Published in final edited form as:
Nat Nanotechnol. 2010 December ; 5(12): 874–877. doi:10.1038/nnano.2010.237.

Hybrid pore formation by directed insertion of alpha hemolysin into solid-state nanopores

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Abstract

Nanopores hold great potential for genomic screening and sequencing technologies. Thus far, most studies have concentrated on the *Staphylococcus aureus* pore-forming protein alpha hemolysin (αHL)[1] and artificial pores in solid-state (SS) membranes[2]. While biological pores offer an atomically precise structure[3] and genetic engineering potential[4], SS-pores offer durability, size and shape control[5] and integratability. Each system, however, also has significant limitations: αHL is difficult to integrate because it relies on delicate lipid bilayers for mechanical support, and the fabrication of SS-pores at precise dimensions remains challenging. Here we show that these limitations may be overcome by inserting a single αHL pore into a SS-nanopore. A double-stranded DNA attached to a protein pore is threaded into a SS-nanopore by electrophoretic translocation. Protein insertion is observed in 30-40% of our attempts and translocation of single-stranded DNA demonstrates that the hybrid nanopore remains functional. The resulting hybrid structure offers a platform to create wafer-scale device arrays for genomic analysis including sequencing[6].

We produce hybrid nanopores by inserting a single, pre-assembled αHL protein pore into a small nanopore fabricated in a SS-membrane. One monomer of the heptameric pore is mutated to include an additional 11-amino acid loop at the tip of the β-barrel (Fig. 1a, arrow). This loop contains a single cysteine residue to which a thiol-derivatized 12-base DNA oligomer is coupled through a disulfide bond, supplying an attachment point for a long dsDNA molecule with a complementary single-strand end. The result is a polyanionic tail that guides entry of the αHL pore into the SS-pore in an orientation-specific manner. This directionality is a crucial advantage. Conceptually, a single αHL pore without a tethered dsDNA could be delivered to the SS-nanopore as well, but such protein pores likely would not end up coaxial with the fabricated pores, resulting in non-functional hybrids.

Insertion of the αHL/dsDNA construct is achieved by electrophoretic translocation (Fig. 1b). First, a single SS-nanopore is fabricated within a thin SiN membrane. This is used as a

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Author Contribution

A.R.H., H.B. and C.D. designed the experiment and wrote the manuscript. D.R. and K.M made the engineered αHL pores. A.R.H and A.S. performed the measurements and analysed data.

Additional Information

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barrier between two reservoirs of ionic solution, with the SS-pore as the only connection between the two sides. Application of an electrical potential across the membrane therefore sets up a field that is highly localized to the pore and is able to pull charged molecules through it. Importantly, these SS-nanopores are fabricated with a diameter of 2.4-3.6 nm: large enough to allow the guiding dsDNA and the stem of the mushroom-shaped αHL protein pore to enter, but too small to allow the αHL cap to pass. Therefore, a dsDNA molecule will be threaded through the nanopore, pulling the attached αHL until it is mechanically stopped by the constriction. This will leave the cap facing the *cis* side of the membrane and the β-barrel facing the *trans* side. The small size of the SS-nanopore also ensures that the dsDNA can not be folded[7] during threading, which would lead to steric hindrance of the seal between the αHL pore and the surface of the SiN membrane.

In a typical insertion experiment, we first observe some transient changes in the measured conductance of the SS-pore (Fig. 2a, I), characteristic of dsDNA translocation through a small SS-nanopore[8] (see Supplementary Fig. S1). These are due to unconjugated dsDNA in the measurement solution. Such events are typically followed by a brief plateau of lower conductance (Fig. 2a, II) and then an irreversible transition to a much lower conductance level (Fig. 2a, III). We interpret the brief (<500 ms) plateau in phase II as the presence of αHL-conjugated dsDNA in the pore while the protein interacts with the surrounding membrane surface and settles into position. This is supported by the fact that this plateau is always observed at about the same conductance level as the preceding dsDNA events (see also Fig. S2). The final (phase III) conductance level has an average value of 1.0 ± 0.5 nS over 21 observed capture events, each in a unique SS-nanopore (See also Fig. S3), in good agreement with the 1.0 nS conductance of αHL in lipid bilayers and under the same solvent conditions[9,10]. The variation in conductance between individual hybrid pores is likely due to a leakage current around the αHL or slight deformation of the protein upon insertion.

The success of insertion is tied to many factors. For instance, to accommodate the protein, the initial SS-nanopore must be stable within the narrow range of diameters given above. With small diameter nanopores, changes in size and shape have been observed in solution over time due to surface rearrangement[11], which sometimes cause the SS-nanopore to grow beyond the nominal size for hybrid pore production. Even so, we have observed protein insertion in 30-40% of our attempts (21 out of ~60 pores). The final configuration of the hybrid pores has been observed to be stable for as long as several hours or even days (Fig. S4). While the stability can likely be improved through the use of covalent cross-linking between the protein and the SS-membrane, the hybrid structures that are realized in bare SS-pores already demonstrates good stability under both positive and negative applied voltages (Fig. 2b).

αHL itself has a slightly positive net charge, as measured by its translocation through a 30 nm wide SS-nanopore (data not shown). Unconjugated proteins therefore never enter the nanopore as they are not electrophoretically transported by the application of a negative potential to the *cis* chamber. Furthermore, the added hydrodynamic drag associated with the bulky αHL ensures that the protein approaches the pore only after the dsDNA has passed through it, in a manner that leaves the protein lodged in the constriction.

Following protein insertion, no further transient conductance changes are observed. This suggests that a single αHL pore has inserted at the SS-pore mouth, since the 1.4 nm inner diameter of the protein pore[3] is insufficiently large to allow dsDNA (2.2 nm diameter) to pass. Combined with the above conductance measurements, this observation is strong evidence that a single αHL pore is introduced into the SS-nanopore rather than, for instance, knotted dsDNA. In order to demonstrate that the αHL pore is functional in the hybrid structure, we investigate the translocation of ssDNA as a test molecule for size selectivity.

As demonstrated previously[9,10], with a diameter of about 1.2 nm, ssDNA molecules are able to translocate through the narrow aperture of the αHL pore, and would result in translocation events if the hybrid structure were functional.

Indeed, upon the addition of single-strand poly(dA)$_{100}$ oligomers (1 ng/μL) to the *cis* side of the chamber, transient conductance blockades are observed (Fig. 3a), indicating the passage of individual nucleic acid molecules and, importantly, demonstrating the presence of a functional, non-denatured αHL protein within the structure. Translocation events through the hybrid pore are easily resolvable with a good signal-to-noise ratio. We note that Fig. 2a shows an increase in the conductance noise after the insertion of αHL, but this is not always the case (cf. Fig. S2). Indeed, we observe that low frequency (1/*f*) noise after αHL insertion can vary slightly compared to that of the original pore, but that high frequency noise (>1 kHz) usually remains unchanged (see Fig. S5). The latter is due to charge noise associated with the large SS-membrane in contact with the measurement buffer[12], which suggests that noise levels approaching those of lipid-bound αHL could be achieved through previously demonstrated methods like PDMS coating of the chip[13].

The distribution of ssDNA events recorded in a hybrid nanopore is shown in Fig. 3b (see also Fig. S6). The measured conductance blockade (ΔG) of these poly(dA)$_{100}$ translocation events (Fig. 3b, top) follows a bimodal distribution, consistent with previous measurements on αHL pores in lipid bilayers[10]. Gaussian fits yield peaks centered at 0.3 and 0.6 nS, which differ somewhat from previous experiments on αHL pores in lipid bilayers, which yielded peaks at 0.8 and 0.9 nS.[10] This again might be attributed to some leakage current around the body of the protein or deformation induced by the insertion. We find a dwell time distribution (Fig. 3b, right) with a peak value of 360 μs, in excellent agreement with the characteristic dwell time of 330 μs reported previously[10].

In summary, we have shown that individual αHL protein pores can be inserted into a SS-nanopore in a controlled manner to form a functional hybrid nanopore that combines the precise structure and protein engineering possibilities associated with a biological pore with the robustness and potential for integration of a fabricated device. We present three main pieces of evidence for this. First, upon the insertion, the measured conductance falls to a level that agrees well with that of the αHL pore in lipid bilayers. Second, at this low conductance level, there are no further signatures of translocation when only dsDNA is present in the solution. Finally, the introduction of ssDNA oligomers once again produces translocation events, demonstrating protein functionality. Without the delicate bilayer, our hybrid structures are found to be durable. For example, as a test of mechanical strength, a large voltage applied across an undrilled SS-membrane yields a failure voltage of about 3 V (see Fig. S7); the highest reported voltage stability for lipid bilayers is ~800 mV in a micropipette system[15].

Importantly, incorporating the biological pore into a solid-state device opens up avenues towards the creation of wafer-scale parallel device arrays that may be useful for genomic sequencing. Future efforts towards this end will concentrate on establishing the techniques proven useful for nucleotide-specific measurements with αHL in lipid bilayers[6] into arrays of individually addressable SS-nanopores. Furthermore, our measurements show that the αHL protein pore can be studied outside a lipid bilayer without lateral diffusion. In such a format, the hybrid system could be used to measure the force[14] on a single molecule within a biological pore; a physical quantity central to an understanding of the translocation process. In addition, the platform that we have established here can potentially be expanded to study different membrane proteins (transporters, receptors, etc.) through solubilization and similar attachment to a polyanionic molecule.

Materials and Methods

Solid-state Nanopores

20-nm thin, free-standing membranes of silicon nitride were formed in silicon wafers using common microfabrication techniques[16]. Following this, the tightly focused beam of a transmission electron microscope (300 kV, beam diameter 3.5 nm) was used to locally ablate the membrane surface and form a single nanopore. The pore size was controlled by blanking the electron beam upon formation of a pore with the desired dimensions. Pore diameters were in the range of 2.4 to 3.6 nm, to ensure an optimal fit to the αHL pore. These diameters correspond to pore resistances of roughly 140 to 190 MΩ for our buffer conditions (see below). Immediately after pore formation, the membrane chip was stored in a 50% ethanol/water solution until use in order to maintain cleanliness.

Engineered α-Hemolysin pores

Monomeric αHL was produced with a cysteine-containing 11-amino acid loop (GGSSGCGSSGG) replacing residue 129 and an extension of 8 Asp at the C terminus. These monomers were mixed with M113N mutant αHL monomers[4] and allowed to form heteroheptameric protein pores on rabbit red blood cell membranes. The membrane-bound proteins were then separated by SDS-polyacrylamide gel electrophoresis[17] to obtain pores with subunits in the ratio of 1:6 (αHL_{Cys}: αHL_{M113N}) that were extracted from the gel into buffer containing 10 mM Tris-HCl, 2 mM EDTA, pH 8.5 (TE 8.5 buffer), 0.1 mM dithiothreitol (DTT) and 0.1 % SDS. Following this, the DTT was removed through buffer replacement by centrifugal ultrafiltration (Microcon YM10). Material was stored at −80°C between subsequent steps. Electrical measurements on these heteroheptamers showed that they form active pores in lipid bilayers.

In a separate preparation, 1 mM thiolated DNA oligomers with the sequence 5′-GGGCGGCGACCT-thiol (Sigma) in 100 μl TE buffer (pH 8.5) were treated with 10 mM DTT for 1 h at room temperature. Excess DTT was removed by five cycles of adding 200 μl ethyl acetate, vortex mixing, centrifuging at 13,000 RPM for 1 min, and removing the organic phase. The oligomers were passed over a Bio-Spin P6 column (Bio Rad) pretreated with TE 8.5 buffer and then incubated with 10 mM 2,2′-dipyridyl disulfide (Aldrich) for 1.5 h at room temperature. Excess dipyridyl disulfide was removed by five cycles of adding 200 μl diethyl ether, vortex mixing, centrifuging at 13,000 RPM for 1 min, and removing the organic phase. Activated oligomers were added to the purified heteroheptamers, followed by a centrifugal ultrafiltration (Microcon YM50) to remove excess DNA. The material was stored at −80°C prior to use.

Protein-DNA Construct

λ-phage DNA (New England Biolabs) was digested with the restriction enzyme SfoI. This enzyme leaves blunt ends, resulting in two fragments (45679 bp *cos*L and 2823 bp *cos*R), each with a 12-nt overhang. Gel purification was performed to select the smaller fragment (overhang sequence 5′-AGGTCGCCGCCC), which was subsequently hybridized with the complementary oligomer attached to the heteroheptamer described above by incubation for 30 min at room temperature.

Ion Current Measurements

A chip with a single solid-state nanopore was mounted in a custom flow cell and measurement solution (1 M KCl, 10 mM Tris buffer, pH 8.0) was added to both sides of the membrane. Electrical measurements were performed using Ag/AgCl electrodes attached to a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Signals were acquired at 200 kHz and low-pass filtered at 20 kHz before digitization. For the $poly(dA)_{100}$ translocation

experiments in Fig. 3 and Fig. S6, the conductance blockade (ΔG) of each event is defined as the difference between the average baseline and the average event level (G_o-G_{event}).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the European Commission project "Revolutionary Approaches and Devices for Nucleic Acid analysis" (READNA), the NanoSci E+ program and the European Research Council. K.M. was supported by a Whitaker Foundation fellowship. The authors wish to thank M. Salichou for assistance with engineering of the αHL proteins.

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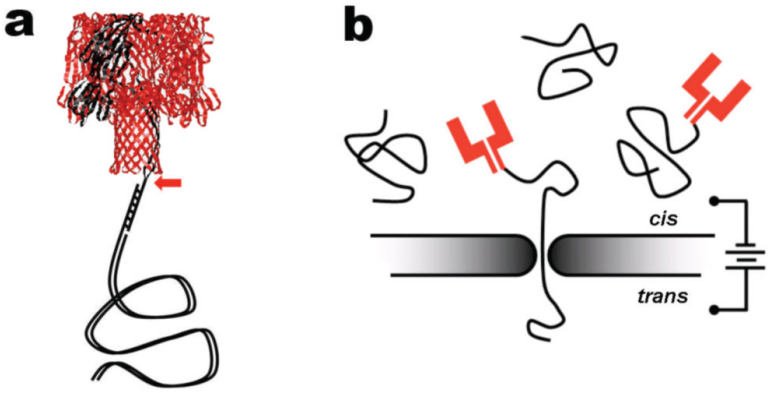

Figure 1. Molecular construct and experimental setup

(a) An αHL heteroheptamer with a 3 kbp dsDNA attached via a 12-nt oligomer to one protein subunit. The arrow indicates the position of the disulfide at the connection point (see text). (b) Experimental setup, in which protein-conjugated dsDNA is electrophoretically translocated through a narrow solid-state nanopore.

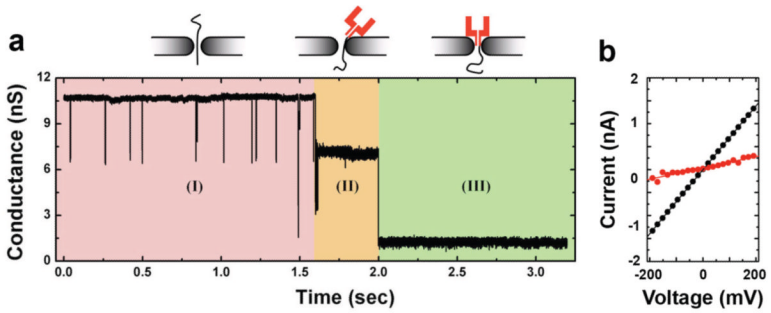

Figure 2. Directed αHL insertion

(a) A typical event wherein an αHL protein pore inserts into a SS-nanopore. The figure shows unconjugated dsDNA translocations (I), followed by a brief plateau indicating "pre-insertion" (II), and finally a stable, low conductance level (III). A voltage of V = −600 mV was applied to the *cis* chamber. Top: sketches of the three phases in the insertion process. (b) I-V response of a SS-nanopore before (black) and after (red) insertion of αHL, demonstrating stability under both positive and negative applied voltage. Linear fits (solid lines) yield a resistance increase from 143 MΩ to 815 MΩ upon insertion.

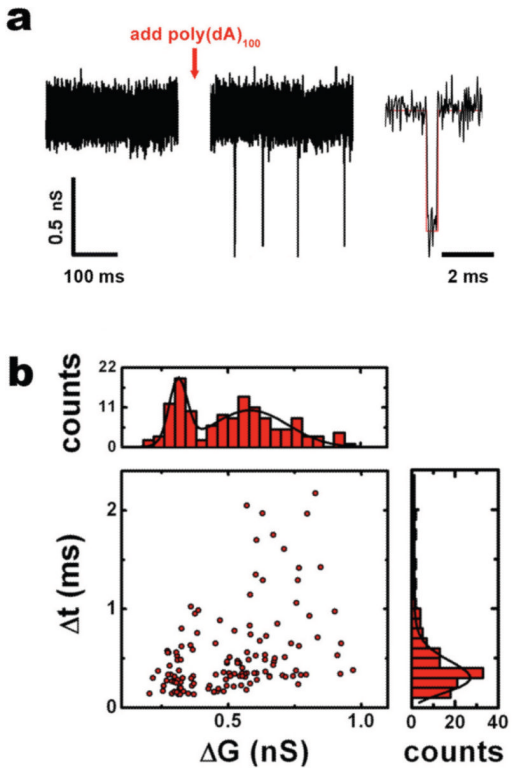

Figure 3. DNA translocation through a hybrid αHL/solid-state nanopore

(a) Recorded current trace through a hybrid nanopore (V = −300 mV applied to the *cis* side), showing the baseline conductance directly after insertion (left) and events upon the addition of poly(dA)$_{100}$ (middle). At right is an expanded view of a typical event (red line indicates a square pulse fit) (b) Event distribution of poly(dA)$_{100}$ translocations, with conductance blockade values (*ΔG*) and dwell times (*Δt*) represented in histograms above and to the right, respectively. Solid lines represent Gaussian fits to the data.