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Tuning protein discrimination through altering the sampling interface formed between the analyte and OmpG nanopore

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Abstract

Nanopore sensors capable of distinguishing homologous protein analytes are highly desirable tools for proteomics research and disease diagnostics. Recently, an engineered outer membrane protein G (OmpG) nanopore with a high-affinity ligand attached to a gating loop 6 showed specificity for distinguishing homologous proteins in complex mixtures. Here we report the development of OmpG nanopores with the other six loops used as the anchoring point to host an affinity ligand for protein sensing. We investigated how the analyte binding to the affinity ligand located at different loops affects the detection sensitivity, selectivity and specificity. Our results reveal that analytes weakly attracted to the OmpG nanopore surface are only detectable when the ligand is tethered to loop 6. In contrast, protein analytes that form strong interaction with the OmpG surface via electrostatic attractions are distinguishable by all seven OmpG nanopore constructs. In addition, the same analyte can generate distinct binding signals with different OmpG nanopore constructs. The ability to exploit all seven OmpG loops will aid the design of a new generation of OmpG sensors with increased sensitivity, selectivity and specificity for biomarker sensing.

Graphical Abstract

Nanopore-based sensing techniques have been used to characterize analytes ranging from small chemicals^{1–3} to large biomolecules^{4–6} and even viruses.⁷ Nanopores have been engineered from naturally occurring pore-forming proteins^{2, 8–14} or derived from synthetic materials^{4, 15–17} such as silicon, silicon nitride, graphene, molybdenum disulfide or

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Conflict of interest

The authors declare no conflicts of interest.

DNA.^{18, 19} Despite having various sources for nanopores, analytes can be detected through four main mechanisms. Analytes can (i) directly interact within the walls or constriction site of the nanopore^{8, 14, 20}, (ii) directly bind to the nanopore's exterior surface¹¹, (iii) bind to external adaptors²¹, and (iv) alter nanopore formation²². Interactions between the analyte molecules and the nanopore modulate the ionic flow through the pore and thus generate characteristic current signals that can be used for analyte identification.

Over the past few years, nanopore-based nucleic acid sequencing has been successfully developed²³ and has made a strong impact on genomic research.^{24, 25} Compared to nucleic acids, proteins are more complex targets as proteomics research is not limited to identifying and quantifying proteins. Instead, proteomics also seeks to understand the structure, function, dynamics, modifications and their inter-relationships of the vastly diverse protein components in cells. Thus, proteomics presents tremendous analytical challenge. Recently, impressive progress has also been made to advance nanopore-based protein analysis for proteomics research.²⁶ For example, nanopores have been explored for peptide/protein sequencing,^{27, 28} protein identification,^{29–31} and monitoring enzymatic activities^{32–34}, structural dynamics^{35–38}, protein folding and unfolding,^{39–42} and protein-protein interactions.⁴³

Recently, we have used an outer membrane protein G (OmpG) nanopore to distinguish protein homologues in complex mixtures.^{11, 32, 44, 45} Distinct from other nanopores with rigid structures, OmpG consists of seven flexible loops with varying lengths and charge distribution (Figure 1).^{46, 47} When a high-affinity ligand is attached to the most flexible loop, loop 6, OmpG can detect analyte binding via changes in loop 6 dynamic conformations.⁴⁸ Different analytes, when bound to the ligand, generate different characteristic current gating patterns. Instead of using the current blockages as the criteria, OmpG nanopore relies on the current patterns for protein recognition. A simple biotin-tethered OmpG construct exhibited the ability to distinguish six different biotin-binding proteins that have similar shape and size.^{11, 44, 45, 48} The remarkable specificity of OmpG nanopore sensing was attributed to the ability of loop 6 to adopt distinct conformations induced by analyte/OmpG interaction. In this work, we explored whether anchoring the ligand to a different loop affects OmpG detection specificity and sensitivity. We created seven OmpG sensor constructs, each containing a biotin ligand bound to one of the loops and assessed the ability of these constructs to resolve various biotin-binding protein analytes.

Materials and Methods

Materials.

All reagents were purchased from Fisher Scientific unless otherwise stated. Biotin-PEG₂-maleimide ligand (PEG: polyethylene glycol) and anti-biotin antibody clone BTN.4 were purchased from ThermoFisher Scientific. Urea was purchased from Boston Bioproducts. Tris (2-carboxyethyl) phosphine (TCEP) and octyl-glucoside (OG) were purchased from Gold Biotechnology. Mutagenic oligonucleotides were purchased from Eurofins MWG Operon. 1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DPhPC) lipid was purchased from

Avanti Polar Lipids. Streptavidin was purchased from Promega. Avidin was from Sigma-Aldrich and anti-biotin antibody SB58C clone from Southern Biotech.

Cloning of OmpG cysteine mutants.

Single cysteine mutations were introduced into the wild type OmpG by mutagenesis polymerase chain reaction (PCR) using a previously described protocol.¹¹ The primers used in the study are listed in Table S1 (Supplemental Information). The oligonucleotides were paired in a single PCR reaction with the pT7-OmpGwt plasmid as the template. After the PCR was complete, the template was digested with DpnI enzyme and the PCR products were transformed into chemically competent XL-Blue cells. The resulting plasmids were extracted from the XL-Blue cells and the mutations were confirmed by sequencing.

Expression and purification of OmpG proteins.

All OmpG proteins were prepared using a previously described protocol.¹¹ Briefly, 250 ml culture of BL21 (DE3) cells transformed with the desired plasmid were grown in Luria Broth media at 37°C. When the OD₆₀₀ reached 0.6, the culture was induced for 3 hours by adding a final concentration of 0.5 mM β -D-1-thiogalactopyranoside. The induced culture was harvested by centrifugation and the pellet was resuspended in 50 mM Tris-HCl (pH 8.0), 3.0 mM TCEP. The cells were lysed via sonication and the lysate was centrifuged at 20,000g for 20 min to pellet the inclusion body (IB). The insoluble IB was resuspended for 10 min in 50 mM Tris-HCl (pH 8.0), 1.5 M urea, 3.0 mM TCEP and then centrifuged. The IB was then solubilized for 30 min with constant stirring in 50 mM Tris-HCl (pH 8.0), 8 M urea, 3.0 mM TCEP. It was then centrifuged, filtered and the cleared inclusion supernatant was applied to a Q sepharose anion exchange column. The column was washed with 50mM Tris-HCl (pH 8.0), 75 mM NaCl, 8 M urea, 3 mM TCEP and the proteins were eluted with 50mM Tris-HCl (pH 8.0), 200 mM NaCl, 8 M urea, 3 mM TCEP.

Biotinylation of OmpG proteins.

The eluted proteins were then desalted into 50 mM HEPES (pH 7), 8 M urea, 150 mM NaCl and mixed in a 1:10 protein to ligand mole ratio with Biotin-PEG₂-Maleimide. Samples were incubated at 22°C for 2 hours with gentle shaking. After that, excess chemicals were removed by desalting. Labeled OmpG was then diluted with refolding buffer containing 50 mM Tris-HCl (pH 9.0), 3.25% (w/v) octylglucoside in a 2:3 protein-to-buffer (v/v) ratio. Samples were then incubated at 37°C for three days. The refolding was determined by heat mobility shift and the labeling efficiency by streptavidin-complex mobility shift using SDS-polyacrylamide gel electrophoresis (Figure S1).

Single-channel recording of OmpG proteins.

Single-channel recording of OmpG was performed as previously reported.¹¹ A chip containing two chambers separated by a Teflon film with a 100 μ m aperture was used. Hexadecane dissolved in pentane (10%, v/v) was added to the Teflon film. After the pentane was evaporated, 300 mM KCl, 20 mM sodium phosphate pH 6 buffer (350 μ l) was added to each chamber, followed by dropping ~70–100 μ g of DPhPC lipid dissolved in pentane (10 μ g/ μ l) onto the surface of the buffer to form a monolayer. The solution was pipetted up

and down below the aperture to allow the formation of the bilayer. A silver/silver chloride electrode was immersed into each chamber with the *cis* chamber grounded. OmpG proteins (1–5 nM) were added into the *cis* chamber and a high voltage ± 200 mV was used to induce single pore insertion. After a single OmpG pore inserted into the lipid bilayer, the voltage was reduced to ± 50 mV. Four different protein analytes were each added into the loop facing chamber: two biotin monoclonal antibody clones BTN.4 (10 nM) and SB58C (1 nM); as well as streptavidin (1 nM) and avidin (1 nM). Current traces were obtained for each protein analyte from at least three independent pore measurements. Current signals were filtered by a 2 kHz Bessel filter and acquired at a sampling rate of 100 μ s after digitization with a Digidata 1320A/D board (Axon Instruments).

Single-channel gating analysis.

To characterize the OmpG current recordings in the absence and presence of protein analyte, we generated all-points histograms from at least 30 s of the raw trace in Clampfit 10.7 (Molecular Devices). The all-points histograms revealed the distributions of the current states of OmpG in the unbound and analyte-bound conditions. We derived the open pore current and the relative current of the unbound and bound states from the all-points histogram by fitting to a Gaussian curve. The dwell times for the analyte-bound states (τ_{off}) and inter-event times (τ_{on}) were collected manually. At least 300 bound and 300 unbound dwell times and inter-event times from each pore were fit with an exponential function to derive the average τ_{off} and τ_{on} . The τ_{off} and τ_{on} values were used to calculate the association ($k_{\text{on}} = 1/(C \cdot \tau_{\text{on}})$) and dissociation ($k_{\text{off}} = 1/\tau_{\text{off}}$) rate constants as well as the dissociation constant $K_d = k_{\text{off}}/k_{\text{on}}$.

Results

OmpG loop constructs show differential sensitivity toward streptavidin homologues

To test how the location of the affinity ligand at different loops affect OmpG's sensitivity to detect protein analytes, we generated six single cysteine mutations on loops 1 to 5 and loop 7 in addition to the previously utilized loop 6 cysteine mutant (Figure 1b). The mutants generated were – E24C for loop 1 (L1), S58C for loop 2 (L2), E101C for loop 3 (L3), T143C for loop 4 (L4), S182C for loop 5 (L5), D224C for loop 6 (L6) as previously characterized and G264C for loop 7 (L7) (Figure 1b). We then conjugated Biotin-PEG₂-Maleimide to seven single-cysteine constructs and confirmed their labeling and refolding efficiency (Figure S1). Here we will refer to the attached ligand as Ln-biotin and the OmpG nanopore constructs as OmpG^{Ln-biotin}, with n indicating the loop number.

We tested all seven loop-biotin constructs with two homologous proteins streptavidin and avidin, both of which bind to biotin irreversibly. For simplicity we only show the results at the positive voltage, which is defined as the voltage felt by the loops. Data acquired at negative voltages are provided in the Supporting Information. OmpG gating is visualized as rapid fluctuations between high and low current states in single-channel recording traces. Upon streptavidin binding, only the OmpG^{L6-biotin} construct generated a significant decrease in gating activity ($n = 5$) while other loop-biotin constructs showed little or no significant change after streptavidin binding ($n = 3$) (Figure 2a,b).

Overall, the surface of the OmpG nanopore is negatively charged due to the multiple acidic residues on loops 1, 6 and 7 (Figure 1b). Although streptavidin and avidin are homologues they differ in the surface charge. Under the experimental condition, streptavidin is negatively charged while avidin carries positive charge.⁴⁸ Previously, we observed that strong electrostatic interactions between the positively charged avidin and OmpG^{L6-biotin} correlated with a large reduction in the open pore current.^{44, 48} Here, avidin binding to OmpG^{Ln-biotin} reduced the open pore current of all OmpG pores at both positive and negative voltages, albeit to varying degrees (Figure 2c,d, Figure S2). The open current of OmpG^{L1-biotin}, OmpG^{L2-biotin}, OmpG^{L3-biotin}, OmpG^{L6-biotin} and OmpG^{L7-biotin} decreased to ~60–65% of the current in unbound state, while the open pore current of OmpG^{L4-biotin} decreased to $25 \pm 9\%$. In contrast, avidin binding to OmpG^{L5-biotin} merely decreased the open pore current to $89 \pm 0.3\%$ (Table S2, S3) suggesting that avidin may be in an orientation that prevents it from forming a close interaction with the OmpG surface. In summary, the results suggest that once avidin binds to the biotin, it can form a close contact with the OmpG loops independent of the attaching point of the ligand. However, the specific position and orientation of the bound avidin may result in distinct avidin-OmpG interfaces that obstruct the ionic current to different degrees.

Although streptavidin and avidin are homologues, streptavidin is essentially undetected when the biotin ligand is attached to loops other than L6. This observation can be explained as follows: because of streptavidin's negative character under the buffer conditions,⁴⁸ a repulsion between streptavidin and the OmpG loops may prevent the streptavidin from forming a close contact with OmpG. Loop 6 is the most dynamic loop of OmpG and is responsible for more than 90% of the gating signals. Once streptavidin is bound to the L6-biotin, streptavidin's mass slows down loop 6's motion which causes a notable reduction in gating. Streptavidin would restrict the motion of any loop it is bound to, but bound to loops 1, 2, 3, 4, 5, or 7 does not significantly reduce the gating readout because these six loops together contribute less than 10% of OmpG gating activity.

Anti-biotin antibody SB58C generates a subtly different binding signal with each OmpG loop

Although avidin binding to OmpG generates a significant change in gating, the binding is irreversible. Therefore, we asked whether a protein analytes showing reversible binding and significant changes in OmpG^{L6-biotin} current behavior as avidin could also trigger any distinguishable current signals with other OmpG^{Ln-biotin}. We thus tested an anti-biotin antibody, SB58C, with a dissociation rate of $8.13 \pm 1.65 \times 10^{-3} \text{ s}^{-1}$ as shown in a previous study.⁴⁵ Binding of SB58C resulted in significant changes in the current traces with all loop-biotin constructs (Figures 3 & S3). In particular, SB58C binding induced three distinct current sub-states which were previously observed in OmpG^{L6-biotin}.⁴⁵ State 1 is characterized by a 5–15% decrease in current. All loop constructs except L3 generated current state 1 (Figures 3 & S3). Constructs OmpG^{L1-biotin}, OmpG^{L3-biotin}, OmpG^{L6-biotin} and OmpG^{L7-biotin} generated state 2 which is characterized by a 31–44% decrease in current. The third distinct state is characterized by a 58–67% decrease in current and is observed in constructs OmpG^{L1-biotin}, OmpG^{L2-biotin}, OmpG^{L3-biotin} and OmpG^{L4-biotin} (Figures 3 & S3, Tables S2 & S3). Previously, we have demonstrated that the decrease of open pore currents

always correlated with the electrostatic interactions between the negatively charged OmpG surface and positively charged protein analytes.⁴⁸ Because the sequence and structure of this antibody is unknown, we presume that it may contain positively charged areas near the biotin-binding site to induce the electrostatic attraction between SB58C and OmpG, which would explain its reduced open pore current signals. We also speculate that SB58C has multiple positive surface areas that bind to OmpG and form different interaction interfaces with each obstructing the pore entrance to a different degree and resulting in three current sub-states (Figure S4). Neighboring clusters of loop constructs such as L1, L2, L3 and L4, or L6 and L7 exhibited similar SB58C binding sub-states. These results support that SB58C has formed a similar interaction/sampling interface with specific loop clusters of OmpG constructs when it is anchored to the pore via biotin. Interestingly, SB58C binding causes less current with OmpG^{L5-biotin} than it does with other loop constructs, a trend that also occurred with avidin binding.

OmpG loops have variable sensitivity towards a lower affinity antibody clone BTN.4

Although SB58C binding is reversible, its slow dissociation rate makes it time-consuming to investigate the kinetics of analyte-nanopore interaction.⁴⁵ Here, we tested another biotin-antibody clone, BTN.4, which has a faster dissociation rate ($0.25 \pm 0.04 \text{ s}^{-1}$), to determine if the binding kinetics are loop-dependent. When the BTN.4 antibody was added to the OmpG^{L1-biotin} and OmpG^{L5-biotin} constructs, neither pore exhibited any noticeable changes in the current recording ($n = 5$). However obvious changes in gating were observed for the other five OmpG^{Ln-biotin} constructs ($n = 3$) (Figure 4). Binding of BTN.4 induced a similar ~10% decrease in open pore current for constructs OmpG^{L2-biotin}, OmpG^{L4-biotin}, OmpG^{L6-biotin} and OmpG^{L7-biotin}. This decrease in open pore current, although to a lesser degree for BTN.4, is similar to that observed with avidin and SB58C binding. Therefore, we presume that BTN.4 antibody may have positive character near its biotin-binding Fab region. Surprisingly, the open pore current of OmpG^{L3-biotin} increased by 4% when BTN.4 was bound (Tables S2 & S3). The calculated dissociation rates, k_{off} were similar for the five constructs except OmpG^{L2-biotin} which had a slightly faster k_{off} (Table 1 & Figure S5). The OmpG^{L6-biotin} construct had the fastest association rate k_{on} while OmpG^{L2-biotin} and OmpG^{L4-biotin} had the slowest (Table 1 & Figure S5). These results suggest that the binding kinetics of the analyte to different OmpG^{Ln-biotin} constructs are slightly loop-dependent. The minor variation in the dissociation and association rates is likely due to subtle orientation and steric effect on analyte-nanopore interaction when the analyte protein is anchored to different loop locations.

The low affinity analyte BTN.4 revealed sensitivity limitations for some loop constructs that were not observed with avidin or SB58C. For example, OmpG^{L1-biotin} and OmpG^{L5-biotin} constructs are insensitive to BTN.4 binding. This result suggests that the orientation in which BTN.4 is attached to these loops hinders the antibody interactions with the OmpG surface, which results in no current change. On the other hand, the OmpG^{L3-biotin} construct generates a unique signal with an increased pore current upon BTN.4 binding as opposed to the decreased current for constructs OmpG^{L2-biotin}, OmpG^{L4-biotin}, OmpG^{L6-biotin} and OmpG^{L7-biotin}. We speculate that BTN.4 binding to loop 3 may cause loop 3 and possibly other loops to bend outward from the lumen, which would increase the open pore current.

Thus, the results reveal that the anchoring point of the ligand can affect the analyte-pore interaction therefore leading to distinct characteristic signals for the same analyte.

Discussion

Previously, we found that the positively charged avidin, being captured by the ligand, interacted with the OmpG^{L6-biotin} surface by electrostatic attractions, a process called “sampling”. During the sampling, avidin generates a reduction in open pore current presumably by obstructing the pore entrance.⁴⁸ In this work, we observed that avidin could reduce the current regardless of the ligand anchoring location. Such a result is not surprising as the electrostatic attraction between the two oppositely charged surfaces is expected to occur as long as the avidin is anchored in close proximity to the nanopore. Importantly, the characteristics of the current signal through the “sampling” process are the decrease of the open pore current, and an intricate gating pattern that is dependent of the ligand location. Not only avidin could sample the OmpG loops but also the antibodies SB58C and BTN.4 based on their similar reduction in open pore current upon binding with OmpG. Because the current signal depends on the orientation and position of the analyte when it comes in close proximity to the pore, the sensitivity and specificity of OmpG can be further tuned by changing the ligand anchoring loop. For example, to resolve three biotin-binding proteins such as avidin, SB58C and BTN.4, using any of OmpG^{L2-biotin}, OmpG^{L3-biotin}, OmpG^{L4-biotin}, OmpG^{L6-biotin} and OmpG^{L7-biotin} constructs would offer good performance because they generated three sets of highly distinguishable signals for the three analytes (Figure 5). In the future, we can engineer OmpG sensors with the same ligand incorporated into multiple loops simultaneously, which could increase the avidity, sensitivity and specificity toward a particular analyte.

For neutral and negatively charged molecules, detection currently can only occur when the analyte binds to the ligand located on the gating loop 6 and thus slows the loop’s dynamic motion. The characteristic signal is a decrease in gating activity, which limits OmpG’s ability to resolve similarly charged analytes that bind the same ligand. For example, our previous study has shown OmpG^{L6-biotin} was unable to discriminate between the negatively charged streptavidin and extravidin, a neutral streptavidin homologue derived from avidin (Figure 5). To overcome this limitation, it is possible to create open OmpG pores with positively charged loops, so that negatively charged analytes may also enter the “sampling” process to generate distinct current signals.

Conclusion

In summary, we have demonstrated that the sensitivity and specificity of the OmpG nanopore can be tuned by altering the ligand anchoring point. The ability to utilize all seven loops to harbor the affinity ligand will allow the OmpG nanopore to evolve new features with enhanced sensitivity and specificity for protein biomarker sensing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Braha O; Gu LQ; Zhou L; Lu X; Cheley S; Bayley H, Simultaneous stochastic sensing of divalent metal ions. *Nat Biotechnol* 2000, 18 (9), 1005–7. [PubMed: 10973225]
2. Li T; Liu L; Li Y; Xie J; Wu HC, A universal strategy for aptamer-based nanopore sensing through host-guest interactions inside alpha-hemolysin. *Angew Chem Int Ed Engl* 2015, 54 (26), 7568–71. [PubMed: 25966821]
3. Zernia S; van der Heide NJ; Galenkamp NS; Gouridis G; Maglia G, Current Blockades of Proteins inside Nanopores for Real-Time Metabolome Analysis. *ACS Nano* 2020, 14 (2), 2296–2307. [PubMed: 32003969]
4. Talaga DS; Li J, Single-molecule protein unfolding in solid state nanopores. *J Am Chem Soc* 2009, 131 (26), 9287–97. [PubMed: 19530678]
5. Ivankin A; Carson S; Kinney SR; Wanunu M, Fast, label-free force spectroscopy of histone-DNA interactions in individual nucleosomes using nanopores. *J Am Chem Soc* 2013, 135 (41), 15350–2. [PubMed: 24079416]
6. Kasianowicz JJ; Henrickson SE; Weetall HH; Robertson B, Simultaneous multianalyte detection with a nanometer-scale pore. *Anal Chem* 2001, 73 (10), 2268–72. [PubMed: 11393851]
7. Gurnev PA; Oppenheim AB; Winterhalter M; Bezrukov SM, Docking of a single phage lambda to its membrane receptor maltoporin as a time-resolved event. *J Mol Biol* 2006, 359 (5), 1447–55. [PubMed: 16697410]
8. Derrington IM; Butler TZ; Collins MD; Manrao E; Pavlenok M; Niederweis M; Gundlach JH, Nanopore DNA sequencing with MspA. *Proc Natl Acad Sci U S A* 2010, 107 (37), 16060–5. [PubMed: 20798343]
9. Mohammad MM; Iyer R; Howard KR; McPike MP; Borer PN; Movileanu L, Engineering a rigid protein tunnel for biomolecular detection. *J Am Chem Soc* 2012, 134 (22), 9521–31. [PubMed: 22577864]
10. Soskine M; Biesemans A; Moeyaert B; Cheley S; Bayley H; Maglia G, An engineered ClyA nanopore detects folded target proteins by selective external association and pore entry. *Nano Lett* 2012, 12 (9), 4895–900. [PubMed: 22849517]
11. Fahie M; Chisholm C; Chen M, Resolved single-molecule detection of individual species within a mixture of anti-biotin antibodies using an engineered monomeric nanopore. *ACS Nano* 2015, 9 (2), 1089–98. [PubMed: 25575121]
12. Wloka C; Mutter NL; Soskine M; Maglia G, Alpha-Helical Fragaceatoxin C Nanopore Engineered for Double-Stranded and Single-Stranded Nucleic Acid Analysis. *Angew Chem Int Ed Engl* 2016, 55 (40), 12494–8. [PubMed: 27608188]
13. Stefureac R; Long YT; Kraatz HB; Howard P; Lee JS, Transport of alpha-helical peptides through alpha-hemolysin and aerolysin pores. *Biochemistry* 2006, 45 (30), 9172–9. [PubMed: 16866363]
14. Huang G; Willems K; Soskine M; Wloka C; Maglia G, Electro-osmotic capture and ionic discrimination of peptide and protein biomarkers with FraC nanopores. *Nat Commun* 2017, 8 (1), 935. [PubMed: 29038539]
15. Li J; Stein D; McMullan C; Branton D; Aziz MJ; Golovchenko JA, Ion-beam sculpting at nanometre length scales. *Nature* 2001, 412 (6843), 166–9. [PubMed: 11449268]
16. Farimani AB; Min K; Aluru NR, DNA base detection using a single-layer MoS₂. *ACS Nano* 2014, 8 (8), 7914–22. [PubMed: 25007098]
17. Schneider GF; Kowalczyk SW; Calado VE; Pandraud G; Zandbergen HW; Vandersypen LM; Dekker C, DNA translocation through graphene nanopores. *Nano Lett* 2010, 10 (8), 3163–7. [PubMed: 20608744]
18. Burns JR; Stulz E; Howorka S, Self-assembled DNA nanopores that span lipid bilayers. *Nano Lett* 2013, 13 (6), 2351–6. [PubMed: 23611515]

19. Bell NA; Engst CR; Ablay M; Divitini G; Ducati C; Liedl T; Keyser UF, DNA origami nanopores. *Nano Lett* 2012, 12 (1), 512–7. [PubMed: 22196850]
20. Sexton LT; Horne LP; Sherrill SA; Bishop GW; Baker LA; Martin CR, Resistive-pulse studies of proteins and protein/antibody complexes using a conical nanotube sensor. *J Am Chem Soc* 2007, 129 (43), 13144–52. [PubMed: 17918938]
21. Movileanu L; Howorka S; Braha O; Bayley H, Detecting protein analytes that modulate transmembrane movement of a polymer chain within a single protein pore. *Nat Biotechnol* 2000, 18 (10), 1091–5. [PubMed: 11017049]
22. Majd S; Yusko EC; MacBriar AD; Yang J; Mayer M, Gramicidin pores report the activity of membrane-active enzymes. *J Am Chem Soc* 2009, 131 (44), 16119–26. [PubMed: 19886696]
23. Branton D; Deamer DW; Marziali A; Bayley H; Benner SA; Butler T; Di Ventra M; Garaj S; Hibbs A; Huang X; Jovanovich SB; Krstic PS; Lindsay S; Ling XS; Mastrangelo CH; Meller A; Oliver JS; Pershin YV; Ramsey JM; Riehn R; Soni GV; Tabard-Cossa V; Wanunu M; Wiggin M; Schloss JA, The potential and challenges of nanopore sequencing. *Nat Biotechnol* 2008, 26 (10), 1146–53. [PubMed: 18846088]
24. Charalampous T; Kay GL; Richardson H; Aydin A; Baldan R; Jeanes C; Rae D; Grundy S; Turner DJ; Wain J; Leggett RM; Livermore DM; O'Grady J, Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol* 2019, 37 (7), 783–792. [PubMed: 31235920]
25. Giesselmann P; Brandl B; Raimondeau E; Bowen R; Rohrandt C; Tandon R; Kretzmer H; Assum G; Galonska C; Siebert R; Ammerpohl O; Heron A; Schneider SA; Ladewig J; Koch P; Schuldt BM; Graham JE; Meissner A; Muller FJ, Analysis of short tandem repeat expansions and their methylation state with nanopore sequencing. *Nat Biotechnol* 2019, 37 (12), 1478–1481. [PubMed: 31740840]
26. Robertson JWF; Reiner JE, The Utility of Nanopore Technology for Protein and Peptide Sensing. *Proteomics* 2018, 18 (18), e1800026. [PubMed: 29952121]
27. Ouldali H; Sarthak K; Ensslen T; Piguet F; Manivet P; Pelta J; Behrends JC; Aksimentiev A; Oukhaled A, Electrical recognition of the twenty proteinogenic amino acids using an aerolysin nanopore. *Nat Biotechnol* 2020, 38 (2), 176–181. [PubMed: 31844293]
28. Asandei A; Rossini AE; Chinappi M; Park Y; Luchian T, Protein Nanopore-Based Discrimination between Selected Neutral Amino Acids from Polypeptides. *Langmuir* 2017, 33 (50), 14451–14459. [PubMed: 29178796]
29. Liu L; Li T; Zhang S; Song P; Guo B; Zhao Y; Wu HC, Simultaneous Quantification of Multiple Cancer Biomarkers in Blood Samples through DNA-Assisted Nanopore Sensing. *Angew Chem Int Ed Engl* 2018, 57 (37), 11882–11887. [PubMed: 29697902]
30. Li W; Bell NA; Hernandez-Ainsa S; Thacker VV; Thackray AM; Bujdoso R; Keyser UF, Single protein molecule detection by glass nanopores. *ACS Nano* 2013, 7 (5), 4129–34. [PubMed: 23607870]
31. Chavis AE; Brady KT; Hatmaker GA; Angevine CE; Kothalawala N; Dass A; Robertson JWF; Reiner JE, Single Molecule Nanopore Spectrometry for Peptide Detection. *ACS Sens* 2017, 2 (9), 1319–1328. [PubMed: 28812356]
32. Pham B; Eron SJ; Hill ME; Li X; Fahie MA; Hardy JA; Chen M, A Nanopore Approach for Analysis of Caspase-7 Activity in Cell Lysates. *Biophys J* 2019, 117 (5), 844–855. [PubMed: 31427065]
33. Harrington L; Alexander LT; Knapp S; Bayley H, Single-Molecule Protein Phosphorylation and Dephosphorylation by Nanopore Enzymology. *ACS Nano* 2019, 13 (1), 633–641. [PubMed: 30588793]
34. Zhao Q; de Zoysa RS; Wang D; Jayawardhana DA; Guan X, Real-time monitoring of peptide cleavage using a nanopore probe. *J Am Chem Soc* 2009, 131 (18), 6324–5. [PubMed: 19368382]
35. Hu R; Rodrigues JV; Waduge P; Yamazaki H; Cressiot B; Chishti Y; Makowski L; Yu D; Shakhnovich E; Zhao Q; Wanunu M, Differential Enzyme Flexibility Probed Using Solid-State Nanopores. *ACS Nano* 2018, 12 (5), 4494–4502. [PubMed: 29630824]

36. Lu B; Stokes C; Fahie M; Chen M; Golovchenko JA; Hau LV, Protein Motion and Configurations in a Form-Fitting Nanopore: Avidin in ClyA. *Biophys J* 2018, 115 (5), 801–808. [PubMed: 30122294]
37. Japrun D; Dogan J; Freedman KJ; Nadzeyka A; Bauerdick S; Albrecht T; Kim MJ; Jemth P; Edel JB, Single-molecule studies of intrinsically disordered proteins using solid-state nanopores. *Anal Chem* 2013, 85 (4), 2449–56. [PubMed: 23327569]
38. Galenkamp NS; Biesemans A; Maglia G, Directional conformer exchange in dihydrofolate reductase revealed by single-molecule nanopore recordings. *Nat Chem* 2020, 12 (5), 481–488. [PubMed: 32251371]
39. Si W; Aksimentiev A, Nanopore Sensing of Protein Folding. *ACS Nano* 2017, 11 (7), 7091–7100. [PubMed: 28693322]
40. Lee JS, Nanopore analysis of the effect of metal ions on the folding of peptides and proteins. *Protein Pept Lett* 2014, 21 (3), 247–55. [PubMed: 24370255]
41. Li J; Fologea D; Rollings R; Ledden B, Characterization of protein unfolding with solid-state nanopores. *Protein Pept Lett* 2014, 21 (3), 256–65. [PubMed: 24370259]
42. Oukhaled A; Pastoriza-Gallego M; Bacri L; Mathe J; Auvray L; Pelta J, Protein unfolding through nanopores. *Protein Pept Lett* 2014, 21 (3), 266–74. [PubMed: 24370253]
43. Thakur AK; Movileanu L, Real-time measurement of protein-protein interactions at single-molecule resolution using a biological nanopore. *Nat Biotechnol* 2018.
44. Fahie MA; Yang B; Pham B; Chen M, Tuning the selectivity and sensitivity of an OmpG nanopore sensor by adjusting ligand tether length. *ACS Sens* 2016, 1 (5), 614–622. [PubMed: 27500277]
45. Fahie MA; Yang B; Mullis M; Holden MA; Chen M, Selective Detection of Protein Homologues in Serum Using an OmpG Nanopore. *Anal Chem* 2015, 87 (21), 11143–9. [PubMed: 26451707]
46. Liang B; Tamm LK, Structure of outer membrane protein G by solution NMR spectroscopy. *Proc Natl Acad Sci U S A* 2007, 104 (41), 16140–5. [PubMed: 17911261]
47. Retel JS; Nieuwkoop AJ; Hiller M; Higman VA; Barbet-Massin E; Stanek J; Andreas LB; Franks WT; van Rossum BJ; Vinothkumar KR; Handel L; de Palma GG; Bardiaux B; Pintacuda G; Emsley L; Kuhlbrandt W; Oschkinat H, Structure of outer membrane protein G in lipid bilayers. *Nat Commun* 2017, 8 (1), 2073. [PubMed: 29233991]
48. Fahie MA; Chen M, Electrostatic Interactions between OmpG Nanopore and Analyte Protein Surface Can Distinguish between Glycosylated Isoforms. *J Phys Chem B* 2015, 119 (32), 10198–206. [PubMed: 26181080]

Figure 1. OmpG nanopore design. (a) Schematic representation of OmpG nanopore sensing. (b) Top view of the OmpG structure (pdb: 2IWV). Charged residues at the loops are highlighted in red for negative amino acids and blue for the positive ones. Locations of the cysteine mutations on each of the OmpG loops are presented as spheres.

Figure 2.

Avidin and streptavidin binding to the OmpG^{Ln-biotin} nanopores. (a) Top view of the OmpG structure with cysteine mutants indicated by yellow balls. (b) Representative current traces in the absence and presence of streptavidin. OmpG^{L6-biotin} is highlighted in a yellow box. (c) Representative current traces in the absence and presence of avidin. (d) All-points histograms before and after avidin binding to OmpG^{Ln-biotin}. Experiments were performed at +50 mV in 20 mM sodium phosphate pH 6 buffer containing 300 mM KCl.

Figure 3.

Anti-biotin antibody SB58C binding to the OmpG^{Ln-biotin} constructs. (a) Current traces of OmpG^{Ln-biotin} before and after SB58C binding. The various sub-current states at the SB58C bound state are indicated by states 1,2,3 while state 0 represents the unbound state. (b) The all-points histogram of the OmpG^{Ln-biotin} pore at the unbound and SB58C bound states. Single-channel current recording experiments were performed in buffer 300 mM KCl, 20 mM sodium phosphate pH 6 at +50 mV.

Figure 4. BTN.4 antibody binding to OmpG loop constructs at +50 mV. (a) Current traces before and after BTN.4 binds to the loop constructs. (b) The all-points histograms before and after BTN.4 binds to the loop constructs. Experiments were performed at +50 mV in buffer 20 mM sodium phosphate pH 6 containing 300 mM KCl.

Figure 5. Model of OmpG and analyte interaction affecting detection sensitivity. Analytes that interact with the OmpG loops via electrostatic attractions undergo multiple loop sampling, whereas other analytes with weak interactions may alter the dynamics of a single loop.

Table 1:

Kinetic parameters of BTN.4 binding to OmpG loop constructs

Construct	k_{on} ($\times 10^7 \text{ M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_d ($\times 10^{-8} \text{ M}$)
L1-biotin	N/A	N/A	N/A
L2-biotin	1.30 ± 0.05	0.490 ± 0.005	3.85 ± 0.15
L3-biotin	1.90 ± 0.07	0.260 ± 0.007	1.42 ± 0.06
L4-biotin	1.20 ± 0.03	0.260 ± 0.007	2.21 ± 0.08
L5-biotin	N/A	N/A	N/A
L6-biotin	2.30 ± 0.05	0.230 ± 0.005	1.02 ± 0.03
L7-biotin	1.60 ± 0.03	0.290 ± 0.009	1.80 ± 0.06