Electronic Supplementary Information (ESI)

Measuring anion transport selectivity: a cautionary tale

Xin Wu,[a] and Philip A. Gale*[a,b]

[a] *School of Chemistry, The University of Sydney, NSW 2006, Australia*

[b] *The University of Sydney Nano Institute (SydneyNano), The University of Sydney, NSW 2006, Australia*

*Corresponding author. Email: philip.gale@sydney.edu.au

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S1 General

POPC was purchased from Corden Pharma GmbH. We have deliberately used the batches of POPC that contained ~1 mol% of free fatty acid impurities, to saturate the fatty acid-dependent H^+ transport pathway.[1] Fluorescence measurements were performed using an Agilent Cary Eclipse Fluorescence Spectrophotometer equipped with a stirrer plate and a temperature controller. The syntheses of compounds **1**[2], **2**[3], **3**[4], and **4**[5] have been reported previously.

Abbreviations

DMSO	Dimethyl sulfoxide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPTS	8-Hydroxypyrene-1,3,6-trisulfonic acid
ISE	Ion-selective electrode
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

S2 Theoretical analysis of the "anion gradient assay"

We here derive the relationship between the anion permeability ratio (P_{X^-}/P_{Cl^-}) and the pH gradient (ΔpH) for the equilibrium condition of the "anion gradient" assay (Fig. 1c) under the approximation that anion exchange is negligible.

Assuming that H^+, Cl^- and X^- are the only membrane permeable ions in the presence of an anion transporter (i.e., permeabilities of other charged species including Na^+, HEPES and HPTS are negligible), the membrane potential V_m can be expressed as follows according to the Goldman-Hodgkin-Katz voltage equation:

$$V_m = \frac{RT}{F} ln (\frac{P_{H^+}[H^+_{out}] + P_{Cl^-}[Cl^-_{in}] + P_{X^-}[X^-_{in}]}{P_{H^+}[H^+_{in}] + P_{Cl^-}[Cl^-_{out}] + P_{X^-}[X^-_{out}]}) \quad \text{Eq.S1}$$

At H^+ electrochemical equilibrium,

$$\Delta G_{H^+} = RT \ln \frac{[H^+_{in}]}{[H^+_{out}]} + FV_m = 0 \quad \text{Eq.S2}$$

Combining Eq.S1 and Eq.S2 gives,

$$\frac{P_{X^-}}{P_{Cl^-}} = \frac{[H^+_{in}][Cl^-_{in}] - [H^+_{out}][Cl^-_{out}]}{[H^+_{out}][X^-_{out}] - [H^+_{in}][X^-_{in}]} \quad \text{Eq.S3}$$

(1) In the case of $P_{X^-} > P_{Cl^-}$, dominant HX influx leads to acidification of vesicle interiors:

Initial conditions:

$[Cl^-_{in}] = 100\ mM$, $[X^-_{in}] = 0\ mM$, $pH_{in} = 7.0$;

$[Cl^-_{out}] = 0\ mM$,$[X^-_{out}] = 100\ mM$, $pH_{out} = 7.0$

Equilibrium conditions (assuming that anion exchange is negligible):

$[Cl^-_{in}] = 100\ mM$, $[X^-_{in}] = \Delta[X^-_{in}]$, $pH_{in} < 7.0$;

$[Cl^-_{out}] = 0\ mM$,$[X^-_{out}] = 100\ mM$, $pH_{out} = 7.0$

where $\Delta[X^-_{in}]$ is the amount of X^- influx.

By applying the equilibrium conditions to Eq.3,

$$\frac{P_{X^-}}{P_{Cl^-}} = \frac{[H^+_{in}]100\ mM}{[H^+_{out}]100\ mM - [H^+_{in}]\Delta[X^-_{in}]} = 10^{\Delta pH}\frac{100\ mM}{100\ mM - 10^{\Delta pH}\Delta[X^-_{in}]}$$

Eq.S4

where $\Delta pH = pH_{out} - pH_{in} > 0$ (i.e., inside more acidic than outside)

(2) In the case of $P_{Cl^-} > P_{X^-}$, dominant HCl efflux leads to basification of vesicle interiors:

Initial conditions:

$[Cl^-_{in}] = 100\ mM$, $[X^-_{in}] = 0\ mM$, $pH_{in} = 7.0$;

$[Cl^-_{out}] = 0\ mM$,$[X^-_{out}] = 100\ mM$, $pH_{out} = 7.0$

Equilibrium conditions (assuming that anion exchange is negligible):

$[Cl_{in}^-] = 100\ mM - \Delta[Cl_{in}^-]$, $[X_{in}^-] = 0\ mM$, $pH_{in} > 7.0$;

$[Cl_{out}^-] = 0\ mM$,$[X_{out}^-] = 100\ mM$, $pH_{out} = 7.0$

where $\Delta[Cl_{in}^-]$ is the amount of Cl^- efflux.

By applying the equilibrium conditions to Eq.S3,

$$\frac{P_{X^-}}{P_{Cl^-}} = \frac{[H_{in}^+](100\ mM - \Delta[Cl_{in}^-])}{[H_{out}^+]100\ mM} = 10^{\Delta pH}\frac{100\ mM - \Delta[Cl_{in}^-]}{100\ mM} \quad \text{Eq.S5}$$

where $\Delta pH = pH_{out} - pH_{in} < 0$ (i.e., inside more basic than outside)

S3 ISE assay

An ISE assay was conducted using POPC vesicles (mean diameter 200 nm) loaded with NaCl (100 mM) and suspended in $NaNO_3$ (100 mM), mimicking the conditions in HPTS assays. The vesicles were prepared as follows. A chloroform solution of POPC was evaporated in a round-bottom flask and the lipid film formed was dried under vacuum for at least 6 h. Then, the lipid film was hydrated by vortexing with an internal solution of NaCl (100 mM) buffered at pH 7.0 with HEPES (10 mM). The lipid suspension was subjected to nine freeze/thaw cycles and then extruded 25 times through a 200 nm polycarbonate membrane. The unentrapped NaCl was removing by a Sephadex G-25 column using an external solution of $NaNO_3$ (100 mM) buffered at pH 7.0 with 10 mM HEPES as the eluent. A $NaCl^{in}/NaNO_3^{out}$ vesicle stock suspension (with POPC concentration of ~10 mM) was then obtained.

For each test, the vesicle stock suspension was diluted using the external solution to obtain a 5 mL sample containing 1 mM of POPC. A DMSO solution (10 μL) of an anion transporter was added to the vesicle suspension to initiate the anion exchange process. Cl^- efflux as appearance of Cl^- in the external solution was monitored using a chloride ISE over 30 minutes. At the end of the experiment, the vesicles were lysed with detergent (50 μL of 11% (w%) Triton X-100 in 7 : 1 (v/v) H_2O-DMSO) to release all Cl^- and calibrate Cl^- efflux to 100%.

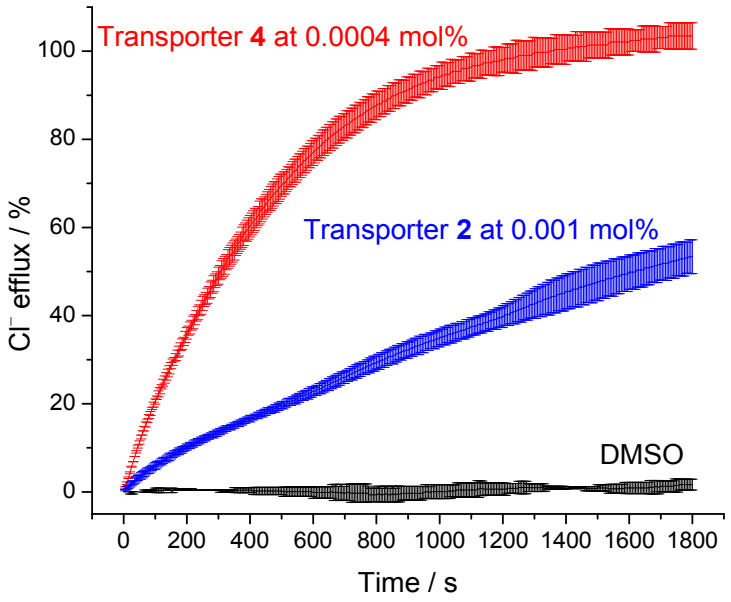


Fig. S1 Cl^-/NO_3^- exchange facilitated by transporters **2** (0.001 mol%), **4** (0.0004 mol%) and DMSO control added at time 0. Initial conditions: In: NaCl (100 mM), HEPES (10 mM), pH 7.0; Out: $NaNO_3$ (100 mM), HEPES (10 mM), pH 7.0; [POPC] = 1 mM, [DMSO] = 0.2 vol%. Transporter concentrations are shown as transporter to POPC molar ratios. Error bars represent standard deviations from two runs.

S4 HPTS assay

S4.1 Vesicle preparation

HPTS assays[6] were conducted using POPC LUVs (mean diameter 200 nm) loaded with the pH-sensitive fluorescence dye HPTS (1 mM). The HPTS-loaded POPC LUVs were prepared as follows. A chloroform solution of POPC was evaporated in a round-bottom flask and the lipid film formed was dried under vacuum for at least 6 h. Then, the lipid film was hydrated by vortexing with an internal solution containing HPTS (1 mM) and NaX (100 mM, X = Cl^-, Br^-, NO_3^-, I^-, or ClO_4^-) buffered at pH 7.0 with 10 mM HEPES. The lipid suspension was subjected to nine freeze/thaw cycles and then extruded 25 times through a 200 nm polycarbonate membrane. The unentrapped HPTS was removed by a Sephadex G-25 column using an external solution of NaX buffered at pH 7.0 with 10 mM HEPES, to obtain NaX^{in}/NaX^{out} vesicle stock suspensions (with POPC concentration of ~10 mM). The lipid concentration was calculated based on the weight of the round-bottom flask before and after lipid addition and the volume of the lipid suspension after the Sephadex column, taking into account the loss of lipid suspensions during the extrusion.

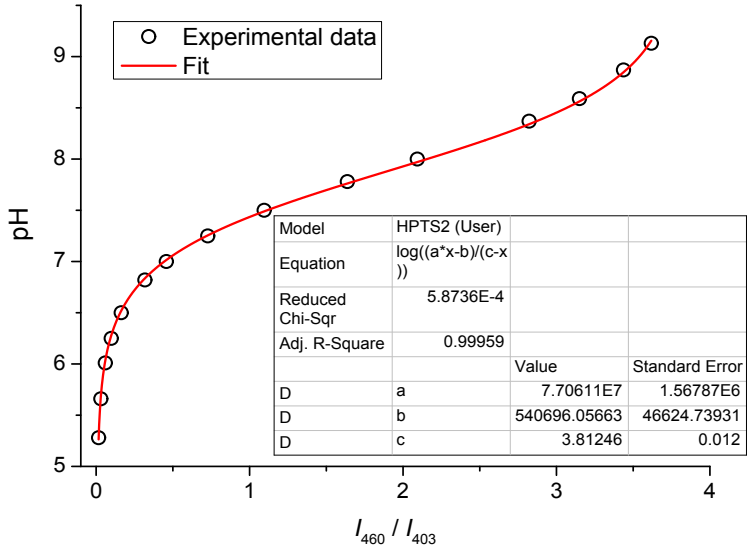
S4.2 HPTS calibration

A calibration has been completed for HPTS assays conducted using $NaCl^{in}$ vesicles. The pH of the external NaCl (100 mM) solution with 10 mM HEPES was adjusted from 5.3 to 9.2 using NaOH, with the solution pH measured by a pH glass electrode. NaCl-containing vesicles prepared following the general procedure were diluted to a POPC concentration of 0.1 mM and a volume of 2.5 mL, using the

NaCl external solutions with adjusted pH. The pH inside vesicles (pH_{in}) was equilibrated to pH_{out} by adding monensin (2.5 µL of a 0.5 mM solution, final concentration 0.1 mol% with respect to POPC). The ratiometric fluorescence response of HPTS I_{460} / I_{403} (λ_{ex} = 460 nm, λ_{em} = 510 nm, base form divided by λ_{ex} = 403 nm, λ_{em} = 510 nm, acid form) at different pH was recorded. The pH was plotted as a function of I_{460} / I_{403}, and the curve was fitted to the following equation derived from the Henderson-Hasselbalch equation, to obtain a calibration for converting I_{460} / I_{403} values to pH:

$$y = lg\frac{ax - b}{c - x} \qquad \text{Eq. S6}$$

where x = I_{460} / I_{403}, y = pH



Model	HPTS2 (User)		
Equation	log((a*x-b)/(c-x))		
Reduced Chi-Sqr	5.8736E-4		
Adj. R-Square	0.99959		
		Value	Standard Error
D	a	7.70611E7	1.56787E6
D	b	540696.05663	46624.73931
D	c	3.81246	0.012

Fig. S2 Calibration for the HPTS assay using $NaCl^{in}$ vesicles.

S4.3 "Dual gradient" assay

Following vesicle preparation according to the general procedure, the NaCl-containing vesicles were diluted using NaX (100 mM) external solutions to obtain $NaCl^{in}/NaX^{out}$ vesicles suspended in 2.5 mL samples containing 0.1 mM of POPC. The samples were stirred at 298 K.

A base pulse of NaOH (25 µL of 0.5 M NaOH solution, final concentration 5 mM) was added to the vesicle samples to generate a pH gradient with pH 7.0 inside and pH 8.0 outside vesicles. Subsequently, an anion transporter (added in 5 µL of DMSO) was added at time 0 and the rate of the pH gradient dissipation was monitored by the ratiometric fluorescence response of HPTS I_{460} / I_{403} (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm). The I_{460} / I_{403} values were converted to pH using the calibration shown in Fig. S2. The results for transporters **2** and **4** are shown in Fig. 4 and the DMSO control data shown in Fig. S3 below.

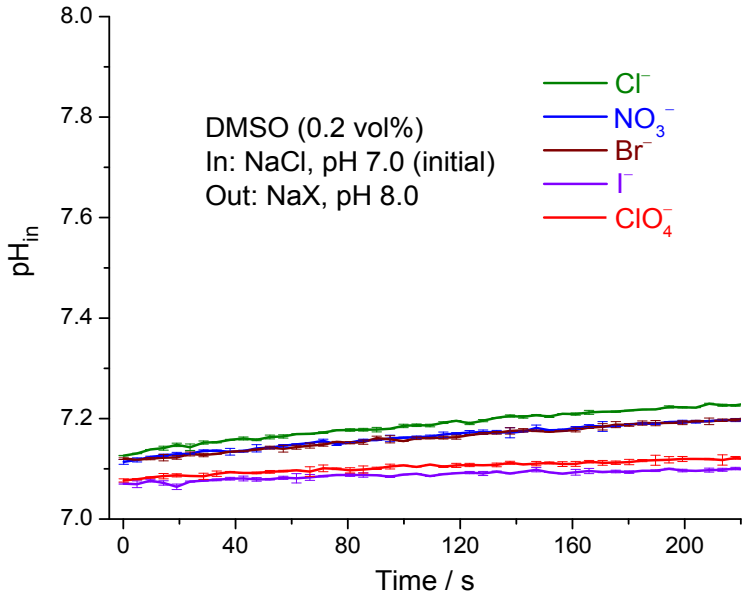


Fig. S3 DMSO control data for the "dual gradient" assay. Initial conditions: In: NaCl (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaX (100 mM), HEPES (10 mM), pH 8.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. DMSO (0.2 vol%) was added at time 0. Error bars represent standard deviations from two runs.

S4.4 "pH gradient" assay

Following vesicle preparation according to the general procedure, the NaX-containing vesicles were diluted using NaX (100 mM) external solutions to obtain NaX^{in}/NaX^{out} vesicles suspended in 2.5 mL samples containing 0.1 or 1.0 mM of POPC. The samples were stirred at 298 K.

A base pulse of NaOH (25 μL of 0.5 M NaOH solution, final concentration 5 mM) was added to the vesicle samples to generate a pH gradient with pH 7.0 inside and pH 8.0 outside vesicles. Subsequently, an anion transporter (added in 5 μL of DMSO) was added at time 0 and the rate of the pH gradient dissipation was monitored by the ratiometric fluorescence response of HPTS I_{460} / I_{403} (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm). The I_{460} / I_{403} data were converted to fractional fluorescence (I_f) values using the following equation:

$$I_f = \frac{R_t - R_0}{R_f - R_0} \qquad \text{Eq. S6}$$

where R_t is the fluorescence ratio at time t, R_0 is the fluorescence ratio at time 0, and R_f is the final fluorescence ratio obtained by curve fitting to the single exponential decay function. Where necessary (e.g., for slow ClO_4^- transport facilitated by transporter **4**), the assay was run for up to 1 hour to ensure precise determination of the R_f value.

We have previously shown that the I_{460} / I_{403} value, instead of the pH_{in}, is proportional to the amount of H^+ efflux.[7] Therefore, here for the purpose of indicating the progress of membrane transport, the I_{460} / I_{403} values were not converted to pH_{in} values.

S4.4.1 Comparison of HPTS and ISE assays

To mimic the conditions of the ISE assay, a POPC concentration of 1 mM was used here instead of 0.1 mM used in most HPTS experiments.

The data from the HPTS assay were overlayed with the ISE data (Fig. S1) to compare the rates of pH gradient dissipation and Cl^-/NO_3^- exchange, shown in Fig. S4 below.

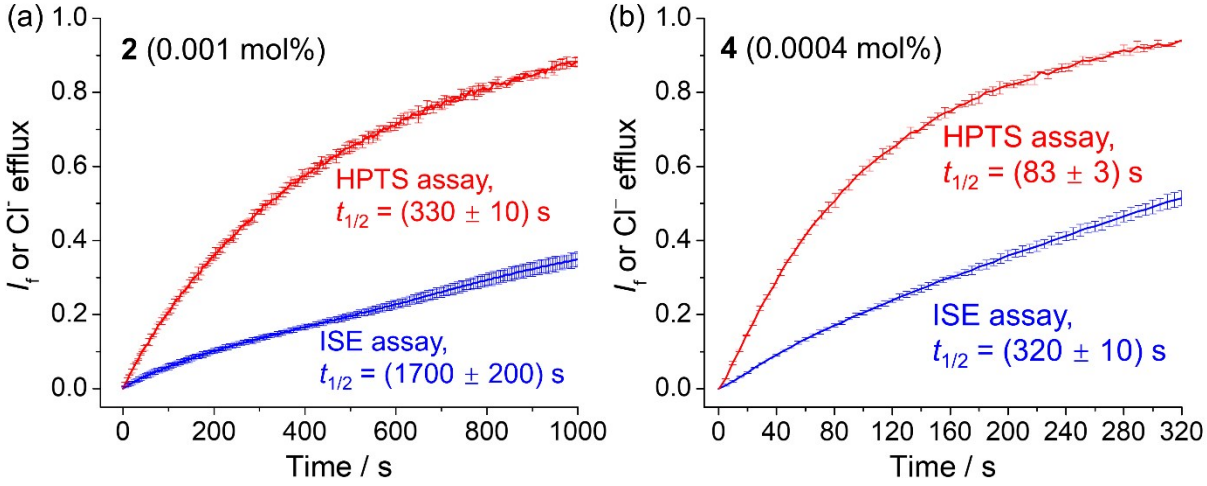


Fig. S4 Comparison of pH gradient dissipation under an HPTS assay and Cl^-/NO_3^- exchange under an ISE assay facilitated by transporters **2** and **4** (added at time 0). In both assays, [POPC] = 1 mM, [NaX] = 100 mM, [HEPES] = 10 mM, [DMSO] = 0.2 vol%. Initial conditions for the HPTS assay: In: NaCl, HEPES, HPTS (1 mM), pH 7.0; Out: NaCl, HEPES, pH 8.0. Initial conditions for the ISE assay: In: NaCl, HEPES, pH 7.0; Out: $NaNO_3$, HEPES, pH 7.0. The anion transporter was added at time 0. Half-lives shown were determined by curve fitting to the single exponential decay function. Transporter concentrations are shown as transporter to lipid molar ratios. Error bars represent standard deviations from two runs.

S4.4.2 Investigation of NO_3^- vs I^- artefact for transporter 4

We previously reported the "pH gradient assay" to give a selectivity sequence of $Cl^- > Br^- > NO_3^- > I^- > ClO_4^-$ for transporter **4**, based on the order of $1/EC_{50}$ values obtained for under different NaX^{in}/NaX^{out} conditions (dilute vesicle suspensions containing 0.1 mM of POPC were used).[8] Here we conducted a single concentration analysis for transporter **4** at 0.0004 mol%, using both dilute ([POPC] = 0.1 mM) and concentrated ([POPC] = 1.0 mM) vesicle suspensions. The results with dilute vesicles (Fig. S5) showed the same selectivity sequence as previously reported based on $1/EC_{50}$ analysis (which contradicts with the $I^- > NO_3^-$ obtained using "anion gradient" assays), but a sequence of $Cl^- > Br^- > I^- > NO_3^- > ClO_4^-$ was produced using concentrated vesicles (Fig. S6).

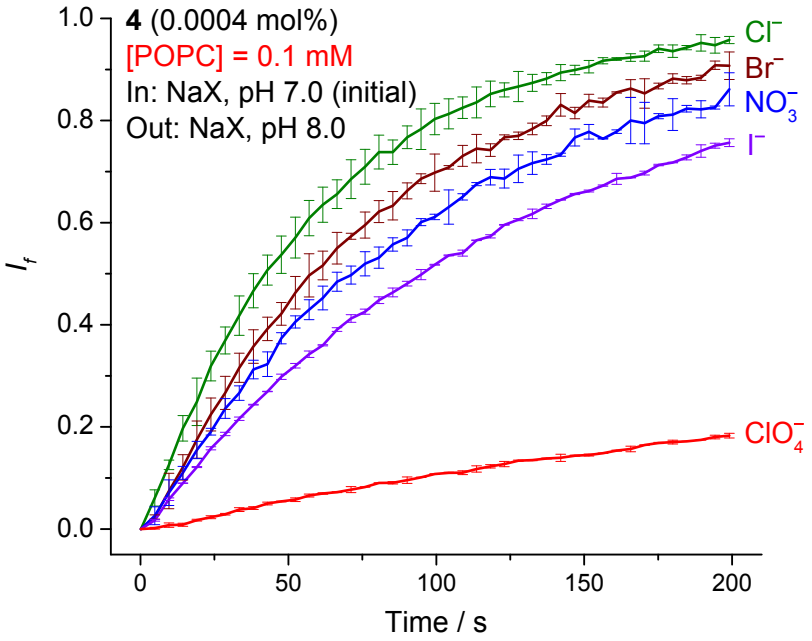


Fig. S5 "pH gradient assay" for transporter **4** (0.0004 mol%, added at time 0) using dilute vesicles. Initial conditions: In: NaCl (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaCl (100 mM), HEPES (10 mM), pH 8.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. Error bars represent standard deviations from two runs.

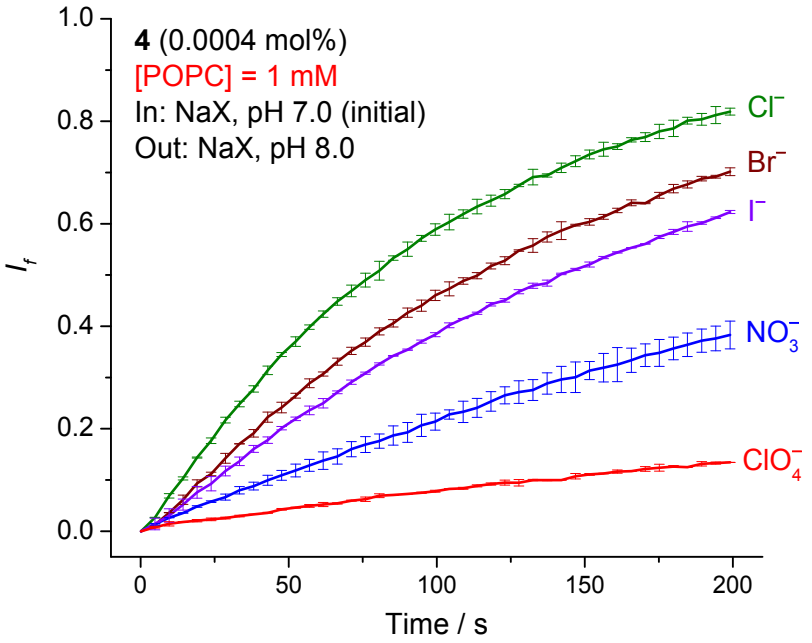


Fig. S6 "pH gradient assay" for transporter **4** (0.0004 mol%, added at time 0) using concentrated vesicles. Initial conditions: In: NaCl (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaCl (100 mM), HEPES (10 mM), pH 8.0. [POPC] = 1 mM, [DMSO] = 0.2 vol%. Error bars represent standard deviations from two runs. Note that the transporter rates here are slower than in dilute vesicles (Fig. S5) presumably because of the higher concentration of transporter **4** (4 nM in Fig. S6 compared with 0.4 nM in Fig. S5, the DMSO content was kept constant at 0.2 vol%), leading to stronger transporter aggregation inhibiting membrane transport.

To compare the amounts of active transporters partitioned in the membrane when transporter **4** was added under $NaNO_3^{in}/NaNO_3^{out}$ and NaI^{in}/NaI^{out} conditions, the following test was conducted.

A DMSO solution of transporter **4** was added to $NaNO_3^{in}/NaNO_3^{out}$ and NaI^{in}/NaI^{out} vesicles prepared following the general procedure to a transporter/POPC ratio of 0.0004 mol%. The vesicles with membrane-partitioned transporters were then separated from the solution-phase transporters by a Sephadex G-25 column using $NaNO_3$ (100 mM, for $NaNO_3^{in}/NaNO_3^{out}$ vesicles) or NaI (100 mM, for NaI^{in}/NaI^{out} vesicles) buffered at pH 7.0 with 10 mM HEPES as the eluent. The isolated vesicles were then diluted with an external solution of NaCl (100 mM) buffered at pH 7.0 with 10 mM HEPES to a POPC concentration of 0.1 mM, with the amount of remaining $NaNO_3$ or NaI being < 5 mol% with respect to NaCl. The diluted $NaNO_3^{in}/NaCl^{out}$ and $NaI^{in}/NaCl^{out}$ vesicles containing transporter **4** were then converted to $NaCl^{in}/NaCl^{out}$ vesicles by stirring overnight to ensure complete anion exchange. Finally, the vesicles were subject to fluorescence kinetic studies, with NaOH base pulse (5 mM) added to generate a pH gradient and initial the membrane transport process. With the final $NaCl^{in}/NaCl^{out}$ condition being identical for the two batches of vesicles, the transport rates indicate the amounts of active transporters that remained after the Sephadex column.

As shown in Fig. S7, a slower kinetics of pH gradient dissipation can be observed for the vesicles originally suspended in NaI compared with those originally suspended in $NaNO_3$, supporting the

hypothesis that the NaI^{in}/NaI^{out} vesicles contained fewer membrane-partitioned active transporters than the $NaNO_3^{in}/NaNO_3^{out}$ vesicles.

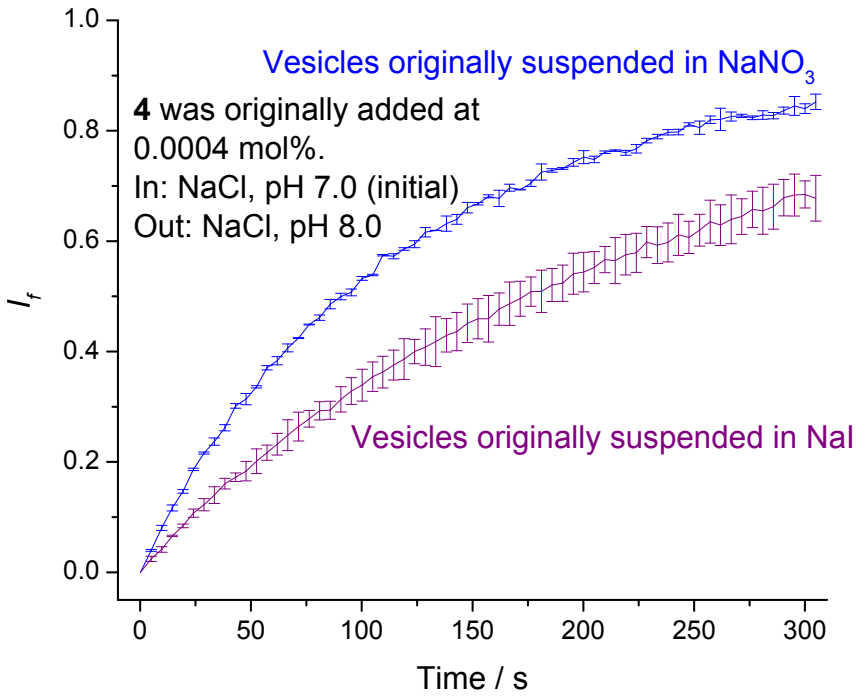


Fig. S7 "pH gradient assay" for transporter **4** originally added at 0.0004 mol% under $NaNO_3^{in}/NaNO_3^{out}$ (blue) and NaI^{in}/NaI^{out} (purple) conditions. After transporter addition, the vesicles were isolated, resuspended in NaCl, and stirred overnight to convert to the $NaCl^{in}/NaCl^{out}$ condition. NaOH (5 mM) was added to initiate the membrane transport. The initial steep increase of HPTS fluorescence ratio due to response from membrane-bound or unentrapped HPTS was removed from the data. Initial conditions of kinetic study: In: NaCl (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaCl (100 mM), HEPES (10 mM), pH 8.0. [POPC] = 0.1 mM. Error bars represent standard deviations from two runs.

S.4.5 “Anion gradient” assay

Following vesicle preparation according to the general procedure, the NaCl-containing vesicles were diluted using NaX (100 mM) external solutions to obtain $NaCl^{in}/NaX^{out}$ vesicles suspended in 2.5 mL samples containing 0.1 mM of POPC. The samples were stirred at 298 K.

No base pulse was added so no pH gradient was initially present. An anion transporter (added in 5 μL of DMSO) was added at time 0 to induce pH_{in} changes. The pH_{in} was monitored by the ratiometric fluorescence response of HPTS I_{460} / I_{403} (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm). The I_{460} / I_{403} values were converted to pH_{in} using the calibration shown in Fig. S2.

The results for transporters **1**–**4** are shown in Fig. 4 and the DMSO control data shown in Fig. S8 below. Note that the weak response with I^- in the DMSO control data is due to the presence of traces of I_2 that facilitated selective I^-/OH^- antiport leading to pH_{in} decrease.

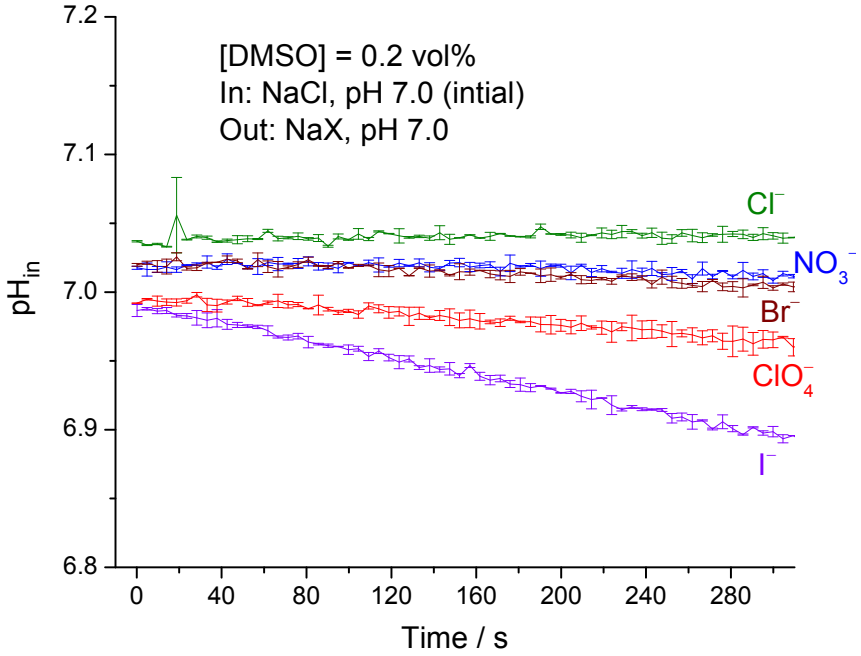


Fig. S8 DMSO control data for the “anion gradient” assay. Initial conditions: In: NaCl (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaX (100 mM), HEPES (10 mM), pH 7.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. Error bars represent standard deviations from two runs.

S.4.6 "Competitive" assay

Following vesicle preparation according to the general procedure, the NaX-containing vesicles were diluted using NaY (100 mM) external solutions to obtain NaX^{in}/NaY^{out} vesicles suspended in 2.5 mL samples containing 0.1 mM of POPC. The samples were stirred at 298 K.

No base pulse was added so no pH gradient was initially present. An anion transporter (added in 5 μL of DMSO) was added at time 0 to induce pH_{in} changes. The pH_{in} was monitored by the ratiometric fluorescence response of HPTS I_{460} / I_{403} (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm).

Transporter **4** has been previously tested under this assay[8] giving a selectivity sequence consistent with that obtained based on ΔpH_{max} comparison under $NaCl^{in}/NaX^{out}$ "anion gradient" assay (Fig. 3d).

To further demonstrate the robustness of the "competitive" NaX^{in}/NaY^{out} assay, we used this assay to re-investigate the I^- vs ClO_4^- selectivity of transporters **1**–**3**. Under $NaClO_4^{in}/NaI^{out}$ condition (Fig. S9), transporter **1** induced a pH_{in} increase whereas transporters **2** and **3** induced pH_{in} decrease (with stronger responses than the DMSO baseline). The results indicate the $ClO_4^- > I^-$ selectivity of transporter **1**, and the opposite $I^- > ClO_4^-$ selectivity of transporters **2** and **3**, consistent with the ΔpH_{max} comparison under the $NaCl^{in}/NaX^{out}$ "anion gradient" assay (Fig. 3). The $NaI^{in}/NaClO_4^{out}$ condition, however, failed to generate a significant response for all transporters (Fig. S10). The cause for the lack of responses under this combination is currently unclear and is possibly related to complex interactions of the membrane with hydrophobic I^- and ClO_4^- ions[9] leading to transport activity inhibition. These results highlight the benefit of using both NaX^{in}/NaY^{out} and NaY^{in}/NaX^{out} conditions when comparing two anions X^- and Y^-.

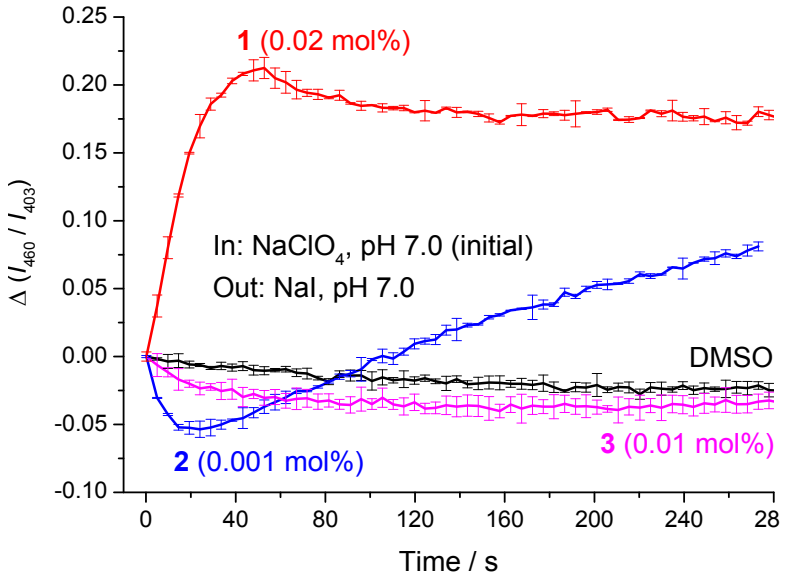


Fig. S9 $NaClO_4^{in}/NaI^{out}$ assay to determine I^- vs ClO_4^- selectivity for transporters **1**–**3** (added at time 0). Initial conditions: In: $NaClO_4$ (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaI (100 mM), HEPES (10 mM), pH 7.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. The DMSO control is included. The weak response in the DMSO control is due to the presence of traces of I_2 that facilitated selective I^-/OH^- antiport leading to pH_{in} decrease. Error bars represent standard deviations from two (transporters **1** and **2**) or four (DMSO and transporter **3**) runs.

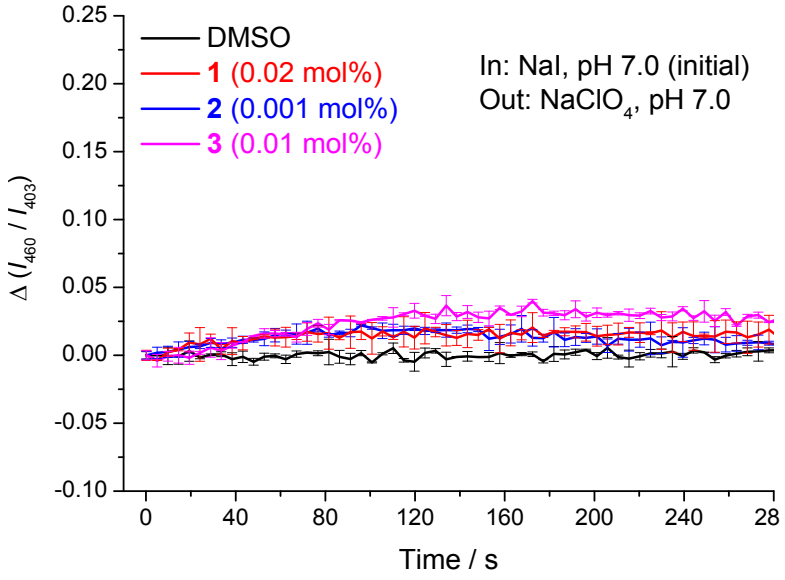


Fig. S10 $NaI^{in}/NaClO_4^{out}$ assay to determine I^- vs ClO_4^- selectivity for transporters **1**–**3** (added at time 0). Initial conditions: In: NaI (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: $NaClO_4$ (100 mM), HEPES (10 mM), pH 7.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. The DMSO control is included. Error bars represent standard deviations from two runs. The initial I_{460} / I_{403} value was higher for NaI^{in} vesicles than other NaX^{in} vesicles because of I^- selectively quenching the fluorescence of the acid form of HPTS when co-entrapped with HPTS inside vesicles.

Since Br^- and NO_3^- produced identical kinetic curves under the $NaCl^{in}/NaX^{out}$ "anion gradient" assay for transporter **2** (Fig. 3b), we also attempted to distinguish between these two anions using the "competitive" assay. The results in Fig. S11 indicate no significant pH gradient induction under asymmetric ionic condition, as the weak HPTS responses induced by transporter **2** observed under asymmetric ionic condition were identical to the responses under symmetric condition (possibly due to interaction of transporter **2** with fatty acids[1] or membrane-bound HPTS; similarly, transporter **2**–**4** also induced weak HPTS responses under symmetric NaCl/NaCl condition as shown in Fig. 3). Therefore, we conclude that transporter **2** facilitated H^+/Br^- symport and H^+/NO_3^- symport at very similar rates.

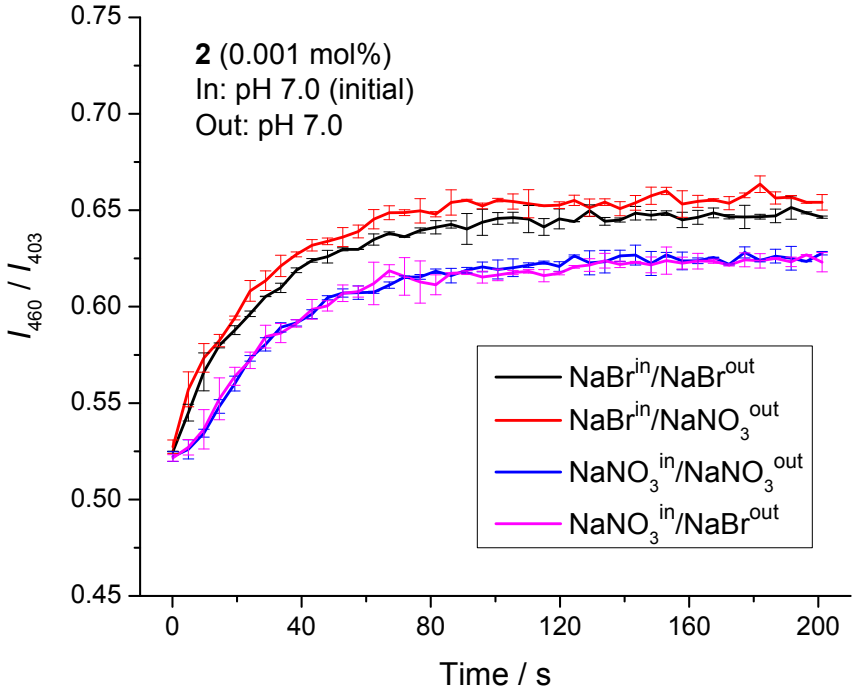


Fig. S11 "Competitive" assay to determine Br^- and NO_3^- selectivity for transporter **2** (0.001 mol%, added at time 0). Initial conditions: In: NaBr or $NaNO_3$ (100 mM), HPTS (1 mM), HEPES (10 mM), pH 7.0; Out: NaBr or $NaNO_3$ (100 mM), HEPES (10 mM), pH 7.0. [POPC] = 0.1 mM, [DMSO] = 0.2 vol%. Error bars represent standard deviations from two runs.

S5 Discussion of high affinity anion transporters

We noted that a different concept of anion selectivity could be applied for extremely high affinity anion transporters. Compounds **1**–**4** are examples of anion transporters that presumably operate far from anion binding saturation in water-lipid biphasic systems. For the strongest anion receptor **4** showing sub-μM apparent Cl^- affinity in MeCN (Fig. 1), we demonstrated that the selectivity sequence determined by the "pH gradient" assay (Fig. 1b) agreed with the "anion gradient" assays (Fig. 1c and 1d) after addressing the NO_3^- vs I^- artefact. In comparison, Valkenier, Šindelář and coworkers have reported extremely high-affinity fluorinated bambusuril anion transporters with NO_3^- affinities > 10^9 M^{-1} and Cl^- affinities about an order of magnitude lower in MeCN.[10] These transporters can be described as selective Cl^-/HCO_3^- exchangers and inactive Cl^-/NO_3^- exchangers because the presence of NO_3^- even at mM concentrations suppressed the transport due to saturation of the transporters by NO_3^- binding. We hypothesise that were these transporters capable of facilitating H^+ or OH^- transport, a "pH gradient" assay by comparing pH gradient dissipation rates in $NaCl^{in}/NaCl^{out}$ and $NaNO_3^{in}/NaNO_3^{out}$ vesicles would demonstrate a "Cl^- selectivity" because anion transport is inhibited in the presence of NO_3^-. By contrast, "anion gradient" assays based on the H^+ transport direction in $NaCl^{in}/NaNO_3^{out}$ or $NaNO_3^{in}/NaCl^{out}$ vesicles would reveal a "NO_3^- selectivity" because more NO_3^- complexes are formed than Cl^- complexes and both complexes should be membrane permeable (with the NO_3^- complex likely to be more permeable). Although the bambusurils are not suitable for testing this hypothesis because they do not possess H^+ or OH^- transport activity, similar behaviour has been found for a cation transporter.[11] This provides another possible cause of the discrepancy between different ion selectivity assays which requires the concept of selectivity (i.e., the faster, the more selective) to be revised for transporters with saturated ion binding behaviour.

S6 References

1. X. Wu and P. A. Gale, *J. Am. Chem. Soc.*, 2016, **138**, 16508-16514.
2. N. Busschaert, I. L. Kirby, S. Young, S. J. Coles, P. N. Horton, M. E. Light and P. A. Gale, *Angew. Chem. Int. Ed.*, 2012, **51**, 4426-4430.
3. S. J. Moore, C. J. E. Haynes, J. González, J. L. Sutton, S. J. Brooks, M. E. Light, J. Herniman, G. J. Langley, V. Soto-Cerrato, R. Pérez-Tomás, I. Marques, P. J. Costa, V. Felix and P. A. Gale, *Chem. Sci.*, 2013, **4**, 103-117.
4. N. Busschaert, P. A. Gale, C. J. E. Haynes, M. E. Light, S. J. Moore, C. C. Tong, J. T. Davis and W. A. Harrell, Jr., *Chem. Commun.*, 2010, **46**, 6252-6254.
5. X. Wu, P. Wang, P. Turner, W. Lewis, O. Catal, D. S. Thomas and P. A. Gale, *Chem*, 2019, **5**, 1210-1222.
6. S. Matile and N. Sakai, in *Analytical Methods in Supramolecular Chemistry*, ed. C. A. Schalley, Wiley-VCH, Weinheim, 2012, pp. 711-742.
7. H. J. Clarke, X. Wu, M. E. Light and P. A. Gale, *J. Porphyr. Phthalocya*, 2020, **24**, 473-479.
8. X. Wu, J. R. Small, A. Cataldo, A. M. Withecombe, P. Turner and P. A. Gale, *Angew. Chem. Int. Ed.*, 2019, **58**, 15142-15147.
9. J. R. Rydall and P. M. Macdonald, *Biochemistry-us.*, 1992, **31**, 1092-1099.
10. H. Valkenier, O. Akrawi, P. Jurček, K. Sleziaková, T. Lízal, K. Bartik and V. Šindelář, *Chem*, 2019, **5**, 429-444.
11. Z. Zhao, B. Tang, X. Y. Yan, X. Wu, L. Zhao, P. A. Gale and Y.-B. Jiang, *Front. Chem. Sci. Eng.*, 2021, in press.