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Chapter VI

Targeted anion transporter delivery by coiled-coil driven membrane fusion

Abstract.

Synthetic anion transporters (anionophores) have potential as biomedical research tools and therapeutics. However, the efficient and specific delivery of these highly lipophilic molecules to a target cell membrane is non-trivial. Here, we investigate the delivery of a powerful anionophore to artificial and cell membranes using a coiled-coil-based delivery system inspired by SNARE membrane fusion proteins. Incorporation of complementary lipopeptides into the lipid membranes of liposomes and cell-sized giant unilamellar vesicles (GUVs) facilitated the delivery of a powerful anionophore into GUVs, where its anion transport activity was monitored in real time by fluorescence microscopy. Similar results were achieved using live cells engineered to express a halide-sensitive fluorophore. We conclude that coiled-coil driven membrane fusion is a highly efficient system to deliver anionophores to target cell membranes.
**Introduction**

There is urgent interest in Drug Delivery Systems (DDSs), such as cell penetrating peptides or liposomes, which are non-invasive and cause no damage to cellular membranes. Liposomes of less than 1 µm in diameter have been used as models for studying biological and biophysical membrane properties, as well as DDSs, due to their biocompatibility and low toxicity. Delivery of molecules into the cytoplasm of cells can be achieved by functionalizing liposomes with positively charged lipids, polymers, antibodies or cell penetrating peptides. Whilst the encapsulation of water-soluble drugs into liposomes is one of the most used tools for drug delivery, the incorporation of lipophilic drugs into the phospholipid bilayer of liposomes has been less exploited. Alternative approaches to delivering drugs with low water-solubility include the use of solubilizing agents or vehicles, such as micelle forming amphiphiles, cyclodextrins or cucurbiturils, although these are not targeted and tend to have limited stability.

One class of lipophilic compounds that could benefit from novel DDSs are transmembrane anion transporters (anionophores). These molecules have potential as tools for biomedical research, and might also replace the function of anion channels which are defective or deficient in genetic diseases. There is particular interest in bypassing the cystic fibrosis transmembrane conductance regulator (CFTR) whose dysfunction causes *cystic fibrosis*. Anionophores require sufficient lipophilicity to partition exclusively into the membrane and to carry anions such as chloride across the apolar membrane interior. They therefore tend to be water-insoluble, with low intrinsic deliverabilities using conventional DDS.

Inspired by the specific molecular recognition of native SNARE proteins, we have developed a DDS employing a synthetic model system to induce targeted membrane fusion. Our membrane fusion system consists of the use of two complementary peptide amphiphiles located in different membranes. The formation of a dimeric coiled-coil by these peptides brings the two opposing membranes into close proximity, thereby inducing efficient membrane fusion. In previous work, we successfully used this coiled-coil motif to modify surfaces of cancer cells and one-day-old zebra fish embryos *in vivo*.
Herein, we report the use of this DDS as a highly specific recognition system for delivering a lipophilic anion transporter to both giant unilamellar vesicles (GUVs) and the plasma membrane of cells. The pair of complementary lipopeptides employed in this study is presented in Scheme 1. This synthetic model system is constructed from two complementary amphiphilic coiled-coil peptides K₄ [(KIAALKE)₄] (1) and E₄ [(EIAALEK)₄] (2) coupled to a cholesterol anchor through a flexible polyethylene glycol linker. The heterodimeric coiled coil acts as a molecular zipper by the binding of two α-helical peptide strands, while the cholesterol anchor allows the insertion of the peptides into the lipid membrane of vesicles or cells.

The anionophore bis-(thioureido)decalin (3) has remarkable ability to transport anions across lipid bilayers, promoting rapid chloride-nitrate exchange even when operating as single molecules. Recently, we evaluated the activity of 3 in individual GUVs by its direct incorporation into the lipid mixture prior to GUV formation. The average initial rate of chloride transport per molecule was determined by analyzing the quenching of the halide-sensitive fluorophore lucigenin encapsulated in the GUVs. Transporter 3 showed exceptional chloride/nitrate exchange activity (820 ± 260 Cl⁻/s) when incorporated a priori into the lipid membrane of liposomes or GUVs at different concentrations. However, the high lipophilicity of 3 limits its deliverability. Not only is it poorly delivered when added in methanol, but the use of simple vesicles as delivery vehicles is also ineffective (Figure 4 in the Experimental Section).

The poor deliverability of 3 and similar anionophores is a critical barrier to future applications. When rates of anion transport were studied in cells, the best performance was obtained by an anionophore with excellent deliverability, but modest intrinsic activity (two orders of magnitude lower than 3 in liposomes). Thus, solving the deliverability problem of 3 has great potential for applications in biophysics and perhaps therapeutics. Here we present a facile method to deliver the highly lipophilic transporter 3 pre-incorporated in liposomes by simple incubation with GUVs and cells.

Figure 1 outlines the protocol for delivering transporter 3 to the lipid membrane of cell-sized GUVs using our synthetic membrane fusion system. The experimental design comprises three main components: (i) GUVs functionalized with lipopeptide 1 as the
target membrane and biophysical cell model; (ii) the halide sensitive fluorophore lucigenin encapsulated in the GUVs as a sensing dye and (iii) the DDS which uses liposomes decorated with lipopeptide 2, and with transporter 3 incorporated \textit{a priori} into the lipid membrane. Following targeting and incorporation of anionophore 3 into the lipid membrane of GUVs by membrane fusion, the quenching of lucigenin fluorescence by chloride is used to measure the chloride transport activity.

**Scheme 1.** Chemical structures of the synthetic membrane fusion model: lipopeptide CP$_4$K$_4$ (1), lipopeptide CP$_4$E$_4$ (2) and the anionophore bis-(thioureido)decalin (3).

**Results and discussion**

Liposomes were formed from the lipid mixture 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol in a 7:3 molar ratio by sonication method in the presence of 10 mol \% transporter 3 and 1 mol \% lipopeptide CP$_4$E$_4$ (see \textit{Experimental Section} for details). The hybrid lipid film was hydrated in an aqueous solution of 225 mM NaNO$_3$, 10 mM TRIS and 200 mM glucose (pH = 7, adjusted with
H$_2$SO$_4$) and sonicated for 4 minutes at 50-55 °C. Liposome formation was confirmed by dynamic light scattering measurements (Figure 5 and 6 in the Experimental Section) and the peptide functionalized liposomes were characterized by cryo-transmission electron microscopy (cryoTEM) in the absence and presence of transporter (Figure 7 in the ESI). Electron microscopy showed that the lipid membrane of the CP$_4$E$_4$ functionalized liposomes was not altered by the incorporation of 3.

In parallel, GUVs were grown by lipid film hydration (POPC and cholesterol, 7:3 molar ratio) on chemically crosslinked dextran (polyethylene glycol) hydrogel (DexPEG) substrates. The hydration of the lipid film was performed with a solution of 225 mM NaNO$_3$, 10 mM TRIS, 200 mM sucrose and 0.8 mM lucigenin at room temperature. The DexPEG hydrogel allows both the efficient encapsulation of the lucigenin fluorophore and the growth of GUVs under the high ionic strength conditions which are required to perform chloride/nitrate exchange.

Lucigenin-loaded GUVs were subsequently functionalized with CP$_4$K$_4$ by incubation in a solution containing lipopeptide 1. Even though it is possible to grow GUVs with lipopeptide 1 pre-incorporated directly into the lipid mixture, we chose to make plain GUVs and modify them a posteriori with lipopeptide 1 because our aim is to deliver the transporter to cellular membranes, which do not contain 1 as a specific recognition motif. As mentioned above, we showed previously that similar cholesterol modified lipopeptides can be inserted efficiently into liposomal membranes and the plasma membrane of cancer cells by simple incubation. In the present work, we followed the same procedure for the peptide functionalization of GUVs.

Briefly, after the formation of lucigenin loaded GUVs, 300 µL of the solution containing the GUVs was transferred to 700 µL of solution containing 225 mM NaNO$_3$, 10 mM TRIS, 200 mM glucose and 1 µM CP$_4$K$_4$ (1). GUVs were incubated for one hour at room temperature to allow the incorporation of molecule 1 into the membrane of GUVs. The higher density of the sucrose lucigenin solution encapsulated in GUVs compared to external glucose solution caused the GUVs to sink to the bottom of the micro centrifuge tube. Transfer of sedimented GUVs to fresh external solution in further steps also enabled the removal of the excess of non-encapsulated lucigenin fluorophore.
Figure 1. Schematic of the targeted delivery of lipophilic transporter 3 by membrane fusion. GUVs (70% POPC and 30% cholesterol) encapsulating 0.8 mM lucigenin fluorophore are incubated with lipopeptide 1 to functionalize the lipid membrane of the GUV. Subsequent formation of a dimeric coiled-coil allows the fusion of liposomes containing 10 mol % transporter 3 and the complementary lipopeptide 2, resulting in targeted delivery of 3 to the membrane of the GUV. Finally, upon the addition of NaCl to the exterior solution, transporter 3 exchanges external chloride for internal nitrate, resulting in the quenching of the encapsulated lucigenin fluorophore.
Liposome-GUV membrane fusion was initiated by treating peptide 1-functionalized GUVs (~20 µm diameter) containing the chloride-sensitive lucigenin fluorophore with peptide 2-decorated liposomes (~150 nm diameter) containing 3 pre-incorporated in the lipid membrane (Figure 1). Briefly, CP4K4 membrane-functionalized GUVs (200 µL) and CP4E4 membrane-functionalized liposomes (100 µL) were combined in 700 µL of 225 mM NaNO3, 10 mM TRIS and 200 mM glucose solution. After gentle mixing for 15 minutes using a rotary shaker, the mixture was incubated for 105 minutes more at room temperature to allow fusion of liposomes with GUVs and concomitant delivery of transporter 3 to the lipid bilayer of GUVs. Finally, 200 µL of the sedimented sucrose-containing GUVs were taken from the bottom of the micro centrifuge tube and transferred to a chamber on the stage of a confocal microscope with 100 µL of 225 mM NaNO3, 10 mM TRIS and 200 mM glucose. The integrity of GUVs following membrane fusion was verified by confocal fluorescence (excitation at 488 nm) and bright field imaging.

To test for delivery of transporter 3 to the GUV membranes, the chloride-permeability of the GUVs was assayed through lucigenin fluorescence. NaCl (25 µL, 1 M solution) was added with a microsyringe to the microscope chamber containing the GUVs, and the lucigenin emission intensity was observed to decay markedly over a period of ~3 minutes (Figure 2, blue triangles). The quenching of lucigenin fluorescence after delivery of transporter 3 (76% after 3 minutes) was significantly stronger than the effect of photobleaching (9% after 3 minutes; Figure 2, black squares). This result agrees well with previous experiments where transporter 3 was pre-incorporated into the lipid bilayer of GUVs for direct visualization of chloride transport into GUVs.29 Thus, membrane fusion efficiently delivered transporter 3 to the membrane of peptide-functionalized GUVs.

As a control, plain GUVs without CP4K4 were mixed with CP4E4-functionalized liposomes containing 10 mol % of transporter 3. After the addition of the NaCl solution, the lucigenin emission intensity inside the GUVs did not decrease (Figure 2, red circles), proving that transporter 3 was not delivered to the GUV membrane. Instead, there was a small increase in the fluorescence intensity. We attribute this increase to the difference in osmotic pressure between the inside and the outside of the GUVs following NaCl addition. This results in a decrease in the diameter of the GUVs (as detected by
bright field microscopy) and hence, an increase in lucigenin concentration. We conclude that omission of the lipopeptide CP₄K₄ from the membrane of GUVs inhibits the delivery of transporter 3 to GUVs. Thus, membrane fusion induced by the lipopeptides 1 and 2 is required for the targeted delivery of transporter 3 to the membrane of GUVs.

**Figure 2.** Averaged normalized lucigenin emission intensity after the addition of NaCl (t = 40 s) to: CP₄K₄-functionalized GUVs treated with CP₄E₄ liposomes containing the transporter 3 (blue triangles); plain GUVs treated with CP₄E₄ liposomes containing the transporter 3 (red circles); CP₄K₄-functionalized GUVs treated with CP₄E₄ liposomes without transporter 3 (green diamonds). The background photobleaching of CP₄K₄-decorated GUVs (no NaCl added) is shown as black squares. The normalized fluorescence traces plotted are the averages of three independent membrane fusion experiments on three different individual GUVs. Data are means ± SEM. For individual experiments, see Figures A1 – A4, Annex Chapter VI. The arrow indicates the addition of NaCl after 40 seconds of time lapse imaging.
In a second control experiment, we omitted transporter 3 from the CP₄E₄ liposomes. After membrane fusion, we added the NaCl solution and monitored the lucigenin emission intensity. Again the fluorescence of GUVs increased (Figure 2, green diamonds), presumably due to GUV shrinkage. This result suggests that the fusion of peptide-decorated liposomes and GUVs neither makes the lipid membrane permeable to chloride ions nor induces leakage of the encapsulated lucigenin fluorophore from GUVs. We conclude that coiled-coil driven membrane fusion is a specific and highly efficient system to deliver anionophores to GUVs.

To determine whether the lipopeptides 1 and 2 can also be used to deliver the lipophilic transporter 3 to live cells, we used cells engineered to express a halide-sensitive fluorophore. We selected for this study Fischer Rat Thyroid (FRT) cells expressing the halide-sensitive fluorophore yellow fluorescent protein (YFP) variant H148Q/I152L, which is highly sensitive for iodide vs. chloride (hereafter termed YFP-FRT cells); FRT cells are a model system used to investigate epithelial ion transport. We demonstrated recently that YFP-FRT cells can be used to study chloride/iodide exchange by anionophores, by monitoring iodide-induced fluorescence quenching. Herein, we use our membrane fusion system for the targeted delivery of transporter 3 to the plasma membrane of YFP-FRT cells. Using the same protocol as that presented in Figure 1 to deliver 3 to GUVs, the plasma membrane of YFP-FRT cells was functionalized with CP₄K₄ by incubating the cells for 2 hours at 37 °C with the CP₄K₄ lipopeptide 1 in phosphate-buffered saline (PBS), followed by the addition and incubation with CP₄E₄ liposomes containing 10 mol % anion transporter 3 for 1 h at 37 °C. The YFP-FRT cells were then transferred to a perfusion chamber mounted on the stage of a fluorescence microscope and perfused with PBS.

After several minutes the PBS flow was changed to a PBS solution containing NaI (10 mM) for 5 minutes. This change of the external buffer led to a rapid and robust quenching of cell fluorescence (Figure 8 in the Experimental Section). This decrease in cell fluorescence was almost completely reversed when NaI was washed from the extracellular solution with fresh PBS, indicating the efficient and reversible exchange of chloride and iodide by anionophore 3. Repeating the exposure to NaI after an interval of 20 minutes elicited a further rapid quenching of cellular fluorescence followed by a recovery after washing NaI from the extracellular solution once more with PBS. We
performed two control experiments by treating the YFP-FRT cells with either plain POPC liposomes containing transporter 3, the delivery method used in our previous work,\textsuperscript{19} or with the targeted delivery system without 3 (Figure 3).

**Figure 3.** Targeted delivery of the anion transporter 3 to the plasma membrane of YFP-FRT cells by membrane fusion.  A) Representative time courses of normalized cell fluorescence and B) Anion transport activity determined from the initial slope of the fluorescence decay for the indicated experimental conditions.  Dashed lines in A indicate the fit of exponential functions to the first two minutes of the fluorescence decay following NaI (10 mM) addition.  Data are means ± SEM ($n = 25 – 45$ cells from 5 independent experiments); **, $P < 0.01$ vs. lipopeptides 1 and 2.
The magnitude of the fluorescence decay in both control experiments was significantly smaller than that elicited by the use of the membrane fusion lipopeptides 1 and 2 for the delivery of anionophore 3. The result of the first control experiment is in agreement with the inability of 3 to be exchanged between membranes without membrane fusion (Figure 4 in Experimental Section). The data are also consistent with the control experiments performed using liposomes and GUVs (Figure 2). Thus, the highly lipophilic anion transporter 3 can be successfully delivered to CP4K4-functionalized YFP-FRT cells via coiled–coil-driven membrane fusion, where it efficiently transports anions across the plasma membrane of YFP-FRT cells.

Conclusion

In conclusion, we demonstrate that the lipidated coiled-coil forming peptides 1 and 2 function as a highly specific molecular recognition system that facilitates membrane fusion. This synthetic model system can be applied as a fast and efficient tool in drug delivery studies. We use a supramolecular approach to solve the deliverability problem of a lipophilic anionophore, with powerful anion transport activity by leakage-free membrane fusion between cell-sized GUVs and liposomes. Similar results were observed using cells engineered to express a halide-sensitive fluorophore. We envisage the delivery of the fusogenic lipopeptides and anionophore by inhalation method, firstly by delivering peptide 1 and subsequently peptide 2 and anionophore 3. This raises the hope that the system can be used to deliver anionophores to the apical membrane of airway epithelia, the key target tissue in cystic fibrosis. There is also potential for extending the method to deliver other poorly soluble molecules to biological membranes.
Experimental Section

Materials

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, 28 (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) were a generous gift of A. S. Verkman (University of California, San Francisco). YFP-FRT cells were cultured as described previously 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$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$; pH 7.40.


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### 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$_4$K$_4$ (1) and CP$_4$E$_4$ (2) were synthesized and utilized following procedures previously reported. 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. A lipid solution (10 µL) with the lipid composition 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$_4$K$_4$ 1 (relative to lipids). A stock solution of CP$_4$K$_4$ 1 (15 µL, 50 µM in CH$_3$OH: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
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micro centrifuge tube containing the CP₄K₄ 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 NaNO₃ (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 % CP₄E₄ 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 CHCl₃) was mixed with CP₄E₄ stock solution (20 µL for GUVs or 100 µL for cells, 50 µM in CH₃OH:CHCl₃ 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 NaNO₃ (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. CP₄E₄ decorated LUVs (200 µL) and CP₄K₄ decorated GUVs (200 µL) were transferred into a micro centrifuge tube with the buffer solution (600 µL) containing NaNO₃ (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 NaNO₃ (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 NaNO₃ 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, 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 \((F_0)\).

*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 CP₄K₄ stock solution (50 µM in CH₃OH:CHCl₃ 1:1) was dried by evaporating the solvent under a gentle stream of air and subsequently placed in high vacuum for 1 hour. The CP₄K₄ 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 CP₄K₄ 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/CP₄E₄ 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/CP₄E₄ 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). For this second control experiment, the cells were not treated with CP₄K₄, 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. 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 (F₀). 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).
**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 N₂ 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 NaNO₃, 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 NaNO₃. The liposomes were collected and diluted with 225 mM NaNO₃ 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 N₂ 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 NaNO₃, 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 NaNO₃ 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 NaNO₃) 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 ($F_0$).

Figure 4 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 4 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.
Figure 4. 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.
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**DLS of LUVs and CP4E4 peptide decorated LUVs with 10 mol % transporter.**

**Figure 5.** Dynamic Light Scattering of LUVs without CP$_4$E$_4$ and transporter 3.

**Figure 6.** Dynamic Light Scattering of CP4E4-decorated LUVs with 10 mol % transporter 3.
Cryo-TEM of 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 CP₄E₄-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.

Figure 7. Representative cryo-transmission electron micrographs of CP₄E₄-decorated LUVs A) without transporter and B) with 10 mol % transporter 3. The scale bars are 200 nm.
Time lapse and frames from an experiment showing anion transport in YFP-FRT cells upon targeted delivery of transporter 3.

**Figure 8.** 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.
References


Annex

Chapter VI

Datasets for the average curves presented in Figure 2 of the Chapter VI.
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Photobleaching

Figure A1. 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.

CP₄E₄-decorated LUVs and CP₄K₄-decorated GUVs, 10 mol % transporter 3 is included in LUVs.

Figure A2. 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.
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Control experiment: CP₄E₄-decorated LUVs and CP₄K₄-decorated GUVs, transporter 3 is excluded from LUVs.

Figure A3. 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: CP₄E₄-decorated LUVs and plain GUVs, CP₄K₄ is excluded from GUVs.

Figure A4. Fluorescence and bright-field microscopy images before (top) and after (bottom) addition of NaCl to GUVs previously incubated with LUVs (10 mol % transporter). CP₄K₄ is excluded from GUVs. The normalized fluorescence intensity over time is given for individual GUVs from independent experiments.