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Electrophoretic separation of DNA sequencing extension products using low-viscosity entangled polymer networks

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Abstract

A low-viscosity (*ca.* 150 cP) polymer solution is used to resolve DNA sequencing fragments up to 580 bases long using the capillary electrophoresis format. Separation performance is compared qualitatively and quantitatively to that of a cross-linked polyacrylamide sequencing slab gel. Both the resolution and length-of-read are essentially equivalent, while the capillary separation is approximately 10 times faster than the slab gel analysis. In addition, separation selectivity and efficiency are examined individually.

1. Introduction

Recently much effort has been directed at the separation of DNA sequencing reaction products by capillary gel electrophoresis [1–6]. The perceived advantages of the capillary format include speed of analysis, increased separation efficiency, on-line real-time detection and automation of sample loading. However, important obstacles to the widespread practical application of this technology still remain: difficulty in capillary gel preparation and instability of the gel matrix during prolonged runs at high voltages [1,2,4,7–9]. The currently reported lifetime of gel-filled capillaries used for DNA sequencing analysis is approximately four injections [10]. Because of the difficulty of gel preparation, this is an unacceptably short lifetime. These problems become particularly serious when one imagines the use of multiple capillary arrays [10] and would almost certainly preclude automation of such systems.

Capillary gel lifetime is limited by three major effects: electroosmosis, bubble formation and capillary inlet fouling with high-molecular-mass sequencing template. Because of the large electroosmotic forces present in microcapillaries [11], the gel can extrude out of the capillary upon application of the electric field, resulting in the formation of a void at the anodic (positive) end of the tube [7]. To counter this effect, gels have been covalently attached to the inside wall of the capillary [1,3,7,12–14]. However, covalent attachment can lead to severe stresses within the gel due to the volume change of the monomer upon polymerization [7,15], leading to the formation of periodic "bubbles", or voids, in the network, causing a devastating loss of separation performance and even loss of electrical continuity along the capillary [4,7,16,17]. This problem has been greatly reduced by using non-cross-linked networks [10,18]. By removing the cross-links, internal stresses within the network can more easily relax, resulting in a "stress-free" network. The use of DNA sequencing mixtures causes additional problems [1–5], further reduc-

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ing gel stability. This effect presumably arises from the large amount of high-molecular-mass sequencing template present in the reaction mixture. In fact, to solve this problem, some workers have had to resort to trimming off a portion of the injection end of the capillary subsequent to sample injection [2]. Thus, capillary fouling after repeated injections of DNA sequencing product remains a key obstacle to the extended lifetime, and thus the practical utility, of capillary gels.

Low-viscosity polymer solutions are a recently developed alternative to rigid gels as electrophoretic sieving networks. Because of the flowability of these systems, the separation network can be easily replaced after each run simply by pumping fresh medium into the capillary, eliminating the need for reusability. To date, polymer solutions have been used as sieving networks for the electrophoretic separation of native double-stranded DNA [19–24], chromosomes [25] and micro particles [26]. Furthermore, the theoretical foundation for these systems has been reported [27].

This report describes the application of low-viscosity polymer solutions to the separation of single-stranded DNA sequencing reaction products, and compares the performance of this system to traditional sequencing slab-gel electrophoresis.

2. Experimental

2.1. Capillary electrophoresis apparatus

A length of polyimide-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 55 cm long (40 cm to the detector) with a 50 μm internal diameter connects the anodic reservoir to the electrically grounded cathodic reservoir. A high-voltage power supply capable of producing up to 30 000 V (Gamma High Voltage Research, Ormand Beach, FL, USA) was used to drive the electrophoretic process. On-column fluorescence detection was performed using the optical system described below. Data were collected using a commercial chromatography data system (Model 600 Data Analysis System; Applied Biosystems, Foster City, CA, USA). Samples were introduced into the capillary using electrokinetic injection by applying a voltage of 6 kV for a period of 5 s while the cathodic end of the capillary and the cathodic electrode were immersed in the sample solution. After the sample was introduced into the capillary, the cathodic end of the capillary and the cathodic electrode were placed back into the electrophoresis buffer, and the electrophoresis voltage was applied.

The inside wall of the capillary was coated with a covalently attached layer of non-cross-linked polyacrylamide according to Cobb *et al.* [28]. A 2-mm wide detection window was formed in the capillary prior to coating by burning off the polyimide coating with a hot wire.

2.2. Capillary electrophoresis fluorescence detector

Excitation light from an argon ion laser (488 nm) (Model 221-40MLA; Cyonics, San Jose, CA, USA) was passed through a 0.5 O.D. neutral density filter (FNG 085; Melles Griot, Irvine, CA, USA) and into a set of focusing optics composed of a 64 mm focal length (F.L.), 7 mm diameter positive lens and a 85 mm F.L., 5 mm diameter negative lens, resulting in a 100 μm laser spot size incident onto the capillary. The fluorescence was collected at right angles by a 12 mm F.L. 14 mm diameter aspheric collector lens and passed through a 530 nm RDF bandpass filter (Omega Optical, Brattleboro, VT, USA). Next, the emitted light was passed through a 48 mm L.L. 19 mm diameter aspheric Fabry lens and a 17 mm F.L. 10 mm diameter spherical Fabry lens before imaging on a photomultiplier tube (R928-21; Hamamatsu, San Jose, CA, USA).

2.3. Preparation of linear polyacrylamide

Linear polyacrylamide was synthesized by standard procedures using isopropanol as a chain transfer agent to control the molecular weight of the product. 222 ml of water and 6.55 ml of

isopropanol were added to the reaction vessel, were degassed with helium, and heated to 35°C. A 25-g amount of acrylamide was dissolved in the solution. Next, 1.25 ml of 10% (v/v) N, N,N′,N′-tetramethylethylenediamine (TEMED) in water solution and 1.25 ml of a 10% (w/v) ammonium persulfate in water solution were added. The mixture was allowed to react for 1.5 h.

To remove any unreacted monomer and other small-molecule contaminants, the reaction mixture was dialysed extensively (four changes over two days) against water using a 12 000 to 14 000 molecular mass cut-off dialysis membrane (Spectra/por 2; Spectrum Medical Industries, Los Angeles, CA, USA). This product was then freeze dried to give 18 g of a white solid.

The molecular mass of the polymer was determined by gel permeation chromatography (GPC). Samples and standards were run on a Waters 590 solvent-delivery system (Milford, MA, USA) equipped with two Waters Ultrahydrogel Linear columns connected in series, and a Waters R401 differential refractometer for peak detection. Other conditions: injected sample, 80 μl of a 4 mg/ml stock; flow-rate, 0.6 ml/min; mobile phase, 100 m*M* potassium phosphate, pH 7.0. The weight-average molecular mass, M_w, and the number-average molecular mass, M_n, of the final product were determined to be 339 000 and *ca.* 100 000, respectively. Four standard polyacrylamide standards were used to calibrate the GPC (Polysciences, Warrington, PA, USA). The M_n and the M_w/M_n values of each of the standards were 465 300 and 2.45, 141 000 and 2.6, 44 400 and 1.8, 13 700 and 1.6.

The polymer solution used as the sieving network was prepared by mixing 3.5 ml buffer [220 ml water, 40 ml methanol, 5.6 g Tris titrated to pH 8.00 with 85% (w/w) phosphoric acid in water], and 2.4 g urea, giving a total volume of 5 ml. To this solution was added 0.310 g linear polyacrylamide resulting in a 6.2% (w/v) polymer solution (6.2% T)[a]. The solution was mixed for approximately 3 h and filtered through a 0.45-μm pore size syringe filter (Acrodisc; Gelman Sciences, Ann Arbor, MI, USA). Both electrode reservoirs and the capillary were then filled with the polymer solution. The viscosity of the solution used in these studies was approximately 150 cP at 25°C.

[a] T = (g acrylamide + g N,N′-methylenebisacrylamide)/100 ml solution.

2.4. Preparation of single-color DNA sequencing ladder

A single-color sequencing ladder of fragments terminating at C was prepared by the dideoxy sequencing method using a sequencing kit and accompanying protocols from Applied Biosystems (part No. 401119). An M13mp18 DNA template (m13mp18 (+) strand, 0.1 pmol) was annealed to a fluorescent dye primer [FAM M13 (−21) primer], and primer extension was carried out using *Taq* polymerase, with dideoxycytidine provided as the 3′-terminating base. The resultant mixture was stored as a dried ethanol precipitate in the dark at −20°C.

Shortly before sample was to be loaded into the filled capillary tube, the dried sequencing reaction mixture from above was resuspended in a mixture of 5 m*M* aqueous EDTA (disodium salt) (0.5 μl) and formamide (6 μl). The solution was heated at 95°C for 2 min, and then transferred to an ice bath. The cooled solution was then directly injected onto the capillary.

2.5. Slab-gel system

A fluorescence-based gel scanner was assembled on an optical bench by using components from an Applied Biosystems Model 373 DNA sequencer. The separation distance was 33 cm (40 cm between the electrode reservoirs), the gel thickness was 250 μm and the laser spot size was 73 μm. The gels were run at a constant voltage of 1000 V (25 V/cm). 1 × TBE (see below) buffer was used in both electrode chambers.

The polyacrylamide gels contained 4% total polyacrylamide (4% T) and 5% bisacrylamide (5% T). The gels were prepared by mixing 25 g urea, 1.9 g acrylamide, and 0.1 g bisacrylamide with 5.38 g 10 × TBE (900 m*M* Tris, 900 m*M*

boric acid, 10 m*M* ethylenediaminetetraacetic acid) and water. This solution was then vacuum filtered through a 0.45-μm pore size sterile filter. To the filtered solution was added 35 μl TEMED and 250 μl of a 10% (w/v) solution of ammonium persulfate. The gel was used immediately.

3. Results and discussion

Fig. 1 compares electropherograms obtained using the 6.2% T low-viscosity entangled polymer media in the capillary format to those obtained using a conventional 4% T, 5% C[a] cross-linked polyacrylamide slab gel. Each peak in Fig. 1 corresponds to a C residue in the known M13mp18 sequence. Resolution of fragments differing by a single base is evident out to 585 bases and is comparable between the two systems. However, it should be noted that there are several regions in the electropherogram where single-base resolution is lost (65, 66; 194, 196, 198; 354–356; 397, 399; 541, 543, 544). In the case of the 65, 66 pair, this loss of resolution is seen in both the CE and the slab gel data. These "compression" regions are most likely a result of secondary structure of the DNA and the lower operating temperature (25 *vs.* 30°C) used in the CE analysis, resulting in reduced denaturant efficiency leading to reduced resolving power.

Fig. 2 graphs the reduced mobility against fragment size for the entangled polymer data and for a 3% and a 4% cross-linked polyacrylamide slab gel. (The reduced mobility is defined as the ratio μ/μ_0 where μ is the mobility of the fragment in the sieving network and μ_0 is the free solution mobility derived from a plot of log μ *vs.* fragment size [29]. The reduced mobility is a convenient parameter to use to correlate electrophoretic migration data from different runs because it eliminates the influence on mobility of solvent properties such as viscosity, ionic strength, and denaturants and reflects only differences resulting from the sieving network.) The mobility *vs.* size behavior of the 6.2% polyacrylamide polymer solution system follows that of the 3% gel almost exactly up to about 200 bases, at which point the CE curve flattens out more quickly. This difference between curves probably arises from the influence of biased reptation on the CE data due to the much higher electrical fields used in CE (218 *vs.* 25 V/cm). This deviation becomes apparent at a μ/μ_0 of approximately 1/2, the point at which the migration mechanism transitions from the Ogston regime to the reptation regime [30]. Thus the sieving properties of the entangled polymer solution resemble those of a 3% cross-linked polyacrylamide gel. This is not an unexpected result. Because of the relatively broad molecular mass distribution of the linear polyacrylamide, some fraction of the polymer will be too small to participate in the entangled network [27]. Thus, the effective concentration of the network-forming polyacrylamide will be less than the bulk concentration.

A quantitative measure of separation performance is the resolution. For electrophoretic separations, the resolution, R, can be expressed as [11]

$$R = \frac{1}{4} \cdot \frac{\Delta\mu_{1,2}}{\bar{\mu}_{1,2}} \bar{N}_{1,2}^{1/2} \quad (1)$$

where $\Delta\mu_{1,2}$ is the difference in mobility between species 1 and 2, $\bar{\mu}_{1,2}$ is the average value of the mobility of species 1 and 2, and $\bar{N}_{1,2}$ is the average value of the number of theoretical plates. (The number of theoretical plates, N, is defined as $t^2/\sigma(t)^2$ where t is the migration time and $\sigma^2(t)$ is the variance of the peak in time units. The variance is related to the peak width at 1/2 the maximum peak height by the relation $2.35\sigma = w_{1/2}$.) Fig. 3 shows the resolution per base for a number of nearest-neighbor fragments in each system, where the resolution per base is calculated by dividing Eq. 1 by the difference in fragment size between the two fragments. It is clear from Fig. 3 that by this measure, the separation performance of the 6.2% entangled polymer system is essentially equivalent to that of the 4% cross-linked gel.

[a] C = g N,N'-methylenebisacrylamide/% T.

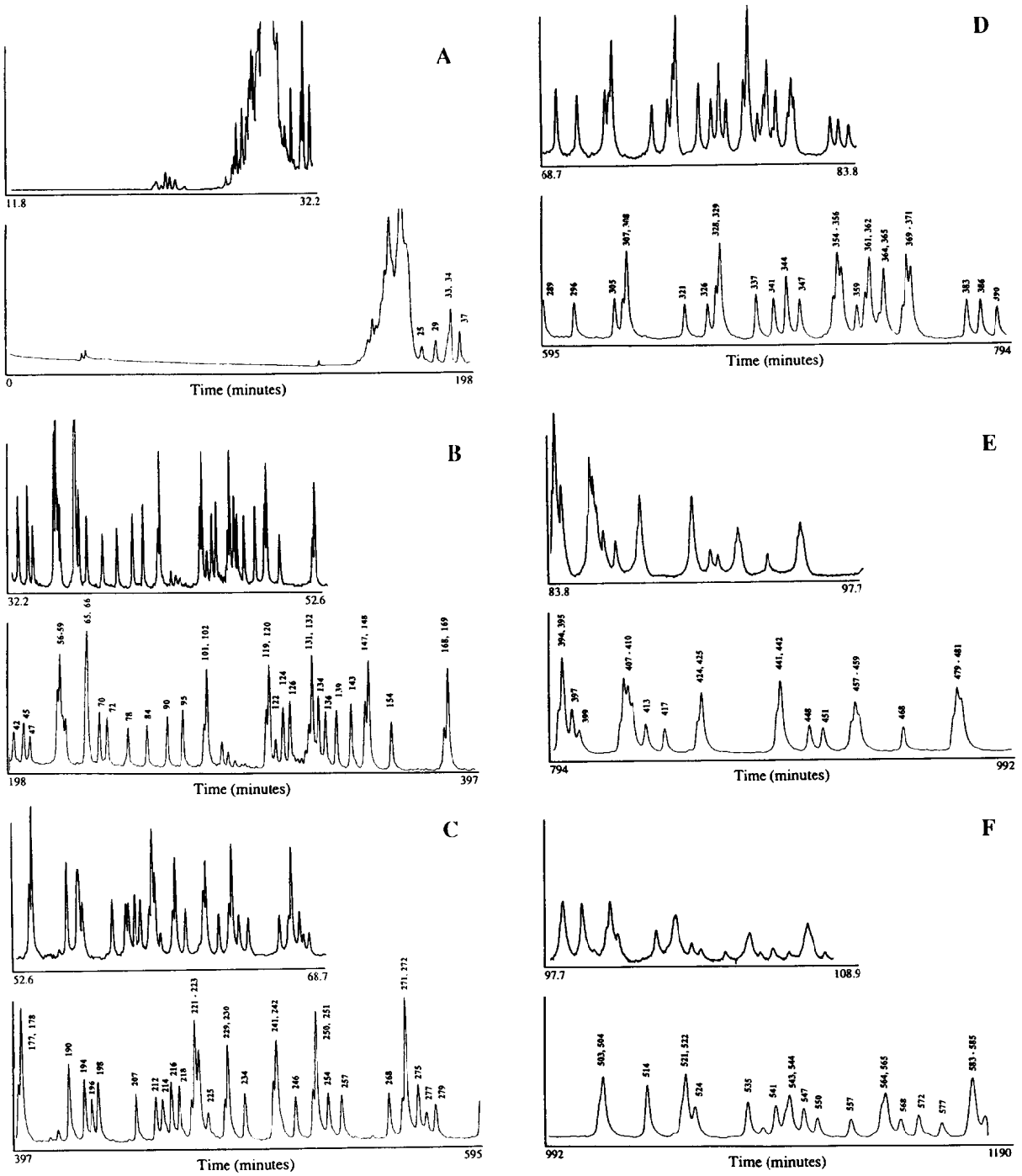

Fig. 1. Comparison of electropherograms showing the separation of C-terminated, fluorescently labeled DNA sequencing reaction products. (Top) Entangled-polymer CE. The polymer concentration was 6.2% T, 0% C linear polyacrylamide, the field strength was 218 V/cm, the current was 7.18 μA, the capillary length from the inlet to the detector window was 40 cm, and the total capillary length was 55 cm. Electrokinetic sample injection was performed at 6 kV for 5 s. (Bottom) 4% T, 5% C cross-linked polyacrylamide slab gel. The field strength was 25 V/cm, the gel length from the top of the gel to the detector window was 33 cm, and the total gel length was 40 cm. The size of each fragment in bases is indicated in the bottom panels.

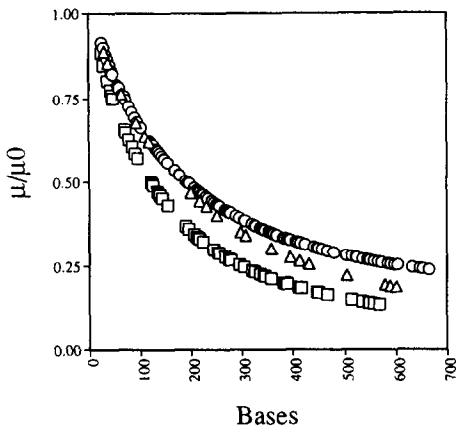

Fig. 2. Reduced electrophoretic mobility, μ/μ_0, as a function of fragment length for the entangled polymer CE system (○), a 3% cross-linked polyacrylamide slab gel (△) and a 4% cross-linked polyacrylamide slab gel (□). (μ_0 = size-independent free solution electrophoretic mobility of the fragments in each system. μ_0 is evaluated by determining the y-intercept of the linear portion of a plot of log μ *vs.* fragment length [29]. The mobilities in the entangled polymer and the 4% polyacrylamide gel system are taken from the electropherograms shown in Fig. 1. The raw data for the 3% polyacrylamide gel are not shown.

An alternative quantitative measure of the performance of a sequencing separation is the length-of-read, LOR. The LOR is defined as the point at which the peak spacing per base equals the half-height peak widths, or

$$\frac{(t_2 - t_1)}{n_2 - n_1} = \overline{w_{1,2}(t)}_{1/2} \qquad (2)$$

where t_2 and t_1 are the migration times of fragments of size n_1 and n_2, respectively, and $w_{1,2}(t)$ is the average value of the peak width at 1/2 the maximum peak height in time units. This is a useful parameter because it allows one to compare quantitatively different electrophoresis experiments performed under greatly different conditions using a single parameter. Comparing the value of the LOR for the data in Fig. 1, LOR $\approx$ 450 bases for the 6.2% polyacrylamide polymer solution CE system, while LOR $\approx$ 450 bases for the 4% cross-linked polyacrylamide slab-gel system. Thus, it again appears that the overall performance of the 6.2% polyacrylamide polymer solution is quantitatively comparable to that of a traditional 4% cross-linked polyacrylamide slab system.

A more detailed description of the factors controlling the separation performance requires that the influence of peak width and peak spacing on resolution be independently quantified. The term $\Delta\mu_{1,2}/\bar{\mu}_{1,2}$ in Eq. 1 is a measure of the selectivity or peak spacing in a separation and is a measure of the sieving power of the network, while the term $\bar{N}_{1,2}$ in Eq. 1 is a measure of the separation efficiency or peak width and is a measure of the dispersion characteristics of the electrophoresis apparatus.

Fig. 4 shows the selectivity per base as a function of fragment size for the 6.2% polyacrylamide polymer solution CE system and the 4% cross-linked polyacrylamide slab gel. The selectivity per base is calculated by dividing the selectivity term in Eq. 1, $\Delta\mu_{1,2}/\bar{\mu}_{1,2}$, by the difference in size of the two fragments. According to Fig. 4, the selectivity per base for the 6.2% entangled polymer solution system is significantly smaller than that of the 4% polyacrylamide gel over the full spectrum of fragment sizes. The reduced selectivity is probably due to the lower effective polymer concentration in the entangled-polymer solution system re-

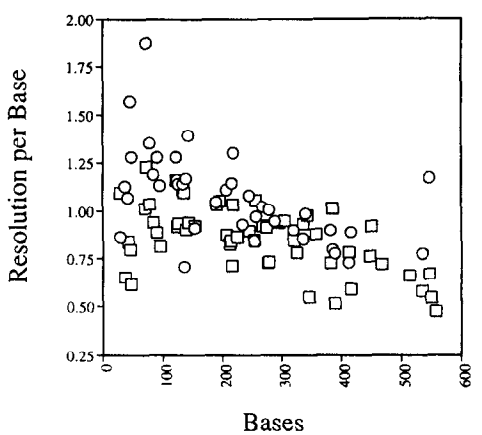

Fig. 3. Plot of resolution per base *vs.* fragment length for the entangled polymer CE system (○) and the cross-linked polyacrylamide slab gel system (□).

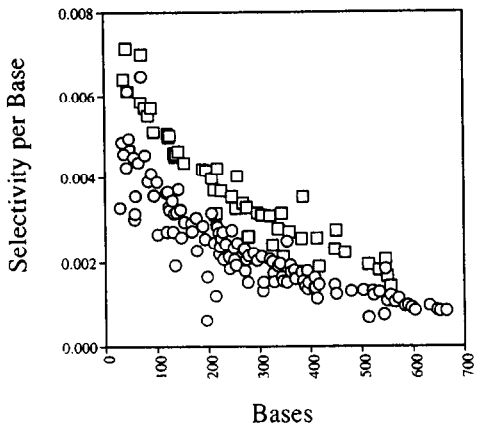

Fig. 4. Selectivity per base as a function of fragment length for the entangled polymer CE system (○) and the 4% cross-linked polyacrylamide slab gel system (□). Mobility data were taken from electropherograms in Fig. 1.

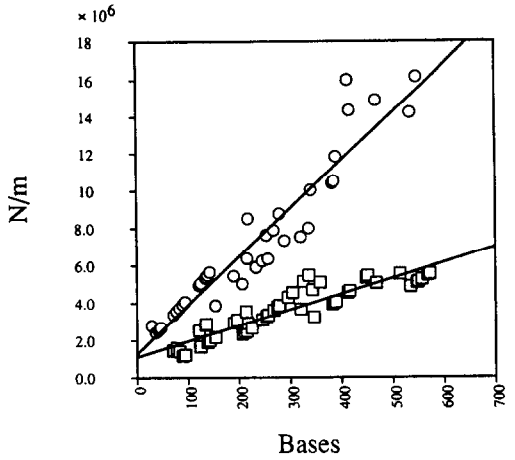

Fig. 5. Number of theoretical plates per meter as a function of fragment length for the entangled polymer CE system (○) and the crosslinked polyacrylamide slab system (□). The lines through each data set results from a linear least squares best fit of the data. Entangled polymer: slope = 25 900, y-intercept = 1 270 000, $r^2 = 0.91$; polyacrylamide gel data, slope = 8370, y-intercept = 1 120 000, $r^2 = 0.84$.

sulting from the molecular weight distribution of the polyacrylamide. Thus, the resolution achieved in Fig. 1 must be due to very sharp peaks, or high efficiency, relative to those obtained in traditional cross-linked polyacrylamide slab-gel electrophoresis.

The next aspect of the resolution to consider is the peak width, or peak efficiency. By dividing N by the separation distance, L, one gets the number of plates per unit separation distance, N/m, a parameter independent of migration distance. Fig. 5 shows a plot of the peak efficiency as a function of fragment length for the 6.2% polyacrylamide polymer solution system and the cross-linked polyacrylamide slab gel system. First, in both the CE and the slab data, it is important to note the linear relationship between N/m and the fragment size. This relationship implies that the time-dependent component of band broadening is due primarily to longitudinal diffusion [29]. This assumption will be exploited in the following analysis.

The next aspect of Fig. 5 to consider is the superior separation efficiency of the CE system. From the ratio of the slopes of the curves in Fig. 5, the efficiency of the CE separation is a factor of 3.1 times higher than the slab system. The superior efficiency of the CE system can be described quantitatively. The ratio of the efficiencies of the two systems is given by the expression

$$\frac{N/\mathrm{m}_{\mathrm{CE}}}{N/\mathrm{m}_{\mathrm{slab}}} = \left(\frac{L_{\mathrm{CE}}}{L_{\mathrm{slab}}}\right) \cdot \left(\frac{\sigma^2_{\mathrm{slab}}}{\sigma^2_{\mathrm{CE}}}\right) \tag{3}$$

Assuming diffusional band broadening,

$$\sigma^2 = 2Dt = \frac{2DL}{v} = \frac{2DL}{\mu E} \tag{4}$$

where D is the longitudinal diffusion coefficient, v is the electrophoretic velocity and E is the electrical field strength. Furthermore, from the Stokes–Einstein relationship and the definition of the electrophoretic mobility,

$$D = \frac{kT}{f} \tag{5}$$

and

$$\mu = \frac{q}{f} \tag{6}$$

where k is Boltzmann's constant, f is the translational friction coefficient, and q is the net charge

of the analyte. Combining Eqs. 4, 5 and 6 results in the expression

$$\sigma^2 = \frac{2LkT}{qE} \tag{7}$$

Therefore, the expected ratio of separation efficiencies of the capillary and the slab systems is

$$\frac{N/\mathrm{m}_{\mathrm{CE}}}{N/\mathrm{m}_{\mathrm{slab}}} = \left(\frac{T_{\mathrm{slab}}}{T_{\mathrm{CE}}}\right) \cdot \left(\frac{q_{\mathrm{CE}}}{q_{\mathrm{slab}}}\right) \cdot \left(\frac{E_{\mathrm{CE}}}{E_{\mathrm{slab}}}\right) \tag{8}$$

Each of the parameters in Eq. 8 can be easily determined except the value of q. The evaluation of q is complicated by the fact that the buffer ions used in the two systems are different, resulting in different degrees of charge shielding. However, the value of q for each fragment can be determined from the measured values of σ and μ. Combining Eqs. 5 and 6 results in the expression

$$q = \frac{\mu kT}{D} \tag{9}$$

Furthermore, substituting Eq. 4 into Eq. 9 to eliminate D gives

$$q = \frac{2\mu kTt}{\sigma^2} \tag{10}$$

Therefore, by measuring μ, t and σ for each fragment, a value of q can be determined. Based on measurements of over 30 fragments ranging in size from 100 to 500 bases, the average values for the net electrical charge per base is 0.177 e^- (R.S.D. = 19%) for the slab system and 0.069 e^- (R.S.D = 5.2%) for the CE system. The larger scatter in the slab data is probably due to the changing ionic composition and temperature of a slab gel during a run.

Insertion of the above values for q, and measured values for T_{slab} (30°C) and T_{CE} (25°C) into Eq. 8 results in an expected value of 3.4 for the ratio of efficiencies. Given the uncertainties of the measured parameters, this value agrees well with the measured value of 3.1 determined from Fig. 5.

4. Summary and conclusion

In summary, we have demonstrated that low viscosity entangled polymer solutions used in the capillary electrophoresis format can serve as an alternative sieving matrix for the electrophoretic separation of DNA sequencing fragments. Overall performance is comparable to that of a traditional cross-linked gel in a slab format in terms of both resolution and length of read. Although the separation selectivity of the entangled polymer preparation used here is inferior to that of the cross-linked gel, it is made up for by superior separation efficiency compared to a 0.4 mm thick slab. The enhanced separation efficiency achieved in the CE system agrees with theoretical predictions.

Low-viscosity, flowable media coupled with the capillary electrophoresis format make possible the opportunity for truly continuous and automated electrophoretic analysis of DNA sequencing extension products. By eliminating the time involved in the pouring of gels and automating the sample loading step, this approach will have a substantial impact on the overall throughput of DNA sequencing operations. Coupled with multi-capillary detection schemes [10], this type of medium should provide the basis for very-high-throughput sequencing technologies. In addition, the reproducibility of these systems should be superior to individually prepared gels. Further increases in the speed and resolution of these systems should be possible through optimization of the polymer solutions in terms of reduced solution viscosity, increased separation selectivity and reduced ionic strength.

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