Garcia-Celma et al., http://www.jgp.org/cgi/content/full/jgp.201210927/DC1

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A detailed tutorial describing the experimental setup and the measurement protocol of a SSM experiment is included as a PDF.

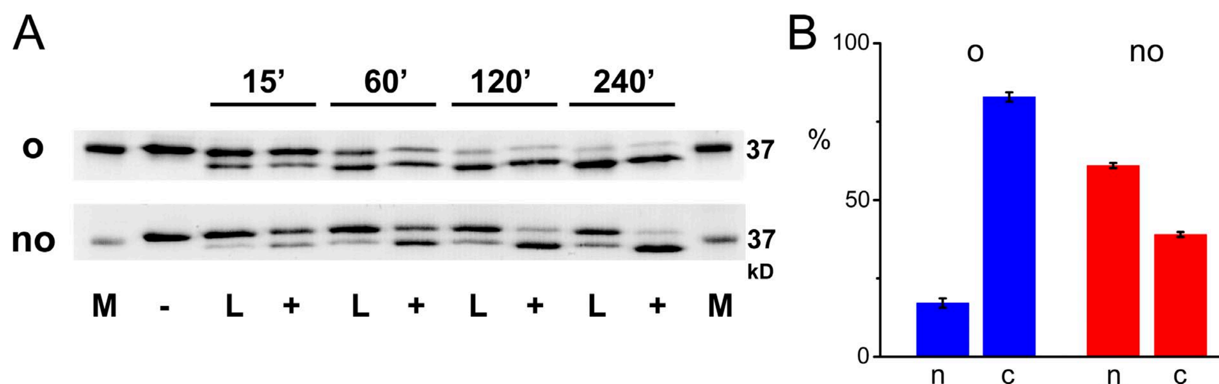

Figure S1. Reconstitution into liposomes. (A) Accessibility of the C terminus of EcClC in proteoliposomes prepared by destabilization of preformed vesicles followed by rapid dilution (o) and by dialysis after mixing with detergent-solubilized lipids (no). The SDS-PAGE gels show a time course of proteolysis in liposomes after addition of the protease LysC (L). Control lanes (+) show the progress of proteolysis in samples containing protein extracted from the liposomes by addition of 2% decyl-β-D-maltoside (DM). The undigested protein is shown in the lane labeled "−," the molecular weight marker of 37 kD is shown in lane M. (B) Quantification of proteolysis. Bars show averages of four different gels, errors bars indicate SEM. Non-cleaved samples after 3–4 h of digestion are labeled as "n"; "c" shows the fraction of cleaved protein. Nearly quantitative removal of the C terminus seen in the reconstitution by rapid dilution (o) used in this study suggests a predominant outside-out orientation of the protein. Two bands of about equal intensity of cleaved and noncleaved protein in proteoliposomes prepared by dialysis indicate the presence of two populations of transporters with inside-out and outside-out orientations. This method was previously described to lead to nonoriented incorporation of the protein.

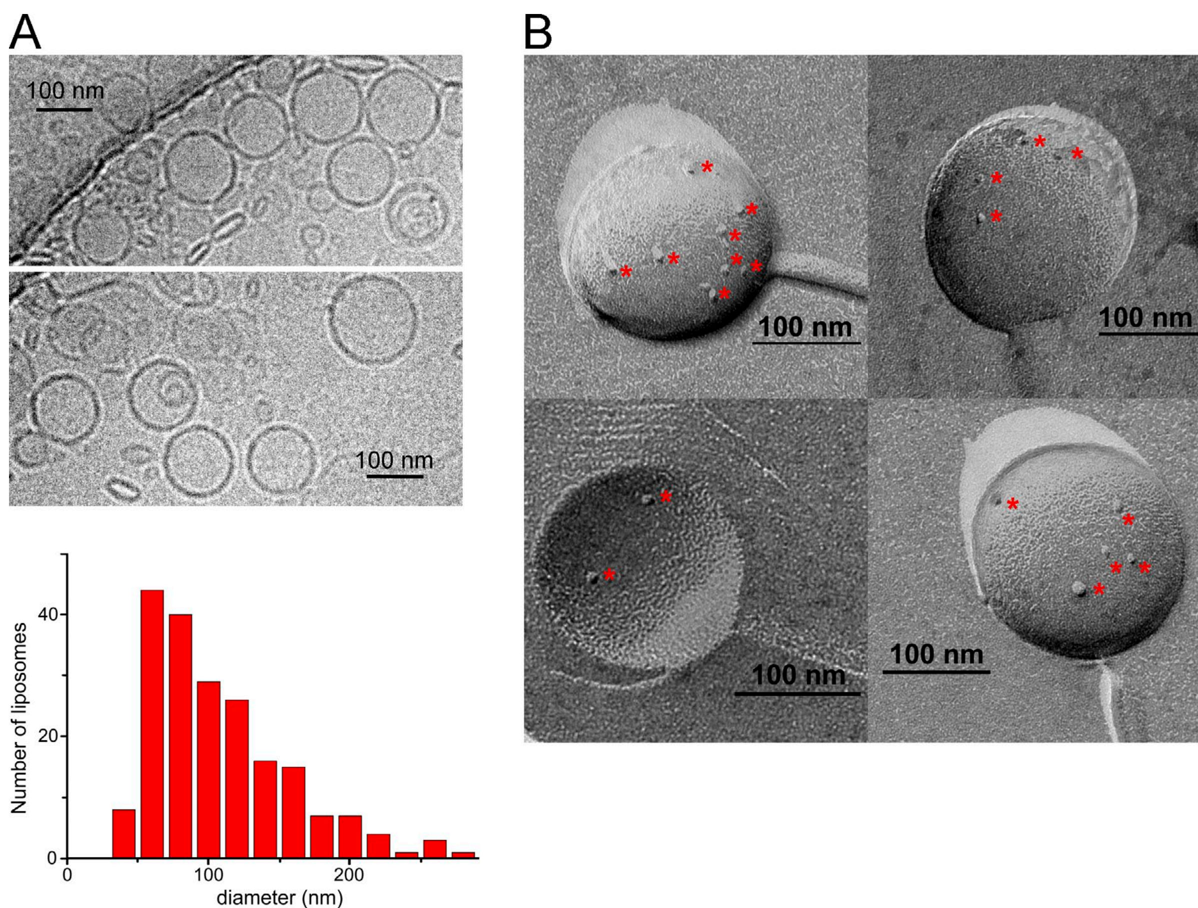

Figure S2. Vesicle size and protein density. (A) Distribution of liposome diameters obtained from cryo-electron microscopy. Sections of electron micrographs (top) and a histogram of the size distribution of 201 vesicles (bottom) are shown. (B) Freeze-fracture electron micrograph of vesicles reconstituted at an LPR of 25:1. Reconstituted transporters are indicated by asterisks.

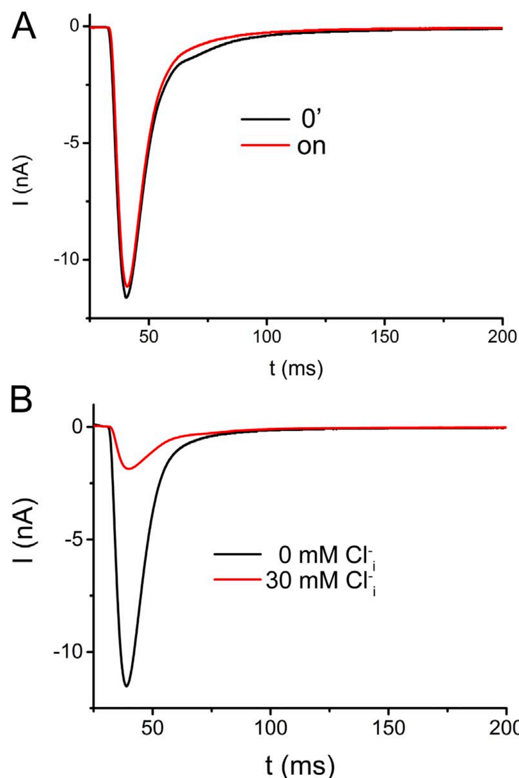

Figure S3. SSM recordings. (A) Stability of the vesicles adsorbed on the SSM electrode. Transient currents recorded upon a 300 mM Cl^- concentration jump at pH 4.0 immediately after adsorption of the vesicles (0') and after an overnight incubation with nonactivating solution (on) show a quantitatively similar response. (B) Recording from chloride-containing vesicles. Transient currents recorded upon a concentration jump to 300 mM Cl^- at pH 4.0. The red trace shows the current response from liposomes reconstituted in 30 mM Cl^- and incubated with a nonactivating solution containing the same chloride concentration. The black trace shows data obtained from proteoliposomes reconstituted and recorded under standard conditions (0 mM Cl^-). Proteoliposomes were reconstituted in parallel with the same batch of protein. To maintain osmotic balance, the concentration of mannitol of the nonactivating solution was decreased accordingly.

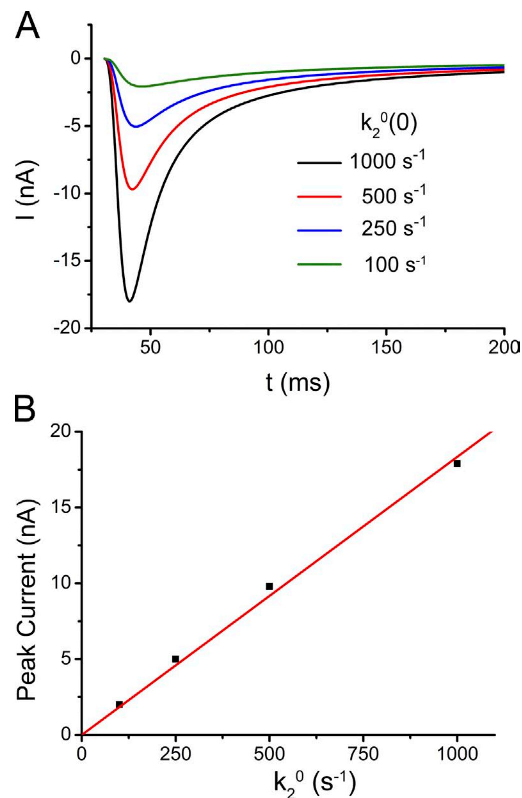

Figure S4. Influence of rate constants on transient currents calculated from a transport model. (A) Time dependence of currents obtained from a numerical simulation of a three-state kinetic model with different values of the rate limiting step. (B) Approximated linear relationship (red) between the simulated current maxima and the rate constants.

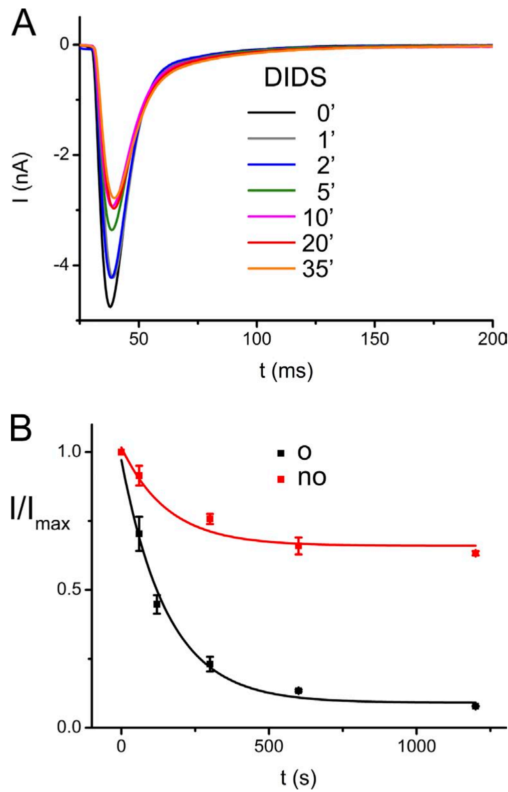

Figure S5. Irreversible inhibition of EcClC by DIDS in nonoriented samples. The current response was measured after incubation of vesicles with 1 mM DIDS for different periods. Experiments were recorded at pH 4.0 from the same sample. Proteoliposomes were prepared by dialysis of detergent solubilized lipids and contain transporters in both orientations. (B) Time course of inhibition by DIDS in oriented (o) and nonoriented samples (no). Both lines show a fit to an exponential decay with a time constant of 156 ± 16 s. Error bars indicate SEM calculated from three independent experiments. Currents were quantitatively inhibited in "o" vesicles and only by ~40% in "no" vesicles.

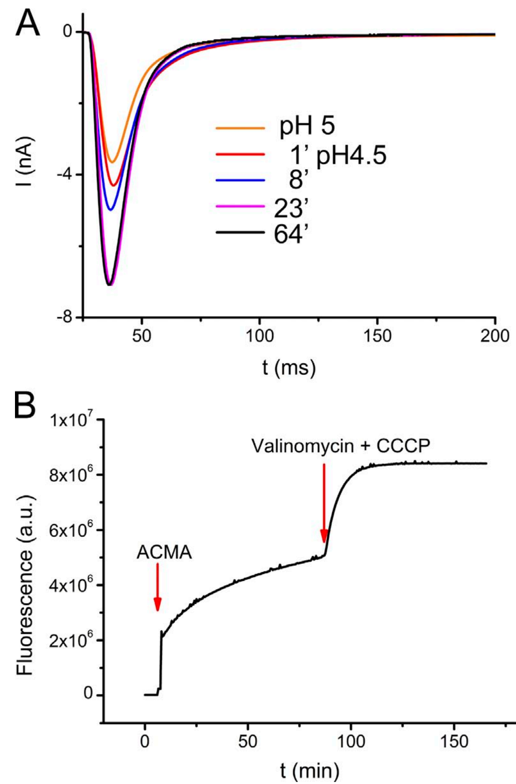

Figure S6. pH equilibration in liposomes. (A) Time dependence of the transient currents measured from vesicles adsorbed to the SSM after a pH change from 5.0 to 4.5. The pH was changed in both activating and nonactivating solutions. The current increase saturates after 20 min of incubation at lower pH. (B) Proton permeability of liposomes. The time-dependent diffusion of protons out of liposomes upon a change from pH 4.0 to pH 7.8 was monitored by the increase in the fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA). Arrows indicate the addition of ACMA and the addition of FCCP and valinomycin, which dissipates the pH gradient and the membrane potential.

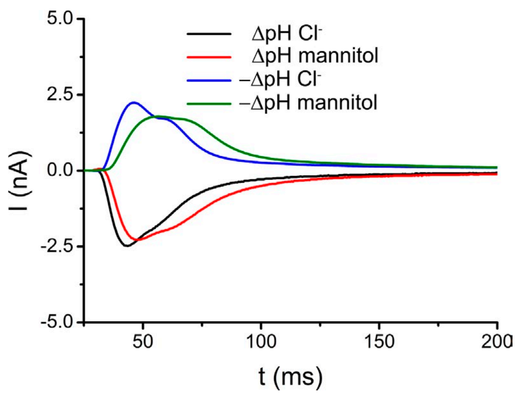

Figure S7. Proton-induced currents in the mutant E148A. The traces were recorded upon pH jumps from pH 3.8 to 6.0 (black) and from pH 6.0 to 3.8 (blue). Activating and nonactivating solutions contained 300 mM Cl^-. Background currents induced by pH jumps were recorded from solutions where Cl^- was replaced by 600 mM mannitol.

Recording of EcClC by SSM Electrophysiology

The tutorial provides a detailed protocol of the SSM experiments. It is divided into four chapters: describing the reconstitution of EcClC by rapid dilution, the preparation of the lipid solution for the SSM, a measurement protocol and a description of the setup.

1. Reconstitution of EcClC by rapid dilution

1) Transfer 500 µl of *E. coli* total lipid extract (Avanti Polar Lipids, 100500C, 20 mg/ml in chloroform) into a glass vial.
2) Dry the lipids under a nitrogen stream while rotating the vial. Lipids adhere to the glass wall as thin film.
3) Add 1 ml of reconstitution buffer (100mM potassium phosphate, 25 mM citrate adjusted with KOH to pH 7.6) to the vial to reach a final lipid concentration of 10 mg/ml.
4) The suspension is sonicated until clarity.
5) Extrude the suspension 21 times thorough a 400 nm filter with a LipoSofast-Basic extruder (Avestin, Inc.).
6) Prepare 200 µl aliquots of pre-formed liposomes. The aliquots can be "flash-frozen" in liquid nitrogen and stored at -80° or used immediately for the next step.
7) Take 200 µl of pre-formed liposomes and add 7 µl of 15% OG solution to destabilize the pre-formed liposomes with 0.5% OG. Mix well with the help of a pipette while avoiding the formation of air bubbles.
8) Incubate the mixture for 30 minutes on ice.
9) For the reconstitution at a LPR of 25, add 80 µg of EcClC (concentrated to 10 mg/ml) to the liposome-OG mixture. Mix well with the help of a pipette and incubate for 5 min on ice.

10) Dilute the mixture in 100 ml of ice-cold reconstitution buffer under gentle stirring. The lipid-OG-protein mixture is added slowly to the ice-cold buffer in the cool room.

11) Centrifuge the diluted sample at 504'000 g for 1 h with a Type 70 Ti Rotor (Beckman Coulter).

12) The liposomes will appear as a very small pellet. Remove the supernatant and suspend all the pellets in 8 ml of fresh reconstitution buffer.

13) Pellet the liposomes again at 287'000 g with a MLA-55 Rotor (Beckman Coulter).

14) Resuspend the pellet in 400 µl of fresh reconstitution buffer and transfer the sample to a 1.5 ml microcentrifuge tube.

15) Mixing with a pipette and sonication are used to homogenize the sample.

16) Prepare 43 µl aliquots and store at -80° after “flash-freezing” in liquid nitrogen.

2. Preparation of the lipid solution for the SSM

1) Add 375 µl of a stock solution of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, 850356C, 20 mg/ml in chloroform) together with 25 µl of octadecylamine (Sigma-Aldrich, 74750-25G, 5 mg/ml in chloroform) to a glass vial.

2) Dry the mixture under a nitrogen stream while rotating the vial.

3) Add 500 µl of n-decane to the dried film to obtain a lipid solution of 15 mg/ml and 1:60 (w/w) octadecylamine.

4) Store at -20° in an air-tight glass vial (no longer than one month).

3. SSM Setup and measurement procedure

The SSM setup is shown in Fig 1. It is composed of the following items: air pressure source, manometer, current amplifier, function generator, valve drivers, computer and a faraday cage containing the cuvette and the solutions for the measurement.

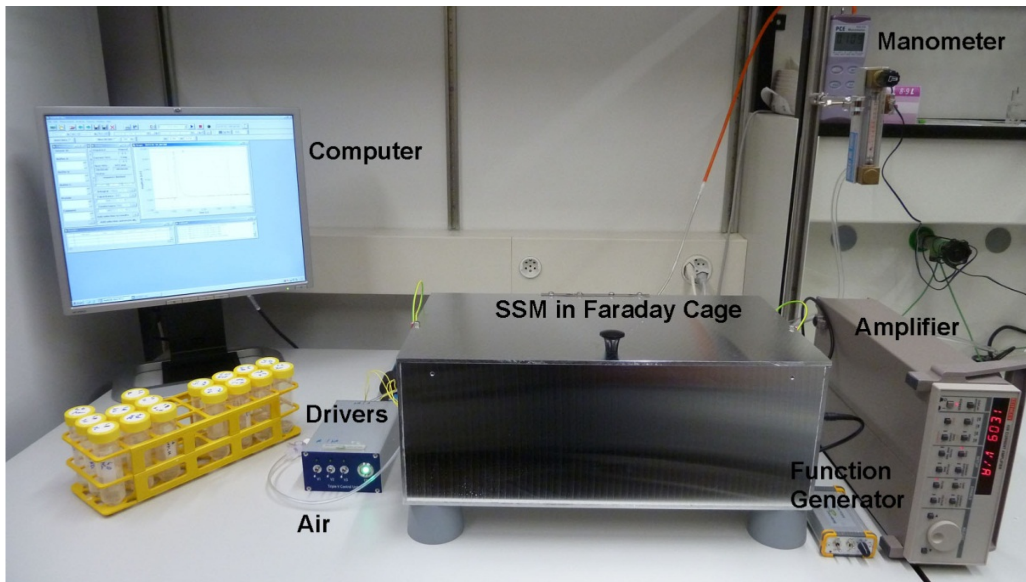

Fig. 1. SSM Setup. The components of the setup are labeled (from top left to bottom right: computer, manometer, air tubing from the air pressure source, drivers of the valves, faraday cage, function generator and current amplifier).

Fig. 2 provides a view into the faraday cage. Two 50 ml conical tubes are filled with the non-activating and the activating solution respectively (placed in the green support). The flow of solutions is controlled by two valves that are usually closed but that can be activated by drivers placed outside the faraday cage. Two three-way stopcocks pass the solutions either to the SSM or to the waste container. The cuvette is mounted on plastic support plate and contains the SSM. Connections: (i) The air pressure source is connected to the 50 ml conical tubes to provide the pressure to initiate the flow of solutions; (ii) The outlet of the tubes is connected the cuvette via valves and the solutions passing the SSM are directed to the waste; (iii) Yellow cables connect the valves with their corresponding drivers; (iv) Reference- and measurement

electrodes are connected to the ground and current amplifier (Keithely, 428-PROG/E) respectively via coaxial cables. The measuring electrodes were purchased from the Fraunhofer Institute for Surface Engineering and Thin Films (IST).

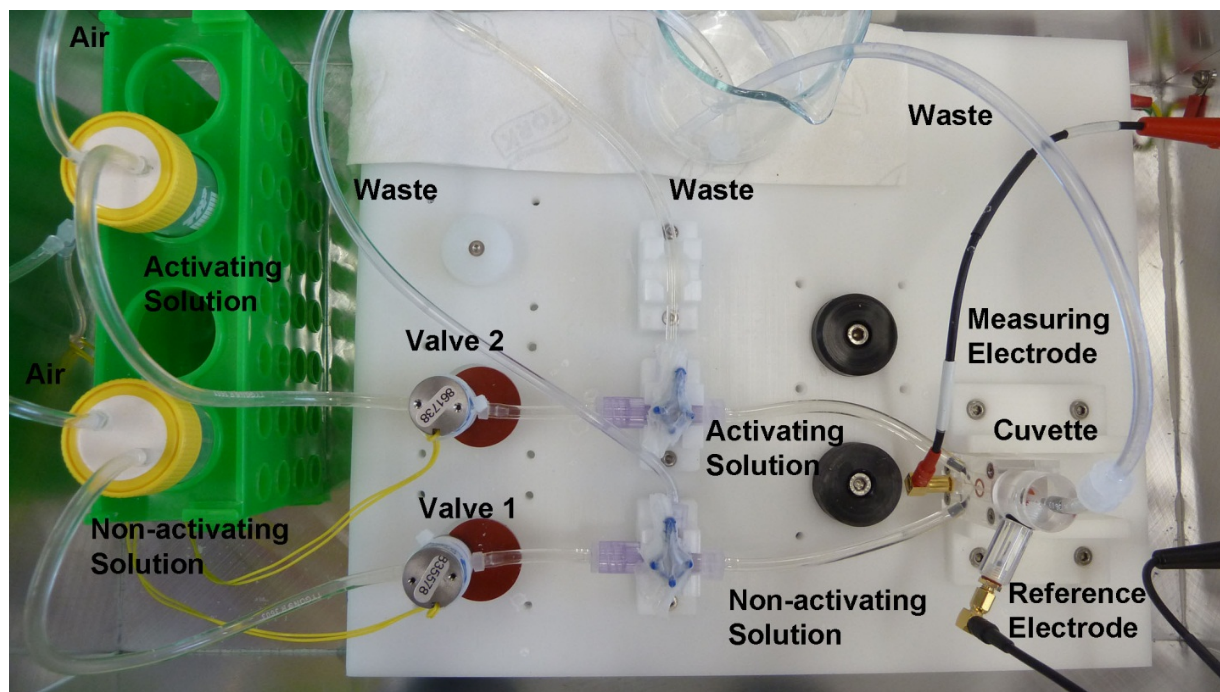

Fig. 2. View into the faraday cage. The components of the setup are labeled. The cuvette contains the SSM electrode.

3.1. Preparation of the setup

1) Fill each 50 ml tube with the corresponding non-activating and activating solutions.
2) Mount the reference electrode into its electrode assembly. Prepare the agar bridge and screw-in the bleached reference electrode. Fill the entire reference electrode assembly with buffer to avoid the presence of air-bubbles (Fig. 3A)
3) Take gold electrode from its storage solution (10 mM octadecanethiol (Sigma, 74731-50G) in ethanol) and clean the electrode with ethanol. Dry the ethanol under nitrogen stream.
4) Place the gold electrode on the rear part of the cuvette and add a small amount (~3 µl) of the lipid solution to the circular active area of the electrode (Fig. 3).

5) Screw the front and rear parts of the cuvette together. Be careful to place the circular active area of the measuring electrode below the inlet bore. The measuring chamber is defined by the gold electrode and the O-ring and has a volume of 17 µl. Connect the reference electrode assembly (Fig. 3).
6) To remove the excess of lipids, place the cuvette in the flow pathway and perfuse the measuring chamber with non-activating and activating solutions. Use a pressure of 0.1 bar which corresponds to a flow rate of 0.46 ml/s in our experimental setup.
7) Use the function generator to apply a triangular wave function on the SSM. In that way the formation of the lipid monolayer is monitored by the measurement of the capacitance of the electrode.
8) Let the membrane incubate for at least 30 minutes to allow the thinning process to complete.
9) Re-check the capacitance as described in 7. A value of around 3 nF is to be expected. If the value is considerably higher the membrane has to be re-prepared.

3.2. Addition of the vesicles

1) The cuvette is filled with non-activating solution and the reference electrode assembly is removed from the cuvette.
2) 43 µl of vesicle suspension are thawed and gently sonicated (3x for few seconds).
3) The three-way stopcocks are rotated by 45° to close the flow pathway.
4) The liposome suspension is taken up with a pipette and the pipette is placed at the outlet of the cuvette (Fig. 3 middle).
5) One of the three-way stopcocks is rotated to open the connection between the waste container and the pipette.
6) The vesicle suspension is added to the cuvette. Before releasing the plunger of the pipette, the flow pathway has to be closed again.

7) The liposomes are allowed to adsorb for 1 hour.

8) After the incubation, the reference electrode assembly is screwed again to the cuvette and the sample is ready for measurement.

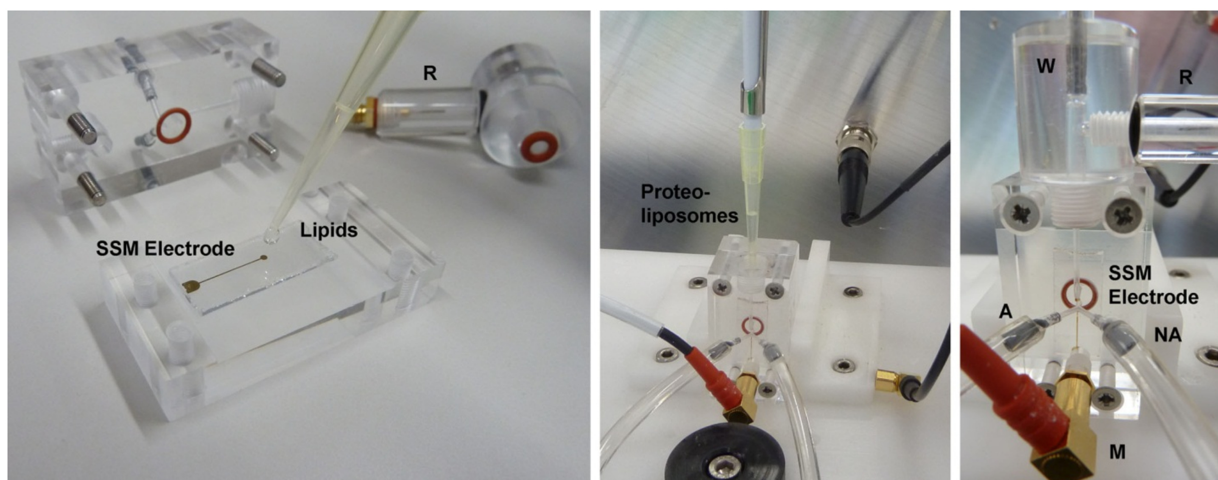

Fig 3: Preparation of the SSM. (left) Explosion view of the Plexiglas cuvette. The gold electrode is placed on the rear part of the cuvette. A few microliters of the lipid solution are applied on to the circular active area of the electrode. The front part (containing the O-ring) and the rear part of the cuvette are screwed together. The reference electrode assembly (R) and the gold pin connecting to the SSM electrode are inserted afterwards. (center) For addition of the proteoliposomes, the reference electrode assembly is removed from the cuvette. (right) Horizontal view of the cuvette once mounted on the setup. The labels indicate the tubes connecting active and non-active solutions as (A and NA); the measuring electrode (SSM-electrode); the connection to the current amplifier (M); the reference electrode assembly (R), and the outlet to waste (W).

3.3. Measurement protocol

Please note that “Valve 1” and “Valve 2” (Fig 1) are normally-closed valves, which block the flow pathways of the non-activating and activating solutions, respectively. Unless otherwise specified, the non-activating (NA) solution is a chloride-free solution and the activating (A) solution is a chloride containing solution. An experiment consists of three measurements (steps 1 to 3) and each experiment is repeated several times (see below):

1) “Valve 1” opens for 2.5 seconds to allow the flow of the non-activating solution.
2) “Valve 1” is closed and “Valve 2” opens for 2 seconds. During this time the liposomes are flushed with activating solution.
3) “Valve 2” closes and “Valve 1” opens again to allow the flow of the non-activating solution. In this way, the liposomes are left in contact with the chloride-free (non-activating) solution.
4) The liposomes are left for 10 seconds in contact with the non-activating solution between the single measurements of the experiment.
5) Steps 1 to 3 are repeated a total of four times to check the reproducibility of the transient current (Fig. 4A measurements #01, #02, #03, #04)

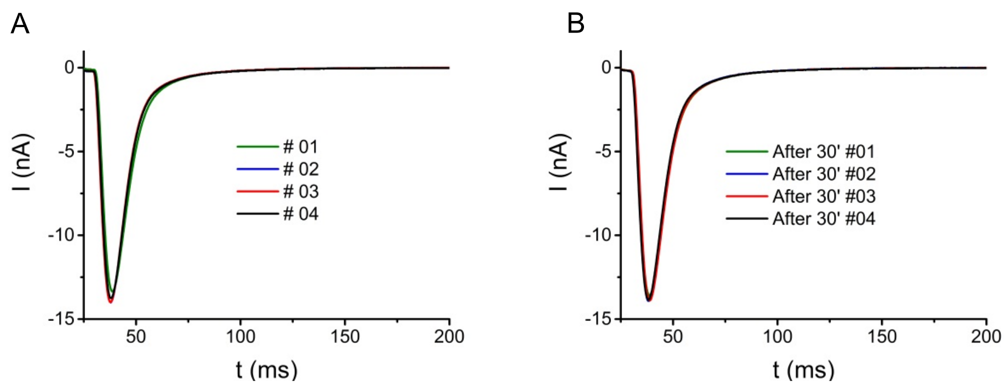

Fig 4. Measurement sequence. (A) Traces labeled as “#1, #2, #3, and #4” correspond to four consecutive single measurements interrupted by a period of 10 seconds. During this time liposomes are in contact with the non-activating solution. The four traces correspond to the first sequence of the measurements. (B) After 30 minutes of incubation with the non-activating solution, the sequence of measurements was repeated. Traces labeled as “After 30’ #1, After 30’ #2, After 30’ #3, and After 30’ #4” correspond to another four consecutive single measurements interrupted by a waiting time of 10 seconds.

6) After an incubation time (typically 30 minutes, Fig. 4B), the sequence of four measurements is repeated again.
7) The last three measurements of the first and the second sequence are compared. If the difference between the recordings is less than 5%, the last two files are averaged. Otherwise, the sequence is repeated until the signals are stable. (see also Remark 3).

3.4. Remarks

1) Care should be taken when exchanging the solutions stored in the 50 µl tubes. During the exchange, air may enter the fluidic pathway and damage the sample. Therefore, every time that the 50 ml tubes are exchanged the three-way stopcocks should be rotated to direct the solution to the waste container while closing the pathway towards the cuvette. By manually operating the valve drivers, solution will be flushed to the waste to remove any air bubble. Afterwards, the three-way stopcocks will be rotated again to re-open the pathway to the cuvette.
2) Check that the pressure is kept constant during the measurements.
3) It may happen that the magnitude of the transient currents is not stable but decreases over time. Under those circumstances, the measurements should be discarded.

4. Equivalent circuit

Figure 5 depicts the measurement principle and the equivalent circuit of the SSM experiment. The gold-electrode (Au) is functionalized with a self-assembled monolayer (SAM) of octadecane thiol. The solid-supported membrane consists on a monolayer of 2-diphytanoyl-sn-glycero-3-phosphocholine (PC) supported by the SAM on the top of the Au-electrode, which is connected to the current amplifier. The immobilized liposomes are adsorbed to the immobilized monolayer of the SSM.

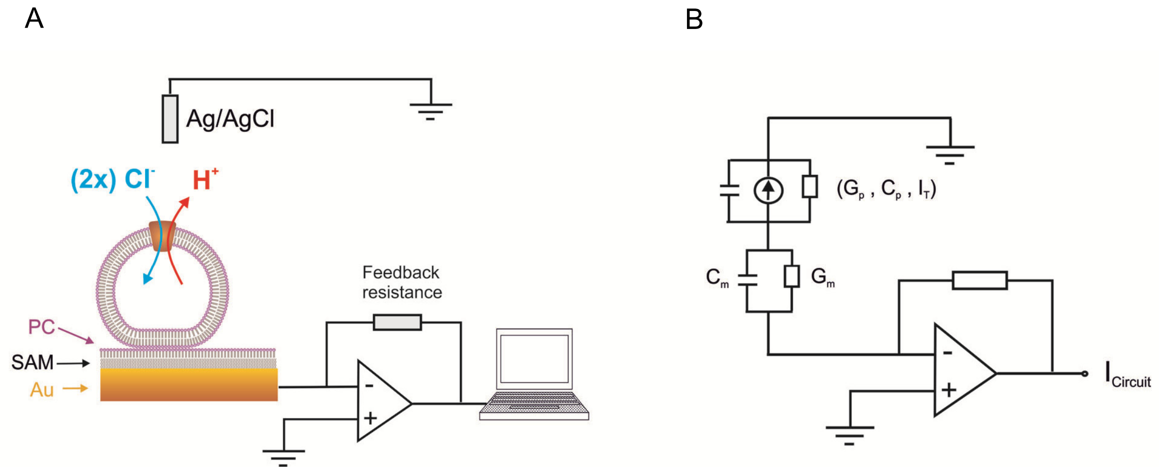

Fig 5.: SSM electrophysiology (A) Putative adsorption geometry of the immobilized liposomes. Abbreviations: gold-electrode (Au), self-assembled monolayer (SAM), monolayer of 2-diphytanoyl-sn-glycero-3-phosphocholine (PC). (B) Equivalent circuit of setup described in (A). The equivalent circuit consists of two membranes, each represented by a certain conductivity G and capacitance C: the proteoliposome membrane (Gp and Cp); and the contact membrane between the gold electrode and the proteoliposomes (Cm and Gm). The current generated by the transporter (I_T) is represented as a current source.