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Tuning the selectivity and sensitivity of an OmpG nanopore sensor by adjusting ligand tether length

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Abstract

We have previously shown that a biotin ligand tethered to the rim of an OmpG nanopore can be used to detect biotin-binding proteins. Here, we investigate the effect of the length of the polyethylene glycol tether on the nanopore's sensitivity and selectivity. When the tether length was increased from 2 to 45 ethylene repeats, sensitivity decreased substantially for a neutral protein streptavidin and slightly for a positively charged protein (avidin). In addition, we found that two distinct avidin binding conformations were possible when using a long tether. These conformations were sensitive to the salt concentration and applied voltage. Finally, a longer tether resulted in reduced sensitivity due to slower association for a monoclonal anti-biotin antibody. Our results highlight the importance of electrostatic, electroosmotic and electrophoretic forces on nanopore binding kinetics and sensor readout.

Keywords

nanopore; OmpG; sensing; biotin; antibody; streptavidin

Nanopores sensing is a single molecule approach by which analytes are sensed when they interfere with ionic current flowing through the pore during an applied potential.¹⁻⁵ Nanopores are broadly grouped into two classes: solid state nanopores and protein nanopores^{1, 4}. Solid-state nanopores are fabricated from silicon nitride, glass or polymer substrates⁶⁻⁸. Solid-state nanopores possess several advantages including the physical and chemical robustness, tunable pore size and shape. On the other hand protein nanopores always have the same, atomically precise structure. Generally, nanopores detect proteins by one of two mechanisms: translocation-dependent or translocation-independent. For translocation-dependent sensing, the analyte is drawn into the pore by electrophoresis, which partially blocks the flow of ionic current. Both solid-state and protein nanopores applied this strategy to detect targets including BSA,⁹⁻¹¹ streptavidin/avidin,^{12, 13} antibodies,^{12, 14} and polyarginine peptides.¹⁵ Alternatively, some proteins may be sensed via binding to an auxiliary polymer that threads through the nanopore.¹⁶⁻¹⁸ Because ionic current is sensitive

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Supporting Information: Supporting Information Available: The following files are available free of charge. Supporting information.pdf: the file contains detailed data analysis, supporting figures and a summary table.

to the size, shape, and surface properties of the analyte protein, as well as analyte-lumen interactions, nanopores are able to distinguish complex protein isoforms,¹⁹⁻²² e.g. ubiquitin dimeric isoforms²⁰ and two configurations of aptamer-thrombin complex.²² In addition, the amino acid composition of a protein may be revealed as an unfolded polypeptide is threaded through a nanopore.²³⁻²⁷ Nanopores were also used as a single molecule analytical approach to investigate the protein folding,^{28, 29} unfolding,^{23, 30} aggregation^{12, 31} and enzymatic activity.^{32, 33}

Despite the utility of these methods, the translocation-dependent approach is generally not suited for directly analyzing proteins that are too large to fit into the pore. While it is relatively straightforward to control the size of solid-state nanopores during fabrication, adjusting the size of protein nanopores is challenging due to our limited understanding of the connection between amino acid sequence and protein structure, i.e. the folding problem.³⁴ To overcome this limitation, large proteins have been detected by protein nanopores via a translocation-independent mechanism. Typically, an analyte binds to a high affinity site located on the exterior of the nanopore. The binding site can be (i) a peptide sequence introduced into a region of the protein nanopore,³⁵⁻³⁸ or (ii) a covalently attached ligand in the lumen³⁹ or on the rim of the nanopore.^{7, 40, 41} Using this approach, a single nanopore can detect proteins of any size, eliminating the need to search for pores of suitable dimensions. Recently, we have used outer membrane protein G (OmpG) from *Escherichia coli* (*E. coli*) to detect large proteins. For example, we distinguished several structural homologues and isoforms of biotin-binding proteins in complex mixture.⁴¹⁻⁴³ OmpG contains seven flexible loops on the extracellular side.⁴⁴⁻⁴⁶ Dynamic loop movement induces current fluctuations (called gating) in ionic current measurements. Our engineered sensor had a biotin covalently attached to the long loop 6 of OmpG.^{47, 48} Proteins were detected via changes in the gating pattern when they bound to biotin. Eight biotin-binding proteins, including four streptavidin homologues and four anti-biotin antibodies were differentiated by their characteristic current gating signals.⁴¹⁻⁴³ Compared to other multimeric protein nanopores,^{21, 37, 39, 49} such as α HL,⁵⁰ MspA,⁵¹ ClyA⁵² and phi29 motor,⁵³ OmpG is a monomeric protein nanopore with flexible loops at the entrance. OmpG sensing relies on the interaction of its flexible loops with the analyte protein which generates distinct characteristic signal for each analyte. The high selectivity of the OmpG nanopore was attributed to the ability of its loops to undergo distinct conformational changes when interacting with each analyte. Such a high selectivity is rarely observed among other nanopores because their rigid structures are unable to undergo large conformational changes when bound by an analyte.

Electrostatic attractions between the nanopore loops and the analyte played an important role in OmpG selectivity.⁴² Such interactions only occurred when the analyte proteins were bound to the tethered ligand and confined in close proximity to the OmpG nanopore. Here, we investigated how the proximity between the ligand and nanopore might affect the sensitivity and selectivity of our detection system. We increased the length of the polyethylene glycol (PEG) biotin linker and measured changes in gating signals as well as binding kinetics. While a longer linker allowed a greater range of motion and therefore new gating states induced by biotin-bound proteins, this also adversely impacted the kinetics and impaired sensitivity. This work shows that the linker length of a tethered ligand influences

the selectivity and sensitivity of the OmpG sensor and is important in the design of future sensors.

Materials and Methods

Materials

All reagents and materials were purchased from Fisher Sci. or Boston Bioproducts unless otherwise noted. Mouse anti-biotin monoclonal antibody BTN.4 clone (MS-1048-P1) was purchased from Thermo Scientific. Avidin (A9275) was purchased from Sigma. Streptavidin (Z7041) was purchased from Promega. Maleimide-PEG45-biotin molecule was purchased from Creative PEG works (Biotin-PEG-MAL, MW 2k (PJK-1930)). Maleimide-PEG2-biotin and maleimide-PEG11-biotin were purchased from (ThermoFisher Scientific). Diphytanoylphosphatidylcholine (DPhPC) were from Avanti polar lipids. Tris(2-carboxyethyl)phosphine (TCEP) and octyl-glucoside (OG) and were purchased from GoldBio Technology.

Cell culture and protein purification

OmpG mutant D224C was created and expressed in *E. coli* cells as previously described.⁴³ OmpG was purified in 50 mM Tris-HCl, 5 mM TCEP, 8 M urea using anion exchange chromatography. Prior to labeling with the MAL-PEG_n-biotin, OmpG-containing fractions were desalted into a HEPES buffer pH 7 to remove TCEP and Tris-HCl. OmpGD224C and MAL-PEG_n-biotin were mixed in 1:10 molar ratio and incubated for 2 hrs at room temperature (~23°C) with constant shaking. The mixture was desalted using HiTrap 5 ml desalting column (GE Healthcare) to remove unconjugated MAL-PEG_n-biotin. The labeled OmpG was then diluted in a 2:3 v/v ratio in refolding buffer (110 mM octyl glucoside, 20 mM Tris-HCl, pH 9) and incubated at 37°C for 3 days. Labeling and refolding efficiency were analyzed by gel-shift assay. Labeling and refolding percentages were both determined to be > 80% for all constructs (Figure S1). Proteins were aliquoted and stored at -80°C in 20% glycerol. Freshly thawed aliquots were used for the experiments.

Single Channel recording

Single channel recording experiments were performed as previously described.⁴³ For recording OmpG-PEG_n-biotin behavior in planar lipid membranes, signals were filtered with a 2 kHz Bessel filter (unless otherwise stated) and acquired at a sampling rate of 100 μ s after digitization with a Digidata 1320A/D board (Axon Instruments). The current was amplified with an Axopatch 200B integrating patch clamp amplifier (Molecular Devices). To create the bilayer, 2 μ L of a 10% v/v hexadecane/pentane mix was applied to both sides of a 25 μ m Teflon film with a 100 μ m aperture separating two chambers. Buffer (900 μ L) was added to each chamber and approximately 15 μ L of 10 mg/ml 1,2-Diphytanoyl-sn-glycerol-3-phosphocholine (DPhPC) dissolved in pentane was dropped onto the surface of the buffer. One Ag/AgCl electrode was inserted into each chamber. The liquid level was raised to form the lipid bilayer in the aperture. Once a bilayer formed, ~1 nM (final concentration) OmpG proteins were added to the *cis* chamber, which was electrically grounded. A positive voltage of 200 mV was applied to induce pore insertion. The voltage was lowered to 50 mV after a single pore inserted. The orientation of nanopore was determined based on its asymmetric

gating behavior at positive and negative potentials.⁵⁴ OmpG behavior was recorded in the absence and presence of analyte proteins that were added to the chamber facing the loop side.

Single channel data analysis

To determine the open probability of OmpG pores, traces were analyzed in Clampfit 10.3 using single channel search. The open probability was calculated as the sum of the dwell time of open states divided by the total time analyzed. To determine the kinetics of mouse monoclonal antibody bound state, the dwell time of 300-500 bound events and unbound events were extracted manually and fit with a single exponential to derive the averaged inter-event time (τ_{on} from unbound events) and dwell time (τ_{off} from bound events). The decrease of open pore conductance and increase in gating frequency of the analyte bound state was used as criteria for data extraction. The dwell times of the binding substates of avidin B1 and B2 were also extracted manually and similarly analyzed as the anti-biotin mAb.

Results and Discussion

Effect of PEG linker length on analyte recognition

A single cysteine mutant, OmpG (D224C) was labeled with one of the following: maleimide-PEG_n-biotin, where n is 2, 11 or 45, respectively. The resulting constructs - OmpG-PEG2-biotin, OmpG-PEG11-biotin and OmpG-PEG45-biotin - could extend the biotin group approximately up to 30, 60 and 170 Å from the cysteine on loop 6 (Figure 1) as estimated by chemical linkage. The dynamic movement of the loop 6, which moves in and out of the OmpG entrance, contributes to more than 95% of gating signal.^{47, 48} Chemical modification of the OmpG proteins with biotin reagents had minimal effect on the gating behavior (Figures S2-3).

Control experiments showed that none of the analytes triggered a signal change with OmpG-D224C pore indicating that non-specific binding of streptavidin and avidin to OmpG was negligible (Figure S4). We then examined how the different OmpG-biotin nanopore constructs responded to the two biotin-binding proteins. Streptavidin resulted in a permanent increase in open probability ($n = 10$, $p = 0.05$) for OmpG-PEG2-biotin as previously seen (Figure 2a, d, Figure S5) but did not induce any quantifiable change in the activity of more than 15 OmpG-PEG11-biotin pores and 13 OmpG-PEG45-biotin pores (Figure 2b-d).^{41, 42}

In contrast, significant changes in current traces of all three constructs were observed using avidin (Figures S6, S7). Avidin binding caused a ~45% decrease of the OmpG-PEG2-biotin and OmpG-PEG11-biotin open pore current (Figure 3a,b). The open current decreased from 24.8 ± 4.4 pA to 14.6 ± 1.7 pA ($n = 6$) for OmpG-PEG2-biotin while for OmpG-PEG11-biotin it decreased from 27.4 ± 0.9 pA to 14.4 ± 3.4 pA ($n = 4$). Interestingly, when avidin bound to OmpG-PEG45-biotin, it induced two distinct current patterns during the bound state (Figure 3c). In the predominant pattern, termed B1, the open pore current decreased from 23.6 ± 1.0 pA to 21.1 ± 1.3 pA ($n = 6$), an 11% decrease in the open state. The minor pattern B2, had an open pore current of 14.9 ± 2.5 pA ($n = 6$) which was 37% decrease of the unbound state (UB) (Figure 3c). Notably there were no significant changes to the open

probability of either the three OmpG-PEG_n-biotin pores after avidin was bound (Figure 3d), unlike the significant change in open current (Figure 3e).

Previous studies using OmpG-PEG2-biotin revealed that analyte proteins could be classified into two categories based on their current signatures. Specifically, some analytes interact with the OmpG pore surface while others do not. We found that non-interacting proteins were either neutral or negatively charged molecules under the experimental conditions used.⁴² Typically, a decrease of the gating activity was observed for these proteins which may hinder the dynamic movement of loop 6 through drag or friction forces. One example was streptavidin. The fact that streptavidin binding inhibited gating in OmpG-PEG2-biotin but not OmpG-PEG11-biotin or OmpG-PEG45-biotin suggest that a linker with 11 or more PEG units is flexible enough to allow loop 6 to freely move even with a ~60 kDa protein attached.

On the other hand, proteins that interacted with OmpG's loops usually induced a reduction in the open pore current by occluding ionic flow.⁴² Such protein-protein interactions were dominated by electrostatic attraction between the negatively charged OmpG loops and the positively charged analyte protein surface, such as avidin.⁴² Avidin binding to OmpG-PEG11-biotin triggered an almost identical effect to that of the OmpG-PEG2-biotin, suggesting that the avidin bound states of these two constructs were similar (Figure S6, S7, Figure 3e). The avidin bound state of OmpG-PEG45-biotin exhibited two distinct gating signatures with relative open pore current of 87% and 60%. We presume this shows that avidin interacted with OmpG-PEG45-biotin in two different configurations (Figure S6, S7, Figure 3e).

Our results show that OmpG lost its ability to detect neutral or negatively charged molecules with an elongated linker but was able to recognize the positively charged protein analytes. This study also suggests that we could adjust the PEG linker to optimize the selectivity of OmpG toward one specific analyte such as a protein like avidin, while removing any interfering signals from related homologues, like streptavidin.

Effect of voltage on OmpG-PEG-biotin - avidin interaction

The OmpG-PEG45-biotin is the first variant to show multiple unique bound states, specifically with avidin. Since we suspect that electrostatic forces govern the interactions between the loops and analyte, we tested the effect of applied voltage on the bound states to see if they responded to electric fields. Here, positive potential was defined as the potential of the chamber in which the OmpG loops were facing was positive relative to the other chamber (Figure 4). The relative open current of OmpG-PEG2-biotin and that of OmpG-PEG11-biotin showed a significant voltage dependence (Figure a,b,d). The relative open pore current of the B1 state to the unbound state showed a weak voltage dependence (Figure 4c,d). Increasing the potential from -75 mV to 75 mV caused the open pore current to decrease slightly. On the other hand, the relative open current of the B2 state showed a strong dependence. Because the open pore current and voltage dependence of the B2 state were similar to the avidin bound states of OmpG-PEG2-biotin and OmpG-PEG11-biotin (Figure 4d), we speculated that avidin interacted with OmpG surface in a similar manner in each scenario (Figure S8). The data suggests that a longer linker would allow the analyte to

sample more than one binding area of the OmpG surface once it bound the tethered ligand. The open pore current of the avidin bound B1 and B2 states of OmpG-PEG45-biotin was likely due to the proximity of the bound avidin molecule to the entrance of OmpG under an applied potential.

We measured the dwell times of the B1 and B2 states of OmpG-PEG45-biotin at voltages ranging from -75 to +100 mV (Figures S9, S10). The B1 dwell time was not voltage dependent. However, the B2 dwell time increased exponentially with increasing positive potential (Figure 4e). Because avidin (positive) and OmpG's surface (negative) are oppositely charged, we expect a strong electrostatic attraction between them. In addition, electrophoretic (EP) and electroosmotic (EO) forces act on the bound avidin in a voltage dependent manner. The OmpG lumen is decorated with 14 positively (12R and 2K) and 16 negatively (13E and 3D) charged residues. At pH 6.0, the lumen had a -2 net negative charge. Thus, at positive potential, EP and EO aligned to push avidin into the surface of the OmpG nanopore. At negative potential, the two forces push in the opposite direction, weakening the electrostatic attraction. In nanopores such as α HL and glass, simulations have shown that the strength EO and EP fields peak at the constriction site and diminish rapidly between the nanopore and bulk solution.^{55, 56} Because the B2 state is voltage dependent, we surmise that bound avidin must be close enough to the pore to be influenced by EO and EP forces. On the other hand, avidin in the B1 state might be further away from the pore entrance as it is not affected by voltage. Here, we have shown that the characteristic gating pattern used to identify analytes and homologues can be tuned and possibly optimized to trap desired states by manipulating the voltage (Table S1).

Effect of salt concentration on the interaction between OmpG-PEG45-biotin and avidin

The electrostatic interactions between OmpG-biotin and avidin, EP and EO are sensitive to salt concentration.⁴² We expected that the avidin-bound complex might respond to changes in salt concentration. For the following experiments, B1 and B2 states of OmpG-PEG45-biotin were examined at +100 mV (Figure S11-S12). At 150 mM KCl, only the B2 state was observed (Figure 5a). At higher salt concentrations, the bound avidin switched between B1 and B2 states (Figures 5b-d). The dwell time of the B2 state decreased rapidly with increasing salt concentration with a corresponding increase in B1 dwell time (Figure 5f, Figure S12). In addition, the relative open current of the avidin B1 and B2 states increased with increasing salt concentration. At 750 mM KCl, the B2 state was still recognizable, while the open current of the B1 state was indistinguishable from that of the unbound state (Figure 5d, e). This suggested that the avidin molecule occluded the ionic flow through OmpG-PEG45-biotin to a lesser degree at higher salt concentration. At 1 M KCl, we did not observe any significant change in gating after the addition of avidin indicating that avidin no longer interacted with the nanopore surface.

Because salt screens the surface charge of the bound avidin and the OmpG, increasing salt concentration could essentially weaken all three forces that act on avidin. This explains why at low salt concentration (150 mM KCl), avidin stayed tightly bound (B2) while the loosely-bound state (B1) became dominant when salt concentration increased. Thus, the salt

concentration of the recording buffer is an important factor to modulate the detection of the binding signature of the chosen target (Table S1).

Effect of linker length on the binding kinetics

To assess the effect of PEG linker length on binding kinetics, a reversible-binding mouse monoclonal anti-biotin antibody (mAb) was used in place of streptavidin and avidin. The recording buffer salt was lowered to 150 mM because the gating activity of OmpG-PEG45-biotin pores did not change in the presence of mAb in 300 mM KCl buffer ($n = 10$). The mAb bound all three nanopore constructs and generated gating signatures differing in open pore current and gating frequency compared to the unbound state (Figure 6). Although mAb binding increased gating frequency for all OmpG-PEG_n-biotin constructs (Figure S14a), the relative open current was $71 \pm 15\%$ ($n = 15$), $77 \pm 10\%$ ($n = 15$) and $94 \pm 7\%$ ($n = 15$) as the length of PEG increased from 2 units to 45 units (Figure S14b). Additionally, the three constructs varied in the mAb association as the longer PEG linker resulted in a slower k_{on} (Table 1, Figure S14c, S15). The linker length showed less effect on dissociation rates (k_{off}) as the differences among the three constructs were insignificant (Table 1, Figure S14d, S15). As a consequence, the observed dissociation equilibrium constant K_d for OmpG-PEG45-biotin was five times weaker than the OmpG-PEG2-biotin. This result shows the apparent binding affinity of mAb to OmpG-PEG_n-biotin constructs decreased with the increasing length of the linker (Tables 1, 2 and Table S1).

In nanopore sensing, the electroosmotic flow, electrophoretic force and electrostatic forces strongly influence the molecule's motion within the nanopore and near the entrance.^{57, 58} Steep potential gradients and strong flow velocity that occur at the constriction site of a nanopore can create an absorbing force to facilitate the capture of the analyte by the nanopore.^{59, 60} All three forces, when acting on the analyte in the same direction, can potentially promote stronger binding with OmpG-PEG_n-biotin by enhancing the mAb association and inhibiting dissociation. Since these forces were strongest in the pore entrance and decrease rapidly with increasing distance, increasing the PEG linker length reduced the binding affinity. Thus, although a longer linker may potentially improve the selective detection, it would compromise the detection sensitivity.

Conclusion

By varying the length of the tether connecting biotin to OmpG, we showed how distance influenced OmpG's detection selectivity and sensitivity. Our data demonstrated that electrostatic interactions, electroosmotic and electrophoretic forces not only strongly modulate the signal characteristics of the bound state but also affect their binding kinetics. Increasing the linker length weakened these forces resulted in the decrease in sensitivity. On the contrary, a longer linker allowed a bound analyte to sample multiple interactions at the interface with the nanopore, which may enhance analyte identification and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The design of the OmpG-PEG_n-biotin constructs. The biotin attached to OmpG nanopore via PEG2, PEG11 and PEG45 linkers could extend up to 30, 60 and 170 Å away from loop 6.

Figure 2. Streptavidin binding to OmpG-PEG_n-biotin constructs. Current recordings of (a) OmpG-PEG2-biotin (b) OmpG-PEG11-biotin and (c) OmpG-PEG45-biotin before and after streptavidin binding at +50 mV. Buffer used was 300mM KCl, 10mM sodium phosphate pH 6. (d) Comparison of the open probability of OmpG nanopores before and after streptavidin binding. Error bars represent standard errors from at least 5 pores. Statistical analysis was performed with an unpaired two sample t-test ($n > 5$, $p = 0.05$).

Figure 3.

Avidin binding to OmpG-PEGn-biotin constructs. Current recordings and the corresponding histograms of (a) OmpG-PEG2-biotin (b) OmpG-PEG11-biotin and (c) OmpG-PEG45-biotin before and after avidin binding at +50 mV. Buffer used was 300mM KCl, 10mM sodium phosphate pH 6. The relative open pore current of the avidin bound states (blue and red dash lines) to the unbound state (UB, green dash line) are indicated on the histograms. (d-e) Comparison of open probability (d) and open pore current (e) of OmpG nanopores before and after avidin binding. Error bars represent standard errors from at least 4 pores. Statistical analysis were performed with an unpaired two sample t-test ($n > 4$, $p = 0.05$).

Figure 4.

Voltage effect on avidin binding to OmpG-PEG_n-biotin. Current recordings of (a) OmpG-PEG₂-biotin (b) OmpG-PEG₁₁-biotin and (c) OmpG-PEG₄₅-biotin before and after avidin binding at various voltages. (d) Effect of voltage on the relative open pore current of the avidin bound states of all three OmpG-PEG-biotins. (e) Effect of voltage on the dwell time of B1 and B2 states of OmpG-PEG₄₅-biotin. The B1 and B2 avidin binding states are indicated by the red and blue bars respectively. Buffer used was 300mM KCl, 10mM sodium phosphate pH 6.

Figure 5.

Salt effect on OmpG-PEG45-avidin binding states. Typical current recording of a single OmpG-PEG45-biotin pore at unbound and traces of avidin bound states (B1 and B2). Single channel recording were carried out in 10mM sodium phosphate buffer pH 6 containing KCl ranging from (a) 150mM (b) 300mM (c) 450mM and (d) 750mM KCl. (e) The effect of salt concentration on the relative open pore current and (f) the dwell time duration of avidin B1 and B2 states.

Figure 6.

Detection of an anti-biotin mouse monoclonal antibody (mAb) by the three OmpG-PEG_n-biotins. Current recordings and all events histograms of anti-biotin mAb binding to (a) OmpG-PEG2-biotin (b) OmpG-PEG11-biotin (c) OmpG-PEG45-biotin. Green dashed lines indicate the open current of the pores in the unbound state and red dashed lines indicate the open current in the avidin bound state. Buffer used is 150mM KCl, 10mM sodium phosphate pH 6. The applied voltages were +50 mV.

Table 1
The binding kinetics of an anti-biotin mouse monoclonal antibody

Biotin construct	k'_{on} ($\times 10^6 \text{ M}^{-1}\text{s}^{-1}$)	K_{off} (s^{-1})	K'_D ($\times 10^{-8} \text{ M}$)
OmpG-PEG2	20.3 ± 6.7	0.30 ± 0.08	1.5 ± 0.6
OmpG-PEG11	16.6 ± 5.0	0.48 ± 0.06	2.9 ± 0.6
OmpG-PEG45	7.5 ± 1.2	0.47 ± 0.07	6.3 ± 1.2

Table 2

Summary of the current characteristics of analyte bound states.

Target	OmpG Sensor	Buffer	Binding Signal		Binding Characteristic
Streptavidin	PEG2	300 mM KCl pH 6		•	Open probability increases by 17%
	PEG11	300 mM KCl pH 6		•	No change in gating activity
	PEG45	300 mM KCl pH 6		•	No change in gating activity
Avidin	PEG2	300 mM KCl pH 6		• •	Current decreases by 39.6% No change in open probability
	PEG11	300 mM KCl pH 6		• •	Current decreases by 42.8% No change in open probability
	PEG45	300 mM KCl pH 6		• • •	B1 current decreases by 10.8% B2 current decreases by 36.8% No change in open probability
Anti-biotin mAb	PEG2	150 mM KCl pH 6		• • •	Current decreases by 28.6% Open probability decreases by 27.6% $K_d = 14.6 \pm 5.77$ nM
	PEG11	150 mM KCl pH 6		• • •	Current decreases by 22.5% Open probability decreases by 17.4% $K_d = 29.2 \pm 6.3$ nM
	PEG45	150 mM KCl pH 6		• • •	Current decreases by 6.0% Open probability decreases by 9.9% $K_d = 62.6 \pm 11.6$ nM