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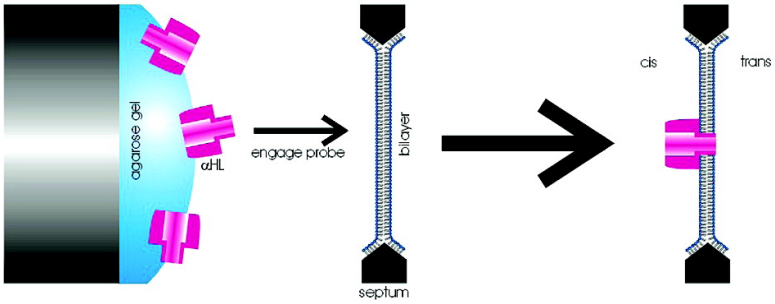
J. Am. Chem. Soc., **2005**, 127 (18), 6502-6503• DOI: 10.1021/ja042470p • Publication Date (Web): 16 April 2005



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Published on Web 04/16/2005

Direct Introduction of Single Protein Channels and Pores into Lipid Bilayers

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The study of single molecule chemistry by using designed protein pores as nanoreactors has become a powerful tool for investigating both noncovalent[1−3] and covalent chemistry.[4−6] For example, the α-hemolysin (αHL) pore has been used to examine host−guest interactions,[2,7,8] covalent bond making and breaking,[6,9] and multistep reaction pathways.[10] The approach can also be used for the single molecule detection, stochastic sensing,[11−13] of a variety of analytes, including ions,[1,3,14] organic molecules,[2,15] DNA,[16−19] and proteins.[20−22] Despite the versatility of the approach, practical application is cumbersome, time-consuming, and requires the hands of a skilled experimentalist. A significant difficulty lies in the techniques used to transfer engineered protein pores into planar lipid bilayers where they are investigated by electrical recording. In some cases (including αHL), oligomeric pores can be assembled in situ from monomers.[23] Preformed pores will also partition into bilayers from dilute detergent solution.[1,24,25] Other approaches include the fusion of lipid vesicles containing membrane proteins to bilayers[26] or the formation of bilayers from monolayers containing the desired protein.[27] For both fundamental research and stochastic sensing, it is preferable that a bilayer contains a single pore, as multiple pores prevent or at least complicate kinetic analysis. Therefore, low concentrations of protein in solution are used to prevent the insertion of multiple pores. However, waiting for a single pore to enter a bilayer by diffusion can be agonizingly slow and unreliable. For newly engineered proteins, it is unclear whether a lack of activity might be due to the inability to function as a pore or other factors, such as altered adsorption or insertion characteristics. Although it is possible to perfuse the bilayer apparatus to remove excess proteins from solution after a single pore has inserted, this is a laborious and unreliable step, which often results in the rupture of the membrane. The field of single channel recording would greatly benefit from a method for introducing proteins that bypasses diffusional transport and reliably delivers single protein pores to planar bilayers upon demand.

Figure 1 illustrates a new approach which fulfills this need. A dome of molten 5% low-melt agarose gel (1.5 mm radius) was formed in a small cup at the end of a hand-operated probe. After the gel cooled, the surface was dried briefly under a stream of N_2. A solution of preformed wild-type (WT) αHL pores (∼0.1 μL of 40 ng/mL) was pipetted onto the dome, which absorbed the liquid, leaving the protein concentrated at the surface. The probe was submerged in the bilayer chamber and engaged with the bilayer/septum during an applied transmembrane potential. When a single pore entered the planar bilayer, a characteristic increase in current was observed after which the probe was disengaged and electrical measurements were performed.

Whether the gel physically touches the bilayer during engagement or is simply extremely close is unclear. Before beginning an experiment, the tip of the gel was carefully aligned to the aperture under a microscope. During engagement, the flexible gel does touch the septum and probably conforms to the aperture. The process is highly reproducible (Figure 2). After a fresh planar bilayer was formed, the probe was engaged to insert a single pore. The bilayer could then be broken with an electrical as pulse, reformed, and a new pore inserted from either the same probe or a new probe. It is important to note that when the loaded probe was simply left in the bilayer chamber for several hours, no pores were inserted. Only engaging the probe caused pores to appear.

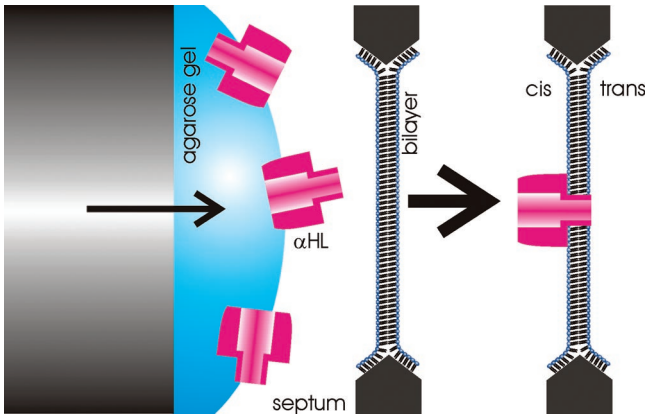

Figure 1. Schematic illustration of the insertion of a single protein pore into a lipid bilayer with a mechanical probe. Apertures, ∼100 μm in diameter, are prepared from 25 μm thick polycarbonate septa (see Supporting Information). Protein pores are deposited on the gel surface, and the probe is introduced into the cis (grounded) chamber of a planar bilayer recording apparatus, which contains a preformed folded[28] bilayer of pure diphytanoylphosphocholine (DPhPC). The current at −50 mV is monitored as the probe is engaged by hand. Pore formation is registered by a stepwise jump in current. The number of steps corresponds to the number of pores inserted. Single insertions are achieved by briefly touching the bilayer/aperture surface and immediately withdrawing the probe after a single current step.

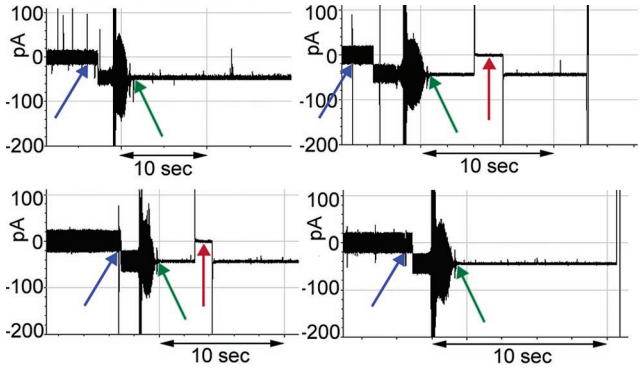

Figure 2. Four examples show the typical lag time between probe engagement and insertion of a single αHL pore into the bilayer. Probe engagement is shown by the blue arrow, while pore insertion is visible as a sharp change in current from 0 to about −50 pA under an applied potential of −50 mV in 1 M KCl, 10 mM MOPS, pH 7.0. The steps to 0 pA in two examples (red arrow) were a check of the electrode balance at 0 mV. During probe engagement, the metal box (which shields the bilayer apparatus from ambient electrical noise during recording) was open to allow for the operation of the probe by hand. After pore insertion, the noise briefly increased as the box lid was closed. The increase was followed by a sharp reduction in noise to the normal value (green arrows).

10.1021/ja042470p CCC: $30.25

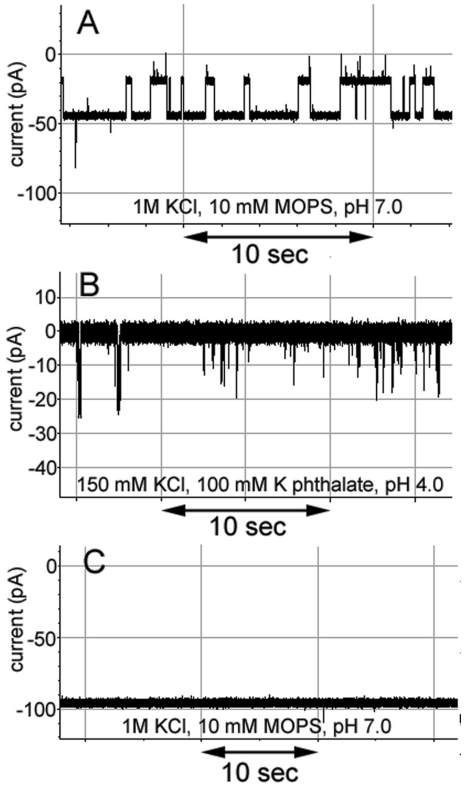

Figure 3. Current recordings from single pores and channels inserted into folded lipid bilayers with a probe. (a) WT αHL at −50 mV. The current blockades were due to the reversible binding of γ-cyclodextrin (15 μM, trans) inside the lumen of the pore. (b) WT KcsA (a K^+ channel) gating under an applied potential of −150 mV with the probe engaged throughout. (c) WT leukocidin pore open at −40 mV. For the αHL and leukocidin pores, the probe was briefly engaged to introduce a single pore and was then immediately withdrawn.

It was paramount to establish whether the probe's surface chemistry or the mechanical impact of the probe with the bilayer caused the properties of a pore to change. The current versus voltage (IV) relationships for single WT αHL pores inserted with the probe were indistinguishable from those obtained with pores inserted by conventional means. Further, single WT αHL pores inserted with the probe were subject to blockade by 15 μM γ-cyclodextrin[2] (Figure 3a). As expected, the current attenuation and kinetics of γ-cyclodextrin binding were closely similar for αHL pores inserted with the probe and those inserted from solution.

We found that probe insertion also works for other channels and pores. Figure 3b shows KcsA[29] (a K^+ channel from *Streptomyces lividans*) gating under an applied potential of −150 mV. Since the opening events of these channels are rare, the probe was left engaged during electrical recording to maximize the number of channels inserted into a bilayer. As opposed to pore introduction, where the insertion was clearly visible as an immediate and stable change in current, the insertion of individual KcsA channels (in the closed state) was not visible. Therefore, the events recorded in Figure 3b show channel gating rather than insertion. In previous work, KcsA was reconstituted into "painted" bilayers by vesicle fusion.[26] In a separate experiment, a single leukocidin[30] pore (from *Staphylococcus aureus*) was inserted into the planar bilayer with the probe, under a potential of −40 mV (Figure 3c). Thus, we have shown that both major classes of membrane protein, β barrels (αHL and leukocidin) and α-helix bundles (K^+ channel), can be introduced directly into bilayers by using a hydrogel probe.

The implications of this method are far reaching. Probes might be used to quickly fabricate arrays of engineered protein pores, each designed to recognize a particular analyte. In fact, it should be possible to add several different species to the same bilayer to construct a rudimentary cell membrane from building blocks. The direct nature of the technique may allow the investigation of membrane proteins that previously have been difficult to reconstitute in artificial membranes. Since the proteins are adsorbed onto the surface of the gel, the local concentration is likely to be increased. Therefore, the diameter (and thus the intrinsic electrical noise) of bilayers that accept pores might be reduced. The probe might also be used to carry analytes to engineered pores, including poorly soluble or short-lived species.

Acknowledgment. This work was supported by University of Oxford start-up funds. The αHL pores, KcsA channels, and leukocidin pores were kind gifts of S. Cheley, A. Mason, and L. Jayasinghe, respectively. We thank O. Braha for valuable comments and suggestions.

Supporting Information Available: A diagram of the bilayer apparatus, procedures for bilayer preparation, and a description of the electrical recording procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA042470P