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Commentary and Perspective

The potential of nanopore technologies toward empowering biophysical research: Brief history, basic principle and applications

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Nanopore technology has emerged as an attractive scientific tool with various potential applications in genomics, proteomics, diagnostics, and beyond. As featured by the symposium "The fundamental and applications of single-molecule nanopore sensing for biophysical studies" of the 61st Annual Meeting of the Biophysical Society of Japan held in November 2023, in this commentary, we briefly introduce the history of nanopore technology, the basic principle of nanopore, and the potential applications.

The origins of nanopore technology date back to the 1980s, with early conceptualization to nano-sized pore in membrane protein for the detection of biological molecules, such as DNA, RNA, and proteins [1]. The basic concept of nanopore sequencing emerged from the idea passing a single-stranded DNA through a nano-sized pore in a membrane driven by electrophoresis. In an early experiment, Kasianowicz *et al.* identified that the bacterial pore-forming protein, staphylococcal α-hemolysin (αHL) with -1.2 nm aperture, serves the ideal sensor to detect the single-stranded DNA and RNA by observing their translocation through a nanopore, resulting in the reduction of ionic current (Figure 1a) [2]. The discovery of αHL led to significant advancement since it showed the sensitivity to distinguish different polynucleotides and the direction of translocation (3' to 5' or 5' to 3') [3,4]. Another crucial development for nanopore sequencing was the establishment of processive enzyme control for DNA translocation. In this process, DNA polymerase tightly binding to the DNA regulate the nucleotides through a nanopore, one nucleobase at a time (Figure 1b) [5]. Additionally, engineered protein nanopores enhanced sensitivity for nucleotide identification by introducing recognition sites (Figure 1c) [6]. Also, Oxford Nanopore Technologies (ONT) played an important role in manufacturing nanopore sequencing device. ONT developed a compact and portable sequencer "the MinION" with improved chemistry and data analysis, leading to sequencing accuracy at practical level and long reading length. This long reading sequencing has provided various applications such as
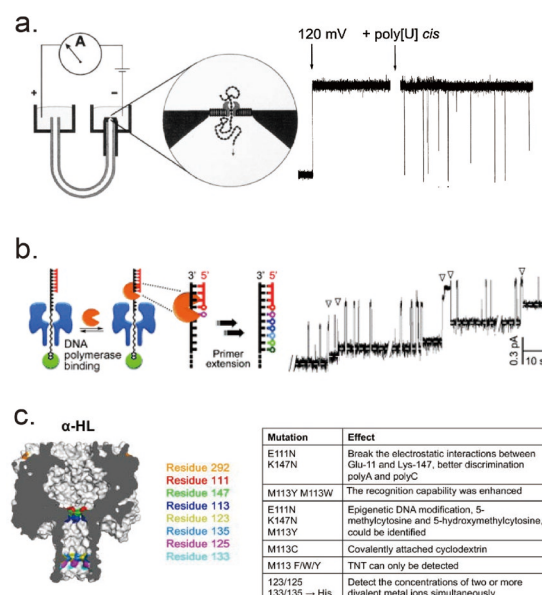

Figure 1 Brief history of nanopore technologies a. DNA and RNA detection using αHL b. Regulation of DNA translocation using DNA polymerase c. Examples of engineered protein pore (αHL) All figures adapted with permission from ref. 2, 5, 6.

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epigenetic/transcriptomics/genomic research, clinical usage, and on-site pathogen detection [7].

The nanopore technology is based on measurement of changes in electrical resistance produced by molecular translocation similarly to sensing principle of Coulter counter. Typically, nanopore sensing employs the electrical model in Figure 2a. When the electrolyte solution is filled within a fluidic cell, the current density into nanopores is described by Eq. (1). Readers can find the summary of symbols in all equations in Table 1.

$$J = \frac{z_i^2 \cdot F^2}{RT} [D_+ + D_-]cE = \sigma_s E \tag{1}$$

In the case where the diameter of the nanopores are significantly smaller than the pore height, the nanopore resistance dominates to the total electrical resistance. When the potential drops between the electrodes and the electrolyte solvent are small, the potential drop in nanopores becomes approximately equal to the applied voltage. Nonpolarizable electrodes, such as Ag/AgCl electrodes, can be used to reduce the potential drop between the electrodes and the electrolyte solvent. Furthermore, the potential drop in the electrolyte solvent is reduced when salt concentration is high. Since typical nanopore sensing use Ag/AgCl electrodes and the electrolyte solvent with high salt concentration (typically 0.1-4M KCl, NaCl or LiCl), the resistance at electrodes and electrolyte solvent are minimal. Thus, the ion current expressed as Eq. (2).

$$I = \frac{A}{h} \sigma_s \Delta V = \frac{\pi d^2}{4h} \sigma_s \Delta V \tag{2}$$

Since strong electric field are formed at vicinity of nanopores, the access resistance should be considered, which is described as $R_{access} = 1/d$ [8]. Therefore, the current obtained from the total resistance can be described by Eq. (3).

$$I_{total} = \sigma_s \Delta V \left(\frac{4h}{\pi d^2} + \frac{1}{d} \right)^{-1} \tag{3}$$

This equation tells that nanopore geometry (diameter and height) modulates the total resistance, resulting in the ion current value. If molecules with known size passed through nanopores, one can estimate the pore geometry from baseline ion current and blockade current using Eq. (3) and (4).

$$\Delta I = \sigma_s \Delta V \left(\frac{4h}{\pi d_m^2} + \frac{1}{d_m} \right)^{-1} \tag{4}$$

In addition, the enhancement of the molecular capture rate into nanopores is also crucial factor for the high-throughput measurement. Therefore, the molecular capture process has been extensively studied [9-11]. The molecular capture process can be explained as a Poisson process ($P(t) = e^{-R_c t}$). The molecular capture probability is significantly influenced by the voltage distribution outside nanopores, and the voltage at certain distance (r) from nanopores is described as Eq. (5) [11].

$$V(r) = \frac{d^2}{8hr} \Delta V \tag{5}$$

In the voltage distribution described by Eq. (5), in regions where $r > r*$, molecules can freely diffuse within the solvent. In contrast, in regions where $r < r*$, the drift motion of molecules becomes dominant over diffusion, causing molecules to gradually be drawn into the nanopore (Figure 2b). The probability of molecular capture can be derived from the Smoluchowski equation, and using this effective distance ($r*$), it can be expressed as Eq. (6) [12].

$$C_R = 2\pi P r^* \tag{6}$$

Since voltage at $r*$ is defined as $V(r^*) = P/\mu$, capture radius and the molecular capture frequency can be expressed as Eq. (7) and (8).

$$r^* = \frac{d^2 \mu}{8hP} \Delta V \tag{7}$$

$$C_R = \frac{2\pi d^2 \mu}{8h} \Delta V \tag{8}$$

Since the effective distance $r*$ includes the diffusion constant and electrophoretic mobility of molecules, it depends on the length of molecules and the total charge of molecules. Furthermore, $r*$ is expected to increase linearly with the applied

voltage, but the capture probability increases exponentially [10]. This suggests that some molecules approaching nanopores may not be able to pass through a nanopore, since the coiled structure of DNA need to unwind before passing through a nanopore.
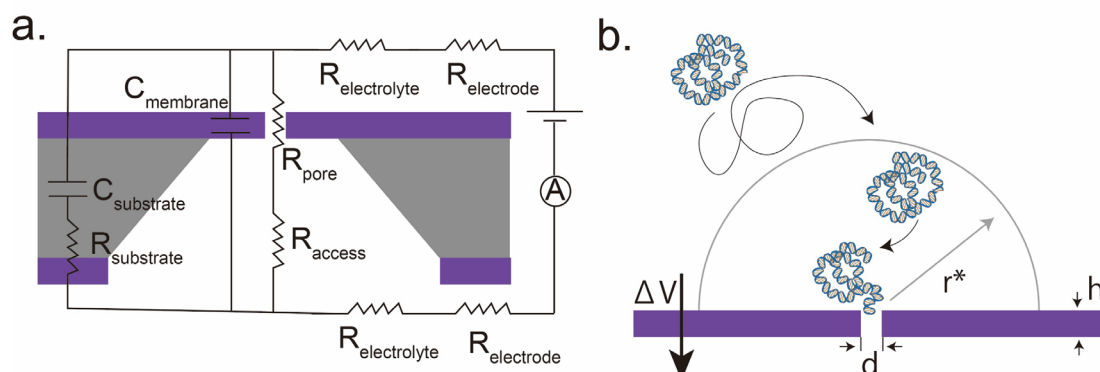

Figure 2 Basic principle of nanopore technologies a. The electrical model of nanopore sensing. b. Schematic illustration of DNA capture into the nanopore under the electrical field.

Table 1 Symbol names

Symbol	Name	Symbol	Name
D_+	Diffusion constants of cations	d	Diameter of the nanopore
D_-	Diffusion constants of anions	h	Pore height
σ_s	Conductivity of the electrolyte solvent	R_{pore}	Nanopore resistance
F	Faraday constant	d_m	Diameter of molecule
R	Gas constant	A	Area of the nanopore
T	Temperature	R_c	Time interval of DNA translocation events
z_i	ionic charge	c	Ion density
E	Electric field	ΔI	The ammount of blockade current
ΔV	Applied voltage	C_R	Capture rate
P	Diffusion constant	μ	Electrophoretic mobility

    The understanding of molecular translocation through nanopores is necessary for analyzing data correctly. Previous study found that molecular translocation through a nanopore is not consistent and is influenced by nanopore size due to the interaction between molecules and the nanopore interface [13]. The nonlinear relationship is attributed to interactions between the nanopore interface and molecules, as well as the contraction of coiled DNA near the nanopore [13-15]. Also, molecular translocation is influenced by buffer condition such as salt concentration, salt type, pH, viscosity and so on [16-19]. Thus, the propriate pore geometry and buffer conditions for target experiments is important to enhance the detection modalities [20].

    Since nanopore sensing works the simple principle and promise the high sensitivity, this sensing approach can be integrated into varieties of applications. The followings are examples of some notable applications. Nanopores can be used to analyze not only sequence of DNA and RNA, but also size, shape and conformation of DNA, RNA, and proteins as well as nanoparticles [21-23]. This means it can be used for understanding molecular folding, interactions, and structural changes. For examples, recent works showed that solid-state nanopore can detect the detailed conformational state of protein during translocation through a nanopore under high electric fields, allowing the investigation of metastable intermediates and the unfolding/folding state of proteins [24,25]. In addition, this strategy can be employed in drug discovery and development since it can detect the conformation change due to the interactions between small molecules and biological molecules. Some recent works showed that protein trapping on nanopores can be used to monitor transition of drug binding kinetics by looking at current value changes [26,27]. Finally, nanopore technology can be integrated into the interface with biological systems, thereby offering cybernetic control systems and enabling a synergy between the fields of genomics and cybernetics. For instance, toward development of chemical artificial intelligence, protein or *de*

novo designed pores were employed to exchange the small molecules outside and inside of live and artificial cells composed by giant unilamellar vesicles (GUVs) [28-31].

In conclusion, we provided a brief introduction of the history, basic principle, and some applications of nanopore technology. Since this technology can analyze single molecules at high sensitivity and adapt to various sensing approaches, it is a promising tool for both fundamental research as well as an extensive range of applications in the field of biophysics. With continuing to explore and develop nanopore technology, we hope that this technology will unlock new research approaches and applications in the realm of biophysics.

Acknowledgements

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