The Origin of Conductive-Pulse Sensing Inside a Nanopore and the Role of Electro-Hydrodynamics

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Abstract

Despite the highly negatively charged backbone of DNA, electroosmotic flow (EOF) within a nanopore can lead to DNA travelling opposite to electrophoretic forces (EPF) at low ionic strengths. However, EOF pumping and its role in producing current-enhancing events is ambiguous due to the complicated interactions between nanopore walls, DNA grooves, ion mobility, and counterion clouds. Here, we discuss how current-enhancing DNA events could be the result of a flux imbalance between anions and cations. The contributing factors for driving a flux imbalance within a nanopore include pore size, voltage bias, and type of alkali chloride electrolyte. Once the mechanism behind conductive events is established, the physics of transducing a DNA translocation into an electrical signal can be further exploited for improving DNA sequencing and, more broadly, bio-sensing.

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 invaluable real-time, single-molecule, electrical read-out platforms. Although the translocation of new biological entities is now a routine practice in labs across the world, the high electrolyte concentrations in which experiments are performed is rather unchanged since nanopores were first utilized in 1996[1]. The popularity associated with high electrolyte solution is largely due to the high signal-to-noise ratio (SNR) 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 has been studied extensively[2–4]. However, 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 wherein the DNA-occupied pore conducts more ions than when the pore is empty[7]. Therefore, events can be categorized as a current-reducing event (i.e. resistive event, RE), or a current-enhancing event (i.e. conductive event, CE). The question, "Why does ionic current *increase* during transient DNA occupancy of a nanopore?", remains unanswered and warrants further investigation.

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 EOF strengthens, sometimes leading to the translocation of molecules opposite of EPF (i.e. negative DNA traveling towards the negatively biased electrode).

Although EOF and CEs often coincide, it is important to note that they are not mechanistically linked. Reports of CEs occurring in nanopores where EOF is reduced to allow EPF-driven events also produced CEs[9]. Despite the large number of experiments describing CEs, the origins of CEs in the presence of low ionic strength has been elusive.

It has been generally accepted that CEs stemming from low ionic strength conditions occur because the introduction of additional DNA counterions (i.e. K^+) within the nanopore is greater than the number of ions within the empty pore[7]. Once electrolyte concentration decreases below roughly 0.02 M, mostly counterions are present within the pore, which explains the current enhancement[17,18]. Interestingly, at approximately 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 evidence for one of these hypotheses[6,19]. One recent experiment in particular conflicts with these hypotheses and may lead to a third potential theory; namely that current enhancement is not only a low salt phenomena but can also be observed at high, asymmetric salt conditions[20]. Above 1 M KCl, counterions should contribute very little to current modulation, which motivated us to explore event properties within this regime. Since a cohesive theory for the nature of conducting events is still elusive, we studied the transport of DNA within a nanopore using various monovalent salts.

Herein, we characterize EOF-driven events (anti-electrophoretic, or anti-EPF) with Lambda DNA (λ-DNA) and neutral polymers (i.e. polyethylene glycol, PEG) using quartz nanopores. Interestingly, we found that current enhancements can be observed using PEG; casting doubt on counterions being the dominant explanation for CEs. Furthermore, DNA CEs are extremely cation-, pore size-, and voltage-specific and may be the result of an imbalance of ionic fluxes. We will discuss the electrokinetic and hydrodynamic phenomena that affect event shape such as counterion cloud, ion mobility, pore size, and electrolyte composition. This report elucidates some of the fundamental prerequisites for observing CEs when DNA translocates 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. membrane thickness), nanopipettes have a gradual taper length (Fig. 1a) that increases the sensing region of the device[21]. We fabricate nanopipettes by laser pulling glass nanocapillaries, producing two identical quartz nanopores. With this technique, we can achieve <10 nm inner pore diameters, Figure 1a. This process is fast, inexpensive, and does not require a clean-room environment[22].

Current-voltage (I/V) analysis reveals that the conductance of the pores (Fig. 1b) varies between 0.58 and 5.35 nS; as well as the presence of ionic current rectification[23]. These conductance values are consistent with pore diameters between 5 (± 0.5) and 48 (± 4) nm,

respectively. Specifically, the relationship between pore conductance (G) and inner diameter (d_i) allows us to estimate the size of the aperture[24,25]:

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

Where l is the length of the conical pore (taper length), g is the measured conductivity of the buffer, and d_b is the diameter of the capillary at the beginning of the conical taper. The initial, inner capillary diameter is constant in our experiments (0.7 mm) and the buffer conductance depends on concentration and alkali chloride used. The taper length was measured using transmission electron microscopy and I/V analysis is used to measure the pore conductance. Pore sizes are also occasionally confirmed using transmission electron microscopy.

After retrieving I/V information, translocation experiments with λ-DNA at 500 pM were performed in 10 mM Tris-EDTA (pH 7.4). When EPF dominates, the capture volume outside the nanopore assumes a nearly spherical shape surrounding the pore's oriface[26–30]. As ionic strength decreases, EOF can dominate as the primary means for DNA entering the pore but is pH dependent since a high surface charge on the glass is required[31]. According to the EOF streamlines, the capture volume adopts a shape confined along the sides of the pore[32]. 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[32].

Finite element analysis was performed to determine the fluid flow rate at different voltages (Fig. 1c). As the applied voltage decreases from 0 mV, the mean fluid velocity increases *into* the glass pore. The same is true for positive voltages, however, the fluid flow direction switches (flow reversal) from towards the pore at negative voltages to away from the pore at positive voltages. Notably, these fluid velocities can influence DNA dwell time inside the pore and have been described using hydrodynamic drag[33,34].

DNA proceeds to diffuse around the solution until it enters the EOF capture volume, where it is then transported through the pore. This method of translocation is further illustrated (Fig. 1d) with KCl as the electrolyte and K^+ ions responsible for the movement of water carrying λ-DNA. Under these conditions, it is possible to see differing DNA configurations: linear, partially folded, and fully folded (Fig. 1d). Reports of different DNA configurations have been witnessed using high ionic strength conditions and with both planar nanopores[35–38] and nanocapillaries[24]. The ability to discriminate folding states using DNA CEs does not directly help uncover the nature of CEs, but it is important to recognize the existence and understand the effects of having various DNA configurations upon translocation.

To show that this finding is not limited to low ionic strength phenomenon, we employed salt concentration gradients as previously described[20]. As shown in Figure 1e, our experimental set-up involved having a solution of 1 M KCl + λ-DNA inside the nanopore with 4 M KCl outside. With an applied voltage of -600 mV, λ-DNA was driven outside the pore through EPF, resulting in CEs. An additional salt gradient was implemented (Fig. 1f) with 4 M KCl inside the nanopore and 1 M KCl + λ-DNA outside. In this situation, EPF drove λ-DNA to translocate through the pore, again resulting in CEs. Based on these results, a working hypothesis was made that the existence of CEs stem from a flux imbalance between anions and cations. 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. EOF pumping of water

into the pore, for example, can change the relative fluxes of ions. Since the electric field acts equally on both chloride and potassium ions, the net movement of water only provides a moving frame of reference which favors one ion over another. Nevertheless, total ionic current is constant regardless of EOF velocity. For the data shown where CEs are observed (Fig. 1 d-f) we speculate that there is a net flux that favors potassium ions. Figure 1g illustrates how K^+ ions are pumped into the pore under low ionic strength (EOF; Fig. 1d) and concentration gradient (asymmetric) conditions (EPF; Fig. 1e).

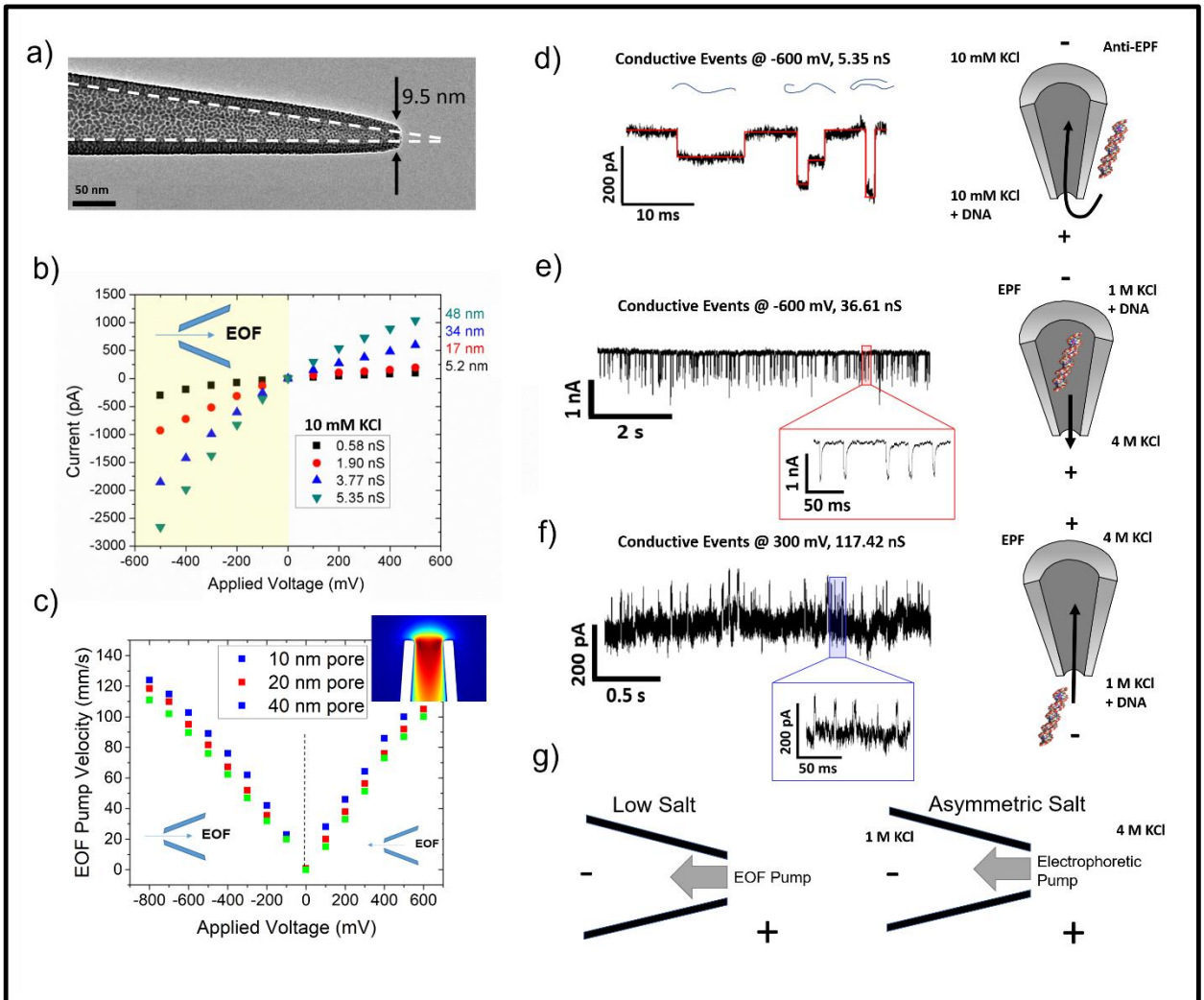

Figure 1: Experimental set-up and characterization of quartz nanopores. (**a**) TEM of quartz nanopore; scale bar, 50 nm. (**b**) I/V curves for quartz nanopores for four differently sized pores. (**c**) Simulations of EOF pump velocity at various voltages within quartz nanopores; pore diameter was 20 nm and was symmetrically filled with 10mM KCl. EOF reversal also shown. Inset: fluid velocity distribution near the pore. (**d**) Schematic and corresponding current trace of linear, partially folded, and fully folded DNA configurations for EOF-driven translocations. (**e**) Current trace of EPF-driven events at -600 mV under influence of salt gradient. Red inset: close-up of above current trace to show event signature and SNR. (**f**) Current trace of EPF-driven events with salt gradient at 300 mV. Blue inset: Magnified region of above current trace to show event signature and SNR. (**g**) Low ionic strength, EOF pumping yields high current-enhancing events as well as electrophoretic pumping of cations from the 4 M KCl solution into the nanopore.

Validation using finite element methods was undertaken to further explain the potential impact that EOF has on DNA sensing and the unique capture dynamics of EOF-driven events. A 20 nm pore was modeled with 10 mM and 2 M salt to demonstrate the difference in capture kinetics of DNA. At low ionic strength conditions, fluid velocity is plotted with colored streamlines indicating where the fluid entering the pore is coming from within the bath solution. Since DNA events only occur anti-EPF, mapping the fluid motion is indicative of the capture zone. To experimentally validate the finite element analysis (Fig. 2a), λ-DNA was tagged with YOYO-1 and the nanopipette tip placed in the focal plane of a water immersion objective (Nikon, NA=1.2). A stacked timeseries of images (acquired from a Princeton Instruments ProEM EMCCD) allowed us to observe λ-DNA capture at -700 mV (Fig. 2a inset reveals that fluid motion along the sides of the pore is responsible for λ-DNA translocation). At high ionic strength conditions, DNA travels through the pore via EPF and so the electric field lines are plotted (Fig. 2b) and represent the capture zone.

Realizing that the capture volume in EOF-driven translocations surrounds the outer walls of the nanopipette, we chose to expand and shrink the capture volume via a depth-dependent study to witness any changes in event frequency (Fig. 2c). By submerging varying lengths of the taper length inside the salt solution containing λ-DNA, the capture volume is controlled (Supplementary Fig. 1). The nanopore was suspended at 0, 0.26, 0.53, 1.1, and 4.0 mm below the electrolyte solution surface containing 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 2.53 nS pore. Capture rate was calculated at each depth. 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.

In order to understand how electro-hydrodynamics influences ionic flux, particularly at low salt conditions, three monovalent salts were modelled by altering the cation diffusion coefficient and electrophoretic mobility (Fig. 2d and e). Although the pore's total ionic flux was not altered significantly by EOF since K^+ flux increased and Cl^- flux decreased by the same amount, EOF does significantly impact the flux imbalance between cation and anion. This finding was particularly noteworthy since CEs have been observed at high asymmetric salt conditions which would also change a pore's ionic flux imbalance. These results predict that a flux imbalance in favor of Cl^- transport leads to REs and a flux imbalance in favor of K^+ leads to CEs. This is based on the experimental results that the 10mM KCl electrolyte always produces CEs. In Figure 2d, anion-dominant flux only occurs with small pore sizes, 20 nm and less, and an applied negative voltage between -300 and -400 mV. It is important to note that no events could be recorded at these conditions to find out whether resistive events are observed. For a nanopore suspended in LiCl, we observed more opportunities for the pore to be Cl^- selective, which we predict will result in REs upon translocation of λ-DNA. As the pore increases in size or an increasingly negative voltage is applied, the pore can become cation selective, which we speculate can give rise to CEs.

Likewise, a comparison between symmetric low salt conditions and asymmetric high salt conditions was performed using 20 nm pores (Fig. 2f). As previously stated, a 20 nm pore under low ionic strength KCl conditions has few opportunities to have a flux imbalance in favor of Cl^-

transport. This can occur at positive applied voltages as well as small negative voltages for KCl (between 0 and 400 mV). In order to improve on our experimental design and use conditions that favor cations (or anions), asymmetric salt conditions can be useful and have already proved to yield CEs. This was also modelled, in which 1 M KCl is inside the pore and 4 M KCl is outside, and the result was that the pore favored K^+ transport due to electrophoretic pumping of the cation.

This computational finding further supports the previous experimental observations of CEs occurring under asymmetric salt conditions as DNA translocates the pore.

Although the ionic diffusion coefficients and electrophoretic mobilities encapsulate basic transport properties, all the while being utilized as variables in the finite element simulations, they neglect the geometric size of the ions and therefore the packing density/strength on oppositely

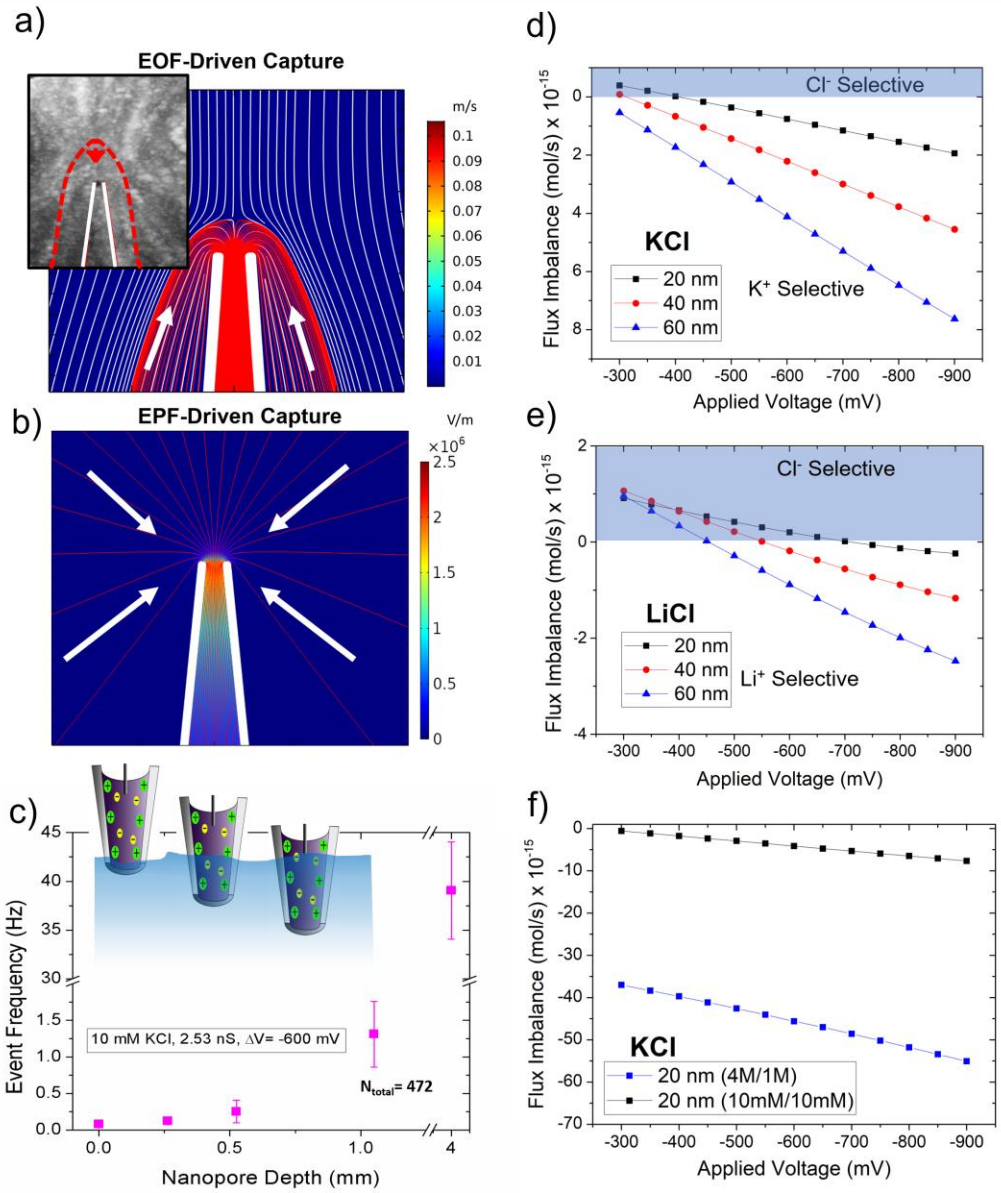

Figure 2: Finite-element modeling and experimental characterization of EOF. Two-dimensional axisymmetric modeling of (**a**) EOF dominant capture in which white and red lines show fluid streamlines (inset: YOYO-labelled λ-DNA sample with an applied voltage of -700 mV). Red lines depict EOF-capture volume for a 20nm pore at a negative 600 mV voltage bias. (**b**) Electric field lines depicting the expected trajectories of charged molecules during EPF capture. Direction of electric field is shown with white lines at -600 mV. (**c**) Frequency of events as the pore is increasingly submerged into the 10 mM KCl + λ-DNA solution. Error bars made by using the standard deviation of event frequencies. Figures showing ion mobilities of (**d**) 10 mM KCl and (**e**) 10 mM LiCl for various pore sizes. (**f**) Flux imbalance comparison between pores (20 nm diameter) with symmetric low salt conditions (10 mM/10 mM) and asymmetric high salt conditions (4 M outside/1 M inside).

charged surfaces. In order to understand the link between electro-hydrodynamics and Debye layer screening of the quartz surface charge, streaming current measurements were used as a proxy for cation mobility within the diffuse ion layer. Contrary to EOF, where mobile ions drag fluid, streaming currents measure the fluid's ability to drag along ions co-axial to the fluid motion[39]. A pressure bias was used to generate a streaming current and the resulting data can be seen in Supplementary Figure 2. Negative pressures generate a flow into the nanopore and in the same direction as EOF in our experiments. We see that larger pressures create larger streaming currents. Interestingly, LiCl has significantly higher streaming currents compared KCl and CsCl at negatively biased pressures. Overall, these results indicate LiCl-filled nanopores can be Cl^- flux dominant at low voltages, and secondly, Li^+ has a higher flux under pressure biased fluid flow compared to other cations (K^+ and Cs^+). The same pore (1.30 nS in 10 mM KCl) was used for all measurements to reduce variability due to different pore sizes.

DNA and Neutral Polymers in Potassium Chloride

Under high ionic strength conditions, pores with a diameter slightly larger than the analyte molecule yield greater SNR values when compared to larger pores[19]. Because of this, we were motivated to explore SNR values under low ionic strength conditions. A typical conductive DNA event can be seen in Figure 3a in 10 mM KCl. Potassium chloride was chosen as the electrolyte because it is most frequently utilized in nanopore research due to similar ion mobilities of anions and cations. We incorporated differently sized pore diameters to witness any effect that pore size may have on event shape and size. The depth of each nanopore was kept consistent for all recordings as well as the voltage (-600 mV).

As λ-DNA translocates through the nanopore, we witness a current-enhancing event. For all SNR calculations, we omitted all configurations except for linear DNA translocations (Fig. 3b). DNA has the ability to translocate linearly, folded[8,24], or in knots[35], in which the latter two increase the current change. To ensure DNA configuration had no effect on SNR, we applied only linearly translocating DNA to our calculation.

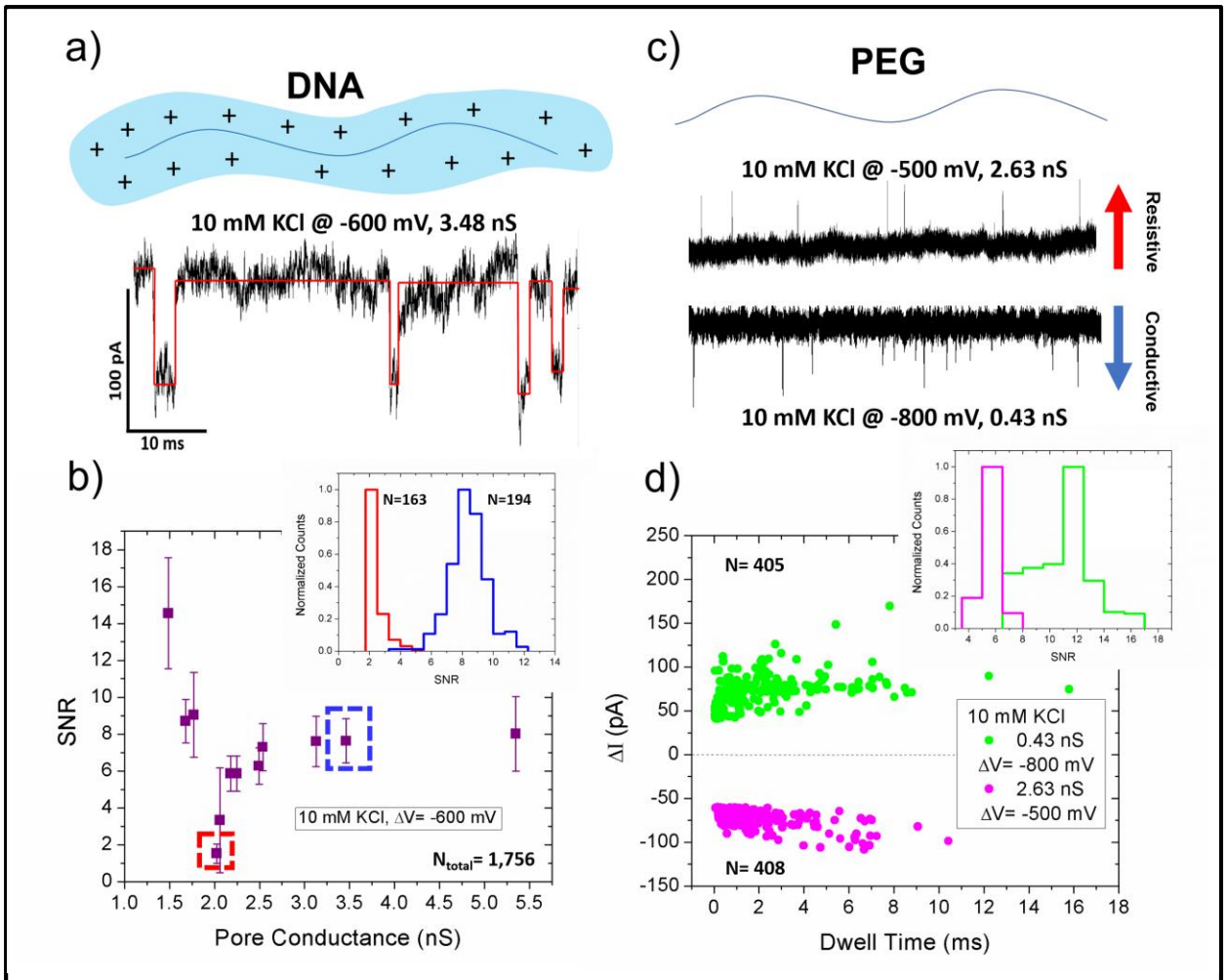

Figure 3: Capture of λ-DNA and PEG using EOF. (**a**) Top: depiction of DNA and its counterion cloud. Bottom: current trace of λ-DNA translocation in 10 mM KCl. (**b**) SNR calculations for various pore sizes (inset: normalized histogram of data sets from red and blue dotted squares). Each symbol and error bar represent the mean and standard deviation of SNR for one pore. (**c**) PEG illustration (no counterions present; top) along with current traces of resistive and conductive events (bottom) (**d**) Scatter plot showing the relationship between current change and dwell time for two different pore sizes (inset: normalized histogram showing SNR between the two aforementioned data sets).

We witness an increase in SNR starting at 2.00 nS and saturating around 3.00 nS. To determine whether the current enhancement or the noise of the signal is the major contributor to the increase in SNR, we acquired the median current change of all events and the root mean square (RMS) noise of a data segment lacking events. We witness that the RMS noise maintaining values of 15 ± 7 pA, whereas the current change increases from 30 to 140 pA as pore size increases. For the left side of the graph, we witness a sharp increase in the SNR as the pore size decreases. This can be explained by a decrease in the noise associated with smaller pores. As seen in Supplementary Figure 3, the RMS noise is extremely low (< 10 pA) whereas the median current change is approximately 100 pA. Therefore, the higher SNR values for smaller pores stem from lower noise. On the right side of the graph, we speculate that the rise in SNR (and current enhancement) is a result of greater EOF pumping as a function of pore size (Supplementary Fig.

4). Owing to larger fluid velocities, the flux imbalance highly favors potassium rather than chloride.

The common hypothesis that DNA counterions are the sole mechanism of CEs led us to explore PEG under low ionic strength conditions with an applied negative voltage[40]. PEG 20,000 was diluted to 15% (w/w) in 10 mM KCl and voltage was applied from -100 to -1000 mV, in increments of 100 mV. PEG events were independently observed with 5 different pores. Interestingly, CEs were most notable at extremely small pore sizes (e.g. 0.43 nS); a pore size regime that we could not observe DNA events. Since EOF flow rate (e.g. fl/s) decreases with smaller pore sizes and EPF increases, we believe DNA could not energetically overcome the barrier at the pore entrance for translocations to occur. Since PEG is neutral, we were able to observe EOF-driven events at very small pore sizes (Supplementary Fig. 5). The results indicated that smaller pore sizes resulted in CEs whereas larger pore translocations yielded REs (Fig. 3c and d). SNR calculations showed that the smaller pore diameter yielded higher SNR values in comparison to larger pore diameters. In both pores, the median current change was 71 ± 1 pA; whereas the RMS noise increased from 7 to 18 pA as the pore size increased from 4 to 25 nm in diameter, respectively. Based on these results, the nature of the event (CEs versus REs) seems un-coupled from the analyte counterions (or lack thereof, in the case of PEG) but rather linked to the pore size and/or voltage in which translocations occur. For the same conductance (i.e. nanopore size) of 2.6 nS, DNA yielded CEs and PEG yielded REs. Two other possibilities exist for why PEG and DNA produce different event signatures at this pore size: (1) PEG could be interacting with the charged glass surface far from the pore and could impact EOF pumping[9] and therefore could be impacting the pore's flux imbalance or (2) the size of PEG (150-fold smaller in length compared to λ-DNA) limits the extent in which EOF pumping is blocked. Nevertheless, it is clear that the analyte counterions do not seem to play a significant role in generating CEs for extremely small, negatively charged pores.

Voltage Dependence with Lithium Chloride

Lithium chloride was chosen as an electrolyte because it has been previously shown to "slow-down" DNA translocations under high ionic strength conditions[41]. This can be attributed to Li^+ having a smaller atomic radius than K^+ and therefore, Li^+ binds to DNA stronger than K^+ [41]. Additionally, LiCl had a significantly higher streaming current (Supplementary Fig. 2) compared to both KCl and CsCl. Finite element simulations indicated a voltage and pore size dependence for flux imbalance that was within the voltage range: -400 to -1000 mV, where events are typically observed. The nanopore containing 10 mM LiCl was inserted inside a solution containing 10 mM LiCl + λ-DNA and current changes were recorded at various voltages (Fig. 4a). The same series of steps were repeated to calculate the SNR at each voltage.

Using the same pore (1.20 nS), we witnessed the crossover point that is independent of salt concentration, which is something not previously observed. At voltages of -300 and -500 mV, λ-DNA translocations resulted in REs and at voltages of -700 and -900 mV, DNA translocations resulted in CEs, as shown in Figure 4b. Interestingly, at an applied voltage of -600 mV the event current shape assumes both a resistive and conductive spike (Supplementary Fig. 6). For this pore, we see an increase in the amplitude of the REs as the voltage applied is reduced to -600 mV. Less than -600 mV (i.e. more negative), the CE amplitude continues to increase as the voltage decreases

to -900 mV. The events recorded at -900 mV and -500 mV yielded higher SNR values in comparison to -700 mV and -300 mV, respectively (Fig. 4c and d). Supplementary Figure 7 shows how the median current change is the main contributor to the SNR fluctuation, the RMS noise for each voltage remains relatively constant. The transition from REs to CEs can be understood by the pore being anion selective at low voltages and cation selective at higher voltages. The respective flux imbalances for each voltage applied can be seen in Supplementary Figure 8. As the applied voltages increase in negativity, the change in current switches to a CE. The biphasic nature of the events at the transitional voltages (-500 mV and -700 mV) suggests that there may be two mechanisms of current modulation (hydrodynamic flow and pore occupancy) that can occur when the DNA molecule is near or entering the pore. DNA entering the flow field of the pore during EOF pumping may cause current modulations that occur immediately prior to translocation.

Another comparison was done using two pores with inner diameters of 33 ± 3 nm. One pore contained 10 mM KCl and was suspended in 10 mM KCl + λ-DNA while the other contained 10 mM LiCl and was suspended in 10 mM LiCl + λ-DNA. Both had an applied voltage of -600 mV and we witnessed CEs for the pore containing KCl and REs for LiCl (Fig. 4e). At -600 mV with the aforementioned pore size, finite element simulations predicted that the nanopipette is cation selective in KCl and anion selective in LiCl, which may be a possible explanation for the event types observed. We also note that KCl and LiCl have similar event durations at these low salt conditions, however, KCl has a much larger variation in the degree of current-modulation (in the case of KCl: current enhancement). The current-reductions observed for LiCl are much more tightly clustered together compared to KCl CEs. The source of the variability observed in KCl CEs is still not fully understood and requires further investigation. The data seems to suggest that CEs are more variable regardless of the cation. The LiCl events in Fig. 4b, for example, show a much greater degree of scatter for CEs compared to REs.

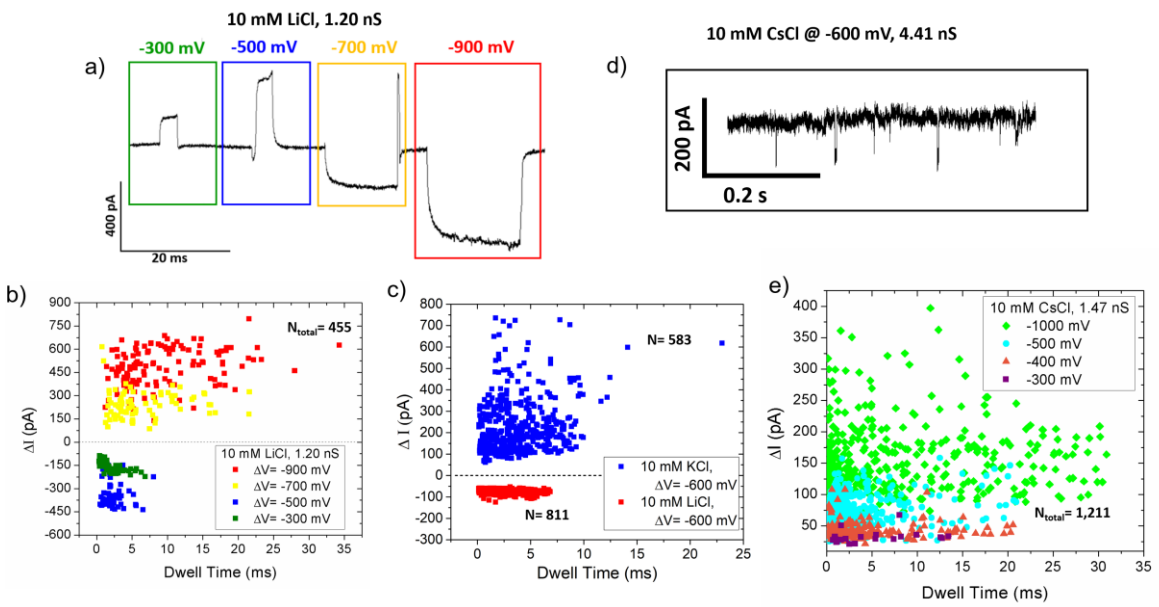

Figure 4: Event shape transition from resistive to conductive as the voltage applied increases in negativity. (**a**) Current traces of each typical event. (**b**) Scatterplot showing current change and dwell time relationship with applied voltage for λ-DNA in 10 mM LiCl for a pore with conductance of 1.20 nS. (**c**) Comparison of current change and dwell time between 10 mM KCl and LiCl (both 33 ± 3 nm pore diameters) at the same voltage showing that LiCl produced resistive events under the same conditions as KCl's CEs. (**d**) Current trace of λ-DNA in 10 mM CsCl for a pore with conductance of 4.41 nS (representative trace of 4 pores). (**e**) Current change versus dwell time for various applied voltages showing that CEs were observed for all voltages ranging from 300 mV to 1000 mV.

Alkali Chloride Dependence on Event Characteristics

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 10 mM CsCl inserted into 10 mM CsCl + λ-DNA. The typical current trace and event signature is displayed in Figure 4d.

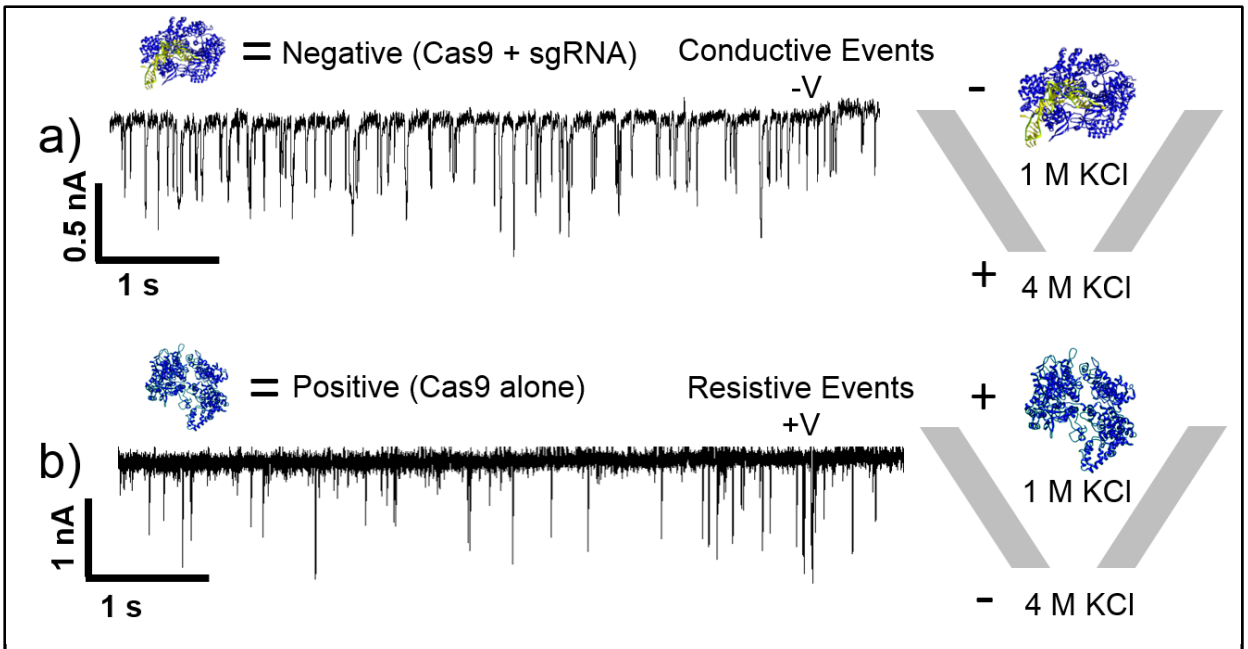

Figure 5: Event characteristics for Cas9 and Cas9 + sgRNA under asymmetric salt conditions (1M/4M KCl). (**a**) Cas9 was pre-incubated prior to dilution in 1 M KCl where the Cas9 and sgRNA were in equimolar ratio (1:1). Events were conductive when Cas9 + sgRNA was inside the nanopipette and a negative voltage was applied inside the nanopipette. (**b**) Current trace of Cas9 in asymmetric salt conditions (1 M KCl inside and 4 M KCl outside). Events were resistive when Cas9 was inside the nanopipette, and a positive voltage is applied inside the nanopipette.

Similar to KCl, we do not see a voltage dependence on event shape with CsCl, which is not surprising considering that K^+ and Cs^+ have nearly the same diffusion coefficient[42]. For confirmation, a pore with a conductance of 1.47 nS (14 ± 2 nm diameter) was used with λ-DNA. Under low ionic strength conditions, we applied voltages of -300 mV, -400 mV, -500 mV, and -1000 mV to witness any transition in event shape (Fig. 4e). All voltages resulted in CEs, which was predicted based on finite element analysis under the assumption that cation selective conditions yield CEs. Simulation results for CsCl can be found in Supplemental Figure 9, but were nearly identical due to the diffusion coefficients for KCl and CsCl being 2.02×10^{-5} and 2.00×10^{-5} cm^2/s, respectively[42].

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. A reversed voltage polarity would therefore generate a flux imbalance that favors chloride ions. We chose to study the Cas9 protein because alone it carries a net positive charge (at pH 7.4) and once bound to sgRNA, the complex becomes highly negatively charged[43]. The pH was not changed since this may have an unwanted effect on the nanopore's surface charge. 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 Cas9 protein was placed inside the nanopipette (in 1 M KCl) with and without sgRNA (Fig. 5a and b, respectively). Similar to DNA, Cas9 + sgRNA produced CEs at negative voltages. Under this condition, potassium from

the outside (4 M KCl) is driven into the pipette. After pre-incubating Cas9 with sgRNA (equimolar for 1 hour at room temperature), the complex was diluted in 1 M KCl and placed inside the nanopipette. At positive voltages, Cas9 produced REs. At this condition, chloride from the outside (4 M KCl) is driven into the pipette. The events at positive voltage could indeed be from either Cas9 + sgRNA or sgRNA alone since both are negatively charged. However Cas9 binding of sgRNA is typically fast with slow reversible reaction kinetics[44].

How a flux imbalance yields CEs has yet to be addressed. The working hypothesis currently is that stored charges can accumulate at the nanopipette tip effectively acting as a capacitor in series with the highly resistive nanopore. Since the voltage at the extreme ends of the fluidic reservoirs are clamped, charge build-up (i.e. potassium) tends to generate a voltage that, in turn, lowers the effective voltage at the pore. Importantly, this effect does not occur with chloride since a negatively charged ion will avoid the negatively charged surfaces within the nanopipettes taper. We speculate that a DNA-occupied pore transiently stops EOF pumping and thereby lowers the stored charge inside the nanopore and that the capacitor discharges current proportional to the blocked EOF. Finite element methods demonstrate the accumulation of charge inside the glass pore (Fig. 6a). The increase in stored charge with applied voltage is a characteristic trait of an ionic capacitor. Upon solving for the effective capacitance, we obtain a value of 4×10^{-17} Farads, as described in Figure 6b. The timescale of charging and discharging accumulated charge is also fast (3-5 µs to reach steady state space charge density; Fig. 6c). The occupied pore will always have a higher resistance to ion flux as shown in Figure 6d. In this scenario, the only way to increase current is to change the voltage to a higher absolute value. We believe that charge storage and dissipation impacts the voltage in a dynamic way throughout the translocation process. A circuit model (Fig. 6d inset) is proposed where in translocation events turns a switch that blocks the charging of the nanopore capacitor. For a charged capacitor (i.e. open pore), the applied voltage will be distributed over the capacitor and the resistive nanopore. As a translocation event occurs, not only is the full applied voltage transferred to the resistive nanopore, but stored charge may also contribute to the overall current measured by the electrodes.

Outlook

Ionic-generated potentials are typically named according to the principle in which they are generated. For example, diffusion potentials, streaming potentials, and exclusion potentials[45]. 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 supports the hypothesis that a flux imbalance plays an important role in the generation of CEs. The existence of CEs with PEG (e.g. using a 0.43 nS pore) further demonstrated that charged analytes are not a pre-requisite for CEs, but may indeed have an important role depending on the pore size. For example, a 2.63 nS pore filled with 10mM KCl produced CEs when DNA was the analyte, and REs for PEG at the same conditions. We speculate that the analyte and its concentration in the reservoir can transiently impact a pore's flux imbalance via translocation, or indirectly via interactions with the glass surface (i.e. outside the pore). For example, adsorbed molecules on the glass surface will hinder EOF pumping velocities and therefore the flux imbalance. Nevertheless, the evidence here demonstrates the importance of the pore's charged surface, voltage-bias, and associated electro-hydrodynamics in generating CEs.

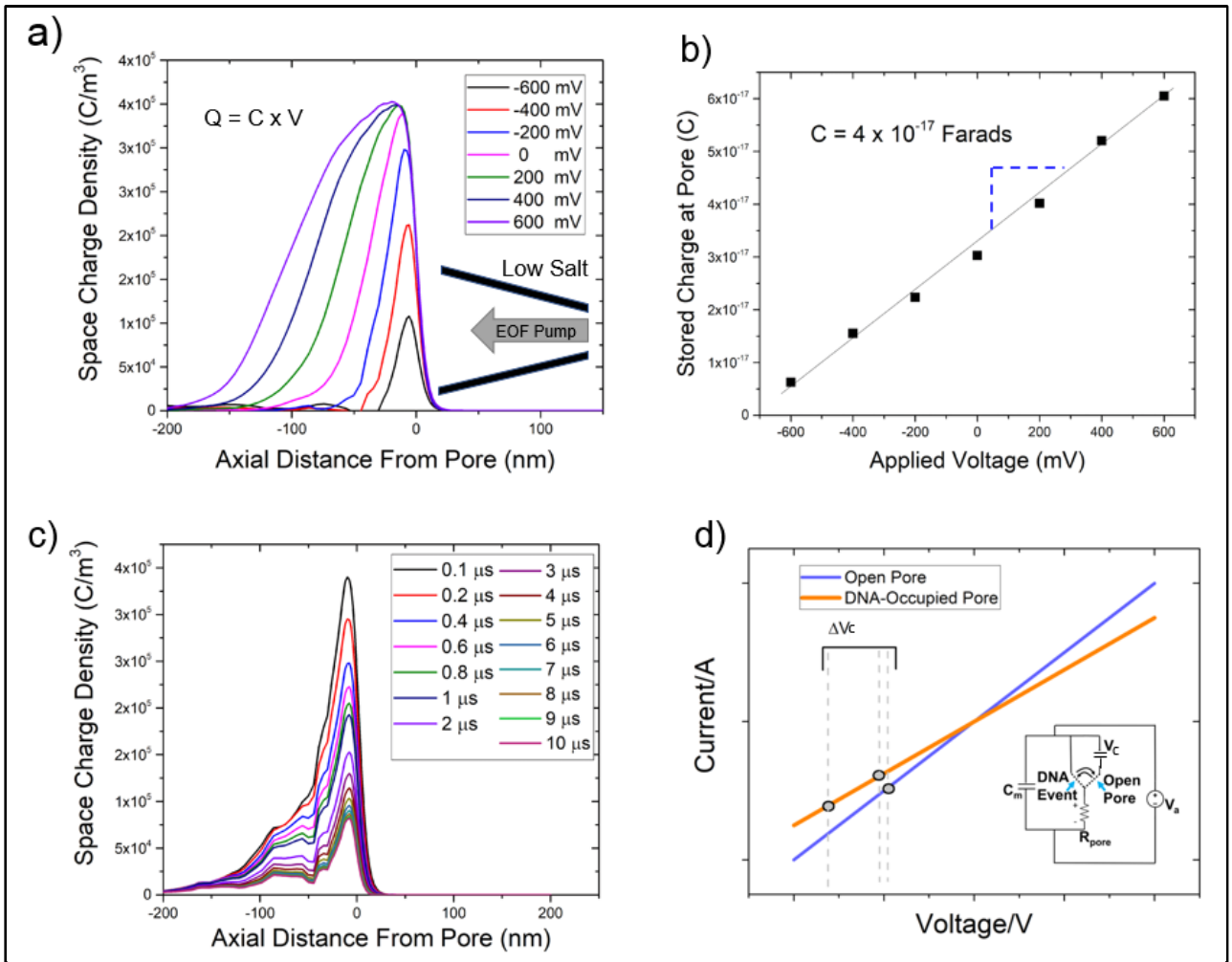

Figure 6: Stored charge model for flux imbalances. (**a**) Space charge density within a glass nanopore; axial distance of zero corresponds to the tip of the nanopipette. Pore diameter was 20 nm and was modelled with 10 mM KCl. (**b**) Integrated stored charge within the nanopore as a function of applied voltage; capacitance is equal to the slope (4×10^{-17} F). (**c**) Timescale of charge dispersion once EOF pumping is removed (voltage bias= -600 mV). (**d**) Proposed mechanism of how a nanopipette with potassium ions stored within the taper can yield CEs. An occupied pore will always act to reduce the current-voltage characteristics of the pore however the translocation event itself can cause transient blockage of potassium ions which act to charge the nanopore capacitor. The net result is that the pore experiences a voltage transient that shifts the voltage to a higher absolute value. Small voltages may yield resistive events (such as λ-DNA in LiCl) but will transition to conductive events at higher voltages (λ-DNA in LiCl).

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 and be used to translocate (un)charged molecules. 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 crossover point, 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. Finally, by utilizing salt gradients to generate a flux imbalance, extremely high signal to noise ratios were achieved. Such information is valuable in the pursuit of using solid-state nanopores to sequence polynucleotides and as a diagnostic test.

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 prior to laser assisted machine pulling to remove any surface contaminations. After, quartz capillaries were placed within the P-2000 laser puller (Sutter Instrument Co.) machine where a CO_2 laser heated the center of the capillary while the ends were pulled away from each other. A one-line protocol was used: (1) HEAT: 630; FIL: 4; VEL: 61; DEL: 145; PUL: between 135 and 195. This resulted in two identical, conical nanopores. The heat duration was approximately 4.5 s.

Electrodes were constructed using silver wire dipped in bleach for 30 minutes and then rinsed. Nanopores were then backfilled with either 10 mM KCl, LiCl, or CsCl. An optical microscope was used to inspect the nanopores at this stage for any irregularities. Once the nanopore had been inspected, it was secured in our Axopatch set-up. Electrodes were then placed inside the pore and the solution containing λ-DNA. The Axopatch 200B patch-clamp amplifier (Molecular Devices, USA) was used in voltage clamp mode to measure the ionic current changes. The gain was optimized prior to each experiment and the signal was filtered with a low-pass filter at 5 kHz. Data analysis for DNA translocations and folding were performed using a custom MATLAB code.

For the microscopy experiments, λ-DNA with a stock concentration of 500 $\mu g ml^{-1}$ was purchased from New England Biolabs. Dilutions were performed in either 10 mM KCl to create a 500 pM concentration of λ-DNA. Afterwards, λ-DNA was incubated with YOYO-1 (Molecular Probes) for 30 minutes. Videos and images were captured using a 60X water-immersion objective.

COMSOL Multiphysics was used for modelling 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 computational resources required. Once the geometries were created in COMSOL, the physics that were utilized included: laminar flow, transport of diluted species, and electrostatics. The electrostatics boundary condition for the glass was set at a surface charge density of -1×10^{-2} C/m^2. In order 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). Diffusion coefficients and mobility values were obtained from Lee et. al.[42]. All models were tested with different solvers, solving conditions, and reservoir sizes to ensure accuracy of results.

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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 guidance of K.J.F. M.N. performed preliminary experiments. N.F. simulated experiments using COMSOL. L.S.L 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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