SINGLE-STRANDED DNA DETECTION VIA CHEMICALLY MODIFIED ALAMETHICIN NANOPORE AT SINGLE MOLECULE LEVEL

R. Kawano1, D. Noshiro2, T. Osaki1,3, K. Kamiya1, K. Asami2, S. Futaki2, and S. Takeuchi1,3

1Kanagawa Academy of Science and Technology, 2Kyoto University, 3Institute of Industrial Science, The University of Tokyo, JAPAN

ABSTRACT

This paper describes the electrical detection of single-stranded DNA (ssDNA) at the single molecule level using an engineered alamethicin (Alm) channel embedded in bilayer lipid membranes (BLMs). Nanopore sequencing has the potential to become a direct, fast and inexpensive DNA sequencing methodology (Fig. 1). We propose here the chemically covalent dimmers of Alm (di-Alm) in which monomers were linked at their N-terminal ends and the di-Alm forms mainly the 6-mers (1.3 nm dia.). In addition, the conductance states were stabilized with lifetimes up to 200-fold longer than the same states observed with monomers. Using the di-Alm nanopore, we can observe the slow ssDNA translocation and this finding highlight the importance of the di-Alm in the future of nanopore sequencing.

KEYWORDS: Droplet contact method (DCM), Alamethicin, DNA Detection

INTRODUCTION

A nanopore-based device provides single-molecule detection and analytical capabilities that are achieved by electrophoretically driving molecules in solution through a nano-scale pore.[1-3] When a small (~100 mV) voltage bias is imposed across a nanopore in a membrane separating two chambers containing aqueous electrolytes, the resulting ionic current through the pore can be measured with standard electrophysiological techniques. α-hemolysin(αHL) has performed for the ssDNA detection in the most studies as the biological nanopore. Especially, analyzing the fluctuations in ionic current as single-strand DNA (ssDNA) or RNA translocate through an αHL ion channel, reconstituted in an electrically insulating lipid bilayer, is being actively investigated as an inexpensive means to determining the nucleic acid sequence.

Ideally, the current vs time trace recorded during the translocation of an individual ssDNA molecule will exhibit four distinct levels, each level corresponding to one of the four bases (adenine (A), thymine (T), guanine (G), and cytosine (C)). As the biopolymer translocates the channel, the electrical readout of the four current levels provides the nucleotide sequence. However, the small difference in blockade currents associated with the different nucleotides (<10%) coupled with the high translocation velocity of ssDNA at typical electrophoretic driving forces (100 mV) has prevented single-based identification using present state-of-the-art electronics. ssDNA or RNA translocation though the pore occurs at 2 to 20 μs per base at room temperature, limiting the use of averaging methods to improve the electrical signal-to-noise ratio.[4, 5] Since the αHL pore is slightly oversized to ssDNA (ca. 1 nm dia.), the translocation time is too short to discriminate the single nucleotides. Therefore, we have considered that other channel-forming peptides, alamethicin (Alm), will be one of the precise candidate for the ssDNA detection because of the 6-mer of Alm provides 1.3 nm (in diameter) pore.

Alm is a well-studied channel forming peptide of 20 residues, which forms voltage-gated ion channels in lipid bilayers. The multi-conductance behavior in the single-channel recordings is interpreted in terms of the helix-bundle model, which is a basic design for the pore region of intrinsic ion channels. Because of the structural resemblance to intrinsic

---

Figure 1 Conceptual illustration of single DNA detection using biologically engineered nanopore embedded in membrane lipid bilayers (BLMs). a) The schematic diagram of the ssDNA translocation via the engineered alamethicin (Alm) nanopore. b) Wild-type Alm nanopore has the equilibrium pore states and gives the multiple pore open conductance. c) The Alm dimer forms the rigid pore states and gives the long-lived open conductance.
ion channels as well as the simple sequence, Alm has provided a good model channel to study the structure-function relationships. Hence, effects of structural modifications in Alm on the channel properties have been extensively studied, and various channel forming peptides based on Alm have been designed to mimic the function of intrinsic ion channels, such as ion-selectivity, gating and sensing. Although the Alm pore will be thought to be suitable for the ssDNA detection, the pore state of Alm cannot be maintained for the enough time, at least more than several sec, because the wild-typed Alm shows the short (<500 ms) and multiple (4, 5, 6, 7, 8-mars) open pore states (Fig. 1b). In this study, we proposed the chemically modified Alm which monomers are connected each other forming Alm dimer (di-Alm) for ssDNA detection (Fig. 1c).

**EXPERIMENTAL**

**Peptide synthesis.**

Di-Alm was synthesized by a solid-phase technique with Fmoc amino acid fluorides. The details were described in a previous paper.[6] After completion of the assemblage of amino acids on the resin, the N-terminal Fmoc-group was deprotected and the resin was separated into two portions. One portion of the resin was treated with acetic anhydride to protect the N-termini of the peptides with acetyl group and then the peptides were cleaved from the resin to obtain Alm. Peptides on the other portion of the resin were further condensed with S-acetamidomethyl-β-mercaptopropionic acid purchased from Peptide International (Louisville, KY) and then were cleaved from the resin. This peptide (acm-Alm) was purified by a gelchromatography and a reverse-phase HPLC. Treatment of the purified the acm-Alm with iodine removed the acm-group and made a disulfide linkage of the peptides to obtain di-Alm. The di-Alm was purified by gelchromatography. The purified di-Alm were characterized by electrospray ionization mass spectroscopy (ESI-MS).

**Channel current recordings.**

Planar lipid bilayer was prepared by the droplet contact method on the parylene-double well chip (DCM, Fig. 2a and b) as we reported previously.[7, 8] The channel current signals were detected with a 1 kHz low-pass filter at a sampling frequency of 5 kHz (unless otherwise noted) at 23 ± 1°C. Current analysis was performed using pCLAMP ver. 10.6 (Molecular Devices, Sunnyvale) and Igor Pro 6.2 (Wavemetrics, Oregon).
RESULTS AND DISCUSSIONS

The two Alm monomers were linked by their N-terminal ends using a disulfide bonding. Channel current recordings were performed by droplet contact method as shown in Fig. 2a and b. As the result, the long-lived open time was observed and even number of monomer assembly state was also observed in the current-time trace (Fig. 2c-f). Next, we tried to obtain the ssDNA translocation via the di-Alm nanopore. Fig. 3 shows the translocation signals of the adenine homo polymer (50-mer, dA50) through the di-Alm pores. The dwell time was around 0.4 ms under applied 100 mV voltage (Fig. 3b). The dwell time and the translocation event frequency had strong voltage dependency. The duration was longer than that of the using αHL pore.

CONCLUSION

Using di-Alm nanopore, we can observe the slow ssDNA translocation and this finding highlight the importance of di-Alm in the future of nanopore sequencing.

ACKNOWLEDGEMENTS

We thank Yoshimi Nozaki, Yumi Kagamihara, Utae Nose, and Maiko Uchida for technical assistance in device preparation and arranging the experimental environment. This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 25107736), Challenging Exploratory Research (No. 24655072), Young Scientists (A) (No. 25708024), and Scientific Research (A) (No. 25246071) from MEXT (KAKENHI) in Japan.

REFERENCES


CONTACT

*R. Kawano, tel: +81-44-819-2037; rjkawano@iis.u-tokyo.ac.jp