Investigation of Self-assembled Protein Dimers through an Artificial Ion Channel for DNA Sensing

Siqi Zhang[a,b], Ting Sun[a], Erkang Wang [b], Jiahai Wang[a,b*]

a College of Science, Northeastern University, Shenyang, 110819, China

b State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Science, Changchun, Jilin, 130022, China.

*Corresponding author

E-mail: jhwang@ciac.jl.cn

Tel.: +86-0431-85262334

Homepage: http://nanopore.weebly.com

Abstract

Artifical nanopores have become promising tools for sensing DNA instead of their biological counterparts. Here we report a new technique to sense DNA through a conical-shaped nanopore embedded in track-etched PET membrane. Two streptavidin conjugated monovalent DNA probes based on formation of biotin and streptavidin moncovalent bond can bind two distinct segments of target DNA. The size of target DNA linked two components is double that of each streptavidin conjugated monovalent DNA probe. By precisely controlling the tip diameter of conical nanopore embedded in PET polymer, the events due to translocation of the streptravidin conjugated monovalent DNA probes through the nanopore can be filtered and undetected on purpose; the current-pulses due to translocation of the target DNA-induced self-assembled complex will be detected. The two streptavidin conjugated DNA probes cannot be linked by multi-mismatched DNA. So the multi-mismatched DNA will not induce any current-pulse signatures. The current-pulse signatures for the self-assembled complex can be used to confirm the existence of target DNA. The concept of size dependent detection of self-assembled complex on molecule level shows strong promise for detection of biomolecules without interference of probes.

Keywords: nanopore, ssDNA, resistive-pulse sensing, self-assembled, protein conjugated probe

1 Introduction

Resistive-pulse [1] sensors, which while applied to the detection of molecule [2-16] are referred to as stochastic sensors [1, 16], use a nanopore in a polymer or biological membrane as the sensor element. In resistive pulse sensing, the membrane was embedded in two chambers of a conductivity cell so that different electrolyte solution could be placed on each side of the PET membrane. The resulting ionic current flowing through the nanopore is measured and as the analytes translocate through the nanopore downward current pulses are produced by the transient blocking of ion current. The frequency of such current pulses is relevant to the concentration of the analyte, and the magnitude and duration of the current pulse indicates the identity of the analyte.

Most ideal resistive pulse sensing work used the biological protein nanopore such as α-hemolysin and porin MspA, which were embedded in a supported lipid-bilayer membrane as the sensing element. The α-hemolysin nanopore can be made selective by biological or chemical preparation, and has been applied to various analytes assay such as metal ions [13], oligonucleotides[15], proteins [9], and small molecules [16]. However, the supported lipid bilayer membrane that houses the nanopore was frail [16] and it is a key impediment to developing practical sensors based on this biological technology.

Recently, artificial nanopores embedded in polymer, silicon or Si_3N_3 membrane [17, 18] have been prepared. A microlithographic method was utilized to prepare artificial nanopores, which uses a focused ion[2] or electron[3] beam to bear the

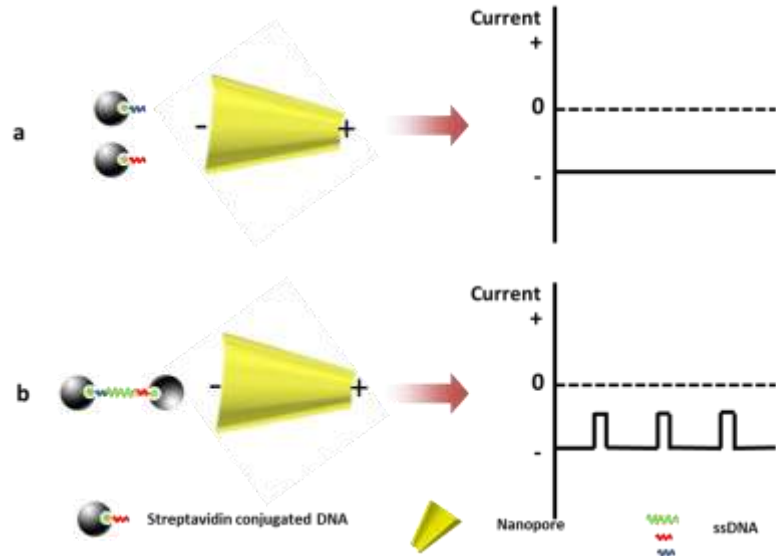
nanopore into a silicon or Si_3N_3 membrane. With the purpose of detection of analytes such as DNA, the functions of the single nanopores have been utilized [19]. Siwy had been exploring an alternative technology, named the track-etching method[20-23] and pointed out nanopores would be developed into a general platform technology to investigate the biophysics, physicochemistry, and chemistry of individual molecules[24-26].

In this study, we foucus on selective resistive-pulse sensing of single stranded DNA (40 bases) through a conical nanopore embedded in PET membrane. Many researches have been done before to explore the behavior of translocation of a single stranded DNA and double stranded DNA through a nanopore [2, 27, 28]. With the purpose of improving the selective translocation of DNA through a nanopore, the functionalization of the nanopore [19] was necessary. Nevertheless, the lifetimes of these selective nanopores will inevitably decrease after immobilizing probe DNA due to translocation of the target DNA. Despite the advantageous features offered by external functionalization of nanopore, one persistent limitation is that one membrane grafted with certain molecule probe is only suitable for one specific target and the regeneration of nanopore functionality may be impeded by the further tedious modification.

Here we provide another strategy of using a conical nanopore without modifications embedded in a PET polymer. The tip diameter of the nanopore is 9 nm and the base diameter of the nanopore is 520 nm. The target DNA could selectively hybridize with the two Streptavidin conjugated DNA probes. The size of the final

self-assembled complex was much larger than that of each Streptavidin conjugated DNA probe and can be detected. The size of Streptavidin conjugated DNA probes (4.5 nm×4.5 nm)[29] is smaller than the tip diameter of the nanopore, so the probes cannot effect the analysis results. Unspecific DNA which cannot induce the assembly of the two Streptavidin conjugated DNA probes also has little interference with the analysis results. So we can successfully distinguish the target DNA from unspecific DNA, using the conical nanopore in the polymer membrane.

The analyte was driven electrophoretically through the conical nanopore (from base to tip), and the translocation could be found as transient pulses in the current. Without the interference of Streptavidin conjugated DNA probes, the current-pulse signal can be related to the assembled complex induced by the target DNA. Since the size of single-stranded DNA (40 bases) was too small for the nanopore to detect, the Streptavidin conjugated DNA probes was used in the experiment. In the previous study, only proteins and large DNAs could be detected in the resistive-pulse sensing. Meanwhile, the strategy could detect various target DNA by changing the sequence of the probe using one certain nanopore. What's more, the advantages of the strategy is that it provides a simple method without further pretreatment processes [30] and functionalization procedures [19].



Scheme 1. Nanopore-based biosensor embedded in PET membrane. (a) In the absence of target DNA, the translocation of Streptavidin conjugated DNA probes cannot be detected by the biosensor. (b) In presence of the target DNA, the target DNA induced self-assembled DNA-protein complex. The translocation of the complex can be detected by the biosensor.

2 Materials and methods

2.1 Materials

12 μm thick polyethylene terephthalate (PET) which was irradiated with swift Au ion of 11.4 GeV/nucleon at UNILAC linear accelerator to create a single damage track through the membrane were obtained from GSI, Darmstadt, Germany. Ultrapage purified oligonucleotides were obtained from Alpha DNA, Inc. Streptavidin and bis-tris propane were purchased from Sigma-Aldrich. The oligonucleotide sequence for the Biotin-labeled oligonucleotide probe 1 is 5'Biotin-ACACACACACTCATCTGTGA-3', and the oligonucleotide sequence for probe 2 is 5'-AGAGAACCTGGGATATATAT-Biotin3'. The sequence for target

DNA is 5'-ATATATATCCCAGGTTCTCTTCACAGATGAGTGTGTGTGT-3'. The sequence for unspecific DNA is 5'-ACACAAAACCTATGTACACATGACAGAT GAGTGTGTGTGT-3'. Oligonucleotides were stored at -20 ^{o}C and were heated to 95 ^{o}C for 5 min and gradually cooled to room temperature before use.

2.2 Nanopore preparation

Single conical nanopore was prepared in 12 μm thick polyethylene terephthalate (PET) membrane by the track-etching technique. Either side of the tracked PET membrane was independently exposed to the UV light (365 nm and 254 nm) for 1 h, and then the membrane was embedded between two chambers of a conductivity cell so that different electrolyte solution could be placed on each side of the PET membrane. The procedure of chemical etching included two steps. An etching solution (9 M NaOH) was placed on one side of the membrane and the stopping solution (1.0 M formic acid plus 1.0 M KCl) was placed in the other side of the membrane. Each side of conductivity cell was added with a Pt electrode and A Keithley 2536A picoammeter/voltage-source (Keithley Instruments, Cleveland, OH) was employed to measure the ion current while chemical etching using a transmembrane potential of 1 V. The procedure of the etching lasted 2 h in the room temperature in the first step. The tip diameter obtained after the first etching was normally in the range of 1 to 7 nm [31] after 2 hours of the first step, a second step was applied to modulate the tip diameter of the conical nanopore. In the second step, 1M NaOH was was placed on both sides of the membrane. A Pt electrode was placed

into the electrolyte to measure the nanopore ion current. The etching lasted for 30 min and then rinsed with purified water before measuring the current-voltage curve.

2.3 Assembly of DNA-Protein Complex

The Streptavidin solution (100 μL, 33 μM) was mixed with probe 1 (10.7 μL, 28μM) and probe 2 (25 μL, 12 μM), the solution was diluted to 3 mL and the final concentration for each component was 1.1 μM for Streptavidin, 100 nM for probe 1 and 100 nM for probe 2. As previous research reported [32], the abundance of Streptavidin was monovalent when the ratio exceeds up to 2.5. In this experiment, a far beyond ratio (5.5) was adopted to make sure the main product is monovalent. In the control experiment, the unspecific DNA was added into the Streptavidin conjugated probes solution with a final concentration of 100 nM in 3 mL. For the present study of target DNA sensing, the target DNA was added into the Streptavidin conjugated probes solution with the final concentration 100 nM in 3 mL. The self-assembly reaction lasted for 5 h, then the solution was added into the base side of the nanopore. Streptavidin (pI ~7.0)[33] and DNA were highly negatively charged in the buffer (20 mM bis-tris propane, pH 9, 1 M KCl), therefore the assembled complex would be driven from the base side to the tip side while applying a negative potential on the base side of the nanopore.

2.4 Currents-pulse measurements of nanopore

The membrane was embedded in a two chambers of a conductivity cell filled with 3 mL of 20 mM bis-tris propane buffer solution (pH =9) that was also 1 M in

KCl to measure the current-pulse. Ag/AgCl electrodes were placed in each side of the conductivity cell and connected to an axopatch 200B (Molecular Devices Corporation, Union City, CA) patch-clamp amplifier. The Axopatch was used to apply the desired transmembrane potential and to measure the resulting ion current flow through the electrolyte-filled nanotube. The current was recorded in the voltage-clamp mode with a low-pass Bessel filter at 2 kHz bandwidth. The signal was digitized using a Digidata 1233A analog-to-digital converter (Molecular Devices Corporation), at a sampling frequency of 10 kHz. Data were recorded and analyzed using pClamp 9.0 software (Molecular Devices Corporation).

3 Results and Discussion

3.1 Characterization of Nanopore Diameter in Track-Etched Membrane

The protocol for etching PET membrane with track is described in the experimental section. The tip diameter and base diameter were the critical parameters for a conical nanopore. Electrochemical technique is used to measure the tip diameters [7]. The I-V curve for pore size measurement was obtained by applying transmembrane potential from -0.2 V to 0.2 V and the single nanopore membrane was sandwiched between two half chamber filled with conductive solution (100 mM NaH_2PO_4, 1 M KCl and pH=3.0). Keithley 6487 picometer voltage source and Ag/AgCl electrodes placed on each side of the nanopore were used to obtain a current-voltage curve. The diameter of the tip opening was calculated by the following equation [34]

$$d = 4LI/\pi DkV \quad (1\text{-}1)$$

Where I/V is the slope of the current-voltage, L is the thickness of the membrane after etching, k is the ionic conductivity of measuring solution, d is the diameter of tip opening and D is the diameter of base opening.

The base opening of a single symmetric nanopore was considered equivalent to the average pore size embedded in PET membrane (1×10^6 tracks/cm^2), which was acquired via SEM after etched under the same conditions as the single pore. The base diameter of the pore observed from SEM images (Figure 1) is 530 nm. And the tip diameter calculated from the aboving equation was 9 nm (Figure 2).

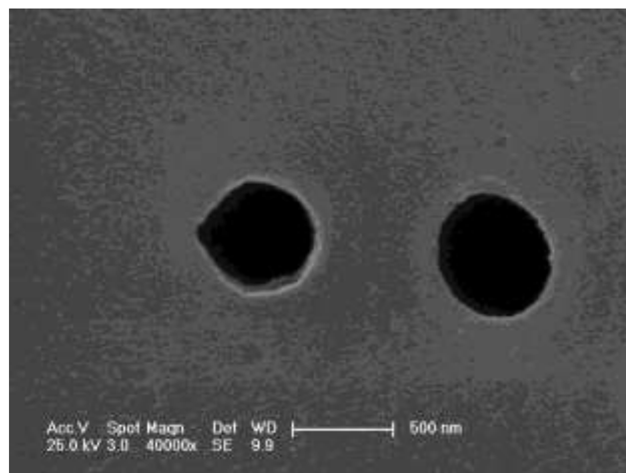


Fig 1. Scanning electron micrograph (SEM) of multipore (base diameter =530 nm) in PET polymer (1×10^6 tracks cm^{-2}) under the same etching conditions as that for single track-etched nanopore.

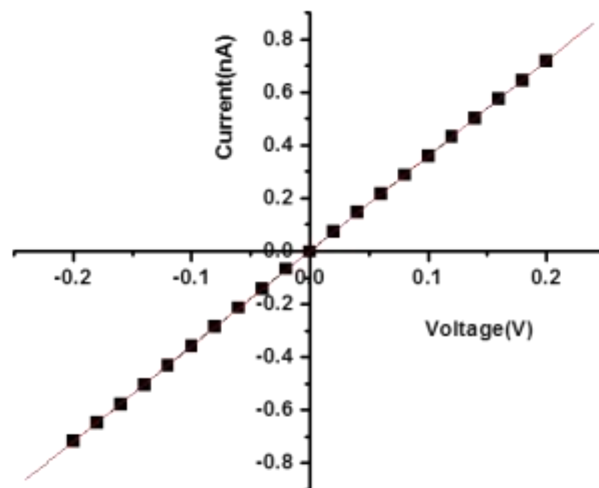


Fig 2. The current-voltage curve of conical pore in 1M KCl with applied -1.0 V voltage. .

3.2 Current-Pulses of the Self-Assembled Complex

Steady-state current and current-pulse data for target DNA linked protein dimers. As a control experiment, a steady-state ion current of -3.7 nA (Figure 3a) was observed for the bare conical nanopore with tip diameter of 9 nm under transmembrane potential -1 V and a buffer solution with 20 mM bis-tris propane, 1 M KCl and 3mM $MgCl_2$). The current was slight decreased to -3.5 nA after adding 1.1 μm streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM unspecific DNA into the half cell facing the base opening of nanopore. The decreases of the currents are due to the unspecific absorption of protein and DNA. No current-pulses was observed. Since the size of single streptavidin conjugated probe (4.5 nm) is much smaller than the tip diameter (9 nm) of conical nanopore and no link occurs between the two

streptavidin probes with the presence of unspecific DNA, no current-pulse was observed in such condition.

The current-pulse was observed after 100 nM target DNA was added to the solution that contained 1.1 μM streptavidin, 100 nM probe 1 and 100 nM probe 2 in the half cell facing the base opening of nanopore. Figure 3c depicts a typical currents-pulse that generated from target DNA linked stretavidin dimers. The continuous currents pulse indicates that the translocation of target DNA-linked protein dimer.

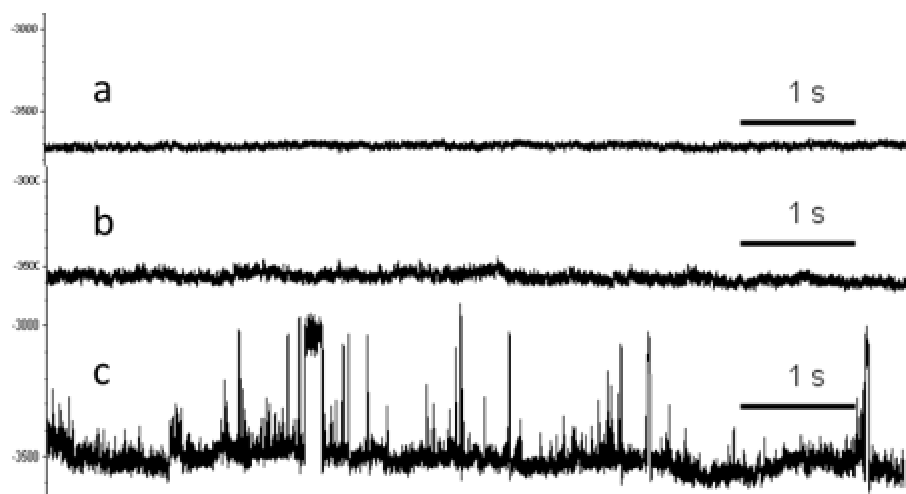


Fig 3. Current-time transients for conical nanotube sensor (tip diameter =9 nm, base diameter =530 nm) (a) buffer solution. (b) Buffer solution plus 100nM unspecific DNA, 1.1 μM Streptavidin and 100 nM probe 1 and 100 nM probe 2. (c) Buffer solution plus 100 nM target DNA, 1.1 μM Streptavidin and 100 nM probe 1 and 100 nM probe 2. Applied transmembrane potential for A, B and C was -1000 mV.

3.3 Optimization of ratio of streptavidin to biotin-labeled DNA

Histogram of current-pulse amplitude for solution that contains 1.1 μM streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM target DNA shows the average current amplitude(400pA)(Figure 4a).

The statistic of the currents-pulse duration predicts the distribution of the sizes of the mass transfer through the pore. A typical histogram current-pulse duration of solution containing 1.1 μM streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM target DNA are depicts in Figure 4b, a broadly distributed current-pulse duration was observed. The majority of these counts of the current-pulse durations are below 100ms. Normally, the current-pulse blow 100 ms are generated from the dimer, and the current-pulse up 100 ms are generated from the multimer. These data are in accordance with our previous data[32].

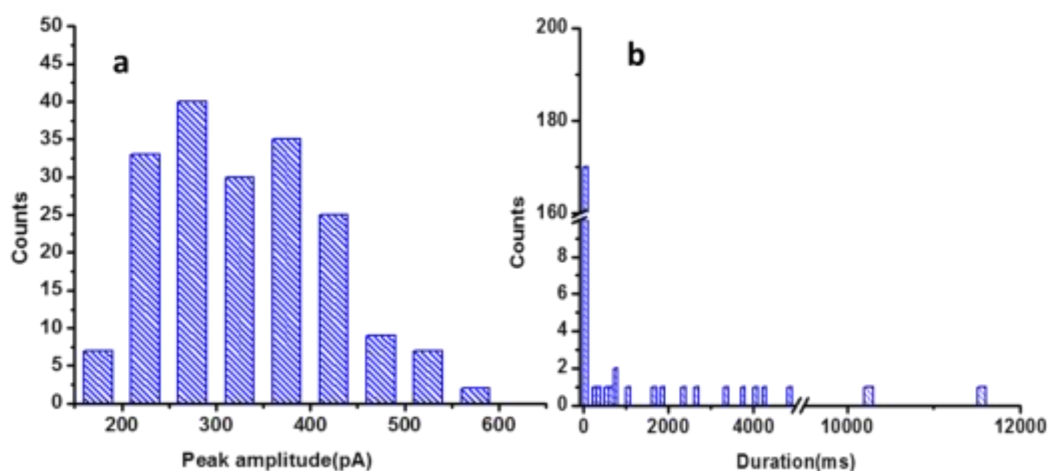


Fig. 4 Histograms of DNA-protein complex current-pulse-peak amplitude data for nanotube with tip diameter =9 nm. Solid curve are Gaussian. (a) Buffer plus 100nM target DNA , 1.1 μM streptavidin and 100 nM probe 1 and 100 nM probe 2. (b)

Buffer plus 100nM target DNA , 1.1 μM streptavidin and 100 nM probe 1 and 100 nM probe 2. Applied transmembrane potential was -1000 mV

The above phenomenon can be explained through the structure of streptavidin. Streptavidin has four binding site available for binding biotins, and four possible complexes can be formed: mono-, di-, tri- and tetra- complex. By increasing the ratio of streptavidin to biotin-labeled DNA up to 2.5, the majority of conjugates will be bi-valent complex with less tri-valent complex. In this project, we use the ratio up to 5.5 to ensure that majority of the conjugates are dimer and to minimize trimer. .

3.4 Distribution of target DNA linked complex

Scatter plots of current-pulse amplitude, Δi, vs. current-pulse duration, t, are often used to summarize resistive-pulse data [2, 6, 24, 35]. The scatter plot for a solution that was 1.1 μM Streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM target DNA shows that the majority of pulses was located below 100 ms and other current-pulses were broadly distributed (Figure 5a). These data also confirm above conclusions that a current-pulse signature can be obtained for the protein dimer induced by target DNA; even in this case, we still cannot define the current-pulses for the protein timer. In addition, no current pulses were observed for a control solution containing unspecific DNA. These data proved that unspecific DNA does not bind protein conjugated probes.

In order to reproduce the results, we etched another conical nanopore with a tip diameter of 10 nm. From the scatter plot (Figure 5b) of a solution that contained 1.1

μM Streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM target DNA, data showed the same trend that we had concluded earlier. Under the transmembrane potential difference -1 V, very long current pulses of duration lasting for more than 12 s exist. The very long current-pulse duration event can be stopped by an increase of the voltage to -3 V. The protein mutimers (or multimers) causing these long current-pulse durations was forced to translocate through the sensing zone by increasing the trans-membrane potential difference. In order to get the current-pulses continually without interruption, we increased the voltage to -2 V. The scatter plot (Figure 5c) of a solution that was 1.1 μM Streptavidin, 100 nM probe 1, 100 nM probe 2 and 100 nM target DNA reinforce our point that this current pulse was caused by protein dimer or multimer.

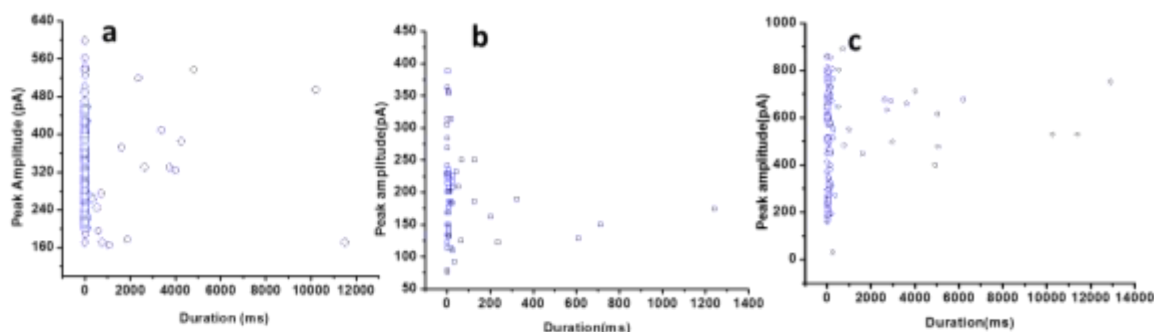


Fig. 5 Scatter plot of current-pulse magnitude (Δi) vs. current-pulse duration (t) for DNA-protein complex. (a) Tip diameter =9 nm. Buffer plus 100 nM target DNA, 1.1 μMStreptavidin and 100 nM probe 1 and 100 nM probe 2. Applied transmembrane potential was -1000 mV. (b) Tip diameter =10 nm. Buffer plus 100 nM target DNA, 1.1 μMStreptavidin and 100 nM probe 1 and 100 nM probe 2. Applied transmembrane potential was -1000 mV. (c) Tip diameter =10 nm. Buffer plus 100

nM target DNA, 1.1 μM Streptavidin and 100 nM probe 1 and 100 nM probe 2. Applied transmembrane potential was -2000 mV

4 Conclusion

We have presented here a new approach to sense DNA through conical shaped nanopore embedded in PET polymer. The translocation of each protein conjugated probe is undetectable due to the size much smaller than the tip opening of bare conical nanopore. On contrary, the translocation of target DNA linked two components is detectable because the size of target DNA linked two components is double the size of each component and also comparable to the diameter of the tip opening of nanopore. The sensing of target DNA can be accomplished by analyzing current-pulses due to translocation of the complex linked by target DNA. The concept of target analyte induced self-assembling on molecular level can be extended to detect small molecule and protein based on nanopore technique.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 21275137), The authors would like to thank the supervisor Charles R. Martin in University of Florida. Thanks for GSI providing track-etched membranes.

References

1 Bayley H, Martin CR (2000) Resistive-pulse sensing - from microbes to molecules. Chemical Reviews 100: 2575-2594

2 Li J, Stein D, McMullan C, et al (2001) Ion-beam sculpting at nanometre length scales. Nature 412: 166-169

3 Storm AJ, Chen JH, Ling XS, et al (2003) Fabrication of solid-state nanopores with single-nanometre precision. Nature materials 2: 537-540

4 Mara A, Siwy Z, Trautmann C, et al (2004) An asymmetric polymer nanopore for single molecule detection. Nano Lett, 4: 497-501

5 Heins EA, Siwy ZS, Baker LA, et al (2005) Detecting single porphyrin molecules in a conically shaped synthetic nanopore. Nano Lett 5: 1824-1829

6 Harrell CC, Choi Y, Horne LP, et al (2006) Resistive-pulse DNA detection with a conical nanopore sensor. Langmuir 22(25): 10837-10843

7 Siwy Z, Trofin L, Kohli P, et al (2005) Protein biosensors based on biofunctionalized conical gold nanotubes. J Am Chem Soc 127: 5000-5001

8 Bayley H, Jayasinghe L (2004) Functional engineered channels and pores - (review). Mol Membr Biol 21: 209-220

9 Braha O, Walker B, Cheley S, et al (1997) Designed protein pores as components for biosensors. Chem Biol 4: 497-505

10 Bean CP, Doyle MV, Entine G (1970) Etching of submicron pores in irradiated mica. J Appl Phys 41: 1454-1459

11 Gu LQ, Braha O, Conlan S, et al (1999) Stochastic sensing of organic analytes

by a pore-forming protein containing a molecular adapter. Nature 398: 686-690

12 Han AP, Schurmann G, Mondin G, et al (2006) Sensing protein molecules using nanofabricated pores. Appl Phys Lett 88: 093901

13 Kasianowicz JJ, Burden DL, Han LC, et al (1999) Genetically engineered metal ion binding sites on the outside of a channel's transmembrane beta-barrel. Biophysical Journal 76: 837-845

14 Gu LQ, Bayley H (2000) Interaction of the noncovalent molecular adapter, beta-cyclodextrin, with the staphylococcal alpha-hemolysin pore. Biophysical Journal 79: 1967-1975

15 Mathe J, Aksimentiev A, Nelson DR, et al (2005) Orientation discrimination of single-stranded DNA inside the alpha-hemolysin membrane channel. Proc Natl Acad Sci U S A 102: 12377-12382

16 Gu LQ, Cheley S, Bayley H (5504) Capture of a single molecule in a nanocavity. Science 291: 636-640

17 Lee S, Zhang YH, White HS, et al (2004) Electrophoretic capture and detection of nanoparticles at the opening of a membrane pore using scanning electrochemical microscopy. Anal Chem 76: 6108-6115

18 Kasianowicz JJ, Brandin E, Branton D, et al (1996) Characterization of individual polynucleotide molecules using a membrane channel. Proc Natl Acad Sci U S A 93: 13770-13773

19 Iqbal SM, Akin D, Bashir R (2007) Solid-state nanopore channels with DNA selectivity. Nat Nanotechnol 2: 243-248

20 Apel PY, Korchev YE, Siwy Z, et al (2001) Diode-like single-ion track membrane prepared by electro-stopping. Nucl Instrum Methods Phys Res Sect B-Beam Interact Mater Atoms 184(3): 337-346

21 Nilsson J, Lee JRI, Ratto TV, et al (2006) Localized functionalization of single nanopores. Advanced Materials 18(4): 427-431

22 Spohr R (2005) Status of ion track technology - prospects of single tracks. Radiation Measurements 40: 191-202

23 Yang L, Zhai Q, Li G, et al (2013) A light transmission technique for pore size measurement in track-etched membranes. Chemical Communication 49: 11415-11417

24 Sexton LT, Horne LP, Sherrill SA, et al (2007) Resistive-pulse studies of proteins and protein/antibody complexes using a conical nanotube sensor. J Am Chem Soc 129: 13144-13152

25 Menestrina J, Yang C, Schiel M, et al (2014) Charged particles modulate local ionic concentrations and cause formation of positive peaks in resistive-pulse-based detection. Journal of Physical Chemistry C 118: 2391-2398

26 Liu N, Jiang Y, Zhou Y, et al (2013) Two-way nanopore sensing of sequence-specific oligonucleotides and small-molecule targets in complex matrices using integrated DNA supersandwich structures. Angew Chem Int Ed Engl 52(7): 2007-2011

27 Chang H, Kosari F, Andreadakis G, et al (2004) DNA-mediated fluctuations in ionic current through silicon oxide nanopore channels. Nano Lett 4: 1551-1556

28 Smeets RMM, Keyser UF, Krapf D, et al (2006) Salt dependence of ion transport and DNA translocation through solid-state nanopores. Nano Lett 6: 89-95

29 Weber PC, Ohlendorf DH, Wendoloski JJ, et al (1989) Structural origins of high-affinity biotin binding to streptavidin. Science 243: 85-88

30 Thomas NE, Coakley WT, Winters C (1996) Contact formation in polylysine-mediated membrane-glass interaction. Colloids and Surfaces B-Biointerfaces 6: 139-147

31 Wharton JE, Jin P, Sexton LT, et al (2007) A method for reproducibly preparing synthetic nanopores for resistive-pulse biosensors. Small 3: 1424-1430

32 Funabashi H, Ubukata M, Ebihara T, et al (2007) Assessment of small ligand-protein interactions by electrophoretic mobility shift assay using DNA-modified ligand as a sensing probe. Biotechnology Letters 29: 785-789

33 Haeuptle MT, Aubert ML, Djiane J, et al (1983) Binding-sites for lactogenic and somatogenic hormones from rabbit mammary-gland and liver - their purification by affinity-chromatography and their identification by immunoprecipitation and photoaffinity-labeling. J Biol Chem 258(1): 305-314

34 Hou SF, Wang JH, Martin CR (2005) Template-synthesized protein nanotubes. Nano Lett 5: 231-234

35 Movileanu L, Howorka S, Braha O, et al (2000) Detecting protein analytes that modulate transmembrane movement of a polymer chain within a single protein pore. Nat Biotechnol 18: 1091-1095