The lucigenin assay:

measuring anion transport in lipid vesicles

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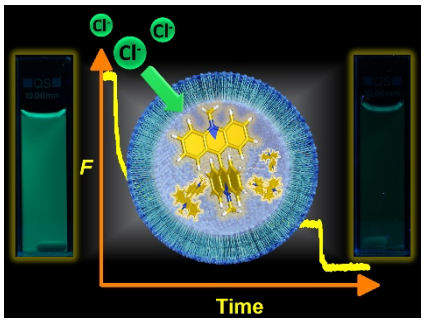

Abstract

Synthetic anion transporters are developed to transport anions across lipid membranes with the long-term perspective of biological applications. The lucigenin assay is a popular tool to study their transport of chloride and other anions in liposomes. It relies on the quenching of the fluorescence of encapsulated lucigenin by anions, which can be monitored by fluorescence spectroscopy. This article provides a tutorial introduction to the practical use and understanding of the lucigenin assay. It describes in detail how to use this assay to monitor chloride/nitrate antiport in liposomes, process and interpret the data, and solve common issues. Variations of the assay enabling the investigation of the transport of other anions and transport mechanisms are discussed. Furthermore, a zwitterionic analogue of lucigenin is demonstrated to have advantages for use in specific cases.

Key words: Anion transport, Fluorescent probes, Fluorescence spectroscopy, Liposomes, Supramolecular Chemistry

1 Introduction

Artificial anion transporters present an attractive field of research with possible applications as either therapeutics for channelopathies or anticancer agents.[1–3] In recent years, a growing number of researchers started to investigate new anion transporters.[4–11] For the screening of promising compounds as potential anionophores artificial model systems are used, in particular large unilamellar vesicles (LUVs, also referred to as liposomes) made of phospholipids to mimic cell membranes.

To assess the activity of anionophores, several types of assays were developed, involving different methods to monitor ion concentrations, such as the use of ion selective electrodes or fluorescence spectroscopy. For the latter, emissive probes are encapsulated inside liposomes and give a response upon transmembrane transport. The change of interior anion concentration during the transport process can be monitored by probes responding to the anion of interest. To directly monitor chloride concentrations, the probes SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium)[12] and lucigenin (*N*,*N*'-dimethyl-9,9'-biacridinium dinitrate) are used.[4] The first use of lucigenin to measure chloride transport in artificial vesicles was by the Verkman laboratory[13] and the method was further adapted by McNally *et al.*[14] to the current form of the lucigenin assay. Since then, the lucigenin assay became an attractive way to directly monitor transport of chloride (and other anions[15,16]) through lipid bilayers, which enables the study of anionophore activity.

In the standard lucigenin assay, lucigenin is encapsulated in liposomes prepared in a 225 mM $NaNO_3$ solution, ensuring the presence of $NaNO_3$ in the interior and the exterior of the liposomes (Figure **1**). Before the transport experiment, a solution of anion transporter (anionophore) is added to the liposome suspension to post-insert the anionophore into the membrane. After that, a pulse of chloride solution is added to the exterior of liposomes resulting in a 25 mM exterior Cl^- concentration, to create a gradient across the membrane. The dissipation of this gradient by the anionophore is monitored by measuring the decrease of fluorescence intensity over time, due to the quenching of the fluorescence of lucigenin by chloride (see Section 2). The advantages of the lucigenin assay are a great time resolution, versatility, and easy utilisation.

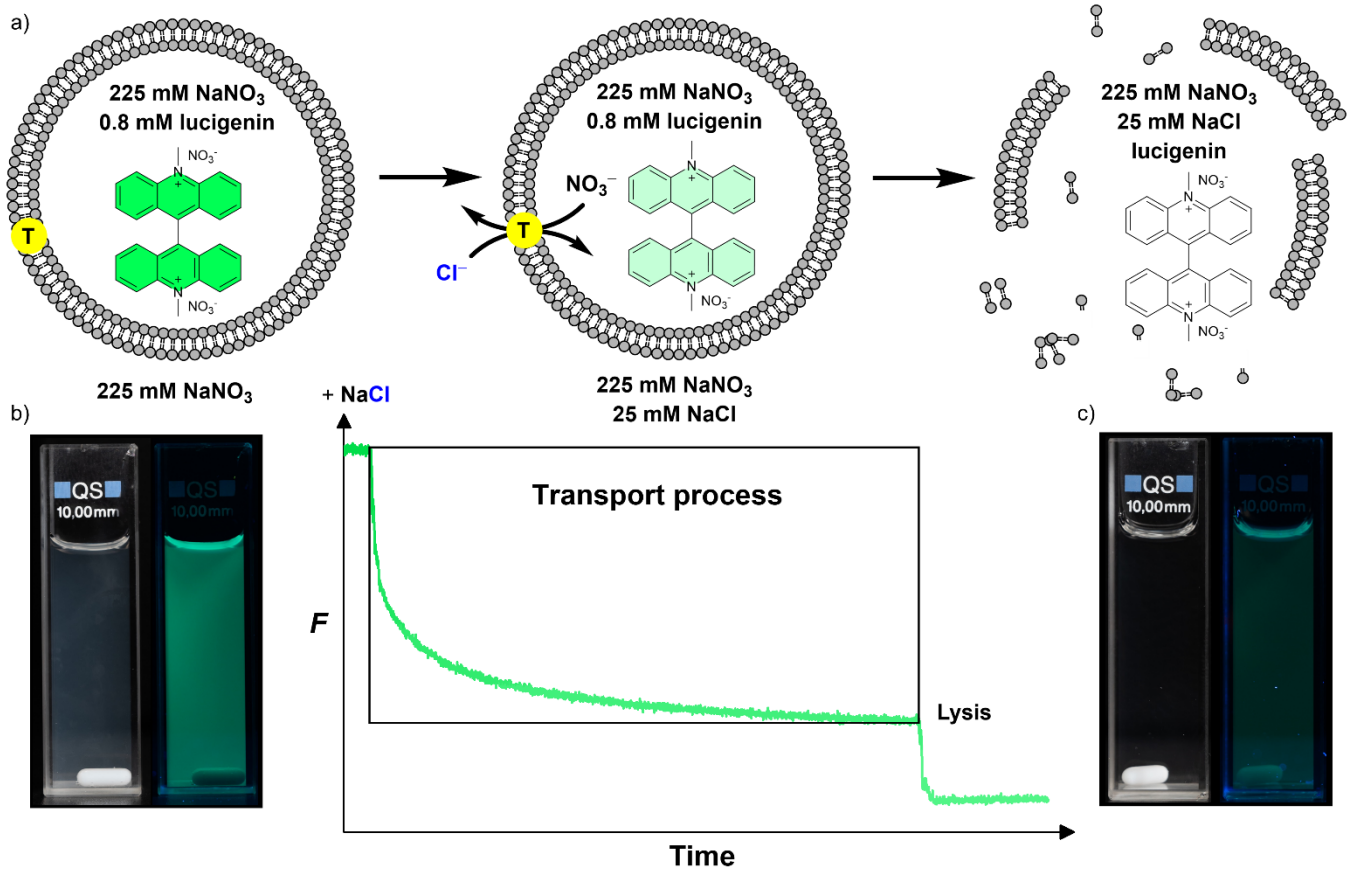

Figure 1. a) Schematic representation of the standard lucigenin assay to study Cl^-/NO_3^- antiport by a transporter (**T**). The photos show: b) the liposome suspension at the start of the transport experiment and c) the sample after NaCl addition and lysis (left: regular image; right: under irradiation with 365 nm light).

Alternative experiments started with lucigenin encapsulated together with a high concentration of a quencher such as NaCl, and the increase of fluorescence intensity caused by the efflux of the quencher was monitored.[17–21] Compared to the assay in Figure 1, such experiments would in principle give a poorer signal-to-noise ratio, are more difficult to quantify, and do not allow to test transport of non-deliverable transporters.

In this tutorial article, we describe the optical properties of lucigenin, the areas of its use, the experimental procedure for the lucigenin assay, problems commonly encountered when using the assay, and solutions to these problems. The standard lucigenin assay to study Cl^-/NO_3^- antiport will be described in detail, followed by a discussion of other variations on the lucigenin assay. Additionally, the use of the closely related probe SPBA (bis(3-sulfopropyl)-9,9'-biacridine) for anion transport studies will be demonstrated.

2 Optical properties of lucigenin

Lucigenin is a commercially available aromatic bisacridinium compound, which has the appearance of an orange powder and forms a yellow-green solution when dissolved in water. The maximum of the main absorption band of lucigenin is observed at 368 nm (ε = 34200 M^{-1} cm^{-1}),[13] and minor bands are present at 352 nm, 430 nm, and 455 nm (Figure 2a). Lucigenin exhibits blue-green fluorescence in solution. The emission spectrum has a broad band of which the maximum is located around 505 nm (Figure 2b). The shape of the emission spectrum does not change when using different excitation wavelengths. It is noteworthy that the fluorescence intensity of lucigenin is temperature dependent, being higher at the lower temperatures (see Section 3.5 and Figure 7).

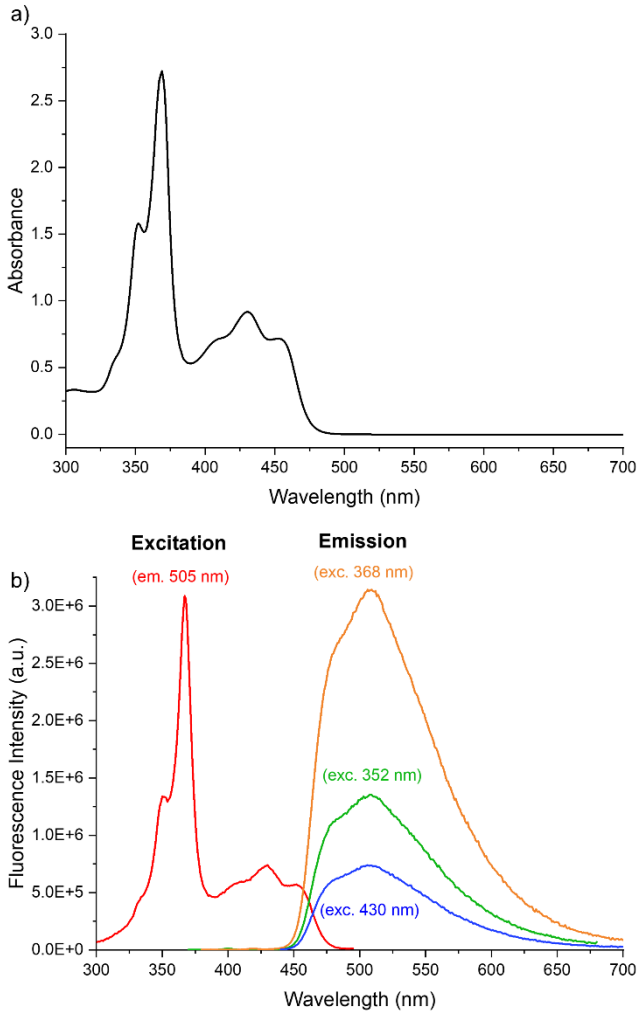

Figure 2. a) Absorption spectrum of 200 µM lucigenin in 225 mM $NaNO_3$; b) excitation-emission spectra of 1 µM lucigenin in pure water.

Lucigenin was reported by Decker and Dunant in 1906[22] and was first investigated for its chemiluminescence.[23–25] The chemiluminescent properties of lucigenin are currently used for the detection of various biologically relevant substances[26–28] and the detection of superoxide.[29–31]

Applications of lucigenin as chloride-sensitive probe emerged originally in the study of anion transport in cells[32,33] and later for the optical sensing of chloride.[34–36] The fluorescence of lucigenin is quenched in presence of chloride, bromide, and iodide, and several other anions. The quenching is very rapid and faster than other processes taking place during transport experiments.

In the literature, two main mechanisms of quenching have been described. For smaller anions such as chloride and reducing anions, the quenching is predominated by the formation of a charge transfer complex between excited lucigenin (L^{2+})* and the anion:[37]

$$L^{2+} \xrightarrow{h\nu} (L^{2+})^* \quad (1)$$

$$(L^{2+})^* + Cl^- \rightarrow (L^{\cdot+}Cl^{\cdot}) \rightarrow L^{2+} + Cl^- \quad (2)$$

For other anions, the quenching is caused by the increase of the intersystem crossing rate constant due to the heavy atom effect (bromide, iodide, *etc.*) and non-radiative relaxation from the triplet state. This mechanism may contribute to the quenching by lighter atoms such as chloride as well.[38]

$$(L^{2+})^* + I^- \rightarrow {}^1(L^{2+}I^-) \xrightarrow{ISC} {}^3(L^{2+}I^-) \rightarrow {}^3(L^{2+}) + I^- \quad (3)$$

The quenching of the fluorescence intensity of lucigenin can be described using the Stern-Vollmer relationship:

$$\frac{F_0}{F} = 1 + K_{SV}[\mathrm{Q}] \quad (4)$$

where F_0 is the fluorescence intensity without the quencher, F is the fluorescence intensity in the presence of a quencher, K_{SV} is the Stern-Volmer constant and [Q] is the quencher concentration. The Stern-Volmer quenching constants of various anions in pure water were determined by fluorescence measurements using a lucigenin dinitrate salt concentration of 1 µM and increasing concentrations of anions. Data for chloride are shown in Figure 3 and for other anions in the SI. The Stern-Volmer constants obtained and the literature values are listed in Table 1.

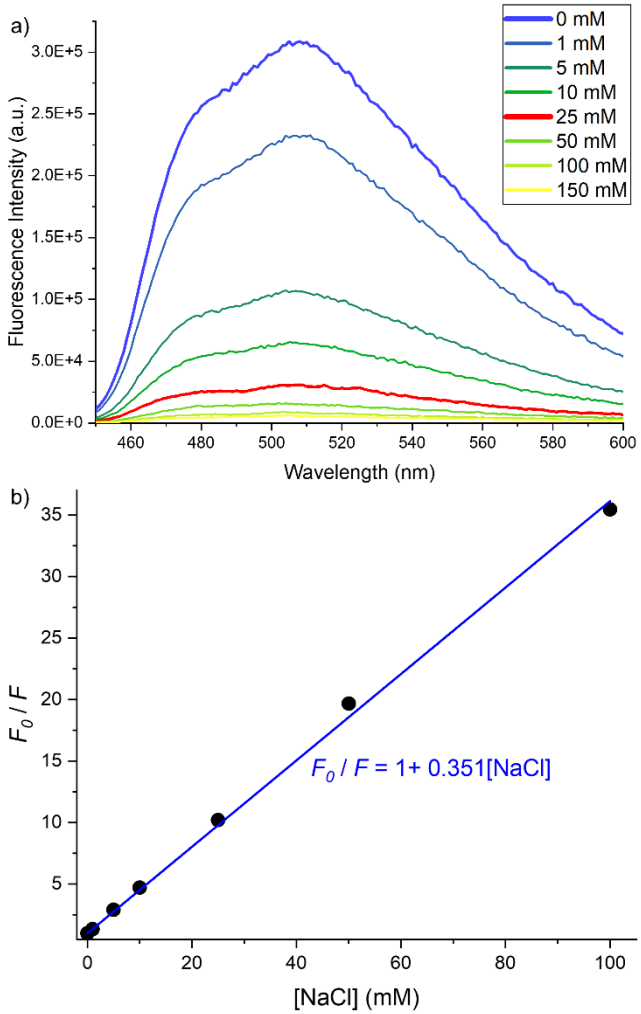

Figure 3. a) Emission spectra (excitation wavelength 430 nm) of 1 µM lucigenin dinitrate salt in water upon addition of increasing sodium chloride concentrations and b) the corresponding Stern-Volmer plot of the fluorescence at 505 nm.

Table 1. Stern-Volmer constants for the quenching of lucigenin dinitrate salt by various anions in water or in aqueous buffer solutions.

Anion	K_{SV} (M^{-1})	Buffer	Reference
Chloride	351	-[a]	This work
	372	-[a]	[37]
	390	Phosphate[b]	[13]
	192	Mannitol-tricine-BTP[c]	[32]
Bromide	556	-[a]	This work
	585	Phosphate[b]	[13]
	357	Mannitol-tricine-BTP[c]	[32]
Iodide	777	-[a]	This work
	750	Phosphate[b]	[13]
	496	Mannitol-tricine-BTP[c]	[32]
Nitrate	3	-[a]	This work
	0.5	Mannitol-tricine-BTP[c]	[32]
Sulphate	13	-[a]	This work
	13	-[a]	[37]
	5	Mannitol-tricine-BTP[c]	[32]
Acetate	45	-[a]	This work
	45	-[a]	[37]
	31	Mannitol-tricine-BTP[c]	[32]
Bicarbonate	28	-[a]	This work
HEPES	39	-[a]	This work
Hydroxide	150	-[a]	This work

[a] pure water without buffer; [b] 5 mM sodium phosphate buffer (pH 7.2)[13]; [c] 500 mM mannitol, 10 mM Tricine–BTP (pH 7.4), 1 mM DTT.[32]

The data reported in the literature exhibit a variation, depending on which medium or buffer system was used for determining K_{SV}. Usually, at higher buffer concentrations, the reported K_{SV} values are lower, as buffer salts can already lead to partial quenching of the lucigenin fluorescence. Similarly, in presence of 225 mM $NaNO_3$, the K_{SV} values for chloride are ~3.5-fold lower than in pure water (Figure S10).

The lucigenin assay is commonly used to study the exchange of chloride with another anion via an antiport mechanism, to avoid the build-up of a potential gradient across the membrane. For this reason, $NaNO_3$ or other salts are encapsulated together with lucigenin (see Figure 1), and the relatively high concentration of 225 mM is used to minimise differences in osmolality upon addition of 25 mM NaCl (a concentration that gives a strong quenching response, while avoiding full quenching). Figure S22 shows the extent to which 25 mM NaCl causes additional quenching in $NaNO_3$, $NaHCO_3$, Na_2SO_4, and NaOAc solutions as used in transport studies. The additional quenching caused by the NaCl addition is lower when the K_{SV} of the other anion is higher. The sensitivity of lucigenin to chloride is thus the best in $NaNO_3$ and NO_3^- is also considered relatively easy to transport.

There are several advantages in using lucigenin (and possibly other acridinium compounds) over quinolinium compounds (in particular SPQ) to monitor transmembrane anion transport in liposomes. Firstly, lucigenin has a higher sensitivity to chloride (K_{SV} = 118 M^{-1} for SPQ vs. 351 M^{-1} for lucigenin). Secondly, lucigenin also has a significantly higher extinction coefficient than SPQ with a similar quantum yield (0.67), which thus leads to higher emission intensities.[13] Another limitation of quinolinium probes is that the excitation wavelength for SPQ is in the UV range (322 and 350 nm), where the scattering by the liposomes (with diameters of 130-200 nm) results in less efficient excitation. The use of the additional excitation band of lucigenin at 430 nm mitigates this issue.

3 Procedure for the standard lucigenin assay to measure Cl^-/NO_3^- transport

3.1 Materials

Research on synthetic anionophores has the long-term perspective of therapeutic applications in human medicine, and the lipid composition of the liposomes as model system should thus reflect that of mammalian cells. Commonly used lipids are EYPC (egg yolk phosphatidylcholine),[39] POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine),[40] and POPC/cholesterol[14] mixtures (for structures of lipids see Figure S24). We prefer to use the mixture of POPC with cholesterol in a 7:3 molar ratio since this is a good mimic for epithelial membranes and the combination of POPC and cholesterol gives more stable membranes and better reproducibility than when POPC is used alone. Cholesterol decreases the membrane fluidity and can thus reduce the rate of leaking of lucigenin into the external solution (see Section 3.5). Experiments with liposomes made of POPC/cholesterol are also more attractive economically, due to the lower price of cholesterol. Because the choice of lipids may affect the diffusion of the anionophore through the membrane, the lipid compositions used should be considered when comparing data for different anionophores. Lipids are susceptible to oxidation and hydrolysis and should be stored according to the manufacturer's recommendations; we store POPC in a freezer at -25 °C under argon atmosphere and cholesterol at room temperature. The stock solution of lipids needs to be prepared in deacidified chloroform, which is obtained by passing it through a column with basic alumina just before use. The stock solutions of lipids are again stored at -25 °C. Considering the relatively low price of cholesterol and previously encountered problems with older cholesterol solutions (see Section 3.5), we are in the habit of preparing fresh solutions weekly. The most convenient concentration range for the lipid stock solutions is 10-30 mM.

Stock solutions of anionophores are prepared in water-miscible solvents such as MeOH or MeCN (not DMSO, see Section 3.5). Concentrated solutions (2.4 mM) are prepared, if possible, which can be further diluted for efficient transporters, so that 5-10 µL would be added to the liposome suspension before the transport experiment to reach the desired transporter:lipid ratio.

Salts with high purity should be used to prepare the aqueous solutions, since even small impurities of other ions might influence the results of the transport experiments. For the same reason, milli-Q grade water is to be used. Salt or buffer solutions are generally stable for longer periods of time at room temperature. A 5% (w/w) solution of Triton X-100 in water is used to lyse the liposomes and it is advisable to prepare this at least one day before the experiment.

A 0.8 mM lucigenin solution is prepared in 225 mM $NaNO_3$ in water and it is stable for several months. The use of this stock solution and a lipid concentration of 0.4 mM gives a global lucigenin concentration in the order of 1 µM in the final liposome suspension. Lucigenin takes time to dissolve, thus sonication and gentle heating can be used to accelerate the process. If any solid particles are still present, the solution should be filtered. The exact concentration of lucigenin is not crucial for the quantification of transport (in contrast to the concentration of NaCl and the transporter:lipid ratio).

An extrusion kit is required to obtain liposomes of the desired diameter. Commonly used are the kits from Avestin or Avanti, equipped with 1 mL syringes and using polycarbonate membranes with 200 nm etched pores.

To separate liposomes from unencapsulated lucigenin, size-exclusion chromatography is used. This can be done with pre-packed commercial columns (for instance with 8.3 mL Sephadex G-25 Medium, also referred to as 'PD-10 desalting columns'), which are time-efficient, or with manually packed columns (for instance by hydrating 2.2 g Sephadex G-50 Fine for a few hours with the eluent and then pouring the suspension into a thin glass column with cotton wool or a sintered glass frit and slowly draining the excess eluent). Sephadex columns should be run using gravity alone, without applying any pressure. Columns can be re-used upon rinsing with ~3 column volumes of salt solution. If maintained well, the column can be used for several months and numerous batches of liposomes. Ideally, a column is only used for a single salt solution. However, if it is required to use another salt solution, the column should be eluted with at least 3 column volumes of this salt solution prior to use.

3.2 Liposome preparation

The stock solutions of lipids are allowed to warm up to room temperature prior to opening the vials, to avoid water condensation. The adequate lipid solution volumes required to obtain a 0.4 mM final lipid concentration are added into a 5 mL round bottomed flask using pipettes for organic solvents. For example, to prepare 20 mL liposomes with 0.4 mM concentration, 8 µmol of lipids is needed, corresponding to 5.6 µmol of POPC and 2.4 µmol of cholesterol for a POPC/cholesterol 7:3 mixture. The solvent is evaporated by applying a gentle nitrogen or argon flow (bringing lower risks of contamination compared to the use of a rotary evaporator). The dried lipid film (Figure 4a) is then placed in high vacuum for at least 1 hour, but this could also be done overnight. Ideally, the lipid film should be protected from UV light.

After drying the lipid film, a small magnetic stir bar is placed into a flask, the lucigenin solution is added (0.5 mL), and the flask is sealed with a closed vacuum adapter (or stopper). The flask is then sonicated for approximately 30 seconds to obtain a homogenous mixture (vortexing could also be used). No more lipids should be stuck on the wall of the flask after the sonication. The mixture is further stirred for 1 hour on a magnetic stirrer to produce heterogeneous vesicles (Figure 4b).

In the next step, 10 freeze-thaw cycles are performed to disrupt the multilamellar vesicles. The vacuum adapter on the flask is closed and the lipid suspension is placed in liquid nitrogen (or dry ice) until the contents of flask are completely frozen (Figure 4c). After several seconds of silence during the initial cooling of the flask and its contents in liquid nitrogen, a bubbling/sissing sound of liquid nitrogen evaporation indicates the phase transition of the lipid suspension. This sound ceases when the content of the flask is completely frozen. To thaw the liposomes, the flask is then placed in a water bath heated to 30-40°C on a magnetic stirrer, until a homogenous suspension is obtained. This freeze-thaw process is repeated 9 additional times to produce unilamellar vesicles.

In the meanwhile, the extrusion kit can be assembled, and the membrane is wetted by passing the 225 mM $NaNO_3$ solution a few times through it (this washing solution is discarded after use). The membrane is fragile and must be handled with care using tweezers; the part through which the liposomes will be extruded needs to remain undamaged (ideally untouched). Pressing only air through the extrusion kit should be avoided.

After the freeze-thawing cycles are completed, the vesicle suspension is transferred to one of the syringes of the extrusion kit (Figure 4d). Small amounts of $NaNO_3$ solution (a few drops) are used to rinse

the remaining vesicles from the flask and are added to the rest of the solution in the syringe, which is then filled up to 1 mL with the $NaNO_3$ solution. The mixture is then passed 29 times through the membrane to produce monodisperse large unilamellar vesicles. Excessive pressure on the pistons should be avoided. The extrusion cycles are ended on the opposite side of the membrane, so that the solution has effectively been filtered.

The suspension of liposomes is then transferred to the top of a size-exclusion column without disturbing the stationary phase. The column should be pre-eluted with the $NaNO_3$ solution. A small volume of eluent is used to rinse the extrusion kit and to transfer all remaining liposomes to the column. The liposomes elute first and give the drops a cloudy appearance, well observable against a dark background (Figure 4e). The liposomes can be collected into a volumetric flask or measuring cylinder for the final dilution. The collection should be stopped as soon as the drops do not have a cloudy appearance anymore to avoid contamination with the unencapsulated lucigenin. Shortly after this, the unencapsulated lucigenin is eluted as a yellow solution (Figure 4f).

The collected liposomes are diluted to the final volume to reach 0.4 mM lipid concentration, assuming that no lipids were lost during the liposome preparation (Figure 4g). The transport experiments should be started right after the liposome preparation, due to slow leaking of lucigenin out of the liposomes over time (see Section 3.5). The size of obtained liposomes can be measured by DLS. Following the above method, liposomes of 130-200 nm mean hydrodynamic diameter are obtained (Figures S25).[41]

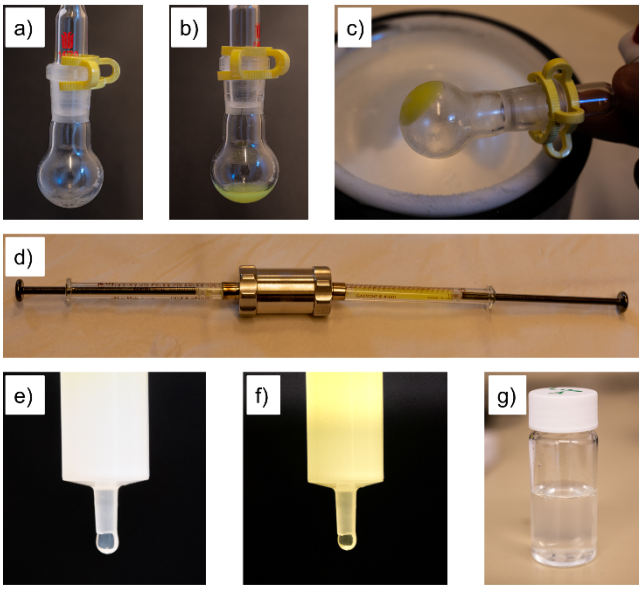

Figure 4. a) Lipid film at the bottom of the flask; b) heterogeneous vesicles dispersed in the lucigenin solution after hydration, sonication, and stirring for 1 hour; c) vesicles after freezing in liquid nitrogen; d) an extrusion kit with vesicles loaded; e) liposomes eluting from the size exclusion column; f) external lucigenin eluting from the column; g) a vial with a final liposome suspension.

3.3 Transport Experiments

A fluorimeter equipped with magnetic stirring, an injection port, and a temperature-controlled cell holder is required to carry out transport experiments. For the lucigenin assay, the samples are excited at 430 nm and the emission at 505 nm is recorded over time (see Section 2). A time resolution of 1 datapoint per second is sufficient, but a higher resolution has advantages during the data analysis (see below).

3 mL of the liposome suspension is transferred into a quartz cuvette with a small magnetic stirrer, which is then placed in the fluorimeter. The stirring should be moderate, so that the stir bar would not jump or produce bubbles, which would interfere during the measurement. The solution of the anionophore (5-10 µL) is added (with a Hamilton syringe or pipette for organic solutions) into the liposome suspension (just above the stir bar) while stirring. After the addition of the anionophore, the lid of the fluorometer is closed and the sample inside should be equilibrated for approx. 3 minutes before the run to ensure that it has a constant temperature of 25°C and that the added transporter is incorporated into the liposomal membranes. At 30 s after start of measurement (use of a stopwatch is recommended), the pulse of 25 mM NaCl is added to the liposomes (75 µL of 1 M NaCl in 225 mM $NaNO_3$) using a pipette. The data is recorded for at least 10 min after the addition of the NaCl pulse. After that, a pulse of detergent (Triton X-100, 5% w/w in water, 50 µL) is added to lyse the liposomes. The recording of data is continued until the emission intensity reaches a stable level, which may take 2 to 5 minutes (making a total run last ~12-15 minutes). For experimental consistency and to make the data treatment easier, the NaCl pulse should be added exactly at 30 s, and the detergent at 630 s after the start of the experiment. For each experiment, runs are done in triplicate to ensure the reliability and minimise noise by averaging the data.

3.4 Data treatment

The data treatment will be demonstrated on transport runs obtained in a typical experiment using anionophore **1**[42] (Figure 5). Data processing can be done in software such as Origin or Excel and includes several steps:

1. Raw data obtained from experimental runs are plotted.
2. Datapoints before the point of the addition of the NaCl pulse (first 30 seconds of the experiment) are removed (red box in Figure 5).
3. Datapoints belonging to the initial drop caused by rapid quenching of unencapsulated lucigenin in the external solution are removed. Usually, these are at most the first 3 seconds after the addition of the external chloride pulse, depending mainly on the time it takes for the NaCl

solution to get mixed into the liposome suspension (Figure 5a, approximately the datapoints in the yellow box).

4. Data are normalized by dividing all datapoints by the maximum of the fluorescence intensity (normally the first datapoint, F_0), giving the F/F_0 values.
5. These operations are done for each run separately. The normalized data after initial drop removal should show similar trends as the unprocessed data, including the final fluorescence levels after lysis. If this is not the case, revising the initial drop removal usually helps.
6. The normalized data are then averaged to give the final transport curve (Figure 5b).

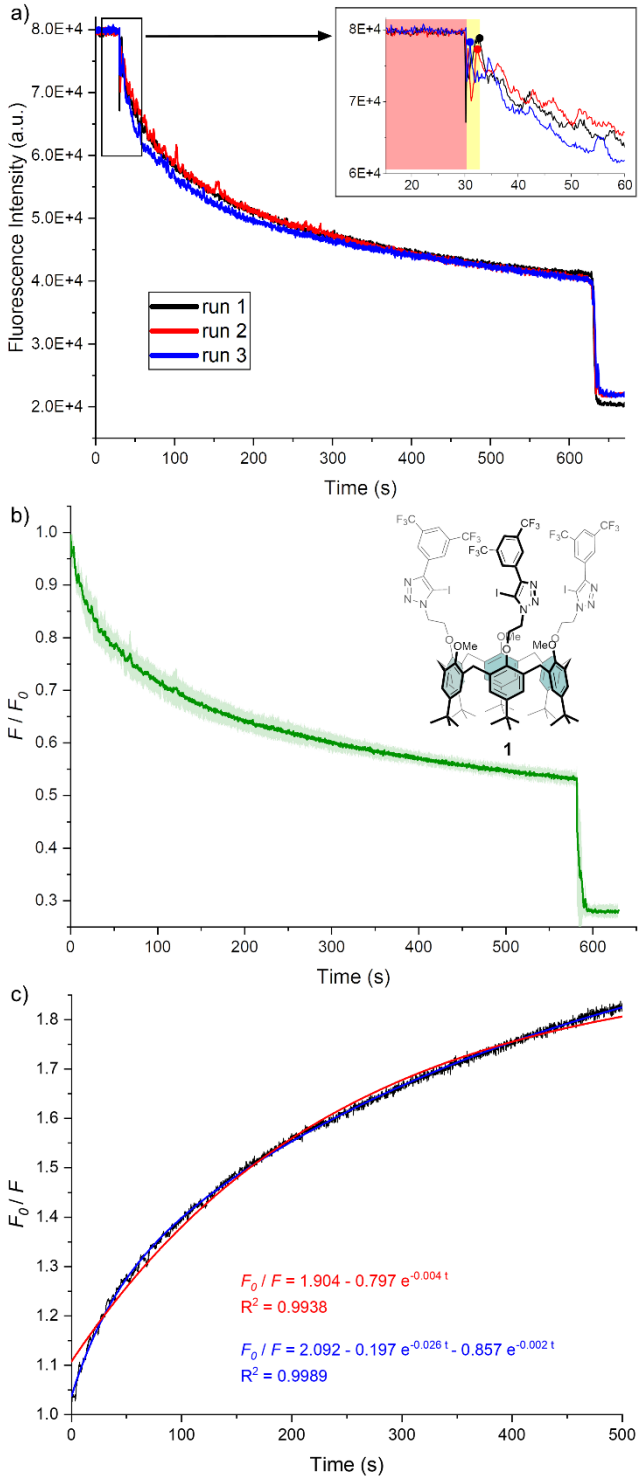

Figure 5. a) Unprocessed data obtained from transport measurements using **1**. The insert shows a zoom of the first part of the curve with the data to be removed during processing in the red (fluorescence before the addition of the NaCl pulse) and yellow boxes (the rapid quenching of external lucigenin); for each curve, the first none-removed datapoint is labelled with a dot; b) averaged curve for all three processed and normalised experimental runs with the shaded area representing the standard deviation; c) inverse of the averaged and normalised data from 0-500 s (black), fitted with a single (red line) and a double exponential function (blue line).

The anionophore activity can be analysed by calculating the initial transport rate and the half-life (or rate constant).[41] When the data from transport experiments are plotted as F_0/F, the curves correspond to

the chloride concentration inside the liposomes according to the Stern-Volmer relationship. This is in contrast with plotting the curves as F/F_0, which allows a visual comparison, but where the y-axis is not linearly correlated to the chloride concentration. Thus, the inverse of the final averaged curves is taken and all data after 500 s are removed (Figure 5c). The data from 0 to 500 s of the F_0/F curve are fitted with a single exponential function:

$$\frac{F_0}{F} = y_\infty - a \cdot e^{-bt} \tag{2}$$

Here t is the time and y_∞, a, and b are fitting parameters. The half-life ($t_{1/2}$) is calculated from the fitting parameter b (which can be considered as a rate constant for the transport process):

$$t_{1/2} = \frac{\ln 2}{b} \tag{3}$$

The curve can be fitted more accurately using double exponential function:

$$\frac{F_0}{F} = y_\infty - a \cdot e^{-bt} - c \cdot e^{-dt} \tag{4}$$

Upon differentiation by t and substituting $t = 0$, the equation for the initial transport rate is obtained:

$$I = ab + cd \tag{5}$$

These values for the initial rate I and half-life $t_{1/2}$ depend on the concentration of anionophore used in the experiments. For the comparison of compounds that were tested at different concentrations, the specific initial rate [I] could be used, which is obtained upon dividing the initial rate by the transporter to lipid concentration. This specific initial rate [I] is based on the assumption that the initial rate increases linearly with the anionophore concentration.[41] The deviation from this linearity can be observed and might have different causes. In some cases, transport at high concentrations of anionophore is so fast that other processes, such as the homogenisation of the sample after NaCl pulse addition or the integration time of the spectrometer become rate limiting. This would give lower specific initial rate values at higher concentrations. On the other hand, at very low anionophore concentrations, little curvature is observed and the impact from the initial quenching typically dominates the curvature, giving values that do not represent the true transport rate. Thus, the described quantification of transport via fitting and calculation of $t_{1/2}$, I, or [I] is only valid if clear transport is observed and transport is not too fast. Alternatively, data from the lucigenin assay can also be analysed via a Hill plot analysis.[43,44] Experiments using the lucigenin assay with giant unilamellar vesicles (GUVs, ~20 µm diameter) and

confocal fluorescence microscopy made it possible to analyse the transport of chloride into individual GUVs (rather than over the ensemble of LUVs), confirming a linear relationship between the half-life of transport and the diameter of the GUVs, and allowing to estimate the rate per individual transporter.[15,45]

3.5 Troubleshooting

The lucigenin assay can potentially give false-positive results, where quenching of the fluorescence of lucigenin is observed without transport taking place. Such results can be obtained either if the quantity of lucigenin outside the liposomes is large compared to encapsulated lucigenin or if the membranes of the liposomes have a lower integrity. Therefore, the quality of liposomes with encapsulated lucigenin must be verified in absence of transporters (blank experiments).

- When the initial drop of unprocessed data is larger than 20-30% of the total intensity (5-10% is normal), too much lucigenin is present outside of the liposomes. This might be caused by a poor separation during gel filtration, or by lucigenin leaking out of the liposomes over time (see next point). In the first case, the collection of liposomes from the size exclusion column should be stopped earlier, or a longer column should be used. Alternatively, two commercial pre-packed columns can be placed in series one above the other. We use this strategy when working in Na_2SO_4 instead of $NaNO_3$ (see Section 4.5) or with SPBA instead of lucigenin as a probe (see Section 4.1).
- One of the disadvantages of the lucigenin assay is the intrinsic ability of lucigenin to permeate through lipid membranes. Therefore, once the external lucigenin is removed from the liposomes using gel filtration, a concentration gradient of lucigenin is present and the lucigenin starts to slowly leak from the liposomes into the external solution. Due to this effect, each prepared batch of liposomes has limited-shelf life, which can be extended a bit by storing the suspension in the fridge (not the freezer). Practically, the leaking of lucigenin can result in an increasing initial drop of fluorescence intensity over time in the individual transport experiments (see Figure S26), which could be misinterpreted to be very fast transport. Therefore, the prepared batch of liposomes should be used as soon as possible (within 2-3 h).
- An observed downward slope of lucigenin fluorescence intensity over time after addition of the chloride pulse during transport experiments in absence of anionophores suggests a poor quality of the liposomal membrane or the presence of an anionophore as pollution. The latter can be picked up from the extrusion kit or a size-exclusion column previously used for the preparation

of liposomes with a high concentration of highly active transporter pre-incorporated (see Section 4.2) after insufficient cleaning. Alternatively, in some cases the membrane is not completely impermeable to the chloride ions. A lower membrane quality is usually caused by a poor lipid quality, arising from using too old lipids, poor storage, not using freshly deacidified chloroform for lipid stock solution preparation, or inadequate handling of the lipids. Figure S27 shows examples of poor 'blank' transport curves due to problems with the lipids. Preparing new lipid solutions and replacing the size-exclusion column are recommended in this case.

- The solvent used for post-insertion of the anionophores can also have an impact on the integrity of the liposomal membrane or on the fluorescence of lucigenin. Therefore, the amount of organic solvent added should be rather small: preferably 5 µL is added to 3 mL of liposomes, while 10-20 µL can be added if necessary. While DMSO is commonly used in other transport assays,[46,47] it cannot be used in the lucigenin assay as it substantially quenches the fluorescence of lucigenin.[32] DMF also gives some quenching, while other solvents (MeOH, THF, acetone, and MeCN) do not cause any significant effect (Figure 6). None of the mentioned solvents did cause a higher permeability of the membrane towards chloride ions when used at 0.3% (v/v), see Figure S28. Note that water-immiscible solvents such as chloroform cannot be used for the post-insertion of anionophores.

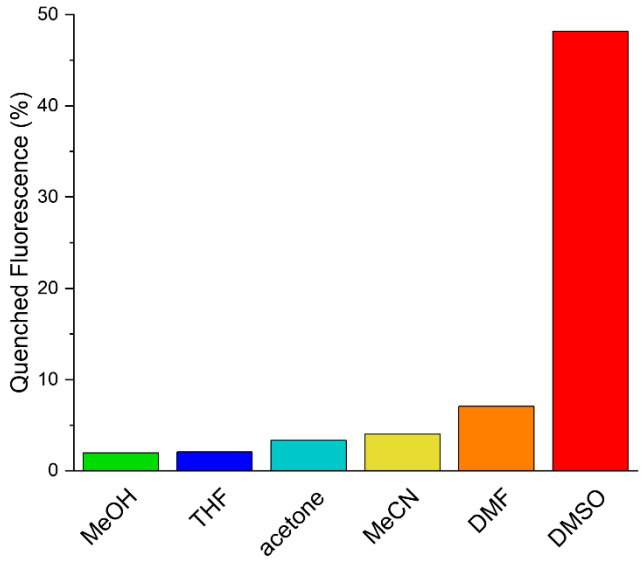

Figure 6. Effect of solvents on the fluorescence of liposomes without anionophore in the lucigenin assay. To 3 mL of liposome suspension in 225 mM $NaNO_3$, 10 µL of solvent was added and the fluorescence intensity after 150 s was compared to a control experiment in which no solvent was added.

- The fluorescence of lucigenin is temperature-dependent and therefore it is necessary to control the temperature inside the spectrometer and to equilibrate the temperature of the samples for 3-5 minutes before starting the experiment to avoid any impact on the results. The fluorescence

decreases linearly when the temperature increases as demonstrated in Figure 7. This means that if the temperature of the liposome suspension would increase during the run, the fluorescence intensity would slightly decrease, which may be misleading at low transporter loadings or when testing transporters with low activity. The first 30 s of the unprocessed transport curve (prior to addition of NaCl) should be constant when the temperature is equilibrated.

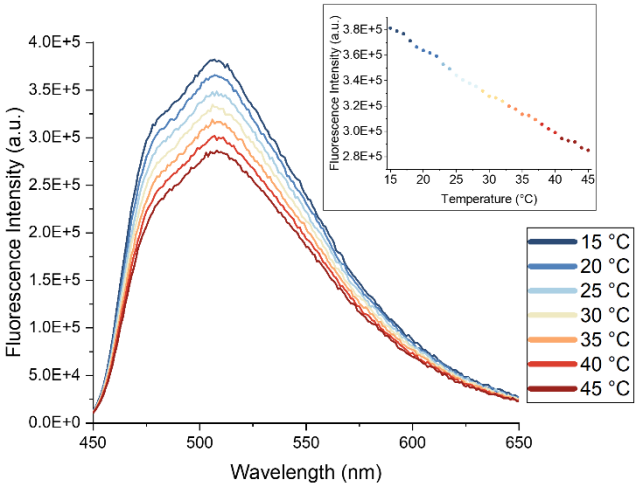

Figure 7. Effect of temperature on the fluorescence of lucigenin. Emission spectra of 1 µM lucigenin in mili-Q water at temperature range 15-45°C and the fluorescence intensity at 505 nm as function of the temperature (excitation at 430 nm).

- Strangely shaped transport curves can be obtained if the mixing of the NaCl solution with the liposomes is not optimal, because the stirring bar is absent, the stirring bar is spinning too slow or too fast, or if the added NaCl solution has ended up on the edge of the cuvette rather than in the liposome suspension. An alternative cause for strangely shaped curves can be the slow incorporation of a transporter. In this case, it is recommended to wait at least 3-5 minutes between the addition of the transporter and the addition of NaCl.
- Sometimes a strange or inconsistent behaviour can be observed after lysis of the liposomes. This is usually caused by a contamination present in the Triton X-100 solution or when the solution of the detergent was freshly prepared and is not yet fully homogeneous.

4 Variations on the standard lucigenin assay

The lucigenin assay as described in Section 3 can be adapted in different ways to gain additional insight into different aspects of the transport process, including the transport mechanism. In this section, different variations will be discussed.

4.1 Novel bis(3-sulfopropyl)-9,9'-biacridine (SPBA) assay

To study the activity of different transporters or to compare activities of a transporter at different concentrations, it is convenient to do the experiments using a single large batch of liposomes. However, in the lucigenin assay, this is not possible due to gradual leaking of the dye from the liposomes (see Section 3.5). In order to avoid leakage from the liposomes, we propose the use of an alternative more hydrophilic bis-acridinium probe that would not partition into and diffuse through the lipid bilayers. Complete removal of external probe could thus in principle be achieved, while the good fluorescence properties of lucigenin should be preserved. Our attention was caught by the lucigenin derivative bis(3-sulfopropyl)-9,9'-biacridine (SPBA), prepared by Werner *et al*.[48,49] The presence of two charged sulphonate groups, its three-step synthesis, and Stern-Volmer constants only slightly lower than for lucigenin, made it an ideal candidate for testing. When prepared and tested, the measured fluorescence properties were found to be in agreement with the literature (

Table **2** and Figure S13-S20). An SPBA solution (in 225 mM $NaNO_3$) was successfully encapsulated in liposomes following the procedure described for lucigenin in Section 3. When tested without transporter, no leaking of SPBA was observed, even after 2-7 days following the preparation of the liposomes (Figure 8). Transport results obtained with SPBA were identical to those obtained with lucigenin (Figure S29), making SPBA a good alternative to lucigenin for experiments over longer time scales (24 h instead of 2-3 h).

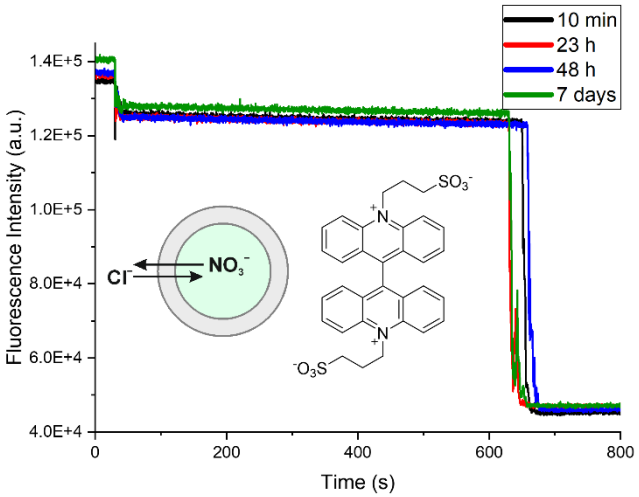

Figure 8. Unprocessed data of blank experiments in conditions to monitor chloride/nitrate antiport. The liposomes were prepared in 225 mM $NaNO_3$ solution with 0.8 mM SPBA encapsulated and a 25 mM NaCl pulse was added at t = 30 s. Runs were measured at different times after the preparation of the liposomes (indicated in the legend) and the liposome suspension was stored in the fridge.

Table 2. K_{SV} values for various anions for SPBA or lucigenin in pure water (1 µM dye concentration).

Anion	K_{SV} (M^{-1}) (SPBA, this work)	K_{SV} (M^{-1}) (SPBA, Werner *et al.*[48])	K_{SV} (M^{-1}) (lucigenin, this work)
Chloride	129	124	351
Bromide	181	209	556
Iodide	333	298	777
Nitrate	3	2	3
Sulphate	4	3	13
Acetate	19	-	45
Bicarbonate	13	-	28
HEPES	27	-	39
Hydroxide	80	-	150

4.2 Pre-incorporation and deliverability

Some transporters have a high lipophilicity, which results in a poor deliverability. Even when very efficient in anion transport, their activity will appear to be poor when post-inserted from an organic solvent into pre-formed liposomes.[50,51] To assess the transport activity of lipophilic transporters without bias from their deliverability, they can be pre-incorporated during the formation of the liposomes. This is done in the very first step. A transporter solution is added together with the lipid solutions in the round bottomed flask during the preparation of the lipid film (Section 3.2). For this, the transporters can be added as a solution in chloroform, or in another appropriate organic solvent that is miscible with chloroform and easily evaporated, such as MeOH, MeCN, THF, hexane, or small amounts of acetone, or mixtures of solvents. DMSO and DMF are more difficult to remove from lipid film afterwards but have been used if necessary. The subsequent steps of liposome preparation are to be performed as described in the main procedure. It should be noted that the pre-incorporation of high concentrations (>0.1 mol%) of large or poorly soluble compounds can cause problems in the extrusion step, which can in certain cases limit the maximal concentration of transporter that can be studied via pre-incorporation. After performing experiments using post-insertion and pre-incorporation at identical transporter to lipid ratios, the deliverability of an anionophore can be quantified by dividing the initial rate of transport upon post-insertion by the initial rate obtained by pre-incorporation.[50,52,53]

4.3 Lucigenin assay with different M^+ and at different pH

The lucigenin assay can be employed to study the effect of different metal cations on the transport process, which can aid to elucidate possible co-transport of M^+ and Cl^- by the studied anionophore.[54,55] The assay can be adapted by replacing the $NaNO_3$ solution for $LiNO_3$, KNO_3, $RbNO_3$ or $CsNO_3$ (and by

using the corresponding MCl salts).[56] A clear change of the rate of Cl^- transport indicates that the cation plays a role in the transport process. On the other hand, similar transport rates in all salt solutions (see for instance Figure S30), suggest that the tested cations are not co-transported with chloride or that the transporter is not selective to any of them.

The effect of pH on the transport process can also be studied using the lucigenin assay, as the fluorescence of lucigenin and $K_{SV}(Cl^-)$ remain approximately unchanged in a range of pH 4-10 (see Figures S9-S11). Considering the K_{SV} for OH^-, the fluorescence of lucigenin would start to decrease significantly at hydroxide concentrations higher than 0.1 mM (pH >10) and the sensitivity towards to Cl^- would be lower because of fluorescence quenching by hydroxide (see Figure S8). Thus, anionophoric activity can be studied conveniently by the lucigenin assay at pH 4-9.[57–59]

4.4 Cl^-/HCO_3^- antiport

The lucigenin assay can also be adapted to monitor chloride/bicarbonate antiport, by using a $NaHCO_3$ solution rather than a $NaNO_3$ solution. This, however, has certain disadvantages that are to be considered. Firstly, the quenching of lucigenin fluorescence is higher by bicarbonate than by nitrate. Thus, transport of chloride into liposomes produces a smaller response: after lysis of the liposomes, the final level of normalized fluorescence intensity (F/F_0) is higher by 0.15-0.20 in bicarbonate solution than in nitrate solution. Secondly, bicarbonate solutions are not stable over time unless stored under a CO_2 atmosphere, thus these should be prepared freshly. The pH value of freshly prepared 225 mM $NaHCO_3$ solution is around 8.3, and therefore a small amount of H_2SO_4 is added, to decrease the pH to 7.5-7.8, to better reflect physiological conditions. However, release of CO_2 over time leads to a decrease of the bicarbonate concentration and a basification of the solution, a process that is faster at lower pH. Due to quenching of lucigenin fluorescence by bicarbonate, using the same $NaHCO_3$ solution over multiple days/weeks, results in different values of initial and final fluorescence levels. Therefore, the best practice is to prepare a fresh bicarbonate solution daily, implying the preparation of a fresh lucigenin solution for lipid film hydration as well.

The importance of fresh $NaHCO_3$ solutions and working at a consistent pH is demonstrated in Figure 9. The transport assay with pre-incorporated anionophore **2**[60] was performed under extreme conditions. Either no acid was added (black curves) or a large volume of H_2SO_4 was added (which results in bicarbonate decomposition). Since there was only a small concentration of bicarbonate left in the acidic solution, the transport started at much higher levels of fluorescence than in the experiment without acid. This causes different overall changes in fluorescence intensities over the course of the experiments. After

removal of the initial drop and normalization of the data, there appears to be more chloride transport at lower pH. However, the shapes of the curves are similar, as confirmed by the close values obtained for the half-lives (32 s at pH 6.3 and 41 s at pH 8.4, calculated as described in the Section 3.4). Similar effects have been observed when running the experiments with new vs. old bicarbonate solutions. We note that other mechanisms can also play a role in the apparent chloride/bicarbonate antiport observed in the lucigenin assay.[61]

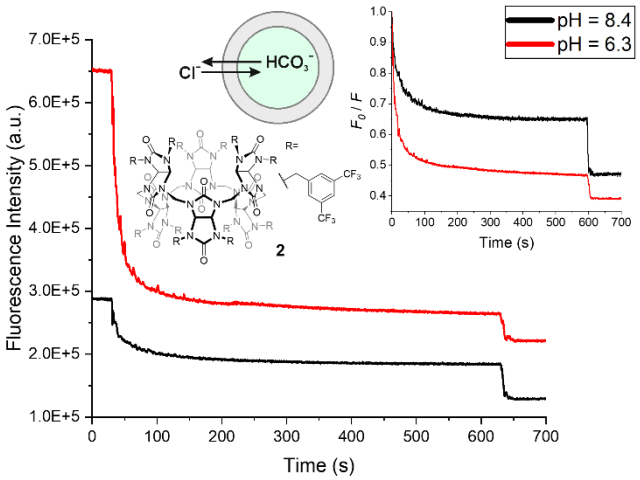

Figure 9. Chloride/bicarbonate antiport by **2** pre-incorporated at 1:25000 transporter:lipid ratio. The liposomes were prepared in $NaHCO_3$ solution with 0.8 mM lucigenin encapsulated. The transport was initiated with a 25 mM NaCl pulse. The curves are an average of 3 individual runs.

4.5 Cl^- transport in sulfate solutions

The lucigenin assay can be used in Na_2SO_4 or K_2SO_4 solution to study chloride/sulfate antiport or other processes. The concentration of M_2SO_4 solution is usually 112.5 mM to have 225 mM M^+ concentration. The sulfate anion is highly hydrated and most of the reported compounds are therefore not able to transport it, and thus do not show any chloride transport when working in Na_2SO_4 solutions.[39,62–64] However, some compounds have been reported to act as sulfate transporters,[65] and we found that bambusuril **2** shows some chloride transport in Na_2SO_4 as well (Figure S31).

The absence of sulfate transport by most transporters allows the study of alternative transport mechanisms. For example, co-transport of propylammonium cations and chloride anions was demonstrated in Na_2SO_4.[66] Furthermore, chloride uniport by anionophores can be studied in a K_2SO_4 solution in presence of the cationophore valinomycin. When either valinomycin or a chloride uniporter is present, no decay in fluorescence intensity should be observed, as a potential gradient would build up upon transport of cations or anions alone. The valinomycin is typically post-inserted into the membrane before the experiment at 0.1 mol% concentration with respect to the lipids (by addition of 5 µL of a 0.24

mM solution in methanol), ensuring fast transport of K^+, so that chloride uniport by the anionophore would be rate-limiting.

The use of the lucigenin assay to measure chloride uniport is demonstrated in Figure 10 using anionophore **1**, which was previously shown to be an effective chloride uniporter when studied in the HPTS assay.[42] In the absence of an anionophore, no transport of chloride is observed upon addition of a KCl pulse, even in the presence of valinomycin (black and red curves in Figure 10a). When **1** is pre-incorporated and valinomycin is added to facilitate potassium transport, equilibration of intra- and extravesicular chloride concentration caused by uniport of chloride by **1** is clearly observed (green curve in Figure 10a). In contrast, when **1** is pre-incorporated but no valinomycin is added, a smaller decrease in fluorescence intensity is observed (blue curve in Figure 10a). This decrease in fluorescence is larger compared to the blank experiments (without anionophore), which might be caused by a very small amount of chloride uniport (before the potential gradient stops the transport), as well as some Cl^-/NO_3^- antiport, due to the presence of nitrate counter anions in commercial lucigenin salt. Substituting lucigenin for SPBA as a probe for this type of experiments eliminates the Cl^-/NO_3^- antiport and allows for a clean observation of chloride uniport activity, as shown in Figure 10b.

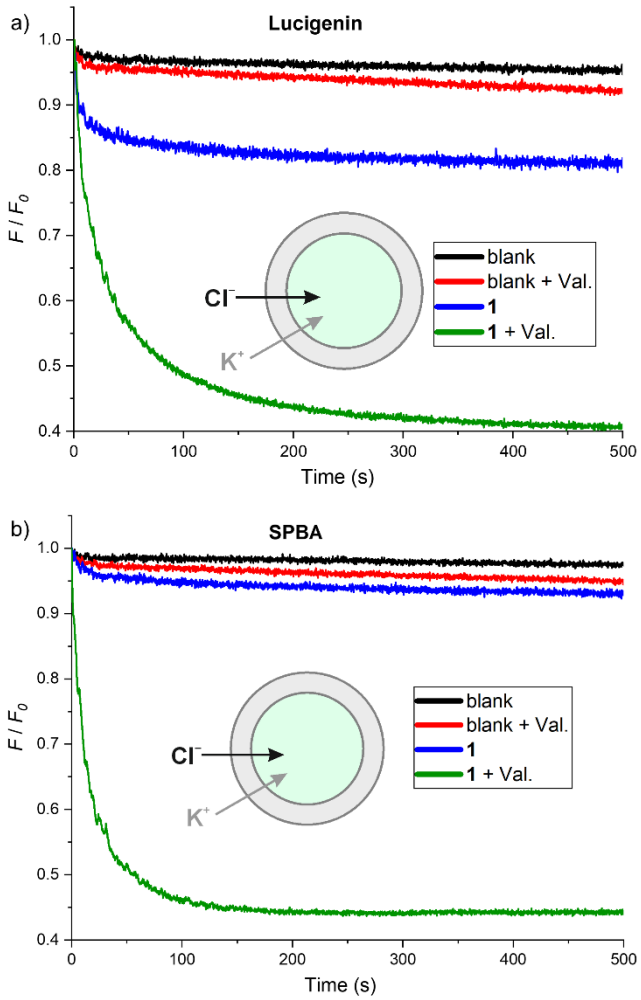

Figure 10. Chloride uniport by **1** pre-incorporated at 1:1000 transporter:lipid ratio. Liposomes were prepared in 112.5 mM K_2SO_4 solution with 0.8 mM lucigenin (a) or SPBA (b) encapsulated. Valinomycin (Val.) was post-inserted at 1:1000 transporter:lipid ratio. The transport was initiated with a 25 mM KCl pulse. The curves are an average of 3 individual runs.

4.6 $RCOO^-/NO_3^-$ exchange

The interest in studying the transmembrane transport of carboxylates is founded on their essential role in diverse cellular processes,[48,49] as well as the presence of this functional group in many active pharmaceutical ingredients (APIs).[16] The fluorescence of lucigenin is quenched by carboxylates, which can be used to monitor the transport of carboxylates through phospholipid membranes, typically in an antiport process with nitrate.[15,16]

As the quenching of the fluorescence of lucigenin depends on the organic moiety of the carboxylate, it is crucial to record the emission spectra of lucigenin in the presence of the selected carboxylate before performing the transport experiments. For instance, aromatic carboxylates such as benzoate quench the lucigenin emission more than aliphatic carboxylates such as acetate (Figure 11a). The carboxylate transport experiments can be performed following the standard protocol for liposome preparation as described in Section 3.2. However, the lucigenin solution and salt solution must be prepared using a

buffer at the selected pH value (such as 225 mM $NaNO_3$, 5 mM HEPES, pH 7), to control the acid-based speciation of the carboxylate appropriately. To perform the transport experiment, the fluorescence intensity decrease is recorded after the anion pulse (25 mM $RCOO^-$). It should be noted that spontaneous diffusion of carboxylates has been observed in liposomes prepared in $NaNO_3$ solution in absence of a transporter.[15,16] However, the emission intensity of lucigenin decreases significantly faster when an anionophore assists the transport of the carboxylate, as shown for transporter **3**[67] at 1:1000 transporter:lipid ratio (Figure 11b). Measuring the pH of the liposome suspension before and after the anion pulse is recommended as it could be significantly affected by the addition of the carboxylate solution. Finally, some carboxylates have displayed unusual behaviour on the lucigenin emission (Figure S33), which limits the employment of this assay for certain carboxylate compounds.

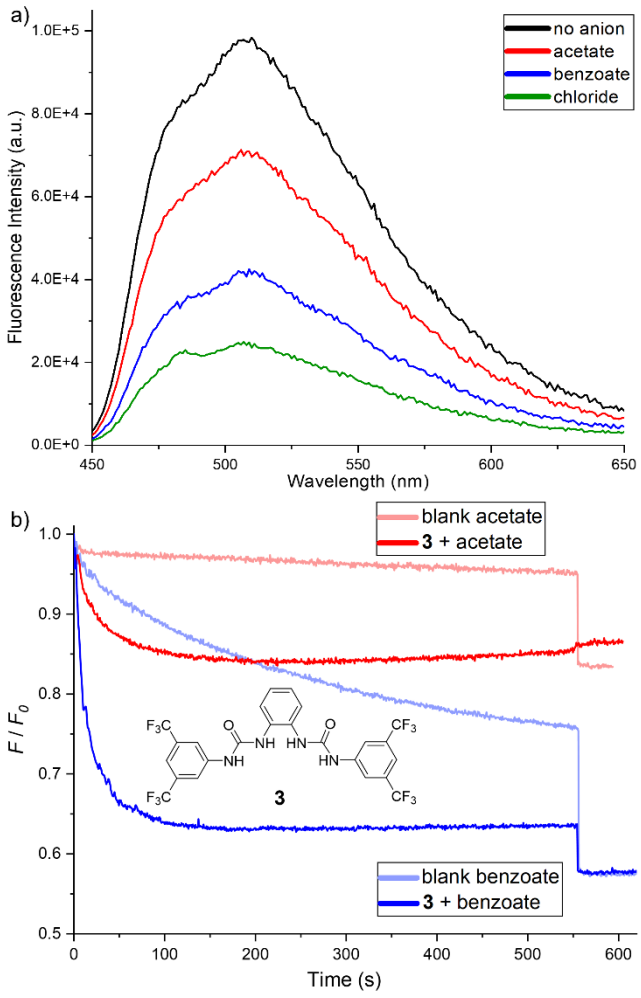

Figure 11. a) Emission spectra of lucigenin (0.8 µM) in 225 mM NaNO3 and 5 mM HEPES at pH = 7 upon addition of various carboxylates or chloride (25 mM). b) Carboxylate/nitrate antiport by 3 post-inserted at a 1:1000 transporter:lipid ratio. Liposomes were prepared in 225 mM NaNO3 and HEPES 5 mM at pH = 7, with 0.8 mM lucigenin encapsulated. The transport was initiated with a 25 mM RCOONa pulse. The curves are an average of 3 individual runs.

4.7 Varying the lipid composition

Using a different lipid composition for the liposome preparation can help to elucidate the transport mechanism. To test whether the transporter acts as a channel or a mobile carrier, DPPC (1,2-dipalmitoyl-*sn*-glycerophosphatidylcholine) can be used as lipid. The DPPC has a transition temperature between the fluid and gel phase at a temperature (T_m) of 41 °C. The working principle here is that, if the channel is a stable assembly and spans the membrane, the transport process should still function, even in the gel phase.[68] On the other hand, mobile transporters need to diffuse through the membrane to perform transport and hence transport will be hindered by the rigid membrane.[43,60,66] Note that care has to be taken when interpreting the results, as certain synthetic transporters can be 'squeezed' out of the membrane at temperatures below T_m.[69]

When using DPPC, liposomes must be prepared at a temperature above T_m, and therefore all the operations (sonication, stirring, freeze-thawing and extrusion) are done at elevated temperature (45-50°C). An extrusion kit with a heating block at 50°C must be used, while the gel filtration can be done at room temperature. The quality of the blank experiment at 45°C is usually worse than the one at 25°C or when using POPC/cholesterol 7:3, due to the faster leaking of lucigenin out of liposomes at higher temperatures.

Another way to test for the channel versus mobile carrier mechanism is to use a higher cholesterol content. Increasing the amount of cholesterol in the POPC/cholesterol membrane decreases the fluidity of the membrane, which should decrease the mobility of carriers.[64] A transmembrane channel, however, should not be affected, as it spans the whole membrane. Usually, a dramatic change from 30% to 40-50% cholesterol is observed,[62,60] thought to be caused by the "super-toughening" effect[70] of the vesicle membrane when increasing the cholesterol content above 30%.

In addition to the experiments with DPPC or higher cholesterol concentrations, the lucigenin assay can also be used with DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine)[71,72] and analogous lipids of different lengths. This allows to study the impact of the diffusion on the transport process.[73] Furthermore, anionic lipids can be used, such as POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)) or POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine). Incorporating 35% of these lipids (with 35% POPC and 30% cholesterol) results in a repulsion of anions at the aqueous/lipid interface and thus in lower rates of anion transport.[66]

4.8 Limitations

As described in Sections 4.3-4.5, the $NaNO_3$ solution in the lucigenin assay can be readily replaced by solutions of other salts to study transport processes different from Cl^-/NO_3^- antiport. However, certain salt solutions cannot be employed in the lucigenin assay. For example, sodium gluconate and *N*-methyl-D-glucamine-based salts (such as $NMDGH^+NO_3^-$) are commonly used in transport studies,[74] but are not compatible with lucigenin. When dissolved in solutions with these carbohydrate-based salts, the lucigenin changes colour and loses its fluorescence over time, most likely due to decomposition following oxidation. Lucigenin can be dissolved in a solution of sodium phosphate rather than $NaNO_3$ without any problem, but this system cannot be used to study $Cl^-/H_2PO_4^-$ antiport, because the buffering capacity of the solution is high enough for Cl^-/OH^- antiport or H^+Cl^- symport to take place. This results in a transport response by any compound that can perform Cl^-/OH^- or H^+Cl^- transport and does not inform on the ability of a compound to perform $Cl^-/H_2PO_4^-$ antiport.

5 Conclusions

In conclusion, we have explained and demonstrated that the lucigenin assay is a robust and versatile method to study anion transport by fluorescence spectroscopy, relying on the quenching of the fluorescence of lucigenin. We have described in detail how to study chloride/nitrate antiport reliably. Additionally, many different transport processes can be studied, such as chloride/bicarbonate antiport, chloride uniport, and carboxylate/nitrate antiport. This assay furthermore permits to study lipophilic compounds via pre-incorporation or to change the composition of the lipids.

However, the lucigenin assay has certain limitations, as the lucigenin probe can slowly leak out of liposomes and because it is commercially available as the dinitrate salt. For these reasons, we have employed the more hydrophilic lucigenin analogue SPBA and demonstrated that this does not leak out of liposomes, making it suitable for transport studies over longer times. Furthermore, the absence of nitrate ions allows to study chloride uniport without interference from chloride/nitrate antiport.

The methods described here open the way to further applications of the lucigenin assay to identify and study novel synthetic anion transporters.

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Conflict of Interest

The authors declare no conflict of interest.

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