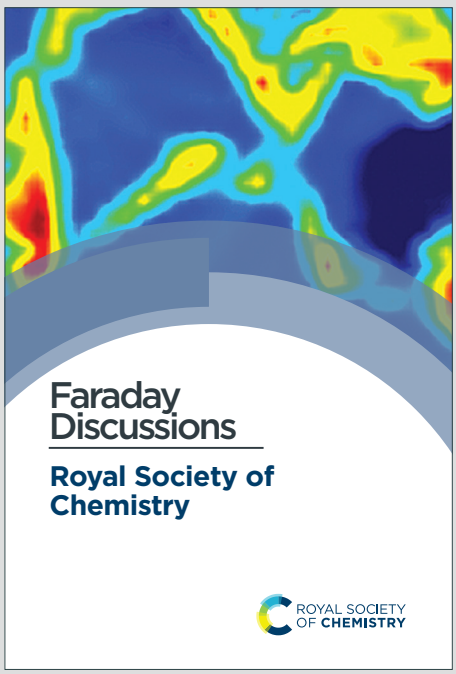
Faraday Discussions

Accepted Manuscript



This article can be cited before page numbers have been issued, to do this please use: L. Wang, S. Zhou, Y. Wang, Y. Wang, J. Li, X. Chen, D. Zhou, L. Liang, B. Yin, Y. Zhang and L. Wang, *Faraday Discuss.*, 2024, DOI: 10.1039/D4FD00146J.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/faraday_d

View Article Online
DOI: 10.1039/D4FD00146J

ARTICLE

Molecular sandwich-based DNAzyme catalytic reaction towards transducing efficient nanopore electrical detection for antigen proteins

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Lebing Wang,[a, b] Shuo Zhou,[c] Yunjiao Wang,[b] Yan Wang,[b] Jing Li,[a, b] Xiaohan Chen,[b] Daming Zhou,[b] Liyuan Liang,[b] Bohua Yin,*[b] Youwen Zhang*[d] and Liang Wang*[b]

Despite significant advances in nanopore nucleic acids sequencing and sensing, proteins detection remains challenging due to the complexity of inherent protein molecular properties (*i.e.*, net charges, polarity, molecular conformation & dimension) and sophisticated environmental parameters (*i.e.*, biofluids), resulting in unsatisfied electrical signal resolution for proteins detection such as poor accessibility, selectivity and sensitivity. The selection of an appropriate electroanalytical approach is strongly desired which should be capable of offering easily detectable and readable signals regarding proteins particularly depending on the practical application. Herein, a molecular sandwich-based DNAzyme catalytic reaction cooperated nanopore detecting approach was designed. Especially, this approach is given the easy use of Mg^{2+} catalyzed DNAzyme (10-23) toward nucleic acids digestion for efficient antigen protein examination. Its applicability within the proposed strategy operates by initial formation of a molecular sandwich containing capture antibody-antigen-detection antibody for efficiently entrapment of target proteins (herein taking HIV p24 antigen for example) and immobilized on magnetic beads surface. After that, the DNAzyme was linked to the detection antibody *via* biotin−streptavidin interaction. In the presence of Mg^{2+}, DNAzyme catalytic reaction was triggered to digest nucleic acids substrates and release unique cleavage fragments as reporters capable of transducing easier detectable nucleic acids as substitute of complicated and difficulty-yielded protein signals, in a nanopore. Notably, experimental validation confirms the detecting stability and sensitivity for target antigen referenced with other antigen proteins, meanwhile demonstrates the detection efficacy in human serum environment at very low concentration (LoD ~ 1.24 pM). This DNAzyme cooperated nanopore electroanalytical approach denotes an advancement in protein examination, may benefit in vitro test of proteinic biomarkers for disease diagnosis and prognosis assessment.

1. Introduction

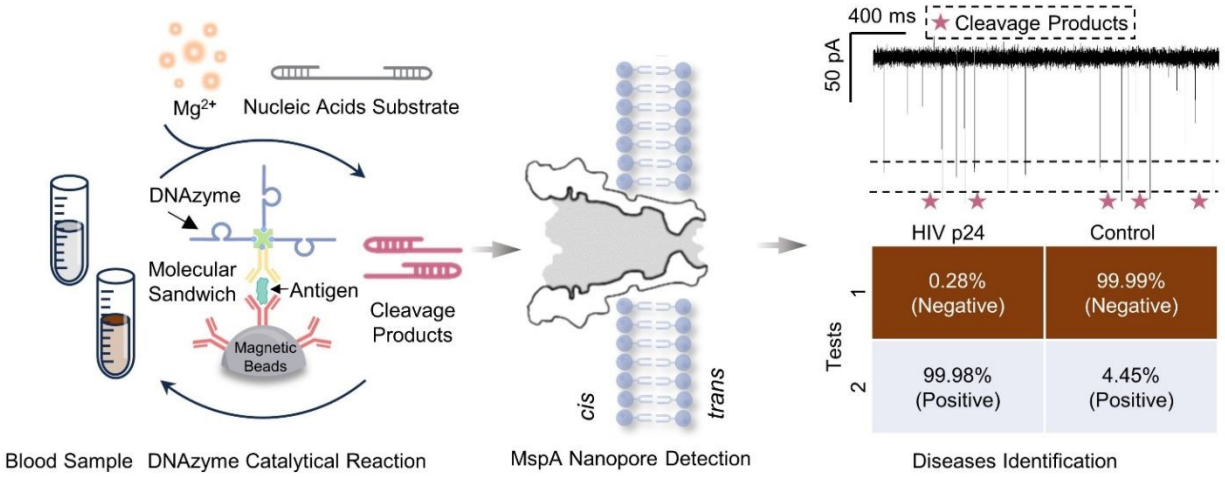
The Covid-19 pandemic facilitates the development of easily accessible and cost-effective antigen protein detections in preventing viral transmission [1]. Nanopore sensing (both solid-state nanopores[2, 3] and biological nanopores[4, 5]) has been demonstrated as one of the most pioneering single-molecule detection techniques[6, 7], which enables to identify individual molecules (*e.g.*, amino acids[8-10], proteins[11-13], and nucleic acids[14, 15]) among a variety of other single entities *via* decoding changes of ionic currents at the moment of molecules communicating with a nanopore channel[16]. Based on this property, nanopore has become a versatile electroanalytical tool for exploring a variety of applications, such as drug development[5, 17], environmental monitoring[18, 19] and disease diagnosis[20-22].

Nanopore is inspired in DNA sequencing and has also contributed greatly to base modifications identification using the most detectible and readable current signals[23-28]. This success has aroused interests in exploring nanopore protein sequencing and examinations. Despite the most recent breakthroughs[9, 29, 30], there are still challenges in nanopore protein sequencing. Compared with the four nucleotide bases and related algorithm combinations in DNA base-calling, the 20 amino acids as well as the chiral isomers and post-translational modifications (PTMs) have led to an exponential increase in the amount of nanopore signal decoding[31-35]. In terms of protein detection, several parameters need to be considered as well, for instance, the complexity of inherent protein molecular properties (*i.e.*, net charges, polarity, molecular dimensions). Meanwhile, each protein has a unique structure and the natural protein conformation is only marginally stable, while the rigid protein structure will unfold into random curls once the sophisticated environmental parameters change (*i.e.*, biofluids, physical and chemical conditions)[36-38]. Therefore, nanopore examination on proteins is more complicated due to the unsatisfied electrical signal resolution, such as poor accessibility, selectivity and sensitivity, compared to those of nucleic acids. In response to the dilemma of direct proteins detecting, the selection of an appropriate electroanalytical approach is strongly desired which should be capable of offering easily detectable and

[a] School of Optoelectronic Engineering, Chongqing University of Posts and Telecommunications, Chongqing 400065, China.
[b] Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences & Chongqing School, The University of Chinese Academy of Sciences, Chongqing, 400714, China.Email:wangliang83@cigit.ac.cn;yinbohua@cigit.ac.cn
[c] Department of Chemistry, University of Missouri, Columbia, MO 65211, USA.
[d] Department of Chemistry & Center for Computational and Integrative Biology, Rutgers University, Camden, NJ 08102, USA.Email:youwen.zhang@rutgers.edu
Supplementary Information available: See DOI: 10.1039/x0xx00000x

readable signals regarding proteins particularly depending on the practical application[39, 40], such as the use of enzymes[41, 42] and host-guest interactions[43, 44], or optimize electrolyte conditions[45].

The DNAzyme (10–23) is the one of the best examples of metal-dependent DNA enzyme that cleaves on RNA sites containing-DNA sequence[46]. It comprises 15-nucleotide (nt) catalytic domain that is flanked on both sides by sub a strate-binding arms that vary in length depending on the sequence of the substrate (Fig. S1a)[47]. DNAzyme (10–23) cleaves substrate at specific sites (Between rG-rU) in the presence of Mg^{2+}, exhibiting a high sequence specificity and catalytic efficiency (Fig. S1b and S1c)[48, 49]. Hence, DNAzyme can be used in various ways to achieve an improved efficacy of detecting biomolecules. In this discussion, we design a DNAzyme cooperated nanopore electroanalytical approach for specific antigenic protein detection (taking HIV p24 antigen as an example) with high sensitivity and selectivity. The approach uses a molecular sandwich containing capture antibody-antigen-detection antibody to chemically entrap target antigens and immobilized on magnetic beads surface. Followed by a DNAzyme (10–23) attached to the detection antibody *via* biotin–streptavidin affinity, the catalytic reaction is triggered to attack on the nucleic acids substrates to yield enzymatic cleavage fragments as reporters. Instead of directly detecting the antigen protein itself, this well-established method is capable of transducing easier detectable nucleic acids as a substitute for the complicated and difficult-yielded protein assay, in a nanopore (Scheme 1). Our results demonstrate here a stable detecting sensitivity for target antigen even interfered with other antigen proteins, meanwhile provide a confidence of detecting low antigen concentrations (LoD : 1.24 pM) in human serum environment. Notably, depending on the selection of DNAzyme-substrate sequences and diseases related antigens-antibodies, these functional oligonucleotides can behave as molecular identification reporters, offering high detection specificity to other protein analytes by transducing easier detectable and readable signals, in nanopores.



Scheme 1 Molecular sandwich-based DNAzyme catalytic reaction enables efficient nanopore electrical detection for antigen proteins. The catalytic reaction is triggered to attack on the nucleic acids substrates to yield enzymatic cleavage fragments as reporters. Instead of directly detecting the antigen protein itself, this well-established method is capable of transducing easier detectable nucleic acids as a substitution of difficulty-yielded protein signals for efficient protein detection in complicated biological matrix, in a nanopore. MspA (PDB data:1UUN).

2. Methods

2.1 Materials and reagents

Recombinant HIV-1 p24 protein (Expressed from Escherichia coli, > 95% SDS-PAGE, ab43037), anti-HIV-1 p24 antibody (capture antibody, ab63958), biotin anti-HIV-1 p24 antibody (detection antibody, ab68617), were purchased from Abcam (USA). Bovine serum albumin (BSA, lyophilized powder, ≥ 98%, ST023) was purchased from Beyotime (China). Streptavidin modified magnetic beads were synthesized at concentration of 4 mg/ml (NEB, Beijing, S1420S). Biotin-labeled DNAzyme and Substrate were purchased from Sangon Biotech, China. Dynabeads™ M-270 carboxylic acid (2×10^9 beads mL^{-1}, supplied in purified water, 14305D) was obtained from Thermo Fisher Scientific (USA). The mutant MspA protein nanopore (M2MspA:D90N/D91N/D93N/D118R/E139K/D134R) is kindly provided by Prof. Jia Geng at Sichuan University, China. All other chemicals were procured from Sigma-Aldrich.

2.2 DNAzyme catalytical reaction

30 μL prepared Sandwich complexes (see Supplementary Methods) were mixed well with $MgCl_2$ solution (20 mM) and 20 μL nucleic acids substrate(seq: AGGCCAAATGGCCTAACCrGrUCATGAGGTCAAGACCTC, 10 μM). Cleavage Products were subsequently released by reacting with a thermal cycler (T100, BIO-RAD, USA) at 95 °C for 5 mins, followed by a temperature cycle of 37 °C for 15 s and 95 °C for 2 s for two hours.

2.3 Nanopore electrical detection

a 150 μm hole in a Teflon membrane was made using a universal spark generator (DAEDALON, SC-EM-09) in order to construct a nanopore, followed by fixing the Teflon membrane in a customized chamber to divide into two compartments (*cis* and *trans*). The formation of bilayer was achieved using the Montal–Mueller method. For nanopore assays, the *cis* side was added with 2 mL 1 M [BMIM]Cl buffer solution, containing 10 mM Tris, pH 7.4 and 2 mL 1.5 M [BMIM]Cl buffer solution, containing 10 mM Tris, pH 7.4 in *trans*

side. Both the analyte sample and MspA protein were added to the *cis* side for nanopore electrical detection. Ion current recordings were performed using clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, America) with a Digidata 1550B A/D converter. Signals were filtered at 2 kHz, recorded at a sampling rate of 100 kHz using Clampfit software (Molecular Devices, Sunnyvale, America), and data were analysed using OriginLab 2021 (OriginLab Corporation, Northampton, Mass. America). All nanopore experiments were performed at room temperature at least three times.

View Article Online
DOI: 10.1039/D4FD00146J

3. Results and Discussion

3.1 Nanopore detection of DNAzyme catalytical reaction

Briefly, our approach makes use of the DNAzyme cooperated nanopore electroanalytical approach for specific antigenic protein detection—that can be divided into two key steps—which we term: (1) DNAzyme catalytic reaction releases detectable nucleic acids

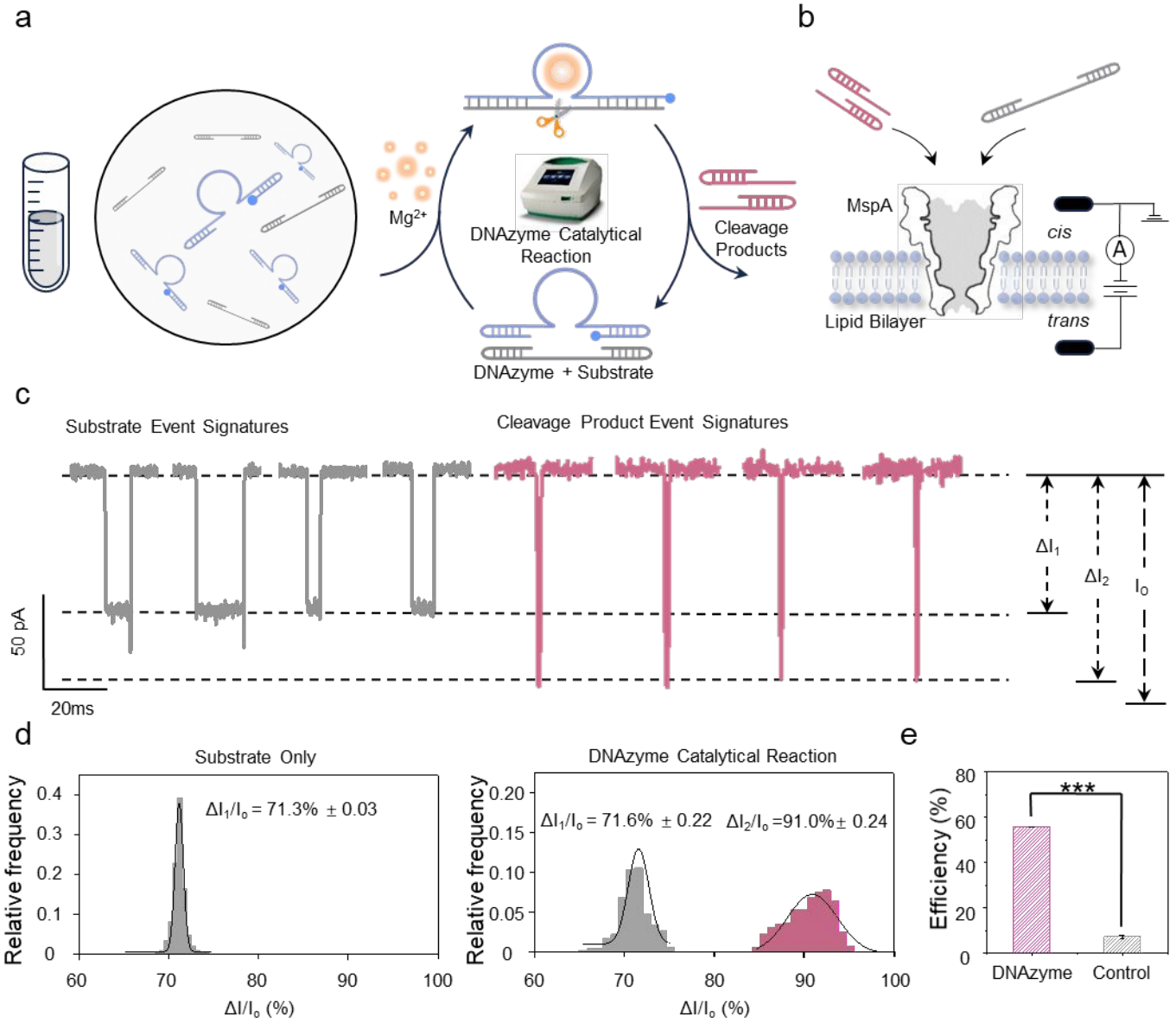


Fig. 1 Nanopore detection of DNAzyme catalytical reaction. (a) DNAzyme catalytical reaction in the presence of Mg^{2+}. (b) Schematic illustration of the detection of nucleic acids reporters released by DNAzyme catalytical reaction by MspA nanopore. The potential across the bilayer is applied to the *trans* side through Ag/AgCl electrodes, while the *cis* side is ground connected. (c) Representative individual current events of nucleic acids substrate and cleavage fragments by MspA nanopore recording. (d) Corresponding statistical analyses of scatter plots of $\Delta I/I_o$, of the nucleic acids substrate and DNAzyme catalytical reaction. (e) Calculation of the catalytical reaction efficiency ($P < 0.001$). All nanopore experiments were performed in asymmetric electrolyte solution (*cis*:1 M [BMIM]Cl & *trans*:1.5 M [BMIM]Cl, 10 mM Tris, pH 7.4). The final concentration of the DNAzyme was 10 nM, nucleic acids substrate was 100 nM. Number of experiments n = 3.

molecular sandwich. Given a high specificity of molecular affinities, the entire approach spontaneously provides the efficient solutions to antigen protein capture and enzyme catalysis for nucleic acids release, as a substitution of difficulty-yielded protein signals for efficient nanopore protein detection in complicated biological matrix, which can further confer antigen proteins quantification.

To confirm the nanopore with reliable detection capability, in the first, we conducted experiments on detecting the nucleic acids reporters in response to DNAzyme catalytical reaction using MspA nanopore, which is extraordinary on DNA/RNA sensing. The specific sequences of the designed DNAzyme and substrate are shown in Fig. S1. As mentioned before, DNAzyme (10-23) recognizes the nucleic acids substrate by complementary Watson–Crick base pairing in the presence of Mg^{2+}, temperature cycling was used to make sure a complete DNAzyme catalytical reaction at the specific sites before nanopore detection (Fig. 1a and b). A constant potential voltage was applied between the two ends of the nanopore to ensure an efficient detection of the target molecules. The target molecules block the nanopore causing a decrease in the current amplitudes, resulting in current blockages (ΔI) in contrast to the open pore current (I_o) by free ion flows through the nanopore without any molecules capture[50, 51]. The representative current traces (Fig. 1c) clearly show the different types of individual event signals. Upon control experiments of detecting the synthesized nucleic acids substrate and cleavage fragments (Fig. S2), the signals of cleavage fragments have a shorter blockage duration as well as a larger current blockage rate ($\Delta I/I_o$) compared to those of substrates. All the current events were statistically analyzed, as shown in Fig. 1 d a n d S 3 . S u b s t r a t e h a s

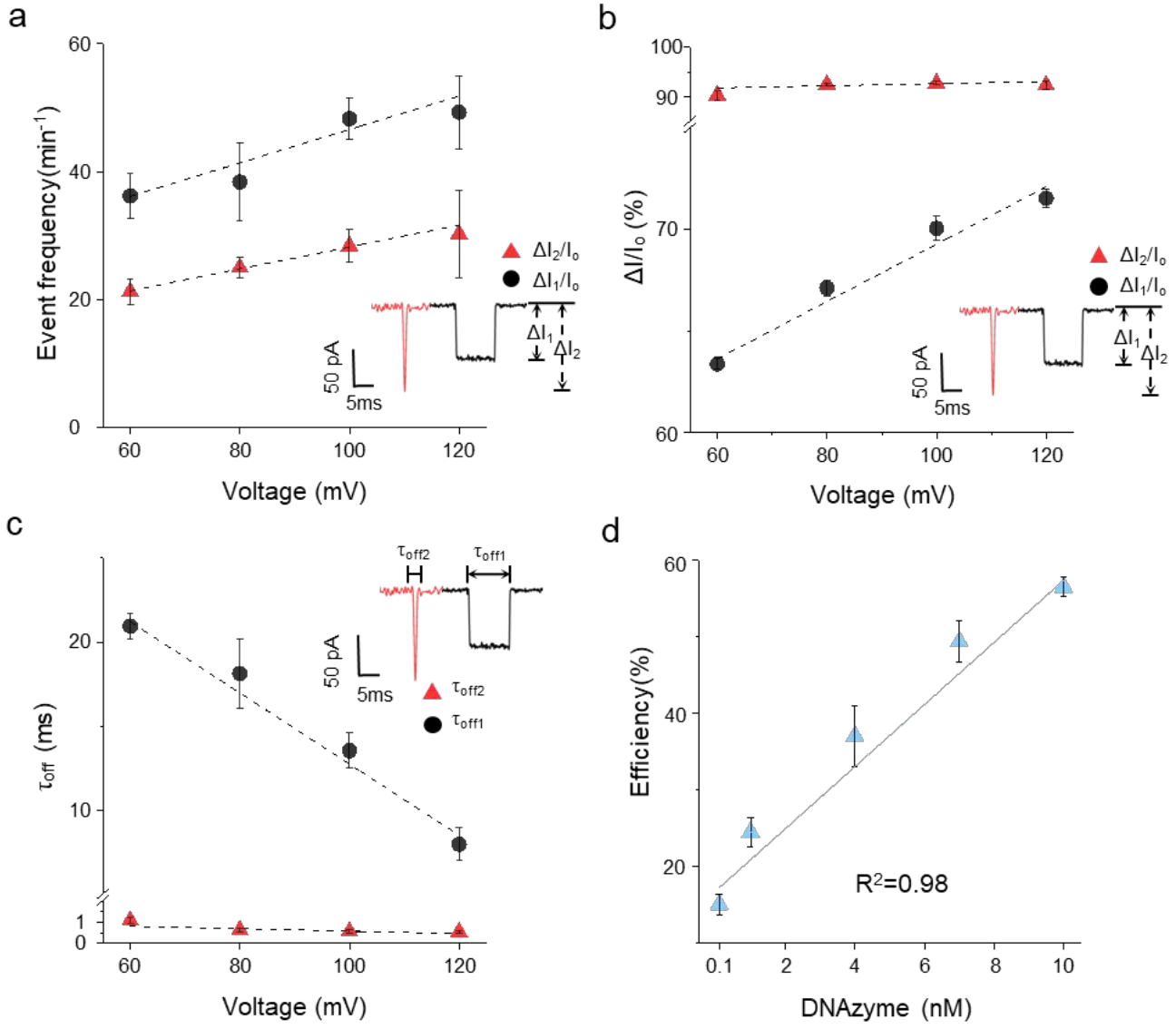


Fig. 2 High-efficiency detection of DNAzyme catalytical reaction. (a) Event frequency (b) $\Delta I/I_o$ (c) τ_{off} (d) DNAzyme concentration-dependent catalysis efficiency. The concentration of the DNAzyme was 10 nM, nucleic acids substrate was 100 nM in a-c. Inset shows the representative individual event signals. Solid line indicates the linear function of cleavage efficiency versus DNAzyme concentrations (R^2 = 0.98). Number of experiments n = 3.

View Article Online
DOI: 10.1039/D4FD00146J

ARTICLE

specific spike-like events showing $\Delta I_1/I_o$ = 71.30 ± 0.03 and τ_{off1} = 7.93 ms. Cleavage fragments produced in DNAzyme catalytical reaction have new specific signals with $\Delta I_2/I_o$ = 91.00 ± 0.24 and τ_{off2} = 0.51 ms, while the same spike-like signals ($\Delta I_1/I_o$ = 71.60 ± 0.22 and τ_{off1} = 7.90 ms) represents the undigested nucleic acids substrates. As implied by the analysis of significant differences for current blockages (Fig. S3, $P < 0.001$), the events signatures confer the effective detection of DNAzyme catalytical reaction, whereas the events differences are attributed to the structure's variation between substrate and cleavage fragments. We presume that the hairpin structure at both ends of substrate (dumbbell-like) resulted in difficulty unravelling the substrate molecules passing through the MspA nanopore. The stay in the vestibular region of MspA makes the substrate interact longer with the nanopore under the action of electric field and consequently caused partially blockades. In contrast, cleavage fragments (two fragments in same length) have single hairpin at one end, which is much easier to unwind and pass through the pore quickly by the electric force, resulting in deeper blockages. Upon the statistical analysis, we hereafter define the signals of $\Delta I/I_o$ 0.65 ~ 0.75 (τ_{off} > 2 ms) as the substrate signals, and those with $\Delta I/I_o$ greater than 0.85 (τ_{off} < 2 ms) as the signals of cleavage fragments. Meanwhile, the efficiency of the catalytical reaction was determined (Fig. 1g, quantified by the ratio of the amount of fragment signals to the total amount of signals, 56.51 %, $P < 0.001$). In the meantime, electrophoresis experiments confirmed the DNAzyme catalytical reactions (Fig. S4). Therefore, the obtained results evidenced that the proposed DNAzyme catalysis can be determined using MspA nanopore.

3.2 High-efficiency detection of DNAzyme catalytical reaction

We then endeavored to determine the high-efficiency detection of DNAzyme catalytical reaction with environmental parameters changes, whereas the external voltage was adjusted from 40 mV to 120 mV. (Fig. S5). As expected, the stable and reliable of nanopore detection of DNAzyme catalytical reaction was observed. The considerably abundant signals of corresponding nucleic acids were sensitively recorded implementing the nanopore detection process (Fig. 2a, maximum at 120 mV, 49.29 events / mins and 30.26 events / mins, respectively). Despite the apparently sensitive detection of DNAzyme catalytical reaction with the strength changes of the electric forces, hereafter, we chose 120 mV as the optimized voltage at the meantime to maintain the stability of the detection system.

The statistical results show that the $\Delta I_1/I_o$ of substrate gradually increases with applied voltages elevated, while the $\Delta I_2/I_o$ of cleavage fragments remains unchanged (Fig. 2b). Meanwhile, τ_{off1} of substrate gradually decreases, while τ_{off2} produced by cleavage fragments remains constant (Fig. 2c). Such drastic changes in the $\Delta I/I_o$ and τ_{off} of substrate is reasonable. Because the limitation of the MspA pore size is extremely difficult for the dual-hairpin substrate to unwind and pass through the nanopore, so it is usual to result in shortening dwell time and deeper current blockages with voltage elevated ($\Delta I_1/I_o$ & τ_{off1}). However, the spike-like events by fragments (much shorter and single-hairpin contained structures) have demonstrated the quick translocation behavior through the nanopore, hence the changes of strength of electric force led to inconsiderable changes in the event signatures ($\Delta I_2/I_o$ & τ_{off2}).

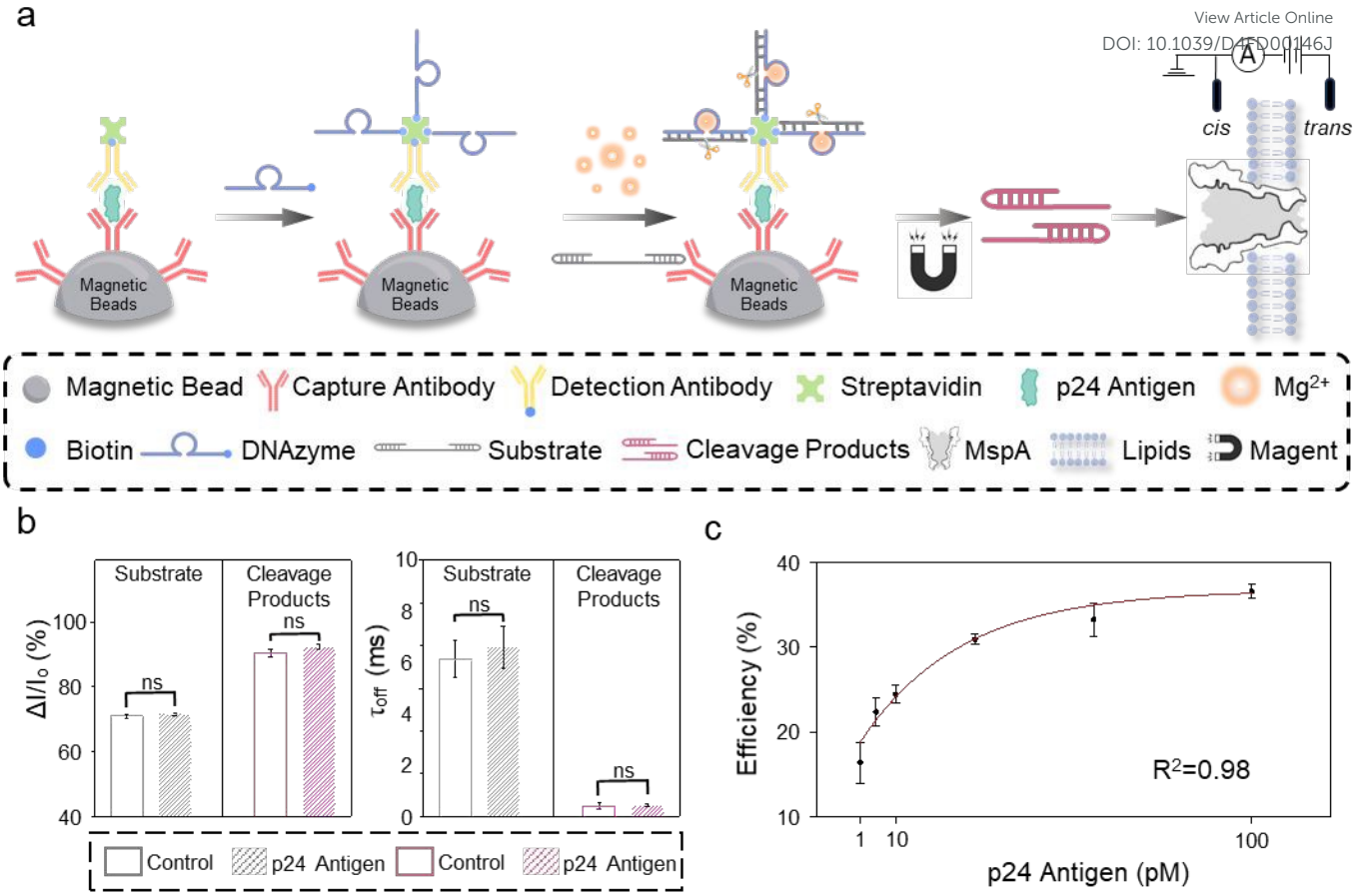
Furthermore, DNAzyme concentration-dependent determination was performed. We found that the event frequency of the cleavage fragments signals (red stars) varies closely with the changes of DNAzyme concentration (Fig. S6). In addition, the calculated cleavage efficiency shows a good linear correlation with DNAzyme concentration (R^2 = 0.98, Fig. 2d). Both the observed event frequencies of fragments and digestion efficiency increases sensitively with more DNAzyme used in the reaction. Therefore, these functional oligonucleotides can behave well as molecular identification reporters, offering high detection stability, specificity and sensitivity by producing more noticeable signals of nucleic acids in DNAzyme catalytical reaction, which benefited from the occurrence nature of current events by the nanopore electrical detection.

3.3 Molecular sandwich-based DNAzyme catalytic reaction towards transducing efficient nanopore electrical detection for antigen proteins

As mentioned previously, the detection of DNAzyme catalytic reaction has been refined through optimizing the electrical and concentration conditions, we next confer the entrapment of target antigens *via* assembly of DNAzyme-functionalized capture antibody-antigen-detection antibody sandwich for nucleic acids release, as a substitute for the complicated and difficult-yielded protein assay. Because the molecular sandwich is used to chemically entrap the target antigens and immobilized on magnetic beads surface for the following catalytic reaction in complicated matrix assay. This high accuracy rate of mag-beads based approach compares favorably to early nanopore experiments, that has been demonstrated with significantly higher efficacy in complicated matrix assay. Therefore, we initially performed additional experiments to verify the reliability of magnetic beads immobilized DNAzyme cleavage (without antigen entrapment) in nanopore detection (Fig. S7). The results showed no variations on signals signatures that evidence the same detectible events as previous nanopore detection for DNAzyme catalysis (Fig. S8).

Next, the molecular sandwich-based DNAzyme catalytic reaction towards transducing efficient nanopore electrical detection for antigen proteins was examined (Fig. 3a). In the case of the antigen detection potentially satisfied the quantification needs in clinical situations, p24 antigen protein was tested at concentrations

View Article Online
DOI: 10.1039/D4FD00146J

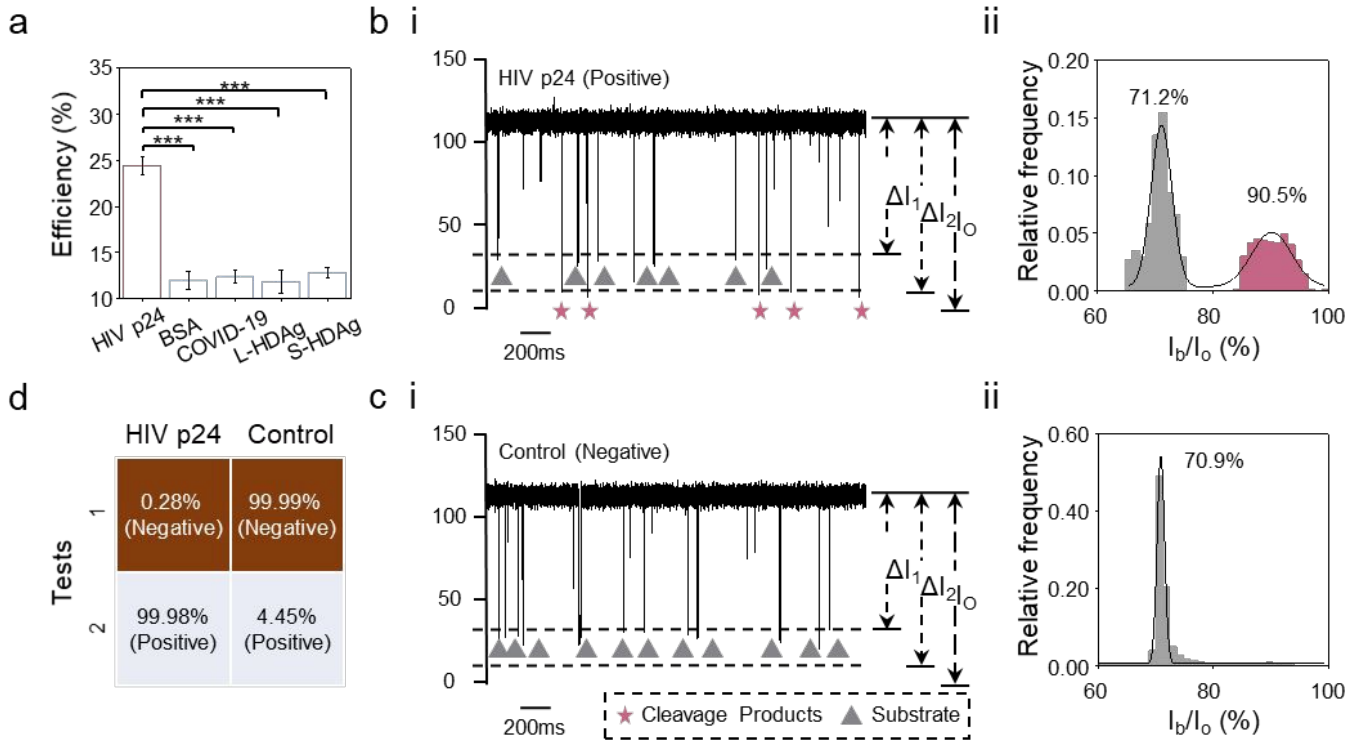


between 1 ~ 100 pM[52-54]. The representative current traces are shown in Fig. S9. As expected, results verified that the frequency of the cleavage fragments events was proportional to the p24 antigen concentration. Fig 3b shows an example of comparing the event signatures between detected nucleic acids released in free DNAzyme catalytical reaction (ctrl.) and sandwich-based transducing system (Fig. 3b). There was no difference between the two key parameters in terms of $\Delta I/I_o$ and τ_{off} ($P > 0.05$). Results confirm that this well-established method is capable of transducing easier detectable nucleic acids as a substitute for the complicated and difficult-yielded antigen protein assay, in a nanopore, instead of directly detecting the target antigen itself. Meanwhile, for sensitivity determination, a calibration curve was established by calculating the cleavage efficiency versus p24 antigen concentrations (Fig. 3c), whereas the assay solution doped with different amounts of recombinant p24 antigen. The experimental results showed a closely positive

Fig. 3 Molecular sandwich-based DNAzyme catalytic reaction towards transducing efficient nanopore electrical detection for antigen proteins. (a) Schematic experimental flow of molecular sandwich-based DNAzyme catalytic reaction towards nucleic acids digestion and nanopore detection, as a substitution of difficulty-yielded protein signals for efficient protein detection in complicated biological matrix. (b) Corresponding statistical analysis of the $\Delta I/I_O$ and τ_{off} in nanopore DNAzyme cleavage detection. p24 antigen represents substrate and cleavage fragments detected in molecular sandwich-based DNAzyme catalytic reaction. Control represents substrate and cleavage fragments released in free DNAzyme catalytic reaction. No significant difference obtained. (c) Efficiency of DNAzyme catalytic reaction as a function of p24 antigen concentration. All experiments were performed in *cis*:1 M [BMIM]Cl *trans*:1.5 M [BMIM]Cl solution containing 10 mM Tris at pH 7.4. Number of experiments n = 3.

View Article Online
DOI: 10.1039/D4FD00146J

ARTICLE



correlation between cleavage efficiency and p24 antigen concentration. Based on the calculation, we obtained the LoD (Limit of Detection) for p24 antigen using the Proficiency Assessment of Clinical Laboratory Measurement Procedures[55], which was determined at 1.24 pM.

3.4 Detection specificity and applicability for target antigens in human serum

In addition, to further validate the selectivity for target antigen detection, Covid-19 Virus, Small- and Large Antigens of Hepatitis Delta (S-HDAg & L-HDAg) as well as Bovine Serum Albumin (BSA) at low concentration (10 pM) were examined only in the presence of HIV p24 antibodies (Fig. S10). Notably, there is no detectable signals corresponding to the cleavage fragments, and displays the highest detection efficiency for p24 antigens ($P < 0.001$, Fig. 4a), indicating no interference entrapment of other antigens for the following catalytic reaction and nanopore detection. That means the approach offers high detection specificity depending on the selection of target antigens-antibodies. Moreover, the next assay revealed that the present molecular sandwich-based DNAzyme-cooperated nanopore sensor can well accomplish the effective detection of p24 antigen (10 pM) contained human serum samples (Fig. 4b and 4c), using these DNAzyme catalysis released easier detectable nucleic acids. Next, we used the mean catalytic efficiency of the HIV p24 group and the Control group as t-test means to analyze the catalytic efficiencies for test groups. For example, the mean t-test of group 1 was 0.28 % ($P < 0.01$), showing a significant difference of the catalytic efficiencies from HIV p24 group, indicating a potential negative result. Additionally, the t-test of 99.99 % ($P > 0.05$) indicates no significant difference from the Control group, further confirm the negative result; In contrast, for group 2, t-test of 99.98 % ($P > 0.05$) indicates no significant difference of the catalytic efficiencies from HIV p24 group, indicating a potential positive result. Similarly, this positive result is further evidenced by additional t-test (4.45 %, $P < 0.05$) showing a significant difference from the Control group. Remarkably,

Fig. 4 Detection specificity and applicability for target antigens in human serum. (a) Detection efficiency for p24 antigens with other antigen proteins ($P < 0.001$). (b) Detection of p24 antigen (10 pM) contained human serum samples (Positive test). (c) Detection of human serum samples without p24 antigen (Negative test). (i) Representative current traces. (ii) Corresponding statistical analyses of $\Delta I/I_O$ of signature signals. (d) The reliability analysis for the quantitative tests. All experiments were performed in *cis*:1 M [BMIM]Cl *trans*:1.5 M [BMIM]Cl solution containing 10 mM Tris at pH 7.4. Number of experiments n = 3.

View Article Online
DOI: 10.1039/D4FD00146J

ARTICLE

the reliability of the tests is demonstrated *via* those quantitative analysis between the signatures of nanopore signals of different samples (Fig. 4d).

4. Conclusions

In this paper, we developed and discussed a molecular sandwich-based DNAzyme-cooperated nanopore sensor which is capable of using easier detectable nucleic acids reporters as a substitution of difficulty-yielded protein signals for efficient protein detection in complicated biological matrix. Our results demonstrate the feasibility and effectiveness of this approach through conducting series of experiments, for example, high-efficiency detection of DNAzyme catalytical reaction in different physical and chemical conditions, selectivity and specificity tests against other antigen proteins, the detection accessibility and reliability in human serum assays at very low concentration (~pM level). Upon the selection of DNAzyme-substrate sequences and diseases-dependent antigens-antibodies, these functional biomolecules can behave as molecular reporters, offering high detection specificity and universal applicability to various proteinic analytes in nanopore examination. Besides, clustered regularly interspaced short palindromic repeats (CRISPR) can precisely recognize and cleave foreign genetic targets towards a specific region of interest in genome through hybridization to a complementary sequence associated with diverse Cas enzyme systems. It is subsequently leveraged for target enrichment to amplify the nucleic acids detection signals. Hence, CRISPR cooperated nanopore detection methods would also be more effective and have the potential to fulfil unmet needs (i.e., single-nucleotide polymorphisms, lower detection specificity) in point-of-care diagnosis. In conclusion, this integrated electroanalytical approach is promising for precise detection of antigenic proteins, paving a new way of advancing biomarkers detection in disease diagnosis, prognosis assessment and nanobiomedical research.

Author Contributions

Lebing Wang: Methodology, Investigation, Data curation, Software, Writing-Original draft preparation. Bohua Yin: Conceptualization, Methodology, Supervision, Writing-Reviewing and Editing. Youwen Zhang: Writing-Reviewing and Editing. Liang Wang: Conceptualization, Methodology, Funding acquisition, Supervision, Project administration, Writing-Reviewing and Editing. All authors contribute to the valuable discussion on the manuscript preparation.

Funding Sources

This work was supported by the National Key Research and Development Program of China (2022YFB3205600), Chongqing Talents-Exceptional Young Talents Project (cstc2021ycjh-bgzxm0016), Natural Science Foundation of Chongqing (CSTB2023NSCQ-MSX0071, cstc2021jcyj-jqX0030); the Youth Innovation Promotion Association (2022388) of Chinese Academy of Sciences. Youwen Zhang would like to thank the financial support from Rutgers Startup Funding (304074).

Conflicts of Interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

Acknowledgments

We would like to thank Chongqing Zhongke Dexin Biotechnology Co. Ltd for the support of instruments and experimental resources.

Notes and References

‡ Electronic Supplementary Information (ESI) available. See DOI: XXX

1. M. Cai, Y. Xie, E. J. Topol and Z. Al-Aly, *Nature Medicine*, 2024, **30**, 1564-1573.
2. M. A. Alibakhshi, X. Kang, D. Clymer, Z. Zhang, A. Vargas, V. Meunier and M. Wanunu, *Advanced Materials*, 2023, **35**.
3. X. Li, T. Zhai, P. Gao, H. Cheng, R. Hou, X. Lou and F. Xia, *Nature Communications*, 2018, **9**.
4. M. B. Steffensen, D. Rotem and H. Bayley, *Nature Chemistry*, 2014, **6**, 603-607.
5. Y. Xing, A. Dorey and S. Howorka, *Advanced Materials*, 2023, **35**.
6. R. Kawano, T. Osaki, H. Sasaki, M. Takinoue, S. Yoshizawa and S. Takeuchi, *Journal of the American Chemical Society*, 2011, **133**, 8474-8477.
7. S. Zhang, M. Andreasen, J. T. Nielsen, L. Liu, E. H. Nielsen, J. Song, G. Ji, F. Sun, T. Skrydstrup, F. Besenbacher, N. C. Nielsen, D. E. Otzen and M. Dong, *Proceedings of the National Academy of Sciences*, 2013, **110**, 2798-2803.
8. C. Yuan, X. Wu, R. Gao, X. Han, Y. Liu, Y. Long and Y. Cui, *Journal of the American Chemical Society*, 2019, **141**, 20187-20197.
9. Y. Zhang, Y. Yi, Z. Li, K. Zhou, L. Liu and H.-C. Wu, *Nature Methods*, 2023, **21**, 102-109.

10. W. Peng, S. Yan, K. Zhou, H.-C. Wu, L. Liu and Y. Zhao, *Nature Communications*, 2023, **14**.
11. H. Brinkerhoff, A. S. W. Kang, J. Liu, A. Aksimentiev and C. Dekker, *Science*, 2021, **374**, 1509-1513.
12. A. Sauciuc, B. Morozzo della Rocca, M. J. Tadema, M. Chinappi and G. Maglia, *Nature Biotechnology*, 2023, DOI: 10.1038/s41587-023-01954-x.
13. P. Waduge, R. Hu, P. Bandarkar, H. Yamazaki, B. Cressiot, Q. Zhao, P. C. Whitford and M. Wanunu, *ACS Nano*, 2017, **11**, 5706-5716.
14. L. Mereuta, A. Asandei, I. Dragomir, J. Park, Y. Park and T. Luchian, *Analytical Chemistry*, 2022, **94**, 8774-8782.
15. I. S. Dragomir, I. C. Bucataru, I. Schiopu and T. Luchian, *Analytical Chemistry*, 2020, **92**, 7800-7807.
16. R. J. Yu, Y. L. Ying, R. Gao and Y. T. Long, *Angewandte Chemie International Edition*, 2018, **58**, 3706-3714.
17. O. Tavassoly, J. Kakish, S. Nokhrin, O. Dmitriev and J. S. Lee, *European Journal of Medicinal Chemistry*, 2014, **88**, 42-54.
18. G. M. Roozbahani, X. Chen, Y. Zhang, R. Xie, R. Ma, D. Li, H. Li and X. Guan, *ACS Sensors*, 2017, **2**, 703-709.
19. G. M. Roozbahani, Y. Zhang, X. Chen, M. H. Soflaee and X. Guan, *The Analyst*, 2019, **144**, 7432-7436.
20. M. Wanunu, T. Dadosh, V. Ray, J. Jin, L. McReynolds and M. Drndić, *Nature Nanotechnology*, 2010, **5**, 807-814.
21. Y. Wang, D. Zheng, Q. Tan, M. X. Wang and L.-Q. Gu, *Nature Nanotechnology*, 2011, **6**, 668-674.
22. L. Chalupowicz, A. Dombrovsky, V. Gaba, N. Luria, M. Reuven, A. Beerman, O. Lachman, O. Dror, G. Nissan and S. Manulis‐Sasson, *Plant Pathology*, 2018, **68**, 229-238.
23. S. Benner, R. J. A. Chen, N. A. Wilson, R. Abu-Shumays, N. Hurt, K. R. Lieberman, D. W. Deamer, W. B. Dunbar and M. Akeson, *Nature Nanotechnology*, 2007, **2**, 718-724.
24. E. A. Manrao, I. M. Derrington, A. H. Laszlo, K. W. Langford, M. K. Hopper, N. Gillgren, M. Pavlenok, M. Niederweis and J. H. Gundlach, *Nature Biotechnology*, 2012, **30**, 349-353.
25. Y. Qing, S. A. Ionescu, G. S. Pulcu and H. Bayley, *Science*, 2018, **361**, 908-912.
26. Y. Qing and H. Bayley, *Journal of the American Chemical Society*, 2021, **143**, 18181-18187.
27. Y. Wang, S. Zhang, W. Jia, P. Fan, L. Wang, X. Li, J. Chen, Z. Cao, X. Du, Y. Liu, K. Wang, C. Hu, J. Zhang, J. Hu, P. Zhang, H.-Y. Chen and S. Huang, *Nature Nanotechnology*, 2022, **17**, 976-983.
28. W. Li, Y. Wang, Y. Xiao, M. Li, Q. Liu, L. Liang, W. Xie, D. Wang, X. Guan and L. Wang, *ACS Applied Materials & Interfaces*, 2022, **14**, 32948-32959.
29. K. Wang, S. Zhang, X. Zhou, X. Yang, X. Li, Y. Wang, P. Fan, Y. Xiao, W. Sun, P. Zhang, W. Li and S. Huang, *Nature Methods*, 2023, **21**, 92-101.
30. M. Zhang, C. Tang, Z. Wang, S. Chen, D. Zhang, K. Li, K. Sun, C. Zhao, Y. Wang, M. Xu, L. Dai, G. Lu, H. Shi, H. Ren, L. Chen and J. Geng, *Nature Methods*, 2024, **21**, 609-618.
31. W. Chen, Y. Chen, Y. Wang, L. Chen, S. Zhou, D. Li, B. Yin, Z. Yang, L. Wang and H. Wang, *Materials Today Physics*, 2024, **42**.
32. X. Wei, T. Penkauskas, J. E. Reiner, C. Kennard, M. J. Uline, Q. Wang, S. Li, A. Aksimentiev, J. W. F. Robertson and C. Liu, *ACS Nano*, 2023, **17**, 16369-16395.
33. J. Wang, J. D. Prajapati, F. Gao, Y.-L. Ying, U. Kleinekathöfer, M. Winterhalter and Y.-T. Long, *Journal of the American Chemical Society*, 2022, **144**, 15072-15078.
34. L. Restrepo-Pérez, C. H. Wong, G. Maglia, C. Dekker and C. Joo, *Nano Letters*, 2019, **19**, 7957-7964.
35. P. Cheng, C. Zhao, Q. Pan, Z. Xiong, Q. Chen, X. Miao and Y. He, *Sensors*, 2024, **24**.
36. D. Japrung, J. Dogan, K. J. Freedman, A. Nadzeyka, S. Bauerdick, T. Albrecht, M. J. Kim, P. Jemth and J. B. Edel, *Analytical Chemistry*, 2013, **85**, 2449-2456.
37. C. Plesa, S. W. Kowalczyk, R. Zinsmeester, A. Y. Grosberg, Y. Rabin and C. Dekker, *Nano Letters*, 2013, **13**, 658-663.
38. K. J. Freedman, S. R. Haq, J. B. Edel, P. Jemth and M. J. Kim, *Scientific Reports*, 2013, **3**.
39. X. Chen, S. Zhou, Y. Wang, L. Zheng, S. Guan, D. Wang, L. Wang and X. Guan, *TrAC Trends in Analytical Chemistry*, 2023, **162**.
40. L. Wang, H. Wang, X. Chen, S. Zhou, Y. Wang and X. Guan, *Biosensors and Bioelectronics*, 2022, **213**.
41. L. Wang, Y. Han, S. Zhou and X. Guan, *Biosensors and Bioelectronics*, 2014, **62**, 158-162.
42. M. Kukwikila and S. Howorka, *Analytical Chemistry*, 2015, **87**, 9149-9154.
43. B. Guo, P. Song, K. Zhou, L. Liu and H.-C. Wu, *Analytical Chemistry*, 2020, **92**, 9405-9411.
44. X. Wei, X. Wang, Z. Zhang, Y. Luo, Z. Wang, W. Xiong, P. K. Jain, J. R. Monnier, H. Wang, T. Y. Hu, C. Tang, H. Albrecht and C. Liu, *Nature Communications*, 2022, **13**.
45. D. Song, Y. Wang, S. Zhang, L. Zhao, L. Gong, L. Liang and L. Wang, *Chinese Journal of Chemistry*, 2023, **41**, 1341-1347.
46. S. W. Santoro and G. F. Joyce, *Proceedings of the National Academy of Sciences*, 1997, **94**, 4262-4266.
47. A. Khvorova and J. K. Watts, *Nature Biotechnology*, 2017, **35**, 238-248.
48. J. Liu and Y. Lu, *Journal of the American Chemical Society*, 2003, **125**, 6642-6643.
49. Y. Wang, K. Nguyen, R. C. Spitale and J. C. Chaput, *Nature Chemistry*, 2021, **13**, 319-326.
50. K. Sun, Y. Ju, C. Chen, P. Zhang, E. Sawyer, Y. Luo and J. Geng, *Small Methods*, 2020, **4**.
51. X. Zhang, D. Zhang, C. Zhao, K. Tian, R. Shi, X. Du, A. J. Burcke, J. Wang, S.-J. Chen and L.-Q. Gu, *Nature Communications*, 2017, **8**.
52. A. M. Anderson, W. R. Tyor, M. J. Mulligan, D. Waldrop-Valverde, J. L. Lennox and S. L. Letendre, *Clinical Infectious Diseases*, 2018, **67**, 137-140.
53. R. Sutthent, N. Gaudart, K. Chokpaibulkit, N. Tanliang, C. Kanoksinsombath and P. Chaisilwatana, *Journal of Clinical Microbiology*, 2003, **41**, 1016-1022.
54. D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak, E. P. Ferrell, J. D. Randall, G. K. Provuncher, D. R. Walt and D. C. Duffy, *Nature Biotechnology*, 2010, **28**, 595-599.
55. D. A. Armbruster and T. A. Pry, *The Clinical biochemist. Reviews*, 2008, **29 Suppl 1**, S49-52.

The data supporting this article have been included as part of the Supplementary Information.

View Article Online
DOI: 10.1039/D4FD00146J