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Electrophoretic Separation of Small DNA Fragments in the Presence of Electroosmotic Flow Using Poly(ethylene oxide) Solutions

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A new and simple method was demonstrated for separating ϕX-174/Hae III DNA restriction fragments and DNA markers V and VI, respectively, without filling capillaries with polymer solutions prior to analysis. Using this novel method, poly(ethylene oxide) (PEO) solutions containing ethidium bromide migrated into capillaries by electroosmotic flow (EOF) during the separation. Two DNA fragments (123 and 124 bp) in markers V and VI were well-resolved. RSD values for the separation of ϕX-174/Hae III DNA restriction fragments were less than 0.52% for 3 runs using a single 75-μm capillary and less than 3.96% using three different 75-μm capillaries. A highly viscous polymer solution prepared from 3% PEO was also used for separation of DNA markers V and VI. Theoretical plates up to 11.91 million/m and separation times of less than 7 min were achieved in the separation of ϕX-174/Hae III DNA restriction fragments using a 10-μm capillary and a 2% PEO solution. Advantages of this method include simplicity, short separation times, the ability to use highly viscous polymer solutions for separating small DNA fragments, and the possibility of introducing several different polymer solutions into capillaries to extend the DNA separation range.

INTRODUCTION

Techniques providing advantages in the speed, resolution, throughput, and sensitivity of DNA separation are of value. Capillary electrophoresis (CE) using polymer solutions and capillary gel electrophopresis (CGE) with multiplexed operation modes have been developed during the Human Genome Project, and are promising for high-throughput DNA sequencing.[1−4] CE using polymer solutions has been commonly applied to DNA diagnostics[5] as well as to the separation of DNA restriction fragments[6,7] and PCR products.[8] High-speed, high-resolution DNA separation using small DNA samples in a microchip is also under study.[9,10]

Since the first report of DNA separation with cross-linked PA,[12] several different polymers have been successfully employed in the analysis of DNA.[13−15] Currently, solutions prepared from linear polymers, such as PA,[16] poly(ethylene oxide) (PEO),[17−19] and celluloses,[20,21] are most commonly used for high-speed and high-resolution DNA separation. The separation of larger DNA fragments can also be achieved even in very dilute (unentangled) polymer solutions, where the pore-based separation model is unsuitable.[22]

To provide molecular sieving and to minimize the interaction between DNA and the capillary wall, deactivated capillaries are generally used.[23−25] To date, only a few methods have been reported for separation of DNA using bare fused-silica capillaries. PEO solutions have been used for DNA sequencing in uncoated capillaries pretreated with HCl.[26] On the other hand, in the presence of a high electroosmotic flow (EOF), separations of larger DNA fragments in hydroxyethylcellulose (HEC)[27−29] and PEO[30] solutions using fused-silica capillaries have been achieved.

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(1) Swerdlow, H.; Zhang, J. Z.; Chen, D. Y.; Harken, H. R.; Grey, R.; Wu, S.; Dovichi, N. J.; Fuller, C. *Anal. Chem.* **1991**, *63*, 2835−2841.
(2) Ueno, K.; Yeung, E. S. *Anal. Chem.* **1994**, *66*, 1424−1431.
(3) Bashkin, J.; Roach, D.; Leong, J.; Bartosiewicz, M.; Barker, D.; Johnston, R. G. *J. Capillary Electrophor.* **1996**, *3*, 61−68.
(4) Carrilho, E.; Ruiz-Martinez, M. C.; Berka, J.; Smirnov, I.; Goetzinger, W.; Miller, A. W.; Brady, D.; Karger, B. L. *Anal. Chem.* **1996**, *68*, 3305−3313.
(5) Baba, Y. *J. Chromatogr., A* **1997**, *687*, 271−302.
(6) Valdislav, D.; Novotny, M. *J. Microcolumn Sep.* **1993**, *4*, 515−519.
(7) Kleparnik, K.; mala, Z.; Bocek, P. *J. Chromatogr., A* **1997**, *772*, 243−253.
(8) Wartell, R. M.; Hosseini, S.; Rowell, S.; Zhu, J. *J. Chromatogr., A* **1998**, *806*, 169−185.
(9) Woolley, A. T.; Sensabaugh, G. F.; Mathies, R. A. *Anal. Chem.* **1997**, *69*, 2181−2186.
(10) Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **1996**, *68*, 720−723.
(11) Effenhauser, C. S.; Bruin, G. J.; Paulus, A.; Ehrat, M. *Anal. Chem.* **1997**, *69*, 3451−3457.
(12) Cohen, A. S.; Najarian, D, R.; Paulus, A.; Guttman, A.; Smith, J. A.; Karger, B. L. Proc. Natl. Acad. Sci. U.S.A. **1988**, *85*, 9660−9663.
(13) Harrington, M. G.; Zewert, T. E. *Electrophoresis* **1994**, *15*, 195−199.
(14) Chen, N.; Wu, L.; Palm, A.; Srichaiyo, T.; Hjerten, S. *Electrophoresis* **1996**, *17*, 1443−1450.
(15) Talmadge, K. W.; Zhu, M.; Olech, L.; Siebert, C. *J. Chromatogr., A* **1996**, *744*, 347−354.
(16) Berka, J.; Pariat, Y. F.; Muller, O.; Hebenbrock, K.; Heiger, D. N.; Foret, F.; Karger, B. L. *Electrophoresis* **1995**, *16*, 377−388.
(17) Lu, X.; Yeung, E. S. *Appl. Spectrosc.* **1995**, *49*, 605−609.
(18) Zhang, N.; Yeung, E. S. *Anal. Chem.* **1996**, *68*, 2927−2931.
(19) Chang, H.-T.; Yeung, E. S. *J. Chromatogr., B* **1995**, *669*, 113−123.
(20) Chrambach, A.; Aldroubi, A. *Electrophoresis* **1993**, *14*, 18−22.
(21) Grossman, P. D.; Soane, D. S. *Biopolymers* **1991**, *31*, 1221−1228.
(22) Hammond, R. W.; Oana, H.; Schwinefus, J. J.; Bonadio, J.; Levy, R. J.; Morris, M. D. *Anal. Chem.* **1997**, *69*, 1192−1196.
(23) Chiari, M.; Riva, S.; Celain, A.; Vitale, A.; Turati, E. *J. Chromatogr., A* **1997**, *781*, 347−355.
(24) Liao, J.-L.; Abramson, J.; Hjerten, S. *J. Capillary Electrophor.* **1995**, *4*, 191−196.
(25) Oana, H.; Hammond, R. W.; Schwinefus, J. J.; Wang, S.-C.; Doi, M.; Moris, M. D. *Anal. Chem.* **1998**, *70*, 574−579.
(26) Fung, E. N.; Yeung, E. W. *Anal. Chem.* **1995**, *67*, 1913−1919.

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Under alkaline conditions, DNA migrates upstream against the EOF. However, the EOF, with its greater velocity in the opposite direction, causes all DNA fragments to be swept past the detector near the cathode. Larger DNA fragments, which need to undergo deformation to pass through polymer solutions, are detected earlier because of their smaller electrophoretic mobilities (EPM).

Slight decreases in the EOF may occur as a result of dynamic coating of HEC in the capillary wall.[27] Even small variations in the EOF may affect the reliability of DNA analysis. Thus, pretreating capillaries with NaOH prior to analysis is suggested to prevent the adsorption of DNA to the wall, to refresh the capillary wall for reproducible results, and to maintain a high EOF to sweep DNA toward the cathode.

So far, the separation of DNA in CE has only been demonstrated using capillaries filled with polymer solutions prior to analysis. The high-resolution separation of small DNA fragments in the presence of EOF has also not been reported. Filling very small capillaries with highly viscous polymer solutions (e.g., 3% PEO) for rapid separation of minute amounts of small DNA fragments is not easily achieved.[31]

In this article, we have described a technique for separating small DNA fragments without filling capillaries with PEO solutions prior to analysis. After injecting DNA samples, PEO solutions entered capillaries by EOF. We also demonstrated the advantages of DNA analysis using highly viscous PEO solutions (e.g., 3%) and small capillaries (e.g., 10 μm).

EXPERIMENTAL SECTION

Apparatus. The basic design of the separation system has been previously described.[19] Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL) was used to drive electrophoresis. The entire detection system was enclosed in a black box with a HV interlock. The high-voltage end of the separation system was housed in a plexiglass box for safety. A 1.5-mW He−Ne laser with 543.6-nm output from Melles Griot (Irvine, CA) was used for excitation. The emission light was collected with a 10 X objective (N.A. = 0.25). One RG 610 cutoff filter was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The fluorescence signal was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a PC. Capillaries (Polymicro Technologies, Phoenix, AZ) with 10−75 μm i.d. and 365 μm o.d. were used for DNA separations without coating procedures.

Chemicals. All chemicals used for preparing buffer solutions were obtained from Aldrich (Milwaukee, WI). Ethidium bromide (EtB) was obtained from Molecular Probes (Eugene, OR). The buffer solutions were 1X TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) in the presence and absence of different concentrations of PEO (MW 8,000,000) and EtB. ϕX 174 RF DNA-Hae III digest was purchased from Pharmacia Biotech (Uppsala, Sweden). DNA markers V (pBR 322/Hae III digest) and VI (pBR 328/Bgl I digest and pBR 328/Hinf I digest) were from Boehringer Mannheim Gmbh (Mannheim, Germany).

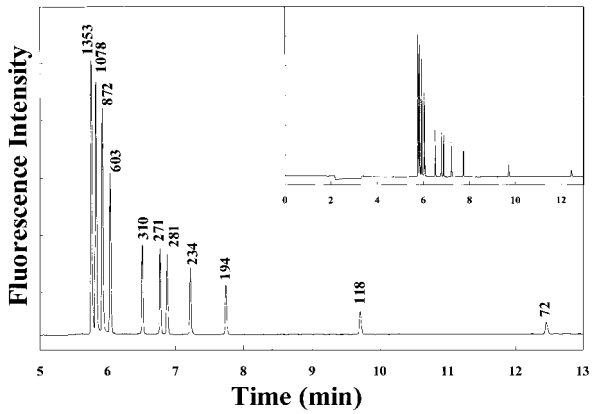

Figure 1. Separation of 5 μg/mL ϕX 174 DNA-Hae III restriction fragments in the presence of EOF at 15 KV using a 75-μm capillary. Capillary length: 40 cm (30 cm to the detector). The capillary was filled with 1X TBE containing 5 μg/mL EtB prior to DNA injection. Buffer vials contain solutions of 2% PEO and 5 μg/mL EtB.

Polymer Solutions. Increasing amounts of PEO were gradually added to TBE buffer solutions in a water bath at 85−90 °C. During the addition of PEO, a magnetic stirring rod was used at high speed to produce a well-homogenized solution. After adding the PEO, the solution was stirred for another hour. Polymer solutions were degassed with a vacuum system in an ultrasonic tank. Polymer solutions stored in a refrigerator at 4 °C were usable for a week.

Treatment of Capillaries. Before separation, capillaries were treated with 0.5 M NaOH for 1 h. Prior to each analysis, capillaries were washed with 0.5 M NaOH at 0.5 kV for 10 min to remove polymer solutions and refresh the capillary wall.

Separation. DNA samples at concentrations of 5 and 25 μg/mL were injected into capillaries filled with 1X TBE buffer containing 1, 5, or 20 μg/mL of EtB at 1 kV for 5 s. Then the ends of the capillaries were immersed in 1X TBE buffer solutions containing different concentrations of PEO. During the analysis, PEO solutions were introduced into capillaries by the high EOF and subsequently served as sieving matrixes for the separation of DNA fragments.

RESULTS AND DISCUSSION

DNA Separation without Prefilling Capillaries with PEO Solutions. Figure 1 shows that 11 DNA fragments were well-separated in the presence of EOF in 13 min at 15 kV using 2% PEO solutions in a 40-cm capillary (75 μm i.d.). We demonstrated the separation of small DNA fragments without using high pressure (e.g., 2% PEO solutions at 600 psi using 75-μm capillaries) to fill capillaries with polymer solutions prior to analysis. After injecting DNA, neutral PEO solutions entered the capillary filled with 1X TBE buffer by EOF. Since DNA migrated upstream against the EOF, DNA entered the PEO zone as PEO solutions were carried into the capillary. Small DNA fragments moved with less retardation (high EPM) and were detected later than larger fragments which underwent deformation in the matrix and moved more slowly against the EOF. It should be noted that Karger et al. have found the reversion of the separation order between 271-

(27) Barron, A. E.; Sunda, W. M.; Blanch, H. W. *Electrophoresis* **1995**, *16*, 64−74.
(28) Mao, D. T.; Levin, J. D.; Yu, L.; Lautamo, R. M. A. *J. Chromatogr., B* **1998**, *714*, 21−27.
(29) Nathakarnkitkool, S.; Oefner, P. J.; Bartsch, G.; Chin, M. A.; Bonn, G. K. *Electrophoresis* **1992**, *13*, 18−31.
(30) Chen, H.-S.; Chang, H.-T. *Electrophoresis* **1998**, *18*, 3149−3153.
(31) Slater, G. W.; Mayer, P.; Grossman, P. D. *Electrophoresis* **1995**, *16*, 75−83.

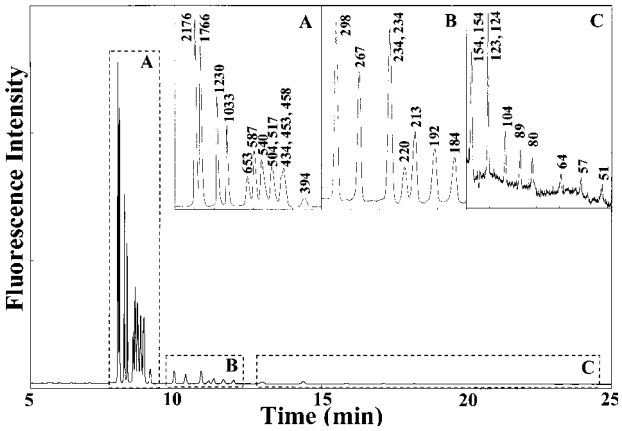

Figure 2. Separation of 5 μg/mL DNA markers V and VI in the presence of EOF at 15 KV using a 75-μm capillary. Capillary length: 40 cm (30 cm to the detector). The capillary was filled with 1X TBE containing 1 μg/mL EtB prior to DNA injection. Buffer vials contain solutions of 3% PEO and 1 μg/mL EtB.

Table 1. Reproducibility of Separations of ϕX-174/Hae III DNA Restriction Fragments Using 2% PEO Solutions and 75-μm Capillaries

DNA (bp)	migration time (min)					mean RSD (%)			
	1[a]	2[a]	3[a,b]	4[b]	5[b]	intra	inter	intra	inter
1353	5.74	5.79	5.76	5.74	5.70	5.76	5.73	0.44	0.53
1078	5.81	5.86	5.82	5.81	5.78	5.83	5.80	0.45	0.36
872	5.90	5.96	5.92	5.90	5.89	5.93	5.90	0.52	0.26
603	6.02	6.07	6.03	6.00	6.02	6.04	6.02	0.44	0.25
310	6.49	6.55	6.51	6.43	6.71	6.52	6.55	0.47	2.20
281	6.86	6.91	6.87	6.76	7.10	6.88	6.91	0.39	2.51
271	6.75	6.81	6.77	6.67	6.99	6.78	6.81	0.45	2.40
234	7.20	7.25	7.21	7.06	7.50	7.22	7.26	0.37	3.08
194	7.72	7.77	7.74	7.52	8.07	7.74	7.78	0.33	3.56
118	9.70	9.75	9.71	9.22	9.97	9.72	9.63	0.27	3.95
72	12.48	12.54	12.45	12.76	12.91	12.49	12.71	0.37	1.85

[a] Data shown in columns marked 1, 2, and 3 are from the same capillary. [b] Data shown in columns marked 3, 4, and 5 are from three different capillaries.

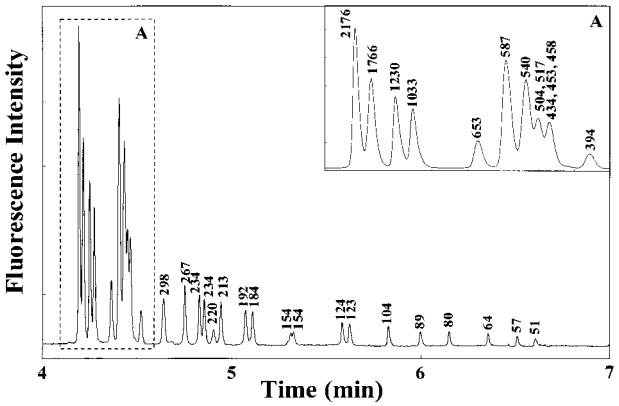

Figure 3. Separation of 25 μg/mL DNA markers V and VI in the presence of EOF at 15 KV using a 10-μm capillary. Capillary length: 35 cm (25 cm to the detector). The capillary was filled with 1X TBE containing 20 μg/mL EtB. Buffer vials contain solutions of 2% PEO and 20 μg/mL EtB.

and 281-bp DNA fragments using CE and mass spectrometry.[32]

The second baseline shift shown in the upper panel of Figure 1 occurred when PEO migrated through the detection point. Using the time until the second baseline shifted, we calculated the mobility of the EOF as 4.04×10^{-4} cm^2 V^{-1} s^{-1}. Using this value, the calculated EPM of a 72-bp DNA fragment was 3.03×10^{-4} cm^2 V^{-1} s^{-1}, which was comparable with the values obtained using capillaries prefilled with matrixes in the absence of EOF.[33] The slight difference between the two techniques was attributed to variations in EOF at the time that PEO entered capillaries. This was supported by Barron et al., who showed slight decreases in EOF in the presence of hydroxyethylcellulose solutions.[27] Reproducible mobility of EOF (RSD = 0.42%, $n = 3$) further suggested that adsorption of the polymer to the capillary wall was trivial if the capillary was electrophoretically washed with 0.5 M NaOH prior to analysis.

It was possible to introduce highly viscous polymer solutions into small capillaries by EOF, avoiding the drawbacks of pressure filling such as the need for low-viscosity polymer solutions, larger capillaries, and high pressure. Figure 2 shows the separation of DNA markers V and VI using 3% PEO solutions containing 1 μg/mL EtB. A 3% PEO solution is gelatinous and cannot be introduced into capillaries by any simple hydrodynamic means. It is worth noting that greater fluorescent intensity and small pore sizes, which are important for separation of small DNA fragments, are obtained using PEO solutions of higher concentration. Our system, using more viscous solutions and lower concentrations of EtB, required longer separation times as a result of lower EOF and higher EPM. DNA migrates slowly against EOF after forming intercalated complexes with cationic EtB molecules, and the reduction in EPM depends on the DNA/dye ratio.[34]

Reproducibility. Table 1 shows the reproducibility of DNA separations using 2% PEO solutions. The RSD values are less than 0.52% for 3 runs using the same capillary and less than 3.96% for 3 runs using three different capillaries. Larger RSD values were found for smaller DNA fragments, possibly resulting from diffusion, a more significant interaction of small DNA with the capillary wall, and slight changes in the EOF.[35] Reproducibility may also be affected by capillary properties and lengths. Electrophoretic washing of capillaries with 0.5 M NaOH at 0.5 kV for 10 min is important to regenerate a fresh and high negatively charged capillary surface for maintaining EOF and minimizing the adsorption of DNA to the capillary wall. Yeung's observation that PEO is unstable under alkaline conditions (hydrolysis occurs above pH 7.4) supports the use of 0.5 M NaOH as a washing solution.[36]

DNA Separation Using 10-μm Capillaries. To exploit the advantages of this new technique, we performed DNA separations using a 10-μm capillary. Figure 3 shows the separation of DNA markers V and VI using 2% PEO solution containing 20 μg/mL EtB. DNA fragments larger than 51 bp were separated and detected in 7 min. Two peaks corresponding to two 154-bp DNA fragments were partially resolved, and 123- and 124-bp DNA fragments were well-separated. The short separation time compared with that shown in Figure 2 supports the idea that higher

(32) Muller, O.; Forest, F.; Karger, B. L. *Anal. Chem.* **1995**, *67*, 2974−2980.
(33) Bocek, P.; Chrambach, A. *Electrophoresis* **1989**, *12*, 1059−1061.
(34) Kim, Y,: Morris, M. D. *Anal. Chem.* **1994**, *66*, 1168−1174.
(35) Iki, N.; Kim, Y.; Yeung, E. S. *Anal. Chem.* **1996**, *68*, 4321−4325.
(36) Preisler, J.; Yeung, E. S. *Anal. Chem.* **1996**, *68*, 2885−2889.

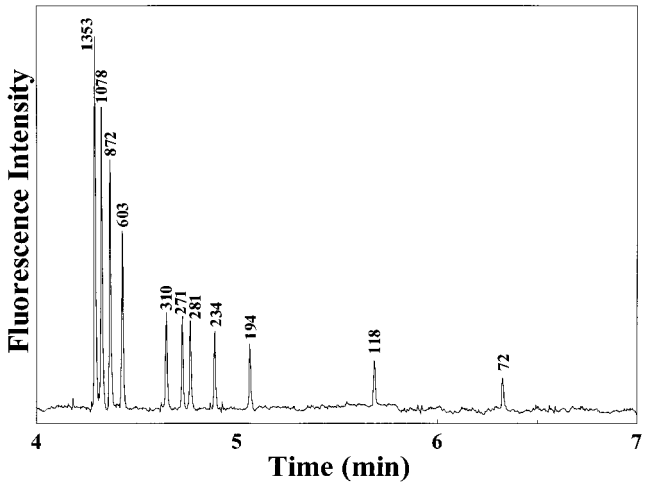

Figure 4. Separation of 5 μg/mL ϕX 174 DNA-Hae III restriction fragments in the presence of the EOF at 15 KV using a 10-μm capillary. Other conditions are as in Figure 3.

EtB concentrations reduce the EPM of DNA. Higher concentrations of EtB also improved resolution of the fragments, possibly because of the decrease in the net charge of the DNA and changes in DNA conformation (elongation and stiffness). The small size of the capillary should also be taken into account. Figure 4 shows the rapid separation of small amounts of ϕX-174/Hae III DNA restriction fragments using a 10-μm capillary. An advantage of using small capillaries is the minimization of cross talking between adjacent capillaries in multiplexed systems. High-resolution and high-speed separation performed at high-electric field strengths can also be achieved using small capillaries because of more efficient Joule heat dissipation. Thus, the throughput of DNA separation may be enhanced using more capillaries and higher electric-field strengths.

Separation Efficiency. Table 2 shows the separation efficiency of ϕX-174/Hae III DNA restriction fragments using 10, 25, and 75-μm capillaries. Higher separation efficiencies were obtained by utilizing a smaller capillary and small amounts of DNA fragments. Theoretical plates (N) up to 11.9 million/m were achieved using a 10-μm capillary (35-cm-long). In these experiments, separation efficiency was also affected by the amount of DNA injected and the concentration of EtB in the buffer solutions.

Smith et al. demonstrated that the width of the DNA band is determined by the four factors of injection, diffusion, thermal gradient, and detection volume, and the variances caused by the latter three are related to the capillary size.[37] Using small capillaries should result in smaller detection variance ($w^2/4$) since the beam waist (w) of the focused laser in the detection window is smaller. Thermal gradient variance (proportional to the square of the inner radius of the capillary and the temperature difference between the center and wall of the capillary ($T_w - T_c$)) is minimized. Diffusion is reduced in a small capillary because of more efficient Joule heat dissipation. The advantages of using small capillaries for DNA analysis, such as DNA sequencing and gene diagnostics, are obvious in terms of resolution and speed. However, a more sensitive method may be required for detecting small amounts of DNA using small capillaries.

(37) Luckey, J. A.; Norris, T. B.; Smith, L. M. *J. Phys. Chem.* **1993**, *97*, 3067−3075.

Table 2. Separation Efficiency of ϕX-174/HaeIII DNA Restriction Fragments Using 2% PEO Solutions and Different Sizes of Capillaries

DNA (bp)	N (million/m)			
	75 μm[a,b]	25 μm[a,b]	10 μm[c,d]	10 μm[a,d]
1353	1.12	7.86	2.86	5.87
1078	1.15	8.07	2.90	9.31
872	1.19	6.39	2.96	9.50
603	1.23	6.67	3.05	6.25
310	2.33	6.10	4.54	6.89
281	2.18	6.76	4.77	7.25
271	2.52	8.31	6.76	7.13
234	2.05	7.41	5.02	11.91
194	2.03	8.45	5.38	8.18
118	1.93	7.43	6.83	7.16
72	1.95	5.03	6.31	8.86

[a] [DNA] = 5 μg/mL. [b] [EtB] = 5 μg/mL. [c] [DNA] = 25 μg/mL. [d] [EtB] = 20 μg/mL.

CONCLUSIONS

We have described a novel technique for high-resolution and high-speed DNA separation using EOF to avoid filling capillaries with PEO solutions prior to analysis. High efficiency (11 million/m) and short separation times (less than 7 min) have been achieved. One drawback of this isocratic technique is the poorer resolution of larger DNA fragments compared with that of smaller ones. This problem may be solved by employing gradient techniques. Pore-gradient CE (PGCE) and/or concentration-gradient (e.g., EtB, electrolytes) CE (CGCE) for separation of DNA and proteins can easily be performed using EOF to fill capillaries with several different polymer solutions.

Separation of DNA over a wide range of sizes using gradient techniques is currently being investigated by this group. Further applications may include single-cell analysis and protein separation. Multiplexed systems and multidimensional separation systems using this new technique are also being developed for the analysis of proteins and DNA. Other polymer solutions, such as HEC, PA, and agarose, are being tested and will be applied to the separation of macromolecules.

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