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Movement of DNA fragments during capillary zone electrophoresis in liquid polyacrylamide

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ABSTRACT

Migration of DNA fragments in the 51–23 130 base pair (bp) size range was investigated by capillary zone electrophoresis in solutions of linear polyacrylamide of concentration from 4 to 10%. Plots of log (mobility) *vs.* log [size (in bp)] clearly indicate three different migration regimes: according to Ogston (*i.e.*, as spherical globules) up to 200 bp, reptation-without-stretching up to 3000–4000 bp and reptation with partial stretching for larger fragments. Guidelines on what percentage of the polymer should be used for optimum resolution can be obtained from plots of peak spacing (in seconds per base pair) *versus* percentage of polymer in solution and from standard plots of peak resolution *versus* percentage of polymer. An optimum linear polyacrylamide concentration, allowing for good resolution of most fragments, from small to large, is found at a level of 6% polymer. It is hypothesized that *in situ* polymerization allows for the formation of a large distribution of polymer sizes (centred at $M_r \approx$ 100 000), thus facilitating simultaneous separation of short and long DNA fragments based on the principle that shorter polyacrylamide chains will sieve shorter DNA fragments and *vice versa*.

INTRODUCTION

It is well known that the electrophoretic mobility of double-stranded (ds) DNA in free solution is independent of molecular size, as the charge to mass ratio is essentially constant. Therefore, in order to effect an electrophoretic separation of dsDNA mixtures, one has to resort to separations in cross-linked, rigid gel matrices which alter the frictional characteristics of DNA in such a way as to introduce a molecular mass dependence on its electrophoretic mobility [1]. While agarose slab electrophoresis has been the preferred technique, in the last few years capillary zone electrophoresis (CZE) has become increasingly popular owing to the speed of separation, the minute amounts of analyte required and the automation of the technique, allowing on-line data acquisition. The earliest reports appeared in 1988 [2,3], and demonstrated the feasibility of both single-stranded DNA analysis (in, *e.g.*, 7.5%T, 3.3%C polyacrylamide gel-filled capillaries in 7 *M* urea)* [2] or dsDNA separations in, *e.g.*, 3%T, 5%C polyacrylamide gels [3]. Such separations were truly unique, in that they proved the possibility of filling troughs of capillary dimensions (typically 75–100 μm I.D.) with a functioning polyacrylamide matrix, while avoiding such frustrating events as air-bubble formation, which would automatically impede electric current flow through the circuit.

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* C = g N,N'-methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution.

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Prior to this application, ultrathin polyacrylamide matrices (as thin as 50 μm) had only been described for isoelectric focusing in flat slabs, where the gelling technique is simple [4]. A host of applications soon followed, a substantial portion of them being centred on the separation of single-stranded oligonucleotides, aimed at single-base resolution in an attempt to confirm CZE as a valid alternative, for human genome sequencing, to the standard flat-slab techniques. All sorts of gel formulations were tried, *e.g.*, 2.5%T, 3.3%C [5], 3%T, 5%C [6,7], 4%T, 3.3%C [8] or 4%T, 5%C [9], 5%T, 5%C [10], 6%T, 5%C [11,12] and even 7.5%T, 3.3%C [13]. Only recently has there been an attempt to evaluate the effect of total percentage of acrylamide (%T) on the resolution, peak spacing and plate count in the sequencing of short DNA fragments [14].

More recently, however, the trend has shifted towards the use of non-cross-linked polyacrylamide or, tout-court, of polymer networks. The reasons are that bubble-free cross-linked gels are extremely difficult to prepare, the life span of gel matrices is short (sometimes limited to only a few runs, notwithstanding the claims of >100 runs from some laboratories!) and gel matrices tend to accumulate sample precipitates at the injection port, with rapid clogging of the pores. The use of polymer networks stems from an original observation by Bode [15,16], who, in 1977, concluded that the mechanism of sieving in liquid linear polyacrylamide is similar to that occurring in cross-linked polyacrylamide gels. Bode's model views macromolecules migrating through "dynamic pores" which are formed by the fluctuating polymer chain network. This thesis found a theoretical support in the classical work of De Gennes [17], who expounded the concept that, once a polymer solution reaches a critical concentration in solution (called overlap or entanglement threshold), it will exert sieving on a particle migrating through it much like a gel does.

Among the earliest reports, Heiger *et al.* [18] described the use of low- and non-cross-linked polyacrylamides for the separation of DNA restriction fragments. Sudor *et al.* [19] reported the advantage of using linear polyacrylamide columns, as the capillary content can be refilled once the polymer network is exhausted (although Chiari *et al.* [20] clearly demonstrated that, owing to the very high viscosity, this is only possible up to 6% polyacrylamide concentrations). Guttman *et al.* [21] demonstrated the possibility of improving separations of dsDNA restriction fragments in 4% linear polyacrylamide by using field strength gradients. Pentoney *et al.* [22] adopted 10% linear polyacrylamide, in manganese buffers, for DNA sequence determination and reported a single-fluor approach offering a sensitivity of 2 zmol ($2 \cdot 10^{-21}$ mol). The use of polymer networks was soon extended to other types of linear macromolecules. Grossman and Soane [23], in reviving De Gennes' theory of entanglement threshold and reptation, demonstrated that hydroxyethylcellulose (HEC), around the entanglement threshold (set at *ca.* 0.4% polymer), could be an excellent medium for DNA fractionation. In a more recent report, the same group were able to derive the relationship between polymer concentration and the mesh size of the entangled network, and found that the average pore radius (R_p) would diminish from 21 nm in 0.5% HEC to 8.5 nm in 2% HEC [24], according to the law $\langle R_p \rangle = 6.0\ C^{-0.68}$ (where C is the polymer concentration), in general agreement with De Gennes' theory. They proposed that the average mesh size of entangled polymer solutions of HEC is about one order of magnitude less than that of agarose gels of comparable concentration [25], the migration mechanism of DNA fragments, however, being much the same as that found in traditional rigid-gel matrices. HEC as a sieving medium was also used by Oefner *et al.* [26], who used 0.5% HEC for DNA separations in both coated and uncoated capillaries (in the last instance, Rb or Cs salts were used for quenching the zeta potential). The same group recently extended their work to a systematic study covering a concentration range of HEC from 0.3 to 0.7% [27]. In a similar approach, other polymers have been proposed, such as 0.5% methylcellulose (MC) [28] and low-melting agarose above its gelling temperature [29,30].

Hydroxypropylmethylcellulose (HMC) was used as a sieving polymer for both proteins and DNAs by Hjertén *et al.* [31] and Zhu *et al.* [32].

As we feel that the future in electrophoretic sieving in CZE will go more and more towards fillings made of viscous solutions, we decided to investigate systematically the properties of one such polymer solution, namely linear polyacrylamide, because, owing to the ease of preparation of such polymers and the possibility of *in situ* polymerization, polyacrylamides appear to be a unique and versatile sieving medium. We give here general guidelines and some basic rules on the optimum %T to be adopted according to the size of the particle to be separated. Such guidelines are based on the evaluation of some basic separation parameters, such as peak spacing and resolution, for given adjacent pairs of DNA fragments, as a function of polymer concentration (%T at 0% C).

EXPERIMENT

Materials

Acrylamide, tris(hydroxymethyl)aminomethane (Tris), ammonium peroxodisulphate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Labs. (Richmond, CA, USA). 3-(Trimethoxysilyl)propyl methacrylate (Bind Silane) and ethylenediaminetetraacetic acid (EDTA) were purchased from Aldrich Chemie (Steinheim, Germany). The DNA molecular mass markers marker V (a mixture of fragments from cleavage of plasmid pBR322 DNA with restriction endonuclease *Hae*III), marker II (a mixture of fragments from cleavage of lambda DNA with restriction endonuclease *Hind*III) and marker III (a mixture of fragments from cleavage of lambda DNA with restriction endonucleases *Hind*III and *Eco*RI) were obtained from Boehringer (Mannheim, Germany) and were available as 250 μg ml^{-1} solutions in 10 m*M* Tris–HCl (pH 8.2). The 1000 base pair (bp) ladder (up to 12 kbp) was supplied by Bethesda Research Labs. (Gaithersburg, MD, USA) at a concentration of 920 μg ml^{-1} in 10 m*M* Tris–HCl (pH 8.2). Fused-silica capillaries (100 μm I.D., 370 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

Capillary zone electrophoresis

CZE was performed in a Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA). For the DNA marker separations, the analysis was run in 100 m*M* Tris–borate–2 *M* EDTA buffer (pH 8.2) at 100 V cm^{-1} in a 40 cm × 100 μm I.D. capillary. The samples were loaded by electromigation for 3 s at 4 kV. The detector was set at 254 nm and the runs were carried out at 24°C under reverse polarity (anode at the detection side).

Coating of the capillary inner wall

The capillary was first cut to the desired length and a 2–3-mm window burned. The capillary was washed and filled with 100 μL of 1 *M* NaOH and allowed to stand for 6 h. The capillary was rinsed and filled with 100 μl of 0.1 *M* HCl, incubated for 30 min and then rinsed with 100 μl of 0.1 *M* NaOH. After incubating the capillary for 1 h, it was rinsed with water and then with acetone. The capillary was filled with Bind Silane–acetone (1:1, v/v) mixture and incubated overnight. The capillary was washed with 100 μl of methanol and then with water, both liquids being pumped through at a slow rate (1 μl^{-1}). The capillary was filled with a 6%T (0% C) acrylamide solution in 100 m*M* Tris–borate–2 m*M* EDTA (pH 8.2) buffer, degassed and containing the appropriate amount of catalysts (4 μl of 10% peroxodisulphate and 2 μl of pure TEMED per millilitre of gelling solution). Polymerization was allowed to proceed for 12 h at room temperature.

Filling of the capillary with polymer networks

The coated capillaries were emptied and then filled with the appropriate solutions of acrylamide (in the absence of cross-linker) in 100 m*M* Tris–borate buffer (pH 8.2) in presence of 2 m*M* EDTA and of the standard amount of catalysts (4 μl of 10% peroxodisulphate and 2 μl of pure TEMED per millilitre of gelling solution). Linear polyacrylamide solutions of 4%, 6%, 8% and 10% concentration were tested.

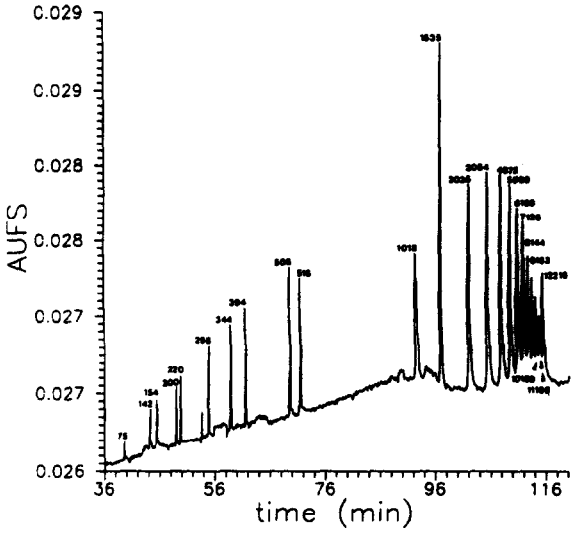

Fig. 1. CZE separation of the 1-kb ladder. Conditions: 47 cm × 100 μm I.D. capillary, in 100 m*M* Tris–borate buffer (pH 8.2)–2 m*M* EDTA, containing 4.5%T polyacrylamide (at O% C). Sample injection, 3 s at 4000 V; run, 4000 V at 10.5 μA; detection at 254 nm. Waters Quanta 4000 capillary unit with forced air cooling. The numbers on each peak represent the fragment length.

Polymerization was allowed to proceed for 12 h. Prior to sample application, the capillaries were conditioned by electrophoresis for 1 h at 4 kV in order to remove the charged catalysts.

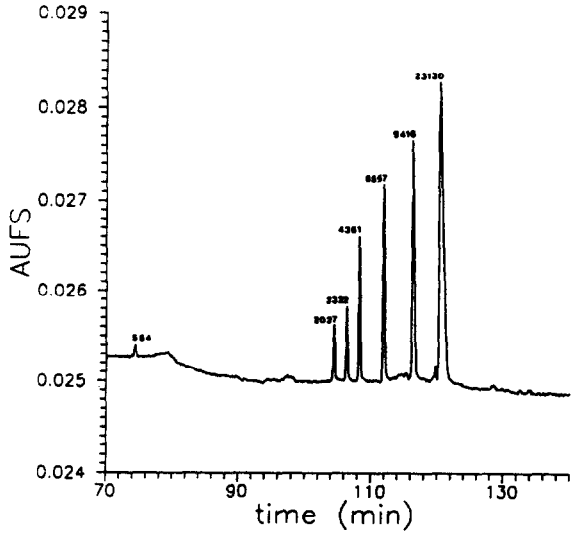

Fig. 2. CZE separation of the marker II DNA fragments. Details as in Fig. 1.

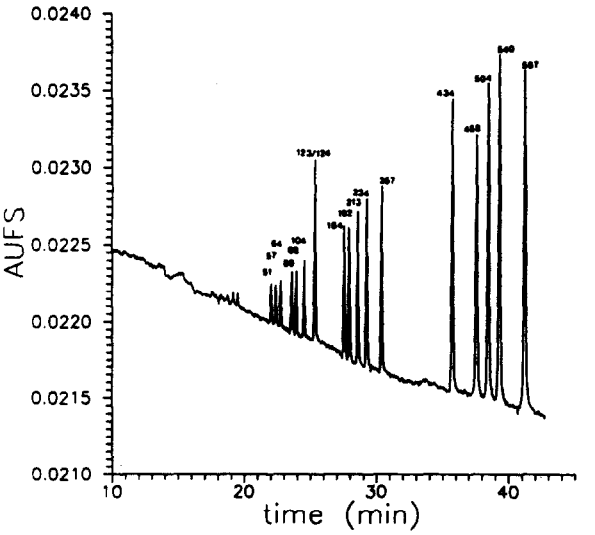

Fig. 3. CZE separation of the marker V DNA fragments. Conditions: 40 cm × 100 μm I.D. capillary, in 100 m*M* Tris–borate buffer (pH 8.2)–2 m*M* EDTA, containing 4.0% T polyacrylamide (at 0% C). Other details as in Fig. 1.

RESULTS

Figs. 1–3 show the electropherograms obtained with the 1-kb ladder (Fig. 1, in a 4.5%T linear polyacrylamide at 4 kV), with marker II (Fig. 2, in 4.5%T linear polyacrylamide at 4 kV) and with marker V (Fig. 3, in 4%T linear polyacrylamide at 4 kV). It is seen that this polymer concentration allows very good separations of relatively small fragments (Fig. 3, from 51 to 587 bp), rather large fragments (Fig. 1, from 75 to 12 216 bp) up to 23 130 bp (Fig. 2). The size selectivity of linear polyacrylamide fillings can be characterized by the well known Ferguson plots, relating the mobility to the %T (at 0% C in our case) as follows:

$$\log \mu = \log \mu_0 - K_R(\%T)$$

where μ is the measured mobility (in $cm^2\ V^{-1}\ s^{-1}$), μ_0 the extrapolated mobility in free solution and K_R the retardation coefficient. We ran a series of fragments in different capillaries containing from 4% to 10% T polymer and assessed the relative mobilities at progressively higher %T. The data are summarized in the Ferguson plot in Fig. 4: it is seen that the relatively smaller

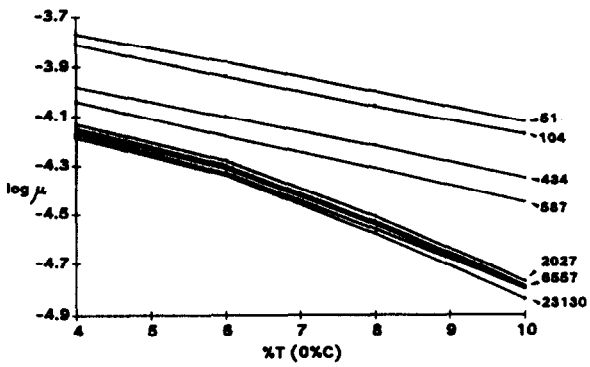

Fig. 4. Ferguson plot of log (mobility) *vs.* percentage of polymer in solution for different DNA fragment sizes. Note the slight downward curvature from fragments longer than 2000 bp.

fragments (*e.g.*, from 51 to 587 bp) exhibit a linear relationship as expected, whereas the larger fragments, *e.g.*, from 2027 up to 23 130 bp) exhibit a downward curvature at the higher %T. In addition, whereas the smaller fragments display progressively steeper slopes, as expected from progressively larger fragment sizes, the set of longer fragments (from 2027 up to 23 130 bp) reveals almost identical slopes, as though size discrimination were progressively lost.

An interesting insight into the migration of DNA fragments can be obtained when the data are expressed in a double-logarithmic plot of μ *vs.* L (the contour length of a DNA molecule, 340 nm per kbp) as suggested by Noolandi [33]. We constructed this plot for 30 DNA fragments, ranging in size from 51 up to 23 130 bp, in four different polymer solutions, of 4%, 6%, 8% and 10%T. The results are summarized in Fig. 5 and it is evident (see also Discussion) that at least three different migration regimes can be evinced: an Ogston dependence, for smaller fragments, reptation without stretching for intermediate sizes and reptation with partial stretching for the upper ladder. These three regimes are particularly evident in the curve pertaining to the 10% T linear polymer, where the transitions to the different migration modes are defined by sharp inflection points. In our case, it appears that the transition from the Ogston regime (when DNAs are considered to migrate as "blobs" or globules) to the reptation mode (where DNAs start slithering head-on through the gel, becoming oriented in the field) occurs already at around 200 bp. Surprisingly, reptation with partial stretching is already apparent at around 3000–4000 bp.

We then tried to obtain some parameters of separation efficiency, in order to allow users to evaluate the proper amount of polymer to be adopted according to the size of the fragments under analysis. One way of expressing the data is to calculate the peak spacing (in seconds per bp increment) as a function of sieving polymer concentration. Fig. 6A and B summarize the data for smaller and intermediate fragments, respectively, using as separation parameter the distance between adjacent peaks in the electropherogram (*e.g.*, 80/89 bp, and so on). It is seen that, for all smaller fragments analysed, the peak spacing strongly increases at progressively higher polymer concentrations (from 4 to 10% T), as it should if they migrate in an Ogston regime (note, however, that for relatively larger fragments, which already start following a reptation regime, such as the pair 458/504 bp, the increment is much less pronounced) (Fig. 6A). For the larger fragments (from 3054 up to 5090 bp, Fig. 6B) two phenomena are evident: (a) the absolute values of peak spacing strongly decrease and (b) little is gained in going from a 4 to a 10%

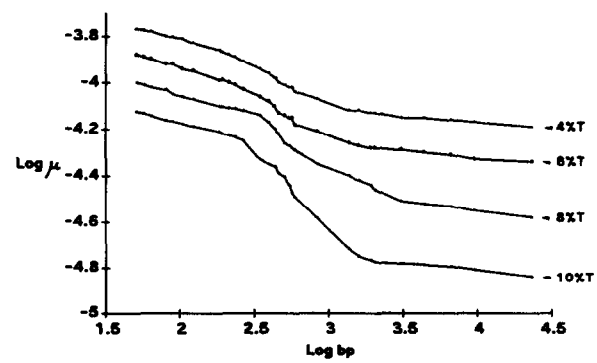

Fig. 5. Double logarithmic plot of mobility *vs.* fragment length (in bp) for 30 DNA fragments (ranging in size from 51 up to 23 310 bp). The plot was calculated for four different polymer concentrations, from 4 up to 10% T. Note the sharp transitions from the Ogston regime to reptation without stretching at *ca.* 200 bp and to reptation with (partial) stretching at 3000–4000 bp.

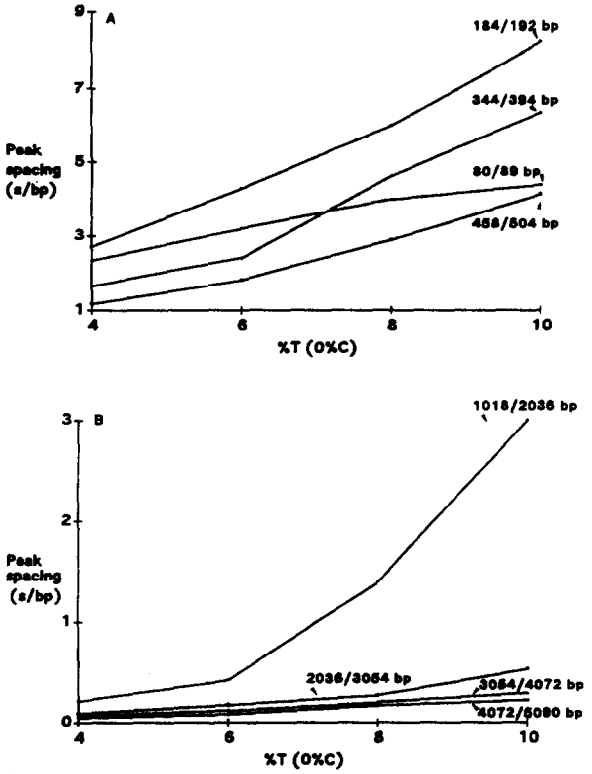

Fig. 6. Plot of peak spacing (in seconds per base pair) *vs.* percentage of polymer in solution. (A) For small fragment pairs (80/89, 184/192, 344/394 and 458/504 bp); (B) for larger fragment pairs (1018/2036, 2036/3054, 3054/4072 and 4072/5090 bp).

T. This is probably due to the fact that, as %T increases, reptation becomes more pronounced, thus diminishing the effective peak distance.

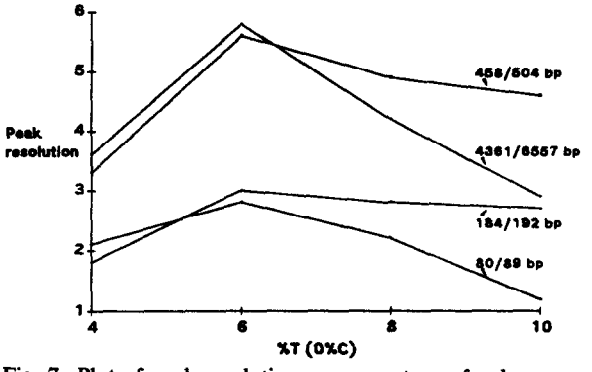

Fig. 7. Plot of peak resolution *vs.* percentage of polymer in solution. Resolution was calculated for the following adjacent pairs: 80/89, 184/192, 458/504 and 4361/6557 bp.

As a final criterion for choosing the optimum polymer concentration, we have plotted in Fig. 7 peak resolution *vs.* %T in the 4–10% T range. It is clearly seen that a sharp resolution optimum is found for most fragments at 6% T, which thus appears to be a good compromise between efficient sieving and reasonable analysis times.

DISCUSSION

Our results begin to shed some light on choosing optimum polymer concentrations when performing DNA fragment analysis in entangled polymer solutions, a field which is rapidly gaining momentum. We shall examine here the different aspects of such sieving polymer solutions.

Sieving of polyacrylamide solutions

We feel that, among all possible polymers listed in the Introduction, polyacrylamide still appears to be the most versatile for the following reasons. First, as several detection methods rely on DNA absorbance at 254 nm, polyacrylamide is completely transparent in this region, thus offering a medium with no background absorbance, which enhances the sensitivity even for minute amounts of DNA by increasing the signal-to-noise ratio at high signal amplification. In principle, this would not apply to polyacrylamide, as it is almost impossible to drive the reaction to 100% consumption of double bonds; however, we have described a scavenging method, based on addition of unreacted double bonds to free SH groups, able to abolish completely the background absorbance of unreacted acrylamide [20]. In addition, polyacrylamide allows complete freedom of selection of sieving ranges, as it can be polymerized *in situ* from non-viscous monomer solutions. With other polymers (*e.g.*, HEC, MC and agarose solutions), concentrated solutions are so viscous as to be unwieldy for any pumping process. In addition, polyacrylamide solutions appear to be surprisingly efficient in sieving mixtures of DNA fragments ranging from small to fairly large (*e.g.*, in the example in Fig. 1, from as little as 75 up to 12 216 bp). It might be asked why this is so. According to Grossman and Soane [23], "in order to create larger pores,

one wants to use a longer polymer and in order to create small pores one wants to use a shorter polymer". We would extend this idea as follows: longer chains will sieve larger objects, whereas shorter chains will act as efficient obstacle toward smaller objects. *i.e.*, we apply to molecular sieving the old adage of organic chemistry: "similar dissolves similar". If this is so, it might be advantageous to use *in situ* polymerization of acrylamide, as it produces a large distribution of chain lengths. We believe that, under our polymerization conditions (as measured by the average viscosity of these solutions), the average chain length is of the order of M_r 100 000, extending from perhaps 50 000 up to as much as 150 000. This provides a vast range of chain lengths for simultaneously sieving both short and long DNA fragments. This is in principle similar to pore-gradient gel electrophoresis, where sharp resolution is obtained for both small and large proteins, owing to the approach to the pore limit [34]. If this is the case, it might be preferable to use *in situ* polymerization rather than dissolve a single-size string (*e.g.*, like the commercially available M_r $5 \cdot 10^6$ polyacrylamide chain, as suggested by some workers), which presumably cannot perform as efficiently for the vast range of DNA sizes commonly found in restriction nuclease fragments (if it does, this would mean that such a chain is highly heterodisperse in size!).

Migration modes of DNA in entangled polymer solutions

In free solution, DNA behaves like a uniformly charged chain and so adopts a fairly open, randomly coiled configuration through which solvent can readily flow. Consequently, the frictional drag it experiences is simply proportional to its length. As the electrical force is also proportional to the length, it follows that the electrophoretic mobility of large DNAs in solution is independent of size [35]. In sieving media, the Ogston model [36] would predict that the mobility of a DNA "globule" would rapidly fall to zero as the diameter of the particle would be slightly larger than the average pore diameter in the gel. However, contrary to this model, it was found that DNA of sizes much larger than the gel pores could also enter the gel and migrate, to a point at which, above 50 kbp, all fragments would migrate at the same speed (*i.e.*, above this size limit, the mobility becomes independent of molecular mass, a most unfortunate behaviour). It was finally understood by Lerman and Frisch [37] that large DNA molecules would start slithering head-on through the gel, a type of motion called reptation, thus becoming oriented by the field [38].

This insight rapidly led to the discovery, by Schwartz and Cantor [39], that periodically changing the direction of the electric field restored the lost sieving mechanism. In addition to a regime of "reptation without stretching", a third regime is prevalent when very high field strengths, or very large fragments, or very small pore size gels are used: "reptation with stretching". When a regime of full stretching is reached, the mobility in a gel becomes again independent of length. Physically this means that for stretched molecules both the electric force and opposing friction scale with length, and the mobility is determined by the force per unit length, which is independent of the actual length, a situation analogous to that experienced by DNA globules in solution.

Noolandi [33] was able to reconstruct these three regimes in a schematic log–log plot of reduced mobility *vs.* molecular size L (see Fig. 2 in ref. [33]). To our knowledge, an actual experimental validation of these three regimes was still not available and we feel that our Fig. 5 (especially in the bottom curve pertaining to 10% polymer) illustrates well these three migration mechanisms. Interestingly, the transition from the Ogston to the reptation without stretching regimes occurs in our polymer solutions at 200 bp, whereas this limit had been set at *ca.* 2000 bp in slab-gel electrophoresis. Moreover, the transition from this last regime to reptation with (partial) stretching occurs in our case at *ca.* 3000–4000 bp, whereas in agarose gel electrophoresis it had been set at around 50 000 bp. The reasons for these discrepancies could be twofold: (a) the higher field strengths (typically extending up to 500 V cm^{-1}) used in CZE as opposed to slab-gel electrophoresis (in fact, in CZE, resolution in DNA analysis decreases above 100 V

cm^{-1}); (b) the smaller pore size of entangled polymer solutions as compared with true gels of similar matrix content. For example, Grossman and Soane [23] estimated an average pore size for entangled polymer solutions *ca.* one order of magnitude smaller than equivalent rigid gels. As the reptation regime begins at a critical value at which $R_g \approx 1.4\ R_p$ (where R_g is the radius of gyration of the particle and R_p is the average pore radius of the gel matrix), this explains why reptation begins in polymer solutions with fragments much smaller than expected. Even this last hypothesis, though, might only be valid when comparing agarose gels with polymer solutions. In polyacrylamide matrices, as commonly used for DNA sequencing, according to Noolandi's group, reptation might already begin at above 500 bases in length, as above this critical length all longer sequences are highly compressed. When pulsed fields were applied to such gels, sequence reading was dramatically improved up to 2300 bases in length [40].

CONCLUSIONS

Polymer networks consisting of linear polyacrylamides appear to be versatile in DNA analysis. Good indicators of the percentage of polymer needed for a given analysis appear to be plots of peak resolution (in seconds per base pair) *vs.* percentage of polymer and also standard plots of resolution [according to the well known equation $(t_2 - t_1)/2(w_1 + w_2)$, where t_2 and t_1 and w_2 and w_1 are the transit times and peak widths (standard deviation), respectively, of bands 2 and 1]. A good compromise appears to be 6% polymer solutions, as they allow for optimum resolution of all fragments in the mixture within reasonable analysis times. However, for very small fragments (up to 100 bp) we have successfully used up to 10% T polymer networks.

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