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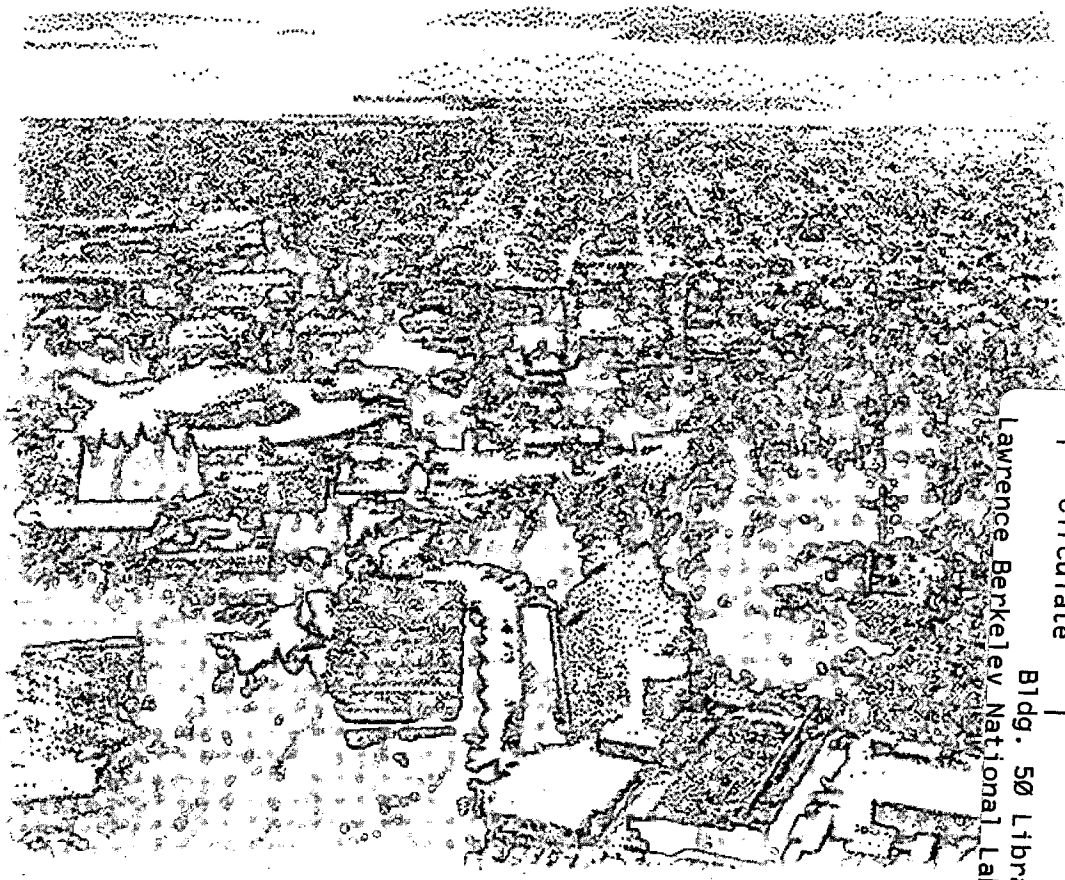

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Capillary Electrophoretic Separation of DNA Restriction Fragments Using Dilute Polymer Solutions

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Abstract

Because the mechanism of DNA separation in capillary electrophoresis is not well understood, selection of polymers is a "trial-and-error" procedure. We investigated dilute-solution DNA separations by capillary electrophoresis using solutions of four polymers that differ in size, shape and stiffness. Hydroxyethylcellulose of high molecular weight provides excellent separation of large DNA fragments (2027 bp - 23130 bp). Polyvinylpyrrolidone separates DNA from 72 bp to 23 kbp and star-(polyethylene oxide), like linear poly(ethylene oxide), provides separation of fragments up to 1353 bp.

Introduction

Capillary electrophoresis using dilute polymer solutions provides a useful technique for rapid separation of DNA fragments to 23000 bp in a constant electric field, and for DNA fragments to several megabase pairs in a pulsed field. Advantages of capillary electrophoresis include on-column detection, suitability for automation, on-column sample loading and easy replacement of the polymer solution; further, separations are usually complete in under 20 minutes. Although many groups (for a review see [1]) have investigated separation of DNA fragments in a variety of dilute polymer solutions, we have little understanding concerning those polymer properties which are required for efficient separation.

In this work we focus on four water-soluble polymers with different properties including molecular mass, polymer shape in solution and stiffness: hydroxyethylcellulose, polyvinylpyrrolidone, star-poly(ethylene oxide), and starburst™ (PAMAM) dendrimer. Barron et al.[2,3,4] established that unentangled hydroxyethylcellulose (HEC) solutions in a wide range of molecular masses provide excellent separation of DNA to 23 kbp. We used HEC with a weight average molecular mass of $2 \cdot 10^6$ g/mol (Barron et al. used HEC with $1.76 \cdot 10^6$ g/mol as highest molecular mass). HEC, a hydrophilic cellulose derivative, has a linear structure and is known to be very stiff and extended in aqueous solutions.

Brown estimated the Porod-Kratky persistence length to be 8.3 nm[1] whereas the persistence length for a typical non-cellulosic polymer is only 0.8-1 nm. Polyvinylpyrrolidone (PVP), in contrast, adopts a loose, random-coil conformation in aqueous solutions typical for flexible chain polymers[5]. The polymer is readily soluble in water due to its amphiphilic character. In addition to a highly polar amide group conferring hydrophilic properties, it contains apolar methylene and methine (CH) groups. Others have reported the separation of diastereomeric derivatives of enantiomers[6,7] and of synthetic cationic dyes[8] using PVP as a buffer additive in capillary-zone electrophoresis.

In contrast to the above linear polymer molecules, star-poly(ethylene oxide) (star-PEO) is a star-shaped polymer with 64 arms, each with M_w = 11000 g/mol, growing linearly from a divinylbenzene core giving a total M_w of 700000 g/mol. Linear poly(ethylene oxide) was used previously in capillary electrophoresis; Chang et al.[9] and Fung et al.[10] obtained separation of DNA fragments to 1353 bp using mixtures of different molecular weights.

The fourth polymer used in this work was starburst™ (PAMAM) dendrimer. Unlike star-PEO, the arms of the dendrimer grow in a highly branched manner from the core. The weight-average molecular mass of the dendrimer was 6909 g/mol.

Experimental

Instrumentation

An uncoated capillary was used in the electrophoresis apparatus. The fused silica capillary is 50 cm in length (35 cm to the detector) with external coating of polyimide (Polymicro Technologies, Phoenix, AZ, USA) and no internal coating with 360 μm O.D. and 51 μm I.D. The capillary connects the anodic reservoir with the electrically grounded cathodic reservoir. A high-voltage power supply with 30 kV capacity (Gamma High Voltage Research, Ormand Beach, CA, USA) drives electrophoresis. Current was measured over a 1 kΩ resistor in the return circuit of the power supply, using a digital multimeter (Model 3465B, Hewlett-Packard, Palo Alto, CA, USA). A modified variable-

wavelength detector (Model 783, Applied Biosystems, Foster City, CA, USA) was used at 260 nm UV absorbance for on-column detection. Data were either recorded using an integrator (Model 3390, Hewlett-Packard, Palo Alto, CA, USA) or acquired and saved for further analysis by a 386 PC equipped with an analog input and digital output (I/O) board (DAS-800 Series board, Keithley Metrabyte, Taunton, MA, USA) connected to the capillary-electrophoresis apparatus.

Materials

A non-stoichiometric mixture of λ-*Hind* III and ϕx174-*Hae* III digest DNA was obtained from Pharmacia LKB Biotechnology (Alameda, Ca, USA). Since λ-*Hind* III restriction fragments of 4361 bp and 23130 bp have cohesive termini, DNA samples were preheated in a water bath at 65 °C for 5 min and then immediately stored on ice. Mesityl oxide was used as a neutral marker. TBE buffer was used for all experiments consisting of 89 mM tris(hydroxymethyl)aminomethane (Tris), 89 mM boric acid, and 5 mM ethylenediaminetetraacetic acid (EDTA) with pH 8.15. All buffer reagents were purchased from Sigma Molecular Biology, St. Louis, MO, USA. HEC 2M ($M_w = 2 \cdot 10^6$), PVP, star-PEO ($M_w = 7 \cdot 10^5$), and the dendrimer (M_w = 6909 g/mol) respectively, were added to the buffer solution, and mixed by tumbling (HEC 2M, PVP) or stirring (star-PEO, Dendrimer) for 24 h. Polymer samples were obtained from Aqualon, Wilmington, De (HEC 2M), Aldrich, Milwaukee, Wis (PVP except PVP 1M and the Dendrimer), Polysciences, Warrington, Pa (PVP 1M), and Shearwater Polymers, Huntsville, Alab (star-PEO). The weight-average molecular weights were determined from intrinsic-viscosity measurements in our laboratory (HEC 2M) or specified by the supplier.

Experimental Procedure

The procedure for preparing each new capillary is given elsewhere[3] (Barron et al. 1994). Each time a new polymer solution was used (a new polymer or a new concentration of the same polymer) the uncoated capillary was first flushed with 1M sodium hydroxide for 15 minutes, then with 0.1M sodium hydroxide for 15 minutes, with distilled deionized water

for 10 minutes, and finally, with the electrophoresis buffer containing the polymer for 10 minutes to one hour depending on the solution viscosity. This protocol was first used (slightly modified) by A.E. Barron[2] and is sufficient to clean out the previous buffer/polymer mixture and to establish the necessary wall condition for uniform separation.

DNA samples were introduced applying a vacuum of 2 to 5 in Hg (7773-19432.5 Pa) for a time depending on the viscosity of the solution, to introduce approximately 3 nl of the sample into the capillary. After this procedure, the capillary was placed back into the polymer solution and the electrophoretic voltage was applied. All experiments were run at 13,282 V (265 V/cm). In all experiments, the capillary was surrounded by convected air at 30.0 ± 0.1 °C.

By applying an electric field, the negatively charged DNA (which would remain at the anodic end of the capillary in the absence of electroosmotic flow) is drawn toward the UV-absorbance detector and the cathode. Thus, the largest DNA fragment pass the detector first, followed by the smaller ones in order of size.

Viscometry

To measure the viscosity of a polymer solution, an automated Ubbelohde-type capillary viscometer (Schott Geräte, Hofheim, Germany) was used, controlled by a desktop PC (Viscosity Measuring Unit AVS 350, Schott Geräte, Hofheim, Germany). The viscometer was placed in a water bath (Model H-1 High Temperature Bath, Cannon Instrument Co., State College, USA) at 30 °C controlled by a thermostat.

Results

Entanglement threshold

The entanglement threshold concentration Φ^* is defined as the polymer weight fraction where interaction between polymer chains begins to affect bulk solution properties such as viscosity. Below the entanglement threshold the polymer chains are hydrodynamically isolated, i.e. there may be some entanglement coupling between isolated molecules, but it does not extend throughout the system[11]. Above Φ^* the solution is said to be entangled. Experimentally, Φ^* can be determined from viscosity data given a log-log plot of specific viscosity η_{sp} versus polymer weight fraction Φ[12]. At low concentrations ($\Phi < \Phi^*$), where polymer molecules do not interact extensively with each other, the specific viscosity scales linearly with Φ. At Φ^* the slope increases because of an entangled polymer network[13]. Figure 1 shows a log-log plot of η_{sp} as a function of Φ in the buffer solution for the PVP 1M sample. The value of Φ^* indicates where the experimental data begin to deviate from linearity. We determined Φ^* as 0.027 %(w/w), 0.85 %(w/w) and 1.2 %(w/w) for HEC 2M, PVP 1M, and star-PEO, respectively. Since separations were not achieved using the dendrimer or lower molecular PVP, we did not determine Φ^* for these polymers.

Hydroxyethylcellulose and Polyvinylpyrrolidone

We used HEC 2M solutions in a concentration range 0.003125 % to 0.1 %(w/w) to separate λ-*Hind* III and ϕx174-*Hae* III DNA restriction fragments. Figure 2 shows the separation performance in a plot of the electrophoretic mobility μ as a function of HEC 2M concentration. For small DNA (< 2027 bp), the separation improves as HEC 2M concentration rises, although neither the fragments 72 bp and 118 bp nor those 271 bp and 281 bp could be separated at any concentration. For larger DNA fragments an optimum polymer concentration exists where the best resolution is achieved. We determined this concentration as 0.025 %(w/w) for HEC 2M. Fragments 2027 bp and 2322 bp could only be separated at this concentration.

Barron et al.[14] suggested that high polymer molecular masses must be used to separate large DNA whereas the separation of smaller fragments could be achieved with lower molecular weights. Indeed, they were able to separate all small DNA fragments using HEC with $M_w = 1.76 \cdot 10^6$ g/mol. Figure 3 demonstrates the improvement of separation for large DNA (> 2322 bp) with increasing polymer molecular weight. The differences in electrophoretic mobilities ($\Delta\mu$) rise using HEC with weight average molecular masses of $1.32 \cdot 10^6$ g/mol, $1.76 \cdot 10^6$ g/mol (both investigated by Barron et al.[3]), and $2 \cdot 10^6$ g/mol, respectively. The best resolution of adjacent DNA fragments is obtained with HEC 2M at the optimum concentration 0.025 %(w/w). $\Delta\mu$ is smaller for the lower molecular masses at their optimum concentrations.

We obtained qualitatively the same results with polyvinylpyrrolidone. Experiments with PVP with molecular masses lower than 100,000 g/mol showed no separations of any size DNA (data not shown). Figure 4 shows the dependence of electrophoretic mobilities μ on the polymer concentration for PVP 1M. In contrast to HEC 2M, fragments 2322 bp and 2027 bp were not separated at any concentration. In general, the separations achieved with HEC 2M solutions are superior to those with PVP 1M solutions. Figure 5 shows the differences in the electrophoretic mobility ($\Delta\mu$) between adjacent DNA fragments in length. $\Delta\mu$ is greater for fragments larger than 1078 bp for HEC 2M solutions. However, the band broadening is much more visible in PVP 1M solutions. Figure 6 shows a plot of the Resolution R_{DNA} for adjacent DNA fragments from 72 to 23130 bp for PVP 1M at 0.75 %(w/w) and HEC 2M at 0.1 %(w/w). The resolution R_{DNA} of two fragments is defined as the distance between the centers of the zones divide by the average width of each zone. When $R_{DNA} > 1.5$, the separation of two bands is essentially complete and the peaks are said to be base-line resolved. Using HEC 2M solutions at 0.1 %(w/w), we find complete separation for almost all fragments, whereas PVP 1M exhibits poor resolution at 0.75 %(w/w), the optimal concentration for PVP 1M to separate DNA restriction fragments to 23130 bp. We tentatively explain these observations by considering experimental conditions and polymer properties.

The viscosity is higher for PVP 1M than for HEC 2M, compared at their optimal concentrations. This high viscosity effects a decrease of the electroosmotic velocity because the force to drag the bulk solution in the cathodic direction is higher. Thus, the overall migration time for the DNA increases as the band width. The higher viscosity also required to double the hydrodynamic injection time which leads to a broader distribution before starting electrophoresis. The polymer properties are also important: whereas HEC is known to be relatively stiff and extended, having a persistence length of 8.3 nm, PVP has the loose, random-coil type of conformation usually possessed by flexible-chain polymers. The radii of gyration, obtained from viscometry measurements, are 96.2 nm for HEC 2M and 36.4 nm for PVP 1M, respectively. We believe that it is more likely for HEC 2M molecules to experience transient entanglement coupling than for PVP 1M because of differences in their conformation in the buffer solution.

Star-Poly(ethylene oxide)

The entanglement threshold concentration Φ^* of star-poly(ethylene oxide) (star-PEO) is 1.2 %(w/w). It is surprising that a separation could only be observed above, but not below, Φ^*. Furthermore, only ϕx174-*Hae* III digest fragments, 72 to 1353 bp, could be separated; and even in this range the resolution was poor. However, consistent with our results using hydroxyethylcellulose and polyvinylpyrrolidone, the resolution of the small DNA fragments improves with increasing polymer concentration (Figure 7). However, notable separation is first seen at the entanglement threshold concentration Φ^* and the best separation was found at 3.2 %(w/w), near the limit of solubility, well above Φ^*.

Most likely the poor separations of star-PEO vs HEC 2M and PVP 1M are due to two effects. The radii of gyration R_g of HEC 2M and PVP 1M are four times and two times, respectively, as large as R_g of star-PEO (23.6 nm). HEC 138k, which has a similar radius of gyration, also produced only separation of small DNA fragments to 1353 bp. The compact conformation of star-PEO compared to the linear structure of PVP and HEC also implies that the separation performance decreases because an entanglement between polymer and DNA is less likely.

Starburst™ (PAMAM) Dendrimer

We did not observe separation of DNA fragments ranging from 72 to 23130 bp. Two effects may explain this result. First, the molecular mass of 6909 g/mol is far too low for capillary electrophoresis. PVP provided DNA separation at 1,000,000 g/mol and star-PEO at 700,000 g/mol. Second, the highly branched structure of the dendrimer may present a surface which is too dense to enable entanglement coupling. Hence, dendrimers are less likely to entangle with DNA fragments.

Conclusion

We have observed that DNA separation in capillary electrophoresis is possible using high-molecular-weight HEC, PVP and star-PEO. Comparing our results with HEC 2M with those of Barron et al.[2,3] we confirmed that the separation improves for large fragments if a higher molecular mass is used. We observed the same effect for PVP solutions. Furthermore, by comparing HEC 2M and PVP 1M, it appears that the stiffness of the polymer has an important influence on resolution.

We found that a linear structure is not imperative. Both, linear and star-shaped poly(ethylene oxide) provided separation of DNA fragments to 1353 bp, but it seems that the high number of arms (64) is detrimental to the DNA separations. Using higher molecular masses of star-PEO with perhaps only five or six arms may improve separations because a few arms of high molecular mass may be able to entangle with DNA.

Acknowledgments

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Figure 1: Determination of the entanglement threshold concentration, Φ^*, for PVP 1M. Φ^* is where the slope of a log-log plot of the specific viscosity η_{sp} vs PVP concentration deviates from linearity. Viscosity measurements were performed using an Ubbelohde viscometer thermostated in a water bath at 25 °C ± 0.1 °C. The buffer was 89 mM tris[hydroxylmethyl]aminomethane, 89 mM boric acid and 5 mM ethylenediamintetraacetic acid (TBE).

Figure 2: DNA electrophoretic mobility μ versus HEC concentration for λ-*Hind* III/ϕx174-*Hae* III fragments ranging from 72 bp to 23130 bp using HEC with M_w = 2,000,000. The entanglement threshold is shown on the abscissa. Data points at each concentration are averaged over 2-4 runs. The electrophoresis buffer is TBE. Uncoated capillary: 50 μm inner diameter, 50 cm total length (35 cm to the detector). Detection was by UV absorbance at 260 nm; hydrodynamic injection. Electric field strength 265 V/cm; current: 6.9 μA.

Figure 3: Comparison of differences in electrophoretic mobilities of large DNA rstriction fragments (2322 bp - 23130 bp) for HEC solutions with weight-average molecular masses of 2,0000,00 g/mol (HEC 2M), 1,760,000 g/mol (HEC 1.76 M) and 1,320,000 g/mol (HEC 1.32M), respectively. The data are for the concentration that gave the optimum separation of large DNA fragments. HEC 1.32M and HEC 1.76M were investigated by Barron et al.[2,3].

Figure 4: DNA electrophoretic mobility μ versus PVP concentration for λ-*Hind* III/ϕx174-*Hae* III fragments from 72 to 23130 bp using PVP with M_w = 1,000,000. The entanglement threshold is shown on the abscissa. Data points at each concentration are averaged over 2-4 runs. The electrophoresis buffer is TBE. Uncoated capillary: 50 μm inner diameter, 50 cm total length (35 cm to the detector). Detection was by UV absorbance at 260 nm; hydrodynamic injection. Electric field strength 265 V/cm; current: 6.9 μA.

Figure 5: Comparison of the differences in electrophoretic mobilities between adjacent DNA restriction fragments for PVP 1M and HEC 2M at their optimal concentrations in capillary electrophoresis. DNA fragments from 1078 - 23130 bp at 0.75 %(w/w) PVP and 0.025 %(w/w) HEC.

Figure 6: Comparison of the dependence of the resolution between adjacent DNA restriction fragments on the polymer. The optimum concentration for HEC 2M is 0.1 %(w/w) for DNA fragments smaller than 1353 bp and 0.025 %(w/w) for fragments from 2027 bp 23130 bp in size. The separation of two fragments is said to be "baseline resolved" if the resolution is larger than 1.5. This line is marked in the diagram.

Figure 7: DNA electrophoretic mobility μ versus PEO concentration Φ for ϕx174-*Hae* III DNA fragments from 72 - 1353 bp. Polymer: star-PEO of M_w = 700,000 g/mol. The entanglement threshold is shown on the abscissa. Data points at each concentration are averaged over 2-4 runs. The electrophoresis buffer is TBE. Uncoated capillary: 50 μm inner diameter, 50 cm total length (35 cm to the detector). Detection was by UV absorbance at 260 nm; hydrodynamic injection. Electric field strength 265 V/cm; current: 7.2 μA.

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PVP 1M

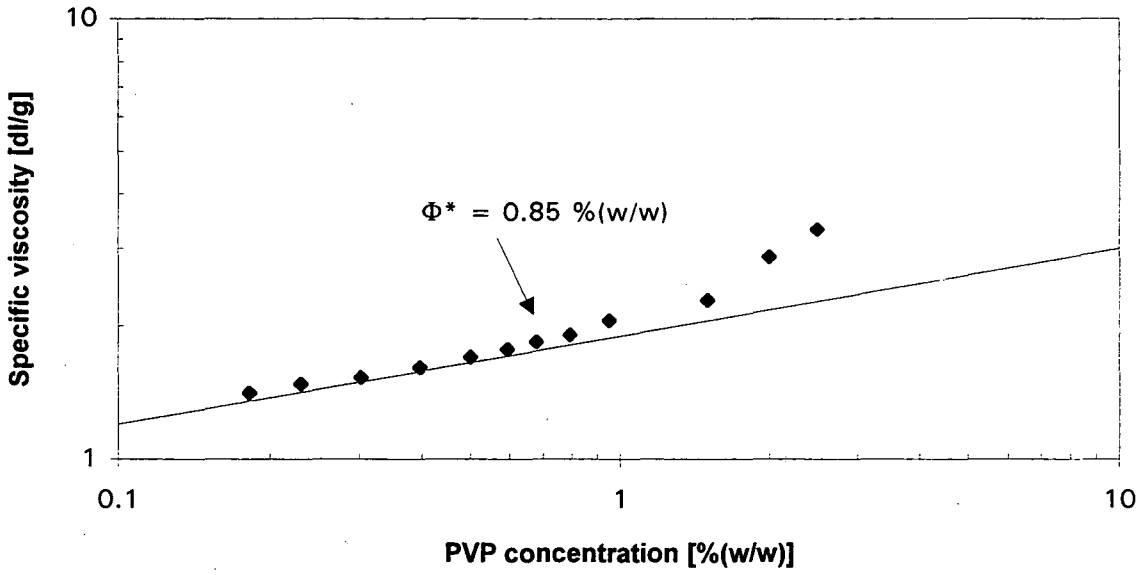

Figure 1

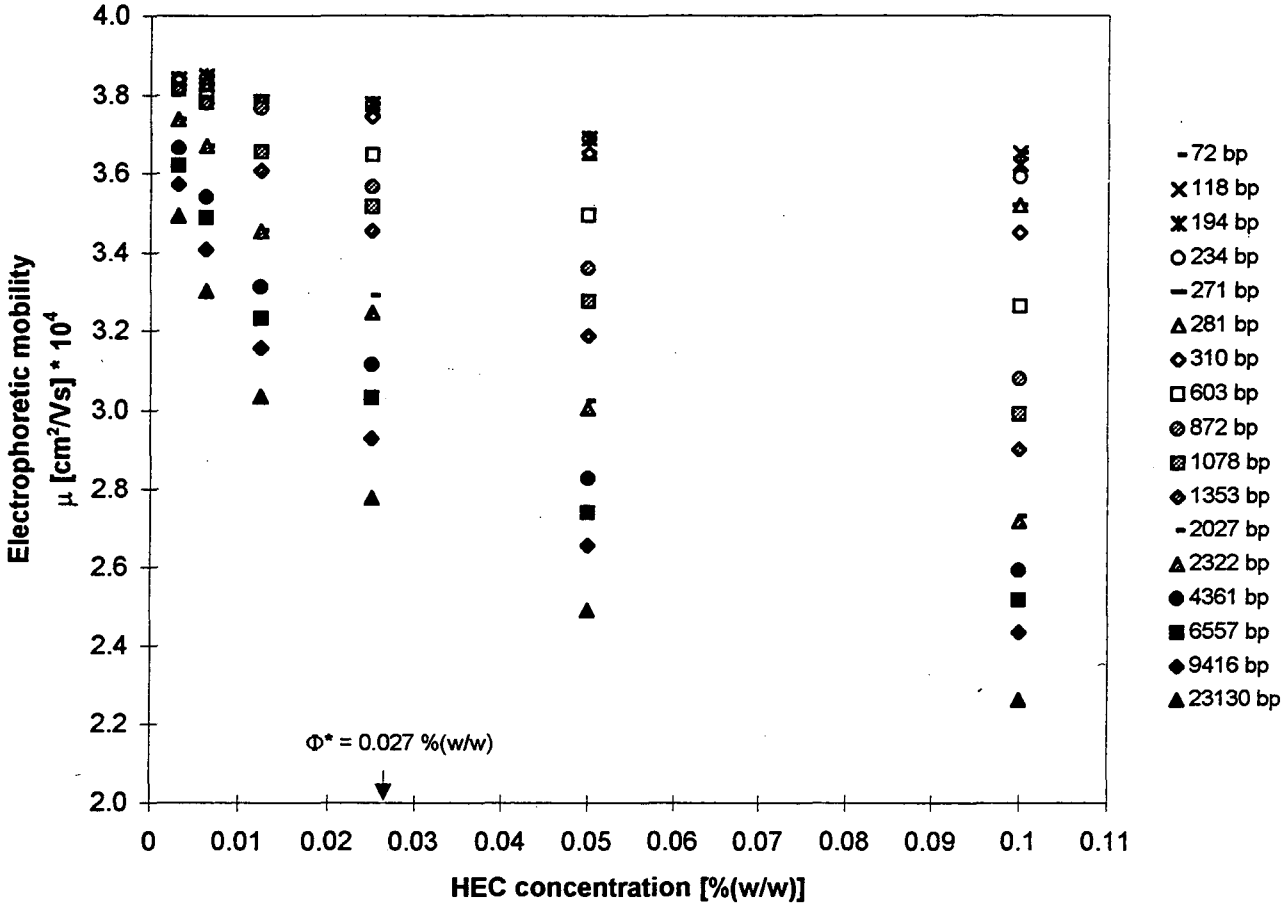
Electrophoretic mobility
μ [cm²/Vs] * 10⁴
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
Φ* = 0.027 %(w/w)
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
0.11
HEC concentration [%(w/w)]
72 bp
118 bp
194 bp
234 bp
271 bp
281 bp
310 bp
603 bp
872 bp
1078 bp
1353 bp
2027 bp
2322 bp
4361 bp
6557 bp
9416 bp
23130 bp

Figure 2

HEC

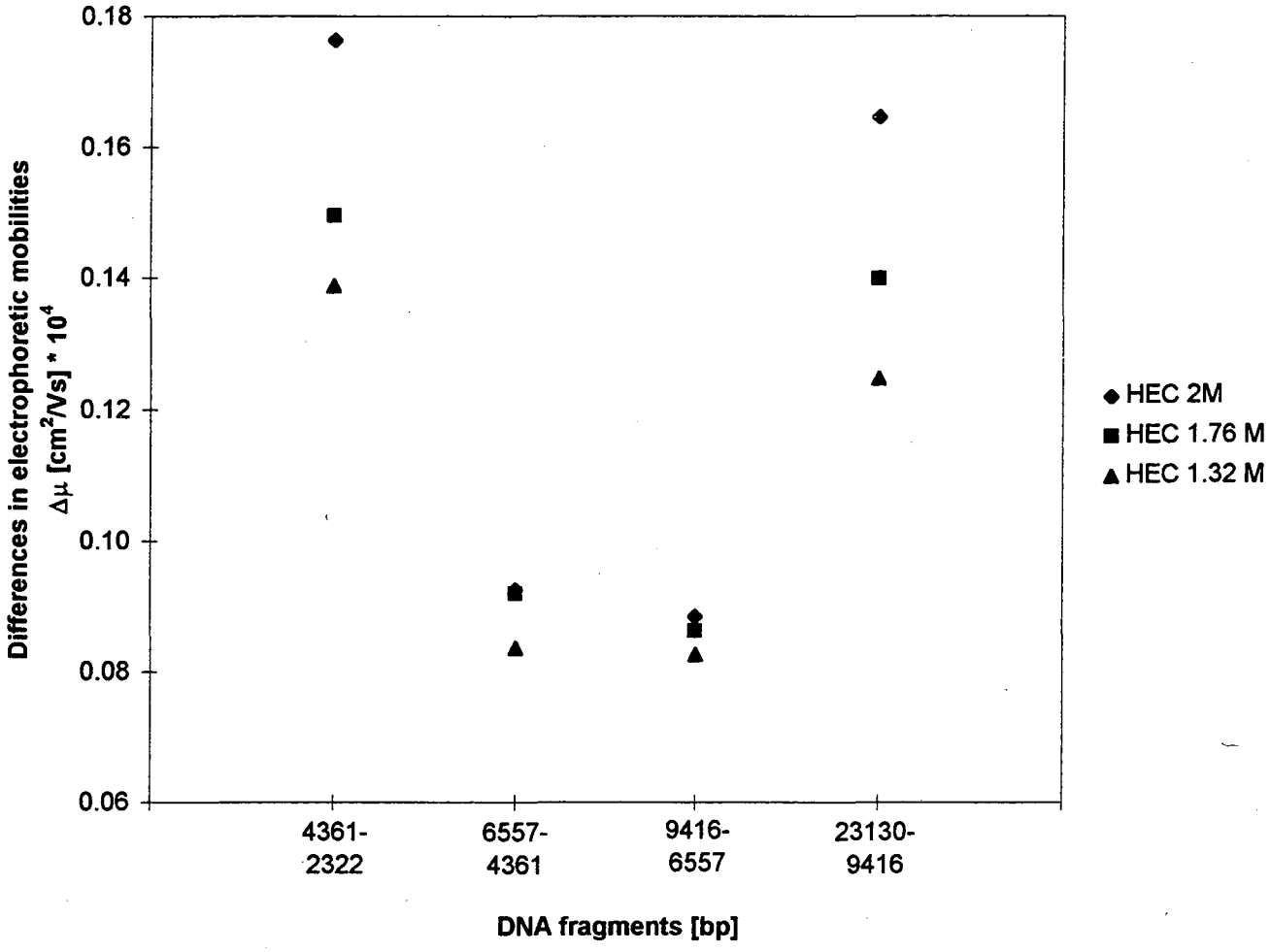
0.18
0.16
0.14
0.12
0.10
0.08
0.06
Differences in electrophoretic mobilities
Δμ [cm²/Vs] * 10⁴
4361-2322
6557-4361
9416-6557
23130-9416
DNA fragments [bp]
HEC 2M
HEC 1.76 M
HEC 1.32 M

Figure 3

PVP 1M

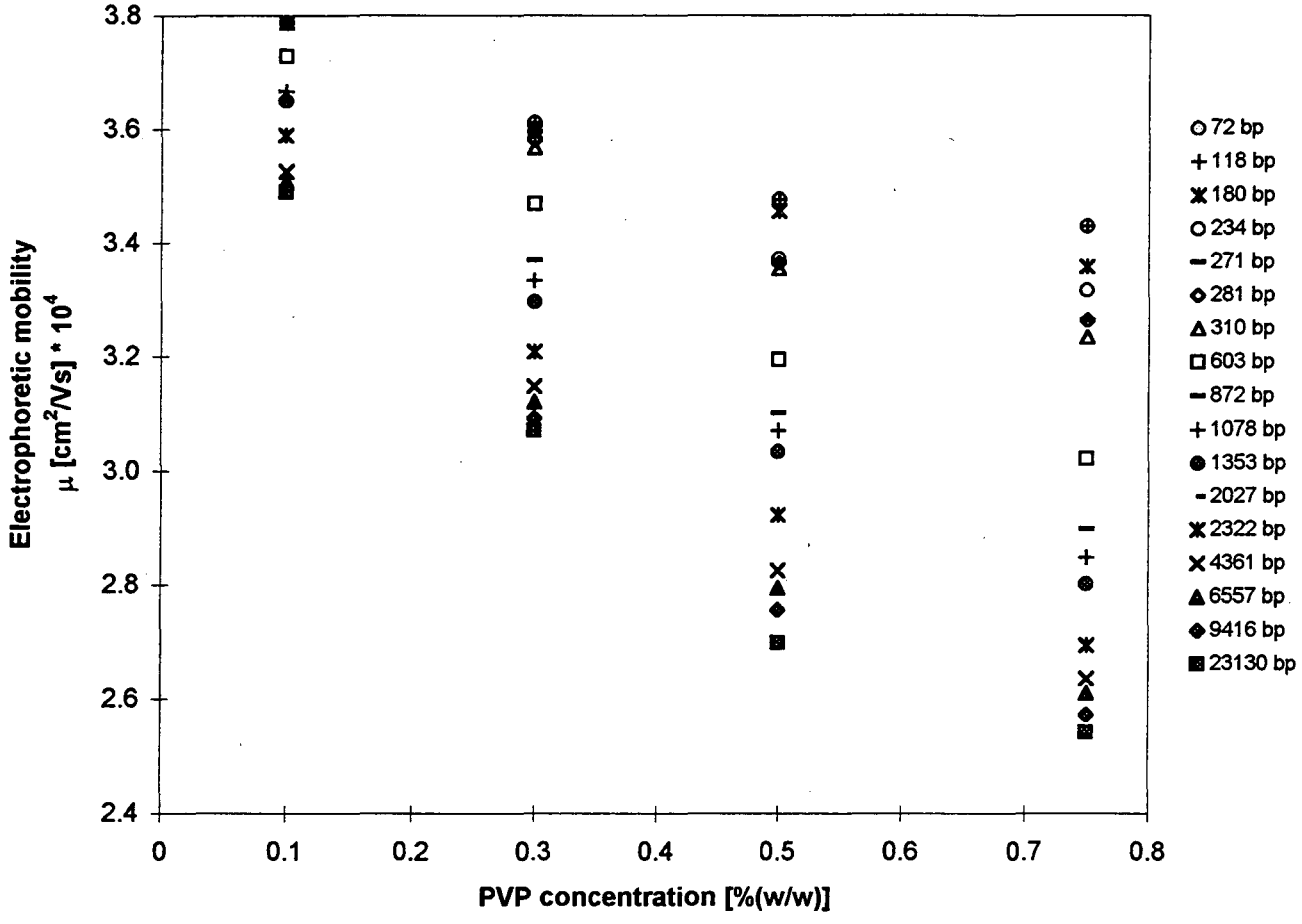

Figure 4

HEC 2M, PVP 1M

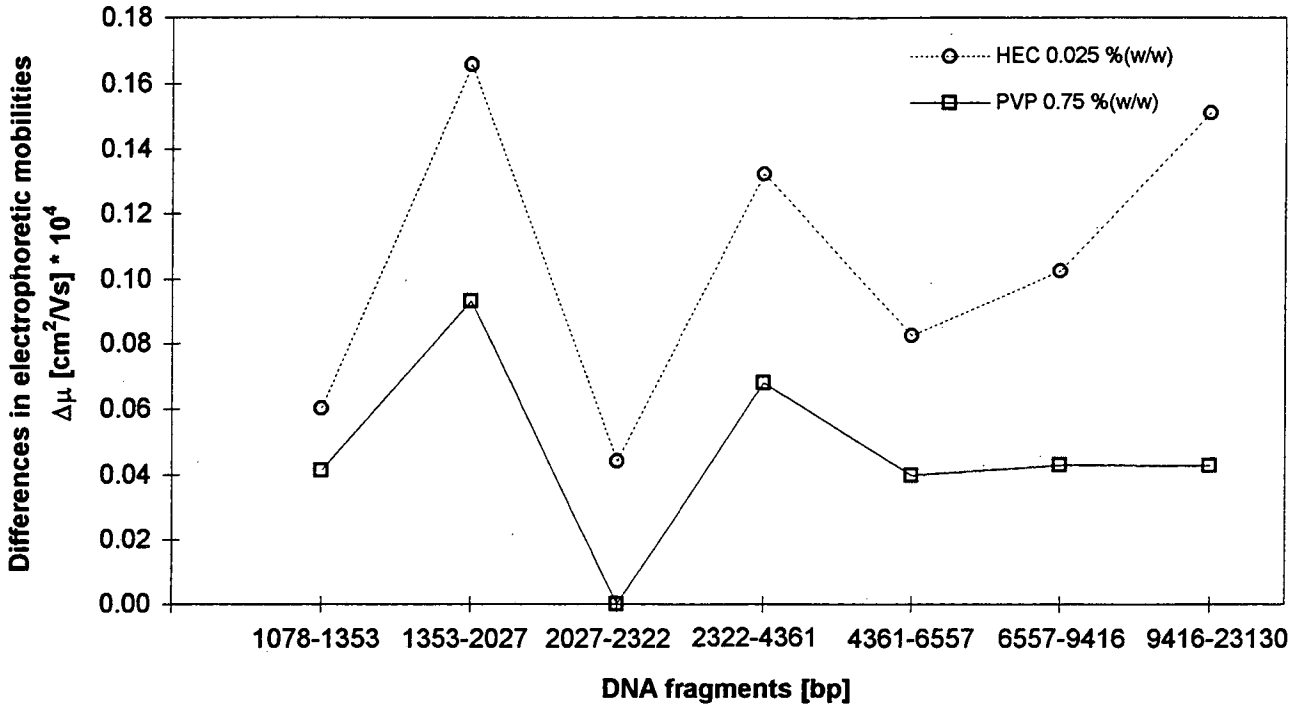

Figure 5

HEC 2M, PVP 1M

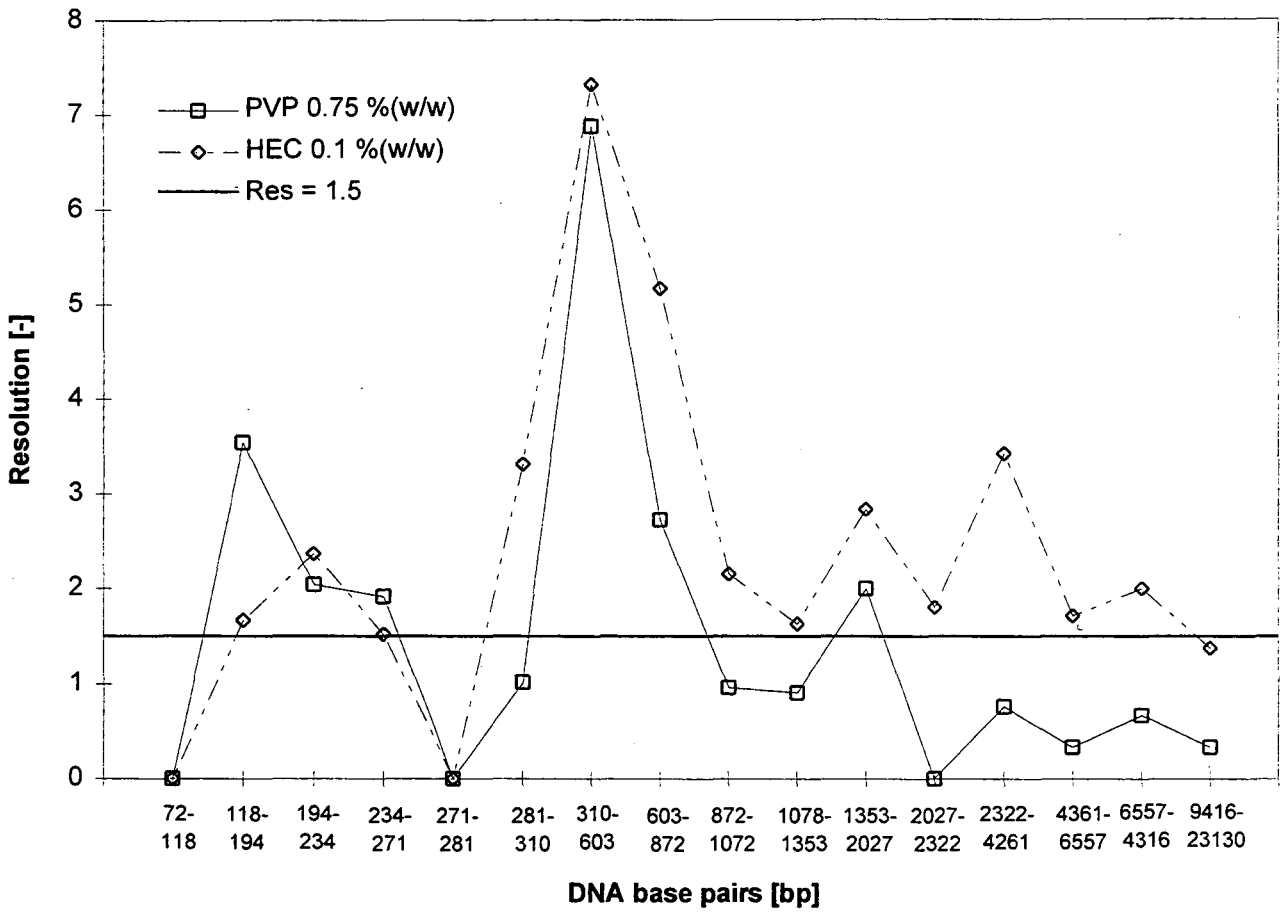

Figure 6

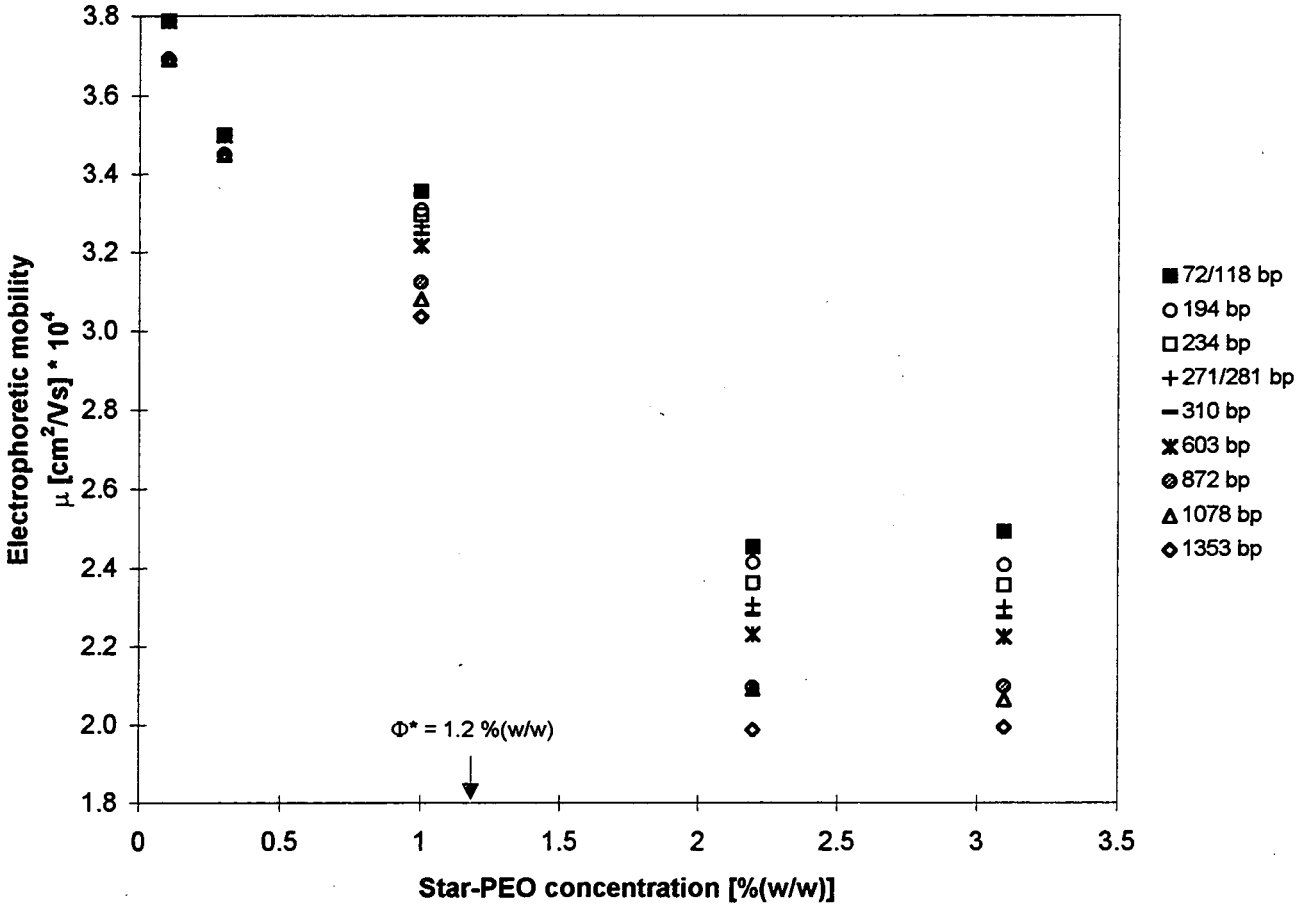
Electrophoretic mobility
μ [cm²/Vs] * 10⁴
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
0
0.5
1
1.5
2
2.5
3
3.5
Φ* = 1.2 %(w/w)
Star-PEO concentration [%(w/w)]
72/118 bp
194 bp
234 bp
271/281 bp
310 bp
603 bp
872 bp
1078 bp
1353 bp

Figure 7

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