Protocol 1

In Vitro Self-Assembly of α-Hemolysin in a Lipid Bilayer

Protein pores, such as α-HL, will self-assemble in vitro within a phospholipid bilayer membrane that has formed over a small aperture (20–40-μm diameter). The aperture can be created in a thin PTFE film or, alternatively, fabricated at the end of a small "U"-shaped PTFE tube, using the method described here. The PTFE tube connects two fluid chambers filled with high-ionic-strength buffer (typically, 1 M KCl), that are in contact with a pair of Ag/AgCl electrodes. This assembly can be used for single-molecule experiments.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

IMPORTANT: H_2O used throughout this protocol should be filtered through an Anotop 0.02-μm filter (Whatman) before use.

Reagents

α-Toxin from *Staphylococcus aureus* (250 μg; Calbiochem) <!>

1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (25 mg; Avanti Polar Lipids) <!>

Divide the lipid into 350-μg aliquots in 6-mm test tubes and store at –20°C.

Ethanol

Filter the ethanol through an Anotop 0.02-μm filter (Whatman) before use.

Hexadecene <!>

Hexane (spectroscopic grade) <!>

KCl (1 M; buffered with 10 mM Tris to pH 8.5, degassed, and filtered using 0.02-μm filter)

Lipid pretreatment solution

Dissolve an aliquot of diphytanoyl phosphocholine <!> in 100 μl of spectroscopic-grade hexane <!>.

Lipid solution

Dissolve an aliquot of diphytanoyl phosphocholine <!> in 15 μl of hexadecene <!>.

Nitric acid (20% aqueous HNO_3) <!>

Equipment

Ag/AgCl electrodes (one pair, shielded; Molecular Devices Corporation)

Analog low-pass filter unit (Krohn Hite)

Data acquisition board and interface unit (NI PCI-MIO 16E [12 bit] or PCI-6251 [16 bit]; National Instruments)

Gel-loading micropipette tips (200 μl)

Hotplate for boiling acid and H_2O baths

Micropipette

N_2 (gas) for drying <!>

Nanopore cell and holder (custom-made) for membrane-embedded proteins (as in Fig. 18-15)

Oscilloscope (digital; Tektronix)

Patch-clamp amplifier (Axopatch 200B [Molecular Devices Corporation] or EPC 10 [HEKA Instruments Inc.])

Stereoscope with swinging arm (~50X total magnification)

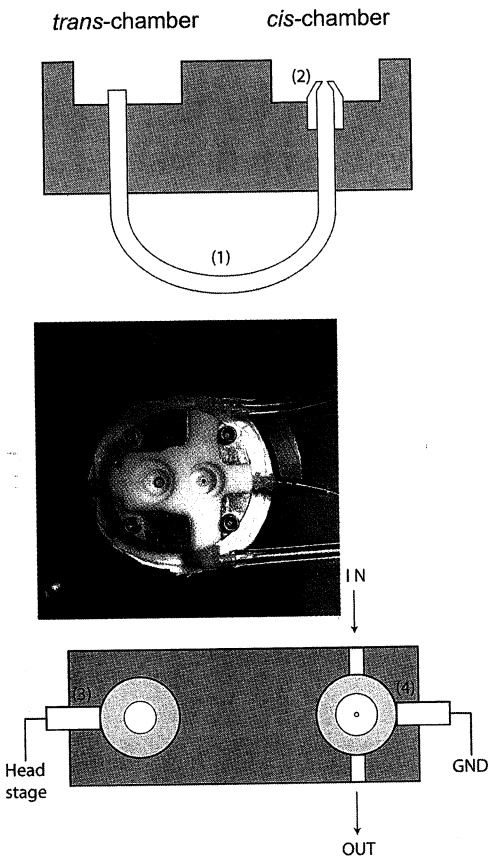

FIGURE 18-15. A PTFE cell used for membrane-embedded α-HL channel recording. (*Top*) Side view of the PTFE U tube (*1*) that connects the *cis* and *trans* chambers, at the end of which a PTFE aperture about 20 μm in diameter (*2*) is used for bilayer support. (*Middle*) Photo of a PTFE cell viewed from above; (*bottom*) top view of the cell, showing the aperture in the *cis* chamber, the IN and OUT ports for buffer flow, and electrode ports for the *trans* (*3*) and *cis* (*4*) chambers.

Syringe pump (Harvard Apparatus)
Thermoelectric temperature controller unit (Newport 3040, Newport Corporation)
Tissues
Vibration isolation table equipped with a Faraday cage (TMC Type II Faraday cage)

METHODS

Refer to Figure 18-15 for guidance in completing this protocol.

1. Working in a chemical fume hood, use a syringe to fill the U tube of the nanopore cell with 20% aqueous HNO_3. Carefully immerse the cell into a beaker filled with 20% aqueous HNO_3.
2. Boil the solution for 15 minutes.
3. Remove the cell and rinse it extensively with H_2O. Boil the cell for 20 minutes in H_2O.
4. Remove the beaker from the hotplate. Rinse the cell with H_2O, and dry it by tapping on a tissue.
5. Place the cell on a holder. Fill the *cis* chamber with ethanol, and connect a vacuum line to the *trans* end of the U tube. Flush about 200 μl of ethanol through the tube.
6. Repeat Step 5 using spectroscopic-grade hexane.
7. Dry the U tube for 1 minute using a vacuum. Thoroughly dry the cell using a dry N_2 stream.
8. Place the cell in the measurement apparatus and connect electrodes as shown in Figure 18-15.

9. Use a micropipette to apply 15 µl of lipid pretreatment solution onto the aperture. Dry by gently pushing air through an air-filled syringe from the *trans* side. Allow the lipid pretreatment solution to dry in air for 10–15 minutes before continuing.
10. Fill the *cis* and *trans* chambers with buffered 1 M KCl. Use a syringe to slowly fill the U tube with the KCl buffer (avoid bubbles in the syringe or U tube).
11. Turn on the patch amplifier and verify that a short circuit is obtained. If not, check that there are no bubbles next to the electrodes or in the U tube that might be blocking the current. If necessary, repeat Step 10.
12. Use a 200-µl gel-loading pipette tip to dispense the lipid membrane solution over the aperture.
    i. Dip a 200 µl gel-loading pipette tip into the lipid membrane solution.
    ii. Dispense most of the solution onto a tissue.
    iii. Use a stereoscope to locate the bilayer aperture in the *cis* chamber. Form a small bubble of lipid solution over the aperture by dispensing air with the pipette.

    CAUTION: Do not touch the aperture with the tip, as this can damage the aperture and ruin the lipid pretreatment.

    iv. The formation of the bubble should create a bilayer over the aperture, which can be verified by observing an abrupt drop in the current to zero.
13. Record the membrane capacitance.

    The capacitance is an excellent indicator of membrane quality. A complete bilayer covering the entire aperture will yield the highest capacitance (~10 pF, although the actual capacitance will depend on the aperture size).
14. If a bilayer was formed, rupture the membrane with a short 300-mV pulse ("zapping"); this should result in a current overload. Use a clean pipette tip to re-form the membrane, as in Step 12iii.

    A good seal is obtained when the dc current is <0.2 pA for 100 mV of applied voltage. The RMS noise should be <0.5 pA at 5-kHz bandwidth. (See Troubleshooting.)
15. Add about 2 µl of the α-HL solution (0.5 g/liter) to the *cis* chamber, and mix gently. Apply a positive voltage of 100 mV.

    A single α-HL insertion should spontaneously occur within 30 minutes, signaled by an abrupt increase in the current to 100 pA at room temperature.

    If several pores are formed, the current will rise in multiples of 100 pA. To obtain a single pore, "zap" the bilayer, form a new one, and wait again for α-HL incorporation.
16. Upon detection that a single α-HL pore has formed, immediately flush the *cis* chamber with 1 ml of buffer by activating the syringe pump.
17. Turn off the voltage, and adjust the pipette offset to null the current. Shield the cell and record an I-V curve. Compare the I-V curve with that shown in Figure 18-3.

    At this point, the setup is ready for SM measurements.
18. Add the analyte with extreme care to avoid disrupting the membrane. After adding analyte, re-shield the cell and wait for temperature stabilization. Continue with analysis.

TROUBLESHOOTING

Problem: The membrane cannot be re-formed.
[Step 14]
Solution: The lipid pretreatment over the PTFE aperature may be inadequate. Carefully reclean the PTFE cell, beginning with Step 1.