Reconstitution of Ion Channels in a Lipid Bilayer in a Microfluidic Device using Cell-Free Protein Synthesis

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Abstract

Ion channels, which are transmembrane proteins, play a crucial role controlling a very wide spectrum of physiological processes. Mutations in genes encoding for ion channels results in the alteration of their function often leading to diseases, making this type of proteins very attractive targets for the development of new drugs.

Nonetheless, the expression and purification of ion channels has been a challenge. Cell-free protein synthesis (CFPS) has emerged in the past years as a promising alternative to overcome the limitations associated with the cellular systems. Additionally, an automable platform with sufficient capacity for high throughput screening and high information content is not currently available. For this reason, miniaturized bilayer lipid membranes (BLMs) platforms have gained more interest.

In this context, this work aimed to expressed ion channels, namely KcsA potassium channel and bacteriorhodopsin (bR) channel, using a CFPS system and their subsequent electrophysiological characterization in a microfluidic BLM device.

The proteins of interest were expressed in a CFPS systems based on an optimized *E. coli* extract, in the presence of nanolipoproteins (NLPs) and in a batch format. The expression of bR was confirmed in a SDS-PAGE gel. However, for KcsA the results of the gel were not conclusive. The inefficient purification procedure not allowed to obtain suitable samples for electrophysiological recordings.

Hence, the results from this experimental work indicate that there is a need to revise the protocol used for the expression of the target proteins in the CFPS system and also to investigate other methods of analysis and purification of the CFPS products.

Keywords: Ion channels; KcsA Potassium channel;, Bacteriorhodopsin channel; Cell-free protein synthesis; Bilayer lipid membranes; Microfluidic

1. Introduction

Ion channels, which are proteins located in cell membranes, play a crucial role controlling a very wide spectrum of physiological processes such as nerve and muscle excitation, hormone secretion, cell and lymphocyte proliferation, learning and memory, salt and water balance, regulation of blood pressure and fertilization or cell death.[1] Mutations in genes encoding for ion channels results in the alteration of the ion channel function often leading to diseases, channelopathies, which include cystic fibrosis, epilepsy, ataxia, myotonia and cardiac arrhythmia.[2][3][4] For these reasons, ion channel have become very attractive targets for the development of new drugs, and approximately 13 % of known drugs have their primary therapeutic action on this class of proteins, representing market of more than 12 billion dollars in worldwide sales. [5]

Nonetheless, the natural abundance of ion channels, and membrane proteins in general, resulting from conventional cellular expression systems is usually too low to purify enough material for high-throughput therapeutic drug studies. Moreover, *in vivo* overexpression of this type of membrane proteins is overtly problematic, resulting in low yield, cell toxicity, protein aggregation and misfolding. Additionally, ion channels are embedded in a complex and dynamic lipid bilayer, which makes their purification difficult, often leading to laborious and hard to handle processes.[6][7]

Cell-free protein synthesis has emerged in the past years as a promising alternative to overcome the previously mentioned obstacles, since target protein expression is s i ndependent of a living cells integrity in these systems.[8] In addition, these systems allow high levels of control, through the direct access and manipulation of the reaction conditions.[6]

However, even if the expression is successful, high-throughput drug screening on ion channels is still challenging. The currently employed pharmacological methods lack in precision. In contrast, the standard technique to study ion channels function, the patch clamp technique, provides high content information but is far from being suitable to multiple compound screening, since it has a very low throughput and is labour intensive.[5][9] Despite automation of the patch clamp technique, the throughput is still not high enough and the associated costs are very high. [10] Furthermore, all these methods use cell models, which is a limitation per se.[11] In this context, cell-free systems like bilayer lipid membranes have gained more interest. The traditional bilayer set up is not suitable for high throughput automated applications since they require skilled operators and the resulting bilayers are not stable. Besides, to perform multiplex experiments large volumes will be needed, which is not favourable. However the fields of microfluidics and microfabrication have contributed to the creation of miniaturized platforms for bilayer lipid membrane experimentation that allow to overcome the aforementioned limitations.[12][13] Decreasing the size of the structures used in these platforms enables the controlled handling of sub-microliter volumes. Also the resulting smaller sized aperture will increase the bilayer stability. Additionally these techniques have the potential for automation and multiplexing. The horizontal aperture allow use of electrophysiological and optical methods as well. Several microfluidic and miniaturized bilayer platforms have been reported in the past years.[12][13][14] However there are still great challenges to overcome; new techniques should be developed and existing ones should be improved in order to develop reliable, automated, high-throughput drug screening platform.

In this context, this thesis aims to help in finding solutions to some of the problems underlined above. More precisely, in this study two ion channels, KcsA Potassium channel and bacteriorhodopsin channel, were expressed in a *E. coli* based cell-free system, in the presence of nanolipoproteins and using a batch format. Additionally, the cell-free synthesized channels were submited to various purification strategies. The resulting products were inserted into bilayer lipid membranes formed on a microfluidic device, previously developed in BIOS Lab on a Chip group. Electrophysiological analysis were used to investigate their functionality.

2. Meterials and Methods

2.1. Cell-Free Protein Synthesis

2.1.1 *E. coli* strains and vectors used

One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) were used for DNA amplification and cloning reaction of KcsA into a new plasmid.

2.1.2 Transformation of competent cells

A vial of One Shot TOP10 chemically competent *E. coli* cells was thawed on ice and DNA solution (1 μg) was added. The tube with cells was incubated on ice for 20 min and then heat-shocked for 30 s in a 42^{o}C water bath. Afterwards, the cells were incubated another 2 min on ice. Then, 250 μL of S.O.C Medium was added and the vial was placed in a shaking incubator (37^{o}C, 225 rpm) for 1 h. At the end of the incubation period, aliquots of 25-50 μL of cells were plated out on a pre-warmed LB-agar selective plate containing 100 μg/mL ampicillin. The LB-agar plates were incubated overnight at 37^{o}C.

2.1.3 Purification of plasmid DNA

A single colony, from one LB-agar plate, was isolated and inoculated with 10 mL of LB medium containing 100 μg/mL ampicillin. Then the tube was incubated in a shaking incubator (37^{o}C, 225 rpm) overnight. The plasmid DNA was purified resorting to QIAprep Spin Miniprep Kit (Qiagen). Bacterial culture was pelleted by centrifugation (8000 rpm) for 5 min at room temperature and resuspended in 250 μL of Buffer P1, previously supplemented with LyseBlue reagent (ratio 1 to 1000) and RNase A solution, then transferred to a microcentrifuge tube. Successively, 250 μL of Buffer P2 and 350 μL of Buffer N3 were added, and gently mixed. The solution was centrifuged for 10 min at 13000 rpm and the supernatant was applied to the column and centrifuged for 45 s at 13000 rpm, the flow-through was discarded. Afterwards, 500 μL of Buffer PB were added followed by 750 μL of Buffer PE. Each addition step was followed by a centrifugation one (13000 rpm) for 45 s. To eluate the DNA, 50 μL of Buffer EB (10 mM Tris.Cl, pH 8.5) were added to the center of the column and centrifuged for 1 min.

2.1.4 Primers design

Taking into account the results of the sequencing of the vector pQE60/KcsA two pairs of primers were designed, Forward primer 1 (A) - 5' ACCATGGCACCCATGCTG 3' and Reverse primer 1 (B) - 5' CCAAGCTCAGCTAATTAAGC 3', Forward primer 2 (C) - 5' **GTTTCTT**ACCATGGCACCCATGCTG 3' and Reverse primer 2(D) - 5' **GTTTCTT**GCTCAGCTAATTAAGC 3'.

2.1.5 Polimerase chain reaction (PCR)

The pQE60/KcsA plasmid (10 ng) were mixed with 10X PCR Buffer (100 mM Tris-HCl, pH 8.3 (at 42^{o}C), 500 mM KCl, 25 mM $MgCl_2$, 0.01% gelatin) (5 μL), dNTP Mix (12.5 mM dATP, 12.5 mM dCTP, 12.5 mM dGTP, 12.5 mM dTTP, neutralized at pH 8.0 in water) (0.5 μL), PCR primers (1 μM each), Taq Polymerase (1 U/μL) and water

to a final volume of 50 μL. The mixture was preheated at 95^{o}C for 5 min and then subjected to a PCR cycle, denaturation - 30 s at 95^{o}C, annealing - 30 s at 53^{o}C, and extension - 1 min/kb at 72^{o}C, repeated 30 times. After the last cycle a final extension step was performed at 72^{o}C for 7 min. The resulting PCR product was then placed at 4^{o}C for short-term storage.

2.1.6 TOPO Cloning

Fresh PCR product (2 μL), salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) (1 μL), water to a final volume of 5 μL and pEXP5-CT/TOPO vector (1 μL) are added in this order to the reaction vessel and gently mixed and incubated for 5 minutes at room temperature (22-23^{o}C).

2.1.7 Concentration measurements

The concentration of DNA template after the purification of the plasmid vector (1.5 μL of the sample) was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.1.8 Cell-Free Protein Synthesis expression

E. coli-based cell-free protein expression reactions were set up using MembraneMax Protein Expression Kit (Life Technologies). To synthesize the recombinant KcsA and the bacteriorhodopsin positive and negative control, three separated reactions were performed. First *E. coli slyD* Extract (20 μL), 2.5X IVPS Reaction Buffer (20 μL), 50 mM Amino Acids (1.25 μL), 75 mM Methionine (1 μL), MembraneMax Reagent (2 μL) for KcsA and for bR positive control, T7 Enzyme Mix (1 μL), pEXP5-CT/KcsA (1 μL) for KcsA, pEXP5-CT/bR (2 μL) for both bR positive and negative control and DNase/RNase-Free Water to a final reaction volume of 50 μL, were added to the reaction vessel. The sample was incubated in the thermomixer (Thermomixer confort, Eppendorf) at 1200 rpm for 30 min at 34^{o}C.

During the incubation, the Feed Buffer composed by 25 μL of 2X IVPS Feed Buffer, 1.25 μL of 50 mM Amino Acids, 1 μL of 75 mM Methionine, and DNase/RNase-Free Water to a final volume of 50 μL, was prepared in a sterile, RNase-free microcentrifuge tube. Addtionally 0.5 μL of 10 mM all-trans retinal were added to the Feed Buffer solutions for the bR expression reactions.

After the 30 min of sample incubation, 50 μL of Feed Buffer were added to the reaction vessel (total volume of 100 μL) and the vessel returned to the thermomixer (Thermomixer confort, Eppendorf) (1200 rpm) to incubate 2 h at 34^{o}C.

2.1.9 Purification of CFPS products

Acetone Precipitation

An aliquot of protein reaction product from -80^{o}C was gently defrosted on ice. Acetone was added in a ratio of 4 to 1 and mixed well. The mixture was centrifuged (12500 rpm) for 5 min at room temperature and the supernatant was carefully removed.

Solubilization in buffer with detergent

An aliquot of protein reaction product from -80^{o}C was gently defrosted on ice and then solubilized in two different buffers. The first solubilization buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole and 10 mM of decyl maltoside (DM) at pH 8 and the second one containing 150 mM KCl, 10 mM HEPES, 4 mM DM at pH 7. The mixture was kept in low agitation for 1 h.

Protein purification using Ni-NTA Spin Column

Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) (540 μL) was added to the protein reaction product (75 μL) and incubated for one hour with low agitation at room temperature. The Ni-NTA spin column was equilibrated with 600 μL of Lysis Buffer through a centrifugation of 2 min at 3700 rpm. The cleared lysate was loaded to the the Ni-NTA spin column and centrifuged for 5 min at 2000 rpm. The spin column was washed by adding 600 μL of Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) and centrifuged for 2 min at 3700 rpm. The protein of interest was eluted twice from the column through the addition of 300 μL of Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8) and centrifugation of the column for 2 min at 3700 rpm.

2.1.10 SDS-PAGE

Polyacrylamide NuPAGE 4-12% Bis-Tris gels (1.0 mm thick, 10-well) (Invitrogen) were used for the SDS-PAGE. Purified protein (10 μL) was mixed with 10 μl of SDS sample buffer (1X NuPAGE LDS Sample Buffer, Invitrogen) and loaded into the gel after 15 min of boiling at 95^{o}C (or without boiling). The gel was placed in an electrophoresis chamber (Invitrogen) and the electrophoresis was performed at 160 mV in running buffer (NuPAGE MES SDS Buffer, Invitrogen) for 50 min. Novex protein standard (Invitrogen) was used as a molecular weight reference. The gel was stained in the microwave for 15 min in the staining buffer (SimplyBlue SafeStain, Invitrogen) and then destained in water.

2.2. Bilayer Lipid Membrane Experiments in a microfluidic device

2.2.1 Fabrication of the Microfluidic Device for Bilayer Lipid Membrane Experimentation

The microfluidic device contains three layers: two glass substrates, each one with three independent microfluidic channels, and a Teflon foil (12.5 μm thickness) between them including three microapertures, located at the channels intersection.

First, the microfluidic channels were wet-etched in Borofloat glass wafers (100 mm diameter, 500

μm thickness). Chromium and gold layers (Cr - 30 nm, Au - 150 nm) were sputtered on the glass wafers and patterned using photolithography (positive photoresist OiR907 - 1.7 μm layer) and dedicated etchants (Cr etchant - Ceric (IV) Ammonium nitrate, Perchloric Acid, and home-made Au etchant - 1 part I_2, 4 parts KI and 40 parts H_2O). The microfluidic glass channels (100 μm height, 300 μm width) were wet-etched using the chromium-gold layer as a mask and hydrofluoric acid (33% in concentration).

Powder-blasting was used to fabricate the access holes in the top glass layers. The reservoirs were defined in the photosensitive foil, which act as a mask, using a standard photolithography technique and then developed with a 2% sodium carbonate solution at 40^{o}C for approximately 6 min. Next, the substrate wafer was exposed to Al_2O_3 powder (29 μm grain size) to create the reservoirs, and the foil was then manually removed.

Next, the three layers were assembled together at the wafer level using a UV-curable glue. Before starting the bonding process all the PDMS, glass and Teflon wafers were cleaned with oxygen plasma, to create a hydrophilic surface and increase the wettability of the glue on it. In order to obtain a thin and uniform layer of glue over the whole surface, UV-curable glue NOA81 (Norland Optical Adhesive) was spin-coated on 1.6 mm thick PDMS wafer, and then transferred to the glass bottom wafer by carefully rolling the PDMS substrate over it. The Teflon foil was placed on top of a wafer holder. The glue-coated glass layer was applied on the Teflon foil and cured with UV-light for 2 min.

Hereafter, the apertures (100 μm diameter) and access holes were dry-etched (50 sccm O_2, 20 sccm N_2, and 2 sccm CHF_3 at 100 mTorr and 60 W for 55 min) in the Teflon foil using RIE (reactive ion etching) and a silicon-based shadow mask.

Following this, the top glass substrate was bonded to the Teflon-glass stack using the procedure previously described for the Teflon-bottom glass assembly. The layers were aligned using dedicated equipment (Electrovision mask aligner EVG 620 and Electrovision Anodic Bonder EV-501) and then NOA81 was cured with UV-light.

The final individual devices were obtained after the assembled structure was diced into 3.5 cm x 1 cm pieces using appropriate equipment (Loadpoint MicroAce 3).

2.2.2 Bilayer Lipid Membrane Experimental Setup

For lipid bilayer experiments, the microfluidic chip was assembled with a PDMS holder with holes corresponding to the reservoirs to insert the fluidics (3 mm diameter - input reservoirs; 1.2 mm diameter - output reservoirs) which fits in the stage of a microscope (Leica DMI 5000M, Rijswijk, The Netherlands). Ag/AgCl electrodes (Molecular devices, Sunnyvale, CA, USA) were inserted in the reservoirs of the PDMS and connected to an Axopatch 200b amplifier equipped with a CV 203 BU headstage (Molecular devices, Sunnyvale, CA, USA). A Faraday cage was placed on the top of the microscope, blocking the interference of external electric fields. The conversion of the data from analog to digital was done by LIH 8+8 Data Acquisition Interface (HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany). Data for electrical measurements were acquired using PatchMaster (HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany).

2.2.3 Bilayer Lipid Membrane Formation and Characterization

Bilayers were formed by introducing buffer solution (150 mM KCl, 10 mM HEPES, pH 7.0, 130 μL) in the bottom microchannel using a conventional micropipette, followed by flushing the lipid solution in n-decane (DPhPC, DOPC/CL and DOPE/CL, 25 mg/mL) in the top microchannel and then replacing it by buffer solution (130 μL). The process of membrane formation was monitored electrically (1 kHz low-pass Bessel filter, 10 kHz sampling rate, dc voltage 100 mV). Bilayer lipid membranes were characterized in terms of noise, seal resistance and capacitance.

2.2.4 Ion Channels Recording

Gramicidin

For the gramicidin experiments, the protein was added to the phospholipid mixture before the BLM formation (2 nM gramicidin in 25 mg/mL DPhPC in n-decane). The bilayer formation and characterization was performed as described before except for the buffer solution used (1 M KCl, 10 mM HEPES, pH 7.0).

KcsA Potassium Channel

First a previously purified KcsA sample in 4 mM DM, kindly provide by Dr. Elwin van der Cruijsen (Utrecht University), was added with a ratio 1 to 300 to the buffer solution (150 mM KCl, 10 mM HEPES, pH 7.0) added to the top channel. The bilayers were formed as mentioned but the buffers and lipids compositions were changed. In this experiments the lipids used were DOPC/CL (1:1) or DOPE/CL (7:3) with a final concentration of 25 mg/mL. In the bottom channel a buffer with acidic pH was applied (150 mM KCl, 10 mM Succinic acid, pH 4.0) The single channel current was recorded as previous described. Then the CFPS products for KcsA expression were measured in the same conditions.

Bacteriorhodopsin Channel

Bilayers were formed by inserting the buffer in the bottom channel (0.1 M $MgCl_2$, 0.5 mM Tris, pH 7),

then the lipids (DPhPC in n-decane, 25 mg/mL) were flushed in the top channel and replaced by the buffer containing the CFPS bacteriorhodopsin samples diluted. For the measurements of light-induced electrical signals, the bilayers were exposed to a mercury lamp filtered with a short-wavelength cutoff filter (560-570 nm - "green light") while a dc voltage of 40 mV (10 kHz sampling rate; 1 kHz low-pass Bessel filter) was applied.

3. Results

3.1. Cell-Free Protein Synthesis

3.1.1 Construction of the pEXP5-CT/KcsA plasmid vector

To perform the cell-free protein synthesis our gene of interest, KcsA gene, was subcloned from the original pQE60 vector (kindly provided by Dr. Abhishek Cukkemane (Utrecht University)) into pEXP-CT plasmid vector, with a T7 promoter, utilized for our cell free expression system. Briefly, pQE60 containing KcsA was amplified, purified and sequenced. Based on the sequencing results, two pairs of primers were designed and a PCR reaction was performed for each pair. The PCR product was cloned into pEXP5-CT vector, which was then amplified and purified. A final sequencing was performed and it was confirmed the proper insertion of KcsA gene when the primers A and B were used for the PCR reaction.

3.1.2 Cell-free protein synthesis and purification of bR

The pEXP5-CT/bR plasmid vector was used as DNA template for CFPS of bacteriorhodopsin. Two reactions were performed: a Positive control were both DNA template and the MembraneMax reagent were added to the reaction mixture and a Negative control where no MembraneMax reagent was added and only DNA template was placed in the reaction vessel. Successful expression of the correctly-folded bR in the presence of its cofactor all-trans retinal causes the reaction mixture to change from a pale yellow to a characteristic pink color (figure 1.a).

In order to optimize the CFPS reaction another expression was performed. Two times the expression volume was incubated in the thermomixer (Thermomixer confort, Eppendorf) at 37^{o}C and 1200 rpm. The feed buffer volume was increased three times and added to the reaction vessel in fractions of 50 μL every hour during three hours. Only the positive control reaction was performed in these conditions.

Bacteriorhodopsin, expressed in standard and optimized conditions, was first purified through an acetone precipitation and followed by analysis by SDS-PAGE (figure 1, b). Under these conditions it is possible to see two distinct bands for the positive bR control and only one for the negative bR control. This results from the fact that the bR-MembraneMax complex is denatured on the SDS gel, resulting in two distinct bands of correct sizes as shown on figure 1, b, bR presents a band around 19 kDa for both, negative and positive expressions, and the scaffold is visible around 28 kDa for the positive control. No significant improvements were observed in the bR expressed in optimized conditions (right) when compared to the standard approach (left).

The second purification strategy followed was a Ni-NTA spin column purification and then analyzed by a SDS PAGE. Figure 1, c presents the gel obtained for the Ni-NTA purification of bR positive control expressed in normal conditions (left). L, FT, W, E1 and E2 represent lysate, flow-through, wash, and eluted fractions, respectively. The analysis of the this gel reveals that the majority of bR is not binding with the nickel column and leaves the column in the flow-through fraction instead of in the elution fractions. To solve this problem, the resulting flow-through fraction of this procedure was submitted to another Ni-NTA spin column purification (data not show), where the centrifuge rotation speed for the binding step was reduced to 1500 rpm and the lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) was also used as washing buffer. This approach was also not effecient and the resulting flow-through fraction was again applied to a Ni-NTA spin column (right). This time, the cetrifugation time for the binding and the washing step was reduced for 1000 rpm and the time increased for 10 min. L1, FT1, W1, and E1 represent lysate, flow-through, wash, and eluted fraction, respectively. Another nickel column purification was run using bR synthesized under optimized conditions (right) The concentration of imidazole in the lysis and washing buffer was reduced to 1 mM and again the centrifugation step for the binding and the washing was performed at 1000 rpm for 10 min. L2, FT2, W2, and E2 represent lysate, flow-through, wash, and eluted fraction.

3.1.3 Cell-free protein synthesis and purification of KcsA

The pEXP5-CT/KcsA plasmid vector was used as DNA template in a concentration of approximately 0.3 μg for the CFPS of KcsA.

KcsA was expressed in standard (figure 2, b) and optimized (figure 2, c) conditions, as described for bR. Three purification strategies were used for KcsA. First an acetone precipitation was performed. Second KcsA was solubilized using a buffer containing the detergent DM. Finally, a Ni-NTA spin column purification was used. All the samples were then analyzed by SDS PAGE. For the KcsA expressed in standard conditions, DM was added to the buffers used in the nickel column,

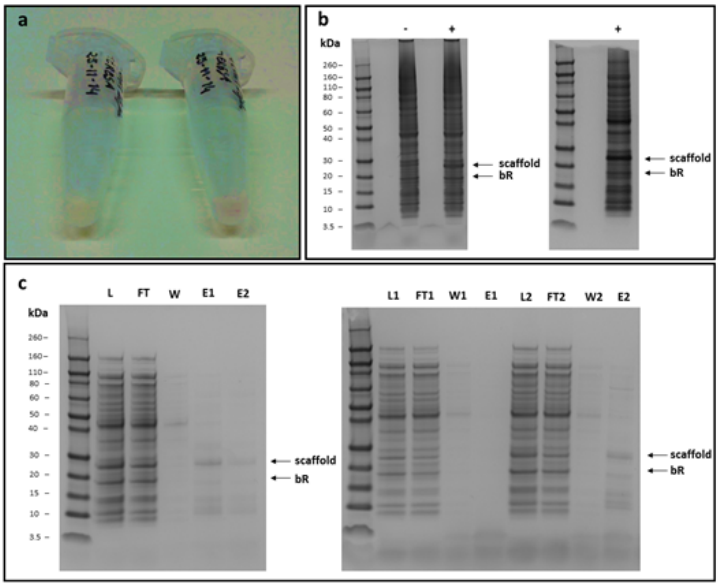

Figure 1: bR expression in the presence (+) or absence (-) of MembraneMax Reagent and All-trans retinal. (a) The characteristic pink color indicate correctly folded bR for the (+) sample. SDS-PAGE of (b) acetone purified bR expressed in *left* - standard conditions, and *right* - optimized conditions; (c) *left* - bR (+) expressed in standard conditions and subsequently purified over a Ni-NTA spin column *Right* - flow-through sample from a previous nickel column applied again to a Ni-NTA column in optimized conditions, and bR expressed and purified in optimized conditions. Samples were separated on a 4-12% Bis-Tris polyacrylamide gel and stained with Coomassie blue.

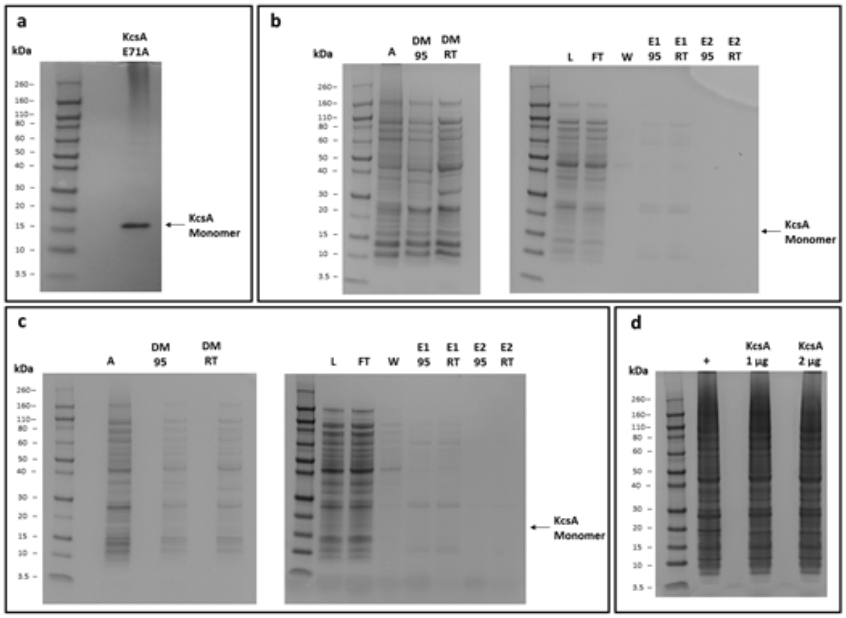

Figure 2: SDS-PAGE KcsA Potassium channel: (a) control sample expressed in cell culture, purified and solubilized in DM; (b) cell-free synthesized KcsA expressed in standard conditions, purified with acetone (A), detergent DM heated (DM 95) and unheated (DM RT) (*left*), and purified over a Ni-NTA spin column (*right*); (c) cell-free synthesized KcsA expressed in optimized conditions, purified with acetone (A), detergent DM heated (DM 95) and unheated (DM RT) (*left*), and purified over a Ni-NTA spin column (*right*); (d) cell-free synthesized control bR (+) and KcsA expressed in optimized conditions for the amount of DNA template used, 1 μg and 2 μg. Samples were separated on a 4-12% Bis-Tris polyacrylamide gel and stained with Coomassie blue.

to an amount of 10 mM in the lysis buffer, and 4 mM to the washing and elution buffers. The KcsA tetramer (≈52kDa) is very stable, and the KcsA monomers (≈17kDa) can only be obtained under harsh denaturing conditions like prolonged heating at 95^{o}C. [15][16][17][18] The purified KcsA samples with acetone (A) were heated at 95^{o}C for 20 min. The samples solubilized with detergent were run untreated (DM RT) and heated (DM 95). For the products of the Ni-NTA column, L, FT, W, E1 and E2 represent lysate, flow-through, wash, and imidazole eluted fractions, respectively. Untreated (RT) and heated (95) samples were used for the elution fractions. Comparing the results with the control experiment (figure2, a) (KcsA E71A in DM4 heated at 95^{o}C for 20 min) it is not possible to conclude that the cell-free expression for KcsA was successful. Since the previous CFPS reactions did not show any conclusive results for the KcsA expression, another two reactions of optimization were performed. This time the final amount of DNA template was increased to 1 and 2 μg. bR synthesized in the same conditions was used as a positive control. No improvements were detected following these alterations and no specific bands for KcsA are found in the SDS PAGE gel (figure 2, d).

3.2. Bilayer Lipid Membrane Experiments in a microfluidic device

3.2.1 Bilayer Lipid Membrane Formation and Characterization

The process of membrane formation was monitored both electrically and optically. The presence of a BLM was observed with bright field microscopy by the observation of a dark area (BLM) surrounded by an annulus (figure 3). Simultaneously, a tight seal is formed by the bilayer across the Teflon aperture, which prevents the flow of ions and results in an increase of the resistance seal (R_m). This process can be monitored through the Ag/AgCl electrodes inserted within the reservoirs and the amplifier used. The membranes were characterized for three types of lipids, DPhPC, DOPC/CL and DOPE/CL, in n-decane (25 mg/mL). After the formation of the gigaohm seal, that demonstrate the device potential for single ion channel recordings, the membrane capacitance (C_m) was measured to confirm the bilayer formation. The specific capacitance (C_s) was determined by taking into account the bilayer capacitance and surface area (A_{BLM}). An applied voltage (up to 100 mV) was used to observe the bilayers stability and 3 out of 14 bilayers collapse while this voltage was applied. Additionally the shelf life of the bilayers was observed for up to 2 hours. The results of membrane characterization for all the lipid solutions are summarized in table 1.

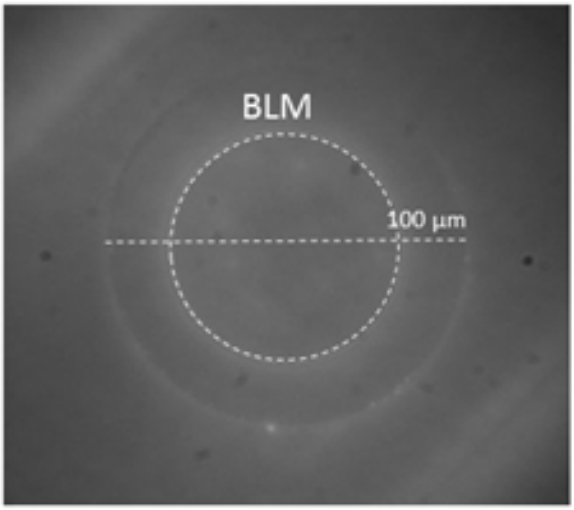

Figure 3: Bilayer lipid membrane. Image of a BLM formed over the 100 μm diameter-size aperture.

Table 1: Membrane properties for control experiments. Bilayers are formed with DPhPC (25 mg/mL) in n-decane. The Buffer used was 150 nM KCl, 10 nM HEPES, at pH 7. The measurements were recorded using a gain of 20 mV/pA. The BLMs are characterized in terms of seal resistance (R_m), capacitance (C_m), surface area (A_{BLM}) and specific capacitance (C_s). n - number of experiments performed; N - number of devices used.

Lipids (in n-decane)	**R_m (GΩ)**	**C_m (pF)**	**A_{BLM} (%)**	**C_s (μF/cm^2)**
DPhPC	37 ± 24^a	17 ± 9^a	41 ± 11^a	0.53 ± 0.19^a
DOPE/CL (7:3)	42 ± 21^b	16 ± 5^b	31 ± 10^b	0.50 ± 0.12^b
DOPC/CL (1:1)	30 ± 11^c	19 ± 3^c	47 ± 18^c	0.53 ± 0.11^c

^{a} - n=14 and N=3, ^{b} - n=6 and N=3, ^{c} - n=6 and N=2

3.2.2 Ion Channels Recording

Gramicidin

Single ion channel recordings were demonstrated by measuring gramicidin, well-known pore forming peptide. [19] The monitorization of the current through the lipid bilayer when a dc voltage of 100 mV are applied (10 kHz sampling rate; 1 kHz low-pass Bessel filter), show the continuous assembly and disassembly of gramicidin monomers in the membrane (figure 4). Gramicidin exhibited a well-defined single channel behavior with single channel amplitudes of 2 pA for an applied voltage of +100

mV. These results are in good agreement with those previously reported in the literature [20]

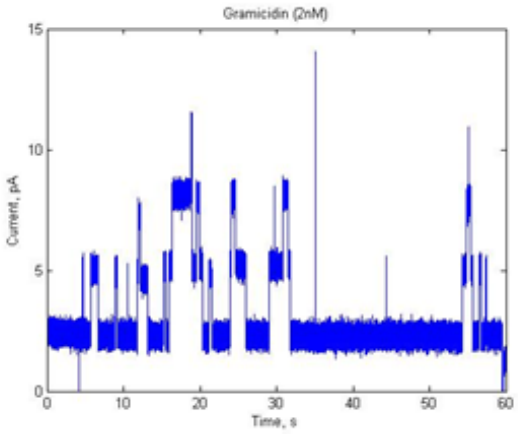

Figure 4: BLM recordings for pore forming gramicidin. Gramicidin channel activity recorded in DPhPC bilayers (25 mg/ml in n-decane suplemented with 2 nM of gramicidin, 100 m apertures, applied DC voltage +100 mV, sampling rate - 10 kHz, Filter - 1 kHz, Gain - 20 mV/pA, Buffer solution: 1 M KCl, 10 mM HEPES, pH 7.0; Sample application: top layer)

KcsA Potassium Channel

Before starting with the electrophysiological experiments for the KcsA samples resulting for the CFPS expression, a control experiment was performed. Under an applied voltage of + 100 mV it is possible to see the alterations between the open and close states for the KcsA channel (figure 5).

For the cell-free synthesized KcsA products, were analysed the samples resulting from three different experiments. First, Ni-NTA elution fraction of a CFPS reaction mixture expressed in standard condition Several dilutions were tested (from 1:10 to 1:100) but only with a 1:100 (sample A.7) dilution stable bilayers were observed. Second, samples from the CFPS reaction mixture expressed in optimized conditions and without purification. Experiments with different dilutions (from 1:10 to 1:100) were carried out but no successfull bilayers formation was observed. Finally, the previous CFPS optimized sample was applied to a Ni-NTA purification, without DM added to the buffers. The KcsA sample was added to the buffer in a dilution of 1:10 (sample B.1)and stable bilayers were achieved However no KcsA activity was recorded in these experiments (figure 5), corroborating the results from the SDS PAGE gels that KcsA portassium channel was not expressed properly in CFPS reaction for this conditions.

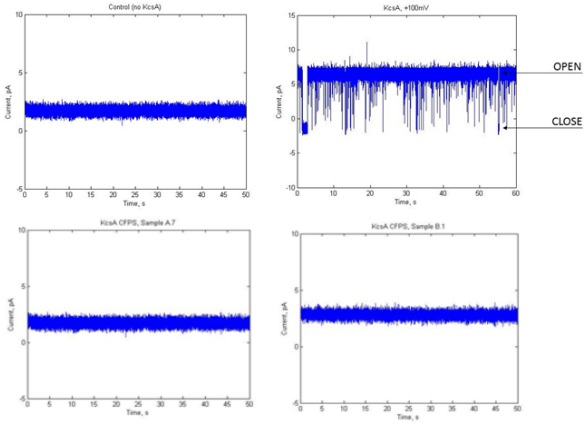

Figure 5: BLM recordings for KcsA Potassium. KcsA channel activity recorded in DOPE (50%):CL (50%) bilayers (25 mg/ml in n-decane, 100 μm apertures, applied DC voltage +50 mV, sampling rate - 10 kHz, Filter - 1 kHz, Gain - 20 mV/pA, Recording mode: On Cell, Buffers solutions: Bottom channel - 150m M KCl, 10 mM HEPES, pH 4.0, Top channel - 150m M KCl, 10 mM HEPES, pH 7.0; Sample application: diluted in top channel buffer, Sample KcsA (from cell culture) - 1/300, Sample A.7 - 1/100, Sample B.1 - 1/10)

Bacteriorhodopsin Channel

Bacteriorhodopsin measurements were performed as described before. As a control experiment, bilayers were formed with the elution fraction resulting from the Ni-NTA purification of the standard CFPS expression of negative bR control with a dilution of 1:10 (sample D.1)in the buffer solution.

For the bacteriorhodopsin experiments, samples from the elution fraction and the flow-through fraction of the Ni-NTA spin column purification of the positive bR control CFPS expression for standard conditions were used to form the bilayers lipid membranes. Stable bilayers were achieved for the elution fraction (dilution 1:10, sample E.1) but no channel activity was observed when then sample was exposed to light from a mercury lamp filtered with a short-wavelength cutoff filter (560-570 nm - "green light"). This result is in agreement with the SDS PAGE gel (figure 1, c) where it is possible to see that all the bR left the column in the flow-through fraction. Considering this, the flow-through fraction was used in the next bilayer lipid membrane formation experiments. Three dilutions of the sample were tested. The first dilution, 1:1, does not allow the formation of stable bilayers. For the other two dilution, 1:5 (sample F.3) and 1:10 (sample F.3), stable bilayers were achieved but no bR activity was recorded (figure 7), probably due to the interaction of other constituents present from the CFPS expression kit that are still in a considerable amount in the flow-through fraction. Additionaly a sam-

ple from the elution fraction of NI-NTA spin column performed in optimized conditions (1000 rpm, 1mM imidazole)for bR also expressed in opttimized conditions was tested. The sample was diluted 1:1 (sample G.1) but again no ion channel activity was recorded.

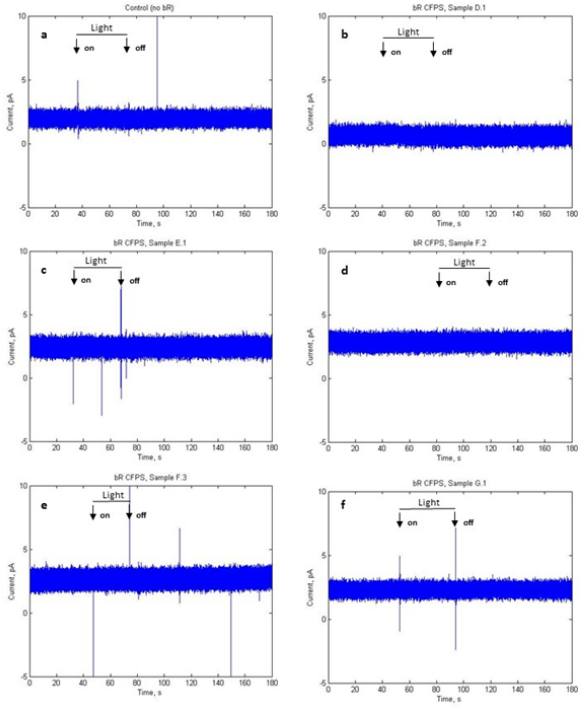

Figure 6: BLM recordings for bacteriorhodospin for CFPS products. bR channel activity recorded in DPhPC bilayers (25 mg/ml in n-decane, 100 μm apertures, applied DC voltage + 40 mV, sampling rate - 10 kHz, Filter - 1 kHz, Gain - 20 mV/pA, Buffers solutions: Bottom and Top channels - 0.1 M $MgCl_2$, 0.5 mM Tris, pH 7; Sample application: diluted in top channel buffer according to sample description in the title of each graph

4. Discussion

For the first CFPS experiments, bR was utilized and successfully expressed in NLPs without any major problems. Bacteriorhodopsin has been extensively studied and is known to be expressed at relatively high successfull yields in cell-free protein systems.[22][23][24][25][26] Despite the protein yield not having been determined, when the reaction was optimized to a "fed-batch" system with an increase of reaction and feed buffer volumes, no significant alteration was observed in the SDS-PAGE gel. This probably results from the increase of the reaction time that could lead to protein degradation.

The CFPS system was then used for the KcsA expression in the same conditions. KcsA was also previously expressed in cell-free systems using both *E. coli* and insect extracts. [15][27][28][29] Through the analysis of SDS-PAGE gel for the CFPS product compared with the control gel for KcsA synthesized in a cell-culture, purified and solubilized in detergent, the gel did not revealed cleared bands for the KcsA monomer (sample heated 10 min at 95^{o}C) or for the KcsA tetramer (unheated sample). Since the sample has a wide background, even after the purification step, it should have been done a Western Blot using a His-tag mouse monoclonal antibody, for example, to confirm the expression or not of KcsA protein.

Considering that KcsA was not expressed at all, several factors might have contributed to this. The next paragraphs aim to provide solutions to overcome this in the future.

First, the space between the RBS and the ATG initiation condon was not in the optimal range (10 nucleotides instead of 7-9 nucleotides as recommended by the manufacturer). This sequence is necessary for optimal translation efficiency of the protein of interest. To surpass this problem two strategies could be utilized. In a first approach, a site directed mutagenesis can take place, and one, two or three nucleotides between the RBS and ATG codon, will be deleted and the optimal spacer will be achieved. Another strategy that could be employed is based on the fact that the initial plasmid with the KcsA, pQE60/KcsA, was successfully used to express the protein of interest in a cell culture. Therefore it is possible to use restriction enzymes to cut out the T5 promoter and lacO operator as well as the T0 terminator of the pQE60/KcsA and replaced them by T7 promoter and terminator from the pEXP5-CT plasmid.

Secondly, some alterations could be tried in order to have a successful expression of KcsA. Our experiments were performed utilizing a modified *E. coli* extract which has proved to be successful for cell-free expression of KcsA, however insect based eukaryotic cell-free systems have also been employed for this purpose with promising results. The major advantage of these systems is the post-translational modifications that the expressed proteins suffer and are commonly critical for their correct folding and consequently for their functional activities. Besides this, insect cell-free lysate contains native microsomes which will mechanically insert the expressed membrane protein into a lipid bilayer in its correct functional form. [29]

A 5'-UTR upstream from an open reading frame of the expression plasmid can be important to increase the protein expression yield. This could be achieved, for example, by placing the Shine-Dalgarno sequence, a ribossomal binding site, at eight nucleotides (typical distance) from the AUG start codon, which will interact with 3'-end of the 16S ribosomal RNA to allow an efficient initiation.

[30][31]

A high throughput assay to measure the effect of supplement the CFPS kit with small molecules, ions, proteins, and/or nucleic acids can also be performed to a better understanding of the process. Although 37^{o}C is the general default temperature for CFPS and typically results in high yields, for some cases a large fraction of the target protein is inactive, so an essay to test different temperatures can also be employed. [32]

Lastly, in relation with the operation mode, some alterations could be performed to improve the batch mode limitations, due to the depletion of reactants and accumulation of inhibitory by-products. In the "thin film" approach the batch reaction mixture is placed on a thin film that alllows a continuous oxygen transfer and a large hydrophobic, this last one facilitating the protein expression by binding inhibitory hydrophobic molecules. An alternative approach is the compartement method, one containing the extract and one containing the feeding solution that is renewed by continuous flow, enabling the refilling of the substrate and the removal of by-products. The two compartments could be separated by a semi-permeable membrane, a dialysis bag immersed in the feeding solution or by a bilayer.

During the optimization process, a "fed-batch" system was implemented and the feeding buffer was partially supplied to the reaction vessel during the reaction time instead of the all volume being add in only one time. However the expression of KcsA was not successful with the alteration probably because the reaction time was also increased which may have led to a degradation of the protein.

After the CFPS experiments the samples were submitted to different purification strategies. Bacteriorhodopsin was first purified with acetone precipitation as suggested by the manufacturer, but the background smearing was not complete removed which could be prejudicious for the further experiments with BLMs. To achieve better results in bR purification a Ni-NTA spin column was employed. Through the observation of the results it was possible to see that the majority of bR elutes in the flow-through fraction, meaning that the 6xHis tag of the protein is not binding to the Ni-NTA Silica that constitute the spin column. To try to overcome this question, another set of purification experiments was performed with variations in the buffers imidazole concentration and in the rotation speed during the centrifugations, and with the addition of benzonase to the lysis buffer. None of these processes proved to be efficient and larger amount of bacteriorhodopsin continue to elute in the flow-through fraction. In the purification of KcsA both strategies applied to bR were also utilized, showing the same weakly results. Additionally a solubilization with a buffer containing a detergent (DM) was tested to improve the stabilization of KcsA but no significant improvement was observed. In the bR case, considering that the CFPS was proved to be successful as well as the insertion of the protein in the NLPs that are tagged with a 6xHis tail, the problem faced in the Ni-NTA spin column, could result from the loss of specific binding of the resin to the 6xHis tag, due to a long time storage, that probably had damaged the ligation between the nickel ions and the NTA resin.

To address this limitation another immobilized metal-affinity chromatography method should be tested, like Pro-Bond Purification System (Life Technologies) also with nickel ions or TALON Metal Affinity Resins (Clontech) charged with cobalt ions. Furthermore, the integration of a Glutathionine S-transferase (GST) tag into expression vectors and the respective purification methods (GST affinity chromatography) should be consider.

The microfluidic device was fabricated according to the protocols developed in our group, and the results for the bilayer lipid membrane formation and characterizations are in good agreement with the ones obtained previously, [33][34] showing stable bilayers and the ability for single channel recording.

KcsA previously expressed in a cell-culture and solubilized in DM was used as a control reference for the electrophysiological measurements. It was possible to observe the open and close states of the channel, when an dc voltage of + 100 mV was applied.

Resorting the CFPS expression of KcsA no ion channel activity was detected during the BLM experiments. This probably confirms that the expression was not successful, however it is worthy to refer that the protein could had been expressed but not correctly folded. KcsA K+ channel must be folded into a tetramer with typically four identical subunits to be functional active as an ion channel. [35]

For bR recordings no ion channel activity was detected. Here the main reason probably is the inefficient purification of the CFPS product.

Additionally, Sachse et al. have stated that nanodiscs could were probably not be the best strategy for ion channel characterization, because these particles are accessible from both sides and the incorporation of the CF-produces MPs into these entities is orientation-independent. [8] It should be important to study the CFPS of our proteins of interest, KcsA and bR, in the presence of other solubilizing agents, like liposomes, lipid-detergent systems or biological membranes.

5. Conclusion

bR channel was successful expressed in the CFPS system used and it was also possible to observe the

correct folding of the protein. However the purification procedure chosen was not efficient and the samples obtained for the electrophysiological studies were highly contaminated with compounds of CFPS reaction, which did not allow the recording of the ion channel activity.

In the KcsA K+ channel, the CFPS expression was not conclusive. The analysis method used, SDS-PAGE, did not show clear bands in the expected molecular weighs for KcsA, but again the purification procedure was not effective and a lot of background was observed in the gel analysis. Besides, no ion channel activity was detected during the electrophysiological measurements. However this did not means that the KcsA was not expressed.

It is possible that the protein was expressed and that a strong background prevented the correct insertion of the KcsA molecules in the bilayer, or it is plausible that the protein was not correctly folded, since KcsA only acquires it functional channel activity when it is folded in a tetrameric form.

Therefore, there is a need to improve the protocol applied and explore the use of different techniques in order to be able to correctly expressed and purified the target ion channels and then reconstitute them in the microfluidic BLM device for further electrophysiological and optical characterization.

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