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Electrokinetic transport properties of deoxynucleotide monophosphates (dNMPs) through thermoplastic nanochannels

Colleen O'Neil^{a,e,1}, Charuni A. Amarasekara^{c,e,1}, Kumuditha M. Weerakoon-Ratnayake^{c,e}, Bethany Gross^{c,e}, Zheng Jia^{e,f}, Varshni Singh^{b,e}, Sunggook Park^{e,f}, and Steven A. Soper^{c,d,e,g,*}

^aDepartment of Chemistry, The University of North Carolina, Chapel Hill, NC, USA

^bDepartment of Biomedical Engineering, The University of North Carolina, Chapel Hill, NC, USA

^cDepartment of Chemistry, Department of Mechanical Engineering, The University of Kansas, USA

^dDepartment of Cancer Biology, Kansas University Medical Center, USA

^eNIH Biotechnology Resource Center of BioModular Multiscale Systems for Precision Medicine, USA

^fDepartment of Mechanical Engineering, Louisiana State University, Baton Rouge, LA, USA

^gUlsan National Institute of Science and Technology, Ulsan, South Korea

Abstract

The electrokinetic behavior of molecules in nanochannels (<100 nm in length) have generated interest due to the unique transport properties observed that are not seen in microscale channels. These nanoscale dependent transport properties include transverse electromigration arising from partial electrical double layer overlap, enhanced solute/wall interactions due to the small channel diameter, and field-dependent intermittent motion produced by surface roughness. In this study, the electrokinetic transport properties of deoxynucleotide monophosphates (dNMPs) were investigated, including the effects of electric field strength, surface effects, and composition of the carrier electrolyte (ionic concentration and pH). The dNMPs were labeled with a fluorescent reporter (ATTO 532) to allow tracking of the electrokinetic transport of the dNMPs through a thermoplastic nanochannel fabricated via nano-imprinting (110 nm × 110 nm, width × depth, and 100 μm in length). We discovered that the transport properties in plastic nanochannels of the dye-labeled dNMPs produced differences in their apparent mobilities that were not seen using microscale columns. We built histograms for each dNMP from their apparent mobilities under different operating conditions and fit the histograms to Gaussian functions from which the separation resolution could be deduced as a metric to gage the ability to identify the molecule based on their apparent mobility. We found that the resolution ranged from 0.73 to 2.13 at pH = 8.3. Changing the carrier electrolyte pH > 10 significantly improved separation resolution (0.80

*Corresponding author. The University of Kansas, Lawrence, KS, USA. ssoper@ku.edu (S.A. Soper).

¹Authors contributed equally.

Appendix A. Supplementary data

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–4.84) and reduced the standard deviation in the Gaussian fit to the apparent mobilities. At low buffer concentrations, decreases in separation resolution and increased standard deviations in Gaussian fits to the apparent mobilities of dNMPs were observed due to the increased thickness of the electric double layer leading to a partial parabolic flow profile. The results secured for the dNMPs in thermoplastic nanochannels revealed a high identification efficiency (>99%) in most cases for the dNMPs due to differences in their apparent mobilities when using nanochannels, which could not be achieved using microscale columns.

GRAPHICAL ABSTRACT

Keywords

Nanofluidics; Electrokinetic transport; Thermoplastic nanochannels

1. Introduction

Electrokinetic transport of molecules in nanochannels is influenced by several physical parameters unique to the nanoscale such as electric double layer (EDL) overlap and the high surface-to-volume ratio of nanochannels. In particular, surface mediated effects can affect the electrokinetic transport properties of charged molecules through nanochannels that is not observable in microchannels, such as surface roughness. Also, partial overlap in the EDL can occur in nanochannels. The EDL thickness (λ_d), is defined by;

$$\lambda_d = \sqrt{\frac{\epsilon_0 \epsilon_r RT}{2F^2 C}} \quad (1)$$

where R is the gas constant, ϵ_r is the dielectric constant of the medium, F is the Faraday constant, T is the temperature, and c is the concentration of the electrolyte solution [1–5]. As λ_d approaches the channel dimensions, EDL overlap can occur, which introduces a parabolic flow profile rather than the classically observed plug-like flow typically seen in microscale channels creating interesting transport properties [4–6]. For example, it has been shown that double stranded DNA (dsDNA) fragments have different mobilities in nanochannels in the absence of a sieving medium when the channel dimensions are on the order of λ_d [4]. By comparison, dsDNA have the same mobility irrespective of their size in microscale channels.

The increased surface area-to-volume ratio in nanoscale channels allows for solute/wall interactions that can influence electro-kinetic transport as well. Potential interactions of solutes with walls include intermittent motion, which can cause mobilities that are electric field strength dependent, and is produced by surface roughness [7–9]. These intermittent motions can be induced by dielectrophoretic trapping generated by surface roughness on the nanoscale producing an inhomogeneous electrical field. Additional wall effects include molecular retardation, friction, and relaxation forces [7]. Viscous retardation forces depend on the solute's radius and Debye length and arise from solute interactions with a non-conducting boundary [10]. Frictional forces are those arising from drag between the direction of bulk fluid flow and the channel wall. The relaxation effects, or EDL distortions, arise from a greater charge density behind the solute compared to the front as the ions comprising the EDL “lag” as the solute is transported through the nanochannel [11,12].

Another phenomenon that can be observed in nanochannels is adsorption/desorption events between the solute and wall, which can be more prevalent in nanochannels compared to micro-channels due to scaling effects (*i.e.*, smaller diffusional distances in the case of nanochannels). Electrostatic, van der Waals, and hydration interactions between molecules and channel walls contributes to the interactions of molecules with channel walls that will affect the transport properties of the molecules. Depending on the magnitude of the interaction, adsorption events can be classified as reversible or irreversible. In a computational study by Moldovan and coworkers, the adsorption/desorption behavior of deoxynucleotide monophosphates (dNMPs) traveling through polymer nanochannels was found to depend on the hydrophobicity of the dNMPs [13–15].

The electrokinetic transport of silver nanoparticles (AgNPs) through poly (methylmethacrylate), PMMA, nanoslits (only one dimension in the nanoscale) was shown recently [7]. The authors were able to demonstrate size-dependent mobilities of AgNPs in buffer only, which was not possible using microscale columns. Optimal differences in the mobilities were observed for 60 and 100 nm AgNPs at high electric field strengths, where no intermittent motion was evident. O'Neil et al. showed differences in the electromigration of 20 nm polystyrene (PS) beads traveling through PMMA nanoslits at varying electric field strengths [8]. At low electric field strengths (200 V/cm), the PS beads showed intermittent transport properties resulting in field-dependent apparent mobilities and high dispersion in the mobilities, a result of this intermittent motion. Further, the standard deviations in the mobilities in general were found to be higher at lower electric field strengths due to flow recirculation that resulted from non-uniform surface charges when using thermoplastic nanochannels [8]. In both cases, the apparent mobilities were found to be electric field dependent until a “critical” field strength was reached at which point, the apparent mobilities were electric field independent as classically seen in microchannels.

Due to the unique transport properties offered by nanochannels, we sought to explore the electrokinetic transport behavior of dNMPs through polymer nanochannels. To undertake this, we used fluorescence tracking of the dNMPs by labeling the dNMPs with a common reporter such that the transport properties could be associated with differences in the chemical properties of the dNMPs only. The ability to efficiently identify dNMPs for applications such as single-molecule sequencing (SMS).

One approach for SMS is nanopore sequencing, which uses naturally occurring pores such as α -hemolysin [16]. For nanopore sequencing, an intact DNA molecule is electrically transported through the pore and produces a characteristic modulation of ionic current through which the nucleotides can be identified [17]. However, base calling accuracy is ~80% for several reasons, including the fact that 10–15 nucleotides are resident within the nanopore at any one time [18–21].

An alternative approach for SMS involves disassembling an intact DNA molecule into its discrete monomer units (*i.e.*, mononucleotides or dNMPs) using an exonuclease immobilized at the inlet of the nanopore, which addresses the issue of multiple nucleotide occupancy within a single pore [22,23]. A challenge with this approach is that each nucleotide is still identified using the amplitude of a current blockage event only and thus, generates high error rates.

To address these issues, we are developing an innovative SMS strategy that enzymatically cleaves DNAs using a surface-immobilized processive exonuclease to generate the dNMPs [24–26]. The released dNMPs are electrokinetically transported through a nanochannel and identification of the nucleobases is carried out using the unique apparent mobility (*i.e.*, mobility matching) of each dNMP electrokinetically traveling through a nanochannel. Thus, a thorough understanding of the electrokinetic properties of dNMPs through nanochannels along with parameters that affect separation resolution between the dNMP apparent mobilities, which is correlated to the base call accuracy, will be required to realize this SMS approach. We investigated various experimental parameters that may differentially affect the electrokinetic transport properties of the dNMPs, such as electrolyte pH and λ_D values as well as the electric field strength to optimize the ability to identify dNMPs based on their apparent mobilities. To thoroughly understand the electrokinetic properties of the dNMPs, fluorescence detection was used by labeling each dNMP with the same fluorescence reporter (ATTO 532) and thus, any differences in the mobilities resulted from the dNMP.

2. Experimental methods

2.1. Reagents and materials

Silicon (100) (Si) wafers were purchased from University Wafers (Boston, MA). Poly (methylmethacrylate), PMMA, substrates and cover plates were purchased from Good Fellow (Berwyn, PA) and cyclic olefin copolymer (COC 8007 and 6017) was purchased from TOPAS Advanced Polymers (Florence, KY). An anti-adhesion monolayer of (Tridecafluoro-1,1,2,2-tetrahydrooctyl) Trichlorosilane (T-Silane) was purchased from Gelest, Inc. (Morrisville, PA). Tripropylene glycol diacrylate (TPGA), trimethylolpropane triacrylate (TMPA), Irgacure 651 (photo-initiator), 1-ethyl-3-[dimethylaminopropyl] carbodimide hydrochloride (EDC), thymidine 5'-monophosphate disodium salt, 5-methylcytosine monophosphate disodium salt, cytidine 5'-monophosphate disodium salt, adenosine 5'-monophosphate disodium salt and guanosine 5'-mono-phosphate disodium salt, triethylammonium acetate (TEAA), cetyltrimethylammonium bromide (CTAB), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2-amino-2-methyl-1-propanol (AMP), and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO). Tris-borate buffer and sodium hydroxide (NaOH) was obtained from Fisher Scientific (Houston, TX). ATTO

532 dye was purchased from Atto-Tec (Siegen, Germany). All required dilutions were performed using 18 M Ω /cm MilliQ water (Millipore technologies) and all measurements were performed at 25 °C unless specified otherwise.

2.2. Fluorescent labeling of dNMPs

The procedure for conjugation of ATTO 532 to each dNMP was modified from Cornelius et al. [27] Full details can be found in the SI material (Fig. S1).

2.3. Fabrication of nanochannel devices

The fabrication of nanochannels in thermoplastics has been detailed elsewhere [28,29]. See the SI for further information and Fig. S2 for SEMs of example devices. In all cases, the nanochannel electrokinetic columns were 100 μ m in length and 110 nm $\times$ 110 nm in width and depth.

2.4. Imaging system for nanochannel electrokinetic

All fluorescence imaging for the nanochannel electrokinetic was performed using an Axiovert 35 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a 100 $\times$ oil immersion objective (NA 1.3) [8]. Images were acquired by EMCCD camera (Andorr iXon 897, Andorr Technologies, Connecticut, USA) at high frame rate and EM gain mode using Metamorph software. A schematic of the microscope can be found in Fig. S3 in the SI.

2.5. Nanochannel electrokinetic behavior of ATTO 532-labeled dNMPs

Assembled nanochannel devices were activated using 21.85 mW/cm² UV/O₃ through a 100 μ m COC cover plate for 15 min. After activation, chips were primed using 50% MeOH for 5 min. The MeOH was evacuated from the nanofluidic device and it was then filled with 50 mM Tris-Borate (TB) buffer at the desired pH and allowed to equilibrate for 10 min. ATTO 532-labeled dNMPs samples (200 nM) were prepared in the carrier electrolyte and introduction of the sample was done by removing the priming carrier electrolyte from the reservoir on chip and filled with the dNMP solution. The carrier electrolyte was then removed from the opposite reservoir to allow for pressure injection of dye-labeled dNMP solution into the access microchannels. Once the access microchannels were filled, all reservoirs were filled with equal volumes of the carrier electrolyte. Electrodes were placed in opposite access reservoirs to allow for a drive voltage to drop across the nanochannels. A square wave voltage (V_{pp}) was applied using an ATTEN ATF20B function waveform generator for a period of 5 s to allow for injection of the dye-labeled dNMPs into the nanochannel. Events were recorded for 5000 frames allowing for multiple events to be analyzed.

2.6. Nanochannel electrokinetic separation data analysis

Fiji (a version of ImageJ) software [30] was used to analyze the nanochannel electrokinetic separation data. Videos of events were imported into Fiji and 1 μ m² “detection windows” were placed at the entrance and exit of the nanochannel. In the Fiji software, these detection windows recorded the fluorescence intensity over time. This data was exported into Origin

8.5 and the first derivative was taken of each data set to produce two peaks indicating the time at both the entrance and exit of the nanochannel. The time between peak 1 and peak 2 was assigned a t value for each dNMP and the apparent mobility of the dye and dye-labeled dNMPs could be determined from t , the applied voltage (V) and the distance between the input point and output points of the column (see SI and Fig. S4).

3. Results and discussion

Theory and experimental studies for the electrokinetic transport of charged molecules through nanoslits has appeared in recent reviews [1,31,32]. For ion transport with d/λ_D ratios (d = channel width/depth) ranging from 1 to 10, anomalous transport behavior has been observed, such as charge-dependent ion speeds due to transverse electromigration resulting from solute/wall electrostatic effects [4,6,33,34]. Pennathur and Santiago determined that electrokinetic transport in nanoslits made from glass was dependent on ion valence, ζ (zeta potential), ion mobility, and λ_D [4,6]. For example, Garcia et al. illustrated the electrokinetic transport of the fluorescent dyes Alexa 488 (negatively charged) and rhodamine B (neutral) in Si nanoslits of various depths ranging from 35 to 200 nm [3]. The apparent mobilities of the dyes were dependent on their charge and extent of interaction(s) with channel walls.

Here, we explored the use of nanochannels (2 dimensions in the nanometer domain), specifically thermoplastic nanochannels, as opposed to nanoslits (only 1 dimension within the nanometer domain) on the electrokinetic transport properties of fluorescently labeled dNMPs. We also investigated how the carrier electrolyte composition would affect the transport properties of the four naturally occurring nucleotide monophosphates, dGMP, dAMP, dCMP, and dTMP, and the methylated cytosine (mdCMP) residue. The monophosphate form of these molecules was selected because these are generated upon cleavage of DNAs by λ -exonuclease, which is the focus of our SMS approach [24,25].

3.1. Nanochannel electrokinetic behavior of ATTO 532 dye

To initiate this study, fluorescence tracking of the ATTO 532 dye was undertaken. Because we intended to use ATTO 532 as a reporter for tracking the electrokinetic motion of the dNMPs through the polymer nanochannels, we sought to understand the mobility of this dye alone. Nanochannel electrokinetic experiments of the dye at varying electrolyte concentrations were performed, because changing the carrier electrolyte concentration modulates the EDL thickness and can introduce different EOF profiles under certain conditions. With a carrier electrolyte concentration of 44.5 mM, λ_d was 1.25 nm and at an electrolyte concentration of 0.45 mM, λ_d was 12.5 nm (see Equation (1)). To determine the extent of EDL overlap, κa was used as an operational metric, where a is the channel radius and κ is the inverse of λ_d ;

$$\kappa = \sqrt{\frac{8\pi n e^2}{\epsilon K T}} \quad (2)$$

where n is the bulk ion concentration [35], e is the elementary charge of an electron, ϵ is the dielectric constant, K is Boltzmann's constant and T is temperature. If $\kappa a \gg 1$, there is negligible EDL overlap and thus, the EOF profile should be classically plug-like; however, for cases where $\kappa a \approx 1$, significant EDL overlap occurs and the EOF profile is predicted to resemble Poiseuille-like flow [5]. For a buffer concentration of 44.5 mM and λ_d of 1.25 nm with the nanochannels possessing a width and height of ~ 110 nm ($a = 55$ nm), the κa value was ~ 44 indicating primarily plug-like flow. For a buffer concentration of 0.45 mM and $\lambda_d = 12.5$ nm, the κa value was 4.4 indicating partial EDL overlap and thus, more parabolic-like flow (see Equations (1) and (2)).

As can be seen in Fig. 1, for both carrier electrolyte concentrations a field-dependent apparent mobility of the dye was observed until a "critical" electric field strength was reached, where the apparent mobility became field independent. Microscale electrokinetics would predict that the mobility of the solute be independent of field strength (e.g. we assumed that the EOF is constant for each electrolyte concentration) and thus, changes in the apparent mobility, which is the sum of the EOF and the electrophoretic mobility of the dye, are due to changes in the electrophoretic mobility of the dye [36]. The field strength dependency of the apparent mobility has been reported for the electrokinetic transport of molecules in nanochannels and arises from intermittent motion [7–9]. We also note that the thermoplastic nanochannels we are using have non-uniform surface charges generating solute recirculation due to inhomogeneous charge distribution of the channel walls, which is also electric field strength dependent [8].

At a "critical" electric field strength, the apparent mobility becomes independent of the applied electric field strength. As seen in Fig. 1, this critical field strength was dependent on the electrolyte concentration. For a carrier electrolyte concentration of 44.5 mM, the critical field strength for the ATTO 532 dye was 479 V/cm, while for an electrolyte concentration of 0.45 mM, the critical field strength was 273 V/cm. Above these critical electric field strength values, the apparent mobility of ATTO 532 was $\sim 5.0 \times 10^{-5} \text{ cm}^2/\text{V s}$ for both electrolyte concentrations. Thus, as the electric field is increased, the effects of intermittent motion and recirculation become less, resulting in an increase in the electrophoretic mobility of the dye and as seen in Fig. 1, a decrease in the apparent mobility. Above the critical field strength, the effects of intermittent motion and recirculation become inconsequential to the apparent mobility and a field independent mobility is observed.

As we have seen in our previous work on nanoscale electrokinetics [7–9], when intermittent motion and recirculation occur, the standard deviations in the measured mobilities are large, consistent with the data displayed in Fig. 1a and b. As seen in Fig. S5, at electric field strengths > 250 V/cm for the ATTO532 dye, the standard deviations in the measured apparent mobilities were smaller compared to those below 250 V/cm.

3.2. Nanochannel electrokinetics of the ATTO 532-labeled dNMPs

Because the same dye reporter was covalently attached to each of the dNMPs, differences in the apparent mobilities of the dNMPs were assumed to arise from the identity of the nucleobase. This was necessary to study, because in our SMS approach, we will be identifying the dNMPs from their characteristic apparent mobility in a label-free approach.

Labelling is not possible post-cleavage due to reaction time limitations and building full complements of DNA strands using modified nucleotides is problematic as well.

Fig. 2a shows the apparent mobility versus electric field strength for the four dNMPs using a 110 nm × 110 nm nanochannel with a carrier electrolyte consisting of 44.5 mM TB at pH 8.3. At pH 8.3, all ATTO 532-labeled dNMPs have a net charge of -3 ; the ATTO-532 dye contributes a -2 charge for all dNMPs and the phosphate is -1 (see SI and Table S1 for more information) with all nucleobases not carrying a charge at this pH. The apparent mobility is a sum of the EOF (μ_{eof}) and the electrophoretic mobility of the dNMP (μ_{ep}). In this case, the electrophoretic mobility of the dye/dNMP conjugate is opposite in direction to the EOF and an increase in μ_{ep} results in a decrease in the apparent mobility.

We observed a smaller apparent mobility of the dye-labeled dNMPs compared to the free dye due to the increased negative charge of the conjugates compared to the free dye arising from the additional negative charge of the phosphate group. From Fig. 2a, there were differences in the apparent mobilities of the 4 dNMPs in spite of the fact that they all carried the same net charge at this pH. Moreover, all of the nucleotides showed an apparent mobility that was dependent on the electric field. Because the EOF mobility does not change as a function of the electric field strength [7,8], changes in the apparent mobility were assumed to arise from differences in the apparent mobility of the dNMPs at different field strengths due to intermittent motion and/or recirculation as was seen for the free dye.

Fig. 2b shows histograms of the apparent mobility for the ATTO 532-labeled dNMPs at 342 V/cm, which was selected because it provided optimal separation resolution between the dNMPs. The resolution for these dNMPs was determined from the Gaussian fits to the histograms. Table 1 shows the separation resolutions between dNMP Gaussian fit pairs at pH 8.3 using 44.5 mM TB buffer. The apparent mobility order, dAMP < dTMP < dGMP < dCMP was in close agreement with the hydrophobicity order of the dNMPs (dGMP < dCMP < dAMP < dTMP) [25,37]. The hydrophobic nature of dAMP and dTMP likely generates more solute/wall interactions and will thus show a lower apparent mobility, while the less hydrophobic dGMP and dCMP will have larger apparent mobilities. This suggests that the apparent mobility order using these conditions (pH 8.3, $\lambda_d = 1.25\text{nm}$), may not only depend on the differences in charges and/or sizes of the dNMPs, but also on the extent of solute/wall interactions; the walls of these devices consist of PMMA/COC carrying a partial negative charge resulting from O_2 plasma activation, which also generates nanoscale surface roughness giving rise to intermittent motion. As we have shown, specific surface areas lack charges following activation creating unmodified polymer with a low surface energy that can promote hydrophobic/hydrophobic interactions as well as recirculation [8].

Taylor dispersion resulting from Joule heating can contribute to variances in the apparent mobilities of the dNTPs. This variance shows a strong dependency on column radius (R^6) and results in the optimum field strength for microscale experiments to be only a few hundred V/cm [36,38,39]. However, nanochannels with their reduced channel dimensions allows for the use of much higher electric field strengths without observing deleterious effects arising from Joule heating. The consequence of this is also reduced variances due to longitudinal diffusion [38–40].

Variances in the observed apparent mobilities (see Fig. 2) for thermoplastic nanochannels can also arise from non-uniform distribution of surface charged groups following O₂ plasma activation giving rise to recirculation [8]; control of the dose used for activation can lead to an optimal surface charge density with higher charge densities correlated to lower surface charge heterogeneity and thus, lower variances in the apparent mobility.

Furthermore, utilizing COMSOL simulations to reconstruct fluid flow profiles in nanochannels with a heterogeneous distribution of point charges, the simulations indicated that there were regions of fluid recirculation leading to both positive and negative velocities. The results indicated that when the electric field strength was high (> 300 V/cm), intermittent motion as well as recirculation were significantly reduced, which was observed when the apparent mobility became independent of the electric field strength, Fig. 2a [8]. Also, in general the variances in the apparent mobilities became less when the mobilities became independent of field strength due to minimal contributions of intermittent motion and recirculation on the solute's mobility.

We calculated the separation resolution between the ATTO 532-dNMPs at a field strength of 342 V/cm and pH 8.3 TB (44.5 mM), the result of which are summarized in Table 2. For these conditions, we noted the separation resolution ranged from 0.73 to 2.13. These were determined from the average mobility values and the standard deviations in the Gaussian fits (Fig. 2b) to the histograms of apparent mobilities for the dNMPs.

3.3. Effect of pH on the nanochannel electrokinetics of ATTO 532- labeled dNMPs

As a matter of comparison between microscale electrophoresis of the dNMPs and the nanoscale electrokinetics in terms of pH effects, we carried out microscale separations of the dNMPs using a capillary (id = 50 μ m; effective length = 56 cm) and those results are shown in Fig. S6. As can be seen, irrespective of carrier electrolyte pH, dAMP and dCMP co-migrated.

Fig. 3 shows histograms of the apparent mobilities for the ATTO 532-dNMPs within a nanochannel (44.5 mM TB) at pH 10.3 and a field strength of 342 V/cm. In Fig. 4 a summary of the pH-dependent mobility of the ATTO 532-dNMPs is shown. Over the pH range investigated (pH 8.3–10.3), the EOF remains constant due to the low pK_a (~ 5) of the surface-confined carboxylic acids [9,41–44], and thus, the observed changes in the apparent mobilities arise from differences in the electrophoretic mobilities of the ATTO 532-dNMPs conjugates. In addition, the reporter fluorescent dye (ATTO 532) does not change its charge state within the investigated pH range indicating that the change in apparent mobility is associated with the chemical properties of the nucleobases. The increase in the pH could have a two-fold effect on the apparent mobilities of the dNMPs: 1) Changing the negative charge state of the dNMP nucleobase due to the presence of weak acid/ base groups; and/or 2) altering potential solute/wall interactions due primarily to changes in the charge states of the nucleobase.

All nucleotides, except for dGMP, showed a larger apparent mobility at pH 9.3 and 10.3 compared to pH 8.3 when using a 44.5 mM carrier electrolyte concentration. The different apparent mobility order compared to pH 8.3 arose from differences in the electrophoretic

mobility of dGMP as a result of pH changes. The mobility for dGMP decreased with an increased pH from 8.3 to 10.3 resulting from an increase in the net negative charge of dGMP from -3 (pH 8.3) to -4 (pH 10.3; see Table S1). Additionally, we show a histogram of the apparent mobilities for mdCMP (Fig. 3), which showed a larger apparent mobility compared to the other nucleotides. Fitting of a Gaussian function to the histogram in this case was poorer ($R^2 > 0.94$) compared to the non-methylated dNMPs ($R^2 > 0.999$), which may have been due to different adducts present in the methylated cytosine sample. In spite of this observation, the large differences in the apparent mobility of the mdCMP compared to the other dNMPs suggests that mobility matching could be used as an identification approach for detecting epigenetic modifications in genomic DNA without requiring bisulfite conversion.

Fig. 4 shows the relative apparent mobilities of the nucleotides as a function of pH. At lower pH values, larger differences in the apparent mobilities were observed. However, at higher pH values, we observed an increase in the overall separation resolution between the nucleobases (see Table 2). The poorer overall resolution observed at lower pH values was a result of the higher standard deviations seen in the measured apparent mobilities for all of the dNMPs as compared to the higher pH values (see Fig. 4). One trend that emerged from this data is that dCMP was not very sensitive to pH changes while the other three nucleotides were.

Another important metric for the utilization of mobility matching for the identification of mononucleotides is the identification or base call accuracy, which is related to the separation resolution between the nucleotides. We define the “identification accuracy” as the amount of overlap of two adjacent Gaussian fits to the histograms of the ATTO 532-dNMPs' apparent mobilities. With a pH value of 10.3 and a field strength of 342 V/cm ($\lambda_d = 1.25$ nm), the separation resolution between the dNMPs ranged from 0.80 to 4.84 (Table 2). A resolution of 0.5 has a classification accuracy of 68%, which is based on the degree of overlap in the Gaussian fits. A resolution of 1.0 has a classification accuracy of 96%. Table 3 shows the calculated identification accuracies for the nucleotides at pH 10.3. As can be seen from this data, the base call accuracy is $>95\%$ in all cases, except for the dTMP/dCMP pair, which was determined to be 87%. There are a number of experimental conditions that can be altered to potentially improve the base call accuracy, such as using higher electric field strengths, increasing the channel length or removing the dye reporter.

3.4. Effect of EDL overlap on the nanochannel electrokinetics of ATTO 532-labeled dNMPs

In the region of EDL overlap, the flow profile becomes more parabolic [4–6]. Due to high surface-to-volume ratios on the nanoscale, electrostatic forces induced by a charged wall can place charged particles within a particular flow streamline allowing for mobility differences of molecules that may show no difference on the microscale; this has been called Transverse Electromigration, TEM [45–47]. EDL overlap may also prevent the injection of co-ions into the nanochannel due to concentration polarization [5]. To observe how EDL thickness affected the electrokinetic transport properties of the dye-labeled dNMPs, studies on the transport of the ATTO 532-dNMPs with a carrier electrolyte concentration of 0.45 mMTB

(pH 8.3) under varying electric field strengths was undertaken (Fig. 5). We did not observe concentration polarization of the dye-labeled nucleotides at any EDL thickness evaluated herein. The overall magnitude of the apparent mobility was higher for the dNMPs when compared to the case of the 44.5 mM carrier electrolyte, which possessed a thinner EDL. dCMP did show a field strength dependence on its apparent mobility while the other dNMPs did not; the cause for this is currently being investigated.

Furthermore, a decrease in separation resolution of the mononucleotides was observed with the thicker EDL (0.45 mM TB) as compared to the carrier electrolyte consisting of 44.5 mM and a more compressed EDL. As seen in Fig. 5, the separation resolution dropped to as low as 0.25 with the highest being 2.33 (Table 4). In addition, increases in the standard deviation in the Gaussian fits to the apparent mobility histograms was also observed.

4. Conclusions

In all cases and consistent with our previous papers dealing with nanoslit electrokinetics of charged particles, higher electric field strengths provided lower standard deviations in the apparent mobilities (see Fig. 1) [7,8]. This was due to the prevalence of intermittent motion and recirculation at low electric field strengths, both of which can cause higher variances in the apparent mobilities. The effects of intermittent motion and recirculation could be minimized when employing higher electric field strengths.

We determined that electrokinetic transport of the dNMPs was sensitive to the pH and ion concentration of the carrier electrolyte. Separation resolutions ranging from 0.73 to 2.13 were achieved at pH = 8.3 and at a pH = 10.3; resolutions to 4.84 were observed. While higher pH values may further improve mobility-based identification, it was not possible to carry out these experiments at pH values > 10.3 due to delamination of the nanofluidic device cover plate from the substrate.

Furthermore, it was determined that buffer concentrations that affect the degree of EDL overlap can affect separation resolution as well. For example, decreased resolution of the dNMPs was observed for cases where the EDL was significantly overlapped compared to the case with a lower degree of EDL overlap. For cases where there is partial EDL overlap, more parabolic flow would be expected that can, under the action of TEM, improve resolution. However, we did not observe for our studies most likely due to the fact that the devices used herein were hybrid devices (PMMA-to-COC) that have different surface chemistries and thus, would have different EOF values [9]. For hybrid-type devices, the flow profile can be distorted, which may cause higher variances in the apparent mobilities compared to monolithic devices (e.g., COC-to-COC), especially with thicker EDLs [48].

In these studies, we utilized dye-labeled dNMPs to allow for tracking of the molecules during their transport through the nanoscale channel. However, in our envisioned single-molecule sequencing approach, labeling of the mononucleotides either before or after exonuclease digestion would be problematic [49]. Therefore, single-molecule detection of the dNMPs using a nonlabeling approach would be preferable to allow for identification of single nucleotides following exonuclease cleavage using mobility matching. We are

currently developing such a strategy that employs in-plane pores poised at the input and output ends of a nanochannel column and are fabricated via nanoimprinting in polymers [50]. While this detection format is desirable for our proposed single-molecule sequencing strategy, it cannot track the transport properties of the dNMPs through the nanochannel. Thus, the use of dye-labeled dNMPs and fluorescence tracking provided a viable approach to carefully understand the transport properties of the dNMPs to guide optimization of experimental conditions and nanochannel geometry for optimal identification of the dNMPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Baseline separation resolution of deoxynucleotide monophosphates (dNMPs) was demonstrated using nanoscale electrokinetics.
- The separation resolution of the dNMPs depends on the ratio of the channel dimensions (width and depth) to the electrical double layer thickness.
- At low electric field strengths, the electrokinetics of the dNMPs showed field-dependent mobilities.
- The high identification accuracy of the dNMPs will provide a viable approach for single-molecule sequencing.

Fig. 1.

Apparent mobility versus field strength for ATTO 532 injected into a nanochannel that was 110 nm × 110 nm (width and depth; L = 100 μm) that were fabricated in PMMA and possessed a COC cover plate. The nanochannel electrokinetic used two different carrier electrolytes consisting of; a) 44.5 mM TB with a pH 8.3 (λ_d = 1.25 nm); and b) 0.45 mM TB pH 8.3 (λ_d = 12.5 nm). Errors bars show ± standard deviations for the apparent mobilities.

Fig. 2.

A) Apparent mobility versus the electric field strength for ATTO 532 conjugated to dCMP, dGMP, dTMP and dAMP injected into a nanochannel that was $110 \times 110 \text{ nm}$ (width and depth; $L = 100 \text{ }\mu\text{m}$) and used PMMA as the substrate with a COC cover plate. The electrokinetic used a buffer of 44.5 mM TB at pH 8.3 ($\lambda_d = 1.25 \text{ nm}$). b) Histograms of the apparent mobility for the dye-labeled dNMPs using a field strength of 342V/cm in a 100 μm total length nanochannel. Histograms were fit to a Gaussian function with of the apparent mobility histogram (each bin represents $2 \times 10^{-5} \text{ cm}^2/\text{Vs}$ apparent mobility units). The error bars represent the standard deviations in the measurements.

Fig. 3. Histogram of apparent mobilities for the dye-labeled dNMPs at field strength of 342 V/cm in a 100 μm long nanochannel. The nanochannel electrokinetic conditions are the same as in Fig. 2, but using a carrier electrolyte of pH = 10.3. Gaussian functions were fit to the histograms as discussed in Fig. 2b.

Fig. 4. Relative apparent mobility of the ATTO 532-labeled dNMPs with respect to carrier electrolyte pH values. Apparent mobilities have been normalized to the highest observed apparent mobility for pH 8.3, 9.3 and 10.3. (Note: data lines are connected only for ease of interpretation). The error bars represent the standard deviations in the measurements.

Fig. 5.

A) Apparent mobility versus the electric field strength for ATTO 532 conjugated to dCMP, dGMP, dTMP, and dAMP. The nanochannel electrokinetic conditions are the same as shown in Fig. 2 except that the carrier electrolyte concentration was 0.45 mM TB pH 8.3 ($\lambda_d = 12.5$ nm). b) Histogram of apparent mobility for the dye labeled dNMPs with a field strength of 342 V/cm in a 100 μm long nanochannel. The error bars represent the standard deviations in the measurements.

Table 1

Separation resolution between ATTO 532-dNMP pairs at pH 8.3 and 44.5mMTB (taken from Fig. 2b) were calculated using $R = \mu/\text{wavg}$ where μ is the difference in the average apparent mobility (determined from the centroid of the Gaussian fit to each histogram) for each peak pair and wavg is the average full width of the two peaks, which is related to the standard deviation of the Gaussian.

Resolution	dAMP	dTMP	dGMP	dCMP
dAMP	–	0.97	2.13	1.47
dTMP	0.97	–	0.85	0.98
dGMP	2.13	0.85	–	0.73
dCMP	1.47	0.98	0.73	–

Table 2

Resolution between ATTO 532-dNMPs pairs at pH 10.3 and 44.5 mM TB (taken from Fig. 3) and calculated as in Table 1.

Resolution	dAMP	dTMP	dGMP	dCMP
dAMP	–	2.98	2.94	1.14
dTMP	2.98	–	4.84	0.80
dGMP	2.94	4.84	–	2.42
dCMP	1.14	0.80	2.42	–

Table 3

Identification accuracy for all ATTO 532-dNMPs at pH 10.3 and were calculated using the peak overlap between Gaussian fits to the histogram of apparent mobilities at pH 10.3

Identification Accuracy	dAMP	dTMP	dGMP	dCMP
dAMP	–	>99.9%	>99.9%	97.0%
dTMP	>99.9%	–	>99.9%	87.0%
dGMP	>99.9%	>99.9%	–	>99.9%
dCMP	97.0%	87.0%	>99.9%	–

Table 4

Resolution of ATTO 532-dNMPs pairs at pH 8.3 and 0.45 mM TBE (from Fig. 5b) and were calculated the same procedure as described in Table 1.

Resolution	dAMP	dTMP	dGMP	dCMP
dAMP	–	2.33	0.25	0.51
dTMP	2.33	–	1.64	0.75
dGMP	0.25	1.64	–	0.29
dCMP	0.51	0.75	0.29	–