On The Origins of Conductive-Pulse Sensing Inside a Nanopore

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Abstract

Nanopore sensing is nearly synonymous with resistive pulse sensing due to the characteristic reduction of ionic flux during molecular occupancy of a pore; particularly at high salt concentrations. However, conductive pulse sensing is widely reported at low salt conditions wherein electroosmotic flow (EOF) can be quite significant. Aside from transporting molecules like DNA, we investigated whether EOF has other potential impact on sensing attributes such as pulse direction. The over-whelming majority of literature report counterions as the dominant mechanism of conductive events (CE). CEs are not well understood due to the complex interplay between (charged) nanopore walls, DNA grooves, ion mobility, and counterion clouds. However counterion theories do not fit well with CEs at high salt (>1M) or proteins which have a heterogeneously charged surface, as shown here. Herein we demonstrate theory and

experiments underpinning the translocation mechanism (i.e., electroosmosis or electrophoresis), pulse direction (i.e., conductive or resistive) and shape (e.g., monophasic or biphasic) through fine control of chemical, physical and electronic parameters. Results from these studies predict strong EOF pumping plays a role in EOF-driven DNA events and generating CEs due to polarization effects. We believe these findings will stimulate a useful discussion on the nature of CEs and their impact on molecular sensing in nanoscale pores.

Introduction

Since their first use as a biosensor, solid-state nanopores continue to explore new biophysical phenomena and have cemented their place in history as high-throughput, low-cost overhead, real-time, single-molecule resolution electrical read-out platforms. Although the translocation profiling of biochemically, biomedically, and pharmaceutically impactful new molecules and particles has gained tremendous traction in laboratories across the world, the high electrolyte concentration paradigm in which experiments are performed has been rather unchanged since the sensing inception of nanopores in 1996[1]. The attractiveness associated with high electrolyte solutions is largely due to the high signal-to-noise ratio (SNR), high electrophoretic throughput, and reliable generation of resistive pulses stemming from DNA transiently blocking ions (typically potassium and chloride). The physical principles in which DNA modulates the flow of ionic current within a nanopore have been studied extensively[2–4]. Although nanopore sensing is synonymously associated with resistive pulse sensing due to transient ionic current perturbation by the molecule, the resistive nature of events is not consistent across all DNA translocation experiments[1,5,6]. In 2004, Chang et. al., reported on current–enhancing events at low electrolyte concentrations wherein the DNA-occupied pore conducted more ions compared to the DNA-free pore[7]. Therefore, pulses generated through

translocations can be categorized as current-reducing (i.e., resistive event, RE), or current-enhancing (i.e., conductive event, CE) events.

As electrolyte concentration decreases, CEs are often observed in both planar membrane nanopores as well as conical nanopipettes, suggesting that CEs are not pore geometry specific[8–16]. It is also at this regime where electroosmotic flow (EOF) strengthens, sometimes leading to the translocation of molecules opposing the more conventional electrophoretic flow (EPF). Although EOF and CEs often coincide, it is important to note that they are not mechanistically linked. For example, CEs are seen in nanopores where EOF is reduced to allow EPF-driven events[9]. Despite the large number of experiments describing CEs, the origins of CEs in the presence of low ionic strength have been elusive. The leading hypothesis is that CEs stemming from low ionic strength conditions occur because the introduction of additional counterions by the charged DNA (i.e. K^+) within the nanopore is greater than the number of ions within the DNA-free pore[7]. Once electrolyte concentration decreases below ~0.02 M, mostly counterions are present within the pore, which explains the current enhancement[17,18]. Interestingly, at ~0.4 M, counterions are thought to precisely compensate for the DNA-occupied regions of the pore and yields no current modulation[19]. A second hypothesis has been that frictional forces (i.e. ionic friction with the grooves on DNA) are influential in generating CEs[3,9]. Although these hypotheses can predict the well-known crossover point in which events transition from resistive to conductive (via decreasing salt concentrations), the cation-specific, voltage-specific, and pore size-specific dependence of CEs have not been studied and should provide confirming or countering evidence for one of these hypotheses

Indeed, the results presented herein conflict with the conventional hypotheses and may lead to a third potential theory; namely that current enhancement is due to a flux imbalance and

charge density polarization. We predict that high asymmetric salt conditions can yield both cation-selective and anion-selective condtions simply by changing the polarity of the applied voltage. *Zhang et al* observed CEs with asymmetric salt conditions (using planar nanopores) and used a multi-ion model composed of Nernst-Plank and stokes equations to explain their observations where they attributed to EOF enhancement in the outer section (i.e., the nanopore section free for ion flow in the presence of DNA) being significantly higher than the ions bought into the pore by the double layer[20]. Our experimental observations with PEG (a natively neutral polymer that functions as a polycationic polymer through cation adsorption) cannot be explained through this model where conductive events were seen with smaller diameter pores where EOF is thought to be meager (SI section 1). Thus, the more intriguing question, "Why does ionic current *increase* during transient DNA occupancy of a nanopore?", remains under-examined and warrants further investigation. Since a cohesive theory for the nature of conducting events is still elusive, we studied the transport of DNA within a nanopipette using various monovalent salts.

A second fundamental question that remains debated is, "can low salt conditions promote EOF-driven DNA transport?' Although it may seem ostensibly obvious, remarkably, electroosmosis dominant transport of DNA is hardly reported (only a couple of works exist to the best of our knowledge) and therefore less known in the nanopore community[21,22]. On the other hand, electrophoretic transport of DNA through nanopores is well-reported and almost unanimously used mode of transport. While electroosmosis has seen widespread adoption in protein and glycan characterization, its use in DNA experiments is meager largely due to the high linear charge density associated with DNA and (high) salt conditions typically used in experiments[23,24]. However, tuning of electroosmosis has been used to, for example, promote single file translocation, improve throughput, and tune translocation time[23,25,26]. To the best of

our knowledge, no previous reports exist outlining the electroosmotic DNA transport through nanopipettes. Thus, herein, we characterized EOF-driven events (anti-electrophoretic, or anti-EPF) with Lambda DNA (λ-DNA)—the gold standard of the nanopore community to benchmark new developments due to its well-known physicochemical parameters—using quartz nanopipettes. In summary of our findings, DNA CEs are extremely cation-, pore size-, and voltage-specific and potentially the result of an imbalance of ionic fluxes, which lead to charge density polarization. We will discuss the electrokinetic and hydrodynamic phenomena that affect event shapes such as counterion cloud, ion mobility, pore size, and electrolyte composition. This report elucidates some of the fundamental prerequisites for observing CEs when DNA translocate through a nanopore and paves the way for harnessing CE mechanisms for DNA sequencing and biophysical discoveries.

Results

While most nanopore-based, single-molecule sensing is performed using planar membranes, which have a well-defined pore length (i.e. effective membrane thickness), nanopipettes have a gradual taper length (Figure 1a) that increases the sensing region of the device[27]. We fabricated nanopipettes by laser pulling glass nanocapillaries, producing two identical quartz nanopores (see *Methods* for fabrication details). With this technique, <10 nm inner pore diameters can be achieved as shown in Figure 1a. This process is fast, inexpensive, and does not require a clean-room environment[28]. The pore conductance (G) was evaluated using the slope of a current-voltage (I-V) curve (Figure 1b) and thereafter used to estimate the size of the aperture using[29,30]:

$$d_i = \frac{4Gl}{\pi K d_b} \tag{1}$$

where l is the length of the conical pore (taper length), K is the measured conductivity of the buffer, and d_b is the diameter of the capillary (0.7 mm) at the beginning of the conical taper. The taper length was measured using transmission electron microscopy. The *G* varied between 0.58 and 5.35 nS and the I-V curve showed ionic current rectification which is consistent with the previous reports[31]. The tabulated *G* values yield pore diameters between 5 (± 0.5) and 48 (± 4) nm, respectively. The pore sizes were also occasionally confirmed using transmission electron microscopy (see SI section 2 for further details).

<u>λ-DNA Translocation in symmetric low salt conditions</u>

After retrieving the I-V information, translocation experiments with λ-DNA at a final concentration of 500 pM were performed in 10 mM Tris-EDTA buffered at pH ~7.4. We opted for very a low salt concentration (i.e., 10 mM) to maximize the EOF while maintaining a high enough SNR for pulse extraction (see discussion in SI section 3 for more details on SNR). The pH was maintained at the physiological pH which renders the glass to be negatively charged (~20 mC/m^2) and therefore EOF and EPF to be opposing in the case of λ-DNA. For electroosmotic capture to take place, it should outweigh the electrophoretic force (provided the two forces are opposing rather than complementing) exerted on the DNA molecule by the applied voltage. For a molecule to translocate, it must first diffuse to the capture zone, drift to the pore opening and overcome electrostatic and free energy barriers (e.g., entropy). The shape and extent of the capture volume are exceedingly crucial as they would govern the transport dynamics of the device. It is known, when EPF dominates, the capture volume outside the nanopore assumes a nearly spherical shape surrounding the pore's oriface[32–36]. EOF on the other hand depends on the fluid flow profiles. According to the EOF streamlines, the capture volume adopts a shape confined along the sides of the pore[37]. There also lies a crossover concentration

point in which EOF reverses direction, where EOF is generated along the glass surface and radiates away from the pore aperature[37]. Finite element analysis was performed to determine the fluid flow rate at different voltages (Figure 1c). Herein, we adopted the conventional operational configuration where the anode/cathode electrode is placed inside the pipette side and grounded electrode in the bath. Since the glass surface is negatively charged at the operational pH, at negative applied voltages, the resultant fluid flow would be towards the taper region (i.e., from the bath to the tip). At positive biases, the fluid flow direction switches. In brevity, the simulation depicted in Figure 1c was carried out in the following manner: Poisson-Nernst-Planck-Stokes equations were solved simultaneously to account for ionic species spatial concentrations, electrostatic forces on ions and convective forces on ions. EOF was imposed as a force on the surrounding liquid by integrating the spatial accumulation of ions into a volume force that acts on the liquid (boundary conditions can be found and simulations details can be found in the SI Section 10). The fluid velocity acts as a moving frame of reference for the DNA and can be compared directly with the electrophoretic drift velocity imposed by the electric field. Electrophoretic drift velocity was calculated by extracting the electric field and multiplying by the electrophoretic mobility of DNA (μ=3.2 × 10^4 cm/Vs). Simulated results shown in Figure 1c indicate, with the operational conditions adopted in this work, the EOF velocity is greater than the EPF drift velocity rendering the net velocity to be in the same direction as the EOF profile.

Given the inherent differences associated with capture volume shapes associated with EOF and EPF dominant mechanisms, the next step was to elucidate the entrance trajectory of DNA. To do this, λ-DNA was added to the bath and a negative voltage bias was applied to the other electrode to ensure if translocations were to happen (i.e., from the bath to the tip side; forward translocation direction), it would be caused by electroosmosis rather than by the

conventional electrophoresis. The fluid flow profiles around pore-tip were simulated to further understand the EOF-driven capture of DNA. The simulated results are shown in Figure 1d and indicate DNA proceeds to diffuse around the solution until it enters the EOF capture volume, where it is then transported through the pore. To reiterate, this transport is fundamentally possible when the EOF velocity is greater than the EPF drift velocity.

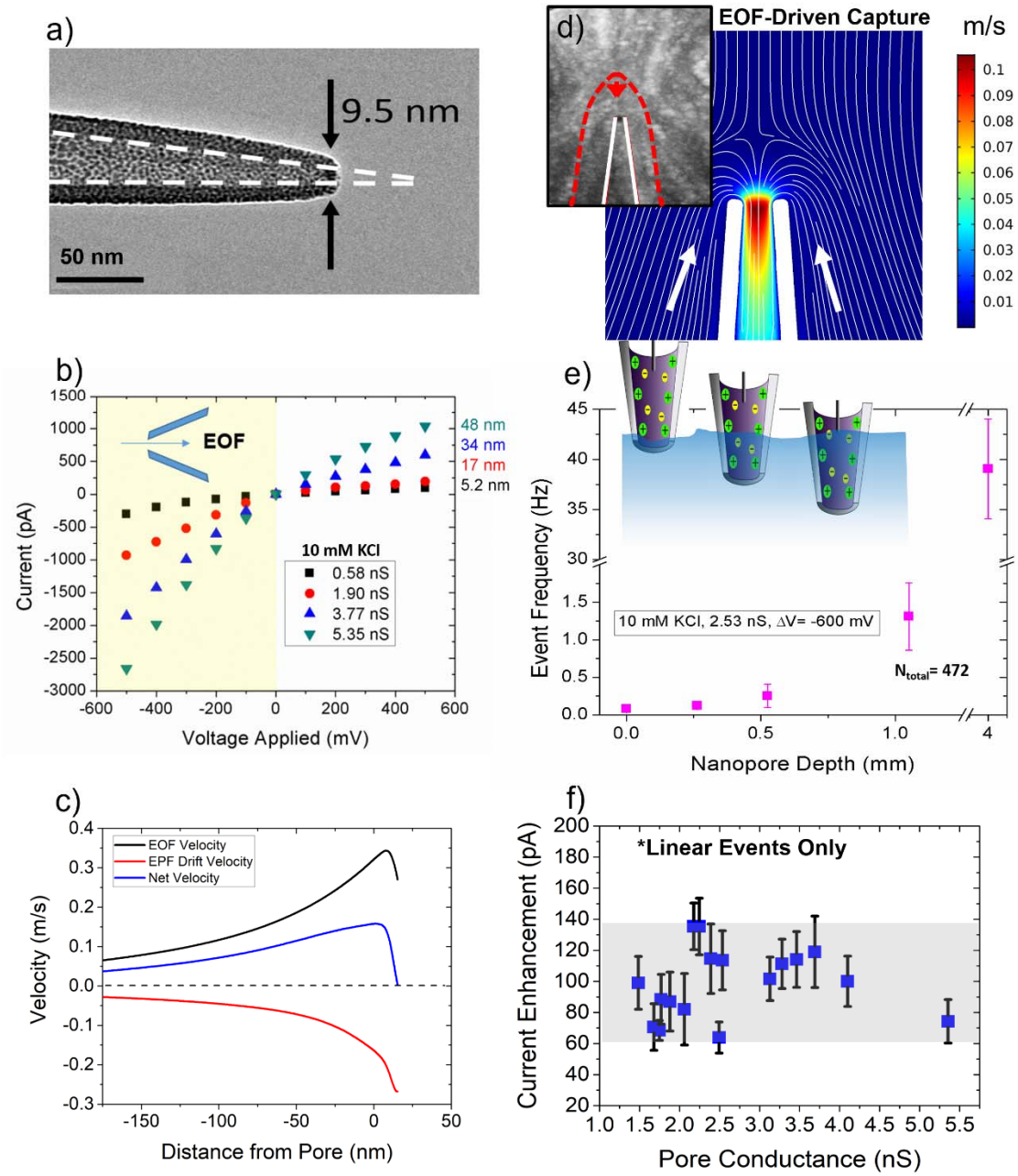

Figure 1: Experimental set-up and characterization of quartz nanopores. (**a**) TEM of quartz nanopore; scale bar, 50 nm. (**b**) I-V curves pertaining to four differently sized nanopipette orifices. (**c**) EOF, electrophoretic drift, and net velocities of λ-DNA along the pore's axis of symmetry (μ=3.2 × 10^4 cm/Vs). (**d**) Simulations of EOF pump velocity at various voltages within quartz nanopores; pore diameter was 20 nm and was symmetrically filled with 10mM KCl. EOF reversal is also shown. Inset: fluid velocity distribution near the pore (**e**) Event

frequency with depth of the pipette inside the bath solution. (**f**) Current enhancements for nanopore sizes (i.e. diameters) ranging from approximately 5-50 nm. All events were observed under EOF-dominant conditions.

Under high salt conditions, DNA transport has been categorized to adopt a range of configurations including linear, looped, partially folded, and knotted: reported with both planar nanopores[38–41] and nanocapillaries[29]. However, reports on the DNA conformations under EO dominant transport are yet to be published. Thus, after confirming the mode of dominant transport, we looked at the conformations adopted by translocating DNA molecules. Realizing that the capture volume in EOF-driven translocations surrounds the outer walls of the nanopipette, we first optimized the throughput of the device by adjusting the pipette position with respect to the bath liquid surface as shown in Figure 1e. The capture volume can be controlled by submerging varying lengths of the taper length inside the salt solution containing λ-DNA. The nanopore was suspended at 0, 0.26, 0.53, 1.1, and 4.0 mm below the electrolyte solution surface containing λ-DNA. For exact measurements, the nanopore was suspended from a micrometer. Translocations were obtained for voltages between -100 and -1000 mV, in increments of 100 mV. Recording at -600 mV yielded the most consistent translocations without clogging the pore. Events were recorded at -600 mV and the I-V relationship yielded a 2.5 nS pore. The capture rate was calculated at each depth (see SI section 4 for capture rate calculation details). As nanopore depth increases, capture volume also increases, leading to higher event frequency with larger depth values. As more of the nanopore is exposed to the λ-DNA solution, the capture volume enlarges, leading to an increase in event frequency and corroborates the EOF capture mechanism more strongly than the electrophoretic capture mechanism. Finally, using a custom-coded MATLAB script, translocation conformations of DNA was examined which

revealed, that DNA adopts the widely seen conformations: linear, partially folded and fully folded (see SI section 5 for further details). By solely selecting linear events, we were able to evaluate the relationship between CE amplitude and pore size; a relationship that may be hidden by multiple conformations of DNA. If counterions become sheared off the negatively charged DNA or overlapped with the counterions from the pore wall to enhance ion concentrations inside the pore, CE amplitude should increase with decreasing pore size. However, as seen in Figure 1f, no observable trends were seen in CE amplitude with pore conductance (a proxy for pore size).

λ-DNA Translocation in asymmetric high salt conditions

To show that CE phenomenon is not limited to low ionic strength configuration, we used salt concentration gradients where the pipette was filled with 1M KCl and the bath was filled with 4M KCl. λ-DNA was either added to the pipette (Figure 2b: case I) or bath (Figure 2c: case II) and a voltage bias consistent with the conventional electrophoretic transport direction of DNA was applied to the pipette. Note that EOF is deemed negligible under the high salt conditions that these experiments operate. In case I, with an applied voltage of -600 mV, λ-DNA was driven outside the pore through EPF, resulting in CEs. This contradicts the conventional expectation of REs under high salt conditions and CEs under low salt conditions. On the contrary, in case II, with an applied voltage of +600 mV, λ-DNA was driven into the pipette resulting in REs. Although directional dependence of DNA transport has been reported previously with nanopipettes[42], a change in the conductive nature of the pulses has not been previously observed. The conductive pulse observations shown here showcase the shortcomings of theory used for nearly two decades which presume excess charge introduced by DNA compensates for the ionic current blockade by DNA to eventually yield conductive pulses. Furthermore, with asymmetric salt conditions (1 M inside, 4M outside), both the forward and

reserve translocations produced anti-conventional event shapes. It is well known that the translocation direction of a particle is reflected through its event shape with tapered pore geometries unlike their cylindrical counterparts[43]. Moreover, shapes analogous to that shown in Figure 2b are typically observed for reverse translocations (i.e., when a molecule enters the pipette through the bath and travels along the confined tapered region). In other words, the geometry of the pore determines the electric field profile and by extension the sensing zone of the pore. Despite the DNA exiting the pore, there is a transient decay back to the baseline current level (Figure 2b). Conversely, reverse translocations under resistive pulse conditions produced a square pulse rather than a pulse with a decaying tale (Figure 2c). By fitting the current to an exponential decay, the decay constant of forward translocations was found to be approximately $1150 \pm 243\ s^{-1}$ which corresponds to a 10% to 90% rise time of ~1.2 ms. This is substantially longer than the rise time associated with the 10 kHz lowpass filter used while recording the data (~33 μs)[44]. Although a definitive mechanism as for what produces the observed waveform shapes is not completely known, since the DNA has already exited the pore, we speculate that the electrochemical gradients require time to relax back to equilibrium.

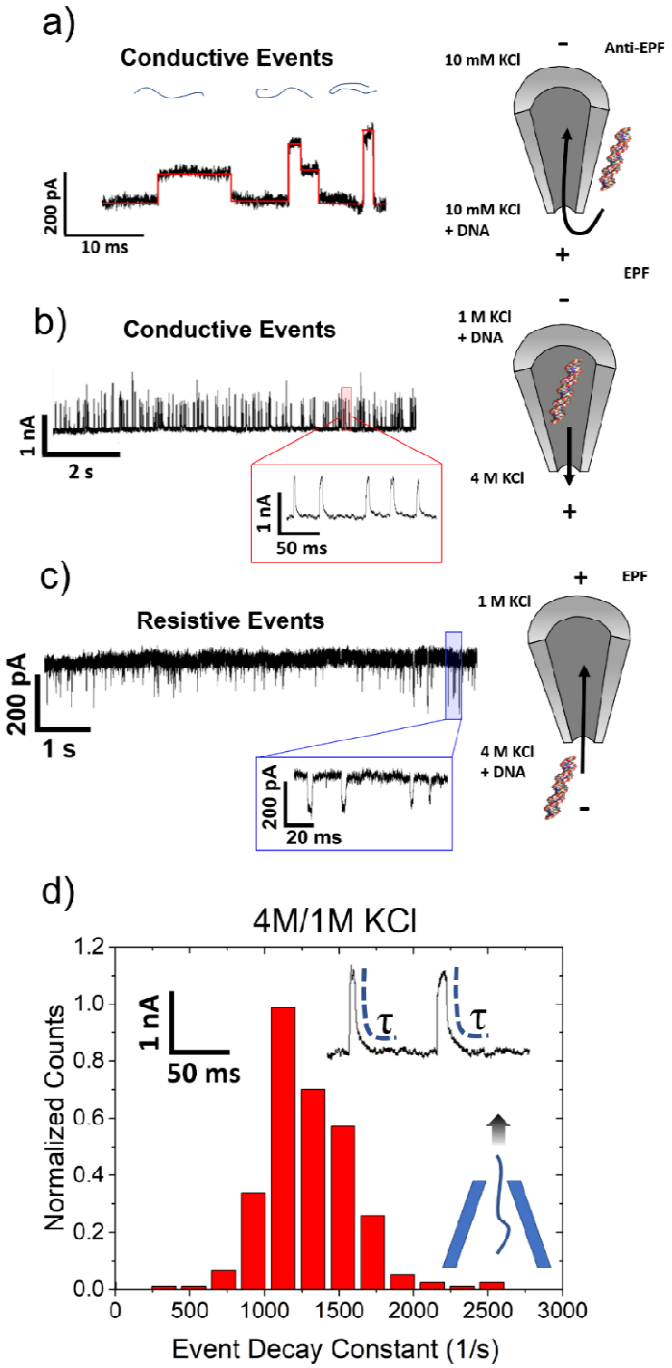

Figure 2: Observations on the nature of events based on salt symmetry or salt asymmetry. (**a**) Typical event structures observed with λ-DNA translocation experiments under low salt conditions. (b) Observation of CEs in asymmetric salt conditions when λ-DNA (-600 mV) was added into the pipette. (c) Observation of REs in asymmetric salt conditions

when λ-DNA (+600 mV) was added into the bath. All buffers were prepared at pH 7.4. (d) Event decay to equilibrium for the case experiments shown in (b). DNA is exiting the pore and thus should immediately leave the sensing zone of the pore as opposed to the reverse translocation direction.

Conceptually, a pore can become ion-selective depending on its surface charge. If the pore is charge neutral, it would not exhibit any selectivity whereas if it is negatively charged, the pore would be cation-selective (Figure 3a). Simulations with a negatively charged pore submerged in 10 mM KCl solution showed that although the pore's total ionic flux was not altered significantly by EOF (K^+ flux increased and Cl^- flux decreased by the same amount), it does significantly impact the flux imbalance between cation and anion (see SI section 6 for simulation details). Flux imbalances can be generated through externally applied conditions and parameters: flux imbalance increases with both the pore diameter and the applied voltage (Supporting Information Figure S9). This finding will be important when discussing other monovalent salts wherein transitions between REs and CEs occur. Nevertheless, the finding that EOF can increase the counter-ion (K^+) flux imbalance is particularly noteworthy since (i) CEs were observed at high asymmetric salt conditions which would also cause ionic flux imbalance and (ii) further supports the previous experimental observations of CEs occurring under asymmetric salt conditions as DNA translocate through the pore[20]. With a salt gradient, in addition to the electrical potential gradient, ions could move as result of the chemical potential gradient. Thus, for the asymmetric salt cases, assuming the same spatial voltage distribution, one ion will outweigh the flux of the oppositely charged ion as shown in Figure 3c. In case I, K^+ moves along both the electrical and chemical potential gradients opposing the DNA translocation direction whereas in case II, due to the positive bias, Cl^- ions move along both the gradients cooperative with DNA translocation direction. This is also reflected through the translocation

time (Δt) where case I produced events that were ~3× slower compared to case II (Δt were 3.2 ms and 1.1 ms respectively for case I and case II). Taken together, these results imply a flux imbalance in favor of Cl^- produces REs whereas CEs stem from a flux imbalance in favor of K^+. This is notably different than ion selectivity which is typically a characteristic of the pore itself. Rather, flux imbalances can be generated through externally applied conditions and parameters. This computational finding further supports the previous experimental observations of CEs occurring under asymmetric salt conditions as DNA translocate the pore.

The impact of the flux imbalance seems to play a role in re-distributing the voltage drop inside the nanopipette; in particular, the taper region where there is a confining negative surface. Using finite-element simulations, and varying the surface charge density incrementally, it is shown that higher surface charges leads to two main effects. First, EOF flow velocity increases, and secondly, the excess charge inside the taper length of the pipette causes ion polarization effects. For example, as surface charge is increased, the electric potential drops significantly between -100 and -400 nm inside the nanopipette (Figure 3d). Under asymmetric salt conditions, the impact is highly voltage dependent based on the flux imbalance. Under positive voltages, the Cl- is rejected from the pore to the tapered region decreasing the voltage drop occurring inside the taper of the nanopipette. That is, as seen in Figure 3, compared to a neutral pore surface, the tapered region become more conductive (i.e. less voltage drop occurs). On the other hand, if is a negative bias is applied inside the pore, K^+ ions are accepted to the pore interior causing the voltage drop inside the taper to increases compared to a neutral pore since the tapered region become more resistive (i.e. larger voltage drop compared to a neutral pore). Thus, the net positive or net negative charges stored inside the pipette changes the voltage distribution and therefore the sensing zone of the nanopipette sensor. The decrease is charge storage at low salt is

observed in Figure 3e wherein there is always positive charge accumulation but it is lessened or exacerbated by EOF pumping. While EOF is the mechanism of charge transport, it is the flux imbalance that ultimately determines the degree of polarization. While charge density polarization effects are commonly taken into account on electrode-electrolyte interfaces, it seems rarely considered for nanoscale confinements until relatively recently[45,46].

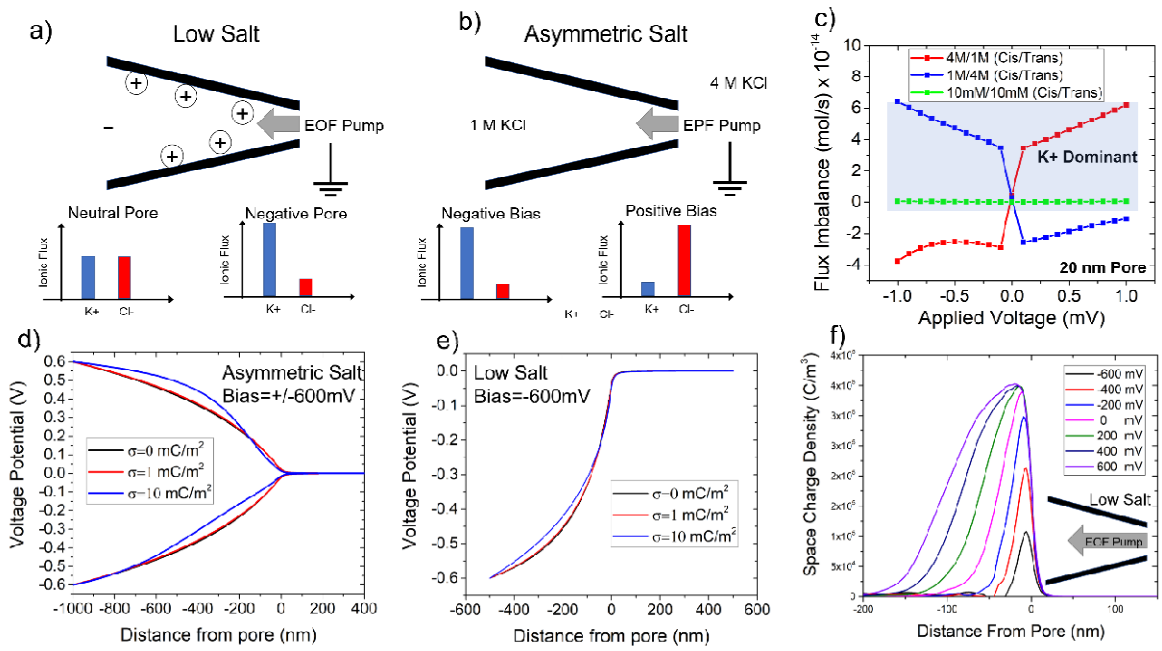

Figure 3: **Conceptual and computational model of symmetric low salt conditions and asymmetric salt conditions**. (a) Schematic of flux imbalance when a negatively charged pore has a negative potential inside the nanopipette. (b) Schematic of EPF ion pumping with negative and positive voltages applied inside the nanopipette. (c) Flux imbalance calculations for symmetric and asymmetric salt conditions (the location of the higher salt concentration is switched from 1 M (cis)/4 M (trans) to 4 M (cis)/1 M (trans)). (d-e) The potential distribution under asymmetric salt conditions and low salt conditions for three surface charge densities (electric potential is plotted along the axis of symmetry). (f) Space charge density (C/m^3) for the

voltage range of -600mV to +600 mV (axial distance of zero corresponds to the tip of the nanopipette) The pore diameter was 20 nm and was modeled with 10 mM KCl.

Alkali Chloride Dependence on Event Characteristics

Now that a relationship between the ion flux imbalance and pulse direction is apparent, the question of whether the nature of the monovalent cation would have any effect on the transport properties was examined. For example, LiCl is known to shield the charge of DNA and slow it down compared to KCl since the former can bind more covalently to charged moieties compared to the latter. Additionally, LiCl had a significantly higher streaming current compared to both KCl and CsCl (see SI section 6 for more details). In this section, first, we draw comparisons between the translocation properties of λ-DNA in symmetric 10 mM KCl and 10 mM LiCl salts followed by 10 mM CsCl.

The nanopipette containing 10 mM LiCl was inserted inside a solution containing 10 mM LiCl and λ-DNA (buffered at pH~7.4) and current traces were recorded from -300 mV to -900 mV in 200 mV increments (Figure 4a). As seen in Figure 4a, a crossover from Res to CEs that is independent of salt concentration was observed. At voltages of -300 and -500 mV, λ-DNA translocations resulted in REs and at voltages of -700 and -900 mV, it resulted in CEs (also see Figure 4c). Intrigued by this observation, we explored the pulse behavior at -600 mV where the event current shape assumed both a resistive and conductive region resembling a biphasic waveform (SI Figure S10). The biphasic nature of the events at the transitional voltages (-500 mV, -600 and -700 mV) suggests that both resistive and conductive modulation mechanisms can conjointly act and perhaps act at different timescales in relation to the translocation event. For example, in the moments before or after the DNA enters the pore, DNA would still exist within the EOF flow field of the pore leading to current modulations on a potentially longer timescale.

Another comparison was done using two nanopipettes with inner diameters of 33 ± 3 nm. One nanopipette contained 10 mM KCl while the other contained 10 mM LiCl. Both were submerged in 10 mM LiCl with 500 pM λ-DNA (all buffered at pH ~7.4). Interestingly, at -600 mV, CEs were observed for the pore containing KCl whereas REs were observed for LiCl (Figure 4e). At -600 mV, finite element simulations (for the 33 ± 3 nm nanopipette) predicted that the nanopipette is strongly cation-selective in KCl and weakly cation-selective in LiCl, which may be a possible explanation for the event types observed. If the transition to CE occurs at a flux imbalance of 2×10^{-16} mol/s as shown in Figure 4c, the same discriminating line appears to be valid for predicting KCl and LiCl current modulation (Figure 4e). The stronger flux imbalance observed with KCl (under the same pore size, voltage, and salt concentration) led to CEs while LiCl produced REs (Figure d-e). The critical value of the flux imbalance has no clear meaning at this time but is extracted from a combination of experimental and numerical approaches.

Intestingly, KCl had longer event durations at these low salt conditions (3.1 ± 1.5 ms compared tp 1.9 ± 0.7ms in LiCl): a counterintuitive observation if DNA was electrophoretically driven since LiCl is known to slowdown DNA trasnlocation through charge shielding compared to KCl[47]. Since translocations in both KCl and LiCl are EOF driven, we suspect the effective charge shielding ability of LiCl allows EOF to transport the DNA with less oposig force. Other than the differences in Δt, as seen in Figure 4d, the *Δ*I of REs observed for LiCl are much more tightly clustered together compared to *Δ*I of CEs observed with KCl (-70 ± 8 pA versus 200 ± 122 pA, respectively). The source of the variability of CEs observed in KCl is still not fully understood and requires further investigation. Once LiCl events trasition to become CEs (Figure

4b), current modulations become more scattered compared to REs. Additional current traces from λ-DNA translocating in 10 mM LiCl can be seen in SI Figure S10.

Recently, CsCl was shown to have an advantage over KCl in respect to sequencing using solid-state nanopores[11]. This publication used CsCl because it disrupts the hydrogen bonding between guanines, therefore denaturing the G-quadruplex into single-stranded structures. Although we are not working with ssDNA, we aimed to compare KCl event properties with another alkali metal chloride that holds promise in the nanopore community. Therefore, we performed experiments using nanopipettes filled with 10 mM CsCl inserted into 10 mM CsCl with λ-DNA (Figure 4f). Similar to KCl, pulse direction in CsCl is expected to be voltage independent since K^+ and Cs^+ have nearly the same diffusion coefficient[48]. To confirm this, a pore with a conductance of 1.47 nS (14 ± 2 nm diameter) was used under low ionic strength conditions and voltages of -300 mV, -400 mV, -500 mV, and -1000 mV were applied. All voltages resulted in CEs. To further strengthen this observation, flux imbalance for CsCl was simulated (SI Section 8) which revealed the pore to be cation-selective across the experimentally viable voltage range. Simulated results of KCl and CsCl were nearly identical due to nearly identical diffusion coefficients for K^+ and Cs^+ (2.02×10^{-5} and 2.00×10^{-5} cm^2/s, respectively[48]).

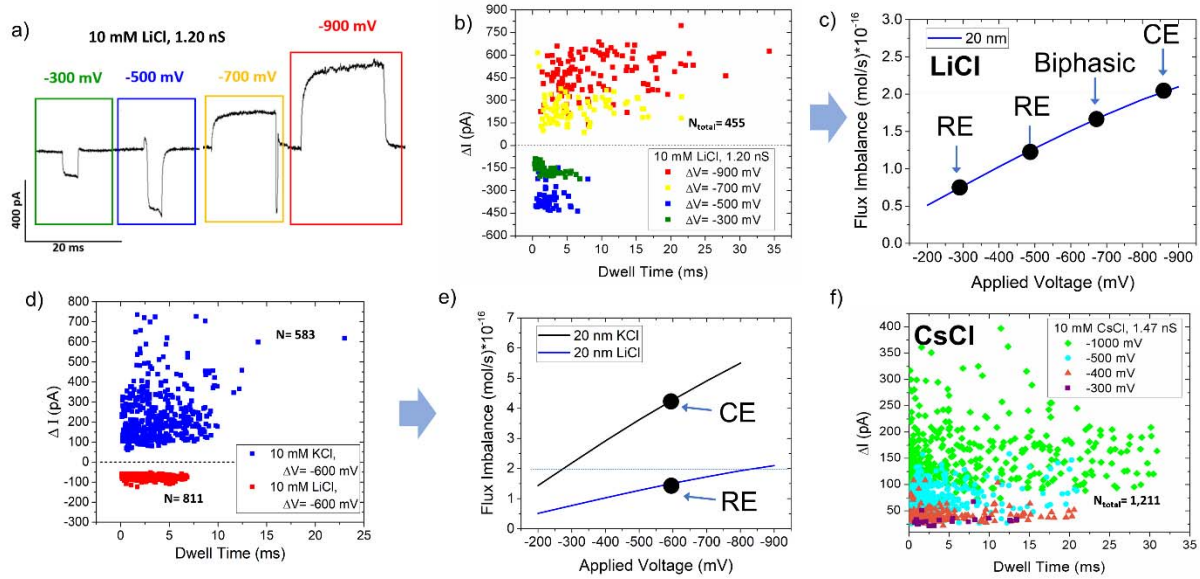

Figure 4: Event shape transition from resistive to conductive as the voltage applied increases in negativity. (**a**) Representative waveforms observed in 10 mM LiCl from λ-DNA translocations in response to negative voltages. (**b**) Scatter plot showing current change and dwell time relationship with applied voltage for λ-DNA in 10 mM LiCl through a pore with a conductance of 1.20 nS. (**c**) Flux imbalance as a function of (negative) applied voltage in 10 mM LiCl and its influence on the waveform generated through λ-DNA translocations. Both experiments and simulations were with a pore size of 20 nm. (**d**) Scatter plot corresponding to λ-DNA translocation in 10 mM KCl (REs) and 10 mM LiCl (CEs) in response to -600 mV. (**e**) Flux imbalance with (negative) applied voltage in 10 mM KCl and 10 mM LiCl for a pore size of 20 nm. The horizontal dashed line corresponds to the flux imbalance corresponding to the transition of REs to CEs. (f) Scatter plot of current change versus dwell time corresponding to λ-DNA translocation in 10 mM CsCl at various applied voltages. All voltages produced CEs similar to KCl.

According to the experimental and numerical evidence, flux imbalance seems to play a role in producing CEs. Using the asymmetric salt conditions, we showed that a flux imbalance can be generated that favors potassium ions (i.e., case I). A reversed voltage polarity would therefore generate a flux imbalance that favors chloride ions (i.e., case II). We further wanted to

investigate whether this would hold for protein structures since they notably have a heterogeneously charged surface. If analyte counterions played a role in CEs, we would expect cation and anion counterions would cancel out and there would be no observation of CEs. Therefore both the high salt conditions and the charge characteristics of a protein should unequivocally prevent CEs from being observed. We chose to study the Cas9 mutant, Cas9d10a, because unbound it carries a net positive charge at pH ~7.4, and once bound to sgRNA, the complex becomes highly negatively charged[49]. For added specificity, amino acid sequence calculations were performed on the Cas9d10a complex alone and bound to sgRNA, providing net charges of both (Figure 4a). The pH was not changed to be consistent with the previous set of experiments. Furthermore, the same asymmetric salt conditions were employed, as before, where 1 M KCl was inside the nanopipette and 4 M KCl was outside the nanopipette. The Cas9d10a protein was added inside the nanopipette (in 1 M KCl) with and without sgRNA (resulting current traces are shown in Figure 5b and 5c, respectively). The Cas9d10a-sgRNA complex was achieved by incubating Cas9d10a with sgRNA (equimolar amounts) for 1 hour at room temperature. Voltages were applied to be consistent with the expected electrophoretic transport directions: negative bias for the Cas9d10a-sgRNA complex and a positive bias for the Cas9d10a protein. Like λ-DNA, Cas9d10a-sgRNA complex produced CEs (Figure 5b). Under this condition, K^+ from the outside bath (4 M KCl) is driven into the pipette and therefore, if we adopt the proposed theory, Cas9d10a correctly produced CEs. The events at negative voltage could indeed be from either Cas9d10a-sgRNA complex or sgRNA alone since both are negatively charged. However, Cas9d10a binding of sgRNA is typically fast with slowly reversible reaction kinetics[50]. For Cas9d10a alone, a positive voltage drives the protein through the pore and also Cl^- from the outside (4 M KCl) is driven into the pipette. Under these

conditions, we observed REs. The pulse direction is consistent with our previous observations where cation selectivity yielded CEs and anion selectivity yielded REs. As observed in our simulations, polarization effects do not seem to occur when a flux imbalance favors chloride, and may be partly due to the confined, negatively-charged taper of the nanopipette preventing excess negative charge from accumulating.

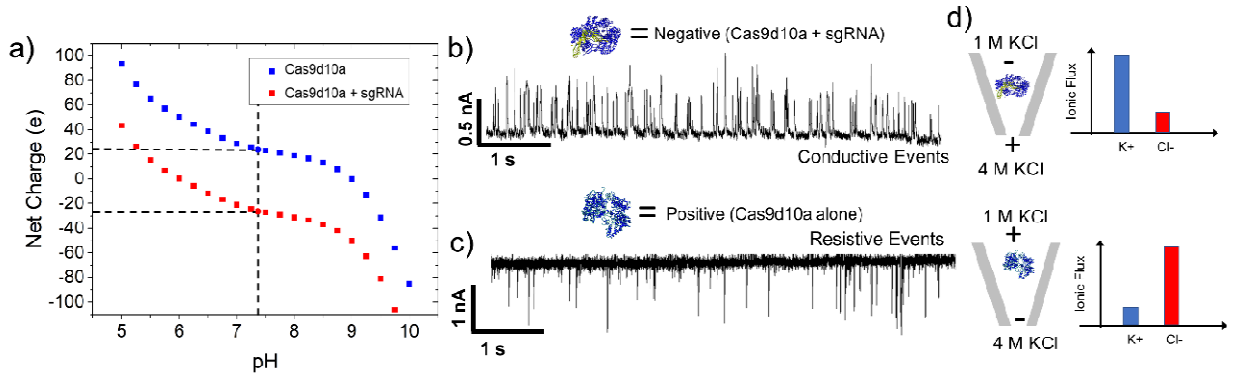

Figure 5: Event characteristics for Cas9d10a and Cas9d10a + sgRNA under asymmetric salt conditions (1M/4M KCl). (**a**) Graph of net charge based on calculations of amino acid composition of Cas9d10a alone and Cas9d10a + sgRNA at various pH values. The black dotted line represents the pH value of our working conditions (7.4) and the circles are the corresponding charges of both molecular complexes. (**b**) Cas9d10a was pre-incubated before dilution in 1 M KCl where the Cas9d10a and sgRNA were in an equimolar ratio (1:1). Events were conductive when Cas9d10a + sgRNA was inside the nanopipette and a negative voltage was applied inside the nanopipette. (**c**) Current trace of Cas9d10a in asymmetric salt conditions (1 M KCl inside and 4 M KCl outside). Events were resistive when Cas9d10a was inside the nanopipette, and a positive voltage is applied inside the nanopipette.

<u>Mechanistic Insight into Conductive Events</u>

How a flux imbalance yields CEs is yet to be addressed to date despite being a commonly observed phenomenon. Since the voltage at the extreme ends of the fluidic reservoirs is clamped, charge build-up (i.e., potassium) tends to generate a voltage that, in turn, lowers the effective

voltage for ion conduction at the pore. That is, especially with low electrolyte conditions, the ion selectivity of the charged pore surface could either increase or decrease the voltage drop through the tapered region by accepting (i.e., K^+) or rejecting (Cl^-) ions through EOF. In the case of asymmetric salt conditions, the ion flux is also dependent on the chemical potential gradient where ions move from high salt to low salt generating a charge density polarization effect. Depending on the space charge density distribution within the taper length of the nanopipette, the voltage drop in the tapered region could either increase or decrease leading to a corresponding electric field decrease or increase at the nanopore. Thus, there would either be charge accumulation or depletion within the pore through EOF pumping. When DNA enters the pore in a case where Cl^- flux is dominant, both charge depletion in the pore region and DNA volume exclusion would increase the pore resistance. However, on the contrary, when K+ flux is dominant, the charge accumulation and volume exclusion are opposing factors that would determine the final nanopore resistance during DNA occupancy. For low salt conditions, we speculate that a DNA-occupied pore transiently stops EOF pumping (i.e., the effective pore size decrease during DNA occupation which would result in diminishing EOF pumping) effectively lowering the charge stored inside the pore. Indeed, finite element methods demonstrate the accumulation of charge inside the glass pore (Figure 3f). Interestingly, the increase in stored charge with applied voltage is a characteristic trait of an ionic capacitor[51]. We believe that charge storage and dissipation dynamically impact the voltage at the pore therefore indirectly measures the occupancy of the molecule inside the pore. Under high salt conditions, resistive events also *indirectly* measure the DNA molecule through a reduction in ion flow. Low salt and asymmetric salt conditions may also *indirectly* measure the DNA's presence inside the pore, however, through flux imbalance and charge polarization effects. The voltage dependence and pore size

dependence of CEs was evaluated and described here which will help to further enhance the signal-to-noise of CEs for different molecules. In summary, a DNA occupied pore will always reduce ionic flux. In the case of CEs, the reduction in ion flux due to the pore being occupied by DNA is less than the flux due to the transient cessation of charging/polarization.

Concluding Remarks

Ionic-generated potentials are typically named according to the principle in which they are generated; for example, diffusion potentials, streaming potentials, and exclusion potentials[52]. Nevertheless, charge separation is a commonality of these potentials as well as our capacitor model which ultimately could generate voltage and current transients. Data thus far support the hypothesis that a flux imbalance plays an important role in the generation of CEs and the evidence here demonstrates the importance of the pore's charged surface, voltage-bias, and associated electro-hydrodynamics in generating CEs. In this study, we described multiple electro-hydrodynamic effects that influence EOF-driven DNA translocations under low ionic strength conditions. We have found that EOF can be used in various alkali chlorides. Confirmation that EOF capture volume resides along the sides of the tip aperture and directs flow inward has been shown. The resulting current enhancement or reduction dependence on pore size can be explained by a pore's flux imbalance. Secondly, we discovered a pulse crossover point from CEs to REs, independent of salt concentration and specific to LiCl, by scanning the applied voltage from -300 mV to -900 mV. We show that changing the electrolyte influences the event shape, SNR values, and event frequency. The pulse nature was also explored for proteins with Cas9 mutant, Cas9d10a, in both free form and bound to sgRNA wherein CEs were observed for the Cas9d10a- sgRNA complex and REs were observed for the free Cas9d10a protein. The pulse direction results were in good agreement with the flux imbalance theory

proposed for DNA. Evaluating polarization effects and its role in producing CEs will provide a framework for understanding experimental results at these low salt and asymmetric salt conditions. Therefore, we propose an additional possible theory for conductive events based on charge density polarization where accumulation of positive charge (for a negatively charged pore), via a flux imbalance, appears to effectively lower the voltage bias at the pore during open pore conditions and enhances the current when the equilibrium conditions are altered.

Methods

Nanopore fabrication began with quartz capillaries (Sutter Instrument Co.) of 7.5 cm in length, 1.00 mm in outer diameter, and 0.70 mm in inner diameter. Capillaries were plasma cleaned for five minutes before laser-assisted machine pulling to remove any surface contaminations. Afterwards, quartz capillaries were placed within the P-2000 laser puller (Sutter Instrument Co.) and a one-line protocol was used: (1) HEAT: 630; FIL: 4; VEL: 61; DEL: 145; PULL: between 135 and 195. This resulted in two identical, conical nanopores. The heat duration was approximately 4.5 s.

Electrodes were constructed using silver wires dipped in bleach for 30 minutes followed by thorough rinsing with water to remove any residual bleach. Freshly pulled nanopipettes were then backfilled with either 10 mM KCl, LiCl, or CsCl buffered at pH~7.4 using the Tris-EDTA buffer. The conductivities of each alkali chloride were recorded using an Accumet AB200 pH/Conductivity Benchtop Meter (Fisher Scientific). The results were as follows: 10 mM KCl= 0.26 S/m, 10 mM LiCl= 0.23 S/m, and 10 mM CsCl= 0.26 S/m at room temperature. An optical microscope was used to inspect the nanopipettes at this stage for any irregularities. Once the nanopipettes had been inspected, electrodes were connected to the head stage of the Axopatch 200B (Molecular Devices, USA). The Axopatch 200B patch-clamp amplifier was used in

voltage-clamp mode to measure the ionic current changes. The gain was optimized before each experiment and the signal was filtered with the inbuilt low-pass Bessel filter at 5 kHz and digitized using Digidata 1550B(Molecular Devices, USA). The data was acquired at a frequency of 250 kHz. Data analysis for DNA translocations and folding were performed using a custom MATLAB code.

COMSOL Multiphysics was used for modeling nanopipette geometries that were based on SEM and TEM images acquired from the same pipette pulling protocols that were used in sensing experiments. A 2D axisymmetric model was employed to reduce the computational resources required. Once the geometries were created in COMSOL, the physics that were utilized included Poisson–Nernst–Planck–Stokes equations: laminar flow, transport of diluted species, and electrostatics. The electrostatics boundary condition for the glass was set at a surface charge density of -2×10^{-2} C/m^2. To model electroosmotic flow, a volume force on the fluid was set to the space charge density of the ions in solution multiplied by the electric field vectors (r and z vectors). An in-built EOF boundary condition was also tested and yielded similar results. Diffusion coefficients and mobility values were obtained from Lee et. al.[48]. All models were tested with different solvers, solving conditions, and reservoir sizes to ensure the accuracy of results. The Stokes flow boundary conditions were no-slip, and the inlet and outlet were kept at the same 1 atm of pressure which is consistent with experiments. The z-component of the flux was extracted for each model from a 2D line that spans the width of the pore. The flux was then integrated across this 2D line to obtain the flux in moles/s.

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Contributions

K.J.F. formulated the idea to explore low ionic strength solutions using DNA and nanopores. L.S.L. performed the experiments, devised analysis protocols, and carried out data analysis under the guidance of K.J.F. M.N. performed preliminary experiments. N.F. simulated experiments using COMSOL. L.S.L, Y.M.N.D.Y.B, and K.J.F. wrote the manuscript with contributions from all other authors.

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Ethics Declarations

The authors declare no competing interests.

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