Published in final edited form as:

Curr Opin Chem Biol. 2016 October ; 34: 117–126. doi:10.1016/j.cbpa.2016.08.005.

Engineered transmembrane pores

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Abstract

Today, hundreds of researchers are working on nanopores, making an impact in both basic science and biotechnology. Proteins remain the most versatile sources of nanopores, based on our ability to engineer them with sub-nanometer precision. Recent work aimed at the construction and discovery of novel pores has included unnatural amino acid mutagenesis and the application of selection techniques. The diversity of structures has now been increased through the development of helix-based pores as well as the better-known β barrels. New developments also include truncated pores, which pierce bilayers through lipid rearrangement, and hybrid pores, which do away with bilayers altogether. Pore dimers, which span two lipid bilayers, have been constructed and pores based on DNA nanostructures are gaining in importance. While nanopore DNA sequencing has received enthusiastic attention, protein pores have a wider range of potential applications, requiring specifications that will require engineering efforts to continue for years to come.

Introduction

Since our last review in 2004 [1], the impact of engineered transmembrane pores has been felt in biotechnology. The development of the MinION portable long-read DNA sequencer by Oxford Nanopore Technologies, based on substantial academic effort, is an outstanding achievement (see e.g. reference [2]). Sequencing has been a massive effort and given similar drive there is no doubt that additional areas can be commercialized, including the stochastic sensing of small molecules [3,4], the detection of reactive substances [5], and membrane permeabilization [6] for drug delivery and the eradication of selected cells. Most of these applications require pores with precision engineering and therefore the present review focuses on protein pores rather than the alternatives, which include pores in thin solid-state films (e.g. silicon nitride) [7], plastics [8], glass [9] and graphene [10], and carbon nanotubes [11]. These structures cannot at present be fabricated with sub-nm precision according to rational designs. Pores made by chemical synthesis are also yet to reach the level of refinement and adaptability offered by protein pores [12]. While all of these approaches are likely to be furthered in the future, we have focussed here on recent work on redesigned (rather than de novo designed) protein pores and their applications, with some diversions notably into DNA origami structures.

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α -Helix-based pores

Much of the focus on engineered pores has been on β barrels. While channel proteins often include four or five tightly packed α helices around the transport pathway and other membrane proteins, such as G protein-coupled receptors comprise say seven closely apposed helices, there are relatively few cases of pores built from transmembrane helix barrels [13]. The Ca-ATPase regulator phospholamban forms narrow pentameric channels from parallel helices [14,15]. True pores include the outer membrane D4 domain of the Wza polysaccharide transporter (an 8-helix barrel) [16], the open state of mechanosensitive channels (five or seven helices) [17] and the sea-anemone pore-forming toxin FraC (an 8-helix barrel) [18].

Recently, interesting work has been performed by the Maglia group on the pore formed by the *E. coli* toxin ClyA, which was crystallized in the form of a 12-mer (Figure 1a) [19]. The size of protein nanopores cannot be readily varied. Therefore, the ability to separate ClyA pores of different apparent diameters by electrophoresis in native gels after oligomerization in the presence of detergent is of considerable interest [20]. Pores most likely corresponding to 12-, 13- and 14-mers were purified and shown to differ markedly in their interactions with small water-soluble globular proteins, which penetrated the larger pores more deeply. Additional work on ClyA has included the assembly of protein-bounded rotaxanes [21] and stochastic sensing with protein adapters trapped inside the pore that respond to ligand binding [22]. In a remarkable study, a DNA transporter was built with which a DNA is recognized and captured by hybridization on one side of a bilayer, transported to the opposite compartment and released by toe-hold strand displacement (Figure 1a) [23].

New work on the de novo design of α -helix barrels is likely to aid in the construction of transmembrane pores. For example, the Woolfson group has built parallel 5-, 6- and 7-helix bundles surrounding 5.7 to 7.6 Å-diameter water-filled channels. These structures have been fully defined by X-ray crystallography [24,25]. The conversion of the exteriors of such structures to hydrophobic surfaces will be a tricky next step towards membrane compatibility, and it is encouraging that a 4-helix Zn(II)-transporter has been constructed [26]. The de novo design and assembly of a transmembrane β barrel has also not yet been achieved.

Applying protein engineering technology to pores

Much of the engineering of protein pores falls into the category of redesign in which an existing protein is modified in such a way that the basic scaffold is largely unchanged. For example, α -hemolysin has been modified by a multitude of techniques including direct mutagenesis, targeted covalent and non-covalent modification (with small molecules, host structures and polymers), unnatural amino acid mutagenesis, and in heteromers the variation of subunit ratios and their organization around the central axis [1,27].

The ability to introduce unnatural amino acids with side chains that project into the lumen of a protein pore is of interest for sensing applications and the exploration of single-molecule covalent chemistry. We have introduced unnatural amino acids into α -hemolysin pores by

using cell-free protein synthesis in the presence of chemically aminoacylated tRNAs [28]. This approach has been further developed (by others) for expression in *E. coli*, but neither technique readily allows the production of proteins with multiple unnatural side chains. With that in mind, we have recently used native chemical ligation (NCL) to construct α -hemolysin polypeptides (Figure 1b) [29]. Solid-phase peptide synthesis was used to make the central segment of the 293-amino-acid polypeptide chain, which forms the transmembrane β barrel. The full-length α -hemolysin polypeptide was obtained by ligation of the central peptide to flanking recombinant polypeptides. In an initial study, an amino acid with an alkyne side chain that projects into the lumen of the pore was used to investigate alkyne/azide click chemistry at the single-molecule level and revealed an intermediate with a mean lifetime of 4.5 s [29].

Screening approaches in protein engineering require a convenient and informative assay, and have been used rarely with pore-forming proteins. α -Hemolysins with an improved ability to form heptamers on giant lamellar vesicles (GUV) have been obtained by “liposome display” [30]. α -Hemolysin was expressed with a cell-free system trapped inside GUV. Randomly mutagenized genes were encapsulated at roughly one gene per vesicle. When active pores were formed, a penetrating fluorophore was trapped inside the GUV by covalent attachment to a HaloTag protein. After 20 rounds of selection, an α -hemolysin with two mutations in the transmembrane domain was obtained that showed a 30-fold increase in activity. It would be of considerable interest to use a similar approach to screen for differential transport of various substrates. It is not obvious how this approach would work for other pores, which might not incorporate spontaneously into bilayers.

In future developments, the use of adapters, such as cyclodextrins, will be enhanced by the availability of structural information [28,31]. It was predicted that the engineering of protein pores would be facilitated by the steadily increasing number of X-ray structures of membrane proteins and by computational approaches [1]. Such developments have been slow in coming, with the exception of α -helix barrels (see above), which are likely to profit first.

Truncated pores and lipid bilayer reorganization

Most protein pores comprise a ring of oligomerized subunits, which form a protein-lined water-filled channel through the lipid bilayer [32]. However, it has long been suggested that certain incomplete rings might be completed by a wall lined with lipid headgroups and that other complete rings might have lipids in their transmembrane domains [18,33].

We made the remarkable observation that α HL pores, so severely truncated that they cannot span a bilayer, elicit well-defined ionic currents at higher applied transmembrane potentials (Figure 2a) [34]. We suggested that the truncated proteins float on the surfaces of lipid bilayers and induce the formation of toroidal lipid pores aligned with the central axes of the donut-shaped structures. The truncated pores were produced by pairwise removal of amino acids from the 14 β -strands to shorten the transmembrane barrel by 2, 4, 6 and 8 amino acids. The truncated pores may mimic the membrane penetration step, subsequent to pre-pore formation, in the assembly of transmembrane pores [34]. The truncated pores may be

useful in improving sequencing technologies, as short pores with a single constriction are optimal for base discrimination [35].

Soon afterwards, similar α HL pores with barrels shortened by 2, 3, 4, 7 and 12 amino acids were constructed (Figure 2b) [36]. The pore shortened by 7 residues showed very weak hemolytic activity, while that missing 12 residues was inactive. Interestingly, the hemolytic activity of the former was enhanced after chemical modification. In all the mutants, four amino acid residues located at the β -turn of the barrel domain had been replaced with the sequence Cys-Pro-Asp-Gly, which allowed site-specific modification. For example, reaction with *N*-(1-pyrenyl)maleimide partly restored hemolytic activity. It is possible that the modified structures also act through a toroidal pore mechanism. This study suggests that other ring-shaped proteins, too thin or too hydrophilic to span a lipid bilayer, might act as scaffolds and be converted to pore-forming structures by similar means.

The experiments on truncated barrels suggest that protein pore formation does not always require a transmembrane component, and instead can be accomplished by lipid bilayer reorganization [33]. Thus, pore formation induced by the truncated α -hemolysin constructs suggests that proteins without obvious hydrophobic domains such as amyloid aggregates [37] and antimicrobial peptides [38] might act by a similar mechanism [34].

Dimeric pores spanning two bilayers

Protein complexes that span two bilayers occur in nature and allow ions and molecules to travel between cells or subcellular compartments. For example, the nuclear pore is an elaborate structure that mediates the movement of RNAs and proteins between the nucleus and the cytoplasm [39]. The gap junction is a far simpler structure comprising one class of subunit, connexin, which forms a hexamer, the connexon (or hemichannel), in each bilayer. Two connexons in apposition form a gap junction, which provides cellular communication by allowing ions and small molecules to pass between cells [40]. We have engineered a structure that mimics a gap junction by covalently coupling two α HL pores in an aligned cap-to-cap orientation (Figure 2c). Each subunit of each α HL heptamer contributed a single cysteine side chain to the flat top of the cap. The original intention was to modify the cysteines in a manner that would allow coupling of two pores. Fortunately, when the α HL mutant was expressed in *E. coli*, dimers of the heptamer formed spontaneously [41]. As desired, each end of the dimer could insert into a different bilayer, albeit inefficiently in this initial study.

Gap junction mimics are worth pursuing because of their potential in tissue engineering where they might be used to couple together tissues or tissue-like materials [42]. One untested approach is to make extended helix barrels (see above). DNA origami pores (see below) and carbon nanotubes [11] are also attractive possibilities for this purpose. Mimics of nuclear pores based on the decoration of solid-state pores with intrinsically disordered nucleoporins have been described [43,44]. The engineering of large protein pores might provide a means to obtain homogeneous simplified nuclear pore mimics.

Transmembrane pores built from DNA and RNA

DNA nanostructures are a recent addition to the field of nanopores [45]. By using assembly techniques, including DNA origami [46], complex 2D and 3D structures can be built rapidly and reproducibly, and in quantity, by annealing long biologically-or enzymatically-derived DNAs and RNAs and (or) shorter synthetic oligonucleotides. DNA nanopore structures, for the most part barrels resembling protein pores or holes in flat plates, can be designed by using freely available software. In principle, DNA nanostructures can encompass a wider range of forms than known protein pores. Unlike the situation with, for example, solid-state pores, the internal diameter of DNA pores can be controlled, although the architecture of double-stranded DNA is such that the distance between engineered groups cannot be as precisely determined as it can in proteins.

Another advantage of working with DNA is that existing means for the chemical modification of nucleic acids are already quite sophisticated. This has been critical for the development of membrane-spanning DNA barrels, such as those constructed by the Howorka group, in which the charged phosphodiester bonds of DNA have been neutralized by alkylation [47] or nucleobases with attached porphyrins incorporated to enhance hydrophobicity [48]. In a recent example, a cholesterol-decorated six-dsDNA-helix barrel of ~2 nm internal diameter was built so that a partly closed form could be opened with a DNA oligonucleotide key that complements and releases a DNA lock (Figure 3a) [49]. Upon opening, the DNA pore is able to transport a weakly charged dye molecule of 559 Da through the bilayer, while a strongly negatively charged dye is excluded.

While transmembrane DNA barrels have intriguing potential, several issues remain unresolved. First DNA nanostructures are flexible [50]. Second ions can move along and through DNA structures [50,51]. The pores also exhibit low insertion frequencies, a variable level of noise and often a range of conductance levels [45].

Not yet exploited, RNA structures or structures built from a combination of DNA and RNA provide a potential alternative to DNA pores [52,53]. Intriguingly, RNA can be genetically encoded and expressed inside cells [54]. RNA duplexes are more stable than DNA duplexes and functional RNA motifs in the form of ribozymes and aptamers are well known.

Hybrid pores

A second means to form pores with DNA nanostructures is to deposit DNA plates (tiles) with central holes onto solid-state apertures in silicon nitride or glass. The translocation of ssDNA, dsDNA and proteins through 5×7 nm and 9×14 nm rectangular DNA pores has been detected and the transient hybridization of ssDNA to DNA overhangs at the mouth of a 5×7 nm pore observed [55].

In an earlier related study, the α -hemolysin pore was equipped with a dsDNA tail and pulled into apertures in silicon nitride to form hybrid pores. The pores remained functional and able to support ssDNA translocation events [56]. Later, a similar approach was used to incorporate a DNA origami pore into a silicon nitride aperture (Figure 3b) [57]. These

studies suggest that a wide variety of hybrid pores might be made. The hybrid pores would exist in a robust environment and be readily elaborated into arrays [58].

Functional pores

While natural pores generally remain in an open state, narrow channel proteins exhibit a range of functional activities including selective transport, rectification (one way transport) and gating (opening and closing). Attempts are being made to introduce these properties into protein pores.

While ion channels can be highly selective, based on the intimate interactions of dehydrated ions with the conductive pathway, e.g. K^+ with the carbonyl groups of the selectivity filter of potassium channels, pores generally transport hydrated ions with comparatively little selectivity. Therefore, the interest lies in the selectivity for larger molecules, for example it has been shown that the ClyA pore is capable of transporting double-stranded DNA [23], while the α HL pore transports only single-stranded DNA.

Voltage-dependent rectification of ion transport has been noted in a mutant of the α HL pore [59]. This phenomenon is likely to require collapse of the transmembrane β barrel, and therefore it can be seen as a form of gating. Futaki and colleagues have used extramembranous leucine zippers to bring a defined number of alamethicin peptides together to form a pore. Modified zippers with chelating groups allow gating with Fe(III) ions [60], most likely mediated by expansion of the pore with additional subunits. Similarly, a calmodulin-derived peptide confers Ca(II) gating [60]. Transmembrane peptide association driven by the non-covalent interaction of extramembranous domains is a useful extension of the template-assisted synthetic approach employed earlier [1,61].

Several attempts have been made to construct light-gated channels and pores [62–65], which would be widely useful for applications in nanotechnology, cell biology and drug delivery. The reversible light-mediated gating of large transmembrane pores with fully open- and off-states, which might require a mobile plug or cap, remains an elusive and prized goal.

Applications of engineered pores

The hottest technology that relies on engineered protein pores is single-molecule nanopore DNA sequencing in which a single strand of DNA is ratcheted through a pore by an enzyme that handles fragments of double-stranded DNA [66]. The order of the DNA bases is revealed from sequence-dependent fluctuations in the ionic current passing through the pore. Based on beginnings in academia, Oxford Nanopore Technologies (www.nanoporetech.com) have over ten years developed a portable nanopore sequencer, the MinION, containing a chip with hundreds of addressable pores [67]. The resilience and portability of the technology has been demonstrated by the sequencing of 142 Ebola virus strains in Guinea, West Africa [2].

Efforts to improve nanopore sequencing include rapid sample preparation, faster base turnover by individual pores and improved data processing. From the viewpoint of the protein engineer, there is still room for improved pores. Indeed, Oxford Nanopore have

recently revealed the adoption of an engineered CsgG pore [68] for future MinION chips (Figure 4a) [69]. CsgG mediates the secretion of polypeptides from gram-negative enteric bacteria. The polypeptides form amyloid-like fibers that facilitate biofilm formation. The CsgG pore, a nonamer of 262-residue subunits, features a 36-stranded β barrel in the bacterial outer membrane with a highly constricted periplasmic entrance that is likely to be involved in base recognition when the pore is applied in nanopore sequencing. There is also a pressing need for chips with a very large numbers of pores, for which separate electrical addresses are not feasible. An approach that engages the optical detection of pore activity has been evaluated with which $>10^4$ pores mm^{-2} can be observed, but as yet in a limited field of view (Figure 4b) [70].

The next frontier in nanopore sensing may be protein analysis with the goal of identifying and counting proteins in cells and tissues, and the detection of variations and modifications, such as the products of alternative splicing and postranslational modification. Activity has begun in this area of nanopore proteomics [71,72], and there is a pressing need for improved means to translocate polypeptide chains through pores. A long-term goal is to characterize proteins from single cells. Because the rate of access to the mouth of a nanopore is diffusion controlled, data acquisition would be slow from any reasonable sample volume (say 100 nL), and means to concentrate the analyte at the pore mouth should be explored, e.g. dielectrophoretic trapping [73].

Conclusion

Over the last 25 years, nanopore research has become an extensive effort involving hundreds of researchers. Numerous additional applications of nanopores are being examined including the detection of small molecules, molecular filtration, single-molecule covalent chemistry and catalysis, cell permeabilization and drug delivery. Progress in these areas will continue to depend on inventive protein engineering.

Acknowledgments

We acknowledge the financial support of the NIH, ERC and BBSRC

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- of special interest
- of outstanding interest

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Highlights

- Nanopores are making a strong impact in both basic science and biotechnology
- Protein nanopores can be engineered with precision and are preferred for most applications
- Recent years have seen an increase in the diversity of pores through the exploitation of new structures
- Diversity has also been increased by the application of a wider variety of engineering techniques
- Emerging applications require continued efforts to obtain pores with specified properties

Figure 1.

Functional pores. **(a)** *Top*, side and top views of the 12-subunit ClyA pore. ClyA spans the lipid bilayer as an α -helix barrel [19]. The presumed boundaries of the bilayer are shown. *Bottom*, an engineered ClyA pore acts as a DNA transporter. DNA is captured by hybridization on one side of a bilayer, transported in an electric field and released by toe-hold strand displacement [23]. **(b)** *Top and bottom left*, a heptameric α -hemolysin pore with one engineered subunit containing an unnatural amino acid with an alkyne side-chain that projects into the lumen of the transmembrane β barrel. *Bottom right*, the engineered subunit was prepared by native chemical ligation. An engineered pore with one such subunit was used as a nanoreactor to monitor alkyne/azide click chemistry at the single-molecule level. An intermediate (S₁) was revealed with a mean lifetime of 4.5 s [29].

Figure 2.

Truncated pores and a pore dimer. **(a)** Truncated versions of α -hemolysin's transmembrane β barrel. All seven subunits were truncated as shown. TBM 2 to TBM 8 formed stable conducting states in lipid bilayers (NN, full-length). The most drastic truncations cannot cross a bilayer suggesting that conduction must involve toroidal lipid pores (*top right*) [34]. **(b)** One subunit of the STM5-FIM α -hemolysin pore. The barrel is shortened by 7 residues and a fluorescein molecule is attached at the turn of the β hairpin. The construct has weak hemolytic activity [36]. **(c)** A head-to-head dimer of the α -hemolysin pore [41]. Two heptameric pores are connected by disulfide bonds. *Left*, model of the dimer spanning a planar bilayer and a liposome (not to scale). *Center*, electron microscopy of negatively-stained α -hemolysin dimers. Class averages are shown: side view (*top*); top view (*bottom*). *Right*, electron micrograph showing α -hemolysin dimers spanning negatively-stained liposomes.

Figure 3.

DNA nanopores. **(a)** Triggered opening of a DNA nanopore [49]. The pore is built from six interlocking DNA strands, with three covalently attached cholesterol molecules, and locked closed with a seventh strand. The locking strand can be removed by hybridization to a “key” strand doubling the unitary conductance and allowing the passage of a weakly charged dye molecule of 559 Da. **(b)** A DNA origami pore embedded in a silicon nitride aperture [57]. The current trace shows the drop in current as the DNA pore enters the aperture after threading with a 2344 bp double-stranded DNA extension.

Figure 4. Recent developments in nanopore DNA sequencing. **(a)** Oxford Nanopore uses the CsgG pore [68] in the MinION sequencing device [69]. The constriction involved in base recognition can be seen in the top view. **(b)** Optical detection of miRNA translocation events by α -hemolysin pores in a droplet interface bilayer [70]. The pore density reaches $>10^4$ pores mm^{-2} (red box). 24 signals collected simultaneously are shown.