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Maximization of injection volumes for DNA analysis in capillary electrophoresis

We report concentration and separation of DNA in the presence of electroosmotic flow (EOF) using poly(ethylene oxide) (PEO) solution. DNA fragments migrating against EOF stacked between the sample zone and PEO solution. To maximize the injection volume, several factors, such as concentrations of Tris-borate (TB) buffer and PEO solution, capillary size, and matrix, were carefully evaluated. The use of 25 mM TB buffers, pH 10.0, containing suitable amounts (less than 10 mM) of salts, such as sodium chloride, sodium phosphate, and sodium acetate, to prepare DNA is essential for the concentration of large-volume samples. In the presence of salts, the peaks also became sharper and the fluorescence intensity of DNA complexes increased. Using 2.5% PEO and a 150 μm capillary filled with 400 mM TB buffer, pH 10.0, up to 5 μL DNA samples (ϕX 174 RF DNA-*Hae*III digest or the mixture of pBR 322/*Hae*III, pBR 328/*Bgl*II, and pBR 328/*Hinf*I digests) have been analyzed, resulting in more than 400-fold improvements in the sensitivity compared to that by conventional injections (ca. 36 nL). Moreover, this method allows the analysis of 3.5 μL PCR products amplified after 17 cycles without any sample pretreatment.

Keywords: Capillary electrophoresis / DNA / Laser-induced fluorescence / Polymer solution / Concentration / Polymerase chain reaction
EL 4638

1 Introduction

Although capillary electrophoresis (CE) has emerged as an important technique for the rapid and effective separation of DNA over the last few years [1–4], its use for preparation purpose has been limited because of a small sample volume injected (nL range). To recover DNA, separations have been simultaneously performed using a number of capillaries [5, 6]. The other problem commonly found in CE is poor concentration sensitivity compared to high-performance liquid chromatography because of a relatively short optical path length (e.g., 75 μm). To improve the sensitivity, a number of on-line electrophoretic concentration techniques in CE that are easily performed in most laboratories have been developed. Isotachopheresis (ITP) and field amplification are the two most common concentration techniques and have been applied to improve the sensitivity for DNA analysis [7–11]. However, loss of resolution, irreproducibility, and low concentration factors are problematic when injecting a large volume of high-ionic-strength samples [12–14]. To overcome these disadvantages, the conductivity of DNA samples was decreased by titrating tris(hydroxy-

methyl)aminomethane (Tris) cations with hydroxide ions [15]. Recently, the use of a microporous membrane-mediated loading has been developed for increasing the sample loading in ultrathin slab-gel electrophoresis [16].

Alternatively, we have developed methods for the concentration and separation of DNA and proteins in the presence of electroosmotic flow (EOF) using poly(ethylene oxide) (PEO) solution [17, 18]. After DNA injection, PEO entered the bare fused-silica capillary filled with Tris-borate (TB) buffer by EOF. With small migration velocities (EOF mobility minus electrophoretic mobilities), DNA fragments stack at the interface between the PEO solution and sample zone due to reduced electrophoretic mobilities. In contrast to conventional methods (in the absence of EOF), large DNA fragments are detected earlier at the cathode end. To obtain run-to-run reproducibility (RSD of the EOF < 2.0%), washing the capillary wall with 0.5 M NaOH at 25 V/cm for 10 min between runs is needed when PEO solutions are used [19, 20]. Although a 66-fold sensitivity improvement has been demonstrated for the analysis of DNA, the injection volume is limited, especially when samples were prepared in very low- or high-conductivity media. To maximize injection volumes while maintaining resolution, the conductivities of different zones in the system should be kept as similar as possible and PEO adsorption must be minimized. Thus, it is our goal to evaluate the impacts of sample matrix, PEO solution, and TB buffer on concentration factor (CF), resolution, and migration time for DNA analysis.

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Abbreviations: CF, concentration factor; PEO, poly(ethylene oxide); TB, Tris-borate

2 Materials and methods

2.1 Apparatus

The basic design of the separation system has been previously described [21]. Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL, USA) was used to drive electrophoresis. The entire detection system was enclosed in a black box with an HV interlock. The high-voltage end of the separation system was put in a laboratory-made plexiglass box for safety. A 1.5 mW He-Ne laser with 543.6 nm output from Melles Griot (Irvine, CA, USA) was used for excitation. The light was collected with a $10\times$ objective (numeric aperture = 0.25). One RG 610 cutoff filter was used to block scattered light before the emitted light reaches the photomultiplier tube (Hamamatsu R928). The amplified currents were transferred directly through a 10 k Ω resistor to a 24 bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a personal computer. Capillaries (Polymicro Technologies, Phoenix, AZ, USA) from 25 to 150 μ m ID and 365 μ m OD were used for DNA separations without any further coating process. The capillary lengths were either 60 cm (50 cm effective length) or 45 cm long (35 cm effective length).

2.2 Materials

PEO a with molecular weight of 8 000 000 and other chemicals for preparing the buffer solutions were from Aldrich (Milwaukee, WI, USA). Ethidium bromide (EtB) was obtained from Molecular Probes (Eugene, OR, USA). Please note that wearing gloves is required when handling EtB because it is a highly carcinogenic compound. TB buffers prepared from Tris were adjusted with boric acid to pH 10.0 and 9.0, respectively. Unless otherwise noted, a $\times$ mM TB buffer herein means a buffer containing $\times$ mM Tris adjusted with suitable amounts of boric acid. PEO solutions were prepared from the TB buffers containing 5 μ g/mL EtB. ϕ X 174 RF DNA-*Hae*III digest (500 μ g/mL) was purchased from Pharmacia Biotech (Uppsala, Sweden). DNA markers V (pBR 322/*Hae*III digest) (250 μ g/mL) and VI (pBR 328/*Bgl*I digest and pBR 328/*Hinf*I digest) (250 μ g/mL) were from Boehringer Mannheim (Mannheim, Germany). QIAamp DNA blood mini kit was purchased from Qiagen (Hilden, Germany). PCR kits were obtained from Promega (Madison, WI, USA).

2.3 Stacking and separation

Prior to analysis, capillaries were treated with 0.5 M NaOH overnight. After each run, capillaries were washed with 0.5 M NaOH at 25 V/cm for 10 min to remove PEO solu-

tions and refresh the capillary wall. DNA samples were injected into the capillary filled with TB buffers by hydrodynamic for certain times. During the separation, PEO solutions entered the capillary by EOF and acted as sieving matrices.

2.4 DNA extraction and PCR products

The blood sample was from a normal male. Human genomic DNA from buffy coat was extracted using the QIAamp DNA blood mini kit in accordance with manufacturer's instructions. Amplification of the DNA sample was conducted as suggested by the manufacturers. Briefly, 22.5 μ L PCR master mix component was prepared by mixing 17.45 μ L sterile water, 2.50 μ L STR 10 $\times$ buffer, 2.50 μ L TH01 10 $\times$ primer pairs, and 0.05 μ L *Taq* polymerase (5 U/ μ L). To the PCR master mix, 2.5 μ L of human genomic DNA (25 ng) was added. PCR was conducted as follows: Initial incubation at 96°C for 2 min; cycling for the first 10 cycles at 94°C for 1 min, at 64°C for 1 min, and at 70°C for 1.5 min; cycling for the last 7 and 22 cycles, respectively, at 90°C for 1 min, at 64°C for 1 min, and at 70°C for 1.5 min.

3 Results and discussion

3.1 Effect of capillary size and PEO concentration

To maximize the injection volumes of DNA samples while maintaining resolution, band broadening should be minimized when using a large size of the capillary [22–24]. Although all 11 DNA fragments of ϕ X 174 RF DNA-*Hae*III digest were completely resolved in a number of capillaries, ranging from 25 to 100 μ m ID, filled with 400 mM or 1.5 M TB, pH 10.0, using 1.5% PEO prepared in 100 mM TB, pH 9.0, at 375 V/cm, they were not completely resolved using a 150 μ m capillary. We also found that resolution decreased with increasing the capillary size and the concentration of TB buffer. With increasing PEO concentration to 2.5%, the 11 DNA fragments were well resolved at 125 V/cm using a 150 μ m capillary filled with 400 mM TB buffer, pH 10.0. This is because diffusion coefficient was smaller in more viscous PEO solution and the electric field dependence of DNA electrophoretic migration [25–27] and of Joule heating was less profound at low electric field strengths.

3.2 Matrix dependence of stacking

We have learned that PEO adsorption on the capillary wall decreased with increasing the ionic strength of DNA samples, thereby affecting CF, resolution, reproducibility, and

Table 1. Comparison of parameter for the analysis of DNA

Matrix		Peak height (mV)			Band width (min)			Peak asymmetry			Resolution			Migration time (min)		
		1350	271	72	1353	271	72	1353	271	72	1353/1078	271/281	118/72	t ₀ ^{a)}	t ₁₃₅₃	t ₇₂
Sodium chloride (mM)	1.0	690	66	12	0.02	0.03	0.04	0.7	0.8	0.7	4.7	4.9	53	44.3	47.1	60.5
	10.0	840	110	13	0.04	0.02	0.07	2.0	0.50	1.2	1.5	7.6	44	35.0	38.2	50.5
	50.0	1190	390	14	0.01	0.01	0.04	1.0	0.7	1.2	3.5	10	68	27.5	32.2	44.7
Sodium phosphate (mM)	1.0	440	57	9	0.03	0.02	0.06	0.5	0.8	0.9	2.6	19	48	41.0	43.7	57.0
	10.0	1220	330	25	0.01	0.02	0.04	0.3	1.5	1.2	3.1	8.9	69	35.1	39.6	55.4
Sodium acetate (mM)	1.0	380	334	9	0.03	0.06	0.04	2.0	0.4	1.0	2.6	3.3	43	36.8	39.2	49.3
	10.0	880	3240	23	0.02	0.01	0.04	1.2	1.0	0.8	2.5	9.0	55	32.6	36.2	47.3

a) The time at which the baseline shifted.

Separation at 333 V/cm, injection of 0.62 μ L of 1 μ g/mL DNA prepared in 25 mM TB, pH 10, containing salts; 60 cm capillary (75 μ m ID) filled with 400 mM TB, pH 10

speed for DNA analysis [18–20]. To reveal salt effects, we injected 0.62 μ L DNA samples prepared in 25 mM TB buffers, pH 10.0, containing different concentrations of sodium chloride, sodium phosphate, and sodium acetate, respectively, using a 60 cm capillary with 75 μ m ID. Table 1 clearly shows that the separation was faster when injecting DNA samples prepared in high-conductivity media. With increasing ionic strengths, greater peak heights and narrower band widths were observed. Possible reasons include: (i) the DNA structure became more compact; (ii) the electrophoretic mobility of the DNA fragments were small in the sample zone, leading to effective stacking at the interface between the sample zone and PEO solution; (iii) the diffusion coefficients of the DNA fragments were small; and (iv) diffusion was less problematic with shorter separation times [4, 19]. When adding the same amounts of these salts, matrices containing 10 mM sodium phosphate provided greater concentration factor and resolution, but caused peak splitting. The same problem was also found when matrices containing high amounts of sodium acetate and sodium chloride. Overall, we suggest the matrix containing 10 mM NaCl is optimum.

The results show that it is possible to inject large volumes of DNA samples prepared in TB buffer containing sodium chloride into a 150 μ m capillary using 2.5% PEO. Figure 1 demonstrates the effect of NaCl on the analysis of 5 μ L DNA samples prepared in 10 mM TB, pH 10. DNA prepared in 10 mM TB buffer containing 2.5 mM NaCl provided the best result. It is important to note that, to the best of our knowledge, this is the first example for the analysis of 5 μ L DNA in CE. The difference in optimum conditions from that shown in Table 1 was due to Joule heating, which is further supported by the fact of relatively broader bands shown in Fig. 1C (10 mM NaCl).

Figure 1. Effect of NaCl on DNA concentration at 333 V/cm using 2.5% PEO solution prepared in 150 mM TB buffer, pH 9.0, containing 5 μ g/mL EtB. Capillary, 45 cm in total length, 35 cm in effective length, 150 μ m ID, filled with 400 mM TB, pH 10.; sample, 1.0 μ g/mL ϕ X 174 RF DNA-HaeIII digest prepared in 10 mM TB buffer, pH 10, containing (A) 0, (B) 2.5, and (C) 10 mM NaCl. Hydrodynamic injection was performed at 30 cm height for 150 s (5 μ L).

3.3 Buffer dependence of stacking

Although Fig. 1 shows the successful analysis of 5 μ L DNA, the separation time (about 76 min) was long. In order to shorten the separation time, separations were carried out at different electric field strengths using 2.5% PEO prepared in 120, 150, and 200 mM TB buffers, pH 9.0, respectively. Compared the results shown in Figs. 2A to C, the concentration and separation of DNA was successful when using PEO solution prepared in 200 mM TB buffer. A better stacking is probably due to greatly reduced electrophoretic mobilities of the DNA fragments

Figure 2. Effect of TB buffer used to prepare 2.5% PEO solution and electric field strength on separating 5 μ L ϕ X 174 RF DNA-*Hae*III digest. (A) 120 mM TB buffer at 444 V/cm, (B) 150 mM TB buffer at 444 V/cm for 30 min and subsequently at 333 V/cm for the rest, (C) 200 mM TB buffer at 444 V/cm for 30 min and subsequently at 222 V/cm for the rest. Other conditions were as in Fig. 1.

and a small difference in the conductivity between PEO solution and that of the sample zone at higher ionic strengths. In addition, small diffusion coefficients and less DNA interactions with the capillary wall are possible contributors. The theoretical plate numbers for 11 DNA fragments obtained in 2.5% PEO prepared in 200 mM TB buffer, pH 9.0, were all above 10^7 even when injecting 5 μ L DNA sample using a 150 μ m capillary. From the peak height for the 72 bp fragment shown in Fig. 2C, the limit of detection (LOD) at a signal-to-noise ratio (S/N) of 3 was 3.10 ng/mL, which was 450-fold in sensitivity improvement compared to that obtained by a conventional method (ca. 36 nL). A good reproducibility (relative standard deviation (RSD) values for the EOF mobility and the electrophoretic mobility of the 72 bp fragment were 2.87% and 3.12%, respectively, in three different runs) was obtained, indicating that the performance was not seriously dependent upon the history of the capillary after treated with 0.5 M NaOH.

3.4 Stacking performance

As shown in Fig. 3, the 123 and 124 bp fragments and two 234 bp fragments were well resolved when injecting 5 μ L of 0.1 μ g/mL DNA markers V and VI. The LOD (S/N = 3) for the sample was 9.05 ng/mL (based on the peak height for the 51 bp fragment), which was 400-fold in sensitivity improvement compared to that obtained by a conventional method (ca. 36 nL). Furthermore, we have obtained linear relationships between the peak height and concentration over a range of 0.05–2.0 μ g/mL for 5 μ L DNA fragments

Figure 3. Separation of 5 μ L 0.1 μ g/mL DNA markers V and VI prepared in 10 mM TB, pH 10, containing 2.5 mM NaCl at 444 V/cm for 30 min and subsequently at 222 V/cm for the rest using 2.5% PEO prepared in 200 mM TB, pH 9.0. Other conditions were as in Fig. 1.

(80–1230 bp), with linear regression coefficients (R^2) all above 0.994. Drawbacks of this method for the analysis of DNA markers V and VI include that the two big DNA fragments (2176 and 1766 bp) were not resolved and the DNA fragments smaller than 52 bp were not detected. Possible reason for not separating the two large DNA fragments is due to small pore size of 2.5% PEO, which is more suitable for low DNA fragments. The problem for separating small DNA fragments is due to small bulk EOF mobility and stronger interactions with the capillary wall [19]. To overcome these disadvantages, gradient techniques of using different PEO solutions should be useful [28].

3.5 Stacking of PCR products

To show the feasibility of this method for analysis of real biological samples, we injected 3.5 μ L PCR products amplified after 17 cycles. Figure 4 clearly shows that there were two peaks between 40 and 41 min. The RSD values ($n = 3$) of the peak height and migration time for the first one were 3.1 and 2.2%, respectively. The PCR cycle performed here was much less than the one (32 cycles) suggested by the manufacture, which needs longer amplification time and large amounts of primers and reagents [29, 30]. To identify the two peaks shown in Fig. 4, we spiked ϕ X 174 RF DNA-*Hae*III digest into the PCR product and performed analysis (result not shown). From the migration times for these two peaks and 234 and 194 bp fragments from the standard, we assigned these two peaks were TH01 (9, 8) allele, with 195 and 191 bp fragments, respectively. This method has apparently been proved to be promising for recovery of DNA by CE.

Figure 4. Separation of 3.5 μ L PCR products amplified after 17 cycles at 222 V/cm using 2.5% PEO prepared in 200 mM TB solutions. Hydrodynamic injection was performed at 30 cm height for 105 s (3.5 μ L). Other conditions were as in Fig. 3.

4 Concluding remarks

To maximize the injection volumes of DNA samples, we have optimized the concentration of TB buffers used to prepare PEO solution and to fill the capillary, PEO concentration, applied electric field strength, capillary size, and matrix. We have found that matrices containing salts like NaCl are necessary for large-volume injection and rapid analysis due to reduced PEO adsorption. Concentration and separation efficiency can be optimized by regulation of EOF, which is impossible by conventional methods (in the absence of EOF). Without any sample pretreatment, this method has been applied to the analysis of 3.5 μ L PCR products amplified after 17 cycles. Compared to methods without concentration (32 cycles) this method is faster (the total analysis time including PCR, concentration and separation is shortened from 122.7 min to 105 min) and possibly more cost-effective. With its high resolving power, the capability for large-volume injection, and simplicity (no sample pretreatment), this method has the potential for recovering DNA such as genes and PCR products from biological samples. This may have an important impact on the role of CE in life science, wherein it is generally used for analysis purpose.

This work was supported by the National Science Council of the Republic of China under contract number NSC 89-2113-M002-062.

Received April 3, 2001

5 References

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