**Reconstitution of a Kv channel into lipid membranes for structural and functional studies**

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**Short Abstract:**

Procedures for complete reconstitution of a prototype voltage-gated potassium channel into lipid membranes are described. The reconstituted channels are suitable for biochemical assays, electrical recordings, ligand screening and electron crystallographic studies. These methods may have general applications to the structural and functional studies of other membrane proteins.

**Abstract:**

To study the lipid-protein interaction in a reductionistic fashion, it is necessary to incorporate the membrane proteins into membranes of well-defined lipid composition. We are studying the lipid-dependent gating effects in a prototype voltage-gated potassium (Kv) channel, and have worked out detailed procedures to reconstitute the channels into different membrane systems. Our reconstitution procedures take consideration of both detergent-induced fusion of vesicles and the fusion of protein/detergent micelles with the lipid/detergent mixed micelles as well as the importance of reaching an equilibrium distribution of lipids among the protein/detergent/lipid and the detergent/lipid mixed micelles. Our data suggested that the insertion of the channels in the lipid vesicles is relatively random in orientations, and the reconstitution efficiency is so high that no detectable protein aggregates were seen in fractionation experiments. We have utilized the reconstituted channels to determine the conformational states of the channels in different lipids, record electrical activities of a small number of channels incorporated in planar lipid bilayers, screen for conformation-specific ligands from a phage-displayed peptide library, and support the growth of 2D crystals of the channels in membranes. The reconstitution procedures described here may be adapted for studying other membrane proteins in lipid bilayers, especially for the investigation of the lipid effects on the eukaryotic voltage-gated ion channels.

**Introduction:**

Cells exchange materials and information with their environment through the functions of specific membrane proteins ([*1*](#_ENREF_1)). Membrane proteins in cell membranes function as pumps, channels, receptors, intramembrane enzymes, linkers and structural supporters across membranes. Mutations that affect the membrane proteins have been related to many human diseases. In fact, many membrane proteins have been the primary drug targets because they are important and easily accessible in cell membranes. It is therefore very important to understand the structure and function of various membrane proteins in membranes, and make it possible to devise novel methods to alleviate the detrimental effects from the mutant proteins in human diseases.

Lipids surround all membrane proteins integrated in bilayers ([*2*](#_ENREF_2)*,* [*3*](#_ENREF_3)). In eukaryotic membranes, the various different types of lipids are known to be organized into microdomains ([*4*](#_ENREF_4)*,* [*5*](#_ENREF_5)). Many membrane proteins were shown to be distributed among these microdomains as well as the bulky fluid phase of membranes ([*3*](#_ENREF_3)*,* [*6*](#_ENREF_6)). The mechanism underlying the organization of the microdomains and the delivery of membrane proteins into them and the physiological significance of such distributions are clearly important but remain poorly understood. One major technical difficulty in studying the lipid effects on membrane proteins is the reliable reconstitution of biochemically purified membrane proteins into membranes of well-controlled lipid composition so that almost all reconstituted proteins are functional ([*7*](#_ENREF_7)). In the past few years, we developed methods to reconstitute the prototype voltage-gated potassium channel from *A. pernix* (KvAP) into various membrane systems for structural and functional studies ([*8-10*](#_ENREF_8)). The data from others and us together showed that the lipids are likely a determinant in the conformational changes of the voltage-sensing domains of a voltage-gated ion channel and may shape the structures of some of these channels ([*11*](#_ENREF_11)). In the next, we will provide a detailed description of our methods and will offer critical technical tips that will likely ensure the successful reproduction of our results as well as the extension of our methods to the studies of other membrane proteins.

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1. **Expression and Purification of KvAP channel (Fig. 1)**
   1. Preparation Work – Day 0
      1. Rinse the glass flasks for the bacterial culture with deionized water (diH2O) and MilliQ H2O (MQH2O) to remove trace of detergent from general dishwashing.
      2. Autoclave 1000 ml LB medium in 2.8 L Erlenmeyer flasks (total two-liter culture as an example here). Low hardness of the water was found to be important for the successful culture of the transformed bacteria.
      3. Autoclave 100 ml LB medium in 500 ml flasks
      4. Transform 60 µl of XL1-Blue competent cells with 200 ng of the pQE60 plasmid containing the gene for KvAP with a thrombin cutting site and a His6 tag at its C-terminus, plate the bacteria on two LB-agar plates containing 100 µg/ml ampicillin, and incubate them overnight in a 37 ºC incubator.
   2. Expression of KvAP – Day 1
      1. Check the appearance of the bacterial colonies on the plates after overnight incubation. We do not want the plates that harbor a lot of satellite colonies.
      2. Add 5.0 ml LB medium to each LB-agar plate incubated overnight and scrap off the colonies. Transfer the bacteria suspension into 100 ml LB medium autoclaved in a 500ml flask. Add ampicillin to a final concentration of 100 µg/ml, incubate the small culture for ~1 hour at 37 ºC or until OD600 reaches 0.60.
      3. In the meantime, place two flasks with 1.0 L medium into a 37 ºC shaking incubator to warm up the medium, and prepare 20 ml of BaCl2 (1.0 M; 10 mM final concentration in each 1.0 L culture), 2.0 ml 0.4 M IPTG (isopropyl-thio-β-galactoside), and 2.0 ml of 100 mg/ml ampicillin stock in water.
      4. Once the small culture is ready, add 10 ml of BaCl2, 1.0 ml of ampicillin stock, and 50 ml of small culture from step 1.2.2 to the pre-warmed LB media. OD600 should be around 0.05. Watch for possible precipitation due to high hardness of the water.
      5. Take OD600 every hour until it reaches 0.70, and every fifteen minutes until it reaches 0.8 to 0.9. In our set-up, it usually takes ~5 hours to reach 0.8.
      6. Add 0.40 mM IPTG to start the induced expression of channel protein, and incubate the culture for another 4.0 hours at 37 ºC with 225 rpm shaking.
      7. Harvest bacteria in 1.0-liter centrifuge bottles by spinning at 4,000 x g, 4.0 ºC for 15 min. Decant the supernatant as much as possible into a waste beaker, add 10 ml 1.0 M Na-Phosphate buffer to precipitate all Ba2+, and then add a small volume of bleach to kill bacteria. Keep the harvested bacteria in the centrifuge bottles buried in ice in a 4.0 ºC cold room overnight.
   3. Purification of KvAP protein (Day 2 and 3; **Fig. 1B**)
      1. Resuspend the bacteria pellet in 15 ml of IMAC lysis buffer per 1.0 L culture. Add ~1.0 U DNase I, and three protease inhibitors of leupeptin, aprotinin and pepstatin A at 1.0 µg/ml. The total volume for two-liter culture was ~35 ml.
      2. Sonicate the resuspended bacteria in a metal beaker buried in ice for a total 10 minutes of ON-time. The microprobe sonicator was set in a 5 seconds ON / 10 seconds OFF cycle with a 40 % output power in a 4.0 ºC cold room. The output power setting was empirically determined so that most of the bacteria were lysed without much heating in the solution.
      3. Add 0.50 g of n-Decyl-β-D-Maltoside (DM; Sol-Grade from Anatrace) in dry powder to the sonicated cell lysate and incubate the mixture for 3.5 hours at room temperature (RT) with constant horizontal shaking (~100 rpm) in order to extract as much channel proteins as possible. It is important to make sure that the detergent powder is fully dissolved over a period of 30-50 minutes.
      4. After detergent extraction, remove cell debris from the lysate by centrifugation at 20,000 x g for 30 minutes at RT. While waiting for the centrifugation to finish, prepare a His-tag-based LC (low-pressure liquid chromatography) column as detailed in **Box 1**.
      5. Load the supernatant from step 1.3.4 onto the pre-packed IMAC (**i**mmobilized **m**etal ion **a**ffinity **c**hromatography) column at a flow rate of 1-2 ml/min that is driven by a peristaltic pump. Alternatively the extracted His-tagged protein can be incubated with the IMAC resin for batchwise binding.
      6. Wash the IMAC resin by running 5 bed volumes of IMAC wash buffer, and 10 bed volumes of IMAC wash buffer plus 20 mM imidazole. An in-line UV monitor is used to make sure that the washing is clean.
      7. Elute the KvAP protein by applying IMAC elution buffer containing 300 mM imidazole. Most of the bound KvAP is eluted within 6 ml of elution buffer through the column. Add thrombin (1.0 U per 2-3 mg of protein) into the pooled elution fractions and incubate the protein overnight on the bench to cleave the His6 tag.
      8. On next day, concentrate the thrombin-digested protein solution in a Centricon (MWCO = 30K) down to 600 µl for size-exclusion FPLC (fast protein liquid chromatography). During centrifugation, mix the sample every five minutes to minimize protein aggregation.
      9. Run the concentrated KvAP through a Superdex 200 column that is pre-equilibrated with FPLC equilibration buffer. Typically the peak of the tetrameric KvAP channel in DM elutes with a retention volume of 12.3 ml. Pool the fractions containing KvAP together and concentrate the protein solution down to 0.5 ml. Determine the concentration of the sample using the extinction coefficient of KvAP at 280 nm.
2. **Ion channel reconstitution**
   1. **Liposome preparation and detergent-induced fusion of vesicles**

Prior to the lipid preparation, wash a 14 ml disposable glass test-tube, a screw-capped glass tube, and a 250 µl glass syringe with chloroform. Pour out ~10 ml chloroform into the testtube.

* + 1. Preparation of Palmitoyl-oleoyl-phosphatidyl-ethanolamine (**POPE**) and palmitoyl-oleoyl-phosphatidyl-glycerol (**POPG**) liposomes
       1. Transfer 3.75 mg of POPE and 1.25 mg of POPG (POPE: POPG=3:1 weight ratio) in chloroform into the pre-cleaned screw-capped glass tube and dry the lipids under a continuous stream of argon gas. When no visible chloroform is left, dry the lipid further under room vacuum for one hour.
       2. Add 440 µl of low salt-buffer (10 mM HEPES, pH 7.4) or water into the dried lipid and vortex the tube to hydrate the lipids. The lipid suspension looks whitish and turbid.
       3. Sonicate the lipid suspension in an ice-cold bath sonicator until the vesicle solution becomes translucent (OD410 < 0.2).
       4. Add 50 µl of 3.0 M KCl and 10 µl of 0.50 M DM to the mixture so that the final lipid suspension has 300 mM KCl and 10 mM DM. Incubate the solution with horizontal rotation for 2 hours at RT to form lipid/detergent mixed micelles. After the incubation, the suspension should become very turbid (**Fig. 2A**), which is due to the detergent-induced fusion of the small unilamellar vesicles.
       5. When the protein is concentrated to more than 2.0 mg/ml, add 0.50 mg KvAP protein, 50 µl of 3.0 M KCl, 16 µl of 0.50 M DM stock in water, and 10 mM HEPES, pH 7.4 to the lipid/detergent mixture to make 1 ml of final volume. The final lipid : protein weight ratio is 10:1 and the final concentration of DM is 18 mM (**Fig. 2A**). Incubate the protein/lipid/detergent mixture in the glass tube with horizontal rotation for another 2 hours.

The selection of the detergent concentration is guided by the detergent-induced vesicle fusion and vesicle solubilization ([*12*](#_ENREF_12)). When the vesicles are formed by strong sonication, the average diameter of the vesicles is in the range of 30-50 nm, which has very low scattering at 410 nm. At low concentrations, detergents are first inserted into the vesicles, destabilize the vesicles, and induce the fusion of detergent-saturated vesicles (OD410 goes up; see Figure 2A). Vesicle fusion leads to the increase of the OD410 nm. For 10 mg/ml POPE/POPG vesicles, the OD410 peaks at around 20 mM DM. When more detergents are introduced, the vesicles start to break into pieces and the lipids become partitioned into detergent/lipid mixed micelles. This latter process is accompanied by the decrease of OD410 down to a final low level ( <0.1).

Because of the active fusion events in the rising phase of the optical density due to the detergent-inducing fusion of small vesicles, it is high likely that the protein/detergent micelles will be actively fused with the detergent-saturated lipid vesicles. That is the reason behind the selection of 18 mM DM at the time of preparing the protein/lipid/detergent mixture. Alternatively the protein can be mixed with fully solubilized lipids, and can be used for efficient reconstitution after the lipids reach an equilibrium distribution among the protein-lipid-detergent micelles and the lipid-detergent micelles. A long incubation is usually needed to reach such equilibrium (see next).

* + 1. Preparation of the KvAP in mixture with detergent-solubilized 1,2-dioleoyl-3-trimethylammonium-propane (**DOTAP**) or 1,2-dioleoyl-sn-glycero-3-succinate (**DOGS**) --- an example of complete lipid solubilization for reconstitution
       1. The lipid preparation is the same as for POPE/POPG vesicles. The sonication of hydrated lipids into small unilamellar vesicles needs longer time than for POPE/POPG lipids. The DOGS vesicles may slowly fuse with each other and form small oily droplets.
       2. To solubilize the DOTAP or DOGS completely, the sonicated vesicles are mixed with 10 mM DM and 40 mM n-octyl-β-D-glucoside (β-OG). The lipid/detergent mixture is incubated overnight (> 15 hours) at room temperature, and the mixture should not have any small particles or droplets. But instead it is fairly clear. Failure to reach complete equilibrium in this step will lead to the formation of a significant fraction of multilamellar vesicles.
  1. **Removal of the detergents to form proteoliposomes**
     1. Dialysis –-- slow removal of the detergents, such as DM and β-OG, that have relatively high critical micelle concentrations (CMC ≥ 1.0 mM)

Load the protein-lipid-detergent mixture into a dialysis tubing (MWCO = 10K; 0.70 cm wide and pre-cleaned with a lot of water and dialysis buffer). Tie both ends of the tubing with minimal space left above the solution. Put the loaded tubing in 1 L dialysis buffer, and use a stirring plate so that the tubing rotates slowly in solution. Change the dialysis buffer once every 8 hours for five times. The vesicles are usually ready after two days.

* + 1. Use of polystyrene beads to help remove detergents that have low CMC

In many cases, we have to use low CMC detergents, such as DDM (CMC ~0.17 mM in water). Beads with tiny hydrophobic pores are used to help remove these detergents. To remove 8.7 mg of DDM (dodecyl-maltoside) in 1.0 ml solution, equivalent to ~18 mM, add 8.7 mg of buffer-equilibrated polystyrene beads, such as Bio-Beads SM2, directly to the protein-lipid-detergent mixture in every hour at RT ([*13-15*](#_ENREF_13)). The beads need to be washed sequentially with methanol, ethanol, water, and then dialysis buffer before being used in detergent removal. The supernatant after the first treatment is incubated with freshly washed beads for 9 more times. The interval between two treatments could be shortened after the first 3 treatments. To remove the trace amount of detergents after the last step, add 35 mg Bio-beads SM2 beads and incubate with the vesicles for 4 hours.

* 1. **Storage of the proteoliposome and the quality control**
     1. Once the vesicles are ready, prepare them into 50 µl aliquots and flash-freeze the aliquots by direct plunging into liquid nitrogen. The frozen vesicles are then stored in a -80 ºC freezer. For biochemical assays, we do not use frozen vesicles. For electrical recordings, we only use vesicles that are stored in -80 ºC for less than 6 months.
     2. Floatation of the vesicles in a density gradient (**Fig. 2B**)
        1. Load 50 µl of the vesicle suspension on top of the layers of sucrose gradient made of 0.3 ml each of 10, 35 and 55 % sucrose made in 10 mM HEPES and spin at 200,000 g for 4 hours at 4 ºC. Accelerate and decelerate slowly to avoid the disruption of the interface between layers.
        2. Collect 100 µl fractions from the top to bottom and run SDS-PAGE (**Fig. 2B**). The band of KvAP should be found at the interface between 10 and 35 % sucrose. No heavy aggregates of proteins are seen at the high density range, indicating that almost all proteins are in the membranes.

2.3.3 Check the proper conformation of the KvAP channel in vesicles.

Our previous data established that a single cysteine mutant (L125C) KvAP, when properly integrated in bilayers, is fully buried in membranes, and cannot be accessed by cysteine- specific reagents ([*8*](#_ENREF_8)). The reconstitution procedures are tested with this mutant channel, and checked by a cys-reagent, MTS-PEG5000. Our reconstitution procedures lead to no detectable reaction for the L125C mutant channels in phospholipid membranes, suggesting nearly complete insertion of all channels in bilayers.

1. **Applications of the reconstituted channel-containing vesicles**
   1. **Functional study of the ion channel activities in black lipid membranes.**

**Preparations of needed materials.**

* + 1. Lipid preparation
       1. Clean a glass test-tube, an amber vial with Teflon-surfaced screw cap, and a set of glass syringes with chloroform. Dry the amber vial under a stream of argon gas.
       2. Transfer 0.75 mg POPE and 0.25 mg POPG in chloroform into the amber vial and evaporate the chloroform with argon gas.
       3. Wash the dried lipids with 0.20 ml pentane, and dry completely to remove residual chloroform. Finally dissolve the lipids in 50 µl decane. The lipids in decane (20 mg/ml) will be used for painting a lipid bilayer across a 150-250 micron hole (Fig. 3B) drilled in the thinned portion at one side of an experimental bilayer cap (**Fig. 3A**).
    2. Solution preparation
       1. Intracellular solution: 10 mM HEPES/KOH, pH 7.4, 15 mM KCl
       2. Extracellular solution: 10 mM HEPES/KOH, pH 7.4, 150 mM KCl
    3. Salt bridge: Bend glass capillaries into U-shaped bridges, and fill them with 1.0% molten agar dissolved in extracellular solution.

**Electrical recordings from KvAP channels in lipid bilayers**

* + 1. Pre-paint the round hole (diameter ~0.25 mm) that is drilled at the cylindrical surface of the bilayer cup (Fig. 3B). The lipids are transferred with a capillary pestle that is made in the lab. The round head of the capillary pestle is polished and does not scratch the surface of the cup. The small amount of lipids carried by the capillary pestle is spread around the hole in the bilayer cup, and air-dried.
    2. Insert the cup into the recording chamber, and put in the salt bridges and connect the electrodes to the two sides of the recording cup (Fig. 4A).
    3. Adjust the potential offset between two sides of the cup to ~0 (usually < 2 millivolts). Use the capillary pestle to transfer a small amount of lipid mixture in decane, and paint the lipid across the hole until a bilayer membrane forms. The formation of the bilayer is detected by recording the capacitance current during the delivery of a ramp pulse.
    4. Wait till the membrane thins out and becomes relatively stable. The general thinking about the planar bilayer formed across a large hole is that the very middle portion of the membrane is close to be ~4.0 nm thick as a regular bilayer, and the membrane become thicker when it gets close to the edge of the whole, where there is an annulus around and most of the decane solvent is. It is inevitable that there is residual decane left in the central bilayer portion of the membrane, especially in the space between the two leaflets. But this does not affect the successful recording of channel activities. From the capacitance current, we can roughly estimate the size of the true bilayer at the center of the hole.
    5. As soon as the membrane becomes stable, shoot 0.5-1.0 µl channel-containing vesicles by positioning the fine end of a P2-pipette tip right above the hole. The vesicles fall down across the hole to the bottom of the cup. During this process, multiple vesicles become attached to the membrane across the hole. Given enough time, some are fused into the bilayer portion so that a small number of KvAP channels are properly inserted into the planar bilayer in the center part of the membrane.
    6. To test the KvAP channels in the bilayer, a short pulse of 80 mV is delivered from the holding potential of -80 mV. Due to the fast inactivation and slow recovery from inactivation, a long interval (~120 seconds for channels in PE/PG membranes) is given between two pulses. A typical current recording from the channels in a POPE/POPG membrane is showed in Fig. **3C**. If the current is small, shoot more vesicles. Once the current looks good in size, balance the ion concentrations in the solutions on both sides of the membrane. The channels are ready for electrophysiological experiments.
  1. **Screening for conformation-specific ligands against channels in vesicles**
     1. Introduction of the phage-displayed library.

The phage-displayed 20-mer peptide library is displayed to the N-terminus of the five-copies of pIII proteins at one end of the filamentous bacteriophage *fd-tet* ([*16*](#_ENREF_16)). The library presents approx. 1 x 108 different types of random 20-mer peptide sequences, and was kindly provided to us by Dr. Kathlynn Brown’s laboratory at UT Southwestern Medical Center ([*17*](#_ENREF_17)). The phages infect *E. coli* K91, and make the bacteria resistant to 12 µg/ml tetracycline.

A detailed procedure for the bacterial culture, the amplification and titering of the phages and the sequencing of the phage colonies has been described by McGuire et al. ([*17*](#_ENREF_17)). We will not reiterate the details in these operations, but instead will focus on the isolation of specific clones that bind tightly to the ion channels reconstituted in vesicles (Fig. 4A).

Our study of the conformational changes of KvAP voltage sensors in different lipid membranes([*8*](#_ENREF_8)) showed that it is possible to keep the voltage sensors in either “activated” or “resting” states by switching the lipids from regular phospholipids to those that do not contain phosphates in the headgroup regions (DOTAP and DOGS in our experiments). These two lipid-determined conformations are utilized in our screening of conformation-specific ligands.

* + 1. Panning of the phages against KvAP channels in lipid vesicles.

3.2.2.1 The peptide-displaying phages need to be amplified and titered before our experiments so that the number of phages per unit volume is known. KvAP was reconstituted into POPE/POPG (3:1) plus 0.50% Biotin-DOPE vesicles as negative control, and into DOGS : biotin-DOPE (199:1; same was done for DOTAP vesicles) is used as the selection target. The final concentration of KvAP in vesicles is 0.50 mg/ml, and the lipids are 5.0 mg/ml. The panning buffer contained 500 mM KCl, 100 mM HEPES/KOH pH 7.4, and 0.10 mg/ml BSA.

For the first run, ~1010 phages (100 copies for each type) were diluted to 0.050 ml LB medium. For the subsequent panning steps, the starting phage was adjusted to be within 106 to 108.

3.2.2.2 Negative selection:

Incubate the diluted phages in 50 µl LB with 100 µl NeutrAvidin agarose beads (capable of binding 1-2 mg biotinylated BSA per mL of resin; Pierce) in 1.0 ml panning buffer. After 10 min incubation, separate the beads by spinning them at 100 x g spin for 1.0 minute. Repeat this step twice to remove any phages that bind directly to the beads.

Mix the left-over phages from the previous step with 50 µl empty vesicles (POPE/POPG plus 0.5% biotin-DOPE) that contain no proteins. Add 100 µl NeutrAvidin Beads that have been washed with the panning buffer to the phage-vesicle mixture. After rotating the mixture at room temperature for 5 minutes, remove the beads by 100 x g spin for 30 seconds. This step is repeated for empty vesicles of DOGS: biotin-DOPE (199:1) as well as for KvAP channels in POPE/POPG vesicles (with 0.5% biotin-DOPE). The supernatant after these treatments contains the fully-cleared phages. After the first two screen cycles, the preclearance could be performed only against KvAP in POPE/POPG vesicles.

3.2.2.4 Positive selection

Incubate the fully-cleared phages with KvAP channels in DOGS : biotin-DOPE (199:1) vesicles (the same for the DOTAP vesicles) in the presence of 100 µl NeutrAvidin beads at room temperature for 10 minutes. Bring the volume of the mixture to 15 ml using the panning buffer before the centrifugation at 100 x g for 10 minutes. Collect the beads, and wash them three times with 45 ml panning buffer.

3.2.2.5 Resuspend the beads in 500 µl LB medium containing 5.0 g/ml biotin, and incubate the mixture at room temperature for two hours in order to release some of the bound phages from NeutrAvidin, which has lower affinity than the native lectins. Separate the beads by 100 x g spin for one minute. Collect the supernatant and the beads into two different tubes for titering.

3.2.2.6 To amplify the selected phages, mix 200 µl of the supernatant or the resuspended beads in the last step with 500 µl K91 bacterial culture (O.D.600 ~ 0.4-0.6, cultured ~ 4 hours before it is used). After incubation at 37ºC for 15 minutes, plate 50 µl of each on tetracycline plates for overnight culture.

During the first two screen cycles (**Fig. 4A**), collect all 500 µl supernatant and the beads from the last step, mix them with the 5 ml bacteria culture, and plate them in 9.5 inch square plates in order to recover and amplify all phage colonies. This step is important for recovering those phages that make the bacteria grow slower than others.

3.2.2.7 The colonies from the plates are amplified and titered before the next cycle of panning and selection (see McGuire *et al.*, ([*17*](#_ENREF_17))).

3.2.2.8 Examine the activities of the positively selected phages on channels.

After 10-12 cycles of selection, test the total amplified phages on the KvAP channels in lipid bilayers made of POPE/POPG. If there are a significant fraction of positive clones, the selected phages at 100-500 nM should block a significant fraction of channels in the bilayer (**Fig. 4B**) as we were selecting against channels stabilized in the resting state. The positive data suggest that there are clones that will show strong binding to the channels in the resting state. After 16 cycles of selection, pick 50 positive colonies for single-colony sequencing. The identified dominating phage clones are selected from comparing the sequences, and are amplified and tested against channels in bilayers. Once the positive clones are confirmed, synthesize the peptides carried by the strong positive clones and test them on the channels as well as to confirm their conformation-specific activities.

* 1. **Crystallization of KvAP channels in membranes for structure determination (**[***18-20***](#_ENREF_18)**)** 
     1. To stabilize the conformation of the channel, a conformation-specific binder of the channel, 33H1 Fv protein, is used to form the KvAP/Fv complex. Purify the complex in a Superdex 200 column.
     2. For the initial screen, mix the protein with DMPC or POPC that is completely solubilized in DM or β-OG. The protein/lipid/detergent mixture is mixed for more than 15 hours in order to reach a thermodynamic equilibrium in the distribution of the three components among the mixed micelles. Usually we first test three different lipid/protein ratios (LPR=0.5, 1.5, 2.5) and three different pH levels (6.0, 7.0, and 8.0). The dialysis buffer contains 20 mM K-phosphate buffer, 100 mM KCl, and 3.0 mM NaN3. A tabloid list of LPR vs. pH is used to determine the behavior the protein in the two different types of lipids. We want to obtain relatively large-sized (more than 150 nm) vesicles or membrane sheets when the dialysis samples are examined by negative-stain EM. A small regular pattern in membranes suggests a hit and will be used to guide further optimization.
     3. The screen is then expanded to examine smaller steps of LPR in order to reach a correct LPR. If the proteins are suitable for crystallization, a small lattice of proteins in membranes may become visible. Around these initial conditions, further optimize the crystallization by varying the salt types and concentrations, the temperature, the speed and methods of detergent removal, divalent cations, detergents used to prepare the proteins, precipitants, lipids composition etc.

The optimized 2D crystals of KvAP∆36/Fv complex grow into large single-layer sheets that range from a few microns to 20-30 microns (**Fig. 5A**). Under cryoEM conditions, the crystals show clear square lattice with obvious four-fold symmetry as expected from the four-fold symmetric channels (**Fig. 5B&C**).

**Representative Results:** Please give the reader an idea of what to expect from a successful execution of this protocol. You may include sub-optimal results to help guide the reader with successful replication. This should be written in paragraph form.

The general flow of the experiments for purifying the KvAP channel into biochemical homogeneity is described in **Fig. 1A**. Typical samples during the expression and purification of the protein is showed in the SDS-PAGE gel in **Fig. 1B**. The protein after the IMAC purification is relatively pure. The yield of the KvAP channel is about 1.0 mg/Liter culture.

Solubilization of lipid vesicles with detergents needs to be worked out for each pair of lipid vs. detergent. The solubilization of small unilamellar vesicles of POPE/POPE by DM is presented in **Fig. 2A**, the results from the vesicles floatation are usually fairly straightforward. Typical results in the SDS-PAGE assay of the fractions from the gradients are showed in **Fig. 2B**. In the sucrose density gradient, the channel-containing POPE/POPG vesicles usually are concentrated at the interface between the 10% layer and the 35% layer. The KvAP-containing DOTAP or DOGS vesicles are lighter and usually at the interface tween 5% and 10% (slightly penetrated into the 10% layer). If there are a significant fraction of multilamellar vesicles, they usually are whitish and concentrated below the 10-35% interface.

Electrical recordings from KvAP channels incorporated in planar lipid bilayers are shown in **Fig. 3C**. The typical current trace shows that at -80 mV, the channels are quiet. Switching to +80 mV leads to a quick capacitance peak (the sharp one at the time of voltage switching). A slow rising phase of the current suggests that the channels become active and are able to conduct outward potassium current. After the peak of the rising phase, the current starts to decrease, a step named inactivation. The inactivation takes a few hundred milliseconds to complete. Once the voltage is switched back to -80 mV, there is a returning phase after the downward capacitance peak, which reflects the closing of the open channels and is called the deactivation (**Fig. 3C**).

In the middle of the phage screen, we tested the activity of the amplified phages on the KvAP channels in the bilayers of POPE/POPG. After 12 selection cycles, the amplified phages inhibited the channel activities (**Fig. 4B**, selected phage). But the starting phage-displayed library did not show any effect on the channels (**Fig. 4B**, control phage). Even though the activities of the selected phages were not very high because only a low concentration of the positive phages were around, the clear, reproducible effects suggested that there are active, positive clones that exhibit high-binding affinity, should be selectively enriched during the remaining screening cycles and once enriched, are likely to have strong inhibitory activities on channels in membranes.

In the early stage of screening the 2D crystals, we saw small lattices in many small vesicles. With optimization, some large sheets showed up with a lot of small vesicles around (**Fig. 5A**). The cryoEM images of these crystals always showed a lot of local defects (**Fig. 5B**), suggesting that further optimization is required to obtain better crystals. Once the crystals were well optimized, they exhibited sharp edges, and appeared as single sheets. At high magnification, the individual units are clearly much better ordered (**Fig. 5C**).

**Tables and Figures:** Notes: At least one figure is required for submission. A Table of Materials is also required for submission. Additional tables are optional but preferred.

Please upload each figure and table as a separate document during submission. Please give full descriptions and legends here. This enables easy integration of figures and tables into the manuscript PDF and/or video, if necessary.

**Tables:**

**Table 1: List of reagents and materials**

**Table 2: Equipments needed**

**Table 3: Buffer name and contents**

**Figure legends:**

**Figure 1: Preparation of KvAP for reconstitution**

**A.** General work flow of protein expression, purification and reconstitution.

**B.** Biochemical purification of the protein. Induced culture of *E. coli* XL1-blue expressing KvAP was processed and KvAP purified. Samples before (1) or after (2) induction, detergent extract (3), flow-through from IMAC chromatography (4), two washing steps (5, 6), 300 mM imidazole elution (7) and after size exclusion FPLC (8) were subjected to 12% SDS-PAGE and Coomassie blue staining.

**Figure 2: Reconstitution of KvAP in POPE/POPG vesicles**

**A.** Vesicle fusion and solubilization as a function of detergent concentrations. Absorbance at 410 nm was used to monitor the formation of scattering of vesicles (baseline subtracted to no detergent fraction). Lipids (POPE/POPG 3:1) were 10 mg/ml. The small unilamellar vesicles after strong sonication, is monodispersed and has weak scattering due to their sizes of 30-50 nm in diameter. When the detergents were introduced, they distributed between solution phase and the lipid membrane phase. Due to the strong curvature in the small unilamellar vesicles, the introduced detergents trigger vesicle fusion and the release of the curvature (thus the low surface potential energy). The fused vesicles are larger in size and show stronger scattering at 410 nm. The rising phase of the peak (gray-colored area) therefore reflects the detergent-induced fusion, a good regime for protein micelle fusion to the vesicles. The concentration of the detergent (DM as an example here) right before the absorption peak was indeed chosen for our reconstitution process.

**B.** Samples of 100 µl fractions from top to bottom (1~9) of sucrose gradient were subjected to 12% SDS-PAGE and Coomassie blue staining.

**Figure 3: Electrical activity of KvAP channel in reconstituted bilayers**

**A.** Recording set-up inside a Faraday cage, 1; two electrodes, 2; *trans* side, 3; *cis* side, 4; salt bridge.

**B.** Magnified section of the thinned portion at one side of the bilayer cup showing a hole (5), which is used for making membranes.

**C.** Electrical activity of KvAP channels that were fused into the black lipid membrane was recorded. While being held at -80 mV, the membrane potential was pulsed to 80 mV for 150 ms, and then changed back to holding potential. The ionic current was recorded in the voltage-clamp mode using an Axopatch 200B amplifier.

**Figure 4: Screening for conformation-specific ligands from a phage-displayed library**

**A.** Scheme behind the screening against ion channels reconstituted in vesicles. The vesicles are doped with biotin-DOPE, and they can be pulled out of solution by NeutrAvidin beads.The conformation of the KvAP channel is controlled by specific lipids. Negative selections against beads, empty vesicles and vesicles with channels in a different conformation are done before the phages are incubated with the channels in the target conformational state (in DOTAP or DOGS as an example here). The selected phages can be amplified and tested against channels in bilayers.

**B.** Testing of the selected phages in the middle of the screen.

*Top:*The starting phage library (total ~1010 phages added to the bilayer) was tested on channels in bilayer, and was found to have no detectable activity because each phage clone has only about 100 copies. *Bottom:* After 12 selection cycles, the phages were still a mixture of different clones. Adding about 1010 phages to the channels in the bilayer lead to the clear inhibition of channel activity, suggesting that there are positive clones that should have high affinity.

**Figure 5: Two-dimensional crystallization of KvAP in membranes**

**A.** Image of negatively stained single layer 2D crystals in the middle of crystal optimization. The crystals were stained with 6.0% ammonium molybdate, pH 6.4 plus 0.50% trehalose. Due to the sugar, they sometimes piled up with each other. The blue box designates an area that gives rise to the diffraction pattern shown on the right side with diffraction spots go to about 20 Å.

**B.** CryoEM image of a 2D crystal from a sample similar to the one used in (**A**). The specimen was mixed with 3% trehalose and frozen by direct plunging, and imaged under a cryoEM. The image was obtained at 50,000 x. It was clear that there were local defects in the crystal packing.

**C.** CryoEM image of afurther-optimized crystal. The specimen was embedded in 0.75% tannin and 10% trehalose and the image was taken at 50,000 X. The straight lines and the tight packing suggested that the channels were well ordered in this type of crystals. The two black arrows mark the square lattice.

**Boxes:**

**Box 1. Preparation of IMAC column**

1. Resuspend the IMAC Resin thoroughly.
2. Immediately transfer the required amount of resin in suspension to a 50ml tube. We use 2 ml bed volume of resin for the purification of KvAP from 2 L culture.
3. Centrifuge the tube at 700 x g for two minutes to pellet down the resin.
4. Remove and discard the supernatant.
5. Add 10 bed volumes of MQH2O and mix briefly to rinse the resin.
6. Recentrifuge at 700 x g for 2 min to pellet down the resin. Discard the supernatant.
7. Add 10 bed volumes of MQH2O and pour into empty column.
8. Wait until resin settles down, and close the column with a flow adaptor for low-pressure liquid chromatography.
9. Run 5 bed volumes of MQH2O and 5 bed volumes of equilibration buffer through the column.

**Box 2. Purification of Fv**

**Fv Transformation – Day 1**

1. Transform 100 ng of the plasmid containing Fv-His6 to 60 µl of JM83 competent cells, plate the bacteria on four LB-agar plates containing 100 µg/ml ampicillin, and incubate overnight in a 37ºC incubator.
2. Prepare 6 X 1L LB for bacterial culture.

**Expression of Fv – Day 2**

1. Scrap all the colonies from the plates into LB medium. Add this suspension of bacteria to 6 X 1L LB in the presence of ampicillin (100 mg/L) and incubate until OD600 reaches 0.5.
2. When OD reaches 0.5, decrease the temperature to 20**°**C and RPM to 115. When the temperature drop to ~ 20**°**C, add anhydrotetracycline (100ul of 1mg/ml in DMF to 1L) for initiating the induction of protein expression and incubate another 5 hours at 20**°**C with normal shaking.
3. Harvest bacteria in 1 L centrifuge bottle at 4,000 x g for 15 min. Pour out the supernatant as much as possible. Keep the harvested bacteria on ice in a 4**°**C cold room overnight.

**Releasing the Fv molecules from the bacteria – Day 3**

1. Resuspend bacteria in 150 ml of Fv-releasing buffer (50 mM Tris pH 8.0, 20 % sucrose, 1.0 mM EDTA).
2. While stirring with a magnetic stirring bar, add lysozyme to 0.1 mg/ml and keep the bacteria on ice for 30 minutes. Afterwards, add MgCl2 to 2.0 mM and keep on ice for another 10-15 minutes.
3. Centrifuge the treated bacteria at 20,000 x g for 30 minutes at 4**°**C. the bacterial spheroblasts should all go down to the pellet, and most of the Fv molecules are released in the supernatant.
4. Dialyze the supernatant against 4.0 L of wash buffer (20 mM Tris pH 8.0, 100 mM NaCl) in cold room for at least 4 hours. At the end of day, change to fresh dialysis buffer and dialyze overnight. Due to the sucrose in the solution, the volume of the dialysate will increase by about 50%. Excess of dialysis tubing should be used.

**IMAC purification and FPLC – Day 4**

1. Equilibrate 10 ml bed volume of IMAC purification resin (see Box 1) with the dialysis buffer in 50 ml tube.
2. Transfer the dialysate from the tubing, add MgCl2 to 10 mM and increase the volume to 200 ml. Mix with the pre-equilibrated resin and incubate for 30 minutes at 4**°**C.
3. Pack the empty column with the incubated resin, and wash the resin with 5 bed volumes of washing buffer, followed by 5 bed volumes of washing buffer with 10 mM imidazole and 30 mM imidazole each.
4. Elute the bound protein with 20 ml of washing buffer plus 300 mM imidazole.
5. Run the concentrated Fv through Superdex 200 column pre-equilibrated with washing buffer. Pool the fractions containing Fv (typical peaks shows up at 12.3 ml retention volume) together and concentrate it. Determine the concentration of the sample using the extinction coefficient of Fv at 280 nm.

**Discussion:** Points to include: What was accomplished? What are future directions? What are the limitations? What are alternative and/or opposing methods and how is your technique inferior or superior to these?

The reconstitution of the KvAP channels into different membranes has been used in several studies ([*8-10*](#_ENREF_8)). Following the idea of ensuring the distribution of lipids between detergent/lipid mixed micelles and the protein/detergent/lipid mixed micelles, we are able to reach nearly complete reconstitution of the KvAP into membranes made of very different lipids. Each tetrameric KvAP channel needs ~100 lipid molecules to fully cover its transmembrane domain. The essential requirement is to allow enough lipid molecules to fuse into the protein/lipid/detergent micelles before the detergents are removed. Our standard conditions (0.5 mg protein and 5.0 mg lipids) ensure that there are on average ~1000 lipid molecules per protein molecule. Our floatation experiment and biochemical experiments confirmed that the reconstitution is almost complete. Electrical recordings from channels inserted into black lipid membranes, the screening of the reconstituted channels against a phage-displayed peptide library, and the growth of 2D crystals of the channels in membranes all demonstrate the successful applications of membrane reconstitution for various purposes.

The lipid-dependent conformational changes of the KvAP channels and the screening against a phage-displayed peptide library showcase a new avenue to screen for channel blockers or channel openers by biochemical methods instead of relying on electrophysiology to keep the channels in specific conformations ([*8*](#_ENREF_8)). The success in our screen for conformation-specific binders suggests that the same strategy can be applied to find specific binders for the activated conformation. It is foreseeable that the reconstituted channels in vesicles can be used against single chain Fv libraries, Fab libraries, etc. Likewise, other membrane proteins can run through these operations and find their tight binders that may be useful for various purposes. We believe that this new method will see more general applications in the future.

Reconstituted membrane systems will allow the elucidation of the chemical details behind the lipid effects on membrane proteins ([*11*](#_ENREF_11)). Lipid-protein interaction has been known to be important for many membrane proteins, and has been subjected to multiple studies in the past ([*3*](#_ENREF_3)). In the cell-based studies, manipulations can be implemented to change the specific components in the membranes and then the functional changes in the membrane proteins are associated with the structural and compositional changes in the membranes. Such connections are indirect and might result from multiple factors in the cell membranes that are not well characterized. In a reconstituted homogeneous membrane, it is more definitive in making connections between the structural and functional changes of the membrane proteins and the changes in lipid composition and membrane properties. Ultimately, to understand the chemical principles behind the lipid-protein interaction, we need to delineate the distribution of lipids around the transmembrane domain, and to understand the dynamic changes of these lipids right next to the proteins. A reconstituted system appears to be a reliable way toward such an understanding.

Reconstitution of membrane proteins requires the controlled removal of detergents from the protein/lipid/detergent mixed micelles, and the fusion of the mixed micelles into large ones that eventually turn into vesicles ([*21*](#_ENREF_21)). Three different methods are being used for removing detergents, dialysis, beads, and cyclodextrin ([*15*](#_ENREF_15)*,* [*22*](#_ENREF_22)*,* [*23*](#_ENREF_23)). But it remains difficult to achieve a well-controlled, gradual removal of detergents from a small volume ([*24*](#_ENREF_24)*,* [*25*](#_ENREF_25)). An ideal method for detergent removal would take the detergents out of the aqueous phase evenly across the whole volume in a controllable pace, and should not exert strong interference on the reconstitution of bilayer membranes. Such a method might be able to change the speed and efficacy of reconstitution, and will likely enable the reconstitution in a small volume. A combination of slow-dilution and any of three conventional methods for detergent removal may approach this goal. Slow-dilution by introducing small amount of water into the protein/detergent/lipid mixture is a controllable way to evenly decrease the detergent concentration down to its CMC. The detergent removal afterwards is less critical for the vesicle formation, although still important for the fusion of small vesicles into large ones. Other ways to achieve controlled detergent removal still need to be conceived and developed.

Our reconstitution procedure takes consideration of lipid distribution among mixed micelles and the detergent-induced fusion of vesicles as well as the mixed micelles. Its success paves the way leading to a broader spectrum of applications of the reconstituted vesicles, much more than the three directions we presented. The adaptation of our procedure to other membrane proteins should not encounter major technical limit. Even though many membrane proteins have been reconstituted one way or the other, it has been difficult to achieve near complete reconstitution and to evaluate the functionality of the proteins from multiple different perspectives. Our efforts in the KvAP reconstitution suggest that our methods may allow full reconstitution and will be suitable for these purposes.

**Disclosures:**

The authors have nothing to disclose.

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