Hybrid pore formation by directed insertion of α-haemolysin into solid-state nanopores

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Most experiments on nanopores have concentrated on the pore-forming protein α-haemolysin (αHL)1 and on artificial pores in solid-state membranes2. While biological pores offer an atomically precise structure3 and the potential for genetic engineering4, solid-state nanopores offer durability, size and shape control5, and are also better suited for integration into wafer-scale devices. However, each system has significant limitations: αHL is difficult to integrate because it relies on delicate lipid bilayers for mechanical support, and the fabrication of solid-state nanopores with precise dimensions remains challenging. Here we show that these limitations may be overcome by inserting a single αHL pore into a solid-state nanopore. A double-stranded DNA attached to the protein pore is threaded into a solid-state 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 hybrid structure offers a platform to create wafer-scale device arrays for genomic analysis, including sequencing6.

We produced hybrid nanopores by inserting a single, pre-assembled α-haemolysin (αHL) protein pore into a small nanopore fabricated in a solid-state (SS) membrane. One monomer of the heptameric pore was mutated to include an additional 11-amino-acid loop at the tip of the β-barrel (Fig. 1a, arrow). This loop contained a single cysteine residue to which a thiol-derivatized 12-base DNA oligomer was coupled through a disulphide bond, supplying an attachment point for a long double-stranded DNA (dsDNA) molecule with a complementary single-stranded end. The result is probably a result of leakage currents around 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,1), characteristic of dsDNA translocation through a small SS nanopore8 (Supplementary Fig. S1). These events arise from un conjugated 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 a result 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 (Supplementary Fig. S2). The final (phase III) conductance level has an average value of 1.0±0.5 nS (taken over 21 observed capture events, each in a unique SS nanopore; Supplementary Fig. S3), in good agreement with the 1.0 nS conductance of αHL in lipid bilayers under the same solvent conditions9,10. The variation in conductance between individual hybrid pores is probably a result of leakage currents around the αHL or slight deformation of the protein upon insertion.

Successful insertion is affected by 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 to allow the connection point (see text).

Figure 1 | Molecular construct and experimental setup. a, αHL heteroheptamer with a 3 kbp dsDNA attached via a 12-nucleotide oligomer to one protein subunit. The arrow indicates the position of the disulphide at the connection point (see text). b, Experimental setup, in which protein-conjugated dsDNA is electrophoretically translocated through a narrow SS nanopore.

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in solution over time due to surface rearrangement\(^1\), which sometimes cause the SS nanopore to grow beyond the nominal size for hybrid pore production. Regardless, we observed protein insertion in 30–40% of our attempts (21 of ~60 pores). The final configuration of hybrid pores has been observed to be stable for as long as several hours, or even days (Supplementary Fig. S4). Stability could probably be improved through the use of covalent crosslinking between the protein and the SS membrane, but the hybrid structures realized in bare SS pores already demonstrate good stability under both positive and negative applied voltages (Fig. 2b).

\(\alpha\)HL itself has a slightly positive net charge, as measured by its translocation through a 30-nm-wide SS nanopore under a positive potential (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 \(\alpha\)HL 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 \(\alpha\)HL pore has been inserted in the SS pore mouth, because the 1.4-nm inner diameter of the protein pore\(^3\) is insufficiently large to allow dsDNA (diameter, 2.2 nm) to pass. Combined with the above conductance measurements, this observation is strong evidence that a single \(\alpha\)HL pore is introduced into the SS nanopore rather than, for instance, knotted dsDNA. To demonstrate that the \(\alpha\)HL pore is functional in the hybrid structure, we investigated the translocation of single-stranded DNA (ssDNA) as a test molecule for size selectivity. As demonstrated previously\(^9,10\), with a diameter of ~1.2 nm, ssDNA molecules are able to translocate through the narrow aperture of the \(\alpha\)HL pore, and would result in translocation events if the hybrid structure were functional.

Indeed, upon the addition of single-stranded poly(dA)\(_{100}\) oligomers (1 ng \(\mu\)l\(^{-1}\)) 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 \(\alpha\)HL 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 current noise following insertion of \(\alpha\)HL, but this is not always the case (cf. Supplementary Fig. S2). Indeed, we observe that low-frequency (1/f) noise after \(\alpha\)HL insertion can vary slightly compared with that of the original pore, but high-frequency noise (>1 kHz) usually remains unchanged (Supplementary Fig. S5). The latter is due to charge noise associated with the large SS membrane in contact with the measurement buffer\(^13\), which suggests that noise levels approaching those of lipid-bound \(\alpha\)HL could be achieved through previously demonstrated methods such as polydimethylsiloxane (PDMS) coating of the chip\(^13\).

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

In summary, we have shown that individual \(\alpha\)HL 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 required for the fabrication of an integrated device. We present three main pieces of evidence for this. First, upon insertion, the measured conductance falls to a level that agrees well with that for \(\alpha\)HL pores 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 ~3 V (Supplementary Fig. S7); the highest reported voltage stability for lipid bilayers is ~800 mV in a micropipette system\(^14\).

Figure 2 | Directed \(\alpha\)HL insertion. a. A typical event, in which an \(\alpha\)HL 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 \(\alpha\)HL, demonstrating stability under both positive and negative applied voltages. Linear fits (solid lines) yield a resistance increase from 143 M\(\Omega\) to 815 M\(\Omega\) upon insertion.
to ensure an optimal fit to the αHL pore. These diameters correspond to pore resistances of ~140–190 Ω under our buffer conditions (see below). Immediately after pore formation, the membrane chip was stored in a 50% ethanol/water solution until use so as to maintain cleanliness.

Engineered αHL pores. Monomeric αHL was produced with a cysteine-containing 11-amino-acid loop (GGSSGCGSGG) replacing residue 129, and an extension of 8 Asp at the C-terminus. These monomers were mixed with M113N mutant αHL monomers and allowed to form heteroheptameric protein pores on rabbit red blood cell membranes. The membrane-bound proteins were then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) to obtain pores with subunits in a ratio of 1:6 (αHL$_{cis}$/αHL$_{trans}$), which 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. DTT was then removed by means of buffer replacement using centrifugal ultraltrafiltration (Microcon YM10). The material was stored at ~80 °C between successive steps. Electrical measurements on these heteroheptamers showed that they form active pores in lipid bilayers.

In a separate process, 1 mM thiolated DNA oligomers with the sequence 5’-GGGGCGGACCT-thiol (Sigma) in 100 μl TE buffer (pH 8.5) was 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 r.p.m. for 1 min, and removal of the organic phase. The oligomers were then mixed into a Bio-Spin P6 column (BioRad) pretreated with TE 8.5 buffer and then incubated with 10 mM 2,2′-dipyridyl disulphide (Aldrich) for 1.5 h at room temperature. Excess dipyridyl disulphide was removed by five cycles of adding 200 μl diethy ether, vortex mixing, centrifuging at 13,000 r.p.m. for 1 min, and removal of the organic phase. For the poly(dA)100 translocation experiments in Fig. 3 and Supplementary Fig. S6, the conductance blockade (ΔG) of each event was defined as the average blockade of the baseline and the average blockade change of αHL pores.

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Materials and Methods

Solid-state nanopores. Thin (20 nm), freestanding membranes of silicon nitride were formed in silicon wafers using common microfabrication techniques. The tightly focused beam of a transmission electron microscope (300 kV; beam diameter, 3.5 nm) was then used to locally ablate the membrane surface and form a single nanopore. Pore size was controlled by blanking the electron beam upon formation of a pore with the desired dimensions. Pore diameters ranged between 2.4 and 3.6 nm.

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 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 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, and so on) through solubilization and similar attachment to a polyanion molecule.

References

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Author contributions
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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