Slowing DNA Translocation through Nanopores Using a Solution Containing Organic Salts

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One of the key challenges to nanopore DNA sequencing is to slow down DNA translocation. Here, we report that the translocation velocities of various DNA homo- and copolymers through protein pores could be significantly decreased by using electrolyte solutions containing organic salts. Using a butylmethylimidazolium chloride (BMIM-Cl) solution instead of the commonly used KCl solution, DNA translocation rates on the order of hundreds of microseconds per nucleotide base were achieved. The much enhanced resolution of the nanopore coupled with different event blockage amplitudes produced by different nucleotides permits the convenient differentiation between various DNA molecules.

Introduction

Development of a high-throughput and cost-effective DNA sequencing method provides invaluable information on the biological and biomedical fields and also makes vital contributions to many areas of high priority research such as forensics, archeology, and anthropology.[1,2] Although significant reductions in DNA sequencing costs have been achieved in the past 25 years, fundamentally different approaches will be required to drastically reduce the cost and increase the speed of routine complete genome sequencing.[2] Among the various DNA sequencing methods under development, the nanopore approach has emerged as one of the most promising technologies to achieve the "$1000 genome" goal set by the U.S. National Institutes of Health.[2,3] In the nanopore method, single-stranded DNA (ssDNA) molecules are electrophoretically driven through a nanochannel, and the discrimination of polynucleotides might be achieved based on their different current signatures, represented by residence times and/or current blockage amplitudes in the pore.[4] However, one of the major hurdles of utilizing nanopores to sequence ssDNA molecules is that DNA polymers translocate through the nanopore very rapidly.[5] For instance, the translocation rate of polydeoxycytosine was ~1 μs/base, whereas that of polydeoxyadenine was ~3 μs/base.[6] This rate requires a high temporal resolution for the accurate detection of single bases, which cannot be provided by the currently available single-channel recording technique.[3] To increase the nanopore resolution for nucleotide differentiation, many attempts have been made to slow down DNA translocation. It has been shown that a decrease in the experimental temperature allowed ssDNA molecules to be electrophoretically driven through the pore more slowly.[6,7] Further, DNA translocation could be manipulated by changing the applied potential.[8] Other approaches include sequence-specific detection of individual DNA strands,[9] formation of DNA-hemolysin rotaxane,[10] differentiation of nucleotide bases in a host β-cyclodextrin compound,[11] and immobilization of DNA polynucleotides with streptavidin.[12] In addition, it was reported that the detection of DNA sequences could be achieved by using an alternating electric field in a nanopore capacitor.[13]

In this study, we investigate the feasibility of utilizing aqueous solutions of ionic liquids to slow ssDNA translocation in the α-hemolysin pore. The study of ionic liquids is currently an active research area. They have been used in various applications, including organic synthesis,[14] extraction,[15] separation,[16] catalysis,[17] and electrochemical studies.[18] In previous work, a solution containing ionic liquid butylmethylimidazolium chloride (BMIM-Cl) was used as a supporting electrolyte in the nanopore stochastic detection of liquid explosives and monovalent cations.[19] The results suggested that the use of BMIM-Cl solution instead of the commonly used NaCl/KCl solutions could improve nanopore resolution.

Experimental Methods

Materials and Reagents. ssDNA samples, including $(dA)_{20}$, $(dC)_{20}$, $(dT)_{20}$, $(dCdT)_{10}$, and $(dC)_{10}(dT)_{10}$, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Lipid 1,2-diphytanoylphosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). Teflon film was purchased from Goodfellow (Malvern, PA). All of the other reagents including butylmethylimidazolium chloride (BMIM-Cl) and tetramethylammonium chloride (TMA-Cl) were purchased from Sigma Aldrich. All the ssDNA polymers were dissolved in HPLC-grade water (ChromAR, Mallinckrodt Baker). The concentrations of the stock solutions were 4 mM for each of the DNA samples. All the three electrolyte solutions used in this work, i.e., 1 M BMIM-Cl, 1 M TMA-Cl, and 1 M NaCl, were prepared in HPLC-grade water and buffered with 10 mM Tris (pH = 6.0).

Preparation and formation of wild-type and mutant protein pores has been described elsewhere.[20] Briefly, the mutant αHL M113F gene was constructed by site-directed mutagenesis. Then, the wild-type and mutant M113F αHL monomer were first synthesized by coupled in vitro transcription and translation (IVTT) using the *E. coli* T7 S30 Extract System for Circular DNA from Promega (Madison, WI). Subsequently, they were assembled into homoheptamers by adding rabbit red cell membranes and incubating for 1 h. The heptamers were purified

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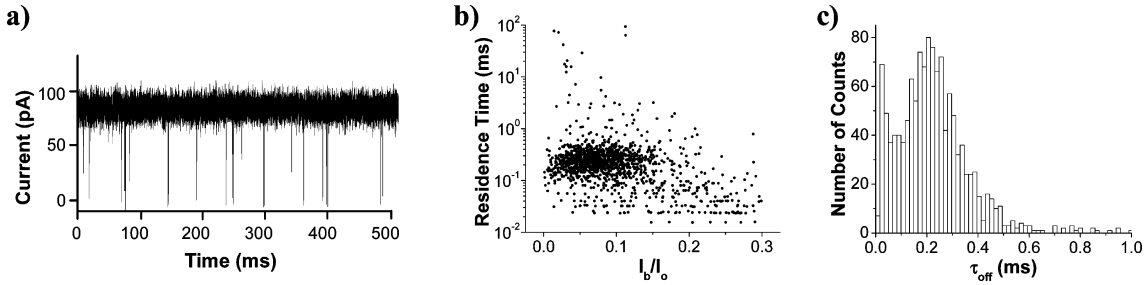

Figure 1. Translocation of $(dA)_{20}$ in the mutant $(M113F)_7$ αHL pore in 1 M NaCl solution. (a) Representative single channel current recording trace; (b) scatter plot of event amplitude vs residence time; and (c) event residence time histogram. I_b/I_o in Figure 1b is normalized blockage residual current, which was obtained by dividing the average blockage residual current of an event by the average open channel current.

by SDS-polyacrylamide gel electrophoresis and stored in aliquots at −80 °C.

Planar Bilayer Experiments. The single-channel recording procedure has been described elsewhere.[21] Briefly, a Teflon septum was used to divide the planar bilayer chamber into two compartments, cis and trans. A lipid bilayer of 1,2-diphytanoyl-sn-glycero-3-phosphacholine was formed on the aperture in the Teflon film by using the Montal−Mueller method.[22] The experiments were carried out under symmetrical buffer conditions with a 2.0 mL solution comprising 1 M BMIM-Cl, and 10 mM Tris·HCl (pH 6.0) at 22 ± 1 °C unless otherwise stated. Both the αHL protein (with the final concentration of 0.2−2.0 ng·mL^{-1}) and the ssDNA sample were added to the cis chamber compartment, which was connected to "ground". The applied potential was +120 mV. Currents were recorded with a patch clamp amplifier (Axopatch 200B, Axon instruments, Foster City, CA). They were low-pass filtered either with an external four-pole Bessel filter at 30 kHz and sampled at 125 kHz or with a built-in four-pole Bessel filter at 10 kHz and sampled at 20 kHz by a computer equipped with a Digidata 1440 A/D converter (Molecular Devices). The final concentrations of ssDNA samples were 10 μM each for the experiments performed at the 30 kHz filter, while those were 4 μM each for the experiments carried out at the 10 kHz filter. At least three separate experiments were carried out for each DNA sample.

Data Analysis. Only the events with at least 70% of full blockage were included in the analysis. It is believed that the events with the blockage amplitudes less than 70% of the open channel current are not associated with the translocation of ssDNA polymers through the αHL pore but instead may be caused by collision with the pore opening or residence only in the channel vestibule.[23] Two significantly different types of events were observed for DNA's transit in the αHL pore in the BMIM-Cl solution, short-lived events with mean residence times of ∼50−100 μs and long-lived events with mean residence times of milliseconds or larger. Data were analyzed with the following software: pClamp 10.0 (Molecular Devices) and Origin 6.0 (Microcal, Northampton, MA). Conductance values were obtained from the amplitude histograms after the peaks were fit to Gaussian functions. Mean residence time (τ_{off}) values of the short-lived events were obtained from the dwell time histograms by fitting the distributions to Gaussian functions,[6] while those of the long-lived events were obtained by fitting the dwell time distributions to single exponential functions by the Levenberg−Marquardt procedure.[24] It should be noted that the dwell time histograms of the short-lived events could also be fitted to single exponential functions by using larger bin widths, and we found that the mean τ_{off} values obtained using these two different approaches were not significantly different. To obtain the mean residence times of the long-lived events for $(dA)_{20}$, $(dT)_{20}$, $(dCdT)_{10}$, and $(dC)_{10}(dT)_{10}$, the events with duration less than 1 ms were not included, while in the analysis of $(dC)_{20}$ events with duration less than 0.5 ms were ignored to minimize the potential interference from the short event signals. Between 1330 and 20 000 events were recorded in each of the single channel recording experiments performed at the 30 kHz filter, while between 280 and 10 000 events were collected in each of the single channel recording experiments carried out at the 10 kHz filter. All the results were reported as mean values ± standard deviation.

To obtain the streaming potentials[25] of protein pores, single-channel current recording experiments were performed under asymmetric conditions. The cis chamber compartment contained a 2.0 mL solution comprising either 1 M NaCl or 1 M BMIM-Cl, 10 mM Tris·HCl (pH 6.0), while the trans compartment contained 2.0 mL of the same buffer solution plus 1 M urea. Streaming potential $\Delta\phi$ for the protein pore was obtained by linearly fitting the $I-V$ curves, which were recorded from ±5 to ±50 mV.

Results and Discussion

To investigate the effect of the ionic liquid solution on DNA translocation, the initial experiment was performed at +120 mV with $(dA)_{20}$ in the mutant α-hemolysin (αHL) $(M113F)_7$ pore and using a 1 M BMIM-Cl solution as the supporting electrolyte. The current was low-pass filtered with a four-pole Bessel filter at 30 kHz and sampled at 125 kHz. As a control, the experiment was repeated under the same conditions with the exception that NaCl was substituted for BMIM-Cl as the background electrolyte. The $(M113F)_7$ protein was constructed by replacing the Met residues at position 113 of the wild-type (WT) αHL with Phe amino acids, and has been shown to provide an enhanced resolution for peptide detection compared with that observed with the WT αHL pore.[20] The experimental results showed that in 1 M NaCl solution, $(dA)_{20}$ produced only a major type of rapid translocation events, although these events could be separated into two subgroups (Figure 1). The mean residence times for the two subgroup events were 190 ± 20 μs and 40 ± 5 μs, respectively (Figure 1). These events might be attributed to the translocation of $(dA)_{20}$ in two different orientations, 5′-first and 3′-first. Note that the observation of two subgroup translocation events with different residence times has been previously reported by Kasianowicz and co-workers in the experiment with the translocation of 210-nt-long poly[U] through the WT αHL channel.[4] Hence, the translocation velocity of $(dA)_{20}$ in the mutant $(M113F)_7$ αHL pore are not significantly different from those well-documented values obtained for the translocation of polydeoxyadenine through the WT αHL pore.[6,7] Similar to the observation made for the translocation of polynucleotides through the WT αHL channel,[4] events with much longer residence times at milliseconds or larger were also

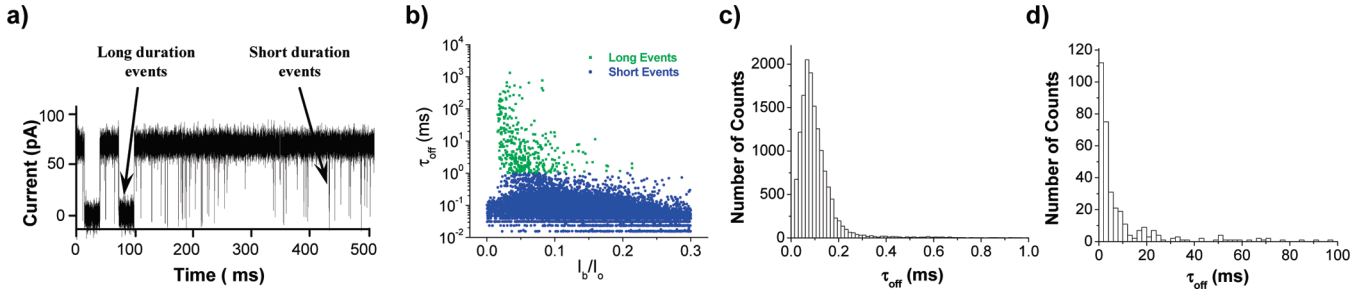

Figure 2. Translocation of $(dA)_{20}$ in the mutant $(M113F)_7$ αHL pore in 1 M BMIM-Cl solution. (a) Representative single channel current recording trace; (b) scatter plot of event amplitude vs residence time; (c) residence time histogram of short-lived events; and (d) residence time histogram of long duration events. I_b/I_o in Figure 2b is normalized blockage residual current, which was obtained by dividing the average blockage residual current of an event by the average open channel current.

occasionally observed for $(dA)_{20}$ in the $(M113F)_7$ αHL pore (Figure 1). These events are believed to be caused by the tangling of polynucleotides to the αHL channel.[4]

In contrast, in 1 M BMIM-Cl solution two major types of current blockage events were observed for $(dA)_{20}$ (Figure 2). One type of events shows a small mean residence time ($\tau_{off\text{-}short}$) and a wide range of current blockage amplitudes (from ~70% to almost full channel blockage), while the other type of events presents a narrow range of current blockage amplitudes and a large mean duration value ($\tau_{off\text{-}long}$) but with a broad distribution of residence times. The mean $\tau_{off\text{-}short}$ and $\tau_{off\text{-}long}$ values were 78 ± 5 μs, and 3.93 ± 0.12 ms, respectively. Since the risetime (= ~0.33/f_c) is ~11 μs at 30 kHz,[26] the vast majority of the rapid DNA translocation events should have been detected under the experimental conditions employed in this work. It should be mentioned that these long-lived current modulations occurred very frequently (at ~5 events per second), although they only accounted for a small portion (~2.5%) of the total current blockages. In part, this was attributed to a ~2 fold increase in the frequency of the $(dA)_{20}$ events when the electrolyte BMIM-Cl was substituted for NaCl. In addition, we noticed that with the change of the electrolyte from 1 M NaCl to 1 M BMIM-Cl the current value of the open state of a single αHL $(M113F)_7$ channel decreased from 90 ± 4 to 62 ± 5 pA at +120 mV. The extent of decrease (i.e., 31%) in our experimental open channel conductance was in agreement with that (i.e., 39%) in the measured conductivities of the bulk solutions (note that the conductivities of 1 M NaCl and 1 M BMIM-Cl solutions were 81.6 and 49.9 mS/cm, respectively[19]).

To investigate whether these long-live events are caused by $(dA)_{20}$'s threading through the αHL pore or rather they are attributed to the sticking of these DNA polymers to the channel, a series of polydeoxyadenine polymers with different lengths were examined with the mutant αHL $(M113F)_7$ protein channel in 1 M BMIM-Cl solution. Our experimental results show that with an increase in the DNA length the mean residence time of the long-lived events increased linearly (Figure 3a). This clearly suggests that these long-lived events were not due to the tangling of the $(dA)_{20}$ molecule to the channel or binding of one or more bases of the polymer to the protein pore for long periods of time with intermittent short periods of rapid translocation but rather caused by the slower translocation of the DNA molecule as a whole. And hence, the long duration events are suitable for the analysis of the length and structure of a polynucleotide molecule. In terms of the short-lived events, we noticed that $(dA)_5$ (with a mean $\tau_{off\text{-}short}$ value of 22.5 ± 3.1 μs) was still in the linear range of the plot of the event residence time versus DNA length (Figure 3b). In contrast, a linear relationship between the event residence time and polymer length was

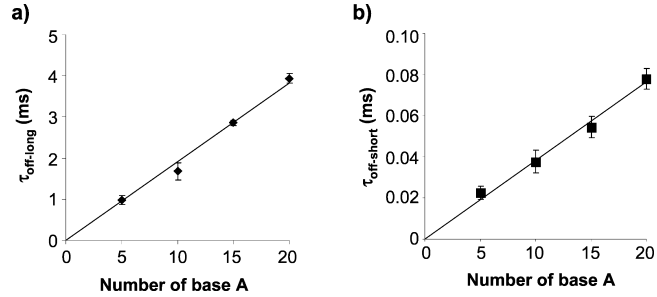

Figure 3. Effect of DNA length on the mean residence time of (a) long-lived events; and (b) short-lived events, suggesting that the long duration events are caused by DNA's threading through the αHL pore. Experiments were performed at +120 mV with the mutant αHL $(M113F)_7$ pore in 1 M BMIM-Cl solution. The currents were low-pass filtered with a four-pole Bessel filter at 30 kHz and sampled at 125 kHz.

observed only when DNA polymers longer than ~12 bases were electrophoretically driven through the WT αHL pore in the KCl solution.[7,8] Therefore, this suggests that the use of ionic liquid solutions coupled with the engineered protein pores provides a potential means to improve the resolution of the nanopore to the nucleotide differentiation, especially in the analysis of short DNA polymers.

It should be noted that such long duration translocation events of polydeoxyadenine have not been previously reported,[6] although $(dT)_{50}$ produced events with duration at ~3.7 ms in the WT αHL pore in 1 M KCl solution.[23] In this work, we focus on the pertinent long residence time events and investigate whether they can be employed to differentiate between various nucleotides. The large duration events have a significant advantage over the short-lived events since high measurement bandwidths are not necessary and hence significantly reduced measurement noise could be achieved. For this purpose, five ssDNA samples, including $(dA)_{20}$, $(dC)_{20}$, $(dT)_{20}$, $(dCdT)_{10}$, and $(dC)_{10}(dT)_{10}$, were examined with the same mutant $(M113F)_7$ pore in the BMIM-Cl solution at a filter frequency of 10 kHz and sampled at 20 kHz. As was found for $(dA)_{20}$, all of the four additional DNA molecules produced large residence time events (Supporting Information, Figure S1). The mean residence times and amplitudes of these long-lived events for the five different DNA samples are summarized in Table 1. The translocation rates for various DNA polymers (201 μs/base for $(dA)_{20}$, 98 μs/base for $(dC)_{20}$, 150 μs/base for $(dT)_{20}$, 256 μs/base for $(dCdT)_{10}$, and 320 μs/base for $(dC)_{10}(dT)_{10}$) obtained were ~2 orders of magnitude larger than the well-documented rates of ~1−3 μs/base with the translocation of 100-mer DNA polymers through the WT αHL channel in the KCl solution at room temperature.[6,7] This clearly shows that the use of BMIM-Cl solution instead of NaCl/KCl solutions significantly slows

TABLE 1: The Residence Times and Current Blockage Amplitudes of Five ssDNA Samples in the $(M113F)_7$ Protein Pore[a]

ssDNA sample	residence time (ms)	residual current (pA)	current blockage (%)
$(dA)_{20}$	4.02 ± 0.17	2.9 ± 0.2	95.3 ± 0.4
$(dC)_{20}$	1.96 ± 0.22	7.0 ± 0.4	88.7 ± 0.6
$(dT)_{20}$	3.00 ± 0.25	2.8 ± 0.2	95.4 ± 0.3
$(dCdT)_{10}$	5.13 ± 0.91	4.0 ± 0.3	93.6 ± 0.5
$(dC)_{10}(dT)_{10}$	6.40 ± 0.19	4.9 ± 0.2	92.2 ± 0.4

[a] Each experimental value represents the mean of three replicate analyses ± one standard deviation. The experiments were performed at +120 mV in 1 M BMIM-Cl solution.

TABLE 2: The Residence Times and Current Blockage Amplitudes of Five ssDNA Samples in the Wild-Type αHL Protein Channel[a]

ssDNA sample	Residence Time (ms)	Residual Current (pA)	Current Blockage (%)
$(dA)_{20}$	2.37 ± 0.20	2.8 ± 0.2	95.6 ± 0.3
$(dC)_{20}$	1.65 ± 0.23	4.1 ± 0.3	93.5 ± 0.4
$(dT)_{20}$	2.17 ± 0.10	0.8 ± 0.1	98.6 ± 0.1
$(dCdT)_{10}$	3.00 ± 0.20	1.3 ± 0.1	98.0 ± 0.2
$(dC)_{10}(dT)_{10}$	4.79 ± 0.71	3.3 ± 0.3	94.8 ± 0.5

[a] Each experimental value represents the mean of three replicate analyses ± one standard deviation. The experiments were performed at +120 mV in 1 M BMIM-Cl solution.

DNA translocation and provides a much enhanced resolution/sensitivity. This increased nanopore resolution coupled with the different event blockage amplitudes produced by different nucleotides (Table 1) permits the convenient differentiation between the five DNA molecules examined.

To further document the utilization of ionic liquid solutions as an effective means to slow the translocation of DNA polymers in nanopores, the WT αHL pore was used instead of the mutant $(M113F)_7$ protein to examine the same series of DNA samples in 1 M BMIM-Cl solution. Similar to the observation made for the DNA translocation through the $(M113F)_7$ pore in the BMIM-Cl solution, all the five tested DNA samples produced long duration events (see Supporting Information, Figure S2). The mean residence times and amplitudes of these long-lived events for the five different DNA samples are summarized in Table 2. Although the residence time values were smaller than those obtained in the $(M113F)_7$ protein pore, the translocation rates of various polynucleotides at 82.5−240 μs/base were still $\sim 10^2$ fold larger than those in KCl or NaCl solutions. The difference in the residence times for the DNA translocation through two different protein pores may be attributed to the change in the van der Waals volumes of amino acids at position 113 of the αHL channels (V_M (124 Å^3) < V_F (135 Å^3)).[27] All together, these results suggest that the BMIM-Cl solution is essential to obtaining the long duration DNA events in the αHL pore, while mutant protein pore plays a smaller role.

The significant increase in the residence time of DNA translocation in the ionic liquid BMIM-Cl solution over the NaCl/KCl solution might be attributed to several possible reasons, for example, the changes in the viscosity of the medium and in the charge selectivity of the pore. Under a specific applied voltage bias, for example, +120 mV in this work, the DNA translocation process is mainly determined by the interaction of the polynucleotides and the αHL pore, and the migration rate of the nucleotide molecules. The latter is primarily dependent on the diffusion of the polymer and the charge selectivity of the pore. Our previous research showed that the

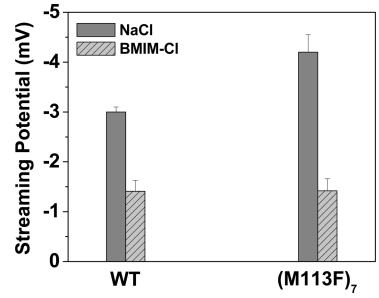

Figure 4. Effect of BMIM-Cl solution on the streaming potentials of αHL pores.

viscosity of the BMIM-Cl solution was only 71% greater than that of the NaCl solution.[19] Hence, such a small change in the viscosity of the medium could not explain the observed large DNA residence times[28] (note that the diffusion coefficient of an ion is inversely related to the viscosity of the medium). On the other hand, the actual charge selectivity of the pore could not be obtained due to the lack of data for the activity coefficient of BMIM-Cl solution. However, our experiments showed that, when BMIM-Cl solution was used instead of NaCl, the values for the streaming potentials in both the WT and mutant $(M113F)_7$ αHL pores reduced significantly (Figure 4). For example, in 1 M NaCl solution the streaming potentials in the wild-type and mutant $(M113F)_7$ αHL pores were −3.00 ± 0.10 and −4.20 ± 0.35 mV, respectively (Supporting Information, Figure S3). In contrast, in 1 M BMIM-Cl, those values were −1.41 ± 0.22 and −1.43 ± 0.24 mV, respectively (Supporting Information, Figure S4). It should be noted that a smaller streaming potential of the pore indicates a more even transport of solvent by cations and anions, leading to a drop in the preferential charge selectivity to either cation or anion.[29] Thus, our results suggest that the weakly anion selective WT or mutant $(M113F)_7$ αHL pore in the NaCl/KCl solution is becoming more neutral in the BMIM-Cl solution. However, previous work suggests that although the frequency of DNA translocation events could be significantly affected by the charge selectivity of a protein pore, the translocation time is not greatly altered.[30] In addition, the constant impact of the charge selectivity of the protein pore on molecular transport (e.g., the event residence time)[29,31] could not explain our observation that the long-lived events only accounted for a small portion of the total events.

Contrary to the observation that poly(dA) translocates through the αHL pore more slowly than poly(dCdT) in the KCl solution,[7] the residence time of $(dCdT)_{10}$ events were larger than that of $(dA)_{20}$ with the BMIM-Cl solution. This suggests that the interaction between nucleotides and the protein pore could be significantly influenced by the presence of the imidazolium cation. It is known that the diameter of the constriction region of the αHL pore is only slightly larger than the diameter of a ssDNA polymer.[32] Thus, in order for a ssDNA molecule to translocate through the αHL pore, the counterions need to be squeezed in the narrow water-filled space surrounding the DNA.[33] Compared with the naked K^+ (radius, 1.33 Å) or Na^+ (radius, 0.97 Å),[34] the bulky $BMIM^+$ (length, 11.0 Å; width, 5.8 Å)[35] is much larger. Furthermore, recent studies have suggested that the interaction between $BMIM^+$ and DNA is very strong, so much so that DNA could be extracted by ionic liquid BMIM-PF_6 solution.[36] The strong interaction between $BMIM^+$ and DNA might be attributed to the interaction of the bulky organic $BMIM^+$ and P−O bonds of phosphate groups in the DNA molecule,[36] and/or the electrostatic interaction between $BMIM^+$ and DNA.[37] Therefore, it is not unreasonable that it

would be much more difficult to squeeze DNA molecules through the narrow αHL pore in BMIM-Cl than in KCl or NaCl solution. Considering that the long-lived $(dA)_{20}$ events only accounted for 2.5% of the total events and the significant difference in the residence times of two types of events, it is likely that the large duration events were attributed to the threading of the DNA-$BMIM^+$ complex through the pore. In contrast, the short-lived events were due to the translocation of uncomplexed DNA molecules or the rapid entrance/exit of the DNA-$BMIM^+$ complex at the cis opening of the channel (e.g., the DNA-$BMIM^+$ complex enters the vestibule, moves toward the β-barrel but does not traverse through the limiting aperture, but instead retracts backward to the cis side and exits). We are leaning toward the latter interpretation of the short-lived events. Further experiments are required to resolve the origin of these events.

To further demonstrate the bulky cation effect on DNA translocation, $(dA)_{20}$ was examined with the $(M113F)_7$ pore using other common organic salt solutions, and the same prolonged DNA translocation phenomenon was observed. For example, in a solution containing 1 M tetramethylammonium chloride the mean residence time of $(dA)_{20}$ in the $(M113)_7$ αHL pore was ∼4.1 ms (Supporting Information, Figure S5).

Conclusions

In summary, we have demonstrated that by using electrolyte solutions that contain organic salts instead of NaCl/KCl, a ∼2 order of magnitude reduction in the velocity of DNA translocation through protein pores can be achieved. Compared with other physical conditions, such as temperature, ionic strength, viscosity, etc.,[38] the effect of organic salts on DNA translocation was far more significant. It is likely that the strategy used in this work could be employed together with other experimental conditions by synthetic nanopores[39−41] to substantially decrease the rapid DNA translocation velocity. Further experimental, theoretical, and computational research is necessary to understand and clarify how the organic salt solutions slow down the DNA translocation in the nanopores.

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Supporting Information Available: Additional graphs. This material is available free of charge via the Internet at http://pubs.acs.org.

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