Targeted anion transporter delivery by coiled-coil driven membrane fusion

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ELECTRONIC SUPPLEMENTARY INFORMATION (*ESI*)

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1. Experimental Details

Materials

Chemicals. Fmoc-protected amino acids were purchased from Novabiochem and Biosolve. Sieber amide resin was purchased from Agilent Technologies. Cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), bovine serum albumin (BSA), biotin labeled bovine albumin (biotin-BSA), and streptavidin from streptomyces avidinii were purchased from Sigma Aldrich. 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Biotinyl)(Sodium Salt) was purchased from Avanti Polar Lipids. 10,10'-dimethyl-9,9'-biacridinium nitrate (lucigenin) was purchased from Tokyo Chemical Industry UK Ltd. Chloroform was deacidified by passage through a column containing activated basic alumina before the preparation of the lipid solutions. The buffer solution for all GUV studies was prepared with $NaNO_3$ (225 mM) and TRIS (10 mM) in Millipore grade water and the pH was adjusted to 7 with sulfuric acid. The lucigenin (0.8 mM) and NaCl (1 M) solutions were prepared with the buffer solution. Lipid solutions of POPC and cholesterol (70 : 30 molar ratio, 1 and 14 mM) were prepared in deacidified chloroform. The transporter octyl t-(2,7)-bis(3-(3,5-bis(trifluoromethyl)phenyl)thioureido)-t-8a-decahydronaphthalene-r-4a-carboxylate,[1] (**3**, 84 μM solution in methanol) was added to the lipid solution at 10 mol % (relative to total lipid). Experiments are performed at room temperature, unless stated otherwise.

Cells and cell culture. Fischer Rat Thyroid (FRT) cells stably expressing the halide sensor YFP-H148Q/I152L (YFP-FRT cells)[2] were a generous gift of A. S. Verkman (University of California, San Francisco). YFP-FRT cells were cultured as described previously[3] with the exception that media contained 10% fetal bovine serum, 2 mM glutamine and the selection agent G418 (0.5 mg/mL). YFP-FRT cells were plated onto glass coverslips and used 4 – 5 days later.

The PBS buffer used for the experiments with YFP-FRT cells was composed of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$; pH 7.40.

General methods for liposomes and GUV studies

Synthesis of lipopeptides. The spacer N_3-PEG_4-COOH, cholesteryl-4-amino-4-oxobutanoic acid, and the lipopeptides CP_4K_4 (**1**) and CP_4E_4 (**2**) were synthesized and utilized following procedures previously reported.[4,5] The peptide segments E: NH_2-$(EIAALEK)_4$-$CONH_2$ and K: NH_2-$(KIAALKE)_4$-$CONH_2$ were synthesized using standard Fmoc chemistry on a peptide synthesizer (CEM-Liberty 1), then the spacer N_3-PEG_4-COOH was coupled to the N-terminus of the peptide segment. The azide terminal group on the spacer was reduced to an amine to obtain an N-terminal free amine for coupling to cholesteryl-4-amino-4-oxobutanoic using 5 eq. of DIPEA and 4 eq. of PyBOP in DMF over 72 h. Finally, the lipopeptides were purified by RP-HPLC with a Gemini C4 column to yield a pure product (Yield: 20-25%) The identity and purity of the peptides and lipopeptides was determined by LC-MS.

Formation of GUVs. Giant Unilamellar Vesicles (GUVs) were grown on Dex-PEG hydrogel (1:1 molar ratio) coated microscope glass slides as described previously.[6] A lipid solution (10 μL) with the lipid composition

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[1] H. Valkenier, L. W. Judd, H. Li, S. Hussain, D. N. Sheppard, A. P. Davis, *J. Am. Chem. Soc.* **2014**, *136*, 12507–12512.

[2] a) A. S. Verkman, L. J. V. Galietta, *Nat. Rev. Drug Discov.* **2009**, *8*, 153-171; b) L. J. V. Galietta, S. Jayaraman, A. S. Verkman, *Am. J. Physiol. Cell Physiol.* **2001***, 281***,** C1734–C1742. c) L. J. V. Galietta, P. M. Haggie, A. S. Verkman, A. S. FEBS Lett. **2001**, *499*, 220-224

[3] D. N. Sheppard, M. R. Carson, L. S. Ostedgaard, G. M. Denning, M. J. Welsh, *Am. J. Physiol. Lung Cell. Mol. Physiol.* **1994**, *266*, L405-L413.

[4] J. Voskuhl, C Wendeln, F. Versluis, E. C. Fritz, O. Roling, H. Zope, C. Schulz, S. Rinnen, H. F. Arlinghaus, B. J. Ravoo, A. Kros, *Angew. Chem. Int. Ed.*, **2012**, *51*, 12616-12620.

[5] a) H. R. Marsden, N. A. Elbers, P. H. H. Bomans, N. Sommerdijk and A. Kros, *Angew. Chem Int. Ed.*, **2009**, *48*, 2330-2333. b) F. Versluis, J. Voskuhl, B. Kolck, M. Bremmer, T. Albregtse and A. Kros, *J. Am. Chem. Soc.*, **2013**, *135*, 8057-8062.

POPC and Cholesterol (70:30 molar ratio, 14 mM) and DOPE-Biotin (0.2 mol %) was deposited on a hydrogel coated glass slide, then the lipid solution was dried by evaporating the chloroform under a gentle stream of air and subsequently it was placed in a vacuum oven overnight. A liquid chamber was made by placing a 15 mm (OD) glass O-Ring on top of the hydrogel, sealed with high vacuum silicon grease. The lipid film was hydrated by adding 400 μL of an aqueous solution that contained lucigenin (0.8 mM), $NaNO_3$ (225 mM), TRIS (10 mM) and sucrose (200 mM) into each chamber and the GUVs were formed overnight at room temperature.

Decoration of GUVs with CPK. GUVs were decorated with 1 mol % CP_4K_4 **1** (relative to lipids). A stock solution of CP_4K_4 **1** (15 μL, 50 μM in CH_3OH:$CHCl_3$ 1:1) was dried by evaporating the chloroform under a gentle stream of air. Subsequently the stock solution was placed in a vacuum oven overnight. The lipopeptide film was hydrated by adding 700 μL of buffer solution that contained $NaNO_3$ (225 mM), TRIS (10 mM) and glucose (200 mM), vortexed and transferred to a micro centrifuge tube. Subsequently, 300 μL of the solution with free floating GUVs was transferred into the micro centrifuge tube containing the CP_4K_4 aqueous solution. The GUVs were incubated for 60 minutes in the lipopeptide solution and finally 300 μL of GUVs were transferred to 700 μL of buffer solution that contained $NaNO_3$ (225 mM), TRIS (10 mM) and glucose (200 mM).

Formation of Large Unilamellar Vesicles LUVs with CPE and transporter. Peptide decorated LUVs were formed with 10 mol % transporter and 1 mol % CP_4E_4 **2** both relative to total lipids. Lipid solution (100 μL for experiments with GUVs or 500 μL for experiments with cells) with the lipids POPC and Cholesterol (70:30 molar ratio, 1 mM in $CHCl_3$) was mixed with CP_4E_4 stock solution (20 μL for GUVs or 100 μl for cells, 50 μM in CH_3OH:$CHCl_3$ 1:1) and transporter **3** (118 μL, 84 μM in methanol for GUVs or 267 μL, 187 μM in methanol for cells). Then, the lipid solution was dried by evaporating the chloroform under a gentle stream of air and subsequently, it was placed in a vacuum oven overnight. The lipid film was hydrated by adding 1 mL of buffer solution that contained $NaNO_3$ (225 mM), TRIS (10 mM) and glucose (200 mM) for experiments with GUVs or 2 mL of PBS for experiments with cells. Finally, the LUVs were formed by sonication at 50-55 °C for 4-5 minutes and the final size distribution was determined by DLS (Zetasizer Nano-S, Malvern) with sizes circa 100-180 nm.

Delivery of transporter to GUVs. The transporter was delivered to the membrane of GUVs by targeted membrane fusion with peptide-decorated LUVs containing the transporter molecule. CP_4E_4 decorated LUVs (200 μL) and CP_4K_4 decorated GUVs (200 μL) were transferred into a micro centrifuge tube with the buffer solution (600 μL) containing $NaNO_3$ (225 mM), TRIS (10 mM) and glucose (200 mM), then the vesicles were mixed for 15 minutes using a tube rotator and incubated for 120 minutes.

Transport experiments in GUVs. The visualization of GUVs after targeted membrane fusion was achieved with a microscopy chamber which was pre-treated first with an aqueous mixture of BSA (0.9 mg/mL) and biotin-BSA (0.1 mg/mL) for one hour and then with streptavidin for another hour. The solution with GUVs (200 μL) was transferred into the microscopy visualization chamber with the buffer solution (100 μL) containing $NaNO_3$ (225 mM), TRIS (10 mM) and glucose (200 mM). The GUVs were left to sediment for at least 30 minutes before imaging. During imaging of the GUVs in a time lapse experiment, 25 μL NaCl (1 M, in $NaNO_3$ and glucose solution) was added to the well after 30-40 seconds with a microsyringe, giving a final NaCl concentration of ~80 mM.

Imaging of the GUVs and data analysis. The imaging of GUVs was performed on a Leica TCS SPE confocal microscope system. Illumination was provided by a solid state laser using the 488 nm laser line (15% laser power) for irradiation of lucigenin. Fluorescence confocal microscopy was carried out using a 63× water objective. The analysis of the images was performed in ImageJ software,[7] by measuring the average intensity of an area corresponding to one GUV for the series of time lapsed microscopy image frames. These fluorescence intensity values (F) were normalized to the fluorescence intensity at the start of the time lapse (F_0).

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[6] N. López Mora, J. S. Hansen, Y. Gao, A. A. Ronald, R. Kieltyka, N. Malmstadt, A. Kros, *Chem. Commun.* **2014**, *50*, 1953–1955.

[7] Schneider, C.A., Rasband, W.S., Eliceiri, K.W. "NIH Image to ImageJ: 25 years of image analysis". *Nature Methods 9*, 671-675, **2012**.

Methods for cell studies

Decoration of YFP-FRT cells with CPK and delivery of the transporter. Modification of YFP-FRT cell membranes was performed by two sequential incubations. 200 μL of the CP_4K_4 **1** stock solution (50 μM in CH_3OH:$CHCl_3$ 1:1) was dried by evaporating the solvent under a gentle stream of air and subsequently placed in high vacuum for 1 hour. The CP_4K_4 film was hydrated with 2 mL of PBS and sonicated for 1-2 minutes at 50 - 55 °C.

YFP-FRT cells plated on glass coverslips (confluency, 80%) were washed 3x with PBS and exposed to the CP_4K_4 solution (5 μM in PBS) for 2 hour at 37 °C. After this first treatment the cells were washed again with PBS and subsequently treated with a solution of liposomes (POPC/cholesterol/CP_4E_4 **2**/transporter **3)** in PBS (in which the total concentration of transporter **3** was 25 μM, see above for details) for 1 hour at 37 °C. For the first control experiment, cells were treated in an identical way, but transporter **3** was omitted from the POPC/cholesterol/CP_4E_4 liposomes. For the second control experiment liposomes were made using POPC with 10 mol % transporter **3**, analogous to our previously reported studies on the activity of anion transporters in cells, albeit the incubation period was longer (previous study, 10 minutes; current study, 1 hour).[8] For this second control experiment, the cells were not treated with CP_4K_4, but solely treated with the **3**/POPC liposomes in PBS (250 μM POPC, 25 μM **3**) for 1 hour at 37 °C.
On completion of the incubation periods, the YFP-FRT cells were transferred to a perfusion chamber mounted on the stage of a Leica DM IRB microscope for cell fluorescence measurements. Any anionophore not incorporated into YFP-FRT cell membranes was removed from the chamber by perfusion with PBS.

Transport studies with YFP-FRT cells. Anionophore-mediated anion transport by YFP-FRT cells was quantified by measuring I^--induced quenching of YFP fluorescence.[8] In brief, a field of view with bright YFP-FRT cells was selected for fluorescence measurements and the cells were perfused with (i) PBS for 5 minutes, then (ii) PBS containing NaI (10 mM) (made by preparing PBS with 127 mM NaCl to maintain osmolarity) for 5 minutes and finally (iii) PBS for 20 – 30 minutes to remove thoroughly NaI from the perfusion chamber. In some experiments, if fluorescence had recovered sufficiently, anionophore-treated YFP-FRT cells were perfused a second time with PBS containing NaI (10 mM) for 5 minutes before cells were again washed with PBS. During all interventions, the rate of solution perfusion was 8 – 10 mL min^{-1}; temperature was 37 °C.

Fluorescence microscopy and data analysis. For cell fluorescence measurements, we used the Volocity (Improvision) data acquisition and analysis system and a cooled CCD camera (Hamamatsu ORCA ER firewall) with the Leica DM IRB inverted fluorescence microscope equipped with an oil objective (x65, numerical aperture 1.32), excitation filter wheel and multiple band dichroic and emission filters (YFP: excitation, 500 ± 10 nm; emission, 545 ± 25 nm). Cell fluorescence data were sampled every 6 seconds.

Fluorescence data from 5 – 9 cells per coverslip were analysed with 4 coverslips tested per intervention. Cell fluorescence values (F) are expressed relative to the fluorescence value immediately before iodide (10 mM) addition to YFP-FRT cells (F_0). By fitting exponential functions to the first 2 minutes of the fluorescence decay following NaI (10 mM) addition, we determined the initial slope to quantify anion transport by compound **3**. Results are expressed as means ± SEM of N observations. To compare sets of data, we used Student's unpaired t-test. Differences were considered statistically significant when $P < 0.05$. All tests were performed using SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

[8] H. Li, H. Valkenier, L. W. Judd, P. R. Brotherhood, S. Hussain, J. A. Cooper, O. Jurček, H. A. Sparkes, D. N. Sheppard, A. P. Davis, *Nature Chem.* **2015**, *7*, accepted for publication.

2. Liposome experiments showing the inability of transporter 3 to exchange between liposomes.

We tested the ability of transporter **3** to exchange between lipid bilayer membranes without the aid of membrane fusion using a fluorescence assay with liposomes. We mixed receiver liposomes which contain lucigenin, but no transporter and delivery liposomes with transporter **3** (but no lucigenin) and used fluorescence spectroscopy to test whether any transport of chloride into the receiver liposomes occurred.

Receiver liposomes. POPC and cholesterol solutions in deacidified chloroform were combined in such a way that the resulting solution contained 7.0 µmol POPC and 3.0 µmol cholesterol. The chloroform was removed by a flow of N_2 and the resulting lipid film was dried for 1 h in vacuum. The lipid film was hydrated with 500 µL of an aqueous solution of 0.8 mM lucigenin and 225 mM $NaNO_3$, sonicated 30 s, and stirred for 1 h. The resulting mixture was frozen and thawed 10x and subsequently extruded 29x through a polycarbonate membrane (200 nm pore size). The external lucigenin was removed by size exclusion chromatography over a column of Sephadex G-50 eluted with an aqueous solution of 225 mM $NaNO_3$. The liposomes were collected and diluted with 225 mM $NaNO_3$ solution to 25 mL, to obtain a total lipid concentration of ~ 0.4 mM.

Delivery liposomes. Solutions of POPC and cholesterol in deacidified chloroform and a solution of transporter **3** in methanol were combined to obtain a solution containing 1.4 µmol POPC, 0.6 µmol cholesterol, and 0.8 nmol **3**. The organic solvents were removed by a flow of N_2 and the resulting lipid film was dried for 1 h in vacuum. The lipid film was hydrated with 150 µL of an aqueous solution of 225 mM $NaNO_3$, sonicated for 30 s, and stirred for 1 h. The resulting mixture was frozen and thawed 10x, subsequently extruded 29x through a polycarbonate membrane (200 nm pore size), and diluted with 225 mM $NaNO_3$ solution to 5 mL (0.4 mM total lipid concentration, ratio **3**:lipid = 1:2500).

Transport experiments. 2.7 mL of receiver liposomes and 300 µL of delivery liposomes were combined in a cuvette and stirred at 25 °C. After a set amount of time (10 min, 1 h, 2 h or 3 h), 75 µL of a solution of 1.0 M NaCl (in 225 mM aqueous $NaNO_3$) was added while the fluorescence was monitored over time (using a PerkinElmer LS45 spectrometer, excitation at 450 nm, emission at 535 nm). The resulting fluorescence traces were normalized by dividing the fluorescence (F) by the fluorescence level just before the addition of NaCl (F_0).

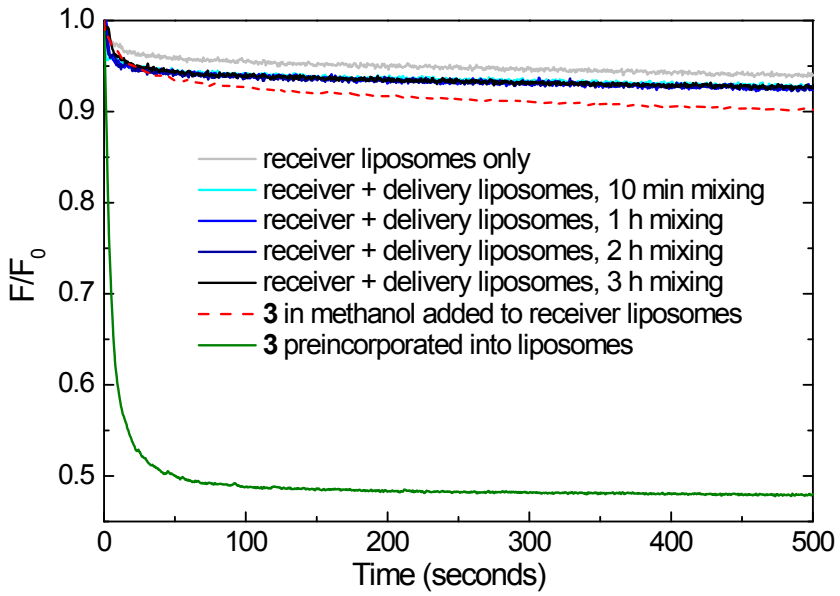

Fig. S1 Normalized fluorescence curves of receiver liposomes (gray) and receiver liposomes mixed with delivery liposomes (different shades of blue for different mixing times) as a function of time after addition of NaCl. For comparison the fluorescence traces from liposomes with transporter **3** pre-incorporated (**3**:lipid = 1:25,000, green) and **3** added as solution in methanol (red dashed) are shown.[1]

Figure S1 shows that upon mixing of delivery and receiver liposomes and subsequent addition of NaCl, no quenching of fluorescence (and thus no transport of Cl^- into the receiver liposomes) takes place. The fluorescence curves obtained from this experiment are very similar to the blank curve from the receiver liposomes without added delivery liposomes. In contrast, when transporter **3** is preincorporated into the liposomes (which also contain lucigenin), fast quenching and thus rapid anion transport is observed.

If transporter **3** would have been able to exchange between liposomes, then the receiver liposomes with lucigenin (which make up 90% of the total of liposomes in the mixture) should have obtained transporter molecules, and these would have carried chloride into the liposomes to quench the fluorescence of lucigenin. If a full equilibrium situation would have been reached (with all transporter molecules spread evenly over all liposomes), the final transporter **3**:lipid ratio would have been 1:25,000 and the resulting fluorescence trace should have been identical to the experiment shown in Figure S1 with the transporter preincorporated. The observation that no transport occurs between 10 minutes and 3 h after mixing receiver and delivery liposomes demonstrates that transporter **3** is not likely to be capable of exchanging between POPC/cholesterol (7:3 ratio) liposomes.

2. DLS of LUVs and CP_4E_4 peptide decorated LUVs with 10 mol % transporter.

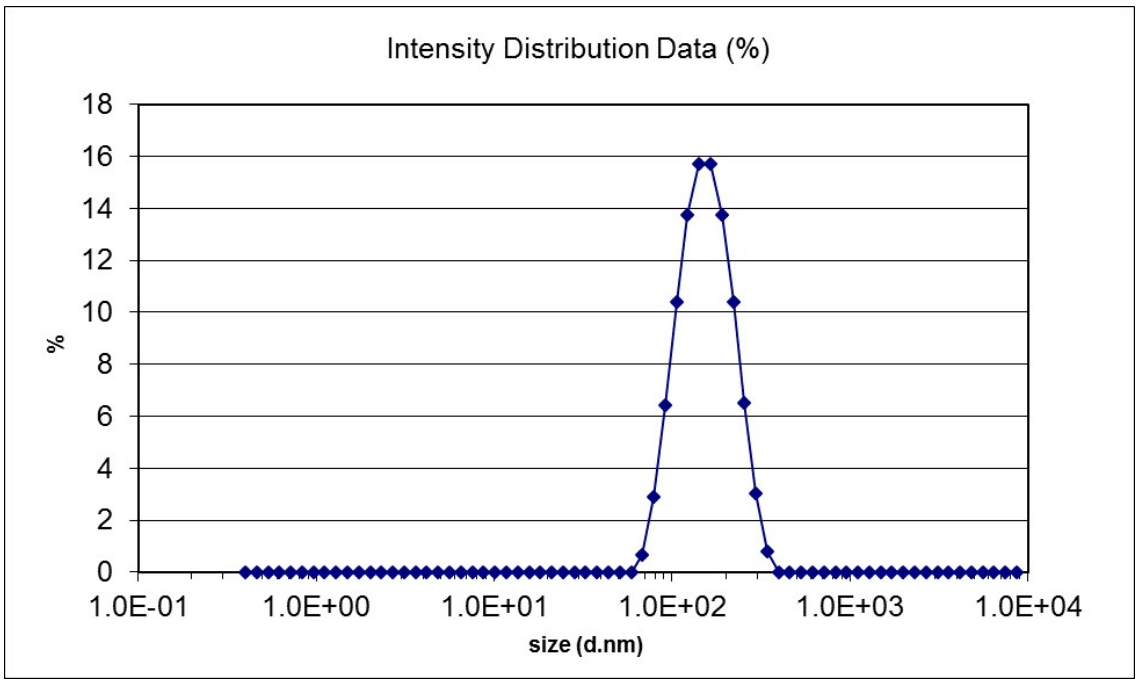

Fig. S2 Dynamic Light Scattering of LUVs without CP_4E_4 and transporter **3.**

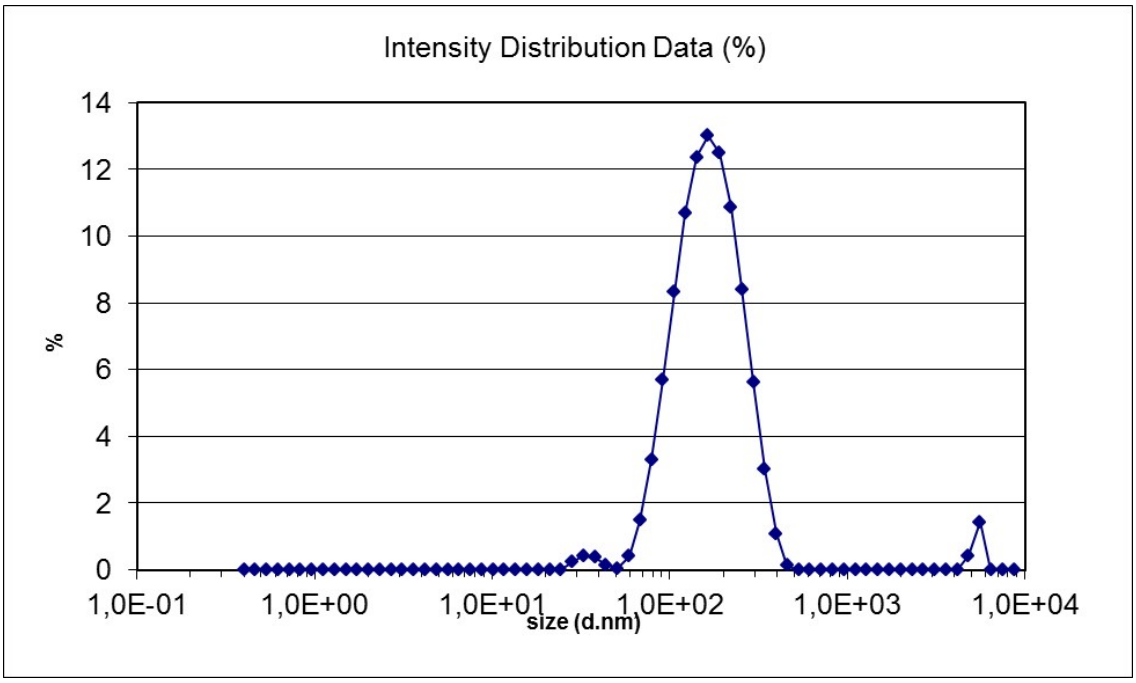

Fig. S3 Dynamic Light Scattering of CP_4E_4-decorated LUVs with 10 mol % transporter **3**.

4. Cryo-TEM of CP_4E_4 peptide decorated LUVs containing 0 mol % and 10 mol % transporter.

Cryo-transmission electron microscopy. Liposome samples were prepared as detailed in the section "Preparation and study of liposomes and GUVs" of the Supplementary Methods. A droplet of 5 μL CP_4E_4-decorated LUVs either with or without 10 mol % transporter **3** was applied to freshly glow-discharged lacey-carbon grids (Electron Microscopy Sciences) in a chamber with 95 % humidity at 21°C, blotted for 2 seconds and plunge-frozen in liquid ethane at -181 °C using a Leica EM GP (Leica Microsystems, Germany). Grids were mounted in a Gatan 626 cryo holder (Gatan, Pleasanton, USA) maintained at -178 °C with liquid nitrogen and imaged using a Tecnai 20 FEG (FEI Company) operated at 200 keV. Images were recorded at -1.5 μm underfocus with a Gatan Ultrascan 4000 camera (Gatan) using low-dose software at a nominal magnification of ×29k. Total dose was less than 10 electrons/Å2.

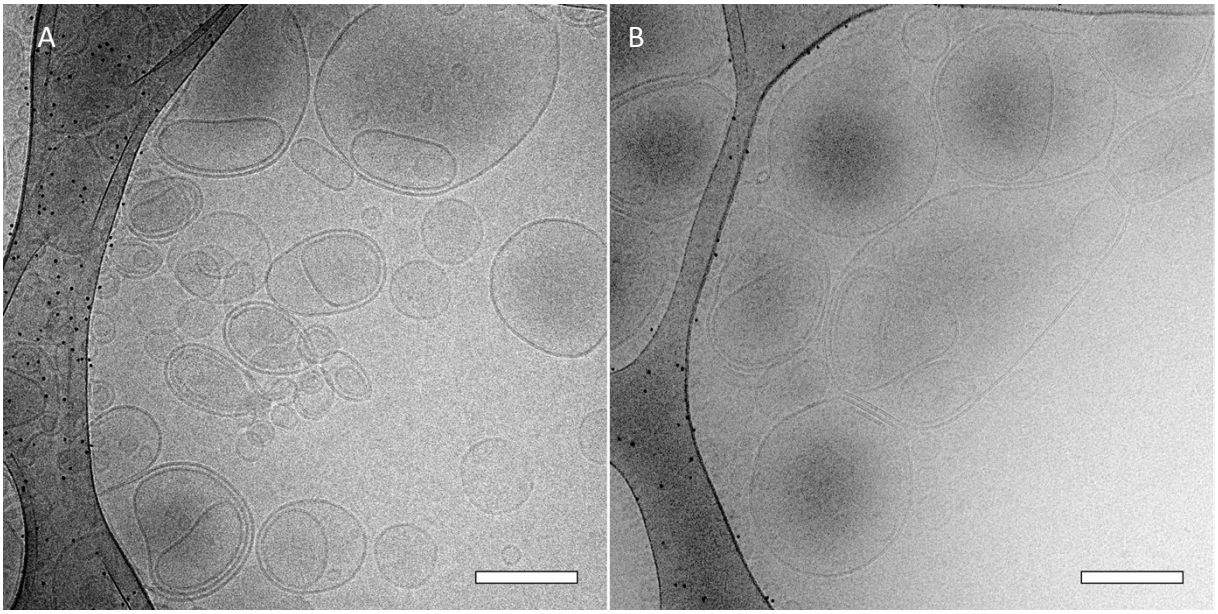

Fig. S4 Representative cryo-transmission electron micrographs of CP_4E_4-decorated LUVs A) without transporter and B) with 10 mol % transporter **3**. The scale bars are 200 nm.

5. Datasets for the average curves presented in Figure 2 of the main manuscript

Photobleaching

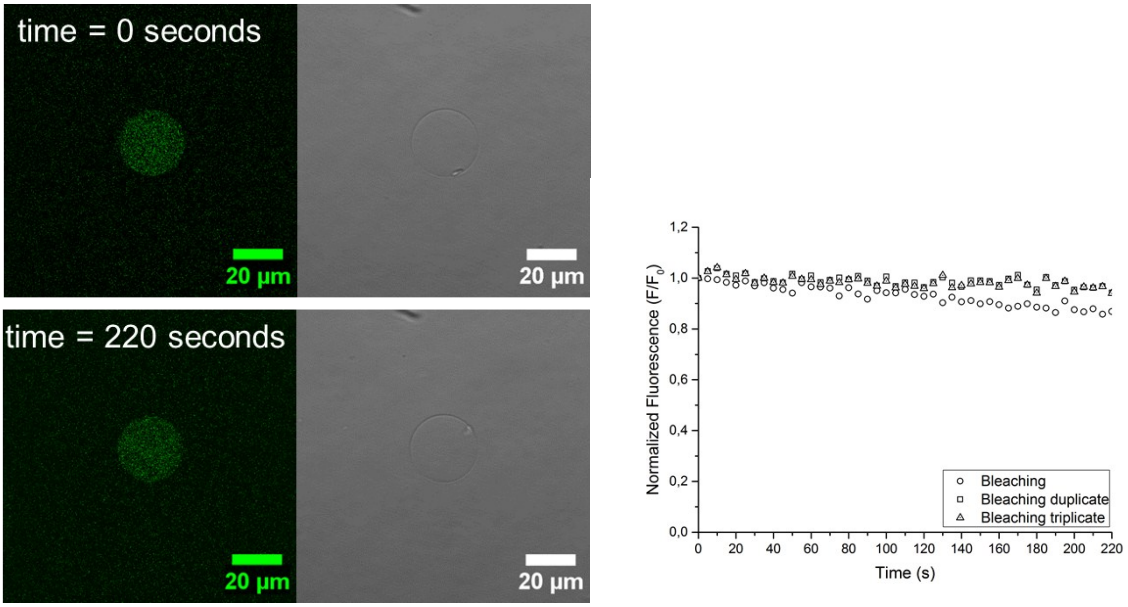

Fig. S5 Fluorescence and bright-field microscopy images at the beginning and end of a 3 minute time lapse measurement to quantify the photobleaching of lucigenin dye during the experiment (no NaCl added). The normalized fluorescence intensity over time is given for individual GUVs from independent experiments.

CP_4E_4-decorated LUVs and CP_4K_4-decorated GUVs, 10 mol % transporter 3 is included in LUVs.

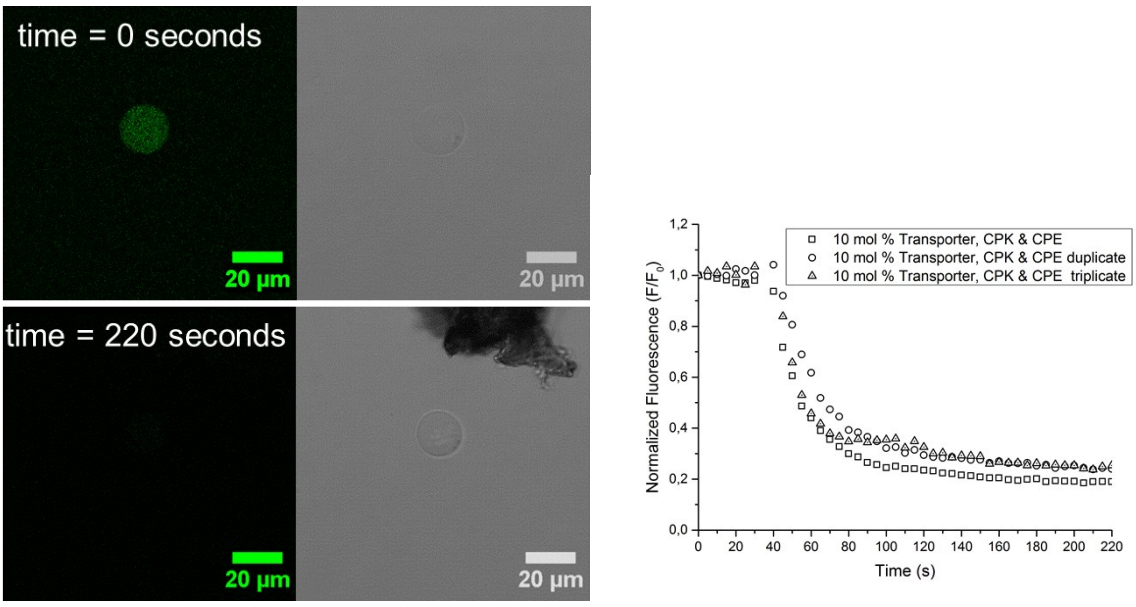

Fig. S6 Fluorescence and bright-field microscopy images before (top) and after (bottom) addition of NaCl to GUVs previously incubated with LUVs (10 mol % transporter). The normalized fluorescence intensity over time is given for individual GUVs from independent experiments.

Control experiment: CP_4E_4-decorated LUVs and CP_4K_4-decorated GUVs, transporter 3 is excluded from LUVs.

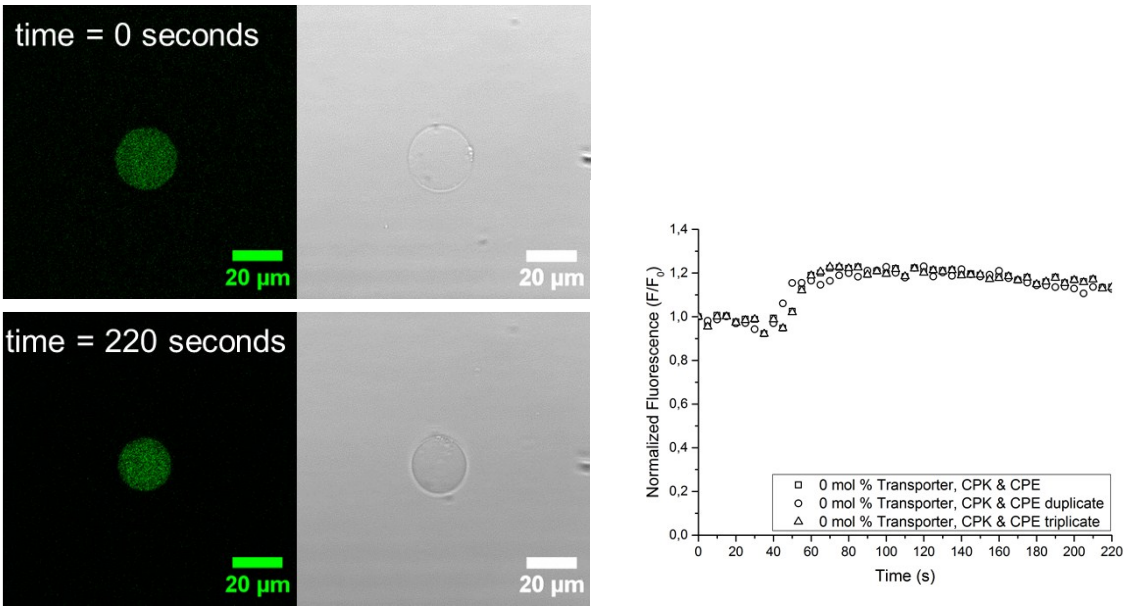

Fig. S7 Fluorescence and bright-field microscopy images before (top) and after (bottom) addition of NaCl to GUVs previously incubated with LUVs (0 mol % transporter). The normalized fluorescence intensity over time is given for individual GUVs from independent experiments.

Control experiment: CP_4E_4-decorated LUVs and plain GUVs, CP_4K_4 is excluded from GUVs.

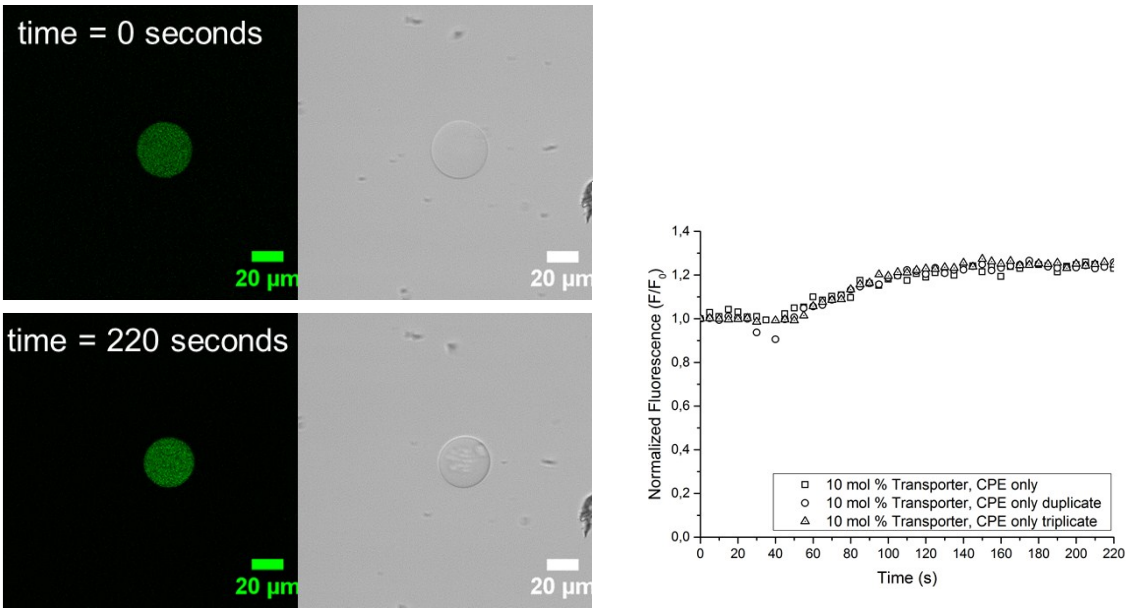

Fig. S8 Fluorescence and bright-field microscopy images before (top) and after (bottom) addition of NaCl to GUVs previously incubated with LUVs (10 mol % transporter). CP_4K_4 is excluded from GUVs. The normalized fluorescence intensity over time is given for individual GUVs from independent experiments.

6. Time lapse and frames from an experiment showing anion transport in YPF-FRT cells upon targeted delivery of transporter 3.

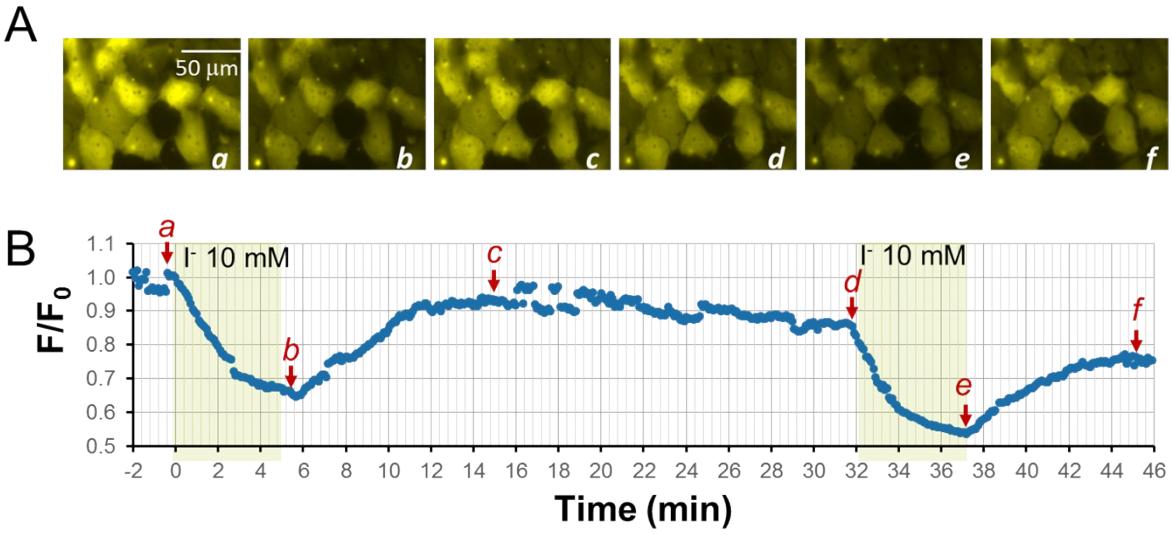

Fig. S9 A) Images of YFP-FRT cells and B) Representative time courses of normalized cell fluorescence after delivery of transporter **3** to YFP-FRT cells using the lipopeptides **1** and **2**. YFP-FRT cells were perfused with PBS for 5 minutes, then (a) PBS containing NaI (10 mM) for 5 minutes leading to a rapid and robust quenching of cell fluorescence. This decrease in cell fluorescence was almost completely reversed when NaI (10 mM) was washed with (b) PBS for 20 – 30 minutes to remove thoroughly NaI from the perfusion chamber. The images of cells in A were taken at the time points labeled a – f on the time course shown in B.