Salt-Mediated Nanopore Detection of ADAM-17

Xiaohan Chen, Youwen Zhang, Golbarg Mohammadi Roozbahani, and Xiyun Guan

Department of Chemistry, Illinois Institute of Technology, Chicago, IL, 60616, USA

Supporting Information

ABSTRACT: ADAM-17 (a disintegrin and metalloproteinase 17) plays an important role in various physiological and pathophysiological processes. Overexpression/underexpression of ADAM-17 could lead to various diseases. In this work, by taking advantage of ionic strength and salt gradient, and monitoring the cleavage of a substrate peptide by ADAM-17 in a nanopore, we developed a label-free sensor for the rapid detection of ADAM-17. The sensor was highly sensitive and selective: picomolar concentrations of ADAM-17 could be detected within minutes, while structure similar proteases such as ADAM-9 and MMP-9 did not interfere with its detection. Our developed nanopore sensing strategy should find useful applications in the development of nanopore sensors for other proteases of biological, pharmaceutical, and medical importance.

Key words: Nanopore, ADAM-17, Salt Effect, Biomarker, Cancer Diagnosis

1. Introduction

A disintegrin and metalloproteinase (ADAM) enzymes are Zn^{2+}-dependent proteins that mediate cell surface proteolysis and modulate cell-cell and cell-matrix interactions.[1] They play an important role in various physiological and pathophysiological processes. Maintaining an appropriate amount of ADAM expression in human body is necessary to guarantee their proper function. Either ADAM overload or ADAM deficiency my cause serious health problems. For example, overexpression of ADAMs has been found in various cancers, such as lung cancer, liver cancer, and prostate cancer.[2, 3] On the other hand, deficiency of ADAM-8, ADAM-9, and ADAM-12 could lead to physiological decks, such as infertility, retinal degeneration, and musculoskeletal disorder,[4, 5] while underexpression of ADAM-10 and ADAM-17 might cause early embryonic death.[1] Thus far, the human genome contains 21 ADAM family members.[6] Among them, ADAM-17 has attracted the most research. ADAM-17, also known as a tumor necrosis factor-alpha converting enzyme, is one of the major enzymes responsible for ectodomain shedding. In addition to cancer, ADAM-17 is also involved in various other serious diseases, including inflammation, diabetes, heart disease, and Alzheimer's disease.[7] Although the biological function of ADAM-17 is far from being clear, ADAM-17 is a promising target for disease treatment.[8] To this end, it is highly important to have analytical capability to detect ADAM-17 sensitively and accurately. At present, three major methods have been developed for ADAM-17 detection, including enzyme-linked immunosorbent assay (ELISA), western blotting, and fluorescence assay. These methods, although sensitive, either involve complicated sampling processes or require the use of labels.[9,10] Therefore, development of other fundamentally different techniques, especially nanosensors, which are label-free and easy to operate for ADAM-17 detection, remains a high priority.

Nanopore sensor has attracted substantial interest as the fourth generation DNA sequencer because of its high accuracy, low cost, long read length, and rapid analysis.[11] By monitoring the ionic current modulations produced by the interaction between analyte molecules and a nano-scale sized pore (either a protein ion channel[12-14] embedded in phospholipid bilayer or an artificial nanopore[15-17] / nanochannel[18] fabricated in a solid-state membrane) under an applied voltage bias, nanopore

technology has been utilized to explore a wide variety of applications, including biosensing, environmental monitoring, pharmaceutical screening, medical diagnosis, and homeland security.[19-25] In this work, by monitoring the cleavage of a peptide substrate by ADAM-17 and taking advantage of ionic strength and salt gradient, we developed a sensitive and selective method for ADAM-17.

2. Material and Methods

2.1. Materials and reagents

ADAM-17 peptide substrate (sequence: LAQAVRSSSARLVFF) and its two cleavage fragments (sequences: LAQAV and RSSSARLVFF) were synthesized by WatsonBio (Houston, TX). ADAM-17 and ADAM-9 were ordered from R&D Systems (Minneapolis, MN), while MMP-9 was obtained from Sigma-Aldrich (St. Louis, MO). All the other chemicals, including sodium chloride, zinc chloride, Trizma base, hydrochloric acid, pentane, hexadecane, HPLC-grade water, and DNase, RNase free water, were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-diphytanoylphosphatidylcholine was bought from Avanti Polar Lipids (Alabaster, AL). The stock solution of ADAM-17 was prepared in DNase, RNase free water at a concentration of 100 μg/mL and stored at -80 °C before and immediately after use. A stock solution of 10 mM for each peptide was also prepared with DNase, RNase free water and kept at -20 °C before and immediately after use.

2.2. Bilayer experiment and data analysis

The procedure for single channel recordings has been described previously.[26] Briefly, two Teflon chamber compartments were separated by a Teflon film (Goodfellow Malvern, PA) containing a 150-μm diameter hole. Planar bilayer was formed according to the Montal-Muller method.[27] Unless otherwise noted, the experiments were performed at 24 ± 1 °C using the wild-type αHL protein nanopore. The αHL protein was added to the *cis* compartment, while the peptide substrate and its enzymatic digestion products were added into the *trans* compartment. Two buffer solutions were used in this study, including: 1) 1.0 M NaCl and 10 mM tris with the pH value adjusted to 7.5 using HCl; and 2) 3.0 M NaCl and 10 mM tris with the pH value adjusted to 7.5 using HCl. Ionic currents were recorded with Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered with a four-pole low-pass Bessel filter at 5 kHz, and then digitized with a Digidata 1440A converter (Molecular Devices) at a sampling frequency of 10 kHz. The event blockage amplitude, residence time, and number of occurrences (i.e., event counts) were obtained by using Clampfit 10.6 software (Molecular Devices).

2.3. Enzyme digestion

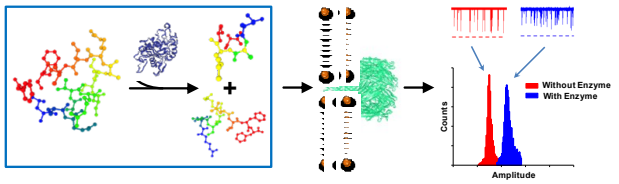
Enzymatic reactions were carried out by incubating ADAM-17 or other proteases such as ADAM-9 and MMP-9 with 45 μL DNase, RNase free water, 5 μL $ZnCl_2$ solution (containing 5 μM $ZnCl_2$, 0.15 M NaCl, and 50 mM Tris, pH 7.5), and 1 μL 10 mM peptide substrate at 37 °C for a period of time ranging from 15 min to 120 min. In the control (non-activated ADAM-17) experiment, reaction was performed by incubating ADAM-17 with 50 μL DNase, RNase free water and the peptide substrate. All the digestion products were stored at -80 °C before nanopore analysis.

3. Results and discussion

3.1. Principle for nanopore detection of ADAM-17

The concept of ADAM-17 detection by nanopore is shown in Scheme 1, where the translocation of an ADAM-17 peptide substrate in a nanopore is monitored both in the absence and presence of the target analyte. Without ADAM-17, the peptide substrate produces only one major type of current blockage events. In contrast, in the presence of the analyte, it would cleave the substrate into two shorter fragments, thus producing new types of events in the nanopore. By determining the frequency (or number of counts) of these new events, the activity of ADAM-17 could be measured. To demonstrate this

detection strategy, translocation of peptide LAQAVRSSSARLVFF in the wild-type αHL protein nanopore was initially investigated at +120 mV under symmetric electrolyte conditions with both the *cis* and *trans* chamber compartments filled with 1 M NaCl buffer solution (pH 7.5). Note that this peptide was a well-documented substrate for ADAM-17, and its cleavage by ADAM-17 would produce two shorter length peptides, LAQAV and RSSSARLVFF[28]. Our experimental results (Fig.1) showed that, in the absence of ADAM-17, the peptide substrate events were rarely observed (event frequency: ~0.05 s^{-1}; normalized event residual current: 30.7 ± 0.3 % of full channel block). One likely interpretation is that, under our experimental condition, the substrate peptide molecules could form stable secondary structure, which had a larger molecular size than the *trans* entrance of the protein pore so that it was difficult for the peptide molecules to be captured by the nanopore. However, after addition of ADAM-17 to the peptide solution, a new type of events with a mean residual current of 47.3 ± 0.5% of full channel block and having a much larger frequency (~0.44 s^{-1}) appeared. Since ADAM-17 (molecular weight: 70 kDa) alone didn't produce current modulations in the nanopore (Supporting Information, Fig. S1), the appearance of new events indicated that the substrate was being digested by the protease.



Scheme 1. The cleavage of a peptide substrate by ADAM-17 produces a new type of events in a nanopore, which could be utilized to measure the protease's activity.

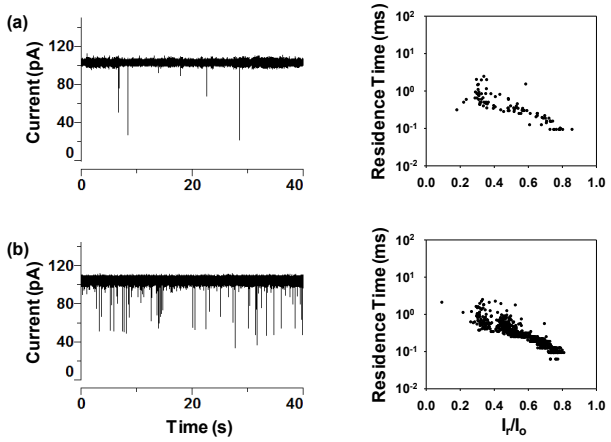


Figure 1. Detection of ADAM-17 in the wild-type α-hemolysin protein nanopore. (a) Without; and (b) with ADAM-17. (*Left*) Typical 40-s trace segments; (*Right*) the corresponding scatter plots of event residence time vs. current blockage amplitude. I_r/I_o in Figs. 1a and 1b is normalized blockage residual current, which was obtained by dividing the average blockage residual current of an event by the average open channel current. The experiments were performed at +120 mV under a symmetric electrolyte condition with both the *cis* and *trans* chamber compartments filled with a solution containing 1.0 M NaCl and 10 mM Tris HCl (pH7.5) and in the presence of 5 μM peptide substrate or a reaction mixture of peptide substrate (5 μM) and ADAM-17 (100 ng/mL).

3.2. Optimization of experimental conditions

Previous studies including ours have shown that electro-osmotic flow through the nanopore plays a significant role in the event frequency.[29,30] In particular, with an increase in the concentration of the background electrolyte solution or employing a salt gradient (i.e., using asymmetric electrolyte solution instead of symmetric electrolyte solution),[29,31] an increase in the event frequency could be observed. In order to improve the performance and the sensitivity of the nanopore sensor, nanopore detection of ADAM-17 were then investigated in 3 M NaCl (*cis*) / 3M NaCl (*trans*) and 3 M NaCl (*cis*) /1 M NaCl (*trans*) buffer solutions. The experimental results were summarized in Fig. 2. Similar to the observation we made with 1 M NaCl (*cis*) / 1 M

NaCl (*trans*) buffer solutions, in 3 M NaCl (*cis*) /3 M NaCl (*trans*), the interaction between the peptide substrate and the nanopore didn't present a significant number of events in the absence of ADAM-17, but produce a new type of current modulations with a much larger frequency (event frequency: ~1.1 s^{-1}; event residual current: 42.4 ± 0.1 % channel block) in the presence of ADAM-17. On the other hand, in 3 M NaCl (*cis*) /1 M NaCl (*trans*), the peptide substrate events with a mean residual current of 30.0 ± 0.1% channel block were clearly observed in the absence of ADAM-17, suggesting that the salt gradient could facilitate peptide translocation in the nanopore. After addition of ADAM-17 to the solution, the peptide substrate events became less frequent, while a new major type of events (mean residual current: 48.6 ± 0.1 % channel block) appeared in the current trace and event histogram. Although the event mean residence time (3.35 ± 0.27 ms vs. 0.83 ± 0.02 ms) of the peptide fragments in 3 M NaCl (*cis*) /3 M NaCl (*trans*) was larger than that of 3 M NaCl (*cis*) /1 M NaCl (*trans*), the salt gradient (i.e., 3 M NaCl (*cis*) / 1 M NaCl (*trans*)) was used in the remaining experiments since more events and hence a better sensor sensitivity could be achieved under this condition (Fig. 2d). Specifically, the frequency of the peptide fragment events

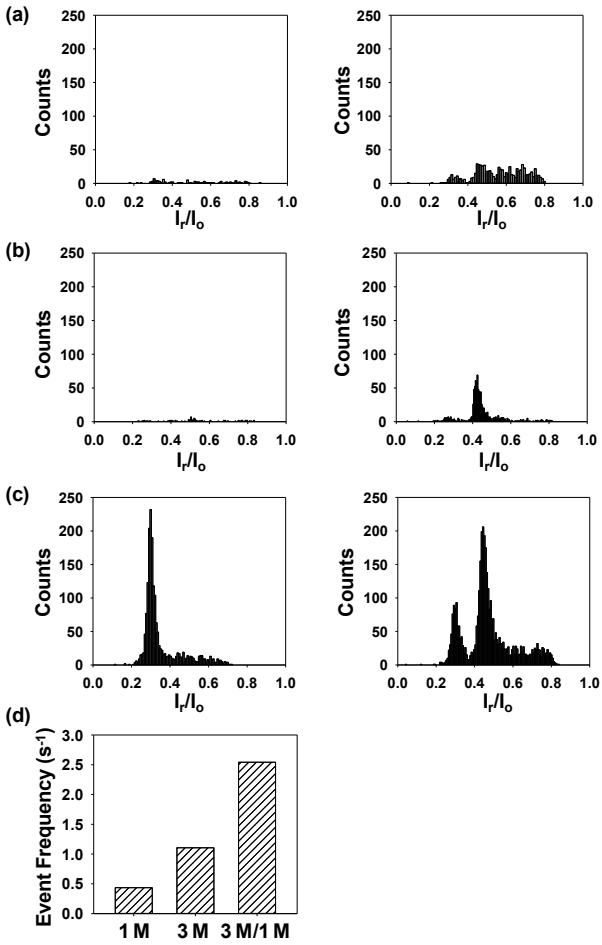


Figure 2. Salt effect on nanopore detection of ADAM-17. (a) 1 M NaCl (*cis*) / 1 M NaCl (*trans*) ; (b) 3 M NaCl (*cis*) / 3 M NaCl (*trans*) ; (c) 3 M NaCl (*cis*) / 1 M NaCl (*trans*); and (d) Plot of event frequency as a function of salt concentration and gradient. (*Left*) without ADAM-17; (*Right*) with ADAM-17. The experiments were performed at +120 mV with the wild-type αHL protein nanopore in the presence of 5 μM peptide substrate or a reaction mixture of peptide substrate (5 μM) and ADAM-17 (100 ng/mL).

in 3 M NaCl (*cis*) / 1 M NaCl (*trans*) was ~5.8 and ~2.3 folds larger than those of 1 M NaCl (*cis*) / 1 M NaCl (*trans*) and 3 M NaCl (*cis*) / 3 M NaCl (*trans*), respectively. It should be noted that, in theory, after cleavage of the peptide substrate by ADAM-17, two types of new events should be observed, which are corresponding to the produced two peptide fragments. Our experimental results (i.e., appearance of only one major type of events) suggest that one peptide fragment might translocate through the nanopore too rapidly to produce observable current modulations. To support this hypothesis, the standard solutions of the two peptide

fragments (RSSSARLVFF and LAQAV) were analyzed by the nanopore in 3 M NaCl (*cis*) / 1 M NaCl (*trans*) at +120 mV. The experimental results were summarized in Fig. S2 (Supporting Information). As we expected, one peptide (LAQAV) didn't produce a significant number of events. The peptide RSSSARLVFF produced current modulations with a mean residual current of 48.0 ± 0.1 % channel block, which was similar to what we observed in the experiment with ADAM-17 digestion of the substrate peptide. In addition, translocation of peptide RSSSARLVFF in the nanopore was further investigated in the other two salt conditions, i.e., 1 M NaCl (*cis*) / 1 M NaCl (*trans*) and 3 M NaCl (*cis*) / 3 M NaCl (*trans*). Their event signatures (Supporting Information, Fig. S3) were similar to those obtained in the ADAM-17 digestion experiments as shown in the previous section.

In addition to the salt effect, applied voltage bias also played an important role in the performance of the nanopore sensor.[32] For this purpose, nanopore detection of ADAM-17 was further performed at +80 mV and +100 mV. As shown in Fig. 3, with a decrease in the applied potential from +120 mV to +80 mV, the normalized event mean residual current for the substrate cleavage products was almost unchanged (from 48.6 ± 1.0% to 46.9 ± 0.5 % of channel block), the residence time decreased by 17% (from 0.83 ± 0.02 ms to 0.71 ± 0.05 ms), while the frequency decreased by 115% (from 2.54 s^{-1} to 1.18 s^{-1}). Clearly, a larger applied voltage bias led to a higher resolution and a better performance of the nanopore sensor. However, under the asymmetric electrolyte buffer condition, as the applied voltage bias increased to more than +120 mV, the protein nanopore sensing system became less stable. Therefore, +120 mV was deemed as the optimum voltage and used for subsequent experiments. It should be noted that, unlike DNA, which generally has a smaller event residence time with an increasing applied potential bias, peptides usually show a biphasic voltage-dependence, as reported by Movileanu et al.[33]

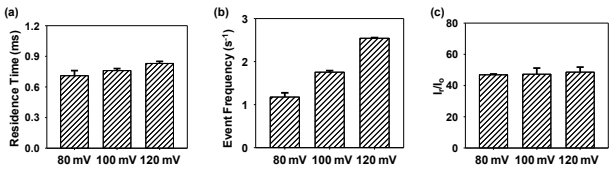


Figure 3. Effect of applied voltage bias on current blocking events. (a) Residence time; (b) event frequency; and (c) blockage amplitude. The experiments were performed in the presence of 5 μM peptide and 100 ng/mL ADAM-17 under asymmetric electrolyte conditions, where the *cis* chamber compartment contained a solution comprising 3 M NaCl and 10 mM Tris (pH 7.5), while the *trans* compartment contained a solution comprising 1 M NaCl and 10 mM Tris (pH 7.5). Only the events attributed to the substrate cleavage products were included in data analysis.

It should be noted that, in a proteolytic reaction with given conditions of substrate and enzyme concentration, the longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. However, the product formation rate is not a simple linear function of the incubation time since all proteins suffer denaturation and hence loss of catalytic activity with time. To achieve sensitive and rapid detection of ADAM-17, a series of peptide substrate / ADAM-17 digestion experiments was performed with incubation time ranging from 15 min to 120 min. The results were summarized in Fig. 4. We found that the event frequency for the digestion products increased with an increase in the incubation time until 60 min, after which the frequency began to saturate. Hence, 60 min was deemed as the optimum incubation time and used for subsequent experiments.

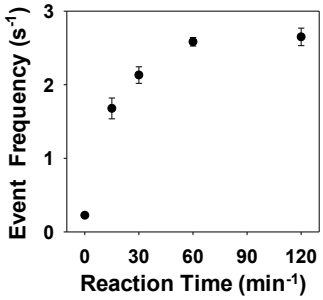


Figure 4. The effect of incubation time on the event frequency of the substrate digestion products. The experiments were performed at +120 mV in a salt

gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate and 100 ng/mL ADAM-17.

3.3. Dose-response curve

Under the current experimental conditions (in 3 M NaCl (*cis*) / 1 M NaCl (*trans*) solutions, at an applied voltage bias of +120 mV, and with 60 min incubation time), dose response curve for ADAM-17 was constructed by monitoring the cleavage of the peptide substrate by ADAM-17 at various concentrations, ranging from 2 ng/mL to 100 ng/mL. Our experimental results (Fig. 5) showed that the event frequency of the cleavage products linearly increased with the ADAM-17 concentration. The detection limit of the nanopore sensor (defined as the concentration of ADAM-17 corresponding to three times the standard deviation of the blank signal) in a 5-minute electrical recording was 0.15 ng/mL (equivalent to 2.88 pM). As far as we are aware, such a detection limit is much better than that (2 ng mL^{-1}) obtained with ELISA[34]. This detection limit is more than good enough for analyzing ADAM-17 in clinical samples (note that the serum level of ADAM-17 in patients with colorectal cancer ranged from 0.4 to 9.8 ng/mL[35]).

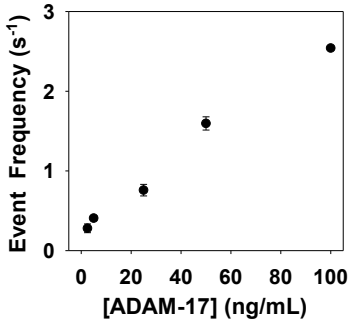


Figure 5. Plot of event frequency as a function of ADAM-17 concentration. The experiments were performed at +120 mV in a salt gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate.

3.4. Selectivity

Two proteases, including ADAM-9 and matrix metalloproteinases 9 (MMP-9), which have similar structures to that of ADAM-17, were selected as potential interfering species to examine the cross-reactivity of the nanopore sensor since they share similar structures to ADAM-17.[36] In addition, ADAM-17 (in the absence of Zn^{2+}) was used as a control (note that ADAM-17 needs metal ions such as Zn^{2+} to be activated). The experimental results were summarized in Fig. 6. We found that all the three protease samples (100 ng/mL each) produced significantly smaller event frequency than ADAM-17, thus suggesting the high selectivity and specificity of our nanopore sensor.

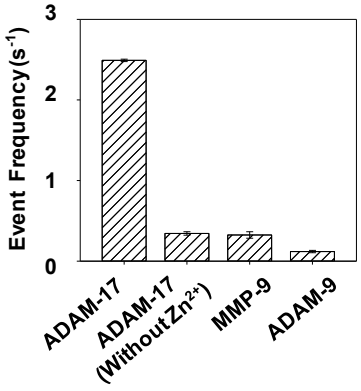


Figure 6. Selectivity of the nanopore ADAM-17 sensor. The experiments were performed at +120 mV in a salt gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate. The concentrations of ADAM-17, MMP-9, and ADAM-9 used were 100 ng/mL each.

4. Conclusions

In summary, by monitoring the cleavage of a substrate peptide by ADAM-17 and taking advantage of ionic strength and salt gradient, we developed a highly sensitive and selective nanopore sensor for the detection of ADAM-17. In spite of the good limit of detection (2.88 pM), the performance of the nanopore sensor has the potential to be further improved by employing engineered αHL protein nanopores such as $(M_{113}F)_7$ [37] instead of the wild-type pore. It can be visualized that, with the same sensing strategy, we can readily develop a variety of

nanopore sensors for other ADAMs and MMPs by varying the substrate peptides. Moreover, by taking advantage of our pioneered nanopore sensor array technique,[38] these individual ADAM / MMP sensors can be further utilized to construct a multiplex nanopore sensor to profile ADAMs and MMPs in human serum. Given the importance of MMPs/ADAMs as valuable biomarkers and potential therapeutic targets for the early detection and treatment of human cancers, our developed nanopore sensing strategy should find useful applications in disease diagnosis and drug screening.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Additional figures, including a typical trace segment of ADAM-17 in the wild-type α-hemolysin protein nanopore, translocation of peptide RSSSARLVFF and peptide LAQAV in the wild-type α-hemolysin protein nanopore, and nanopore analysis of peptide RSSSARLVFF in various salt solutions.

AUTHOR INFORMATION

Corresponding author

*Tel: 312-567-8922. Fax: 312-567-3494. E-mail: xguan5@iit.edu.

Notes

The authors declare no competing financial interests.

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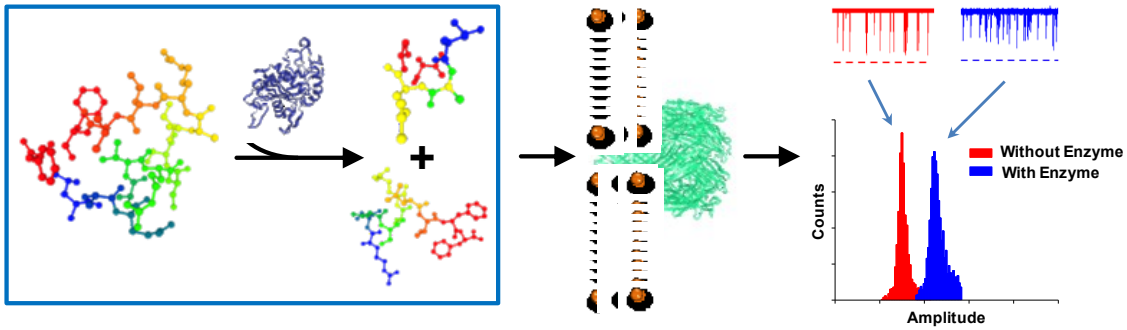

Scheme 1. The cleavage of a peptide substrate by ADAM-17 produces a new type of events in a nanopore, which could be utilized to measure the protease's activity.

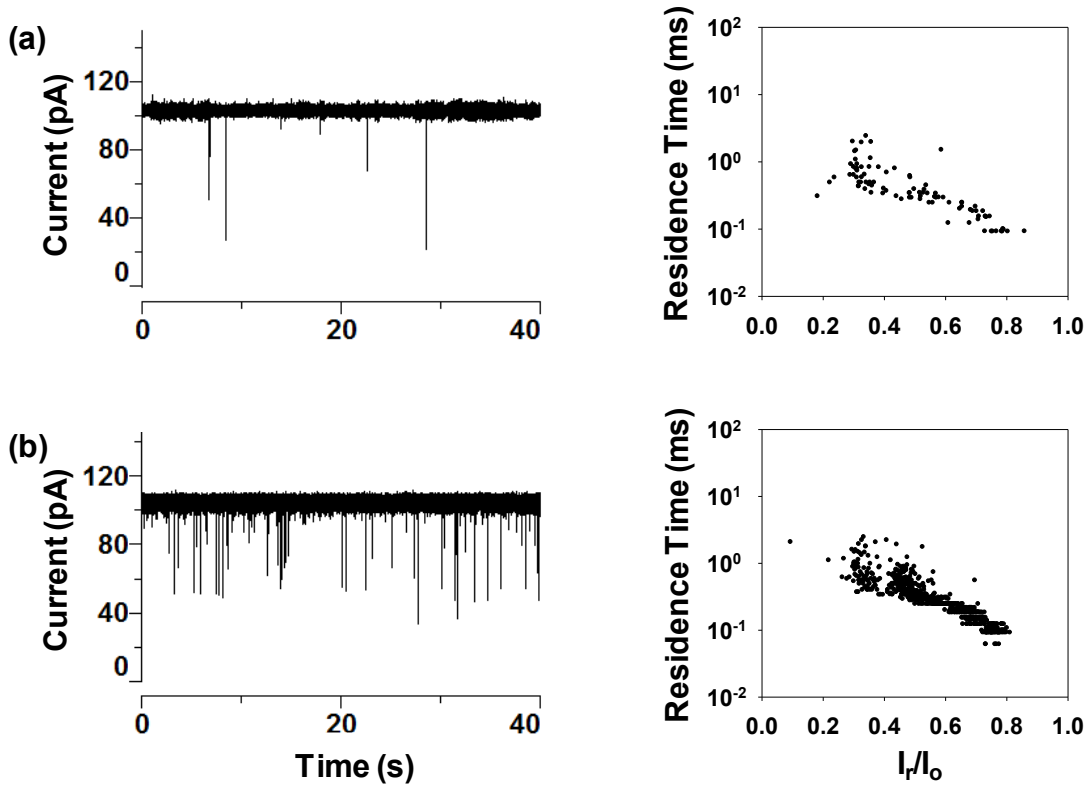

Figure 1. Detection of ADAM-17 in the wild-type α-hemolysin protein nanopore. (a) Without; and (b) with ADAM-17. (*Left*) Typical 40-s trace segments; (*Right*) the corresponding scatter plots of event residence time vs. current blockage amplitude. I_b/I_o in Figs. 1a and 1b is normalized blockage residual current, which was obtained by dividing the average blockage residual current of an event by the average open channel current. The experiments were performed at +120 mV under a symmetric electrolyte condition with both the *cis* and *trans* chamber compartments filled with a solution containing 1.0 M NaCl and 10 mM Tris HCl (pH7.5) and in the presence of 5 μM peptide substrate or a reaction mixture of peptide substrate (5 μM) and ADAM-17 (100 ng/mL).

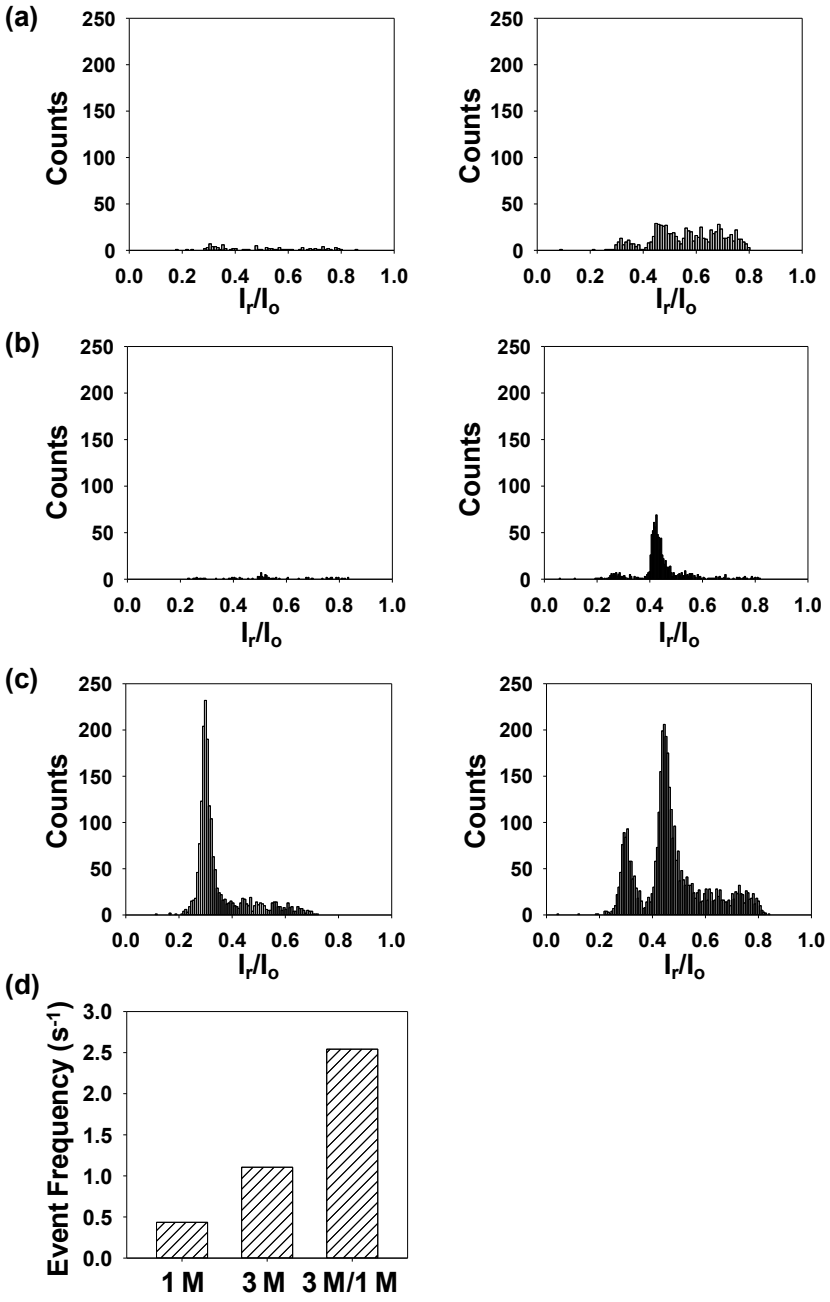

Figure 2. Salt effect on nanopore detection of ADAM-17. (a) 1 M NaCl (*cis*) / 1 M NaCl (*trans*) ; (b) 3 M NaCl (*cis*) / 3 M NaCl (*trans*) ; (c) 3 M NaCl (*cis*) / 1 M NaCl (*trans*); and (d) Plot of event frequency as a function of salt concentration and gradient. (*Left*) without ADAM-17; (*Right*) with ADAM-17. The experiments were performed at +120 mV with the wild-type αHL protein nanopore in the presence of 5 μM peptide substrate or a reaction mixture of peptide substrate (5 μM) and ADAM-17 (100 ng/mL).

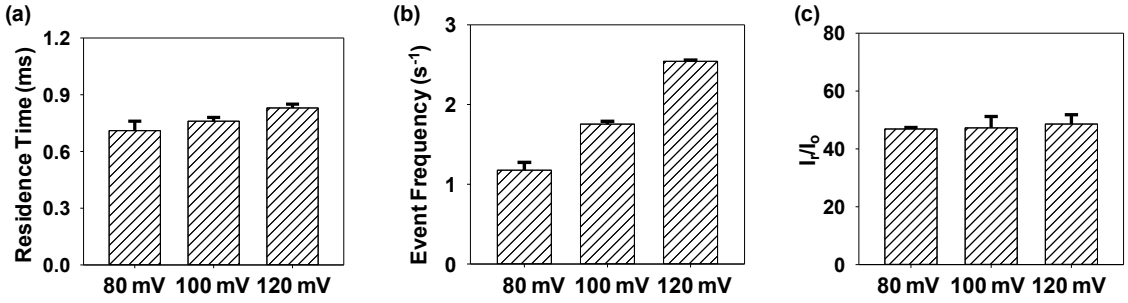

Figure 3. Effect of applied voltage bias on current blocking events. (a) Residence time; (b) event frequency; and (c) blockage amplitude. The experiments were performed in the presence of 5 μM peptide and 100 ng/mL ADAM-17 under asymmetric electrolyte conditions, where the *cis* chamber compartment contained a solution comprising 3 M NaCl and 10 mM Tris (pH 7.5), while the *trans* compartment contained a solution comprising 1 M NaCl and 10 mM Tris (pH 7.5). Only the events attributed to the substrate cleavage products were included in data analysis.

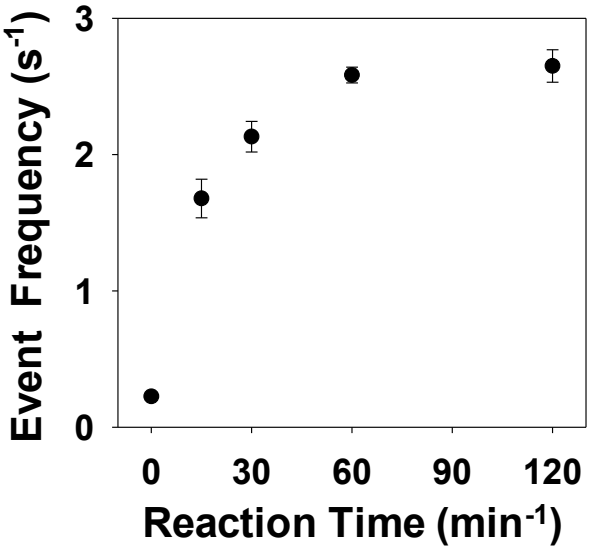

Figure 4. The effect of incubation time on the event frequency of the substrate digestion products. The experiments were performed at +120 mV in a salt gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate and 100 ng/mL ADAM-17.

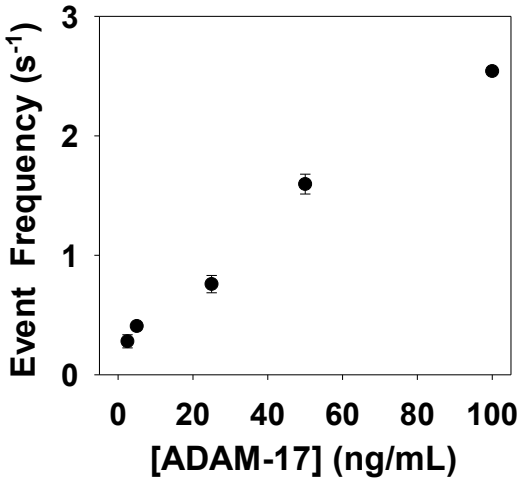

Figure 5. Plot of event frequency as a function of ADAM-17 concentration. The experiments were performed at +120 mV in a salt gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate.

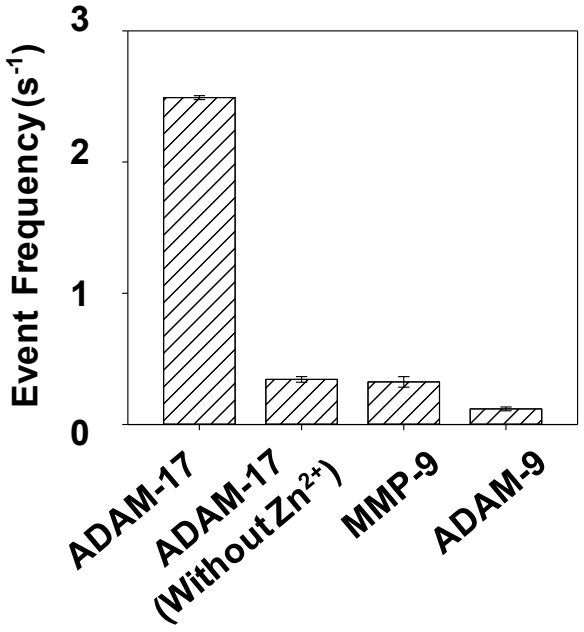

Figure 6. Selectivity of the nanopore ADAM-17 sensor. The experiments were performed at +120 mV in a salt gradient of 3 M NaCl (*cis*) / 1 M NaCl (*trans*) in the presence of 5 μM peptide substrate. The concentrations of ADAM-17, MMP-9, and ADAM-9 used were 100 ng/mL each.

TOC Graphic