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Sequence-Based Separation of Single-Stranded DNA by using Nucleotides in Capillary Electrophoresis: Focus on Phosphate

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Abstract

DNA analysis has widespread applicability in biology, medicine, biotechnology and forensics. DNA separation by length is readily achieved using sieving gels in electrophoresis. Separation by sequence is less simple, generally requiring adequate differences in native or induced conformation or differences in thermal or chemical stability of the strands that are hybridized prior to measurement. We previously demonstrated separation of four single-stranded DNA 76-mers that differ by only a few A-G substitutions based solely on sequence using guanosine-5'-monophosphate (GMP) in the running buffer. We attributed separation to the unique self-assembly of GMP to form higher order structures. Here we examine an expanded set of 76-mers designed to probe the mechanism of the separation and effects of experimental conditions. We were surprised to find that other ribonucleotides achieved the similar separation to GMP, and that some separation was achieved using sodium phosphate instead of GMP. Potassium phosphate achieved almost as good separations as the ribonucleotides. This suggests that the separation medium provides a physicochemical environment for the DNA that effects strand migration in a sequence-selective manner. Further investigation is needed to determine whether the mechanism involves specific interactions between the phosphates and the DNA strands or is a result of other properties of the separation medium. Phosphate generally has been avoided in DNA separations by capillary gel electrophoresis because its high ionic strength exacerbates Joule heating. Our results suggest that phosphate compounds should be examined for separation of DNA based on sequence.

1 Introduction

Rapid separation of single-strand DNA (ssDNA) has been central to advances in biology, medicine and forensics. DNA separation by length using sieving gels is a mature field that has been applied in slab gel electrophoresis, capillary gel electrophoresis (CGE), and more recently, in micro- or nano-fluidic chips. Separation by length is adequate for DNA sequencing, which uses four different tags to identify the terminal base in position and thereby recreate the sequence. However, in other applications such as those requiring analysis of complex mixtures of DNA fragments from different organisms, detection of genetic variants, or forensic fingerprinting, separation by length alone is often insufficient. In those cases, the same-length fragments must be sequenced or at least separated by sequence. Although sequencing is the “gold standard”, it is not always necessary or practical and separation by sequence would suffice.

Techniques for DNA separation by sequence generally rely on the established methods of Single-Stranded Conformation Polymorphism (SSCP) [1–3] and Heteroduplex Analysis (HA) [3,4], which separate DNA based on intrinsic or induced (through homoduplex vs. heteroduplex hybridization) differences in secondary structure. Alternatively, separation may be achieved using Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE) [5,6], which separate dsDNA based on differences in resistance to chemical or thermal denaturation. Drawbacks of these techniques include the

need for sufficient differences in conformation or in resistance to thermal or chemical denaturation, and in some cases, knowledge of a “wild type” or target sequence in order to design hybridization probes. Additionally, DGGE and TGGE require stable gradients and the use of DNA primers to prevent complete melting of the dsDNA.

Because of these considerations, DNA analysis would benefit from a new approach to separation by sequence that would be as simple as separation by length and that would require no DNA primers or probes, no gradients, and no prior knowledge of a wild-type or target sequence.

Previous work in our group demonstrated the separation of four ssDNA 76-mers differing by only a few A/G base substitutions using guanosine-5'-monophosphate (GMP) in CE [7,8]. The circular dichroism spectra of the four strands were essentially identical and corresponded to random strands without secondary structure, which indicated that the separation was based on sequence alone rather than conformational differences. We attributed the separation to the tendency of guanosine compounds including GMP to reversibly self-associate through hydrogen bonding into “G-tetrad” structures, in which each of four guanines has two hydrogen bonds to each of its two neighboring guanines [9–11]. The G-tetrads can further associate into columnar aggregates and, at higher total monomer concentrations, into reversible gels. These structures are stabilized by cations such as K⁺ and Na⁺ [12].

To further investigate the sequence selectivity of GMP and the basis of the DNA separation by sequence, in the present work we designed a set of ten ssDNA 76-mers, including the four strands used in the previous work [7,8], variations of the four in which G was randomly replaced by A, T or C to create G-free sequences, a scrambled sequence variant of one of the original strands, and a random, unrelated sequence. We investigated the effects of treatments to denature the ssDNA sample prior to CE injection and of experimental conditions on the separation of the ten 76-mers using GMP. In order to investigate the basis of the separation, we replaced GMP in the running buffer with other reagents including AMP, UMP, sodium phosphate, ribose and dGMP. We also investigated potential interactions among the ssDNA strands in the sample that could influence the separation.

2 Materials and Methods

2.1 Chemicals and reagents

The ten 76-mers with fluorescent dye (6-FAM) labeled at 5' end were synthesized and purified by Integrated DNA Technologies (Coralville, IA, USA). Guanosine-5'-monophosphate disodium salt (GMP, 99%), deoxyguanosine monophosphate (dGMP), guanosine (Guo, 98%), trizma hydrochloride (Tris), 10× concentrate Tris-Borate-EDTA (TBE) buffer, ribose and salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenosine 5'-monophosphate (AMP, 99%) and uridine 5'-monophosphate disodium salts (UMP, 98%) were purchased from Acros Organics (Pittsburgh, PA, USA). Certified A.C.S. urea (60.06 g/mol), glucose and NaOH (1M) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Genetic analysis grade Hi-Di™ formamide was purchased from Applied Biosystems (Warrington, UK). Ultrapure water from an Ultrapure Milli-Q system (Millipore, Bedford, MA, USA) was used in all solution preparations.

2.2 Buffer and sample preparation

Stock solutions of Tris (200 mM), sodium phosphate (200 mM), KCl (200 mM) and urea (10 M) were prepared in water and stored at −5 °C. Running buffers for CE were prepared by adding the appropriate amounts of reagent to the appropriate volumes of stock solutions. GMP and UMP were disodium salts and could be directly dissolved in the buffer. AMP and

d-GMP were in the acid form and were dissolved by adding NaOH at 2x the AMP or d-GMP concentration, followed by pH adjustment as needed using NaOH or HCl. Ribose and glucose were added directly to the running buffers. Unless otherwise noted, the running buffer was 15 mM Tris, pH 8, with 40 mM KCl and 0.14 M separation agent. All stock solutions and running buffers were stored at -5°C and used within one week.

A 50 μM stock solution of each 76-mer was prepared in water and stored at -20°C . Unless otherwise noted, the mixtures of the ten 76-mers used for the separations contained 0.5 μM of each 76-mer in water, 0.01M NaOH or Hi-Di formamide, for a total concentration of 5 μM DNA in the sample. For the spiking experiments, the concentration of the spiked 76-mer was 1.5 μM and the other 76-mers were 0.5 μM each. Samples were either used directly after mixing or thermally denatured by placing the sample in a water bath at 95°C for 5 min and then immediately cooling it in ice water where it remained until use.

2.3 CE separation

Separations were performed using a Beckman Coulter P/ACE MDQ system with fluorescence detection (488 nm excitation and 520 nm emission). Data acquisition and analysis were performed using 32 Karat software (version 7.0). Bare fused-silica capillaries (25 μm I.D., 375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). The separation capillaries were 50.2 cm in total length and 40.2 cm to the detector. Samples were maintained at 20°C in the CE sample holder and buffers were kept at room temperature in the buffer holders. Every day before use the capillary was equilibrated with 0.1 M NaOH for 20 min. Before each run, the capillary was rinsed successively with 0.1 M NaOH, water and buffer, each for 5 min. The sample was then injected using pressure injection (0.5 psi for 5 s). Unless otherwise noted, separations were performed at 27 kV forward voltage. The capillary cartridge was maintained at temperatures ranging from 25 to 55°C . When using a new capillary, stable results were not obtained until after the second run, and so the results for the first two runs on a new capillary were not used. The compositions of the buffers at the capillary inlet and outlet gradually changed over the course of multiple runs, resulting in a shift of migration time, and so buffers were replaced after no more than 10 runs. At the end of each day, the capillaries were rinsed successively with 0.1 M NaOH and water, each for 10 min, and the ends of the capillaries were immersed in water for storage.

3 Results

3.1 Separation of 76-mers using GMP

3.1.1 Sequences of the ten DNA strands—The sequences of the ten single-stranded 76-mers are shown in Table 1. Strands 1.1–1.4 are the original four strands of human STR polymorphisms used in previous work [7,8]. These tetranucleotide repeat polymorphisms are composed of TCTA/TCTG repeats and have many natural variants. The four selected strands differ by only a few A/G base substitutions [13]. Strands 2.1–2.4 correspond to Strands 1.1–1.4 but with G randomly replaced by A, C, or T. The purpose of these strands was to determine if interactions between GMP in the buffer and Gs in the strands are required for separation, in which case strands with no G should not be separated. Strand 3.1 is a random rearrangement of Strand 1.1, designed to test if strands with the same base composition but in different sequences could be separated. Strand 4.1 is a randomly generated sequence that is unrelated to the other nine strands. The random nucleotide replacements in Strands 2.1–2.4, the randomization of Strand 1.1 to form Strand 3.1, and the random design of Strand 4.1, were all performed using an in-house Visual basic program.

3.1.2 Spiking experiments to identify peaks—Figure 1 shows the results of spiking experiments to identify the peaks in the separation of the ten 76-mers at 55 °C using 0.14 M GMP. The DNA sample was prepared in formamide and thermally-denatured prior to injection into the CE. Strands 1.1–1.4 were separated in the same order as previously reported [7,8], with migration time increasing with increasing A/decreasing G in the strands. Strands 2.1–2.4, in which other nucleotides were substituted for G, were separated into three peaks. The strands had longer migration times than 1.1–1.4 but appeared in the same order, with the exception that Strands 2.2 and 2.4 were not resolved. This indicates that G needn't be present in the strand to achieve separation and that the separation mechanism does not rely on interactions between G in the DNA strands and G in the buffer. The overlap of Strands 1.4 and 2.3 is attributed to the overall shifting of Strands 2.1–2.4 to longer migration times compared to Strands 1.1–1.4. Strands 1.1 and 3.1, which have the same base composition but in different sequences, were well resolved, indicating that the separation is sensitive to sequence and not just overall base composition. The randomly generated Strand 4.1, which is unrelated to the other strands and has a high content of G and C bases, appeared at much longer migration times. Over the course of several runs, the Strand 4.1 peak would move to longer migration times, broaden, and eventually disappear. We attribute this to a return to stable conformations over time following the denaturing of the sample pre-injection, as discussed in the following section.

3.1.3 Effects of Denaturation—Since we set out to determine if GMP can separate ssDNA based solely on sequence, it was important to minimize other contributions to a separation such as intramolecular conformation or intermolecular interactions. This is generally achieved by running the separation under denaturing conditions using high concentrations of a denaturant such as urea in the running buffer. However, these denaturants would likely interfere with the self-assembly of monomeric GMP in the running buffer. Additionally, we found that the viscosity of the GMP medium increased dramatically in the presence of urea, leading to difficulties with heating, reproducibility and even with filling the capillary. An alternative is to increase the run temperature so that it is above the melting temperature of intramolecular conformations and intermolecular aggregates and apply thermal and/or chemical denaturation to the sample prior to injection into the CE. These experiments are described in this section.

The fact that ssDNA could form hairpin structures in free solution is well known and has been applied in SSCP. The addition of various cations at moderate concentration could stabilize hairpin structures and thereby affect the separation of the 76-mers [14–16]. In order to determine the likelihood of hairpin formation by the 76-mers, we calculated the melting temperatures T_m of possible hairpin structures using IDT OligoAnalyzer 3.1. The limitation of this analyzer is that it only provides the calculation model for DNA in solutions containing Na^+ or Mg^{2+} . However, predication of the upper limit of hairpin T_m is still possible since the stabilizing effects of cations decrease in the order of $\text{Na}^+ \sim \text{K}^+ > \text{Tris}^+$ [15]. In our experiments, the most concentrated buffer contained 0.14 M GMP (0.28 M Na^+), 0.04 M KCl and 15 mM Na-PO_4 (pH = 7.6). We estimated the total concentration of cations to be 340 mM. The DNA sample has a total concentration of 5 μM and will be diluted during the separation. To calculate the highest possible T_m , the Na^+ concentration was set to 340 mM and DNA concentration was set to 5 μM . The values for the highest T_m for Strands 1.1–1.4 and Strands 2.1–2.4 were calculated to be -2.6°C . Since the CE separations were performed at 25–55 °C, these strands should not exhibit any significant amount of stable hairpins that could influence the separation. The highest T_m s of hairpins for Strands 3.1 and 4.1, however, were calculated to be 31 °C and 51 °C, respectively. Secondary structure could therefore play a role in the separation of these strands.

In order to study the influence of hairpin formation by Strands 3.1 and 4.1 on the separation, we compared the separation of mixtures of the ten strands with and without thermal and/or chemical denaturation prior to injection into the CE. Formamide is a commonly used denaturant that has low ion-solvating power and can increase the hydrophobic character of the solvent [17,18]. NaOH is another denaturant that increases the net charge of DNA [19]. Results for separations performed on samples directly dissolved in water and not denatured prior to injection were compared with results for samples prepared in water, formamide or 0.01 M NaOH and thermally denatured prior to injection. The results are shown in Figure 2. In all cases, the peaks migrated in the same general order as in Figure 1. The non-denatured sample (Figure 2a) was separated into 6 peaks, whereas the thermally denatured samples in water (Figure 2b) and formamide (Figure 2c) were separated into 7 peaks. Several hours after denaturation, the denatured sample in formamide was still separated into 7 peaks. However, the height of the last peak, identified as Strand 4.1, was much lower than before. The sample was re-denatured thermally and the seventh peak increased in height. Treatment with 0.01 M NaOH prior to injection (Figure 2d) did not improve separation compared to the other denaturation methods and yielded longer migration times due to the increased charge on the DNA. The signal was lower than in the other separations, and only 6 peaks were observed.

As mentioned above, denaturation of DNA can also be achieved by adding urea to the running buffer at concentrations of 1 M or greater [20,21]. In our experiments, high concentrations of urea made the running buffer with GMP too viscous to use in the capillary separations. For this reason, we could go no higher than 1 M urea. The separation using Tris buffer containing 1 M urea (Figure 2e) for a sample that was thermally denatured in water prior to injection shows 7 peaks, but unlike the other separations, the seventh peak was close to the sixth peak. This is probably due to the absence of secondary structure of Strand 4.1 during the separation. However, the fluorescence signals were weak and therefore not amenable to detection. For this reason as well as the problem with viscosity that could limit the range of possible experimental conditions, urea was not used in subsequent experiments. However, the similarity among the results under the various conditions and sample treatments do support the conclusion that the separation is not due to conformational differences. Additionally, the preparation of the DNA sample in distilled deionized water in Figure 2b and in the remainder of experiments following this section should further ensure the absence of secondary structure and discourage any intermolecular interactions of the DNA since distilled deionized water is known to be a denaturant for DNA.

The variation in migration times in Figure 2 may be due to differences in migration caused by the different media in which the DNA sample is dissolved, particularly 0.01 M NaOH used in Figure 2d which shows the most discrepancy. Another contributing factor may be irreproducibilities due to capillary history, age and use, capillary-to-capillary variation, etc. Since the experiments varied so much in what was being injected over the course of several runs in a given capillary we did experience these irreproducibilities in many experiments. With the exception of Strand 4.1, however, the profiles of the electropherograms and the migration orders of the strands were consistent among the different experiments.

3.1.4 Effects of Separation Temperature—Separation temperature is a particularly important consideration for running buffers containing GMP since the aggregation and gelation of GMP are temperature dependent and will affect the buffer viscosity as well as the formation of stable, G-quadruplex structures that might be a factor in the DNA separation. Temperature may also affect the formation of stable conformations by Strands 3.1 and 4.1, as discussed in the previous section. To examine these effects, we performed the separations at temperatures ranging from 25 °C to 55 °C. Because the last peak (Strand 4.1 peak) had a relatively low signal, it was spiked to 3x the concentration of the other strands. As shown in

Figure 3, the electropherogram profile changed with temperature. This may be due to reduction of secondary structures as well as changes in GMP aggregation at the higher temperatures. Since the goal is to demonstrate separation based solely on DNA sequence and not strand conformation, subsequent separations were performed at 55 °C.

3.1.5 Effects of pH—The pH is an important consideration in DNA separation. Limitations at the low pH end are imposed by quenching of the fluorescence of the FAM-6 tag at acidic pH and by GMP self-association, which is promoted by acidity and causes the GMP run buffer to become too viscous to use in the capillary separation. At the high pH end, the upper limit is imposed by the deprotonation of guanine and thymine, which have pK_a s around 9.5 and would tend to reduce the interactions of DNA with the negatively charged GMP in the buffer. These considerations led us to restrict the pH range that we examined to pH 7–9.

The effect of pH on the separation of the ten 76-mers is shown in Figure 4. The peaks migrated in the same general order as in Figure 1. At pH 7, the GMP buffer was highly viscous, resulting in long migration times and peak broadening. Results were improved at pH 8, at which the viscosity of the GMP buffer appeared similar to that of the buffer without GMP. At this pH, the peaks were narrower, migration was faster, and the ten 76-mers were resolved into 7 peaks. Migration time was further reduced at pH 9 but the separation was poor. We therefore selected pH 8 for subsequent experiments.

3.1.6 Effects of buffer type—The chemical composition of the buffer to which GMP is added could influence the DNA separation. Among buffers commonly used at pH 7–9, potassium phosphate has relatively high ionic strength and could reduce the EOF. Potassium is also known to stabilize G-quadruplex structures [12]. Tris buffer has lower ionic strength and would decrease Joule heating. TBE buffer contains boric acid, which could form complexes with the DNA [22,23]. A comparison of the separations achieved upon addition of 0.14 M GMP to each of these buffers is shown in Figure 5. Differences among the separations were minor, with overall best separation achieved using GMP in Tris buffer. The presence of boric acid in TBE apparently does not affect the separation. The results indicate that supporting buffer type to which GMP is added is not a major factor in the separation.

3.1.7 Effects of GMP concentration—GMP influences the ionic strength of the solution and increasing GMP concentration drives its self-association into G-tetrads and higher order structures [9–12]. To investigate the influence of GMP concentration, a series of Tris running buffers were prepared with increasing GMP concentration. Sodium phosphate monobasic/dibasic were added to the buffers to maintain constant phosphate concentration. The results (Figure 6) show that migration time and peak resolution both increased significantly with increasing GMP, even though at 55 °C and pH 8 higher order aggregates of GMP are unlikely. Interestingly, there is some resolution of peaks in the Tris buffer containing 0.14 M sodium phosphate but no GMP (Figure 6a). Since the samples were thermally denatured prior to injection and the separations were performed at 55 °C, it is unlikely that the separation in Tris buffer containing sodium phosphate but no GMP is due to conformational differences among the strands. This result therefore suggests that the separation involves interactions between phosphates, whether added to the run buffer as GMP or as sodium phosphate, and the 76-mers. This is supported by results (not shown) for separation of the ten 76-mers in simple Tris buffer without GMP or sodium phosphate, for which there was no separation and the 76-mers migrated as a single peak.

Experiments were also performed using the Tris buffer without GMP containing 0.14 M potassium phosphate instead of 0.14 M sodium phosphate. The results (Figure S1) show separation that is better than for sodium phosphate (Figure 6a) and although not as good as

0.14 M GMP (Figure 6d). This strongly suggests that phosphate is responsible for the separation. We next performed experiments to hone in on the separation mechanism, as described in the following sections.

3.2 Separation mechanism

3.2.1 Effect of Nucleobase—According to the separation results for Strands 2.1–2.4, the interaction of GMP with the 76-mers is not simply based on interactions between GMP in the running buffer and G in the DNA strands. To gain insight into the separation mechanism, we performed the separations using AMP and UMP instead of GMP in the running buffer. As shown in Figure 7, the results for AMP and UMP were similar to those for GMP. Spiking experiments demonstrated that the migration order was essentially the same as for GMP, as shown by the peak assignments in Figure 7. The only exception was that the 3.1 and 2.1 peaks were somewhat resolved using AMP and UMP but not GMP. The similar separation abilities suggest that the separation is based on some common properties shared by AMP, GMP and UMP. All of the nucleotides have a phosphate, a ribose and a nucleobase. We performed experiments to examine each of these.

3.2.2 Effect of Sugar—To investigate the role of the sugar moiety in GMP on the DNA separation, we performed separations using sugars instead of GMP in the Tris running buffer. We also performed a separation using the deoxyribose nucleotide dGMP instead of GMP. Finally, we used sugars in combination with GMP. The results are shown in Figure 8. For the separations using glucose (Figure 8a) or ribose (Figure 8b), the 76-mers were detected within 5 min and no separation was achieved. The separations using dGMP, which differs from GMP only in the presence of the deoxyribose instead of ribose, were essentially the same as those for GMP (Figure 8c). To eliminate the influence of ionic strength and migration time, we then tested 0.14 M GMP in Tris buffer with the addition of 0.14 M glucose or ribose (Figures 8d and 8e). The separations showed some improvement in peak resolution compared to GMP alone and in a shorter time. The results indicate that the sugar alone is not responsible for the separation, but the presence of the sugar improves the separation.

3.2.3 Effect of DNA concentration in the sample—Another possible explanation for the separations is interactions among the 76-mers in the sample. Strand 4.1 has particularly high content of G and C bases and a T_m of 39.7 °C. It could form hairpin structures even at 55 °C and may also be capable of forming hetero-dimers with other strands. Since the 4.1 peak had lower intensity than the other 76-mers and in some cases was not even detected, we considered the possibility that some amount of this strand could remain in the capillary, coating the capillary walls to form a phase that could interact selectively with the other strands in subsequent runs. To test this, we performed the separations in fresh capillaries and eliminated Strand 4.1 from the sample mixture. Separations were performed using GMP, AMP and UMP. In the first few runs, the separation was not as good as results for the separation of the 76-mers including Strand 4.1, but this was generally the case for the first couple of runs on a new capillary. After several runs, the separation was the same without Strand 4.1 as with Strand 4.1 (Supplementary material Figure S2).

Other intermolecular interactions among the 76-mers could also induce separations. These interactions would be reduced by decreasing the total DNA concentration in the sample. To test this, we compared separation of 76-mers at 5 μ M total DNA concentration and 0.5 μ M total DNA concentration. Separation was similar at both concentrations, indicating the absence of effects due to inter-strand interactions (Supplementary material Figure S3).

4 Discussion

The results appear to rule out several possible explanations for the sequence-based separation of the 76-mers in this work. Contrary to our original hypothesis that the separations are due to interactions of the ssDNA with higher-order G-quadruplex structures arising from self-assembly of GMP [7,8], this cannot be the case since AMP and UMP, which do not form such higher order structures, achieved the same results as GMP. Although the sugar in the nucleotides appears to promote the interactions that lead to separation, no separation is achieved using sugar alone.

The results also rule out interactions among the DNA strands in the sample as the basis for the separations. Interactions should increase with increasing concentration. Since 10-fold dilution of the DNA in the sample (Figure S3) had no effect on the separation, we conclude that intermolecular interactions are not involved in the separation. This is further supported by the absence of changes when Strand 4.1 is omitted from the sample (Figure S2). The separations also do not appear to be due to differences in DNA conformation, based on the similarity of the results for the 55 °C separations regardless of thermal or chemical denaturation of the sample prior to injection. Only Strand 4.1 showed any evidence of conformation change and this effect was time-dependent, indicating renaturation over time.

This leaves phosphate as the driver of the separation. Interestingly, it has been reported that AMP, UMP, CMP or GMP can be used in the running buffer for capillary electrophoretic separation of polyphosphates and polyphosphonates [26]. Since DNA is a polyphosphate, similar mechanisms may be involved here. Comparing the separation media prepared in Tris buffer, sodium phosphate achieves some resolution of the 76-mers with short migration times, potassium phosphate improves the separation and shows longer migration times, and ribonucleotides show somewhat better resolution than potassium phosphate with similar migration times. This suggests that the separation medium provides a physicochemical environment for the DNA that effects strand migration in a sequence-selective manner. Further investigation is needed to determine whether the mechanism involves specific interactions between the phosphates and the DNA strands or is a result of other properties of the separation medium.

Because of their comparatively high ionic strength, phosphate buffer systems produce high Joule heating during CE separations, leading to peak broadening and detachment of capillary coatings [24, 25]. This is especially problematic in CGE separations, which are often conducted in 50–100 µm I.D. capillaries, and so phosphate buffer has generally been avoided in DNA separations. This may be why the DNA sequence selectivity of phosphate has not been previously noted. Based on the present work, the use of nucleotides and other phosphate-containing compounds for sequence-based separation of DNA could provide a simple alternative to stability- and conformation-based approaches and warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Results of spiking experiments to identify peaks in GMP separation of ten 76-mers. The DNA sample was prepared in formamide and thermally denatured prior to injection into the CE. The running buffer was 0.14 M GMP, 0.04 M KCl, 15 mM Tris, pH 8. Separations were performed at 27 kV, 55 °C.

Figure 2.

Effects of different methods for denaturation of the ssDNA sample prior to injection on the GMP separation of ten 76-mers. The running buffer was 0.14 M GMP, 0.04 M KCl, 15 mM Tris, pH 8. Separations were performed at 27 kV, 55 °C. (a) DNA sample dissolved in water without denaturation. (b) DNA sample dissolved in water and thermally denatured. (c) DNA sample dissolved in formamide and thermally denatured. (d) DNA sample dissolved in 0.01 M NaOH and thermally denatured. (e) DNA sample dissolved in water and injected without further treatment into the Tris running buffer containing 1 M urea.

Figure 3.

Effect of temperature on GMP separation of ten 76-mers. The DNA sample was prepared in water and thermally denatured prior to injection. Separations were performed at 27 kV. The concentration of each strand was 0.5 μ M, except for 4.1, which was spiked to 1.5 μ M to improve its detectability. The running buffer was 0.14 M GMP, 0.04 M KCl, 15 mM Tris, pH 8. (a) 25 °C. (b) 35 °C. (c) 45 °C. (d) 55 °C. The figures to the right show an expanded time axis.

Figure 4.

Effect of pH on GMP separation of ten 76-mers. The DNA sample was prepared in water and thermally denatured prior to injection. The running buffer was 0.14 M GMP, 15 mM Tris, 40 mM KCl. Separations were performed at 25 kV, 55 °C. Separation voltage was 25 kV. For (a), 0.1 psi pressure was also applied to assist in pushing viscous buffer through the capillary. (a) pH 7. (b) pH 8. (c) pH 9.

Figure 5.

Effect of different buffers on GMP separation of ten 76-mers. The DNA sample was prepared in water and thermally denatured prior to injection. Separations were performed at 27 kV, 55 °C. (a) 0.14 M GMP, 0.04 M KCl, 15 mM potassium phosphate, pH 7.6. (b) 0.14 M GMP, 0.04 M KCl, 15 mM TBE, pH 7.8. (c) 0.14 M GMP, 0.04 M KCl, 15 mM Tris, pH 8.

Figure 6.

Effect of GMP concentration on separation of ten 76-mers. The DNA sample was prepared in water and thermally denatured prior to injection. The run buffer was 15 mM Tris, pH 8, 40 mM KCl. Sodium phosphate was added as needed to maintain 0.14 M total phosphate in (a)–(d). The separations were performed at 27 kV, 55 °C. (a) no GMP, 0.14 M sodium phosphate. (b) 0.06 M GMP, 0.08 M sodium phosphate. (c) 0.10 M GMP, 0.04 M sodium phosphate. (d) 0.14 M GMP, no sodium phosphate. (e) 0.18 M GMP, no sodium phosphate.

Figure 7.

Comparison of separations of ten 76-mers using different nucleotides. The DNA sample was prepared in water and thermally denatured prior to injection. Running buffer was 0.14 M nucleotide, 15 mM Tris, 40 mM KCl, pH 8. Separations were performed at 25 kV, 55 °C. (a) GMP; (b) AMP; (c) UMP. Strand 4.1 is not shown in the UMP electropherogram due to its long migration time.

Figure 8.

Separations of ten 76-mers using sugars, dGMP, or sugar + GMP. The DNA sample was prepared in water and thermally denatured prior to injection. The separations were performed at 27 kV, 55 °C. The running buffer was 15 mM Tris, 40 mM KCl, pH 8, with: (a) 0.14 M glucose; (b) 0.14 M ribose; (c) 0.14 M dGMP; (d) 0.14 M GMP and 0.14 M glucose; (e) 0.14 M GMP and 0.14 M ribose.

The sequences of the ten 76-mers, Nucleotides highlighted in bold in Strands 1.2–2.4 indicate base substitutions relative to Strand 1.1.

Table 1

76-mers differing by only a few A-G substitutions	1.1	TCTATCTATCTATCTGTCGTCTGTCGTCTGTCGTCTATCTATCTATCTATCTATCTATCTATCTA
	1.2	TCTATCTATCTATCTATCT A TCATCTATCTGTCGTCTGTCGTCTGTCGTCTATCTATCTATCTATCTATCTA
	1.3	TCTATCTATCTATCTGTCGTCTGTCGTCTGTCGTCTGTCGTCTATCTATCTATCTATCTATCTATCTA
	1.4	TCTATCTATCTATCTATCT A TCGTCTGTCGTCTGTCGTCTATCTATCTATCTATCTATCTATCTATCTA
76-mers with G replaced by other bases	2.1	TCTATCTATCTATCTATCT T TCTCTCTATCT T TCTATCTATCTATCTATCTATCTATCTATCTATCTA
	2.2	TCTATCTATCTATCTATCT A TCATCTATCT T TCTATCTATCTATCTATCTATCTATCTATCTATCTA
	2.3	TCTATCTATCTATCTATCT T TCTATCTATCTCTCTCTCT T TCTATCTATCTATCTATCTATCTATCTATCTA
	2.4	TCTATCTATCTATCTATCT A CT T TCTATCTCTCTATCTATCTATCTATCTATCTATCTATCTATCTATCTA
Rearrangement of 1.1	3.1	TTTATTCAGTTTACATTGTCGTTTGTCGATCCCTATCTCTACCTTTCTCTGTCCTTACTTCTTTCTTAAACATTT
A random 76-mer	4.1	TATTTTATGTAATCGTACTGGTGAGAGTACATCCCGGTTGAGAGGTACGAGTTATTGGTCGCGGATGGGATATGCGGT