Review

Interrogating single proteins through nanopores: challenges and opportunities

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A single nanopore represents an amazingly versatile single-molecule probe that can be employed to reveal several important features of polypeptides, such as their folding state, backbone flexibility, mechanical stability, binding affinity to other interacting ligands and enzymatic activity. Moreover, groundwork in this area using engineered protein nanopores has demonstrated new opportunities for discovering the biophysical rules that govern the transport of proteins through transmembrane protein pores. In this review, I summarize the current knowledge in the field and discuss how nanopore probe techniques will provide a new generation of research tools in nanomedicine for quantitatively examining the details of complex recognition and, furthermore, will represent a crucial step in designing other pore-based nanostructures and high-throughput devices for molecular biomedical diagnosis.

What is stochastic biosensing with nanopores?

The modulation of ionic current through protein channels, as a result of external biophysical or chemical stimuli, has profoundly impacted the single-molecule science and biosensor arenas. In particular, the field of stochastic biosensing of small molecules and biopolymers using the resistive-pulse technique (see Glossary) has undergone a major expansion [1–4]. Stochastic biosensors are a class of single-molecule detectors that are based upon the alteration of electrical current by distinct analytes that interact transiently or permanently with a functional recognition group located within a nanopore [3,5]. The recognition group is strategically engineered within the pore lumen of a robust protein channel (Figure 1a). Each transient interaction of a single analyte with the recognition group generates a current blockade, the duration of which depends upon the strength of the interaction between the analyte and the reactive group (Figure 1b) [2,6]. This current blockade is produced as a result of the obstruction of the nanopore-mediated ionic flow by the analyte. Therefore, the partitioning of an analyte into the nanopore lumen is probed by the resitive-pulse technique in real time. It is worth mentioning that the electrophysiology techniques, which are nowadays extensively employed in examining the transport of biopolymers through nanopores, have been previously used for investigating the translocation machineries of proteins [7].

Stochastic biosensing has been employed for the detection of not only small molecules but also long biopolymers. In the past few years, a remarkable number of studies with nanopores have been stimulated by the promise of this technique for single-molecule sequencing of nucleic acids [8–10]. Despite numerous obvious challenges in using nanopores for nucleic acid sequencing [10], recent studies have demonstrated that nanopores represent versatile single-molecule probes for a wide range of molecules [11–13]. This review focuses on advancements in this area that are pertinent to the detection of short polypeptides and proteins.

A decisive transformation in the area of stochastic sensing with protein nanopores has occurred in Hagan Bayley's group, which set out the basic principles for the design of protein nanopores – these dictate that a robust protein channel scaffold is a prerequisite for developing a sensitive stochastic sensor element [2]. Recent studies in proteomics and structural biology have revealed that the β-barrel

Glossary

Aptamer: an oligonucleotide or peptide that binds to specific molecular targets, such as small molecules, nucleic acids, and proteins. Aptamers are used in biotechnological applications and therapeutics because they represent molecular recognition elements, which are more robust alternatives to traditonally employed antibodies.
Atomic force microscopy (AFM): atomic force microscopes contain a spring-mounted probe for imaging individual molecules on the surface of a material. The probe is a sharp micro-scale tip attached to a cantilever and is used to scan the surface. When the tip is moved near a surface, various forces between the tip and the sample produce a deflection of the cantilever. AFM can also be used to determine force–distance curves.
Cis chamber (side): the half-side of a cuvette for planar lipid bilayers that corresponds to the extracellular side of the β-barrel protein pores.
Langevin molecular dynamics: a mechanics approach that uses simplified models and stochastic differential equations to justify omitted degrees of freedom.
Optical platform: a device that consists of optical elements and that is used for probing molecules, cells and tissues.
Optical tweezer: an instrument that employs a laser beam to provide an attractive or repulsive force, in the order of piconewtons, to manipulate microscopic or nanoscopic dielectric objects. Optical tweezers have been used extensively in studying the motion and dynamics of biopolymers.
Resistive-pulse technique: a technique that detects conductance fluctuations caused by single fluid-born particles that transit a nanopore. Resistive-pulse devices combine traditional approaches of microfluidics and nanofluidics with electronic detection.
***Trans* chamber (side)**: the half-side of a cuvette for planar lipid bilayers that corresponds to the periplasmic side of the β-barrel protein pores.

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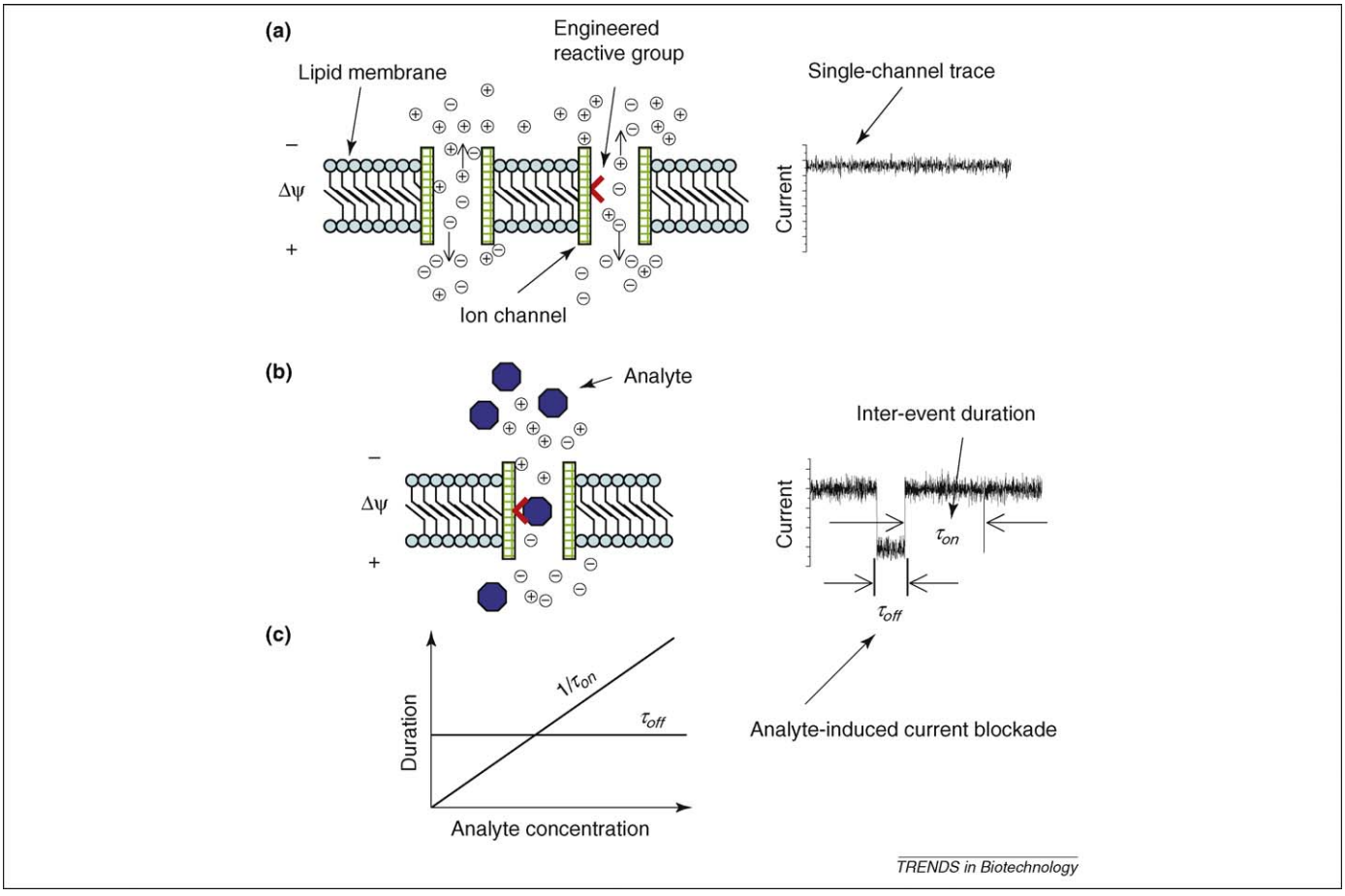

Figure 1. Principle of single-molecule stochastic sensing. A permanently open transmembrane protein pore facilitates the transport of small ions across an insulating bilayer lipid membrane. **(a)** When a voltage bias is applied, a single-channel electrical current is recorded. **(b)** Analytes added to one of the chambers non-covalently interact with the functional reactive group located within the pore lumen, producing transient single-channel current blockades, the nature of which depends on the strength of the interaction between analyte molecules and the reactive group. **(c)** A non-covalent interaction between a single analyte and an engineered reactive group obeys a simple bimolecular kinetic scheme, with a time constant of dissociation τ_{off} that is independent of the analyte concentration. The rate constant of association k_{on} is calculated as $1/(\tau_{on}c)$, where τ_{on} is the inter-event duration, and c is the concentration of analyte. The rate constant of dissociation k_{off} is calculated as $1/\tau_{off}$, where τ_{off} is the event duration.

membrane proteins fulfill such a requirement [14–16]. Most of the β barrels fold into approximately cylindrical pores, with the hydrophilic side chains exposed inside the pore lumen and the hydrophobic residues exposed to the lipid bilayer. More importantly, the structure of these proteins is stabilized by an array of backbone hydrogen bonds between adjacent β strands. If we take the energy of each hydrogen bond as being ~0.5 kcal/mol/residue [17], then, for a small β-strand-containing protein of only 100 amino acids, the total free energy associated with hydrogen bonding is ~50 kcal/mol. This oversimplified calculation reveals that the β-barrel proteins indeed feature an exceptional thermodynamic stability [18–20]. Conversely, membrane proteins consisting of α-helical bundles exhibit much lower free energy (<8 kcal/mol) [17].

Watching single proteins inside the nanopore

Among the many β-barrel proteins, the staphylococcal α-hemolysin (αHL) protein [21] is the most robust nanopore when employed under various harsh conditions of experimentation (Figure 2). This protein nanopore is stable in planar lipid membranes up to 95 °C [19] and exhibits a well-defined single-channel conductance [22,23], permitting extensive genetic engineering throughout the pore lumen [2,5,24,25]. These attributes made the αHL protein a suitable nanopore platform for single-molecule stochastic sensing of small molecules and large biomolecules [2,4–6,8–10,12,13,26].

The pioneering study of John Kasianowicz, Eric Brandin, Daniel Branton and David Deamer [27], which demonstrated the translocation of single-stranded DNA (ssDNA) through the αHL protein pore, inspired more than a decade of fertile single-molecule science with nanopores. In some respects, it is harder to deal with polypeptides than with ssDNAs. An ssDNA homopolymer is a linear, chemically stable and densely but homogenously charged molecule that can be driven through a single nanopore with little effort; the application of a voltage bias across the membrane is sufficient for successful translocation from one side of the membrane to the other. By contrast, polypeptides are not homogeneously charged, containing positive, negative, polar and hydrophobic side chains. Moreover, the polypeptides are folded polymers with a complex free-energy landscape encompassing numerous transition states and conformational sub-states. ssDNA heteropolymers can also form all kinds of structures, including bulges, which can be even more stable than proteins. However, the biophysical and biotechnological applications of

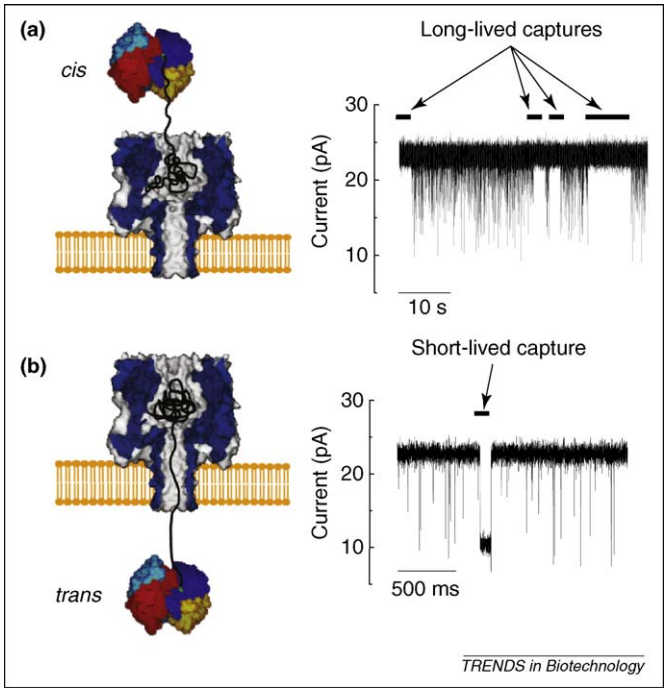

Figure 2. Single-molecule stochastic sensing of a binding protein using a transmembrane protein nanopore. A low-affinity streptavidin mutant is detected outside the pore lumen of a staphylococcal α-hemolysin (αHL) channel at the single-molecule level. The streptavidin size is greater than the diameter of the nanopore, so the protein sensing occurs in the aqueous phase. **(a)** The ligand, a biotinyl group, was attached to the untethered end of the PEG chain (shown in black). The binding protein was detected on the *cis* side through transient current blockades caused by reversible captures of the protein by the tethered PEG chain. **(b)** The binding protein was detected on the *trans* side through very rare and short-lived current blockades. The arrows represent single-molecule transient captures of the binding protein that produce a distinctive conformation of the linker, as observed by single-channel electrical recordings. Reproduced, with permission, from Ref. [35].

polypeptide translocation through a protein nanopore have not been pursued as aggresively as those employing nucleic acids.

Several research teams have already explored the interaction of model polypeptides with the wild-type αHL (WT-αHL) protein pores, including α-helical [23,28,29] and β-hairpin [30] polypeptides. In general, these investigations revealed a kinetic profile of polypeptide translocation through the αHL protein pore that depends on the biophysical characteristics of the interacting polypeptides, such as their length, charge, folding and stability. The simplest possible model of the interaction between a polypeptide and a protein pore can be illustrated by a two-barrier, single-well, free-energy lansdcape [4,23]. To transit the nanopore, a folded polypeptide has a greater energetic penalty than an unfolded polypeptide. In accord with this reasoning, a recent study that combined single-molecule electrical recordings and Langevin dynamics simulations showed that highly unfolded β-hairpin polypeptides entered the pore in an extended conformation, producing fast single-file translocation events. This finding contrasted with that of the translocation of structured β-hairpin polypeptides, which occurred more slowly, producing long-lived current blockades [30].

Loic Auvray and colleagues [31] identified the αHL protein pore as a suitable tool for examining the folding properties of proteins. The experiments with αHL protein pores were accomplished in elevated concentrations of guanidinium hydrochloride (Gdm-HCl, ~1.5 M), a chemical denaturant that is commonly used in protein-folding studies. They tested the ability of the αHL-based nanopore detector to respond to well-defined changes of the unfolding state of the 370-residue maltose-binding protein (MBP) of *Escherichia coli* at different concentrations of Gdm-HCl. In the absence of the denaturing agent, no current blockades were recorded, suggesting a large energetic penalty of the protein to partition into the pore lumen. Remarkably, protein-induced current blockades were observed in the presence of increased concentrations of Gdm-HCl. The frequency and duration of the events was dependent on the concentration of Gdm-HCl in the chamber, indicating that the partioning of the MBP protein into the nanopore lumen is correlated with its folding state in the aqueous phase.

There is no fundamental difficulty in replacing the global (i.e. thermodynamic) unfolding induced by a chemical denaturant or temperature with spontaneous unfolding through single-site mutagenesis of a strategic position of the protein. Jeremy Lee and colleagues [32] have employed the αHL protein pore to show that the transit of an analyte, a histidine-containing protein (HPr), is sensitive to single-site mutations that alter its folding state. The low activation free-energy of unfolding HPr (~5 kcal/mol), which is a typical value for small proteins, partly explains the fragile folding state of HPr, which was revealed by single-channel electrical recordings [32]. This finding contrasted with recent results obtained by the same authors with a tightly folded Zn(II)-finger protein interacting with the WT-αHL protein pore; these results showed very short-lived current blockades that were interpreted as 'bumping' of proteins into the pore entrance [33].

We were also able to engineer an exogenous polypeptide loop within the large vestibule of the αHL protein pore, a cavity with a volume of ~39 500 $Å^3$ [18]. The exogenous polypeptide had the amino acid sequence $(VPGGG)_5$, which is an elastine-like-polymer that undergoes an inverse temperature transition. The elastin-like polypeptides (ELPs) dehydrate and hydrophobically collapse at temperatures above the inverse transition temperature and unfold at temperatures below the inverse transition temperature. Indeed, we were able to observe the hydrophobic collapse of the engineered ELP loop through significant changes in the temperature-dependent amplitude of the ELP-induced current blockades. Below its transition temperature, the ELP loop was fully expanded and blocked the pore completely, but reversibly [18]. At temperatures above the transition temperature, the ELP excursions into the pore lumen did not produce a full blockade, enabling a substantial flow of ions because of the hydrophobic collapse of the ELP. Remarkably, the features of the transient ELP-induced current blockades were dependent on the peptide sequence. A glycine/serine-rich loop with similar length produced a different single-channel electrical signature comprised of highly frequent and very short-lived current spikes. The current spikes were consistent with substantially increased peptide flexibility because of the glycine residues that have less bulky side chains. These results demonstrate the capability of probing the conformational fluctuations and flex-

ibilities of the polypeptides placed permanently within the large vestibule of the nanopore lumen. In addition, other engineered αHL pores with foreign and functional, structured or responsive polypeptides might be developed. Protein pores with stimulus-activated (e.g. photo-responsive) gating mechanisms might be useful for fundamental studies in membrane protein engineering and for the design of new biosensor-based nanostructures. Engineering loops in a robust β barrel would generate exciting findings regarding the gating mechanisms of protein channels by flexible polypeptide chains.

Watching single proteins outside the nanopore

Detection of polypeptides inside the pore lumen has limitations. The most important limitation is probably the inability to capture all the kinetic information of the polypeptides in the aqueous phase. A folded protein is several-fold bulkier (~a few tens of Å) than the inner diameter of the protein pore (~20 Å) and so has to transit the pore while in an unfolded or partly folded conformation [23]. The kinetic transformations of the polypeptides in the aqueous phase are not detectable by single-channel electrical recordings. This daunting limitation has been overcome by covalently attaching a flexible linker, a poly(ethylene glycol) (PEG) chain, to the pore lumen within the large vestibule of the αHL protein pore [34,35]. A ligand, in this case biotinyl, was covalently attached to the untethered end of the linker. The biotinyl group was able to move across the entire region of the pore lumen from one side of the membrane to the other, a distance of ~100 Å. When a binding protein, for example a low-affinity streptavidin mutant, was added to the *cis* chamber, a distinctive alteration in the single-channel current occurred (Figure 2) [35].

The above single-molecule experiment had many consequences. First, it demonstrated that the single-molecule stochastic sensing of proteins can be fundamentally expanded into the aqueous phase. Second, various protein analytes with different binding affinities could be detected, indicating the remarkable power of single-molecule stochastic sensing of large, folded proteins in the aqueous phase. Third, the single-molecule captures of the binding protein occurred with a greater probability in the *cis* side of the membrane (Figure 2a) than on the *trans* side (Figure 2b), revealing a significant energetic penalty for the polymer to undergo conformational transitions under stretched-out configurations. Inspired by this experimental design, Kong and Muthukumar [36] performed Langevin molecular dynamics simulations and Poisson-Nernst-Plank calculations to obtain the current fluctuations produced by a single PEG covalently anchored within the large vestibule of the αHL protein pore. This computational work revealed the dynamics of the single-molecule captures of the binding protein in the aqueous phase. From a practical point of view, this groundwork might be expanded to other situations that employ flexible tethers as tools in single-molecule sensing of proteins.

Based on the same approach, multivalent interactions were examined through the binding kinetics of a lectin protein to one or more ligands [37]. The remarkable feature of this methodology was the ability to detect monovalent and divalent binding events directly. In a natural expansion of these studies, a genetically encoded stochastic sensor element for sensing proteins at the single-molecule level has been reported [38]. In contrast with previous work [39], in which the ligand was covalently attached to the nanopore lumen via a short tetra(ethylene glycol) (TEG) linker, in this investigation an enzymatic peptide inhibitor was engineered in one subunit of the αHL protein nanopore near the *trans* entrance. Individual binding events of the peptide inhibitor to the catalytic subunit of the cAMP-dependent protein kinase (protein kinase A [PKA]) were observed through the modulation of the single-channel electrical current, providing detailed kinetic and thermodynamic information of the peptide–enzyme interactions. In this way, single-molecule stochastic sensing might be expanded to obtain a rapid screening of kinase inhibitors.

Grabbing single proteins through electrostatic traps

One way to reduce the high energetic barrier for a polypeptide to traverse a pore is to place a binding site within the pore lumen. Negatively charged binding sites (or 'traps') have been engineered at the entry and exit of the β-barrel part of the αHL protein pore (Figure 3) [40]. These traps were heptameric rings of aspartic acid residues. The first stage of this study explored how positively charged polypeptides (~25 residues in length) interact with the negatively charged electrostatic traps [40]. The electrostatic trap engineered at the entry of the pore (the *trans* opening of the pore, Figure 3) had a major impact on the rate constant of association (Figure 1b) [40]. By contrast, the rate constant of dissociation was only marginally altered. Surprisingly, when the entry and exit traps were concurrently engineered within the nanopore lumen, significant increases in the rate constants of association and dissociation were observed. By analogy with ion-conducting protein channels, these electrostatic traps functioned as binding sites for the polypeptide analyte. Remarkably, the two traps together catalyzed the net flow of polypeptides from the *trans* to the *cis* side of the membrane more efficiently than either the WT-αHL pore or a single-trap-containing αHL protein pore. The substantial increase in the rate constant of association observed with the single-trap-containing αHL protein pore was caused by the overlap of the transition state of the entry barrier with the energetic minimum created by the engineered trap on the *trans* entrance of the pore [4,41].

Later, a similar design, but with a much larger translocating protein, was employed for exploring the polypeptide–pore interactions. The protein analyte was a positively charged presequence polypeptide (pb_2) fused to folded ribonuclease barnase (Ba) (Figure 3a) [42]. The addition of nanomolar concentrations of pb_2–Ba to the *trans* side of the bilayer produced infrequent and short-lived current blockades with WT-αHL protein pore (Figure 3b,i). The experiments accomplished with the αHL protein pore containing a single electrostatic trap on the *trans* entrance revealed a dramatically changed frequency of the current blockades, increased by almost two orders of magnitude compared with the WT-αHL pore (Figure 3b,ii). Remarkably, when the electrostatic trap was engineered on the *cis*

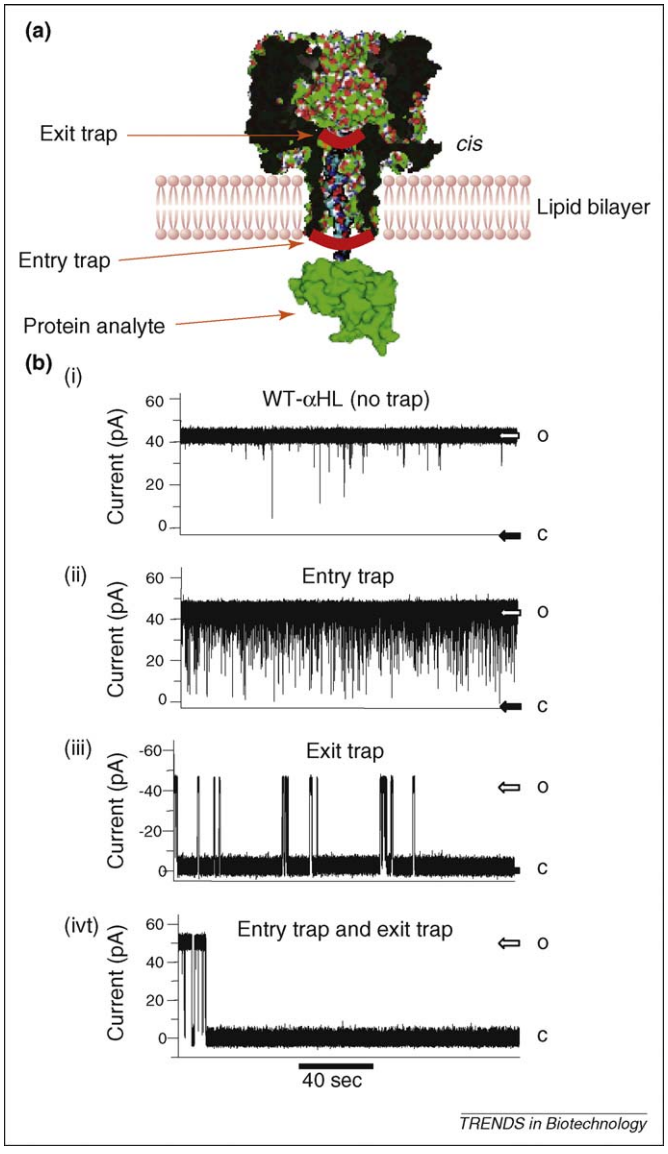

Figure 3. Detection of single proteins using electrostatic traps that have been engineered within the pore lumen. **(a)** The traps were made from rings of aspartic acids on the entry and exit of the β-barrel part of the pore (indicated in red). **(b)** These graphs illustrate representative single-channel electrical recordings of the WT-αHL protein: (i) no trap, WT-αHL; (ii) entry-trap-containing αHL pores; (iii) exit-trap-containing pores; and (iv) double-trap-containing αHL pores (traps at pore entry and exit). All graphs were recorded in the presence of 200 nM pb_2–Ba added to the *trans* side of the bilayer. The white and black arrows indicate the open (O) and closed (C) states, respectively. Reproduced, with permission, from Ref. [42].

end of the β-barrel, the transient current blockades became long lived, with duration in the range of tens of seconds to minutes (Figure 3b,iii). The transient current blockades became permanent when the interaction between double (*cis–trans*) trap-containing αHL protein pore and pb_2–Ba was examined (Figure 3b,iv). These results demonstrate that protein design is instrumental in obtaining major alterations in the single-channel electrical signature as a result of minute changes within the pore lumen.

Can a synthetic nanopore perform as well as a β-barrel pore?

A natural extension of the single-molecule studies of polypeptides that employed transmembrane protein nanopores was the initiation of experiments with synthetic nanopores [43–47]. The first protein biosensor based on a synthetic nanopore was designed in 2005 by Charles Martin's group [43]. Their biosensor consisted of a single conically shaped gold nanotube placed in a mechanically stable polymeric membrane. The strategy was to functionalize the small diameter of the conical nanopore with a reactive group. A permanent current blockade was recorded upon the binding of the analyte with the reactive group. Remarkably, such a detector is versatile to a broad range of applications, including protein–ligand (e.g. streptavidin–biotin) and antibody-binding protein (e.g. IgG–protein-G) complexes. Two years later, the same team expanded their ability to probe selectively for protein analytes with nanopores. Their sensor element was a PEG-functionalized gold conical nanotube. The protein analyte, in this case bovine serum albumin (BSA) and a Fab fragment from a BSA-polyclonal antibody, were detected from the distinctive signature of the current blockades made by the free BSA and the BSA–Fab complex. Although the signatures of the current blockades were well correlated with the size and the stoichiometry of the protein–protein complex, this experimental design does not entail direct determination of the rate constants of association and dissociation between the interacting molecules of the complex. Recently, other groups initiated protein-sensing studies with single nanopores based on silicon nitride [44,45,47]. These explorations have demonstrated the capability of the technique to probe several characteristics of the proteins, such as their size, length, shape and concentration. Using synthetic nanopores for the detection of hormones and of antibodies targeting these hormones in solution has proven to be a powerful technique when compared with traditional immunoassays because costly labeling and amplification steps are no longer necessary [47].

The use of synthetic nanopores will be enhanced in the future by an array of advantageous features, including a greater robustness of the solid-state membranes, the ability to tune the diameter of the nanopore, and the potential for integration into a 'laboratory-on-a-chip' platform or parallelization for high-throughput devices. Therefore, it is conceivable that the single-molecule studies performed with protein nanopores might be expanded to incorporate a much wider spectrum of applications and harsh conditions of experimentation. For example, temperature-dependence experiments with synthetic nanopores would enable the measurement of kinetic and thermodynamic enthalpies and entropies of the complex formation between a protein and a ligand, revealing information about which process (e.g. enthalpic or entropic) in the ligand-binding protein interactions is dominant. One obvious challenge is to attach a single functional reactive group within a strategic position of the nanopore interior. This approach would allow transient bindings of a protein analyte to a ligand engineered within a synthetic nanopore. Moreover, it is likely that such an achievement will revolutionize the area of high-throughput nanopore-based devices for both nucleic acids and proteins.

Employing tweezers: prospects for force measurements of protein translocation

The use of solid-state membranes enables the amalgamation of single-molecule nanopore recordings with other

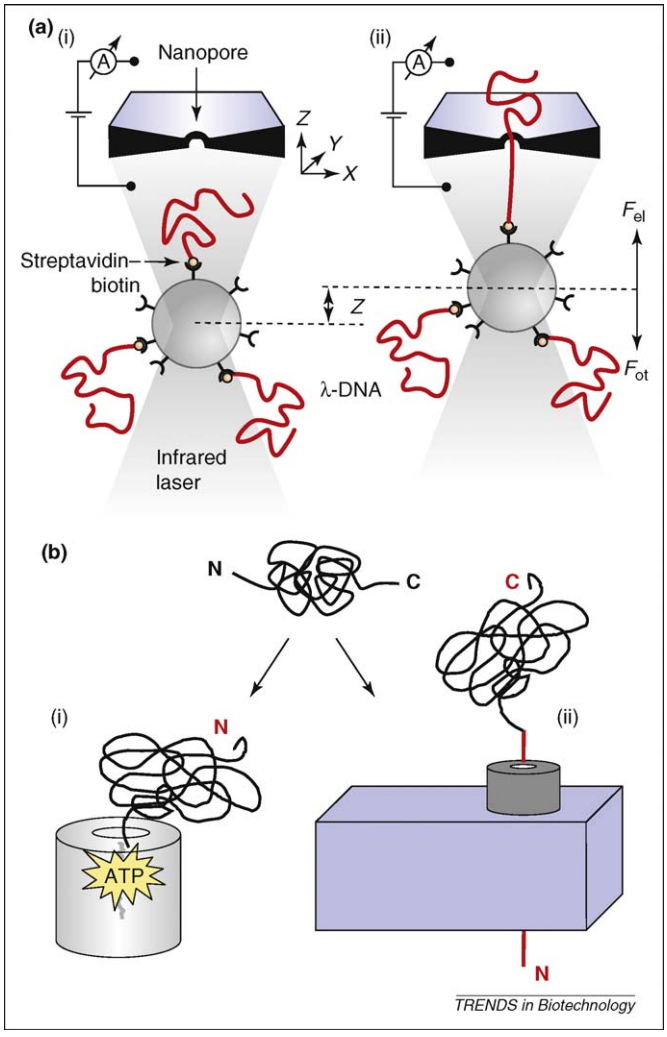

Figure 4. A single nanopore as a versatile tool for probing mechanical stability of biopolymers. **(a)** A focused laser beam is employed to control a ssDNA-coated bead in the proximity of a synthetic nanopore (i). A transmembrane potential applied across the membrane electrophoretically drives the ssDNA into the synthetic nanopore (ii). The transmembrane potential is also used to drive the small ions through the nanopore, producing a well-defined electrical current. As soon as the DNA partitions into the nanopore, a striking change in the current occurs. The electrical force pulls the bead out of the trap center until the optical force exerted on the trap balances the driving (electrical) force. Coupling the single-molecule nanopore measurements with an optical tweezer [48] will enable the measurement of the balance between driving and entropic forces of biopolymers across a synthetic nanopore. Reproduced, with permission, from Ref. [48]. **(b)** Unfolding of single proteins across protein pores is a ubiquitous process in biology. Examples are protein degradation (i) and protein translocation through β-barrel protein pores located in the outer membranes of mitochondria, chloroplasts and Gram-negative bacteria (ii).

molecular probe techniques, such as an optical platform or atomic force microscopy (AFM). One of the most exciting examples is combining the single-channel electrical set-up with an optical tweezer [11]. This remarkable achievement was made for the first time in the group of Cees Dekker at Delft University of Technology, The Netherlands [48]. Their pioneering work with a nanopore–optical-tweezer instrument permitted extensive exploration of the balance between the driving force that pulls on a single piece of negatively charged ssDNA and the corresponding forces caused by the free energy penalty for partitioning this polymer into a single synthetic nanopore (Figure 4a). One year later, Trepagnier and colleagues carried out similar experiments to reduce the translocation speed of nucleic acids through a single synthetic nanopore [49]. Slowing the translocation of nucleic acids is a major step that must be accomplished for single-molecule sequencing using nanopores [10].

How useful is this new technique for our quantitative knowledge of single biopolymers? In principle, one could use an optical trap to insert a biopolymer through a synthetic nanopore and then control the movements of the biopolymer through a trapped microbead. Although challenging, there is no technical limitation in replacing an ssDNA molecule with a polypeptide. Remarkably, many processes in nature resemble those in which a protein is threaded through a nanometer-scale pore or a constriction. Two examples are the protein degradation achieved through the proteasome channel [50,51] and protein translocation through the β-barrel protein pore of the outer membranes of mitochondria and other plastids [52] (Figure 4b). A complete mechanistic understanding of these natural molecular processes is not yet available. A nanopore–optical-tweezer instrument would be a useful tool for probing the underlying kinetics of the nanopore-mediated unfolding of a protein at the single-molecule level.

This type of protein unfolding is different from mechanical stretching occurring during AFM (Figure 5a), and it is more immediately pertinent to other natural processes, such as protein translocation, in which the ATP-dependent protein motor heat shock protein 70 (Hsp70) and transmembrane potential are the crucial mechanisms for pulling on the protein passenger (Figure 5b) [53]. Nanopore-mediated protein unfolding is also different from global (or spontaneous) unfolding, which is induced by temperature jumps or chemical denaturants, with the former process being between two and three orders of magnitude faster than the latter [53]. The striking difference in kinetic rates between these denaturation mechanisms is likely to be determined by different free-energy landscapes for unfold-

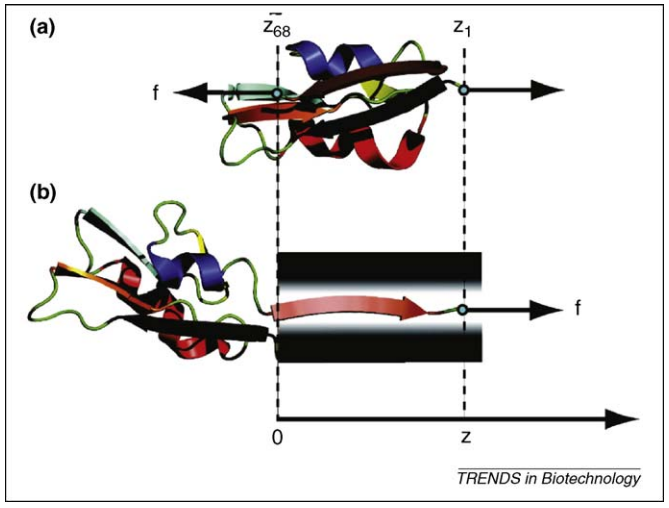

Figure 5. Schematic illustration depicting the differences between mechanical stretching and pore-mediated mechanical unfolding. **(a)** Mechanical stretching by pulling both ends of the protein away from each other. Here, z is a reaction coordinate that is defined as the end-to-end distance projected onto the direction of the stretching force (AFM-type mechanical unfolding). **(b)** Pore-mediated mechanical unfolding. Here, z is a reaction coordinate that defines the position of one chain end along the pore axis. Reproduced, with permission, from Ref. [56].

ing [54–56]. Alternatively, one can perform experiments with AFM-induced mechanical stretching and nanopore-mediated unfolding to examine the relative mechanical stability of proteins when denatured by using atomic force spectroscopy and the nanopore–tweezer approach [57]. One example is the small ribonuclease Ba, which has free-energy (ΔG) of ~10 kcal/mol and activation free-energy for global unfolding of ~20 kcal/mol in dilute buffer at 25 °C [58], indicating a high thermodynamic stability. Remarkably, this protein is mechanically stretched at low forces, suggesting a weak mechanical stability [59]. Since mechanical and thermodynamic stabilities are fundamentally distinct, measurements of pore-mediated protein unfolding that employ a nanopore–tweezer instrument hold promise for furthering our comprehesive understanding of the underlying energetics and kinetics of single proteins in crucial biological processes, such as passage through the proteasomal degradation channel and translocation of proteins through β-barrel pores.

Grabbing single proteins through movable nucleic acid arms

In general, nanopore studies on single-molecule sensing of proteins have employed recognition templates, such as small ligand–protein [35,37,60], peptide–protein [38,39] and antibody–protein [43,46,47] complexes. Very recently, several groups have examined the subtle interactions of the binding proteins with their nucleic acid targets [61–65]. These efforts showed a broad range of opportunities for the quantitative exploration of the biophysical and biochemical characteristics of the complexes between proteins and nucleic acids, such as the voltage threshold for complex dissociation [61], the sequence-specific sensitivity detection of individual DNA polymerase [62], the underlying equilibrium kinetics and thermodynamics of protein–nucleic-acid interactions [63], the motor activity of RNA-processing enzymes [64] and the transit time of the protein–nucleic-acid complex within the nanopore [65]. Reza Ghadiri and co-workers [66] have probed DNA polymerase activity at the single-molecule level, and with single-base resolution, by using a nanopore complex consisting of a DNA–PEG copolymer threaded across the WT-αHL protein pore. In its present formulation, this experimental design is not only a step forward for competitive nucleic acid sequencing when compared with other, well-established sequencing technologies [67] but is also instrumental for a systematic exploration of the subtle mechanisms that govern DNA and RNA polymerases.

The WT-αHL protein pore was also employed in the single-molecule detection of aptamers. Li-Qun Gu's group [68] has examined the single-molecule detection of thrombin-binding aptamer (TBA), a G-quadruplex nucleic acid, in the presence of divalent cations. In this case, the large vestibule of the pore was used to trap single thrombin molecules through the electrophoretic insertion of a single G-quadruplex oligonucleotide. This study demonstrates a remarkable power of the nanopore detector for sensing the DNA and RNA aptamers. There are numerous advantages of the DNA and RNA aptamers as molecular recognition elements in biosensing when compared with traditional detection methodologies using enzymes and antibodies [69]. These include enhanced specificity, selectivity and affinity, ranging from small molecules to folded protein domains. In addition, the DNA and RNA aptamers exhibit an extraordinary chemical stability, thereby offering an exceptional flexibility and convenience in their design.

Concluding remarks

This review has discussed the promise of recent exciting advancements in the area of nanopore sensing of single proteins. These single-molecule measurements with natural and synthetic nanopores hold great promise for discovering unique properties of short polypeptides and folded proteins. The detection mechanisms of folded proteins include chemical modification and genetic engineering of a transmembrane protein nanopore, functionalization of the inner surface of a synthetic nanopore, and electrophoretic capture through movable nucleic acid arms. This methodology enables the determination of the binding affinity and the enzymatic activity of proteins at the single-molecule level without resorting to labeling of the interacting molecules. Nanopores also represent single-molecule probes for examining the flexibility and folding state of single proteins. Coupling a single synthetic nanopore in a silicon membrane with an optical trap will be instrumental for examining the mechanical stability of biopolymers in relation to unusual pore-mediated unfolding.

Despite the complexity of the experiments and inherent complications, I anticipate that the coupling of nanopores with other optical or fluorescence platforms will provide crucial insights into the translocation process, leading to a better understanding of how proteins undergo conformational transitions when they traverse the pore. This is because the translocation and unfolding of a protein through a single nanopore are two intimately related processes. These efforts will help to provide a quantitative understanding of pore-mediated unfolding, which is more immediately pertinent to protein import *in vivo*. Doubtless these experimental efforts will inspire new theoretical [70] and computational studies [56,71,72] on protein dynamics through nanopores.

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References

1 Bezrukov, S.M. (2000) Ion channels as molecular Coulter counters to probe metabolite transport. *J. Membr. Biol.* 174, 1–13
2 Bayley, H. and Cremer, P.S. (2001) Stochastic sensors inspired by biology. *Nature* 413, 226–230
3 Martin, C.R. and Siwy, Z.S. (2007) Chemistry. learning nature's way: biosensing with synthetic nanopores. *Science* 317, 331–332
4 Movileanu, L. (2008) Squeezing a single polypeptide through a nanopore. *Soft Matter* 4, 925–931
5 Howorka, S. and Siwy, Z. Nanopores: generation, engineering and single-molecule applications. In *Handbook of Single-Molecule*

Biophysics (Hinterdorfer, P., ed.), Springer-Verlag, Heidelberg (in press)
6 Bayley, H. and Martin, C.R. (2000) Resistive-pulse sensing – from microbes to molecules. *Chem. Rev.* 100, 2575–2594
7 Simon, S.M. and Blobel, G. (1991) A protein-conducting channel in the endoplasmic reticulum. *Cell* 65, 371–380
8 Bayley, H. (2006) Sequencing single molecules of DNA. *Curr. Opin. Chem. Biol.* 10, 628–637
9 Rhee, M. and Burns, M.A. (2007) Nanopore sequencing technology: nanopore preparations. *Trends Biotechnol.* 25, 174–181
10 Branton, D. *et al.* (2008) The potential and challenges of nanopore sequencing. *Nat. Biotechnol.* 26, 1146–1153
11 Dekker, C. (2007) Solid-state nanopores. *Nat. Nanotechnol.* 2, 209–215
12 Griffiths, J. (2008) The realm of the nanopore: interest in nanoscale research has skyrocketed, and the humble pore has become a king. *Anal. Chem.* 80, 23–27
13 Hayden, T. (2008) Threading proteins through a nanopore needle. *Anal. Chem.* 80, 3955
14 Miles, G. *et al.* (2002) Subunit composition of a bicomponent toxin: staphylococcal leukocidin forms an octameric transmembrane pore. *Protein Sci.* 11, 894–902
15 Paschen, S.A. *et al.* (2005) Biogenesis of β-barrel membrane proteins of mitochondria. *Trends Biochem. Sci.* 30, 575–582
16 Chen, M. *et al.* (2008) Outer membrane protein G: engineering a quiet pore for biosensing. *Proc. Natl. Acad. Sci. U. S. A.* 105, 6272–6277
17 White, S.H. and Wimley, W.C. (1999) Membrane protein folding and stability: physical principles. *Annu. Rev. Biophys. Biomol. Struct.* 28, 319–365
18 Jung, Y. *et al.* (2006) Temperature-responsive protein pores. *J. Am. Chem. Soc.* 128, 15332–15340
19 Kang, X.F. *et al.* (2005) Single protein pores containing molecular adapters at high temperatures. *Angew. Chem. Int. Ed. Engl.* 44, 1495–1499
20 Chimerel, C. *et al.* (2008) Transport at the nanoscale: temperature dependance of ion conductance. *Eur. Biophys. J.* 38, 121–125
21 Song, L. *et al.* (1996) Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science* 274, 1859–1866
22 Movileanu, L. *et al.* (2003) Partitioning of individual flexible polymers into a nanoscopic protein pore. *Biophys. J.* 85, 897–910
23 Movileanu, L. *et al.* (2005) Interactions of the peptides with a protein pore. *Biophys. J.* 89, 1030–1045
24 Movileanu, L. and Bayley, H. (2001) Partitioning of a polymer into a nanoscopic protein pore obeys a simple scaling law. *Proc. Natl. Acad. Sci. U. S. A.* 98, 10137–10141
25 Movileanu, L. *et al.* (2001) Location of a constriction in the lumen of a transmembrane pore by targeted covalent attachment of polymer molecules. *J. Gen. Physiol.* 117, 239–251
26 Howorka, S. *et al.* (2001) Kinetics of duplex formation for individual DNA strands within a single protein nanopore. *Proc. Natl. Acad. Sci. U. S. A.* 98, 12996–13001
27 Kasianowicz, J.J. *et al.* (1996) Characterization of individual polynucleotide molecules using a membrane channel. *Proc. Natl. Acad. Sci. U. S. A.* 93, 13770–13773
28 Sutherland, T.C. *et al.* (2005) Structure of peptides investigated by nanopore analysis. *Nano Lett.* 4, 1273–1277
29 Stefureac, R. *et al.* (2006) Transport of α-helical peptides through α-hemolysin and aerolysin pores. *Biochemistry* 45, 9172–9179
30 Goodrich, C.P. *et al.* (2007) Single-molecule electrophoresis of β-hairpin peptides by electrical recordings and Langevin dynamics simulations. *J. Phys. Chem. B* 111, 3332–3335
31 Oukhaled, G. *et al.* (2007) Unfolding of proteins and long transient conformations detected by single nanopore recording. *Phys. Rev. Lett.* 98, 158101
32 Stefureac, R. *et al.* (2008) Nanopore analysis of a small 86-residue protein. *Small* 4, 59–63
33 Stefureac, R.I. and Lee, J.S. (2008) Nanopore analysis of the folding of zinc fingers. *Small* 4, 1646–1650
34 Howorka, S. *et al.* (2000) A protein pore with a single polymer chain tethered within the lumen. *J. Am. Chem. Soc.* 122, 2411–2416
35 Movileanu, L. *et al.* (2000) Detecting protein analytes that modulate transmembrane movement of a polymer chain within a single protein pore. *Nat. Biotechnol.* 18, 1091–1095
36 Kong, C.Y. and Muthukumar, M. (2005) Simulations of stochastic sensing of proteins. *J. Am. Chem. Soc.* 127, 18252–18261
37 Howorka, S. *et al.* (2004) Stochastic detection of monovalent and bivalent protein–ligand interactions. *Angew. Chem. Int. Ed. Engl.* 43, 842–846
38 Cheley, S. *et al.* (2006) A genetically encoded pore for the stochastic detection of a protein kinase. *ChemBioChem* 7, 1923–1927
39 Xie, H. *et al.* (2005) Single-molecule observation of the catalytic subunit of cAMP-dependent protein kinase binding to an inhibitor peptide. *Chem. Biol.* 12, 109–120
40 Wolfe, A.J. *et al.* (2007) Catalyzing the translocation of polypeptides through attractive interactions. *J. Am. Chem. Soc.* 129, 14034–14041
41 Mohammad, M.M. and Movileanu, L. (2008) Excursion of a single polypeptide into a protein pore: simple physics, but complicated biology. *Eur. Biophys. J.* 37, 913–925
42 Mohammad, M.M. *et al.* (2008) Controlling a single protein in a nanopore through electrostatic traps. *J. Am. Chem. Soc.* 130, 4081–4088
43 Siwy, Z. *et al.* (2005) Protein biosensors based on biofunctionalized conical gold nanotubes. *J. Am. Chem. Soc.* 127, 5000–5001
44 Han, A. *et al.* (2006) Sensing protein molecules using nanofabricated pores. *Appl. Phys. Lett.* 88, 093901
45 Fologea, D. *et al.* (2007) Electrical characterization of protein molecules by a solid-state nanopore. *Appl. Phys. Lett.* 91, nihpa38991
46 Sexton, L.T. *et al.* (2007) Resistive-pulse studies of proteins and protein/antibody complexes using a conical nanotube sensor. *J. Am. Chem. Soc.* 129, 13144–13152
47 Han, A. *et al.* (2008) Label-free detection of single protein molecules and protein-protein interactions using synthetic nanopores. *Anal. Chem.* 80, 4651–4658
48 Keyser, U.F. *et al.* (2006) Direct force measurements on DNA in a solid-state nanopore. *Nat. Phys.* 2, 473–477
49 Trepagnier, E.H. *et al.* (2007) Controlling DNA capture and propagation through artificial nanopores. *Nano Lett.* 7, 2824–2830
50 Inobe, T. *et al.* (2008) How to pick a protein and pull at it. *Nat. Struct. Mol. Biol.* 15, 1135–1136
51 Prakash, S. *et al.* (2009) Substrate selection by the proteasome during degradation of protein complexes. *Nat. Chem. Biol.* 5, 29–36
52 Poynor, M. *et al.* (2008) Dynamics of the preprotein translocation channel of the outer membrane of mitochondria. *Biophys. J.* 95, 1511–1522
53 Prakash, S. and Matouschek, A. (2004) Protein unfolding in the cell. *Trends Biochem. Sci.* 29, 593–600
54 Huang, L. *et al.* (2005) Computer simulations of the translocation and unfolding of a protein pulled mechanically through a pore. *J. Chem. Phys.* 123, 124903
55 Huang, L. and Makarov, D.E. (2008) The rate constant of polymer reversal inside a pore. *J. Chem. Phys.* 128, 114903
56 Makarov, D.E. (2009) Computer simulations and theory of protein translocation. *Acc. Chem. Res.* 42, 281–289
57 Tian, P. and Andricioaei, I. (2005) Repetitive pulling catalyzes co-translocational unfolding of barnase during import through a mitochondrial pore. *J. Mol. Biol.* 350, 1017–1034
58 Matouschek, A. *et al.* (1997) Active unfolding of precursor proteins during mitochondrial protein import. *EMBO J.* 16, 6727–6736
59 Best, R.B. *et al.* (2001) Can non-mechanical proteins withstand force? Stretching barnase by atomic force microscopy and molecular dynamics simulation. *Biophys. J.* 81, 2344–2356
60 Mayer, M. *et al.* (2008) Using ion channel-forming peptides to quantify protein–ligand interactions. *J. Am. Chem. Soc.* 130, 1453–1465
61 Zhao, Q. *et al.* (2007) Detecting SNPs using a synthetic nanopore. *Nano Lett.* 7, 1680–1685
62 Benner, S. *et al.* (2007) Sequence-specific detection of individual DNA polymerase complexes in real time using a nanopore. *Nat. Nanotechnol.* 2, 718–724
63 Hornblower, B. *et al.* (2007) Single-molecule analysis of DNA–protein complexes using nanopores. *Nat. Methods* 4, 315–317
64 Astier, Y. *et al.* (2007) Stochastic detection of motor protein–RNA complexes by single-channel current recording. *ChemPhysChem* 8, 2189–2194

65 Smeets, R.M. *et al.* (2008) Translocation of RecA-coated double-stranded DNA through solid-state nanopores. *Nano Lett. 10. 1021/nl* 803189k
66 Cockroft, S.L. *et al.* (2008) A single-molecule nanopore device detects DNA polymerase activity with single-nucleotide resolution. *J. Am. Chem. Soc.* 130, 818–820
67 Croquette, V. (2008) Sensing single base incorporation with nanopore micromanipulation. *ACS Chem. Biol.* 3, 92–94
68 Shim, J.W. and Gu, L.Q. (2008) Encapsulating a single g-quadruplex aptamer in a protein nanocavity. *J. Phys. Chem. B* 112, 8354–8360
69 Song, S. *et al.* (2008) Aptamer-based biosensor. *Trends Analyt. Chem.* 27, 108–117
70 Kolomeisky, A.B. (2007) Channel-facilitated molecular transport across membranes: attraction, repulsion, and asymmetry. *Phys. Rev. Lett.* 98, 048105
71 Kirmizialtin, S. *et al.* (2006) Computer simulations of protein translocation. *Phys. Status Solidi* 243, 2038–2047
72 Huang, L. and Makarov, D.E. (2008) Translocation of a knotted polypeptide through a pore. *J. Chem. Phys.* 129, 121107