Diversified exploitation of aerolysin nanopore in single-molecule sensing and protein sequencing

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Abstract
Nanopore electrochemistry has emerged as a promising technology to achieve the real-time investigation of nanoscale processes at the single-molecule level in a label-free manner. To further extend the single-molecule sensing versatility of nanopore single-biomolecule interface, mutant aerolysin nanopore is developed followed by exploring the sensing mechanism. The exquisite design enables the site-directed controlling of every amino acid along the aerolysin single-biomolecule interface with the precision of chemical group. Owing to diversified pore-analyte weak interactions between chemical groups (e.g., electrostatic interactions, hydrogen bond, van der Waals’ force) and steric confined effect, the mutant aerolysin displays extraordinarily high sensitivity and selectivity toward individual analytes in the stochastic sensing process. These well-designed nanopores open more opportunities for biophysical, diagnostic applications, and even emerge as promising candidates for single-molecule protein sequencing.

KEYWORDS
mutant aerolysin, nanopore electrochemistry, single-biomolecule interface, single-molecule protein sequencing

Sensing and characterization of individual biomolecules (e.g., nucleic acid, peptides, proteins) are of vital importance in all kinds of bioapplications, because they offer an opportunity to reveal the intrinsic properties and behaviors of single biomolecules that are closely related to the development of various diseases. Among various analytical technologies, nanopore has been well established as a facile tool for in-situ single-molecule analysis in a label-free manner; it begins in earnest as a technology for the next-generation DNA sequencing. For the past few years, a great deal of efforts has been made in expanding the versatility of nanopore; the applications of nanopore in single-molecule sensing beyond sequencing are booming, such as the detection of genetic, proteomic, and epigenetic biomarkers, the evaluation of enzyme activity, as well as the discovery of hidden bio-intermediates. In principle, nanopore sensing is performed by threading target molecules through the nanoscale volume and resolving the electrical perturbations in a current flow through the pore.

Here, we focus on aerolysin biological nanopore, one promising and well-characterized nanopore, which is...
Mutant aerolysin nanopore. Critical sensing regions of aerolysin single-biomolecule interface (left); possible mutation sites at pore-forming, selectivity (middle), and sensitivity (right) regions. Copyright 2020, Springer.

deemed as a single-biomolecule interface with a well-defined confined space. Aerolysin, a β-pore-forming toxin, is first determined and named by Bernheimer and Avigad in 1974. Within a few years, its expression, secretion, purification, and biological properties are successively reported. Aerolysin is then reported as a pore-forming toxin, which can self-assemble to a heptameric structure in lipid bilayer. In the process, an inactive pro-aerolysin is first expressed and purified as the precursor of aerolysin. To achieve the activation of oligomerization, approximately 25 amino acids from the C-terminal of the pro-aerolysin sequence are removed by treating with trypsin. Subsequently, the toxin protein will undergo the oligomerization to form aerolysin heptameric pore. As shown in Figure 1, this heptameric pore is in a mushroom-like structure with a β-barrel stem inserted into the lipid bilayer. The first example of wild-type (WT) aerolysin nanopore on the biomolecule sensing is the determination of α-helix collagens in 2006. Several study are then reported that aerolysin is applied to the studies of transport dynamics of unfolded proteins and identification of polymer size. Until 2016, it is amazing that WT aerolysin is proposed as an ultrasensitive sensor and applied in the direct discrimination of short oligonucleotides with different lengths (2-10 bases) from the mixtures. The unexpected sensing capability of aerolysin is further extended to the real-time monitoring and investigation of stepwise cleavage of oligonucleotides induced by exonuclease I.

As shown in Figure 1, there are two factors contributing to the excellent sensing performance of the aerolysin nanopore to oligonucleotides. The first underlying factor is that the positively charged amino acids locating at cis entrance and trans exits form two unique sensing regions of aerolysin sensing interface, which are around R220 and K238, respectively. The exits of these two sensing regions enrich the single-molecule pore-analyte weak interactions between the single-analyte molecules and the sensing interface including electrostatic force, hydrogen bond (H-bond), and van der Waal forces. The other one is a unique three-dimensional nanoconfinement. In detail, the aerolysin sensor offers a sensing space with the narrowest constriction of approximately 1 nm in diameter. This confined space, thus, can not only accommodate a single molecule, but also enhance the weak interactions via the electrochemically nanoconfinement. As the individual molecules of interest pass through the three-dimensional sensing region of aerolysin, electrochemical confined effects will effectively transfer the properties of single analytes to distinguishable ionic current via the magnified pore-analyte interactions without labeling. Taking advantage of electrochemical confined effect, aerolysin is gradually applied in the measurements of individual disease-related biomolecules via electric read-outs.

The ability to sense targets with high sensitivity and selectivity lies at the heart of biochemical sensor’s performance. With the assistance of electric filed, an aerolysin nanopore can use its confined space to capture and accommodate individual molecules. The current feedbacks respond to the movements of molecules through the inner channel of aerolysin. However, a central question arises: how can aerolysin single-biomolecule interface feature a higher specificity and how to enhance the sensitivity of ionic current responses to the molecules of interest? Our group first indicates the sensing regions inside aerolysin, guiding the rational design of mutant aerolysin nanopore with atomic precision. We design the mutant aerolysin to rapidly modulate the functional aerolysin sensing interface at the chemical group level, marking an important step toward the enhanced sensing performance of aerolysin.
As shown in Figure 1, the combination of nanopore experiments and molecular dynamics simulation determines the critical sensing regions, including pore-forming region, selectivity region, and sensitivity region. The biological technique is subsequently performed to achieve the site-directed mutagenesis of specific amino acid groups at three sensing regions on the aerolysin single-biomolecule interface, effectively tailoring the pore-analyte weak interactions and the steric confined effect of pore itself.\textsuperscript{27}

According to the research, the pore-forming process is largely determined by the glutamine-212 (Q212), because the substitution of Q212 with positively charged arginine restricts the assembly of the aerolysin monomers. To value whether the site-directed mutagenesis method is efficient in nanopore-based sensing, the negatively charged glutamic involved instead of positively charged R220, which situates at the entrance of the aerolysin.\textsuperscript{28} The mutagenesis exerts an electrostatic repulsion between the sensing interface and Poly(dA)\textsubscript{4}, hindering the entrance of oligonucleotides to the aerolysin nanopore in an effective way. As a result, the presence of negatively charged oligonucleotides hardly results in ion current read-outs in R220E mutant aerolysin system, achieving high selectivity toward oligonucleotides. The results suggest that the variations in weak interactions between R220 region and analytes dominate the direct discrimination ability of aerolysin, subsequently achieving high selectivity toward targets.

Prolonging the residence time of molecules within the sensing region would produce the narrow ionic current distribution of the target molecules, which therefore leads to the improvement of sensitivity of nanopore sensors. Similarly, the positively charged K238, one of the significant sensing spots at the $\beta$-barrel region, is designedly replaced with glutamic residue. Accordingly, the K238E aerolysin is capable of effectively governing the translocation speed of oligonucleotides via pore-analyte electrostatic interactions, making duration time three times longer than that of WT aerolysin.\textsuperscript{28} To further probe the key factors determining the sensitivity of aerolysin, K238 is latterly replaced by amino acids with different size and charge (Figure 2A).\textsuperscript{29} It
is found that the blockage level generally depends on the size of residues, whereas the chemical properties of residues obviously affect the duration time. Besides, a recent study shows that the pore-analyte H-bond also has an effect on the translocation behaviors of oligonucleotides. To verify the influence of hydrogen bond on the nanopore sensing process, K238 is mutated to K238C that features sulfhydryl. The mutagenesis surprisingly facilitates six times longer translocation time of oligonucleotides. K238C surprisingly facilitates six times longer translocation time of poly(dA)₄ than that of WT aerolysin. These researches indicate the precise design and mutagenesis to command the pore-analyte weak interactions and geometrical structure of pore availability to ensure the enhanced sensing ability of mutant aerolysin sensing interface. Moreover, the mutant aerolysin sensors are found that always display a long period of stability under the electric field.

Diverse aerolysin mutants are being developed and widely applied in the single-biomolecule measurements owing to their superior sensing performance including excellent sensitivity, selectivity, and stability. With the attempt to realize the direct identification of DNA lesions that is closely associated with genetic diseases, a K238Q from aerolysin is designed with the prolonged residence time for oligonucleotides by two orders of magnitudes. This mutant aerolysin can directly convert the minor chemical structural difference in lesions, including methylcytosine (mC), 8-OXO-guanine (OG), or inosine (I), into distinguishable ionic current feedbacks (Figure 2B). By employing the powerful mutant K238Q aerolysin, one can not only figure out at least three DNA lesions but also quantify the lesions in a mixed sample without labeling via strong noncovalent interactions rather than the steric effect. The critical sensing region of K238Q aerolysin displays more negative charge by replacing the positively charged arginine residue with neutral glutamine residue. In addition, the uncharged glutamine has stronger noncovalent interactions (hydrogen bond and van der Waals force) with nucleotide base. Thus, the limited resolution of WT aerolysin to DNA lesions sensing can be effectively enhanced within K238Q aerolysin via multiplex pore-analyte weak interactions. The focus is then turned to the detection of amino acids as there is a larger demand for the protein analysis rather than the DNA sequencing. The temporal/spatial resolution of mutant aerolysin is sufficiently high to permit the determination of amino acids with the resolution of one chemical group. The power of mutant aerolysin is harnessed to achieve the effective discrimination of cysteine (Cys) and homocysteine (Hcy) in the mixture. In this approach, a powerful K238Q sensor and 50-benzaldehyde poly(dA)₄ (AHA₄) probes are designed and engineered. The strong pore-analyte weak interactions hinder the translocation of AHA₄, enhancing the sensitivity for the identification of Cys and Hcy only with a difference in methylene group. Recently, a label-free screening technique based on T232K/K238Q aerolysin is further developed to observe the phosphorylation of single tau fragments. The enhanced pore-analyte electrostatic interactions at T232K site and efficient repelling barrier for targets at K238Q site allow the low translocation speed of phosphorylated tau peptides through the pore with hundreds of millions of seconds at maximum. After the well-designed mutagenesis, T232K/K238Q effectively discriminates the multiple and adjacent phosphorylation sites in tau fragments with nearly 100% accuracy, whereas the WT aerolysin has limited sensitivity for the accurate differentiation. As shown in Figure 2C, the distinguishable distribution of characteristic events demonstrates the versatility and power of T232K/K238Q aerolysin to scrutinize a posttranslocation modification of single amino acids. In addition, it is possible for aerolysin to be used to monitor the single-molecule reactions. K238C aerolysin is used as an efficient nanoreactor to carry out chemical bonds making and breaking. Cysteine residue is introduced to substitute the lysine at the position of 238 in the nanofield; the presence of cysteine enables the disulfide bonds making and breaking between mutant aerolysin and 5,5’-dithiobis-(2-nitrobenzoic acid). Subsequently, the kinetic processes of bonds making and breaking in the nanoreactor are visualized and analyzed by the ionic current oscillation. The engineered aerolysin nanopore is also challenging and important for biophysics field, several promising mutant aerolysin nanopores for single molecule sensing have been presented at the last two Annual Meetings of the Biophysical Society. Inspired by the site-directed mutagenesis of aerolysin single-biomolecule interface, we have been performing a series mutant experiments to test the structure-function relationship of aerolysin nanopore. While these studies were underway in our group, a conceptually similar result was published.

Mutant aerolysin nanopore marks a vital step toward single-molecule sensing with biological nanopore. The well-designed modification with chemical group precision tunes the pore-analyte weak interactions and geometrical size of aerolysin sensing interface, ensuring controllable and higher resolution toward single-molecule measurements. Although the site-directed mutagenesis is impressive and is an important step in the rational design of advanced sensors, many challenges lie ahead in engineered aerolysin. Much of the focus on mutant aerolysin has been on the modifications of natural amino acid, it could also anticipate the introduction of unnatural amino acids into aerolysin pores. The projection of unnatural
amino acids into the confined space of aerolysin will offer a powerful platform for multiplex biosensing and the analysis of chemical reaction, because the presence of unnatural amino acids can enrich pore-analyte interactions (non-covalent interactions and covalent interactions) by diverse chemical groups such as aldehyde, alkynyl, azide group, and so forth.

The mutant aerolysin is desired to couple with other synthetic nanopore such as nanopipette. The unique combination makes aerolysin sensing interface ready to exist in a robust environment, permitting the possibility to achieve in-situ selective sensing in real biosystems, such as single living cells. These modified pores can be also readily elaborated into nanopore arrays chip with different kinds of mutant aerolysin nanopores. The nanopore arrays chip can be termed as “nanoporelectrics” and further scale up the number of measuring sensors, and accordingly contribute to high-throughput and multiplex single-biomolecule measurements. For instance, such a powerful tool could be developed into efficient drug screening system and might be applied in in-situ point-of-care testing in a fast and accurate manner one day.

The next frontier in nanopore-based single-molecule sensing might be protein sequencing; the direct single-molecule protein discrimination based on biological nanopore still faces lots of hurdles. Threading proteins through the nanopore are restricted by the diversities of charge distribution of residues and amino acids. Disruption of the secondary and tertiary structures of protein also needs to be resolved in the sequencing process. Moreover, the precise control of the translocation speed of proteins is necessary for protein sequencing. A recent study seemingly provides a route to bring protein sequencing via aerolysin nanopore close to fruition; it estimates the residential time of a peptide with 8 amino acids can be slowed down to 200 ms. Given the merits of mutant aerolysin mentioned above, the mutant aerolysin array is expected to be exploited to a pocket-sized protein sequencing device. Every aerolysin sensors in aerolysin array can be precisely engineered to better control the repelling barrier and translocation of proteins by tuning pore-analyte interactions according to the properties of proteins (surface charge, geometric structures, polarity, etc). Last but not the least, to meet the requirement in dynamic single-molecule characterization with high temporal-spatial resolution, advanced data mining algorithm and instrument should be available. Look ahead, the appearance of mutant aerolysin nanopore provides more access to disease diagnosis, biomedicine, and protein sequencing.

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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