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Separations of DNA fragments by capillary electrophoresis in N-substituted polyacrylamides

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Abstract

In spite of its high resolving capability, the use of polyacrylamide as a sieving matrix for capillary electrophoresis (CE) is limited by several factors. The viscosity of polyacrylamide solutions is too high for easy capillary filling and the matrix requires a highly pressurized system to be replaced after each run. The systems currently used to produce short polyacrylamide chains needed to overcome this problem, require a careful optimization of polymerization conditions to produce polymers of reproducible composition. In addition, the polyacrylamide viscosity may vary with time depending on the shear stress to which the solution is subjected. This work proposes some new matrices for DNA separations by CE that overcome these obstacles while maintaining the high resolution offered by polyacrylamide. The properties of dimethylacrylamide and poly(acryloylaminoethoxy)ethyl-β-D-glucopyranoside were investigated. Both polymers were found to be more suitable than polyacrylamide as DNA sieving media. The presence of substituents on the amido nitrogen reduces the viscosity of the polymer solutions without changing separation selectivity as demonstrated by the identical profile of the double-log curves of mobility versus DNA size obtained for the three polymers. In general, an increase in the monomer size leads to the formation of a network with larger pore sizes in which DNA molecules migrate more rapidly, providing shorter analysis times and more efficient separation. © 1997 Elsevier Science B.V.

Keywords: Capillary columns; Polyacrylamide-derived gels; DNA

1. Introduction

Capillary electrophoresis (CE) in uncross-linked polymer solutions allows rapid and efficient separation of DNA restriction fragments up to 23 000 base pairs (bps). A wide variety of polymers have been used as sieving matrices in CE. Most of these polymers can be obtained or synthesized in a wide range of molecular masses and dissolved at various concentrations in the electrolyte buffer. The polymer concentration may be varied to broaden the range of DNA size that can be separated in a specific medium. Operative concentration depends on the characteristics of the polymer which should be employed above its overlap threshold concentration. Solutions of natural polysaccharides such as hydroxyethyl- [1–5], hydroxypropylmethyl- [6–10] and methylcellulose [11,12] and liquified agarose [13,14] have been used with varying degrees of success as have synthetic polymers like uncross-linked acrylamide [15–17], polyvinyl alcohol [18] and polyethylene oxide [19]. The wide selection of matrices suggests that none of them fully satisfy the requirements of an ideal medium. Moreover, the mechanism of DNA separation in polymer solutions is not unequivocally understood as the relationship between polymer structure, size, concentration and sieving properties is not predicted by theory.

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Polyacrylamide was the first synthetic polymer adopted as a sieving matrix in CE on account of its unsurpassable sieving capacity. Although polyacrylamide solutions do provide excellent separation of double and single stranded DNA molecules, there are several factors that limit their large-scale use. One is the high viscosity of polyacrylamide solutions which depends on the high molecular mass of its chains [20]. The molecular mass of a polymer can be adjusted in a number of ways [21], for example, by carrying out the polymerization in the presence of chain transfer agents, such as isopropanol [15], or by increasing the catalyst concentration [17]. Although the polymer molecular mass is considerably reduced in this way, the viscosity of polyacrylamide solutions is still high and the concentration that can be used in practice does not exceed 6.5%. A second method for controlling the molecular mass of a polymer is to fractionate it by a technique such as gel permeation, dialysis or fractional precipitation from water-miscible organic solvents. This is an efficient way of controlling polymer properties but requires careful optimization of fractionation conditions.

There are several reasons for preferring low viscosity polymers as DNA sieving media for CE. First, the time needed to replace the separation medium between successive analyses depends on the rate of solution flow through the capillary, which, in turn, depends on the viscosity of the polymer. Second, DNA moves through less viscous polymers more rapidly, providing faster separation.

Polyacrylamide, like other industrially important fluids, has a complex structure which imparts anomalous flow properties, manifested by changes in viscosity as the shear stress varies in magnitude and time of application. Rheological properties of polyacrylamide might explain the often poor reproducibility in the transit times of DNA fragments separated by CE. Beside these problems, polyacrylamide is sensitive to hydrolytic cleavage in alkaline solutions [22]. When high pH and temperature are employed hydrolysis of the amido linkage occurs, generating carboxyl residues that stay covalently linked to the matrix.

We propose replacing polyacrylamide with polymers obtained by radical polymerization of N-mono- and di-substituted acrylamido derivatives. We used N,N-dimethylacrylamide (DMA) and N-(acryloylaminoethoxy)ethyl-β-D-glucopyranoside (AEG) instead of acrylamide to produce linear polymers. The appropriate substituent on the amido nitrogen influences the viscosity and the hydrolysis of the resulting polymers. Both poly(DMA) and poly(AEG) were found to be more suitable than polyacrylamide as sieving components of electrophoretic separation media. The substituents on the nitrogen favorably change the way viscosity depends on polymer concentration, so the polymer concentration can be varied over a wide range without detrimental effects on the viscosity. Double-log curves of mobility versus DNA size for fragments indicate that the separation selectivity in N-substituted polyacrylamides is similar to that obtained in polyacrylamide. In general, an increase in monomer size leads to the formation of a network with larger pore sizes in which DNA molecules migrate more rapidly achieving much shorter analysis times.

2. Experimental

2.1. Reagents

Acrylamide, ammonium persulfate (AP) and N,N,N′N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Labs., (Hercules, CA, USA); tris(hydroxymethyl)aminomethane, N-tris-(hydroxymethyl)methyl - 3 - amino - propanesulfonic acid (TAPS), ethylendiaminetetracetic acid (EDTA), β-D-glucose and almond β-D-glucosidase were purchased from Sigma (St. Louis, MO, USA). N,N-Dimethylacrylamide and *tert.*-butanol were from Aldrich (Steinheim, Germany), silica 60 F_{254}, silica gel 60 (230–400 mesh) were from Merck (Darmstadt, Germany). N-Acryloyl aminoethoxyethanol (AAEE) was synthesized as described by Chiari et al. [23].

DNA molecular mass marker V, a mixture of fragments from cleavage of plasmid pBR322 with restriction endonuclease HAE III, was purchased from Boehringer (Mannheim, Germany) and was available as a 250 μg/ml concentration in 10 m*M* Tris–HCl pH 8.2. The 1 kilobase pair (kb) ladder was supplied by Bethesda Research Labs. (Gaithers-

burg, MO, USA) at a concentration of 920 μg/ml. Water and all solvents were of analytical grade.

2.2. Capillary electrophoresis

CE separations were performed in a SpectraPhoresis 1000 capillary system Thermo Separation Products (Freemont, CA, USA). Data collection was performed on a personal computer utilizing SW-Phoresis 1000 software. In all the experiments fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 30 cm (23 cm to the window)×100 μm I.D. coated by the method described in Ref. [23] were used. DNA samples were detected at 254 nm. Before each run the capillary was washed for 5 min with water and filled with the polymer solution by applying a pressure of 15 p.s.i. (1 p.s.i.=6894.76 Pa) for 5 min. The samples were loaded electrophoretically by applying 130 V/cm for 1 s. Separation was performed at 130 V/cm, using 100 m*M* TAPS–Tris buffer, pH 8.5, containing 2 m*M* EDTA.

2.3. Enzymatic synthesis of N-(acryloylaminoethoxy)ethyl-β-D-glucopyranose (AEG)

A solution of β-D-glucose (1.9 g, 0.01 mol) in 3.45 ml of water was added to a solution of N-acryloylaminoethoxyethanol (13.6 g, 0.085 mol) and *tert.*-butylhydroquinone (0.2 g) in 5 ml *tert.*-butanol. To the mixture β-D-glucosidase (0.360 g; 2.7 U) was added and the suspension was stirred for 48 h at 50°C. When the reaction had reached its equilibrium, the mixture was filtered to remove the enzyme and the *tert.*-butanol was evaporated under reduced pressure. Flash chromatography of the residue using CH_2Cl_2–MeOH (8:2) as eluent, gave 0.614 g of AEG as an amorphous solid (yield 18%).

The ^{1}H NMR [dimethyl sulfoxide (DMSO)] data were δ: 8.25 (1H, br t, NH), 6.35 (1 H, dd, H_a), 6.18 (1H, dd, H_b), 5.78 (1H, dd, H_c), 4.25 (1H, d, H_1), 3.98 (1H,m), 3.9-3 (12H, m).

Fast atom bombardment mass spectrometry (FAB-MS) *m/z*: 341 $(M+Na)^+$, 319 $(M+H)^+$, 192, 181, 159, 97.

The IR spectrum showed the following bands: 3366 cm^{-1}, ν (OH, NH), 1659 cm^{-1} (amide I), 1626 cm^{-1}, ν (C=C), 1551 cm^{-1} (amide II), 1100 cm^{-1} ν (C–O alcohol), 1050 ν (C–O ether).

2.4. Preparation of linear polyacrylamide, poly(DMA) and poly(AEG)

Linear polyacrylamide and poly(DMA) of reduced chain length were synthesized according to Grossman [15] by using 2-propanol as a chain transfer agent to control the molecular mass of the product.

Acrylamide and freshly distilled N,N-dimethylacrylamide (1 g) were dissolved in 9.7 ml of water and degassed under vacuum for 30 min. Isopropanol (0.3 ml) was then added to the reaction vessel. Next, 100 μl of 10% (v/v) TEMED in water and 10 μl of a 40% (w/v) ammonium persulfate in water were added. The mixture was allowed to react for 1 h at 50°C. To remove any unreacted monomer and contaminants, the reaction mixture was dialyzed against water using a 12 000 molecular mass cut-off dialysis membrane from Sigma. The solution was lyophilized to give 0.8 g of a white solid.

AEG was polymerized in the same manner, except that isopropanol was not added to the polymerizing mixture.

Each polymer was dissolved in the separation buffer (100 m*M* TAPS–Tris, pH 8.5, 2 m*M* EDTA, pH 8.3) resulting in a 7.5% (w/v) polymer solution that was stirred for approximately 30 min and filtered through a 0.45 μm pore size filter.

2.5. Molecular mass determinations

The average molecular masses (M_w) of the different polymers were determined by gel permeation chromatography. Samples and standards were run in a Ultraspherogel SEC 400 TSK column (Beckmann) connected with a Jasco RID 300 refractometer for peak detection injecting 0.2% (w/v) sample solutions. The mobile phase was 0.2 *M* Na_2SO_4. Five dextran standards were used as calibration markers with M_w of 10 000, 40 000, 70 000, 150 000 and 270 000.

2.6. Viscosimetry

The Rheometrics Dynamic Analyzer (Model DSR 200) was used to measure the steady shear viscosity

η as a function of shear stress, with the sample thermostatted at 25°C. The shear stress chosen was in the interval 50–500 dyne/cm^2. A paraplate geometry viscometer was adopted with a 25 mm diameter, and 0.15 mm distance between the plates. Viscosity is expressed in g/(cm) (s) $(10)^2$ (cP).

3. Results and discussion

In a recent article [24] we described the synthesis of AEG, a new monomer bearing glucose units, and its application in the production of coatings for CE. These monomers are used in the synthesis of polymers with an artificial polymer backbone and pendant glucose units with a marked hydrophilic character and stability to hydrolysis. Although very attractive, monomers which have a pendant sugar take many chemical steps to be synthesized. Therefore their large-scale application is limited. On the other hand, CE allows the use of more expensive and exotic chemicals as it only needs minute amounts. In addition, a chemo-enzymatic procedure can be used to shorten the synthetic pathway as shown for allyl glycosides prepared using an approach based on glucosidase action [25]. Fig. 1 shows the scheme of the synthesis of AEG catalyzed by almond β-glucosidase used in a reverse hydrolysis mode. The thermodynamic equilibrium, normally in favor of hydrolysis in an aqueous medium, is shifted towards the synthesis by carrying out the reaction in an organic solvent containing a controlled amount of water. Though the yield is low, only 18%, this approach provides a simple and cost-effective way to produce this glycoside monomer in one step. We extensively investigated its properties in terms of chemical stability and polymerization efficiency [24]. AEG was more stable towards alkaline hydrolysis than acrylamide, whereas its stability in acid was fully compatible with its use in CE. Spectrophotometric measurements of the unreacted amount of conjugated double bonds indicated complete conversion of the monomer into polymer. However, radical polymerization of the monomer leads to the formation of a large number of short chains, as indicated by the recovery after dialysis in a 12 000 molecular mass cut-off membrane, which never exceeded 70% of the monomer mass.

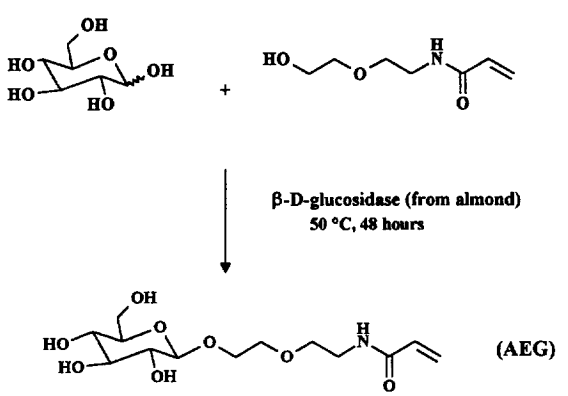

Fig. 1. Enzymatic synthesis of N-(acryloylaminoethoxy)ethyl-β-D-glucopyranoside (AEG).

The second polymer used in the present investigation was poly(DMA). This monomer, together with various cross-linkers, was introduced as an electrophoretic sieving medium in 1989 [26]. A recent patent describes its use in CE [27]. Linear short chains of this polymer have the unique features of being able to reduce electroosmotic flow and to sieve single and double stranded DNA molecules. The chemical stability of cross-linked poly(DMA) was illustrated by Gelfi et al. [28] who extensively investigated the properties of a number of N-mono and di-substituted acrylamido derivatives.

In the present investigation we evaluated the viscosity and sieving capacity of poly(DMA) and poly(AEG) (Fig. 2) and compared their performances as DNA sieving matrices for CE. Fig. 3 shows the relationship between viscosity, extrapolated at zero shear stress and concentration for the three polymers. The viscosities of 5% solutions were similar but polyacrylamide and poly(DMA) were polymerized in the presence of a chain transfer agent in order to reduce the length of their chains, whereas poly(AEG) was polymerized under ordinary conditions. The M_w of the polymers are summarized in Table 1.

The viscosity of a solution is important in CE as it governs the concentrations that can be employed for the separation. Fig. 3 shows how the viscosity of short polyacrylamide chain solutions increases dramatically with the concentration, to the extent that a 10% solution, with a viscosity of 1450 cP, cannot be used as replaceable matrix unless the capillary is

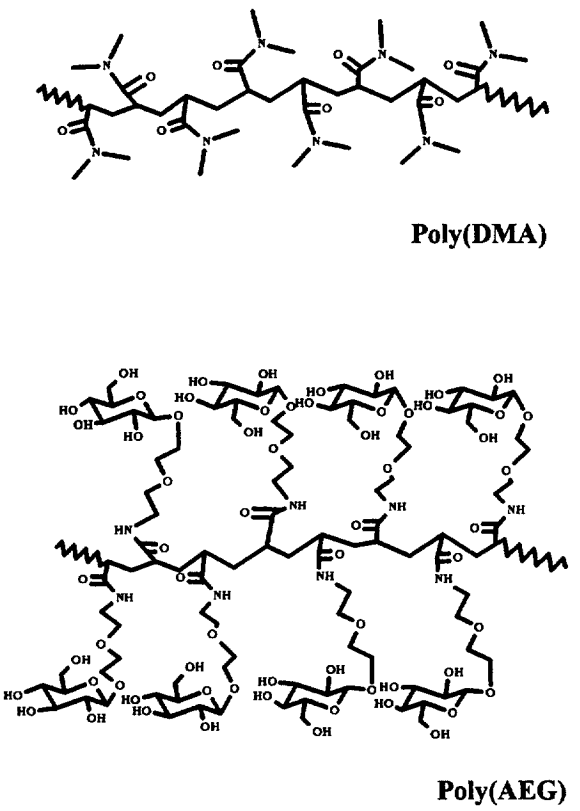

Fig. 2. Chemical structure of poly(DMA) and poly(AEG).

Table 1
Average molecular masses (M_w) and polydispersity of different polymers determined by gel permeation chromatography

Type of polymer	M_w	Polydispersity
Poly(acrylamide)	267 000	1.13
Poly(DMA)	225 000	1.37
Poly(AEG)	240 000	1.40

Dextran standards with M_w values of 10 000, 40 000, 70 000, 150 000 and 270 000 were used as calibration markers.

equipped with a powerful pressurizing system. The viscosity of poly(DMA) and poly(AEG), however, increases little with concentration so that 10% or even more concentrated solutions can be replaced.

The dissolution of polyacrylamide chains in the background electrolyte takes a long time and carefully controlled conditions. Table 2 shows the viscosities of freshly prepared 10% polyacrylamide and poly(AEG) solutions, measured four times at intervals of 3 min, under the conditions described in Section 2.4. The viscosity of polyacrylamide increases steeply after each determination as a consequence of the shear stress applied by the viscometer. This implies that polyacrylamide solutions possess not one single viscosity value but a whole range, depending on the flow conditions and the history of the solution. The viscosity across the capillary wall might be considerably higher than in the bulk solution and the pressure required to clean up the

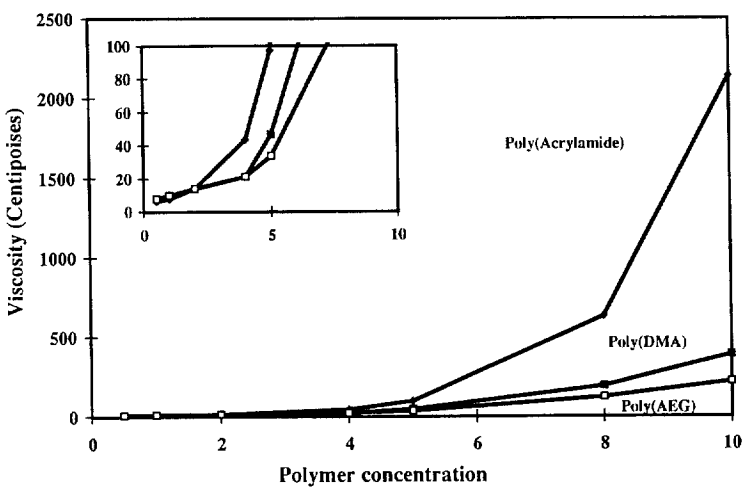

Fig. 3. Viscosity of polyacrylamide, poly(DMA) and poly(AEG) as a function of polymer concentration. The viscosity was measured as described in Section 2.6. The insert shows a more detailed view of the viscosity at low polymer concentration.

Table 2
Viscosities of freshly prepared 10% (w/v) polyacrylamide and poly(AEG) solutions, measured four times at intervals of 3 min under the conditions described in Section 2.4

Type of polymer (10%, w/v)	Viscosity (cP)			
	1st meas.[a]	2nd meas.	3rd meas.	4th meas.
Polyacrylamide	1450	2450	2734	4068
Poly(AEG)	120	133	170	260

[a] meas.=measure.

column higher than expected. The greater viscosity observed might derive from more efficient dissolution of the polymer chains in the aqueous buffer induced by the shearing or by an orientation of the polymer facilitating associations of the different chains. Although a similar increase was found in a poly(AEG) solution the change was less striking than in polyacrylamide.

Despite all its defects, polyacrylamide remains one of the most efficient sieving media for DNA molecules, with unsurpassed resolving capability, towards small fragments and single stranded DNA in particular. Figs. 4 and 5 show separations of DNA size markers, ranging in size from 50 to 12 000 bps, in polyacrylamide and in poly(DMA) and poly(AEG). The concentration was 7.5% in the three cases. Resolution of DNA fragments results from the sieving ability of the medium and from the efficiency of the separation. The three polymers give a similar performance but migration is faster in poly(AEG), as depicted in Fig. 6 by the three double-log plots of mobility in relation to DNA size. The curves have almost the same shape with the plot corresponding to the separation in poly(AEG) shifted to higher mobility values. As shown in Fig. 7, where the efficiency of separating DNA fragments is plotted against their size, the efficiency is generally better in poly(DMA) and poly(AEG) than in polyacrylamide. The presence of a suitable substituent in the amido nitrogen could influence selectivity in several ways. Gelfi et al. [29] showed a remarkable separation of the 123/124 bp achieved in poly(N-acyloylpropanol). The authors stated that the distal –OH group in the N-substituent could be responsible for the high separation power of this matrix. An increase of separation selectivity could result from a transient

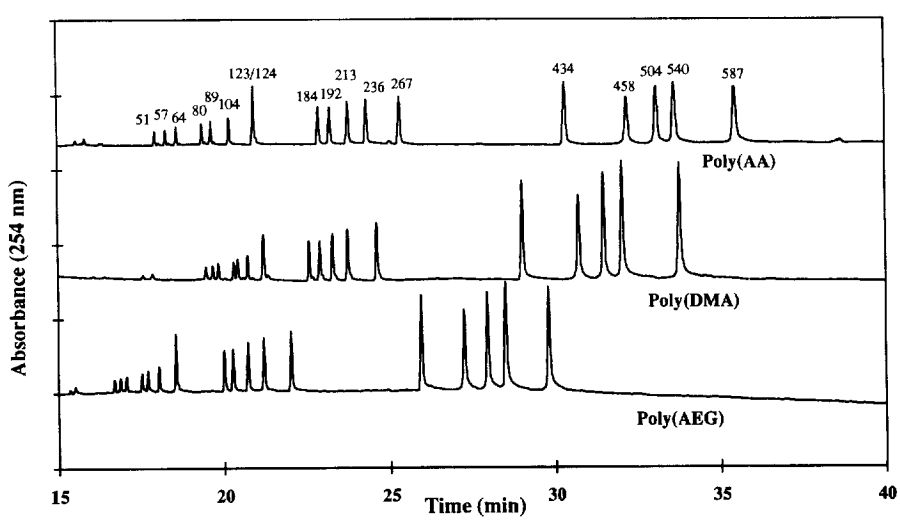

Fig. 4. CE separation of marker V in various 7.5% (w/v) polymer solutions. Conditions, buffer 100 m*M* TAPS–Tris, 2 m*M* EDTA pH 8.5. Applied voltage 130 V/cm. Other conditions as in Section 2.2.

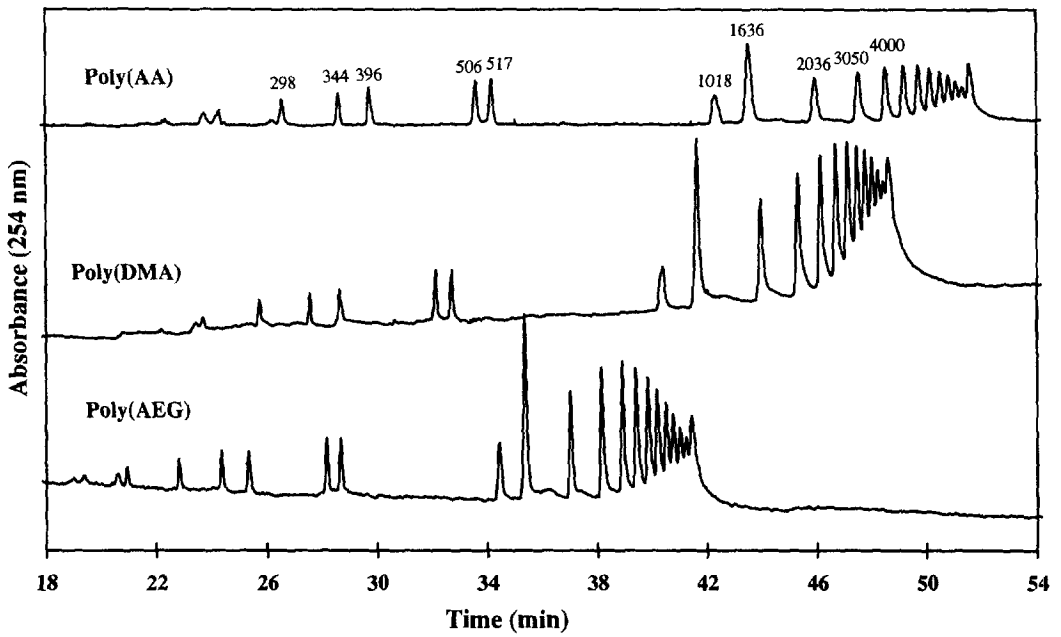

Fig. 5. CE separation of 1 kb ladder in various 7.5% (w/v) polymer solutions. Conditions, buffer 100 m*M* TAPS–Tris, 2 m*M* EDTA pH 8.5. Applied voltage 130 V/cm. Other conditions as in Section 2.2.

hydrogen bonding between the DNA analyte and the distal –OH groups of the sieving liquid polymer. Barron et al. demonstrated [30] that large DNA can be separated in ultra-diluted hydroxyethyl cellulose solutions. To explain their experimental results the authors proposed a mechanism of separation based on the entanglement of DNA and isolated polymer molecules. Stiff extended polymers become entangled more strongly than flexible, random coil, polymers. The presence of nitrogen substituents increases

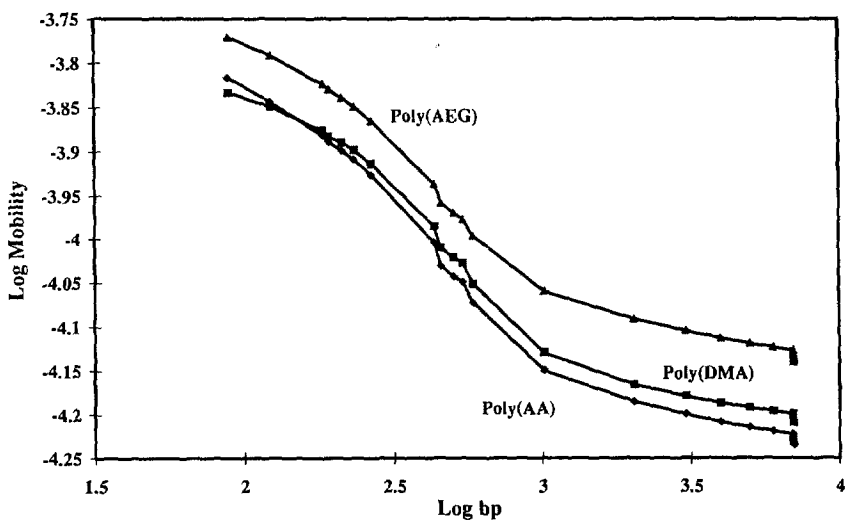

Fig. 6. Double log plots of mobility versus DNA size for fragments separated by CE as described in Fig. 4.

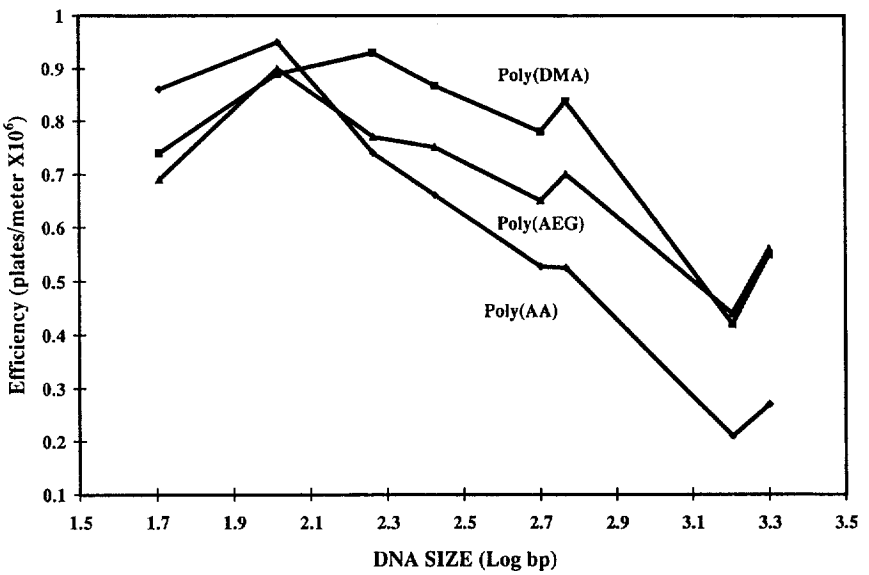

Fig. 7. Efficiency of CE separation of DNA fragments expressed as number of theoretical plates/m plotted as a function of DNA size. Separation conditions as in Fig. 4.

the stiffness of N-substituted polyacrylamides and introduces –OH groups that can interact via hydrogen bonding with DNA molecules.

4. Conclusions

The present work investigated the properties of two polymers obtained by radical polymerization of DMA and AEG, a novel monomer produced by chemo-enzymatic synthesis. Both polymers offer a valid alternative to polyacrylamide as sieving media for DNA molecules in CE, overcoming some of the problems that hinder the large-scale use of this matrix. The introduction of substituents on the nitrogen of the amido group reduces the viscosity of the solutions of these polymers, allowing easy refilling of the capillary between each run using a purge at 15 p.s.i..

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