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Published in:
Acs Nano

DOI:
[10.1021/acsnano.6b03159](https://doi.org/10.1021/acsnano.6b03159)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Franceschini, L., Brouns, T., Willems, K., Carlon, E., & Maglia, G. (2016). DNA Translocation through Nanopores at Physiological Ionic Strengths Requires Precise Nanoscale Engineering. *Acs Nano*, 10(9), 8394-8402. <https://doi.org/10.1021/acsnano.6b03159>

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DNA Translocation through Nanopores at Physiological Ionic Strengths Requires Precise Nanoscale Engineering

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Supporting Information

ABSTRACT: Many important processes in biology involve the translocation of a biopolymer through a nanometer-scale pore. Moreover, the electrophoretic transport of DNA across nanopores is under intense investigation for single-molecule DNA sequencing and analysis. Here, we show that the precise patterning of the ClyA biological nanopore with positive charges is crucial to observe the electrophoretic translocation of DNA at physiological ionic strength. Surprisingly, the strongly electronegative 3.3 nm internal constriction of the nanopore did not require modifications. Further, DNA translocation could only be observed from the wide entry of the nanopore. Our results suggest that the engineered positive charges are important to align the DNA in order to overcome the entropic and electrostatic barriers for DNA translocation through the narrow constriction. Finally, the dependencies of nucleic acid translocations on the Debye length of the solution are consistent with a physical model where the capture of double-stranded DNA is diffusion-limited while the capture of single-stranded DNA is reaction-limited.

KEYWORDS: mechanism, dsDNA, ssDNA, translocation, transport, barrier

The translocation of DNA across specialized proteins is an important biological process. Bacteria use secretin channels in their pili to uptake or transfer DNA, while viruses such as phages use their portal proteins to either pack genomic DNA into the viral capsid or to eject it into target cells. In the three domains of life, β -clamp proteins form a toroid structure that encircles and slides along DNA to aid the function of DNA processing enzymes. Portal proteins in all known bacteriophages (λ , P22, T4, T3, T7, SPP1, P2, and ϕ 29)¹ and many components of secretin channels² form dodecameric rings. The available crystal structures of portal proteins (ϕ 29,³ SPP1,⁴ P22,⁵ and T4⁶) have revealed the presence of a central channel through which double-stranded DNA (dsDNA) must both enter during packaging and exit during injection. While the interior channel lining is mostly negatively charged, a few positively charged amino acids, arranged as rings along the length of the pore, can also be found. A similar arrangement of negative and positive charges can also be observed on β -clamp DNA sliding proteins.⁷ It has been proposed that the net internal negative surface charge is important to allow the smooth sliding of the opposing negatively charged DNA as it passes through the connector^{3,8}

or β -clamp⁷ channels and that the positively charged rings play a direct role in packaging by interacting with the negatively charged phosphate groups in the backbone of the translocating DNA.^{3,9}

The ionic current flowing through biological nanopores reconstituted into lipid membranes has been used to identify small molecules¹⁰ or folded proteins¹¹ and to monitor chemical¹² or enzymatic¹³ reactions at the single-molecule level. The electrophoretic translocation of DNA across nanopores holds great promise for practical applications such as DNA sequencing^{14–22} and biomarker recognition.²³ Although not a membrane protein *per se*, ϕ 29 portal protein was found to insert into black lipid bilayers.^{24–27} Such nanopores electrophoretically translocated dsDNA at 1.0/0.5 M NaCl. Despite this achievement, however, the exact hydrophobic modifications of the nanopore that allowed membrane insertion were not disclosed,²⁷ and the ϕ 29

Received: May 12, 2016

Accepted: August 11, 2016

Published: August 11, 2016

Figure 1. Engineering the ClyA nanopore for DNA translocation. (A) Cross sections of the ClyA-AS (left) and ClyA-RR (right) nanopores imbedded into a lipid bilayer (blue) constructed by homology modeling from the *Escherichia coli* ClyA structure using VMD⁴⁹ and NAMD⁵⁰ (PDB: 2WCD, 90% sequence identity). The inner pore lumen is shown using the solvent-accessible surface area as calculated by PyMOL (version 1.8 Schrödinger, LLC) and colored according to the electrostatic potential in a 150 mM NaCl solution as calculated by APBS.^{51–53} Red and blue regions correspond to negative and positive potentials (range -2 to $+2$ $k_B T/e$ or -51.4 to $+51.4$ mV), respectively. (B) Electrostatic potential at the center of ClyA-AS (black line) and ClyA-RR (red line) nanopores at 150 mM NaCl concentration. (C) ssDNA (1 μ M, 1.0 μ M) and (D) dsDNA (1, 170 nM) translocation through ClyA-RR nanopores at physiological ionic strength at +70 mV. DNA was added in the *cis* compartment. The bottom current traces show a magnification of the DNA translocation events. The current signal was acquired at 10 kHz applying a 2 kHz low-pass Bessel filter. The buffer contained 150 mM NaCl and 15 mM Tris-HCl (pH 7.5) at 22 °C.

nanopores occasionally released from the lipid membranes,²⁷ posing limitations in practical applications. dsDNA has been shown to translocate through artificial nanopores fabricated in solid-state membranes,^{28–31} which, with the exception of atom-thin materials such as graphene^{32–34} or a bilayer of molybdenum disulfide,^{35–37} mostly have a negative internal surface charge.³¹ In such nanopores, with radii comparable to the Debye length of the solution, the diffusive layer on the inner nanopore walls overlaps, resulting in a large electrostatic barrier for the entry of DNA into the nanopore. As a consequence, the translocation of DNA across solid-state nanopores at physiological ionic strength has only been observed using large nanopores (10 nm)³⁸ or using small nanopores (~ 3.5 nm) in 340 mM salt³⁹ or under asymmetric salt concentrations.⁴⁰

Recently, we have described the ClyA nanopore, a dodecameric protein with an internal constriction of ~ 3.3 nm (Figure 1A), as an effective tool to investigate folded proteins.^{11,41–44} Although dsDNA translocation across the nanopore was observed at 2.5 M NaCl solutions,⁴⁵ the strong negative interior of the nanopore (Figure 1A) prevented DNA translocation at lower ionic strengths. In this work, we engineered the ClyA nanopore, enabling it to translocate DNA at physiological ionic strengths. This is useful in many applications where electrostatic interactions between molecules and DNA are important, for example, in DNA sequencing or mapping where enzymes are used to control the translocation of DNA across the nanopore or to study DNA–protein interactions. We could observe the translocation of DNA after two rings of positive charges were introduced at the wider *cis* side of the nanopore, while modification of the more constricted *trans* entry of the nanopore did not improve the

efficiency of DNA translocation. Interestingly, many proteins that slide on DNA display a surface charge similar to the engineered ClyA nanopores,³ suggesting that the alternation of positive and negative charges might provide a general mechanism for improving the translocation of DNA across nanoscale structures.

RESULTS

Engineering ClyA Nanopores To Capture DNA. In this work, we used ClyA-AS (Figure 1A), an engineered version of cytolysin A from *Salmonella typhi* selected for its favorable properties in planar lipid bilayers¹¹ and in which the translocation of ssDNA or dsDNA is only observed above 2.0 M NaCl ionic strengths.⁴⁵ Most likely, at low ionic strengths the strong negative electrostatic potential inside the nanopore (Figure 1B) prevents DNA entry and translocation, while at high ionic strengths, the charges of the nanopore surface are effectively screened. To induce the capture of DNA by the nanopore at physiological ionic strengths, inspired by previous work with the α HL^{46–48} and MspA¹⁹ nanopores, we modified the internal charges of the ClyA-AS nanopore (Table S1 and Figure 1A). We first introduced a single ring of positive charges in the form of arginine residues at the *cis* entry of ClyA-AS (S110R, ClyA-R, Figure 1A), and then we proceeded to modify three sections of the nanopore: the *cis* entry, the midsection, and the *trans* constriction (Figure 1A). The substitution of neutral residues with positive charged residues at the *cis* opening of ClyA-R showed no DNA translocation in 150 mM NaCl (Table S1). Arginine rings in the midsection of the ClyA-R nanopore induced ssDNA (Figure 1C) and dsDNA (Figure 1D) translocation when the negatively charged glutamate residues at position 64 were replaced by arginine (D64R, ClyA-

Figure 2. DNA rotaxane formation in 150 mM NaCl solutions at +50 mV. (A) dsDNA rotaxane was formed by adding 1a/1c (1.0 μ M, black lines) and 1d (1.0 μ M, orange line) to the *cis* and *trans* compartments, respectively. NeutrAvidin (NA, 0.3 μ M, tetramer, red) was also added in both solutions. (B) ssDNA/dsDNA hybrid rotaxane was formed by addition of a 5'-biotinylated ssDNA thread 2a (1.0 μ M, black line) to the *cis* compartment and a 5'-biotinylated ssDNA molecule complementary to the 3' end of 2a (2b, 1.0 μ M, orange line) to the *trans* compartment. NA (0.3 μ M, tetramer) was present on both sides. The graphs on the right-hand side of the current traces show the voltage relationship (I - V curve) for ClyA-RR (blue line) and ClyA-RR in a rotaxane configuration (red line). Experiments were carried out in a buffer containing 150 mM NaCl and 15 mM Tris-HCl (pH 7.5) at 22 $^{\circ}$ C. The DNA sequences are shown in Table S3.

RR) but not when a neutral side chain at a nearby position was substituted with arginine (Q56R). The exchange of negatively charged residues in the transmembrane region with a neutral (ClyA-R-E11S) or a positively charged residue (ClyA-R-Q8K) induced no DNA translocation events in 150 mM NaCl solutions (Table S1 and Figure S1). Surprisingly, the substitution of neutral residues with positively charged residues in both the midsection and *trans* entry of ClyA-R (ClyA-R-Q56R-Q8K) also did not induce DNA translocation events (Figure S1). Despite the observation that only ClyA-RR allowed DNA translocation, ClyA-RR, ClyA-R, and ClyA-AS showed the same ion selectivity ($P_{\text{Na}^+}/P_{\text{Cl}^-} = 1.9 \pm 0.7$, 2.0 ± 1.6 , and 1.9 ± 0.9 , respectively, Table S2), indicating that DNA translocation was not induced by an enhanced electro-osmotic flow through the nanopore.

In order to obtain a greater insight into the changes of the electrostatic potential caused by the two additional arginine rings, full-atom homology models of ClyA-AS and ClyA-RR were constructed using VMD⁴⁹ and NAMD⁵⁰ starting from the *E. coli* ClyA crystal structure. The adaptive Poisson-Boltzmann solver (APBS)^{51–53} was employed to calculate the electrical potential distribution of both pores in 150 mM NaCl (Figure 1B). In ClyA-AS, the potential at the center of the pore was found to be increasingly negative moving from the *cis* entry, through the midsection, and to the *trans* entry (averaging -2.6 , -4.8 , and -15.2 mV, respectively). In the case of ClyA-RR, a rise in the potential could be observed at both the *cis* entry and the midsection of the pore (averaging -0.3 and -1.1 mV, respectively). The potential in the *trans* constriction appeared to decrease further to an average of -17.3 mV. Notice that these values are calculated when no external bias is applied.

DNA Rotaxane as Proof of DNA Translocation. A rotaxane is a dumbbell-shaped molecule formed by a macrocycle that encircles a thread locked by two stoppers. Here, we formed two nanopore/DNA rotaxanes in 150 mM NaCl solutions to prove the translocation of ssDNA and dsDNA through the nanopore. The first rotaxane was formed using a 3'-biotinylated 59 bp dsDNA molecule extended with a 31 bases overhang at the 5' of the biotinylated strand as the initial thread (1a/1c, Table S3). The second rotaxane was formed using a 100mer 5'-biotinylated ssDNA molecule (2a, Table S3) added to the *cis* compartment as the initial thread. The rotaxanes were locked by adding on the opposite side of the nanopore another biotinylated ssDNA molecule, 1d (31mer, 3'-biotinylated) or 2b (50mer, 5'-biotinylated), designed to hybridize with the overhangs of 1a/1c or 2a, respectively. Both *cis* and *trans* solutions contained NeutrAvidin (NA, 0.3 μ M), which in complex with biotin prevented the full translocation of the DNA strands across the nanopore.

At negative applied potentials, the ionic current in the rotaxane configuration was higher than the open pore current ($I_{\text{RES-50}} = 1.16 \pm 0.03$ and 1.11 ± 0.06 for ssDNA and dsDNA/ssDNA threads, respectively, $N = 3$ independent nanopore experiments, Figure 2). This effect was previously observed for the translocation of DNA through 10 nm solid-state nanopores at low ionic strengths³⁸ and was explained by the accumulation of counterions inside the DNA blocked pore.³⁸ Intriguingly, however, at positive applied potentials, the open pore current was higher than the blocked current (Figure 1C,D and Figure 2), suggesting that the accumulation of counterions on the DNA differs at the *cis* and *trans* sides of the nanopore.

DNA Capture and Threading Depends on the Ionic Strength of the Solution. The capture rate k_{on} , which is the

Figure 3. Ionic strength dependence of DNA translocation and threading under +70 mV. (A,B) Debye length dependence of the frequency of dsDNA (A) and ssDNA (B) translocation per 1 μM DNA. The dotted line in (A) depicts the theoretical prediction of translocation frequencies for a diffusion-limited process. The blue line in (B) is an exponential regression indicating a barrier-limited process. (C,D) Debye length dependency of residual current of dsDNA (C) and ssDNA (D). The translocation of free DNA is shown as blue circles, the signal of the NeutrAvidin/DNA complex is shown as red circles. The lines represent linear regressions. dsDNA (1, 170 nM) and ssDNA (1a, 1 μM) were added to the *cis* side. The electrical recordings were carried out in buffers containing the given NaCl concentrations and 15 mM Tris-HCl (pH 7.5) at 22 °C.

inverse of the interevent time τ_{on} (Table S4, +70 mV, 1 μM DNA), increased with the Debye length of the solution (λ_D) for both ssDNA and dsDNA (Figure 3). However, while the dsDNA capture rate increased linearly with λ_D (Figure 3A), ssDNA capture rate increased exponentially with λ_D (Figure 3B). This suggests, therefore, different capture mechanisms for dsDNA and ssDNA (see Discussion). As reported before with solid-state nanopores, the residual current of DNA blocked nanopores increased as the ionic strength of the solution decreased³⁸ (e.g., from 0.78 ± 0.09 in 2.5 M NaCl to 0.92 ± 0.02 in 150 mM NaCl for dsDNA). Interestingly, we found a linear relationship between the I_{RES} of the DNA blockades and the Debye length of the solution (Figure 3C,D). For dsDNA in complex with NeutrAvidin, the residual current was $\sim 10\%$ lower than during free DNA translocation, suggesting that NeutrAvidin contributed to the overall ionic current of the blockade, most likely by interacting with the nanopore lumen. At 150 mM NaCl, ssDNA molecules in complex with NeutrAvidin showed permanent blockades to ClyA-RR nanopores, while at 1 M NaCl or higher, the blockades were transient (Figure S2). A likely explanation for these data is that at high ionic strengths ssDNA enters and escapes the pore from the *cis* side. Confirming this interpretation, at ionic strengths ≥ 1 M, the I_{RES} values for ssDNA and ssDNA/NeutrAvidin were the same (Figure 3D and Figure S2), suggesting that under these conditions ssDNA might not fully thread the nanopore, preventing NeutrAvidin from interacting with the lumen of ClyA.

Unidirectional Entry of DNA into ClyA Nanopores. In 150 mM NaCl solutions, the addition of 1 μM of ssDNA or dsDNA to the *cis* and *trans* compartments of ClyA-RR induced DNA blockades only under positive applied potentials (higher than $\sim +50$ mV, Figure 4A), indicating that (1) DNA cannot enter the nanopore from the *trans* entry of ClyA and (2) there is a voltage threshold for the translocation of ssDNA from the *cis* side of the nanopore. The entry (Figure 4B) and translocation (Figure S3) of DNA from the *trans* compartment, however, was observed in 1 M NaCl solutions. In order to induce the entry of DNA from the *trans* compartment under physiological ionic strengths, we remodeled the charges of the transmembrane region of ClyA-RR nanopores (Figure 1 and Table S1). Although DNA-induced current blockades to the modified ClyA-RR pores were occasionally observed upon the addition of 1 μM of dsDNA 1 to the *trans* chamber under negatively applied potentials (Table S1 and Figure S4), DNA rotaxanes could not be formed, suggesting that dsDNA might not fully translocate through ClyA-RR nanopores under these conditions.

DISCUSSION

Precise Nanopore Engineering Is Required for DNA Translocation at Physiological Ionic Strength. In this work, we engineered ClyA nanopores to allow the electrophoretic translocation of DNA at physiological ionic strengths. DNA translocation was observed when two sets of positive

Figure 4. Unidirectional DNA translocation through ClyA-RR nanopores. (A) In 150 mM NaCl solutions, the addition of $3.0 \mu\text{M}$ of dsDNA 1 to both the *cis* and *trans* sides of a ClyA-RR nanopores induced transient current blockades (red lines) only under positive applied potentials. (B) In 1.0 M NaCl solutions, the DNA blockades were observed under both applied potentials. DNA-induced blockades are shown in red. The applied potential was automatically changed from +70 to -70 mV (A) or from +100 to -100 mV (B) in 21 s. The electrical recordings were carried out in buffer solutions containing the given NaCl concentrations and 15 mM Tris-HCl (pH 7.5) at 22°C . Data were recorded by applying a 2 kHz (A) and 10 kHz (B) low-pass Bessel filter and using a 10 kHz (A) and 50 kHz (B) sampling rate.

charges were introduced at the entry and in the midsection of the ClyA nanopore (Figure 1A). Surprisingly, the *trans* entry of the nanopore, which provides the highest entropic and electrostatic barriers for DNA translocation (Figure 1A,B), did not require modification. Further, despite extensive remodeling to the charge of the *trans* entry of ClyA (Table S1), DNA translocation could be observed only when initiated from the wider *cis* entry of the nanopore. Moreover, the frequency of dsDNA translocation through ClyA-RR nanopores increased with the Debye length of the solution (Figure 3A), confirming that the favorable electrostatic interactions of dsDNA with the *cis* entry of ClyA-RR dominate over the unfavorable electrostatic repulsion of the DNA with the nanopore constriction. Worth noting is that the stiffness of dsDNA does not change significantly over the range of ionic strength tested.⁵⁴ Further, the increased electro-osmotic flow as the ionic strength is lowered cannot account for the increased frequency of DNA translocation because the electro-osmotic flow opposes DNA entry and translocation. These data suggest, therefore, that the *cis* lumen of the nanopore is important to initiate the translocation of DNA through the constriction of the nanopore.

Mechanism of DNA Translocation: dsDNA Capture Is Diffusion-Limited and ssDNA Capture Is Reaction-Limited. The DNA translocation experiments at different salt concentrations revealed two different capture mechanisms for dsDNA and ssDNA (Figure 3A,B, respectively, and Figure 5A,B, respectively). The behavior of dsDNA is consistent with a diffusion-limited capture process.⁵⁵ This is because the dsDNA used in this work is shorter than its persistence length (150 bp) and behaves as a rigid uniformly charged rod. Within the capture radius (about 50 nm from the nanopore center for a λ_D of 0.5 nm, Supporting Information), the electric field attracts the DNA toward the pore and aligns it along the field lines so that it hits the pore entry with one end (Figure 5A,i). Once inside the pore, the engineered charges interact with the DNA, preventing the retraction back to the *cis* solution (Figure 5A,ii–iv). Therefore, the dynamics of DNA capture can be approximated by that of a diffusing particle in a purely attractive potential of electrophoretic origin. In this case, the electrophoretic mobility of the dsDNA is proportional to the Debye length of the solution and the corresponding drift–diffusion equation can be solved exactly⁵⁵ (Supporting Information). By approximating the geometry of the ClyA nanopore with a cylinder of length $l = 13$ nm and a capture diameter $d = 6$ nm (Figure 1A), the capture frequency can be estimated⁵⁵ by the following (see Supporting Information for more details):

$$k_{\text{on}} \sim 14\lambda_D (\text{s nm } \mu\text{M})^{-1} \quad (1)$$

This is in remarkably good agreement with the experimental data for λ_D (at high salt concentrations, Figure 3A). This is striking because no fitting parameters are used. However, some care should be taken in this comparison, as the choice of the pore parameters is to some extent arbitrary since ClyA's geometry deviates significantly from a perfect cylinder. At low salt concentrations (0.15 M NaCl, $\lambda_D = 0.8$ nm), the capture rate is higher than predicted by eq 1 (Figure 3A). Likely, the positive charges at the ClyA-RR entry, which are not taken into account in the model, speed up the capture at low salt concentrations, while at higher salt concentrations, these charges are more effectively screened.

For ssDNA, the relation between k_{on} and λ_D is exponential, which is consistent with a barrier crossing (reaction-limited process). In solution, the ssDNA assumes a coiled conformation while it is pulled toward the nanopore by the electrophoretic force as DNA approaches the nanopore (Figure 5B,i). In the vicinity of the entry of the pore, however, a successful translocation event can only take place if one end of the strand faces the pore entry (Figure 5B,ii) and if the ssDNA is uncoiled (Figure 5B,iii,iv). This additional repulsive force of entropic origin effectively results in an energy barrier that must be crossed prior to translocation. The theory of such barrier-limited translocation has been discussed recently,⁵⁶ and on general grounds, the capture rate is given by

$$k_{\text{on}} = \omega e^{-\Delta F_b/k_B T} \quad (2)$$

Here, ΔF_b is the barrier height and ω is a characteristic attempt rate for barrier crossing. The exponential factor gives the probability of a successful crossing event. Although estimating ΔF_b from model inputs is difficult,⁵⁶ it was shown that the probability of successful translocation contains a term proportional to the electrophoretic mobility, which in turn is proportional to λ_D (Supporting Information). This would

Figure 5. Mechanism of dsDNA and ssDNA translocation through ClyA-RR nanopores. (A) dsDNA translocation is diffusion-limited. (i) dsDNA, which under the experimental conditions is a rigid rod, is aligned by the electric field lines (green) and enters the nanopore with a defined orientation. (ii) dsDNA penetrates inside the nanopore, where it interacts with the second layer of engineered charges. (iii) dsDNA can then translocate the constriction and (iv) exit the pore. The charges at the *cis* entry of the nanopore aid in the initial capture. (B) ssDNA translocation is reaction-limited. (i) ssDNA has a coiled structure with a gyration radius ($R_g \approx 6$ nm), which is about twice the radius of the nanopore. (ii) ssDNA is not yet in the pore, and it searches for the entry. (iii) One end of ssDNA finds the entry of the *cis* lumen and starts to uncoil. Because there is an entropic energy barrier to enter the nanopore, several attempts can be made before a successful translocation event. (iv) In order to translocate the constriction, ssDNA needs to fully uncoil. (v) DNA exits the nanopore and then recoils. The additional charges at the *cis* entry most likely mediate the efficient capture of the DNA inside the nanopore. The DNA molecules and the nanopore are drawn to scale.

explain the exponential dependence of k_{on} on λ_D (Figure 3B). It should be noticed that while k_{on} is obtained from the inverse interevent time, not all measured current blockades necessarily describe a translocation event. Part of these blockades may be due to the entry of a DNA strand followed by a retraction back to the *cis* side (Figure 5B, iii to i). Nevertheless, the formation of rotaxanes shows that at least some molecules successfully translocate. In any case, the argument leading to an exponential dependence of k_{on} on λ_D remains valid.

Biological Significance. In bacteriophages, DNA is transferred into the procapsid by packing proteins that align and push the DNA through portal proteins. Portal proteins have similar dimensions, stoichiometry, internal surface charge, and internal diameters to ClyA nanopores.⁹ A negative internal surface charge appears to be important for the smooth translocation of DNA across the portal proteins,³ and it is observed as well in other proteins that encircle and slide along DNA such as β -clamp proteins.⁷ Portal proteins and β -clamp proteins also have positively charged rings that have been proposed to play a direct role in genomic DNA packaging by interacting with the negatively charged phosphate backbone of

the translocating DNA.^{3,9} In this work, we showed that the electrophoretic translocation of DNA through ClyA nanopores was observed when two rings of positive charged residues were introduced at the *cis* entry and midsection of the nanopore, effectively aligning the DNA for the passage through the narrow and very electronegative constriction. In the absence of such interactions, that is, during the threading from the *trans* side, DNA translocation is not observed. Our results suggest, therefore, that in connector proteins, such rings of positive charges might be important to help initiate the ejection of the DNA out of the capsid into the infected cell.

CONCLUSION

In this work, we have engineered a ClyA nanopore by introducing two rings of positive charges, named ClyA-RR, to translocate dsDNA and ssDNA at physiological ionic strengths. ClyA-RR might be used to study protein–DNA interactions at the single-molecule level and could be employed in DNA mapping and sequencing applications, where an enzyme controls the translocation of the nucleic acid through the nanopore. We found that the introduction of rings of positive

charges at the wider entry (the *cis* side) and midsection of the nanopore is crucial to induce DNA translocation through the negatively charged *trans* constriction. Surprisingly, the constriction itself did not require modifications. These results suggest that attractive interactions at the entry and in the middle of the nanopore are important to “grab” and orient the DNA for effective electrophoretic-driven sliding through the narrow and negatively charged *trans* constriction. Interestingly, the charge distribution in ClyA-RR is mirrored in viral portal proteins, suggesting that the precise engineering of biological nanopores is important for the efficient packing and ejection of DNA in and out the viral capsid. Finally, the linear and exponential ionic strength dependencies of the frequency of dsDNA and ssDNA translocations, respectively, suggest a likely mechanism where the dsDNA capture follows a diffusion-limited process, while the ssDNA capture a reaction-limited process.

MATERIALS AND METHODS

DNA was purchased from Integrated DNA Technologies (IDT), NeutrAvidin from Thermo Fisher, and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine from Avanti Polar Lipids. β -Dodecyl maltoside was purchased from GLYCON Biochemicals GmbH. Enzymes were bought from Fermentas and all other materials from Sigma.

Protein Preparation. Single-point mutations to the ClyA-AS gene were performed by using the “mega primer” method.^{44,57} ClyA was expressed in *E. coli* BL21 (DE3) cells by using a pT7 plasmid. Monomers containing a C-terminal oligo-histidine tag were expressed in *E. coli* BL21 cells, purified by using Ni-NTA affinity chromatography and oligomerized in the presence of 0.5% β -dodecyl maltoside (GLYCON Biochemicals GmbH) as extensively explained before.³⁷

DNA Preparation. dsDNA 1 was prepared by mixing equimolar concentrations of 1a and 1b (Table S3). The mix was brought to 95 °C, and the temperature stepped down at regular intervals. The DNA was then purified from the excess of ssDNA with affinity chromatography using a biotin-binding column containing monomeric avidin immobilized on agarose beads (Thermo Scientific Pierce). The dsDNA was then eluted from the column according to the manufacturer's protocol. The elution fraction was concentrated and further purified using a PCR quick purification kit (QIAGEN). Typically, a DNA concentration of 0.2 μ g/mL was obtained. 1a/1c duplex was annealed as explained for 1 but not further purified.

Ion Permeability. I – V curves under asymmetric conditions (Table S5) were collected by adding ClyA to the *cis* chamber under symmetric conditions (150 mM NaCl, 15 mM Tris-HCl pH 7.5 in both *cis* and *trans* solutions). The electrodes were then balanced, and the electrolyte concentration in *cis* was increased up to 1 M by adding aliquots of 5 M NaCl stock solutions to the *cis* compartment. The volume of the *trans* chamber was adjusted by adding the same volume added to the *cis* side using the same buffer of the *cis* solution (150 mM NaCl).

Permeability ratios ($P_{\text{Na}^+}/P_{\text{Cl}^-}$, Table S2) were calculated using the Goldman–Hodgkin–Katz equation (below) using the measured reverse potential (V_r) values (Table S2), which were extrapolated from the I – V curves.

$$P_{\text{Na}^+}/P_{\text{Cl}^-} = \frac{[a_{\text{Cl}^-}]_{\text{trans}} - [a_{\text{Cl}^-}]_{\text{cis}} e^{V_r F/RT}}{[a_{\text{Na}^+}]_{\text{trans}} e^{V_r F/RT} - [a_{\text{Na}^+}]_{\text{cis}}} \quad (3)$$

R is the universal gas constant (8.314 J K^{−1} mol^{−1}), T the temperature in Kelvin, F the Faraday's constant (96 485 C mol^{−1}), P_{Na^+} and P_{Cl^-} are the relative membrane permeability for the ions Na⁺ or Cl[−], and a_{Na^+} and a_{Cl^-} are their respective activities. The *cis* chamber was the ground. Ag/AgCl electrodes with 2.5% agarose bridges containing 2.5 M NaCl were used to perform all of the experiments.

Electrical Recordings. Ionic currents were measured by recording from planar bilayers formed from diphytanoyl-*sn*-glycero-3-phospho-

choline (Avanti Polar Lipids, Alabaster, AL). The electrical potential was applied by using Ag/AgCl electrodes submerged in agar bridges (3% w/v low-melt agarose in 2.5 M NaCl buffer) using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) as described previously.^{13,58} Single channels were characterized by measuring the current *versus* applied voltage relationship (I – V curve, the potential was applied in 10 mV steps from −100 to +100 mV in 21 s, Figures S1 and S4 and Table S6). In 0.15 M NaCl, ionic currents were recorded by applying a 2 kHz low-pass Bessel filter and using a 10 kHz sampling rate. At higher salt concentrations, ionic currents were sampled at 50 kHz and the low-pass Bessel filter was set at 10 kHz. Current traces at 0.3 and 0.5 M NaCl were filtered postacquisition with a 4 kHz Bessel digital filter (Figures S5 and S6). The use of different filtering frequencies influences the overall number of detected events. For example, applying a 2 kHz digital Gaussian filter to a trace sampled at 50 kHz while applying a 10 kHz Bessel filter increases the interevent time by about 50% (from 221 to 311 ms, 0.17 μ M dsDNA, 1 M NaCl, average dwell time of 0.12 ms). Therefore, to test the effect of excessive filtering on the Debye length dependence of the DNA capture frequency, we plotted the data described in Figure 3A after applying a 1 kHz Gaussian filter to all current traces (Figure S7). We found that the ssDNA and dsDNA blockades fitted well to an exponential and a linear regression, respectively (Figure S7).

Data Analysis. Current blockade events were collected individually by using the “single channel search” function of the Clampfit software (Molecular devices) using a data acquisition threshold of 0.05 ms. Open and blocked pore current values were obtained from Gaussian fitting to all-point histograms. Residual currents were calculated by dividing the blocked pore current values for the open pore current values. The DNA translocation dwell time values (τ_{off}) were calculated from a single exponential fit from event histograms of DNA blockade dwell times, while interevent time (τ_{on}) values were calculated using an exponential logarithmic probability fit from logarithmic histograms of the interevent times (Figure 3, Table S4, and Figures S5 and S6). The errors indicate the standard deviation from the average from at least three independent nanopore experiments, the number of which is indicated by N .

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b03159.

Additional text explaining the derivation of eqs 1 and 2, additional tables showing the electrical properties of engineered ClyA variants, and additional figures showing the details of the translocation of ssDNA and dsDNA across ClyA nanopores (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Misha Soskine for suggesting the use of ClyA-R. We thank the European Research Council and IWT for financial support.

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