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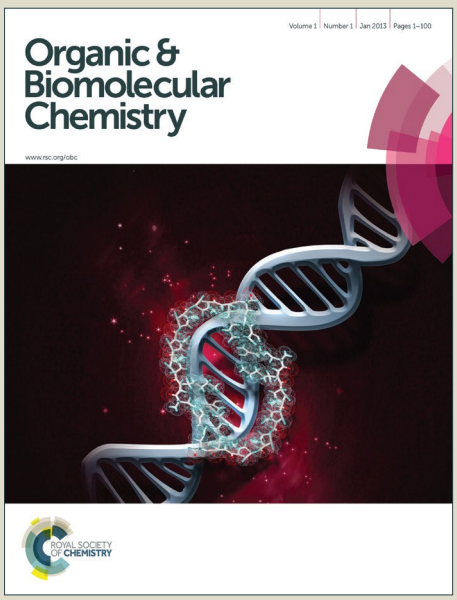

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Cation-Halide transport through Peptide Pores containing Aminopicolinic Acid

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Synthetic pores that selectively transport ions of biological significance through membranes could be potentially used in medical diagnostics or therapeutics. Herein, we report cation-selective octapeptide pores derived from alanine and aminopicolinic acid. The ion transport mechanism through the pores has been established to be cation-chloride symport. Cation-chloride co-transport is biologically essential for the efficient functioning of the central nervous system and has been implicated in diseases such as epilepsy. The pores formed in synthetic lipid bilayers do not exhibit any closing events. The ease of synthesis as well as infinite lifetimes of these pores, provides scope for modifying their transport behaviour to develop sensors.

Introduction

Synthetic peptides capable of biomimetic ion transport are emerging as useful materials or therapeutics.[1-6] In particular, molecules designed to mimic the ion-selectivity and/or gating ability of natural ion channel proteins or carriers are extremely useful.[7-15] Pores such as alpha hemolysin that do not show a transition between open and close states, have been utilized as biosensors by genetic engineering or incorporation of adapters.[16-23] There are few examples of synthetic pores that show open states with infinite lifetimes.[24,25] Such pores, if readily accessible are highly attractive for use as materials. Furthermore, if these robust pores are able to selectively transport ions, one can envision using them in therapeutics, diagnostics or as model systems to standardize ion transport assays.

Reported herein, are pore forming acyclic octapeptides **1** and **2** derived from alanine and aminopicolinic acid. The peptides transport cations *via* an M^+/Cl^- symport mechanism and do not show any closing events. Cation-chloride symporters play a crucial role in maintaining homeostasis and transmembrane chloride potential in neurons in the body. These symporters are associated with diseases such as epilepsy and renal acidosis. [26-29] Synthetic cation-chloride symporters have been previously reported from macrocycles such as crownethers, azacrownethers, calix[4]pyrrole or oxacalix[2]arene[2]triazine.[30-34] Given the fact that our cation-selective pores show infinite lifetimes, provides an additional scope for using them as scaffolds for developing sensors.

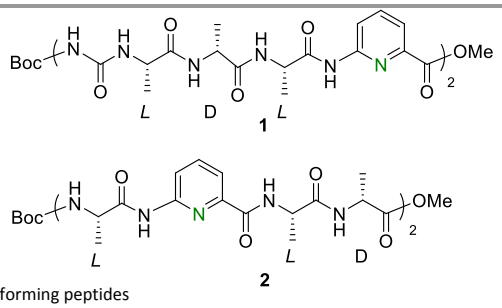

Chart 1. Pore forming peptides

Previously reported peptides **3**-**5** from our research group selectively transported cations and were impervious to halides (Figure 1).[35-37] Replacement of aromatic units in peptide scaffold **4** to amino methyl pyridyl units was shown to significantly enhance its cation transport activity.[37] Inspired by these observations, we designed peptide **2** that incorporates aminopicolinic acid units into our most active peptide scaffold **3** that we believe forms tetrameric pores.[35,36] We envisioned that the pyridyl rings would significantly enhance the cation transport activity of peptide **2**. Peptide **1** containing an aromatic unit at the C-terminus was designed based on our prior hypothesis that peptide **3** formed tetrameric pores with the peptide C-termini at the lipid-water interface.[35] We believed that the closer proximity of the aminopicolinic acid units to the lipid-water interface could enhance its influence on the ion transport properties of the pore. At the outset, we

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had not expected these peptides to co-transport cations and halides.

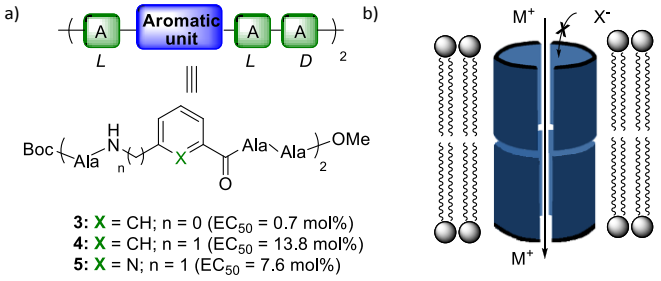
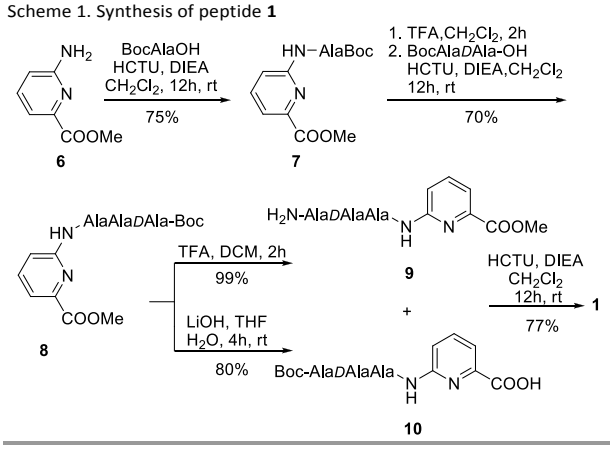


Figure 1 a) Basic design and comparison of peptide derived pores; b) schematic representation of tetrameric pore formed by peptide **3**.

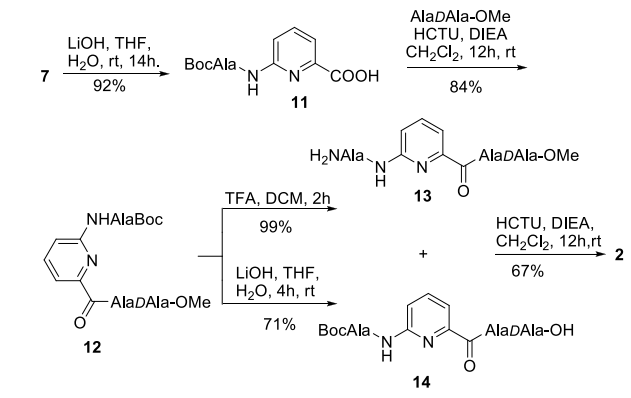
Results and discussion

Octapeptide **1** was synthesized starting from amine **6** (Scheme 1), which is obtained in three steps from 2-amino-6-picoline. Amine **6** was coupled with Boc-Alanine using HCTU to form dipeptide 7 in 75% yield. Boc deprotection and subsequent coupling with Boc-L-Ala-D-Ala-OH afforded tetrapeptide **8** in 70% yield. Tetrapeptide **8** was selectively deprotected to get peptides **9** and **10**, which were coupled to afford octapeptide **1** in 77% yield. Similarly, octapeptide **2** was synthesized in solution from dipeptide **7** as shown in Scheme 2.

Scheme 1. Synthesis of peptide **1**



Scheme 2. Synthesis of peptide **2**



Vesicles entrapped with pH sensitive HPTS dye[38-40] were prepared in HEPES-NaCl buffer to investigate ion transport through peptides **1** and **2**. The vesicles were equilibrated with the peptide, following which NaOH was added to increase the external pH by 0.6 units (Figure 2a). Ion transport was gauged by monitoring an increase in the concentration of the deprotonated $HPTS^-$ due to Na^+/OH^- symport, Na^+/H^+ antiport or OH^-/Cl antiport. The fluorescence intensity was normalized based on the concentration of $HPTS^-$ obtained after adding the detergent Triton X. Surprisingly, a negligible increase in the concentration of $HPTS^-$ in the presence of peptides **1** and **2** was observed after the base pulse (Figure 2b). Rate constants obtained by fitting the curves obtained (with the peptides) to first order exponential equations were marginally higher than the control experiments. Such a drastic decrease in the ion transport activity due to replacement of aminobenzoic acid[36] by aminopicolinic acid units was extremely intriguing.

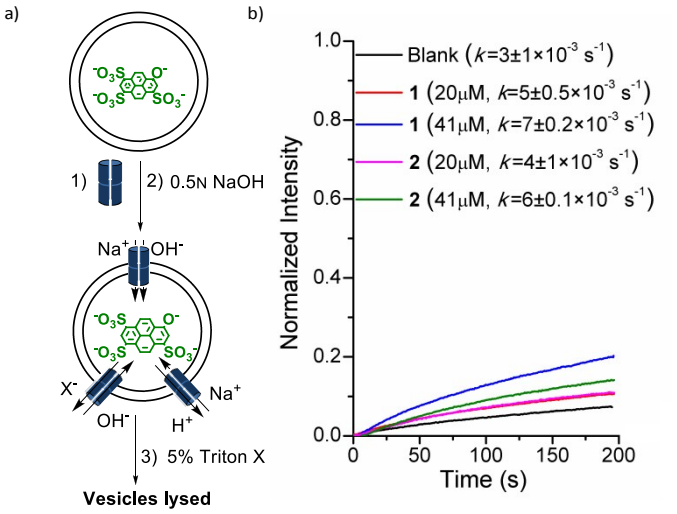


Figure 2 a) Schematic representation of the HPTS assay. b) Change in $HPTS^-$ concentration after NaOH pulse until addition of Triton X (i.e. between Step 2 to step 3 in the schematic represention). 20.4 μM = 5 mol % peptide with respect to lipid. Average *k* values reported in the legend. Origin 8.1 was used to fit the curves.

To investigate whether the peptides transport halides across lipid membranes, vesicles entrapped with lucigenin dye were prepared. The fluorescence of lucigenin is quenched in the presence of halides (Figure 3a).[41] In contrast to peptide **3**, which was impervious to halides, peptides **1** and **2** were transporting Cl^-, Br^- (Figure 3b) and I^- (Figure S7). The results from the lucigenin assay indicated that the presence of the aminopicolinic acid units increased the affinity of the peptides for halides.

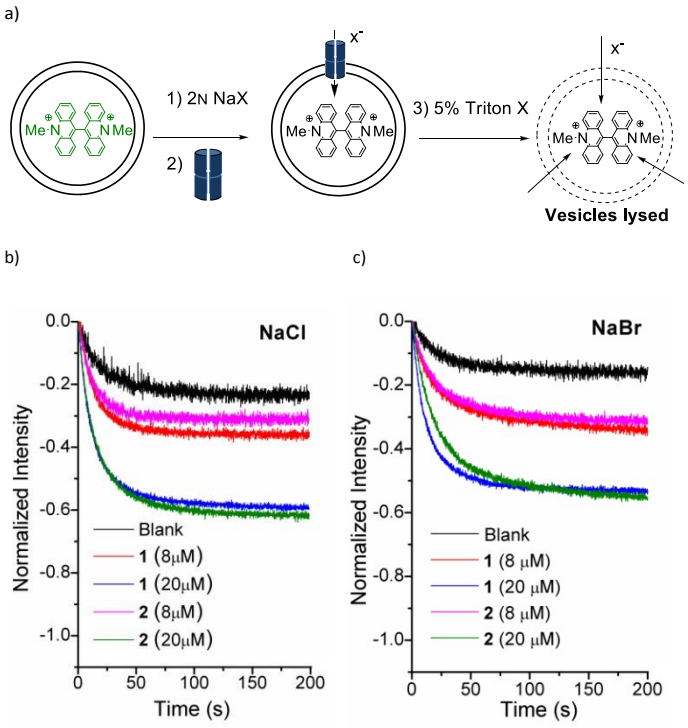


Figure 3 a) Schematic representation of Lucigenin assay. Change in lucigenin fluorescence after addition of peptides (i.e. between step 2 and step 3 of schematic representation) in the presence of b) NaCl; c) NaBr. 20 μM = 5 mol % peptide with respect to lipid.

To gain further insights into the ion transport mechanism of peptides **1** and **2**, the two peptides were incorporated in lipid bilayers (composed of DPhPC – 20 μL, 23.6 mM). Incubation of 40 μL of peptide (0.125 mM) leads to channel formation. Channel incorporation was observed every time the experiment was conducted. The membrane currents increased linearly, proportional to the applied voltages. The current traces recorded at -80 mV holding potential are shown in Figure 4a. No single channel opening and closing events or gating was observed, indicating that the peptides possibly formed pores. The reversal potential was estimated experimentally by creating an ionic gradient (0.5M:1M KCl in cis:trans side respectively with the cis side being held in virtual ground) across the bilayers. Currents were measured at different voltages using step protocol, and current-voltage (I-V) plots were generated. The reversal potential was found to be 17.9 mV for Peptide **1** and 21.6 mV for Peptide **2** (Figure 4b) both of which were close to the theoretical reversal potential of K^+ ions (17.8 mV). This suggests that the peptides preferentially transport cations over anions.

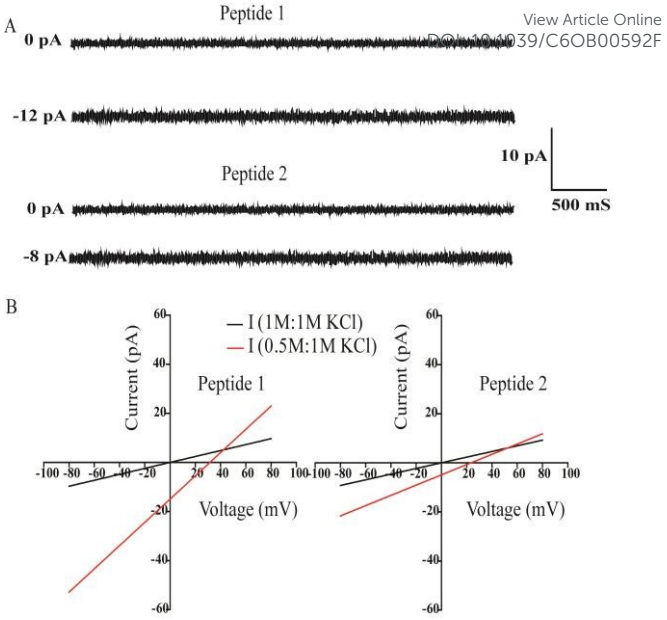


Figure 4. A) Current traces of peptides 1 and 2 recorded at -80 mV. The current traces shown besides '0 pA' is the base line current at 0 mV holding potential. B) I-V Plots of Peptides 1 and 2. The currents generated in symmetric solution of 1 M KCl are shown with black line, while the red line represents currents recorded in 0.5M:1M KCl (cis:trans) gradient. *Cis* chamber was grounded.

The lucigenin assay and the conductance measurements showed that peptides **1** and **2** transported cations as well as halides, with cation transport being the driving force. This observation could be explained by cation transport through the peptides *via* an M^{+}/Cl^{-} symport mechanism. The symport mechanism could also explain the minimal change in intravesicular pH for the HPTS assay with an NaOH pulse (Figure 1b). The HPTS assay uses change in pH as an indirect probe to study ion transport. As suggested by the conductance studies, if the peptides are cation-selective, one can envision the system maintaining charge neutrality *via* the mechanisms shown in Figure 5 (for a HEPES:NaCl buffer). A dominant Na^{+}/Cl^{-} symport mechanism as hypothesized will lead to negligible change in the internal pH of the vesicle as observed.

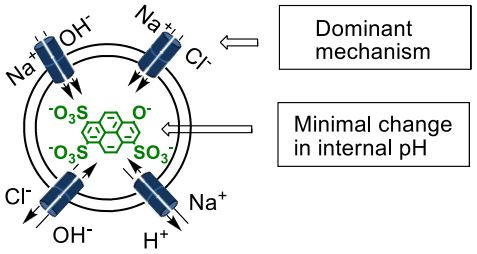


Figure 5 Schematic representation explaining minimal pH change in HPTS assay.

To confirm the M^{+}/Cl^{-} symport mechanism, counter cations (M^{+} = Na^{+}, K^{+}, Ni^{2+}, Co^{2+}) to chloride were varied in the lucigenin assay. In this assay where the vesicles are prepared in a solution of $NaNO_3$, likely ion transport mechanisms are M^{+}/Cl^{-} symport and Cl^{-}/NO_3^{-} antiport (Figure 6a). Chloride transport was only observed with alkali metal ions and not with Ni^{2+} and Co^{2+} that exist as large pore impermeable $[M(H_2O)_6]^{2+}$ complexes (Figure 6b and S9). Had the antiport mechanism been operative, quenching of lucigenin dye would be observed in the presence of all cations. Peptide **1** also showed a marginal preference for K^{+} ion over Na^{+} ion, indicating that M^{+} transport could be the driving force for the M^{+}/Cl^{-} symport. To rule out the possibility of Ni^{2+}/Co^{2+} binding with the peptide prior to membrane insertion, the assay was carried out by premixing peptide and vesicles before addition of MX (Figure S19 and S20). Similar results were obtained with the modified lucigenin assay confirming that the lack of peptide activity in the presence of Ni^{2+}/Co^{2+} was not due to binding of these ions to the peptides. In addition, the absorbance spectra of each peptide, metal salt and peptide-salt mixture were measured in the absence of vesicles (Figures S32 & S33). The peptide to metal salt ratio was same as that used in the Lucigenin assay. The absorbance spectra of the peptide-salt mixtures were similar to the free peptide, which further ruled out metal-binding to peptide.

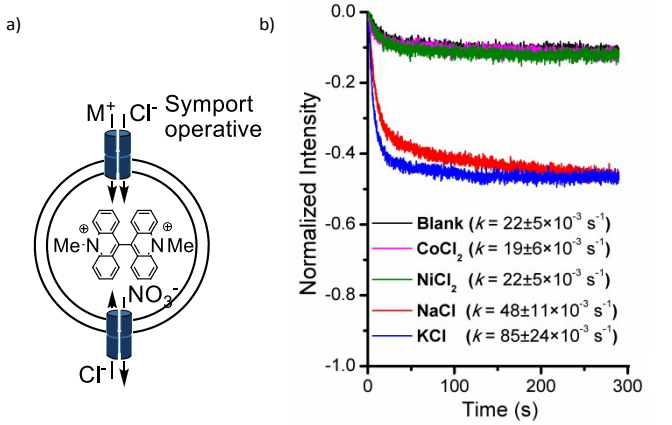


Figure 6 *a)* Schematic representation of ion transport mechanism. *b*) Change in lucigenin fluorescence in the presence of MX salts and peptide **1** (20 μM = 5 mol % peptide with respect to lipid). Average *k* values reported in the legend. Origin 8.1 was used to fit the curves.

Peptides **1** and **2** contain a scaffold of alternating L and D alanine units and two aromatic units similar to our previously reported octapeptides **3**- **5**. In particular, the peptides reported here are the pyridyl analogs of peptide **3**. Peptide **3** was shown to have an S-shaped structure with β-sheet like dihedral angles and formed thermodynamically stable pores in the lipid bilayer.[35] The replacement of phenyl groups with pyridyl units should not drastically change peptide conformation. Therefore, we believe that peptides **1** and **2** self-assemble to form thermodynamically stable pores similar to peptide **3**. The TEM images of peptides **1** and **2** indicate that they form nanotubes or nanofibres (Figure 7a & b). While the TEM image does not directly correlate to the structure of the ion transporting pore, it indicates that these peptides have a propensity to form stable nano-assemblies. The conductance studies corroborate the hypothesis that the peptides form stable pores as they show infinitely open lifetimes. Low ion transport for the HPTS assay rules out non-specific ion transport due to detergent like behaviour of peptides **1** and **2**. Furthermore, dynamic light scattering studies before and after peptide addition to vesicles indicated that the vesicle integrity was maintained after addition of peptide (Figure S30 and S31).

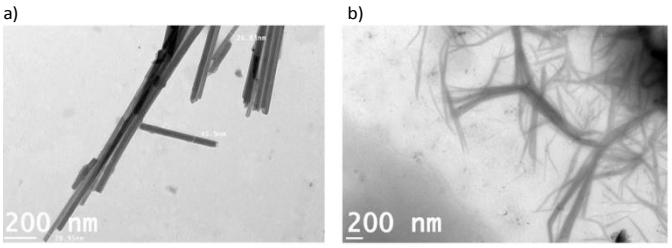


Figure 7 *a)* TEM image of peptide **1**. *b)*TEM image of peptide **2**.

As with the case of our earlier peptides, the ability of these pores to conduct cations could be attributed to the affinity of cations to the carbonyl groups from the peptide backbone. In contrast to our earlier peptides, halide transport is observed with peptides **1** and **2** containing aminopicolinic acid units. Aminopicolinic acid units are known to facilitate halide binding in macrocyclic peptide based receptors.[43, 44] The presence of these units in our peptide scaffolds appears to significantly improve the affinity of the pores for halides.

Conclusions

In conclusion, peptides **1** and **2** derived from aminopicolinic acid and alanine have been developed as ion transporting pores. The ion transport mechanism has been established using vesicles entrapped with pH sensitive HPTS or halide sensitive lucigenin dyes. The assays show that the peptides transport cations through lipid membranes *via* an M^{+}/Cl^{-} symport mechanism. The activity of peptide **1** with the picolinic units at the C-terminus has been found to be marginally higher than peptide **2**. Both peptides form cation selective pores in synthetic lipid bilayers which do not show any transition to the closed state. Such readily accessible cation–selective pores with prolonged open states are highly attractive for the development of sensors and materials.

Experimental Section

Preparation of vesicles for HPTS assay: Cholesterol (1.6 mg, 4.1 μmol, 1 equiv) was added to a solution of EYPC lipids (28.4 mg, 36.9 μmol, 9 equiv) in chloroform (0.284 mL). Chloroform was removed under a stream of nitrogen and further in vacuo for 5 h at 0 ºC to give a lipid film. The lipid film was hydrated with 1 mL of a buffer solution containing HPTS dye (0.1 mM HPTS, 10 mM NaCl, 100 mM, HEPES) at pH 7.2. The resulting suspension was allowed to stir at room temperature for 1 h.

and then subjected to five freeze-thaw cycles. The vesicle solution was placed in an ice bath and subsequently sonicated in a bath sonicator at 0-4 °C for a total time of 2 minutes (30 s on & 15 s off in degas mode). The vesicles were allowed to anneal for one hour in the refrigerator, following which they were extruded 20 times through 100 nm polycarbonate membranes using a mini-extruder from Avanti Polar Lipids. The extra-vesicular dye was removed by size exclusion chromatography using Sephadex G-50 (eluent: HEPES buffer at pH 7.2 (10 mM NaCl, 100 mM HEPES). The milky white solution containing vesicles were collected and used for further study.

HPTS Assay: Vesicle solution (100 µL), HEPES, NaCl buffer (2.9 mL) and an appropriate amount of peptide in methanol (0.5% DMSO) were added to a cuvette equipped with a magnetic stir bar. The solution in the cuvette was stirred for 2 minutes before the fluorescence experiment was started. After 50 s, a solution of NaOH (20 µL, 0.5 N) was added to the vesicles. 5% Triton X (50 µL) solution was added at 250 s to achieve final equilibration of the dye. Fluorescence measurements were done at an emission wavelength of 510 nm and an excitation wavelength of 460 nm.

Preparation of vesicles for Lucigenin Assay: Cholesterol (1.6 mg, 4.1 µmol, 1 equiv) was added to a solution of EYPC lipids (28.4 mg, 36.9 µmol, 9 equiv) in chloroform. Chloroform was removed under a stream of nitrogen and further in vacuo at 0 °C for 2 h to give a lipid film. The lipid film was hydrated with 1 mL of a solution containing Lucigenin dye (1.0 mM Lucigenin, 225 mM $NaNO_3$). The resulting suspension was allowed to stir at room temperature for 5 minutes and then subjected to five freeze-thaw cycles. The vesicle solution was placed in an ice bath and subsequently sonicated in a bath sonicator at 0-4 °C for a total time of 2 minutes (30 s on & 15 s off in degas mode), following which they were extruded 20 times through 400 nm polycarbonate membranes using a mini-extruder from Avanti Polar Lipids. The extra-vesicular dye was removed by size exclusion chromatography (SEC) using Sephadex G-50 (eluent: 225 mM $NaNO_3$). (Note: Carrying out the SEC twice ensured removal of majority of the extra-vesicular dye). The milky white solution containing vesicles were collected and used for further study.

Lucigenin Assay with NaX salts: Vesicle solution (100 µL) and $NaNO_3$ (2.9 mL) was added to a cuvette equipped with a magnetic stir bar. The solution in the cuvette was stirred for 2 minutes before the fluorescence experiment was started. After 50 s, a solution of 2.0 N NaX (35 µL), where X is Cl^-, Br^- or I^- was added to the vesicles. At 100 s, an appropriate concentration of peptide in 0.5 % DMSO-MeOH was added. 5 % Triton X (50 µL) solution was added at 350 s to achieve final equilibration of the dye. Fluorescence measurements were done at an emission wavelength of 505 nm and an excitation wavelength of 455 nm.

Planar Lipid Bilayer Experiment: The ion transport through the peptide channels was also investigated with Planar lipid bilayer (PLB) recording. Synthetic lipid 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) was dissolved in n-decane at a concentration of 20 mg/mL and used to make the lipid bilayer. Briefly, the lipid was painted on to the aperture (pore diameter of 150 µm) of a polystyrene bilayer cuvette (Warner Instruments, USA). Both chambers of the cuvette contained symmetrical solution (1 M KCl). The cis chamber was held at virtual ground and the trans chamber connected to a recording amplifier via a PC501A head-stage (Warner instrument). Ag-AgCl electrodes were used. Currents were low pass filtered at 1 kHz and digitized at 5 kHz using the Digidata 1440A (Axon Instruments). Once a stable bilayer was obtained, the synthetic peptide was added to the cis chamber and mixed with a magnetic stirrer. Membrane currents were frequently checked to monitor channel incorporation. After stable insertion of the peptides in bilayer, membrane current was recorded at different positive and negative voltages. For measuring reversal potential, the solution of the cis chamber was replaced with 0.5 M KCl while the trans chamber had 1 M KCl. Theoretical reversal potential was estimated using the Nernst Equation and compared with the experimentally derived value.

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