A SINGLE MOLECULE DETECTION OF PROTEIN BY APPROPRIATE SIZED BIOLOGICAL NANOPORES

Hirokazu Watanabe and Ryuji Kawano

Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, JAPAN

ABSTRACT

This paper describes the single molecule detection of a protein (granzyme B, ~5 nm in dia.) using human perforin (3~20 nm in dia.) nanopores. Nanopore sensing has already implemented extensive biochemical measurement at single molecule level, such as DNA sequencing and estimating the molecular weight of water-soluble polymers. Although α-hemolysin (1.4 nm in dia.) has been mainly used as the nanopore, the analytes that size is larger than α-hemolysin pore cannot be measured using α-hemolysin. To address this issue, we reconstituted larger sized nanopores by human perforin in bilayer lipid membranes and detected an apoptosis-inducing serine protease, granzyme B at single molecule level.

KEYWORDS: Bilayer lipid membranes, Nanopore, A single molecule detection

INTRODUCTION

Cell membranes separate the inner components of a cell from outer environment, and various types of membrane proteins exist in cell membranes. A part of these membrane proteins form nano-scale pores in the cell membranes, and they control transportation of substrates between inside and outside the cells. Besides, the nano-scale pores are utilized in the field of biosensing, which is well-known as the nanopore sensing [1]. The nanopore sensing is a strong tool for a single molecule analysis of the translocating molecules through a nanopore, for example, sequencing of a single-stranded DNA and estimating the molecular weight of polyethylene glycol (PEG) [2]. An α-hemolysin (αHL) is used in most of previous studies as biological nanopore because the size of DNA and PEG are match with pore diameter of αHL [3]. In this sensing, reconstituting αHL in bilayer lipid membranes (BLMs) separating two chambers filled with buffered electrolyte and applying a voltage across the BLMs, we can detect passing ions through pore as channel current. Moreover, when the comparable sized molecule translocate the pore, they cause blocking of ion passing, and we can examine the molecular volume and translocating velocity from current reduction. Although much attention has been paid to nanopore sensing because of potential for versatile application, it has a problem that only a few type of nanopores has been used and diameter is restricted only near 1 nm. Hence, we cannot detect the larger molecules than αHL because they are enable to translocate the nanopore. In this study, we used human perforin (PFN) which is the protein produced by killer T cell and forms nanopores on infected cells in human immune system. We believe that PFN nanopores enable to detect various-sized target molecules by the nanopore sensing.

EXPERIMENTAL

BLMs were prepared by "droplet contact method" using a device fabricated with microfabrication technology as shown in Fig. 1A. In this method, two lipid monolayers contacted and formed BLMs on the micropore of a thin hydrophobic film (paryleneC) shown in Fig. 1B. 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) were from Avanti Polar Lipids. First, 2.3 μl of DPhPC /n-decane (10 mg/mL) solution was dropped in each chamber. Next, 4.7 μl of the buffer solution was dropped into both chambers. Because it is reported that PFN form nanopores in the presence of Ca^{2+}, we prepared buffer solution consisted of 140 mM KCl, 0.1 mM $CaCl_2$, 10 mM HEPES (pH 7). PFN was added at 1 nM concentrations to *cis* side only (the *cis* side was where the potential of +100 mV was applied and the *trans* side was grounded). Then, two lipid monolayers contacted and formed BLMs and pore-forming proteins were reconstituted in the BLMs. Reconstituted proteins allowed ions to pass through nanopores under the voltage gradient and we obtained the channel current signals. Ionic current through nanopores was measured using a Pico Patch Clamp Amplifier (Tecella, USA) connected to a Ag/AgCl electrode in

each chamber and an applied constant voltage of 100 mV. We calculated pore diameter from channel current [4] and estimated the number of monomers composing the nanopore. Moreover, to detect proteins by PFN nanopore, we added granzyme B (~5 nm, GZB) at 1 μM concentrations to *cis* side and applied +100 mV between two chambers. GZB is serine protease inducing apoptosis of target cells in human immune system, and the size is about 5 nm in diameter. Then, we measured channel current signals to compare the signals of with or without GZB.

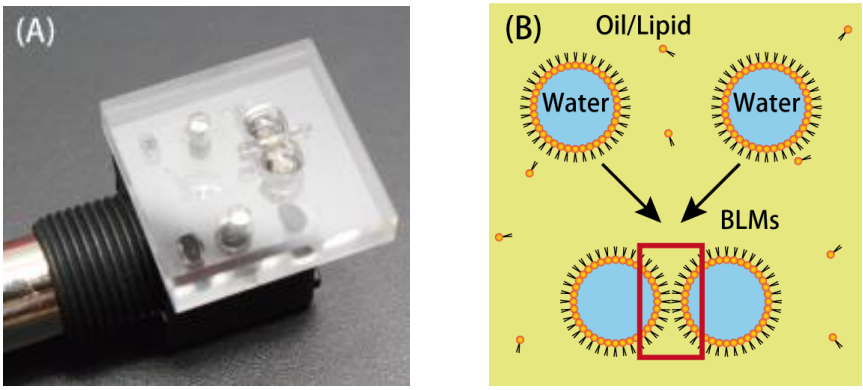

Figure 1: (A) The photograph of a device for preparing BLMs. (B) BLMs were prepared by droplet contact method. Two lipid monolayers contact together and form lipid bilayer.

RESULTS AND DISCUSSION

As the result of the channel recordings, we observed current increase at various levels of which conductance were from 0.1 to 6 nS. From the conductance, we calculated that PFN pore diameter as from 3 to 20 nm. Therefore, we considered PFN formed heterogeneous sized pores by the oligomerization, and estimated the number of assembling monomers. To calculate the pore area and the number of monomers, we assumed the area of pore formed by n-mers as that of regular n-sided polygon, and derived the formula for estimating the number of monomers. As the result, we concluded PFN formed variable-sized pores of which diameter are from 3 to 20 nm by from 4-mer to 22-mer. Next, we tried to detect a GZB, which is serine protease and induces apoptosis of target cells. GZB added into *cis* chamber translocated through PFN, and this event resulted a spike-like current blocking. The analysis of this blocking events revealed that translocation time and molecular volume of GZB from duration time and blocking rate. Using this method, we can analyze the behaviors of larger protein than αHL nanopores by PFN nanopores. We believe that extensive sized PFN pore enable to detect and analyze targeting-sized molecule, and this technique is applicable to numerous proteins such as diagnostic marker and protein cause food poisoning.

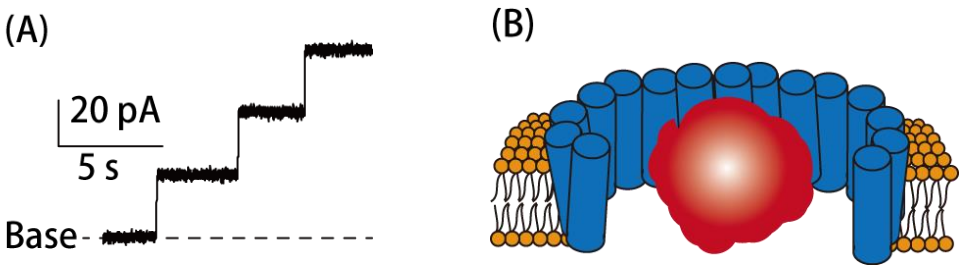

Figure 2: (A) The typical current and time trace of the PFN nanopore at a voltage of +100mV. Reconstituted PFN in BLMs formed nanopores and allowed ions to pass through nanopores under the voltage gradient. (B) GZB added into trans chamber translocate through PFN nanopore from cis side to trans side.

CONCLUSION

In order to detect larger molecule than αHL nanopore, we reconstituted PFN in BLMs and we evaluated the properties of PFN nanopores. PFN formed heterogeneous sized pores which diameter are larger than αHL nanopore. Moreover, we detected larger protein than αHL by PFN nanopore, and analyzed the behaviors of protein molecules at single molecule level. We believe that nanopore sensing by PFN is applicable to numerous proteins such as diagnostic marker and protein cause food poisoning.

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CONTACT

Hirokazu Watanabe; e-mail: 50015641133@st.tuat.ac.jp