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Mobility of single-stranded DNA as a function of cross-linker concentration in polyacrylamide capillary gel electrophoresis

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ABSTRACT

Mobility, theoretical plate count, and resolution are reported for single-stranded DNA in polyacrylamide containing 0, 0.5, 2.5, and 5% cross-linker and 6% total acrylamide. An electric field of 300 V/cm was used with 50 μm I.D. capillaries. Mobility decreases with increasing cross-linker concentration. The transition from normal mobility to the limiting mobility of biased reptation with stretching occurs for longer fragments as the %C is decreased. Theoretical plate counts do not vary significantly between the different polyacrylamide compositions. Resolution is higher for 0%C polyacrylamide, due to the latter onset of biased reptation with stretching.

INTRODUCTION

Since 1989, there have been a number of reports of DNA sequencing by capillary gel electrophoresis [1–9]. The high surface-to-volume ratio of the capillary facilitates the use of high electric fields for fast separations. However, there have been few systematic studies of the effect of gel composition on separation parameters [10]. In this paper, we consider the effect of cross-linker concentration on mobility, plate count, and resolution in the separation of DNA sequencing fragments by polyacrylamide gel electrophoresis.

Bisacrylamide is the most common cross-linking agent used in polyacrylamide gels. The cross-linker concentration (%C) is usually expressed as a mass percentage of the total monomer concentration in the gel. The mass percent of monomer plus cross-linker is denoted as %T, the total acrylamide concentration. Most workers employ 5%C polyacrylamide gels, although there have been reports on the use of much lower %C gels.

Recently, attention has focused on the use of 0%C polyacrylamide in electrophoresis. These entangled polymer solutions have relatively low viscosity and they may be pumped from the capillary after use. There is hope that by refilling the capillary after every separation, it will not be necessary to realign optical systems in automated DNA sequencers. Bode [11,12] apparently performed the first work with non-cross-linked polyacrylamide in the mid-1970s; he combined 0%C polyacrylamide with agarose for separation of proteins and nucleic acids. Mobility was a function of polymer length. Crambach and co-workers [13–15] compared the performance of 0%C and 5%C gels at various total acrylamide concentrations. More recently, Heiger *et al.* [16] compared 0, 0.5 and 5%C gels for separation of

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double-strand DNA; they also studied the effect of electric field on the resolution of fragments 4363 and 7253 bases in length. Sudor et al. [17] reported that 0%C polyacrylamide could be pumped from a capillary through use of a special high pressure syringe; the effect of the total acrylamide concentration was considered. Pentoney *et al.* [18] reported DNA sequencing in 10%T, 0%C polyacrylamide at an electric field of 300 V/cm; sequence could not be determined for fragments longer than 300 bases. Similar results have been reported by Huang *et al.* [19] with 9%T 0%C gels. Chiari *et al.* [20] argued that the high viscosity of 10%T, 0%C polyacrylamide eliminates any hope of refilling capillaries with that material; only low %T polyacrylamide has sufficiently low viscosity for replacement. These authors also pointed out that the polymerization reaction does not go to completion; at least 20% monomer remains after polymerization. Guttman *et al.* [21] reported the use of low total percent linear polyacrylamide for separation of double-strand DNA; they claimed that the capillary could be reused 100 times without replacement of the separation medium.

EXPERIMENTAL

Fluorescently labeled DNA sequencing samples were prepared using 1 μl M13mp18 single-stranded DNA (United States Biochemical, Cleveland, OH, USA), 1 μl of ROX primer (Applied Biosystems, Los Angeles, CA, USA) and 1 μl of Sequenase (United States Biochemical). Only ddATP was used as terminating nucleotide. The samples were ethanol precipitated, washed, and then re-suspended in 4 μl of a mixture of formamide–0.5 *M* EDTA (49:1) at pH 8.0.

Stock solution of 40% acrylamide–X%N,N′-methylenebisacrylamide (Bio-Rad, Toronto, Canada) were prepared monthly. Gels (6%T) were prepared daily in 5-ml aliquots by dilution of the stock acrylamide-bisacrylamide solution. The aliquots also contained 1 × TBE buffer (0.54 g Tris, 0.275 g boric acid, and 0.100 mmol disodium EDTA, diluted to 50 ml with deionized water) and 7 *M* urea. Before polymerization, the solution was carefully degassed for at least 20 min under vacuum provided by a water aspirator. Polymerization was initiated by addition of 2 μl of N,N,N′,N′-tetramethylethylenediamine (TEMED) and 20 μl of 10% ammonium persulfate; polymerization was done at room temperature over night. The solutions were injected into a capillary using a modified syringe. The gels were covalently bound to the capillary wall through use of γ-methacryloxypropyltrimethoxysilane. 0%C polyacrylamide was covalently bound to the full length of the capillary; other gels were bound to the last 5 cm of the capillary. The polyimide-coated, fused-silica capillaries were typically 35 cm long × 50 μm I.D. × 190 μm O.D.

The one-spectral channel DNA sequencing capillary electrophoresis system has been described before [6,8]. In this system, the injection end of the capillary is kept in a Plexiglas box equipped with a safety interlock. The opposite end of the capillary is inserted into the flow chamber of a sheath flow cuvette; the cuvette is held at ground potential. Fluorescence is excited with the green (543 nm) line of a He–Ne laser. Fluorescence is collected at right angles with a microscope objective, imaged onto a pinhole, passed through a band pass filter and detected with a photomultiplier tube (PMT). A Macintosh computer digitizes the signal of the PMT. The samples were injected at 200 V/cm for 60 seconds and eluted at 300 V/cm with a fresh 1 × TBE buffer.

RESULTS AND DISCUSSION

Three to six electropherograms were obtained for each value of %C studied; in all cases, 6%T polyacrylamide was used. The order in which the gels were prepared was randomized, and fresh gels were used for each experiment. The results reported are average values. There were approximately a 5 to 10% variation in retention time for different gels of the same nominal composition. We believe that this variation arises during our vacuum degassing step; evaporation produces *ca.* 5% decrease in solution volume. Because the final volume is not precisely controlled, the concentration of polymer components will have a minor variation. It appears that the length of the polymer is a strong function of the concentration

of catalyst and initiator, which gives rise to the variation in viscosity and electrophoretic mobility.

As in our earlier work, there is a linear relationship between fragment length and retention time for relatively short sequencing fragments ranging from 60 to 240 bases [10]. The slope and intercept are summarized in Table I. The plots have a common intercept, which corresponds to a retention time of 11.7 min and a mobility of $1.43 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} for a vanishingly small fragment. This value is slightly less than observed in our %T study, and presumably results from differences in the fluorescent labeling scheme used in the two experiments.

The mobility (μ) of the DNA sequencing fragments is calculated from the retention time, t_r

$$\mu = \frac{L}{Et_r} = \frac{L^2}{Vt_r} \quad (1)$$

where L is the capillary length, E is electric field and V is the applied potential. Within experimental error, the mobility is identical for 2.5 and 5%C gels. The mobility increases with lower %C, and is maximum for 0%C.

The electrophoretic behaviour of long DNA fragments is described by the biased reptation model [22,23]. Fragments smaller than some threshold are in a random coil configuration; their mobility decreases inversely with fragment length. Fragments longer than that threshold are stretched into a linear configuration; their mobility is independent of fragment length. The biased reptation model predicts that mobility is given by

$$\mu = a\left(\frac{1}{N} + \frac{1}{N^*}\right) = \frac{a}{N} + \mu_\infty \quad (2)$$

where a is the slope of the line, N^* is the fragment length for the onset of biased reptation with stretching and μ_∞ is the mobility of the longest fragments. A plot of μ *versus* N^{-1} is shown in Fig. 1. The biased reptation model seems to apply to all the fragments larger than 125 bases for 0.5, 2.5, and 5%C and 200 bases in the case of 0%C. Eqn. 2 was fit to the linear portion of the plot of mobility *versus* the inverse fragment length. The coefficients obtained are reported in Table II. The intercept, μ_∞, is the mobility of the longest fragments; lower %C gels generate higher mobility for the longest fragments. The slope decreases with %C. The ratio a/μ_∞ is N^*. Fragments with N^* bases have half the mobility compared to the absence of biased reptation with stretching. The 0%C polyacrylamide produces the highest values of N^*, which suggests that the non-crosslinked polymer suffers least from the effects of biased reptation with stretching.

According to the biased reptation model, the slope of eqn. 2 is proportional to the frictional coefficient for migration of an elongated fragment through the gel. The decrease in slope with increasing %C implies that the frictional force experienced by the fragment decreases with decreasing %C. The largest increase in slope between 5%C and 0%C should be related to the fact that 5%C is a gel and 0%C is an entangled polymer.

A Gaussian function was fit to peaks obtained for bases 85, 117, 184, 217, 258, 309, 350 and 396

$$I(t) = a_0 + a_1 \exp[-(t - a_2)^2/a_3^2] \quad (3)$$

where $I(t)$ is intensity as a function of time, a_0 is the baseline signal, a_1 is peak height, a_2 is the elution time for the peak center, and a_3 is the standard deviation of the peak. The number of theoretical plates, N_p, was estimated by

$$N_p = 2 \cdot \left(\frac{a_2}{a_3}\right)^2 \quad (4)$$

TABLE I

LEAST SQUARES SLOPE AND INTERCEPT FROM A PLOT OF RETENTION TIME (min) *VERSUS* THE FRAGMENT LENGTH IN BASES FOR FRAGMENTS RANGING IN SIZE FROM 60 TO 240 BASES IN LENGTH

%C	Slope (min/base)	Intercept (min)	r
0	$1.02 \pm 0.04 \cdot 10^{-1}$	$1.1 \pm 0.1 \cdot 10^{1}$	0.9995
0.5	$1.17 \pm 0.08 \cdot 10^{-1}$	$1.2 \pm 0.1 \cdot 10^{1}$	0.9988
2.5	$1.75 \pm 0.03 \cdot 10^{-1}$	$1.1 \pm 0.5 \cdot 10^{1}$	0.9999
5.0	$1.61 \pm 0.09 \cdot 10^{-1}$	$1.2 \pm 0.2 \cdot 10^{1}$	0.9992

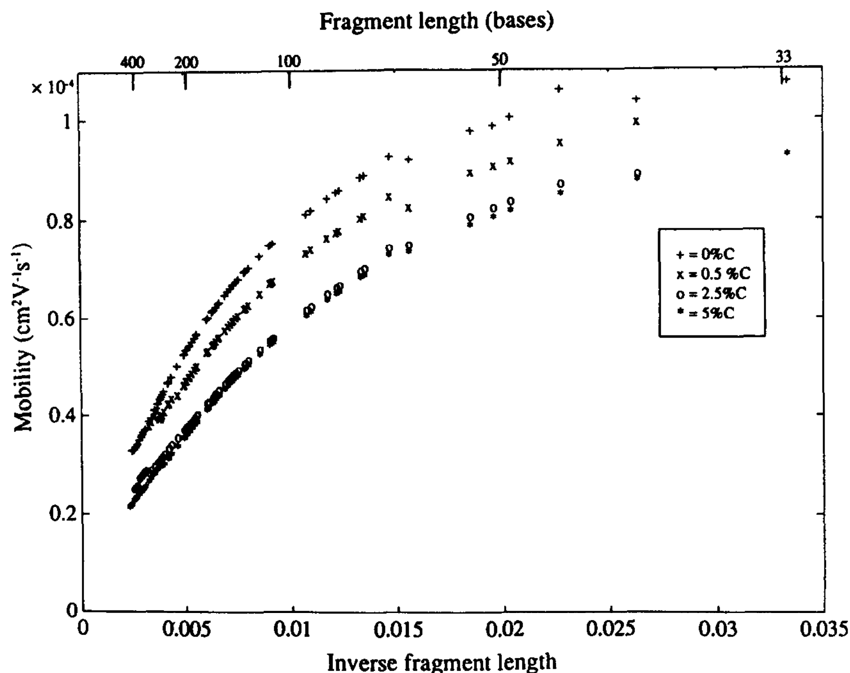

Fig. 1. Mobility *versus* inverse fragment length. Data were obtained with 6%T polyacrylamide in 50 μm I.D. capillaries at 300 V/cm. The %C of the polyacrylamide is noted in the figure. Data were obtained at room temperature.

The plate count (Fig. 2) varied slightly with fragment length; the smallest fragments produced roughly a factor of two higher plate count compared with the longest fragments. Plate counts were lower for lower %C polyacrylamide; however, the correlation between plate counts and cross-linker concentration is poor, 0.62.

TABLE II

LEAST SQUARES SLOPE AND INTERCEPT FROM A PLOT OF MOBILITY *VERSUS* N^{-1}, WHERE N IS THE FRAGMENT LENGTH IN BASES

The least-squared fit was performed for fragments longer than 200 bases. N^* is found by dividing the slope by the intercept, and gives a measure of the fragment size for which biased reptation with stretching becomes significant.

%C	Slope	Intercept	N^*	r
0	$8.11 \pm 0.03 \cdot 10^{-3}$	$1.25 \pm 0.01 \cdot 10^{-5}$	650	0.9998
0.5	$5.77 \pm 0.04 \cdot 10^{-3}$	$1.78 \pm 0.02 \cdot 10^{-5}$	320	0.9994
2.5	$5.25 \pm 0.02 \cdot 10^{-3}$	$0.97 \pm 0.01 \cdot 10^{-5}$	540	0.9997
5	$4.97 \pm 0.02 \cdot 10^{-3}$	$1.23 \pm 0.01 \cdot 10^{-5}$	400	0.9997

In zone electrophoresis, the number of theoretical plates is given by

$$N_p = \frac{E\mu}{2D} \quad (5)$$

where D is the diffusion coefficient of the analyte. Both diffusion coefficient and mobility depend on the product of fragment size and viscosity; the ratio should be independent of those two parameters [24]. Variation in plate count with fragment length probably is associated with the change in the shape of the fragments as they undergo transition from a random coil conformation for small fragments to a linear configuration for longer fragments.

While plate count varies slowly with fragment length, the spacing between adjacent peaks decreases for longer fragments. This phenomenon is a result of biased reptation with stretching, and leads to a degradation in resolution with fragment length. Fig. 3 presents the resolution of adjacent fragments. We found that the resolution of adjacent peaks is inversely proportional to fragment length (Table III). In this case, res-

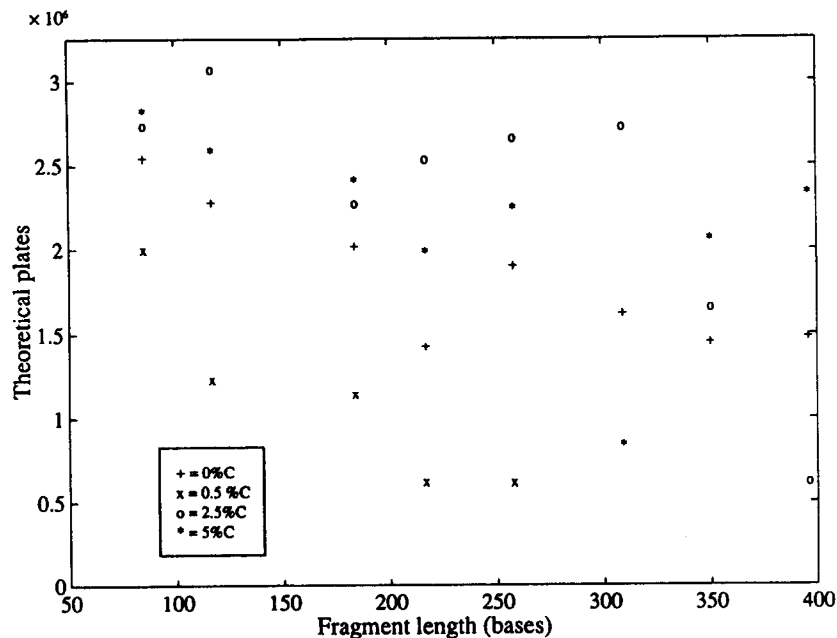

Fig. 2. Theoretical plates *versus* fragment length. Data were obtained with 6%T polyacrylamide in 50 μm I.D. capillaries at 300 V/cm. The %C of the polyacrylamide is noted in the figure. Data were obtained at room temperature. Plate count was estimated by use of a non-linear regression analysis of each peak with a Gaussian function.

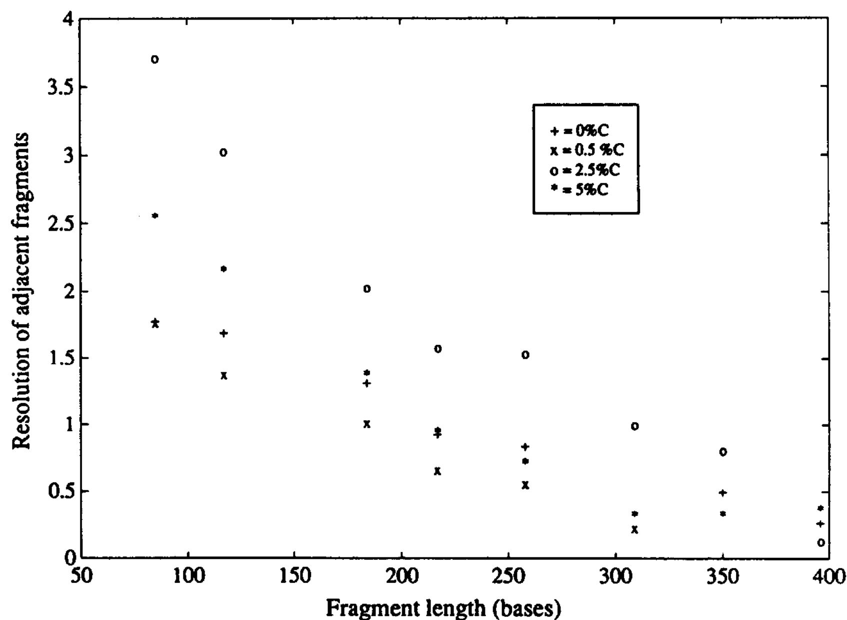

Fig. 3. Resolution *versus* fragment length. Data were obtained with 6%T polyacrylamide in 50 μm I.D. capillaries at 300 V/cm. The %C of the polyacrylamide is noted in the figure. Data were obtained at room temperature. Resolution of adjacent peaks was estimated by peak width determined in Fig. 2 and by the peak spacing determined from mobility data of Fig. 1.

TABLE III

LEAST SQUARES SLOPE AND INTERCEPT FROM A PLOT OF RESOLUTION *VERSUS* N^{-1}, WHERE N IS THE FRAGMENT LENGTH IN BASES

Slope is given to two significant figures.

%C	Slope	Intercept	r
0	160 ± 30	0.1 ± 0.2	0.93
0.5	170 ± 20	−0.1 ± 0.2	0.97
2.5	350 ± 40	−0.2 ± 0.2	0.97
5.0	260 ± 20	−0.3 ± 0.1	0.98

olution asymptotically approaches zero for long fragments as peak spacing goes to zero for long fragments. Resolution may be written as

$$R = \frac{\beta}{N} \quad (6)$$

where β is a proportionality constant. The proportionality constant increases with cross-linker concentration; there is a more rapid decrease in resolution with fragment length for high %C gels compared with low %C gels. This result reflects the earlier onset of biased reptation for higher %C gels.

Resolution drops below 1 for fragments longer than *ca.* 250 bases for low %C gels. As a result, it will be difficult to obtain sequence information for longer fragments. This observation is consistent with the data of both Pentoney *et al.* [18] and Huang *et al.* [19], who were unable to obtain sequence information for fragments longer than *ca.* 300 bases in 0%C gels.

We were unable to reproduce the long column life reported by Guttman *et al.* [21] for 0%C polyacrylamide gels. After two or three injections, our capillaries inevitably failed, producing very low resolution separations. This observation is not surprising. Early reports on capillary gel electrophoresis separations of oligonucleotide standards stated that at least a hundred separations could be performed without replacement of the gel. However, no group has been able to reuse gel filled capillaries for many separations when dealing with DNA sequencing samples. It appears that the high-molecular-mass template present in the sequencing sample leads to rapid degradation of the gel performance [25]. 0%C polyacrylamide is not immune to this phenomenon. It might be argued that gel damage is produced by hot zones formed due to different ionic movement of buffer ions in the gel. However, we found that the polymer is quite stable if a blank solution is loaded onto the capillary; this blank solution contains all of the components used to prepare the sequencing sample, with the exception of the template.

CONCLUSIONS

Mobility decreases with increasing cross-linker concentration. It appears that addition of cross-linker increases the internal viscosity of the polymer, retarding the analyte. Similarly, the transition from normal mobility to the limiting mobility of biased reptation with stretching occurs for longer fragments as the %C is decreased. Both observations are explained by a decrease in pore size produced by higher concentration of cross-linker. This result is at odds with the classic Ogsten [26] sieving model of gels, which anticipates that pore size depends only on the total acrylamide concentration.

The later onset of the deleterious effects of biased reptation with stretching for low %C polyacrylamide implies that the material will be more useful for DNA sequencing applications; longer sequencing runs should be possible. However, it has been frustrating that fragments longer than *ca.* 320 bases can not be separated with high resolution in these polymers at an electric field of 300 V/cm. We anticipate that the use of lower electric fields will delay the onset of biased reptation with stretching, allowing the separation of longer fragments.

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