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Nanopore Technology: When Proteins Analyse Proteins

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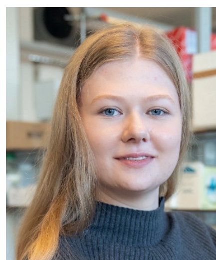
Abstract: Nanopore sensing is an emerging technology that can distinguish subtle differences in molecules and allows the observation of molecular processes. The technique has revolutionized DNA sequencing through long reads of single molecules. Following this success, nanopores are now increasingly applied to protein analysis. Proteins play central roles in cellular function and major diseases, however their analysis using established methods is complicated by the lack of protein-amplification methods. Here, two examples of nanopore-based protein analysis are described: the identification of biomarkers, and the analysis of protein function.

Keywords: Biomarker detection · Hsp70 · Nanopores · Protein analysis · Protein function



Verena Rukes obtained her bachelor's and master's degree in biotechnology in Germany before moving to Switzerland. She is now a PhD student in the program for biotechnology and bioengineering at EPFL and affiliated with the University of Geneva through the group of Prof. Chan Cao. Her research focuses on the development of nanopore sensing technology for applications in the identification and functional analysis of proteins.

1. Introduction

In recent years nanopores have been developed into a powerful single-molecule tool. The technology was commercialised for DNA sequencing and has significantly impacted the field of genomics due to its unique advantages such as portability and long-read sequencing.[1]

In measurements with biological nanopores a transmembrane protein channel is embedded in a lipid bilayer. As voltage is applied across it, ions from the surrounding electrolyte solution travel through the pore, causing a measurable current. When a molecule in solution interacts with, and *e.g.* passes through the nanopore, the measured current is modulated.[2] Such modulations can for example be used to identify the molecule.

Currently, the technology is being further developed and adapted towards the analysis of proteins.[3] Proteins are the main actors in biological systems. They allow our neurons to communicate, defend us as part of the immune system, and enable breathing and energy intake in the metabolism. Unsurprisingly, they are frequently involved in diseases, medications, and technical applications. However, as proteins are complex molecules and cannot be amplified, measuring them is challenging.[4]

This article focuses on two examples of how nanopores can be applied to protein analysis: firstly, the detection of protein-based biomarkers for diagnostic purposes, and secondly the investigation of protein function.

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2. Nanopores for Biomarker Detection

A valuable application of nanopore sensing is the detection of biomarkers,[5,6] meaning to specifically identify the presence of a known disease indicator in patient samples. Proteins are excellent targets for this, due to their important role in all cellular processes. Specifically, the post-translational modifications (PTMs) to proteins can dictate the location, activity or function of proteins.[7] PTMs can thus completely alter a protein's behaviour, which occurs in numerous diseases and makes PTMs important biomarkers. Their detection is commonly done using mass-spectrometry or anti-body-based methods.[8,9] However, identifying neighbouring PTMs remains challenging.[10] As the combinatorial effect of PTMs is highly important,[11,12] there is a need for novel detection methods.

Recently, Cao *et al.* used the nanopore aerolysin (Fig. 1a) to identify pathologically relevant PTMs on a 17 amino acid peptide derived from α-synuclein (α-syn).[13] Eight different combinations of phosphorylation (p), and nitration (n) were measured (Fig. 1b). Using deep-learning, the single-molecule interactions with the nanopore were distinguished with 78.2% accuracy (Fig. 1c).

To translate such results into a method that detects biomarkers in patient samples, further development is necessary. For instance, α-syn is present at 26.2 ± 3.0 µg/ml in red blood cells (RBCs).[14,15] While the nanopore measurements can be carried out at 2% v/v RBC extract, this corresponds to a low expected concentration of only 35 nM α-syn. To cope with such low concentrations, the detection limit of the system can be improved by using asymmetric buffer conditions.[16] The sample is added in a compartment of 0.15 M KCl, while the buffer on the other side of the nanopore contains 3 M KCl. Using this strategy, α-syn peptides interacted with the pore more frequently (Fig. 2), lowering the detection limit to 100 pM.

3. Nanopores Analyse Protein Function

Additionally, beyond the identification of proteoforms, nanopore sensing can be a valuable tool to investigate protein function at the single-molecule level. For instance, DNA processing enzymes can be studied as they feed DNA through a nanopore,[17] and metabolic enzymes can be investigated when trapped inside a nanopore.[18,19]

Recently, nanopore sensing was used to end a longstanding debate about the underlying physical mechanism of the 70 kDa Heat Shock Protein (Hsp70) ATPase family.[20] Among their

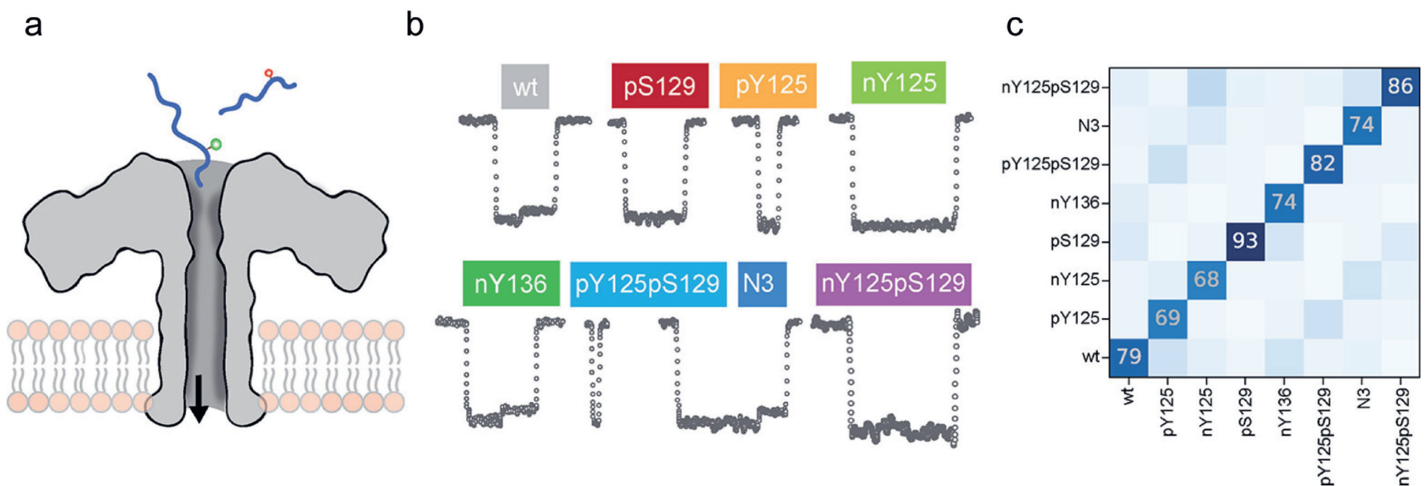

Fig. 1. Identification of PTMs on an α-synuclein derived peptide. (a) Illustration of the aerolysin K238A nanopore system. The translocation direction is indicated. (b) Representative current modulations caused by the peptide with different PTM configurations. N3 stands for nY125nY133nY136. (c) Confusion matrix showing classification accuracies for all eight peptides. Reproduced from Cao *et al.*[13]. 2024 under CC-BY 4.0.

many functions, these chaperones are involved in the trans-membrane-transport of proteins from the cytosol into cellular organelles like the Endoplasmatic Reticulum (ER).[21,22] Hsp70s thereby bind the translocating protein from inside the organelle with the help of an adjacent J-domain protein. What has been under debate, is how exactly this binding can cause the translocation.

To investigate the molecular mechanism of Hsp70s, the authors designed a nanopore system that mimics *in vivo* protein transport, using the nanopore aerolysin (Fig. 3a). A substrate polypeptide was used that can be bound by Hsp70 in solution and has an unstructured, charged tail. With an applied voltage, the tail was trapped inside the pore which resulted in a decrease in current signals (Fig. 3b). The current remained blocked over extended time periods, since the substrate was sterically hindered from passing through the pore, and it could only escape 'backwards' against the applied voltage. The time it takes for such an escape to occur (escape time) allowed to infer information about the energy barrier for the escape.

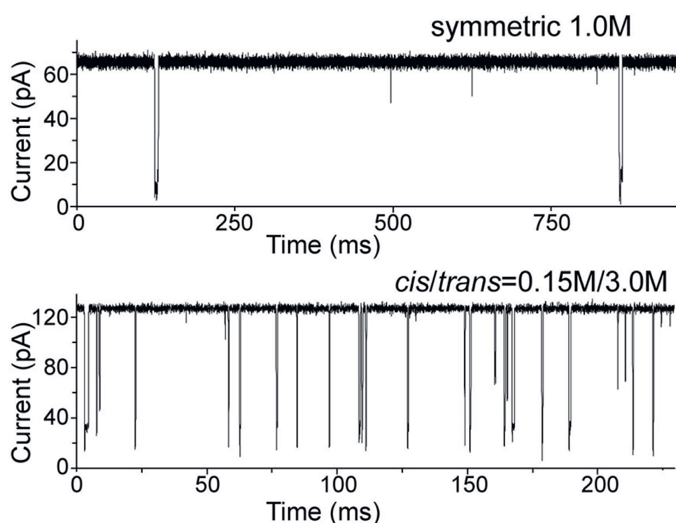

Fig. 2. Example traces of measurements with the wt α-syn peptide in symmetric (top) and asymmetric (bottom) conditions at 100 mV. Each downward spike corresponds to a single-molecule interaction. Reproduced from Cao *et al.*[13] 2024 under CC-BY 4.0.

The effect of Hsp70 binding was demonstrated by comparing escape times of the substrate with and without bound Hsp70. This showed that Hsp70-binding greatly facilitated the escape (Fig. 3c), proving that a force was generated by the chaperone. The Brownian Ratchet[23,24] was therefore ruled out as a possible mechanism of Hsp70s. According to this theory, Hsp70s passively prevent the translocating protein from slipping back, without generating any forces. Two alternative models had been presented: Hsp70s could generate a force either *via* Power Stroke[25] – which relies on a conformational change that mechanically pushes against the pore mouth – or *via* Entropic Pulling[26,27] – which relies on the confinement of the added volume of Hsp70: when very close to the pore mouth, the entropy of Hsp70 is reduced, which generates a pulling force. The authors therefore designed a polypeptide with a folded domain of intermediate size between the substrate and the substrate-Hsp70 complex. This intermediate construct escaped faster than the unbound substrate (Fig. 3c), which could not be explained by a Power Stroke. Instead, the escape times of the different constructs became faster with increasing size of the domain on top of the pore, behaving as expected according to the Entropic Pulling theory. Thus, the nanopore system was able to identify Entropic Pulling as Hsp70's molecular mechanism.

4. Conclusions

Nanopore technology is an elegant approach to single-molecule measurements. As exemplified here, the analysis of proteins and proteoforms benefits from nanopore systems in multiple ways. Nanopore signals can be used for identification, which might for example lead to diagnostic tools. Additionally, nanopores can be used to build systems that investigate protein functions at the single molecule level in ways that are not accessible to other methods.

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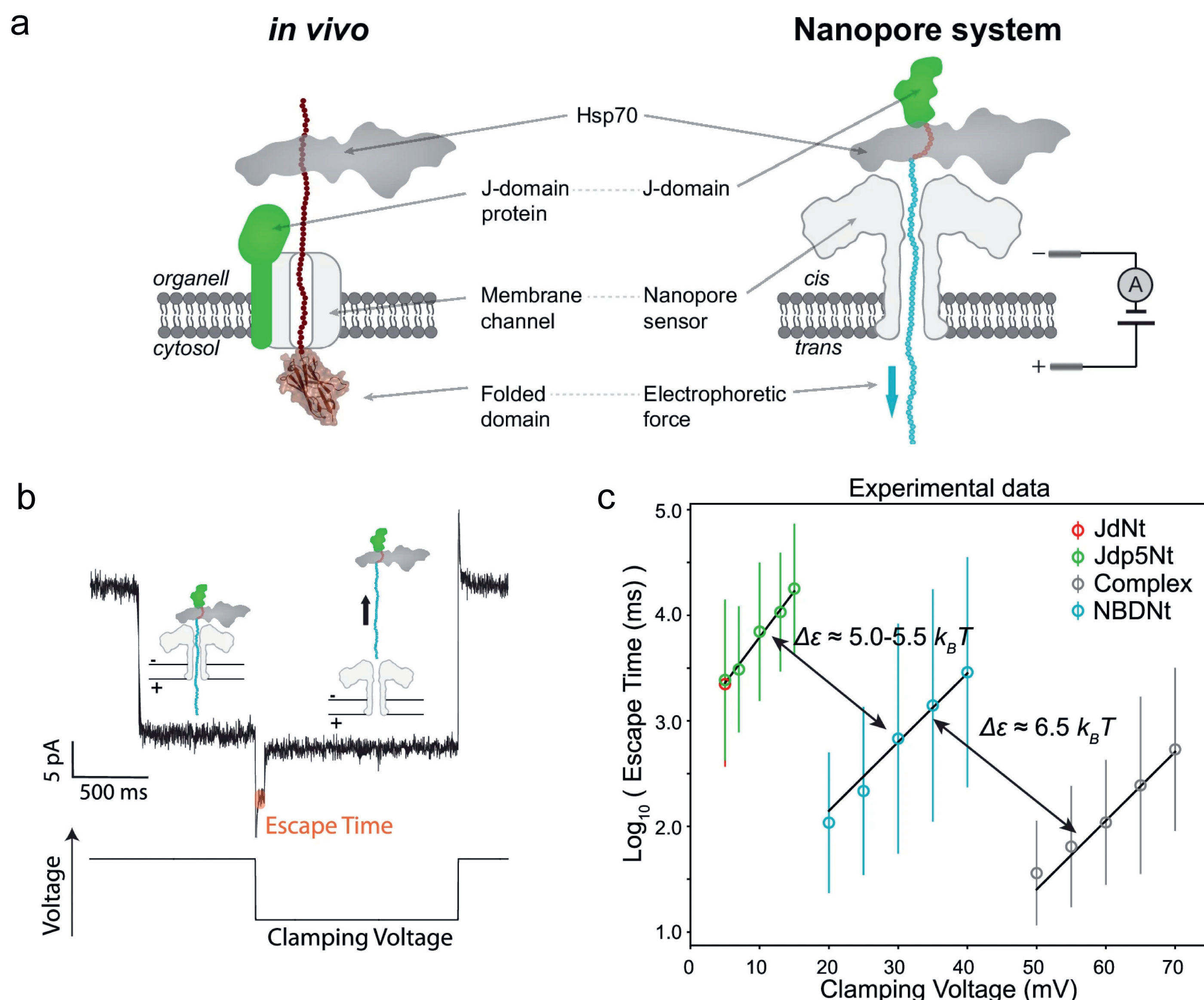

Fig. 3. (a) Illustration of the Hsp70 transmembrane transport *in vivo* compared to the designed nanopore system. The membrane channel (light grey) is replaced by the aerolysin K238A nanopore. Instead of a folded domain as *in vivo* (brown), the chaperone is working against a voltage-induced force in the designed system. The charged, unstructured part of the designed substrate is shown in blue. (b) An example event of Jdp5Nt-Hsp70-complex trapping. Following the trapping at 140 mV the current drops instantaneously. The voltage is then lowered to the clamping voltage (here 50 mV). When the escape occurs, the current increases instantaneously. (c) Logarithmized escape times of the measured analytes. The log values of each system at various clamping voltages were averaged, standard deviation is indicated. Black lines show the fit to a model based on Kramer's theory[19] as described further in.[20] Reproduced from Rukes *et al*.[20] 2024 under CC-BY-NC-ND.

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