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RESEARCH PAPER

A Single-molecule Mycobacterium Smegmatis Porin A Protein Nanopore Sensor for Host-Guest Chemistry

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Abstract: The mycobacterium smegmatis porin A (MspA) protein nanopore was prepared by *E. coil* prokaryotic expression system. The extraction of MspA nanopore using surfactants were carried out under the optimal conditions such as 0.5% extraction agent, 30 min of extraction and at 90 ºC. The interactions of MspA nanopore with a variety of cyclodextrins were examined at single-molecule level. The interaction between MspA and Per-6-amino-β-cyclodextrin (am_7-β-CD) was stronger and had consistent blocking platform. Its retention time in the MspA nanopore decreased with the increasing voltage, whereas the blocking current was not affected by the voltage. So, am_7-β-CD was selected as a non-covalent adapter and lodged within single MspA nanopore to study the single-molecule host-guest chemistry. After adding am_7-β-CD to cis side and displaying a first blocked platform, a low concentration of 50 nM for the amantadine hydrochloride could show a second blocked platform and its residence time decreased with the increasing voltage. This new protein nanopore sensor can be used as a single-molecule detector and identifier for small organic molecules, which will greatly broaden the application range of MspA nanopore.

Key Words: Mycobacterium smegmatis porin A; Nanopore; Single-molecule detection; Cyclodextrin; 1-Amantadine hydrochloride

1 Introduction

Protein nanopore-based analytical technology combines the nanotechnology, biotechnology and single-molecule detection technology, has developed as the simplest and cheapest single-molecule tool with high sensitivity. In stochastic sensing application, α-hemolysin (α-HL) protein is the mostly commonly used model nanopore. It is a mushroom-like heptametrical transmembrane pore assembled from seven monomers and each monomer contains 293 amino acids. The length of α-HL nanopore is approximately 10 nm, with a cis side opening of 2.6 nm in diameter and a vestibule of 4.6 nm in diameter. The constriction of the nanopore is 1.5 nm at the vestibule-transmembrane domain junction, and the transmembrane β-barrel is about 5 nm in length and 2 nm in diameter[1]. α-HL sensor has been widely applied in different fields including peptides/proteins and biomolecular complex[2–14]. The detection of small organic molecules were usually achieved by logging cyclodextrin (CD) adapters within the α-HL nanopore. As is well known, the study on host-gust chemistry of CDs has a history over one hundred, and thousands of organic molecules can be captured into the cavity of CDs through non-covalent hydrophobic interactions. However, the CDs/α-HL system could only identify and detect very limited organic molecules[4,5,15–17]. This is mainly due to the pore shape, spatial size and charge distribution within the pore, which could limit the access of organic molecules. Therefore, it is particularly necessary to develop new protein-based nanopore sensor owning different shapes and properties.

Mycobacterium smegmatis porin A (MspA) is the main component in the outer membrane of mycobacterium smegmatis cells. It is revealed to be a homooctameric goblet-like conformation with a single central channel containing eight monomeric proteins with a molecular weight of 20 kDa[19].

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MspA has a short and narrow channel constriction, with a diameter of about 1.0 nm and a length of about 0.5 nm. This very short β barrel and the goblet shape structure are very suitable to prepare nanopore molecule devices, with a smaller effect of space and charge[19]. MspA has superior thermal and chemical stability. It is very robust and retains channel-forming activity at pH 0–14 after extraction for 30 min at 100 °C and further incubation for 15 min at 80 °C in the presence of 2% SDS[20]. MspA is also ideal for engineering via site-directed mutagenesis. These characteristics suggest that MspA is an excellent nanopore for analytical applications[19,21].

Currently, MspA protein nanopore analyses mainly focus on the DNA sequencing[19,21–24], and there is few reports on the application in host-guest chemistry and small organic molecules detection. In this study, we employed MspA nanopore as the sensing element to develop a new nanopore sensor. Firstly, we explored the interaction between MspA nanopore and per-6-amino-β-cyclodextrin (am$_7$-β-CD), and further investigated individual am$_7$-β-CD as a host molecule to capture and hence sense organic drug molecule. This study is important to understand the biophysical characteristics of MspA nanopore, and develop its potential applications in host-guest chemistry and small organic molecule detection. It may help to expand the application range of nanopore sensor in single-molecule detection.

2 Experimental

2.1 Instruments and reagents

Patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA), function generator (BK Precision, 4040A, USA) and 1440A A/D digital converter (Axon Instruments, USA) were used to record the nanopore current signals. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids Company (USA). Pentane was purchased from Honeywell Burdick & Jackson Company. Hexadecane (> 99%), analytical pure reagents such as α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), carboxymethyl-β-cyclodextrin (CM-β-CD), per-6-sulfo-β-cyclodextrin (S$_7$-β-CD), per-6-amino-β-cyclodextrin (am$_7$-β-CD), per-6-amino-γ-cyclodextrin(per-6-NH_3Cl-γ-CD), per-6-quaternary-β-cyclodextrin (per-6-$N^+(CH_3)_3$-β-CD) and 1-amantadine hydrochloride (1-AdNH_2·HCl) were all purchased from Sigma-Aldrich (USA). All aqueous solutions were prepared with ultrapure water (18.25 MΩ cm).

2.2 Preparation of MspA protein nanopore

The MspA nanopore was prepared by the *in vivo* expression method in *E. coil.* BL21 (DE3) pLysS. The pT7 (α-HL) plasmid was used as the template to give pT7 empty plasmid by double digestion enzyme reaction with HindIII and NdeI enzymes, then the MspA synthetic gene was ligated into this pT7 empty plasmid by ligase reaction to obtain the pT7 (MspA) recombinant plasmid. This recombinant plasmid was transformed into *E. coil* BL-21 (DE3) pLysS strain, and the host bacteria were cultured under suitable conditions at 37 °C. When the bacteria were in the logarithmic phase, the MspA protein in the bacteria was highly expressed by inducing the MspA protein with isopropyl thiogalactoside (IPTG). Based on the super stability of MspA protein, MspA protein was selectively extracted by Triton-100/PBS buffer at high temperature and purified by gel purification method. The channel parameters and pore-forming activity were verified by patch-clamp amplifier.

2.3 Experiment method

Planar lipid bilayer membranes of DPhPC were formed according to the previous literature method[9]. The supporting electrolyte was 1 M KCl-10 mM Tris buffer (pH 9.0). The potential was applied form the cis chamber with two freshly prepared Ag/AgCl electrodes in 1.5% agarose saturated with 1 M KCl. The MspA protein was added to the cis chamber, which was connected to "ground". Once the successful insertion of a single MspA pore occurred, the cyclodextrin molecules and guest molecules were added to the transchamber. The signal was filtered with a low-pass Bessel filter set at 5 kHz and sampled at a frequency of 20 kHz. Data were analyzed with pClamp 10.3 software (Axon Instuments) and Origin 8.5 software.

3 Results and discussion

3.1 Cyclodextrin adapter selection

MspA protein nanopore got a good expression in *E. coil* BL21 (DE3) pLysS under the optimal extraction conditions such as an agent concentration of 0.5%, and a extraction time of 30 min at 90 ºC. Patch-clamp experiments showed that the channel could be quickly formed when adding 1–2 μL of MspA protein with a 1000-fold dilution at *cis* side after the formation of lipid bilayer. What's more, the formation of multi-channel proved that MspA protein nanopore had good channel activity (Fig.1a). In 1M KCl-10 mM Tris-HCl buffer solution, the single-channel current was I = (180 ± 5) pA at + 40 mV voltage and I = (–169 ± 4) pA at –40 mV voltage. Channel current value increased with increase of voltage linearly.

The linearity between the current and the voltage could be expressed as follows: $I = 4.4395V_{DC} - 9.3$, $R^2 = 0.999$ at positive voltage and $I = 3.4165V_{DC} - 11.9$, $R^2 = 0.998$ at negative voltage, which are consistent with the channel parameters related to the literature (Fig.1b). Those results