

Fluidified polyacrylamides as molecular sieves in capillary zone electrophoresis of DNA fragments

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Abstract

In order to optimize the separation of DNA fragments, in the 50–500 base pairs (bp) range, a typical size interval of most polymerase chain reaction-amplified DNA chains produced for analysis of genetic diseases, different ways of preparing liquid linear polyacrylamides were evaluated. Standard linear polyacrylamides (PAA), as prepared with typical levels of catalysts (1 μ l of pure N,N,N',N'-tetramethylethylenediamine and 4 μ l of 10% peroxydisulfate per ml of gelling solution) at room temperature, have extremely high weight-average molecular mass (M_w) values (in excess of $2 \cdot 10^6$) and can be injected or extruded from a capillary at concentrations above 6% only with great difficulty. The same polyacrylamide, if subjected to mastication by ultrasound at 45 kHz for up to ca. 50 h, exhibits much reduced viscosities (e.g. 700 vs. 3600 mPa s, at 8% concentration) and chain lengths (M_w ca. 550 000) and offers increased resolution in the 50–500 bp interval. However, chain rupture by ultrasound produces charged chains, which migrate out of the capillary under the influence of an electric field, thus impairing resolution. Two other ways have been found to produce uncharged, short chains of very low viscosity: chain termination in 2-propanol by polymerization at 35 and 70°C, respectively. The latter process produces chains of M_w as low as 230 000 ($M_n = 55 000$; polydispersity = 4.2) with a viscosity of only 350 mPa s for a 10% polymer solution. In the separation of the seventeen DNA fragments of the marker pBR322/HaeIII (ranging in size from 51 to 587 bp), a 6% solution of “short-chain polyacrylamide (PAA)” affords a resolution of 880 000 theoretical plates, vs. 440 000 for “long-chain PAA”. In a biological sample of a multiplex Duchenne muscular dystrophy containing eighteen DNA fragments, “short-chain PAA” resolves 17 of them, compared with a pattern of only eleven zones in “long-chain PAA”.

1. Introduction

Electrophoretic separation of macromolecules having a constant charge to mass ratio [typically DNA and sodium dodecyl sulfate (SDS)-laden proteins] requires the presence of a sieving gel matrix, in general a polyacrylamide gel for

proteins and agarose matrices for DNA [1]. Although slab-gel techniques are now routine in all life-science laboratories, gel-filled capillaries have so far met with only limited success. This is due to a variety of reasons, such as pore-size limitations, formation and trapping of air bubbles during gel polymerization, denaturation and collapse of the matrix due to local overheating, sample trapping and precipitation at the injec-

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tion port. Soon chemically cross-linked gels were abandoned in favour of viscous solutions of linear (or branched) polymers, according to original observations of De Gennes [2] and Bode [3] that polymer networks, above a critical concentration (the “entanglement threshold”) would be just as efficient in sieving macromolecules as true gels. Sieving dynamic matrices are immune from the noxious problems of air-bubble formation (which would automatically open the electric circuit in such tiny channels) and from sample precipitation at the injection port. Owing to the lack of a fixed-pore geometry, also very large macromolecules (or aggregates) can open a pore in their wake, while they would inevitably precipitate at the deposition site in cross-linked polyacrylamide gels. This allows repeated use of the same matrix (typically >30 runs).

For DNA separations, modern technologies employ viscous solutions of linear polymers such as polyacrylamide (in the absence of a cross-linker) [4–6], methylcellulose [7,8], hydroxyethylcellulose [9–11], liquefied agarose [12] and hydroxypropylmethylcellulose [13]. For protein analysis, the ideal detection system would be by UV absorption at 214 nm, where the molar absorptivities of proteins are 20–50 times larger than at 280 nm. Thus, a search was started for efficient UV-transparent polymers, ideally also exhibiting low viscosity, so as to allow replacement and refilling even after each run, if needed. Ganzler et al. [14] reported successful SDS runs in two such formulations: a 10% solution of dextran (M_r $2 \cdot 10^6$) and a 3% solution of polyethylene glycol (PEG, average M_r 100 000). The applicability of such an approach was later confirmed by Lausch et al. [15]. Soon, several articles appeared reporting efficient protein sieving in other types of polymers. Thus, Guttman et al. [16] described the use of lower M_r dextrans (M_r 72 000, 10% branching, at a 15% concentration) and of polyethylene oxide (PEO, typically a 3% solution of average M_r 100 000). They also studied the influence of temperature on the sieving effect in SDS–protein complexes and found the best separations at 50°C (in dextrans, whereas a deterioration of separation was apparent in the same temperature interval in PEO). Recently, SDS-electrophoresis was reported for

other types of UV-transparent polysaccharides, such as pullulan [a branched polysaccharide composed of α -(1–6)-linked maltotriose] [17]. Typically, separations were carried out in a 7% polymer solution (having average M_r 50 000–100 000), although good resolution was achieved for proteins standards (in an M_r range from 14 400 to 116 000) from as low as 1% up to 7% pullulan.

For DNA analysis, polyacrylamides have proved to have a unique sieving ability, unsurpassed by other types of linear or branched polymers. Unfortunately, under standard polymerization conditions, and in the absence of a cross-linker, long strings (with M_r possibly in the range of a few million) are formed, which results in extremely viscous solutions, unwieldy to any pumping process. We have in fact reported that the concentration limit, for replenishing a capillary, is at around 6% polymer [4]. Above this critical concentration, polyacrylamide has to be polymerized in situ, with all the typical problems connected with radical polymerization: elimination of catalysts, unreacted monomers, etc. In the analysis of polymerase chain reaction (PCR)-amplified products, for the screening of genetic defects, ideally one would want to optimize resolution in a window of DNA sizes ranging from 50 to 500 base pairs (bp) [18,19]. In this size range, one would need much more concentrated polyacrylamides (e.g., 8–10%) for single-base resolution, if needed (although occasionally even 6% polymer solution can afford that). It would therefore be ideal to be able to polymerize low-viscosity polyacrylamides, so as to prepare bulk amounts outside the capillary, purify them by standard means (precipitation in ethanol) and adopt “fill-in and emptying” procedures as needed. In this paper, we propose different methods for achieving this goal.

2. Experimental

2.1. Reagents

Acrylamide tris(hydroxymethylaminomethane) (Tris), ammonium peroxodisulfate and N,N,N',N'-tetramethylethylenediamine (TEMED)

were obtained from Bio-Rad Labs. (Richmond, CA, USA), 3-(trimethoxysilyl)propyl methacrylate (Bind Silane) and poly(ethylene oxide) from Aldrich (Steinheim, Germany) and ethylenediaminetetraacetic acid (EDTA), boric acid, acetic acid and 2-propanol from Merck (Darmstadt, Germany). Fused-silica capillaries (100, 75 and 50 μm I.D., 375 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The DNA marker pBR322/HaeIII was obtained from Boehringer (Mannheim, Germany).

2.2. Polymer preparations

Linear polyacrylamide of reduced chain length was synthesized by the procedure of Grossman [20], by using 2-propanol as a chain-transfer agent for controlling the molecular mass of the product. Acrylamide (11%) was dissolved in 3% 2-propanol and polymerized at 35 or 70°C in a thermostated bath for 2 h. After extensive dialysis against water, the polymer was lyophilized and dissolved in separation buffer [TBE: 89 mM Tris–89 mM boric acid–2 mM EDTA (pH 8.3)] at different concentrations.

Acrylamide (6%) was polymerized (in the absence of cross-linker) in capillaries overnight at room temperature; the capillaries were then conditioned with separation buffer for 20 min at 100 V/cm.

Mechanically degraded linear polyacrylamide was obtained from 10% polyacrylamide, polymerized overnight at room temperature, subjected to a shearing action, for different times, at 45 kHz (output power 120 W, 25°C) in a sonicator. The viscous matrix causes the molecules to rotate in a clockwise direction. The combined effect of this rotation and the flow of the pervading matrix is to stretch and compress the molecule until chain breaking [21].

2.3. Molecular mass determinations

The number-average (M_n) and weight-average (M_w) molecular masses of the different polymer preparations were determined by gel permeation chromatography (GPC). Samples and standard were run using a Waters Model 590 solvent-

delivery system equipped with two Waters Ultrahydrogel linear columns connected in series and with a Waters R401 differential refractometer for peak detection. The injected sample was 100 μl of a 100 mg/ml stock solution, the flow-rate was 0.8 ml/min and the mobile phase was 100 mM NaNO_3 . M_w and M_n of the final products were assessed to be 550 000 for the masticated polyacrylamide (after 47 h of digestion), 230 000 for the polyacrylamide subjected to chain transfer at 70°C and 450 000 for the chain-transfer preparation at 35°C. Five PEO standards were used as calibration markers for GPC, having M_r values of 661 000, 148 000, 73 000, 25 000 and 10 000.

2.4. Capillary electrophoresis

Capillary zone electrophoresis (CZE) was carried out with a Waters Quanta 4000 E apparatus from Millipore (Milford, MA, USA). Capillaries 37 cm long and with different I.D., coated by a slight modification of Hjerten's protocol, utilizing N-acryloylaminoethoxyethanol (AAEE) as monomer [22,23], filled with different viscous polymer solutions, were used. The sample and standard were loaded electrophoretically by applying 100 V/cm for 6 s. Separations were performed at 100 V/cm. Ultraviolet absorbance was monitored at 254 nm. pBR322/HaeIII, covering an 8–587 bp range, and multiplex PCRs in Duchenne muscular dystrophy, covering an 113–547 bp range, were used as markers for evaluating the separation performance.

2.5. Viscosimetry

Viscosity measurements on polymer solutions were performed on a Bohlin VOR rheometer (Bohlin Rheology, Lund, Sweden), with the sample thermostated at 30°C. The shear rate chosen was in the interval 1.86–7.33 s^{-1} . A concentric cylinder measuring system was adopted, with a 14 mm diameter for the inner cylinder and a 15.4 mm diameter for the outer cylinder. Viscosity is expressed in mPa s, the viscosity of water at 25°C being 1.

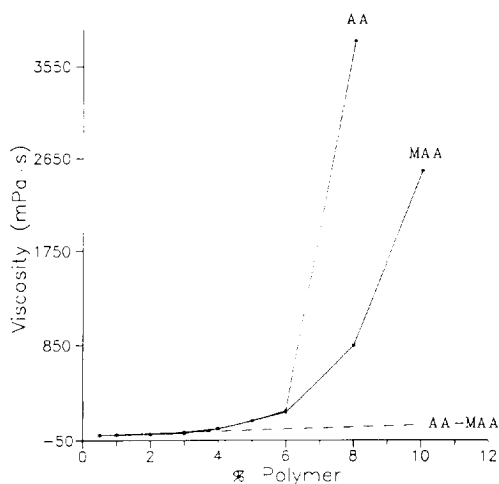


Fig. 1. Viscosity measurements as a function of increasing polymer concentration for conventionally polymerized polyacrylamides (AA) and masticated polyacrylamides (MAA). Measurements performed on a Bohlin VOR rheometer at 30°C. The broken line is an extrapolation of the initial linear slope. In principle, the point of departure from linearity would represent the entanglement threshold, but it is not applicable here owing to the wide range of viscosities explored.

3. Results

Fig. 1 shows the viscosity, at increasing concentrations, of two different polyacrylamide preparations: a standard sample, as polymerized (AA), and the same sample subjected to mastication with ultrasound for 47 h (MAA). It can be seen that, at low concentrations, both samples appear to exhibit very similar viscosities, with a sharp departure at 6% polymer, where the masticated chains show a dramatic decrease in viscosity (e.g., an 8% solution of MAA gives 849 vs. 3800 mPa s for an equivalent 8% concentration of AA). The point of departure from linearity is generally taken to represent the entanglement threshold [11]. However, here the low-range viscosities could not be explored well at the shear rates utilized, so that we consider that 3% polymer (i.e. the apparent departure from linearity) cannot be taken as a threshold value.

Fig. 2 represents the kinetics of chain rupturing by ultrasound, as followed by viscosity mea-

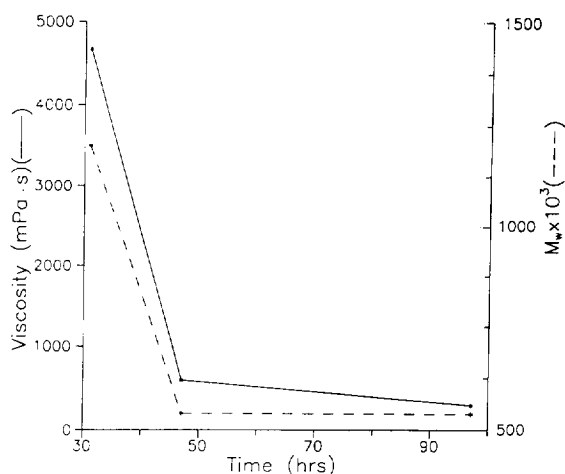


Fig. 2. Kinetics of viscosity (solid line) and M_w (broken line) variations as a function of time of mastication at 45 kHz. Note that, after ca. 50 h of cavitation, no further appreciable viscosity or M_w changes take place.

surements and, in parallel, by assessment of M_w . We have taken as first point a 30-h value since, owing to the limited range of M_r standards we had available and to the high viscosity of undegraded polyacrylamide (in a 10% solution), it was impossible to assess both viscosities and M_w in the starting solutions. However, as a rough estimate of M_w , the starting material exhibited values well above $2 \cdot 10^6$. As expected, viscosity decreases are paralleled by comparable M_w decreases. Mastication appears to reach a plateau after about 50 h ($M_w = 550\,000$; $M_n = 151\,000$; polydispersity = 3.6), after which no substantial decreases in both parameters are experienced. Masticated chains offer a unique increment in the resolution of DNA fragments in the 50–500 bp window we are trying to optimize (not shown); however, after much trial and error, this approach was abandoned, as it seems to produce charged chains, with deleterious effects on the run reproducibility, since the charged polymers migrate out of the separation channel (see Discussion).

In search of other means for reducing chain length and viscosity, we noticed a recent paper by Grossman [20] on polymerization in the presence of chain-transfer agents. We adopted this procedure, in two variants: polymerization

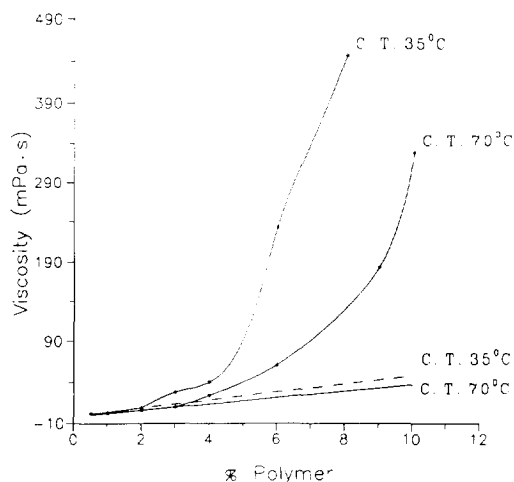


Fig. 3. Viscosity measurements as a function of increasing polymer concentration for polyacrylamides polymerized in the presence of a chain-transfer (C.T.) agent (2-propanol) at 35 and at 70°C. Measurements were performed on a Bohlin VOR rheometer at 30°C. Note that the entanglement threshold, taken as the point of departure from linearity of the experimental viscosity curve, is ca. 1.5% polymer for the reaction at 35°C, but it is at 3% polymer for reaction at 70°C.

at 35 and at 70°C. The results (in terms of viscosity vs. percentage of polymer) are shown in Fig. 3; it is seen that much reduced viscosities can be obtained by chain transfer (C.T.) at 35°C, and even lower if the reaction is conducted at 70°C, as adopted here. As an example, while a 10% solution of masticated chains gives a viscosity of 2540 mPa s, an equivalent solution of polymer produced by chain transfer at 70°C produces a viscosity of only 329 mPa s. These very low viscosities allow easy refilling after each run even at polymer concentrations up to 10%. It is additionally seen (Fig. 3) that the two chain populations exhibit different apparent entanglement thresholds: while this value is ca. 1–1.5% polymer for C.T. at 35°C, it is ca. 3% polymer for C.T. at 70°C. In parallel we examined the decrease in M_w in the two C.T. procedures at the two different temperatures adopted. The final M_w for the lower temperature reaction is centred around 450 000 ($M_n = 115 000$; polydispersity = 3.9), whereas when adopting the 70°C reaction protocol, the final size of the polyacrylamide chains is centred around M_w 230 000 ($M_n =$

55 000; polydispersity = 4.2). This explains the still large differences in viscosities between the two chain populations, as plotted in Fig. 3.

The more homogeneous size population of polymers produced by the chain-transfer technique [here called short-chain polyacrylamide (PAA)] should allow a higher resolution of DNA fragments, even at comparable concentrations, as compared with chains polymerized by the standard protocol (long-chain PAA). Fig. 4 compares the patterns obtained when running the DNA standard in long-chain PAA (A) vs. the same concentration (6% polymer) of short chain PAA (B). The substantially increased resolution in the latter system is immediately evident, e.g., the triplet of chains of 458–540 bp is baseline resolved only in the short-chain PAA. The same applies to the quadruplet of chains of 184–234 bp. Additionally, the last fragment, 587 bp, has an unacceptably high width at the base, compared with a much narrower peak width in the short-chain system. When compared with a real biological sample, the differences were even more dramatic. For example in Duchenne muscular dystrophy, we prepared a multiplex PCR of eighteen exons, covering the 50–400 bp range. The long-chain PAA system, even on optimization of all running parameters, could resolve only eleven of the eighteen fragments. The short-chain system proposed here could resolve seventeen of the eighteen DNA fragments (in preparation). In the case of the separation of the DNA marker pBR322/HaeIII (Fig. 4) we evaluated the number of theoretical plates N under the two different experimental conditions, in an electrophoretic run utilizing a 100 μm I.D. capillary. As shown in Fig. 5, long-chain PAA gives an N value of 440 000, as opposed to 880 000 in short-chain PAA. Curiously, however, the resolution is markedly dependent on the capillary diameter, since in a 50 μm I.D. capillary identical resolution is obtained in both instances. This “wall effect”, which we have found also in the case of solutions of poly(vinyl alcohol) [24], will be discussed below. In any event, it is not so easy to use, in daily practice, a capillary of only 50 μm I.D., as the signal is too low for a correct evaluation of a

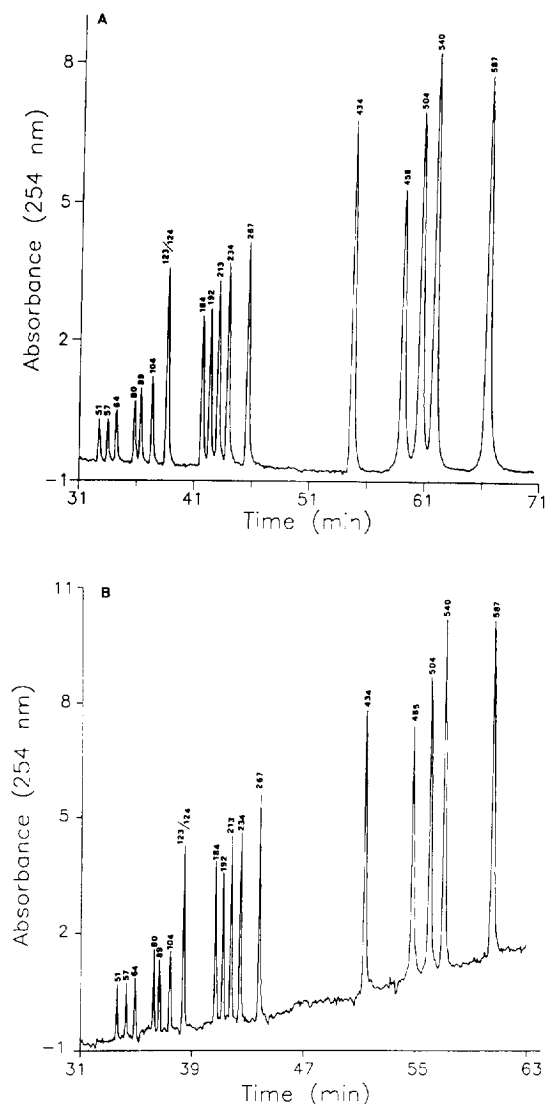


Fig. 4. CZE of DNA marker pBR322/HaeIII in (A) long-chain and (B) short-chain PAA. The standards were loaded electrophoretically by applying 100 V/cm for 6 s. Separations were performed at 100 V/cm in TBE as running buffer and 6% long-chain or short-chain PAA, respectively. Ultraviolet absorbance was monitored at 254 nm. The capillary was 37 cm \times 100 μ m I.D.

DNA peak when exploiting the natural DNA absorbance at 254 nm, as in the present case (however, it has been suggested that high-sensitivity detectors, such as from Spectra-Physics, etc., can pick up a DNA signal from a 50 μ m I.D. capillary). Nevertheless, in general, narrow-

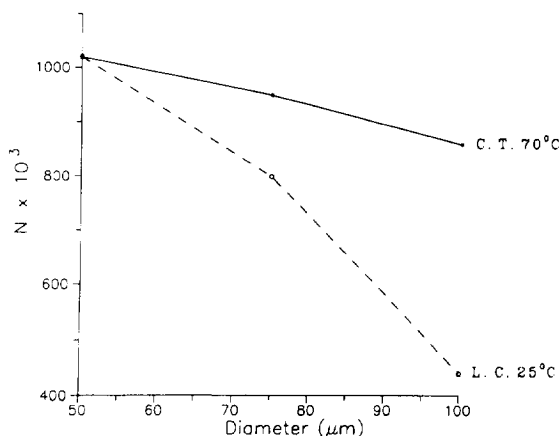


Fig. 5. Evaluation of the number of theoretical plates for the two pBR322/HaeIII separations shown in Fig. 4A and B. L.C. 25°C = long-chain polyacrylamide prepared at 25°C; C.T. 70°C = chain-transfer polymerization at 70°C. The experiments were performed in three different capillaries of 100, 75 and 50 μ m I.D. Note how the resolution increases markedly at progressively lower I.D.s.

bore capillaries can only be used in the laser-induced fluorescence mode, where capillaries as narrow as 5 μ m I.D. can be successfully employed [25]. Moreover, in 50 μ m I.D. capillaries, pumping in and out long-chain, viscous solutions of polyacrylamide represents a difficult task, as it requires high pressures not available in all CZE units.

4. Discussion

4.1. Use of “masticated” chains

The use of mastication (i.e. of cavitation of dilute polymer solutions by ultrasonic radiation) was long ago adopted by the rubber industry for the production of short-chain polymers [26], and is a well standardized procedure. It is based on a chemical reaction (rupture of C–C bonds, in the case of polyacrylamides) induced by direct acquisition of mechanical energy on a molecular scale, and it is conveniently termed a “mechanochemical” reaction. One of the main advantages is that, whereas in thermal degradation of polymers there is an equal probability of scission along the polymer chain, giving a random M_w

distribution, in the process of cavitation non-random scission takes place, producing a much more homogeneous chain size (under optimum conditions approaching the M_w/M_n limit of 1.05) [21]. The reason is that entangled chain structures tend to localize the adsorbed energy in the bonds near the centres of the chain: this non-random rupture restricted to central sections of the molecule leads to a much narrower M_w distribution. It is hypothesized that this concentration of energy near the centre of the molecule is due to the fact that the central region of long molecules in bulk polymer is not able to disentangle sufficiently under shear to dissipate the mechanical work in motion, as instead occurs at the end segments.

On these assumptions, we prepared "long-chain PAA", by standard polymerization conditions, and subjected it to cavitation. The chains obtained after ca. 50 h of mastication indeed exhibited much reduced average lengths and viscosities (see Figs. 1 and 2), but did not offer run reproducibility, although the first few CZE separations offered much increased resolution and N values. We finally understood that chain rupture was accompanied by radical formation at the two new extremities of the shorter chains. In the presence of oxygen, peroxy radicals are formed, which are then terminated by abstraction of hydrogen from the surroundings of the polymer. The end-product of this reaction is the formation of charged carboxylic groups on the ruptured polyacrylamides, which slowly migrate out of the capillary under the electric field. Attempts at quenching this reaction by other means met with limited success. For example, when cavitating under completely anaerobic conditions, no chain degradation took place. Also, the addition of other radical scavengers did not allow proper fluidification of the starting, long-chain polymer solution. Among the different scavengers utilized, we tried also dihydropyrolamide (a kind gift from Professor Citterio, Milan Polytechnic), known to form a very unreactive radical, shielded by the three methyl groups on the nitrogen, but even this procedure was not satisfactory. We also tried fluidifying the long-chain polyacrylamide by using different

frequencies of the ultrasound radiation, up to 20 MHz. However, higher frequencies resulted only in strong heating of the viscous polymer solution, up to boiling, but in no real chain degradation. It turns out that polymer molecules are not stretched enough by higher frequencies to give rise to any sizeable amount of degradation [21]. Owing to all these problems, chain mastication was abandoned in favour of polymerization in presence of chain-transfer agents.

4.2. Use of chain transfer in polyacrylamide production

Grossman's group [20,27,28] has made extensive studies on the use of entangled polymer solutions for DNA separation. Originally, they advocated hydroxyethylcellulose, but the solution finally adopted was the production of short-chain, low-viscosity polyacrylamides obtained by chain termination in the presence of chain transfer agents, such as 2-propanol [20]. Under these polymerization conditions, a final product exhibiting an M_w value of 339 000 was obtained, with a viscosity, for a 6.2% polymer solution, of only 150 cP at 25°C. Although, with these reaction conditions, we also obtained a product with similar physico-chemical properties and electrophoretic performance, in order to optimize further the separation of DNA in the 50–500 bP range, we adopted different polymerization conditions leading to an additional decrease in the viscosity and molecular size of the final product, i.e., reaction in presence of 2-propanol, as a chain transfer agent, but at much higher temperatures (70°C as opposed to room temperature). Under these conditions, our final product exhibits an apparent chain length of M_w 230 000 (M_n 55 000, polydispersity = 4.2) and still lower viscosities for comparable concentrations (barely 80 mPa s for a 6.2% polymer, see Fig. 3). At such low viscosities, we can easily empty and refill capillaries after each run even with a polymer solution of up to 10% concentration. In turn, this produces a drastic increment in resolution in the 50–500 bp DNA size window we wanted to optimize. The idea of using temperature and modulating the catalyst concentration

for varying the average chain length of polyacrylamides was in fact proposed long ago by our group [29–31]. Note that an absolute comparison of M_w between our and Grossman's data cannot be made, since the latter was able to calibrate his column with polyacrylamide standards, whereas we only had PEO available. Thus, whereas he gives an M_w of 339 000 for C.T. at 35°C, under our experimental conditions we obtain, for the same reaction product, an apparent M_w of 450 000. However, on a relative scale, it is clear that C.T. at 70°C generates a product of ca. half size and with a much lower viscosity.

4.3. Optimization of DNA separation according to size windows

It is now clear that, for optimizing DNA separations, one would have not only to select some given polymers (polyacrylamide being the best in our hands), but also to choose the right polymer concentration and appropriate chain length. We therefore consider that the best that can be achieved is the optimization of a given "window" of DNA chain intervals, at which a proper polymer concentration will offer the highest performance. Diluting or concentrating the polymer solution will result in a shift of this optimum window towards higher or lower DNA chain lengths, respectively. This is analogous to SDS electrophoresis, where, for achieving optimum size discrimination over a large protein size interval, one has to resort to a porosity gradient (typically extending from 4% up to 20%T) [32]. At a single polyacrylamide concentration, the range of molecular mass values covered is limited. Although it might be difficult, but not impossible, to fill a capillary with a concentration gradient of sieving linear polymers, for broadening the macromolecular size interval explored, one would still be faced with the impossibility of using standard CZE units commercially available. In such porosity gradients, where optimum separation will be obtained only by driving DNA fragments of appropriate lengths in selected regions of the gradient, one would have to resort to rigid, stretched and fully transparent capillaries, which could be

scanned by a moving detector, as demonstrated long ago by Hjertèn [33].

As a final remark, we observed a strong wall effect in performing separations at progressively lower capillary I.D.s (see Fig. 5), as a result of which resolution in long-chain or short-chain PAA becomes indistinguishable at 50 μm I.D. A similar phenomenon was recently discovered by us when attempting separations of SDS-laden proteins in entangled poly(vinyl alcohol) (PVA) solutions. This phenomenon was attributed to the strong tendency of PVA to form hydrogen bonds not only among the polymer chains in solution, but also with the free silanols on the fused-silica wall [24]. Perhaps a similar type of phenomenon is also operative here as well [note additionally that our capillary is coated with poly(AAEE), which offers a free distal OH group on every other carbon along the chain]. Work is in progress to elucidate these aspects.

Acknowledgements

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