Resolved Single-Molecule Detection of Individual Species within a Mixture of Anti-Biotin Antibodies Using an Engineered Monomeric Nanopore

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Supporting information

Materials:

The DNA oligos and all chemicals were purchased from Fisher Scientific unless stated otherwise. Streptavidin (Z7041) was purchased from Promega. The biotin-maleimide reagents, Maleimide-PEG2-Biotin and Maleimide-PEG11-Biotin were purchased from Piercenet (Thermo Scientific). Mouse anti-biotin monoclonal antibody (MS-1048-P1) was purchased from Thermo Scientific. Goat anti-biotin polyclonal antibody (B3640) was from Sigma Aldrich. Mouse anti-His_6 (BSM-0287M) and mouse anti-GAPDH (BSM-0978M) antibodies were obtained from Bioss antibodies. LB media and DL-dithiothreitol (DTT) were purchased from Boston BioProducts. Diphytanoylphosphatidylcholine (DPhPC) were from Avanti polar lipids. Octyl-glucoside (OG) and *tris*(2-carboxyethyl)phosphine (TCEP) were purchased from GoldBio Technology.

Methods:

Construction of OmpG D224C mutant: Single cysteine was introduced to replace the aspartic acid 224 by mutagenesis PCR based on the plasmid pT7-OmpG wt.[1] The primers for D224C were
5'- GGGACTGGCAGTGTGATATTGAACGTGAAG (forward) and 5'-GTTCAATATCACACTGCCAGTCCCAGTTAC (reverse). These two primers were used in a pair with the primer SC47: 5'-CAG AAG TGG TCC TGC AAC TTT ATC (reverse) and SC46: 5'-ATA AAG TTG CAG GAC CAC TTC TG (forward) which annealed to the middle of the plasmid. The two PCR products were mixed in a 1:1 molar ratio and subjected to DpnI digestion for three hours to degrade the parental plasmid. *E. coli* DH5α cells were then co-transformed with the PCR mixture and colonies containing the desired mutant construct pT7-OmpG D224C was identified by DNA sequencing.

Cloning, expression and purification of OmpG D224C: The OmpG D224C was prepared by following an established protocol.[1] The pT7-OmpG D224C was transformed into the BL21(pLys) *E. coli* cells and cells were grown in LB medium at 37°C until the OD600 reached 0.6. IPTG (0.5 mM) was added to the culture to induce the protein expression. Cells were harvested 3 hours after induction and lysed in lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 200 µg/ml lysozyme, 1 mM EDTA, 1mM TCEP). Cells were sonicated on ice to break the bacterial membranes. DNAase I (5ul, 2,000 U/µl) and 2 mM $MgCl_2$ were then added to the mixture to decrease the viscosity. The lysate was centrifuged at 13,000 rpm for 30 min. The pellet was washed once with 30 ml 50 mM Tris·HCl, pH8.0, 1.5 M Urea, 1mM TCEP. Then the OmpG-containing inclusion body was dissolved in 50 ml 50 mM Tris·HCl, pH 8.0, 3 mM Tris(2-carboxyethyl)phosphine (TCEP), 8 M Urea and passed through a 0.45 µm filter before FPLC purification. All OmpG proteins were purified using a 5ml Q-ionic exchange column (GE Healthcare).

Biotinylation and refolding of OmpG proteins: The purified OmpG D224C was incubated

with 10 mM freshly prepared DL-dithiothreitol (DTT) for 30 min on ice to reduce the thiols. The DTT was then removed using a desalting column equilibrated with buffer 50 mM HEPES, pH7.0, 150 mM NaCl, 8M Urea. To label the OmpG D224C with biotin, the protein was incubated with maleimide-PEG-biotin in a molar ratio 1:20 (protein to biotin) at room temperature (~23°C) for 2 hours and then at 4 °C overnight. DTT (10 mM) was added to quench the reaction. The reaction mixture was passed through the desalting column once again to remove the unreacted chemicals. The biotin labelled OmpG was then diluted with the refolding buffer 50 mM Tris·HCl, pH 9.0, 3.25% OG until the final concentration of urea reached 3.0 M. Samples were then incubated at 37 °C for 3 days. The biotinylation and refolding efficiency was determined by SDS-PAGE (Fig. S1).

Supplementary figures

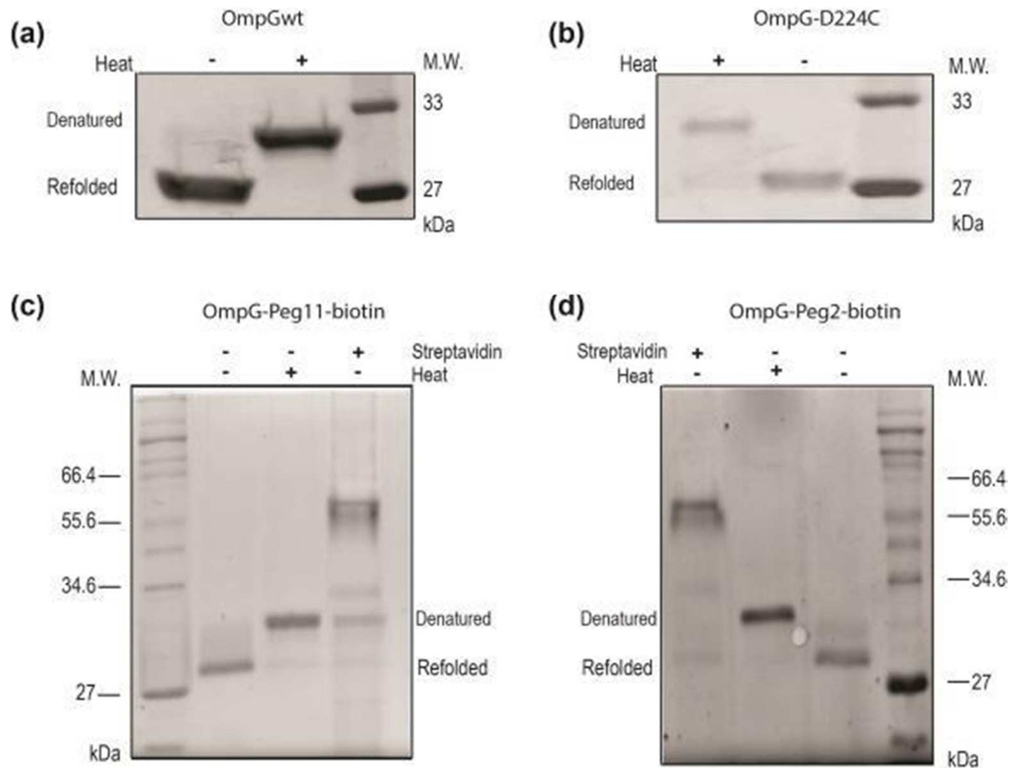

Figure S1: SDS-PAGE analysis of OmpG variants. The refolded OmpG variants were either pre-heated at 95ºC for 15 min or directly loaded on a 12.5% SDS-PAGE. Heating denatures the OmpG protein which migrates slower in SDS-PAGE as previously demonstrated.[1-3] To determine the labelling efficiency, the OmpG-Peg11-biotin and OmpG-Peg2-biotin were incubated with streptavidin for 5 minutes which forms an SDS-resistant complex with biotin. Consequently, OmpG shifts to higher-molecular weight and labeling percent can be calculated by the disappearance of the OmpG refolded or denatured band.

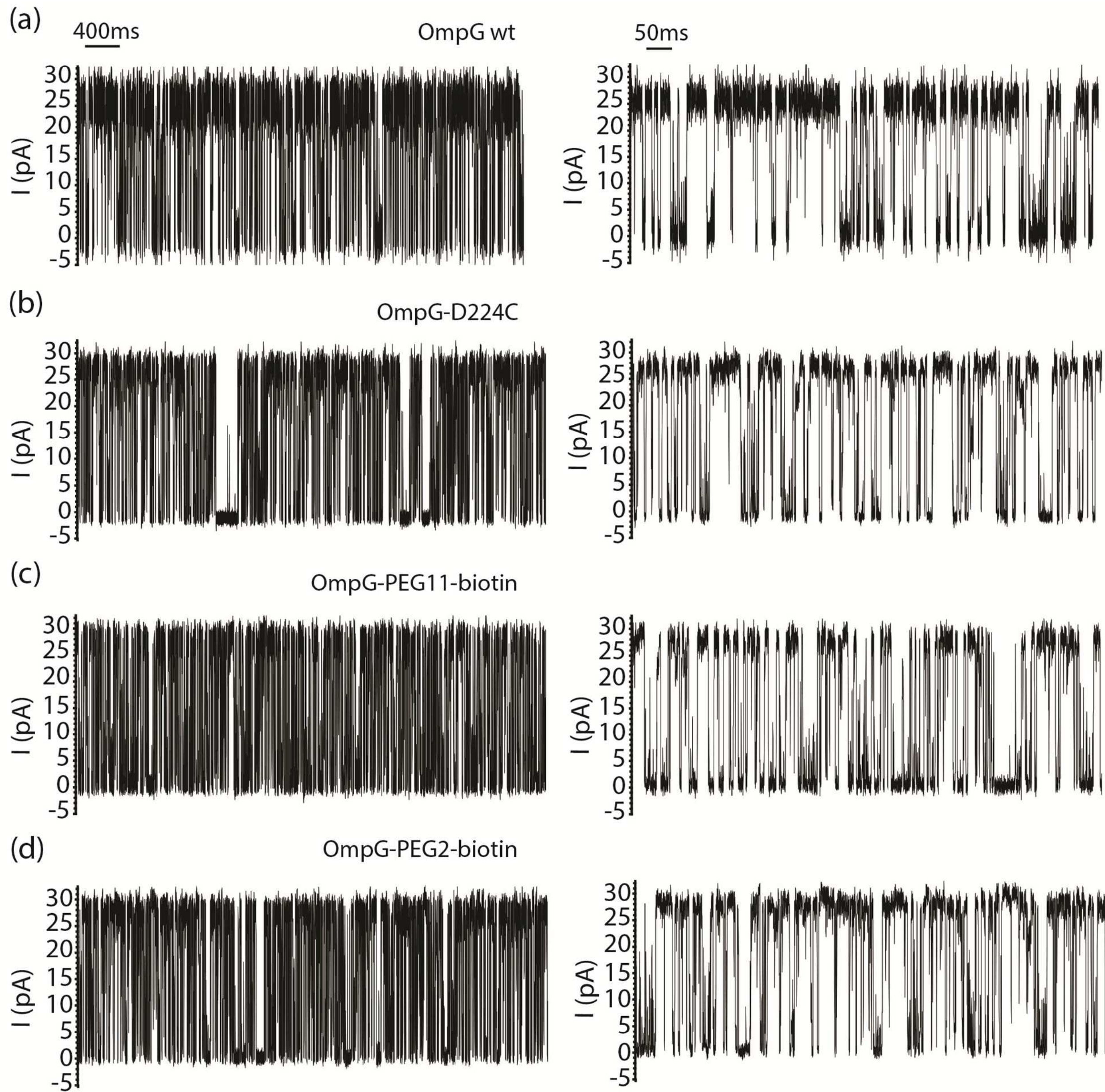

Figure S2: Single point mutation or biotinylation of OmpG does not affect its inherent gating behavior. Single channel recording traces of (a) OmpG wt, (b) OmpG D224C (unlabeled), (c) OmpG-Peg11-biotin and (d) OmpG-Peg2-biotin. Buffer used was 300mM KCl, 10mM sodium phosphate pH 6 and the applied potential was +50 mV.

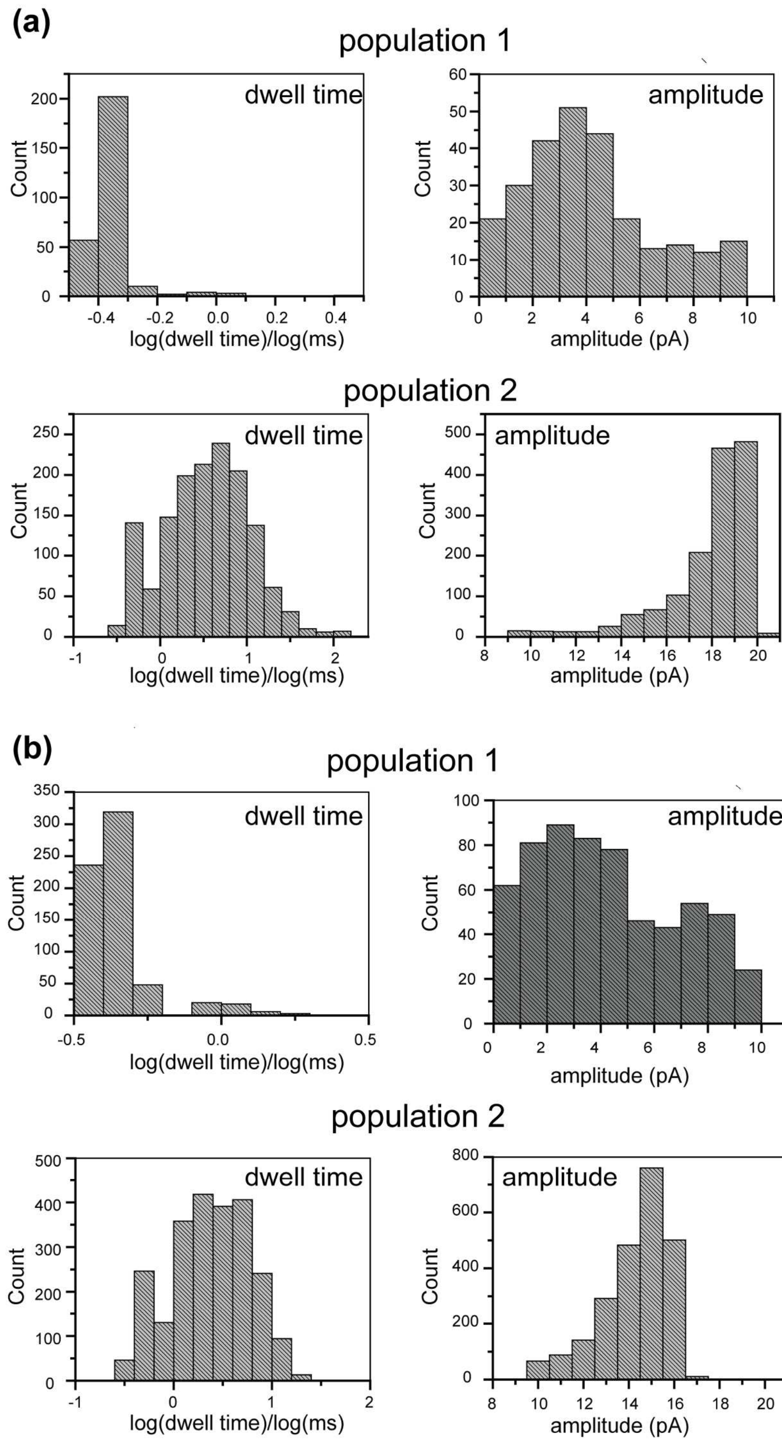

Figure S3: Characteristic of the two gating event populations in Fig. 2d. Histogram of amplitude and dwell time of gating events before (a) and after streptavidin binding (b).

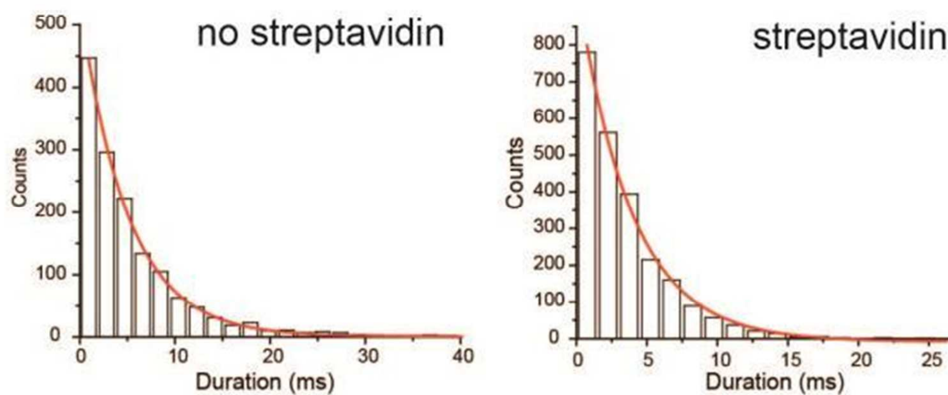

Figure S4: Effect of streptavidin binding on the bending events. Histogram of the duration time (τoff) of the gating events before and after streptavidin binding to OmpG-Peg11-biotin. Data were fitted with single exponential function yielding an average τ_{off} of 5.0 ms before and 3.9 ms after streptavidin binding.

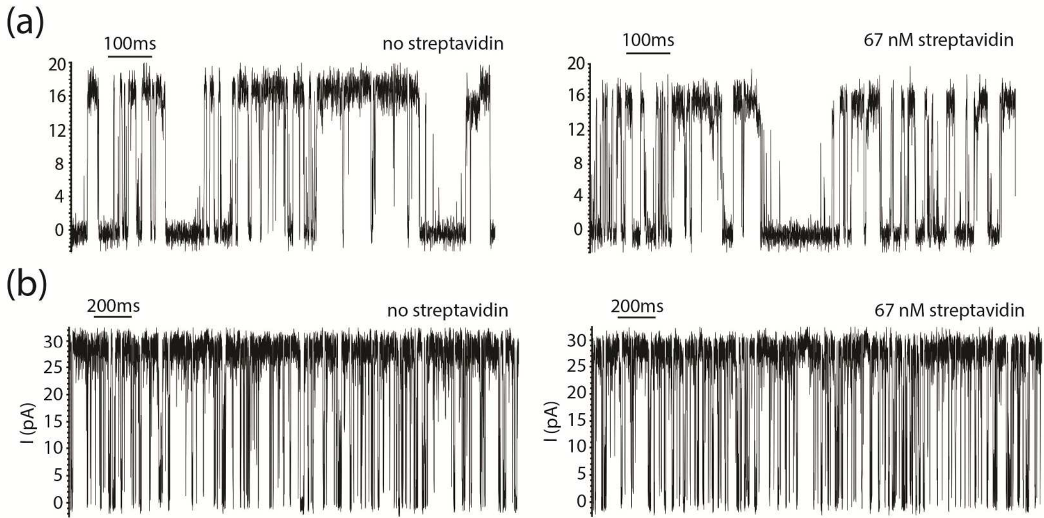

Figure S5: Gating behavior due to streptavidin binding is specific. The addition of streptavidin to OmpG D224C does not change the behavior of the pore at either (a) 150mM KCl, 10mM sodium phosphate pH 5.7 or (b) 300mM KCl, 10mM sodium phosphate pH 6.

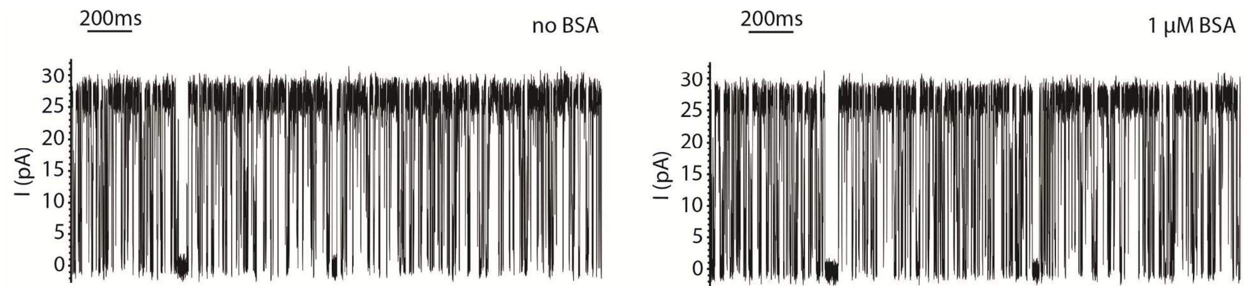

Figure S6: BSA does not elicit change in OmpG behavior. The addition of BSA to OmpG-Peg2-biotin does not change its gating pattern. This condition was performed in 300mM KCl, 10mM sodium phosphate pH 6 buffer.

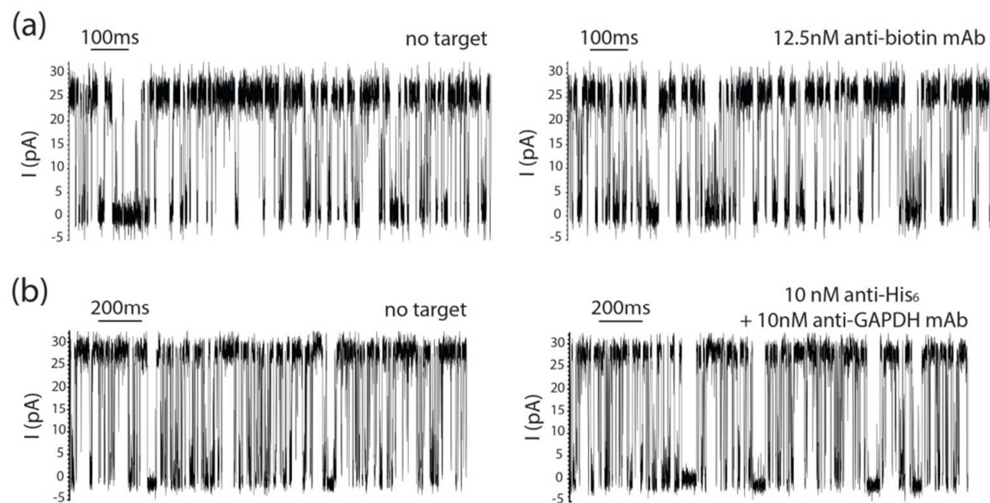

Figure S7: Monoclonal anti-biotin antibody (mAb) binding is specific. (a) Representative trace of OmpG D224C pore in the absence and presence of mAb. (b) Representative trace of OmpG-Peg2-biotin in the absence and presence of control antibodies anti-GAPDH and anti-His_6. Buffer 300mM KCl,10mM sodium phosphate pH 6 was used in both experiments. The applied potential was +50 mV. Neither the addition of mAb to (a) OmpG D224C nor the addition of anti-His, anti-GAPDH mouse mAb to (b) OmpG-Peg2-biotin resulted in a change in a characteristic binding pattern.

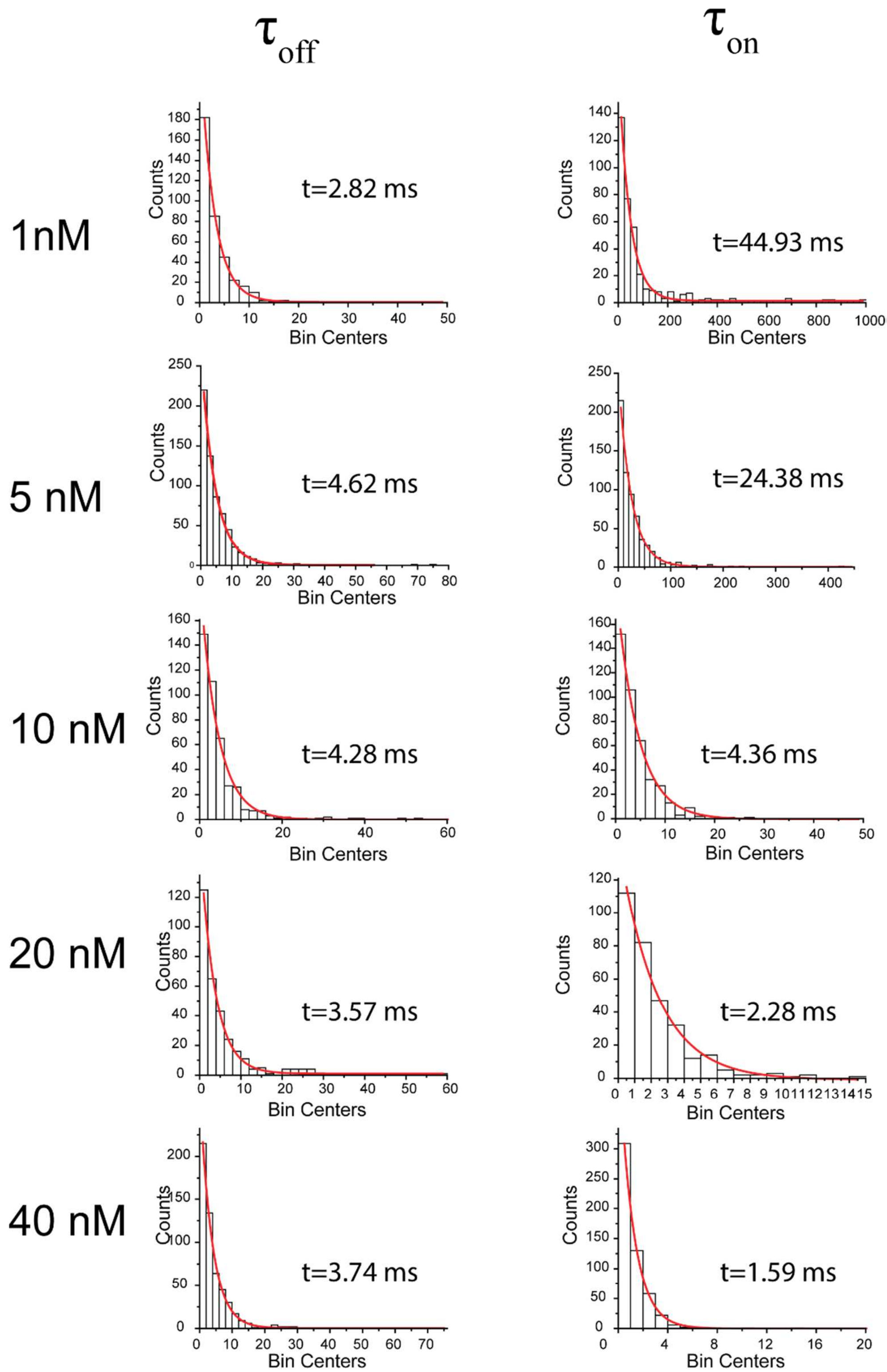

Figure S8: Analysis of kinetic parameters of mAb binding to OmpG-Peg2-biotin. Histograms of the dwell time and the inter-event duration of these events were fitted with single exponential decay function to derive the average time constants.

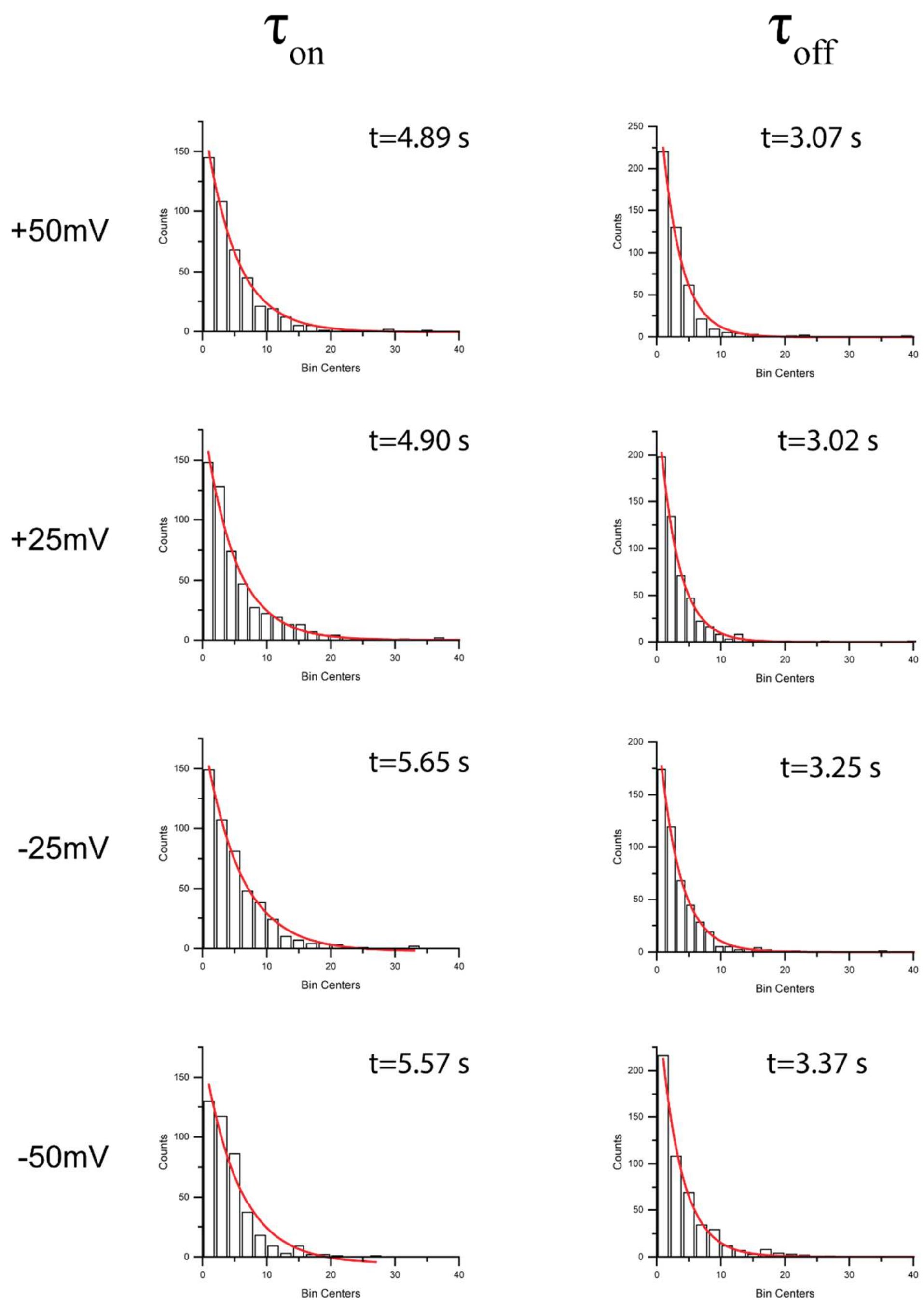

Figure S9: Analysis of kinetic parameters of mAb binding to OmpG-Peg2-biotin at various applied voltages. Histograms of the inter-event duration (τ_{on}) and dwell time (τ_{off}) of mAb binding events were fitted with single exponential decay function to derive the average time constants.

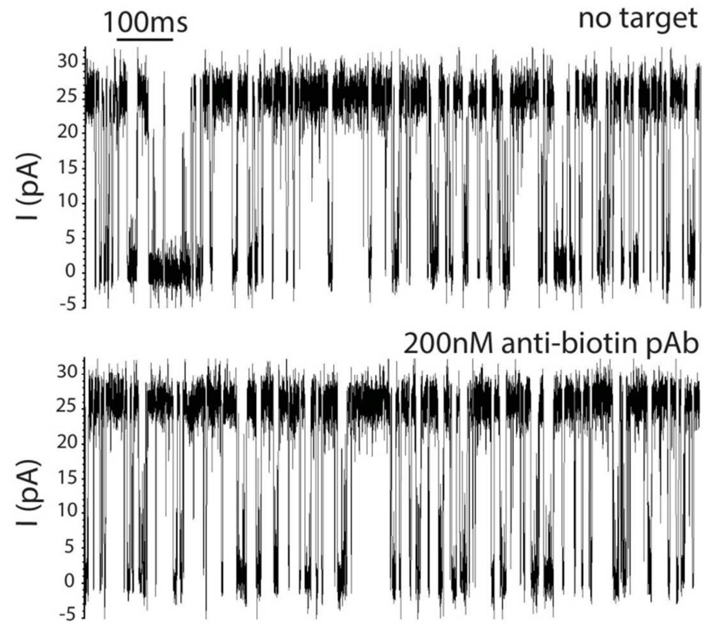

Figure S10: Representative traces of single channel recording of OmpG D224C in the presence of polyclonal anti-biotin antibody (pAb). OmpG D224C pores were recorded for 2 hours after the addition of 200 nM pAb at +50 and -50 mV. Buffer 300mM KCl, 10mM sodium phosphate pH 6.0 was used. No detectable change in the gating pattern of the pore was observed.

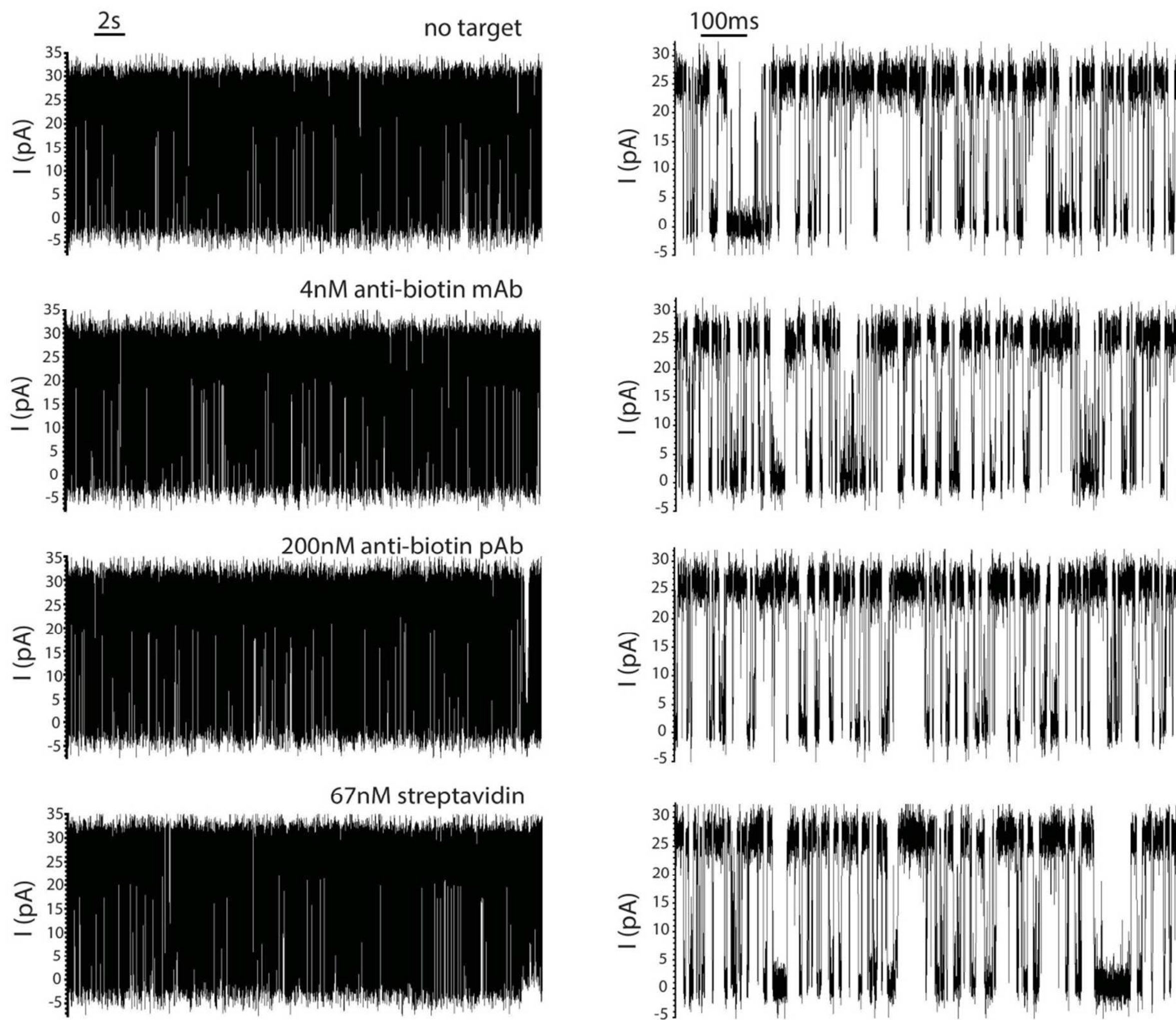

Figure S11: A complex mixture of biotin binding proteins elicits no effect on the gating of OmpGwt. The gating behavior of OmpGwt was recorded after each biotin binding protein was subsequently added to the chamber in which loop 6 was located. As shown in the figure, the gating behavior of OmpGwt never changes even after adding several biotin binding proteins into the same chamber. The behavior was recorded for 1 hour. Buffer 300mM KCl, 10mM sodium phosphate pH 6.0 was used.

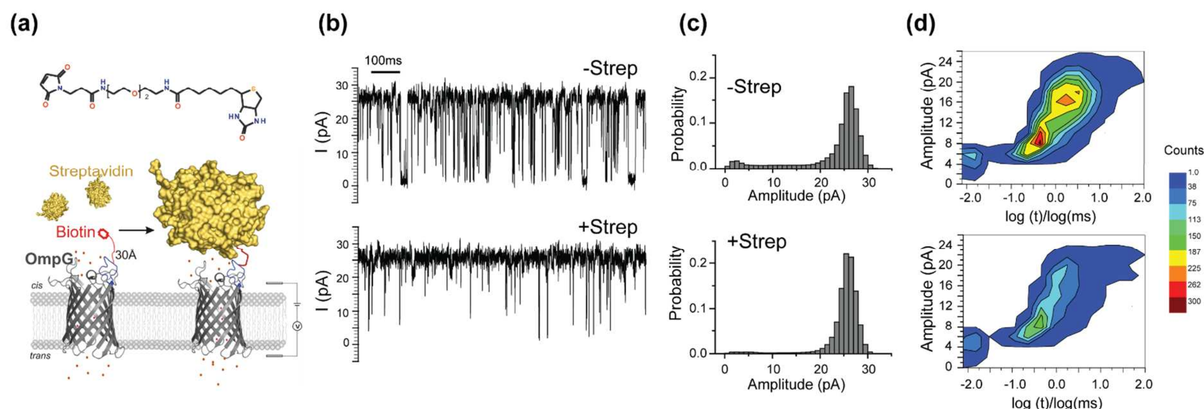

Figure S12: Detection of streptavidin by OmpG-PEG2-biotin pore. (a) Schematic model showing the OmpG nanopore chemically modified with a maleimade-PEG2-biotin. The streptavidin is placed around 30Å away from the OmpG pore in the model of the bound state. (b) Representative single channel recording traces of the OmpG pores before and after the addition of the streptavidin (3 nM). The measurements were performed in buffer 10 mM Na_2PO_4, pH 6.0, 300 mM KCl at +50 mV. (c) All current histogram of the corresponding traces in (b). (d) Two dimensional histogram of the gating events. Gating events collected from 60s recording traces of OmpG pore with and without streptavidin bound were distributed based on their intensity versus duration. The color scale indicates the number of the events.

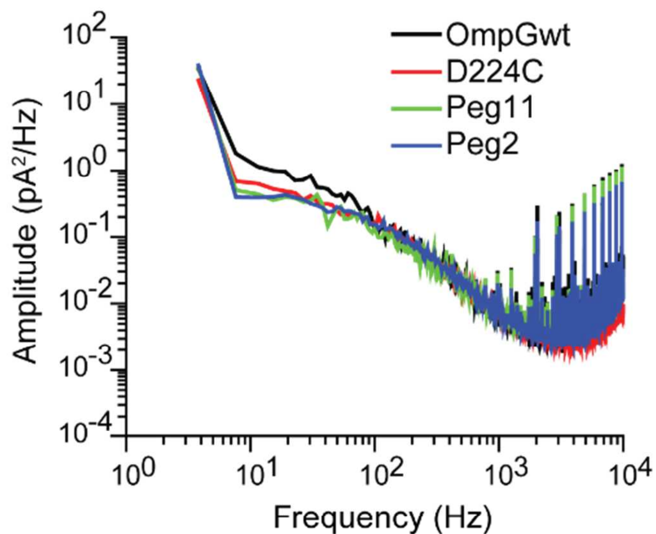

Figure S13: Power spectra of OmpG proteins. Electrical recording traces of OmpGwt, OmpG D224C, OmpG-Peg11-biotin and OmpG-Peg2-biotin were obtained in 10 mM Na_2PO_4, pH 6.0, 300 mM KCl at +50 mV.

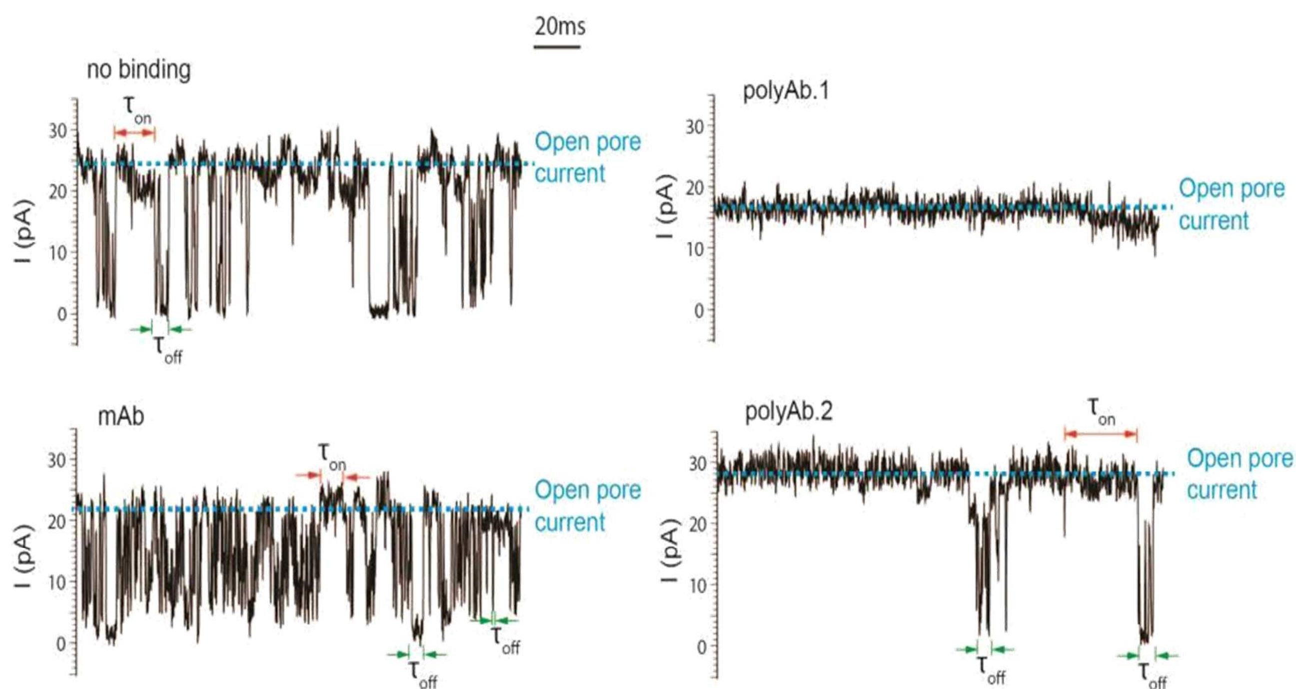

Figure S14: Gating events analysis. The current recording traces were analyzed by Clampfit 10.3 using single channel search to identify gating events which were defined as current blockages larger than 2pA (4% of fully open pore current). The open probability was calculated as the time the pore stays in the open state divided by the recording time. The gating frequency was calculated as the number gating events divided by the recording time. The average inter-event duration (τ_{on}) and event duration (τ_{off}) were obtained by fitting the histogram of these duration values with single exponential function the same as in Fig. S4 and S8. The open pore current is indicated with a blue dashed line.

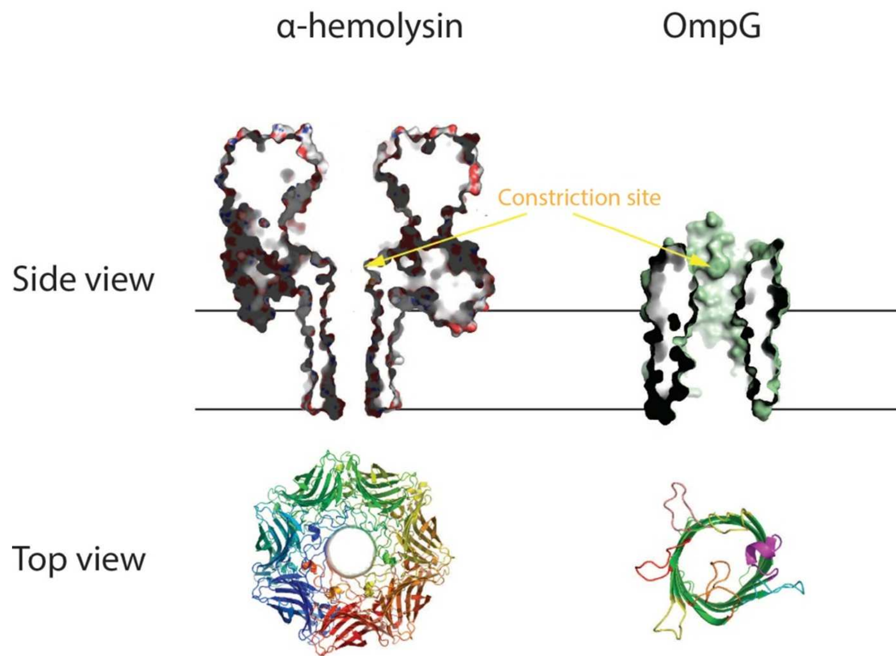

Figure S15: Structures of αHL and OmpG. The side view shows a cross-section of both proteins to reveal the constriction site. For αHL, the constriction site is located in the middle of the pore. In contrast, the narrowest site of the pore is located at the top entrance of OmpG. The top view structures show that αHL has β-strands packing into a highly ordered structure at its entrance while OmpG contains seven flexible loops.

References:

1. Chen, M.; Khalid, S.; Sansom, M. S.; Bayley, H. Outer Membrane Protein G: Engineering a Quiet Pore for Biosensing. *Proc Natl Acad Sci U S A* **2008,** 105, 6272-7
2. Conlan, S.; Zhang, Y.; Cheley, S.; Bayley, H. Biochemical and Biophysical Characterization of Ompg: A Monomeric Porin. *Biochemistry* **2000,** 39, 11845-54.
3. Liang, B.; Tamm, L. K. Structure of Outer Membrane Protein G by Solution Nmr Spectroscopy. *Proc Natl Acad Sci U S A* **2007,** 104, 16140-5.