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Label-free Sensing of Main Protease Activity of SARS-CoV-2 with an Aerolysin Nanopore

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Abstract: The main protease (M^{pro}), which is highly conserved and plays a critical role in the replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a natural biomarker for SARS-CoV-2. Accurate assessment of the M^{pro} activity is crucial for the detection of SARS-CoV-2. Herein, we report a nanopore-based sensing strategy that uses an enzyme-catalyzed cleavage reaction of a peptide substrate to measure the M^{pro} activity. The peptide was specifically cleaved by the M^{pro}, thereby releasing the output products that, when translocated through aerolysin, quantitatively produced the signature current events. The proposed method exhibited high sensitivity, allowing the detection of M^{pro} concentrations as low as 1 nM without the use of any signal amplification techniques. This simple, convenient, and label-free nanopore assay may expand the diagnostic tools for viruses.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-caused coronavirus disease 2019 (COVID-19) pandemic continues to pose a threat to the world's economy and health.[1] To facilitate early intervention and treatment, which in turn may lower the risk of disease transmission, rapid and early detection of this virus is essential.[2] Currently, quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) is the criterion diagnostic standard for SARS-CoV-2 infection.[3] However, this method usually calls for trained personnel, specialized laboratories, and a protracted turnaround time.[4] Other methods, such as the lateral flow assay and enzyme-linked immunosorbent assay (ELISA), have also been developed; however, these methods yield moderate sensitivity and are prone to false positives.[5] To overcome these limitations, new strategies, such as fluorescent and colorimetric methods, have recently been developed by employing the main protease (M^{pro})-catalyzed cleavage reaction of peptide substrates.[6] The open reading frames of the coronavirus RNA genome specifically encodes the M^{pro}, also known as $3CL^{pro}$, which is also encoded by essential non-structural proteins, for converting the viral precursor polyprotein into functional proteins during viral replication.[7] Notably, the M^{pro} can act as a natural SARS-CoV-2 biomarker, because it is not closely linked to any human protease.[8] While the fluorescent method enables the sensitive measurement of M^{pro} activity and the colorimetric assay enables the visual detection of SARS-CoV-2,[9] they both require tedious labeling and cautious probe designs and have weak anti-interference capabilities. In this context, the accurate, sensitive, and rapid evaluation of M^{pro} activity will offer an efficient tool for the detection of SARS-CoV-2.

Nanopore technology is an emerging label-free technique for single-molecule analyses.[10] The basic principle behind nanopore sensing involves monitoring the fluctuations in the ionic current flowing through nanopore that occur when an analyte binds within a pore.[11] Moreover, the identity of the analyte is revealed by the characteristic current signature of the binding, and the analyte concentration is revealed by the frequency of the binding events.[12] Furthermore, the target identity and quantity can be reported by analyzing the resulting single-molecule signatures.[13] To date, nanopore sensing has been extensively employed to detect DNA/RNA,[14] peptides,[15] proteins,[16] enzymes,[17] and host-guest molecules.[18] Because of their small nanocavity volume (approximately 1 nm), aerolysin nanopore in particular are naturally advantageous for capturing and analyzing peptide probes.[19] In recent years, aerolysin has been used to investigate peptides with different charges and to monitor the kinetics of enzymatic degradation.[20] These advancements indicate that the aerolysin nanopore has great potential for application in SARS-CoV-2 detection.

In this study, we developed a facile, sensitive, and label-free strategy for detecting SARS-CoV-2 by using aerolysin nanopore to probe M^{pro} activity. A probe containing an enzyme cleavage site was designed to act as a protease substrate. The substrate peptide and digestion fragment by M^{pro}-catalyzed cleavage reaction can yield characteristic events when translocated through the aerolysin nanopore. By monitoring the specific transient ionic current modulations, we quantitatively analyzed the enzyme activity. The proposed strategy exhibits exceptional sensitivity and high specificity, and it can be used to detect SARS-CoV-2 in human exhaled breath condensates.

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Results and Discussion

Assay Principle

The principle of aerolysin nanopore detection of M^{pro} is illustrated in Scheme 1. The substrate peptide molecules pass through the nanopore in the absence of the M^{pro}, thereby producing a single signal reading, which is a type of characteristic current modulation event with a unique residence time and blockage amplitude. In contrast, if the M^{pro} is present in the solution, it functions like a pair of scissors and splits the peptide molecules into two fragments, resulting in entirely distinct current modulations than those produced by the substrate peptide. By analyzing the current signal frequency of the digestion product, the M^{pro} enzymatic activity can be detected.

Nanopore-Based Sensing of M^{pro} activity

To test the aforementioned hypothesis, we designed a peptide probe named S1 that contains a specific cleavage site, the Leu–Gln (LQ) amide bond, for the target enzyme, M^{pro}.[21] The translocation of S1 through the aerolysin nanopore generated a large number of short single-level events, as represented in Figure 1A. To analyze the current blockage, I_0 and I were defined as the open pore current and the blockage current,[22] respectively, when the analyte was still within the pore. Figure 1B depicts the scatter plot of the current blockage versus the duration, indicating that a majority of blocks possess a wide range of $1\text{-}I/I_0$ from 0.5 to 0.9 and duration time ranging from 0.2 to 2 ms. Presumably, because the volume of M^{pro} is considerably larger than the aerolysin channel diameter,[23] compared to the probe S1, the addition of the enzyme M^{pro} alone produces a few noise-like blocks as a result of the collision of the enzyme molecules with the pore, as illustrated in Figure 1C. Unexpectedly, the simultaneous addition of S1 and M^{pro} allows for short single-level events identical to those observed in the sole presence of S1 solely (Figure 1D). We hypothesized that either no enzymatic reactions occurred or that the current events of the digestion products were identical to and indistinguishable from those of the substrate probe S1.

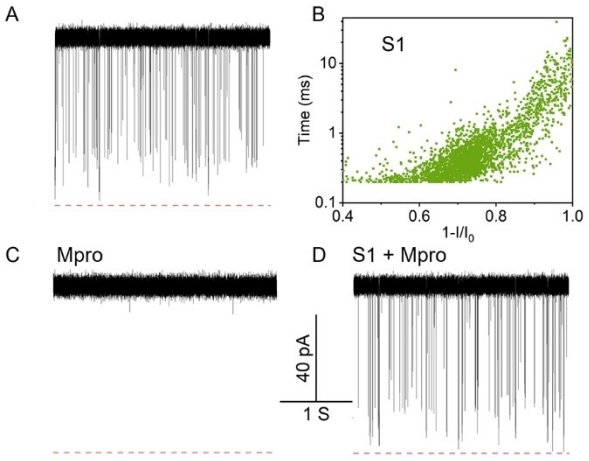
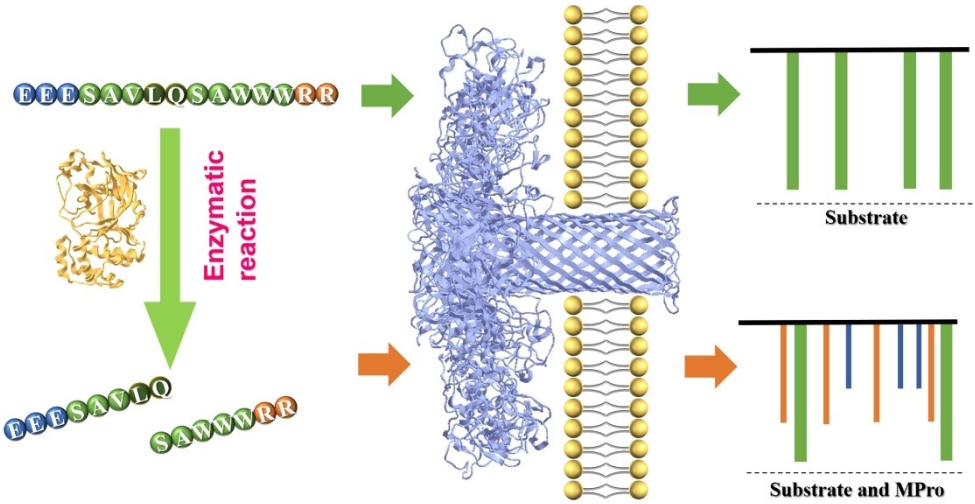


Figure 1. Detection of M^{pro} enzymatic reaction via S1 probe. (A) The current traces of the probe S1. (B) The scatter plot of S1. (C) The current traces of M^{pro}. (D) The current traces of S1 and M^{pro}. Current traces were recorded at +50 mV. The final concentration of S1 was 10 μM.

We addressed this issue by adjusting amino acids in the P3 and P4 regions of the substrate peptide's core cleavage sites (the position of amino acids in substrates named from N- to C-terminal as follows: -P4-P3-P2-P1↓P1′-P2′-P3′P4′).[24] The probe, named S2, was tested in the same experiment under identical conditions. As illustrated in Figure 2A, the addition of S2 caused a large number of moderately long events with higher current blockage: the most probable current blockage and the corresponding duration were approximately 90% and 0.8–3 ms, respectively. As expected, the simultaneous addition of S2 and M^{pro} allowed for the emergence of two new types of single-level events, named S2R and S2E, which are entirely distinct from



Scheme 1. Schematic Illustration of the Nanopore-Based Assay for the Detection of M^{pro} Activity.

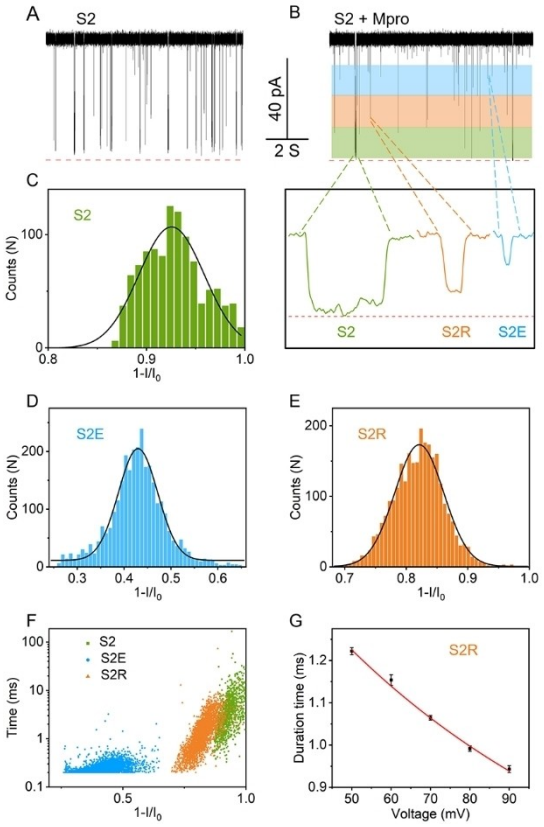


Figure 2. Detection of M^{pro} activity with the nanopore. (A) The current traces of the probe S2. (B) The current traces of the products of M^{pro} digestion reaction. (C–E) 1-I/I_0 histogram of S2E, S2R and S2. Current traces were recorded at +50 mV. The final concentration of S2 was 100 nM. (F) The scatter plot of the products of M^{pro} digestion reaction. (G) Duration time versus applied voltage for the enzyme-catalyzed product S2R. A single-exponential function was used to fit the durations from +50 to +90 mV. Number of individual experiments n = 3.

those observed in the presence of S2 alone (Figures 2B and 2C). Although all of the abolve nanopore assays were performed in 3 M KCl, we also did experiments with 1 M KCl as electrolyte. We found that positively charged peptide (S2R) produced only a few current events at 1 M KCl, far less than that produced at 3 M KCl. Therefore, a high concentration (3 M KCl) was used in the following experiments. According to the results of the statistical analysis, S2 exhibited an 1-I/I_0 of 0.925 ± 0.02 that was fitted to the Gaussian distribution, whereas S2R and S2E exhibited 1-I/I_0 values of 0.821 ± 0.01 and 0.429 ± 0.01, respectively (Figures 2D and 2E). The statistically determined current amplitude was consistent with that of the spiked free S2R and S2E samples (named S2R′ and S2E′), respectively (Figure S1). The positively charged S2R′ peptide is driven through the nanopore at an applied voltage of +50 mV. Generally, there are two driving forces for the peptide to move through the nanopore, including electrophoresis and electro-osmotic flow. Herein, electro-osmotic flow is possibly the main driving force leading to the capture of the positively charged peptide.[25] These results attest to the existence of released S2R and S2E and thus the successful recognition and digestion of S2 via M^{pro} catalysis.

The scatter diagram illustrating the current blockage and duration of probe S2 and the M^{pro}-digested products is depicted in Figure 2F. The S2R and S2E populations were located in regions distinct from that of S2 and were easily distinguishable. Furthermore, as the applied voltage increases from +50 to +90 mV, the duration of the S2R current events displays a strong voltage dependence (Figure 2G), which is consistent with the behavior anticipated for translocation events (Figure S2). The current duration of the S2E events decreases consistently as the voltage increases from +50 to +70 mV (Figure S3). When the voltage continued to increase, its duration time dropped to the level of the transient bumping event (Figure S4), most likely because the S2E strand was too short to be sensed by the nanopore. Accordingly, the current S2R and S2E signals can serve as output signatures for the identification of the M^{pro}.

M^{pro} Detection Sensitivity

An essential factor affecting the sensitivity of the assay is the reaction time of the enzymatic reaction, the products of which are used to quantify the concentration of the M^{pro}. Thus, the change in the frequencies of the signature events in the nanopore test with the reaction time was investigated. The frequency of the signature events of S2R and S2E was divided by the frequency of all translocation events to construct the substrate digestion curve. As demonstrated in Figure 3A and Figure S5, the digestion increases gradually with the reaction time until it plateaus at 60 min. Thus, the optimum incubation time for the enzymatic reaction in subsequent experiments was selected as 60 min.

Under optimal conditions, the sensitivity of the proposed assay was assessed by monitoring the variance in signal events at various M^{pro} concentrations. Using a single nanopore for each analyte, continuous recordings were performed for 5 min to eliminate the effect of time-dependent data. Upon increasing the target concentration from 1 nM to 10 μM, the characteristic current events increase consistently (Figure S6). In this wide range, the corresponding calibration plots exhibit a strong linear correlation between the digestion and logarithm of the M^{pro} concentration (Figure 4B). The linear equation can be expressed as the function $y = 1.879 + 0.199 \log \times (R^2 = 0.991)$,

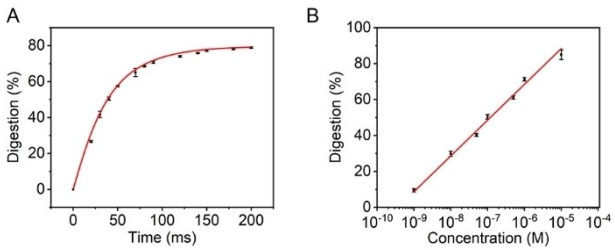


Figure 3. Time and concentration-response curve for M^{pro} detection. (A) Correlation of the digestion with the duration of M^{pro} enzymatic reaction. (B) Correlation of the digestion with the concentration of M^{pro}. Data were recorded at +50 mV. S2 final concentration was 100 nM. Number of individual experiments n = 3.

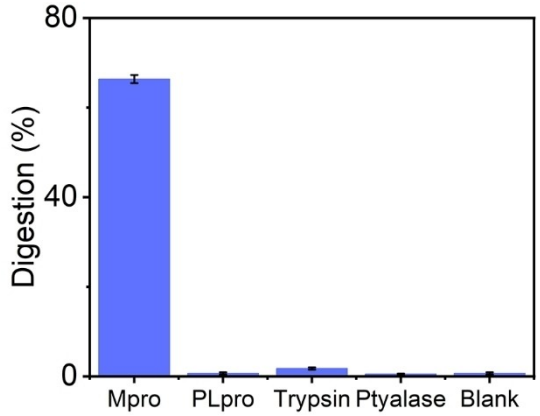


Figure 4. Investigation of the selectivity of the assay. Comparison of the digestion generated by different enzymes. The final concentration of enzymes was 1 μM. Number of individual experiments n = 3.

where y represents the digestion and × denotes the M^{pro} concentration. Notably, the M^{pro} was detected in this assay at concentrations as low as 1 nM. The high rate of peptide capture by the aerolysin nanopore, in addition to the suitable substrate peptide, is likely responsible for the remarkable sensitivity. Thus, their effective combination allows for the trace detection of the M^{pro}.

M^{pro} Detection Selectivity

Different proteases, such as papain-like protease (PL^{pro}), ptyalase, and trypsin, were detected to assess the selectivity of the proposed assay for M^{pro}. PL^{pro} is essential non-structural proteins (NSPs) for processing the viral precursor polyprotein to form functional proteins during viral replication and can cleave polypeptides containing RLRGG/K.[8] Ptyalase promotes starch digestion in the human mouth.[26] Trypsin can specifically digest peptide that contain R or K. As indicated in Figure 4, S2R and S2E translocation blocks are solely produced in response to the M^{pro}. In contrast, the addition of other enzymes generates a few signature events at a frequency that is comparable to that of the control group. As for the trypsin, it can specifically digest peptide on the position of R. However, in our work, the amino acid R is located at the end of the peptide substrate S2. When cleaved by the trypsin, only one amino acid R is released, which cannot be detected by the nanopore and therefore does not produce interfering current signals.[15b] Thus, the adopted approach exhibits extraordinary selectivity for the M^{pro}, which is attributed to the high specificity of the M^{pro}-catalyzed reaction (Figure 4).

Real Sample Assay

To further evaluate the applicability of the strategy in practical scenarios, a series of different amounts of M^{pro} were spiked into the condensate of exhaled breath, mixed with 10 μM peptide substrate and incubated at 37 °C for 60 min. 10 μL of the reaction product was added to the cis chamber. Substrate digestibility was obtained by dividing the characteristic event frequencies of S2R and S2E by the frequencies of all translocation events. Recovery was constructed by dividing the actual digestibility by the corresponding value of the digestibility curve. According to the results presented in Table 1, the recovery ranges from 89.82% to 96.55%, which is within the acceptable range for a real sample assay. Therefore, the nanopore sensing platform has enormous potential for use in complex biological samples.

Conclusion

In conclusion, we developed a nanopore-based strategy for the sensitive evaluation of SARS-CoV-2 M^{pro} activity. By leveraging the efficient cleavage of the peptide probe via M^{pro} catalysis, the information concerning enzyme activity was converted to a measurable current signal recorded in the aerolysin. To the best of our knowledge, this study offers the first example of SARS-CoV-2 M^{pro} activity detection using a nanopore sensor. This assay opens a new window for SARS-CoV2 diagnosis by enabling simple, label-free analysis with enhanced sensitivity as opposed to the previously approaches.

Experimental Section

Reagents and Chemicals: 1,2-Diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, ≥ 99%) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA), and Decane (anhydrous, ≥ 99%), papain-like protease (PL^{pro}), ptyalase, trypsin, and the main protease (M^{pro}) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The aerolysin was kindly provided by Professor Yi-Tao Long (School of Chemistry and Chemical Engineering, Nanjing University, China). All the peptide samples were synthesized and purified via high-performance liquid chromatography at GL Biochem Ltd. (Shanghai, China). The peptide sequences used in this assay are listed in Table 2. All the solutions were prepared using ultrapure water (18.2 MΩ·cm at 25 °C) obtained from a Milli-Q Academic A10 system (EMD Millipore, Billerica, MA, USA). Unless otherwise stated, all the chemicals were of analytical grade.

Table 1. Recovery Tests of M^{pro} in Human Exhaled Breath Condensate Samples by the Nanpore-Based Strategy.

Sample	Added [M]	Digestion [%]	Recovery [%]
1	5×10^{-7}	60.37	96.55
2	1×10^{-7}	44.53	91.63
3	5×10^{-8}	38.29	89.82

Table 2. Sequences and properties of the peptides used in this work.

Name	Sequence (N′→C′)	Net charges	Volume
S1	EEEEEGLQSAGGWWRR	-3	2322 A^3
S2	EEESAVLQSAWWWRR	-1	2339 A^3
S2E′	EEESAVLQ	-3	1094 A^3
S2R′	SAWWWRR	+2	1276 A^3

The activation protocol for aerolysin: 500 μl of the proaerolysin solution (2.0 mg/mL) was mixed with 10 μl of tryp-sin–agarose. The obtained mixed solution was slowly rotated at RT for 4 h to convert the inactive proaerolysin (dimer) to the aerolysin monomer protein. Next, the mixture was centrifuged at 10,000 g for 15 min at 25 °C to pellet the trypsin-agarose. Finally, the supernatant was transferred to a new tube and this step was repeated three times to collect the final supernatant.

Preparation of Probe and Detection of M^{pro} activity: First, peptide substrates (S1 and S2) for the M^{pro} and fragments (S2E′ and S2R′) were dissolved in ultrapure water to obtain stock solutions of 100 μM. Subsequently, all the products were stored at −20 °C prior to the nanopore analysis.

Thereafter, peptide substrates dissolved in 20 mM Tris-HCl buffer (pH = 8.0) were diluted with the M^{pro} assay buffer (20 mM Tris-HCl buffer (pH = 8.0) with 150 mM NaCl, 1 mM DTT, and 5% glycerol) to obtain a 100 μL working solution.

Finally, the enzymatic reaction was conducted in 100 μL of a reaction mixture containing 10 μM peptide substrates and 10^{-9} to 10^{-5} M M^{pro}. The reaction mixture was thereafter incubated at 37 °C for 60 min. Finally, the obtained sample was prepared for nanopore analysis.

For experiments with human exhaled breath condensate, normal human exhaled breath condensate was centrifuged at 5000 rpm for 5 min. Then, the supernatants were collected. Exhaled breath condensate supernatants were mixed with 10 μM peptide substrates and M^{pro} for incubation at 37 °C. Other procedures are the same as those described above.

Nanopore Electrical Recording and Data Analysis: The lipid bilayer membrane was formed by spanning a 150 μm orifice in a Delrin bilayer cup (Warner Instruments, Hamden, CT, USA) that was partitioned into cis and trans chambers. Both the chambers were filled with 1 mL buffer solution (3 M KCl, 25 mM HEPES, and 1 mM EDTA; pH = 7.0). The peptide samples were subsequently added 10 μL to cis chamber. The final concentration of the peptide substrate was 100 nM. Furthermore, the current trace was recorded using an integrated patch clamp amplifier (Axon Instruments, Forest City, CA, USA) equipped with a DigiData 1440 A converter (Axon Instruments). The signals were collected using a 5 kHz low-pass Bessel filter at a sampling rate of 10 kHz using a PC running PClamp™ 10.6 software (Axon Instruments). The data analysis was performed using MATLAB® software (R2013b, MathWorks, Natick, MA, USA) software and OriginLab 2019 (OriginLab Corp., Northampton, MA, USA).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords: enzyme · M^{pro} · nanopore · SARS-CoV-2 · sensors

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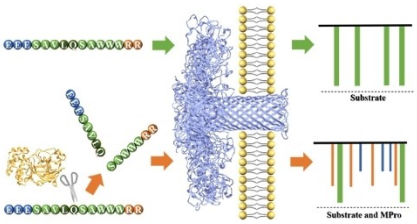
RESEARCH ARTICLE

A nanopore-based sensing strategy was developed that used an enzyme-catalyzed cleavage reaction of a peptide substrate to measure the M^{pro} activity. The peptide was specifically cleaved by M^{pro}, thereby releasing the output products which would quantitatively produce the signature current events upon translocation through aerolysin.



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