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Detecting SNPs using a Synthetic Nanopore

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Abstract

We have discovered a voltage threshold for permeation through a synthetic nanopore of *dsDNA* bound to a restriction enzyme that depends on the sequence. Molecular Dynamic simulations reveal that the threshold is associated with a nanoNewton force required to rupture the *DNA*-protein complex. A single mutation in the recognition site for the restriction enzyme, i.e. a single nucleotide polymorphism (SNP), can easily be detected as a change in the threshold voltage. Consequently, by measuring the threshold voltage in a synthetic nanopore, it may be possible to discriminate between two variants of the same gene (alleles) that differ in one base.

Restriction enzymes are used prevalently in recombinant *DNA* technology for cleaving double-helical *DNA* segments containing a specific target sequence. Another use is genotyping. Because the binding to the target is extraordinarily sequence specific, restriction enzymes can be used to identify single nucleotide polymorphisms (SNPs) that occur when variants of the same gene (alleles) differ in one base.

We have discovered a method for discriminating between alleles that uses a synthetic nanopore to measure the binding of a restriction enzyme to *DNA*. When a voltage is applied across a membrane containing a nanopore, polyanionic *DNA* immersed in electrolyte at the cathode diffuses toward the anode and is driven across the membrane by the electric field in the pore. The force due to the field acting on the strand during the translocation impels *DNA* to bend and stretch within the pore.^{1–4} At low fields $\mathcal{E} < 500\text{mV}/10\text{nm}$, double-stranded *DNA* (*dsDNA*) easily permeates pores with diameters $\geq 2.4\text{nm}$ because the double helix ($\sim 2\text{nm}$ diameter) is smaller than the pore.⁵ But the permeability of *DNA* through the pore changes dramatically if it is bound to a restriction enzyme.

To study the binding of a restriction enzyme like *EcoRI* to *DNA*, we introduced an excess of the enzyme in solution with *DNA* without the Mg^{+2} cofactor that is required for cleaving the nucleic acid. Under these conditions, *EcoRI* is thought to bind and diffuse along *DNA*.^{6,7} The diffusive motion along the strand is arrested at the cognate site, i.e. —GAATTC— for *EcoRI*. Bulk measurements of the binding at the cognate site indicate a free energy of formation $\Delta G = -15.2\text{kcal/mol}$.^{6–9} However, the introduction of any mutation among the cognate sites produces a position-dependent reduction in the binding energy that ranges from 6–13kcal/mol.^{8,9} Site-specific *DNA*-binding proteins also have an affinity for nonspecific *DNA*. In contrast with site-specific binding or binding to a non-cognate site with a single nucleotide mutation, a nonspecifically bound complex is not localized to a particular site. For *EcoRI*, sites that differ from the cognate sequence by two or more base-pairs(bps) are considered nonspecific since they are not cleaved and show low binding constants. For a nonspecifically bound *EcoRI*-*DNA* complex, the free energy of formation is reduced to -4.8kcal/mol .^{8,9}

We measured the permeability of *dsDNA* in solution with *EcoRI* through nanometer-diameter pores in nominally 10nm thick Si_3N_4 membranes as a function of the applied voltage. With an enzyme-*DNA* complex trapped in the nanopore, the electric field in the pore pulls on the *DNA* introducing a shear force between the enzyme and the cognate sites in *DNA*. We observe

a threshold in the field required to rupture the bond that depends on the sequence and enzyme. Molecular dynamics (MD) simulations reveal that the rupture occurs within several nanoseconds with a force ~ 1 nN.

METHODS

The fabrication of the synthetic nanopores in inorganic membranes has been described elsewhere.¹⁰ A single nanopore is created in a Si_3N_4 membrane by stimulated decomposition and sputtering using a tightly focused electron beam as small as 0.5 nm (Gaussian width) in diameter in a JEOL 2010F transmission electron microscope (TEM) operating at 200 keV. Figure 1(a) shows transmission electron micrographs (TEM) of three pores in Si_3N_4 membranes nominally 10 nm thick. The pores are $1.8 \times 2.5 \pm 0.2$ nm (blue); $2.4 \times 3.2 \pm 0.2$ nm (green) and 3.6 ± 0.2 nm (red) in diameter—the shot noise observed in the area identified as the pore is indicative of perfect transmission of the electron beam through the membrane. The thickness of similarly processed 10 nm membranes was inferred from TEM to be 12 ± 2 nm, which is consistent with results 12 ± 3 nm obtained from using electron energy loss spectroscopy. The TEM images shown in Figure 1 represent a two-dimensional projection through the membrane. To investigate the three-dimensional structure, we tilted the membrane in the specimen about the pore axis and explored various defocus conditions. We have developed models for the pore geometry. Although it is not unique, a simple model for the three-dimensional structure of the pore consists of two intersecting cones each with a cone angle of $\sim 10^\circ$.¹⁰

To further characterize the pore, we measured the electrolytic current as a function of the electrochemical potential applied across the membrane at $23.5 \pm 1^\circ\text{C}$. All experiments were carried out in a membrane transport bi-cell made from acrylic using silicone O-rings with PDMS coatings to seal the membrane to the acrylic (with $>5.0 \pm 0.2$ T Ω resistance.) The membrane separates two reservoirs: one with a 140 μl volume on the *cis*-side; and the other on the *trans*-side with 15 ml volume. Each reservoir contains a $Ag/AgCl$ electrode which are connected to an Axopatch 200B amplifier used in resistive feedback mode. LabView software is used to record the ionic current and to apply the voltage across the membrane. All data is low-pass filtered at 10 kHz and digitized at 50 kHz for analysis. The current-voltage (*I-V*) characteristics of the pores shown in Figure 1(a) were measured over a range of ± 1 V in microfiltered buffered 100 mM *KCl* (10 mM Tris, pH 8.0) after >55 hours of immersion in de-ionized water. The characteristics are approximately linear—line fits to the data yields the conductances: 89.5 ± 3 pS for the $1.8 \times 2.5 \pm 0.2$ nm (blue) pore, 0.49 ± 0.03 nS for the $2.4 \times 3.2 \pm 0.2$ nm (green) pore, and 1.46 ± 0.03 nS for the 3.6 ± 0.2 nm (red) pore shown in Figure 1(a); and 1.18 ± 0.03 nS for 3.4×4.7 nm pore shown in Figure 3(a), and 2.12 ± 0.03 nS for the 3.4×4.5 nm pore shown in Figure 3(b). It is apparent that the conductance does not scale linearly with the diameter derived from TEM.^{10–12} We have analyzed similar measurements using molecular dynamics to estimate the ion mobility, and then solving coupled Poisson-Nernst-Planck and the Stokes equations self-consistently for the ion concentration, velocity, and electrical potential. We find that the measurements are consistent with the presence of a small (relative to *DNA*) fixed negative charge in the pore, and a reduction of the ion mobility due to the fixed charge and the ion proximity to the pore wall.¹⁰

Following the conductance measurements, we tested the permeability of *dsDNA* in solution with *EcoRI* through nanometer-diameter pores of Figure 1 as a function of the applied voltage. We used two separate *dsDNA* strands:

1. a 105 bp *dsDNA* which is extracted from the pUC19 (NEB part # N3041S) vector with the sequence:

5'-TAAGT TGGGT AACGC CAGGG TTTTC CAGT CACGA CGTTG TAAAA
CGACG GCCAG TGAAT TCGAG CTCGG TACCC GGGA TCCTC TAGAG TCGAC

CTGCA GGCAT-3' where the cognate site for *Eco*RI is highlighted in red (and sites with double substitutions are indicated in green); and

2. a 900bp *DNA* strand, which is extracted from a pTWIN1 vector (NEB part # N6951S) using forward primer GAATGACATCATTGTACACA and reverse primer GCCAACTCAGCTTCCTTTTCG to amplify a sequence that spans both *Eco*RI and *Bam*HI cognates; the length between the start of the *Eco*RI recognition site and start of *Bam*HI site is 815bp. Selected portions of the sequence are given below:

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GAATGACATCATTGTACACAACGGAAGAGCCATGGGCGGCCGCGAATTC
CTCG
AGGGCTCTTCTGCATCACGGGAGATGCACTAGTTGCCCTACCCGAGGG
CGAG
TCGGTACGCATCGCCGACATCGTGCCGGGTGCGCGGCCCAACAGTGAC
AACG
CCATCGACCTGAAAGTCCTTGACCGGCATGGCAATCCCGTGCTCGCCGA
CCGG
CTGTTCCACTCCGGCGAGCATCCGGTGTACACGGTTCGTACGGTCAAG
GTCT
GCGTGTGACGGGCACCGCGAACCACCCGTTGTTGTGTTTGGTCGACGTC
GCCG
GGGTGCCGACCCTGCTGTGGAAGCTGATCGACGAAATCAAGCCGGGCG
ATTAC
GCGGTGATTCAACGCAGCGCATTACGCGTCACTGTGCAGGTTTTGCC
GCCG
AAAACCCGAATTTGCGCCCAACAACCTACACAGTCGGCGTCCCTGGACTG
GTGC
GTTTCTTGGAAGCACACCACCGAGACCCGGACGCCCAAGCTATCGCCG
ACGA
GCTGACCGACGGGCGGTTCTACTACGCGAAAGTCGCCAGTGTCACCGA
CGCC
GGCGTGCAGCCGGTGTATAGCCTTCGTGTGACACGGCAGACCACGCGT
TTAT
CACGAACGGGTTTCGTACGCCACGCTACTGGCCTCACCGGTCTGAACTCA
GGCC
TCACGACAAATCCTGGTGTATCCGCTTGGCAGGTCAACACAGCTTATAC
TGCG GGACAATT
GGTCACATATAACGGCAAGACGTATAAATGTTTGCAGCCCCACACC
TCCTTGGCAGGATGGGAACCATCCAACGTTCTGCCTTGTGGCAGCTTC
AATG ACTGCAGGAAGGGGATCC
GGCTGCTAACAAAGCCCCGAAAGGAAGCTGAGTTGGC
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where the cognate site for *Eco*RI is highlighted in red while the site for *Bam*HI is highlighted in blue.

A membrane including a pore was mounted in a bi-cell, and then either *Eco*RI or *Bam*HI in solution with *DNA* containing the cognate sequence was injected into the electrolyte at the cathode. We introduce either commercial grade *Eco*RI (100,000U/ml stock concentration, 62kD dimer, 2×10^6 U/mg specific activity; (NEB part# R0101Q) or *Bam*HI (100,000units/ml, 52kD dimer, 1.7×10^6 U/mg specific activity; (NEB part# R0136Q) without purification into the *cis*-chamber of the membrane bi-cell. To a solution containing microfiltered 100mM KCl and 10mM Tris, we added HCl to adjust the pH value to 7.9. The 100mM KCl electrolyte concentration was chosen because *Eco*RI has a tendency to aggregate at lower ionic strengths.² We then mixed 400μl of this buffer with 40 μl of *Eco*RI (or *Bam*HI) solution and 2μl of 105bp *DNA* solution (diluted to 88pg/ μl for 105bp, and 10ng/ μl for 900bp), and incubated at

37°C for 10 hours to ensure that the enzyme binds to the *DNA*. None of the buffers contained Mg^{2+} , which is required for catalytic activity of the restriction endonucleases. The solution injected into the *cis*-reservoir of the bi-cell showed pH 7.9.

With a voltage applied across the membrane, we observed an electrolytic current through the pore. Superimposed on the open pore current, I_0 , we detected transients associated with the *EcoRI*-*DNA* complex interacting with the pore, as illustrated in the inset to Figure 1(b). We have previously reported^{11,12} that electric field-driven translocations of unbound *DNA* cause temporary blockades of I_0 , but the duration of the transients was generally short (<100 msec). In contrast, we observe transients >10 sec associated with the *EcoRI*-*DNA*, which is unprecedented for a synthetic pore. The translocation velocity of unbound *DNA* in a synthetic pore is estimated to be >1 bp/nsec at these voltages^{3,4,11,13}—so the duration of these events must represent an extended residence time of the complex over the pore that terminates either through dissociation and subsequent translocation of the *DNA*, or with the complex uneventfully exiting the pore due to thermal agitation. The same inset also shows transient currents greater than the open pore current. Currents > I_0 have been observed before^{11,13,14} and are attributed to a local enrichment of electrolyte near the pore due to counter-ions responding to the molecular charge. The narrow bandwidth (10–100 kHz) of the current amplifier we used precludes the observation of transients shorter than 10–100 μ sec. Hence, to establish unequivocally if *DNA* injected at the cathode permeates the membrane through the pore, the *DNA* near the anode was analyzed using quantitative PCR (*qPCR*).

A *PCR* reagent system obtained from Invitrogen (Carlsbad, CA) was used in conjunction with the sequence-specific primers synthesized by IDT-DNA (Ames, IA) to amplify the *DNA*, which was analyzed by agarose gel electrophoresis. Finally, we used a Qiagen gel extraction kit to purify the *DNA* and remove primers, nucleotides, enzymes, salts, agarose, ethidium bromide, and other impurities. The final solution from the extraction had a concentration of about 44 ng/ μ l for the 105 bp and 10 ng/ μ l for the 900 bp.

We systematically investigated *DNA* permeability through nanopores in the membrane as a function of pore diameter. Corresponding to the gel arrays shown in the supplementary materials, Figure 1(b) represents the results of three *qPCR* analyses showing the number of *DNA* copies permeating as a function of the applied potential. SYBR Green I, a commonly used fluorescent *DNA* binding dye, was included in the *qPCR* reaction mix. SYBR Green I binds all *dsDNA* and the increase in fluorescence of it can be detected after each *PCR* cycle, which is proportional to the amount of *DNA*.

Figure 1(b) illustrates *DNA* permeability through the three pores shown in Figure 1(a) as a function of the voltage. When the diameter is small enough to preclude the translocation of the complex, while at the same time allowing *DNA* to easily permeate the membrane through the pore ($2.4 < d < 4.9$ nm, see supplement), we observe a voltage threshold for permeation of *dsDNA* that depends on the pore. Apparently, *dsDNA* can be forced through the pores only if the voltage is $V > 1.75$ V, which corresponds to an electric field $\mathcal{E} > 2.1 \pm 0.2$ MV/cm that is estimated by assuming a cylindrical pore geometry and a uniform voltage drop. The threshold must depend on either the geometry or charge in the pore¹⁰ since the thresholds for the 1.8×2.5 nm and 3.6 nm pores are comparable, while the threshold for the 2.4×3.2 nm pore is $V > 2.5$ V.

Assuming that the bound protein frustrates *DNA* permeability through the pore, a large force corresponding to ΔG will be required to dissociate the complex and induce the translocation of *DNA*. To investigate the microscopic mechanics of the rupture and assess the forces involved, we used MD to simulate the rupture of the complex under the influence of an applied voltage. A microscopic model of an *EcoRI*-*DNA* complex was built based on a 0.18 nm-resolution

crystal structure (Protein Data Bank code 1CKQ).¹⁵ The first 15 residues of the protein not resolved in the X-ray structure were not modeled. The 12-bp fragment of DNA resolved in the X-ray structure (CGCGAATTCGCG) was extended to 62 bps by adding two pre-equilibrated DNA fragments (20- and 30-bps long) to the ends of the resolved fragment. A Si_3N_4 membrane was built by replicating a unit cell of $\beta\text{-Si}_3\text{N}_4$ crystal to form a 10.3-nm-thick hexagonal prism of a 117.23-nm^2 cross-section. A 2.7-nm-diameter double-cone pore was produced by removing silicon and nitrogen atoms. The surface of Si_3N_4 was modified to produce a uniform surface charge of 100 mC/m^2 (about 0.6e/nm^2).¹⁰ The Si_3N_4 structure was merged with the *EcoRI*-DNA complex, having the DNA molecule oriented perpendicular to the membrane and its longer tail inserted by 2.6 nm (system I) or 8.6 nm (system II) into the pore. The resulting structures were then solvated in a volume of pre-equilibrated TIP3P water molecules. Potassium and chlorine ions were added, corresponding to a concentration of 0.1M. The final systems included about 415,000 atoms. To build a system for SMD simulations (system III), the 12-bp DNA fragment resolved in the X-ray structure was extended to 34 bps by adding 11 bps to either end of the resolved fragment. The *EcoRI*-DNA complex was then solvated in 100mM aqueous solution of *KCl*. The final system measured $12\times 12\times 18\text{ nm}^3$ and included about 248,000 atoms.

Each system constructed underwent 2000 steps of minimization followed by gradual heating from 0° to 295°K in 4ps. Systems I and II were equilibrated in the NpT ensemble (i.e., with particle number N , pressure p , and temperature T fixed) for 1 ns each, having all backbone atoms of *EcoRI*-DNA restrained. System III underwent 8.4ns equilibration. For the first 2.2ns all backbone atoms of *EcoRI*-DNA were restrained, while in the rest 6.2ns only α -carbons of the protein were subject to weak harmonic restraints ($k_{\text{spring}} = 10\text{kcal}/(\text{mol nm}^2)$).

Our MD simulations were performed using the program NAMD2,¹⁶ periodic boundary conditions, particle mesh Ewald full electrostatics and multiple time stepping,¹⁷ the CHARMM27¹⁸ force field describing DNA, protein, water, and ions, and a custom force-field describing the Si_3N_4 membrane. The integration time step chosen was 1fs. The equilibration in the NpT ensemble was performed using the Nose'-Hoover Langevin piston pressure control.¹⁹ Van der Waals energies were calculated using a smooth (1–1.2nm) cutoff. During the equilibration, the temperature was kept at 295°K by applying Langevin forces¹⁹ to all heavy atoms; the Langevin damping constant was set to 5ps^{-1} , the backbone atoms of the *EcoRI*-DNA complex were restrained applying harmonic forces with the spring constant of $100\text{kcal}/(\text{mol nm}^2)$, unless stated otherwise. Other simulation conditions and protocols are described in Aksimentiev et al.¹³ To simulate an electric field-driven rupture of an *EcoRI*-DNA complex, a uniform electric field was applied to all atoms in systems I and II. Induced by this field rearrangement of ions and water focused the electric field to the vicinity of the membrane and produced a transmembrane bias equivalent to $-\mathcal{E}L$, where L is the system size in the direction of the applied field (in our case, normal to the Si_3N_4 membrane).¹³ All simulations under external electric field were carried out in the *NVT* (constant N , T , and volume V) ensemble. The damping constant of the Langevin thermostat was set to 1ps^{-1} .

All SMD simulations were carried out on system III according to standard protocols.^{20–22} A harmonic restraint, applied to the center of mass of a terminal 10-bp DNA fragment ($k_{\text{spring}} = 50,000\text{ kcal}/(\text{mol nm}^2)$), was moved with a constant velocity away from *EcoRI* in the direction of the DNA end. Each α -carbon of *EcoRI* was harmonically restrained using a weak spring ($k_{\text{spring}} = 10\text{kcal}/(\text{mol nm}^2)$).

The displacement of the 6bp cognate fragment from its equilibrium position in the *EcoRI*-DNA complex was calculated using the following algorithm. First, we identified all atom pairs (ij) such that, in each pair, atom i belonged to a cognate nucleotide and atom j belonged to the protein, and their distance $\langle d_{ij} \rangle$, averaged over the 6.2-ns equilibration of system III, did not

exceed 0.4nm. This procedure yielded 80 such atom pairs, 40 for each DNA strand. Next, for each pair, the distance d_{ij} was computed along the simulated trajectory. Finally, the displacement of the cognate sequence from its binding pocket in *EcoRI* was determined as an average change of the pair distance, i.e., $R = \sum_{ij} (d_{ij} - \langle d_{ij} \rangle) / n$, where $n = 80$.

The effective force between *DNA* and *EcoRI* in the nanopore simulations was determined by computing the total nonbonded force on the 12bp DNA fragment adjacent to *EcoRI*. Using a custom version of the NAMD2 program, a sum of *all* van der Waals, short-range electrostatic (Coulomb), long-range electrostatic (PME), and the external electric field forces on the 12bp DNA fragment were computed for every time step (1fs) of a 25-ps post-processing MD run, restarted from a conformation of interest. The instantaneous forces were averaged during the post-processing run; the standard error of the mean was determined using 20fs mean values of the force. All analysis and visualization were done using VMD.²³

In the 9.6ns MD simulation shown in Figure 2(a), 4V caused the complex to dissociate and induced the translocation of *DNA*. On the other hand, no rupture was observed in a 38ns simulation carried out at 2V, as illustrated in Figure 2(b). However, by restarting this simulation near the midpoint (16.8ns) with both 3V and 4V, we were able to dissociate the complex. (The rupture occurred at 4V sooner than at 3V.) Figure 2(c) shows the force on the complex associated with the trajectories in (b). At 2V, the force fluctuates around 0.7nN, while at 3V and 4V, coinciding with the rupture of the complex, the force >1.2 nN. In supplementary materials we show that the rupture force, f^* , depends logarithmically on the loading rate, r : i.e. $f^* = 1.710 + 0.364 \ln(r)$, and we conclude that for loading rates from 0.1–1N/s, the dissociation of the complex occurs in <10 ns with a force ~ 1 nN. We find that rupture occurs when a ~ 1 nN force stretches the bonds associated with the first two base-pairs from the 5' end in the 6bp cognate site and an adjacent flanking nucleotide by more than ~ 0.15 – 0.2 nm, such that $\Delta G = f^* a \sim 13$ – 17 kcal/mol.

We also find that threshold voltage depends on the *DNA* sequence. Figure 3(a) represents three *qPCR* analyses of the permeation of *DNA* variants bound to *EcoRI*. The pore used to test the permeability (see inset) is $2.7 \times 3.4 \pm 0.2$ nm and the corresponding threshold for disrupting the bond between the *EcoRI* and the cognate sites on the *DNA* is $V = 1.75$ V. However, the threshold voltage collapses to $V = 0.5$ V with a single base mutation from G to T on the first cognate site from the 5' end, in correspondence with the change in the bulk dissociation energy for the same mutation. An estimate for the free energy of formation for the first-base substitution with —TAATTC— is -8.6 kcal/mol (with a different flanking sequence and strand length).^{6,8} The ratio of the bulk dissociation energies, $8.6/15.2$, is comparable to the ratio of the thresholds, $0.5/1.75$. For a two-base substitution among the cognate sites (the red trace in Figure 3(a)), the *EcoRI*-*DNA* bond is nonspecific and correspondingly, we observe no threshold (i.e. $V < 200$ mV). From the ratio of the nonspecific bulk binding energy to the bulk dissociation energy for the cognate sites, we expect a threshold $V < 500$ mV.⁸

Restriction enzymes specific to hundreds of distinct sequences have been identified, and corresponding cognate sites can be found in almost any gene. Consequently, measurement of the threshold voltage might ultimately be used for genotyping *DNA* without sequencing it completely. However, the efficacy of using different enzymes will depend on the ability to discriminate between the threshold voltages. As a test we measured the threshold for permeation through a nanopore of a longer strand of *dsDNA* (900bp) that incorporates cognate sites for both *EcoRI* (GAATTC) as well as *BamHI* (GGATCC). Since the respective cognate sites differ by two base-pairs, we expect only nonspecific binding of one enzyme to the other site. Three *qPCR* analyses are shown in Figure 3(b): the first measures the threshold associated with the *EcoRI*-*DNA*; the second measures the threshold for *BamHI*-*DNA*; and the third measures *EcoRI*-*DNA* again to establish reproducibility. The inset shows the nanopore used

for this test ($3.4 \times 4.5 \pm 0.2 \text{ nm}$)—it has a threshold voltage of $V = 1.80 \text{ V}$ for rupturing the *EcoRI*-DNA bond. Notice that the threshold for rupturing *BamHI*-DNA complex is 220 mV lower, i.e. $V = 1.58 \text{ V}$, and the ratio of the thresholds ($1.58/1.80$) corresponds to the ratio of the dissociation energies ($13.2/15.2$). The 220 mV difference is easily resolved since a second measurement of *EcoRI*-DNA after measuring the *BamHI*-DNA threshold reports the original threshold.

In summary, these results demonstrate a voltage threshold for permeation through a synthetic nanopore of *dsDNA* bound to a restriction enzyme that depends on the sequence and on the enzyme—a SNP in the recognition site can easily be detected. Since SNPs are used to predict susceptibility to various therapeutic regimes, and identify viral and bacterial infections, this simple detection strategy prospectively offers enough sensitivity to make single point-of-care diagnostics a reality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The threshold voltage depends on pore diameter

(a) TEM micrographs taken at a tilt angle of 0° of three nanopores, 1.8×2.5nm (blue), 2.4×3.2nm (green) and 3.6nm (red) diameter, sputtered with a tightly focused high energy electron beam in nominally 10nm thick Si_3N_4 membranes. The shot noise observed in the area identified as the pore is indicative of perfect transmission of the electron beam through the membrane. 105bp permeates these pores only for $V > 1.75\text{V}$ (b) *qPCR* results indicating the number of 105bp DNA copies from *EcoRI*-DNA complex that permeates the membrane through the pores in (a) found at the positive (+) electrodes in a bi-cell as a function of the voltage across the membrane. The inset shows the electrolytic current through the 2.4×3.2nm pore as a function of time with the membrane voltage as a parameter. Long duration transients >10sec and transient currents greater than the open pore current $>I_0$ are observed.

Figure 2. MD simulations of EcoRI-DNA complex encountering synthetic nanopore
 (a) Snapshots illustrating simulated dissociation of an EcoRI-DNA complex due to the electric field in a nanopore. The Si₃N₄ membrane is shown in grey, two strands of DNA in blue and green, a protein dimer in pink and purple, water and ions are not shown. Time elapsed from the moment a 4V transmembrane bias was applied is indicated. (b) The protein-DNA displacement versus time in four MD simulations carried out at different transmembrane biases. The displacement is defined as the change of the contact distance between the cognate nucleotides and the binding site of *EcoRI*. The insets illustrate the starting (0 ns) and the midway (16.8 ns) conformations of the EcoRI-DNA complex in a simulation carried out at a 2V bias. Two additional simulations, at 3 and 4V, were initiated starting from the midway conformation

of the 2V simulation. Another simulation at 4V (denoted as 4V*) began from a different conformation and is illustrated in (a). (c) The effective force of the DNA-protein interaction, defined as a sum of all nonbonded forces acting at the 12-bp DNA fragment adjacent to the protein. The effective force plots correspond to the displacement plots shown in (b), color-coded accordingly. Each data point was obtained by averaging the instantaneous force over 8-25 ps; the error bars are the standard errors of the mean.

Figure 3. The threshold voltage depends on sequence and the enzyme

(a) *qPCR* results indicating that the number of 105bp copies from *EcoRI*-DNA that permeates a pore with a threshold voltage that depends on the DNA sequence. The inset shows a TEM micrograph of the a 2.7×3.4nm pore. The cognate sequence GAATTC (blue) has a threshold voltage of about 1.5V, but in contrast, with a substitution for the first-base, TAATTC (green) corresponding to star activity has a threshold of only 0.5V. There is no threshold apparent for the two-base substitution GGATCC (red), which is associated with non-specific binding. (b) *qPCR* results indicating that the number of 900bp DNA copies from either *EcoRI*-DNA or *BamHI*-DNA that permeates a pore depends on the enzyme. The inset shows a TEM micrograph of the 3.4×4.5nm pore. The threshold for the *EcoRI*- and *BamHI* rupture scales with the bulk dissociation energies.