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Templated Assembly of Pore-forming Peptides in Lipid Membranes

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Abstract: Pore-forming peptides are of interest due to their antimicrobial activity and ability to form gateways through lipid membranes. Chemical modification of these peptides makes it possible to arrange several peptide monomers into well-defined pore-forming structures using various templating strategies. These templated superstructures can exert antimicrobial activity at significantly lower total peptide concentration than their untemplated equivalents. In addition, the chemical moieties used for templating may be functionalized to interact specifically with targeted membranes such as those of pathogens or cancer cells. A range of molecular templates has been explored, including dimerization of pore-forming monomers, their covalent attachment to cyclodextrin, porphyrin or fullerene scaffolds as well as attachment of amino acid linkers or nucleic acid constructs to generate assemblies of 4 to 26 peptides or proteins. Compared to free peptide monomers, templated pore assemblies showed increased membrane affinity, prolonged open-state lifetimes of the pores and more frequent pore formation due to higher local concentration. These constructs are useful model systems for biophysical studies to understand porin and ion channel proteins and their mechanisms of insertion into lipid membranes. Recently designed DNA-templates are expanding the usefulness of templated pore assemblies beyond applications of cell killing and may include targeted drug delivery and accelerate the emerging field of single-molecule detection and characterization of biomolecules by nanopore-based resistive pulse sensing.

Keywords: DNA oligonucleotides · Lipid membrane · Pore-forming peptide or protein · Resistive pulse sensing · Template

Michael Mayer obtained a PhD in biophysical chemistry with Horst Vogel at the Swiss Federal Institute of Technology in Lausanne (EPFL), followed by postdoctoral research with George M. Whitesides at Harvard University. In 2004, he started a faculty position in Biomedical Engineering at the University of Michigan. In 2015 his group moved to the Adolphe Merkle Institute at the University of Fribourg, where he holds the chair of Biophysics. His research takes inspiration from nature to solve problems in biophysics ranging from understanding signaling and transport processes in biological membranes as well as detecting protein complexes relevant for neurodegenerative diseases and engineering biocompatible electrical power sources.

1. Introduction

During the past three decades, pore-forming antimicrobial peptides (AMPs) have gathered increasing attention as potential therapeutics[1] to address the resistance of pathogens to current antibiotics.[1c,d,2] As AMPs are often cationic, they interact preferentially with bacterial membranes, which are negatively charged due to the presence of phosphatidylglycerol or cardiolipin lipids.[3] In the case of Gram-positive bacteria, AMPs bind directly to the negatively charged plasma membrane.[3a,e] Gram-negative bacteria on the other hand exhibit an additional membrane, the outer membrane, which displays a high content of negatively charged lipopolysaccharides; AMPs thus bind first to the outer membrane *via* electrostatic interactions, penetrate into the periplasmic space and then form pores in the negatively charged plasma membrane.[3a,e] Eukaryotic cell membranes, on the contrary, are mainly composed of zwitterionic lipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, leading to weak electrostatic interactions with most antimicrobial peptides.[3b–e] Cancer cells, which possess only slightly more negatively charged lipids (< 9% of the total lipid content) than regular mammalian cells, additionally contain O-glycosylated mucins – a glycoprotein – increasing the negative charge of their membranes and facilitating the binding of AMPs.[3b,4] Cancer cells also display an increased number of microvilli, increasing the surface area for binding of AMPs compared to regular mammalian cells.[3b,4a,c,d] In addition to the attractive electrostatic interaction between AMPs and bacterial or cancer cells, the high transmembrane potentials of pathogen cells (typically –140 mV versus –15 mV for most eukaryotic cells) facilitates dipole- or charge-driven insertion of pore-forming peptides into pathogen membranes.[1b,2f,3a,5] Finally, eukaryotic cells have cholesterol-rich membranes, unlike the membranes of most pathogens, altering the membrane fluidity and reducing the propensity for pore formation by many AMPs.[3b,c,e]

Altogether, these properties of cell membranes lead – in some cases – to selective toxicity of AMPs against pathogen cells over host cells by forming membrane defects or pores.[1b,c,2a,3b,4a,d,6] In contrast to traditional small molecule antibiotics, this broad mechanism of targeting negatively charged membranes reduces the likelihood for the development of resistance of the pathogen to AMPs.[2f–h]

In order to develop AMPs that are potent and selective for killing pathogens, several groups have studied the pore-forming activity of native AMPs and developed variants for potential cell killing applications using high-throughput screening methods.[7] Another strategy involved chemical modification of the peptide termini with reactive groups in order to link multiple peptide

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monomers into pore-forming structures. To enhance antimicrobial activity, various groups are employing a range of strategies for templating to minimize the peptide concentration required for pore formation. Recent advances using nucleic acid templates suggest applications of these templated assemblies beyond killing pathogens such as targeted drug delivery or resistive pulse sensing of biomolecules.[8] Triggered assembly of pores with diameters that may be tuned to the size of the cargo or analyte of interest may on the one hand enable delivery of membrane-impermeant therapeutic molecules such as siRNA[9] and on the other hand enable nanopore sensing of specific biomarkers.[10] This article provides a brief review of the field of templated assembly of pore-forming peptides to transmembrane pores and ends with a short description of the ongoing work in our group on DNA-mediated assembly of pore-forming peptides to pores with programmable diameter.

2. Assembly of Pore-forming Peptides by Dimerization or by Amino Acid Templates

One of the simplest modifications of pore-forming peptides in the context of triggered assembly to a pore consists in the dimerization of two monomers *via* disulfide bridges or other chemical moieties acting as linkers (Fig. 1A). Most of these approaches employed the 20-amino acid AMP alamethicin as it forms pores by the so-called barrel-stave mechanism that grow and shrink in diameter with defined single-channel conductance levels as a function of the number of peptides in the assembly.[5,11] A few studies were also conducted with the melittin,[12] amphotericin B[13] or magainin 2 peptides[14] and with gramicidin.[15] All these studies report an improved stabilization of the pores in lipid membranes compared to alamethicin monomers, leading to increased open pore lifetimes from a few tens of milliseconds for the monomers to a few seconds for the dimers (Fig. 1). Some studies also report improved antimicrobial activity up to 60-fold for the dimerized peptides compared to the peptide monomers.[16] Other pore assemblies took advantage of small individual molecules such as cyclodextrins[17] (Fig. 1C), fullerenes[18] or porphyrins[19] as templates to form pores of defined sizes with up to seven peptide monomers. The peptide of choice in these studies was alamethicin.[17,18,19b] These strategies led to insertion of well-defined structures in lipid membranes and to a 10- to 100-fold decrease in peptide concentration required for observing single-channel activity or for monitoring the release of the membrane-impermeant fluorophore calcein compared to the control experiment without template. Similar to the dimerization strategy, using small molecules as a template also improved the stability of the resulting pores: open-pore lifetimes increased from a few tens of milliseconds to a few seconds compared to pores formed by non-templated alamethicin monomers.[17,18,19b]

Instead of using small molecules as templates, Manfred Mutter's group developed a strategy based on biopolymers as templates, called Template-Assembled Synthetic Proteins (TASP)[20] (Fig. 1D). In these constructs, polypeptide chains covalently linked amphiphilic helices, such that the resulting structure formed a pore once inserted into a lipid membrane. The amino acid sequences of these helices were carefully constructed to optimize the helical content of the peptide and to tune their hydrophobic and hydrophilic sectors.[20] The TASP concept was further employed to template existing pore-forming peptides in order to improve their antimicrobial activity or to better understand their structural properties.[19a,21] For example, a four-helix bundle of a short segment from the L-type calcium channel was designed to resemble the calcium channels. Horst Vogel's group similarly templated four melittin monomers, achieving pore formation at concentrations 100 times lower than non-templated versions of the same peptide.[21f,22] Taken together, these studies allowed a better understanding of the structure and function of pore-forming peptides and improved their efficiency as potential antibiotic agents by increasing their antimicrobial activity compared to the native peptides. So far, these strategies however did not permit the formation of large structures comprising more than seven peptide monomers.[17]

3. Extension of the TASP Concept by Programmable Assembly of DNA Strands

Recently an expansion of the TASP concept emerged that takes advantage of oligonucleotide sequences rather than amino acid sequences to form large pores in order to extend the number of assembled peptides from seven[17] to more than twenty.[23] For instance, Henning-Knechtel *et al.* presented the use of DNA templates to assemble the natively heptameric α-hemolysin (αHL) protein into pores containing twelve, twenty or twenty-six monomers[23] (Fig. 2A). To do so, the authors attached single-stranded DNA (ssDNA) to K237C-αHL mutants using oligonucleotides that were activated with an N-ε-maleimidocaproyloxysulfosuccinimide ester. The resulting DNA-modified αHL monomers were mixed with DNA templates with a ratio of 5 αHL monomers for 1 binding site on the template. While previous studies templated the assembly of relatively small peptides that could often be produced by peptide synthesis, Henning-Knechtel *et al.* employed

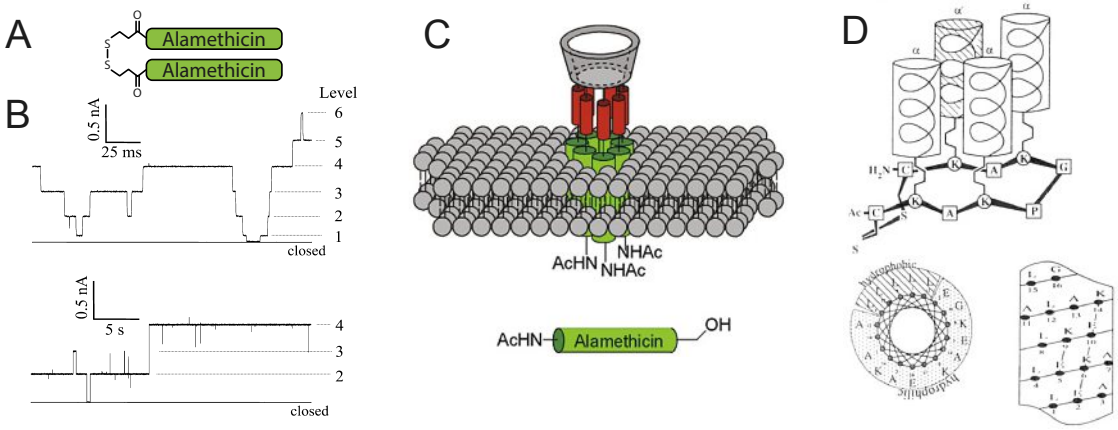

Fig. 1. Strategies for templating pore-forming peptides to pores in lipid membranes. (A) Simple dimerization of two alamethicin monomers *via* a disulfide bridge. (B) Comparison of current versus time recordings from alamethicin monomers (upper current trace) and dimers (lower trace). In the case of dimeric alamethicin peptides, long-lived pores were formed predominantly from even numbers of peptides. Reprinted with permission from ref. [11d]. (C) Templating up to 7 alamethicin peptides with cyclodextrin molecules. Reprinted with permission from ref. [17]. (D) Template-Assembled Synthetic Proteins consisting of membrane-inserting helices and an amino acid chain as template. Hydrophobic and hydrophilic residues were grouped in sectors as shown in the helical wheel. Reprinted with permission from ref. [20c].

the protein toxin αHL, which was recombinantly expressed and purified. In contrast, Spruijt *et al.* used a ring-shaped DNA scaffold to assemble stable pores of the octameric polysaccharide transporter Wza[10] (Fig. 2B). In this case the authors attached Wza peptides on both ends of amino-C6-functionalized ssDNA sequences consisting of four segments; two central segments to form the circular template and two outer segments connected to the peptides. Both studies arranged pore-forming proteins or peptides using nucleic acid templates, but the resulting pores behaved drastically differently. Scaffolding Wza monomers turned natively short-lived octameric pores into pores that remained in an open state for hours, while the size of the pores was limited to the octameric assembly. Assemblies with fewer or more than eight Wza monomers either were unstable or gave only the same conductance as the octamers.[10] In contrast, DNA-mediated scaffolding αHL, which intrinsically forms heptameric and permanent pores in lipid membranes apparantly made it possible to form assemblies of αHL with drastically increased diameter that remained stable after insertion in lipid membranes.[23] In both studies, the DNA templates were circular and consisted of segments with a length of 21 base pairs capable of hybridizing two monomers with covalently attached single-stranded DNA. While Spruijt *et al*. constructed the DNA scaffold structure from twelve different oligonucleotides that had previously been coupled to the peptide, Henning-Knechtel *et al.* employed a long circular template strand. In order to do so, they ligated multiple short oligonucleotides to form one long single-stranded template followed by a second ligation step to circularize the DNA strand.

Our group has been working on triggered formation of an artificial membrane attack complex by DNA-mediated assembly of pore-forming peptides to pores with programmable diameter since 2016 after this project received funding by the National Center of Competence in Research (NCCR) on Bio-Inspired Materials by the Swiss National Science Foundation (SNSF). In contrast to work by Henning-Knechtel *et al.* and Spruijt *et al.*, we explored two pore-forming peptides for DNA-mediated pore assembly: first the peptide MelP5,[24] which is a derivate of melittin[25] with five amino acid replacements and second, ceratotoxin A (CtxA), which is produced by the medfly *Ceratitis capitata* to protect its eggs against microbial attack.[26] We ultimately conducted most of our work with CtxA[27] as this peptide is intrinsically able to form pores by the barrel-stave mechanism from various numbers of monomers and therefore assembles to pores with various diameters, similar to alamethicin.[26c,d] CtxA and its modified version with a covalently attached single-stranded DNA are furthermore available from commercial sources through solid-phase peptide synthesis, avoiding tedious chemical modification and purification steps and enabling straightforward sequence optimization of the peptide if desired. So far, we have been able to trigger the assembly of pores with four different and predictable pore diameters. To do so, we added ssDNA templates with programmable numbers of hybridization sites to which CtxA peptides with covalently attached, complepepmentary ssDNA moieties could hybridize. Currently our efforts are focused on stabilizing the pores in lipid bilayer membranes and to increase the lifetime of the desired open state of the pores from milliseconds to minutes or hours. To do so, we are taking advantage of molecular dynamics simulations and are exploring various chemical modifications and strategies for anchoring the peptides in the membrane. In addition to these efforts, we are investigating the cytotoxicity of these DNA-templated pores to cancer cells. First results indicate a more than 10-fold increased cytotoxic effect of the templated structures compared to the native peptide at the same total peptide concentration. Future work will explore the potential of these designed pores for targeted drug delivery,[9] extraction of cellular components, targeted cell killing,[8,28] and resistive pulse sensing.[29]

4. Conclusion

Templated assembly of pore-forming peptides provides compelling opportunities to generate membrane pores with at least four advanced functionalities: First, enhancing the pore-forming and antibiotic activity at low total peptide concentration, which may be beneficial for reducing unwanted side effects. Second, incorporating moieties for targeting specific membranes such as those of pathogen or cancer cells. Third, designing pores whose internal diameters can be tuned in sub-nanometer increments by precise control of the number of peptide monomers in the templated assembly. And fourth, stabilizing these designer pores such that they exist only in one open state with defined ionic conductance and a stable, low noise ionic current through the pore for hours. The ability to generate nanopores with diameters that can be tuned to the size of analytes or cargo of interest would address one of the critical limitations of resistive pulse sensing experiments and would accelerate progress in the emerging field of nanopore-based single-molecule analytics.

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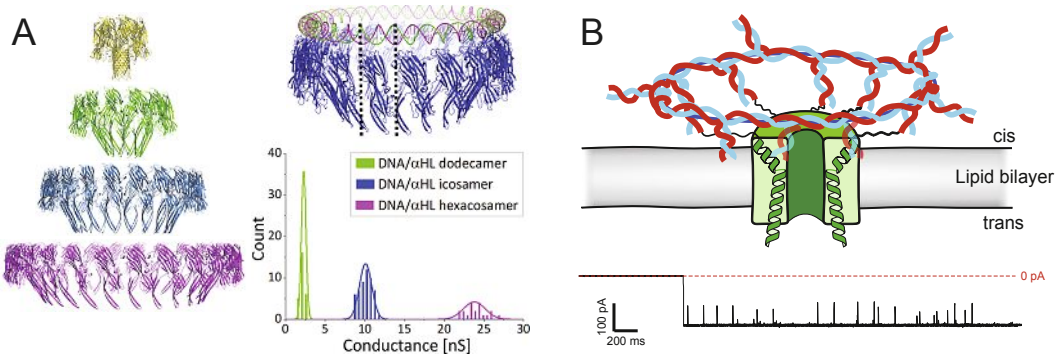

Fig. 2. DNA-mediated assembly of pore-forming peptides or proteins makes it possible to assemble pores with large diameter. (A) Templating of α-hemolysin to pores with defined numbers of monomers to various sizes as represented by distinct conductance populations in the event histogram. Reprinted with permission from ref. [23]. (B) DNA-mediated scaffolding of Wza peptide monomers dramatically increased the open-state lifetime of octameric pores. Reprinted with permission from ref. [10].

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