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Nano-channel of Viral DNA Packaging Motor as Single Pore to Differentiate Peptides with Single Amino Acid Difference

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Abstract

Detection, differentiation, mapping, and sequencing of proteins are important in proteomics for the assessment of cell development such as protein methylation or phosphorylation as well as the diagnosis of diseases including metabolic disorder, mental illness, immunological ailments, and malignant cancers. Nanopore technology has demonstrated the potential for the sequencing or sensing of DNA, RNA, chemicals, or other macromolecules. Due to the diversity of protein in shape, structure and charge and the composition versatility of 20 amino acids, the sequencing of proteins remains challenging. Herein, we report the application of the channel of bacteriophage T7 DNA packaging motor for the differentiation of an assortment of peptides of only a single amino acid difference. Explicit fingerprints or signatures were obtained based on current blockage and dwell time of individual peptide. Data from the clear mapping of small proteins after protease digestion suggests the potential of using T7 motor channel for proteomics including protein sequencing.

Keywords

nanopore; T7 DNA packaging motor; protein sequencing; single pore sensing; biomotor; bacteriophage DNA packaging

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Author contributions

P.G. and Z. J. conceived and designed the experiments in this study; Z. J. and X. K. conducted the experiments; S. W. designed the cloning; Z. J. analyzed data; Z. J. and P. G. wrote the manuscript.

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Competing financial interests

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Introduction

Proteomics, a challenging but highly significant field, is imperative in understanding the biological function and role of certain proteins in the onset of various genetic diseases, immunological aberrations, metabolic disorders, and cancer ailments. [1–3] In its current stage, the fingerprinting of polypeptides or proteins is mainly dependent on mass spectrometry (MS), which currently has not reached the sensitivity of single molecules. [4;5] In order to advance the field of proteomics, nanopore technology provides an alternative approach for peptide fingerprinting at a single molecule level. The principle of nanopore detection is based on the resistive pulse technique, that is, analytes are driven through a pore by electric force to produce a profile of pico-ampere scale current signatures characteristic of each analyte in interest. [6–8]

Currently, the nanopore technology primarily focuses on nucleic acid characterization. [9–13] A few studies have shown great potential for peptide or protein sensing. [14–18] For example, the protein size, fluctuation, and conformational changes have been investigated by solid-state nanopore. [19–22] In a particular study α -hemolysin was used in real-time monitoring of peptide cleavage. [23;24] Other studies utilizing other portals such as Cly A for protein folding, [25;26] Phi29 connector for the distinguish of peptides, [27] and SPP1 connector for peptide oligomerization [28;29] have also been reported.

Biological motors are very common in living systems, facilitating various functions. [30–36] In dsDNA animal or bacterial viruses such as Phi29, SPP1, T3, T4, T5, and T7, their genome enters and exits the bacteriophage capsid during replication and infection, respectively, through a portal protein channel called the connector. [37–43] Bacteriophage T7 belongs to the *Podoviridae* family, which infects *Escherichia coli* (*E.coli*). Data from cryo-electron microscopy reveals that the T7 connector is composed of twelve subunits encoded by gene 8 (gp8) with a molecular weight of 59 k Da. [44;45] In this study, the T7 connector gene was cloned and expressed in *E. coli*. The purified T7 connector was inserted into a lipid bilayer membrane as a biosensor for the fingerprinting of different peptides. Their blockage or dwell time was then used as an explicit signature of each peptide. Clear mappings of several peptides were achieved after digestion with protease, suggesting the potential of using T7 nanopore technology for proteomics and protein sequencing.

Material and methods

Materials.

Adenosine triphosphate (ATP) was obtained from VWR. Imidazole was purchased from Acros. 1, 2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids. The peptides in this study are synthesized by Genescript. The trypsin was purchased from Sigma and dissolved in the dd H₂O. Other chemicals were from Fisher Scientific, if not specified. The sequences of peptides in this study are:

- (1) R8: RRRRRRRR;
- (2) R9: RRRRRRRRR;
- (3) R10: RRRRRRRRRR;

- (4) R11: RRRRRRRRRRRR;
(5) R12: RRRRRRRRRRRR.

Cloning, expression and purification of T7 connector.

The plasmid to express T7 connector was synthesized and constructed by Genescript. The gene 8 (gp8) encoding single subunit of T7 connector with the deletion of ADSVGLQPGL in the C-terminal, and the DNA fragment encoding six histidine in the C-terminal, were inserted between NdeI and BamHI of the pET-3c vector. [46;47] The newly-constructed plasmid was transformed to *E.coli* HMS 174 (DE3). The expression and purification procedure are similar to phi29 connector. [10;27;48] The *E.coli* containing the plasmid for T7 expression was cultured in 500 ml standard Luria broth (LB) medium at 37 °C until OD₆₀₀ reached 0.5–0.6. The T7 protein expression was induced with the addition of IPTG (final concentration: 0.5 mM). The cultures were grown for additional 3 hours before harvest by centrifugation (5000 rpm for 30min). The pellets were resuspended in buffer (Tris-HCl 0.1 M, NaCl 0.5 M, ATP 10 mM, Glycerol 14.4%, Imidazole 5 mM) and then cells were lysed by French Press. The soluble protein was recovered by centrifugation (11000 rpm for 30min) and supernatant was passed through a 0.45 µm membrane filter before loading to purification column (Thermo Fisher Scientific) filled by His•Bind[®] resin (EMD Millipore). The T7 connector protein was eluted using elution buffer (Tris-HCl 0.1 M, NaCl 0.5 M, ATP 50 mM, Glycerol 14.4%, Imidazole 1 M). The purified T7 protein was analyzed by 12% SDS-PAGE.

Incorporation of T7 connector into liposomes.

The incorporation process contains dehydration and hydration steps. [10;48;49] Briefly, 100 µl 10 mg/ml DPhPC in chloroform was dried under vacuum by the evaporator (Buchi). The T7 connector (final concentration 100–500 µg/ml) and liposome buffer (Liposome buffer: 3 M KCl, 250 mM sucrose, 5 mM HEPES, pH 7.4) were added and vortexed thoroughly. Then the mixture was filtered through 0.4 µm polycarbonate membrane for around 30 times using the extruder (Avanti Polar Lipids) in order to produce homogenous proteoliposomes.

Electrophysiological assays.

The lipid bilayer membrane was formed on the Teflon partition membrane (pore size: 200 µm) which separates the whole chamber into two compartments, cis- and trans-chamber. [48] A pair of Ag/AgCl electrodes were placed in both chambers. Bilayer Clamp Amplifier BC-535 (Warner Instruments) or Axon 200B (Molecular Devices) was connected to the Axon DigiData 1440 A analog-digital converter (Molecular Devices). Data was recorded at 1 K Hz bandwidth with a sampling frequency 20 KHz. The Clampex 10 (Molecular Devices), Clampfit 10 (Molecular Devices), MOSAIC [50] and Origin 8 (Origin Lab Corporation) were used to collect and analyze data. The events that last <0.4 ms were excluded for translocation data analysis.

Peptide translocation assays.

The peptide was added into chambers after single channel insertion or peptide was premixed with conductance buffer (0.15 M KCl, 5 mM HEPES, pH 7.4) for the detection of single

type of peptide (63 nM). The mixture of R8, R9, R10 and R12 peptides was kept the same concentration (21 nM) in buffer.

Peptide cleavage assay.

The R11 (RRR RRR RRR RR) or R12 (RRR RRR RRR RRR) was premixed with conductance buffer. After single connector was inserted and then the translocation signals of R11 or R12 peptide were observed under -50 mV, the trypsin was added and mixed into the cis-chamber while recording the data until the majority of R11 or R12 was digested. [51]

Results and discussion

Cloning and expressing the T7 connector in *E. coli* and insertion of the purified connector into lipid bilayer membrane.

The gene gp8, which codes for the connector protein of the bacteriophage T7 DNA packaging motor was cloned into the plasmid pET-3c through the fusion of a six-aa His-tag into the C-terminus, facilitating protein purification. [46;52] After induction by IPTG, the overexpressed gp8 gene production was spontaneously assembled into its channel complex (Figure 1a, b). The connector was purified into homogeneity based on 12% SDS-PAGE result (Figure 1c). Unlike other membrane proteins, viral motor channels cannot be inserted into a lipid bilayer membrane without mediating vesicle liposome or porphyrin. [10;53] For this reason, the purified T7 connector was incorporated into the liposome to form the proteoliposome which was then inserted into a lipid bilayer membrane by fusion as reported previously. [10;48] Single channel characterization (Figure 1d) revealed that the conductance of T7 connector was 0.73 ± 0.10 nS, based on the measurement of 117 channels under 50 mV (Figure 1e). Cloning and expression of the T7 connector gene and subsequent connector insertion in the lipid bilayer membrane resulted in an assembled nanopore that will ultimately be utilized for characterization and mapping of peptides.

Differentiation of peptides of varying residues by current blockage

Considering the principle of nanopore sensing, we first investigated five different lengths of arginine peptides consisting of 8 (R8; Figure 2a, b, c), 9 (R9; Figure 2d, e, f), 10 (R10; Figure 2g, h, i), 11 (R11; Figure 2j, k, l), and 12 (R12; Figure 2m, n, o) residues, which are positively charged. The blockage and dwell time were used to characterize the peptides. The blockage percentage was calculated using the equation $(I_o - I_b)/I_o \times 100\%$, in which I_o is the channel current in the absence of peptide translocation (open channel current), and I_b is the current during peptide translocation (block current). Presently, we only focus on the differentiation of peptides based on blockages, which were fit by Gaussian distributions. The blockages of R8, R9, R10, R11, R12 were $44.58\% \pm 1.74\%$, $50.70\% \pm 0.93\%$, $54.61\% \pm 0.81\%$, $57.77\% \pm 1.20\%$, $60.55\% \pm 0.77\%$, respectively (Figure 2b, e, h, k, n). Each blockage peak for each peptide had relatively good separation, at least $\sim 3\%$. The electrical signal resulting from peptide passing through the channel is related to the length of the narrow region of the channel. Unsurprisingly, the longer peptides have larger blockage percentages and longer dwell time. This indicates that the length of peptides with twelve amino acids is shorter than the length of the narrow region of T7 connector and each amino acid contributes to the blockage and dwell time. Peptides were driven to translocate through

T7 connector by electric force, and the differences of amino acids in mass, charge and size could affect current blockage and dwell time in principle.

Discriminating peptides of varying size in mixture

The T7 motor channel was investigated for its potential to differentiate peptides in mixtures of biological samples. As shown in Figure 2, the blockage peaks for each peptide showed adequate separation. The R8, R9, R10, and R12 peptides were kept at a constant concentration (21 nM) for the nanopore translocation experiment (Figure 3). From Figure 3a, b, there were four distinct peaks of blockages at $45.20\% \pm 1.55\%$, $50.80\% \pm 0.96\%$, $54.65\% \pm 0.75$, and $59.89\% \pm 0.64\%$ as a result of fitting the data by Gaussian distributions. These peaks matched the blockage parameters of individual peptides measured separately. Although current blockage has been extensively used as a reliable and sensitive signature for a variety of analytes, dwell time parameters have been reported to be heterogeneous with large variation. [54–56] Surprisingly, clear and distinct dwell time signatures were obtained with discrete order when R8, R9, R10, and R12 peptides were present in a mixture (Figure 3c).

Mapping of 11-aa and 12-aa peptides by real-time sensing *via* trypsin cleavage

In the interest of assessing the potential of T7 channel for protein mapping, two peptides were digested by trypsin. R11 or R12 peptides (63 nM) were premixed with buffer in chambers, and translocation signals were recorded after single channel insertion. Subsequently, trypsin was added into the cis-chamber and mixed while translocation events were continuously recorded. As shown in Figure 4a, b, c, the blockage of R12 was $60.57\% \pm 0.45\%$. After the addition of trypsin, three new peaks appeared, $45.29\% \pm 0.82\%$, $51.23\% \pm 0.54\%$, and $54.91\% \pm 0.48\%$; these results suggested that peptide R12 was cut into multiple fragments, R8, R9, and R10 (Figure 4d, e, f). However, peptides composed of less than 8 amino acids were not detectable by T7 connector.

Trypsin is considered to be an endopeptidase, which means that it does not digest arginine at the C-terminal. Mapping of R12 *via* protease digestion did not reveal the blockage peak corresponding to R11 (Figure 4e). To confirm that the missed peak of R11 was not due to the issue of overlap of the blockage peaks between R11 and R12, R11 cleavage was carried out in parallel. Before adding trypsin to the chamber, there was a single blockage peak of $57.68\% \pm 1.22\%$ representing R11 (Figure 4 g, h, i); however, with the addition of trypsin to the cis-chamber, the translocation events corresponding to R11 decreased with time, while two new peaks with $45.06\% \pm 1.38\%$ and $51.59\% \pm 2.27\%$, respectively, appeared (Figure 4j, k, l). These two peaks matched the two peaks representing R8 and R9 in Figure 2. There was no R10 blockage peak in R11 digestion by trypsin. The peptide mapping of R11 and R12 support a notion that trypsin is an endopeptidase and at least two amino acids at the C-terminal are required for cleaving.

A variety of protease assays have been used for the diagnosis of different diseases. [57–59] For example, trypsin has been used as the most reliable marker for the diagnosis of pancreatitis. Therefore, the peptide mapping methods reported here could also be used to monitor serum enzyme activity or diagnosis of various diseases utilizing proteases as

markers. The results from this study suggest that nanopore technology has a potential for diseases diagnosis, in addition to protein sequencing.

Conclusions

Reengineered T7 connector was used as single pore sensing instruments to discriminate peptides with a difference of one amino acid residue. Individual signatures of current variation and dwell time of four peptides were clearly distinguished through use of the nanopore. The mapping of proteins based on fingerprints of small peptides after trypsin digestion reveals the potential to detect serum protease abnormality in diseases diagnosis. In furtherance of the field of proteomics, our results pave the way to protein mapping and sequencing using nanopore technology, and demonstrate additional potential of protein pore in biomedical application.

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Figure 1. Overview of bacteriophage T7 connector.

(a-b) The top view (a) and side view (b) of T7 connector (PDB:3J4A), showing the dimension, the positively charged (blue), negatively charged (red), and neutral (white) amino acids. (c) 12% SDS-PAGE showing the purified C-His tagged T7 connector (lane 2, 59 k Da) overexpressed in *E. coli*. Lane 1: molecular weight marker; lane 3: phi29 connector (37 k Da) as a control. (d) Current trace of single T7 connector insertion with polarity voltage switch of 50 mV. (e) Histogram of conductance of T7 connector based on 117 insertion events. Buffer: 0.15 M KCl, 5 mM HEPES, pH 7.4.

Figure 2. Differentiation of peptides with 8, 9, 10, 11, 12 arginine residues.

The blockage is defined as $(I_0 - I_b)/I_0 \times 100\%$ in which I_b is the block current during translocation and I_0 is the current of open channel without translocation, and fitted by a Gaussian distribution. Peptide concentration: 63 nM. Applied voltage: -50 mV. Buffer: 0.15 M KCl, 5 mM HEPES, pH 7.4.

Figure 3. Discrimination of R8, R9, R10 and R12 peptides in a mixture.

(a-c) The electric profiles of the mixture were shown in the blockage histogram (a), current trace (b) and the scatter plot (c). R represents arginine. Different colors represent different peptides. The histograms of blockages were fitted by four Gaussian distributions. Applied voltage: -50 mV. Concentration of each peptide in the mixture is 21 nM. Buffer: 0.15 M KCl, 5 mM HEPES, pH 7.4 . More than $10,000$ events were analyzed in the histogram or scatter plot.

Figure 4. Mapping of the 11-aa and 12-aa peptides cleaved by trypsin.

(a-f) The electric profiles of R12 before (a-c) and after (d-f) addition of trypsin. Before trypsin digestion, only one peak of $60.57\% \pm 0.45\%$ blockage was found for R12. With the addition of trypsin (final concentration: 25 ng/ml) into the cis-chamber, multiple peaks that represent the cleaved products were shown in the trace (d), the blockage histogram (e), and the scatter plot (f). The blockage levels of $45.29\% \pm 0.82\%$, $51.23\% \pm 0.54\%$, and $54.91\% \pm 0.48\%$ represent R8, R9 and R10, respectively. The blockage level of $60.24\% \pm 0.42\%$ matches the R12 blockage level before adding trypsin. (g-l) The electric profiles of R11

before (g-i) and after (j-l) addition of trypsin. The trace (g), blockage histogram (h), scatter plot (i) of R11 showed single blockage peak of $57.68\% \pm 1.22\%$. Two additional peaks of $45.06\% \pm 1.38\%$ and $51.59\% \pm 2.27\%$ were generated after addition of trypsin (final concentration: 50 ng/ml), matching the blockage level of R8 and R9. Arrows indicate the cleavage sites of each R11 and R12 by trypsin. The histograms of blockages were fitted by single or multiple Gaussian distributions. e and f were recorded 10 min after the addition of trypsin, and k and l were recorded 8 min after adding trypsin. The recording time for the plotted data (b, c, e, f, h, i, k, l) was 5 min. The R11 or R12 peptide concentration: 63 nM. Applied voltage: -50 mV. Buffer: 0.15 M KCl, 5 mM HEPES, pH 7.4.