Electronic Supplementary Information

# Single-channel electrophysiology of cell-free expressed ion channels by direct incorporation in lipid bilayers

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## 1. Plasmids and cell-free protein expression

A DNA fragment encoding the KcsA channel was ligated into a pEcoli-Cterm 6xHN vector with a T7 promoter sequence (Clontech Laboratories, CA, USA) and DNA encoding the $hERG_{S5\text{-}S6}$ pore domain (UniProtKB entry Q12809, residues 540-673) was inserted into a pProEX HTa vector with a Trc promoter sequence (Invitrogen Life Technologies, UK). Both plasmids allow for the expression of His-tagged protein, enabling purification with Ni-NTA affinity chromatography. A modified pEcoli plasmid was constructed for the production of non-tagged KcsA. The Promega L1130 system (Promega, WI, USA) was used to express KcsA, while the Promega L1110 system was used to express the hERG construct. Both systems are based on S30 lysates from *E. coli*. The reaction was usually performed in 50 μL volumes, as directed by the manufacturer, with the exception that the distilled water component in the reaction mixture was replaced with electrophysiology buffer pH 7 (150 mM KCl, 10 mM HEPES, pH 7.0). KcsA was expressed for 2 hours at 37 °C without agitation and $hERG_{S5\text{-}S6}$ was expressed for 1 hour at 37 °C with agitation. The reactions were terminated by placing the mixtures on ice. Completed expression reactions were used immediately for experiments.

## 2. Preparation of unilamellar vesicles

The desired amount of lipid in chloroform was added to a glass vial. The lipids were a 1:1 (w/w) mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (PG) (Avanti Polar Lipids, AL, USA). The chloroform was evaporated under a stream of nitrogen to form a dry lipid film, which was placed inside a vacuum desiccator for ~1 hour to remove any residual solvent. Subsequently, 1 mL of electrophysiology buffer pH 7 was added to the lipid film, which was then resuspended using a vortexer. The resulting dispersion of multilamellar vesicles was freeze-thawed five times and then passed 21 times through a polycarbonate filter with 100 nm pores (Avanti Polar Lipids, AL, USA) to obtain large unilamellar vesicles. For cell-free expression reactions in the presence of vesicles, the electrophysiology buffer in the reaction mixture was replaced with this extruded vesicle dispersion.

## 3. Protein purification from reaction mixtures

Expressed channels were purified because gel electrophoresis of cell-free reaction mixtures, with their abundance of *E. coli* lysate proteins, could not give an indication of KcsA or $hERG_{S5\text{-}S6}$ expression. Ni-NTA agarose beads (Qiagen, UK) from 40 μL of as-supplied bead slurry were equilibrated and resuspended in 50 μL wash buffer (20 mM imidazole in phosphate buffered saline (PBS)) according to the instructions from the manufacturer. The affinity bead dispersion was then added to 100 or 150 μL of a completed cell-free reaction mixture, followed by overnight incubation under agitation at 4 °C. Beads were then washed with four bead volumes of wash buffer, before elution with stepwise increases in imidazole concentration: 0.1, 0.5 and 1 M imidazole in PBS with 5% glycerol. Finally, the beads were resuspended in 50 μL PBS. For purifications where the pooled eluates were to be used as an electrophysiology sample (see section 6), all elution buffers were supplemented with electrophysiology buffer pH 7 and the 1 M imidazole elution step was replaced with more 0.5 M imidazole elutions. 15% SDS-PAGE gels were prepared using the mini-PROTEAN system (Bio-Rad Laboratories, UK). The samples (molecular weight standard, bead wash fractions, bead imidazole eluates and the final bead suspension itself) were mixed with β-mercaptoethanol-containing loading buffer, denatured at 99 °C for 3 minutes, and then loaded on the gel for gel electrophoresis. The gels were stained with Coomassie Brilliant Blue, imaged with a Gel Doc XR imager (Bio-Rad Laboratories, UK) and analyzed with Image Lab software (Bio-Rad Laboratories, UK).

## 4. Expression of KcsA

His-tagged KcsA was expressed, purified and analyzed by gel electrophoresis as described above. Initially, the expression was performed with 3 μg of plasmid encoding His-tagged KcsA in 150 μL of reaction mixture. The SDS-PAGE gel in Figure 1 shows many different

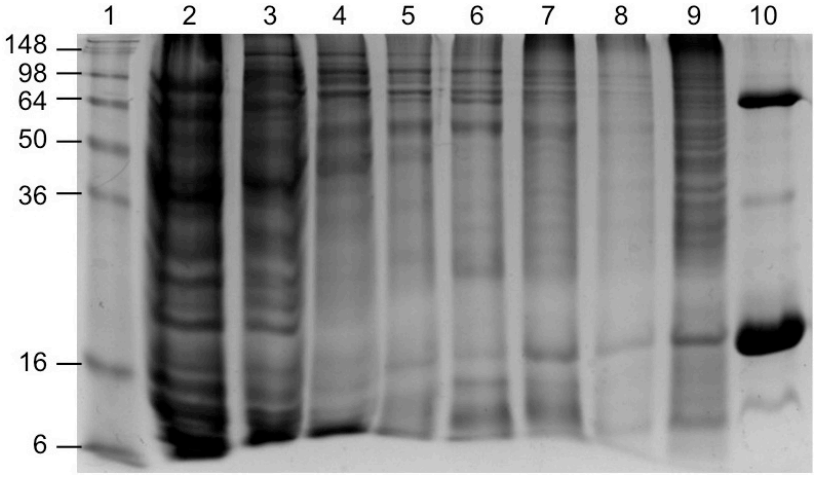


**Fig. 1** SDS-PAGE analysis of purified cell-free expressed His-tagged KcsA. The 150-μL reaction mixture contained 3 μg of the KcsA-encoding plasmid. The molecular weight standard was loaded into lane 1 (labels are kDa units), subsequent bead wash fractions are in lanes 2-5, subsequent bead eluates with 0.5 M imidazole are in lanes 6-8, and proteins dissociated from the beads by boiling in loading buffer are in lane 9. A reference sample of 57 μg of purified His-tagged KcsA (19 kDa) expressed in *E. coli* culture is loaded in lane 10, with the monomer, dimer and tetramer migrating near the 16, 36 and 64 kDa markers, respectively.

protein bands in the wash fractions (lanes 2-4), reflecting the large number of proteins in the cell-free reaction mixture. Some of these bands, particularly at higher molecular weight positions, are also present in the imidazole elution fractions (lanes 5-8), indicating that some non-specific binding of reaction mixture proteins to the affinity beads has occurred. However, with subsequent elutions, the intensity of the reaction mixture bands decreases while a new band emergences near the position of the 16 kDa protein marker. This band is clearly visible in lane 7, which is the second eluate with 0.5 M imidazole, and in lane 9, which contains the proteins that have been dissociated from the affinity beads by boiling in loading buffer. Because this bands migrates at the same position as pure His-tagged KcsA monomer (expressed in *E. coli* culture as described in reference S1), we assign this band to cell-free expressed His-tagged KcsA. From the applied amount of KcsA and the intensity ratios of the KcsA tetramer, dimer and monomer bands, it follows that the KcsA monomer band in lane 10 contains ~36 µg KcsA, implying that the amount of cell-free expressed KcsA eluted/dissociated from the affinity beads (equivalent bands in lanes 5-9) is ~8 µg. Hence the yield of cell-free expressed KcsA was ~50 ng/µL reaction mixture.

The result of varying the amount of plasmid in the reaction mixture and supplementing the reaction mixture with vesicles is depicted in Figure 2. Note that in all four gels, the second lane shows *E. coli* lysate proteins that did not bind to the affinity beads, while lanes 4-10

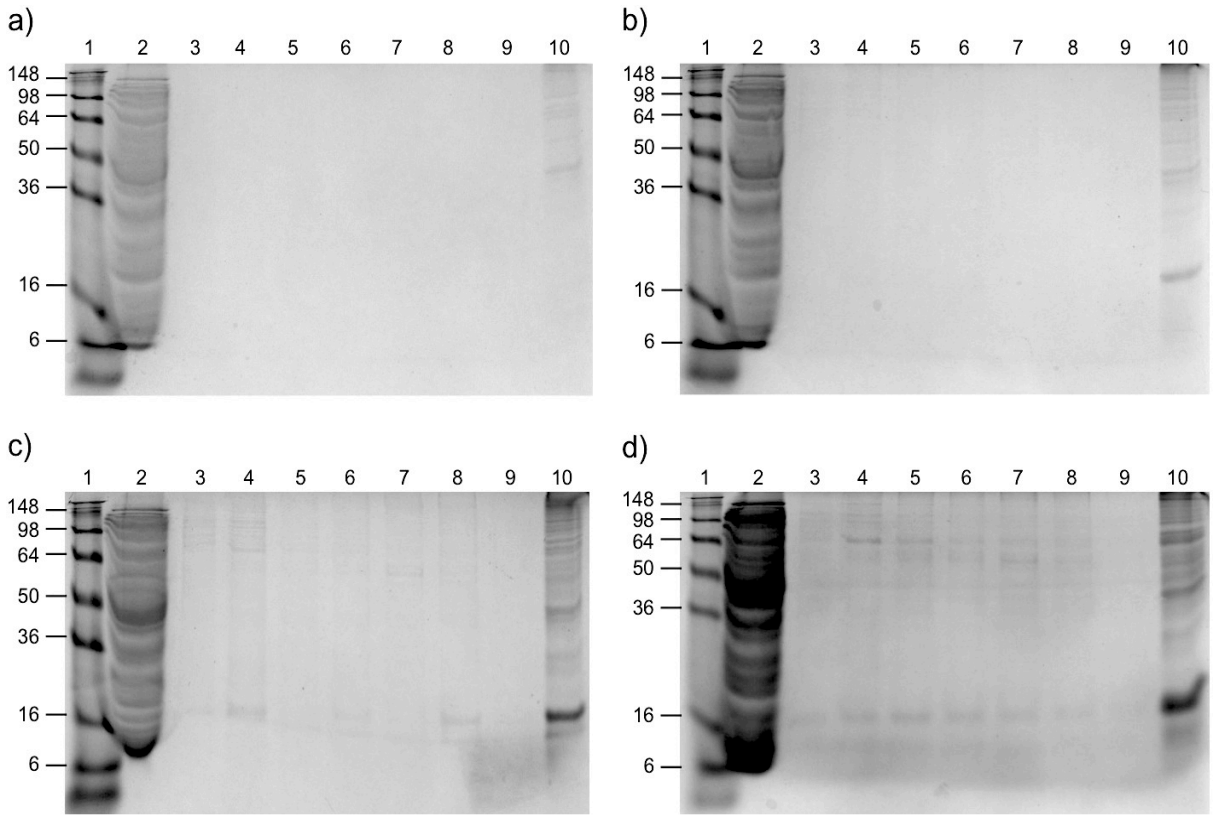


**Fig. 2** SDS-PAGE analysis of purified cell-free expressed His-tagged KcsA. Four different 100-µL reaction mixtures contained: a) no KcsA-encoding plasmid, b) 6 µg of DNA, c) 12 µg of DNA, and d) 2 µg of DNA and PC/PG vesicles (8 µg total lipid). For each gel the molecular weight standard was loaded into lane 1, the first bead wash in lane 2, the final bead wash in lane 3, the bead eluates with 500 mM imidazole in lanes 4-6 and the eluates with 1 M imidazole in lanes 7-9, and proteins dissociated from the beads by boiling in loading buffer are in lane 10. The approximate molecular weight for the protein ladder is given in kDa. His-tagged KcsA monomer has a molecular weight of 19 kDa.

contain samples that were eluted with increasingly strong detachment conditions for bead-bound proteins. Analysis of a control reaction, without KcsA-encoding plasmid, resulted in no detectable protein bands in the bead eluate lanes, except for some bands in lane 10, which were assigned as abundant *E. coli* proteins from the reaction mixture non-specifically bound to the affinity beads (Fig. 2a). In contrast, a reaction mixture containing 6 μg of plasmid resulted in an additional band in the last lane, with an apparent molecular weight of ~16 kDa (Fig. 2b). Increasing the amount of DNA to 12 μg gave an increased intensity of this band while low-intensity bands at the same position were now also visible in earlier eluate fractions (Fig. 2c). This was also the case when a smaller amount of plasmid (2 μg) was present in a vesicle-supplemented reaction mixture (Fig. 2d). By normalizing the intensities of the protein ladder bands of the gels in Figure 2 to the ladder bands of the gel in Figure 1, we could approximate the amount of protein that had dissociated from the affinity beads by boiling in sample buffer (lanes 10 in Fig. 2b-d). It was estimated that the reaction mixtures incubated with 6 μg KcsA plasmid, 12 μg plasmid, and 2 μg plasmid with vesicles, contained 1, 4 and 8 μg protein per 100 μL, respectively. From these experiments, which were performed simultaneously with the same batch of lysate, it can be concluded that the expression yield of KcsA is higher when more DNA is present in the reaction mixture, and that the presence of vesicles in the mixture also leads to an increased expression efficiency.

## 5. Expression of $hERG_{S5\text{-}S6}$

The $hERG_{S5\text{-}S6}$ pore domain was expressed and purified in duplicate and the expression product was run on two SDS-PAGE gels, as described above. One of the gels was Coomassie stained and destained, resulting in a band in lane 10 at a position that corresponds to proteins with an apparent molecular weight of approximately 16 kDa (Figure 3a), while $hERG_{S5\text{-}S6}$ has a theoretical molecular weight of 18 kDa. The second gel was used for Western transfer of the proteins onto nitrocellulose paper, which was then incubated with a monoclonal anti-polyhistidine primary antibody (Sigma-Aldrich, UK). This was subsequently labelled using the Quick Western Kit IRDye 680RD (LI-COR Biotechnology, UK) and imaged in a LI-COR

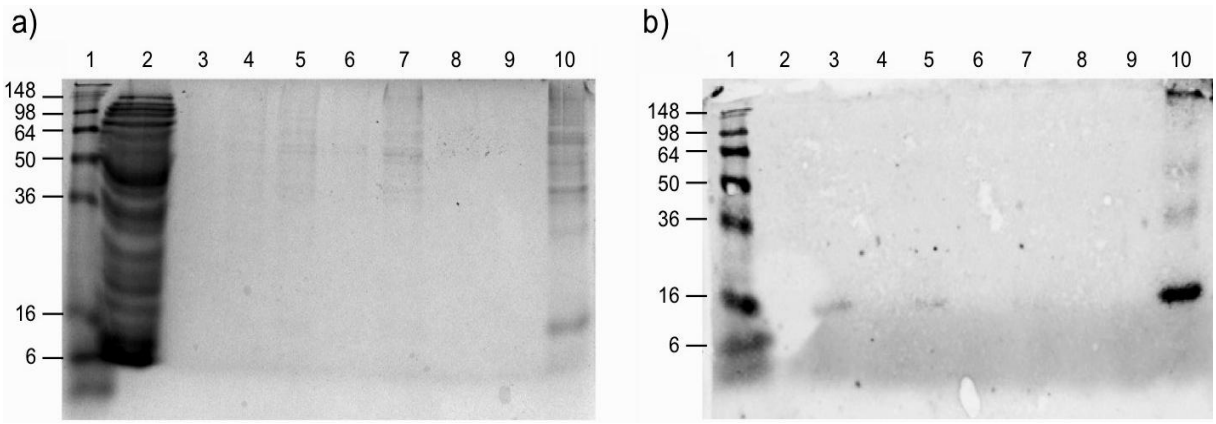


**Fig. 3** Analysis of CF expressed and purified $hERG_{S5\text{-}S6}$. a) SDS PAGE analysis of cell free expressed and purified hERG. b) Western blot of CF expressed and purified hERG. The lane assignments are the same as Figure 1. The approximate molecular weight for the proteins contained within the standard ladder in lane 1 of the gels is indicated in kDa. His-tagged $hERG_{S5\text{-}S6}$ monomer has a molecular weight of 18 kDa.

Odyssey infrared imager. Figure 3b shows the Western blot: the pre-stained molecular weight markers are visible in lane 1 while additional bands can be observed in lanes 2-10 (clearly visible in lane 10). These bands were labelled with the anti-polyhistidine antibody and had migrated with an apparent molecular weight of ~16 kDa and were therefore assigned as His-tagged $hERG_{S5\text{-}S6}$ monomer.

## 6. Bilayer current measurements and analysis

Interdroplet bilayers were formed on an SU-8 coated glass surface fitted with a reservoir containing 200 μL of 60 mg/mL lipid in decane. Lipids were the asolectin lipid extract from soybean (~25% phosphatidylcholine content, product 11145 from Sigma-Aldrich, UK). A 2 μL droplet of the completed reaction mixture was placed on an agar coated (5% w/v) Ag/AgCl wire electrode and lowered into the lipid-oil phase. A second 2 μL droplet containing electrophysiology buffer (150 mM KCl, 10 mM HEPES, pH 4 for KcsA measurements or pH 7 for hERG measurements) was then lowered into the well on a second Ag/AgCl electrode. The two droplets were brought into contact by manual manipulation of the electrodes. Bilayer formation was confirmed by capacitance measurements, which were obtained by applying a triangle waveform with a linear voltage ramp of 1 V/s such that the measured current signal can be interpreted as the bilayer capacitance. The bilayer current was measured by connecting the Ag/AgCl electrodes to a CV-203BU headstage of an Axon Axopatch 200B amplifier (Molecular Devices, CA, USA) with the 4-pole Bessel output signal filter of the amplifier set at 5 kHz.

Bilayer current traces were sampled at 50 kHz with an Axon Digidata 1440A digitizer. The data was subsequently processed with a 1 kHz low-pass filter for data analysis and with a 0.5 kHz filter for display purposes. Histograms were from 10-second current traces, while the open probability $P_o$, dwell time and the average amplitude of channel openings was calculated using Clampfit software (Molecular Devices) from bursts of single-channel activity.

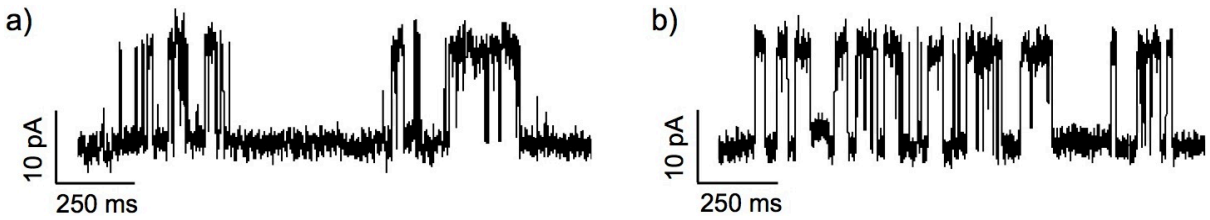


**Fig. 4** Comparison of purified and non-purified cell-free expressed His-tagged KcsA single-channel activity in interdroplet bilayers with the second droplet containing electrophysiology buffer of pH 4, and the first droplet containing a) the complete cell-free reaction mixture with expressed His-tagged KcsA, and b) affinity-bead purified His-tagged KcsA solution in 0.5 M imidazole.

Examples of single-channel current traces and analysis are shown in Figures 4-7. Figure 4a depicts single-channel activity of His-tagged KcsA that has inserted into the interdroplet bilayer from a complete cell-free reaction mixture, as described in the main paper. The average amplitude of the channel openings was 11.8 ± 1.8 pA, the average dwell time was 16.3 ms and the open probability was 0.60. Figure 4b shows single-channel activity of His-

tagged KcsA, where the first droplet consisted of a solution of affinity-purified His-tagged KcsA in 0.5 M imidazole (see section 3). For purified His-tagged KcsA, the average amplitude of the channel openings was 13.0 ± 1.0 pA, the average dwell time was 17.7 ms and the open probability was 0.46. Histograms generated from the current traces of non-tagged KcsA at +100 mV and +75 mV, shown in the main paper, are displayed in Figure 5. The peaks centered at ~1.5 pA represent the current baseline (i.e. closed channels) while the peaks centered at ~16 pA (Fig. 5a) and ~11 pA (Fig. 5b) represent single open channels. Note that these histograms are for 30-second bilayer current traces and hence depict a lower open probability then intra-burst histograms. Details of the gating properties of non-tagged KcsA are given in the main paper.

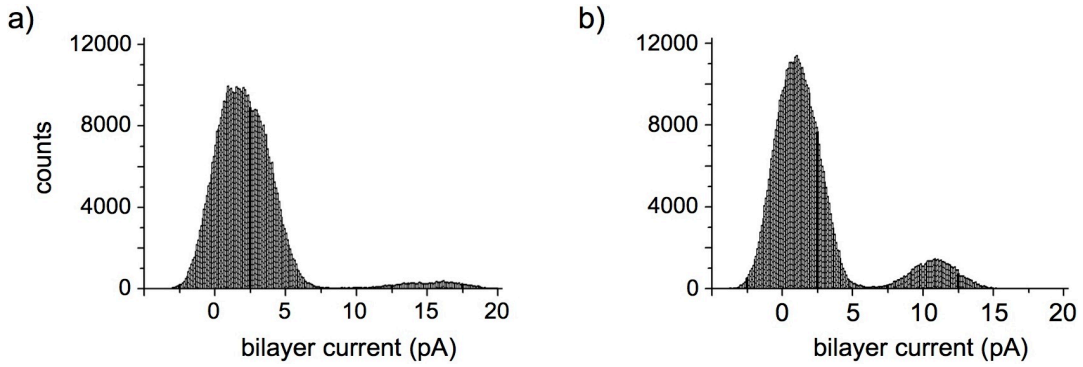


**Fig. 5** Histograms of bursts of non-tagged KcsA channel activity in an interdroplet bilayer, with a cell-free expression reaction mixture in the first droplet and electrophysiology buffer of pH 4 in the second droplet, recorded with a voltage clamp potential of +100 mV (a) and +75 mV (b). Note that the current baseline is centered at approximately +1.5 pA.

A current-voltage plot for non-tagged KcsA, inserted from a reaction mixture in an interdroplet bilayer, is given in Fig. 6 and current traces obtained at +100 mV with 25 or 50 mM of the potassium channel blocker tetraethylammonium (TEA) are shown in Fig. 7 and are discussed in the main paper. The average open amplitude of the KcsA channels was 8.2 ± 1.7 pA in the presence of 25 mM TEA and 6.2 ± 1.1 pA in the presence of 50 mM TEA.

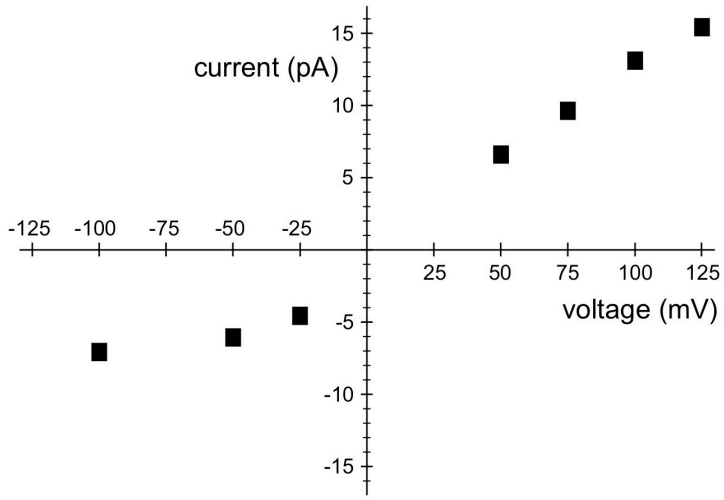


**Fig. 6** Current-voltage relation for non-tagged KcsA in interdroplet bilayers, with the reaction mixture containing the KcsA plasmid as the first droplet and a 150 mM KCl solution at pH 4 as the second droplet.

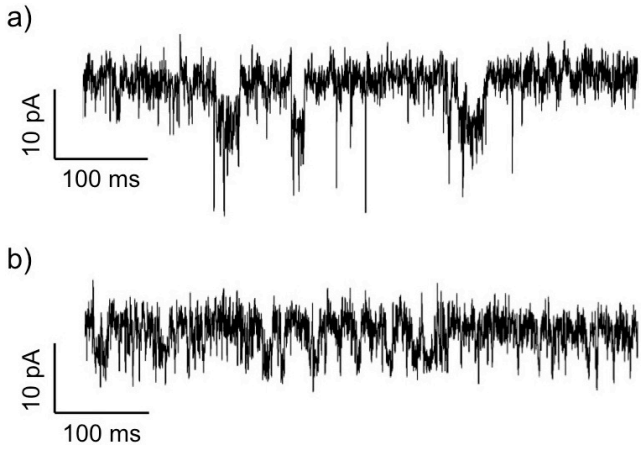


**Fig. 7** Bilayer current traces for non-tagged KcsA in inter-droplet bilayers, with the reaction mixture containing the KcsA plasmid as the first droplet and a 150 mM KCl solution at pH 4, which also contained 25 mM TEA (a) or 50 mM TEA (b), as the second droplet.

## Reference

S1 P. Marius, M. R. R. de Planque and P. T. F. Williamson, *Biochim. Biophys. Acta*, 2012, **1818**, 90−96.