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Free-solution electrophoresis of DNA

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Abstract

We report the first experimental evidence that double-stranded DNA fragments can be electrophoretically separated in free-solution (i.e., in the absence of a sieving matrix) when either a single or two streptavidin molecules are attached to the end(s) using biotinylated nucleotides. As previously predicted, higher resolution is obtained at higher electric fields or when two streptavidin molecules are attached to each DNA fragment. The resolution is also affected by the diameter and coating of the capillaries. © 1998 Elsevier Science B.V.

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1. Introduction

DNA fragments are commonly separated using slab gel electrophoresis. In recent years, high electric-field capillary electrophoresis (CE), using cross-linked polyacrylamide gels [1,2] or entangled polymer solutions [3] as a sieving matrix, has led to 10–100 fold increases in the speed at which DNA fragments can be separated [4,5]. Unfortunately, high electric fields decrease resolution [6,7] and lead to gel degradation [8–11]. High-viscosity, entangled, polymer solutions have been used to resolve DNA sequencing ladders up to at least 600 bases when relatively low (~100 V/cm) electric fields are used [5,12]. Recently, entangled polymer solutions [2% (w/v) linear polyacrylamide with a molecular mass in excess of $5.5 \cdot 10^6$] have been used to sequence more than 1000 bases in 80 min [13]. Dilute, non-entangled, polymer solutions have also been used to separate restriction fragments, but they have yet to achieve the single base resolution necessary for DNA sequencing [12,14,15].

DNA fragments cannot normally be separated in free solution because each fragment has the same friction to charge ratio, hence the same electrophoretic velocity [16–18]. However, it has been proposed that adding an uncharged molecule (or label) at the end(s) of the DNA fragments could break this friction/charge symmetry and lead to efficient separations [19–21]. We called this new method ELFSE for end-labelled free-solution electro-

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phoresis [21]. Since the charge-to-mass ratio of labelled DNA increases with the size of the DNA fragments, larger DNA fragments are expected to migrate faster, and hence to elute earlier, than smaller DNA fragments in the absence of electroosmotic flow (EOF). Conversely, larger DNA fragments are expected to migrate effectively slower, and hence to elute later, than smaller DNA fragments in the presence of EOF. Higher resolution was predicted for systems using larger uncharged labels and higher electric fields [21]. The experimental results presented below, using one or two streptavidin label(s), confirm these predictions and demonstrate that ELFSE can indeed be used to separate relatively large DNA fragments in the 100–900 base pair (bp) range.

Although we present the first experimental evidence that ELFSE can be used to separate relatively large DNA fragments, other examples of ELFSE separations have already been reported. An ELFSE separation of short single-stranded oligonucleotides was recently reported by Völkel and Noolandi [22]. Oligonucleotides labelled with a biotin or trityl group at their 5′-end were observed to elute first in the presence of EOF because these neutral groups decreased the mobility relative to the corresponding unlabelled oligonucleotides. ELFSE separations of oligosaccharides labelled with fluorescent tags have also been reported by Sudor and Novotny [23].

2. Data analysis

Following Lerman and Sinha [24], we define the separation factor $S=1/2(w_1+w_2)/(\Delta t_m/\Delta M)$, where w_1 and w_2 are the widths (at half height) of the peaks corresponding to the two DNA fragments to be separated, Δt_m is the separation between the peaks, and $\Delta M=M_2-M_1$ is the difference in size (in base pairs, or bp). Note that S gives the smallest difference in size that can be resolved (in bp). DNA sequencing requires approximately $S\leq 1$. We also give the number of theoretical plates $N=8\ \ln\ 2(t_m/w)^2$, where t_m is the migration time, for a few cases as a measure of the separation efficiency, although S is a more relevant parameter for separation purposes.

3. Experimental

3.1. Capillary electrophoresis (CE)

The DNA separations shown in Figs. 1–3 were performed at 25°C using an ABI PRISM 310 Genetic Analyzer, using either 75 μm I.D. (inner diameter) DB-210, 50 μm I.D. DB-17, or 100 μm I.D. DB-17 capillaries (J and W Scientific, Folsom, CA, USA). The capillaries were 47 cm long and the distance to the detector was 36 cm. They were rinsed with 0.5× TAPS buffer [1× TAPS buffer is 100 m*M* N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid adjusted to pH 8.0 with NaOH] containing 0.25% hydroxyethyl-cellulose for 3 min, followed by a 2 min rinse with 0.5× TAPS buffer, and a 1 min rinse with 0.5× TAPS buffer containing 0.1 n*M* TOPRO-1 (Molecular Probes, Eugene, OR, USA). A 1 min rinse corresponds to approximately 5 μl of solution (i.e., 1.4 to 5 capillary volumes). Injection was performed at an electric field of 65.2 V/cm for 10–30 s and the running buffer was 0.5× TAPS containing 0.1 n*M* TOPRO-1 as the intercalating fluorescent dye. Data were collected using the ABI GeneScan Analysis software 2.0.2.

The DNA separations shown in Fig. 4 were performed in separate consecutive experiments in 1× TBE buffer (89 m*M* Tris, pH 8.0, 89 m*M* boric acid, 2 m*M* EDTA) at 400 V/cm using an in-laboratory designed CE system [11] which allowed to reverse the polarity of the electrophoresis. A 50 cm×50 μm I.D. capillary (Polymicro Technologies, Phoenix, AZ, USA) was coated with a thin layer of polyacrylamide which was sufficient to reduce the binding of the analytes to the capillary walls, but only reduced the EOF by 50% (results not shown). This coating was prepared using the method described by Hjertén [25] except that the activation of the inner wall with γ-methacryloxypropyltrimethoxysilane was performed for only 5 min instead of 1 h. Note that the higher sensitivity of this sheet-flow cuvette detection CE apparatus allowed us to detect DNA fragments labelled only with fluorescein (see Section 3.2), i.e., the addition of TOPRO-1 dye to the running buffer was not required.

Analyses of streptavidin were performed at 25°C using a Waters Quantus 4000 CE system. Electro-

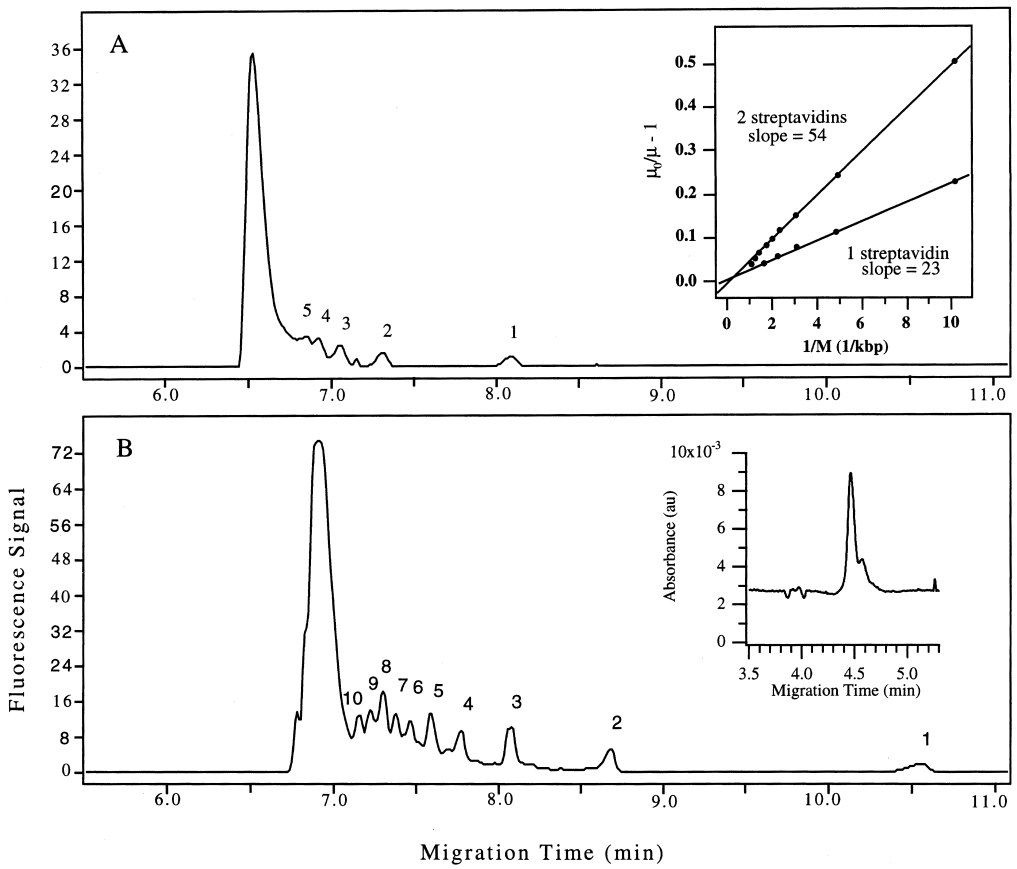

Fig. 1. Separation of a 100 bp ladder labelled with (A) one and (B) two streptavidin molecules. Electrophoresis was performed at 213 V/cm using a 75 μm I.D. DB-210 capillary. The peaks marked 1 to 10 represent the 100 to 1000 bp DNA fragments, respectively. Insert of A: plot of $\mu_0/\mu - 1$ vs. $1/M$. Insert of B: polydispersity of the recombinant streptavidin as measured by CE.

phoresis was performed in an uncoated 57 cm×50 μm I.D. capillary (Polymicro Technologies), with a 50 cm distance to the detector, at 400 V/cm in 1× TBE buffer at room temperature. 10^{-4} *M* solutions of recombinant streptavidin (Boehringer Mannheim, catalog No. 1 721 666) or streptavidin (Boehringer Mannheim, catalog No. 973 190) in 1× TBE were hydrodynamically injected for 20 s. Detection was performed by UV absorbance at 214 nm.

3.2. DNA samples

A 100 bp DNA ladder (Pharmacia) was used for the experiments shown in Fig. 1. This ladder contains a 2-fold greater concentration of the 800 bp fragment. It was labelled with biotin at one end by adding biotin-16-dUTP (Boehringer Mannheim) using Sequenase V2.0 (United States Biochemicals), or at both ends by adding biotin-16-dUTP, biotin-14-dATP (Life Technologies), dCTP and dGTP using Sequenase V2.0. The peaks were identified by the fact that larger DNA fragments, having a greater mass, show a higher relative intensity, and that the 800 bp fragment has a relative intensity twice its expected value.

In Figs. 2 and 3, a DNA sample with 98, 127, 140, 225, 237, 271, 356, 367, 878 bp fragments having biotin at both ends was prepared by digesting the pBlueScript KS+ plasmid vector (Stratagene, La Jolla, CA, USA) with the restriction enzyme MseI

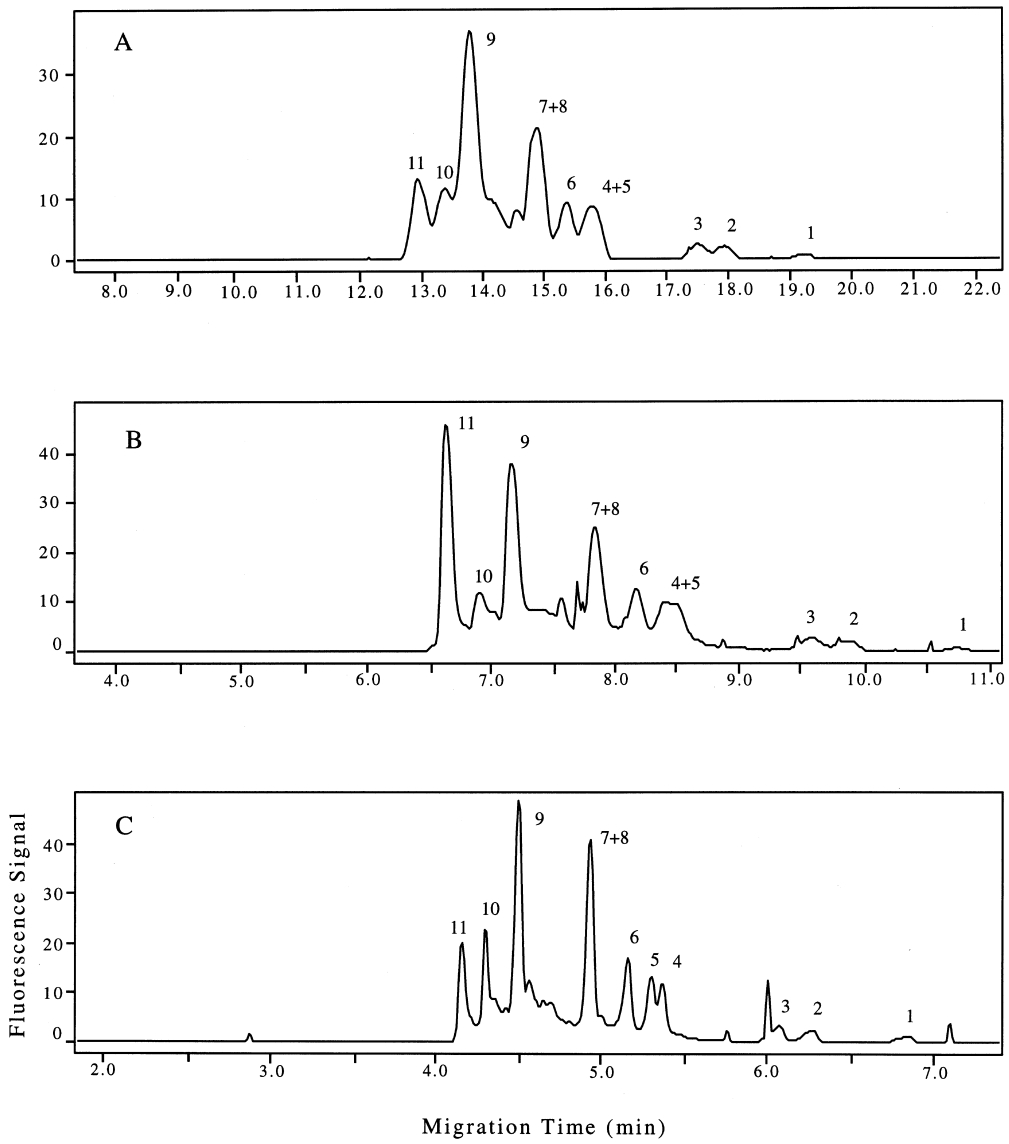

Fig. 2. Influence of the electric-field intensity on the resolution of DNA fragments. A recombinant streptavidin molecule was attached to both ends of these DNA fragments. Electrophoresis was performed at (A) 106 V/cm, (B) 213 V/cm and (C) 319 V/cm using a 75 μm I.D. DB-210 capillary. Peaks 1 to 9 represent DNA fragments of 98, 127, 140, 225, 237, 271, 356, 367, 878 bp, respectively.

(New England Biolabs) and filling the ends with biotin using biotin-16-dUTP, dATP and Sequenase V2.0. The same DNA ladder was used in the electropherograms shown in Fig. 4, except that these DNA fragments were labelled with fluorescein by replacing the dATP by fluorescein-15 dATP (Boehringer Mannheim). These DNA fragments were also isolated using a 3% NuSieve GTG agarose (FMC, Rockland, ME, USA) gel, purified using GeneClean (BIO 101, La Jolla, CA, USA), and used to identify

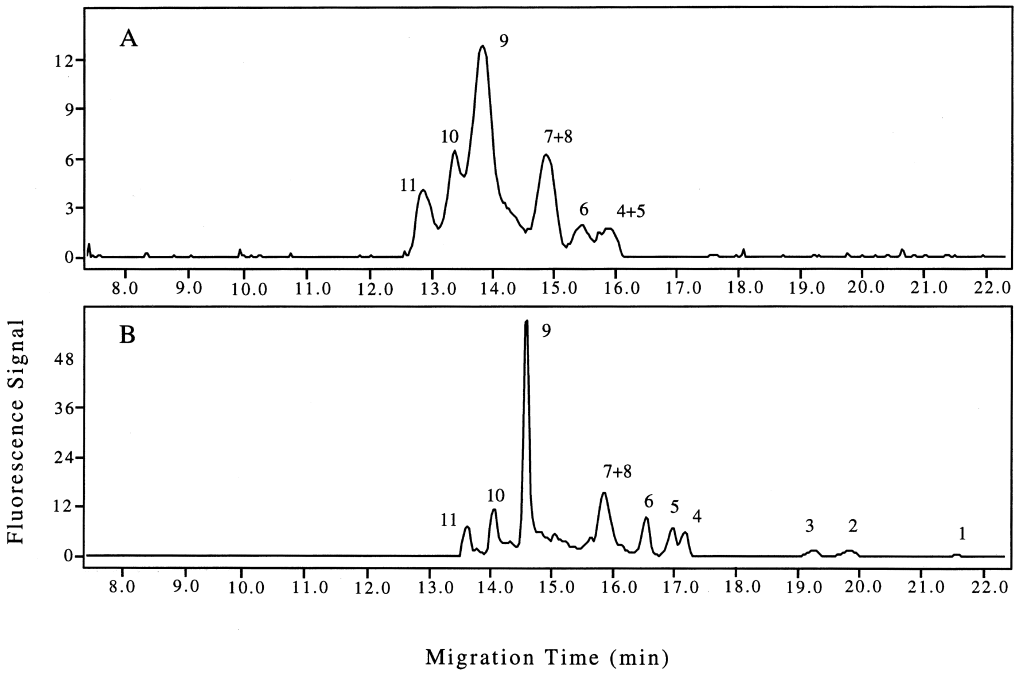

Fig. 3. Effect of the internal diameter of the capillaries on the resolution. Electrophoresis of DNA fragments performed at 106 V/cm in DB-17 capillaries having (A) an I.D. of 100 μm and (B) an I.D. of 50 μm. DNA fragments were as described in Fig. 2.

the peaks observed in the electropherograms shown in Figs. 2 and 3, and to perform the experiments shown in Fig. 4.

Streptavidin was attached to the biotinylated ends of DNA fragments by adding a 100 molar excess of recombinant streptavidin (Figs. 1–3) or streptavidin (Fig. 4) to the DNA samples before loading.

4. Results and discussion

4.1. ELFSE separations using one or two streptavidin molecules

Fig. 1 shows ELFSE of a 100 bp ladder labelled with either one (Fig. 1a) or two (Fig. 1b) streptavidin molecules. The peaks marked 1 to 10 correspond to the 100 to 1000 bp DNA fragments, respectively. The large peak at the left of the figure contains unresolved larger fragments of the ladder as well as the cloning vector. As predicted [21], larger DNA fragments elute first (in the absence of EOF). Furthermore, using two streptavidin molecules instead of one improves the separation factor of the 100 and 200 bp DNA fragments from $S=12$ to $S=8$. The separation factor of the 200 and 300 bp DNA fragments goes from $S=28$ to $S=12.5$, while the efficiency is $N \approx 9 \cdot 10^4$ for the 300 bp fragment.

Although the DB-17 and DB-210 capillaries are coated and show little EOF, the EOF still present was enough to lead to the loss of samples after electrokinetic injection. In fact, rinsing these capillaries with 0.5× TAPS buffer containing 0.25% hydroxyethyl-cellulose for 3 min, followed by a 1 min rinse with 0.5× TAPS buffer containing 0.1 n*M* TOPRO-1, proved to be necessary in order to successfully inject the DNA samples (results not shown). Under these conditions, the hydroxyethyl-cellulose molecules do not act as a sieving matrix, but as dynamic coating agents that suppress the EOF

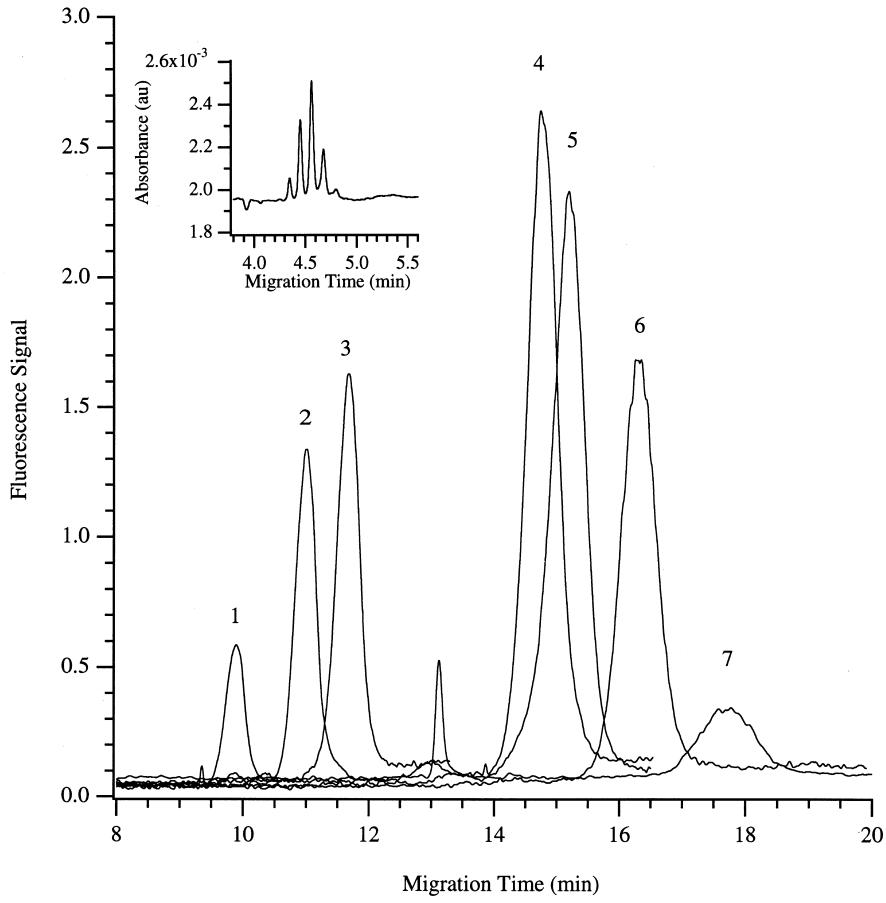

Fig. 4. Effect of the EOF on the order of migration of DNA molecules. A streptavidin molecule and a fluorescein-15-dATP molecule were attached to both ends of these DNA fragments. Electrophoresis was performed at room temperature using an electric field of 400 V/cm and a 50 μm I.D. capillary prepared as described in Section 3.1. Peaks 1 to 7 represent DNA fragments of 98, 127, 140, 225, 237, 271, 356 bp, respectively. Insert: polydispersity of the streptavidin as measured by CE.

still present in these capillaries. It should be noted that a dilute solution of free polymers would actually reduce the resolution of the ELFSE separations since it would compete with the ELFSE drag process.

4.2. ELFSE mobility of DNA fragments

According to Ref. [21], the ELFSE mobility μ of a DNA fragment should be given by

$$\mu(M) = \frac{\mu_0}{1 + \alpha/M} \quad (1)$$

where μ_0 is the free-solution mobility of a bare DNA fragment, M is the molecular size of the fragment (in bp), and α is the ratio of the friction coefficient of the label to that of one DNA bp. Therefore, the relative friction coefficient for streptavidin, α, can be estimated by the slope of the straight line obtained from plotting $\mu_0/\mu - 1$ vs. $1/M$. Using the data of Fig. 1, we estimate that $\alpha \approx 23$ bp for one streptavidin, and $\alpha \approx 54$ bp for two streptavidins (insert, Fig. 1a).

According to the recent theory of Long and coworkers [18,26], Eq. (1) is valid only if the DNA fragments are almost entirely stretched when pulling the friction-generating label(s). They point out that, at low velocities, the DNA may retain a random coil conformation and that one would then have to

replace M by $M^{3/5}$ in Eq. (1). The fact that Eq. (1) does provides an excellent fit of the data shown in Fig. 1 (insert of Fig. 1a) suggests that our DNA fragments were indeed almost entirely stretched during the electrophoresis.

4.3. Effect of the electric field intensity

Parts A–C of Fig. 2 demonstrate that increasing the voltage improves the resolution. For example, the 225 and 237 bp DNA fragments are not resolved at 106 V/cm, but show a $S=12$ separation in less than 5.5 min at 319 V/cm. The nature of peak 10 is unknown but could represent multimers (i.e., streptavidin molecules linked to more than one DNA fragment), and peak 11 corresponds to co-migrating DNA fragments not labelled with streptavidin (which is likely due to the absence of biotin). The peaks were identified by comparing our data to electrophoretic runs performed on single fragments (results not shown). At high electric field, Joule heating is expected to lead to a parabolic velocity profile inside the capillary, which increases the effective diffusion coefficient. This effect, however, should be partially compensated by the shorter separation times. Our results suggest that Joule heating is not yet a major limiting factor since higher voltages improve resolution, but we were unable to test higher electric fields due to the fact that 15 000 V is the maximal usable voltage of the ABI PRISM 310 Genetic Analyzer.

4.4. Effect of the internal diameter of the capillaries

Using a capillary with a smaller internal diameter increases the resolution (Fig. 3). For instance, the 225 and 237 bp fragments show a $S=18$ separation in a 100 μm I.D. capillary ($N\approx 3\cdot 10^4$), compared to $S=9$ for a 50 μm I.D. capillary ($N\approx 5\cdot 10^4$). This may indicate that Joule heating does have some effect on resolution because smaller I.D. capillaries are expected to provide a more uniform heat distribution and less pronounced parabolic velocity profiles than capillaries with a larger I.D.. Alternatively, the better separation observed with the smaller I.D. capillary might be due to the smaller transverse path that the DNA fragment migrate through when one uses curved capillaries. Testing this alternative explanation will require the use of a capillary apparatus which does not need bent capillaries.

4.5. ELFSE separations in the presence of EOF

Fig. 4 shows that EOF does not have to be suppressed for ELFSE to be useful. The thin polyacrylamide coating used in these experiments reduced the EOF by about 50%. The EOF in this system is typically in the order of $1\cdot 10^{-7}$ m^2/V s. Under these conditions, the labelled DNA fragments were pulled towards the detector by the EOF, whereas the (weaker) electrophoretic force acted in the opposite direction. Since small DNA fragments offer less electrophoretic retardation, separation is still achieved, with the small molecules now eluting first. However, the separations obtained in these experiments were not as good as those observed in the absence of EOF. For example, at 400 V/cm, we observe an $S=16.5$ separation for the 225 and 237 bp DNA fragments, whereas an $S=12$ separation was observed for the same two DNA fragments at 319 V/cm in the absence of EOF (Fig. 2c). Obviously, the fact that the experimental conditions of these two sets of experiments were widely different (i.e., they differed in electric fields, streptavidin, buffers and fluorescent molecules used for detection) make this conclusion only tentative. On the other hand, these results show that ELFSE separations can be achieved with or without EOF. This should afford some flexibility to future ELFSE-based technologies.

5. Conclusions

A number of factors may affect the resolution of the ELFSE separation of DNA molecules. Among these, the monodispersity of the analytes is critical. Although DNA fragments of a given size are monodisperse by definition, the streptavidin labels we used were not. The fact that recombinant streptavidin (insert, Fig. 1b) is less polydisperse than non-recombinant streptavidin (insert, Fig. 4) suggests that this polydispersity is mainly due to size variations, and not to molecules of the same size having different three dimensional conformations (and hence, friction

coefficients). In fact, matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) experiments have shown that the two peaks of recombinant streptavidin have molecular masses of 13 248 and 13 419, while the four peaks of non-recombinant streptavidin have molecular masses of 16 035, 16 186, 16 465 and 16 623 (results not shown). Therefore, size purification of this protein will be required in order to optimize ELFSE separations. This can be achieved by polyacrylamide gel electrophoresis [27], and could also possibly be achieved by isoelectric focusing and high-performance liquid chromatography, among other techniques.

Using streptavidin to implement ELFSE of DNA fragments is attractive because double-stranded DNA fragments can easily be labelled with this protein using biotinylated nucleotides and a DNA polymerase. It could also be easily implemented in the separation of products of PCR reactions or of DNA sequencing reactions using biotinylated primers. Furthermore, the fact that sharp peaks are observed in the inserts of Fig. 1b Fig. 4, and that the number of peaks observed in these experiments correspond to those observed in MALDI-TOF-MS experiments, indicate that there are minimal interactions of the streptavidin with the capillary walls. Streptavidin would therefore make for a useful label to implement ELFSE separations of DNA molecules. On the other hand, alternative friction generating labels, such as uncharged oligonucleotide primers or other synthetic molecules, could prove to be even more practical.

Our results demonstrate that DNA ELFSE is possible when streptavidin is used as a label. The factors affecting the resolution include the size of the label, the ionic strength of the buffer (results not shown), the intensity of the electric field, as well as the I.D. and coating of the capillaries. Other factors, such as the pH of the buffer, the injection volume and the length of the capillary are likely to affect the resolution as well. It should be possible to achieve single base resolution with ELFSE by using large monodisperse labels, optimized buffer composition and capillaries, stacking to minimize the initial loading width of the sample and higher electric fields.

Contrary to current electrophoretic methods, ELFSE requires no sieving matrix, and provides better results at higher voltages. ELFSE thus leads to less preparation time and faster separations. Therefore, ELFSE could become the separation method of choice for fast sizing of DNA fragments in mapping and diagnosis applications, and for massively parallel capillary (or microchannel) apparatuses [28,29] where the loading and stability of the sieving matrix could be a problem.

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