Optimization of a High-throughput Liposomes Fluorescence Assay for the screening of drug libraries with potential therapeutic use for ion channel dysfunction related diseases.

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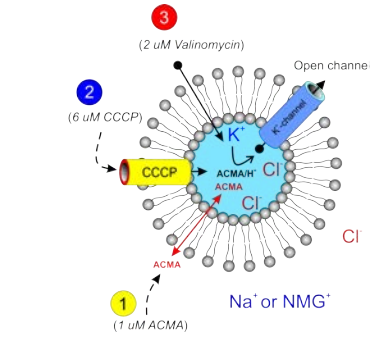
Abstract:

Voltage-gated sodium and potassium channel dysregulation and overexpression have been found to be the leading cause of many channel-related diseases, including epilepsy, Alzheimer's disease, schizophrenia, and most recently, several cancer types. In relation to cancer, potassium channels can become dysregulated, allowing an increased efflux of positively charged potassium ions out of the cell, overriding many checkpoints within the cell cycle, and permitting the cell the ability to divide uncontrollably as a result. It has also been found that overexpression of potassium channels also promotes cell mobility of cancer cells, often leading to metastasis. KcsA (a prokaryotic K^+ channel) is the archetypal potassium channel that would be used in the study of finding therapeutic drugs that could act as a "gatekeeper" (in the case of an inhibitor) to regulate the flow of water molecules as a consequence of positively charged ions (K^+-ions) moving out of the cell. We attempt to do this by employing a liposome fluorescence assay in which the fluorescent decay indicates channel activity. The presence of an inhibitor can therefore "block" (or inhibit) the K^+ channel activity, halting the decay in the fluorescent signal, and potentially identifying a putative therapeutic novel blocker. Finally, electrophysiology will be utilized to track the movement of K^+-ions across the cell membrane, as well as the blocking properties of putative new therapeutic drugs. The development of this novel high-throughput functional assay will provide a robust and reliable first drug screening approach to identify ion channels blockers in general.

1. L. G. Cuello, Elife 6 (2017).
2. J. Ostmeyer, S. et al,. Nature 501, 121-124 (2013).

A Liposome Fluorescence Assay Concept



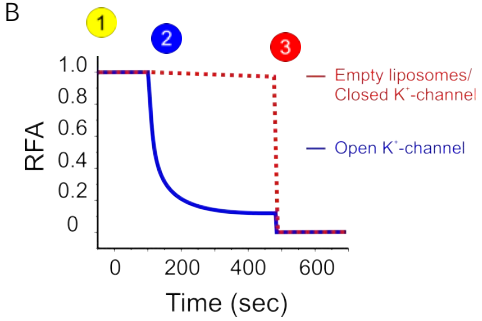


Figure 1. Liposome Fluorescence Assay Concept. **(A)** Open K^+-channel containing liposome (made of 75% of Palmitoyl oleoyl phosphoethanolamine (POPE) and 25% Palmitoyl oleoyl phosphoglycerol) are made in a buffer containing a 150 mM KCl, 20 mM HEPES pH 7.5. Next, the proteo-liposome suspension is diluted in a free-K^+ solution (150 mM NaCl or NMGCl, 20 mM HEPES pH 7.5). In these conditions, a net outwardly flow of K^+-ions generates a large internal negative membrane potential. **(B)** After the subsequently addition of 1 uM of ACMA (9-Amino-6-Chloro-2- Methoxyacridine) and 5 uM of CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) a rapid time dependent quenching of the fluorescence signal develops (**blue trace, upper panel**) as the ACMA that has partitioned into the liposome interior is protonated and quenched by the constant influx of proton inwardly driven through the CCCP and by the strong negative membrane potential. Once the fluorescence signal has plateau 2 uM of Valinomycin is added to assess the number of empty liposomes. If the channels are closed or the liposomes are empty (control condition), the sequential addition of ACMA followed by CCCP will not produce a time dependent fluorescence quenching (**red trace, upper panel**).

KcsA-OM represents a good test open channel for LFA optimization procedure

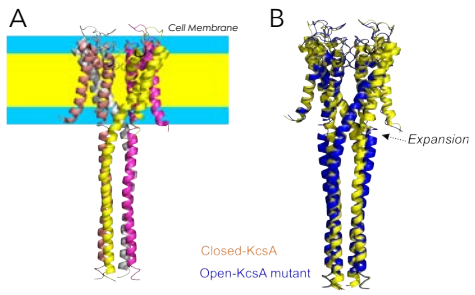


Figure 2. Structure of the closed and the open KcsA channel. A. A KcsA channel is made of four identical subunits (shown in a ribbon representation of four different colors). The four subunits are arranged around a central permeation pathway that allows potassium ions move across the cell membrane. **B.** A structural superposition of KcsA closed (blue) and open states (yellow) highlights the expansion or widening of the channel's activate gate. The activation gate expands and allows potassium ions move through the channel's permeation pathway.

Purification and reconstitution of a constitutively open mutant

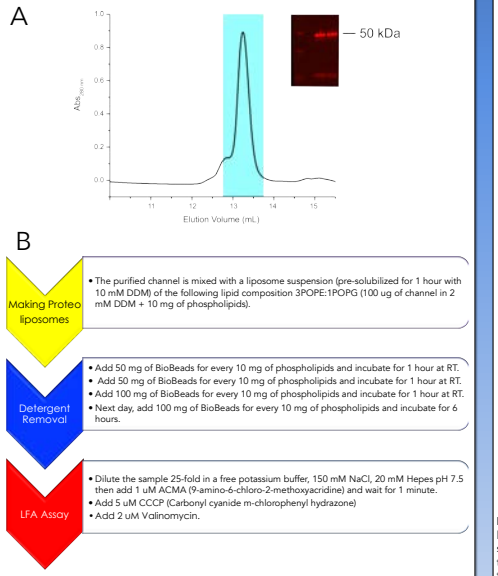


Figure 3. Workflow for protein purification and LFA assay. **A.** The KcsA open mutant was expressed as a recombinant protein in *E. coli* expression system and extracted from the cell membrane with the detergent Dodecyl Maltoside. Next, KcsA was purified by Metal affinity chromatography since the channel has a poly-histidine Tag at the end of its aminoacid sequence. KcsA affinity purified then was applied to a Size Exclusion Chromatography column (SEC column) and further purified by its molecular weight and hydrodynamic properties (KcsA's shape). The pure protein was run in a protein gel to assess its biochemical purity. Since the tetrameric KcsA channel is resistant to the very harsh detergent Sodium Dodecyl Sulfate (SDS) used during the electrophoresis, the channel migrates in the protein gel mostly as a tetramer but a fast migrating faint band indicates the presence of the monomer. **B.** A workflow indicates the main steps to produce proteo-liposomes suitable for LFA Assay. The workflow consists of an initial step (making proteo-liposomes) that involves making the POPE/POPG liposomes in a KCl containing buffer and the addition of the pure channel followed by a detergent removal step, which involves the sequential addition of of polystirene adsorbent beads (Bio-beads SM-2) that effectively remove the detergent from the proteo-liposomes suspension.

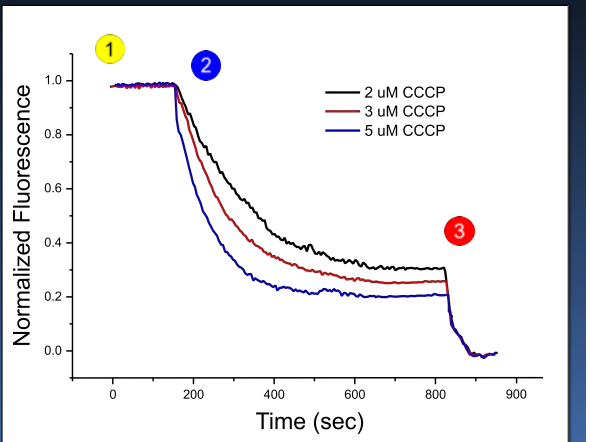


Figure 4. Optimization of the CCCP concentration used in the KcsA-OM LFA Assay. The KcsA-OM containing liposomes were diluted 40-fold in a free-K^+ containing solution (150 mM NaCl) and the ACMA fluorescence signal was recorded using the following parameters (Excitation=410 nm and Emission=490 nm). The concentration of CCCP was titrated aiming to maximize the rate of quenching in an attempt to make the LFA assay suitable for high-throughput screening of drugs.

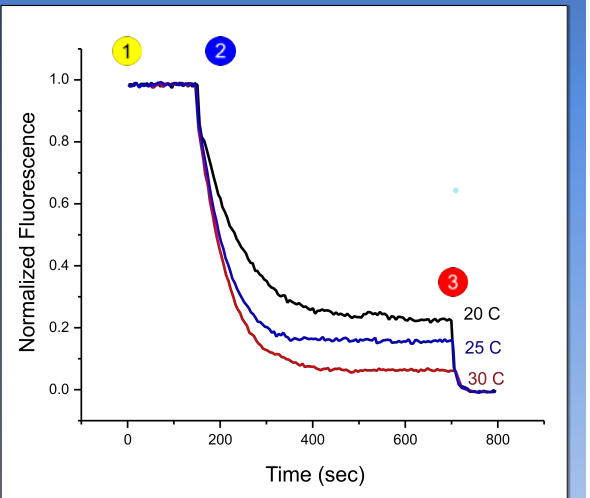


Figure 5. Optimization of the temperature used in the KcsA-OM LFA Assay. The KcsA-OM containing liposomes were diluted 40-fold in a free-K^+ containing solution (150 mM NaCl) and the ACMA fluorescence signal was recorded using the following parameters (Excitation=410 nm and Emission=490 nm). The temperature of the assay was optimized to maximize the rate of the fluorescence quenching aiming to make the LFA assay suitable for high-throughput screening of novel therapeutic drugs.

Summary

We have optimized the CCCP concentration and the temperature of the LFA assay to make it high-throughput suitable for the screening on novel therapeutic drugs to treat diseases associated to the dysfunction of ion channels.

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