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Size exclusive capillary electrophoresis separation of DNA oligonucleotides in small size linear polyacrylamide polymer solution

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Abstract

The mobility of analytes in capillary electrophoresis using polymer gels and solutions is usually described as having an inverse relationship with the molecular size (mobility decreases as molecular size increases). The most commonly used models for predicting such mobility are the Ogston model and the Reptation model. However, in this study a new separation phenomenon was observed in which the mobility of DNA oligonucleotides increased with molecular size in a capillary electrophoresis phase (CEP) coated capillary column. The polymer system used was a 11% linear polyacrylamide ($M_r = 1500$) solution. The log-transformed number of base pairs ($\log N$) of three double stranded oligonucleotides had an inverse linear relationship ($r^2 > 0.9981$) with their migration time in the capillary column. Such a relationship is similar to that observed with size exclusive chromatography.

Keywords: Inverse relationship; Low molecular mass polymers; Oligonucleotides; CE separation; Size exclusive

1. Introduction

Significant progress has been made in the past decades in the separation of bio-molecules such as proteins, polysaccharides, DNA and its fragments using capillary electrophoresis (CE) techniques [1]. The availability of commercial CE instruments has greatly helped the advance of the application of this technique [2]. Since later 1980s, flexible polymer solutions have been used to replace rigid gels in the separation of DNA fragments, and in DNA sequencing [3]. Unlike gels such as agarose and cross-linked polyacrylamide [4], flexible polymer solutions (also referred to as replaceable polymers) in CE columns can be renewed between runs by flushing with polymer-containing running buffer which is more suitable for routine analysis since it avoids such problems as gel shrinkage, bubble formation, and the short life time of the gel columns [5]. Several polymers including hydroxyethylcellulose (HEC), linear polyacrylamide (LPA), poly-*N*,*N*-dimethylacrylamide (pDMA), and poly(ethylene oxide) (PEO), have been used in DNA separation [6]. Capillary CE columns, in which polymer solution is filled during the separation, are either dynamically or permanently coated with polymers to minimize or entirely eliminate the undesired electroosmotic flow (EOF). The polymers used for such coatings include hydrophilic polymers such as poly(vinyl alcohol) (PVA), LPA and PEO.

So far, in almost all reported DNA separations using coated CE columns, the elution order of the DNA fragments is from small (low base number) to large (high base number), when polymer solutions were used [5,7]. These polymer solutions are also referred to as sieving matrices, as the separation of DNA fragments under such conditions could be explained by the sieving mechanisms including the Ogston model and the Reptation model [8,9]. Up to now, all replaceable polymers used in the DNA separation had a molecular mass greater than 50 000 amu, often exceeding 100 000 amu [6]. DNA separation in polymer solutions is a complex process. Many factors such as type, size and concentration of the polymers can influence the migration of DNAs in the polymer solution, especially in a replaceable polymer system [10,11]. In particular, separation of DNAs using lower molecular mass polymers has not been reported.

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In this paper, we report a reversed migration order of DNA oligonucleotides (from large to small molecular size) in a low molecular mass LPA polymer solution (M_r = 1500 amu), using a coated CE column. A high molecular mass polymer (M_r = 600 000–1 000 000 amu) was also tested for comparison.

2. Experimental

2.1. Apparatus and operating conditions

An Agilent Capillary Electrophoresis instrument (3D-CE) with a built-in Diode-Array Detector was used for CE analyses (Agilent Technologies, Waldbronn, Germany). An Agilent CE ChemStation was used for the instrument control, data acquisition, and data analysis. The peaks were monitored at a UV wavelength of 258 nm. An Agilent capillary column covalently coated with a capillary electrophoresis phase (CEP) (72/80.5 cm length, 75 μm i.d.) was used for the separation of oligonucleotides. Separations were carried out at a column voltage of −15 kV. The analytes were introduced into the capillary column by electrokinetic injection of 3 s or 5 s at −15 kV.

2.2. Chemicals and solvents

Three single-stranded DNA oligonucleotides, brain-derived neurotrophic factor (BDNF), serotonin transporter (5-HTT), and half size of BDNF starting from 3′ to 5′ (H-BDNF), and their complements were synthesized and purified by the BIO S&T (Lachine, Canada). BDNF is a 22-mer, 5-HTT a 20-mer, and H-BDNF a 11-mer oligonucleotide. The sequence of these oligonucleotides was: BDNF, 5′-GGACTCTGGAGAGCGTGAATGG-3′5-HTT; 5-HTT, 5′-TGGACCTGGGCAATGTCTGG-3′; and H-BDNF, 5′-GGACTCTGGAG-3′. A commercially available 10 bp step ladder DNA mixture (from 10 bp to 100 bp, catalog no. 4771) was purchased from Promega Corporation (Madison, WI, USA). Tris(hydroxymethyl)aminomethane (Tris, +99.9%), boric acid (99.99%), EDTA (+99%), hydrochloric acid (HCl, high purity grade), and LPA-1500 (M_r = 1500 amu) were obtained from Aldrich (Oakville, Canada). Another LPA (M_r = 600 000–1 000 000 amu) was obtained from Polysciences (Warrington, PA, USA). De-ionized water was generated in-house in the laboratory.

2.3. Preparation of DNA samples and buffer solutions

Double-stranded DNA oligonucleotides, ds-BDNF (22 bp), ds-5-HTT (20 bp) and ds-H-BDNF (11 bp), were prepared by mixing 50 μL of 500 μM of each single-stranded oligonucleotide with 50 μL of 500 μM of its correspondent complement and 400 μL of water in an Eppendorf tube, respectively. The tubes were floated in a 1000-ml beaker containing 700 mL of boiling water and then the water was left cool down naturally to room temperature (in about 4 h). Dilution of the synthesized double-stranded oligonucleotides was made with de-ionized water. All DNA samples were stored at −20 °C.

The polymer buffer solution containing 90 mM Tris, 330 mM boric acid and 2 mM EDTA for CE analysis was prepared as follows. The buffer solution was made of 9.0 mL of 1 M Tris, 2.0404 g of boric acid and 0.07445 g of EDTA in water in a 100-mL volumetric flask. The pH in the water solution was adjusted to 7.4 with 1 M HCl. An appropriate amount of LPA polymer was added slowly to 25 mL of the buffer solution to form a CE polymer buffer solution. The solution was filtered through a 0.45 μm filter prior to instrument use.

3. Results

Since the double stranded DNA oligonucleotides used in the study were relatively small (11–22 bp), low molecular mass polymer LPA-1500 was selected for the separation of the three oligonucleotides, especially the 20 bp and 22 bp oligonucleotides. Under the experimental conditions, the larger DNA oligonucleotides (22 bp) migrated through the column first, followed by the 20 bp one. The 11 bp oligonucleotide came out the last (Fig. 1a). This migration order was opposite to the reported migration order of DNA fragments in polymer solutions [5,7]. When the same type of LPA polymer with high molecular mass (M_r = 600 000–1 000 000 amu) was used, the observed migration order (Fig. 1b) was from small to large oligonucleotides, same as the reported ones. To better understand the newly observed "unusual" migration order, separation conditions of CE such as polymer concentration,

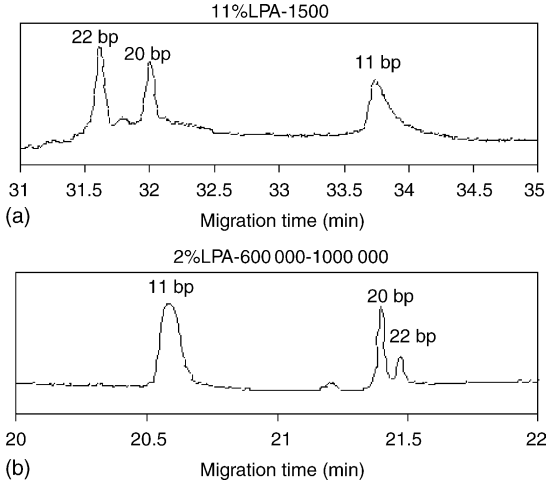

Fig. 1. Migration order of three oligonucleotides (22 bp, 20 bp and 11 bp) in a CEP coated capillary column (75 μm i.d. and 72/80.5 cm long). Conditions: injection: −15 kV/3 s; voltage: −15 kV; cassette temperature: 25 °C; detector wavelength: 258 nm; running buffer: 90 mM Tris, 330 mM boric acid, 2 mM EDTA, adjusted to pH 7.42; polymer solution: (a) 11% LPA-1500 and (b) 2% LPA-600 000–1 000 000.

electrical field strength, and separation temperature were investigated.

The effect of polymer concentrations of the LPA-1500, ranging from 1% to 11% was examined. While increasing the polymer concentration slowed down the migration of the three tested DNA oligonucleotides, it did not change the migration order (Fig. 2). Over the tested range, a linear relationship between migration time of oligonucleotides and the polymer concentration was evident ($r^2 > 0.998$). Interestingly, the intercept of all three oligonucleotides was at around 20.6 min, which represented the migration time without the polymer, and at that point, there was also no separation of the three oligonucleotides.

The effect of polymer concentration was also evaluated on the high molecular mass LPA polymer (M_r = 600 000–1 000 000 amu), in the concentration range from 0.1% to 4%. The high molecular mass polymer was very viscous, which limited the maximal concentration of the polymer in the running buffer to 4%. Similar to the results with LPA-1500, while migration times of each DNA oligonucleotide increased with increased polymer concentration in the buffer solution, the elution order (in this case, from small to large oligonucleotides) did not change.

The effect of temperature was measured by conducting CE at various cassette temperatures. In general, the increase of cassette temperature accelerated the migration of the oligonucleotides in the column resulting in reduced migration time (Fig. 3). The migration order of the three oligonucleotides remained the same under various cassette temperatures.

Finally, the voltage that was applied to the CE column was investigated at three levels: −5 kV, −10 kV and −15 kV (Fig. 4). The CE column used for the voltage experiments was a short column (40 cm effective length instead of 72 cm) of the same type. Again, the migration order remained same for all three voltages. However, the separation efficiency decreased with the increase in voltage.

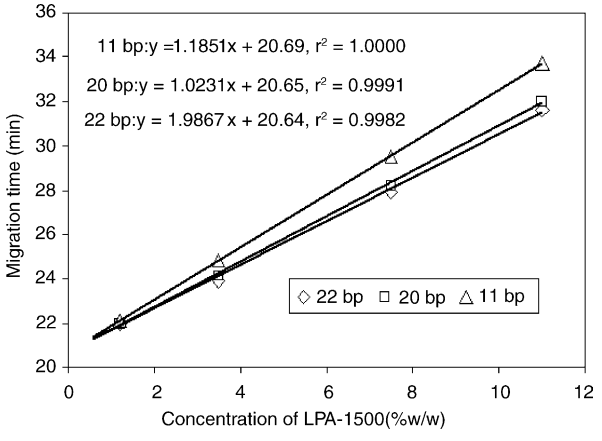

Fig. 2. Effect of polymer concentration of LPA-1500 on migration time. Conditions: capillary column: CEP, 75 μm i.d. and 72/80.5 cm long; injection: −15 kV/3 s; voltage: −15 kV; cassette temperature: 25 °C; detector wavelength: 258 nm; running buffer: 90 mM Tris, 330 mM boric acid, 2 mM EDTA, adjusted to pH 7.42.

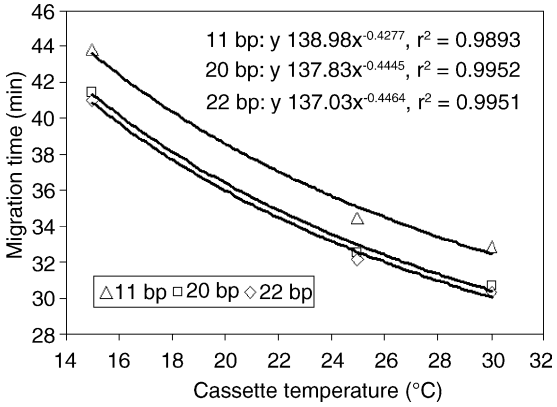

Fig. 3. Migration time of DNA oligonucleotides at different column cassette temperatures. Conditions: capillary column: CEP, 75 μm i.d. and 72/80.5 cm long; injection: −15 kV/3 s; voltage: −15 kV; detector wavelength: 258 nm; running buffer: 90 mM Tris, 330 mM boric acid, 2 mM EDTA, adjusted to pH 7.42; polymer solution: 11% LPA-1500.

4. Discussion

Summarizing the experimental results mentioned above, it is evident that polymer size (M_r = 1500 amu versus M_r = 600 000–1 000 000 amu) was the controlling factor for the migration of the three DNA oligonucleotides in capillary electrophoresis.

It is commonly believed that when a polymer concentration, c, is below the overlapping concentration, c^*, the polymer coils are hydro-dynamically isolated from each other, in other words, each polymer chain is self-tangled to form a global structure in the solution. When $c > c^*$, the polymer solution becomes so concentrated that the chains become entangled with each other forming a dynamic network [8]. In latter case, smaller DNA fragments can move more rapidly than the larger ones through this network and polymer solutions serve as sieving media [12]. Diluted polymer solutions have been used to separate large DNA fragments, up to 23 kbp [13–15]. High molecular mass polymers such as Dextran

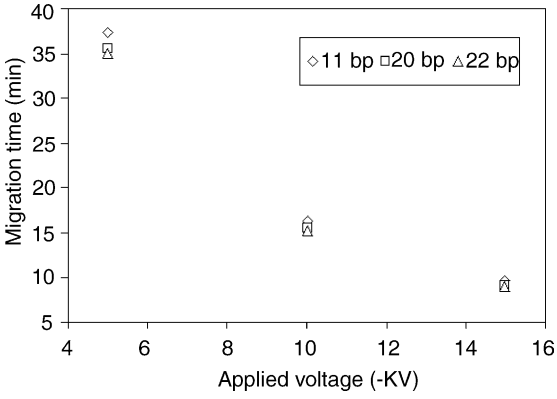

Fig. 4. Migration times of DNA oligonucleotides at three applied column voltages. Conditions: capillary column: CEP, 75 μm i.d and 40/48.5 cm long; buffer: 90 mM Tris, 330 mM boric acid, 2 mM EDTA and 11% PA-1500 at pH 7.4; cassette temperature: 25 °C; injection: −10 kV/5 s.

(M_r = 2 000 000 amu) and HEC (M_r = 90 000–105 000 amu) were used in such cases where the migration order of DNA fragments from small to large sizes was still observed.

In the case of low molecular mass polymer LPA-1500, the migration order of the DNA nucleotides did not change over a concentration range from 1% to 11% (Fig. 2). Under the experimental conditions in this study, the entanglement of the polymers seems to have been insignificant because the migration order observed in Fig. 1a could not be explained by the sieving mechanism [8,10]. An inversed migration order of DNA fragments in bare capillary columns, however, has been reported for DNA fragments using ultra-diluted HEC polymer solutions [16]. This phenomenon can be attributed in large measure to the use of bare column, which generates the electroosmotic flow (EOF) and interacts with the analytes. Indeed, when the same polymer (HEC, 90 000–105 000 amu) was used on a coated column, the "normal" migration order was observed [13,14].

One of the possible mechanisms to explain the lack of entanglement in low molecular mass polymer solutions could be that, at a molecular mass of 1500 amu, each polymer chain, which consists of about 21 units of acrylamide, might have a greater tendency to self-coil and to form a globular structure instead of connecting with other chains leading to entanglement. Lack of entanglement in low molecular mass polymers has been well documented in the literature [17,18]. The viscosity of the polymer, which resulted from the entanglement of polymers, is directly dependent of the polymer size [17]. It was calculated that the chain entanglements are not important to flow behaviour when the polymer molecular mass is below some critical values, which varies from 2000 amu to 60 000 amu, depending on the structure of polymers [18]. Not only the lack of entanglement of the polymers, but also the short chain of DNA oligonucleotides (11–22 bp) may have contributed to the reversed migration order in this study. The short chain of double stranded DNA oligonucleotides would likely form a rod-like structure instead of a coil structure [19]. The migration of the short chain DNA oligonucleotides might be well controlled by the interaction between globular structure of the polymer and rod-like DNA oligonucleotides through diffusion of oligonucleotides in and out of the "porous" structure of the polymer, in a similar manner to that by which macromolecules move in size exclusive chromatography (SEC), where the larger molecules have less probability of being trapped in the pores of the separation medium, and therefore, travel faster than the smaller ones [20]. The diffusion of DNA nucleotides in the polymers has been documented as being independent of their sequences [21]. That also explains the fact that the sequences of the three oligonucleotides tested in this study had little effect on the migration order.

According to SEC theory, the retention times of molecules are inversely proportional to the logarithmic value of their molecular size, or the molecular mass in approximation. The behaviour of oligonucleotides in CE follows such relationship quite well. When the migration time (t) of the three oligonucleotides was plotted against the logarithm of the number of bp (log(N)), an inverse linear relationship was observed (Fig. 5) with a coefficient of determination value r^2 of 0.9981. The dotted line in Fig. 5 is the extension of the fitting curve of the three oligonucleotides. It can be seen that the migration of DNA oligonucleotides from 10 bp up to 40 bp could be predicted well using the three oligonucleotides as the reference. The migration time started deviating from the linear relationship at lengths greater than 40 bp. Similar to SEC, size exclusive CE also has dynamic range limits in which the migration times of the oligonucleotides could be predicted. But compared to SEC, the deviation of migration time in size exclusive CE is rather a gradual process, implying the difference between size exclusive CE and size exclusive chromatography.

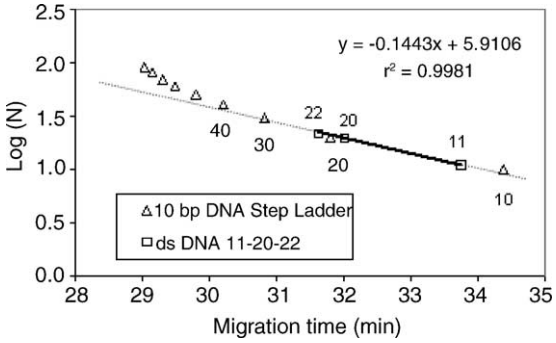

Fig. 5. Relationship between migration time of DNA oligonucleotides and logarithmic value of the number of DNA base pair (log(N)). Conditions: capillary column: CEP, 75 μm i.d. and 72/80.5 cm long; injection: −15 kV/3 s; voltage: −15 kV; cassette temperature: 25 °C; detector wavelength: 258 nm; running buffer: 90 mM Tris, 330 mM boric acid, 2 mM EDTA, adjusted to pH 7.42; polymer solution: 11% LPA-1500.

5. Conclusion

Our study has demonstrated for the first time that an inverse migration order in coated CE capillary columns exists when polymer solutions are used. The inverse migration order might resulted from a mechanism involving size exclusion. More research is needed to further explain this separation phenomenon. The single base pair resolution of this new separation condition is particularly useful for detecting changes in small DNA fragments. If the proposed size exclusive mechanism is verified, it would open a new frontier of size exclusive capillary electrophoresis.

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