A Highly Active Anion Selective Aminocyclodextrin Ion Channel
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Experimental Section

General. All starting materials were obtained from commercial suppliers and were used without further purification unless otherwise stated. All air- or moisture-sensitive reactions were performed using oven-dried or flame-dried glassware under an inert atmosphere of dry argon or nitrogen. Air- or moisture-sensitive liquids and solutions were transferred via syringe or cannula. All vesicle preparations were done under an inert atmosphere of dry argon or nitrogen. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl; methanol was distilled from magnesium methoxide; pyridine, and dichloromethane were distilled from calcium hydride. Dimethylformamide was stored over 4 Å molecular sieves prior to use. β-Cyclodextrin was dried overnight under vacuum at 110 °C prior to use. p-Toluenesulfonyl chloride was purified using standard procedures. Egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar lipids as a solution in chloroform (20 mg/mL). Sodium cholate, gramicidin A and melittin were obtained from Sigma-Aldrich and 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) was obtained from Molecular Probes and used without further purification.

Analytical thin layer chromatography (TLC) was performed using EM Science F254 precoated silica gel 60 plates. Eluting solvents are reported as volume ratios or volume percents. Compounds were visualized using UV light, cerium ammonium molybdate (CAM), p-anisaldehyde, ninhydrin, potassium permanganate, or iodine stains. Flash column chromatography was performed using silica gel 60 (230 - 400 mesh) from Merck. Ion exchange chromatography was carried out using Sephadex
CM-25 resin. Aqueous solutions were concentrated using the lyophilizer from VirTis Research Equipment. During the synthesis of channel 1 dialysis was carried out using Spectra/Por RC membranes with a molecular weight cut-off of 1000. An IEC HN-SII centrifuge from International Equipment Company was used to isolate the crude channel 1. All $^1$H and $^{13}$C NMR spectra were recorded on Varian Unity 400 or Varian Unity 500 spectrometers using CDCl$_3$, D$_2$O, CD$_3$OD, or DMSO-d6 as solvent. The NMR spectra were referenced using residual solvent peaks as the standard. Chemical shifts are expressed in parts per million ($\delta$), coupling constants ($J$) are reported in Hertz (Hz), and splitting patterns are reported as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and apparent (app). High-resolution mass spectra were obtained from the University of Illinois mass spectrometry lab. IR spectra were recorded using a Perkin Elmer Spectrophotometer referenced to a polystyrene standard. Samples were prepared as thin films on a NaCl salt plate by evaporation of chloroform, dichloromethane or methanol solutions. Peaks are reported in wavenumbers (cm$^{-1}$) as strong (s), medium (m), weak (w), and broad (br).

Small unilamellar vesicle preparations were carried out using a Mini Lipo-prep apparatus from Harvard Apparatus. DIACHEMA membranes (63 mm diameter) with a molecular weight cut-off of 5000 from DIANORM were used for the dialysis. The size exclusion chromatography was performed using Sephadex G-50 resin. For visualizing vesicles on TLC molybdenum blue stain for phospholipids was used. Large unilamellar vesicles (LUV) were prepared using the mini-extruder from Avanti Polar lipids. The solutions for LUV preparation were extruded through 0.4 µm polycarbonate membranes. Millipore water from a Milli-Q gradient A10 system was used for vesicle preparations. Unless stated otherwise the buffer (HEPES buffer) used for the fluorescence experiments comprised 100 mM HEPES and 100 mM NaCl. The pH of the buffer solutions was adjusted to 7.0 with conc. NaOH using a $\phi$350 pH meter from Beckman. Fluorescence spectra were recorded on a Fluoromax-2 or a
Fluoromax-3 spectrometer from Jobin Yvon Inc. equipped with an injector port and stirrer, using 10 mm quartz cuvettes from Starna Cells. The emission at 510 nm was monitored for the excitation at $\lambda_{\text{max}}$ of 400 and 450 nm for the protonated and deprotonated HPTS dye respectively. Dynamic light scattering data was obtained using a Dyna Pro DLS instrument from Protein Solutions. A 45 µL sample cell (3 mm) was used to acquire the light scattering data. The $^{23}$Na NMR was acquired on a General Electric GN300WB spectrometer. Transmission electron microscopy (TEM) was performed using a FEI/Philips CM12 instrument, with 200 mesh Cu holey carbon grids from SBI supplies used for sample preparation.

![Chemical Structure](image)

**Pentabutylene glycol monotosylate 3:** To a solution of pentabutylene glycol 2\textsuperscript{[2]} (5.19 g, 13.7 mmol, 1 equiv) in CH$_2$Cl$_2$ (300 mL) at 0 °C was added pyridine (2.30 mL, 27.4 mmol, 2 equiv). While cooling the solution at 0 °C a solution of $p$-toluenesulfonyl chloride (2.62 g, 13.7 mmol, 1 equiv) in CH$_2$Cl$_2$ (300 mL) was added dropwise over a period of 6 h using an addition funnel. After addition of the TsCl was complete, the solution was warmed to room temperature and allowed to stir for 12 h. Water (300 mL) was added to the reaction mixture and the organic layer was separated. The organic layer was washed with saturated aqueous NaCl, dried over anhydrous sodium sulfate, filtered and concentrated in vacuo to afford a white solid. Purification using flash column chromatography (70% hexane/acetone) afforded 3.38 g of sulfonate 3 (46%) as a colorless oil. TLC $R_f = 0.26$ (70% hexane/acetone); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.79 (d, 2H, $J = 8.2$ Hz, $H_{\text{Ar}}$), 7.34 (d, 2H, $J = 7.9$ Hz, $H_{\text{Ar}}$), 4.04 (t, 2H, $J = 6.3$ Hz, $CH_2$OTs), 3.64 (t, 2H, $J = 5.8$ Hz, $CH_2$OH), 3.47-3.33 (m, 16H,
$H_2COCH_2$), 2.45 (s, 3H, $H_3C$), 1.75-1.56 (m, 20H, OCH$_2$CH$_2$CH$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 144.9, 133.4, 130.1, 128.1, 71.1 (overlapping signals), 70.89, 70.86, 70.83, 70.77, 70.72, 70.70, 69.9, 63.0, 30.6, 27.2, 26.69, 26.68, 26.66, 26.63, 26.6, 26.1, 25.9, 21.9; IR (thin film) v 3452 (m, br), 2941 (s), 2860 (s), 2798 (m), 1598 (m), 1447 (m), 1361 (s), 1252 (s), 1189 (s), 1177 (s), 1112 (s), 956 (s), 816 (m) cm$^{-1}$; HRMS (FAB$^+$) calcd for C$_{27}$H$_{49}$O$_8$S (MH$^+$) 533.3148, found 533.3146.

![Diagram 1](image1)

**Pentabutylene glycol monoazide:** The sulfonate 3 (1.59 g, 2.95 mmol, 1 equiv) was heated in DMF (33 mL) at 80 °C with sodium azide (577 mg, 8.87 mmol, 3 equiv) for 1 day. The solvent was removed under reduced pressure to afford a white precipitate. Diethyl ether (50 mL) was added to the precipitate and the resulting suspension was filtered. The flask and the precipitate were washed with ether (3 × 5 mL) and the combined filtrates were concentrated in vacuo to give 1.14 g of the desired azide (96%) as a pale yellow oil. TLC $R_f = 0.5$ (60% hexane/aceton): $^1$H NMR (500 MHz, CDCl$_3$) δ 3.63 (t, 2H, $J = 5.7$ Hz, CH$_2$OH), 3.47-3.4 (m, 16H, $H_2COCH_2$), 3.33 (t, 2H, $J = 6.4$ Hz, CH$_2$N$_3$), 1.69-1.61 (m, 20H, OCH$_2$CH$_2$CH$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 71.08, 71.07, 70.96, 70.94, 70.85, 70.84, 70.79, 70.73, 70.3, 63.0, 51.5, 30.66, 30.65, 27.19, 27.12, 26.69, 26.64, 26.61, 26.1; IR (thin film) v 3452 (br), 2940 (s), 2859 (s), 2096 (s), 1726 (w), 1448 (m), 1368 (m), 1252 (m), 1113 (s); HRMS (ESI$^+$) calcd for C$_{20}$H$_{41}$N$_3$O$_5$Na (M+Na$^+$) 426.2944, found 426.2954.

![Diagram 2](image2)
**Pentabutylene glycol monoamine 4:** A solution of the azide (2.4 g, 6.2 mmol, 1 equiv) in THF (34 mL) was cooled to 0 °C and triphenylphosphine (2.04 g, 7.77 mmol, 1.25 equiv) was added. After the addition, the reaction mixture was allowed to warm to room temperature and stirred for 24 h. Water (10 to 12 drops) was added to the reaction mixture and the solution was allowed to stir for 1 hr and subsequently concentrated in vacuo to remove THF. The solution was diluted with water, neutralized with dil. HCl and purified using cation exchange chromatography (Gradient: 0.05 M to 0.9 M NH₄HCO₃). After concentration in vacuo to 40 mL, the solution was lyophilized to remove the NH₄HCO₃ to afford 1.59 g of the desired amine 4 (71%) as a white solid. TLC Rₚ = 0.59 (5:4:3:1 iPrOH/H₂O/EtOAc/aq. NH₃); m.p. = 32 °C; ¹H NMR (500 MHz, CDCl₃) δ 3.63 (t, 2H, J = 5.9 Hz, CH₂OH), 3.46-3.4 (m, 16H, H₂COC₂H₂), 2.72 (t, 2H, J = 6.8 Hz, CH₂NH₂), 1.79 (br(s), 1H, OH), 1.69-1.49 (m, 20H, OCH₂CH₂CH₂); ¹³C NMR (126 MHz, CDCl₃) δ 71.05, 71.02, 70.88, 70.84, 70.82 (overlapping signals), 70.80, 70.7, 62.8, 42.2, 30.5, 27.3, 27.1, 26.68 (overlapping signals), 26.64, 26.61; IR (thin film) ν 3359 (m, br), 2939 (s), 2858 (s), 2797 (m), 1574 (w), 1484 (m), 1447 (m), 1369 (m), 1113 (s) cm⁻¹; HRMS (ESI⁺) calcd for C₂₁H₄₄NO₅ (MH⁺) 378.3219, found 378.3229.

**Hepta iodo cyclodextrin 5:**[³] To a solution of triphenylphosphine (4.00 g, 15.5 mmol, 22 equiv) in DMF (16 mL) was added iodine (4.00 g, 15.5 mmol, 22 equiv) over a period of 10 min. To the red solution was added dry β-cyclodextrin (800 mg, 0.705 mmol, 1 equiv) and the solution was heated to
70 °C for 8 h. The reaction mixture was concentrated under vacuum to half of its original volume. The concentrated solution was cooled to 0 °C and sodium methoxide, prepared using MeOH (8 mL) and Na\textsuperscript{0} (500 mg), was added. The solution was allowed to stir for 45 min at 0 °C, then warmed to room temperature. Methanol (400 mL) was added and the solution was filtered to afford a yellow precipitate. The residual triphenylphosphine and triphenylphosphine oxide were removed by performing an overnight Soxhlet extraction with MeOH (600 mL) to afford 1.10 g of heptaiodo cyclodextrin \textbf{5} (82%) as an off-white solid. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ 6.04 (d, 7H, J = 6.7 Hz), 5.93 (d, 7H, J = 2.1 Hz), 4.99 (d, 7H, J = 3.2 Hz), 3.8 (d, 7H, J = 8.6 Hz), 3.66-3.57 (m, 14H), 3.46-3.35 (m, 14H), 3.29 (t, 7H, J = 8.9 Hz); MS (ESI\textsuperscript{+}) calcd for C\textsubscript{42}H\textsubscript{63}O\textsubscript{28}I\textsubscript{7}Na (M+Na) 1927.1, found 1926.7

\begin{center}
\includegraphics[width=\textwidth]{channel1.png}
\end{center}

**Channel 1:** To amine \textbf{4} (1.2 g, 3.2 mmol, 76 equiv) at 70 °C, was added heptaiodo cyclodextrin \textbf{5} (80.0 mg, 0.042 mmol, 1 equiv). The reaction mixture was heated until MALDI mass spectrometry indicated displacement of all seven iodides from cyclodextrin (2 d). The solution was cooled to room temperature and acetone (150 mL) was added. The resulting solution was cooled overnight at -20 °C, centrifuged at 2000 rpm for 2 min while cold, following which the supernatant solution was decanted. The residue remaining was washed with cold acetone (2× 3 mL), centrifuged with cooling and isolated as an off-white solid (91 mg). Chloroform (3× 5 mL) was added to the solid in order to extract compound \textbf{1} from unreacted iodo cyclodextrin \textbf{5}. The extract was concentrated in vacuo to afford 84 mg of the desired channel \textbf{1} (55%) as a pale yellow oil. \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD) δ 5.0 (s, 7H, C-
O), 3.85 (t, 14H, J = 9.2 Hz, CH₂OH), 3.57 (t, 14H, J = 6.3 Hz, CH₂NH), 3.45 (m, 112H), 2.95 (s, 14H), 2.78-2.61 (m, 21H); ¹³C NMR (126 MHz, CD₃OD) δ 102.6, 83.2, 73.5, 73.1, 70.8, 70.58, 70.54, 70.49 (overlapping signals), 61.6, 49.9, 49.3, 29.3, 27.6, 27.5, 26.49, 26.42, 26.37 (overlapping signals), 26.1; IR (thin film) ν 3315 (br), 2937 (s), 2857 (s), 1660 (w), 1447 (s), 1367 (w), 1151 (m), 1110 (s), 1044 (s) cm⁻¹; MS (MALDI⁺) for C₁₈₂H₃₅₇N₇O₆₃ (MH⁺) 3652.88, found 3652.45.

²³Na NMR experiments for ion transport studies.

Preparation of Large Unilamellar vesicles.

A solution of EYPC lipids (4.00 mL, 13.2 µmol) was dried under a stream of argon and further in vacuo to give a white solid. To the residue 200 mM aqueous NaCl (4 mL) was added and the resulting suspension was allowed to stand for 30 min. The suspension was then subjected to five freeze-thaw cycles in order to obtain a homogenous solution. Subsequently, the solution was extruded 25 times through a 0.4 µm polycarbonate membrane using an extruder (1 mL at a time) to give the LUV solution.[⁴]

Preparation of shift reagent.

The shift reagent was prepared by mixing 2.0 mL of aqueous sodium tris(polyphosphate) solution (0.2 M) with 1.0 mL of aqueous DyCl₃ solution (0.1 M).

NMR experiments.

Samples for acquiring NMR data were prepared in eppendorf vials (1.5 mL). To the vial was added the LUV suspension (500 µL) followed by an appropriate amount of a solution of channel 1 in methanol (2.17 × 10⁻⁴ M or 6.41 × 10⁻⁴ M) needed for the addition of 5 to 50 nmol of channel 1. The solution was allowed to stand for 45 min following which D₂O (100 µL) and the shift reagent (100 µL) were added. The solution was further allowed to stand for 45 min and then transferred to the NMR
tubes. For comparison with gramicidin A the NMR samples were prepared as before. However, a 20 µL solution of gramicidin A in trifluoroethanol (0.48 mM) was used instead of the channel solution.

For each NMR spectrum 400 FID transients were acquired. The line widths for obtaining rate constants were determined using the line fitting subroutine in the NUTS software for analyzing NMR data. A line broadening of 2 was applied to all the spectra prior to determining the line widths. Average values obtained from 10 line width measurements were used to determine the rates for sodium exchange.

**Fluorescence experiments for ion transport studies.**

**Preparation of small unilamellar vesicles.**[^5]

To the EYPC solution (0.50 mL, 13 µmol, 1 equiv) was added sodium cholate (9.1 mg, 21 µmol, 1.6 equiv) and EtOH (400 µL). The solution was swirled until a clear solution was obtained and then concentrated in vacuo for 3-4 h to give a dry white residue was obtained. To the residue, 1 mL of the HEPES buffer with 0.1 mM of HPTS dye (buffer with dye) was added and swirled until a clear solution was observed. The resulting solution was transferred to the Mini Lipoprep apparatus with a membrane.[^6] The solution was dialyzed using the Lipoprep at a speed of 10 rpm against the buffer with dye (150 mL) for 5 h to ensure entrapment of the dye within vesicles. The Lipoprep apparatus was covered with aluminum foil during the 5 h dialysis period, following which the external dialysis solution was switched to HEPES buffer (400 mL). The solution was dialyzed for 12 h (buffer solution was switched once during this period) in order to remove dye present in the external vesicular solution. After dialysis, the solution was removed from the Lipoprep apparatus to afford the vesicle solution as a pearly suspension. Any dye remaining in the external vesicle solution was removed by size exclusion chromatography using Sephadex G-50 (eluent: HEPES buffer). The solutions containing the vesicles (visualized using TLC) were collected.
Fluorescence studies to determine molecularity of the channel.

For the fluorescence experiments, the SUV solution (200 µL) diluted to 3 mL with HEPES buffer was used in the cuvette with stirring. A solution of 2N NaOH (50 µL) was added to the stirred cuvette solution after a period of 99 s which established a pH shock of 0.6 units. An appropriate amount of a solution of channel 1 in methanol (1.27×10^-4, 4.23×10^-5 or 1.4×10^-5 M) which corresponded to 0.564-7.62 nmol of channel 1 was added to the cuvette solution after a period of 129 s. Finally, a solution of gramicidin A in trifluoroethanol (50 µL, 0.48 mM) was added to the cuvette after 1100 s.

Fluorescence studies to determine ion selectivity of the channel.

a) Alkali Hydroxides (Na^+, K^+, Li^+).

![Figure 1](image_url)

**Figure 1.** Fluorescence vs. time plot of HPTS entrapped in vesicles upon addition of M^+OH^- and 1.1 mol% channel 1.

For the fluorescence experiments, the SUV solution (150 µL) diluted to 3 mL with HEPES buffer was used in the cuvette with stirring. After 90 s either 50 µL of 2N NaOH or 2N KOH or
200 µL of 0.5N LiOH was added to the cuvette. A solution of channel 1 in methanol (50 µL, 9.13 ×10⁻⁵ M) was added to the cuvette solution after 135 s. Finally, a solution of gramicidin A in trifluoroethanol (50 µL, 0.48 mM) was added to the cuvette after 1100 s.

b) Sodium Halides (Cl⁻, Br⁻, I⁻).

For the fluorescence experiments, the SUV solution (150 µL) diluted to 3 mL with HEPES buffer was used in the cuvette with stirring. After 90 s 50 µL of 2N NaX was added to the cuvette. A solution of channel 1 in methanol (50 µL, 3.1×10⁻⁵ M) was added to the cuvette after 135 s. Finally, a solution of gramicidin A in trifluoroethanol (50 µL, 0.48 mM) was added to the cuvette after 1100 s.

The experiment was repeated with melittin wherein a solution of melittin in water (30 µL, 4×10⁻⁵ M) was added to the cuvette instead of the channel.

![Figure 2. Fluorescence vs. time plot of HPTS entrapped in vesicles upon addition of Na⁺X⁻ and 0.4 mol% channel 1.](image-url)
Data obtained upon fitting the curve for NaCl.

**Figure 3.** Fluorescence vs. time plot of HPTS entrapped in vesicles upon addition of Na'X¯ and 0.3 mol% melittin.

**Figure 4.** Fluorescence vs. time plot of HPTS entrapped in vesicles upon addition of NaCl fitted using the Microsoft Excel software

In order to understand the competing processes occurring through channel 1 upon addition of sodium halides, the curve for NaCl (series 1) was fit to the equation $A + Be^{-k_1t} - Ce^{-k_2t}$. The values for
A, B, C, k₁, and k₂ were optimized using the solver tool in the excel software to get the best fit for the NaCl curve. The final equation obtained upon optimization was:

$$I = 2691026 + 26609702e^{-0.0097t} - 26420105e^{-0.0097t}.$$  

**Data obtained upon fitting the curve for NaBr.**

![Figure 5](image_url)

**Figure 5.** Fluorescence vs. time plot of HPTS entrapped in vesicles upon addition of NaBr fitted using the Microsoft Excel software.

The curve for NaBr (series 1) was fit to the equation \(A + Be^{-k_1t} - Ce^{-k_2t}\). The values for A, B, C, \(k_1\), and \(k_2\) were optimized using the solver tool in the excel software to get the best fit for the NaBr curve. The final equation obtained upon optimization was:

$$I = 2670276 + 586799e^{-0.018t} - 389739e^{-0.0066t}.$$
Data obtained upon fitting the curve for NaI.

The curve for NaI (series 1) was fit to the equation $A + Be^{-k_1t} - Ce^{-k_2t} + De^{-k_3t}$. The values for $A$, $B$, $C$, $D$, $k_1$, $k_2$ and $k_3$ were optimized using the solver tool in the excel software to get the best fit for the NaI curve. The final equation obtained upon optimization was,

$$I = 2605747 + 607834e^{-0.071t} - 616755e^{-0.0075t} + 12473e^{-0.023t}$$

**Preparation of grids for acquiring TEM images of vesicles.**

SUVs were prepared without HPTS dye encapsulated following the general procedure for vesicle preparation.

Grids for TEM images without staining:

A drop of the vesicle solution was placed on a sheet of parafilm. The holey carbon grid was placed on the drop such that the grid was floating on the drop with the carbon side down. After 10 min
the grid was removed from the drop and the excess vesicle solution on the grid was removed from the edge of the grid using a filter paper. The grid was dried by placing a CaSO₄ crystal on the grid (the grid was placed vertically in a special container for grids).

Grids for TEM images with negative staining.[7]

The vesicle solution was adsorbed onto the grid using the procedure described above for preparing grids for TEM without staining. After drying the grid with filter paper, the grid was placed on a drop of 2% ammonium molybdate (adjusted to pH 6) placed on the parafilm sheet. After 2 min the grid was removed and dried again with filter paper. The grid was dried further using a crystal of CaSO₄.

**Assay to determine the lipid content in vesicles.**

The concentration of vesicles used for the ion transport studies was determined using a variation of the Bartlett assay[8] for the colorimetric determination of inorganic phosphate. Sodium phosphate monobasic solution (3.26 mM) was used as the phosphorus standard solution. The vesicle samples and the phosphorus standards were converted to the inorganic phosphates using perchloric acid, following which the phosphates were converted to phosphor-molybdic acid by the addition of 4% (w/v) ammonium molybdate solution. The ammonium molybdate was reduced to a blue colored complex (λ_max= 815 nm) by ELON solution (750 mg of a sodium bisulfite-sodium metabisulfite mixture, 250 mg of p-methyl amino phenol sulfate in 25 mL water). The absorbance of the blue complex at 815 nm was measured for all samples. A standard curve was obtained by plotting the absorbance of the phosphate standards against the concentration of phosphorus. The concentration of the vesicle samples were determined from their absorbance values by reference to the standard curve.


[6] Membranes were wetted in H2O for 30 min and the HEPES buffer for 30 min just before adding to the lipoprep.
