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On-column preconcentration and separation of DNA fragments using polymer solutions in the presence of electroosmotic flow

We demonstrated DNA preconcentration and separation in the presence of electroosmotic flow (EOF) using poly(ethylene oxide) (PEO) solutions. After injecting large volumes of DNA samples into a capillary filled with free tris(hydroxymethyl)aminomethane (Tris)-borate (TB) buffers, PEO solutions entered the capillary by EOF and acted as sieving matrices. In contrast to conventional methods (in the absence of EOF), controlling the EOF was also useful for resolution optimization. We have found that PEO adsorption on the capillary wall was more pronounced when low ionic strength buffers were used. Thus, the EOF decreased with increasing injection length, which led to longer migration times and changes in resolution and stacking efficiency. All resolution values were higher than 1.5 when 1.0 μg/mL DNA samples were injected at 240 V/cm for 60 s (0.67 μL). In addition, as low as 0.015 μg/mL DNA samples (an about 66-fold increase in sensitivity) were detected when the injection was performed at 250 V/cm for 60 s.

Keywords: Capillary electrophoresis / Preconcentration / Polymer solution / Electroosmotic flow / DNA
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1 Introduction

Capillary electrophoresis (CE) using polymer solutions has been shown to be an attractive DNA separation technique due to its high resolving power and extremely high speed compared to conventional gel electrophoresis [1–5]. However, the sample concentration detection limits in CE with UV detection at 260 nm have been cited as insufficient for modern analysis [6]. The concentration detection limit of analysis of DNA fragments in CE with laser-induced fluorescence (LIF) has been improved by using a UV argon ion laser at 275.4 nm [7]. More commonly, LIF using relatively low-cost lasers, such as an argon ion laser at 488 nm and an He-Ne laser at 543.6 nm, has been used for detecting DNA molecules intercalated with dyes in running buffers or labeled by fluorophores through covalent bonding [8–11]. However, LIF is sometimes not sensitive enough to detect dilute PCR products and DNA sequencing. Consequently, preconcentration of DNA samples is required [12, 13].

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Abbreviations: EB, ethidium bromide; **PEO**, poly(ethylene-oxide); **TB**, Tris-borate

In order to overcome the poor concentration limits in CE, the large volume of DNA samples injected has been stacked by the isotachophoretic (ITP) preconcentration method [14–16]. The DNA samples are initially stacked between leading and terminating electrolytes prior to CE separation. In ITP-CE, about 20–50% of the column volumes can be injected without loss of resolution. The migration-time shifts due to nonhomogeneous electric field strength distribution, the variation of ITP preconcentration steps, and the effect of matrix, especially salts, are problematic [17, 18]. To overcome these problems, a method called instrinsic isotachophoretic preconcentration has been proposed [19]. By partially filling a capillary with free solutions and choosing suitable terminators, separations of up to 700 nL DNA samples with accurate migration times are possible. However, the injected sample volume (plug length) is limited by the length of the capillary filled with polymer solutions for optimizing resolution. The longer the sample plug, the shorter the separation zone is, which may result in poor resolution.

Alternatively, field-amplified CE originating from the mismatch of the electrophoresis has been shown to be useful for dramatic improvement in the concentration sensitivity [20]. Generally, samples dissolved in a low-conductivity buffer were injected into a relatively high-conductivity running buffer to obtain sharp peaks while stacking. A method of injecting a water plug prior to sample injection to create low field strengths at the beginning of the separation for loading larger volumes of sample and obtaining

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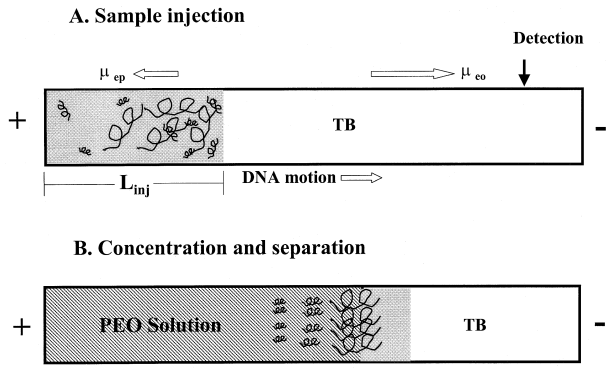

Figure 1. Evolution of zones in the separation of DNA in the presence of EOF. After a plug of DNA sample (L_{inj} was injected into a capillary filled with free buffers, PEO solutions entered the capillary by EOF. The DNA was subjected to stacking in the PEO zones and the large DNA fragments were detected earlier since they migrated slowly against the EOF. μ_{ep}, electrophoretic mobility; μ_{eo}, EOF mobility.

sharper peaks has been demonstrated [21]. Using an untreated capillary, about 50% of the capillary volume of DNA sample dissolved in water was separated in polymer solutions containing *N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid [22]. However, the peaks corresponding to small DNA fragments were not resolved from several ghost peaks and the quantitative results were not provided.

For preconcentration of a DNA sample with high conductivity, a method of pH titration of the CE buffer by acids or bases can be applied. As a result of the decrease in the conductivity, a highly focused electric field is generated. The influence of the field focusing is strong enough to push analytes from the titrated zone to the titration boundary regardless of the salt concentration in the original sample zone. For example, the conductivity of the DNA sample (dissolved in a buffer containing Tris cations) was decreased when the Tris cations were titrated by hydroxide ions [23]. Only a minor loss of resolution was observed when DNA samples were injected at 50 V/cm for 360 s. However at maximum, only 15-fold increases in injection length was demonstrated, presumably because shorter separation lengths and low electric fields increased band broadening and reduced resolution. More recently, a similar idea was developed for ultrathin slab gel electrophoresis by using a microporous membrane-mediated loading [24]. The DNA molecules stack up against the higher-conductivity sieving matrices at the interface of the separation gel.

To prevent the interaction of DNA and the capillary wall, DNA samples were injected into deactivated capillaries filled, or partially filled, with polymer solutions or slab gels using the above methods. In contrast, we have developed DNA separation in the presence of the EOF [25–27]. The advantages over conventional CE methods (in the absence of the EOF) include the possibility of using small capillaries and highly viscous polymer solutions, and performing gradient techniques for optimizing resolution by regulating the EOF during separation. However, the EOF decreased temporally due to the dynamic adsorption of polymer molecules [25–28]. Thus the bulk EOF must be well controlled in order to obtain reproducible results. We have shown that the EOF with reproducibility (RSD < 2%) is easily achieved by washing the capillary wall with 0.5 M NaOH at 1 kV for 10 min between runs when poly(ethylene oxide) (PEO) solutions are used. More recently, we also found that the adsorption of PEO molecules during separation was trivial (the EOF remained fairly constant after the whole capillary filled with PEO solutions) when buffers with high ionic strength, such as 100 mM Tris-borate (TB) buffers, pH 10.0, were used (Tseng and Chang, in preparation). In this study, we attempted to develop a method for preconcentration and separation of DNA samples in the presence of the EOF. Unlike the above method, after large volumes of DNA samples were injected into a capillary filled with free TB buffers, PEO solutions entered the capillary by EOF and acted as sieving matrices.

2 Materials and methods

2.1 Chemicals

All chemicals for preparing buffer solutions were from Aldrich (Milwaukee, WI, USA). Ethidium bromide (EB) was obtained from Molecular Probes (Eugene, OR, USA). A TB-free buffer prepared from Tris was adjusted with boric acid to pH 10.0 and 9.0, respectively. PEO (M_r 8 000 000) was purchased from Aldrich and solutions were prepared from the TB-free buffers containing 5 μg/mL EB, respectively. ϕX 174 RF DNA-*Hae*III digest was purchased from Pharmacia Biotech (Uppsala Sweden). Genescan-500 ROX was purchased from Perkin-Elmer (Forster City, CA, USA).

2.2 Apparatus

The basic design of the separation system was described previously [25]. Briefly, a high-voltage power supply (Gamma High Voltage Research, 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 homemade 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

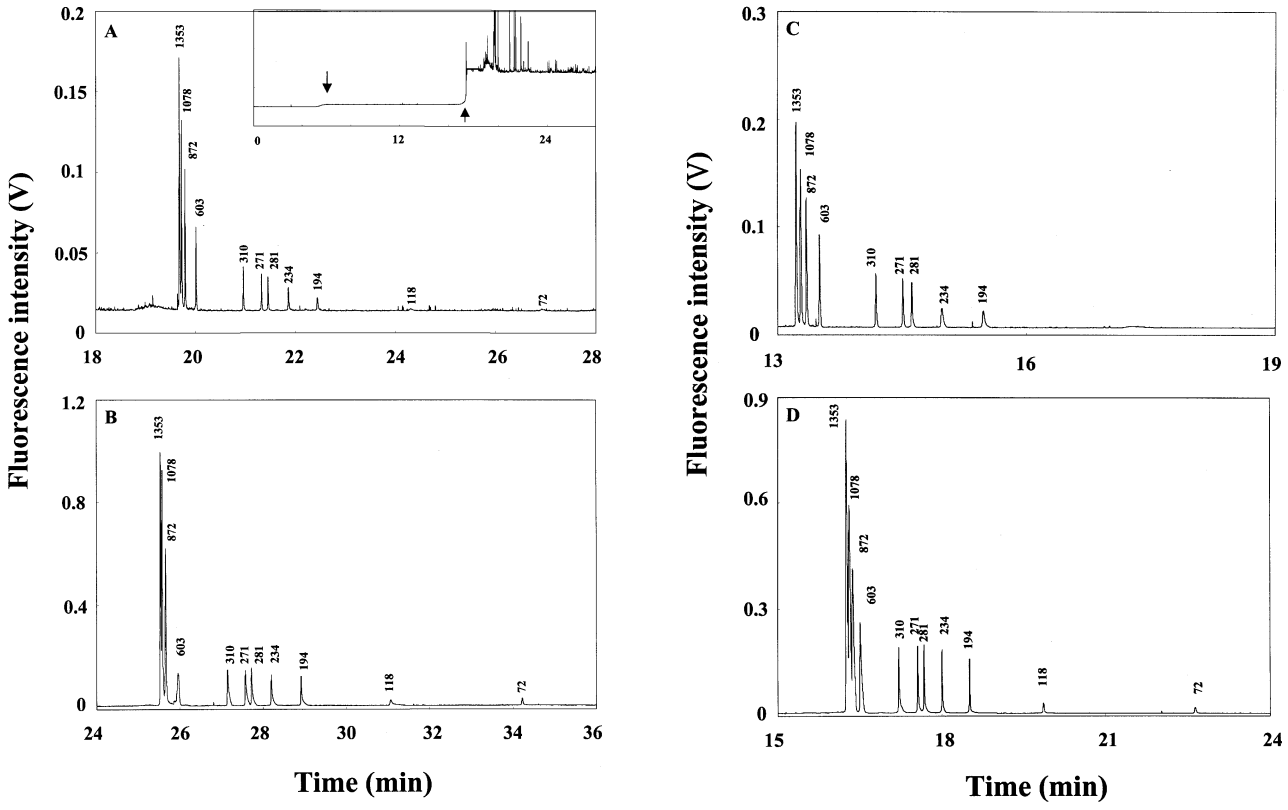

Figure 2. Separation of ϕX 174 RF DNA-*Hae*III digest DNA in the presence of the EOF at 15 kV using 1.5% PEO solutions prepared in 100 mM TB buffers, pH 9.0, containing 5 μg/mL EB. Capillary 40 cm total length and 30 cm effective length; filled with (A), (B) 50 and (C), (D) 100 mM free TB buffers, pH 10.0, respectively. Injections were performed (A), (C) at 25 V/cm for 10 s and (B), (D) at 250 V/cm for 60 s.

collected with a 10 × objective (numerical aperture = 25). One RG 610 cutoff filter was used to block scattered light before the emitted light reaches the phototube (Hamamatsu R928; Hamamatsu, Japan). The amplified currents were 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 (Biotaq.com Inc, Potomac, MD, USA) with 75 μm ID and 365 μm OD were used for DNA separations without any further coating process.

2.3 Preparation of PEO solutions

Certain amounts of PEO were gradually added into the free TB buffer, pH 9.0, in beaker, stirring in a water bath at 85–90°C. During the addition of PEO, a magnetic stirring rod was used at high speed to ensure a homogeneous solution. After addition was complete, the suspension was stirred for at least one more hour. Finally, polymer solutions were degassed with a vacuum system in an ultrasonic tank. Polymer solutions stored in a refrigerator at 4°C were usable for at least one week.

2.4 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 1 kV (to prevent from generating high Joule heating) for 10 min to remove polymer solutions and refresh the capillary wall. Large volumes of DNA samples were injected into the capillary filled with TB buffer at 25–250 V/cm for 10–60 s. During the separation, PEO solution entered the capillary *via* the high EOF for sieving DNA fragments. A scheme of the evolution of zones in the preconcentration and separation of DNA samples is depicted in Fig. 1.

3 Results and discussion

3.1 EOF and separation times *vs.* injection length

Figure 2 shows electropherograms of separating different volumes of 1 μg/mL DNA using 1.5% PEO solutions prepared in 100 mM TB buffers, pH 9.0, in a capillary filled with 50 and 100 mM TB-free buffers, pH 10.0, respective-

Table 1. Effect of injection length and ionic strength on the EOF and migration times

	50 mM TB			100 mM TB		
	Migration time (%RSD)			Migration time (%RSD)		
Injection	t_0[a)]	1353 bp	72 bp	t_0	1353 bp	72 bp
1 kV 10 s	17.42 (0.59)	19.68 (0.53)	26.92 (0.78)	11.15 (0.98)	13.78 (1.03)	ND[b)]
10 kV 10 s	18.23 (0.62)	20.36 (0.70)	26.33 (1.19)	12.87 (1.04)	15.74 (1.53)	25.46 (1.75)
10 kV 30 s	20.57 (0.62)	23.44 (0.71)	31.39 (1.23)	13.47 (1.07)	16.88 (1.67)	29.89 (1.89)
10 kV 60 s	22.07 (0.65)	25.54 (0.78)	34.22 (1.46)	14.37 (1.12)	16.25 (1.73)	22.63 (2.26)

a) t_0 the time at which the baseline shifted due to the entrance of the PEO matrices
b) ND, not detected

ly. Two baseline shifts were observed (shown in the upper panel of Fig. 2A) because EB (cationic) and PEO solutions (neutral) migrated through the detection window, respectviely. Thus, the EOF mobility could be calculated from the time at the second baseline shift. For example, the bulk EOF mobilites were 7.65×10^{-5} and 1.19×10^{-4} $cm^2V^{-1}s^{-1}$ when DNA samples were injected at 25 V/cm for 10 s into the capillary filled with 50 and 100 mM TB-free buffers, respectively. The run-to-run variation of the migration times due to the change in the EOF can be further normalized if the electrophoretic mobilities of the DNA fragments are calculated.

The bulk EOF depends on the fraction of the local EOF mobilities when the separation is performed in a capillary filled with more than two different buffers [20]. Thus, the bulk EOF decreased when the PEO solutions (higher viscosity) entered the capillary. In addition, the PEO adsorption also caused decreases in the bulk EOF mobility. Since the adsorption of PEO molecules is significant when buffers with low ion strengths are used, injection of a long plug of DNA samples dissolved in water is not suitable. On the other hand, DNA samples prepared in buffers with high ionic strengths, leading to the dispersion of the sample zone, are also not recommended. To optimizeing the stacking effect and migration time, the DNA sample was dissolved in a 5 mM TB buffer, pH 7.5. For a short sample plug, the contribution of the local EOF of the sample plug on the bulk EOF should be ignored. However, the effect of the sample plug on the bulk EOF should be significant when the injection volume is large.

From the view of the decrease in the EOF with increasing ionic strength in CZE, the bulk EOF should increase with increasing sample plug during the injection. In contrast, the bulk EOF decreased temporally due to increases in viscosity und adsorption of PEO molecules when PEO matrices entered the capillary through the high EOF during separations. Only the second effect was related to the sample plug since the adsorption was also controlled by the ionic strength of the DNA sample. Table 1 shows that the bulk EOF decreased (t_O increased) with increasing injection length. Strikingly, the migration time was shorter when the DNA sample was injected at 10 kV for 60 s than that at 10 kV for 30 s using the capillary filled with a 100 mM free TB buffer. This may be due to a larger bulk EOF before PEO solutions entered the capillary, or probably results from the effect of borate on DNA conformation, *i.e.*, small electrophoretic mobility (EPM) at low borate concentrations) [29].

3.2 Resolvation *vs.* injection length

Using deactivated capillaries filled with polymer solutions (in the absence of EOF), the mesh sizes of polymer matrices as well as buffer additives such as EB play important

Table 2. Effect of injection length on resolution using a capillary filled with a 50 mM free TB buffer

Injection	Resolution									
	1353/1078	1078/872	872/603	603/310	310/271	271/281	281/234	234/194	194/118	118/72
1 kV 10 s	2.55	4.13	12.98	55.75	21.54	7.67	20.65	18.77	28.76	34.15
10 kV 10 s	2.06	3.73	14.75	60.88	18.59	5.73	14.09	17.40	26.57	30.43
10 kV 30 s	1.69	3.47	11.95	64.21	15.20	3.40	10.70	16.22	35.04	46.20
10 kV 60 s	1.28	2.73	4.97	20.97	15.16	5.70	18.81	25.49	42.48	48.38

Resolution (R_s) was calculated by $R_s = 2\ (t_2 - t_1)/(t_{w1} + t_{ww2})$, where t_1 and t_2 are the migration time, and t_{w1} and t_{w2} are the width of the peak at baseline for fragments 1 and 2, respectively.

Table 3. Effect of injection length on resolution using a capillary filled with a 100 mM free TB buffer

Injection	Resolution									
	1353/1078	1078/872	872/603	603/310	310/271	271/281	281/234	234/194	194/118	118/72
1 kV 10 s	3.24	4.13	9.34	34.39	14.38	4.79	11.64	11.88	ND[a)]	ND
10 kV 10 s	3.02	3.28	7.91	39.67	21.46	7.60	16.91	20.10	59.20	77.20
10 kV 30 s	2.56	3.17	4.93	17.43	8.50	2.53	8.54	20.61	57.85	75.52
10 kV 60 s	1.82	1.87	4.90	32.20	15.34	5.09	16.69	25.62	43.67	57.61

a) ND, not detected

roles in determining resolution [3, 4]. In contrast, controlling the EOF was also useful for resolution optimization in this study since the DNA migrated against the EOF. Tables 2 and 3 show that all adjacent peak pairs were resolved (R_S > 1.25) and a resolution of 271/281 as high as 7.67 was obtained. Compared to our previous studies using 1 × (Tris-borate-EDTA, TBE) or 1 × TB at pH 8.2 (unpublished results), DNA separation at pH 9.0 provided better resolution, indicating the pH effect should be taken into account for higher resolution [30]. For a shorter sample plug, we found the resolution (besides 1353/1078 and 1078/872) obtained from a 50 mM free buffer to be better than that of 100 mM free buffers. This is presumably because the differential migration times were relatively large due to a small bulk EOF (the adsorption of PEO molecules).

Just as the EOF depends on the length of the sample plug, as addressed in Section 3.1, resolution was also related to the injection length. The effect of the injection length on resolution was inconsistent since the variance is proportional to migration time. In the presence of the PEO matrices, the adsorption of the DNA molecules on the capillary wall was trivial, as supported by the result of reproducible migration times (RSD < 3.0%) and symmetrical peaks corresponding to all DNA fragments. Furthermore, thermal variations inside the capillary during the separation were also ignored because low currents (< 10 μA) were observed. Therefore, the variance could be simply divided into two terms: injection length (σ^2_{inj}) and diffusion (σ^2_{dif}). The longer the injection length, the

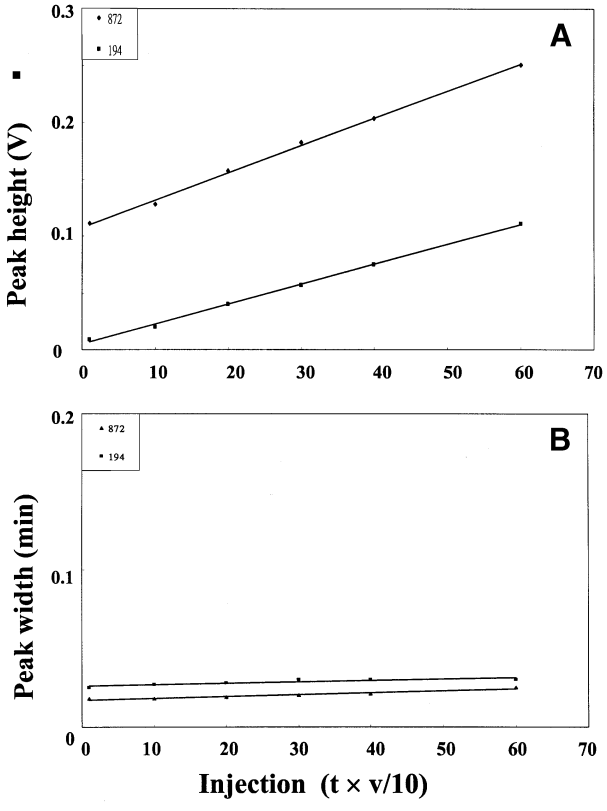

Figure 3. DNA concentration using a capillary filled with 100 mM free TB buffers in the presence of the EOF. (A) Peak height *vs.* injection voltage × injection time. (B) Peak width *vs.* injection voltage × injection time.

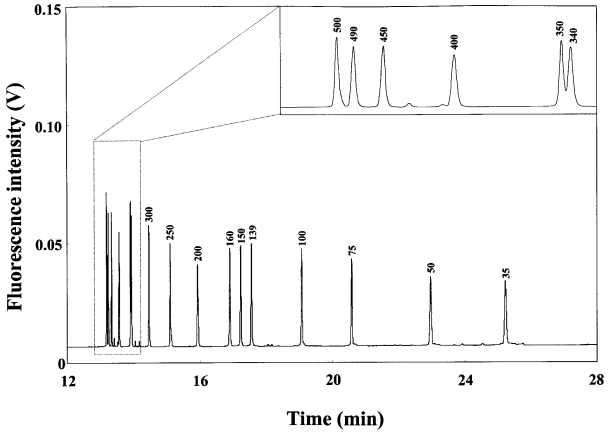

Figure 4. Separation of 0.05 nM Genescan-500 ROX in the presence of the EOF at 15 kV using 1.5% PEO solutions prepared in 100 mM TB buffers, pH 9.0. The DNA sample was injected at 250 V/cm for 45 s. Other conditions as in Fig. 2.

higher the σ^2_{inj} and the longer the migration time were, which led to a higher σ^2_{dif}. In other words, loss of resolution was expected when a longer plug of DNA samples was injected. Although diffusion was expected to be relatively large for smaller DNA fragments (higher diffusion coefficient and longer migration times), resolution generally increased with increasing injection length. This was due to larger differential migration times when the bulk EOF mobility was small.

3.3 Concentration factor *vs.* injection length

Figure 3 shows peak height and peak width against injection voltage × injection time, respectively, using the capillary filled with 100 mM free TB buffers. Good linear relationships between peak height and injection volume were obtained (R^2 = 0.998 and 0.995 for 872 and 194 bp fragments, respectively) and the peak widths at half height of all peaks kept fairly constant (less than 1.8 s). As migration times increased with increasing injection length, diffusion should be taken into account for the variation of the peak width. Due to its slow migration against the EOF in PEO matrices, the large DNA fragment stacked closer to the interface. The stacking efficiency of large DNA fragments was not as efficent as small ones if the stacking time was not long enough because it took much longer for large DNA fragments to migrate from free TB buffers to PEO solutions [31]. For example, using the capillary filled with 100 mM free TB buffers the peak height ratios (PHR) of 1353 and 194 bp fragments obtained between 250 V/cm for 60 s and 10 s were 2.0 and 5.5, respectively. This was further supported by the result of higher PHR of large DNA fragments obtained using 50 mM rather than 100 mM TB buffers. The PHR of the 1353 bp fragment was 2.4 and 2.0 using 50 and 100 mM free TB buffers, respectively. A long capillary should thus be used for a long plug of sample injection if more sensitivity enhancement is needed.

Since the actual bulk EOF during injections was different from that during separations, the actual length of the sample plug could not simply be obtained from the electropherograms shown in Fig. 2. In order to estimate the injected length when DNA samples were injected at 250 V/cm for different times, we monitored the baseline during the DNA injections. The baseline shift occurred at 124 s. From that we estimated that the injection length was about 15 cm (about 0.67 μL) at 250 V/cm for 60 s if we ignored the variation of the bulk EOF as a result of changes in ionic strength during injections. When injecting about 0.67 μL of 0.015 μg/mL DNA samples into the capillary filled with 100 mM TB buffers, the peak height of the 194 bp DNA fragment was close to that obtained using 1.0 μg/mL DNA samples injected at 25 V/cm for 10 s, indicating that the sensitivity improvement was about 66-fold. A similar result was also shown in the separation of 0.5 μL of 0.05 nM Genescan-500 ROX using the capillary filled with 100 mM free TB buffers. The signal is close to that using 8 nM Genescan-500 ROX injected at 25 V/cm for 10 s. Note that the DNA sample was labeled with dyes, and that PEO solutions without EB were used. In addition, we also found this resolution without significant loss when the DNA samples were injected up to 0.6 μL.

4 Concluding remarks

In contrast to injecting DNA samples into a capillary filled with polymer solutions, the DNA sample was concentrated and separated in moving PEO matrices by the EOF. In this study we showed that the ionic strength of free solutions and injection length affected the PEO adsorption on the capillary wall. We also demonstrated that the injection length and ionic strength affected migration time, resolution, and stacking efficiency. Although we only showed about a 66-fold sensitivity improvement, using longer capillaries and choosing suitable buffers or polymer solutions may be useful for futher optimizing resolution and stacking efficiency. The results of high resolution between 271/282 DNA fragments and sensitivity improvement also indicate that his method may be suitable for analysis of dilute PCR products (several hundred bp).

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