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ORIGINAL PAPER

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Capillary electrophoretic separation of dsDNA under nonuniform electric fields

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Abstract Improved sensitivity for the analysis of DNA by capillary electrophoresis has been achieved, based on simultaneous increases in optical path length and injection volume. To increase the optical path length, bubble cells with diameters ranging from 150 to 450 μm have been fabricated and tested. In terms of resolution and sensitivity, a bubble cell of 300 μm diameter is appropriate when using 75-μm capillaries. To allow greater injection volumes, we performed on-line concentration of DNA in the presence of electroosmotic flow (EOF) using 2.0% poly(ethylene oxide) (PEO). With a 300-μm bubble cell, a 170-fold improvement in the sensitivity for the 89-bp fragment has been accomplished when injecting about 0.33 μL DNA. In the presence of the bubble cell, the resolution for the large fragments improves while that for the small ones (<124 base pair) decreases. The effect of bubble cells was further investigated by conducting DNA separation in the absence of EOF, showing that improvements in resolution are mainly due to increased migration differences when DNA migrated at low electric field strengths in the bubble region. We have suggested that such an effect is more profound using shorter capillaries, leading to complete separation of φX 174 RF DNA-Hae III digest in 2 min.

Keywords Bubble cell · DNA separation · Stacking · Capillary electrophoresis · Laser-induced fluorescence

Introduction

The advantages of capillary electrophoresis (CE) over slab gel electrophoresis for DNA analysis have been well demonstrated, including rapidity, high throughput, ease of automation, and less amounts of samples and reagents used [1, 2, 3, 4]. Typical applications of CE include DNA sequencing, single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), and fragment sizing [5, 6, 7, 8]. However, owing to small injection volumes (several nL) and short optical path length (μm) in CE, poor concentration sensitivity has been recognized since its inception.

To improve sensitivity in CE, a number of friendly on-line electrophoretic concentration techniques have been developed, including isotachophoretic (ITP) analysis [9, 10] and field amplification [11, 12, 13]. Alternatively, we have demonstrated the separations of large volumes of proteins and DNA in the presence of electroosmotic flow (EOF) using poly(ethylene oxide) (PEO) solution [14, 15, 16]. When proteins or DNA fragments migrate against the EOF, they slow down due to sieving as well as increases in viscosity, and thus stack at the interface between the sample zone and PEO solution. As a result, the separation of DNA up to 5-μL using a 150-μm capillary has been demonstrated, with a 450-fold sensitivity improvement [17].

Techniques based on extending the optical path length such as Z-shaped and bubble cells have been developed for the analysis of small analytes, with several tens-fold improvements in sensitivity [18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. Although it is easy to make Z-shaped cells, loss of resolution is problematic when analytes migrate too closely. On the other hand, loss of resolution due to imperfect distribution of the electric field strength inside the capillary and a limited extension of the optical path length (generally less than 10-fold) are disadvantages when using bubble cells [24]. As a consequence, techniques based on extending optical path length for DNA analysis have not been reported in CE.

In this report, we tested the possibility of improving the sensitivity for the analysis of DNA by simultaneously increasing injection volumes and optical length. To achieve this goal, one of the aforementioned on-line concentration approaches [16] was applied and capillaries with different sizes of bubble cells were fabricated and utilized. We also found the influence of bubble cells on resolution as a result of changes in electric field strength [28, 29, 30].

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Experimental

Apparatus

The basic design of the separation system has been previously described [16]. 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 a high-voltage interlock. The high-voltage end of the separation system was put in a laboratory-made Plexiglas 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× 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, Hamamatsu Photonics K. K., Shizuoka-ken, 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 (Polymicro Technologies, Phoenix, AZ, USA) of 75 µm i.d. and 365 µm o.d. were used for DNA separation. A 30-cm bare fused-silica capillary (20 cm effective length) without coating was used for on-line concentration of DNA in the presence of EOF. When conducting DNA separations in the absence of EOF, capillaries coated with 5% polyvinyl pyrolidone (PVP) were used. The bare-fused silica capillaries were rinsed with fresh water for 10 min and then filled with 5% PVP. After sitting at room temperature overnight the dynamically coated capillaries were formed mainly through hydrogen bonding and hydrophobic patches between PVP molecules and the capillary wall [31]. Please note that this simple dynamic coating method allows effective suppression of EOF ($<1\times10^{-5}$ cm^2 V^{-1} s^{-1}) under the experimental conditions. An inverse optical microscope I×70 from Olympus (Tokyo, Japan) was used to view the bubble cells.

Materials

PEO with molecular weight 8,000,000 (entanglement threshold concentration 0.07%) and other chemicals for preparing buffer solutions were from Aldrich (Milwaukee, WI, USA). Ethidium bromide (EtBr) was obtained from Molecular Probes (Eugene, OR, USA). Please note that wearing gloves is required when handling EtBr because it is a highly carcinogenic compound. TB buffers prepared from Tris were adjusted with boric acid to pH 10.0 or 9.0. In this study, the molarity of Tris represents the concentration of TB buffer. PEO solutions were prepared in TB buffers containing 5 µg mL^{-1} EtBr [14]. 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/Bg1 I digest & pBR 328/Hinf I digest) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Equal volumes of DNA markers V and VI were mixed and used in this study. 2-Log DNA ladder was a gift from New England Biolabs (Beverly, MA, USA).

Bubble cell

When making a bubble cell, a low pressure (400 psig, 1 psig= 6894.76 Pa) of nitrogen gas was applied from one end of a capillary. The distance between two pieces of aluminium foil (2×2 cm) covering the capillary is 0.2 cm, allowing confinement of the bubble cell length (parallel to the flow direction) to less than 0.1 cm. While making bubble cells, the capillaries were heated under an air–butane torch with a flame size of about 0.08 cm (temperature up to 1537 °C) and were manually rotated at a rate of roughly 10 rpm to achieve even geometries. It took about 5 min to make a bubble cell for an experienced researcher, with a success rate greater than 90%. The radius of the bubble cell (Fig. 1) was estimated by comparing the i.d. (75 µm) of the capillary when viewing under an optical microscope. The relative standard deviation (RSD) values of the diameter and length of five different bubble cells were less than 7.0%.

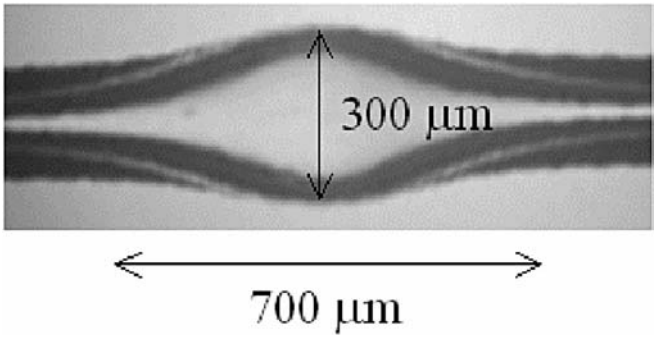

Fig. 1 A 300-µm bubble cell

Stacking and separation

For conducting on-line concentration of DNA in the presence of EOF using a bubble cell, the capillary was treated with 0.5 mol L^{-1} NaOH overnight prior to analysis. After each run, the capillary was washed with 0.5 mol L^{-1} NaOH at 1 kV for 10 min to remove PEO solution and refresh the capillary wall. DNA samples were injected into the capillary filled with TB buffers by hydrodynamic injection at 30-cm height for times ranging from 5 to 90 s. During the separations at 10 kV, PEO solution entered the capillary by EOF from the anodic reservoir and acted as a sieving matrix. On the other hand, prior to DNA separations in the absence of EOF, the capillary dynamically coated with 5% PVP overnight was filled with PEO solution by pressure. Owing to the difficulty of injecting samples by pressure when using the short capillaries (<10 cm), electrokinetic injection was performed at 1 kV for different times. The separations were carried out at applied voltages of 0.5–5.0 kV. After each run, PEO solution was pushed out by nitrogen gas and the capillary was re-filled with 5% PVP to perform dynamic coating. For reproducibility, PVP was kept in the capillary for 5 min.

Results and discussion

Sensitivity improvement

The electric field strength (E) decreases proportionally to the second order of the inside radius (r) according to the equation $E_1/E_2=(r_2/r_1)^2$, where subscripts 1 and 2 represent the separation channel and the bubble cell, respectively. Since the inner radius of the original column is smaller than that of the bubble cell, once the sample plug moves into the bubble region, it will experience lower electric field strengths and slow down. In addition, peak broadening occurs due to the geometric change and eventually counteracts any gain in the sensitivity improvement from the increase in the optical path length. As a result, sensitivity improvement and separation are no longer possible. For example, we have found that a bubble cell with a diameter over 300 µm is inappropriate for DNA separation when using a capillary of 75 µm i.d.

Figure 2A shows a successful example of the separation of 2.0 µg mL^{-1} DNA using a 300-µm bubble cell, with a signal-to-noise ratio (S/N)=6 for the 89-bp fragment. Based on this result, the limit of detection (LOD) at S/N=3 for this method is 1.0 µg mL^{-1}. Fig. 2B shows that the sensitivity improved further when the injection volume was 0.33 µL, with an LOD of 0.03 µg mL^{-1} on the basis of the peak height for the 89-bp fragment. The improvement is

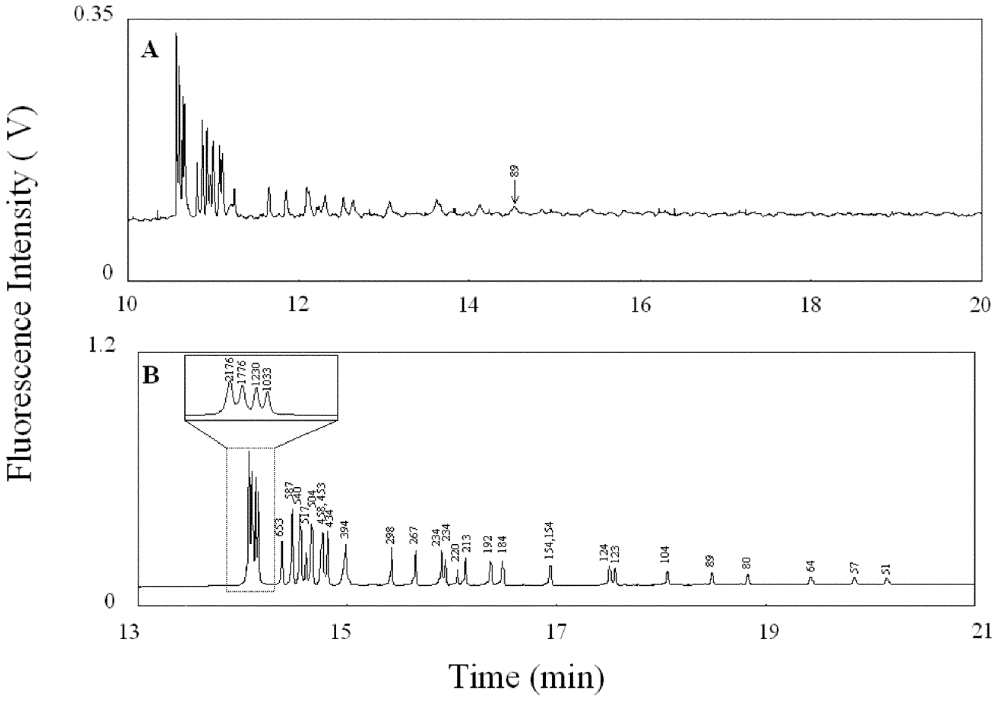

Fig. 2 Analyses of 2.0 µg mL^{-1} DNA markers V and VI in the presence of EOF at 10 kV. Injections were conducted at 30-cm height for 5 and 45 s in (**A**) and (**B**), respectively. Capillary with a 300-µm bubble cell: 30 cm in total length, 20 cm in effective length, and filled with 400 mmol L^{-1} TB, pH 10.0. The buffer vials contained 2.0% PEO prepared in 200 mmol L^{-1} TB containing 5 µg mL^{-1} EtBr, pH 9.0. The numbers in the electropherograms denote the DNA sizes (bp)

Table 1 Comparison of peak enhancement and resolution for the analysis of DNA at 10 kV[a]

Peak enhancement[b] (RSD%, n=5)				Resolution[c]				
Base pairs	150 µm	300 µm	450 µm	Base pairs	Normal capillary	150 µm	300 µm	450 µm
2176	5.3 (2.1)	10 (1.8)	15 (3.3)	2176/1776	1.4	1.4	1.5	1.03
1776	5.2 (2.7)	11 (2.1)	15 (3.2)					
1230	5.0 (2.9)	9.7 (2.0)	12 (3.7)	1230/1033	1.2	1.2	1.6	1.1
1033	3.9 (2.1)	8.5 (2.1)	11 (3.1)					
540	4.3 (2.7)	12 (2.9)	12 (3.5)	540/517	3.0	3.15	2.8	1.3
517	4.4 (2.5)	8.7 (3.1)	9.4 (3.4)					
504	4.6 (3.9)	13 (2.7)	14 (3.1)	394/298	12.0	20.5	19.4	11.0
394	8.5 (1.9)	17 (2.1)	15 (3.8)					
298	13 (3.3)	25 (2.3)	29 (3.1)	298/267	8.3	11.6	13.6	5.2
267	11 (2.3)	45 (2.1)	55 (3.3)					
234	6.3 (2.6)	24 (2.2)	ND[d]	234/234	0.5	0.9	1.3	0
234	10 (2.7)	21 (2.6)	ND					
123	6.1 (2.5)	25 (2.1)	ND	124/123	1.8	1.2	1.6	0
124	5.9 (3.1)	24 (2.7)	ND					
89	4.6 (2.9)	19 (3.5)	10 (2.3)	89/80	9.9	7.1	8.7	2.3
80	4.4 (3.2)	17 (2.9)	9.7 (2.3)					

[a]Injection volume was about 0.33 µL; the concentration of DNA prepared in 25 mmol L^{-1} TB, pH 10, was 2.0 µg mL^{-1}; and the 30-cm capillary was filled with 400 mmol L^{-1} TB, pH 10.0

[b]Peak enhancement was the ratio of the peak area obtained in the presence and absence of bubble cells

[c]$R = \frac{2(t_2 - t_1)}{(w_1 + w_2)}$

[d]Peaks were not resolved

170-fold for the 89-bp fragment when compared to that obtained in the absence of a bubble cell with an injection at 30-cm height for 5 s (LOD=5.1 µg mL^{-1}) (not shown). Although the sensitivity improvement is not great compared to our previous one (450-fold) [17], the analysis is much faster (ca. 20 min vs. 70 min). Based on our previous results [14, 15, 16, 17], it is also our belief that the sensitivity, resolution, and speed can be further optimized by injecting a greater volume of the DNA sample into a longer capillary and conducting the separation at higher electric field strengths.

With the same injection volume, Table 1 shows up to 55-fold improvement in the sensitivity compared to that in the absence of a bubble cell, which is comparable to a reported result [22]. With the same bubble cell, different sensitivity improvements for these DNA fragments are attributed to different stacking efficiency [16]. The improvement greater than the increased ratio of the optical length (6-fold) is due to weaker scattering light (low background) and greater collection efficiency (greater signal) as a result of a thinner capillary wall. Stacking when DNA migrated to the bubble region is also a possible contributor to improved sensitivity. Please note that the reproducibility of this method is reasonable, with relative standard deviation (RSD) values for the peak enhancement (sensitivity improvement) and migration time in five con-

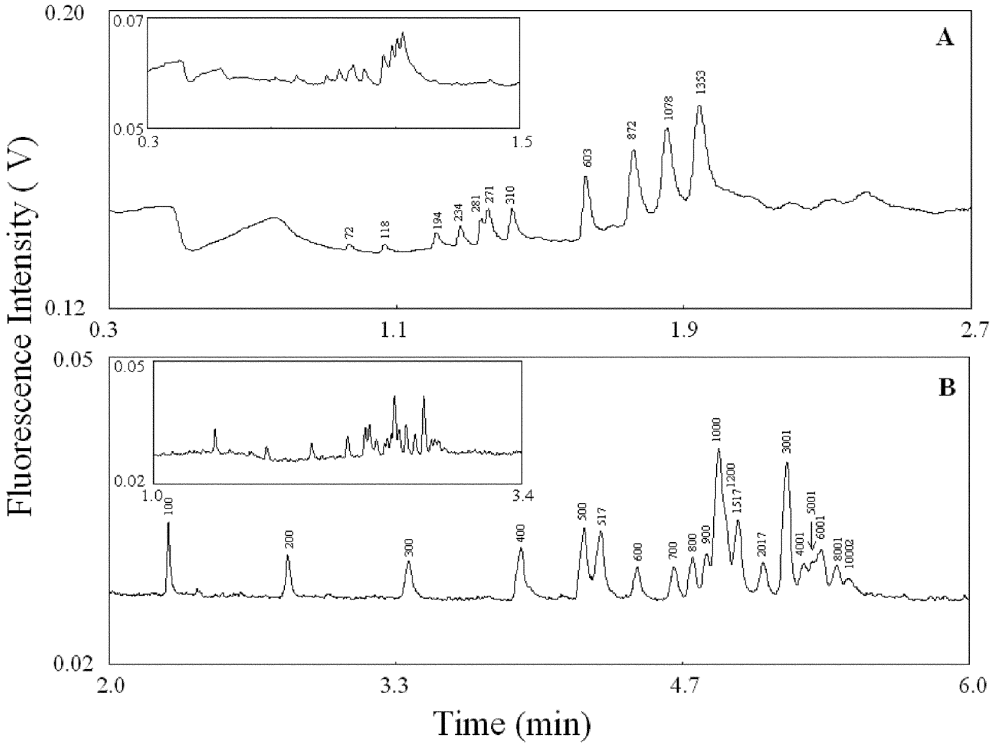

Fig. 3 Effects of bubble cell and electric field strength on separations of DNA in the absence of EOF using 2.0% PEO prepared in 200 mmol L^{-1} TB containing 5 μg mL^{-1} EtBr, pH 9.0. (**A**) Separations of 0.2 μg mL^{-1} φX 174 RF DNA-Hae III digest were conducted at 2 kV with and without (*inset*) a 300-μm bubble cell. (**B**) Separations of 0.2 μg mL^{-1} 2-Log DNA ladder were conducted at 100 V cm^{-1} and 200 V cm^{-1} (*inset*), without a bubble cell. Electrokinetic injections were conducted at 1 kV for 10 s. The capillary length was 5 cm (4 cm effective length). Other conditions were as in Fig. 2

secutive runs less than 3.9 and 3.3%, respectively. Table 1 also clearly shows that the sensitivity improvements for the small DNA fragments are less than that for DNA fragments around 300 bp, mainly due to greater peak broadening. Similar results were also found when using a 450-μm bubble cell. The peak width in baseline for the 51-bp fragment increased from 0.03 to 0.05 min when the bubble cells were varied from 75 to 450 μm diameter. On the other hand, the resolution values for large DNA fragments increase in the presence of bubble cells with i.d. less than 300 μm. The improvement in resolution is mainly because DNA migrated at low electric filed strengths when entering the bubble region, with a support of longer migration times. For example, the migration times for the 2176-bp fragments are 13.3 and 14.2 min, respectively, in the absence and presence of a 300-μm bubble cell.

Separation in the absence of EOF

To further show the impact of bubble cells on high-speed DNA separation we performed DNA separations in the absence of EOF using a short capillary (5.0 cm). Figure 3 A clearly shows better resolution for the large DNA in the presence of a 300-μm bubble cell, at the expense of a longer separation time (1.2 min vs. 2.0 min). Please note that the separation order reverses in the absence of EOF and improvements in the resolution for the large DNA fragments are apparent when compared to that shown in Fig. 2. It should be noted that Karger et al. have found the reversal of the separation order between 271- and 281-bp DNA fragments using CE and mass spectrometry [32]. It is also important to point out that the peaks corresponding to all the DNA fragments are broader in the presence of the bubble cell. Thus, we infer that improved resolution for the large DNA fragments is due to increases in peak spacing (changes in DNA conformation at low electric field strengths) and self-focusing when DNA entered the bubble cell. To support our reasoning, we conducted separations of 2-Log DNA ladder (0.1–10.0 kbp) without using a bubble cell at applied voltages of 0.5 and 1 kV, corresponding to field strengths of about 100 and 200 V cm^{-1}, respectively. The separation was more successful, especially for large DNA fragments (>2.0 kbp), at 100 V cm^{-1}, as shown in Fig. 3B, with a relatively longer separation time (5.5 vs. 2.8 min). Please note that it is essential to perform the separation of the DNA ladder at relatively lower electric field strengths according to the biased reptation model [28, 29, 30, 33].

To further show the features of a 300-μm bubble cell, we conducted the separation of DNA markers V and VI, with sizes ranging from 8 bp to 2176 bp, using a 6-cm capillary. Compared to the result shown in the inset in Fig. 4, the electropherogram shows improved resolution for the large DNA fragment in the presence of the bubble cell, at the expense of a slightly longer separation time. For example, the resolution values for 1033/1230 and 1776/2176 are 0.5 and 0.2, respectively, in the absence of a bubble cell. In the presence of the bubble cell, they are 1.2 and 0.9, respectively. It is also interesting to note that the loss in resolution increased with decreasing capillary length (8 to 5 cm) for most of the DNA fragments, besides the large DNA fragments (>1033 bp) (results not shown). On the other hand, the discrepancy for the large DNA supports our suggestion that the separation of large DNA fragments benefits from the existence of a bubble cell.

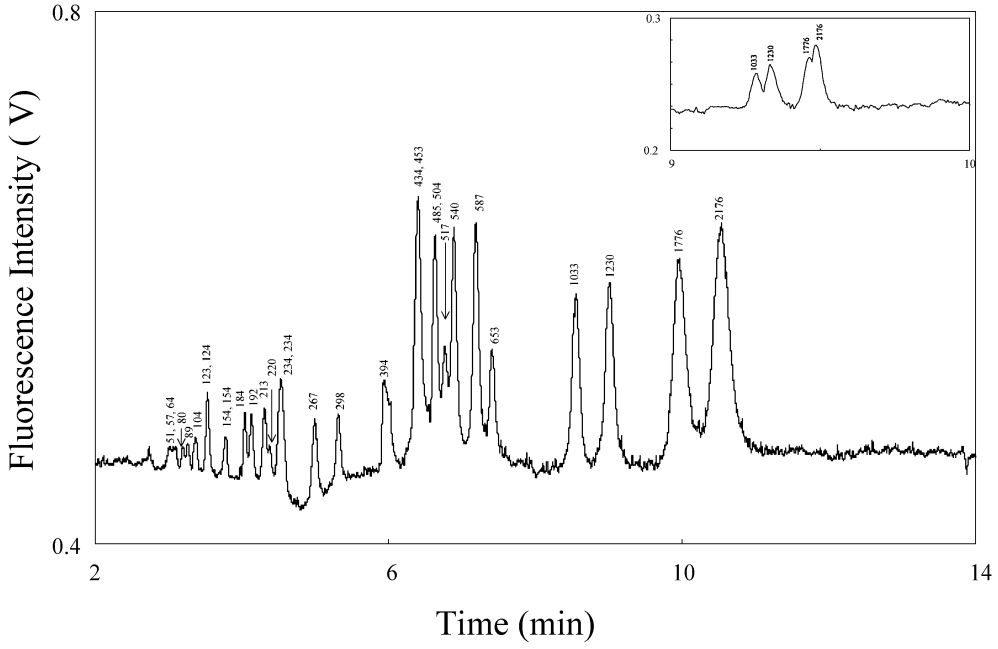

Fig. 4 Separation of a sample containing 0.2 μg mL^{-1} DNA markers V and VI in the absence of EOF using 2.0% PEO prepared in 200 mmol L^{-1} TB containing 5 μg mL^{-1} EtBr, pH 9.0. The separations were conducted at 0.5 kV. The capillary length was 6 cm (5 cm in effective length). The electropherogram shown in the *inset* was obtained in the absence of a bubble cell. Other conditions were as in Fig. 3

Conclusions

We have taken advantage of on-line concentration and bubble cells for sensitivity improvement in the analysis of DNA by CE. Using bubble cells with the diameter less than four times that of the separation channel, we have shown slight loss of resolution for small DNA fragments and improved resolution for large ones. Resolution improves mainly due to increases in peak spacing when DNA migrates at low electric fields in the bubble cell region. In conjunction with on-line concentration, sensitivity improvements up to 170-fold for the DNA fragments have been demonstrated using a capillary with a 300-μm bubble cell. Compared to our previously proposed methods, this new approach allows one to improve sensitivity and resolution for large DNA with a slight cost of analysis time. In this study, high-speed DNA separations using a short capillary with a bubble cell were also developed. With a bubble cell, its merit for the separation of large DNA is significant compared to that of using a longer capillary in the presence of EOF.

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