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Reconstitution of mammalian ion channels in droplet bilayers

Author
Jeurkar, Abha

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Reconstitution of mammalian ion channels in droplet bilayers

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of the requirements for the degree Master of Science

in Biomedical Engineering

by

Abha Jeurkar

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ABSTRACT OF THE THESIS

Reconstitution of mammalian ion channels

in droplet bilayers

by

Abha Jeurkar

Master of Science in Biomedical Engineering

University of California Los Angeles, 2012

Professor Jacob Schmidt

Ion channel studies are important for scientific characterization of cellular processes and for the purpose of drug discovery. The sessile droplet bilayer platform allows for simple, fast, inexpensive and high-yield formation of artificial lipid bilayers and reconstitution of ion channels. We validated this bilayer formation platform with mammalian ion channels like Chloride Intracellular Channel 1 (CLIC1) and Voltage-gated potassium channel 1.2 (Kv1.2). Ion channel synthesis using traditional bacterial expression systems is a complex and time consuming process and it limits the number of eukaryotic proteins that can be expressed therein. We explored the use of *in vitro* protein synthesis and plasma membrane fractionation of commercially available mammalian cells for expressing and reconstituting ion channel proteins in planar lipid bilayers.

The thesis of Abha Jeurkar is approved.

Gerard C. L. Wong

Dino Di Carlo

Jacob Schmidt, Committee Chair

University of California Los Angeles

2012

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Introduction

Ion channels are unique and structurally complex class of membrane proteins that play crucial roles in cell signaling and a myriad of other physiological processes. Study of ion channels is important for understanding their implications in diseases and for pharmacological discovery via drug screening. Commercial state-of-the-art automated patch clamping platforms like QPatch® and Port-a-Patch (Nanion®) are very expensive and require specialized personnel for their operation. IonFlux® and Nanion® provide microfluidic patch clamp platforms and SDx tethraPod and Orbit 16 (Nanion®) provide microfluidic artificial bilayer formation platforms. These microfluidic systems are beneficial as they require minimal sample volume for their operation but they are limited by the need of specialized manufacturing processes, restricted access to individual bilayers and surrounding buffer solutions. The sessile droplet bilayer platform, developed by our group, is easy-to-use, inexpensive, and easy-to-manufacture and provides excellent control on lipid and buffer environment of individual bilayers. This platform is highly amenable to parallelization and automation and has the potential to replace automated patch clamp for drug screening. Chapter 1 describes the method of formation of artificial bilayers using sessile droplets.

Sessile droplet bilayers are especially useful in electrophysiological studies of intracellular ion channels, as patch clamping onto the delicate intracellular membranes is very

challenging. Chapter 2 describes the study of one such intracellular channel- CLIC1, which is purified from *Escherichia Coli* bacteria.

Synthesis of proteins using bacterial expression systems, however, is complex, time consuming and often results in imperfect folding of eukaryotic proteins. Hence, in Chapter 3, we explore the use of *in vitro* expression systems for the formation of voltage-gated potassium channel- Kv1.2 and successfully incorporate it in our droplet bilayer platform.

The *in vitro* expression technique, however, is still in its nascency and limits the choice of ion channel proteins that can be synthesized therein. To address the need for fast, efficient and high-throughput ion channel synthesis and reconstitution, in Chapter 4, we explore various techniques used for the fractionation of plasma membranes of commercially available mammalian cell lines. These membrane fractions containing ion channels can then be directly used in our droplet bilayer platform for bilayer formation and ion channel measurement.

Chapter 1
Sessile Droplet Bilayer platform

1.1 Introduction

Artificial lipid bilayers are simplified models of cellular membranes, which are widely used in research on ion channel proteins because of their ease of use and the ability to control lipid environment and surrounding solutions. However, practical use of artificial lipid bilayers in applications requiring high throughput measurements has been limited in part by their need to be formed individually and manually. Recent work by my group (Biohybrid Microsystems Laboratory, UCLA) and others has aimed to overcome these shortcomings by developing novel platforms for bilayer formation that are highly amenable to automation and parallelization (Baaken G, 2008) (Ide T, 2008) (Le Pioufle B, 2008) (Malmstadt N, 2006) (Osaki T, 2009) (Poulos J, 2009) (Syeda R, 2008) (Thapliyal T, 2011). Such high-throughput artificial bilayer platforms can potentially replace the complex and expensive patch-clamp technology for ion channel screening. The sessile droplet bilayer platform developed by my group uses the principle of mechanically contacting two monolayers to form a bilayer, where the individual monolayers are formed at the interface between aqueous and organic solutions. Recently, the use of masking apertures at the monolayer contact area was demonstrated, which stabilized the bilayers, controlled their areas and also allowed for exchange of solution adjacent to the bilayers at high speeds without

rupturing them or changing their areas (Portonovo S, 2012). This bilayer platform has demonstrated potential in measuring not only bacterial ion channels like gramicidin (Poulos J, 2010), α-hemolysin (Thapliyal T, 2011) and alamethicin (Poulos J, 2008) but also mammalian ion channels like hERG (Portonovo S, 2012) and TRPM8 (El-Arabi A, 2012) in their functional form.

1.2 Bilayer formation technique

A three piece bilayer chamber is fabricated, consisting of a top acrylic piece (McMaster Carr) containing three wells, a bottom acrylic piece with a channel connecting these three wells, and a Delrin film (McMaster Carr), sandwiched between the two acrylic pieces, with large holes cutout to allow direct access to the bottom channel from the top side wells. The Delrin film separating the center well from the bottom channel contains an aperture of a sufficiently small size to prevent flow of aqueous solution through it. The aperture size dictates the bilayer area and typically varies from 50 to 400μm in diameter. Ag/AgCl pins are fabricated from 16 gauge silver wire (0.999 purity, C.C. Silver & Gold). The pins are cut to approximately 1 inch and electrical discharge machining is used to create a blunt tip end and to cut slots 0.05 inches deep and 0.015 inches wide into the ends of the pins. Prior to use, all of the silver pins are immersed in bleach (Clorox) for approximately 1 min to chlorinate their surfaces, followed by a thorough rinse in DI water. A counter-electrode is made using a thin silver wire, which is also bleached and thoroughly washed before use.

The lower chamber typically contains 200μl of liposome solution, which is separated from the middle chamber containing ~80μl organic solution (n-decane or hexadecane) by the masking partition. The aperture in the partition allows for the formation of a monolayer at the aqueous-organic interface. A 2μl aqueous droplet containing liposomes is pipetted onto the base of the Ag/AgCl electrode, which is mechanically lowered into the well containing organic solution using a micromanipulator. As the droplet gets immersed in the organic

solution, another monolayer is formed at the boundary of the droplet. As the droplet is completely lowered into the organic well, it contacts the monolayer at the aperture and these two contacting monolayers form a bilayer. Bilayer formation is monitored by measuring the capacitive current resulting from an applied 8 Hz 20 mV (p–p) triangle wave. The capacitance gradually grows and stabilizes at a higher value (>100 pF for a 200μm aperture), signifying bilayer formation. For the incorporation of ion channels into these bilayers, soluble ion channel proteins can be directly added to the liposome solution. However, for most membrane proteins, a detergent-based procedure needs to be followed for the formation of proteoliposomes. Bilayer formation and incorporation of ion channels in the bilayers is hypothesized to occur concurrently and is confirmed from the fact that ion channel activities can be seen in almost all the bilayers immediately after the bilayers are formed.

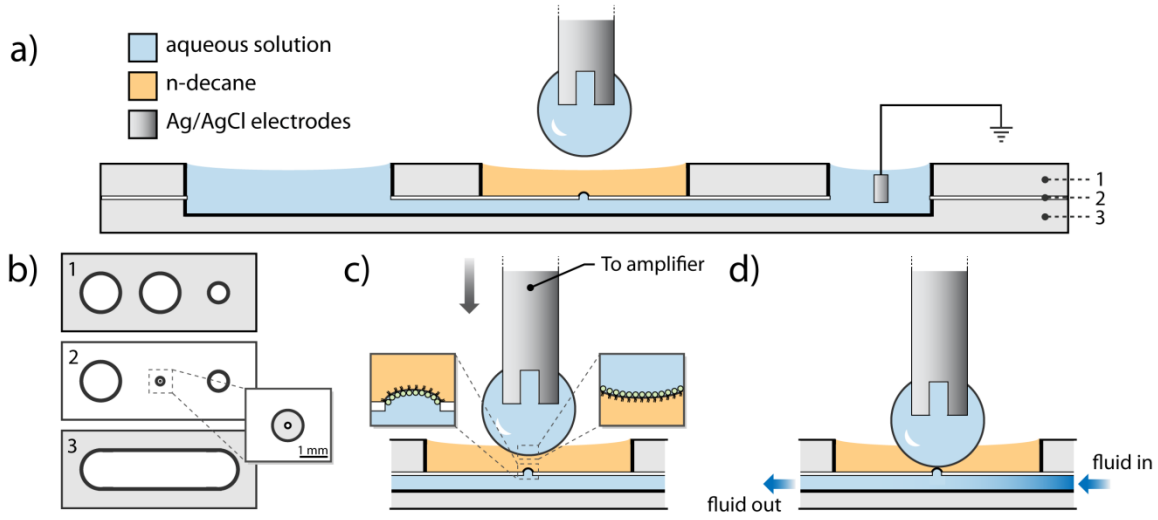


Figure 1-1. **Schematic of bilayer formation apparatus.** (a) The lower aqueous channel is filled with ~200µl of liposome/proteoliposome solution. The central well is filled with ~80µl of n-decane/hexadecane solution. The central well and the lower channel are separated using a Delirin partition containing a 50-400 µm sized aperture, leading to formation of a monolayer at the interface. The top droplet hangs off an Ag/AgCl electrode and consists of ~1µl of liposome/proteoliposome solution. Counter-electrode is placed in the lower aqueous channel. The two electrodes are connected to an Axon 200B amplifier. (b) Exploded view of the bilayer formation chamber (c) The top Ag/AgCl electrode is lowered into the central well using a micromanipulator, leading to the formation of a monolayer at the droplet/organic solution interface. As the electrode is lowered into the central well completely, the two monolayers contact, leading to the formation of a bilayer (d) Use of a partition at the monolayer contact area allows for high-speed exchange of solution in the lower chamber.

1.3 Discussion

Bilayer formation in a sessile droplet bilayer platform is very simple and requires only solution loading and mechanical translation of the center electrode. Ion channels get reconstituted into the bilayers at the time of bilayer formation, allowing for their measurements to be done immediately after the bilayers are formed. Bilayer formation and ion channel measurement require less than 15 minutes on an average. The bilayer chambers are easy to fabricate and inexpensive. The chamber design enables electrical and fluidic access to both sides of the bilayer from the chamber top, allowing quick set up and convenient exchange of chambers between experiments.

With the introduction of masking apertures in the droplet bilayer systems, the lower aqueous solution can be perfused without rupturing the bilayers or changing their areas. The metal Ag/AgCl electrodes can be replaced with electrodes made from hydrogels like agarose, which enable formation of more stable bilayers that support fluid exchange rates as high as 60ml/min. Solution exchange enables increased experimental throughput by allowing for a variety of different experimental conditions to be tested in a short time, as well as enabling measurement of the activity of a large but fixed number of ion channels in the presence of varying concentrations of drug compounds for IC_{50}/EC_{50} determination.

As of now, the solution in the top droplet cannot be exchanged; however, in the future, suitable design modifications can be made to the top electrode fairly easily to enable that. It can be recognized that the large reservoir of organic solution adjacent to the bilayer can

cause structural changes in the reconstituted protein and work is being done to reduce or eliminate the use of organic solution during bilayer formation.

Chapter 2
Purification and reconstitution of Chloride Intracellular ion channel- CLIC1

2.1 Introduction

Ion channels embedded in the outermost plasma membrane of a cell have been extensively studied and characterized, but relatively little is known about ion channels residing in the membranes of intracellular organelles. Most of the electrophysiological characterization of ion channels has been done using patch clamps. In spite of the recent advents in the patch clamping technology, there remain serious technical limitations, which make it very difficult to patch onto intracellular ion channels when they are present in their native form and physiological environment. Intracellular channels can be driven to the plasma membrane by overexpressing them in cells. However, in the presence of specific cellular localization signals, they are often transported back to the intracellular membranes. Engineered protein sequences can also be used to assist the targeting of these ion channels to the plasma membrane and but this approach has its own physiological limitations (Singh H, 2010). Hence, currently the most effective method to study intracellular channels is by reconstituting them in artificial planar lipid bilayers.

Intracellular chloride ion channel proteins (CLICs) are a relatively new class of putative anion channels. In 1987, Landry, Al-Awqati and colleagues isolated the first CLIC protein,

p64 (now known as CLIC5b), which bound to IAA 94- a known chloride channel inhibitor, from bovine tracheal apical epithelium and kidney cortex microsomal membrane fractions (Landry D, 1990). The CLIC family of ion channel proteins has 6 members in vertebrates (CLIC 1-6), 3 in invertebrates (DmCLIC in Drosophila melanogaster, EXC4 and EXL1 in Caenorhabditis elegans) and at least four genes in Arabidopsis thaliana (Singh H, 2010). These channels possess distinct properties such as a single transmembrane domain and a dimorphic existence as either water-soluble globular form or as integral membrane protein form. Almost all the members of the CLIC family share significant similarity in their sequence (60-75%) but they vary in their cellular and subcellular distribution (Warton K, 2002). Among all the CLIC channels, CLIC1 channels are the most extensively studied. CLIC1 is shown to form functional ion channels in the absence of any ancillary protein (Ashley R, 2006; Tulk B, 2002).

Immunofluorescence studies of CLIC1 transfected Chinese Hamster Ovary (CHO) cells show that these proteins predominantly localize to the nuclear membrane, lysosomes, endosomes, secretory vesicles as well as the cytoplasm and nucleoplasm (Warton K, 2002). CLIC1 is shown to play a key role in an Alzheimer's disease (AD) brain, which is characterized by plaques containing β- amyloid (Aβ) protein. It has been demonstrated that Aβ promotes translocation of CLIC1 from cytoplasm to the plasma membrane; thereby upregulating CLIC1 mediated chloride current. Activation of microglial cells by Aβ is shown to produce reactive oxygen species (ROS) by the plasmalemmal NADPH oxidase. The

increased chloride conductance of the microglial cells and the production of ROS are thought to contribute to neurodegeneration in AD. Pharmacological inhibition of CLIC1 channels using anti-CLIC1 antibodies is shown to prevent the transmembrane chloride current and the production of ROS; thereby qualifying CLIC1 as a novel potential therapeutic target for AD (Milton R, 2008). Endogenous CLIC1 channels are shown to relocate to the plasma membranes during the G2/M phase of cellular mitosis and have been shown to contribute towards an increase in anion conductivity of cells. This indicates a role of CLIC1 channels in cell cycles and cell volume regulation (Valenzuela S, 2000). CLIC1 is widely expressed in epithelial and non-epithelial cells, where it exhibits tissue specific distribution and sub-cellular localization. In columnar epithelial cells, CLIC1 is present in apical domains and it is located in the cytoplasm in placental trophoblasts (Singh H, 2010). This vast and diverse distribution of CLIC1 in cells indicates its proposed role in a wide range of physiological processes such as bone resorption, regulation of cell motility, tubulogenesis, angiogenesis, formation of skeletal muscle, beta amelyoid induced neurotoxicity and p53 mediated apoptosis.

CLIC1 is a relatively small protein with a sequence of 241 amino acids and a molecular weight of 27kDa (Valenzuela S, 1997). To form a functional ion channel, CLIC1 undergoes structural transition from its soluble globular form to an integral membrane form and auto-inserts into a phospholipid bilayer. The crystal structure of soluble monomeric CLIC1 was solved at 1.4 Å resolution at pH 5.0 (Harrop S, 2001). CLIC1 has two domains, a mixed α-

helix/β-sheet N-terminal domain and a majority α-helix C-terminal domain (Adamson, 2009). CLIC1 channels share significant structural homology with the members of the omega glutathione S-transferase (GST) superfamily. However, how these soluble proteins unfold, target to and auto-insert into the membranes are complex biological questions, which still need to be answered.

Single and multi-channel electrophysiology recordings of CLIC1 have been obtained in its native environment by patching onto primary rat microglial cells and murine microglial immortalized cell line BV2 (Milton R, 2008) and via plasma membrane and nuclear patches of Chinese Hamster Ovary (CHO) cells overexpressed in CLIC1 (Tonnini R, 2000). It is also measured in artificial bilayers using the tip-dip technique (Warton K, 2002) and painted bilayer technique (Ashley R, 2006). CLIC1 is shown to retain its functionality as a chloride channel when expressed and purified from *E. coli* bacteria and reconstituted in artificial lipid bilayers (Tulk B, 2000). However, the study of biophysical properties of CLIC1 is still in its infancy and the literature is quite conflicting while reporting its selectivity and conductance. CLIC1 conducts chloride ions and also other halides, pseudohalides (SCN-) and bicarbonates. CLIC1 is reported to be anion-selective through its electrophysiology studies in transfected CHO-K1 cells where fluoride and pseudohalides are shown to be more permeable than chloride ions (Tonnini R, 2000). But these channels are still referred to as chloride channels as chloride ion is the most common anion (4 to 10mM) in organisms (Singh H, 2010). In this light, the nomenclature of CLICs as chloride ion channels may need

be revised. CLIC1 is also reported to be a poorly selective or non-selective ion channel in artificial lipid bilayers (Ashley R, 2006) (Singh H, 2007). In these cases, the permeability of cations and anions through the pore is often very similar to each other. In presence of a large cation like Tris, however, the CLIC channels were found to be more anion-selective. From these observations, it can be hypothesized that the CLIC channels may have lost an essential component that decides their selectivity, after reconstitution in artificial bilayers. The single channel conductance of CLIC1 has been reported to lie within a wide range of values between 6pS and 120pS (Singh H, 2010). This difference in measured conductance may be attributed to lipid environment, presence or absence of cholesterol in bilayers, redox conditions, salt concentration or pH of the recording solution. But the exact relationship between the conductance of this channel and its recording conditions has not been properly established yet.

2.2 Methods and Materials

2.2.1 Expression of protein in Escherichia Coli bacteria

Escherichia Coli strain BL21 RIL, containing recombinant CLIC1 plasmid was generously gifted to us by Dr. Harpreet Singh (UCLA, Department of Anesthesiology). 2-3 colonies of bacteria were scraped off from the culture plate using a sterile pipette tip. The bacteria were inoculated in 13mL of LB (Luria Bertani) medium, containing 50 µg/mL of kanamycin. This mixture was incubated overnight on a shaker at 37^{o}C at 250rpm. This overnight grown culture was inoculated in 1L of TB (Terrific Broth) containing 50µg/mL of kanamycin. It was incubated in the orbital shaker at 37^{o}C for approximately 4 hours, until the OD_{600} of the solution (optical density of the solution when 600nm light was passed through it) reached between 0.5 and 0.6. 0.25mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) solution was added and the solution was incubated at about 24^{o}C in the shaker for around 6 hours. IPTG is a molecular mimic of allolactose, which triggers the transcription of the *lac* operon. The culture was then spun at the speed of 5000g at 4^{o}C for 15min and the supernatant was discarded. The pellets were then air dried for about 10 mins to remove any excess medium sticking to the pellets. The pellets were then stored at -80^{o}C overnight. The pellets were resuspended in a 100mL of a buffer containing 150mM NaCl, 20mM Tris-base and 1mM DTT (pH 8.0). Lysozyme was added to this solution at the concentration of 40mg/100mL and was incubated for 1 hour. The solution was sonicated on ice at pulses of 0.5s on and

0.5s off for 1 min. This cycle was repeated 3 times. The sample was allowed to cool down for 3 mins between the consecutive sonication cycles. This sonicated sample was spun down at 28000g for 50mins at 4^{o}C. Both the pellets and the supernatant were saved. Ni-NTA beads were used to isolate the polyhistidine-tagged proteins from the supernatant solution. For each liter of induced cells, 2.5mL of Ni-NTA beads were used. The bottle containing the beads was briefly vortexed before use to form a uniform suspension of beads. The beads were mixed with 5mL the buffer containing 150mM NaCl, 20mM tris-base and 1mM DTT (pH 8.0) and centrifuged down at 1000rpm for 2 mins. This washing step was repeated 3 times. The beads were then mixed with the supernatant and incubated for 4 hours at 4^{o}C. The mixture was then poured through two gravity columns (Biorad) and allowed to drain at 4^{o}C. The beads were washed with 3 volumes of a buffer containing 150mM NaCl, 20mM Tris-base, 20mM imidazole and 1mM DTT (pH 8.0). The beads were then washed with 8mL of elution buffer containing 150mM NaCl, 20mM Tris-base, 250mM imidazole and 1mM DTT (pH 8.0) and the eluate was collected. 5µg/ml of thrombin was added to a part of the eluate to cleave the His-tag. This obtained a fraction of proteins with the His-tag and the rest of it without His-tag. This solution was poured into a dialysis cassette (Qiagen) and left at 4^{o}C in 1 liter of dialysis buffer containing 150mM NaCl, 20mM Tris-base and 1mM DTT (pH 8.0). The dialysis buffer was exchanged three times after 10 hours, 16 hours and 32 hours. The dialyzed proteins were incubated with 150µL of benzamidine sepharose 4B beads for 1.5 hours to remove thrombin from the solution.

The purified proteins were stored at -80^{o}C in storage buffer containing 150mM NaCl, 20mM Tris-base and 5mM DTT (pH 8.0). Thermo Scientific Pierce Coomassie Plus Protein Assay Kit was used to estimate the concentration of the purified protein. Absorbance of the protein sample and the BSA standards was measured at 595nm with a Tecan microplate reader and then fitted on a curve to estimate the unknown protein concentration.

2.2.2 Circular Dichroism

Purified *E.Coli*-derived CLIC1 (diluted to a concentration of 200µg/ml) was analyzed for integrity of its secondary structure by carrying out far-UV Circular Dichroism spectral analysis using Jasco J-270 spectropolarimeter. CLIC1 was stored in a buffer containing 150mM NaCl, 20mM Tris-base and 5mM DTT (pH 8.0). CD data was collected over the wavelength range of 260-195nm with the step resolution of 0.5nm, response time of 4s and at an acquisition speed of 20nm/min. The spectral bandwidth of the collected data was 1nm and sensitivity of data display was 20mdeg.

2.2.3 SDS-PAGE analysis

The protein was stained using Coomassie stain and electrophoresis was performed under denaturing conditions by SDS-PAGE on precast 5-20% acrylamide gels (BioRad) at 100V for around an hour.

2.2.4 Gel filtration

Extracted protein was run down a Superdex-200 10/300GL gel filtration column for both purification and molecular weight characterization. Two samples of protein were made, one under reducing conditions and the other under oxidizing conditions via addition of 20mM H_2O_2. The concentration of the protein was around 1-2 mg/ml and the volume was adjusted to 250µl before running it down the column.

2.2.5 Channel incorporation in painted bilayers

Painted bilayer technique was used to measure single-channel currents of CLIC1. CLIC1 was incorporated into planar artificial lipid bilayers formed from palmitoyl-oleoyl phosphatidylethanolamine (POPE), palmitoyl-oleoyl phosphatidylserine (POPS) and cholesterol (4:1:1 molar ratio) (All the lipids were purchased from Avanti Polar Lipids, Inc.) Lipids dispersed in n-decane at a concentration of 25mg/ml were used to pre-paint an aperture of 200micron diameter, present in a thin Delirin membrane that separated two solution-filled chambers. The chamber in which the protein was subsequently added was designated *cis* (corresponding to the cytosolic side of the cell), whereas the other chamber was designated *trans* (corresponding to the external side of the cell). The *cis* chamber contained 500mM KCl, 10mM Tris-base (pH 7.4) and the *trans* chamber contained 50mM KCl, 10mM Tris-base (pH 7.4). The volume of buffer on both sides was 300µl. Approximately 2 µg of CLIC1 was introduced in the *cis* chamber.

2.2.6 Channel incorporation in droplet bilayers

Droplet bilayers were used measure ensemble CLIC1 channel activity. CLIC1 was incorporated into liposomes consisting of palmitoyl-oleoyl phosphatidylethanolamine (POPE), palmitoyl-oleoyl phosphatidylserine (POPS) and cholesterol (4:1:1 molar ratio). The upper droplet consisted of 500mM KCl, 10mM Tris-base (pH 7.4) and the lower aqueous contained buffer containing 50Mm KCl, 10mM Tris-base (pH 7.4). The detailed procedure of forming these sessile droplet bilayers is explained in Chapter 1 of this thesis.

2.3 Results

2.3.1 SDS-PAGE, Circular Dichroism (CD) and Gel Filtration

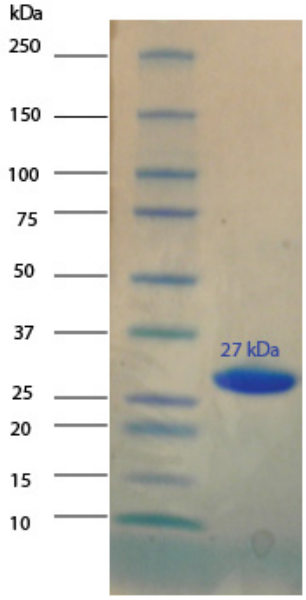


Figure 2-1. **SDS-PAGE analysis of reduced recombinant CLIC1.** The Coomassie-stained SDS-PAGE analysis of *E. coli* purified recombinant CLIC1 shows a single band at approximately 27kDa, with no evidence of multimers under denaturing conditions.

Figure 2-1 shows the results of SDS-PAGE electrophoresis experiments. Molecular weight of monomeric CLIC1 is reported to be around 27kDa and comparable results were found in these experiments consistently.

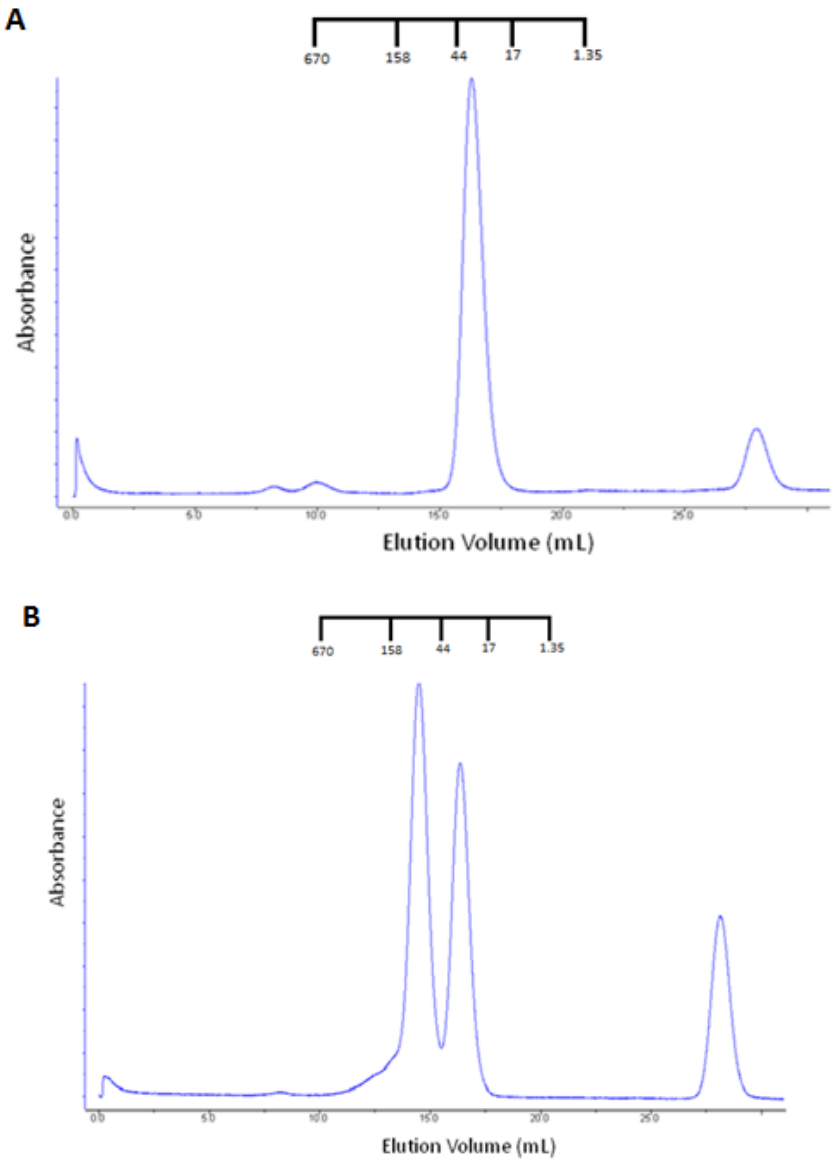


Figure 2-2. **FPLC analysis of CLIC1 under reducing and oxidizing conditions.** The molecular weight values are pre-calibrated using standard protein solutions. (A) CLIC1 is stored in reducing buffer conditions containing 150mM NaCl, 20mM Tris-base and 5mM DTT (pH 8.0). The FPLC peak corresponds to a molecular mass of ~30kDa. CLIC1 exists primarily in its monomeric form under reducing conditions. (B) CLIC1 is stored in an oxidizing buffer containing 150mM NaCl, 20mM Tris-base and 20mM H_2O_2 (pH 8.0). Two peaks are seen at ~30kDa and ~70kDa. The peaks correspond to monomeric and dimeric CLIC1.

The gel filtration plots are calibrated with standards of known molecular weight. The

calibration bar can be seen on top of the plot. CLIC1 is known to be in its monomeric state under reducing conditions and it oxidizes to form a dimer in presence of an oxidizing agent like H_2O_2 (Littler D, 2004). We verified that fact by running the oxidized and reduced protein on a gel filtration column. In Figure 2-2-A, it can be seen that the protein sample is entirely in its monomeric form (molecular weight ~27kDa) under reducing conditions. In Figure 2-2-B, it can be seen that the protein sample contains both monomeric and dimeric forms. The two peaks at ~30kDa and ~70kDa are clearly visible in the plot. It has been hypothesized that oxidation is essential for the transition of CLIC1 from the monomer to the integral membrane chloride channel form (Littler D, 2004). However, we were able to reconstitute CLIC1 in artificial bilayers under both reducing and oxidizing conditions and could not see a significant difference in the efficiency of reconstitution.

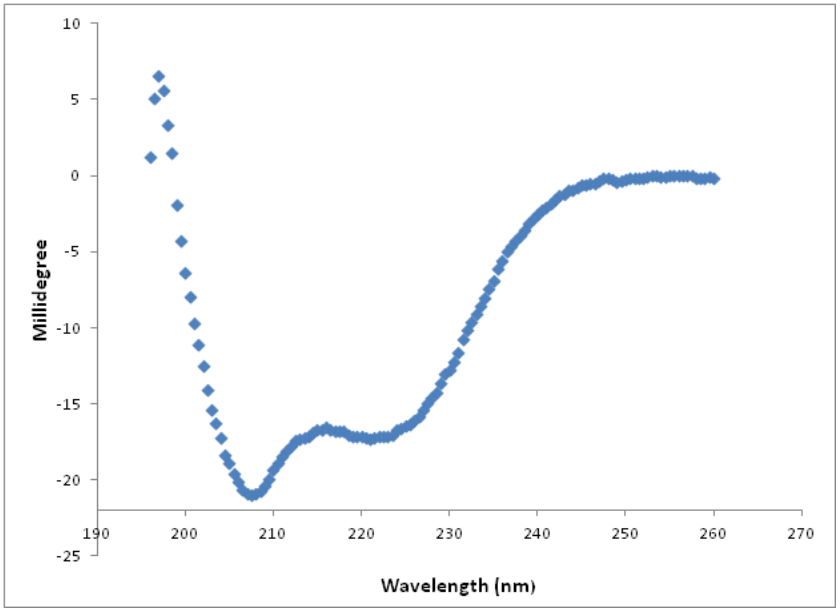


Figure 2-3**. Circular Dichroism of CLIC1.** CLIC1 was diluted in a buffer containing 150mM NaCl, 20mM Tris-base and 0.5mM DTT (pH 8.0) at a concentration of ~200µg/ml. The CD data was collected over the wavelength range of 195-260nm. The spectrum shown is averaged over three scans and is baseline-corrected.

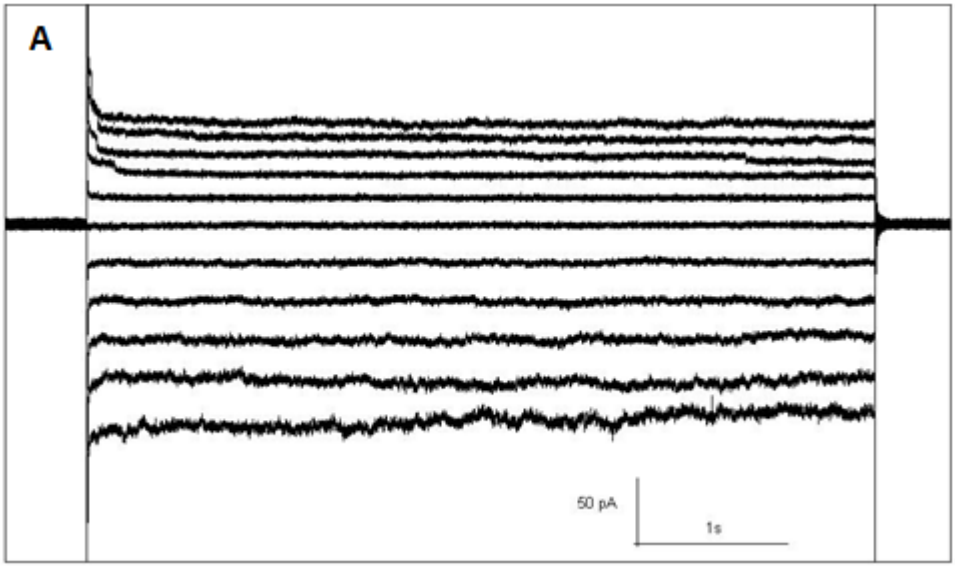
For CD measurements, CLIC1 was suspended in a buffer containing 150mM NaCl, 20mM Tris-base and 0.5mM DTT (pH 8.0) at a concentration of around 167microgram/ml. Clear troughs in the CD spectrum are observed at 298nm and 220nm, as well as a strong positive peak around 195 nm, which are strong indicators of presence of α-helix structures in the protein. This CD data matches very closely with the published literature (Warton K, 2002).

2.3.2 Single-channel and ensemble currents

Ensemble CLIC1 currents were measured using the droplet bilayer platform and single-channel currents were measured using painted bilayer technique. The fraction of CLIC1 that was purified without cleaving the His-tag was used for its preliminary functionality test, where Nickel Chloride ($NiCl_2$) was used to block the CLIC1 mediated ensemble current. The vector used for cloning and expression of CLIC1 was pHis8, a modified pET-28a (+) vector with additional histidines to increase the efficiency of purifications. This pHis8 vector encodes an N terminal octa-His tag and a corresponding thrombin cleavage site. This N-terminus His-tag binds with transition metals like Nickel and Cobalt with micromolar affinity. 10µM $NiCl_2$ was added in both *cis* and *trans* chambers and it consistently caused a complete block of the CLIC1 channel current (Fig. 2-4). The exact mechanism of this

blockage could not be figured out, but it was hypothesized that the nickel ions in $NiCl_2$ bound to the N-terminus His-tag of CLIC1, thereby occluding the transmembrane pore.

Indanyloxyacetic acid-94 (IAA-94) (purchased from Sigma-Aldrich) is a known chloride channel blocker and is shown to block CLIC1 mediated chloride currents with an IC_{50} value of 8.6μM. The CLIC1 mediated currents could be blocked with 100μM of IAA-94 (Fig. 2-5). As CLIC1 also mediates the passage of other halides and cations, some residual channel activity could be seen after blockage.



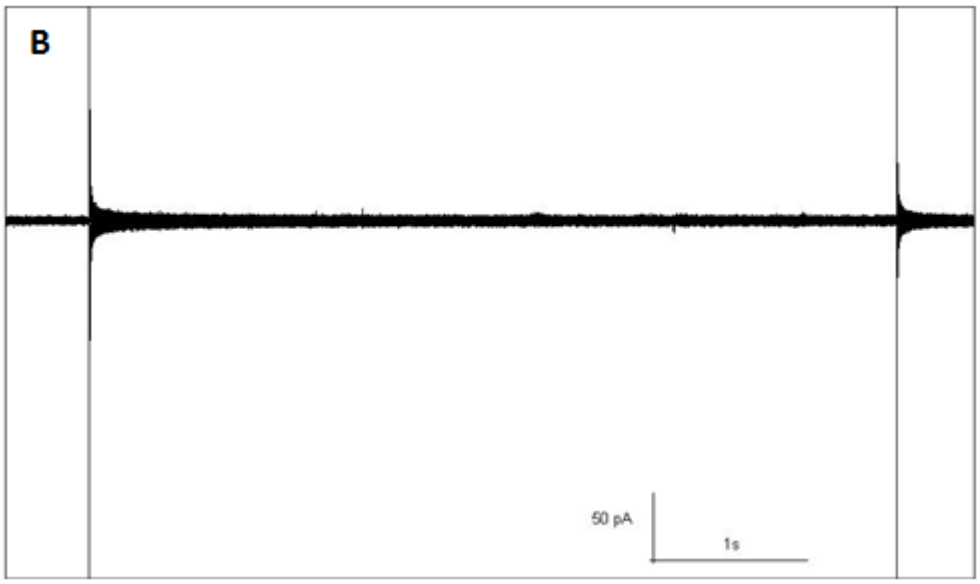
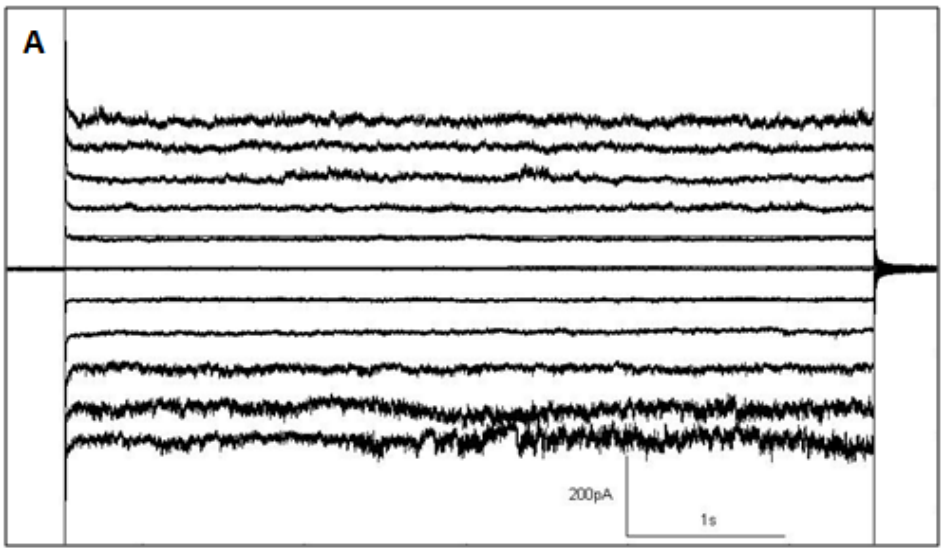


Figure 2-4. **Representative multi-channel CLIC1 currents before and after block with $NiCl_2$.** Artificial lipid bilayers were formed using sessile droplet bilayer platform, with the top and bottom aqueous solutions containing CLIC1 proteoliposomes (500µg/ml) made of POPE, POPS and cholesterol at the molar ratio of 4:1:1. (A) Data represents CLIC1 ensemble currents recorded in asymmetric buffer conditions with a cis:trans gradient of 500:50 mM KCl. Voltage protocol: 0mV holding potential, voltage ladder running from -100mV to +100mV at 20mV steps and step back to 0mV holding potential. (B) CLIC1 channel activity is completely blocked after addition of 10µM $NiCl_2$.



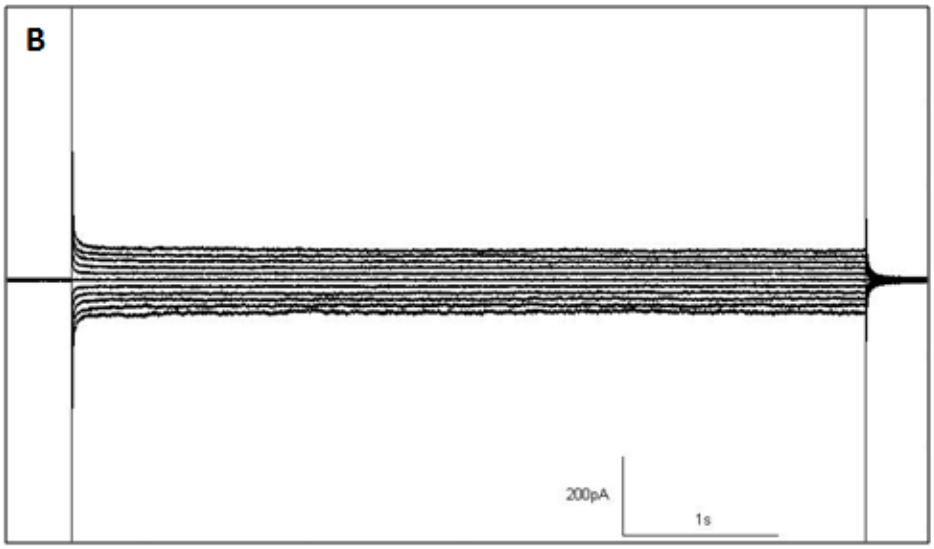


Figure 2-5. **Representative multi-channel CLIC1 currents before and after block with IAA-94.** Artificial lipid bilayers were formed using sessile droplet bilayer platform, with the top and bottom aqueous solutions containing CLIC1 proteoliposomes (500µg/ml) made of POPE, POPS and cholesterol at the molar ratio of 4:1:1. (A) Data represents CLIC1 ensemble currents recorded in asymmetric buffer conditions with a cis:trans gradient of 500:50 mM KCl. Voltage protocol: 0mV holding potential, voltage ladder running from -100mV to +100mV at 20mV steps and step back to 0mV holding potential. (B) CLIC1 channel activity is blocked after addition of 100µM IAA-94.

Single-channel current measurements were carried out in painted bilayers. Under the cis: trans gradient of 500:50 mM KCl, the single channel conductance of CLIC1 was observed to be ~30pS, which is very similar to the published value. In these conditions, the conductance of CLIC1 has been reported to be 24pS, 25pS, 38pS (Singh H, 2010).

Figure 2-6. **Single-channel current of CLIC1.** Current is recorded in the painted bilayer system in asymmetric buffer conditions with a cis:trans gradient of 500:50 mM KCl at +100mV.

2.4 Discussion

CLIC1 is an intracellular ion channel and primarily localizes on cellular nucleosomes. To isolate nucleosomes from mammalian cells and to study the electrophysiology of ion channels present on their delicate inner/outer membranes using patch-clamp is a very challenging process. The demonstration of our ability to reconstitute purified CLIC1 into droplet lipid bilayers signifies considerable potential in easy, inexpensive and high-throughput measurements of intracellular ion channels for their biophysical characterization and also for pharmaceutical screening.

The efficiency of reconstitution of CLIC1 into droplet bilayers was, however, very low (~5%). As the CLIC1 protein is water soluble, we hypothesize that our reconstitution system may be missing a significant physical/chemical component that aids the conversion of the protein from its soluble form to membrane integral form. The overall yield of the formation of bilayers with incorporated CLIC1 can be significantly increased by parallelization and automation of our droplet bilayer platform. Work towards that goal is in progress (Thapliyal T, 2011). Redox conditions, solution composition and pH values can also be optimized to obtain a greater reconstitution rate.

We did not observe strong chloride selectivity in these 'chloride intracellular channels'. Similar results on the observed non-selectivity of these ion channels have been reported by other groups (Ashley R, 2006). This non-selectivity could be an intrinsic phenomenon of CLIC proteins, in which case the 'chloride channel' nomenclature needs to be reconsidered;

or this may be because the reconstituted CLICs were missing a critical cellular component interacting with the protein that plays a key role in determining the selectivity of the channel.

The *in vivo* bacterial expression system used for the synthesis of CLIC1 is a complex and time consuming process, limiting the throughput of our ion channel measurement platform. This recognizes the need for efficient protein synthesis and reconstitution methods.

Further work can be done in the area of obtaining drug response curves and pharmacologically relevant IC_{50} and EC_{50} values by controlled and incremental perfusion of drugs/CLIC1 blockers into the surrounding solution.

Chapter 3
Reconstitution of *in vitro* synthesized voltage-gated potassium channel (Kv1.2)

3.1 Introduction

Synthesis of proteins using bacterial expression systems, such as *Escherichia coli,* is a slow and complex process. Bacterial systems often do not allow for proper glycosylation and post-translational modifications of eukaryotic proteins leading to their misfolding and most complex eukaryotic proteins become insoluble upon expression. The time consuming nature of this *in vivo* bacterial protein expression system also severely limits the throughput of our droplet bilayer system as a drug screening platform for ion channels. However, recent technical developments have revitalized cell free expression systems and have underlined their use to meet the increasing demands for an efficient protein synthesis process. Cell free or *in vitro* expression is the production of recombinant proteins in solution using translation machinery extracted from cells. This process is known as the cell free expression process, as the protein synthesis occurs in cell lysates rather than within live cultured cells. Pioneering studies for cell free expression systems were done more than 4 decades ago (Matthaei M, 1961). *In vitro* translational systems are based on this early demonstration that cell integrity is not required for the synthesis of proteins. Cell free protein production can be achieved outside of a live cell by using several kinds and species

of cell extracts. Cell free systems offer a bunch of advantages over the traditional cell-based expression systems, including easy post-translational modifications that favor protein folding, decreased sensitivity to product toxicity and amenability to high-throughput because of drastic reduction in the reaction volumes and process time (Katzen F, 2005). Improvement in translational efficiency has enabled production of over 1 miligram of protein per 1 ml of reaction mixture (Katzen F, 2005). It must be noted, however, that cell free expression may not be practical for large scale commercial protein production.

In its simplest form, two major components are required to accomplish cell free protein expression; the genetic template in the form of an mRNA (requires only *in vitro* translation) or DNA (requires both *in vitro* transcription and translation) encoding the target protein and a reaction mixture containing cellular machinery required for transcription and translation. The cellular machinery can be obtained by using a crude lysate from any given organism. The lysate provides the translational machinery, RNA polymerase, ribosomes, tRNA, accessory enzymes, amino acids, cofactors and cellular components essential for proper protein folding. An exogenous energy source is often required to carry out these cellular processes. *Escherichia Coli* is the most common source of cellular lysate. *E. coli* based systems provide higher yields and more homogenous samples. The protein yields of *E. coli* based systems range from micrograms to a few milligrams per milliliter of reaction volume, depending on the protein and the reaction format (Katzen F, 2005). More recently, extracts from insect cells, mammalian cells and human cells have been developed and made

commercially available.

Membrane proteins account for nearly a third of the genes encoded by most fully sequenced genomes (Lehnert U, 2004). However only a handful of (<80) integral membrane proteins have been solved to full resolution. This reflects the difficulties faced in expression and preparation of these membrane proteins, especially the eukaryotic or mammalian ion channel proteins. Structural and functional studies of membrane proteins are problematic because unlike soluble proteins, they require the presence of a lipid bilayer for correct folding. *In vitro* translation offers a unique opportunity to use the highly efficient bacterial transcription and translational machinery, while introducing eukaryotic and mammalian lipids. Often mild detergents are used in the process to reduce aggregation and insolubility of expressed proteins. By adjusting the relative amount of coding templates, membrane proteins and their cognate partner proteins can also be co-expressed at a specific molar ratio required for the proper folding and solubility considerations. *In vitro* synthesized ion channel proteins such as G-protein coupled receptors (GPCRs) (Robelek R, 2007), viral potassium channel Kcv (Syeda R, 2008), VDAC and Bak (Liguori L, 2007), Stearoyl-CoA desaturase (Goren M, 2008), bacteriorhodopsin (Kalmbach R, 2007), Cx43 (Moritani Y, 2010), MscA (Berrier C, 2004) have been shown to be incorporated in liposomes and artificial lipid bilayers till date. Voltage gated potassium channels (Kv channels) control the flow of potassium ions (K^+) through the cell membrane in response to changes in the membrane potential. Potassium voltage-gated channel subfamily A member

2, also known as $K_v1.2$ is a protein that in humans is encoded by the *KCNA2* gene. In this chapter, we demonstrate successful reconstitution of *in vitro* synthesized Kv1.2 in our droplet bilayer system.

3.2 Methods and materials

The *in vitro* synthesis of the Kv1.2 proteins and their reconstitution in liposomes (at the concentration of 20mg/ml) was carried out by Synthelis S.A.S® (La Tronche, France). The composition of the liposomes was not disclosed by the concerned party. The concentration of Kv1.2 in the proteoliposome solution was 35-50 µg/ml. The supplied proteoliposomes were further diluted 40 fold using control liposomes (500µg/ml) made up of palmitoyl-oleoyl phosphatidylethanolamine (POPE) and palmitoyl-oleoyl phosphatidylglycerol (POPG) (Avanti Polar Lipids) at a molar ratio of 3:1 (MacKinnon R, 2008). The control liposomes were formed by drying the mixture of required lipids under argon to form a thin film, which was resuspended at 1mg/ml in the reconstitution buffer (150mM KCl, 10mM HEPES, pH 7.4) and by extruding it through 200nm filters (Avanti Polar Lipids). The measurement chamber and bilayer mask were constructed as previously described in Chapter 1. The lower aqueous well of the chamber was filled with 250 µl of the diluted proteoliposomes and a 2 µl droplet of the same solution was deposited on an Ag/AgCl electrode. The central well was filled with 80 µl n-decane solution. A bilayer was formed using the methods/procedures described previously. For electrical measurements, the bilayer apparatus was placed inside a Faraday cage. The Ag/AgCl pin and a counter-electrode were connected to Axopatch 200B amplifier (Axon Instruments).

3.3 Results

3.3.1 SDS-PAGE of the in vitro synthesized Kv1.2

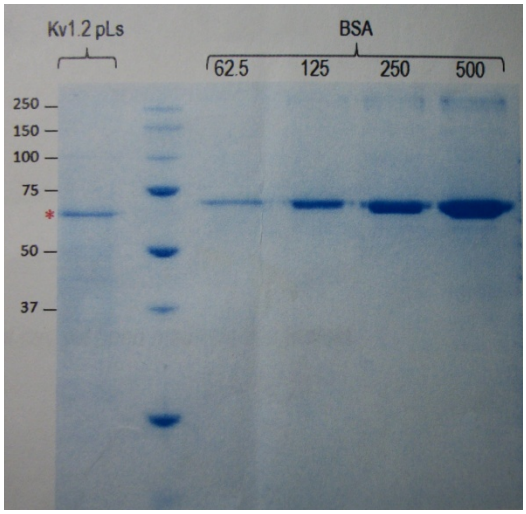


Figure 3-1. **SDS-PAGE analysis of *in vitro* synthesized Kv1.2.** The values of molecular weight markers are indicated on the left in kDa. The Coomassie stained band of full length Kv1.2 is represented by (*). From left to right the lanes represent bands for 10µl of Kv1.2 proteoliposomes, 5µl of BSA solutions at 62.5, 125, 250 and 500 µg/ml respectively.

The SDS-PAGE results of Kv1.2 were sent to us by Synthelis S.A.S® (La Tronche, France). The molecular weight of Kv1.2 is reported to be 57kDa, which is close to the value observed in the gel analysis.

3.3.2 Electrophysiology data of Kv1.2

Multi-channel currents of Kv1.2 of magnitude more than 500pA were recorded in symmetric *cis* and *trans* solutions containing 150mM KCl (Figure 3-2). The ion channel signals obtained were digitized using Digidata 1332A (Axon Instruments) at 5kHz, filtered with a 1kHz 8-pole Bessel filter and were analyzed with Clampfit (Molecular Devices Inc.). Bilayer capacitance was observed to be around 150pF. The bilayers were held at 0mV for

1s, stepped to voltages from -100mV to +100mV in 50mV increments for 7s and were stepped back down to 0mV for 1s.

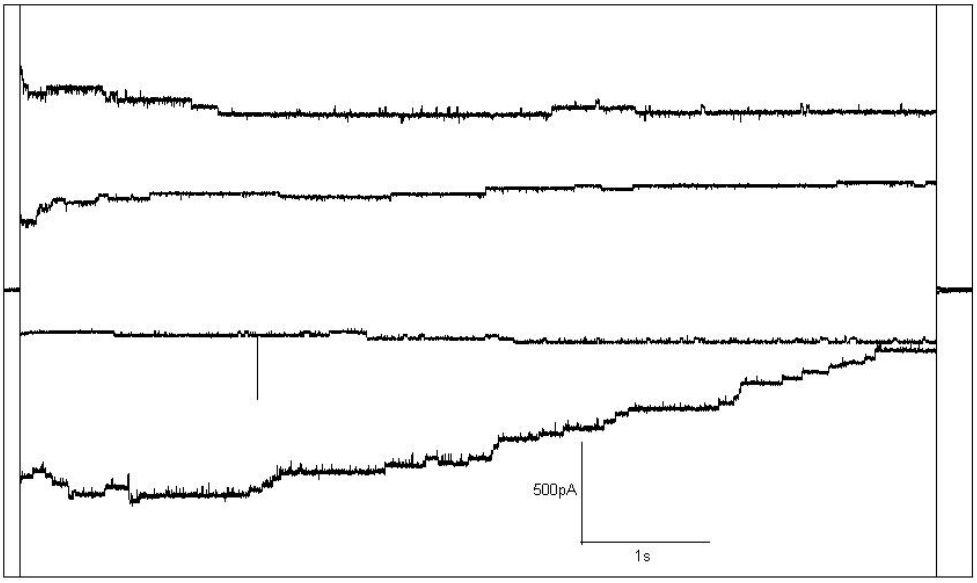


Figure 3-2. **Representative multi-channel current traces of Kv1.2.** Artificial lipid bilayers were formed using sessile droplet bilayer platform, with the top droplet containing Kv1.2 proteoliposomes (500µg/ml) that were synthesized using *in vitro* expression systems. The lower aqueous chamber was filled with liposomes (500µg/ml) containing POPE and POPG at a molar ratio of 3:1. Data represents Kv1.2 ensemble currents recorded in symmetric buffer conditions at 150mM KCl. Voltage protocol: 0mV holding potential for 1s, voltage ladder running from -100mV to +100mV at 50mV steps for 7s each and step back to 0mV holding potential for 1s.

Under symmetric buffer conditions, on application of -100mV, inactivation of the ensemble current was consistently observed. The direction and magnitude of K+ currents during the depolarizing steps depend on both the channel open probability and the electrochemical driving force for K+, which produces inward (downward) current at negative voltages and outward (upward) current at positive voltages under these ionic conditions. Many Kv channels are reported to undergo spontaneous inactivation during the application of long depolarization steps (MacKinnon R, 2008).

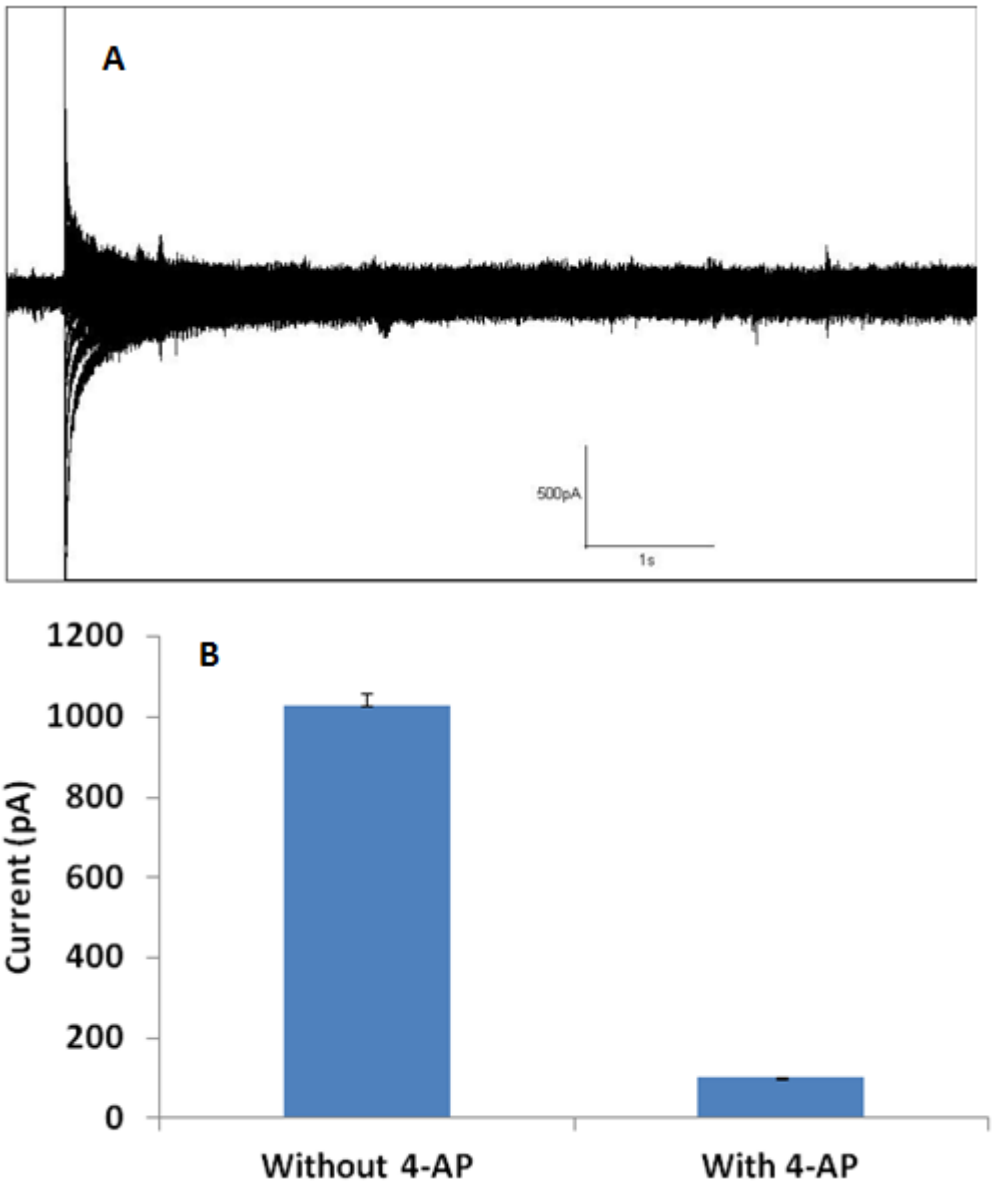


Figure 3-3. **Inhibition of Kv1.2 channel activity with 4-AP.** (A) Kv1.2 proteoliposomes were pre-mixed with 20mM 4-AP and activity was recorded in artificial lipid bilayers in symmetric buffer containing 150mM KCl (B) Bar chart shows normalized ensemble Kv1.2 currents in the presence and absence of 20mM 4-AP

4-Aminopyridine (4-AP) is a classical K^+-channel blocker, with half-blocking concentration for Kv1.2 close to 600μM (MacKinnon R, 2008). In our experiments, the Kv1.2 proteoliposomes were pre-mixed with 20mM 4-AP and incubated for an hour. The Kv1.2

mediated potassium currents drastically went down after treatment with 4-AP. Current magnitudes before and after treatment with 4-AP (at applied voltage of 100mV) were averaged over 5 experiments and plotted (Figure 3-3).

3.4 Discussion

The *in vitro* synthesized Kv1.2 was kindly provided to us by Synthelis S.A.S® (La Tronche, France), however, the stability of the sample during its transportation could not be ascertained. The currents observed in the bilayers formed from these proteoliposomes often degraded steeply within a week, even when they were maintained at recommended storage conditions. Also, as the proteoliposomes were not synthesized in-house, our knowledge of the lipid composition and storage buffers was limited.

As the technology of cell-free protein synthesis is still in its nascency, many complex eukaryotic ion channels have not yet been synthesized using this technique and that limits the choice of ion channels that can be worked with.

Although we could obtain ensemble ion channel currents reliably, the orientation of reconstituted ion channels could not be easily controlled in our droplet bilayer platform, making it difficult to observe voltage rectification of ion channels. However, with the use of ion channel blockers that block only one side of the channel, measurement of oriented channels can be achieved. CTX has been reported to be such a 'sided blocker' for Kv1.2 (Tao X, 2008; Long S, 2007), which can enable measurement of kinetic behavior and rectification of these proteins.

Along with Kv1.2, we also measured the electrophysiological properties of *in vitro* synthesized mammalian intracellular channels like VDAC and ion channels formed by the Hepatitis C virus p7 protein (data unpublished). Measurement of these ion channel proteins

allowed validation of our bilayer platform for measurement of a varied range of channels and it also verified the ability of *in vitro* protein synthesis methods to produce functional proteins.

Chapter 4
Processing mammalian cells for extracting membrane proteins

4.1 Introduction

A high-throughput sessile droplet bilayer platform can potentially replace the complex and expensive patch-clamp technology for ion channel screening. However, the yield and throughput of ion channel measurement in artificial bilayers is often limited due to the complex and time consuming processes required for synthesizing these proteins. *In vivo* bacterial expression systems for protein synthesis are inadequate in carrying out glycosylation and post-translational modifications to eukaryotic proteins leading to their misfolding. Cell-free or *in vitro* expression systems have gained momentum in the area of protein synthesis; however, the technology is still in its nascency. Only a handful of ion channel proteins have been successfully synthesized and reconstituted into artificial bilayers using *in vitro* expression systems. Mammalian *in vivo* expression systems can be used to avoid these problems, but their yield is low, the cost of production is high and their cell culturing is very time consuming. However, mammalian cell lines overexpressed in ion channel proteins are readily available commercially for their use in patch clamp technology. Recently, the cell membrane fragments formed by rupturing the Human Embryonic Kidney (HEK293) cells were shown to be successfully used in reconstitution of ion channels in

artificial droplet bilayers (Leptihn S, 2011). This method requires minimal sample preparation, allowing for greater potential for high throughput measurements of ion channels. Due to the high observed efficiency of protein reconstitution of this method, very little sample volume is required. Usually, less than one cell is required for protein reconstitution in a bilayer, which allows for easy reconstitution of membrane proteins having low expression levels in eukaryotic cell lines. Additionally, live cells are not required, enabling long-term storage of these cells in -80^{0}C refrigerator or by snap-freezing in liquid nitrogen.

In this particular chapter of my thesis, I discuss various techniques that can be potentially used for the formation of plasma membrane fragments or plasma membrane vesicles from eukaryotic cells, which can be used for reconstitution of membrane proteins in artificial lipid bilayers. Bilayers formed using these membrane fragments are characterized by their stability, amount of leak, expression of functional ion channels and the need for external lipids for their formation.

4.2 Methods and materials

In these experiments control (not overexpressed in any ion channels) HEK293 (Human Embryonic Kidney) cells were used for plasma membrane fractionation. The control cells were generously supplied to us by Dr. Zhu Zhang (UCLA, Department of Anesthesiology). All the cell processing was done with 2 million initial cells.

The cell fractionation techniques that were tested out in our experiments were sonication, freeze-thaw, osmotic lysing and other methods employing a combination of these techniques. These methods of cell disruption are host cell dependent. Along with membrane fragments/vesicles, cell disruption also yields other soluble proteins, remaining intact cells, cell debris and other contaminants. The cell membrane fragments can be used as a crude extract or they can be further purified using differential centrifugation.

These processed samples were first observed under the microscope for a preliminary visual characterization and then tested for their ability to form bilayers with ion channels using the droplet bilayer platform, wherein the top droplet consisted entirely of the cellular fragments and the lower aqueous solution was filled with DPhPC (1,2-diphytanoyl-sn-glycero-3-phosphocholine) liposomes (500 μg/ml). It must be noted that the membrane fragments derived from mammalian cells also contain ion channels which are endogenous to the mammalian host cells, in addition to the overexpressed ion channels.

4.2.1 Osmotic Lysing

HEK293 control cells and hERG cells were resuspended in a hypo-osmotic buffer containing 0, 10, 25 and 100 mM KCl and 10mM Tris-base (pH 7.4). Protease Inhibitor tablets were added to the solution to prevent the denaturation of membrane proteins.

4.2.2 Sonication

The pellets from the control HEK293 cells and the hERG cells were placed in isotonic buffer solutions and were placed in an ultrasonic bath solution (VWR 50T, operating at 20-50 kHz, 117V) for 0.3, 1, 5, 10, 60 seconds.

4.2.3 Freeze-thaw

Cell pellet formed with control HEK293 cells was resuspended in an isotonic buffer was frozen slowly in the -80^0C refrigerator and subsequently thawed. The slow freezing of the sample causes the formation of ice crystals and thawing of those crystals causes the cell membranes to shear, leading to disintegration of cells to form cell membrane fragments. As this process is repeated, the size of the cell membrane fragments is predicted to reduce. The size of these membrane fragments determines the amount of protein available for reconstitution into the planar lipid bilayers.

4.2.4 Plasma Membrane Purification Kit

Qproteome® Plasma Membrane Protein kit was purchased from Qiagen®. This kit uses a combination of various cell-lysis and purification techniques to isolate plasma membrane fractions of a cell. The control HEK293 cells were tagged using a ligand that specifically bound to their plasma membranes. A combination of osmotic lysis and mechanical disruption was used to burst the cells. The cell debris, intact cells and other intracellular organelles were removed from the suspension by centrifuging them at 12000g. The separated plasma membrane fractions were made to bind with Strep-Tactin magnetic beads, which were then separated using magnets and the beads were then eluted using an elution buffer to isolate purified plasma membrane fractions.

4.3 Results

4.3.1 Osmotic Lysing

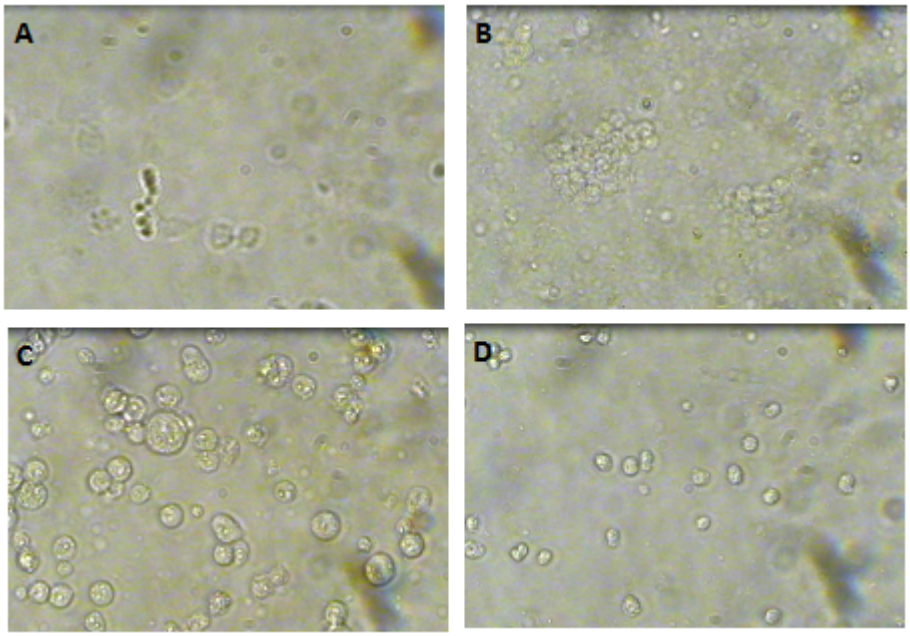


Figure 4-1. **HEK293 cells after Osmotic Lysing.** HEK293 cells when placed for a period of 2 hours in a hypotonic extracellular solution containing (A) 0mM KCl, 10mM Tris-base (pH 7.4) (B) 10mM KCl, 10mM Tris-base (pH 7.4) (C) 25mM KCl, 10mM Tris-base (pH 7.4) (D) 100mM KCl, 10mM Tris-base (pH 7.4)

Osmotic lysing is a mild, simple and inexpensive method of cell disruption. The extracellular hypo-osmotic solution causes the cells to swell and results in consequent membrane disintegration. Osmotic lysing of cells yields large membrane fragments and releases other cytosolic components to the suspension. This method is only effective against less robust cellular membranes, for example, those of animal cells.

The HEK293 cells were osmotically lysed by placing them in extracellular hypo-osmotic solutions containing 0, 10, 25 or 100mM KCl. Placing the cells in 100mM KCl solution did

not create a strong osmotic gradient across the cell membranes, leaving most of the cells intact. In 25mM KCl, the cells were visibly swollen and could also been seen aggregating with each other forming cellular clumps. In 10mM KCl, the individual cell size was much smaller indicating the rupture of cells and the cells formed much larger aggregates. In de-ionized extracellular buffers, almost all the cells ruptured completely, leaving behind very little traces of visible cellular components. The stock of cells lysed with 25mM extracellular solution was used in the droplet to form a bilayer and the lower aqueous solution was filled with DPhPC (1,2-diphytanoyl-sn-glycero-3-phosphocholine) liposomes (500 µg/ml). It was observed that only the cell membrane fragments were sufficient to form the top monolayer and upon its contact with the lower monolayer consisting of DPhPC lipids, an asymmetric bilayer could be formed. Upon the formation of bilayers, leak currents less than 5pA in magnitude could be observed in the system. These currents can be attributed to be mediated by the endogenous ion channels present on the plasma membrane or the intracellular organelles of the HEK 293 cells.

4.3.2 Sonication

Sonication is a simple, easy but harsh method of cell disruption. It may cause overheating of the sample and can compromise the integrity of membrane proteins by shearing. Sonication is also not favorable to use with large sample volumes.

As mentioned in the materials, the control HEK293 cell samples were sonicated for times

varying from 0.3s to 60s. The processed samples were observed under the microscope at 20X magnification. The sample that was sonicated for the least amount of time (0.3s) seemed to have a small number of vesicles (presumably made from the plasma membrane) and a large number of intact cells. With increase in sonication time, the number of intact cells reduced and the number of vesicles (represented by tiny dots in the figures) increased. However, increase in sonication also seemed to cause formation of long and presumably aggregated structures.

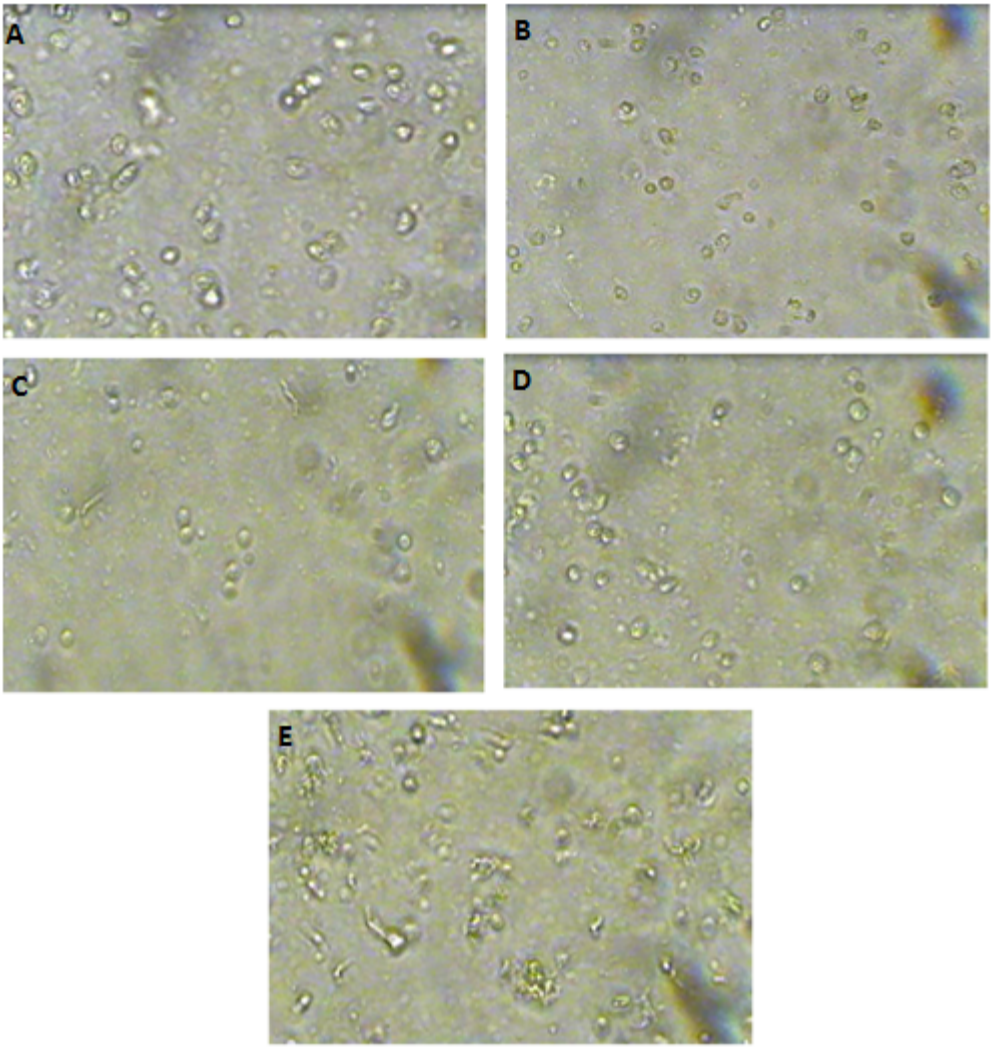


Figure 4-2. **HEK293 cells after Sonication.** HEK293 cells are placed in an isotonic buffer solution (A) Sonicated for 300ms. Most of the cells remain intact. (B) Sonicated for 1s. Number of intact cells reduces considerably and small structures, presumably membrane vesicles appear. (C) Sonicated for 5s. Vesicles seem to undergo aggregation/fusion. (D) Sonicated for 10s. Further aggregation leads to lumps of cellular fragments and cellular debris. (E) Sonicated for 60s. Long and narrow aggregated structures and cellular debris can be seen. This solution presumably also contains membrane vesicles that cannot be observed at this microscope magnification.

The cell membrane fragments formed after a brief period of sonication were insufficient to form a stable lipid monolayer in absence of external auxiliary lipids, but stable bilayers could be formed entirely from the cell fragments that were created after sonicating the cells for >1min. As the cell fragments were formed using control HEK293 cells, which are not overexpressed in any ion channel proteins, the bilayers conducted very little transmembrane current. The small observed transmembrane activity can be attributed to the endogenous ion channels present in the plasma membranes of these mammalian cells.

4.3.3 Freeze-thaw

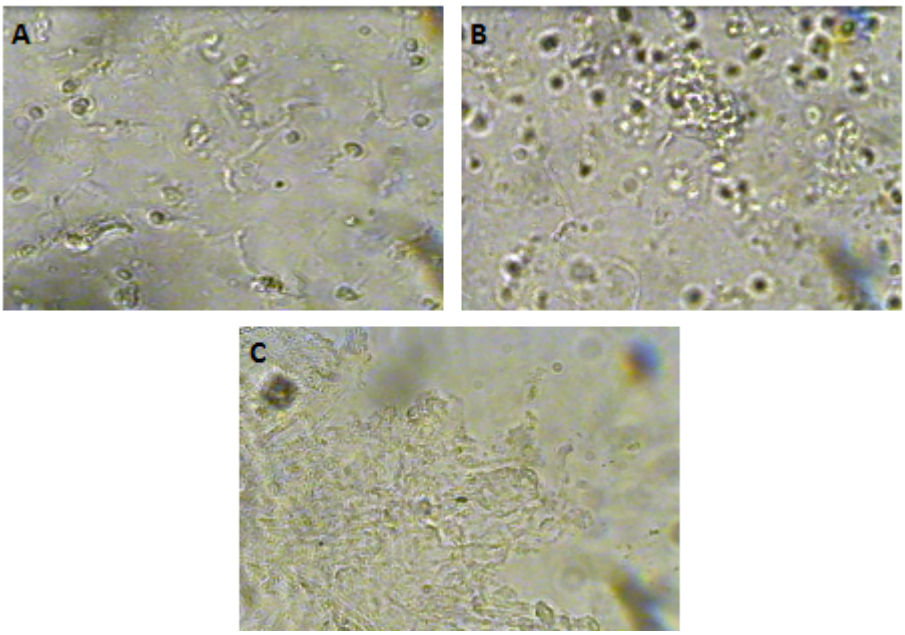


Figure 4-3. **HEK293 cells after Freeze-thaw.** The cells undergo (A) one freeze-thaw cycle. Long and narrow cellular fragments observed. (B) two freeze-thaw cycles. Membrane fragments seem to aggregate. (C) three freeze thaw cycles. The size of the membrane fragments reduces considerably, but larger aggregates of cellular material can be seen.

Freeze-thawing is a very simple and inexpensive technique of cell disruption, which yields large membrane fragments. However, this process is has a relatively low yield, is slow, may damage sensitive proteins and dissociate membrane protein complexes. Cellular fractions were collected after 1, 2 and 3 brief freeze-thaw cycles of HEK293 cells and observed under the microscope with 20X magnification. After the first freeze-thaw treatment, long and narrow cellular fragments were observed. Second freeze-thaw cycle caused the fragments to aggregate, forming clumps of cellular and possibly intracellular material. After the subsequent freeze-thaw cycle, the size of the fragments seemed to have reduced but the fragments formed much larger aggregates. Membrane fragments formed after 2 freeze-

thaw cycles were used in a preliminary interface experiment, wherein the fragments were observed under the microscope and were seen to break open and fuse with the aqueous-organic interface, indicating the formation of a possible monolayer. The freeze-thawed membrane fragments of control HEK293 cells were used in electrophysiology experiments, where they could successfully form lipid bilayers having minimal leak currents.

4.3.4 Plasma membrane purification kit

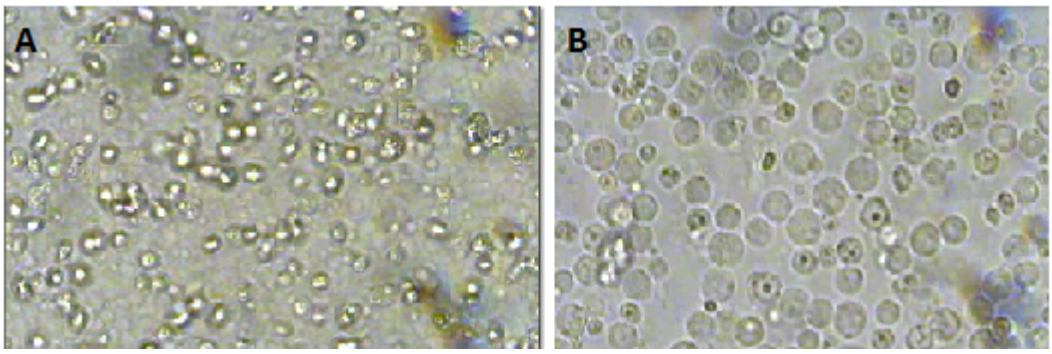


Figure 4-4. **Fractionation of HEK293 cells using Qproteome® plasma membrane kit.** (A) HEK293 cells before processing (B) Qproteome® plasma membrane purification kit uses trademark hypotonic extracellular solution to swell the cells. The cells swell considerably but do not get aggregated.

The Qproteome® plasma membrane purification kit uses osmotic swelling to burst the cells open. The primary difference from the osmotic swelling of cells as observed in Figure 4-1- (C) is the observed lack of aggregation of cells. The swollen HEK293 cells were then passed through a syringe to cause mechanical fractionation of their cell membranes. However, bilayers formed using these cell fragments were very leaky and often needed an

external source of lipid in the form of liposomes or lipid in the organic well for their formation. This method of plasma membrane purification is relatively slower and more expensive than other methods, but is believed to yield highly purified and concentrated plasma membrane fragments.

4.4 Discussion

A key feature of this method is the incorporation of ion channel proteins directly from the cell membranes into artificial lipid bilayers without the need for detergent purification. In absence of detergents, the membrane proteins retain their functional structure and the bilayers formed are more stable.

Our study primarily focused on identifying the most efficient method of cell membrane fractionation and optimizing the process characteristics for the formation of stable and non-leaky bilayers. Work regarding determination of the optimal concentration of the processed fractions to be used for bilayer formation is in progress. Quantitative studies need to be done to correlate the magnitude of leak currents observed in the bilayers and the bilayer size. More electrophysiology studies using hERG overexpressed cells are currently being undertaken.

The physical mechanism underlying the reconstitution of ion channel proteins using this method has not been characterized. However, it can be speculated that the most likely route is via the incorporation of cell membrane fragments containing native protein during bilayer formation (Leptihn S, 2011). Artificial lipids, which presumably act as filler molecules, may be required in conjunction with the plasma membrane fragments for bilayer formation.

Plasma membrane channels, phospholipids and intracellular organelles may need to be

labeled for easy identification and characterization. In this work, we have not characterized the lipid composition of the bilayers, or any potential variation in channel function that may arise due to the artificial lipid environment within these droplet bilayers.

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