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

Xin Zhou, Ruping Tang, Yusen Li, Shusheng Zhang, and Dongmei Xi*[a]

Abstract: The main protease (Mpro), 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 Mpro 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 Mpro activity. The peptide was specifically cleaved by the Mpro, 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 Mpro 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 (Mpro)-catalyzed cleavage reaction of peptide substrates.[6] The open reading frames of the coronavirus RNA genome specifically encodes the Mpro, also known as 3CLpro, which is also encoded by essential non-structural proteins, for converting the viral precursor polyprotein into functional proteins during viral replication.[7] Notably, the Mpro 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 Mpro 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 Mpro 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 Mpro activity. A probe containing an enzyme cleavage site was designed to act as a protease substrate. The substrate peptide and digestion fragment by Mpro-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.

[a] X. Zhou, R. Tang, Y. Li, Prof. Dr. S. Zhang, Prof. Dr. D. Xi
Department Shandong Provincial Key Laboratory of Detection Technology for Tumor Markers
Institution College of Life Sciences
Linyi University, Linyi 276005
(P. R. China)
E-mail: dongmxi@126.com

Supporting information for this article is available on the WWW under https://doi.org/10.1002/asia.202200747

This manuscript is part of a special collection on Nanopore Electrochemistry.
Results and Discussion

Assay Principle

The principle of aerolysin nanopore detection of M\textsuperscript{pro} is illustrated in Scheme 1. The substrate peptide molecules pass through the nanopore in the absence of the M\textsuperscript{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\textsuperscript{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\textsuperscript{pro} enzymatic activity can be detected.

Nanopore-Based Sensing of M\textsuperscript{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\textsuperscript{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\textsubscript{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-I/I\textsubscript{0} from 0.5 to 0.9 and duration time ranging from 0.2 to 2 ms. Presumably, because the volume of M\textsuperscript{pro} is considerably larger than the aerolysin channel diameter,[23] compared to the probe S1, the addition of the enzyme M\textsuperscript{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\textsuperscript{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.

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\textsuperscript{pro} allowed for the emergence of two new types of single-level events, named S2R and S2E, which are entirely distinct from
these results attest to the existence of released S2R and S2E and thus the successful recognition and digestion of S2 via Mpro catalysis.

The scatter diagram illustrating the current blockage and duration of probe S2 and the Mpro-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 Mpro.

Mpro 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 Mpro. 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 Mpro 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 5E). In this wide range, the corresponding calibration plots exhibit a strong linear correlation between the digestion and logarithm of the Mpro concentration (Figure 4B). The linear equation can be expressed as the function $y = 1.879 + 0.199 \log x$ ($R^2 = 0.991$).
proposed assay for \textit{M} \textit{pro} and trypsin, were detected to assess the selectivity of the concentrations as low as 1 nM. The high rate of peptide capture thus, their effective combination allows for the trace detection of the peptide, is likely responsible for the remarkable sensitivity. attributed to the high specificity of the \textit{M} \textit{pro}.

**\textit{M} \textit{pro} Detection Selectivity**

Different proteases, such as papain-like protease (\textit{PL} \textit{pro}), ptyalase, and trypsin, were detected to assess the selectivity of the proposed assay for \textit{M} \textit{pro}. \textit{PL} \textit{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.\textsuperscript{[26]} Ptyalase promotes starch digestion in the human mouth.\textsuperscript{[15b]} 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 \textit{M} \textit{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.\textsuperscript{[15b]} Thus, the adopted approach exhibits extraordinary selectivity for the \textit{M} \textit{pro}, which is attributed to the high specificity of the \textit{M} \textit{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 \textit{M} \textit{pro} were spiked into the condensate of exhaled breath, mixed with 10 \textmu M peptide substrate and incubated at 37 °C for 60 min. 10 \textmu L 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 \textit{M} \textit{pro} activity. By leveraging the efficient cleavage of the peptide probe via \textit{M} \textit{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 \textit{M} \textit{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 (\textit{PL} \textit{pro}), ptyalase, trypsin, and the main protease (\textit{M} \textit{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 \textit{M} \textit{pro} in Human Exhaled Breath Condensate Samples by the Nanopore-Based Strategy. |
| --- | --- | --- |
| Sample | Added [M] | Digestion [%] | Recovery [%] |
| 1 | 5 \times 10^{-7} | 60.37 | 96.55 |
| 2 | 1 \times 10^{-7} | 44.53 | 91.63 |
| 3 | 5 \times 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 | EEEEGQLQAGGWWR | -3 | 2322 A\textsuperscript{f} |
| S2 | EEEAVLQSAWWR | -1 | 2339 A\textsuperscript{f} |
| S2E | EEEAVLQQ | -3 | 1094 A\textsuperscript{f} |
| S2R | SAWWWWR | +2 | 1276 A\textsuperscript{f} |
Collaborative Innovation Center in Universities of Shandong Province (Linyi University). The Open Project Foundation of China (21874062, 22076073, and 21775063), This work was supported by the National Natural Science Foundation of China (21874062, 22076073, and 21775063), E. C. Holmes, Y. Zhang, Nature 2020, 579, 265–269; c) N. Zhu, D. Zhang, W. Wang, X. Li, B. Yang, J. Song, X. Zhao, B. Huang, W. Shi, R. Lu, P. Niu, F. Zhan, X. Ma, D. Wang, W. Xu, G. Wu, G. F. Gao, W. Tan, I. China Novel Coronavirus, T. Research, N. Engl. J. Med. 2020, 382, 727–733.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21874062, 22076073, and 21775063), Collaborative Innovation Center in Universities of Shandong Province (Linyi University), The Open Project Foundation of Shandong (Linyi) Institute of Modern Agriculture, Zhejiang University (No. ZDNY-2021-FWLY0212).

Conflict of Interest

The authors declare no conflict of interest.
[14] a) D. Xi, M. Cui, X. Zhou, X. Zhuge, Y. Ge, Y. Wang, S. Zhang, ACS Sens. 2021, 6, 2691–2699; b) C. Cao, J. Yu, Y. Q. Wang, Y. L. Ying, Y. T. Long, Anal. Chem. 2016, 88, 5046–5049.
[15] a) H. Tang, H. Wang, D. Zhao, M. Cao, Y. Zhou, Y. Li, Anal. Chem. 2022, 94, 5715–5722; b) M. Afshar Bakshloo, S. Yahiaoui, H. Ouldali, M. Pastoriza-Gallego, F. Piguet, A. Oukhaled, Proteomics 2022, 22, e2100056.
[16] M. Afshar Bakshloo, J. J. Kasanowicz, M. Pastoriza-Gallego, J. Mathe, R. Daniel, F. Piguet, A. Oukhaled, J. Am. Chem. Soc. 2022, 144, 2716–2725.
[17] a) X. Chen, Y. Zhang, X. Guan, Nanoscale 2021, 13, 13658–13664; b) Y. Sheng, K. Zhou, L. Liu, H. C. Wu, Angew. Chem. Int. Ed. Engl. 2022, 61, e20220866.
[18] D. Xi, Z. Li, L. Liu, S. Ai, S. Zhang, Anal. Chem. 2018, 90, 1029–1034.
[19] S. Li, C. Cao, J. Yang, Y.-T. Long, ChemElectroChem 2019, 6, 126–129.
[20] H. Ouldali, K. Sarthak, T. Ensslen, F. Piguet, P. Manivet, J. Pelta, J. C. Behrends, A. Aksimentiev, A. Oukhaled, Nat. Biotechnol. 2020, 38, 176–181.
[21] A. Sarkar, K. Mandal, Angew. Chem. Int. Ed. Engl. 2021, 60, 23492–23494.
[22] F. Piguet, T. Ensslen, M. A. Bakshloo, M. Talarimoghari, H. Ouldali, G. Baaken, E. Zaitseva, M. Pastoriza-Gallego, J. C. Behrends, A. Oukhaled, Methods Enzymol. 2021, 649, 587–634.
[23] a) Z. Jin, X. Du, Y. Xu, Y. Deng, M. Liu, Y. Zhao, B. Zhang, X. Li, L. Zhang, C. Peng, Y. Duan, J. Yu, L. Wang, K. Yang, F. Liu, R. Jiang, X. Yang, T. You, X. Liu, X. Yang, F. Bai, H. Liu, X. Liu, L. W. Guddat, W. Xu, G. Xiao, C. Qin, Z. Shi, H. Jiang, Z. Rao, H. Yang, Nature 2020, 582, 289–293; b) C. Cao, Y. T. Long, Acc. Chem. Res. 2018, 51, 331–341.
[24] a) Y. F. Shan, G. J. Xu, Acta Biochim. Biophys. Sin. 2005, 37, 807–813; b) H. T. H. Chan, M. A. Moessler, R. K. Walters, T. R. Malla, R. M. Twidle, T. John, H. M. Deeks, T. Johnston-Wood, V. Mikhailov, R. B. Sessions, W. Dawson, E. Salah, P. Lukacik, C. Strain-Damerell, C. D. Owen, T. Nakajima, K. Swiderski, A. Lodola, V. Moliner, D. R. Glowacki, J. Spencer, M. A. Walsh, C. J. Schofield, L. Genovese, D. K. Shoezmark, A. J. Mulholland, F. Duarte, G. M. Morris, Chem. Sci. 2021, 12, 13686–13703.
[25] M. A. Bakshloo, S. Yahiaoui, F. Piguet, M. Pastoriza-Gallego, R. Daniel, J. Mathé, J. J. Kasanowicz, A. Oukhaled, Nano Res. 2022. https://doi.org/10.1007/s12274-022-4610-1.
[26] A. C. Poole, J. K. Goodrich, N. D. Youngblut, G. G. Luque, A. Ruaud, J. L. Sutter, J. L. Waters, Q. Shi, M. El-Hadidi, L. M. Johnson, H. Y. Bar, D. H. Huson, J. G. Booth, R. E. Ley, Cell Host Microbe 2019, 25, 553–564 e557.
A nanopore-based sensing strategy was developed that used an enzyme-catalyzed cleavage reaction of a peptide substrate to measure the \( \text{M}^\text{pro} \) activity. The peptide was specifically cleaved by \( \text{M}^\text{pro} \), thereby releasing the output products which would quantitatively produce the signature current events upon translocation through aerolysin.