

# Oxyanion Transport across Lipid Bilayers: Direct Measurements in Large and Giant Unilamellar Vesicles

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## 1. Procedures

### 1.1. Materials and instruments

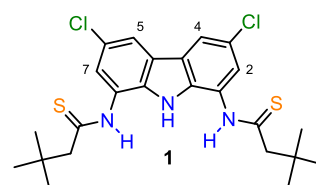
10,10'-Dimethyl-9,9'-biacridinium nitrate (lucigenin) was obtained from TCI. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and rhodamine B labeled lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt were purchased from Avanti Polar Lipids. Anions were purchased from Sigma Aldrich as sodium salts or as corresponding acids. Standard solution of sodium hydroxide (5 M), cholesterol, and chloroform (for HPLC, stabilized with amylene) were purchased from Sigma Aldrich. All reagents for the synthesis of anionophore **1** were obtained from Sigma-Aldrich or Acros Organics (Lawesson reagent) and were used as received. Water was taken from Mili-Q purification system.

Fluorescence spectra were recorded on Hitachi F-7000 spectrophotometer equipped with Peltier temperature controller. Imaging was performed on a Leica TCS SPE confocal microscope system. NMR spectra were recorded using Agilent 400 MHz NMR spectrometer. The residual signal of DMSO- $d_6$  solvent was used as an internal reference standard ( $\delta_H = 2.500$  ppm).

### 1.2. Synthesis

#### 1.2.1. Di(3,3-dimethylthiobutyrylamino)-3,6-dichlorocarbazole

A 50 ml two-neck round-bottom flask was dried in a stream of hot air from heat gun and left to cool down *in vacuo*. 1,8-Bis(trimethylacetyl-amino)-3,6-dichlorocarbazole<sup>1</sup> (0.116 g, 0.25 mmol), Lawesson reagent (0.223 g, 0.55 mmol) and a magnetic stirrer were added to the flask, which was then equipped with a rubber septum and a reflux condenser connected to a vacuum gas manifold. The flask was evacuated and filled with argon. Next,



20 ml of anhydrous DCE was added. The condenser was connected to a check-valve bubbler and the reaction was left under reflux for 72 h. After this time, the reaction mixture was cooled down to room temperature and THF (5 ml) was added to obtain a clear solution. Silica gel (2 g) was added and the solvent was removed on a rotary evaporator and an oil pump. The product was purified by a column chromatography using 40 g RediSep normal phase columns and CombiFlash system.  $CHCl_3/AcOEt$  was used as an eluent, with ethyl acetate content varying from 0 to 5%. Fractions containing the pure product were combined and evaporated to yield 0.086 g (69.2%) of a light-yellow solid.

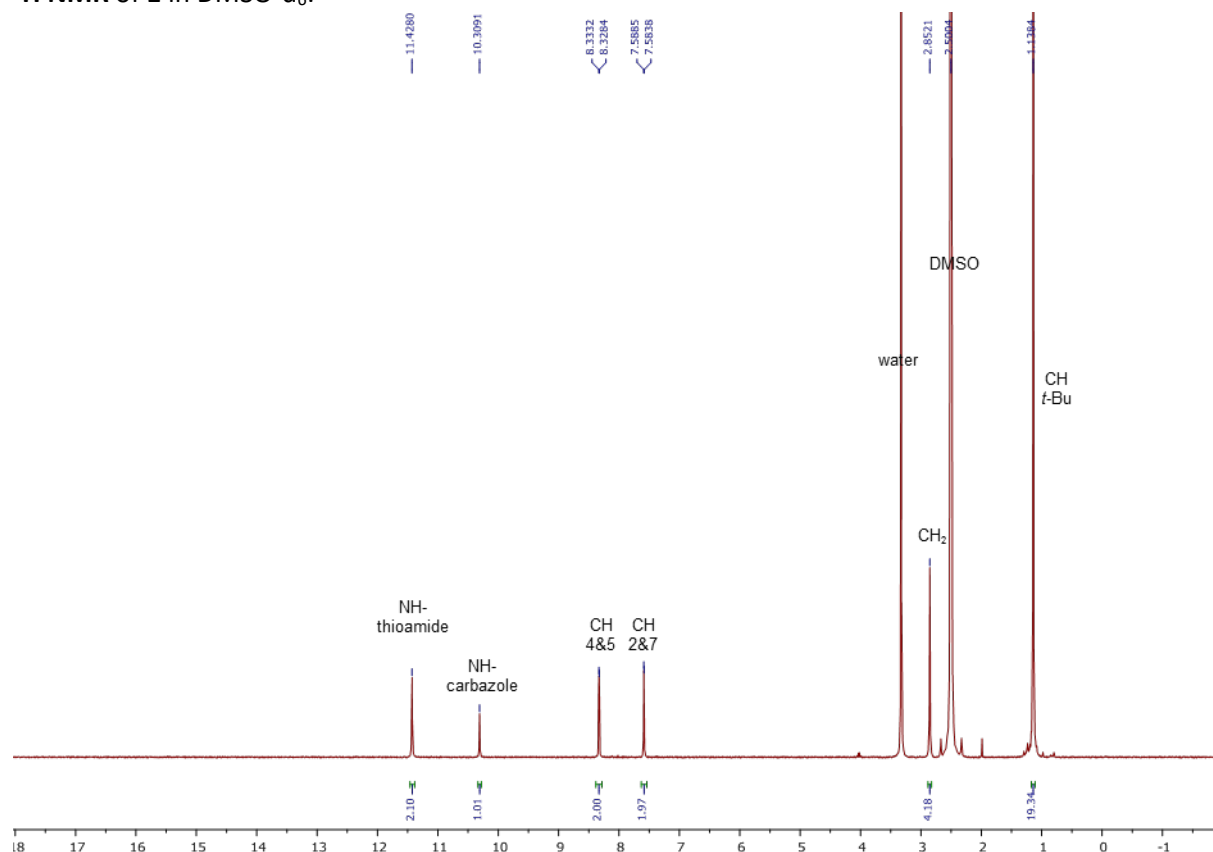
<sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta_{DMSO}$ : 11.43 (s, 2H, thioamide NH); 10.31 (s, 1H, carbazole NH); 8.33 (d,  $J = 1.9$  Hz, 2H, aromatic H-4 and H-5); 7.59 (d,  $J = 1.9$  Hz, 2H, aromatic H-2 and H-7); 2.85 (s, 4H,  $CH_2$ ); 1.14 (s, 18H, *t*-Bu);

<sup>13</sup>C NMR (DMSO- $d_6$ )  $\delta_{DMSO}$ : 203.50; 133.43; 125.04; 124.75; 123.89; 123.17; 119.18; 59.12; 33.02; 29.77;

HR-MS (ESI):  $m/z$  calcd. for  $C_{24}H_{28}Cl_2N_3S_2$  [M]<sup>+</sup>: 492.1102; found: 492.1113;

Elemental Analysis calcd. for  $C_{24}H_{28}Cl_2N_3S_2$ : C 58.29; H 5.91; N 8.50; found C 58.37; H 6.06; N 8.25.

$^1\text{H}$  NMR of **1** in  $\text{DMSO-d}_6$ :



### 1.2.2. Preparation of aspirin sodium salt

Aspirin sodium salt<sup>2</sup> was prepared from acetylsalicylic acid. Acetylsalicylic acid (5.000 g, 0.0277 mol) and water (2 ml, Milli-Q) were mixed in a 100 ml round-bottom flask. Subsequently, solid sodium bicarbonate (2.330 g, 0.0277 mol) was added and the mixture was left for 24 h until the formation of  $\text{CO}_2$  bubbles ceased. Next, isopropanol was added, and the mixture was left at  $5^\circ\text{C}$  for several hours resulting in the formation of white needle-shaped crystals. These were filtered, washed with isopropanol and dried under high vacuum over KOH in a desiccator. During this time the white crystalline needles turned into white powder.  $^1\text{H}$  NMR shows no traces of hydrolysis and powder x-ray diffraction (PXRD) confirms the formation of anhydrous salt. Yield: 1.805 g (8.93 mmol, 32.1%). The aspirin sodium salt was stored over KOH and remained stable in the solid form. In aqueous solution it slowly decomposes: after 24 h 5% of salicylate was present in solution, as inferred from  $^1\text{H}$  NMR spectra.

### 1.3. Lucigenin quenching

To an aqueous solution of lucigenin (2 mL, 0.8 mM) placed in a septum-sealed, screw-capped SUPRASIL Quartz fluorescence cuvette (light path: 10 mm) aliquots of an appropriate salt (0.8 M) dissolved in the aqueous solution of lucigenin (0.8 mM) were added with a gas-tight microsyringe. Fluorescence spectrum was recorded after each addition (excitation wavelength: 455 nm, scan speed: 1200 nm/min, temperature:  $25^\circ\text{C}$ ).

In cases where sodium salts were not commercially available (amino acids, organic phosphates), their solutions were prepared as follows: to a 1 ml volumetric flask 1 mmol of acid was weighed out. Then, stoichiometric amount of aqueous solution of NaOH (5 M) was added (200  $\mu\text{L}$ ) with a gas-tight syringe. Next, the volumetric flask was filled with water to 1 ml to obtain 1 M solution of

sodium salt (solution **A**). In a separate vial, aqueous solution of lucigenin was prepared (4 mM, solution **B**). Solution **A** (800  $\mu$ l) and **B** (200  $\mu$ l) were mixed together to obtain the titrant solution (0.8 M of salt, 0.8 mM of lucigenin).

The addition of aliquots of the hydroxide solution to the lucigenin solution (0.8 mM) during measurements was followed by the addition of the same aliquots of lucigenin solution (1.6 mM) to avoid dilution of lucigenin.

Titration curves were determined by measuring the emission at 505 nm. The obtained signal was normalized against the initial intensity.

#### **1.4. Anion transport studies using GUVs**

The general procedure is a modified version of a previously published protocol.<sup>3</sup>

A lipid solution (POPC and cholesterol in 7:3 molar ratio, total lipid concentration: 14 mM) was prepared in chloroform, which was previously deacidified by passing through a pad of activated basic alumina. Anionophore **1** (3.5 mM, dissolved in  $\text{CHCl}_3$ :MeOH 7:3 v/v) was added to the lipid solution in 0 – 0.5 mol% ratio, relative to the total lipid amount. Next rhodamine B labeled lipid (0.1 mol% with respect to the total amount of lipids) was added.

Giant unilamellar vesicles (GUVs) were grown on Dex-PEG (1:1 molar ratio) coated microscope glass slides as described previously.<sup>4</sup> A lipid solution (10  $\mu$ L) was deposited on a hydrogel coated glass slide and dried under a gentle stream of nitrogen. A glass O-ring (OD = 15 mm) was placed on top of the hydrogel and sealed with a high vacuum silicon grease. The lipid film was rehydrated by adding 400  $\mu$ L of an aqueous solution containing lucigenin (0.8 mM),  $\text{NaNO}_3$  (225 mM) and sucrose (200 mM) dissolved in Milli-Q water. The chamber was covered with a glass slide and protected from light. GUVs were grown for at least 3 hours at room temperature. Next, free floating GUVs were transferred into an Eppendorf tube containing 700  $\mu$ L of  $\text{NaNO}_3$  (225 mM) and glucose (200 mM) and left for ~1.5h. Next, 300  $\mu$ L of the lower part of this solution was transferred to another Eppendorf tube containing 700  $\mu$ L of  $\text{NaNO}_3$  (225 mM) and glucose (200 mM) and left again for 1.5 h. Due to these dilutions the concentration of the unencapsulated lucigenin was reduced to ca. 10% of its initial value. GUVs were transferred into the microscopy visualization chamber (225  $\mu$ L) and left for 1 h before measurements to allow sedimentation of GUVs.

Solutions of anions (1 M) were prepared by dissolving commercially available ( $\text{NaCl}$ ,  $\text{PhCOONa}$ ,  $\text{NaHCO}_3$ ) or freshly prepared (AspirinNa) salts in a 1 ml volumetric flasks or by mixing commercially available acids (diphenylphosphate, phenylalanine) with standard solution of  $\text{NaOH}$  under the control of a pH-meter.

During time lapse imaging of the GUVs, 25  $\mu$ L of the aqueous solution of an appropriate anion (1 M) was added with a microsyringe to the visualization chamber, resulting in a final anion concentration of 0.1 M. Quenching of unencapsulated lucigenin fluorescence marked anion transport across the membrane.

The 488 nm laser line (30% laser power) was used for scanning the fluorescence of lucigenin (emission: 500-550 nm, green channel) and the 532 nm laser line (30% laser power) for scanning the fluorescence of lissamine rhodamine (emission: 550-650 nm, red channel). Image analysis was performed using ImageJ software, by measuring the average intensity of an area corresponding to one GUV for the series of time lapsed confocal image frames (time interval between images: 4.037 s).

### 1.5. Anion transport studies using LUVs

The general procedure is a modified version of a previously published protocol.<sup>5</sup>

In a 5 mL round bottom flask a solution of lipids (POPC and cholesterol in a 7:3 molar ratio, total lipid concentration: 10 mM) was prepared in chloroform which was previously deacidified by passing through a pad of activated basic alumina. Anionophore **1** (0.3 mM or 3 mM in CHCl<sub>3</sub>:MeOH 7:3 v/v) was added to the lipid solution at 0 – 0.5 mol% ratio relative to the total amount of lipids. The organic solvents were evaporated using a membrane vacuum pump and the residue was dried under high vacuum for 1 h. The lipid film was hydrated with 500 µL of an aqueous solution containing lucigenin (0.8 mM) and NaNO<sub>3</sub> (225 mM), sonicated for 30 s and stirred for 1 h resulting in vesicle formation. Next, the mixture was subjected to 10 freeze-thaw cycles, diluted to 1 mL by the addition of 0.5 mL a NaNO<sub>3</sub> (225 mM) solution and extruded (29 times) through a polycarbonate membrane (200 nm pore size). Unencapsulated lucigenin was removed by passing the solution through a size exclusion column (ca. 2 g Sephadex 50G, superfine) using an aqueous solution of NaNO<sub>3</sub> (225 mM) as eluent. The collected vesicles were diluted with NaNO<sub>3</sub> solution (225 mM) to 15 ml (lipid concentration ≈ 0.4 mM). 3 mL of the vesicle solution was placed in a quartz cuvette with a small stirring bar and the fluorescence (excitation: 455 nm, emission: 505 nm) was measured as a function of time. An aqueous sodium salt solution (75 µL, 1 M) was added after 60 seconds and the fluorescence intensity was measured for 14 minutes.

Solutions of anions (1 M) were prepared by dissolving commercially available (NaCl, PhCOONa, NaHCO<sub>3</sub>) or synthesized (AspirinNa) salts in a 1 ml volumetric flasks. Diphenylphosphate solution was prepared by mixing commercially available acid with standard solution of NaOH under the control of pH-meter.

In case of benzoate, aspirin and diphenylphosphate, significant transport of the anions took place in the first 2 seconds during which anions were not fully diffused. This hampers the selection of the starting point and affects values of relative fluorescence. Therefore, the first 2 seconds after anion addition were discarded from the measurement.

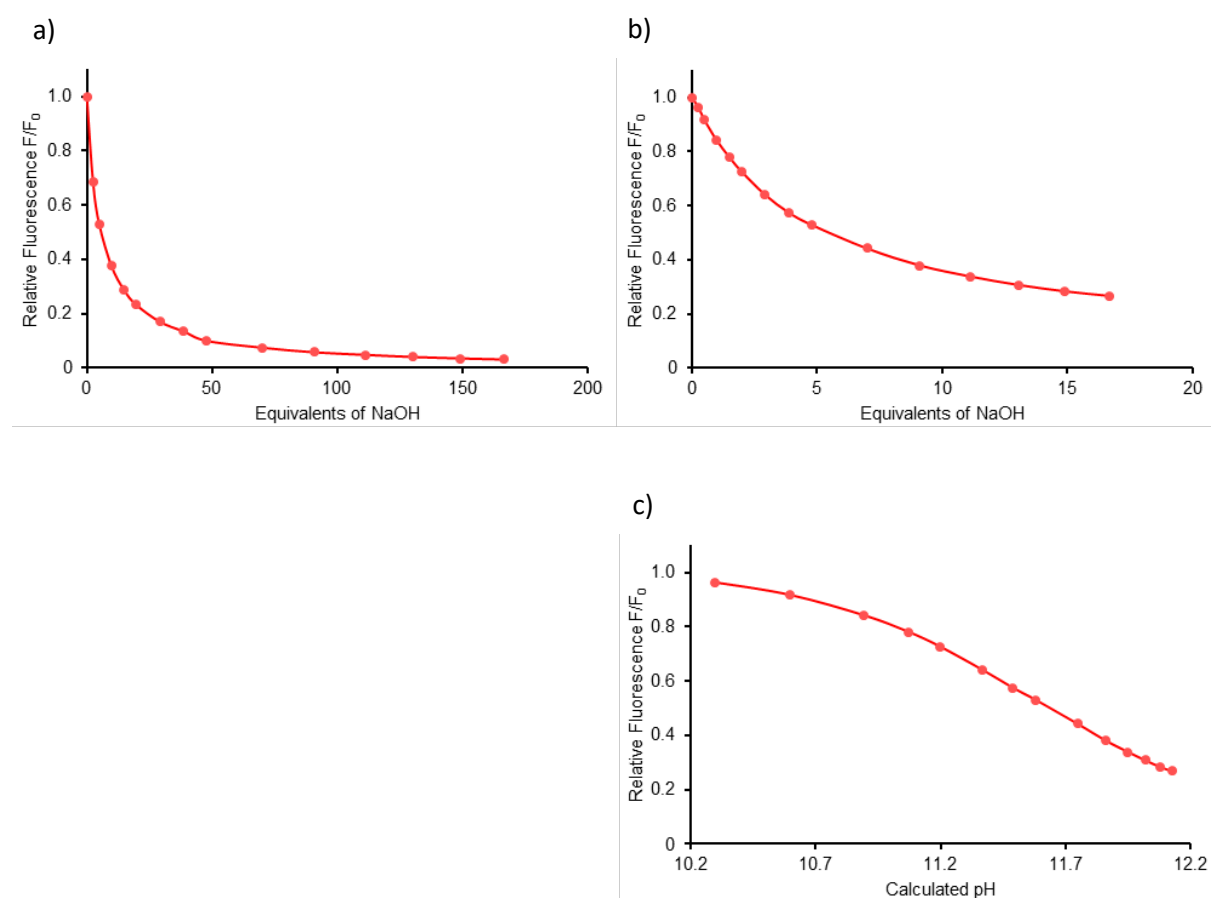
### 1.6. Cryogenic transmission electron microscopy

Liposomes (3µL, 10 mM total lipid concentration) were applied to a freshly glow-discharged carbon 200 mesh Cu grid (Lacey carbon film, Electron Microscopy Sciences, Aurion, Wageningen, The Netherlands). Grids were blotted for 3 s at 99% humidity in a Vitrobot plunge-freezer (FEI Vitrobot<sup>TM</sup> Mark III, Thermo Fisher Scientific). Cryo-EM images were collected on a Talos L120C (NeCEN, Leiden University) operating at 120 kV. Images were recorded manually at a nominal magnification of 13500x or 22000x yielding a pixel size at the specimen of 7.44 or 4.56 Å, respectively.

## 2. Experimental data

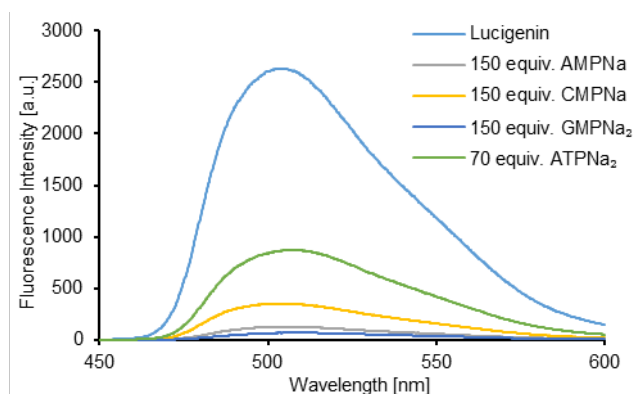
### 2.1. Lucigenin quenching by $\text{OH}^-$ and its influence on transport experiments

Anion transport studies with many oxyanions require high pH to suppress spontaneous diffusion of their protonated forms through lipid bilayers. This increases the probability of concomitant  $\text{OH}^-$  transport and raises concerns about its contribution to the observed fluorescence quenching. Fortunately, lucigenin is poorly quenched at  $\text{pH} < 11$  (see below), although it becomes unstable when the pH exceeds 11.<sup>6</sup> To exclude its influence on the assay, NaOH was added to a suspension of GUVs containing **1** to adjust the external pH to 11 ( $C_{\text{OH}^-} = 1 \text{ mM}$ ). No change in the fluorescence of GUVs was observed over 10 min. Since the pH in our experiments was never higher than 11, we concluded that lucigenin fluorescence quenching inside the vesicles caused by  $\text{OH}^-$  transport is negligible.



**Figure S2.1** Fluorescence titration curves of an aqueous lucigenin solution (0.8 mM) with NaOH at various concentrations: 0.8 mM (a), 0.08 mM (b, c). Chart (c) was obtained by replacing a scale of chart (b) with calculated pH. Excitation wavelength: 455 nm, emission wavelength: 505 nm.

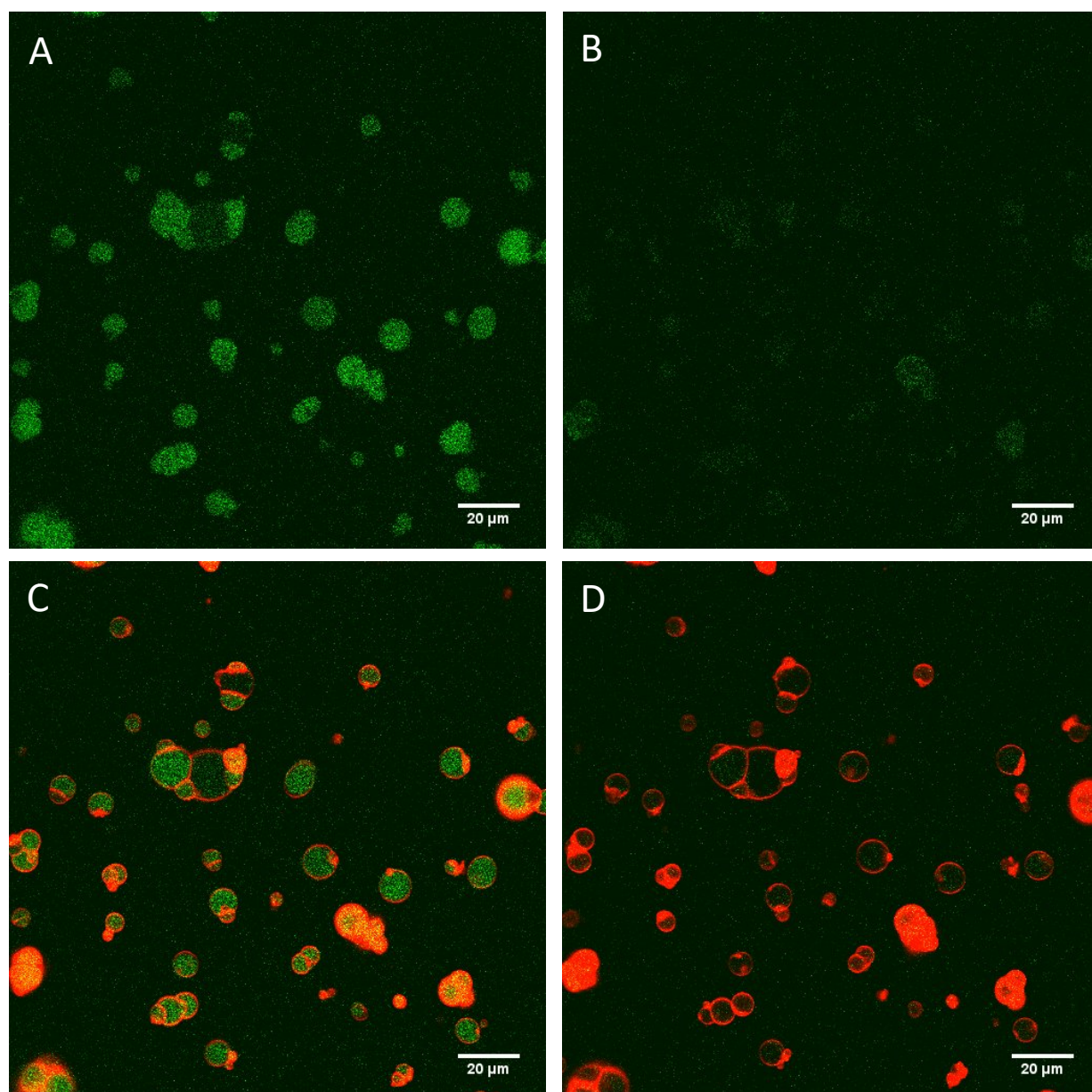
## 2.2. Lucigenin quenching by organic phosphates



**Figure S2.2** Fluorescence spectra of an aqueous lucigenin solution (0.8 mM) in the presence of various organic phosphates. Excitation wavelength: 368 nm.

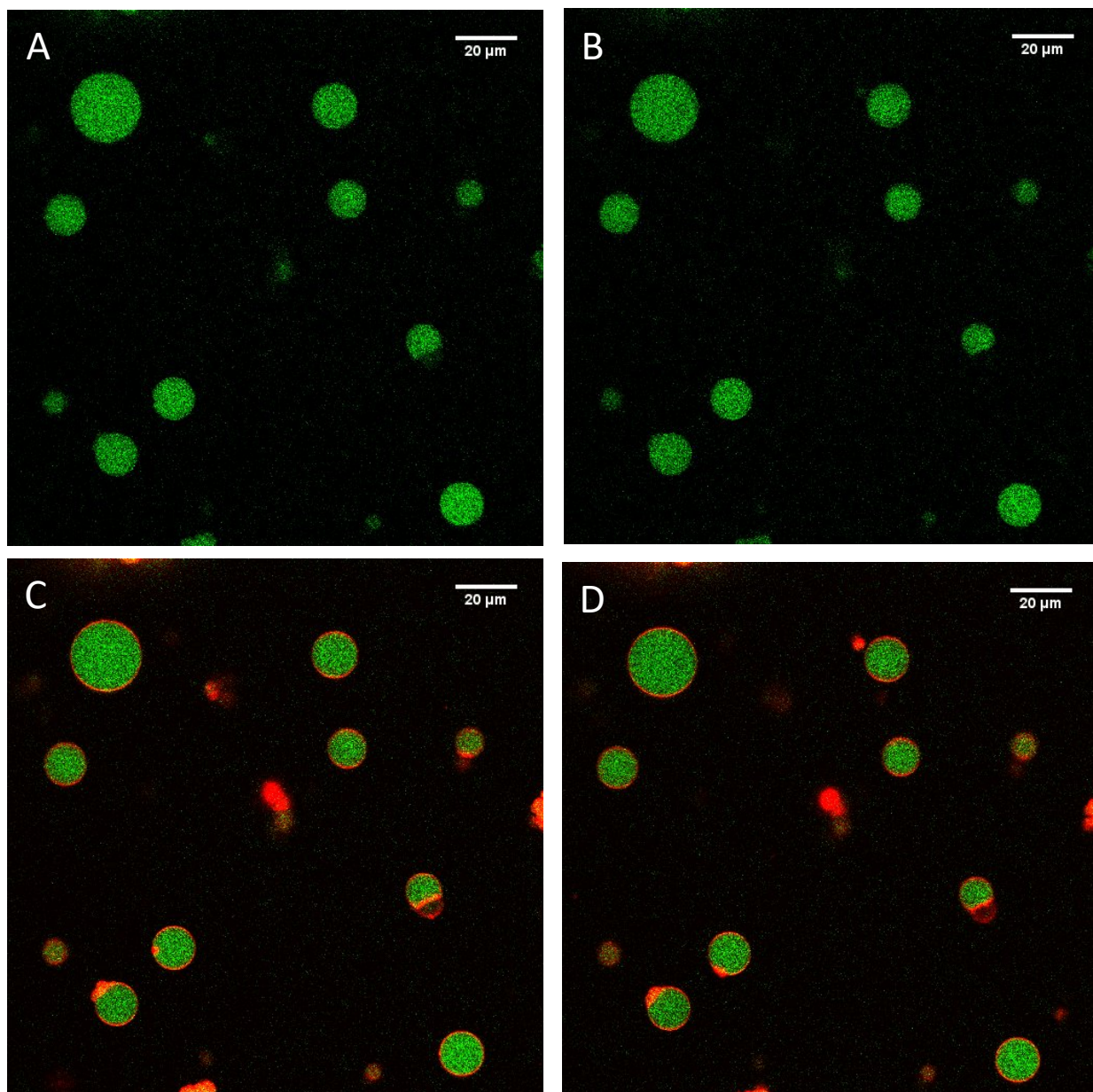
## 2.3. Anion transport using GUVs

### 2.3.1. Chloride transport.

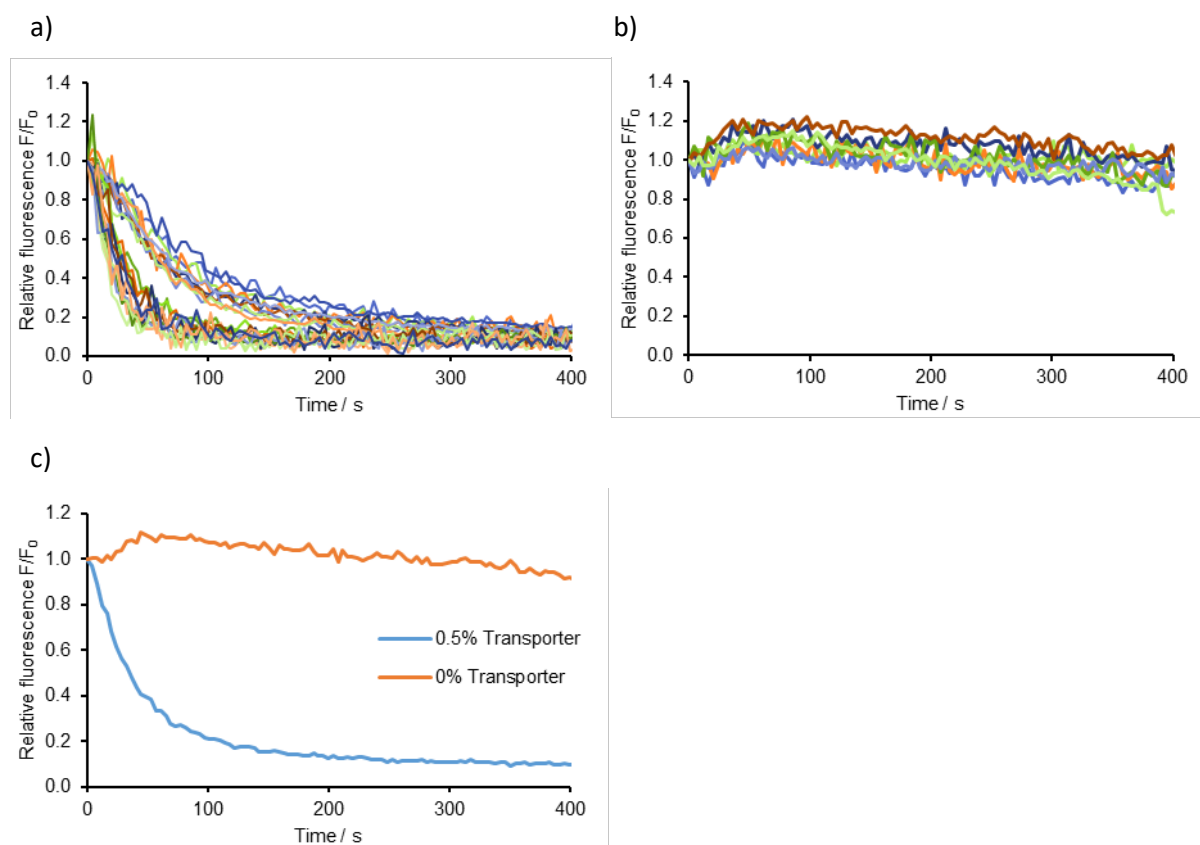


**Figure S2.3** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of NaCl (final concentration 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



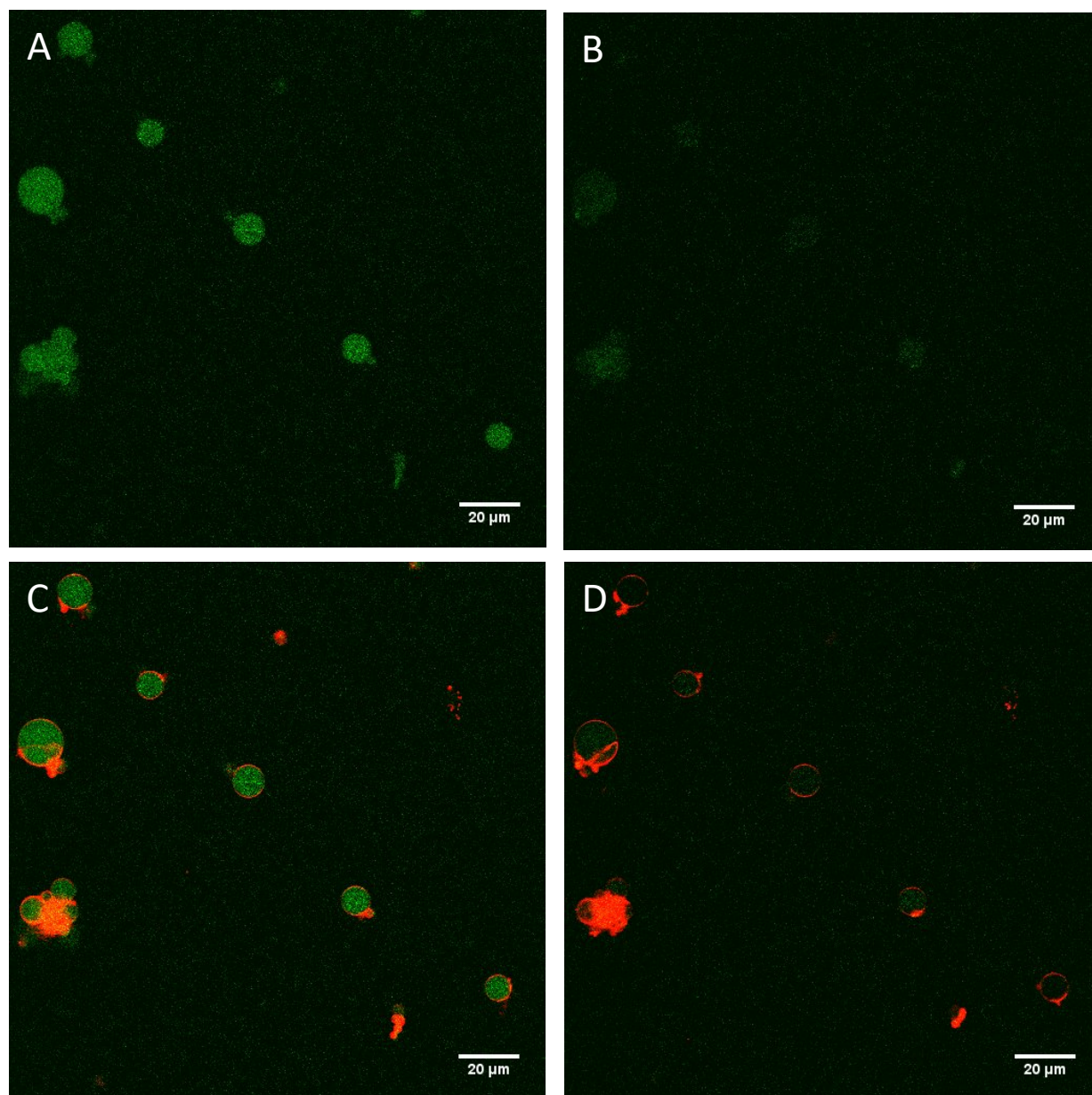


**Figure S2.4** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of NaCl (final concentration 0.1M) to GUVs containing no transporter in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



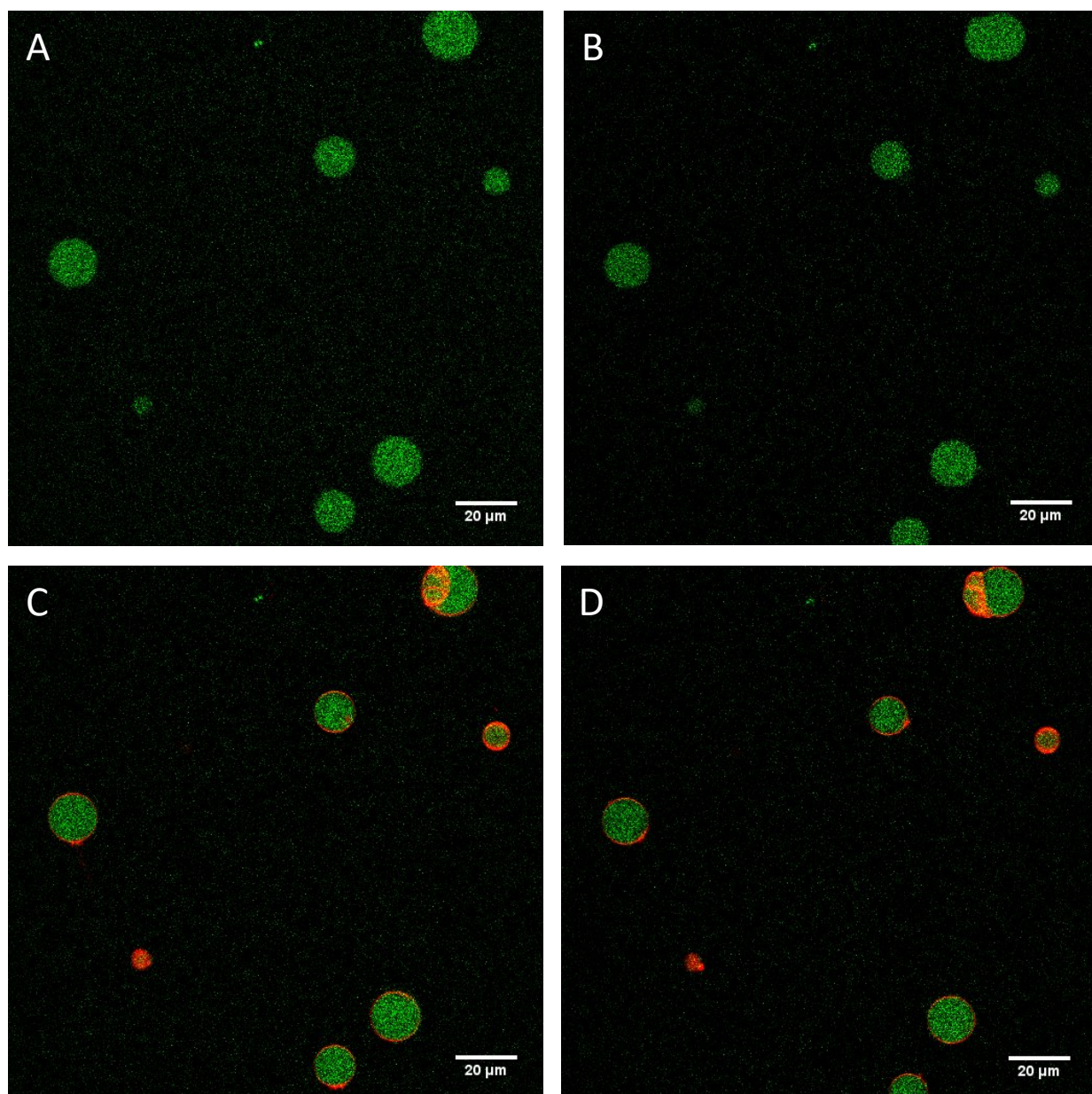
**Figure S2.5** Normalized fluorescence intensity profiles over time for individual GUVs in the presence of 0.5% transporter (a) and in the absence of transporter (b) after addition of NaCl (final concentration 0.1 M). (c) Averaged values of GUVs containing transporter and in the absence of transporter.

### 2.3.2. Benzoate transport.

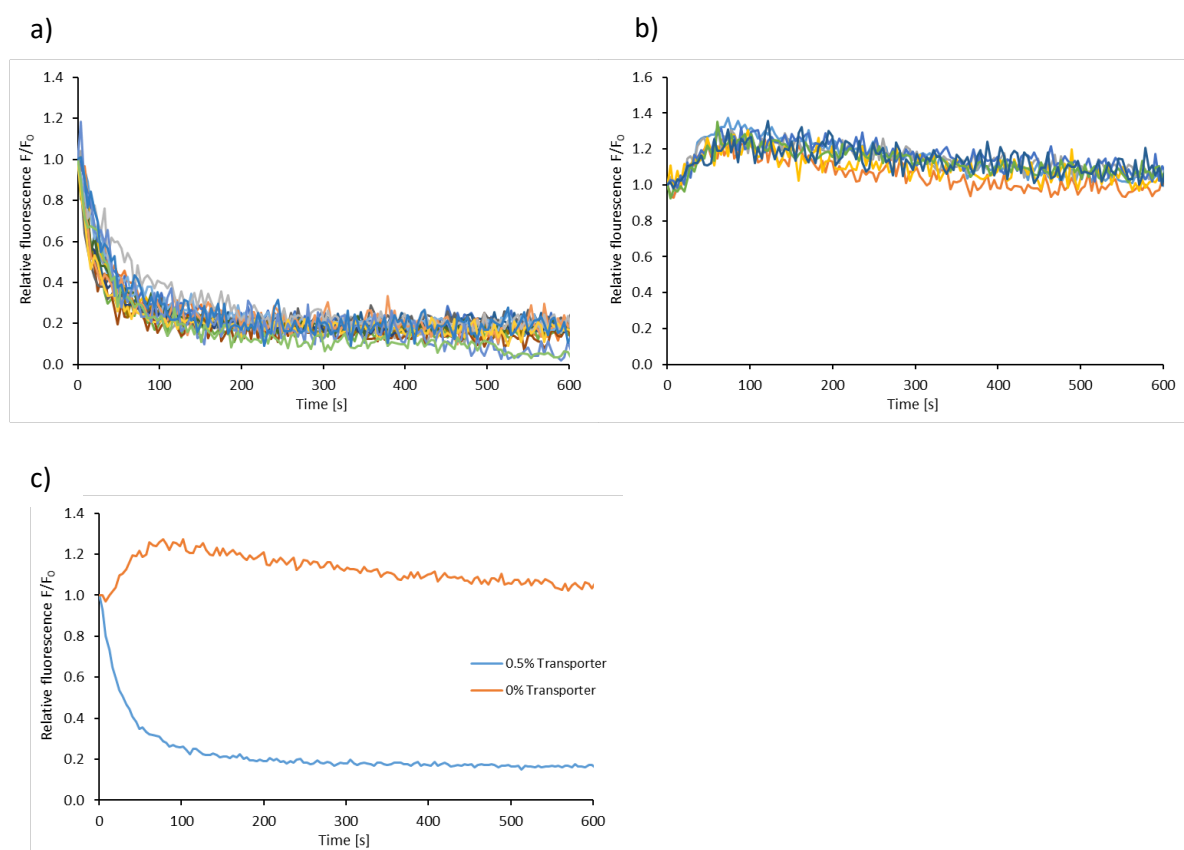


**Figure S2.6** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of PhCOONa (final concentration of 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.





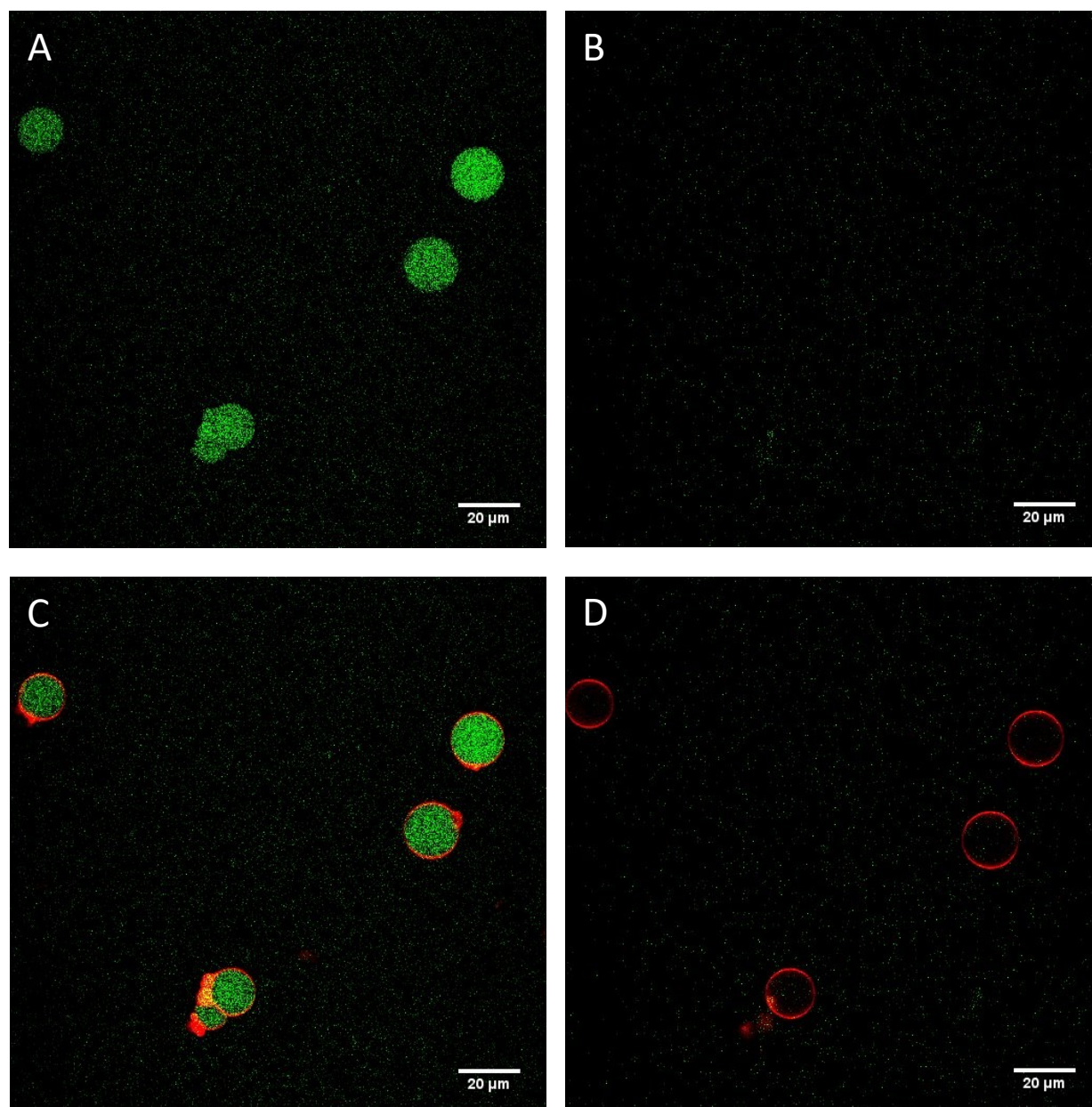
**Figure S2.7** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of PhCOONa (final concentration of 0.1M) to GUVs with no transporter incorporated in membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



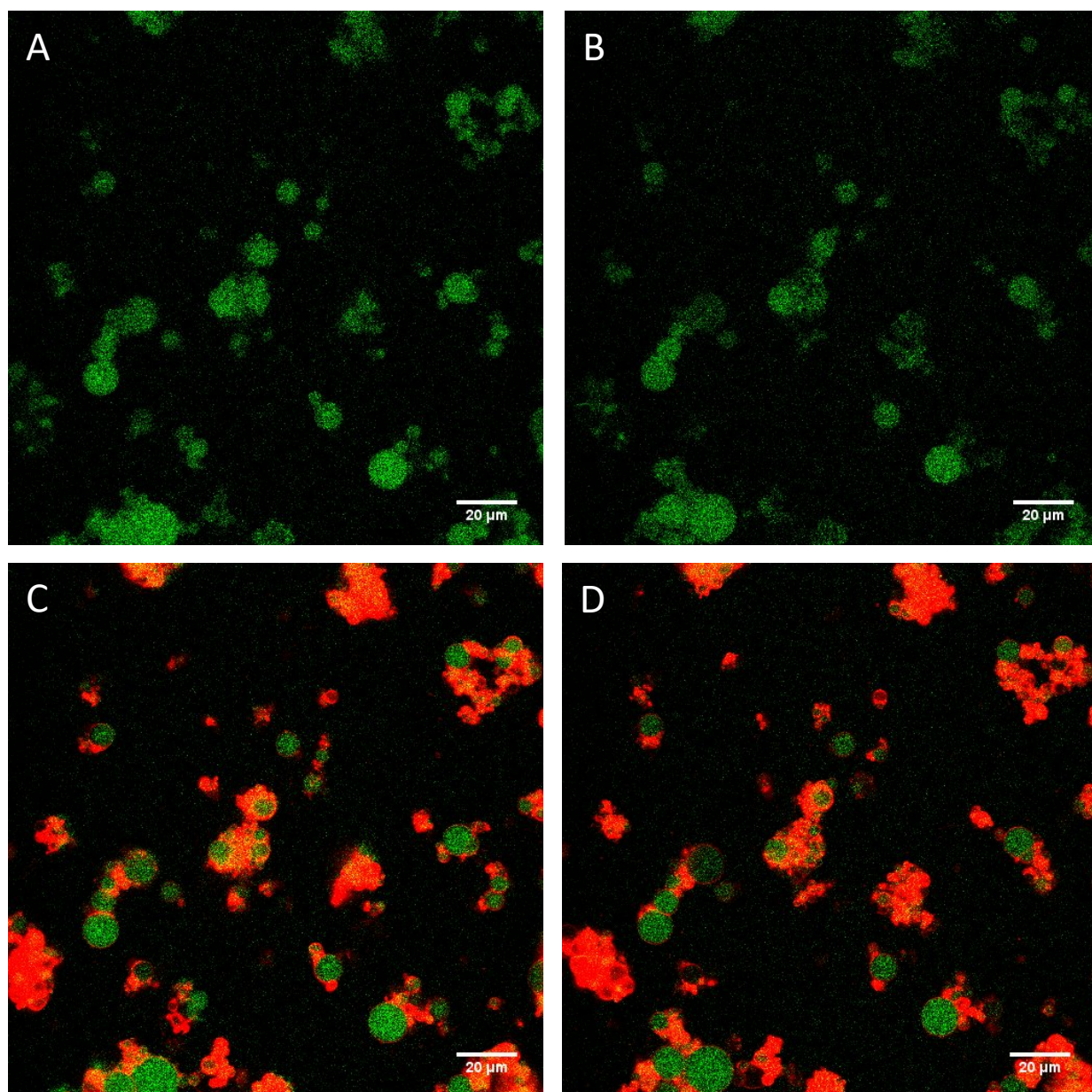
**Figure S2.8** Normalized fluorescence intensity profiles over time for individual GUVs in the presence of 0.5% transporter (a) and in the absence of transporter (b) after addition of PhCOONa (final concentration 0.1 M). (c) Averaged values of GUVs containing transporter and in the absence of transporter.



### 2.3.3. Bicarbonate $\text{HCO}_3^-$ transport.



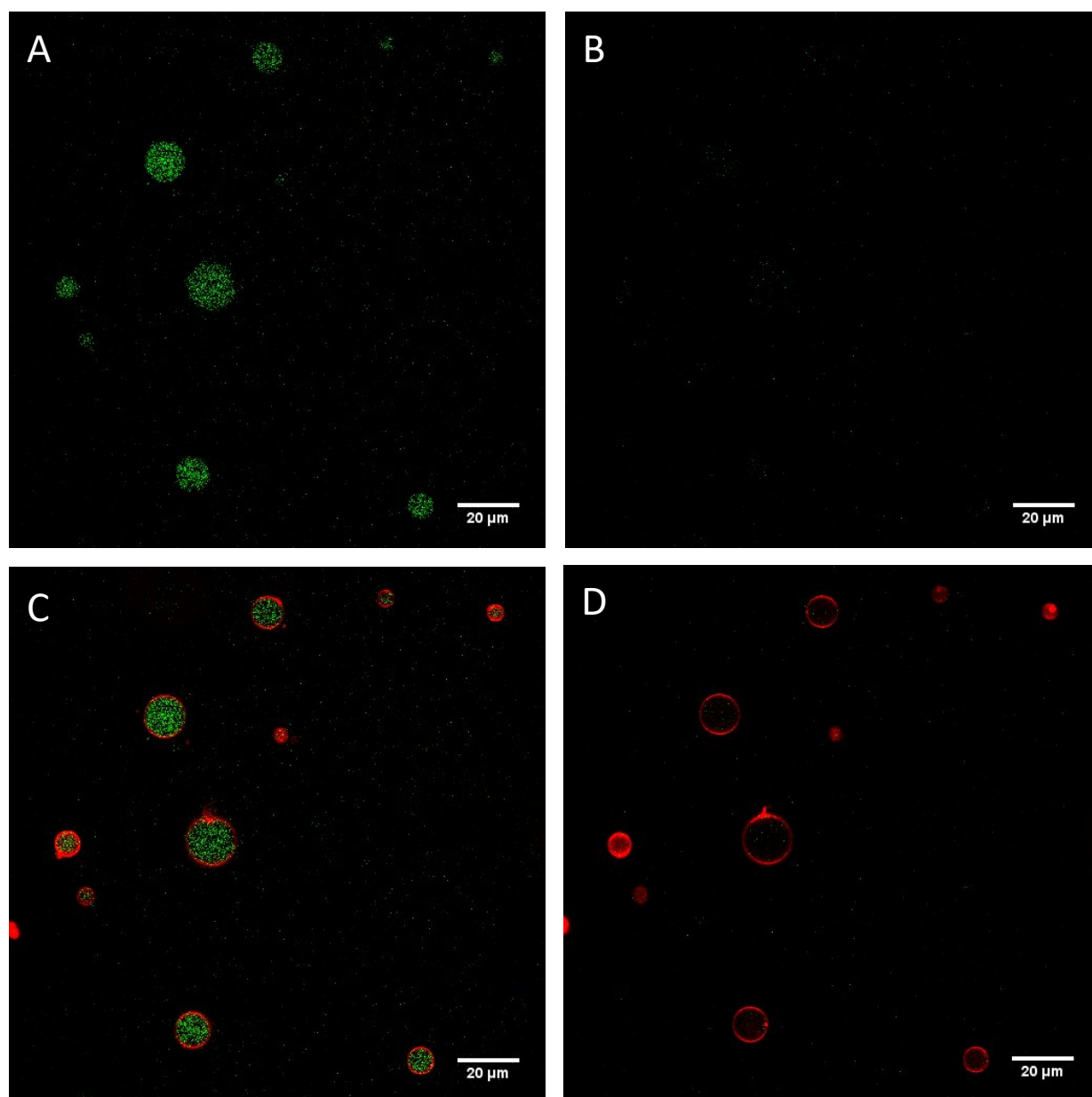
**Figure S2.9** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of  $\text{NaHCO}_3$  (final concentration of 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to that the lipid membranes are intact.



**Figure S2.10** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of NaHCO<sub>3</sub> (final concentration of 0.1M) to GUVs with no transporter incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to that the lipid membranes are intact.

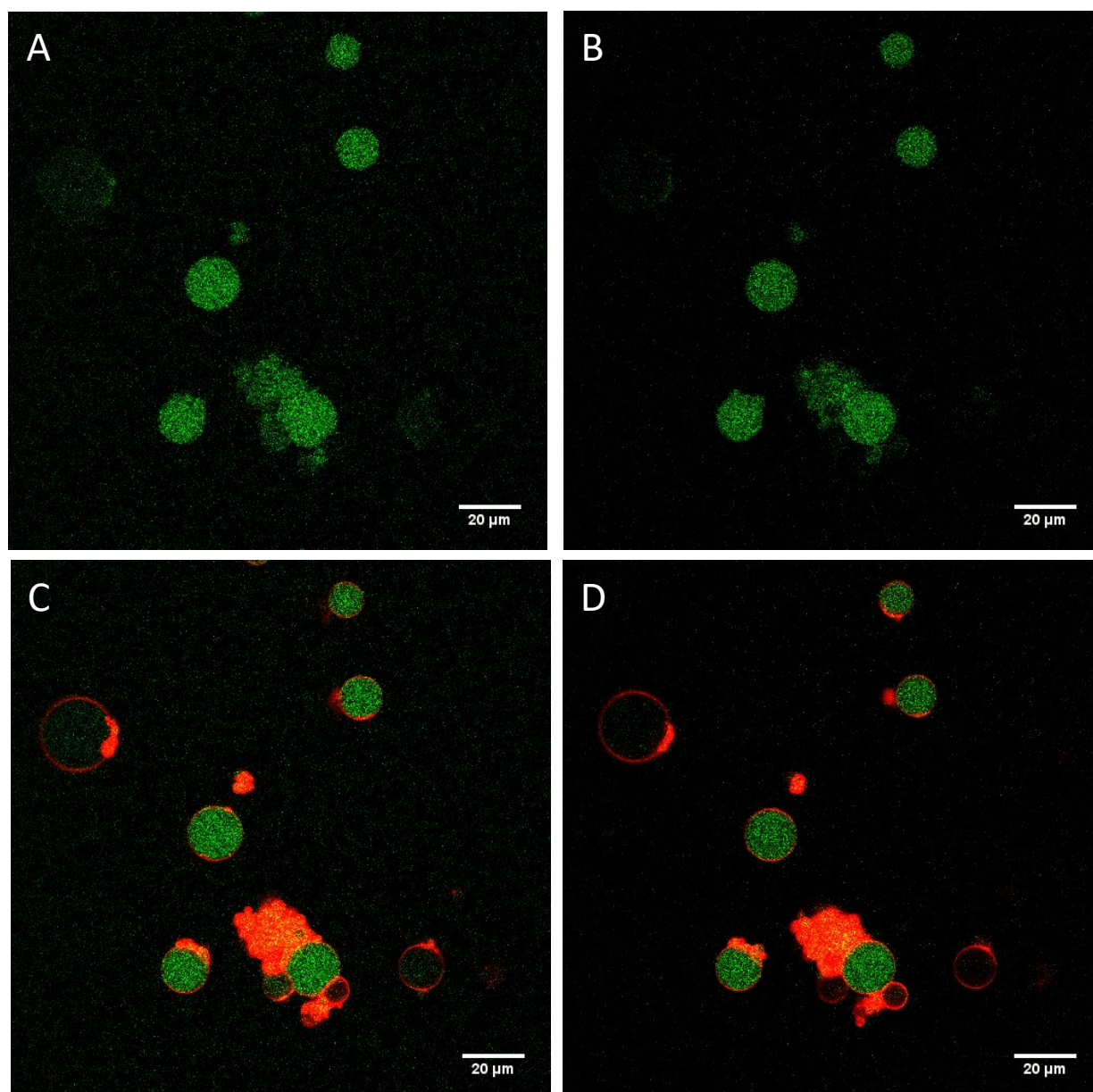


#### 2.3.4. Aspirin (sodium salt) transport.

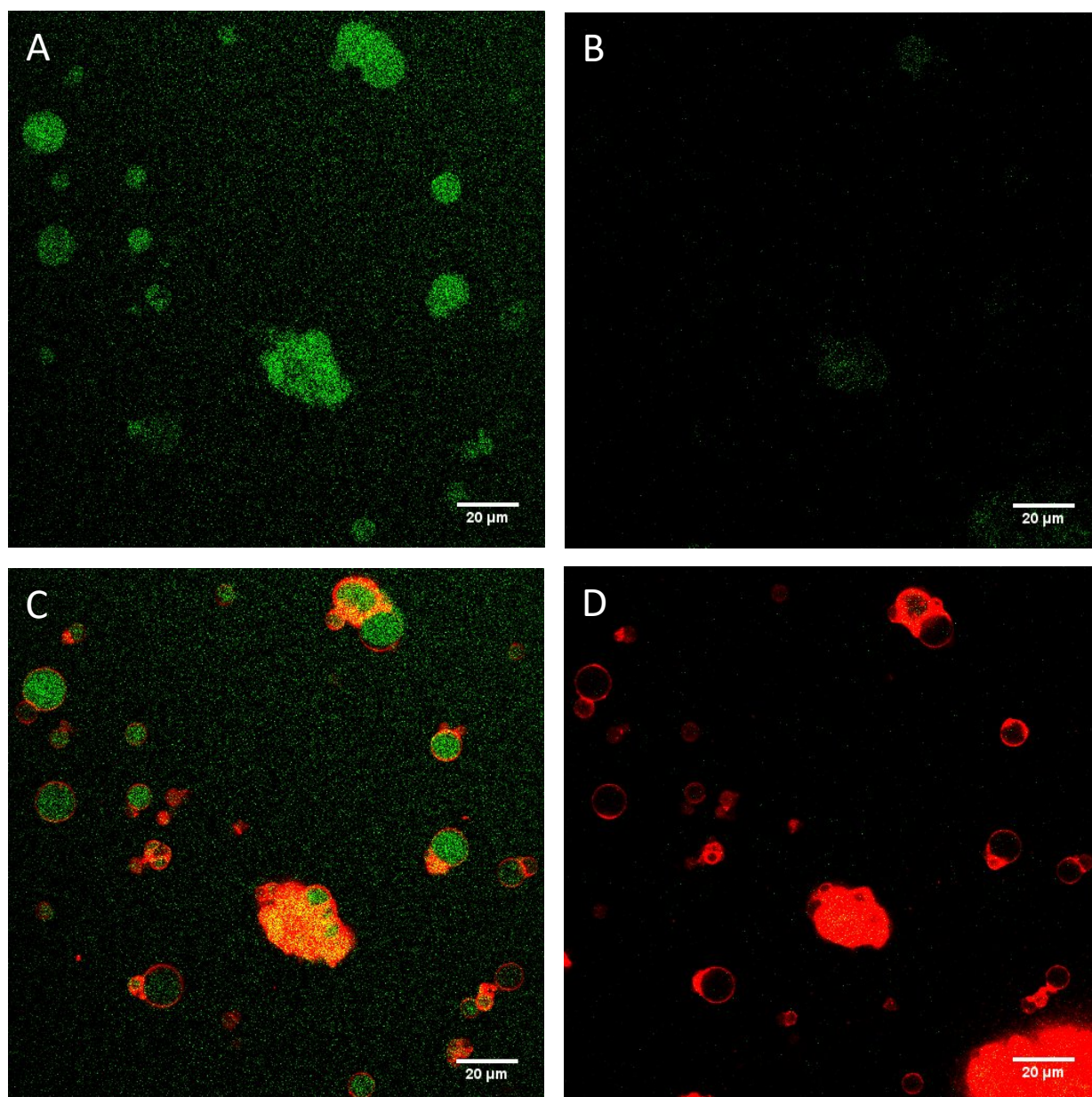


**Figure S2.11** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of sodium salt of aspirin (final concentration of 0.05M) to GUVs containing 0.25% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.

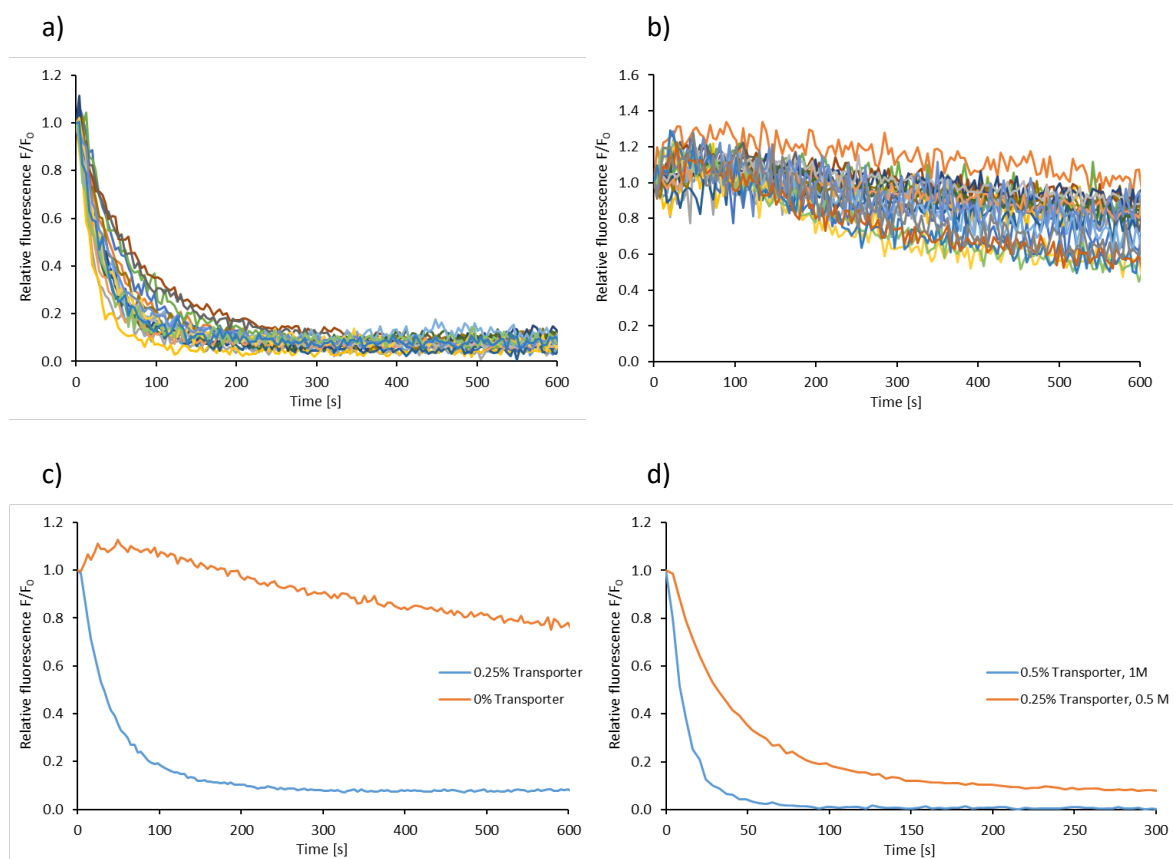




**Figure S2.12** Fluorescence microscopy images before (a, c) and after (b, d) addition of sodium salt of aspirin (final concentration of 0.05M) to GUVs with no transporter incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



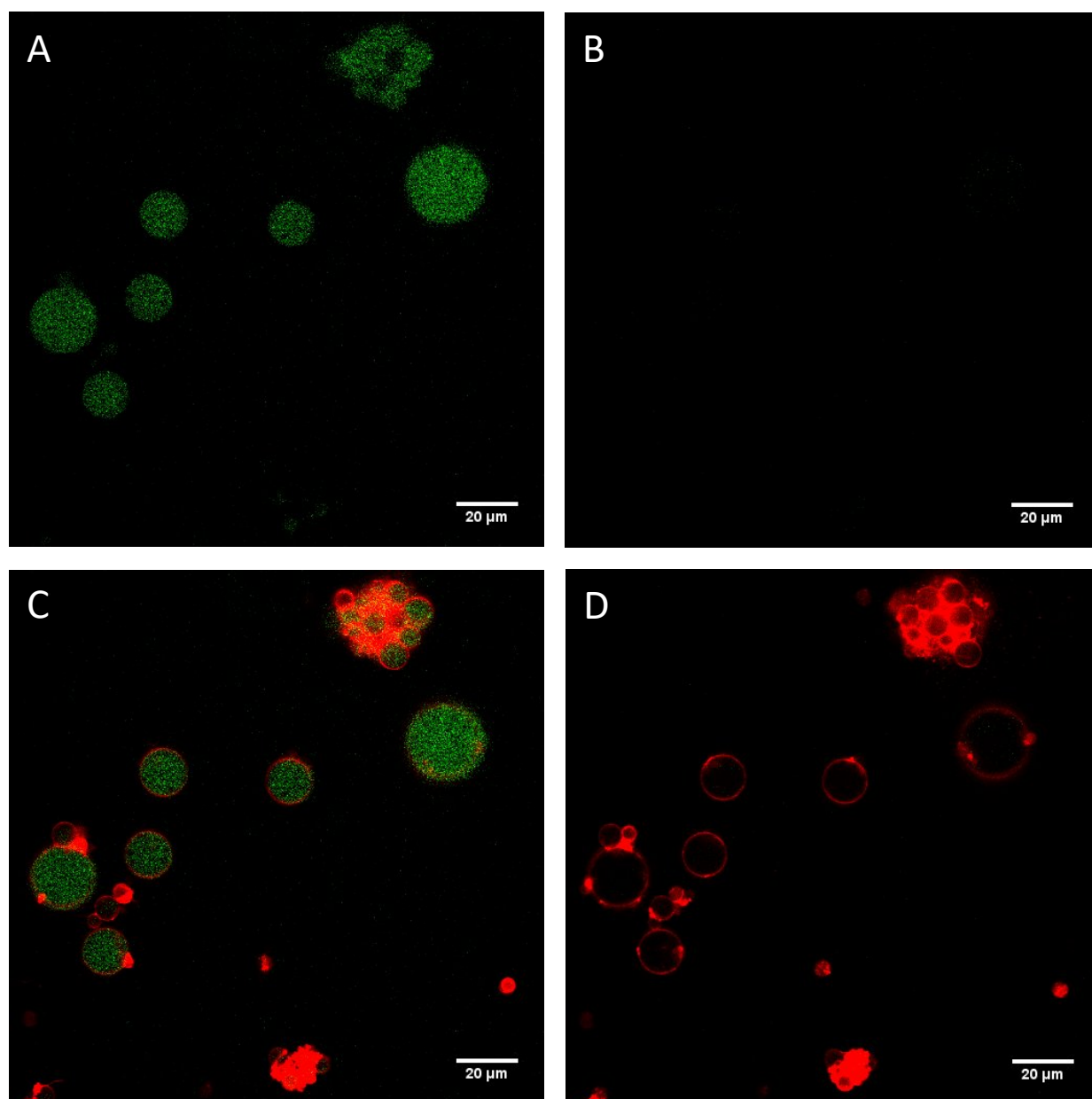
**Figure S2.13** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of sodium salt of aspirin (final concentration of 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



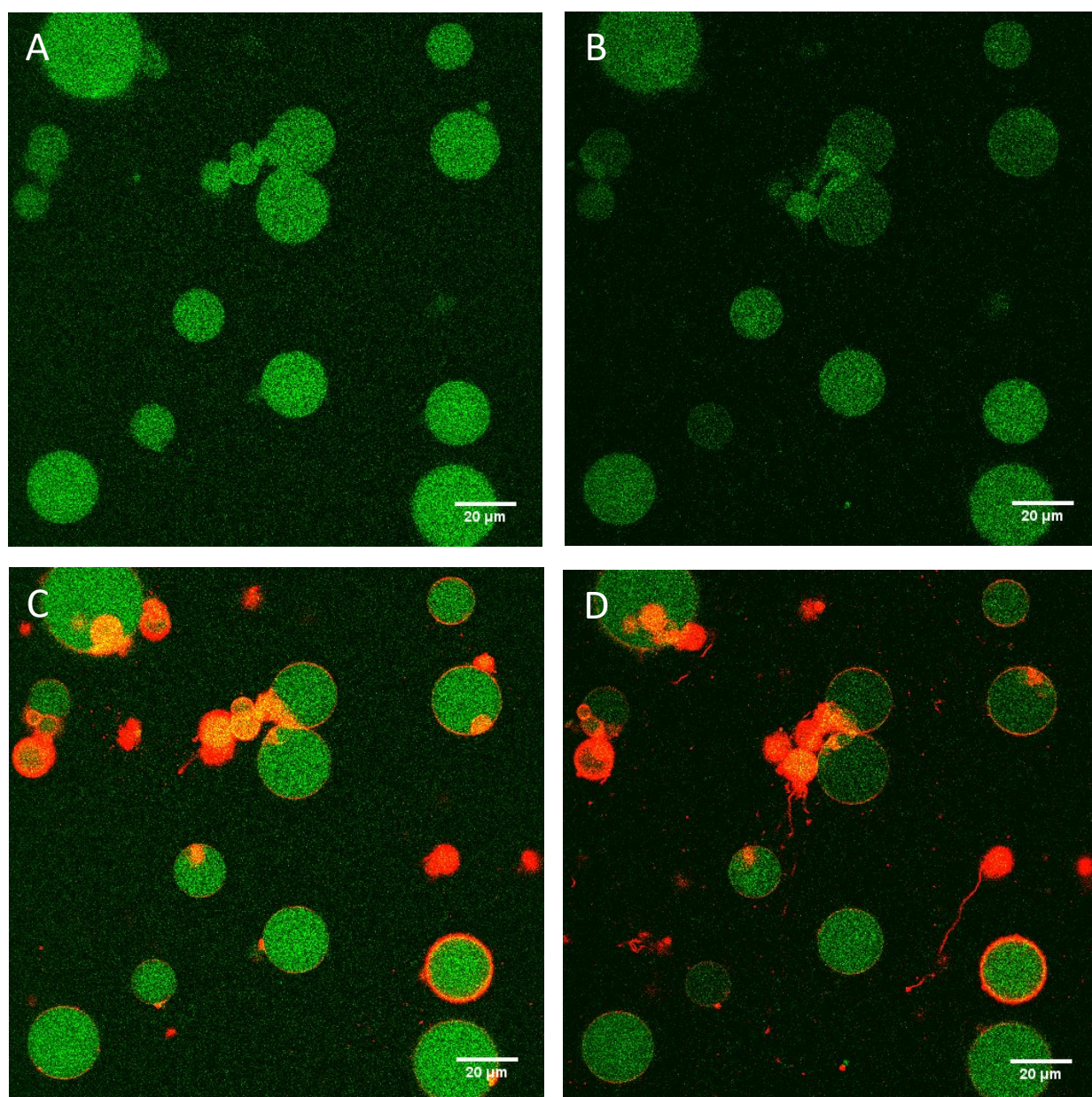
**Figure S2.14** Normalized fluorescence intensity profiles over time for individual GUVs in the presence of 0.25% transporter (a) and in the absence of transporter (b) after addition of sodium salt of aspirin (final concentration 0.1 M). (c) Averaged values of GUVs containing 0.25% transporter and in the absence of transporter. (d) Averaged values of the normalized fluorescence intensity profiles over time for GUVs with 0.25% transporter and 0.5% transporter after addition of sodium salt of aspirin.



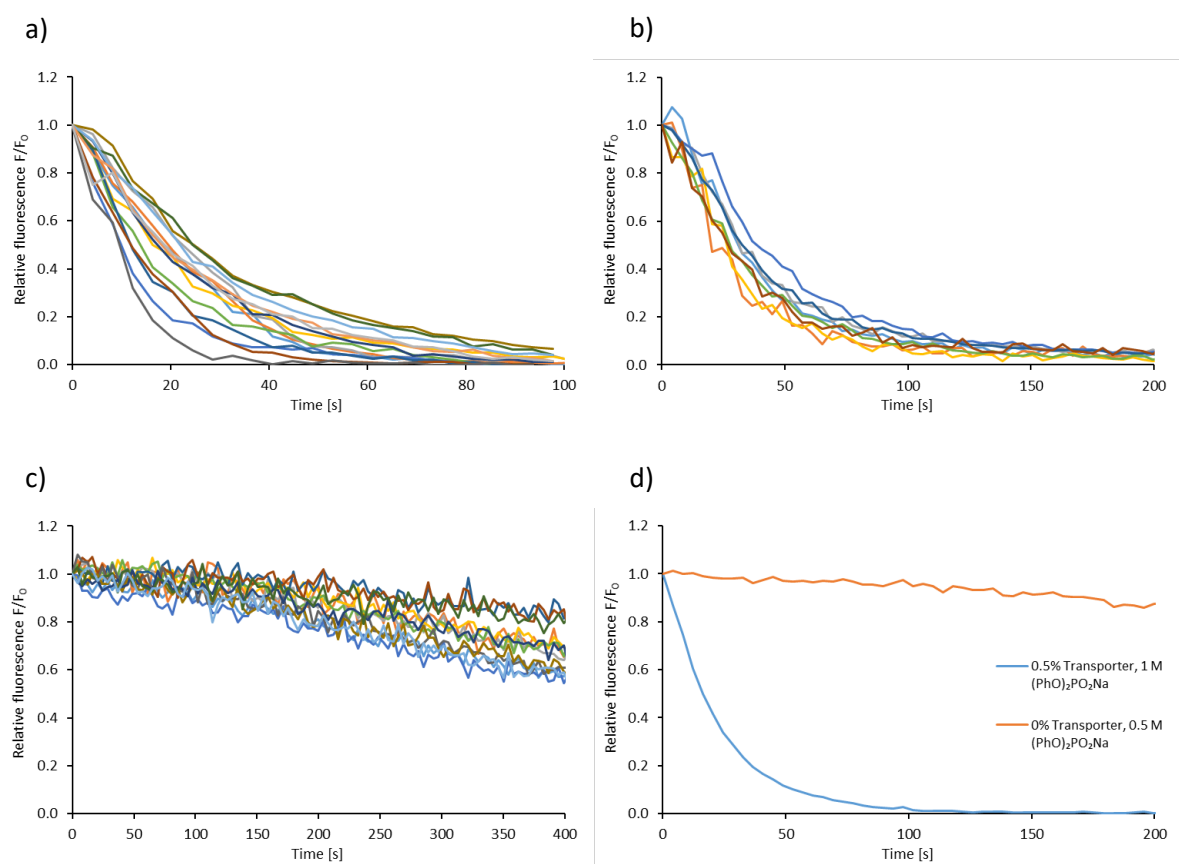
### 2.3.5. Diphenylphosphate $(\text{PhO})_2\text{PO}_2^-$ transport.



**Figure S2.15** Fluorescence microscopy images before (a, c) and 2 minutes after (b, d) addition of  $(\text{PhO})_2\text{PO}_2\text{Na}$  (final concentration of 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.

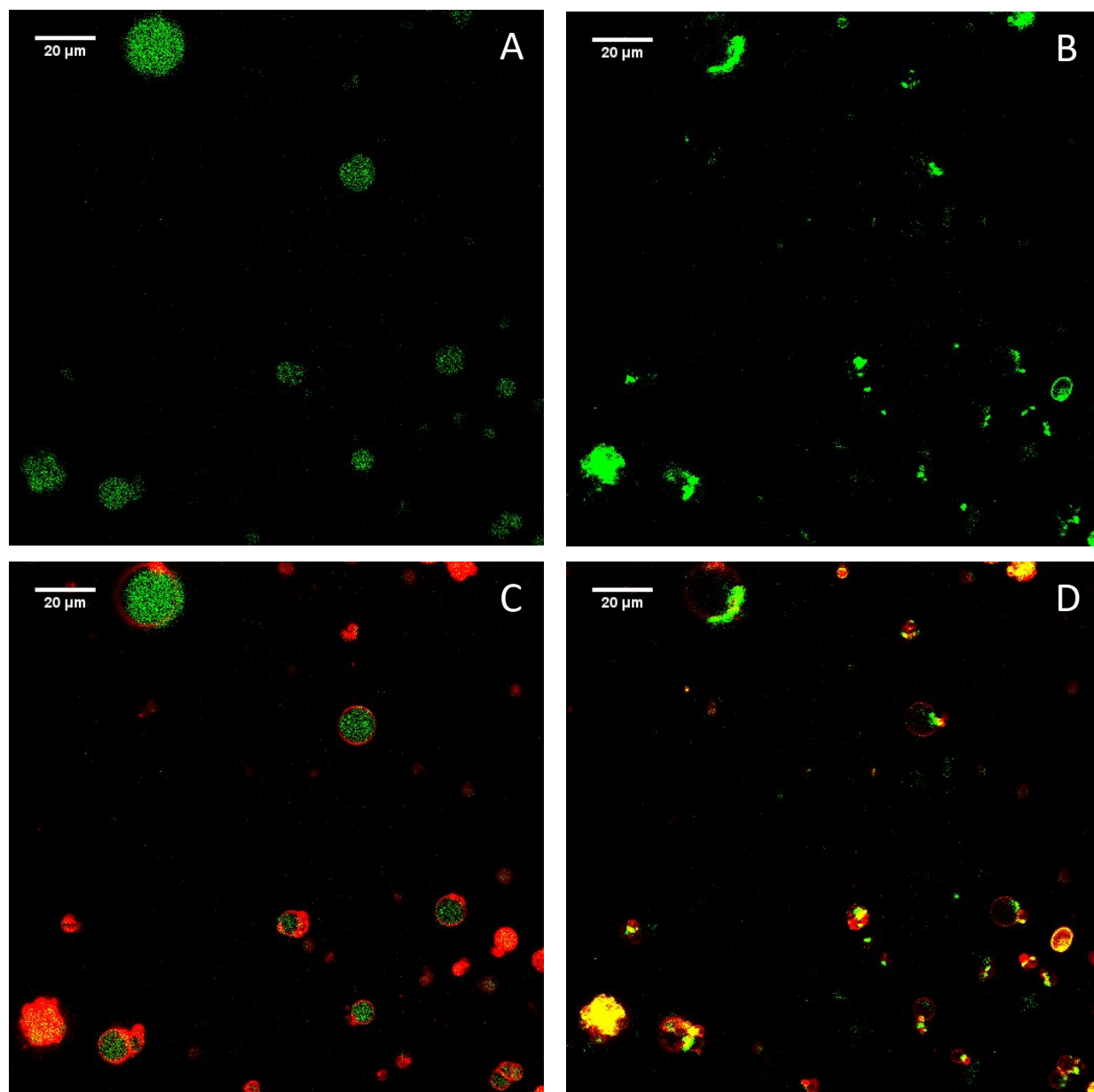


**Figure S2.16** Fluorescence microscopy images before (a, c) and 5 minutes after (b, d) addition of  $(\text{PhO})_2\text{PO}_2\text{Na}$  (final concentration of 0.05M) to GUVs to GUVs with no transporter incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.



**Figure S2.17** Normalized fluorescence intensity profiles over time for individual GUVs in the presence of 0.5% transporter (a,b) and in the absence of transporter (c) after addition of 1 M (a) and 0.5 M (b, c) of  $(\text{PhO})_2\text{PO}_2\text{Na}$  (final concentration 0.1 M and 0.05M respectively). (D) Averaged values of GUVs in the presence of transporter and in the absence of transporter.

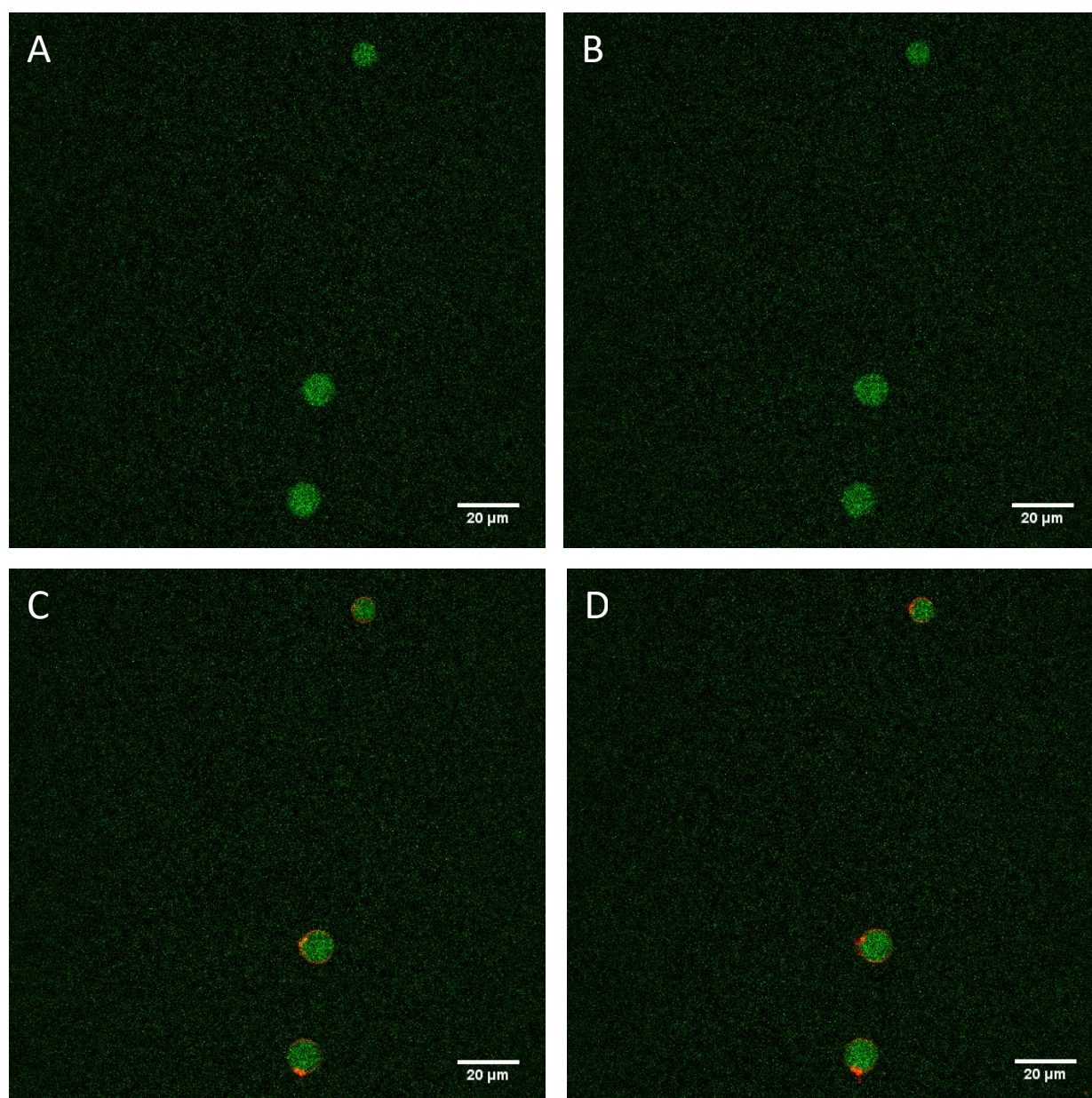
### 2.3.6. Phenylalanine (sodium salt) transport



**Figure S2.18** Fluorescence microscopy images before (a, c) and after (b, d) addition of sodium salt of phenylalanine (final concentration of 0.1M) to GUVs containing 0.5% of transporter **1** incorporated in the membrane. Images (a) and (b) show lucigenin fluorescence while images (c) and (d) consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid) to show that the lipid membranes are intact.

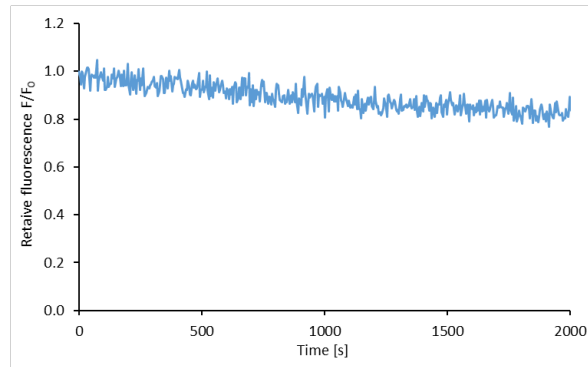


### 2.3.7. Photobleaching



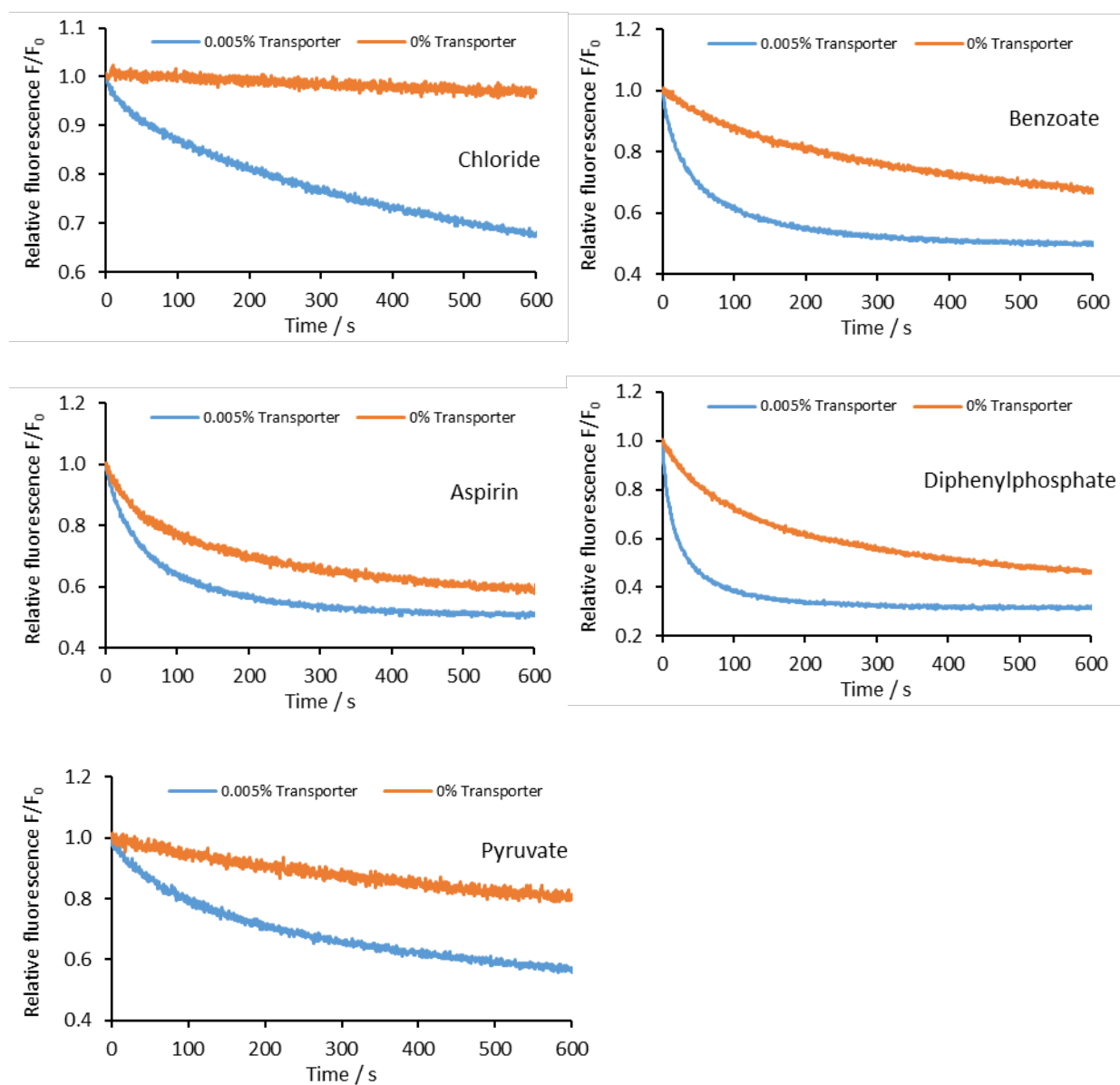
**Figure S2.19** Fluorescence microscopy images of GUVs with no transporter incorporated in the membrane at  $t = 0$  (a) and  $t = 600$  s (b). Images consist of two observation channels merged (green – lucigenin, red – rhodamine B labelled lipid).



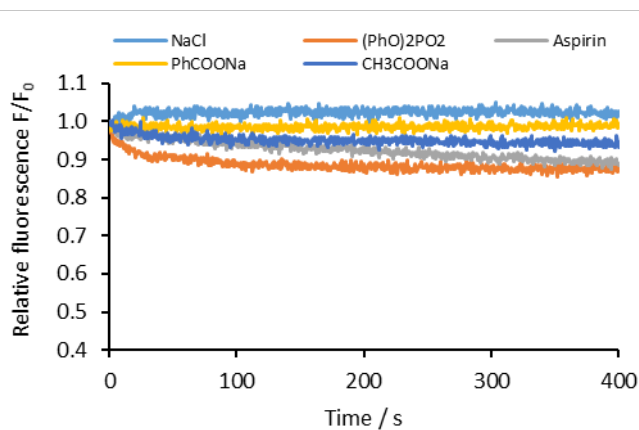


**Figure S2.20** Averaged normalized fluorescence intensity profiles over time without transporter.

## 2.4. Anion transport using LUVs



**Figure S2.21** Comparison of normalized fluorescence intensity over time of LUVs suspension with and without transporter **1** incorporated in the membrane after the addition of sodium salts: (top left) chloride, (top right) benzoate, (middle left) aspirin, (middle right) diphenylphosphate, (bottom) pyruvate.

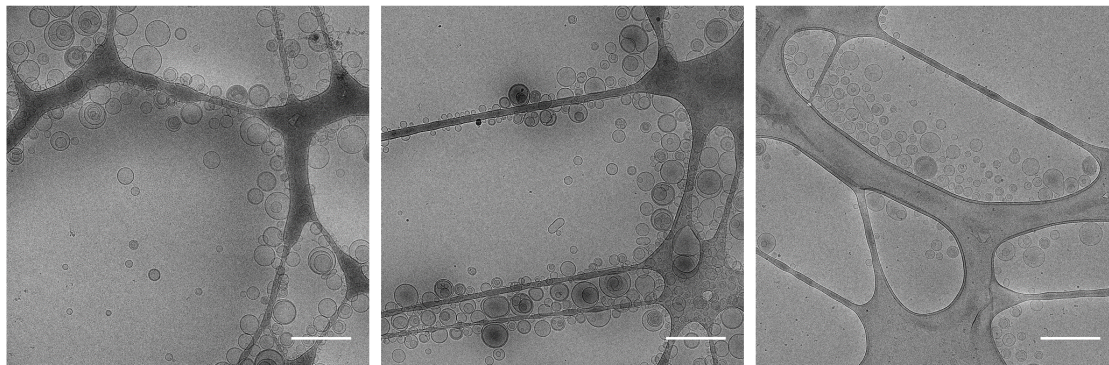


**Figure S2.22** Comparison of normalized fluorescence intensity over time of LUVs suspension containing 0.150 M  $\text{Na}_2\text{SO}_4$  (instead of 0.225 M  $\text{NaNO}_3$ ) with transporter **1** (0.005%) incorporated in the membrane after the addition of sodium salts: chloride, benzoate, aspirin, diphenylphosphate.

**Table S2.1** Results of size and surface charge measurements by Dynamic Light Scattering (DLS) of LUVs (POPC:Cholesterol 7:3) containing transporter **1** (0%; 0.005%; 0.5%) incorporated in the membrane.

Concentration of transporter	Diameter [nm]	PDI	Surface potential [mv]
0%	152.3	0.097	-5.92
0.005%	145.2	0.115	-3.02
0.5%	141.9	0.115	-1.79

## 2.5. Cryo-electron microscopy images of LUVs

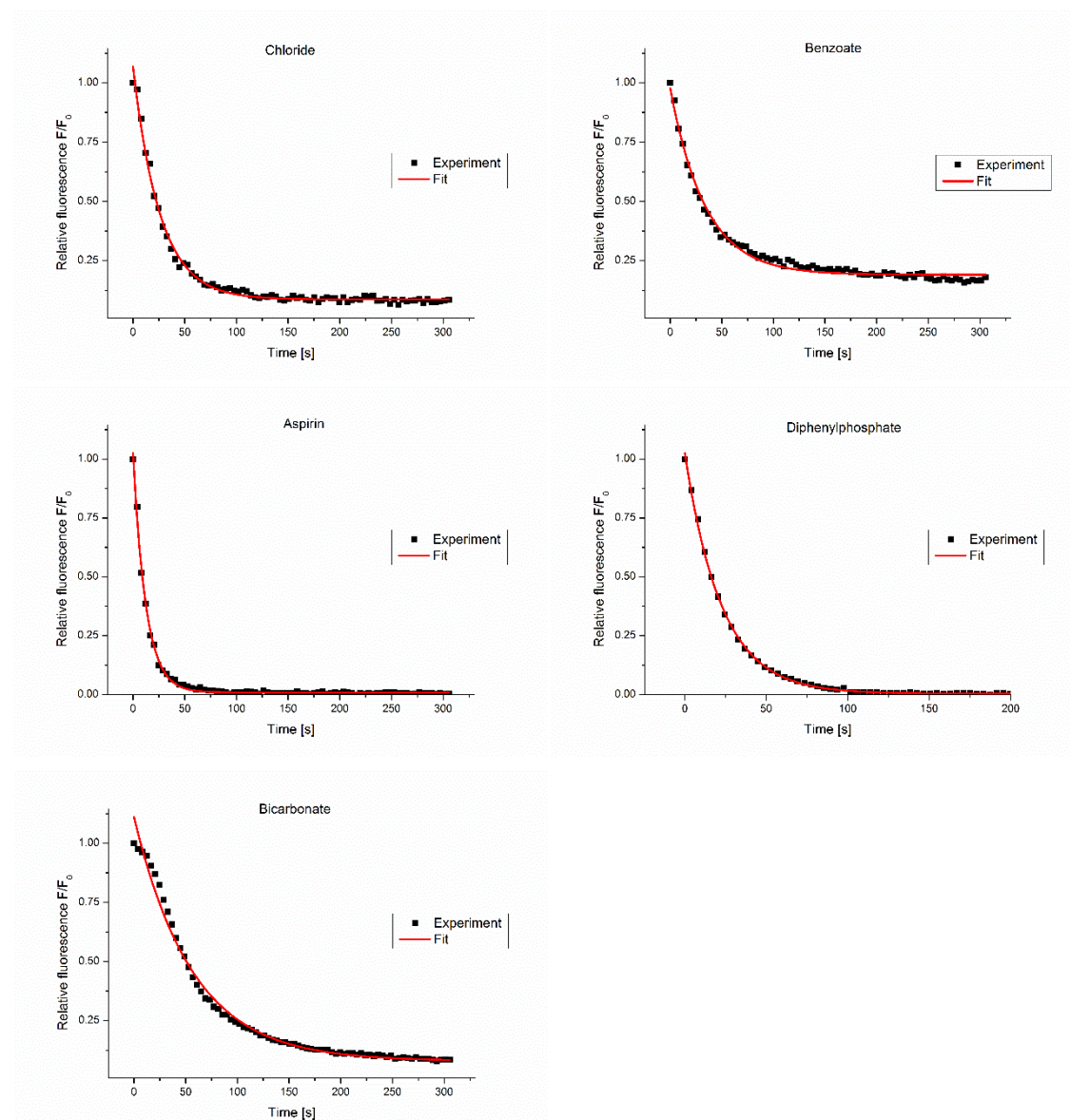


**Figure S2.22** Cryo-electron microscopy images of LUVs (POPC:CHOL 7:3) containing 0% mol (left), 0.005% mol (middle) or 0.5% mol (right) of transporter **1**. Scale bar = 500nm.

### 3. Data fitting

#### 3.1. Fitting of the results of anion transport studies in GUVs.

To compare transport of various anions in GUVs quantitatively we decided to fit single exponential decay ( $y = y_0 + Ae^{-x/k}$ ) to the averaged relative fluorescence  $F/F_0$  from different experiments.  $y_0$ ,  $A$  and  $k$  were treated as fitting parameters. The rate constant  $k$  was used to compare the activities of transporter 1 with respect to different anions.



**Figure S3.1** Results of fitting of exponential decay to averaged relative fluorescence from GUVs transport studies of transporter 1 and chloride(top left), benzoate(top right), aspirin (middle left), diphenylphosphate (middle right), bicarbonate (bottom).

**Table S3.1** Values of rate constants  $k$  obtained from fitting of exponential decay to averaged relative fluorescence from GUVs recorded during anion transport studies facilitated by the transporter **1** (0.5%mol).

Anion	$k$
Chloride	$42.0 \pm 0.6$ s
Benzoate	$34.0 \pm 0.9$ s
Aspirin	$12.3 \pm 0.2$ s
Diphenylphosphate	$22.5 \pm 0.2$ s
Bicarbonate	$56.5 \pm 1.5$ s

### 3.2. Fitting of the results of anion transport studies in LUVs.

In first approximation we can assume that time rate of anion concentration  $[A]$  inside vesicles is proportional to concentration gradient. Since extravesicular concentration  $[A]_0$  remains constant, rate is proportional to anion concentration inside vesicle:

$$\frac{d[A]}{dt} = k([A]_0 - [A])$$

Moreover, we can assume that free diffusion and carrier-mediated transport are independent from each other so that:

$$\frac{d[A]}{dt} = k_{diffusion}([A]_0 - [A]) + k_{transport}([A]_0 - [A])$$

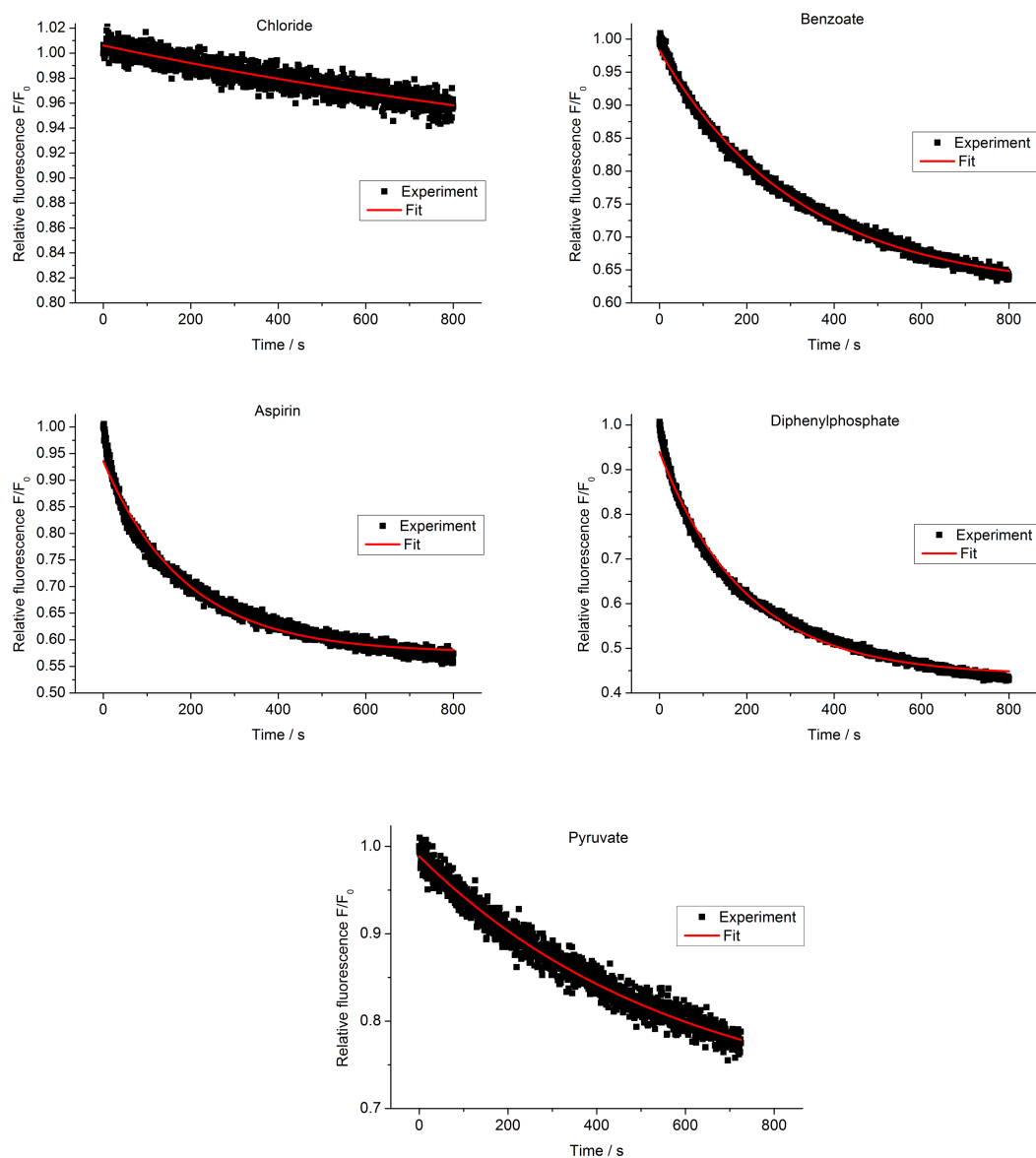
$$\frac{d[A]}{dt} = k_{total}([A]_0 - [A]),$$

$$k_{total} = k_{diffusion} + k_{transport}$$

The solution to this equation is exponential decay:

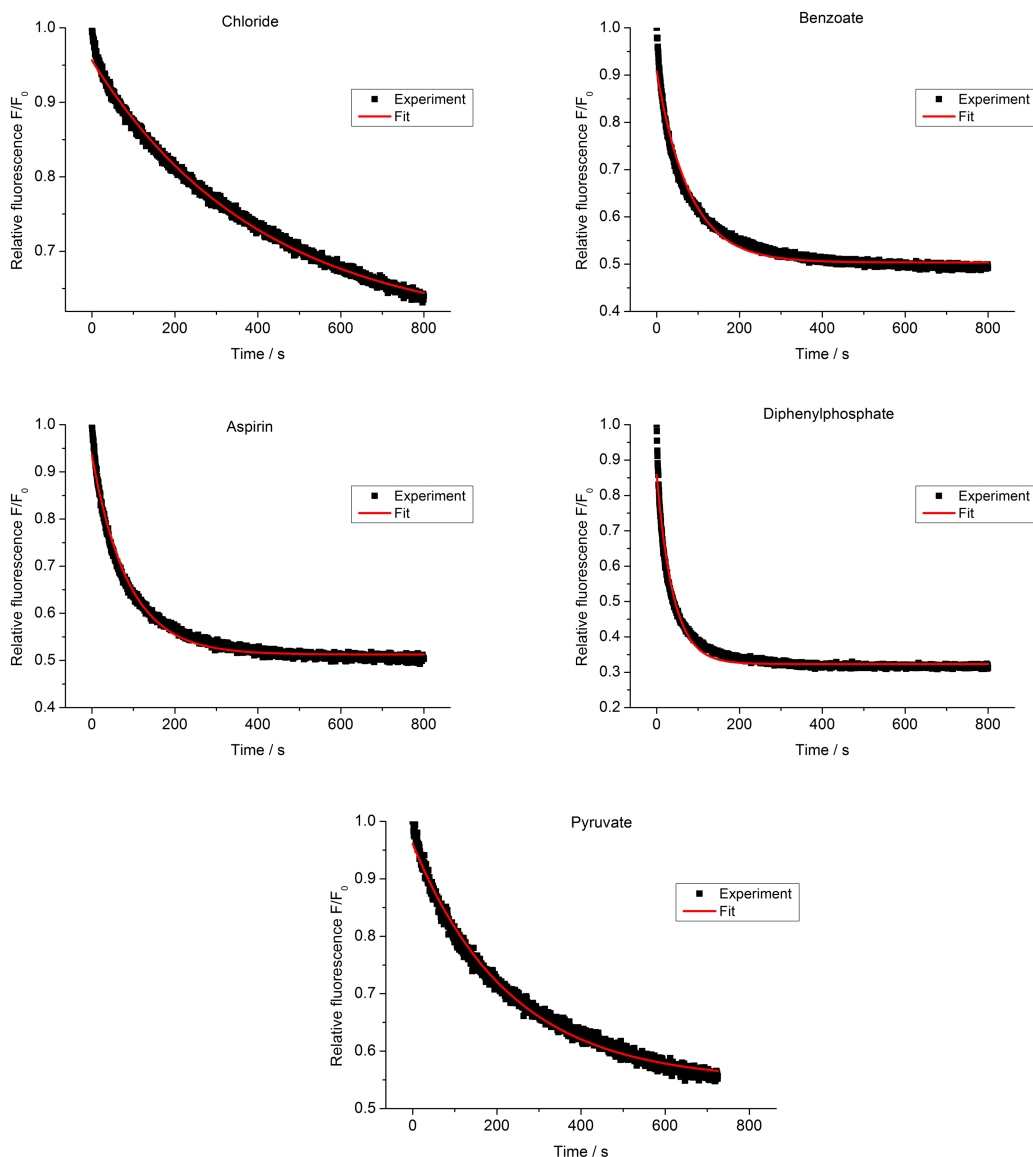
$$[A] = [A]_0(1 - \exp(-kt))$$

Thus, to compare transport of various anions in LUVs quantitatively we decided to fit single exponential decay ( $y = y_0 + Ae^{-kt}$ ) to the averaged relative fluorescence  $F/F_0$  from different experiments with and without transporter in membrane.  $y_0$ ,  $A$  and  $k$  were treated as fitting parameters. The rate constant  $k$  was used to compare the activities of transporter **1** with respect to different anions and  $k_{diffusion}$  can be extracted from the experiment without transporter in the membrane.



**Figure S3.2** Results of fitting of exponential decay to averaged relative fluorescence from LUVs transport studies without transporter 1 in membrane and chloride (top left), benzoate (top right), aspirin (bottom left), diphenylphosphate (bottom right).





**Figure S3.3** Results of fitting of exponential decay to averaged relative fluorescence from LUVs transport studies with 0.005% of transporter **1** in membrane and chloride (top left), benzoate (top right), aspirin (bottom left), diphenylphosphate (bottom right).

**Table S3.2** Values of rate constants  $k$  ( $s^{-1}$ ) obtained from fitting of exponential decay to averaged relative fluorescence of LUVs recorded during anion transport studies with transporter **1** in the membrane (0.005%mol) and without it.

Anion	$k_{\text{total}} [\times 10^{-3} s^{-1}]$	$k_{\text{diffusion}} [\times 10^{-3} s^{-1}]$	$k_{\text{transport}} [\times 10^{-3} s^{-1}]$
Chloride	$2.469 \pm 0.018$	$0.578 \pm 0.101$	$1.891 \pm 0.23$
Benzoate	$12.64 \pm 0.10$	$3.13 \pm 0.02$	$9.51 \pm 0.09$
Aspirin	$11.51 \pm 0.07$	$5.35 \pm 0.06$	$6.16 \pm 0.11$
Diphenylphosphate	$25.06 \pm 0.19$	$5.08 \pm 0.03$	$19.98 \pm 0.80$
Pyruvate	$4.348 \pm 0.038$	$1.700 \pm 0.049$	$2.648 \pm 0.070$

## 4. References

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