doi:10.2533/chimia.2025.18 Chimia 79 (2025) 18–24

Aerolysin Nanopores for Single-Molecule Analysis

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Abstract: Biological nanopores have become powerful tools for single-molecule analysis in many fields, including metal ion detection, single-molecule chemistry, polymer size discrimination, nucleic acid sequencing, and protein/peptide/glycan analysis. Among all biological nanopores, aerolysin is considered one of the most promising nanopores for analytical applications. It is a heptameric β-barrel pore-forming toxin (β-PFT) secreted by *Aeromonas*, featuring a narrow, elongated β-barrel lumen composed of highly charged amino acids. In this review, we summarize the recent advances of biological nanopores in molecular sensing, sequencing, and their applications in solving biophysical questions, with a focus on aerolysin nanopores.

Keywords: Analytical chemistry · Biomacromolecule sensing · Nanopores · Sequencing · Single-molecule analysis

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Chan Cao studied Applied Chemistry at East China University of Science and Technology, and obtained her PhD in Analytical Chemistry. She then worked as a postdoc with Prof. Matteo Dal Peraro at EPFL, on molecular dynamic simulations. She started her independent research in 2020 at EPFL with a SNSF-PRIMA and Synapsis fellowship. In 2023, she was appointed tenure-track Assistant Professor of Analytical Chemistry at the University of Geneva. Her research focuses on the development of novel analytical approaches to study the molecules of interest at the atomic and molecular levels.

1. Introduction

During the past three decades, nanopore sensing has become a powerful method for single-molecule analysis. Fig. 1 illustrates the basic principle of nanopore technology. It contains a nanometer-sized pore embedded in an insulating membrane that separates two electrolyte-filled chambers (*cis* and *trans*). When an electrical bias is applied across the nanopore, ions move through the pore and generate a constant open pore current (OPC, I_0). When an analyte enters the pore, driven by electrophoresis or electroosmosis, the ionic current can be modulated by the interaction between the analytes and the pore. Changes in ionic current, including dwell time (τ_{on}) and residual current (I_b), are used to reveal the analyte features, while the frequency of the events ($f = 1/\tau_{off}$) indicates its concentration.

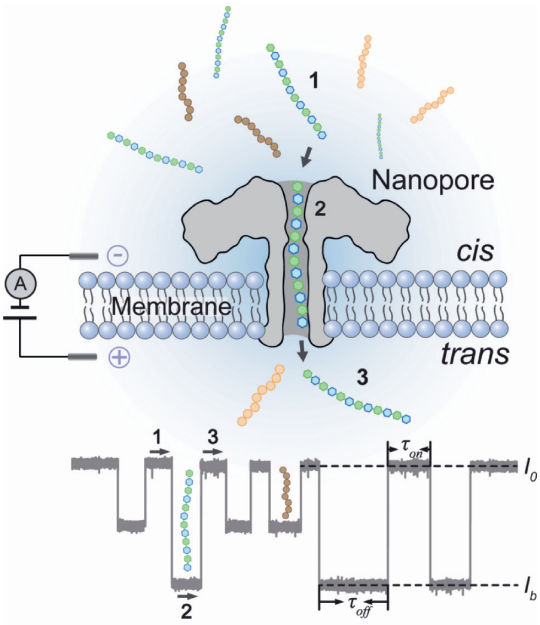

Fig. 1. Schematic of a nanopore set up and ionic current trace. Two electrolyte-filled chambers are separated by an insulating membrane with a nano-sized pore. On applying a constant voltage across the pore, the analytes translocate through the nanopore, generating characteristic ionic current signals. I_0 represents open pore current. I_b is residual current. **1**: Analyte is in solution and the pore is unoccupied. **2**: The analyte enters the pore and ionic current is interrupted. **3**: The analyte exits through the *trans* compartment and the pore returns to its open state.

Since Kasianowicz and coworkers first demonstrated the electrophoretic transport of ssDNA and ssRNA molecules through an α-hemolysin nanopore in 1996,[1] biological nanopores have been widely used for the detection and characterization of diverse molecules, as well as for sequencing DNA at the single-molecule level in a rapid, label-free and highly sensitive manner.[2–4] More importantly, they can be miniaturized into portable devices. Beyond α-hemolysin, a number of pore-forming toxin proteins with different geometries and surface charge properties, such as *Mycobacterium smegmatis* porin A (MspA),[5] aerolysin,[6] and bacterial curli transport lipoprotein (CsgG),[7] have been thoroughly inves-

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tigated and widely utilized as nanopores for sequencing and sensing.[8] Compared to the commonly used α-hemolysin, aerolysin possesses a longer β-barrel, highly charged lumen, narrower constrictions, and lacks a vestibule. All these features provide aerolysin with higher sensitivity and specificity for single-molecule sensing. Therefore, it has been widely used for size discrimination of polymers,[9,10] peptide and protein sensing,[11–14] discrimination of single nucleobases,[15,16] amino acids,[11,12] DNA modifications,[17] polysaccharides,[18] and biomarker detection.[19–22] In this review, we will first focus on recent developments in aerolysin nanopores for sensing and sequencing applications, then summarize their potential in solving biophysics questions.

2. Applications of Aerolysin Nanopore in Macromolecular Sensing

Aerolysin was first used as a biological nanopore sensor to investigate the translocation of α-helical peptides in 2006.[23] Since then, it has been extended to the analysis of oligosaccharides,[18] poly(ethylene glycol) (PEG),[9,10] protein unfolding dynamics,[24] and the kinetics of enzymatic degradation.[25] This section highlights the applications of the aerolysin nanopore in molecular sensing and characterization (Fig. 2).

2.1 *PEG Size Discrimination and Transport Dynamics*

PEGs behave as electrically neutral in LiCl or as positively charged polyelectrolytes in KCl. Additionally, the size versatility of PEGs makes them suitable samples for polymer analysis using nanopores.[9,10,26–28] Krasilnikov and colleagues first demonstrated the size or mass discrimination of PEGs with monomer resolution using the α-hemolysin nanopore as a single-molecule mass spectrometer.[26] The results showed that PEG oligomers could strongly interact with the α-hemolysin nanopore and induce mass-dependent decreases in ionic conductance. Later, in 2015, Baaken *et al*.[9] compared aerolysin with α-hemolysin under high salt conditions (4 M KCl) to explore the capability of aerolysin to discriminate different sizes of PEG molecules (Fig. 2a). Their results showed that aerolysin nanopores provide enhanced mass discrimination, which is attributed to a combination of a high signal-to-noise ratio caused by the large driving force, a steeper mass-conductance relation and prolonged dwell times for small PEGs (20-25 repeat units).[10]

To explore the transport dynamics of macromolecules, Oukhaled's group[10] investigated the interactions of cyclodextrins and PEGs with aerolysin and α-hemolysin under varying applied voltages in two electrolytes (3 M KCl and 3 M LiCl) to elucidate the contributions of electroosmosis and electrophoresis to the translocation dynamics of macromolecules through aerolysin and α-hemolysin nanopores. It was proven that electroosmotic flow (EOF) in the direction of anion flow was the primary force for electrically neutral cyclodextrins in both KCl and LiCl electrolytes. Similarly, non-ionic PEGs in LiCl also exhibited EOF as the main force, with differences between aerolysin and α-hemolysin indicating the influence of pore structure and charge. In contrast, in KCl, PEGs behaved like positively charged polyelectrolytes, with electrophoresis dominating in both aerolysin and α-hemolysin.

2.2 *Applications in Protein and PTMs Analysis*

Proteins and peptides exert crucial roles in all living cells, and importantly post-translational modifications (PTMs), including phosphorylation, glycosylation, methylation, sulfonation, and acetylation, significantly expand the functional diversity of peptides and proteins.[29] Recently, protein PTMs have also emerged as reliable biomarkers for several diseases.[30] Consequently, identifying individual protein species and their PTMs in complex biological samples would greatly benefit cell biology research and clinical diagnostics. Compared to traditional proteome analysis methods, including mass spectrometry and Edman degradation, nanopores offer a powerful tool for protein and PTM detection at the single-molecule level in an inexpensive, label-free manner. Both Maglia's and Oukhaled's groups[13,14] developed a protein

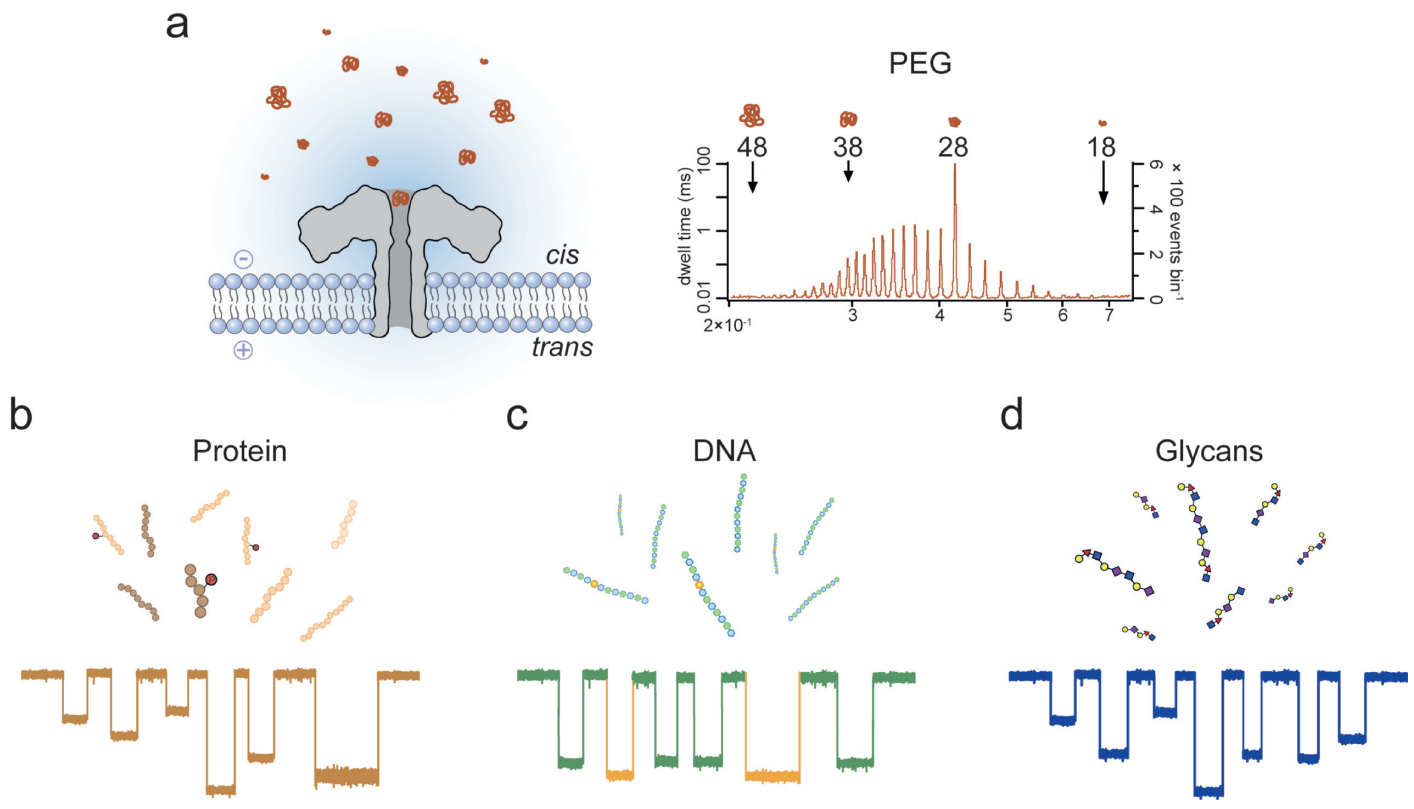

Fig. 2. Macromolecular sensing using aerolysin nanopore. (a) Size discrimination of PEG. Reprinted with permission from *ACS Nano* **2015**, *9*, 6443. Copyright 2015 American Chemical Society. (b) Peptides and PTMs analysis. (c) DNA discrimination. (d) Glycans structural analysis.

fingerprinting method based on nanopore sensing. In their work, protease-digested peptide fragments were converted into current spectra derived from nanopore sensing and then compared to a signal database of known proteins, successfully identifying the original protein. Apart from protein fingerprinting, Pelta and his collaborators also used wild-type aerolysin nanopores to discriminate peptide biomarkers based on their enantiomer or conformation differences.[19,21,22] To enhance the sensing capability of aerolysin nanopore for peptides and nucleic acids, Cao *et al.* designed a series of mutated pores and evaluated them through both molecular simulations and nanopore experiments to study the influence of the mutated sites on pore structure, ion selectivity, and ionic conductance.[31] In 2022, Behrends and Aksimentiev groups developed a method to detect PTMs and their positions by sensing the shape of a fully entrapped peptide using an R220S aerolysin.[32] In our group, we explored an aerolysin mutant to detect various PTMs on proteins associated with neurodegenerative diseases, such as the α-synuclein protein involved in Parkinson's disease.[20] Liao *et al.* and Li *et al.* utilized a mutant aerolysin nanopore for mapping adjacent phosphorylation sites of a single Tau peptide or tyrosine sulfation sites of native peptides.[33,34] In addition to the aerolysin nanopore, Qing and her collaborators utilized electroosmosis in an engineered $(NN\text{-}113R)_7$ α-hemolysin nanopore for the non-enzymatic capture, unfolding, and translocation of individual long polypeptides, aiming to identify and locate serine phosphorylation, cysteine-directed glutathionylation, or glycosylation.[35] They then employed Phos-tag, a molecule that selectively and strongly binds to phosphate monoesters when complexed with zinc ions, to target and locate phosphorylation sites within long polypeptide chains.[36] However, there are still some challenges in transforming biological nanopore platforms for the detection of various PTMs or protein biomarkers in real biofluids. One is that the complex matrix of real biofluids may interfere with the signals of the target molecules. Second, the supporting membranes are fragile and prone to rupture when interfaced with biological samples. The use of more stable membranes, like copolymers[37] and their hybrid forms with lipids,[38] together with robust aperture structures, can provide a stable membrane.[39] Third, it may also be necessary to increase selectivity and sensitivity, as some biomarkers are low-abundance proteins at their physiological concentrations. Therefore, advancements in these research areas could significantly influence the development of new technologies in single-molecule proteomics and diagnostics.

2.3 *DNA Discrimination and Analysis*

In 2016, Cao *et al.*[15] first demonstrated that a wild-type aerolysin nanopore can discriminate the lengths of short oligonucleotides ranging from 2 to 10 nucleotides. They also monitored the stepwise degradation of a dA_5 oligonucleotide by exonuclease I in real-time using the aerolysin nanopore. In subsequent research, Yu *et al.* found that methylated cytosine could also be distinguished from cytosine using the aerolysin nanopore in human serum.[40] The event frequency of methylated cytosine correlates with its relative concentration, facilitating the quantification of methylation. Nevertheless, one obstacle for aerolysin-based nanopore biosensors is the low capture efficiency for long nucleic acids, which significantly lowers the sensitivity of their analytical applications. Wang *et al.*[41] developed a simple strategy to improve the transport of DNA through a remote pH-modulation mechanism. By regulating the pH on one side of the pore, they found the capture rate of target DNA on the opposite side of the pore could be effectively enhanced. At the same time, Liao *et al.*[42] reported a method to directly analyze structured oligonucleotides that are 30 nucleotides long. By replacing the cation K^+ with Mg^{2+} in the electrolyte solution, the structured oligonucleotides are unfolded into a linear form, facilitating their subsequent translocation through the aerolysin nanopore. In this work, the authors utilized Teln (n = 1, 2, 3, 4, and 5) from human telomere sequences, which tend to fold into G-quadruplexes as the model oligonucleotides. The results showed that the length of Teln is proportional to the dwell time, showing a translocation velocity that is hundreds of times slower than that detected with the α-hemolysin nanopore. In addition, they also adopted LiCl to enhance aerolysin's capture ability for long ssDNA.[43] These achievements provide valuable insight into the aerolysin nanopore methodology for genetic and epigenetic biosensing, demonstrating its potential for practical diagnostics involving long and structured nucleic acids.

2.4 *Glycans Structural Analysis*

Glycans and glycoconjugates play vital roles in various biological activities, including signal transduction, cell development, differentiation, immune response, nutrition supply, recognition, structural support, and cancer progression.[44] The structural complexity of glycans makes it challenging to analyze the structure and function of saccharides and glycoconjugates. Nanopore technology offers excellent spatial and temporal resolution for single-molecule analysis. In this context, we present some recent advancements in glycan sensing using aerolysin nanopore.

Glycosaminoglycans (GAGs) are highly anionic linear polysaccharides expressed on the cell surface and in the extracellular matrix.[45] Fennouri *et al.* reported the detection of hyaluronic acid (HA) oligosaccharides and the activity of depolymerization enzymes using the aerolysin nanopores in 2012.[18] They subsequently studied the kinetics of polysaccharide enzymatic degradation by monitoring the enzymatic reaction of HA polysaccharides.[25] Then, Bayat and his colleagues employed a wild-type aerolysin nanopore to characterize different GAG oligosaccharides with various oligomer lengths, sulfate distribution, glycosidic bonds, and uronic acid epimers, which proves the great potential for future development in polysaccharide characterization.[46] Meanwhile, to improve the capability for structural analysis of neutral glycans, Qing's group demonstrated a derivatization strategy to increase the interaction of glycan with the nanopore interface for glycan detection.[47,48] They first used the tag 1-methoxy-4-phenoxybenzene (MPB) to derivatize glycans, which demonstrated its potential to identify diverse glycan isomers, glycans with different monosaccharide numbers, and branched glycans.[47] Then, they introduced an *R*-binaphthyl tag into the glycans. This labeling strategy effectively enhances the cation-π interaction between the derivatized glycan molecules and the nanopore interface, allowing for the effective detection of neutral glycans using a T240R mutant.[48] In addition, they also reported a strategy for discriminating 15 steviol glycosides (SGs) using an EOF-driven capture mechanism and modulating transmembrane voltage, combined with a deep learning method for data analysis.[49] At low voltages, the neutral SG molecules are trapped at the pore entrance by an energy barrier around the R220 sites, allowing for the identification of most SG species. When the voltage exceeds the threshold, the EOF force becomes strong enough for SG molecules to overcome this barrier and pass through the pore, facilitating the identification of several pairs of SGs that differ by only one hydroxyl group. Furthermore, using the nanopore data from 15 SGs, a deep learning-based artificial intelligence model was developed to analyze individual blockage events, which enables rapid, automated, and precise identification and quantification of SGs in real samples. All these results demonstrate the superior capability of the aerolysin nanopore for precise structural analysis of glycans and complex glycosides (Fig. 3).

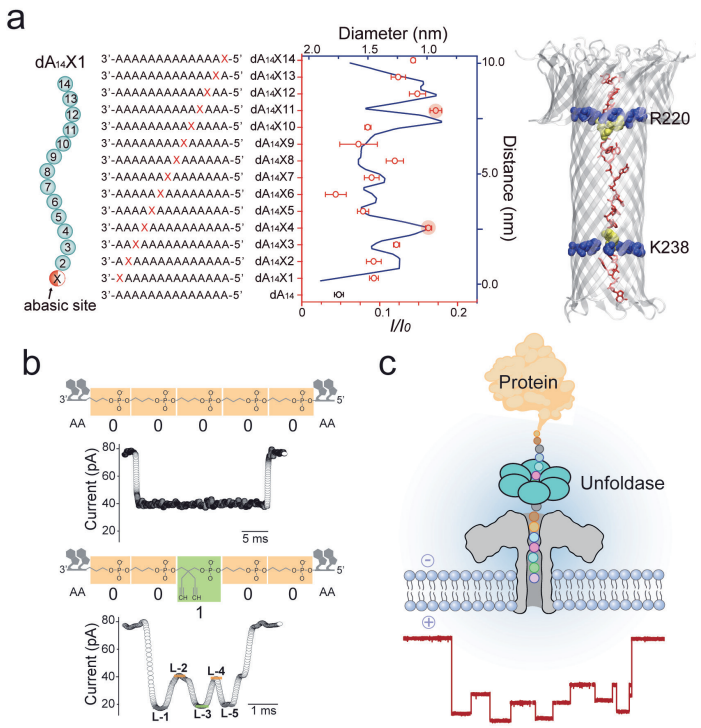

Fig. 3. Applications in polymer sequencing using aerolysin nanopore. (a) Mapping the sensing spots of aerolysin using both experiments and molecular dynamics simulations. Reprinted without modification from *Nat. Commun*. **2018**, *9*, 2823. Copyright 2018 Nature Publishing Group. (b) Aerolysin nanopores decode digital information stored in tailored polymers. From *Sci. Adv*. **2020**, *6*, eabc2661. Reprinted with permission from AAAS. (c) Schematic illustration of single-molecule protein sequencing using aerolysin nanopore and unfoldase.

3. Applications of Aerolysin in Polymer Sequencing

While the nanopore technique was developed to sequence DNA, which is one of the most important biopolymers, to date it has also been applied to sequencing other biopolymers, like polypeptides and proteins, as well as synthetic polymers.

3.1 *Applications in DNA Sequencing*

Since the first nanopore paper on ssDNA and ssRNA,[1] it took several years to realize the identification of a single nucleotide at a specific location on ssDNA,[50] biological nanopore sequencing has undergone significant progress in the accuracy of single-nucleobase identification as well as in controlling the reading speed using molecular motors.[51,52] The first nanopore technology-based commercial device, MinION, was launched by Oxford Nanopore Technologies (ONT) in 2014.[51] Since then, significant advances in DNA nanopore sequencing have led to considerable improvements in accuracy, read length and throughput. The accuracy of nanopore sensing or sequencing largely depends on the geometry and properties of the nanopore. Compared with other commonly used biological nanopores, such as α-hemolysin and MspA, aerolysin has high sensitivity in discriminating very short oligonucleotides due to its small constriction and highly charged β-barrel wall.[6,15] In 2017, Cao *et al.* studied the translocation of four 5'-XAAA-3' oligomers, where X represented A, T, C, or G, through a wild-type aerolysin to explore its capacity for identifying individual nucleobases.[16] The results confirmed that aerolysin can be used to detect single-base variations without any modification, labeling, or enzyme processing steps. To gain a deeper understanding of the high sensitivity of aerolysin for oligonucleotide detection, they combined experimental analysis with molecular simulations to map the sensing spots of aerolysin during ssDNA translocation.[53] The results revealed two critical sensing spots (R220, K238) that generate two constriction points along the pore lumen (Fig. 3a). The narrowest constriction defined by R220, is the most sensitive site for the discrimination of all four types of nucleobases, as well as cytosine methylation and oxidation of guanine in free ssDNA. All these studies demonstrate that aerolysin is a promising candidate to improve the accuracy of DNA sequencing. However, not much work has focused on translating aerolysin in DNA sequencing.

3.2 *Applications in Polymer Sequencing*

DNA molecules are important carriers of genetic information in nature, serving as an ideal medium for next-generation storage systems due to their advantages, such as ultra-high information density and long-term stability. Therefore, strategies for decoding the molecular information in DNA are essential for this application. With the development of nanopore sequencing technology, it has emerged as an effective method for decoding data in DNA storage systems.[54–56] However, DNA is vulnerable to external environmental factors during preservation, such as ultraviolet rays and extreme temperature changes. Synthetic informational polymers present a promising alternative to DNA because of their higher storage density, facile customization synthesis, and stability under harsh conditions.[57] Inspired by these advances, Cao *et al.* demonstrated the ability of engineered aerolysin nanopores to accurately read, with unprecedented resolution, the digital information encoded in tailored informational polymers, both alone and in mixed samples, without compromising information density.[58] As illustrated in Fig. 3b, the biohybrid polymer macromolecules consist of two parts: non-biological monomers, *n*-propyl-phosphate and (2,2-dipropargyl)-propyl-phosphate, representing bit-0 and bit-1, respectively, and the terminal di-deoxyadenosine. The terminal nucleotides in the informational polymer can not only prolong the dwell time but also enhance the resolution of the system to single-bit precision. Combined with deep learning, Cao *et al.* achieved high-accuracy decoding of digital sequences with up to 4 bits of information. These findings open up promising new possibilities to develop writing-reading technologies to process digital data using a bio-inspired platform.

3.3 *Applications in Protein Sequencing*

Inspired by the successful applications in nanopore DNA sequencing, significant efforts have been focused on protein sequencing using nanopores (Fig. 3c). However, compared with DNA, which consists of only four different nucleotides, proteins are more complex, comprising 20 distinct amino acids. The heterogeneous charge distribution along the peptide chains impedes the capture and controlled translocation through nanopores. Furthermore, the degree and type of post-translational modifications increase the challenges of nanopore protein sequencing. In the last decade, significant progress has been made in protein sequencing using biological nanopores.[59,60] Numerous efforts have focused on protein unfolding, controlling the movement of peptides/proteins, and amino acid recognition using nanopores.[11,12,24,37,61,62] Several strategies have been reported to unfold proteins before nanopore translocation, including the use of denaturants,[62] thermal unfolding,[61] or protein unfoldase.[63–65] In order to precisely control their translocation to generate sequence-dependent signals, inspired by the motor enzyme-based control of DNA translocation, two methods have been developed: one is to use established DNA motors to control peptide motion.[66-68] Another is to employ protein motor like ClpX to pull proteins through a nanopore, effectively distinguishing different protein segments. However, these reads are hard to interpret due to ClpX's irregular stepping.[63] Recently, Nivala's group developed a new method for single-molecule reading of long protein strands using nanopores and the ClpX motor protein.[65] By introducing a 'slip sequence', it allows the individual protein to pass through the nanopore multiple times, thereby improving sequencing accu-

racy and signal-to-noise ratio. Apart from electrophoretic forces driving translocation like DNA, EOF caused by the ion selectivity of the pores has also been used to facilitate the unidirectional translocation of peptides or proteins with heterogeneous charge distributions.[35,37,69,70]

However, current biological nanopore techniques still lack sufficient spatiotemporal sensitivity for the discrimination of all 20 proteinogenic amino acids and diverse PTMs. By utilizing the geometry and the highly charged pore wall of the aerolysin nanopore, Piguet *et al.*[11] realized the discrimination of short homopeptides of different lengths at single amino acid resolution. Based on this work, Oukhaled and his colleagues[12] successfully differentiated 13 out of 20 amino acids coupled with a polyarginine carrier using a wild-type aerolysin nanopore, while Xi's group identified 9 amino acids.[71] An alternative approach is sequencing by hydrolysis. In this method, exopeptidase-digested amino acids are read sequentially by a nanopore. Recently, three groups have developed methods for amino acid recognition and short peptide sequencing based on this principle.[72–74] Using this approach, they have been able to distinguish the 20 proteinogenic amino acids and four PTMs with high resolution, as well as successfully sequence short peptides.

4. Applications in Single-Molecule Biophysics Investigation

Beyond sensing and sequencing, the nanopore technique can real-time monitor membrane proteins related biological processes at the single-molecule level, which can offer valuable insights into the function of membrane proteins, and interactions of protein-protein, protein-DNA, or protein-membrane.[75–79] By combining different biochemical and biophysical technologies, we studied the functions of membrane-associated proteins and their interaction with lipids. We have built a high-throughput lipid membrane platform, and together with a second harmonic scattering measurement, we can quantitatively characterize the affinity of pore-forming proteins and lipids.[76] In addition, we showed that Turandot A binds phosphatidylserine on membranes *in vitro* and inhibits the pore-forming activity of Drosophila and human antimicrobial peptides on eukaryotic cells without affecting their microbicidal activity.[78] More recently, we developed an *in vitro* nanopore system to mimic the *in vivo* transmembrane transport of proteins mediated by Hsp70s.[79] These chaperones are central components of the cellular network, however, their underlying physical mechanism remains uncertain. Measurements of the escape time of a substrate polypeptide from the K238A aerolysin nanopore showed that binding of Hsp70 greatly lowered the energy barriers for escape (Fig. 4). Furthermore, we found that this effect relied on the size of the chaperone, providing unambiguous evidence to support the entropic pulling mechanism of Hsp70s. This finding resolved the long-standing debate about whether Hsp70s use entropic pulling, power stroke, or Brownian ratchet for their force generation.

5. Conclusions

After decades of development, nanopore technology has evolved into a powerful tool for sequencing and sensing applications. With the advantages of long read length, high throughput, and portability, it has played significant roles in genomics, and has shown great potential in proteomics, metabolomics, and disease-related studies.[52] Owing to the surface charge distribution and geometry, the aerolysin nanopore has shown superior sensitivity and specificity for discriminating polymer size, single nucleobases, and amino acids, as well as for detecting epigenetic modifications or biomarkers in complex biological samples. However, two common challenges that all biological nanopore sensors still face are their temporal-spatial resolution and platform stability. More efforts and interdisciplinary collaborations are required to develop versatile and accurate stochastic sensors as well as sequencing platforms. With continuing achievements in experimental strategies, microelectronics, and bioinformatics, researchers are expected to make further breakthroughs in developing advanced and functional analytical tools using nanopore devices in the future.

Acknowledgements

We thank Swiss National Science Foundation for their financial support (PR00P3_193090 to C.C.).

Author Contributions

Yun Zhang wrote the manuscript. Chan Cao revised and supervised the manuscript. All authors read and approved the manuscript.

Received: October 31, 2024

1] J. J. Kasianowicz, E. Brandin, D. Branton, D. W. Deamer, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 13770, https://doi.org/10.1073/pnas.93.24.13770.
[2] H. Bayley, P. S. Cremer, *Nature* **2001**, *413*, 226, https://doi.org/10.1038/35093038.
[3] M. Wanunu, *Phys. Life Rev.* **2012**, *9*, 125, https://doi.org/10.1016/j.plrev.2012.05.010.
[4] D. Deamer, M. Akeson, D. Branton, *Nat. Biotechnol.* **2016**, *34*, 518, https://doi.org/10.1038/nbt.3423.
[5] T. Z. Butler, M. Pavlenok, I. M. Derrington, M. Niederweis, J. H. Gundlach, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 20647, https://doi.org/10.1073/pnas.0807514106.
[6] I. Iacovache, S. De Carlo, N. Cirauqui, M. Dal Peraro, F. G. van der Goot, B. Zuber, *Nat. Commun.* **2016**, *7*, 12062, https://doi.org/10.1038/ncomms12062.
[7] P. Goyal, P. V. Krasteva, N. Van Gerven, F. Gubellini, I. Van den Broeck, A. Troupiotis-Tsailaki, W. Jonckheere, G. Péhau-

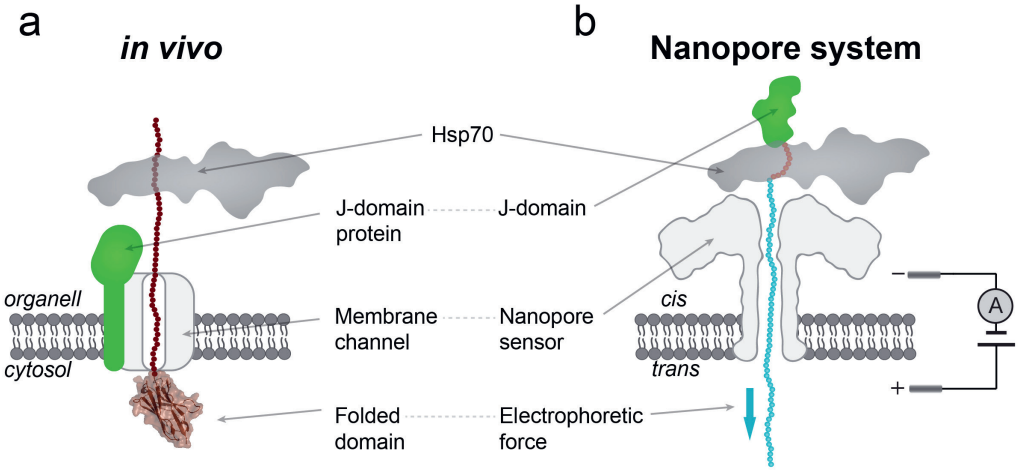

Fig. 4. Studying the force mechanism of Hsp70 chaperones using K238A aerolysin nanopore. (a) Example of *in vivo* protein transport mediated by Hsp70s. (b) The designed system of entropic pulling by Hsp70 chaperones using K238A aerolysin nanopore. Reprinted without modifications from *Nat. Commun.* **2024**, *15*, 8604. Copyright 2024 Nature Publishing Group.

Amaudet, J. S. Pinkner, M. R. Chapman, S. J. Hultgren, S. Howorka, R. Fronzes, H. Remaut, *Nature* **2014**, *516*, 250, https://doi.org/10.1038/nature13768.
[8] W. Shi, A. K. Friedman, L. A. Baker, *Anal. Chem*. **2017**, *89*, 157, https://doi.org/10.1021/acs.analchem.6b04260.
[9] G. Baaken, I. Halimeh, L. Bacri, J. Pelta, A. Oukhaled, J. C. Behrends, *ACS Nano* **2015**, *9*, 6443, https://doi.org/10.1021/acsnano.5b02096.
[10] M. Boukhet, F. Piguet, H. Ouldali, M. Pastoriza-Gallego, J. Pelta, A. Oukhaled, *Nanoscale* **2016**, *8*, 18352, https://doi.org/10.1039/C6NR06936C.
[11] F. Piguet, H. Ouldali, M. Pastoriza-Gallego, P. Manivet, J. Pelta, A. Oukhaled, *Nat. Commun*. **2018**, *9*, 966, https://doi.org/10.1038/s41467-018-03418-2.
[12] H. Ouldali, K. Sarthak, T. Ensslen, F. Piguet, P. Manivet, J. Pelta, J. C. Behrends, A. Aksimentiev, A. Oukhaled, *Nat. Biotechnol.* **2020**, *38*, 176, https://doi.org/10.1038/s41587-019-0345-2.
[13] F. L. R. Lucas, R. C. A. Versloot, L. Yakovlieva, M. T. C. Walvoort, G. Maglia, *Nat. Commun*. **2021**, *12*, 5795, https://doi.org/10.1038/s41467-021-26046-9.
[14] M. Afshar Bakshloo, J. J. Kasianowicz, M. Pastoriza-Gallego, J. Mathé, R. Daniel, F. Piguet, A. Oukhaled, *J. Am. Chem. Soc.* **2022**, *144*, 2716, https://doi.org/10.1021/jacs.1c11758.
[15] C. Cao, Y.-L. Ying, Z.-L. Hu, D.-F. Liao, H. Tian, Y.-T. Long, *Nat. Biotechnol*. **2016**, *11*, 713, https://doi.org/10.1038/nnano.2016.66.
[16] C. Cao, J. Yu, M.-Y. Li, Y.-Q. Wang, H. Tian, Y.-T. Long, *Small* **2017**, *13*, 1702011, https://doi.org/10.1002/smll.201702011.
[17] J. Yu, C. Cao, Y.-T. Long, *Anal. Chem*. **2017**, *89*, 11685, https://doi.org/10.1021/acs.analchem.7b03133.
[18] A. Fennouri, C. Przybylski, M. Pastoriza-Gallego, L. Bacri, L. Auvray, R. Daniel, *ACS Nano* **2012**, *6*, 9672, https://doi.org/10.1021/nn3031047.
[19] A. Stierlen, S. J. Greive, L. Bacri, P. Manivet, B. Cressiot, J. Pelta, *ACS Cent. Sci*. **2023**, *9*, 228, https://doi.org/10.1021/acscentsci.2c01256.
[20] C. Cao, P. Magalhães, L. F. Krapp, J. F. Bada Juarez, S. F. Mayer, V. Rukes, A. Chiki, H. A. Lashuel, M. Dal Peraro, *ACS Nano* **2024**, *18*, 1504, https://doi.org/10.1021/acsnano.3c08623.
[21] S. J. Greive, L. Bacri, B. Cressiot, J. Pelta, *ACS Nano* **2024**, *18*, 539, https://doi.org/10.1021/acsnano.3c08433.
[22] L. Ratinho, L. Bacri, B. Thiebot, B. Cressiot, J. Pelta, *ACS Cent. Sci*. **2024**, *10*, 1167, https://doi.org/10.1021/acscentsci.4c00020.
[23] R. Stefureac, Y. Long, H.-B. Kraatz, P. Howard, J. S. Lee, *Biochemistry* **2006**, *45*, 9172, https://doi.org/10.1021/bi0604835.
[24] M. Pastoriza-Gallego, L. Rabah, G. Gibrat, B. Thiebot, F. G. van der Goot, L. Auvray, J.-M. Betton, J. Pelta, *J. Am. Chem. Soc.* **2011**, *133*, 2923, https://doi.org/10.1021/ja1073245.
[25] A. Fennouri, R. Daniel, M. Pastoriza-Gallego, L. Auvray, J. Pelta, L. Bacri, *Anal. Chem*. **2013**, *85*, 8488, https://doi.org/10.1021/ac4020929.
[26] J. W. F. Robertson, C. G. Rodrigues, V. M. Stanford, K. A. Rubinson, O. V. Krasilnikov, J. J. Kasianowicz, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8207, https://doi.org/10.1073/pnas.0611085104.
[27] C. G. Rodrigues, D. C. Machado, S. F. Chevtchenko, O. V. Krasilnikov, *Biophys. J*. **2008**, *95*, 5186, https://doi.org/10.1529/biophysj.108.140814.
[28] M. F. Breton, F. Discala, L. Bacri, D. Foster, J. Pelta, A. Oukhaled, *J. Phys. Chem. Lett*. **2013**, *4*, 2202, https://doi.org/10.1021/jz400938q.
[29] C. T. Walsh, S. Garneau-Tsodikova, G. J. Gatto Jr., *Angew. Chem., Int. Ed*. **2005**, *44*, 7342, https://doi.org/10.1002/anie.200501023.
[30] J. Hermann, L. Schurgers, V. Jankowski, *Mol. Aspects Med*. **2022**, *86*, 101066, https://doi.org/10.1016/j.mam.2022.101066.
[31] C. Cao, N. Cirauqui, M. J. Marcaida, E. Buglakova, A. Duperrex, A. Radenovic, M. Dal Peraro, *Nat. Commun*. **2019**, *10*, 4918, https://doi.org/10.1038/s41467-019-12690-9.
[32] T. Ensslen, K. Sarthak, A. Aksimentiev, J. C. Behrends, *J. Am. Chem. Soc.* **2022**, *144*, 16060, https://doi.org/ 10.1021/jacs.2c06211.
[33] S. Li, X.-Y. Wu, M.-Y. Li, S.-C. Liu, Y.-L. Ying, Y.-T. Long, *Small Methods* **2020**, *4*, 2000014, https://doi.org/10.1002/smtd.202000014.
[34] H. Niu, M.-Y. Li, Y. Gao, J.-G. Li, J. Jiang, Y.-L. Ying, Y.-T. Long, *Nat. Chem. Biol*. **2024**, https://doi.org/10.1038/s41589-024-01734-x.
[35] P. Martin-Baniandres, W.-H. Lan, S. Board, M. Romero-Ruiz, S. Garcia-Manyes, Y. Qing, H. Bayley, *Nat. Nanotechnol*. **2023**, *18*, 1335, https://doi.org/10.1038/s41565-023-01462-8.
[36] W.-H. Lan, H. He, H. Bayley, Y. Qing, *J. Am. Chem. Soc*. **2024**, *146*, 24265, https://doi.org/10.1021/jacs.4c03912.
[37] L. Yu, X. Kang, F. Li, B. Mehrafrooz, A. Makhamreh, A. Fallahi, J. C. Foster, A. Aksimentiev, M. Chen, M. Wanunu, *Nat. Biotechnol*. **2023**, *41*, 1130, https://doi.org/ 10.1038/s41587-022-01598-3.
[38] E. Vreeker, F. Grünewald, N. J. van der Heide, S.-J. Marrink, K. Tych, G. Maglia, *bioRxiv* **2024**, https://doi.org/10.1101/2024.05.16.594548.
[39] X. Kang, M. A. Alibakhshi, M. Wanunu, *Nano Lett*. **2019**, *19*, 9145, https://doi.org/10.1021/acs.nanolett.9b04446.
[40] J. Yu, C. Cao, Y.-T. Long, *Anal. Chem*. **2017**, *89*, 11685, https://doi.org/10.1021/acs.analchem.7b03133.
[41] Y. Wang, K. Tian, X. Du, R.-C. Shi, L.-Q. Gu, *Anal. Chem*. **2017**, *89*, 13039, https://doi.org/10.1021/acs.analchem.7b03979.
[42] D.-F. Liao, C. Cao, Y.-L. Ying, Y.-T. Long, *Small* **2018**, *14*, 1704520, https://doi.org/10.1002/smll.201704520.
[43] Z.-L. Hu, M.-Y. Li, S.-C. Liu, Y.-L. Ying, Y.-T. Long, *Chem. Sci*. **2019**, *10*, 354, https://doi.org/10.1039/C8SC03927E.
[44] A. Varki, *Glycobiology* **2017**, *27*, 3, https://doi.org/10.1093/glycob/cww086.
[45] A. J. Lepedda, G. Nieddu, M. Formato, M. B. Baker, J. Fernández-Pérez, L. Moroni, *Front. Chem*. **2021**, *9*, 680836, https://doi.org/10.3389/fchem.2021.680836.
[46] P. Bayat, C. Rambaud, B. Priem, M. Bourderioux, M. Bilong, S. Poyer, M. Pastoriza-Gallego, A. Oukhaled, J. Mathé, R. Daniel, *Nat. Commun*. **2022**, *13*, 5113, https://doi.org/10.1038/s41467-022-32800-4.
[47] M. Li, Y. Xiong, Y. Cao, C. Zhang, Y. Li, H. Ning, F. Liu, H. Zhou, X. Li, X. Ye, Y. Pang, J. Zhang, X. Liang, G. Qing, *Nat. Commun*. **2023**, *14*, 1737, https://doi.org/10.1038/s41467-023-37348-5.
[48] W. Lu, X. Zhao, M. Li, Y. Li, C. Zhang, Y. Xiong, J. Li, H. Zhou, X. Ye, X. Li, J. Wang, X. Liang, G. Qing, *ACS Nano* **2024**, *18*, 12412, https://doi.org/10.1021/acsnano.4c01571.
[49] M. Li, J. Wang, C. Zhang, X. Zhao, Y. Xiong, Y. Cao, D. Wang, X. Li, X. Liang, G. Qing, *ACS Nano* **2024**, *18*, 25155, https://doi.org/10.1021/acsnano.4c07038.
[50] N. Ashkenasy, J. Sánchez-Quesada, H. Bayley, M. R. Ghadiri, *Angew. Chem., Int. Ed*. **2005**, *44*, 1401, https://doi.org/10.1002/anie.200462114.
[51] Y. Wang, Y. Zhao, A. Bollas, Y. Wang, K. F. Au, *Nat. Biotechnol*. **2021**, *39*, 1348, https://doi.org/10.1038/s41587-021-01108-x.
[52] E. A. Manrao, I. M. Derrington, A. H. Laszlo, K. W. Langford, M. K. Hopper, N. Gillgren, M. Pavlenok, M. Niederweis, J. H. Gundlach, *Nat. Biotechnol*. **2012**, *30*, 349, https://doi.org/10.1038/nbt.2171.
[53] C. Cao, M.-Y. Li, N. Cirauqui, Y.-Q. Wang, M. Dal Peraro, H. Tian, Y.-T. Long, *Nat. Commun*. **2018**, *9*, 2823, https://doi.org/10.1038/s41467-018-05108-5.
[54] K. Chen, J. Kong, J. Zhu, N. Ermann, P. F. Predki, U. F. Keyser, *Nano Lett.***2019**, 19 1210, https://doi.org/10.1021/acs.nanolett.8b04715.
[55] N. A. W. Bell, U. F. Keyser, *Nat. Nanotechnol*. **2016**, *11*, 645, https://doi.org/10.1038/nnano.2016.50.
[56] Z. Song, Y. Liang, J. Yang, *Nanomaterials* **2022**, *12*, 3135, https://doi.org/10.3390/nano12183135.
[57] M. G. T. A. Rutten, F. W. Vaandrager, J. A. A. W. Elemans, R. J. M. Nolte, *Nat. Rev. Chem*. **2018**, *2*, 365, https://doi.org/10.1038/s41570-018-0051-5.
[58] C. Cao, L. F. Krapp, A. Al Ouahabi, N. F. König, N. Cirauqui, A. Radenovic, J.-F. Lutz, M. D. Peraro, *Sci. Adv*. **2020**, *6*, eabc2661, https://doi.org/10.1126/sciadv.abc2661.
[59] H. Ju, L. Cheng, M. Li, K. Mei, S. He, C. Jia, X. Guo, *Adv. Sci*. **2024**, *11*, 2401877, https://doi.org/10.1002/advs.202401877.
[60] X. Wei, T. Penkauskas, J. E. Reiner, C. Kennard, M. J. Uline, Q. Wang, S. Li, A. Aksimentiev, J. W. F. Robertson, C. Liu, *ACS Nano* **2023**, *17*, 16369, https://doi.org/10.1021/acsnano.3c05628.
[61] L. Payet, M. Martinho, M. Pastoriza-Gallego, J.-M. Betton, L. Auvray, J. Pelta, J. Mathé, *Anal. Chem*. **2012**, *84*, 4071, https://doi.org/10.1021/ac300129e.
[62] G. Oukhaled, J. Mathé, A.-L. Biance, L. Bacri, J.-M. Betton, D. Lairez, J. Pelta, L. Auvray, *Phys. Rev. Lett*. **2007**, *98*, 158101, https://doi.org/10.1103/PhysRevLett.98.158101.
[63] J. Nivala, D. B. Marks, M. Akeson, *Nat. Biotechnol*. **2013**, *31*, 247, https://doi.org/10.1038/nbt.2503.
[64] S. Zhang, G. Huang, R. C. A. Versloot, B. M. H. Bruininks, P. C. T. de Souza, S.-J. Marrink, G. Maglia, *Nat. Chem*. **2021**, *13*, 1192, https://doi.org/10.1038/s41557-021-00824-w.
[65] K. Motone, D. Kontogiorgos-Heintz, J. Wee, K. Kurihara, S. Yang, G. Roote, O. E. Fox, Y. Fang, M. Queen, M. Tolhurst, N. Cardozo, M. Jain, J. Nivala, *Nature* **2024**, *633*, 662, https://doi.org/10.1038/s41586-024-07935-7.
[66] H. Brinkerhoff, A. S. W. Kang, J. Liu, A. Aksimentiev, C. Dekker, *Science* **2021**, *374*, 1509, https://doi.org/10.1126/science.abl4381.
[67] S. Yan, J. Zhang, Y. Wang, W. Guo, S. Zhang, Y. Liu, J. Cao, Y. Wang, L. Wang, F. Ma, P. Zhang, H.-Y. Chen, S. Huang, *Nano Lett*. **2021**, *21*, 6703, https://doi.org/10.1021/acs.nanolett.1c02371.
[68] Z. Chen, Z. Wang, Y. Xu, X. Zhang, B. Tian, J. Bai, *Chem. Sci*. **2021**, *12*, 15750, https://doi.org/10.1039/D1SC04342K.
[69] A. Sauciuc, B. Morozzo della Rocca, M. J. Tadema, M. Chinappi, G. Maglia, *Nat. Biotechnol*. **2024**, *42*, 1275, https://doi.org/ i10.1038/s41587-023-01954-x.
[70] L.-Q. Gu, S. Cheley, H. Bayley, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15498, https://doi.org/ 10.1073/pnas.2531778100.
[71] Y. Ge, M. Cui, Q. Zhang, Y. Wang, D. Xi, *Nanoscale Adv*. **2022**, *4*, 3883, https://doi.org/10.1039/D2NA00190J.
[72] K. Wang, S. Zhang, X. Zhou, X. Yang, X. Li, Y. Wang, P. Fan, Y. Xiao, W. Sun, P. Zhang, W. Li, S. Huang, *Nat. Methods* **2024**, *21*, 92, https://doi.org/10.1038/s41592-023-02021-8.
[73] Y. Zhang, Y. Yi, Z. Li, K. Zhou, L. Liu, H.-C. Wu, *Nat. Methods* **2024**, *21*, 102, https://doi.org/10.1038/s41592-023-02095-4.

[74] 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, J. Geng, *Nat. Methods* **2024**, *21*, 609, https://doi.org/10.1038/s41592-024-02208-7.
[75] A. K. Thakur, L. Movileanu, *Nat. Biotechnol*. **2019**, *37*, 96, https://doi.org/10.1038/nbt.4316.
[76] L. A. Mayse, Y. Wang, M. Ahmad, L. Movileanu, *ACS Nano* **2024**, *18*, 20468, https://doi.org/10.1021/acsnano.4c04857.
[77] T. Roesel, C. Cao, J. F. B. Juarez, M. D. Peraro, S. Roke, *Nano Lett*. **2024**, *24*, 13888, https://doi.org/10.1021/acs.nanolett.4c00035.
[78] S. Rommelaere, A. Carboni, J. F. Bada Juarez, J.-P. Boquete, L. A. Abriata, F. Teixeira Pinto Meireles, V. Rukes, C. Vincent, S. Kondo, M. S. Dionne, M. Dal Peraro, C. Cao, B. Lemaitre, *Curr. Biol*. **2024**, *34*, 1426, https://doi.org/10.1016/j.cub.2024.02.049.
[79] V. Rukes, M. E. Rebeaud, L. W. Perrin, P. De Los Rios, C. Cao, *Nat. Commun*. **2024**, *15*, 8604, https://doi.org/10.1038/s41467-024-52674-y.

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