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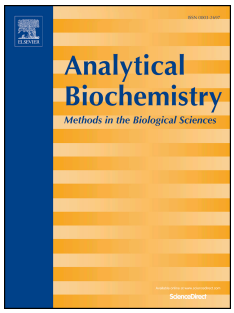

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On-chip Membrane Protein Cell-free Expression Enables Development of a Direct Binding Assay: A Curious Case of Potassium Channel KcsA-Kv1.3

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Abstract

Despite the significant role integral membrane proteins (IMPs) play in the drug discovery process, it remains extremely challenging to express, purify, and *in vitro* stabilize them for detailed biophysical analyses. Cell-free transcription-translation systems have emerged as a promising alternative for producing complex proteins, but they are still not a viable option for expressing IMPs due to improper post-translational folding of these proteins. We have studied key factors influencing *in vitro* folding of cell-free-expressed IMPs, particularly oligomeric proteins (*i.e.*, ion channels). Using a chimeric ion channel, KcsA-Kv1.3 (K-K), as a model IMP, we have investigated several physiochemical determinants including artificial bilayer environments (*i.e.*, lipid, detergent) for K-K *in vitro* stabilization. We observed that fusion of a 'superfolder' green fluorescent protein (sfGFP) to K-K as a protein expression reporter not only improves the protein yield, but surprisingly facilitates the K-K tetramer formation, probably by enhancing the solubility of monomeric K-K. Additionally, anionic lipids (*i.e.*, DMPG) were found to be essential for the correct folding of cell-free-expressed monomeric K-K into tetramer, underscoring the importance of lipid-protein interaction in maintaining structural-functional integrity of ion channels. We further developed methods to integrate cell-free-expressed IMPs directly onto a biosensor chip. We employed a solid-supported lipid bilayer onto the surface plasmon resonance (SPR) chip to insert nascent K-K in a membrane. In a different approach, an anti-GFP-functionalized surface was used to capture *in situ* expressed K-K *via* its sfGFP tag. Interestingly, only the K-K-functionalized capture surface prepared by the latter strategy was able to interact with K-K's small binding partners. This generalizable approach can be further extended to other membrane proteins for developing direct binding assays involving small ligands.

Introduction

Integral membrane proteins (IMPs) comprising receptors, ion channels, enzymes, and transporters are an essential class of proteins in the human genome [1-4]. They modulate numerous cellular processes including signal transduction, cell adhesion, metabolism, and the immune response [5]. Their pharmacological relevance is substantiated by the fact that they are targets of approximately 60% of all pharmaceuticals for the treatment of serious illnesses ranging from cancer and cardiovascular disease to immunological and neuropsychiatric disorders [6-10]. Despite their significant role in the drug discovery process, developing direct binding assays to characterize lead compounds for IMP targets using standard biophysical techniques such as nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) have been challenging [11-13]. IMPs are typically expressed in a cell-based system. However, in order to interface them with a biophysical technique, target IMPs need to be isolated from the membrane and presented in a soluble form, which is a cumbersome multistep process [10, 14].

Cell-free technology has recently emerged as an alternative facile method to cell-based platforms for producing complex proteins such as membrane proteins [15-17]. To circumvent traditional limitations in developing direct binding assay for IMPs, we envisaged combining cell-free technology with a SPR biosensor for performing multiple processes on-chip, including protein expression and lipid reconstitution for the detection of ligand binding with the surface-immobilized IMP.

Here, we used previously developed S30 *E. coli* extract based transcription-translation (TX-TL) cell-free system [18-20]. Our initial target was a previously constructed prototypical chimeric ion channel, potassium channel KcsA-Kv1.3 (K-K). Here, KcsA is a prokaryotic potassium channel of *Streptomyces lividans* [21]. K-K plasmid was constructed by replacing

multiple residues at the KcsA pore regions with corresponding residues from a eukaryotic potassium channel Kv1.3 [22, 23]. Kv1.3 plays a key role in the activation of effector memory T lymphocytes (T_{EM}) by modulating the extracellular calcium influx. It has been a potential therapeutic target for T cell-mediated autoimmune diseases including multiple sclerosis and rheumatoid arthritis [24, 25]. Drug discovery efforts has been focused on developing Kv1.3 selective inhibitors for the treatment of these debilitating disease [26]. The ShK peptide derived from *Stichodactyla* toxin has been known to block the pore region of eukaryotic Kv1.3 by interacting with all the four subunits of tetrameric Kv1.3 [23], and it can suppress the activation of T_{EM} cells. We used Shk and its analogs for testing the binding activity of TX-TL-expressed K-K.

KcsA has a tetrameric structural organization with each monomer comprising two transmembrane domains that form the ion channel through the lipid bilayer. Cellular protein expression systems utilize chaperones and inner membrane for folding and assembling of the newly synthesized KcsA into a tetramer [27]. However, due to the absence of the intrinsic factors responsible for protein folding, cell-free TX-TL expresses oligomeric IMPs such as K-K as a monomeric polypeptide. In this study, we have first investigated biochemical factors governing oligomerization and activity of the K-K in an *ex-situ* TX-TL system. We explored various bilayer mimics (*i.e.*, detergents, bicelle, nanodisc, liposome) [28] for solubilizing/stabilizing TX-TL-expressed K-K. Green fluorescent protein (GFP) is a commonly used reporter to monitor protein expression [29] We used GFP for K-K expression in two different formats: (1) transcriptional reporter fusion (bicistronic expression) and (2) translational reporter fusion (sfGFP directly fused to K-K). It was surprising to observe that GFP fused K-K construct

expressed tetrameric species in TX-TL. However, bicistronic expression yielded only monomeric K-K species. We further explored the role of lipids in obtaining native K-K configuration.

Cell-free expressed membrane proteins require additional steps including detergent/lipid solubilization and purification before it can be used for any biophysical studies, which results in significantly lowering down the overall protein yield [30]. A key objective of the present study is to explore the possibility of performing *in-situ* TX-TL onto the SPR chip, while maintaining the *ex-situ* reaction conditions (i.e., incubation temperature and time) for membrane protein expression. Here, we have investigated two different *in situ* strategies involving SPR functionalized surfaces for concurrently capturing TX-TL synthesized K-K (Figure 1). In the first approach, a solid-supported lipid bilayer was used to incorporate nascent protein directly into a native membrane mimicking environment. Whereas in the second approach, an anti-GFP-functionalized surface was used as a platform for capturing *in situ* expressed GFP-fused protein, and subsequently folding it by introducing a bilayer mimic. There have been few reports utilizing tethered lipid bilayer systems to directly incorporate cell-free IMPs, albeit without mentioning of any biomolecular interactions with binding partners [31, 32]. Here we demonstrated direct binding of small ligands to cell-free expressed K-K, which is correctly folded into a bilayer mimic.

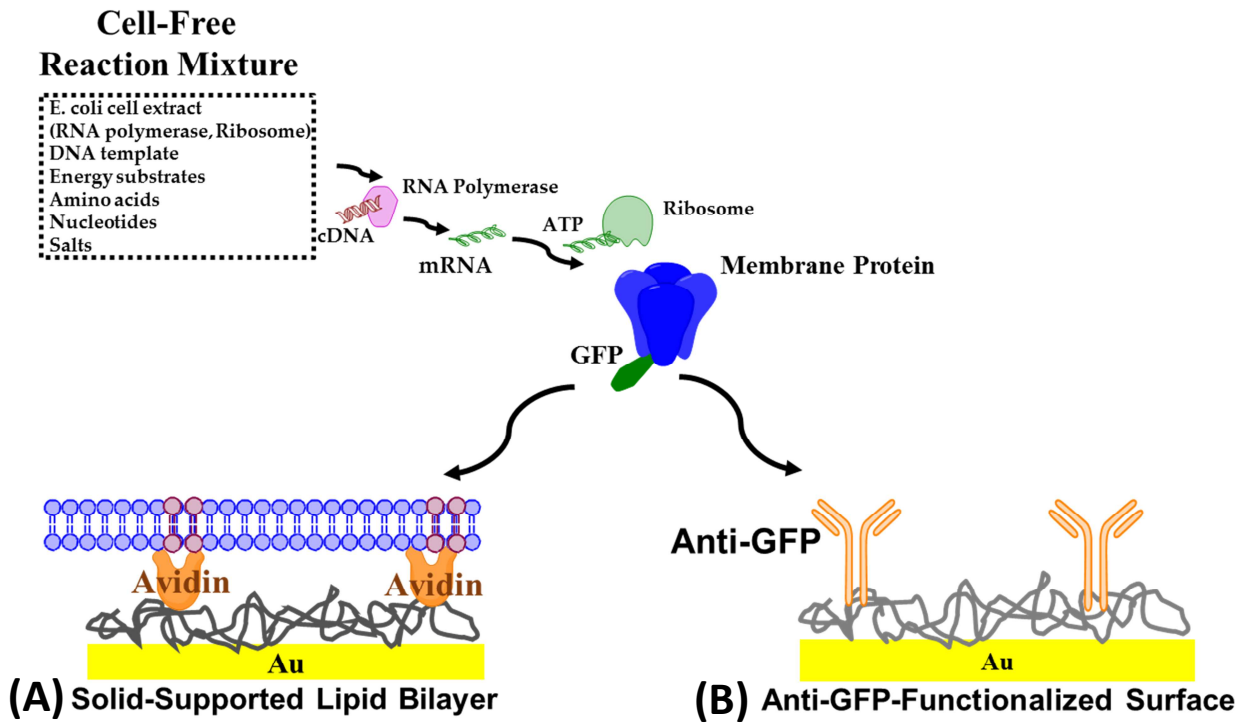


Figure 1. Schematic illustration of membrane protein synthesis via cell-free system onto functionalized surfaces: (A) prefabricated solid-supported lipid bilayer was used to directly reconstitute nascent synthesized protein into bilayer and (B) anti-green-fluorescent-protein (GFP)-functionalized surface to capture GFP-fused protein

Materials and Methods:

Chemicals:

The detergents n-decyl-β-D-maltoside (DM), lauryldimethylamine N-oxide (LDAO), Brij35/58, and n-decyl-β-D-glucoside (DG) were purchased from Anatrace (Maumee, OH). Lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) , 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG), and 1-oleoyl-2-(12-biotinyl(aminododecanoyl))-*sn*-glycero-3-phosphoethanolamine (biotin-labeled PE) were bought from Avanti Polar Lipids (Alabaster, AL). Peptide ShK and its analog anti-dinitrobenzene-fused-ShK (anti-DNP-ShK) were produced in-house at Amgen Inc.

Plasmids and Linear DNAs:

Oligonucleotides primers and DNA were ordered from Integrated DNA Technologies (Coralville, Iowa). Plasmids in this study were designed in Geneious 8 (Biomatters, Ltd.), and were made using standard golden gate assembly (GGA) protocols. BsaI-HF (R3535S) enzyme used in GGA was purchase from New England Biolabs (NEB). Linear DNAs were made by PCRing protein expression related sequences out of GGA constructs using Phusion Hot Start Flex 2X Master Mix (M0536L) from NEB.

Cell-free Reactions (TX-TL):

TX-TL reaction mixture was prepared based on the previously developed protocol [19]. Briefly, *E. coli* BL21 Rosetta cells were grown to an OD600 of 1.5, pelleted via centrifugation, and washed with a buffer at pH 7.7 containing Mg-glutamate, K-glutamate and Tris. Lysis was performed via bead-beating, followed by centrifugation to collect lysed cell extracts. The resulting supernatant extract was incubated at 37 °C for 80 minutes, followed by centrifugation

to remove endogenous nucleic acids before aliquoting and flash-frozen in liquid nitrogen for −80°C storage. Alternatively *E. coli* extract can be purchased from Cube Biotech Inc. (Plymouth Meeting, PA) or Promega Inc. (Madison, WI). The reaction buffer was also prepared according to the previously published protocol encompassing an energy solution (700 mM HEPES pH 8, 21 mM ATP, 21 mM GTP, 12.6 mM CTP, 12.6 mM UTP, 2.8 mg/mL tRNA, 3.64 mM CoA, 4.62 mM NAD, 10.5 mM cAMP, 0.95 mM folinic acid, 14 mM spermidine, and 420 mM 3-PGA) and amino acids (i.e., 5 mM leucine, 6 mM all other amino acids,). TX-TL reactions in 120 μL were conducted for *ex-situ* (Eppendorf tube) *or in-situ* (SPR flow cell) by mixing a calculated ratio of extract, buffer and plasmid at 30°C for 10 hours. Final mixes of extracts and buffers are composed of: 8.9 – 9.9 mg/mL protein, 5 mM Mg-glutamate, 40 mM K-glutamate, 1.5 mM each amino acid except leucine, 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 2% PEG-8000.

SDS-PAGE and Western Blot:

Bolt 4–12% Bis-Tris Plus Gels from ThermoFisher Scientific was used in this work with Bolt MES SDS as a Running Buffer. SDS-PAGE were run without reducing agents. Protein samples were mixed with SDS sample buffer, no sample heating, before loading into gels. SeeBlue Plus2 Pre-stained Protein Standard was used as a protein molecular-weight marker. iBlot 2 Gel Transfer Device and iBlot Nitrocellulose Regular Stacks were used for transfer proteins from gel to membrane. Membrane was then transferred to iBind device and incubated with Penta-His HRP Conjugate in 1:500 dilutions. Blots were detected using SuperSignal Chemiluminescent HRP Substrates from ThermoFisher Scientific.

Ex-situ Protein Folding:

TX-TL reaction mix was spun at 14,000x g for 10 min at 4 °C. Pellet was dissolved in 8 M urea, and transferred to buffer-equilibrated HisPur Ni-NTA Spin Purification column (ThermoFisher Scientific), followed by 1 h incubation with shaking. The column was attached to a pulsatile pump for continuous flow of a folding solution comprising detergent/lipid mixed solution for 12 h. Finally column was eluted by introducing buffer (20 mM Tris pH 7.4, 0.1 M NaCl, 250 mM imidazole) for 3 times in 2 column volumes. Proteins were then concentrated using Amicon Ultra Centrifugal Filter Units (Millipore) with Ultracel-30 membrane.

Fluorescence Assay:

TX-TL samples were read for GFP in a Synergy H1 plate reader (Biotek) using excitation/emission: 485 nm/525 nm. GFP relative fluorescence units (RFUs) were converted to protein conc. (nM) using a purified GFP-His6 standard. Unless otherwise stated, end point measurements were taken after 2 h of expression.

Surface Plasmon Resonance based Binding Assay for *ex situ* TX-TL:

GE Biacore T-200 SPR system was used for all the binding studies involving *ex situ* TX-TL. ShK or anti-GFP antibody functionalized surfaces were created on Biacore CM4 chips. ShK (250 μM in pH 4 sodium acetate solution) was introduced onto the -ethyl-3-(3-dimethylaminopropyl) carbodi-imide (EDC)/*N*hydroxysuccinimide activated carboxy surface for 8 min, followed by injection of 1 M ethanolamine for 7 min to inactivate the remaining carboxyl group. In all the experiments, dual channels were used with one channel as a reference to subtract the background response from blank dextran. Binding response was calculated after 5 min of stabilization. 50 nM anti-DNP-ShK was used for binding studies of folded K-K.

In-situ TX-TL onto Surface Plasmon Resonance Sensor Chip Surface:

GE Biacore 3000 SPR system was used for all *in situ* TX-TL experiments.

(a) Solid-Supported Bilayer

Biacore C1 sensor chip was used for solid-supported bilayer formation. NeutrAvidin (20 μg/ml diluted in 100 mM, pH 4.5 sodium acetate solution) was introduced onto the -ethyl-3-(3-dimethylaminopropyl) carbodi-imide (EDC)/ *N*hydroxysuccinimide activated carboxy surface for 8 min, followed by injection of 1 M ethanolamine for 7 min to inactivate the remaining carboxyl group. Liposome was prepared by first dissolving 2 mg/mL lipids comprising DMPC, DMPG, biotin-labeled PE at the molar ratio of 9/1/0.1 in chloroform. The mixture was dried under a stream of nitrogen, and left for overnight in a vacuum desiccator. This lipid film was resuspended in PBS buffer at 1 mg/ml conc. for liposome formation. Using an automated mini-extruder, we extruded the liposome solution 1000 times through a 100 nm pore size polycarbonate membrane for producing a homogeneous sample comprising ~120 nm diameter liposome. The liposomes were introduced to the NeutrAvidin-functionalized SPR chip at a flow rate of 5 μl/min for 1 h. Captured vesicles were ruptured to lipid bilayer by introducing a solution containing PEG 12% and 500 mM NaCl for 5 min. TX-TL was performed onto the bilayer-functionalized chip at the 30° C flow cell temperature by introducing 120 μl of reaction mixture at a flow rate of 2 μl/min. The flow was stopped for 10 hours to complete the TX-TL process on the chip.

(b) Anti-GFP Functionalized Surface

Biacore CM3 chip was used for creating anti-GFP-functionalized capture surfaces. Briefly, anti-GFP antibody (20 μg/ml diluted in 100 mM, pH 4 sodium acetate solution) was introduced onto the -ethyl-3-(3-dimethylaminopropyl) carbodi-imide (EDC)/*N*hydroxysuccinimide activated carboxy surface for 8 min, followed by injection of 1 M ethanolamine for 7 min to inactivate the remaining carboxyl group. This functionalized surface was used as template for performing TX-TL at the 30 °C flow cell temperature by introducing 120 μl of reaction mixture at a flow rate of 2 μl/min. The flow was stopped for 10 h to complete the TX-TL process on the chip. 8 M urea was introduced to the functionalized surface for 10 min, followed by 2 μl/min flow of folding solution comprising 0.5% DM and 0.05% DMPG onto the functionalized surface for 2 hours. For ligand binding studies, ShK at the top concentration of 100 nM with two-fold dilution was used. Whereas 30 μM PAP1 or tripelenamine from a 10 mM stock solution were used with 0.3% DMSO in the sample solution and running buffer.

Results and Discussion

sfGFP Fusion to KcsA-Kv1.3 Facilitated Protein Solubilization and Oligomerization

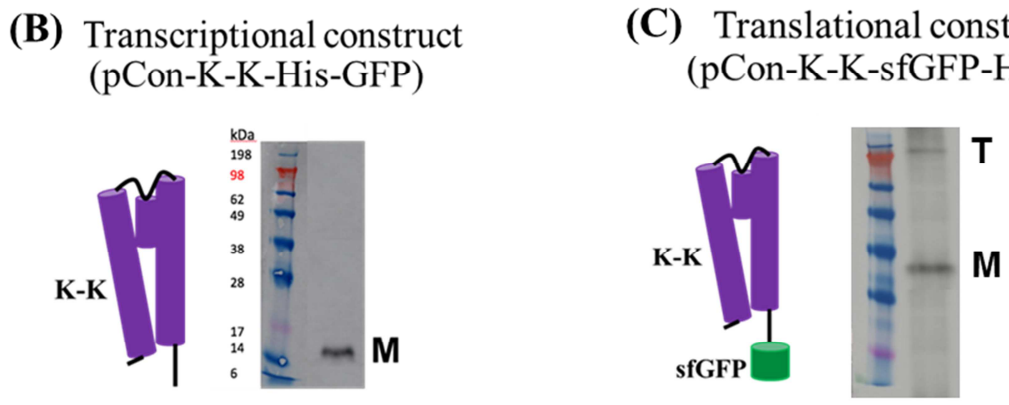
Green fluorescent protein was primarily used in the K-K plasmid to monitor its expression level in TX-TL. However, K-K is predisposed to aggregation during TX-TL expression due to the presence of hydrophobic domains. We reasoned that introducing a water soluble protein such as β-barrel GFP to K-K would enhance the stability of TX-TL-expressed monomeric K-K, and thus GFP may subsequently promote the oligomeric protein assembly. To test this hypothesis, we created a plasmid with K-K fused with superfolder-GFP (sfGFP), which is an aggregation-resistant GFP variant [33]. As shown in Figure 2, Western analysis of the pelleted TX-TL comprising translational-sfGFP-fusion plasmid confirmed the presence of different K-K-sfGFP

oligomeric species including tetramers. In contrast, TX-TL containing transcriptional-GFP-fusion plasmid expressed only monomeric K-K. Additionally, we evaluated K-K expression levels in TX-TL by comparing the Western band intensities of expected TX-TL monomeric species to that of previously expressed *E. coli* K-K (Supplemental Information, Figure S1). Transcriptional-GFP-fusion resulted into ~0.1 mg/ml K-K total expression in TX-TL. In sharp contrast, K-K total expression level increased 10-fold when translational-GFP-fusion (sfGFP) plasmid was used in TX-TL. Therefore, translational-GFP-fusion plasmid (K-K-sfGFP) was used for subsequent studies.

(A)

KcsA	**VL**AERGAPGAQLITYPRA**L**WW**S**V**E**T**A**TTVGYGD**LY**PVT**LW**G**RL**
Kv1.3	**YF**AEADDPTSGFSSIPDA**F**WW**A**V**V**T**M**TTVGYGD**MH**PVT**IG**G**KI**
KcsA-Kv1.3 (K-K)	**VL**AEADDPTSGFSSIPDA**L**WW**S**V**E**T**A**TTVGYGD**LY**PVT**LW**G**RL**

Figure 2. (A) Sequence alignment of potassium channels, KcsA (M1/M2 linker region) and Kv1.3 (S5/S6 linker region), and chimeric KcsA-Kv1.3 (K-K) sequence comprising grafting of S5/S6 sequences of Kv1.3 onto KcsA (grayed). K-K plasmids for TX-TL (B) Transcriptional GFP fusion expresses both K-K and GFP independently, and (C) Translational construct produces a GFP fused version, K-K-sfGFP. Non-reducing Western blot analysis indicates presence of only monomeric K-K (18 kDa, **M**) in the TX-TL samples prepared using plasmid **A**. However, samples comprising plasmid **B** yielded both monomeric (47 kDa, **M**) and tetrameric K-K (188 kDa, **T**).



Anionic Lipid is Critical for KcsA-Kv1.3 Folding

Purified membrane proteins are required for reconstitution into a biomimetic environment for maintaining their functionality outside of a cellular membrane. Detergents, nanodiscs, bicelles, and liposomes are generally used to stabilize membrane proteins after membrane solubilization [28, 34]. To stabilize TX-TL expressed K-K, various nonionic and zwitterionic detergents and lipidic systems (i.e., nanodisc and liposome) were introduced into the TX-TL reaction mixture containing transcriptional K-K plasmid. GFP fluorescence and Western blot analyses indicated that all the tested detergents except Brij-35/Brij-58 inhibited TX-TL reaction resulted into minimal or no protein expression. On the contrary, lipid-based systems such as liposome, bicelle, or nanodisc were compatible with TX-TL system. However, TX-TL-expressed K-K was unable to bind peptide ShK, suggesting that either the protein was not correctly folded (*in liposome/bicelle of DMPC or DMPG lipids*) in tetramer or it was not incorporated in the system (*in nanodisc comprising DMPC or DMPG lipids*). Concurrently, another reconstitution/stabilization strategy involving a two-step process was investigated: (1) TX-TL in the absence of any bilayer mimic; and (2) reconstitution/folding of a precipitated TX-TL reaction pellet on a column. Recently, a similar two-step approach was used to refold a TX-TL expressed KcsA, albeit refolding was done in a liposome at 45 ºC [35].

Refolding of the column-loaded denatured protein was performed with membrane mimics including a detergent and detergent/lipid mixtures (Supplemental Information, Figure S3). Both zwitterionic and anionic lipids were investigated for the probable lipid-mediated correct folding of TX-TL-expressed K-K using translational K-K plasmid. An antibody fusion of peptide ShK (anti-DNP-ShK) was employed to detect the SPR binding response of low-density captured K-K-sfGFP on the SPR surface [~200 pg/mm^2 (based on ~200 RU SPR response]. As shown in Figure 3A, K-K folded/reconstituted in a mixture of anionic lipid DMPG and DM bound to anti-DNP-

ShK, indicating the presence of biologically-relevant configuration of K-K. In contrast, K-K folded in DM alone or with zwitterionic lipid DMPC alone was unable to recognize anti-DNP-ShK.

Previously, Valiyaveetil *et al.* and others have demonstrated that the anionic lipids are essential for maintaining the function of potassium channel KcsA [36]. Our findings further underscore the importance of anionic lipids in preserving the biological activity of KcsA. Surfaces functionalized with ShK were employed to test the binding specificity between the folded K-K and ShK. As illustrated in Figure 3B, TX-TL-expressed K-K, folded in the presence of DMPG, was able to bind ShK with the binding response similar to 100 nM *E.coli* expressed (DM solubilized) K-K. In contrast, K-K either unfolded or folded in DMPC elicited considerably lower binding response. Additionally, pre-incubation of ShK with folded K-K (in DMPG) abolished K-K binding to surface-immobilized ShK, further confirming the specificity of this interaction.

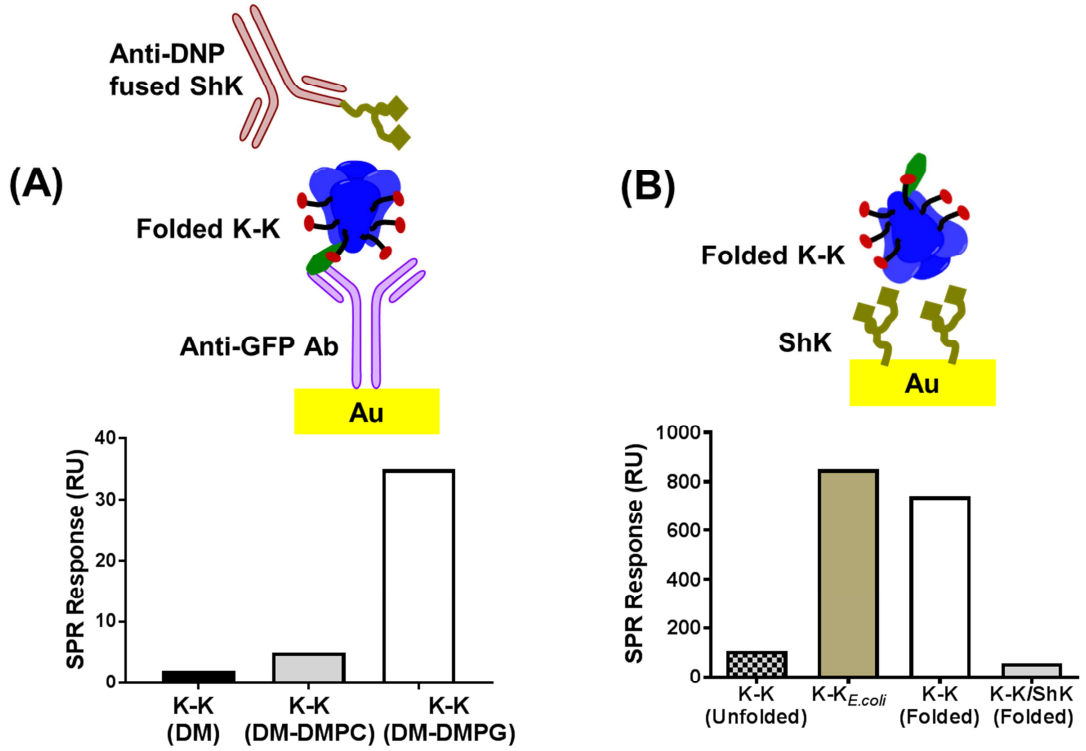


Figure 3. Schematic illustration of KcsA-Kv1.3 (K-K) SPR binding assay. (A) K-K under different folding conditions captured to anti-GFP functionalized surface for anti-DNP-ShK binding (left). 50 nM Anti-DNP-fused-ShK antibody enabled enhancing the SPR binding response of low K-K coverage surfaces. K-K folded in the presence of anionic lipid DMPG illustrated significantly higher binding response to anti-DNP-ShK compared with samples folded either in detergent DM or with lipid DMPC. (B) ShK-functionalized surface for K-K direct binding. TX-TL expressed K-K showed binding response similar to 100 nM *E.coli* expressed K-K after folding in the presence of DMPG. Folded K-K pre-incubated with ShK exhibited limited binding response to Shk-functionalized surface.

In-situ KcsA-Kv1.3 Expression on Functionalized SPR Surfaces via TX-TL

The translational K-K plasmid (K-K-sfGFP), which enabled K-K tetrameric species expression in the *ex-situ* TX-TL was used for performing *in situ* TX-TL on the following functionalized surfaces:

(A) Solid-supported Lipid Bilayer to Stabilize KcsA-Kv1.3

We sought to explore whether nascent TX-TL-expressed protein can be directly integrated to a solid interface for binding analysis. A solid-supported lipid bilayer was fabricated on a SPR chip to stabilize TX-TL-expressed K-K (Figure 1A). The lipid bilayer was created by first capturing a biotin-containing liposome to a NeutrAvidin-functionalized SPR chip. This tri-component liposome comprises an anionic lipid DMPG for facilitating protein folding along with a biotinylated lipid (biotin-PE), and a zwitterionic lipid DMPC. The captured liposome was ruptured by the osmotic shock created by introducing a solution containing high concentration of PEG and NaCl. As illustrated in Figure 4A, after osmotic shock the liposome-captured-surface underwent a drop in SPR response, which suggests the transformation of vesicle-to-bilayer by liposome rupturing. This phenomenon has been previously reported as the loss of entrapped water from a liposome during rupture [37]. This functionalized SPR chip was further used for performing *in situ* TX-TL. SPR system was utilized as an incubator for providing the required temperature (30 °C) for TX-TL in addition to monitoring the entire process in real-time. As shown in Figure 4B, TX-TL resulted in an increase in SPR binding response over the course of reaction indicating reaction species were inserting into the bilayer.

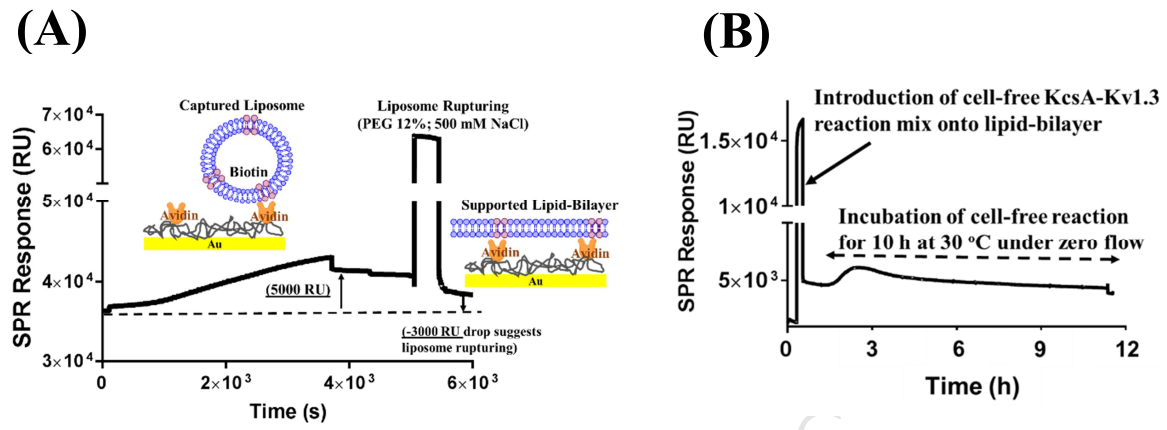


Figure 4. (A) Schematic illustration of supported lipid-bilayer formation from liposome. A biotin containing liposome was captured onto an avidin-functionalized surface followed by liposome rupture using osmotic shock. A drop in SPR response indicates lipid-bilayer formation with the release of water from liposome. (B) Real time monitoring of cell-free reaction (TX-TL) onto lipid-bilayer-functionalized SPR chip.

Additionally, peptide ShK and its analog (i.e., anti-DNP-ShK) were introduced to the lipid bilayer-functionalized surface to verify the presence of tetrameric K-K species formed from the *in-situ* TX-TL reaction. SPR response indicated no binding between ShK and the K-K functionalized surface. We reasoned that K-K probably inserted in an "inside-out" format into the bilayer with soluble GFP domain staying on the outer leaflet, thus ShK cannot access the (tetrameric) K-K binding pockets. To test this hypothesis, we introduced an anti-GFP antibody to these surfaces. As shown in Figure 5, anti-GFP binds to the lipid bilayer containing GFP-fused K-K, thus confirming that GFP is exposed at the bilayer. In a control experiment, TX-TL on a lipid bilayer without K-K plasmid, did not show any binding of anti-GFP (Supplemental

Information, Figure S5). Nevertheless, this method enables insertion of a membrane protein into a solid-supported bilayer in a preferred or known orientation. Recently, a similar strategy was reported to insert proteorhodopsin into a liposome in a known orientation [38].

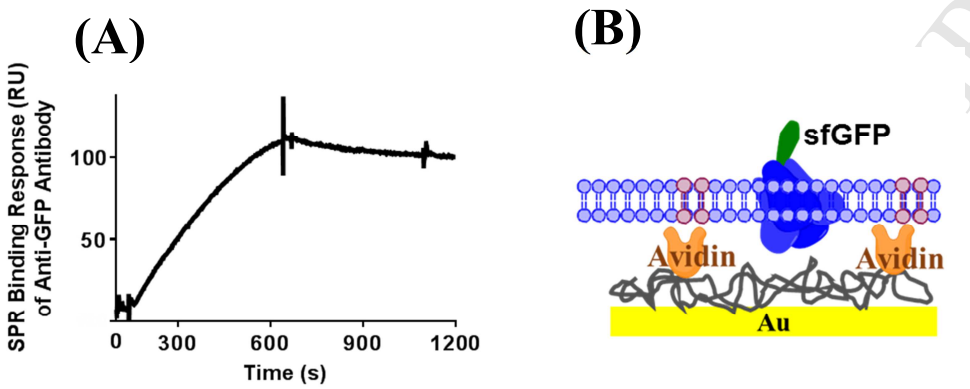


Figure 5. (A) Representative SPR sensorgram of anti-GFP antibody binding to TX-TL containing K-K DNA (K-K-sfGFP) onto lipid-bilayer-functionalized surfaces. (B) Schematic illustration of TX-TL expressed K-K probable orientation into lipid bilayer.

(B) Anti-GFP-Functionalized Substrate to Capture *in situ* Expressed KcsA-Kv1.3

Protein orientation on a surface can be controlled by employing a specific immobilization strategy [39, 40]. In contrast to using a solid-supported lipid bilayer to incorporate TX-TL expressed K-K, an anti-GFP-functionalized surface was employed to capture the protein via its GFP constituent. Additionally, this strategy enables further *in situ* reconstitution of the captured protein for retaining its native functionality. As shown in Figure 1B, TX-TL reactions carried out inside the SPR on an anti-GFP-functionalized chip resulted into an increased SPR response indicating binding of GFP-fused K-K to the surface (Figure 6). In contrast, when an anti-GFP-functionalized chip was exposed to a TX-TL mixture without K-K DNA, it yielded minimal SPR response (~300 RU). This nonspecific binding might have occurred due to the interaction of endogenous constituents of TX-TL with the anti-GFP functionalized surface. Subsequently, *in-situ* reconstitution with lipids was performed to fold the captured K-K. This reconstitution approach is similar to that of the aforementioned on-column two-step process comprising first a brief introduction of urea to unfold the protein followed by a slow pulse of lipid/detergent (i.e., DMPG/DM) solution for protein folding.

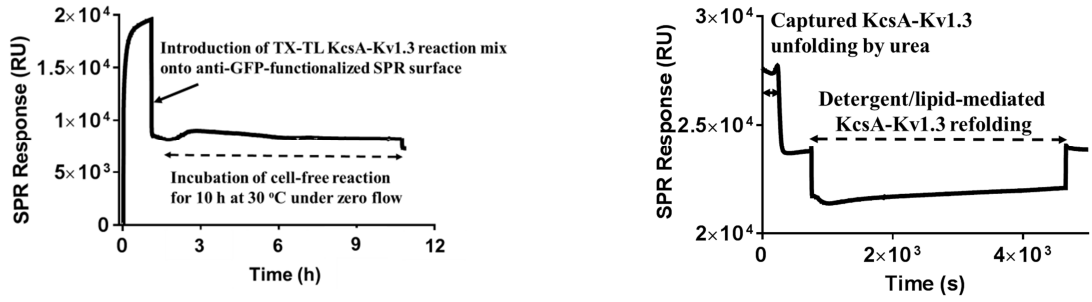


Figure 6. Real time SPR monitoring of TX-TL onto anti-GFP-functionalized SPR chip (left). TX-TL was carried out on the surface for 10 hours at 30 °C. Reconstitution and refolding of captured protein on the functionalized surface (right).

To assess the binding activity of the *in situ* expressed and folded K-K-sfGFP, peptide ShK was introduced to the SPR surface. Figure 7 shows equilibrium binding levels as a function of ShK concentration. The equilibrium binding constant (K_D = 14.8±3.2 nM) was determined by curve fitting of the experimental results using a one-site binding nonlinear regression model. The presence of saturation binding for the interaction between surface-captured K-K and ShK in solution suggests that this is a specific binding event and the K-K-sfGFP tetramer has been folded to its native conformation [23, 26]. In contrast, control functionalized surfaces, either without K-K DNA in TX-TL mixture or using transcriptional reporter fusion (K-K bicistronic expression) did not show any or minimal binding response to ShK. The K_D of ShK binding to TX-TL expressed K-K-sfGFP is ~70-fold lower than that of *E. coli* expressed K-K ($K_D \approx 0.2$ nM) [22]. Molecular modeling of Shk docking with K-K and mutagenesis experiments have indicated that inaccessibility of certain residues in K-K tetramer can impair its binding affinity to ShK [41-43]. Correspondingly the current data suggests that TX-TL expressed K-K-sfGFP tetramer might not have been able to acquire the complete native tertiary/quaternary configuration of tetrameric K-K by *in situ* folding on the captured surfaces. Future experiments focused at optimizing the K-K folding protocol which could enhance the binding affinity to ShK.

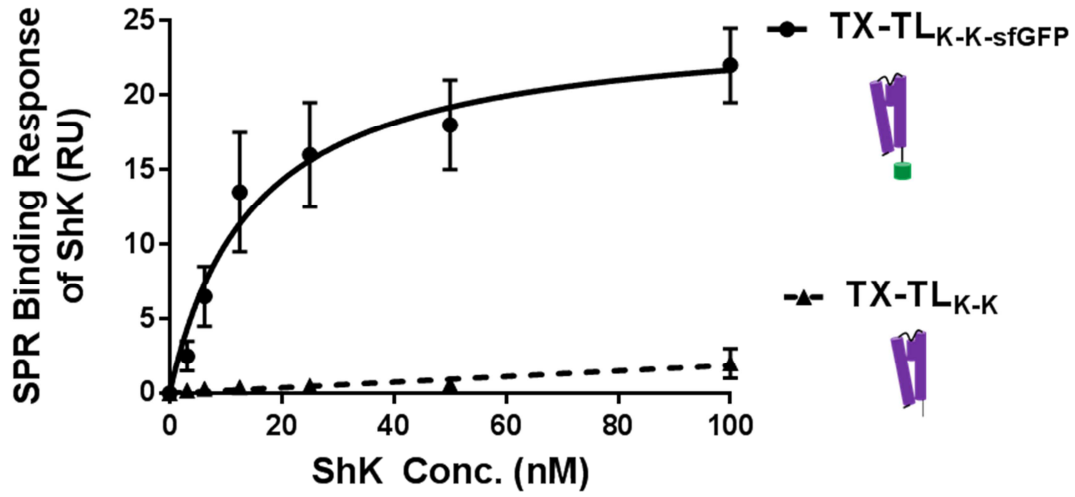


Figure 7. Determination of the equilibrium binding constant of ShK binding to K-K-sfGFP-anti-GFP-functionalized surfaces by monitoring changes in the SPR binding response to different concentrations of ShK. Contrary, TX-TL reaction with K-K plasmid (containing only transcriptional GFP) onto the anti-GFP-functionalized surfaces show significantly lower binding to ShK (filled triangles). Symbols represent the average experimental results with error bars reflect SEMs and the solid curve represents the curve fit.

We also investigated the direct binding of a selective K-K small molecule inhibitor, PAP-1, to the surface-captured K-K [22]. As shown in Figure8, PAP-1 bound to the anti-GFP captured K-K surface with a detectable SPR signal. On the contrary, tripelenamine, a commonly used

antipruritic, did not bind to the captured K-K surface. This tandem strategy, which involves on-demand protein expression using TX-TL and the real-time detection capabilities of SPR, overcomes the traditional limitations of direct binding detection of small molecules with the membrane protein targets by creating a high-density protein surface with a defined orientation.

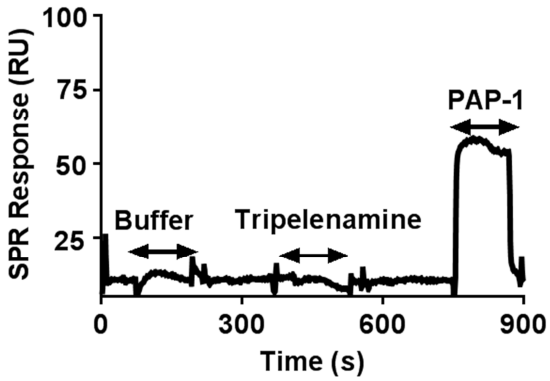


Figure 8. SPR sensorgram illustrating binding of 30 μM PAP-1, a Kv1.3 blocker, to TX-TL expressed and folded K-K onto anti-GFP-functionalized capture surface.

Conclusions:

We have developed a methodology for studying direct-binding interactions involving IMPs by combining the facile protein expression attribute of TX-TL with a sensor chip. For membrane proteins, traditional *in vivo* protein generation methods require multiple cumbersome processes including expression, membrane solubilization, purification, and protein stabilization

to produce the protein for any *in vitro* biophysical studies including SPR. On the contrary, this strategy exploits the flexibility of TX-TL in combination with the integrated microfluidics and real time monitoring feature of a SPR for rapidly performing all the processes *in situ* ranging from expression to folding. Using K-K as a prototypical oligomeric membrane protein for TX-TL, we have demonstrated that the fusion of a water soluble protein, sfGFP, to the monomeric K-K facilitated its tetramer formation. Additionally, we have underscored the importance of anionic lipids in achieving the biologically relevant conformation of tetrameric K-K.

We have explored two different ways to integrate TX-TL with SPR. The first approach involved using a solid-supported lipid bilayer for directly incorporating the TX-TL expressed membrane protein. Although protein was inserted in the bilayer, this method didn't yielded the desired protein orientation in the bilayer; extracellular binding sites probably buried in the bilayer which resulted into inaccessibility of the protein's binding domains to the binding partners. Nevertheless, this strategy might be applicable to the proteins with cytoplasmic binding sites such as transporters for characterizing binding and transport. Using an anti-GFP-functionalized capture surface with *in situ* TX-TL reaction and lipid reconstitution, we were able to achieve high protein coverage along with an optimal control of protein orientation to the surface. Success of this method is particularly important for studying the molecular interactions between membrane proteins with their small binding partners. Our initial studies on a G-protein coupled receptor, β2 adgrenegic receptor, indicated that this generic bottom-up methodology can be straightforwardly extended to other membrane proteins for developing direct binding assays [44]. Future efforts focused on developing a facile one-step protein folding methodology such as a captured nanodisc onto SPR surface for incorporating TX-TL expressed IMPs directly into a

membrane-mimicking platform [35] with access to both sides of the membrane by binding partners for binding analysis.

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Notes

The authors declare no competing financial interest.

REFERENCES:

[1] E. Wallin, G. von Heijne, Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, Protein Sci., 7 (1998) 1029-1038.

[2] V. Katritch, V. Cherezov, R.C. Stevens, Structure-Function of the G Protein–Coupled Receptor Superfamily, Annu. Rev. Pharmacol. Toxicol., 53 (2013) 531-556.

[3] N.J. Harris, P.J. Booth, Folding and stability of membrane transport proteins in vitro, Biochim. Biophys. Acta, 1818 (2012) 1055-1066.

[4] W.A. Catterall, Structure and Function of Voltage-Gated Ion Channels, Annu. Rev. Biochem., 64 (1995) 493-531.

[5] C.R. Sanders, J.K. Myers, Disease-Related Misassembly of Membrane Proteins, Annu. Rev. Biophys. Biomol. Struct., 33 (2004) 25-51.

[6] H.-P. Shih, X. Zhang, A.M. Aronov, Drug discovery effectiveness from the standpoint of therapeutic mechanisms and indications, Nat. Rev. Drug Discov., 17 (2017) 19-33.

[7] H. Yin, A.D. Flynn, Drugging Membrane Protein Interactions, Annu. Rev. Biomed. Eng., 18 (2016) 51-76.

[8] J.P. Overington, B. Al-Lazikani, A.L. Hopkins, How many drug targets are there?, Nat. Rev. Drug Discov., 5 (2006) 993-996.

[9] M. Rask-Andersen, M.S. Almén, H.B. Schiöth, Trends in the exploitation of novel drug targets, Nat. Rev. Drug Discov., 10 (2011) 579-590.

[10] P.M. McNeely, A.N. Naranjo, A.S. Robinson, Structure-function studies with G protein-coupled receptors as a paradigm for improving drug discovery and therapeutic development, Biotechnol. J., 7 (2012) 1451-1461.

[11] J.A. Maynard, N.C. Lindquist, J.N. Sutherland, A. Lesuffleur, A.E. Warrington, M. Rodriguez, S.-H. Oh, Next generation SPR technology of membrane-bound proteins for ligand screening and biomarker discovery, Biotechnol. J., 4 (2009) 1542-1558.

[12] K.S. Mineev, D.N. Kirill, Membrane mimetics for solution NMR studies of membrane proteins, Nanotechnol. Rev., 6 (2017) 15-32.

[13] S.J. Opella, F.M. Marassi, Applications of NMR to membrane proteins, Arch. Biochem. Biophys., 628 (2017) 92-101.

[14] R.M. Stroud, New tools in membrane protein determination, F1000 Biol Rep, 3 (2011) 8.

[15] E.D. Carlson, R. Gan, C.E. Hodgman, M.C. Jewett, Cell-free protein synthesis: Applications come of age, Biotechnol. Adv., 30 (2012) 1185-1194.

[16] M.C. Jewett, K.A. Calhoun, A. Voloshin, J.J. Wuu, J.R. Swartz, An integrated cell-free metabolic platform for protein production and synthetic biology, Mol. Syst. Biol., 4 (2008) 1-10.

[17] C. Roos, L. Kai, D. Proverbio, U. Ghoshdastider, S. Filipek, V. Dötsch, F. Bernhard, Co-translational association of cell-free expressed membrane proteins with supplied lipid bilayers, Mol. Membr. Biol., 30 (2013) 75-89.

[18] J. Shin, V. Noireaux, Efficient cell-free expression with the endogenous E. Coli RNA polymerase and sigma factor 70, J. Biol. Eng,, 4 (2010) 8-8.

[19] Z.Z. Sun, C.A. Hayes, J. Shin, F. Caschera, R.M. Murray, V. Noireaux, Protocols for Implementing an Escherichia coli Based TX-TL Cell-Free Expression System for Synthetic Biology, J. Vis. Exp., (2013) e50762 (50761-50714).

[20] Z.Z. Sun, E. Yeung, C.A. Hayes, V. Noireaux, R.M. Murray, Linear DNA for Rapid Prototyping of Synthetic Biological Circuits in an Escherichia coli Based TX-TL Cell-Free System, ACS Synth. Biol., 3 (2014) 387-397.

[21] R. MacKinnon, S.L. Cohen, A. Kuo, A. Lee, B.T. Chait, Structural Conservation in Prokaryotic and Eukaryotic Potassium Channels, Science, 280 (1998) 106-109.

[22] H. Xu, J.J. Hill, K. Michelsen, H. Yamane, R.J.M. Kurzeja, T. Tam, R.J. Isaacs, F. Shen, P. Tagari, Characterization of the direct interaction between KcsA-Kv1.3 and its inhibitors, Biochim. Biophys. Acta, 1848 (2015) 1974-1980.

[23] C. Legros, V. Pollmann, H.-G. Knaus, A.M. Farrell, H. Darbon, P.E. Bougis, M.-F. Martin-Eauclaire, O. Pongs, Generating a High Affinity Scorpion Toxin Receptor in KcsA-Kv1.3 Chimeric Potassium Channels, J. Biol. Chem., 275 (2000) 16918-16924.

[24] C. Beeton, H. Wulff, N.E. Standifer, P. Azam, K.M. Mullen, M.W. Pennington, A. Kolski-Andreaco, E. Wei, A. Grino, D.R. Counts, P.H. Wang, C.J. LeeHealey, B. S. Andrews, A. Sankaranarayanan, D. Homerick, W.W. Roeck, J. Tehranzadeh, K.L. Stanhope, P. Zimin, P.J. Havel, S. Griffey, H.-G. Knaus, G.T. Nepom, G.A. Gutman, P.A. Calabresi, K.G. Chandy, Kv1.3 channels are a therapeutic target for T cell-mediated autoimmune diseases, Proc. Natl. Acad. Sci. U.S.A., 103 (2006) 17414-17419.

[25] H. Wulff, P.A. Calabresi, R. Allie, S. Yun, M. Pennington, C. Beeton, K.G. Chandy, The voltage-gated Kv1.3 K(+) channel in effector memory T cells as new target for MS, J. Clin. Invest., 111 (2003) 1703-1713.

[26] J.K. Murray, Y.-X. Qian, B. Liu, R. Elliott, J. Aral, C. Park, X. Zhang, M. Stenkilsson, K. Salyers, M. Rose, H. Li, S. Yu, K.L. Andrews, A. Colombero, J. Werner, K. Gaida, E.A. Sickmier, P. Miu, A. Itano, J. McGivern, C.V. Gegg, J.K. Sullivan, L.P. Miranda, Pharmaceutical Optimization of Peptide Toxins for Ion Channel Targets: Potent, Selective, and Long-Lived Antagonists of Kv1.3, J. Med. Chem., 58 (2015) 6784-6802.

[27] M. Raja, The potassium channel KcsA: A model protein in studying membrane protein oligomerization and stability of oligomeric assembly?, Arch. Biochem. Biophys., 510 (2011) 1-10.

[28] E. Serebryany, G.A. Zhu, E.C.Y. Yan, Artificial membrane-like environments for in vitro studies of purified G-protein coupled receptors, Biochim. Biophys. Acta, 1818 (2012) 225-233.

[29] D.E. Drew, G. von Heijne, P. Nordlund, J.-W.L. de Gier, Green fluorescent protein as an indicator to monitor membrane protein overexpression in Escherichia coli, FEBS Lett., 507 (2001) 220-224.

[30] K. Christian, S. Daniel, L. Frank, S. Birgit, D. Volker, B. Frank, Cell-free expression as an emerging technique for the large scale production of integral membrane protein, The FEBS Journal, 273 (2006) 4141-4153.

[31] J.L. Zieleniecki, Y. Nagarajan, S. Waters, J. Rongala, V. Thompson, M. Hrmova, I. Köper, Cell-Free Synthesis of a Functional Membrane Transporter into a Tethered Bilayer Lipid Membrane, Langmuir, 32 (2016) 2445-2449.

[32] R. Robelek, E.S. Lemker, B. Wiltschi, V. Kirste, R. Naumann, D. Oesterhelt, E.-K. Sinner, Incorporation of In Vitro Synthesized GPCR into a Tethered Artificial Lipid Membrane System, Angew. Chem. Int. Ed., 46 (2007) 605-608.

[33] J.-D. Pédelacq, S. Cabantous, T. Tran, T.C. Terwilliger, G.S. Waldo, Engineering and characterization of a superfolder green fluorescent protein, Nat. Biotechnol., 24 (2005) 79-88.

[34] E.N. Lyukmanova, Z.O. Shenkarev, N.F. Khabibullina, G.S. Kopeina, M.A. Shulepko, A.S. Paramonov, K.S. Mineev, R.V. Tikhonov, L.N. Shingarova, L.E. Petrovskaya, D.A. Dolgikh, A.S. Arseniev, M.P. Kirpichnikov, Lipid–protein nanodiscs for cell-free production of integral membrane proteins in a soluble and folded state: Comparison with detergent micelles, bicelles and liposomes, Biochim. Biophys. Acta, 1818 (2012) 349-358.

[35] P.J. Focke, C. Hein, B. Hoffmann, K. Matulef, F. Bernhard, V. Dötsch, F.I. Valiyaveetil, Combining in Vitro Folding with Cell Free Protein Synthesis for Membrane Protein Expression, Biochemistry, 55 (2016) 4212-4219.

[36] F.I. Valiyaveetil, R. MacKinnon, T.W. Muir, Semisynthesis and Folding of the Potassium Channel KcsA, J. Am. Chem. Soc., 124 (2002) 9113-9120.

[37] N.-J. Cho, C.W. Frank, B. Kasemo, F. Höök, Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates, Nat. Protoc., 5 (2010) 1096-1106.

[38] N. Ritzmann, J. Thoma, S. Hirschi, D. Kalbermatter, D. Fotiadis, D.J. Müller, Fusion Domains Guide the Oriented Insertion of Light-Driven Proton Pumps into Liposomes, Biophys. J., 113 (2017) 1181-1186.

[39] V. Früh, A.P. Ijzerman, G. Siegal, How to Catch a Membrane Protein in Action: A Review of Functional Membrane Protein Immobilization Strategies and Their Applications, Chem. Rev., 111 (2011) 640-656.

[40] A. Vaish, V. Silin, M.L. Walker, K.L. Steffens, S. Krueger, A.A. Yeliseev, K. Gawrisch, D.J. Vanderah, A generalized strategy for immobilizing uniformly oriented membrane proteins at solid interfaces, Chem. Commun., 49 (2013) 2685-2687.

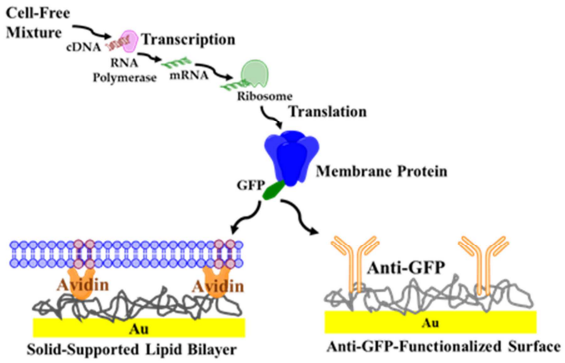
[41] R. Chen, A. Robinson, D. Gordon, S.-H. Chung, Modeling the Binding of Three Toxins to the Voltage-Gated Potassium Channel (Kv1.3), Biophys. J., 101 (2011) 2652-2660.

[42] M.D. Lanigan, K. Kalman, Y. Lefievre, M.W. Pennington, K.G. Chandy, R.S. Norton, Mutating a Critical Lysine in ShK Toxin Alters Its Binding Configuration in the Pore−Vestibule Region of the Voltage-Gated Potassium Channel, Kv1.3, Biochemistry, 41 (2002) 11963-11971.

[43] R. Zhao, H. Dai, N. Mendelman, L.G. Cuello, J.H. Chill, S.A.N. Goldstein, Designer and natural peptide toxin blockers of the KcsA potassium channel identified by phage display, Proceedings of the National Academy of Sciences, 112 (2015) E7013-E7021.

[44] S. Guo, A. Vaish, Q. Chen, R.M. Murray, Expressing Biologically Active Membrane Proteins in a Cell-Free Transcription-Translation Platform, bioRxiv (DOI: 10.1101/104455), (2017).

Graphical Abstract:



Highlights:

1) Developing a generalized strategy for detecting direct binding of small ligands to surface-immobilized membrane proteins

2) Integrating cell-free technology with a SPR biosensor for on-chip protein expression and subsequent ligand binding to a surface-immobilized membrane protein

3) During cell-free expression of a potassium channel, KcsA-Kv1.3, ‘superfolder’ green fluorescent protein fusion not only improves the protein yield, but surprisingly facilitates the tetrameric species formation