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Nanopore sensing: A physical-chemical approach

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ABSTRACT

Protein nanopores have emerged as an important class of sensors for the understanding of biophysical processes, such as molecular transport across membranes, and for the detection and characterization of biopolymers. Here, we trace the development of these sensors from the Coulter counter and squid axon studies to the modern applications including exquisite detection of small volume changes and molecular reactions at the single molecule (or reactant) scale. This review focuses on the chemistry of biological pores, and how that influences the physical chemistry of molecular detection.

1. Introduction

Ion channels and porins are emerging as an important class of biosensor for the detection and characterization of a wide variety of polymers from both synthetic and biological origin. These sensors have a long history originating with the discovery of membrane spanning ion channels [1]. The principle of operation for an ion channel or porin sensor is simple: a dielectric barrier is formed across an aperture [2] (or on a conductive surface [3]) and membrane spanning peptides [4–6], ion channels, or large porins and pore forming toxins [7,8] form a conductive pathway across the membrane. Pore forming proteins, in particular, have been the premier class of membrane proteins used for biosensing [9]. These proteins are characterized by a large, water-filled cavity that spans the dielectric membrane. When these proteins are assembled in a membrane, ionic current can be driven through the pore, and an examination of this current can be used to determine both geometric and surface charge characteristics of these pores [10,11]. In the late 1980s, researchers began using polymer-induced conductivity changes to characterize ion channels and porins [10,12,13]. Two observations published in the early 1990s suggested that these pore-forming proteins could be used as a sensor: ionic current fluctuations could be detected and were shown to be dependent on protonation kinetics [14,15] and single channels isolated in membranes could be used as molecular-scale Coulter counters [16]. These studies were compelling, but the observation that single-stranded DNA could be observed translocating through the water-filled channel of a porin really accelerated the interest in nanopore sensing [17]. The role of DNA sequencing in nanopore sensor development is undeniable, and has been reviewed thoroughly [18,19].

Single-molecule methods for the detection and characterization of biological systems has flourished over the last three to four decades with fluorescence methods and force methods [*e.g.*, optical tweezers and atomic force microscopy (AFM)] being the most common methods [20]. While powerful and wildly successful for untangling complex interactions, including nanoscopic molecular motion or folding pathways that can be lost when only the ensemble average is measured. Most of these single molecule methods require chemical modification of the molecule to be studied which can entail attachment of fluorophores to a protein or anchoring the protein to an AFM tip or polymer bead. Nanopore sensors have a long history of single molecule sensing [21]. Although nanopores are not a panacea for single molecule biosensors, their prevailing advantage is the relative ease of producing high-quality single molecule analyses without the need for complicating labels.

In this review, we will focus on the physical chemistry of nanopore sensing and will review operational principles of these nanopore sensors. This journey will answer fundamental questions, such as: what types of analyte can be detected by nanopore sensors? what are the practical detection limits of nanopore sensing? and how is chemical selectivity achieved in a nanopore-based biosensor? While the paper will focus primarily on pore forming proteins and peptides, examples from structural DNA nanotechnology and solid-state materials will be used to highlight the flexibility of the resistive-pulse approach for chemical detection and characterization, particularly the diversity in geometry, electrostatic barriers, and physisorption that can be achieved with

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careful selection of the pore's properties. Throughout the paper we will highlight some notable examples and stress the significance of approaches that may fall outside the normal sensing strategies to offer a full picture of nanopore biosensors.

2. The physical manifestation of the signal

The principle of operation for nearly all nanopore sensors is conceptually simple. The measurement relies on the time-dependent conductance of ions through a nanochannel formed by proteins, nucleic acids or other means through a dielectric barrier. The origin of this method has its root in the study of ion transport through the squid axon (Fig. 1a), by Cole [22,23], and Hodgkin & Huxley [24,25] (a deeper exegesis can be found in Jan Beherend's fantastic history of ion channels and disease [1], and the exhaustive monograph by Hille [26]). These studies were enabled by the unusually large squid axon, which allowed measurements to be performed across the walls of single cells. Independent of the academic work on cellular transport, Wallace Coulter developed a device for a cell counting that relied on resistive pulses generated by cells passed through a narrow aperture between two reservoirs of conductive fluid (Fig. 1b) [27]. This phenomenon, now known as the Coulter effect, is simply the observation that a particle of sufficient volume reduces the conductance of the fluid passing through the aperture. The Coulter principle was first reduced to the nanoscale in 1970 by DeBlois and Bean, who used track-etched pores through polymer membranes [28]. More critically, they developed a theoretical framework for the magnitude of current interruption and set the resistive-pulse field on a solid theoretical foundation. Although it took nearly 30 years, the discovery that ion channels and porins could be used for nanoscale sensing hinges on these early discoveries.

Quite by accident, these two independent lines of technological development foreshadow the two predominant detection schemes that can be employed for a nanopore sensor. These schemes can be separated conceptually by the physical mechanism through which the ionic current is interrupted (Fig. 2). For the case of porin-based sensing, which is the molecular-scale equivalent of the Coulter counter, the molecule of interest must partition into the central cavity (*i.e.*, the pore) and interrupt the current. This is primarily through volume-exclusion (Fig. 2a), but has secondary effects due to chemical details of the pore and analyte [16,17]. The less utilized, but highly promising alternative relies on gating of the pore due to interactions with the environment (Fig. 2b). In this illustration the transmembrane current is modulated by movements of the pore itself as the channel (usually as unstructured extramembraneous loops) responds to molecules in the solution. This is often called gating, and can be observed in various single-channel studies of potassium ion channels, where current is modulated by fluctuations in the structure of the channel [29,30]. Here we classify any sensor that shows such gating behavior as a result of chemical interactions outside the pore as a gating sensor.

Perhaps the most critical detail for sensing any molecule is that these ion channel sensors are commensurate in size with the single molecules being detected. Typical protein sensors, described in detail in Section 4, have dimensions on the order of 1 nm in radius and 10 nm in length. This has broad implications for sensing as the analyte has to be held in this volume for a time long enough to be interrogated by flowing ions. The current state-of-the-art amplifiers can achieve 1 MHz [33] to 10 MHz [34] bandwidth (*i.e.*, 1 μs to 100 ns), but realizing such high bandwidth requires significant noise reductions through optimization of experimental geometries (*i.e.*, membrane dimensions, electrode interfacing). More common commercial amplifier systems have bandwidths on the order of 50 kHz to 100 kHz [35]. Advanced signal processing can accurately monitor signals with as few as 5 data points [36]. As a practical, rule of thumb this means that resistive pulses (or gating events) should be on the order of 10 μs to 100 μs, for any significant characterization. In the following sections, we highlight some typical pores and their chemical modifications and the efforts to understand the physical chemistry of the processes involved in nanopore sensing. This insight informs the development of a semi-universal sensor for polymers, both synthetic and natural, and provides a roadmap to advance nanopore technology.

3. A wealth of chemistry in protein nanopores

Pore forming proteins, whether porins or toxin-based nanopores, play an important role in molecular transport of ions and molecules across barriers both inside and outside of cells and their organelles. The central cavity of these pores is typically between 1 nm and 6 nm in diameter, and each channel has a specific function which has emerged through evolutionary processes [37–39]. The practical result for the context of biosensing is that the structure (*i.e.*, shape, charge distribution, *etc.*) is dictated by the amino acid sequence and subsequent folding. These porins act like a molecular gateway, trafficking molecules or ions

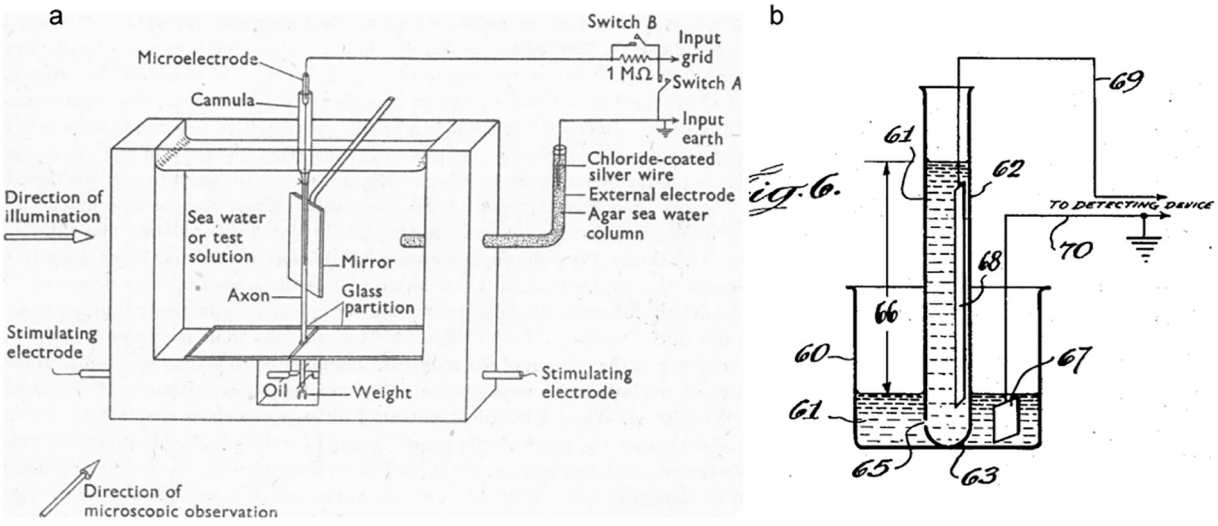

Fig. 1. The development of nanopore sensors have their origin in the discovery of the propagation of electrical signals in squid axon (a) and the Coulter method (b). (a) Hodgkin, Huxley and Katz's single axon measurement apparatus [24], and (b) the cell-counting unit from an early version of the Coulter counter, of note: the electrodes #67 and #68 are used to drive current and measure the conductance through the micropore #65 fabricated in a glass test tube #61. Definitions for the other elements can be found in [27].

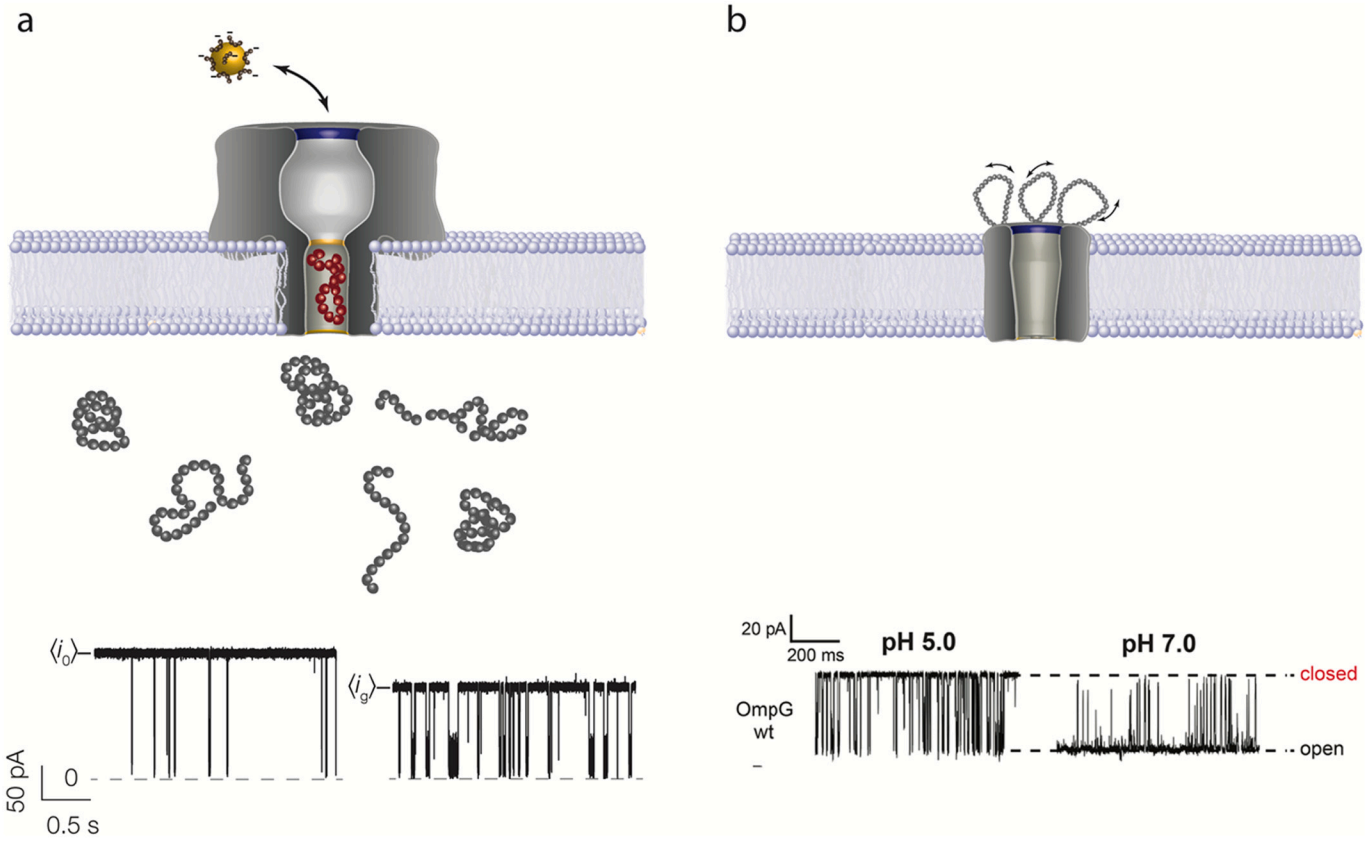

Fig. 2. Nanopore-based biosensors fall in two major categories characterized by how the analyte interacts with the pore. (a) In the most common method, the analyte, or analyte and co-analyte partition into the central cavity of the pore causing current interruptions, and (b) the analyte induces conformational changes in the pore causing the channel to gate.
The images were adapted with permission from [31] Chavis et al. *ACS Sensors* (2017) https://pubs.acs.org/doi/10.1021/acssensors. Further permissions related to the material excerpted should be directed to the ACS, and [32] Perez-Rathke et al. *J. Am. Chem. Soc.* (2018) Copyright 2018 American Chemical Society, respectively.

in healthy systems [40], but they can also destabilize membranes causing disruptions in normal cellular function in disease [41], as well as apoptosis [42]. There are thousands of pore forming proteins found in nature [43] many of which have been, or can be adapted for biosensing applications [44]. The choice of pore for each sensing application depends upon the nature of analytes, and on the structural and chemical selectivity of the channel among other factors. Below, we highlight a few proteins that have been used extensively as biosensors. A selection of their structures is highlighted in Fig. 3. Many of these proteins are used from wild-type preparations, but researchers are not limited by what nature provides. Biochemical and post translational modifications of these proteins, not to mention advances in semiconductor processing and DNA nanotechnology, give us unlimited variability in the chemical nature of the pores that can be applied to these single-molecule sensors [8].

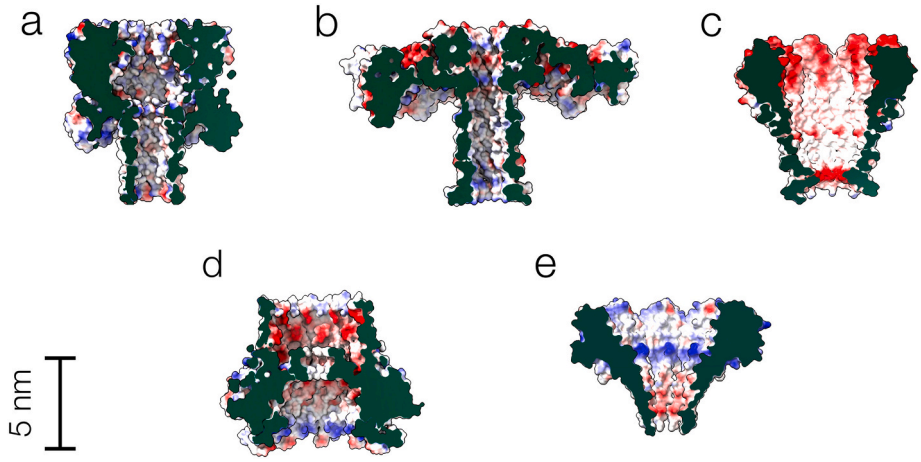

Fig. 3. Structures of pore forming proteins currently used in nanopore sensing. Cross-sectional view of the pores created using the data from RSCB protein databank (PDB). a. α-hemolysin (αHL) PDB ID: 7AHL [45] b. aerolysin PDB ID: 5JZT [46] c. MspA PDB ID: 1UUN [47] d. CsgG PDB ID: 4UV3 [48] and e. FraC PDB ID: 4TSY [49]. Colors represent local charge using the electrostatic color map feature in Chimera X [50].

3.1. α-Hemolysin

The most widely used pore in the community is α-hemolysin (αHL), in large part because of its early emergence in single-molecule sensing, particularly in the early DNA sequencing efforts [14,15,17,51,52]. The protein self-assembles and remains in a stable open configuration under transmembrane potentials up to 150 mV in high ionic strength solutions (*i.e.*, [KCl] > 1 mol/L) [14]. In addition to its excellent stability, reproducibility and electrical properties, the diameter of the pore is commensurate with the dimensions of small polymer molecules (Fig. 3a) [45]. The pore is nominally 10 nm long separated into three regions: a vestibule, a single central constriction and the β-barrel. Each of these regions has been used semi-independently for sensing applications [53,54]. The particular hourglass topology of the pore also facilitates interactions between molecules that are sterically prevented from crossing the constriction [55,56]. The wild-type pore is moderately anion selective [57] with a net charge (Z = +7e) [58].

As we discuss later, controlling analyte-pore interactions is critical for sensing efficiency. The αHL pore provides a platform for engineering these interactions through site-directed mutagenesis, chemical post processing, or by altering the solution conditions to cause changes in the pore structure or internal charge.

With DNA sequencing as the principal goal, several lines of research were undertaken to slow down molecular transport by changing the environmental factors such as lowering the solution temperature [59], or using solution containing organic *versus* inorganic salts [60], decreasing the transmembrane potential, increasing the salt viscosity [17,59,61], or adding crowding agents to alter the osmotic pressure gradients [62]. Other changes were made to the molecular structure of the DNA such as embedding the secondary structures to single-stranded DNA (ssDNA) [51], adding hairpins to the structure [63], chemically tagging the bases [64,65], modifying both ends of the DNA molecule by adding specific antibody or complementary strands to the other side of the pore, which upon hybridization, keeps molecules trapped in the pore for extended periods of time [66,67]. More recently, metal ions ligated with the DNA molecules or attached chemically by metal binding chemicals are detected with high selectivity and sensitivity using αHL nanopores [68–71].

Engineering a pore by substituting different charged molecules into the pore wall significantly changes the pore conductivity. Conductivity plays a vital role in understanding the ion-selectivity of a channel. Merzlyak et al. [72] showed that ion selectivity of a genetically engineered αHL channel could be controlled by placing various charged amino acid residues at different locations along the longitudinal axis of the pore. They found that the ion selectivity depends on the net charge of the pore wall, while the balance of charges between the *cis* and *trans* openings influences the shape of the conductance-voltage curve. This innovative work shows an early approach to modifying a pore based on the charge of the analyte.

Genetic mutations were also used to reduce translocation rates for DNA. Howorka and co-workers modified the pore by attaching an ssDNA to the cis-side entry of a pore (external to the channel) and demonstrated the detection of single-base mismatches using the duplex lifetimes [73] as well as the kinetics of duplex formation [74]. The translocation rate can also be decreased by introducing positive charges at the constriction region of the channel [75]. Blocking translocation by using streptavidin-complexed DNA identified that the β-barrel domain is the region that contributes most of the resistive signal, but changes in the constriction could tune the interactions [76], and adding unnatural amino acids having aromatic side-groups allowed for the detection of epigenetic DNA base modifications [77,78]. While these modifications are typically performed through traditional biochemical techniques, direct chemical modifications have been shown to be equally effective providing nearly limitless chemical modifications [79,80].

Apart from chemical and genetic modifications and changing the external environmental factors, the other prominent way to improve detection is to incorporate molecules and enzymes at various locations within the αHL pore. The combination of DNA polymerases (DNAP) with αHL has been shown to act as a motor to control the transport of DNA through the pore. Enzymes such as E.coli polymerase I Klenow fragment, bacteriophages T7 and phi29 DNA have been widely used for DNA sensing in this way [81–85]. Endonuclease and exonuclease techniques are other ways that can broaden the DNA sequencing approach. The endonuclease enzyme attached to the sensing region of an αHL pore allows the sequencing of cut-off bases during their interaction. Simultaneously, the DNA strands are digested and the cleaved nucleotides are then detected with a non-covalent adapter in the latter approach [86]. For example, an αHL mutant pore $(M113R)_7$ non-covalently linked with a modified cyclodextrin adapter is used for sensing all four 2′-deoxyribonucleoside 5′-monoposphophates (dNMPs) and all four ribonucleoside 5′-monophosphates (rNMPs). The interaction between the monophosphates and the adapter produced a distinct current blockade for each of the four nucleobases with 93% to 98% accuracy [87]. However, the blockades were very short and similar for each of the bases, which limits the discrimination capabilities of the pore. This issue was addressed by covalently attaching a molecular adapter to the β-barrel of a mutated pore. This mutated pore-adapter complex method enabled the identification of all four dNMPs with 99% accuracy [88]. This was a significant step towards nanopore-based sequencing because it showed that biological nanopores can detect exonuclease activity and identify nucleobases. Like a polymerase approach, all single-nucleotides were identified when added to the αHL-tethered DNA strand by an attached DNA polymerase [89]. Moreover, this approach can be used to monitor DNA polymerase activity at a single-molecule level.

Continuous efforts to optimize nanopore sequencing have led to RNA base discrimination as well. Biotin tagged on to the 3′ end of RNA complexed with streptavidin at the entry point of the pore immobilizing the RNA in a mutated αHL nanopore (E11N/K147N/M113Y). Here, both the modified and unmodified individual RNA bases were identified with superior nucleobase discrimination [90]. In a different experiment, all four ribonucleoside diphosphates (rNDPs) and ribonucleoside monophosphates (rNMPs) were continuously detected more efficiently by using mutated M113R αHL pores with non-covalently linked cyclodextrin adapters [91].

More recent progress towards DNA sequencing has utilized sequencing by synthesis (SBS) approaches [92,93]. This process involves tagging of nucleotides with an identifiable polymer that gives rise to continuous distinct current blockades during the DNA polymerase catalytic cycle. First, the phi29 DNA polymerase molecule is covalently linked to the αHL. Polymer tags of four different lengths of polyethylene glycol (PEG), between $n = 16$ and $n = 36$ were attached to each nucleotide's phosphate terminal, which can incorporate with the DNA polymerase. The tags are then released as polyphosphate byproduct after the DNA polymerase reaction, leaving nucleotide on the template DNA to grow further. The byproduct tag of different lengths enters the pore and yields distinct current blockades that identify the nucleotide attached. Importantly, this SBS approach addresses problems associated with long repeats in DNA sequences.

3.2. Aerolysin

Aerolysin is a water soluble cytolytic protein secreted by the gram-negative bacterium and human pathogen *Aeromonas hydrophilia* [94,95]. The aerolysin pore was used for single-molecule analysis, and has been successfully employed to detect DNA [96], single amino acids [97], peptides [58], polymers [98], methylated cytosines [99], and can directly discriminate single nucleobases [100] with high sensitivity.

Unlike αHL nanopores, aerolysin has a central β-barrel that is approximately 10 nm long and 1 nm in diameter (Fig. 3b) [95,101] and is stabilized by a concentric β-barrel bound together by hydrophobic side-chain interactions [46]. Aerolysin is negatively charged (Z = −52 e), and subsequently anion selective [102], with a conductance that is

lower than αHL [103]. The geometry and chemistry of aerolysin makes it a compelling compliment to or replacement for αHL in a number of applications [104,105]. The barrel in aerolysin contains two constriction regions (R282-R220 and K238-K242) that were identified and confirmed by theory. The R220 residue is located near the *cis* entrance, whereas K238 is located deeper in the stem. Cao and colleagues have shown the effect of mutation at constriction regions for both ion selectivity and sensing of the pore using biophysical and computational methods [106]. Alanine substitutes were made (R282A, R220A, K238A, and K242A) to expand the diameter along the pore lumen and tryptophan was substituted to compress the diameter (R282W, R220W, K238W, and K242W). Additionally, the electrostatic properties of the sensing regions were studied by altering the charge using amino acids of comparable side-chain volume (Cap: R220K, R220E, and R220Q, stem: K238N, K238E, K238Q). Molecular dynamics confirmed that the results were in line with their predictions for alanine substitutions (*i.e.*, alanine broadens the pore at the *cis* side and narrows the *trans* side). In contrast to the prediction, the constrictions were enlarged by the tryptophan due to increased repulsion by the hydrophobic residue. These experiments demonstrated control over the constriction from 0.5 nm to 1.5 nm and provide a firm basis for understanding the energetics of sensing with the aerolysin channel.

3.3. *Mycobacterium smegmatis A*

Another promising biological nanopore is *Mycobacterium smegmatis A* (MspA), a water regulating channel found in mycobacterium [107]. Unlike the pore-forming toxins above, MspA (Fig. 3c) is an octameric pore with a goblet-like conformation with a large interior cavity and a thin narrow hydrophobic constriction at one end [47]. The internal diameter varies from 4.8 nm at the *cis* side (external to the cell) and 1.2 nm at the *trans* mouth. Unlike αHL and aerolysin, wild-type MspA does not form an ion-conducting channel that has the necessary properties for biosensing. Rather the channel was rigorously mutated to produce a pore that is both thermally and chemically stable, which was ideally suited for DNA sensing [47,108]. The mutated channel used for sensing is cation selective, and its internal cavity is large compared to both αHL and aerolysin with its conductance higher as a result. However, this pore's most compelling characteristic is its thin, narrow constriction estimated to be 1.2 nm diameter and only 0.6 nm thick near the trans mouth of the pore [109,110]. This feature restricts the sensing location of the pore to this region. Utilizing a polymerase enzyme outside the pore to restrict the motion of DNA, MspA was the first pore to perform sequence reads of the phi X 174 genome up to 4500 bases in lengths [108,111]. In addition to DNA sequencing, Cao et al. modified the narrow constriction with methionine to demonstrate detection of $AuCl_4^-$ directly [112].

3.4. *Curli assembly protein G*

The mechanism by which the *Escherichia coli* transport channel *Curli assembly protein G* (CsgG) promotes the secretion and assembly of amyloid-like fiber proteins across the outer membrane of Gram-negative bacteria remains an open question [48,113]. Nevertheless, the crown-shaped CsgG shows promise as a single molecule nanopore sensor given its symmetrical nonameric structure. This pore consists of a central channel characterized by three regions: the periplasmic lumen, the pore eyelet, and the transmembrane β-barrel. Like MspA, CsgG has a thin narrow constriction which makes it amenable to DNA sequencing. Unlike MspA, the constriction is located in the center of the pore, and it is punctuated by two closely spaced bottlenecks (Fig. 3d) [48]. The interior of the pore lumen is negatively charged and contains several hydrophobic residues [48,114]. The narrow constrictions give CsgG a low conductance compared to similar sized pores but provide a unique double signature ionic current profile for polymers translocating through the pore. This feature gives CsgG and other CsgF family pores, the ability to resolve homonucleotide sequences with high accuracy [115], a previously unattainable goal for strand-based DNA sequencing.

3.5. *Fragaceatoxin C*

Fragaceatoxin C (FraC) is an α-helical pore-forming toxin from an actinoporin protein family, which sets it apart from the primarily β-barrel pores typically used as biosensors. The pore can be formed from 6, 7 or 8 monomer units and each variant takes on a conical shape [49,116], which allows the pore to sense molecules over a much wider range than is typical in most biological nanopores. The spacious vestibule lumen (Fig. 3e) facilitates characterization of small folded molecules like peptides or proteins, while the narrow constriction site is ideally suited for protein sequencing [117]. In contrast to other channels as discussed above, the negative charge lining the pore lumen of FraC creates a cation selective channel. Double mutating wild type FraC (WtFraC) with D10R/K159E (ReFraC) makes the interior surface of the constriction zone positively charged allowing the translocation of negatively charged DNA molecules. The most remarkable characteristic of ReFraC is that it allows sensing of dsDNA (≈2.0 nm) despite having the narrower constriction (1.2 nm) region. This is allowed because the α-helical transmembrane region of the pore can be readily deformed [116].

These pores represent a small sampling of the thousands of pores available for nanopore sensing, and the ability to engineer different chemistry both biochemically and by post translational modification gives an unlimited number of different iterations that can be used to control capture and transport of molecules to and inside the pore. While the main focus of nanopore sensing has been on sequencing-based applications, a large number of more recent studies have considered other applications of nanopore sensing. To provide context for this discussion we will focus the next section on some of the more fundamental aspects of nanopore sensing *via* polymer on-rate and off-rate kinetics. In addition, we will discuss various connections between current blockade distributions and polymer characteristics. Finally, we will focus on the use of nanopores as single molecule "test tubes" where chemical processes can be observed within the nanopore confined volume.

4. The physical chemistry of sensing

Polymer partitioning into the nanopore volume leads to clearly identifiable current blockades and the magnitude of these blockades provides information about the hydrodynamic volume of the molecule in question. In addition to the blockade depth, the corresponding nanopore blockade kinetics, specifically the on-rate and off-rate to and from the pore, yields detailed information about the interaction of the molecules with the pore. The ability to introduce point-mutations into the pore wall enables controlled interactions between the target analyte and the engineered pore surface [118]. This in turn can be used to design and study chemical interactions at the single molecule limit [119]. In addition, increasing the analyte residence time (decreasing the off-rate) improves the prospects of using the pore as a single molecule sensor. In brief, the longer a molecule remains in the pore, the more details can be extracted from each individual current blockade event. Additionally, adjusting the on-rate kinetics of analyte to the pore improves the sensitivity of the detector and reduces the limit of detection for any counting-based nanopore application. These applications motivate our interest in reviewing the development of understanding polymer-nanopore kinetics. A nanopore sensor follows a straightforward reaction (interaction) scheme delineated in Fig. 4. Using a laser-based heating approach, we recently measured the energetics of capture and retention of peptides and PEG partitioning into and out of the αHL nanopore [120]. In this study the free energy for polymer capture was measured with both the barrier for capture and release. Most compelling was that the laser drive temperature oscillation, which allowed for the enthalpic and entropic escape barrier to be unambiguously separated,

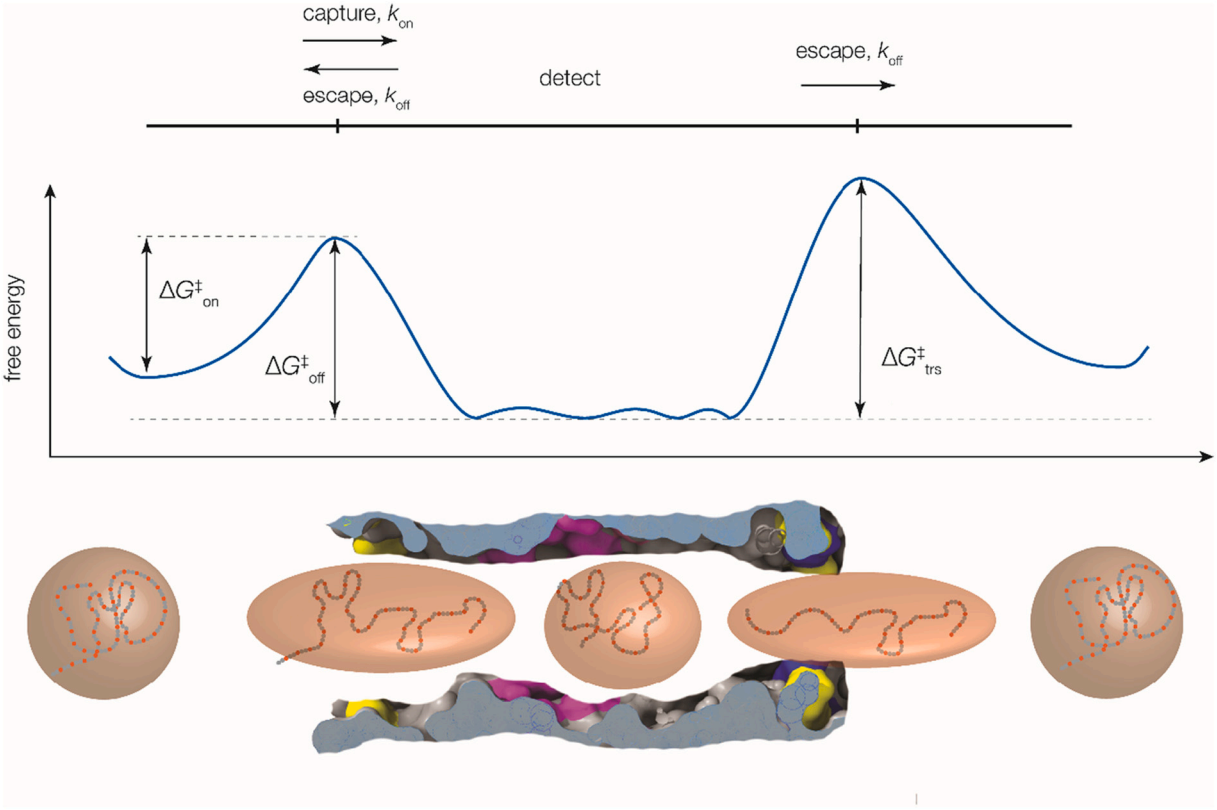

Fig. 4. Nanopore sensors operate according to a simple reaction scheme that requires capture, retention and release either in the forward or reverse direction. The magnitude of the free energy barriers, which can be entropic, enthalpic or both dictate the efficiency and effectiveness of the sensor. In the simplified scheme presented here, a polymer reorganizes to cross a barrier for entry into the pore and is held in the pore by barriers at either end. The event is complete when the polymer exits in either direction. The barriers are dependent on the chemical details of the molecule and the pore. Understanding and manipulating these barriers is a major focus for biosensor development.

with entropy limiting the PEG system and enthalpy limiting the peptide systems.

4.1. Nanopore kinetics

For the case of Coulter-like resistive pulse nanopore sensing, the nanopore dimensions must be on the same scale as individual molecules. While this enables distinct current blockade signals for each individual molecule, it raises questions regarding the capture efficiency of a nanopore sensor. Plainly speaking, for the nanopore sensor to be a viable detector, analyte molecules must both reach and enter the pore with sufficient frequency so as to enable a sufficient number of events for constructing informative single molecule distributions. While mass transport is often the limit for single molecule measurement techniques, it is somewhat in contrast to more traditional electrospray mass spectrometry [121] in which the solution is expelled through the tip of an electrified capillary, where the analyte is ionized and for the most modern tools captured in an magnetic trap which can be operated as an integrating detector. The practical limit of detection (LOD) approaching the low pmol/L range [122]. This LOD is similar to what is observed for an intensity calibrated fluorescence correlation spectroscopy (FCS) microscope, which has a detection volume on the order of 10 fL [123]. Alternatively, fluorescence assays based on molecular recognition reporters such as those found in enzyme linked immunosorbent assays (ELISA) are dependent on the association constant of the reporter antibody to the analyte. As discussed below, the nanopore sensor has limitations that are similar to both of these techniques. The analyte must first be transported and captured by the nanopore and the pore must retain the analyte in its central cavity long enough to be detected.

Analyte transport can be described by either diffusion or drift where diffusive transport follows from the chemoreception work of Berg and Purcell [124] who showed that the arrival rate k of diffusion-based analyte transport to a single isolated circular pore is given by

$$k_{dif} = 4Dc_b a \quad (1)$$

where D is the diffusion coefficient of the analyte molecule, c_b is the bulk concentration of the analyte and a is the radius of the nanopore opening. In addition, charged analyte (*i.e.*, DNA) can undergo drift-dominated transport where the rate of arrival is given by [125,126]

$$k_{drift} = Ac_b V \quad (2)$$

where V is the applied transmembrane potential and A is a proportionality constant dependent on numerous parameters such as mobility, viscosity, *etc.* Numerical studies have expanded on the capture rate kinetics [127], but in most cases it is the combination of both diffusion and drift-based transport that are required to accurately describe the arrival rate kinetics to the pore.

Entry into the pore for single molecule analysis is further limited by a thermodynamic barrier that depends on numerous parameters including the pore dimensions, analyte size, shape and flexibility [125]. Regardless of the details of this barrier, it is important to note that it can be significant and this will lead to a reduction of capture events by up to an order of magnitude [17,128].

Once inside the pore, molecules can either translocate through or diffuse back out the side they entered from. The question of analyte translocation through pores has been developed through the study of protein transmembrane translocation rather than chemical detection. Early studies consider the motion dominated by biased Brownian motion or a thermally driven ratchet model [129,130]. The seminal work by

Kasianowicz et al., that clearly showed DNA translocation across an αHL pore, motivated the development of the nanopore sensor as a sequencer. This motivated the work of Lubensky and Nelson to describe the transport of DNA through a pore with a coarse-grained model to extract first passage time distributions [131]. Given the importance of developing a single-molecule DNA sequencing engine, much effort was subsequently focused on understanding this DNA threading process [132–140]. Generally speaking, understanding polymer transport through a nanopore leads to either a dynamic picture of the transport process (*i.e.*, drift-diffusion) [129,131,136] and/or a free energy barrier [137,139–147] against escape from the pore.

While DNA sequencing motivated much of the development of nanopore sensors, the field has also focused on the analysis of near-neutral polymers and peptides for further single molecule analysis. One molecule of particular interest is PEG, which has shown a strong dependence between ionic strength for several salts (KCl, NaCl, RbCl, CsCl) and pore residence time [148]. It has been proposed that weak binding between these cations and the PEG can modify the interaction between the PEG and an αHL pore. This increases the residence time of PEG polymers to milliseconds, which enables single monomer resolution of the PEG current blockades [149]. This motivated a more detailed study of the PEG-pore interaction, which led to a model of the PEG residence time that combined an electrophoretic-based drift of the cation-charged PEG with a free energy barrier to exit that incorporated polymer confinement and cation binding to the PEG [53,150].

Near-neutral polymer analysis with nanopore sensing was motivated in-part by the interest in protein and peptide analysis. While DNA sequencing is the clear motivation for most of the early results and focus on nanopore sensing, more recent efforts have begun to focus on the development and understanding of peptide analysis. Peptides introduce a number of additional complications (*i.e.*, folding, analyte-pore interactions, solvent interactions) that need to be understood to fully develop the nanopore sensor in this venue. Several reviews have already been written on the subject of peptide and protein analysis with nanopores [151–154]. Here we highlight a few studies that analyze the free energy barrier to peptide and protein escape from the nanopore.

Hoogerheide et al. utilized a drift-diffusion description of α-synuclein through voltage dependent anion channel (VDAC) pores. The free energy barrier against escape depends on an enthalpic component of the α-synuclein binding with the pore wall and an entropic confinement term [155]. Larimi et al. studied the role that molecular crowding outside the pore has on the kinetics of polypeptides inside an αHL pore. The crowding modification affects the entropic component of the free energy barrier to escape, which in turn affects the on and off-rate kinetics of Syn B2 polypeptide with an αHL pore [62]. Mohammad and Movileanu demonstrate a modified free energy barrier to protein escape which incorporates a binding term inside a mutated pore [156]. Asandei et al. modified the on- and off-rate kinetics of polypeptides by modifying the charge at the end terminals of the peptide. This modifies the drift force along with the free energies to capture and escape [157]. Each of these examples illustrates the importance of the free energy barrier description to nanopore kinetics. The last two examples illustrate the flexibility that nanopore sensing provides by allowing for the experimental conditions to be modified either through modifications to the pore, the analyte, the chemical or physical conditions to modify the sensing capabilities of the pore. This degree of control allows for improvements to the nanopore sensor, which we describe in detail in the next subsection.

4.2. Enhanced sensing

Optimizing nanopore sensing requires increasing analyte on-rate to the pore and decreasing analyte off-rate from the pore. In the former case, the nanopore sensitivity is maximized and in the latter case the ability to accurately characterize each capture molecule increases. This has motivated the exploration of a wide range of modifications to optimize the polymer kinetics which we summarize here.

DNA translocation through wild-type αHL was first reported to be on order of 1 μs [17] for each nucleotide in a ssDNA molecule, which is too rapid to enable base-level sequencing. This drove a considerable effort to apply methods to slow down this translocation, such as modifications to the nanopore wall [158–161], solution conditions [60,162–167], temperature [168–170], pressure [171], electrode composition [172,173], adding external reagents that alter the electroosmotic flow in the pore such as β-cyclodextrin [174–177], or gold nanoparticles [178] as well as modifications to the physical environment outside of the pore [179,180] and molecules that bind and slow down transport outside the pore DNA-antibody binding [181].

In addition to slowing DNA translocation through the pore, other efforts have focused on modifications to the free energy barriers to escape for other molecules of interest. These efforts include gold cluster-induced off-rate enhancement of PEG [182,183] and peptides [31] from αHL, pH-induced adjustment to electroosmotic braking for slowing down peptide translocation [184], controlling ionic permeability *via* polymer modifications to nanopore walls [185] and molecular crowding for enhanced detection of beta-galactosidase and α -synuclein amyloid fibrils with a glass nanopipette tip [180].

While the aforementioned results focus on enhancement to the off-rate kinetics from the pore by increasing the time that the analyte spends in the pore, other efforts have focused on enhancement to on-rate kinetics. These include using gold clusters to increase the on-rate of peptides to an αHL pore [31], dielectrophoresis to increase the on-rate of DNA to a glass nanopipette-based sensor [186], and molecular crowding to improve the capture rate of freely diffusing analyte outside the pore entrance [187].

Clearly, understanding and controlling polymer-nanopore kinetics has been an important driving force in the development of nanopore sensing. The ability to modify the pore, environment and/or analyte improves the prospects for sensing across a wide range of targets. In the next section we explore in more detail the connections between the current blockade signatures and the ability to perform size-selection studies on various polymers with an emphasis on the pore's ability to discriminate between polymers differing in size by a single monomer unit.

5. Selection by molecular size

As discussed previously, these nanopore sensors operate under a sensing regime that is controlled largely by the volume occupancy of the molecule in the pore. It was noted early in the development of these sensors that the pores worked as molecular sieves, allowing small molecules to partition into the pore while excluding polymers that were larger than the pore diameter [10,188], and this effect was used to estimate the diameter of a number of different protein channels without solved crystal structures [10,189–191]. Naturally, the problem was reversed, and the current fluctuations were used to investigate the analyte. Bezrukov and Kasianowicz examined the partitioning of polyethylene glycol into the cavity of αHL by analyzing fluctuations in the noise signature as a function of polymer size [188]. This line of research was greatly aided by the discovery that increasing the ionic strength gave rise to current blockades on the order of milliseconds, which allows unambiguous single molecule detection and characterization [192]. By analyzing the resistive pulses obtained under high electrolyte concentration, PEG-induced resistive pulses were used to produce a histogram with polymers sufficiently resolved to the single monomer level [149] to produce a single molecule mass spectrometer (in reality a molecular volume sensitive spectrometer). To understand how to optimize this sensor, Rodrigues and Krasilnikov proposed a mechanism that attributed the long current blockades to the polymer solubility [193]. Reiner and Robertson attributed it to the electrolytication of the PEG through specific interactions with the cation [53], which was later confirmed with molecular dynamics simulations [150]. Regardless of the physical

mechanism, the resolution in the case of αHL can be scaled by optimizing the analytical algorithms used for building the histogram [36,194]. In short, the reported current blockade distributions represent the average magnitude of each current blockade, therefore the fluctuations or relative noise associated with any single blockade event will decrease with time proportional to $t_{off}^{-1/2}$. Thus, increasing the time that the polymer spends in the pore, or filtering out the short, noise limited events improves the size resolution of the technique. The evolution of this size-selectivity is shown in Fig. 5.

PEG resolution has now been shown to be baseline resolved for two different systems, Au cluster-modified αHL [182,183], which increases t_{off} and aerolysin pores which likely has a different polymer retention mechanism [98]. Furthermore, temperature-induced structural modifications can alter the blockade depth shifting the blockades and altering resolution [195,196].

Size-dependent resistive pulses are not only true for PEG, but it extends to peptides and proteins as well (Fig. 6). Chavis et al. demonstrated that despite the difference in the chemical identity, peptides follow the same size-dependent change in the resistive pulse as PEG, and chemical mediators, such as denaturing guanidinium hydrochloride, only serve to alter the dynamics and subsequent noise of the blockade [31]. Aerolysin channels were modified to detect changes as subtle as single amino acid substitutions on a carrier chain [197], which is likely a manifestation of the subtle change in volume between each residue, provided that the molecule is driven through the pore as an unfolded chain [198,199]. These results have clear implications for the ability to sequence peptides. At a less granular level, the size dependent sensing has been extended to large, fully-folded proteins in two novel ways. Huang and colleagues took advantage of the flexible FraC pore which has a funnel shaped cross-section (rather than a rigid β-barrel) to extend the detection and discrimination range to 25 kDa or more [117] with resolution as low as 44 Da for smaller peptides [200]. While this resolution does not approach the sub-part per million resolution of an advanced mass spectrometer, the instrumentation is an order of magnitude or more less expensive. Additionally, nanopore measurements can be made application specific and massively parallel. Recently, the FraC pore was used to monitor post-translational modifications to peptides as they passed through the pore in an unfolded arrangement [201]. Yusko and

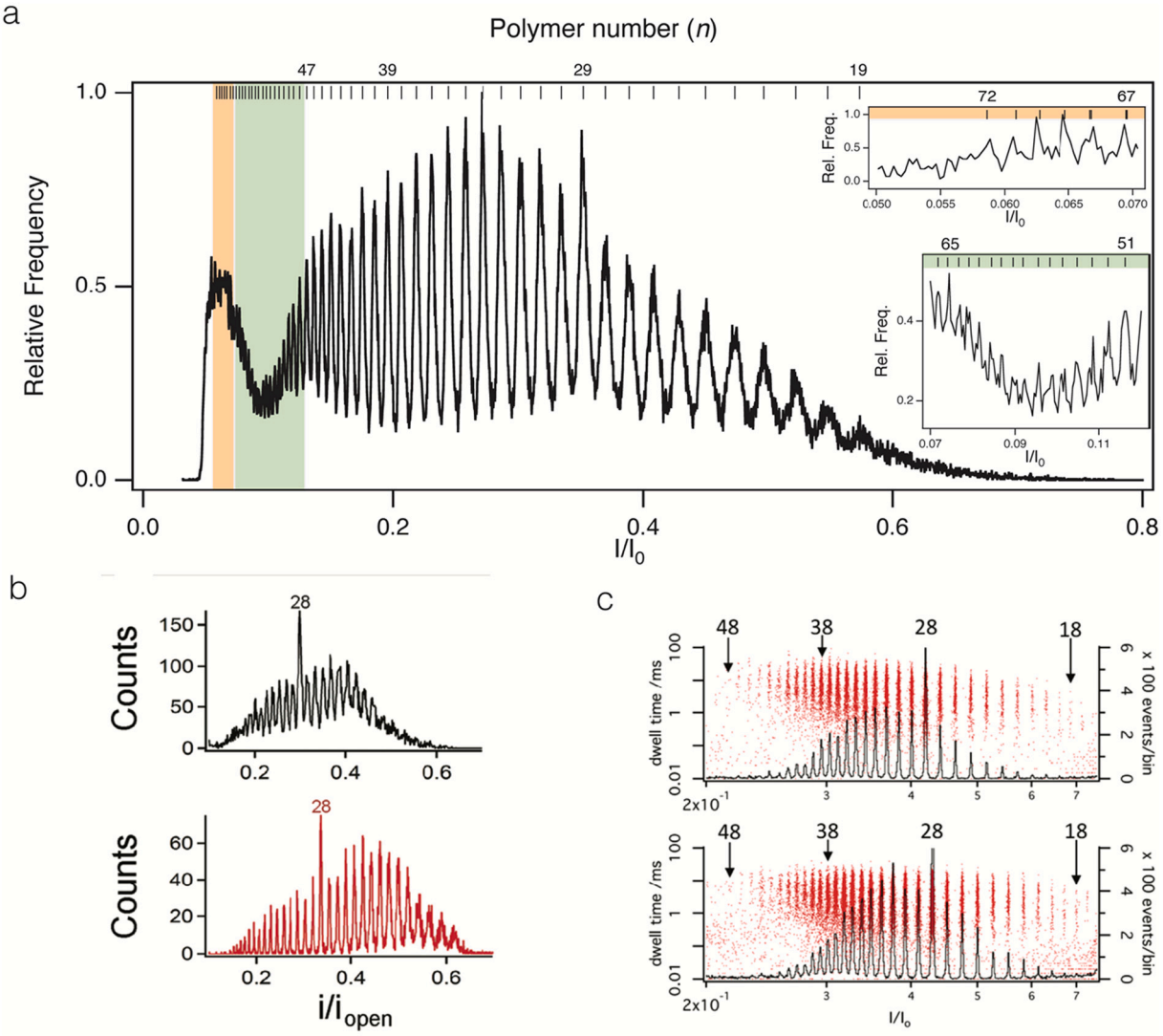

Fig. 5. Nanopores as size-selective sensors. Blockade depth histograms show the evolution of mass resolution of PEG. (a) Recast data from [53] shows the full range of size discrimination of PEG from $n = 18$ to $n = 72$. (b) Increasing the residence time with a pore-modifying gold cluster improves the resolution (red) with respect to the cluster free αHL pore (black) due to higher escape barriers and an order of magnitude increase in residence time.
Reproduced from Ref. [183] with permission from The Royal Society of Chemistry. (c) Aerolysin pores alter the balance of electroosmotic and electrophoretic transport and possibly the dynamics of PEG inside the pore and further improve molecular resolution. Reprinted with permission from [98]. Copyright 2015 American Chemical Society.

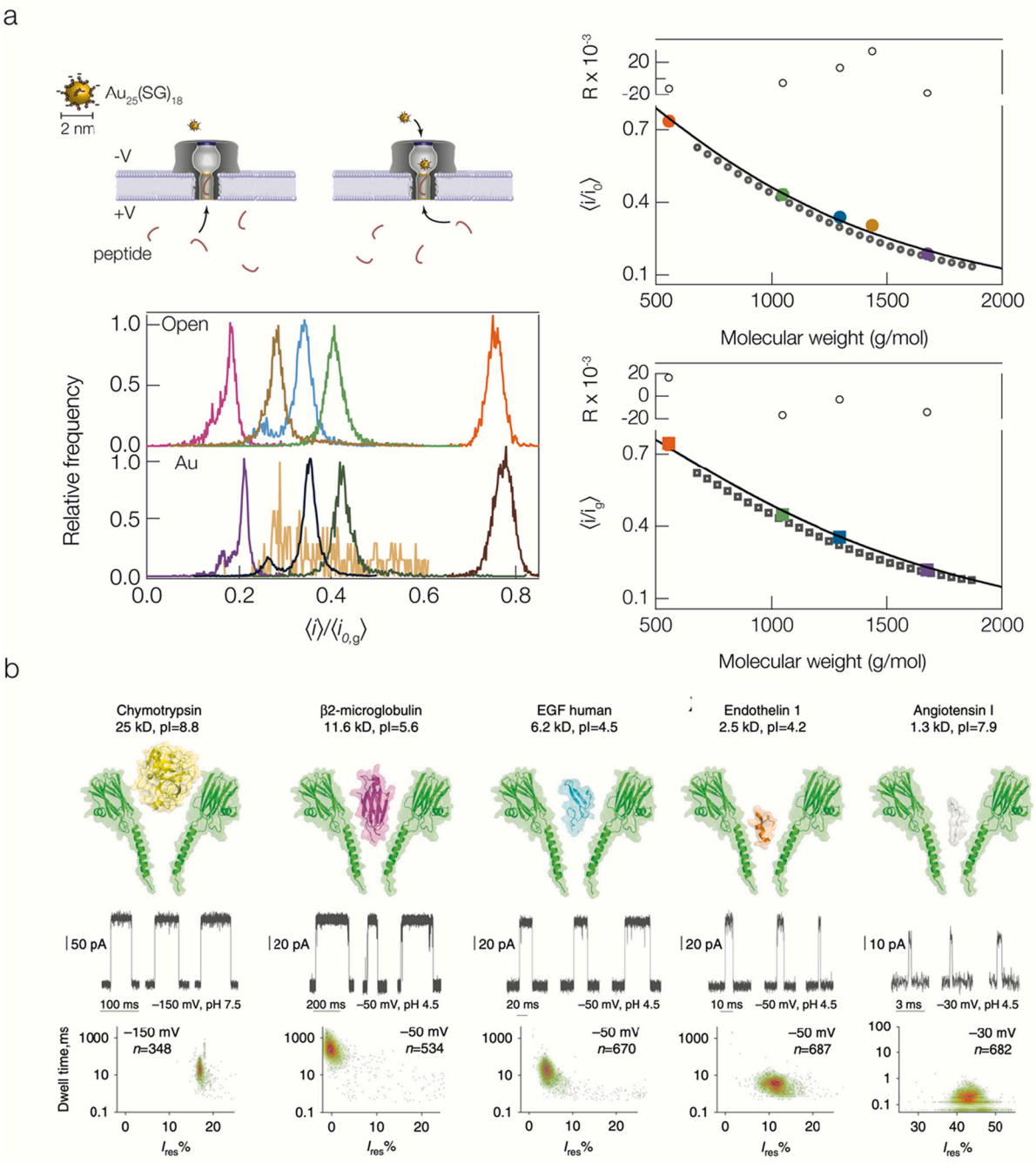

Fig. 6. Size selectivity for peptides in a nanopore sensor shows that volume (mass) of the peptides serves as a reliable discriminant. (a) Small peptides in an αHL pore show that peptide size scales well with PEG blockade depth suggesting a common volume dependent mechanism [31].
Adapted with permission from [31] Chavis et al. *ACS Sensors* (2017) https://pubs.acs.org/doi/10.1021/acssensors. Further permissions related to the material excerpted should be directed to the ACS (b) This selectivity is preserved with the more complex pore geometry of FraC, which provides a gradient selectivity governed by the unique shape of the pore [117]. See text for further details.

colleagues took a different approach of using a bio-like pore created by lining a solid-state pore with a mobile lipid wall [202,203]. By confining the protein to the pore wall with site-specific receptors, both size and orientation of the fully folded proteins could be resolved. There are few easily accessible measurements that can disentangle the biophysical properties of molecules, including shape, and dynamical structural variations. The most commonly used techniques include NMR and EPR spectroscopy (see [9] for examples), and vibrational spectroscopy (*e.g.*, FTIR) [204,205]. While these methods are highly effective and yield detail-rich information, they generally require large amounts of material, or in some cases isotopic labeling, which can add complexity and cost to an experiment.

6. Following chemical reactions: nanopore "test tubes"

Protein nanopores offer more than simple size selectivity for the development of biosensors. They can also be exquisite tools for following chemical reactions *in situ*. The most conceptually simple implementation of this scheme is based upon introducing reactive amino acids into engineered pores [206], and this has been utilized to map the sensitivity of several different pores to analytes [72], including divalent metal ions [207] and polymers with reactive functional groups

[208–210]. These sensors rely on the molecule partitioning into the pore and binding, often covalently, which often only allows the sensor to offer a one-time observation. There are clever exceptions to this rule as demonstrated by Qing et al., who engineered a cystine track in αHL that allows voltage mediated hopping of reversible disulfide bond formation/breaking [211].

Although the above examples typically use biochemical techniques to modify the pore's reactivity to analytes, some pores allow carefully chosen chemical reactions to be followed in real time. Cox et al. demonstrated real-time ligand exchange with thiolate-capped gold nanoclusters confined in an αHL pore (Fig. 7a) [212]. Ligand exchange kinetics in the nanopore were sufficiently rapid (*i.e.*, exchange steps on the order of 0.1 s to 1 s) to permit observation of exchange processes, which were found to be commensurate with previous calculations [213]. In addition, they reported real-time observation of peptide ligand exchange with the tripeptide glutathione exchanging with tiopronin-capped gold clusters. The nanopores can also be used to follow chemical reactions that are best characterized by conformational changes in the molecule. Johnson and colleagues followed the base-flipping in a segment of double stranded DNA [54]. To observe this reaction, DNA was captured in the pore with a segment of ssDNA passing through the pore, which traps the molecule in the cavity. A double stranded segment extends out of the pore through the vestibule. When a single base mismatch is present in the latch constriction at the *cis* mouth of the pore, current oscillations can be attributed to the mismatched base reversibly slipping in and out of the double helix structure, and examination of the kinetics of this process allows the energetics of this reaction to be estimated. Maglia and colleagues have developed a suite of tools for following enzyme reactions using protein pores [7]. They captured a dihydrofolate reductase (DHFR) enzyme in the cavity of cytolytic pore toxin (ClyA) using a c-terminal polypeptide to hold the enzyme in place. With the enzyme immobilized, they were able to resolve up to four ground state conformations of DHFR in the course of its reaction sequence. Together these studies show how carefully chosen nanopore sensors can be used to sense subtle geometric changes in molecules as a result of chemical reactions.

Many of these reaction systems are difficult to study with other measurement modalities. For example, prior to the nanopore example above, base-flipping experiments were only able to be studied indirectly with NMR techniques and through extraordinary crystal preparation methods [214]. While NMR has a much wider dynamic range from picoseconds to minutes or hours, the measurement for base-flipping dynamics is indirect —measured through protonation rates of the amino groups on the DNA bases. In contrast, the nanopore tools offer a narrower bandwidth from microseconds to minutes, but they are direct —measured through conformational changes of the DNA cross section. None of this is to say that nanopore measurements are superior to the alternative, rather that the nanopore can be developed as a complimentary tool that can provide high-quality rate constants through measurements that are orthogonal to the widely used structural biology tools in the biophysicist's toolbox.

It is not critical for the analyte, enzyme or other reactant to partition into the pore for a sensor. Recent work utilizing a different style of sensor relies on the ability of reactions outside the pore to induce gating in contrast to partitioning-based sensors [215–217]. Unlike the partitioning sensors, which are often pore forming toxins, the gating sensors are often made from large β-barrel pores with unstructured segments at the periphery that are not structurally significant and can thus be mutated to selectively bind to analytes. Fahie and Chen developed such a sensor from OmpG with a biotin capture group [218]. Their work highlights the role that electrostatics and steric effects play in both sensitivity and selectivity for the functionality of these sensors [219]. One advantage of these gating sensors is that the significant energy barrier for a large polymer to partition into a narrow pore is eliminated from the sensor reaction sequence. This significantly reduces the energy barrier that is often encountered for a nanopore sensor. Coupled with a highly selective capture loop, this provides a strong platform for the development of clinical biosensors for the detection of antibodies [219] and as a general scheme for biomarker discovery [217].

7. Conclusions and future directions

Nanopore sensors have come of age with the race to develop rapid and inexpensive genome sequencing devices and they appear poised to offer solutions for other sequence-based applications including RNA and proteins. However, these sensors have additional attributes that make them a versatile choice for the development of other clinical biosensors and more fundamental biophysical studies on polymer dynamics, particularly under confinement. There are two significant challenges that must be overcome before nanopore biosensors can become

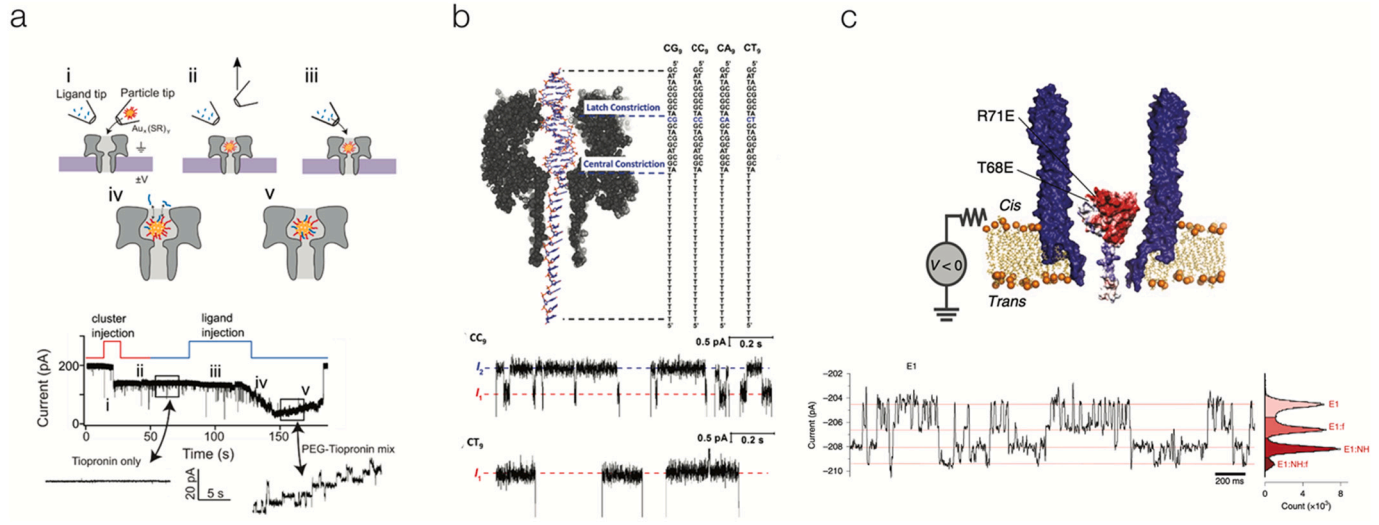

Fig. 7. Protein nanopores have been used to follow a number of different chemical interactions and transformations while trapped inside the pore. These are as varied as (a) observing ligand exchange on a gold nanocluster.
Reprinted with permission from [212]. Copyright 2020 American Chemical Society. (b) pH dependent base-flipping of mismatched DNA. Reprinted with permission from [54]. Copyright 2016 American Chemical Society, and (c) conformational changes of an enzyme during its reaction cycle. Adapted by permission from SpringerNature, Nature Chemistry [220] Copyright 2020.

workhorse tools in molecular sensing. The first affects all single molecule tools. Namely, the analyte must first find the pore which presents a volume that is on the order of yoctoliters (with a square nanometer cross section). These dimensions offer further complication because there will always be an energetic barrier to capture that provides the ultimate limit to the sensitivity. The second is that the thin membrane and support structures have a relatively large capacitance, which when coupled with the high resistance to ion flow, limits the bandwidth to around 1 MHz or a minimum observable retention time of 5 μs to 10 μs [36]. Efforts are currently underway in a number of different laboratories to better understand the chemical processes of these sensors, particularly the kinetic and thermodynamic optimizations that will enable new sensing schemes, improved selectivity for detection of analytes in complicated media and lower practical detection limits. Furthermore, developments in solid-state material processing [221], and schemes to make hybrid pores will further extend the range of these biosensors. Ultimately, the migration of nanopore sensors from the specialist's laboratory into general use will depend on the breadth of measurement that can be developed. The success of portable commercial DNA sequencing tools, such as Oxford Nanopore's Minion instrument has demonstrated a viable path to creating tools that will be used by experts and non-experts alike [222].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Joseph Reiner reports financial support was provided by NSF. Joseph Robertson has patent with royalties paid to Genia Technologies, Inc. Joseph Robertson and Joseph Reiner has patent #9,921,174 issued to United States Government.

Data availability

Data presented are compiled from published sources and cited in the reference section.

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