function is crucial. It could be a pathway to new drugs and could help scientists to cure illnesses such as cancer.

1.2. Membrane structure

Our current picture of the cell membrane has been built up over many years of research. The fluid mosaic model was proposed by Singer and Nicholson (1972) as a two-dimensional lipid-protein bilayer which has a fluid character. The model explains the function and structure of the phospholipid bilayer based on its chemical and physical behaviour.\(^3\)

Cell membranes mainly consist of phospholipids and protein molecules. According to the fluid mosaic model they are in an organised arrangement of flexible sheets. A semi-permeable environment is created by the bilayer
membrane, while proteins are responsible for the dynamic processes throughout the cell membranes, such as ion or molecule transport into or out of the cell.²

![Cell membrane model of Singer and Nicolson](image)

**Figure 1: The cell membrane model of Singer and Nicolson.⁴**

### 1.2.1 The lipid bilayer

There are three types of amphipathic lipids found in the cell membrane, including phospholipids, glycolipids and cholesterol. Phospholipids are amphipathic molecules, meaning they have a hydrophilic (water-loving) head which are present at the intracellular and extracellular faces of the bilayer membrane. Phospholipids have two hydrophobic (water-fearing) tails. These two fatty acid chains will be away from the surrounding polar solution at the centre of the membrane due to the hydrophobic effect.²

In water, phospholipids naturally form a bilayer because this prevents exposure of the hydrophobic groups, which can close to form a spherical structure called a vesicle.
The fluidity of the components of the cell membrane depends on the length and properties of the fatty acid chains in the phospholipid components. There are several different types of phospholipid head groups (Figure 2).

![Figure 2: Head-group structures of phospholipids.](image-url)
1.2.2. Bilayer proteins

The Singer and Nicolson model of the cell membrane also contains two types of protein; peripheral (or extrinsic) proteins which simply adhere to the membrane where they are bound by polar interactions. The other type of cell membrane proteins are integral (or intrinsic) proteins which are located within the membrane between the fatty acid chains.\textsuperscript{5, 6}

![Different types of proteins in the cell membrane.](image-url)
1.2.3 Other components of the membrane

The other components of cell membranes are glycolipids and glycoproteins. These components have carbohydrates linked to lipids or proteins and are often positioned towards the outside of the membrane.

Glycolipids and glycoproteins are very important for organisms to identify cells. For example some changes in glycolipid carbohydrates units occur when a cell becomes cancerous. This allows white blood cells to identify and attack the cancerous cell.²

1.3 Transport across the cell membrane

1.3.1 Types of transport

Cells employ different mechanisms for transporting chemical substances across the membrane. However, transport across the cell membrane is classified according to two major mechanisms, which differ according to their energetic requirements.

1. Passive transport (no energy input required by the cell).

Some substances like small ions, carbon dioxide, oxygen and water can spontaneously cross the membrane, as mentioned previously. This passive transport depends on a concentration gradient, meaning that the concentration of the substance on either side of the membrane is different (water also can flow by a concentration gradient which is set up by osmotic pressure across the membrane).
Passive transport is also sub-classified as either simple diffusion or facilitated diffusion (Figure 4).

Facilitated diffusion also involves diffusion, but of polar and ionic molecules, while simple diffusion is only possible for hydrophobic (non-polar) and small polar molecules through the bilayer.

Diffusion of these polar and ionic molecules is facilitated by proteins, in the cell membrane. These proteins provide channels which can aid the passage of specific molecules. The process also does not require energy input as it is thermodynamically favourable.
2. Active transport: this requires energy from the cell to be put in, which leads to pumping in or out of polar molecules such as sugars, amino acids and ions (which cannot pass readily into the cell). The process requires specific protein pumps and a source of energy which is usually ATP (adenosine triphosphate). Active transport typically involves moving chemical substances against their concentration gradient; therefore it would be entropically unfavourable.\textsuperscript{7}

1.4 Synthetic ion channels

Ion channels are natural proteins that allow ions to move from one to other side of the cell membrane and regulate key functions of the cells. Supramolecular chemists have been able to create analogues of natural ion channels which can reproduce many of the membrane’s functions.\textsuperscript{8}

1.4.1 History

Synthetic ion channels have a long history, going back to 1970s when they were often based on modified peptidic channels like gramicidin (Figure 5).

Gramicidin is barrel-shaped, and spans a single leaflet of a bilayer membrane. It becomes “active” when two molecules are positioned in opposite leaflets and come together in an end-to-end model.

A successful synthetic example was created by Ghadiri \textit{et al.}, who designed the cyclic $\beta^3$ peptide nanotubes.\textsuperscript{9}
Figure 5: Scheme showing Gramicidin A acting as an ion channel. (a) Gramicidin channels form by the trans-bilayer dimerization of two subunits, (b) side views of a bilayer-spanning gramicidin channel.\textsuperscript{10}

1.4.2 Mechanisms and characteristics of ion channels

Natural ion channels typically consist of four to seven protein helices which span the membrane, but most synthetic channels have much less complex structures.

This project is not focused on creating ionophores that are channels, but it is instead aimed at developing ionophores without a specific mechanism (carrier or channel). However, discussing the behaviour of synthetic ion channel will give a clear picture of ion transport through bilayer membranes. There are some specific criteria for making an artificial ion channel. Firstly, it needs a membrane-spanning structure.\textsuperscript{11,12} The membrane thickness (a
lipid bilayer) is about 4 nm and the thickness of the hydrophobic core is around 3-3.5 nm.

Secondly, this membrane-spanning structure must confine a sufficient volume for the passage of the ions. Effective molecular weights of the channel structure should be around 3-4 kDa. In addition, the channel must produce an interior hydrophobic contact within a bilayer membrane. As a result, an artificial ion channel reduces the surface tension or the interfacial tension of the membrane, much like a surfactant.  

But there is a balance between a synthetic compound that can simply destroy the membrane by detergent action and an ion channel which can behave as a regular membrane component and contribute to the function of the membrane. Therefore, to create an ion channel a special shape and size must be considered. For example, compounds which can fit into the membrane directly and cause no further disruption are more likely to act as channels than detergents.  

1.5 Detection of ion channel activity

1.5.1 Methods for studying ion channels

For the study of ion channels, two experimental methods have been designed; vesicle bilayer membranes and planar bilayer membranes.
1.5.2 HPTS detection of ion transport

The 8-hydroxypyrene trisulfonate (HPTS) assay is a vesicle based experiment. Vesicles, like small unilamellar vesicles and large unilamellar vesicles (SUVs and LUVs), range in size between 20 nm and 1 μm in diameter with an internal volume which is bounded by one or more layer of the membrane. Thus, the volume inside the vesicle is isolated from the outside environment. In general, vesicles can entrap many different species in the interior space.

Ion transporters act as catalysts that increase the rate of ion passage from one side to the other side of the membrane. Consequently, any technique that can measure changing ionic concentration or pH on one or both sides of the bilayer membrane could determine the transporting activity in vesicles. However the internal volume of the vesicles compared to the external volume is very small, so releasing material from the inside can make only a very small change in the external environment. Therefore, it requires a special detection method to follow and monitor ion transport in vesicles.

The HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid) method is a technique that uses a pH-sensitive fluorescent dye to report the pH inside vesicles. In this method vesicles are prepared in the presence of HPTS in a buffer. They are then purified by GPC then the transporter (ionophore) is added to the vesicle solution. At this point, by adding a base to the external buffer, a pH gradient is established. The change of pH inside the vesicle caused by $M^+/H^+$ antiport is recorded by the change in fluorescence of the indicator.
(the HPTS inside the vesicles). The HPTS method was the main ion flux measurement method used in this project.

Figure 6: HPTS method for measuring ion transport across a vesicle.\textsuperscript{14}

1.5.3 Planar bilayer membrane conductance measurements

Another method for assessing transport is using planar bilayer membranes. The technique is reliable and ideal for monitoring ion conductance across the bilayer membrane. In this method a small hole in a Teflon sheet is needed to create a barrier which is positioned between two aqueous electrolyte solutions. The bilayer is formed by painting the lipid across the hole. This should be in a non-polar solvent (e.g. decane) across the opening. A constant transmembrane potential is applied then current changes are monitored and recorded as a function of time.
The membrane is a good resistor itself and therefore, when the ion channel is not open, very little current is observed. However, the signal produced by an open channel, which response to the applied potential, is notable and appears as a step-change in the current on the recorded baseline (Figure 7).

Figure 7: Schematic representation of the PBC cell. (a) is a cuvette which has a small hole in a Teflon sheet on one-side. (b) is the bilayer which is formed by painting (across the hole). (c) is an Agar salt-bridge. (d) is an electrolyte. (e) is the reference electrode.

1.6 Types of ion transporter

There are different modes of transport through the cell membrane. Transport via ion carriers is facilitated by formation of a non-covalent interaction between an ion and a carrier molecule. The process can occur in four-steps; formation of the complex (carrier-ion), diffusion through the membrane, release of the ion (or substrate species), and finally reverse diffusion of the carrier to manage another transfer. The other mode of transport, which is mediated by ion channels,
occurs via a pathway through the membrane. Ion channels are usually long and have a tubular or helical shape.

1.6.1. Ion carriers

Ion carriers have the ability to diffuse through the membrane rather than span the membrane; they are typically macrocyclic species. There are many natural ion carriers such as valinomycin, which is a cyclic peptide antibiotic that increases $K^+$ transport through the membrane.\textsuperscript{16} Nonactin is another antibiotic example of ion carrier which allows ammonium ions to be transported through the bilayer.\textsuperscript{17}

![Figure 8: Valinomycin with $K^+$ in the centre.\textsuperscript{18}](image.png)
Transport by ion carriers has been studied for many years, and there are some examples of artificial anion carriers which have been inspired by prodigiosin, a natural carrier. Prodigiosin is red pigment with anticancer properties that is produced by the bacterium *Serratia marcescens*. It is believed that prodigiosin (Figure 9) has ability to transport $H^+$ and $Cl^-$ into cells.\textsuperscript{19}

![Figure 9: Prodigiosin](image)

### 1.6.2 Ion channels

Ions and molecules can also cross the membrane *via* direct diffusion through ion channels or ion pumps (energy required) (Figure 4). Naturally there are a wide range of molecules can act as ion channels. They are mainly proteins and notably are integral membrane proteins, such as nAChR and hERG channels.\textsuperscript{15}
A well known example of a natural ion channel is the peptide gramicidin which is made from an (alternating D- and L) sequence of amino acids. (Val-Gly-Ala-D-Leu-Ala-D-Val-Trp-D-Lev-Trp-D-Lev-Trp-D-Leu-Trp-NH-CH₂CH₂OH). In membranes or non-polar solvents it can make a β-helix, however, the helix length is not long enough to cover the thickness of a bilayer but is almost half of the bilayer in length. Therefore two gramicidin molecules meet end-to-end, stabilized with hydrogen bonds, to make a channel that is suitable for alkali cations. Another example of an ion channel is the polyene antibiotic amphotericin which forms channels in bilayer membranes that contain sterols. The amphotericin monomer has a mycosamine head group to give it amphiphilic character. The polyene tail is in the hydrophobic region of the membrane, and the hydroxyls aid the passage of ions through the membrane (Figure 10).

Figure 10: Amphotericin
1.6.3 Artificial ion channels

The phenomenal behaviour of natural ion channels has inspired supramolecular chemists to create a significant variety of biomimetic ion transport systems and synthetic ion channels in bilayer membranes.\textsuperscript{22} Many natural ion channels have peptide-based structures but other motifs can be used to create a wide variety of synthetic ion channels. The diversity of structural motifs has enabled researchers to mimic these crucial ion transport processes.\textsuperscript{15}

1.6.4 Target synthetic ion channels for this project

A large number of synthetic molecules have been reported to act as ion transporters in lipid membranes. Many of these have been based upon the cholic acid dimers of Kobuke, such as compound 5 overleaf.\textsuperscript{23} It was hoped that by appending boronic acid groups to the ends of this bis(cholate) scaffold it would be possible to develop ion channels or carriers that were responsive to the presence of sugars. A series of boronate-cholates were synthesised by Dr J. Brown in the Webb group (Figure 11) and were used in this
Figure 11: The series of boronate-cholates used in this project. Kobuke’s methoxycholate dimer 5, boronate-cholate monomer 6, cholate dimer 7, boronate-cholate dimer 8, cholic acid 9.
Chapter 2:
Synthetic ion channels
2.1. Bile acids as motif for transporter species

One of the most important popular compounds that have been used as a motif for designing new transporters are bile acids. Many of these have been based on cholic acid, and cholate dimers have been shown to transport $K^+$.\textsuperscript{23}

2.1.1. Cholic acid as ion channel motif

The structure of cholic acid 9 is similar to cholesterol, the steroid in cell membranes. It has a curved structure with a hydrophobic surface and hydrophilic surface. Cholate spans one leaflet in the bilayer and can be easily functionalised at the 3-position of 9. These features make it an ideal motif membrane to create ion transporters.\textsuperscript{15}

2.1.2 Synthesis of ion channels based on cholic acid

To gain some experience in synthesis it was decided to synthesise one of the small but important intermediates towards 8 (Figure 12).
Figure 12: $N$-[8-(benzyloxycarbonylamino)-3,6-dioxaminoctyl]-3α,7α,12α-trihydroxy-5β-cholan-24-amide.

This synthetic step was developed by James Brown and Craig Wilson. One of the reactants was cholic acid and the other was the amine 11 (1-(benzyloxycarbonylamino)-3,6-dioxa-8-aminoctane). This amine was provided by Dr J. Brown in the Webb group (Figure 13).
2.1.3 Previous work

To synthesise the ion channel 12, the first step was to synthesise the head group “linker” and then attach it to the cholic acid. For this synthesis the diamine 10 was converted to a monoamine using either benzyl chloroformate or di-tert-butyldicarbonate (Figure 13). Dr Brown provided the benzyl protected amine, so the next step was to attach 11 to cholic acid 9.

2.1.4 Synthesis of ion transporter 12

Cholic acid 9 and amine 11 were to be coupled using a HBTU/DIPEA-mediated coupling (Figure 13). \( O-(\text{Benzotriazol-1-yl})-N,N,N',N'-\text{tetramethyluronium hexafluorophosphat} \)e or HBTU is a frequently used coupling reagent for peptide synthesis. It also requires simple reaction conditions, a very short reaction time, and gives high yields. \( N,N'-\text{Diisopropylethylamine} \) or DIPEA is an amine that is a good base but a poor nucleophile, which makes it a useful organic reagent. The synthesis was followed by well-established HBTU coupling chemistry. 24

The synthesis was carried out three times. The first time, a solution of cholic acid 9 was made by adding dry dimethylformamide and HBTU. The solution was stirred for 45 minutes. After that, a solution of the amine 11 in dry dimethylformamide was added to the first solution, and left to stir for 48 hours. Then the solution was diluted with dichloromethane and washed with dilute hydrochloric acid, saturated sodium bicarbonate solution and brine. After that, the organic solution was dried, filtered and the filtrate evaporated in \textit{vacuo}. Flash chromatography was then attempted to obtain compound 12 which was the product of peptide coupling between cholic acid 9 and amine 11.
Unfortunately, $^1$H NMR spectroscopy on the fractions from the column did not reveal pure product (e.g. signals in the aromatic region are too strong relative to the other peaks). The experiment was therefore repeated in the same way but once again no product was found in the NMR spectra of the column fractions. The third time, it was decided to halve the amount of reactants (cholic acid 9, amine 11 and solvent). This was to prevent overloading of the column and to provide a better chance of purifying the compound. The NMR spectra of the crude reaction mix before the column suggested 9 and 11 had been added in the right ratio. However, purification by column chromatography gave fractions that did not show any product in the NMR spectra (Figure 14), and instead only showed unreacted cholic acid.

![NMR spectrum of product 12](image)

**Figure 14: NMR of product 12.** The problem is that the integral for the aromatic region is too strong relative to the other peaks. So this sample is not pure. It also showed no amide signals (5.03-7.28 ppm).
2.1.5 Considering problems in the synthesis of 12

The failure to synthesise this intermediate was disappointing, because it was a straightforward synthesis which had been carried out successfully by both James Brown and Craig Wilson in the group. A possible problem was a practical mistake concerning the ratio of the reactants 9 and 11, including; accuracy of measurements and errors with adding solvents or reagents. A second possible problem was using the wrong size of the column which would only affect purification not the reaction itself. However, the column was changed to get pure peaks. A third concern was about the possible use of wet solvents during the experiment, however, it was made sure that the whole reaction was under nitrogen and dry solvents were used.

TLC analysis showed new spots that possibly corresponded to the product each time the compound but not seen in the NMR spectra.

Unfortunately there was not enough time to try again and consider the other possible mistakes.
Chapter 3:
Ion transport and the HPTS assay
3.1 Boronic acid for membrane transport

Crown boronic acids (Figure 15) have been designed as carriers for catecholamines through membranes, and were studied by Jeffery T. Bien and Bradley D. Smith. Catecholamines are very important compounds associated with stress, heart disease, thyroid, hormone levels. It has been proposed that blood pressure changes could be stabilised by changing catecholamine levels in cells.

![Figure 15: An example of a crown boronic acid.](image)

3.2 Previous work

The HPTS assay method had been already used for compounds 7 and 6 by James Brown, who synthesised and observed ion transport via compound 8 through a lipid bilayer. Following this previous research, it was decided to find out whether boronic acids such as 6 and 8 can facilitate Na⁺/K⁺ transport through the bilayer. The behaviour of compounds which do not include boronic acids in their structures, such as 7, would serve as controls.
3.2.1 The HPTS assay for ion transport

The HPTS assay method was chosen for the detection of ion transport through the bilayer in this project. The method is based on the pH sensitivity of HPTS and is a vesicle-based fluorescence assay. The pH-sensitive fluorescent dye trisodium-8-hydroxypyrene-1,3,6-trisulfonate (HPTS or Pyranine)\textsuperscript{27} has two forms with different fluorescent properties. The protonated form has an excitation maximum at 405 nm and the deprotonated form has a maximum excitation at 460 nm (Scheme 1). The emission of both forms is green fluorescence at 510 nm. By using the ratio of emission intensities from both forms, the ion transport could be monitored via pseudo-simultaneous excitation at those wavelengths.

\begin{center}
\textbf{Scheme 1: Acidic/base forms of HPTS or pyranine.}
\end{center}
HPTS is suitable for monitoring the internal pH of the vesicles because when it is encapsulated inside the vesicles it cannot escape. Transport of Na⁺/K⁺ with H⁺ (antiport), or Na⁺ / K⁺ with OH⁻/Cl⁻/phosphate (symport) can occur across the bilayer, and depends upon the buffer solution that is used during the preparation of the vesicle and the exterior solution (in the cuvette). The transport of these ions effectively changes the pH inside the vesicles. Therefore increasing the pH inside the vesicles increases the concentration of the deprotonated form (Scheme 1) and a change in fluorescence is seen.

The HPTS method used here was a modification of that performed by Winchel et al. A 1 mM suspension of 8:2 EYPC/cholesterol vesicles containing HPTS (100 mM) in a MOPS buffer (pH = 6.5) was placed into a fluorimeter. Addition of a base gave a one unit (approximate) change in pH. Then the ion channel species (e.g. the boronic acid 8) was added (1 mol %) and the assay recorded over 35 minutes. Then to complete the experiment and get the maximum values for normalisation, a 25% (v/v) solution of Triton X-100 detergent in MOPS buffer was added to break the vesicles.

**3.2.2 Preparation of synthetic phospholipid vesicles**

For the HPTS assay, it is very important to prepare good quality phospholipid vesicles. There are many methods for preparing artificial vesicles like sonication and interdigitation-fusion. However, one of the most important methods is extrusion through a polycarbonate membrane, as used in this project. A wide variety of phospholipids can form vesicles using the extrusion method. In this method, a suspension of multilamellar vesicles (formed by dispersion of
phospholipids in aqueous solution) is forced through a polycarbonate membrane in a special extrusion apparatus. The pore size of the membrane, used for this process is normally between 200 nm and 800 nm and the vesicle size depends on the size of the pores in the membrane.

3.3 Detection of ion transport from boronic acid

The boronic acid 8 had been already made by James Brown from the Webb group, so the challenge for this project was to prove the ion transporting ability of 8 using the HPTS assay.

Vesicles were prepared and ion transport analysed using the HPTS method as described in Section 3.2.2. In brief, an appropriate suspension of vesicles in MOPS buffer with pH = 6.5 was incubated before the base pulse was added, the pH and ion gradient was monitored over a period of up to 35 minutes. Ion channel/carrier species, the boronic acid 8 (20 μL, to give 5 mol% in the membrane) was added after 10-15 minutes. To complete the experiment and reach the maximum fluorescence values, which allows data normalisation, a 25% (v/v) solution of Triton X-100 (a detergent in MOPS buffer) was added to destroy the vesicle membranes.

These experiments indicated that compound 8 had the ability to transport Na⁺ through the bilayer. The change in HPTS fluorescence (Figure 16) shows an increase in internal pH due to Na⁺/H⁺ antiport, indicating that the pH gradient was becoming discharged.
Figure 16: HPTS ion transport data for compound 8. (a) Raw fluorescence data showing emission at 510 nm after excitation at 460 nm (—) and 405 nm (—), indicating the addition of the base pulse at the first point (black arrow) and TX-100 at the second point (blue arrow). (b) Normalised data, which is the emission ratio (excitation at 460 nm)/(excitation at 405 nm). The second arrow indicates ionophore addition.

**3.3.1 Ion transport by boronic acid 8 at different membrane loadings**

The HPTS assay was set up as per the previous section and the experiment repeated with three different volumes of vesicle suspension added. Keeping the concentration of 8 constant meant that the membrane loading of 8 decreased as the amount of vesicle suspension was increased.

Ion transport by the boronic acid 8 with these increased amounts of vesicles (lower membrane loadings of 8) was found to be almost the same as the lower concentrations of vesicle. The normalised data in (Figure 17) shows the
concentration of vesicle in solution did not have an effect on transport rate. However, a control for these experiments, the methanol control (Figure 18), showed there was a bit of “leakage” from the vesicles itself, therefore some of the changes in fluorescence were due to H⁺ leakage not ion transport.

Figure 17: Transport at different membrane loadings of 8: (—) 25 μL vesicle suspension (5 mol % boronic acid 8), (—–) 50 μL vesicle suspension (2.5 mol % boronic acid 8), (—–) 100 μL vesicle suspension (1.25 mol % boronic acid 8).
3.3.2 The effect of increasing the concentration of the boronic acid 8 on ion transport

In this experiment different concentrations of the boronic acid 8 were tested, but this time using the same concentration of vesicle suspension (25 μL). This also has the effect of increasing the membrane loading of 8, but with a concomitant increase in overall concentration of 8.

An appropriate amount of vesicle suspension was added to a solution of MOPS buffer with pH = 6.5. After incubation of the suspension for two or three minutes, addition of NaOH (20 μL) created the approximately one unit base pulse. Then the ion channel species (boronic acid 8) was added; either 20 μL of stock solution (to give 5 mol %) or 40 μL (to give 10 mol %). The assay was recorded
over 35 minutes before 25% (v/v) solution of Triton X-100 (a detergent) was added to normalise the data. To improve the reliability of the result, the experiments were repeated three times.

The normalised data (Figure 19) shows there was more transport using the higher concentration of the ion channel 8 (10 mol %). Furthermore this was much higher than the “blank” methanol test (Figure 20).

Figure 19: Transport at different concentration of 8: (—) 20 μL or 5 mol % boronic acid 8 (25 μL vesicle suspension), (—) 40 μL or 10 mol % boronic acid 8 (25 μL vesicle suspension).
3.3.3 Comparing the ion transport ability of compounds 6 and 7 with the boronic acid 8

It seemed that boronic acid 8 acted as an ion channel, and also worked better at a high concentration of approximately 10 mol %. Now, the next challenge was to consider the quality of ion transport of the boronic acid 8 by comparing with the other species (the monomer 6, the cholate acid 7), which had already been synthesised by James Brown in the group.

The other two compounds 6 and 7 were tested for ion transport using the HPTS assay. The usual concentrations were used for each; 25 μL of vesicles in 1.95 mL pH 6.5 MOPS buffer, then adding 20 μL base and 20 μL of the potential ionophoric species (boronic acid 8, cholate 7 or the monomer 6). Initially ion channel species in each experiment were only doped at 5 mol % in the vesicles,
but the experiments were then carried out with a higher concentration of the
ionophore that gave a 10 mol % doping level in the membrane (40 μL of ion
channel/carryer).

Secondly, a series of experiments comparing the ion channel activity for
mixtures of several species were completed, using a stock vesicle solution and
with the same HPTS assay conditions.

Only a slight difference between dimeric boronic acid 8 and monomeric boronic
acid 6 was observed although transport was better with boronic acid 8 (Figure
21). It suggests that this weaker activity was because of the structure of the
boronic acid 8, which can present boronic acids on both sides of the membrane
whereas the monomer 6 has only one boronic acid on one side and it is not long
enough to span the membrane (Figure 23). Furthermore 8 a doping level of 5
mol % puts twice as many boronic acid groups in the membrane compared to 6.

Cholate 7 transported less than the boronic acid 8, but with a very small
difference (Figure 22). However, it was predicted that boronic acid 8 and
cholate acid 7 should have a big difference in ion transporting ability because
the cholate 7 does not have any boronic acid groups.

The experiments were also carried with 10 mol % of each species 6, 7 and 8.
The transport at higher concentration (Figure 24) showed a similar result to 5
mol % of the species. The transport for 8 was better than 7 and very similar to
6.
Figure 21: Comparing (—) 20 μL, or 5 mol % of the boronic acid 8 and (—) 20 μL, or 5mol % of the monomer 6. The data showed a very little difference between 6 and 8, although 8 seemed to produce slightly better transport.

Figure 22: Comparing (—) 20 μL, or 5 mol % of the boronic acid 8 and (—) 20 μL, or 5 mol % of the cholate 7 the graph is shows more transport for 8 which suggests that acid headgroup caused the improvement. Arrow indicates compound addition.
Figure 23: Ion transport with 5 mol % or 20 μL of the monomer 6 (25 μL vesicle suspension). Arrow indicates compound addition.

Figure 24: Ion transport with (—) 40 μL or 10% the monomer 6, (—) 40 μL or 10% the cholate 7 and (—) 40 μL or 10% the boronic acid 8. At the higher concentration transport showed a small difference, although 8 was still better than 7 and very similar to 6. Arrow indicates compound addition.
3.3.4. Ion transport with better pH control

During the HPTS assays, it was felt that controlling the pH was difficult when adding 20 μL of the base (NaOH or KOH 1 mM). As HPTS assay experiments are pH sensitive, having the right pH was key to getting a reliable result. To assess the effect of too little or too much base, a series of experiments were set up with different amounts of the base (KOH, 1 mM) added. The base (KOH, 1 mM) was added in different amounts to vesicles in MOPS buffer and the pH checked each time. Adding between 5 μL and 20 μL gave a pH range between 7 and 12 (with 7 μL appearing to be optimal). Therefore, it was known how much of the base should be added to get a certain pH.

The HPTS assay was setup as detailed previously, using boronic acid 8 (5 mol %). Figure 25 shows that upon adding a smaller amount of the base (KOH, 1 mM), such as 7 μL and 5 μL, the base pulse was smaller, which was expected.

However this method still was not efficient enough because too many mistakes resulted from adding a small volume of the base. Therefore it was decided to increase the capacity of the MOPS buffer and then add a greater volume of the base (KOH).
3.3.5 Increasing the capacity of the MOPS buffer

It was decided to make a new buffer (MOPS) with increased buffering capacity. The old MOPS buffer was 20 mM in concentration (NaH$_2$PO$_4$, KH$_2$PO$_4$, KCl, pH = 6.5) while the new MOPS was made 100 mM in concentration with the same pH = 6.5. The new buffer was used for vesicle preparation (including the HPTS solution inside the vesicles) and throughout the assay (outside the vesicle). The HPTS assay was carried out using compounds 6, 7 and 8.

The data obtained was not promising (Figure 26) because all compounds behaved in the same way as at the old buffer concentration.
Figure 26: Ion transport with (—) 5 mol % of the boronic acid 8, (—) 5 mol % of the cholate 7 and (—) 5 mol % of the monomer 6, at a higher capacity of MOPS (100 mM). First arrow (black) is showing the base pulse and the second one (purple) is showing compound addition.

3.3.6 A new method for the HPTS assay

Due to these problems with pH control, it was decided to create a new HPTS assay method which was based on diluting the vesicles into a buffer with the correct pH (after base pulse) instead of adding base to increase the pH. After discussions in the Dr Webb group, and getting some assistance from James Brown, three different MOPS buffer stock solutions with different pH values were prepared. A stock vesicle solution was provided with the usual MOPS buffer (NaH$_2$PO$_4$ 20 mM, NaCl 100 mM, pH = 6.5 with HPTS 100 µM; used inside the vesicle). Then the appropriate volume of this vesicle solution was added (25 µL) to a solution of MOPS buffer at pH = 8.5 (NaH$_2$ PO$_4$, 20 mM,
NaCl 100 mM). After incubation of the suspension for three minutes, the ionophore (e.g. the boronic acid 8) was added. In this method, there was no “base pulse” as the base adding step was removed by fixing the external MOPS buffer at pH=8.5. To complete the experiment and obtain the maximum fluorescent values for normalisation, a 25% (v/v) solution of the Triton X-100 was added.

The experiments were carried out for all the ion channel species (6, 7, and 8) and repeated three times for each species. The ion transport data for all the species 6, 7, and 8 were better than the old method (Figure 27). This was a satisfactory result, and J. Brown also showed that this adjusted method was better for these compounds, giving more reliable results.

![Figure 27: New HPTS ion transport method, diluting into pH 8.5 buffer. (—) 5 mol % of the boronic acid 8, (—) 5 mol % of the cholate 7 and (—) 5 mol % of the monomer 6.](image-url)
3.4 Measuring ion transport using the U-tube method.

The U-tube method is another common method for measuring ion transport. It was used in this project to determine if the boronic acids operated via carrier or channel mechanism. In this method a U-shaped glass tube was used to provide a tri-phasic system (Figure 28).

![Figure 28: Schematic representation of a U-tube experiment containing a triphasic system.](image)

A chloroform phase which contained a 1 mM solution of the ion transport species (the monomer 6) was placed in the U-tube and was stirred at 300 rpm. The receiving phase on one side of the U-tube consisted of distilled water. To start the experiment, a “source” phase was added to the other side of the U-tube which was a 0.01% (w/v) solution of appropriate metal picrate (436 μM) MOPS buffer. Then, transport of potassium (or sodium as their picrate salt) was monitored and recorded by measuring the picrate absorbance in the receiving phase.
The experiment was first carried out without the monomer 6 as a control. Then in the presence of monomer 6, ion transport was observed (Figure 29).

![Figure 29: Normalised U-tube data showing Na⁺ ion transport by monomer 6, as determined from picrate absorption in UV spectra of the receiving phase.](image)

Transport took place only when 6 was present, suggesting the monomer 6 was an ion carrier (as it carried the alkali metal ion into the receiving phase) not an ion channel. The same experiment with the other potential ionophores (7 and 8) was planned, but unfortunately there was not enough of these compounds, as a large quantity was needed for these U-tube experiments. There was not enough time to synthesis the addition quantities of the ion transporter species required.
Chapter 4:

Boronic acids binding to sugars
4.1.1 Importance of sugar transport through the cell membrane

Glucose is the main sugar (monosaccharide) used as an energy source inside cells and can cross the cell membrane only through a dedicated protein channel. For example, a human cell normally contains 200,000 glucose transport molecules, and any small faults on this process can cause a serious health problem. It has been shown that this monosaccharide is transported across the cell membrane by selective transport.

4.1.2 Previous study

The ability of boronic acids to transport saccharides through bilayer membranes was studied by Bradley D. Smith and Stephen J. Gardiner. They also found that twenty-one different boronic acids could facilitate monosaccharide transport; however, it was not possible for those which were highly hydrophilic. According to their experiments, the transport-selectivity order for monosaccharides was sorbitol > fructose > glucose. There was no transport of disaccharides like maltose and sucrose by the boronic acids they studied.
4.1.3 Transport mechanism

It has been shown that boronic acid can combine with the diol group of monosaccharides reversibly to produce a tetrahedral anionic boronate, which is in equilibrium with its trigonal form.\textsuperscript{32}

There are two possible mechanisms that could explain monosaccharide transport through the bilayer membrane by a boronic acid. The first involves binding of the transported species as the trigonal boronate-ester (Figure 30).

![Figure 30: Formation of a trigonal boronate ester.\textsuperscript{32}](image)
The second mechanism involves a conjugate base with a counterion (Figure 31). It has been proved that both mechanisms depend on experiment conditions such as pH.\(^{32}\)

\[
\begin{align*}
R-B-OH & + \text{Sugar} & \text{Sugar} & + \text{H}_3O^+ \\
& & & \\
& & & \\
& & & \\
& & & \\
& & & \\
\end{align*}
\]

**Figure 31: Formation of a tetrahedral boronate ester.**\(^{32}\)

### 4.2 Boronic acid binding to diols and effect on alkali metal ion transport

The ability of boronic acids to facilitate transport of ions (e.g. K\(^+\)/Na\(^+\)) through the membrane has been shown in Chapter 3. The effect of binding boronic acids to monosaccharides on ion transport was now to be investigated, using compounds 6, 7, and 8. To find the answer, a series of experiments with monosaccharides using the HPTS assay were initiated.
4.2.1 HPTS assay for boronic acid ionophores with fructose

First, the normal HPTS assay method was tried (with a base pulse) which was explained in Section 3.3. The concentration of fructose to use was an important issue. Initially a high concentration of fructose (0.1 M) was used to see whether there was any change in transport, and the new method for the HPTS assay (as described in Chapter 3) was tried with different concentration of fructose.

MOPS buffer solutions (2 mL, pH = 6.5) with fructose (0.1 M) were used. The buffer used into the cuvette and inside the vesicle was the same. One unit of base KOH (1 mM, 8.5 μL) was added to give the base pulse after incubation of the suspension for 2-3 minutes. Compound 8 (5 mol %) was added after 10-15 minutes. The change in fluorescence was then monitored over a 35 minute period. Triton X-100 was added to end the experiment and obtain the maximum values for data normalisation. The experiment was also carried out with no fructose (Figure 32).
Figure 3: Ion transport by (—) 5 mol % of the boronic acid 8 in the presence of fructose (0.1 M) and (—) 5 mol % of the boronic acid 8 with no fructose. Arrow indicates compound addition.

As expected, ion transport in the presence of fructose (0.1 M) was less than in its absence. This suggested that these changes were due to binding of the boronic acid to the diol group of fructose, and therefore destroyed the ion transporting ability of 8 by making 8 a poorer carrier.

To find out over what concentration range fructose can affect ion transport the experiment was attempted with different concentrations of fructose. Half the concentration of fructose (0.05 M) also showed a similar result (Figure 33). There was an improvement in transport rate at this lower concentration of fructose, but the rate was still lower than in the absence of fructose. After considering this result, a smaller concentration of fructose was tried. The effect of fructose was also ascertained on ion transport by the other potential ionophores. Therefore, an experiment with fructose (0.01 M) was carried out with compounds 6, 7 and 8 (Figure 34). It showed that all the species had almost the same transport in present of fructose (0.01 M).
Figure 33: Ion transport by (→) 5 mol % of the boronic acid 8 with no fructose and by (→) 5 mol % of the boronic acid 8 in the presence of fructose (0.05 M).

Figure 34: Ion transport by (→) 5 mol % of the boronic acid 8, (→) 5 mol % of the cholate 7 and (→) 5 mol % of the monomer 6 in the presence of fructose (0.01 M). Arrow indicates compound addition.

The experiment was then carried out with fructose (0.005 M) and comparing the behaviour of 8 and 7 (Figures 35 and 36). Comparing 8 and 7 (Figure 35) showed that transporter was almost the same in the presence of fructose (0.01
M). It suggested that the boronic acid 8 was bound to fructose and the ion transport was decreased, so 8 behaved as an ionophore with no boronic acid such as 7. However, the data presented in Figure 36 shows that ion transport with compound 8 gradually increased with decreasing fructose concentration, from 0.01 M to 0.005 M.

![Graph showing ion transport](image)

**Figure 35:** Ion transport by (—) 5 mol % of the boronic acid 8 and (—) 5 mol % of the cholate 7 in the presence of fructose (0.01 M).
Figure 36: Comparing transport of 8 with different concentrations of fructose: (—) no fructose, (—) 5 mM of fructose and (—) 10 mM of fructose. Transport increased with decreasing fructose concentration.

Overall, from the normalised data shown in Figures 34, 35, and 36, a gradual increase in ion transport with decreasing concentration of fructose (from 0.1 M to 0.005 M) can be observed. The transport process improves because the ratio of free boronic acid (the more active ion transporter) to boronic acid bound to fructose (less active) increases as the concentration of fructose decreases.
4.2.2 HPTS assay for 8 in the presence of sorbitol.

Next the effect of other monosaccharides on the behaviour of 8 was assessed. HPTS assays with different concentrations of sorbitol were planned but unfortunately there was not enough time to do them all. However, sorbitol (0.01 M) gave some increase in ion transport with compound 8 compared to fructose at the same concentration (Figure 37). This result may suggest that fructose bound more tightly to boronic acid 8 than sorbitol, leading to less active transporter and therefore less ion transport.

Figure 37: Ion transport by 5 mol % of the boronic acid 8 in the presence of (—) sorbitol (0.01 M) and (—) fructose (0.01 M).
Chapter 5:
Conclusion and further work
5.1 Conclusions

The aim of this project was to develop and detect boronic acid mediated ion transport across a lipid bilayer. It involved monitoring and measuring the ion flux as well as manipulating the HPTS assay to optimise the conditions for boronic acids.

The experiments were initially carried out with a synthetic ionophore 8 (synthesised by James Brown) which had boronic acids at either end of a bis-cholate core. The transport of Na\(^+\) and K\(^+\) across the lipid bilayer by 8 at a membrane loading of 5 mol % was observed.

These HPTS assays were repeated with the same concentration of 8 with different concentrations of vesicles (lowering the membrane loading), but almost no change in transport rate was observed with 5.0, 2.5 and 1.25 mol % ionophore. However, experiments with higher concentrations of ion channel species 8 showed increases in the transport rate; there was faster transport with upon doubling the concentration 8 (40 μL added, to give a membrane loading of 10 mol %).

The HPTS assay was carried out with analogous compounds 6 (cholate with one boronic acid) and 7 (cholate with no boronic acid). It was observed that compounds 6 and 7 had a similar ability to transport Na\(^+\)/K\(^+\) across a lipid bilayer.
New methods to control the pH during the HPTS assay were developed after first assessing the best pH for measuring the behaviour of 6, 7 and 8. The capacity of the MOPS buffer was increased but the best method was to fix the external pH at 8.5 and not to use a base pulse.

Finally, a U-tube assay was used to prove that compound 6 (the boronic acid-cholate monomer) was an ion carrier and not an ion channel.

The HPTS assay experiments were then carried out to detect the effect of fructose and sorbitol on ion transport via 6, 7 and 8, and to monitor the effect of boronic acid binding with diols on the transport process through the lipid bilayer.

The ion transporter 8 in the presence of fructose (0.01 M) showed very poor transport. Experiments were then carried out with lower concentrations of fructose, to find the highest concentration of fructose which would allow the transport to occur. Transport was higher with 0.005 M fructose; the overall result from these experiments was that decreasing the concentration of fructose from 0.01 M to 0.005 M gave a gradual increase in ion transport, ascribed to less binding between the boronic acid and the fructose at lower concentrations of the sugar.

The series of experiments with sorbitol was planned; however, it was only completed with 8 and 0.01 M sorbitol and there was no more time to continue these experiments.

Finally, the synthesis of an intermediate towards the boronic acid-cholate ion carrier 6 was attempted three times but, unfortunately, it was unsuccessful each time.
5.2 Further Work:

Further work on this project will focus first on boronic acid 8-mediated ion transport in the present of sorbitol (0.005 M), as the initial trial was successful (Chapter 4). Then, the result will be compared with ion transport by 6, 7 and 8. Ion transport assays will also be completed with different concentrations of sorbitol, and then the final result will be compared with the fructose trials of 8, 7 and 6.

Secondly, the synthesis of 12 will be completed. Alternative synthetic strategies or solutions will be considered, such as using other peptide coupling agents (e.g. HATU, HOBT, etc). The other option could be changing the solvents to see whether the reaction mechanism will be different or not as the reaction mechanism is secondary amine coupling.
Chapter 6:
Experimental details
6.1 Instrumentation

All reagents and solvents were obtained from commercial suppliers such as Aldrich, Fluka or Acros. However, boronate ionophore 6, 8 and the cholate 7 were kindly prepared by James Brown in the Webb group. Thin layer chromatography was carried out using Merck aluminium-backed F254 silica gel plates, and visualised under UV light (256 nm or 365 nm), or alkaline aqueous potassium permanganate was used. Column (flash) chromatography was carried out using available normal silica gel (technical grade, pore size 60 Å, 230 – 400 mesh, 40 - 63 μm) from Aldrich.

NMR spectra were measured on Bruker DPX400, instruments for solution in CDCl₃.

Fluorescence spectroscopy was carried out on Perkin-Elmer LS55 fluorimeter, temperature was controlled using a Julabo F25-HE water circulator. UV-visible spectroscopy was carried out using a Jasco V-660 spectrophotometer, temperature was controlled by a Jasco EHC-716 Peltier.
6.2 Synthetic studies towards 12

\[ N-[8-(\text{benzyloxycarbonylamino})-3,6\text{-dioxaminoctyl}]\text{-}3\alpha,7\alpha,12\alpha\text{-trihydroxy}5\beta\text{-cholan-24-amide} \ 12. \]

The synthesis utilised well-established \( N,N,N',N'\text{-}\text{tetramethyl}-O\text{-}(1\text{H}-\text{benzotriazol-1-yl})\text{uronium hexafluorophosphate} \) (HBTU) coupling chemistry.

The first time, a solution of cholic acid 9 (450 mg, 1.1 mmol), was made by adding dry dimethylformamide (10 mL) and HBTU (418 mg, 1.1 mmol, 1 eq). The solution was stirred for 45 minutes. After that, a solution of the amine 11 (1-(benzyloxycarbonylamino)-3,6-dioxa-8-aminoctane)(310 mg, 1.1 mmol, 1 eq) in dry dimethylformamide (4 mL) was added to the first solution, and the mixture left to stir for 48 hours. Then, the solution was diluted with dichloromethane (25
mL) and it was washed successively with dilute hydrochloric acid (25 mL), saturated sodium bicarbonate solution (25 mL), and brine (25 mL). After that, the organic solution was dried by MgSO$_4$, and filtered. The filtrate was evaporated in vacuo, then flash chromatography (silica gel, eluant: 9:1(v/v) ethylacetate/methanol) was attempted to obtain compound 12.

The source of 12 used in the later experiments was donated by Dr James Brown.

The final TLC showed no spots under short-wavelength UV light (254 nm), but yellow or light brown spots were observed with a solution of potassium permanganate (Dissolve 1.5g of KMnO$_4$, 10g K$_2$CO$_3$, and 1.25ml 10% NaOH in 200 ml water).

The synthesis was carried out three times; twice with the same amounts and once with half the amount of reactants and solvents.

Unfortunately, $^1$H NMR spectroscopy on the fractions from the column did not reveal any product (e.g. no signals in the aromatic region). An IR scan was not performed as the NMR did not show any signal of the product 12. A MS scan was planed after the third attempt but unfortunately, time did not permit this to happen.
6.3 Transport studies and analysis:

6.3.1 Preparation of vesicles for transport assays:

EYPC (egg yolk phosphatidylcholine, 64 mmol, 49.28 g) and cholesterol (16 mmol, 6.18 g) was selected as an appropriate lipid composition (EYPC/cholesterol 8:2). The lipids were weighed out and dissolved in (~ 1 mL) spectroscopic grade chloroform.

The solvent (chloroform) was removed by evaporating in vacuo to make a thin film of lipid on the interior side of the round bottomed flask. MOPS buffer (1.2 mL) was then added to the thin film which contained; HPTS dye (100 µM), MOPS (20 mM adjusted to pH = 6.5 by using sodium or potassium hydroxide), and NaCl or KCl (100 mM) as appropriate. After that, vortex mixing was used to detach the lipid from the flask. The suspension (1 mL) was extruded 19 times through an 800 nm pore size polycarbonate membrane or 200 nm, as appropriate, to give a suspension of unilamellar vesicles.\(^\text{15}\)

After that a GPC (gel permeation chromatography) column was prepared by equilibrating with MOPS buffer (or buffer used during extrusion). Buffer (25 mL) was allowed to run through the column to equilibrate the column. (GPC was Sephadex G-25). The extruded lipid suspension (1 mL) was then diluted by MOPS buffer (1.5 mL) and was loaded onto the GPC column. After adding the suspension and eluting the suspension onto the column, MOPS buffer (3.5 mL) was added to the top of the column. The 3.5 mL which came out of the column was collected and labelled as a stock vesicle solution (final concentration of lipids was 19 mM). The point of using the GPC column was to remove any unencapsulated (free) HPTS from the suspension.
Vesicle solutions were prepared freshly each time and individually for the same series of experiments.

6.3.2 Procedure for HPTS fluorescence assay

Typically in these experiments, the resulting stock vesicle solution (25 μL) from (Section 6.3.1) was diluted in MOPS buffer (1.95 mL). The cuvette was then placed in the fluorimeter with normal stirring. The fluorescence emission wavelength was set to 510 nm, and the intensity was monitored after simultaneous excitation at 405 nm and 460 nm. After two or three minutes (60 s-180 s) running, KOH (20 μL, 1 M, aq) solution was added, to provide the base pulse. After ten minutes (600 s), the ion transporter solution (boronic 8, 20 μL 1 mM in methanol) was added. After a further thirty minutes (1800 s), a 25% v/v solution of Triton X-100 detergent in MOPS (20 μL) was added to burst the vesicles and give maximum response.

In some experiments, different concentrations of the vesicle suspension were added (25 μL, 100 μL and 50 μL) to the same amount of the buffer (1.95 mL) and same conditions. Each experiment was repeated three times to prove the reliability of the data. The emission data was collected, I(460 nm) divided by I(405 nm), and the ratio divided by the maximum value after Triton X-100.

The exchange rate though the bilayer was calculated from the normalised data from the ratio I(460 nm)/I(405 nm). Apparent first-order rate constants $k$ were obtained by fitting the normalised data to the equation 1 below:
\[ I_{\text{norm}} = I_{\infty} - \exp(kt + c) \]

Equation 1: \( I_{\infty} \) is the extrapolated normalised fluorescence intensity after infinite time.

**6.3.3 Control experiment for the HPTS fluorescence assay:**

In order to observe whether the vesicles were leaking, a methanol control was used at the end of each series of the experiments. The same procedure was used as detailed in Section 6.3.2, except methanol (20 μL, 1 mM) was added in the place of a solution of 8.

**6.3.4 Procedure for HPTS fluorescence assay using different concentrations of ion transporter**

For the experiments in different concentration of the ion transporter (boronic acid 8), the procedure in Section 6.3.2 was repeated by adding different volumes of the solution of ionophore 8 (20 μL, 40 μL or 5 μL). The methanol control was also measured by following Section 6.3.3 (20 μL, 40 μL or 5 μL methanol). For each addition of 20 μL, 40 μL, 5 μL of boronic acid 8 the experiments were repeated three times.

These experiments also were carried out with monomer 6 (20 μL, 40 μL or 5 μL, of 1 mM 6 in methanol) and the cholate 7 (20 μL, 40 μL or 5 μL, of 1 mM 7 methanol).
6.3.5 Procedure for HPTS fluorescence assay using different base pulses

A vesicle solution was prepared according to the method described in Section 6.3.1. The HPTS method described in Section 6.3.2 was repeated but a different amount of the base KOH (1 mM) was added to the cuvette, which included; 8 μL, (pH was 9.69), 7 μL, (pH was 7.65), 5 μL (pH was 6.68) and 20 μL, (pH was 11.78). At each different pH the activity of the ionophore 8 was measured three times and methanol control was measured.

6.3.6 Procedure for HPTS assay for different ionophores (boronic acid cholate dimer 8, boronic acid cholate monomer 6, cholate 7)

A vesicle solution was prepared according to the method in Section 6.3.1. The HPTS assay was carried out as detailed in Section 6.3.2, but the following volumes of the ionophore solutions were added to give 10 mol % in the membrane; boronic acid 8 (40 μL), the cholate 7 (40 μL), and the monomer 6 (40 μL). Each experiment was carried out three times for each. The methanol control was measured according to Section 6.3.3.

6.3.7 Procedure for HPTS fluorescence assay with higher capacity buffer

For better control of pH during the assay, the capacity of the MOPS buffer was increased from 20 mM MOPS to 100 mM MOPS. The vesicle stock solution was made using the new MOPS buffer (100 mM). All experiments from Section 6.3.6 were repeated using the new MOPS buffer (100 mM); 25 μL of 1 M KOH was added. In these experiments different pH values following the base pulse were
measured: for the cholate 7, pH = 9.00; the monomer 6, pH = 9.09; boronic acid 8, pH = 8.18, although the same amount of the base (25 μL) was added.

6.3.8 Procedure for HPTS fluorescence assay in the presence of sorbitol

Vesicle solutions was prepared according to Section 6.3.1 but this MOPS buffer contained: HPTS dye (100 μM), MOPS (20 mM) and sorbitol (1 M, 4.55 g in 25 mL), and adjusted to pH = 6.5 (by using sodium or potassium hydroxide, and NaCl or KCl (100 mM). The fluorimeter was set up, the vesicle solution was diluted and a base pulse was created as detailed in Section 6.3.2, but the sorbitol containing MOPS buffer was used. These experiments were repeated using different volumes of KOH as the base pulse (1 mM; 20 μL or 9 μL or 8.5 μL or 8.7 μL) and the pH of each checked (pH = 11.8, 10.36, 7.45, 9.60 respectively). The experiments were repeated three times for each of volume of added KOH, and a control methanol test was carried out as per Section 6.3.3.

6.3.9 Procedure for HPTS fluorescence assay with presenting of fructose.

The vesicles were prepared according to Section 6.3.1, but the MOPS buffers used contained; HPTS dye (100 mM), MOPS (20 mM), and fructose (0.0025 M or 0.005 M or 0.05 M or 0.1 M), pH = 6.5. The HPTS assay was carried out according to Section 6.3.2, but the appropriate fructose containing MOPS buffer was used.
These experiments were also carried out with the boronic acid monomer 6 and the cholate 7. The methanol control assay took place according to Section 6.3.3 at the end of each series of experiments.

6.3.10 HPTS fluorescence assay without the base pulse

In this method, the vesicle preparation method and the GPC column all were followed according to the procedure in Section 6.3.1. However the buffer used to dilute the vesicle suspension (20 μL) in the cuvette was changed from pH = 6.5 to pH = 8.5 (1.95 mL, MOPS, NaH$_2$PO$_4$ (20 mM), NaCl (100 mM)), which meant a base pulse did not need to be added. Then the procedure detailed in Section 6.3.2 was followed, using boronic acid 8, the cholate 7 or the monomer 6 was added (20 μL addition). Then after 30 minutes (1800 s), a 25% v/v solution of Triton X-100 detergent in MOPS (20 μL) was added to complete the experiment and to lyse the vesicles. The experiment was repeated three times for each of the ion channel species (6, 7 and 8).

This new pH = 8.5 buffer was used to repeat all fructose experiments ([fructose] =0.0025 M, 0.005 M, 0.1 M and 0.05 M), and with the three ion channel species (6, 7, and 8). Then all were repeated three times. At the end a methanol control was taken for each series.
6.4. U-tube sodium picrate transport experiment.

A glass U-tube (14 mm internal diameter) was placed in a water bath at 25 °C. A solution of the transporting species (compound 6, 1 mM, 4 mL) was made in chloroform (4 mL) then placed in the U-tube to form the bottom layer. Stirring at 300 rpm during the whole of experiment, distilled water was added to one side of the U-tube as a receiving phase. To the other side of the U-tube was added a solution of 0.01% (w/v) sodium picrate in phosphate buffer (2 mL, buffer adjusted to pH = 8.5, [picrate] = 436 mM) as a source phase. Addition of the picrate solution started the experiment, so immediately after adding the source phase, 0.5 mL of the receiving phase was taken and analysed via UV-visible spectroscopy to give the concentration of picrate (measurement range 700-250 nm).

After the measurement, the sample was replaced back in the receiving phase immediately. After one hour the measurement was repeated by taking 0.5 mL of the receiving phase and analysing via UV-visible spectroscopy. The measurements were made every hour for the next ten hours.
References
References:


