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Journal of Chromatography A, 853 (1999) 511–518

JOURNAL OF CHROMATOGRAPHY A

Effect of linear polymer additives on the electroosmotic characteristics of agarose gels in ultrathin-layer electrophoresis

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Abstract

Electroosmotic properties of agarose gels with low, medium, high and super high electroendosmosis (EEO) were evaluated based on the apparent electric field mediated mobility of a neutral, fluorescent marker under constant field strength using ultrathin-layer separation configuration. Electroosmotic flow mobility values were measured in different gel concentrations and also in the absence and the presence of various linear polymer additives. Under ultrathin-layer separation conditions, a slight decrease in electroosmotic flow mobility was observed with increasing agarose gel concentration of 1 to 3% for all agarose gels investigated. When linear polymer additives, such as linear polyacrylamide, hydroxyethyl cellulose or polyethylene oxide were added to 1% low electroendosmosis agarose gel, significant reduction of the electroosmotic flow properties were observed with increasing additive concentration. Effect of the intrinsic electroosmotic properties of the various electroendosmosis agaroses on the apparent mobilities and separation performance of double-stranded DNA fragments during automated ultrathin-layer agarose gel electrophoresis was also investigated.

Keywords: Ultrathin-layer electrophoresis; Agarose gels; Electroosmotic flow; DNA

1. Introduction

Agarose gels were one of the first matrices used in electrophoresis separations to avoid convection-related problems, otherwise characteristic of free solution electrophoresis [1]. Superior to other mediums, e.g. paper or cellulose acetate, agarose has an excellent ability to size separate biopolymers with very similar mass to charge ratios, such as nucleic acids and sodium dodecyl sulfate (SDS)-protein complexes. Agarose is a polysaccharide, consisting of 1,3-β-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactose units, building up the average molecular mass of 120 000 agarbiose polymer units [2]. Due to the presence of charged residues, such as sulfate, pyruvate and carboxy groups in natural agar, agarose gels exhibit electroosmotic properties when exposed to electric field. At basic pH values commonly used during electric field mediated separation of biopolymers (pH 7–9), these groups on the agarose chains carry negative charges. These negatively charged domains attract positively charged ions from the surrounding aqueous buffer solution, creating an electrical double layer [3]. Under the influence of the electric field, these positively charged ions in the diffuse portion of the double layer migrate towards the cathode, dragging water

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00578-6

with them. As a result, a bulk flow is formed with a magnitude depending on the composition and the nature of the background electrolyte within the gel matrix. During the separation of DNA molecules in agarose gels under regular basic pH conditions, the bulk electroosmotic flow (μ_{eof}) is moving from the positive electrode towards the negative electrode, therefore, it decreases the electrophoretic mobility (μ_{ef}) of the oppositely migrating negatively charged solute molecules, resulting in an apparent electrophoretic mobility (μ_{app}) of:

$$\mu_{app} = \mu_{ef} - \mu_{eof} \quad (1)$$

Electroosmotic flow (EOF; also known as electroendosmosis, EEO) in agarose gels is historically characterized by subjecting the mixture of albumin and dextran to regular agarose slab gel electrophoresis [4]. Measuring the respective distances of these two markers from the injection point, electroendosmosis is calculated by dividing the migration distance of the neutral dextran by the sum of the migration distances of dextran and albumin. The resulting number referred to as m_r, usually ranges from 0.06–0.3 for commercially available agarose gels, and used to characterize the electroosmotic properties of the given agarose [5].

Features like high mechanical strength even at low (several percent) gel concentrations, biological inertness and stability in a wide pH range of 4–9 make agarose a popular separation matrix for the analysis of DNA fragments [6]. Composite gels containing agarose mixed with other polymers have also been reported in the literature as high resolution separation matrices [7]. One of the most frequently used composite additives is polyacrylamide, which can be employed in both crosslinked [8] or non crosslinked (linear) formats [9]. Other linear polymers such as derivatized celluloses have also been proved to be advantageous as additives to composite agarose gel based separation of double stranded (ds) DNA fragments [10].

Ultrathin layer agarose gel electrophoresis is a novel combination of agarose slab gel electrophoresis and capillary agarose gel electrophoresis [11]. The relatively large temperature difference between the gelling and melting temperature of the agarose matrix (35–40°C versus 70–90°C) makes possible its use as an effective separation medium even at high electric fields (>40 V/cm) under ultrathin separation conditions where extra Joule heat dissipation is efficient [12]. In this paper, we evaluate the effects of several linear polymer additives, such as linear polyacrylamide, hydroxyethyl cellulose and polyethylene oxide, on the electroosmotic properties of low EEO agarose gel in ultrathin layer electrophoresis. The influence of the agarose induced electroosmotic flow on the migration behavior of dsDNA fragments during ultrathin layer agarose gel electrophoresis in the presence and absence of linear polymer additives is also discussed.

2. Materials and methods

2.1. Chemicals

In all the experiments agarose gels with low ($-m_r = 0.1$), medium ($-m_r = 0.17$), high ($-m_r = 0.24$) and special high ($-m_r = 0.33$) EEO values (Sigma, St. Louis, MO, USA) were dissolved in 45 m*M* Tris, 45 m*M* borate, 1 m*M* EDTA·Na_2 buffer, pH 8.3 (0.5×TBE). Tris, boric acid, EDTA·Na_2 and ethidium bromide were obtained from ICN (Costa Mesa, CA, USA), all in electrophoresis grade. The linear polyacrylamide (LPA; M_r 700 000–1 000 000) and hydroxyethylcellulose (HEC; M_r 90 000–105 000) were obtained from Polysciences (Warrington, PA, USA), the polyethylene oxide (PEO; M_r 600 000) was from Aldrich (Milwaukee, WI, USA). The neutral, fluorescent EOF marker, Bodipy FL hydrazide was from Molecular Probes (Portland, OR, USA). During the separation experiments, the λ Hind III DNA restriction fragment mixture (Life Technologies, Gaithersburg, MD, USA) was used, diluted with de ionized water to the working concentration of 5 ng/μl and was stored at −20°C until use.

2.2. Instrumentation

The electroosmotic properties of the various agarose and composite agarose gels were evaluated by automated ultrathin layer agarose gel electrophoresis (see description below) using various concentration agarose and linear polymer–agarose composite ma-

trices. The electroosmotic mobility values of the agarose and composite agarose gels were defined based on the migration time of a neutral fluorescent EOF marker, Bodipy FL hydrazide under constant applied electric field (42 V/cm) using reversed polarity separation mode, i.e., anode at the injection side.

The automated ultrathin layer agarose gel electrophoresis system with real time laser induced fluorescent detection was described earlier [13]. This particular system employed a fiber optic bundle based scanning detection system using a 532 nm frequency doubled Nd–YAG laser excitation source and avalanche photodiode (APD) detection with a 585±15 nm wide band interference filter. The lens set scanned across the multilane separation platform by means of a translation stage and collected the emitted fluorescent light. The analog signal from the APD was digitized in a micro controller then acquired by a personal computer. The ultra thin layer separation platform was an 18 cm×7.5 cm×190 μm float glass cartridge with built in 15 ml buffer reservoirs at both ends. The λ Hind III DNA restriction fragment mixture was injected by previously discussed membrane mediated loading technology [14]. The injection side of the ultrathin separation slab gel had a straight edge with no individual wells. Small amounts of samples (0.2–0.5 μl) were spotted onto the tips of the 32 tab membrane loader (Genetic BioSystems, San Diego, CA, USA) and carefully inserted in close proximity to the straight edge of the separation gel. A layer of high viscosity and low ionic strength well solution was used to interface between the membrane and the separation matrix, to assure the best possible sample transfer. After the insertion of the spotted loading membrane, the electric field was applied and the DNA fragments were quantitatively loaded into the separation matrix from the membrane within 5 s.

The dsDNA molecules were fluorescently labeled during the separation process by ''*in migratio*'' intercalation with ethidium bromide dissolved in the separation gel buffer. The used gels were simply replaced by pumping fresh melted agarose into the separation cartridge after each run. Preheating of the separation cassette to 50–60°C helped to prevent premature solidification of the freshly poured gel during replacement process.

2.3. Procedures

The appropriate amounts of agarose powder were suspended in 0.5x TBE buffer, boiled repetitively in a microwave oven until clear, and was kept at 60°C until use. Linear polymer additives were dissolved in the melted agarose in the concentration range of 0.2 to 1% with 0.2% (w/v) increments. For ultrathin layer agarose gel electrophoresis, the preheated separation cassettes (40–45°C) were filled with the melted agarose and after several minutes of cooling/solidification, the gel filled cassette was ready to be used. The effective separation length of the agarose gel filled ultrathin layer cassette was 2 cm for the electroosmotic flow measurements, and 6 cm for the dsDNA fragment analysis. For the EOF correction experiments, 2% (w/v) agarose gels with various electroosmotic characteristics were used in 0.5x TBE buffer containing 50 n*M* ethidium bromide. For the separation performance experiment 1% (w/v) low EEO agarose and 1% (w/v) low EEO agarose mixed with 1% (w/v) LPA; 1% (w/v) HEC and 1% (w/v) PEO were used, respectively. In all the separations, the applied voltage was 750 V, generating 5–7 mA current. An aluminum heat sink was employed to hold the separation cartridge and dissipate extra Joule heat. The temperature of the heat sink was regulated by a thermostated air bath with a precision of ±1°C. The actual separation temperature was measured at the middle of the heat sink.

3. Results and discussion

3.1. Electroosmotic flow as a function of gel concentration

First the effect of the agarose concentration on the electroosmotic properties of the various agarose gels was examined in ultrathin layer electrophoresis. Gels were prepared in 1, 2 and 3% concentrations, using low, medium, high and super high EEO agaroses. Electroosmotic properties were evaluated by measuring the electric field mediated mobility of the uncharged, low molecular weight fluorescent marker Bodipy FL hydrazide. After the injection, the ultrathin layer separation gel was subject to 42 V/cm constant applied electric field strength. The migration

time of the neutral, fluorescent marker was measured at 2 cm form the injection site. The electroosmotic mobility values (μ_{eof}) were determined by using Eq. (2):

$$\mu_{eof} = l/(E \times t_M) \quad (2)$$

where, l is the actual migration distance of the neutral, fluorescent marker from the injection point (2 cm), E is the applied electric field strength (42 V/cm) and t_M is the measured migration time. Table 1 shows the EEO values of the various agaroses used during the experiments defined by the vendor ($-m_r$) and the experimentally measured μ_{eof} values at various gel concentrations. Apparently, under ultrathin layer electrophoresis conditions, all the gels exhibited approximately 8–12% decrease in EOF values with every percent increase in the gel concentration between 1 and 3%. Fig. 1 depicts the relationship between the vendor supplied $-m_r$ values and our measured μ_{eof} data in ultrathin-layer agarose gel electrophoresis. The slopes of the individual plots are very similar to each other: 11.7; 11.5 and 11.2 for the 1, 2 and 3% gels, respectively, suggesting excellent correlation between the published EEO values and our EOF data. Similar good correlation was reported earlier by Stellwagen [15] in conventional agarose slab gel electrophoresis.

3.2. Effect of linear polymer additives on the electroosmotic flow

Next, the effect of the addition of non-crosslinked polymers, such as (LPA; M_r 700 000–1 000 000), (HEC; M_r 90 000–105 000) and (PEO; M_r 600 000) on the electroosmotic characteristics of 1% low EEO agarose were evaluated. Linear polyacrylamide was reported earlier as providing good hydrophilic surface coating for electroosmotic flow reduction [16]. Derivatized cellulose polymers [17] and polyethylene oxides [18] have also been utilized to reduce electroosmotic flow. As a first approximation, we considered that these linear, uncharged, hydrophilic polymers were capable of appropriate entanglement with the agarose polymer chains. In this way they could provide appropriate shielding of the charged residues and, therefore, suppress the formation of the electric double layer. Fig. 2 shows the effect of the concentration of each of these linear polymers on the electroosmotic characteristics of the agarose–linear polymer composite gels in ultrathin layer electrophoresis. Linear polyacrylamide, hydroxyethyl cellulose and polyethylene oxide were added from 0.2% to 1.0% concentration in 0.2% increments to 1% low EEO ($-m_r = 0.1$) agarose, respectively. The electroosmotic mobilities of the various composition linear polymer–agarose gels were calculated from the migration time of the uncharged, low molecular weight fluorescent marker Bodipy FL hydrazide using Eq. (2), and plotted in Fig. 2. Please note that all mobility values were corrected to a common temperature of 20°C according to the procedure of West [19] with respect to the actual temperature of the thermostated side of the separation compartment (heat sink). In Fig. 2, the LPA plot exhibits a shallow, linear decrease in electroosmotic flow mobility with increasing polymer concentration. Addition of HEC and PEO both resulted in more significant, in the case of HEC even exponential decrease in μ_{eof} with increasing additive concen-

Table 1
Observed electroosmotic mobilities of the various concentration agarose gels in ultrathin layer electrophoresis format[a]

Agarose	EEO ($-m_r$)	1% ($\mu_{EOF} \times 10^{-5}$) ($cm^2/V\ s$)	2% ($\mu_{EOF} \times 10^{-5}$) ($cm^2/V\ s$)	3% ($\mu_{EOF} \times 10^{-5}$) ($cm^2/V\ s$)
Low EEO	0.10	2.93	2.53	2.33
Medium EEO	0.17	4.12	3.61	3.49
High EEO	0.24	4.44	3.61	3.49
Super high EEO	0.33	5.73	5.41	4.93

[a] Abbreviations: EEO ($-m_r$): electroendosmosis; μ_{EOF}: electroosmotic flow mobility.

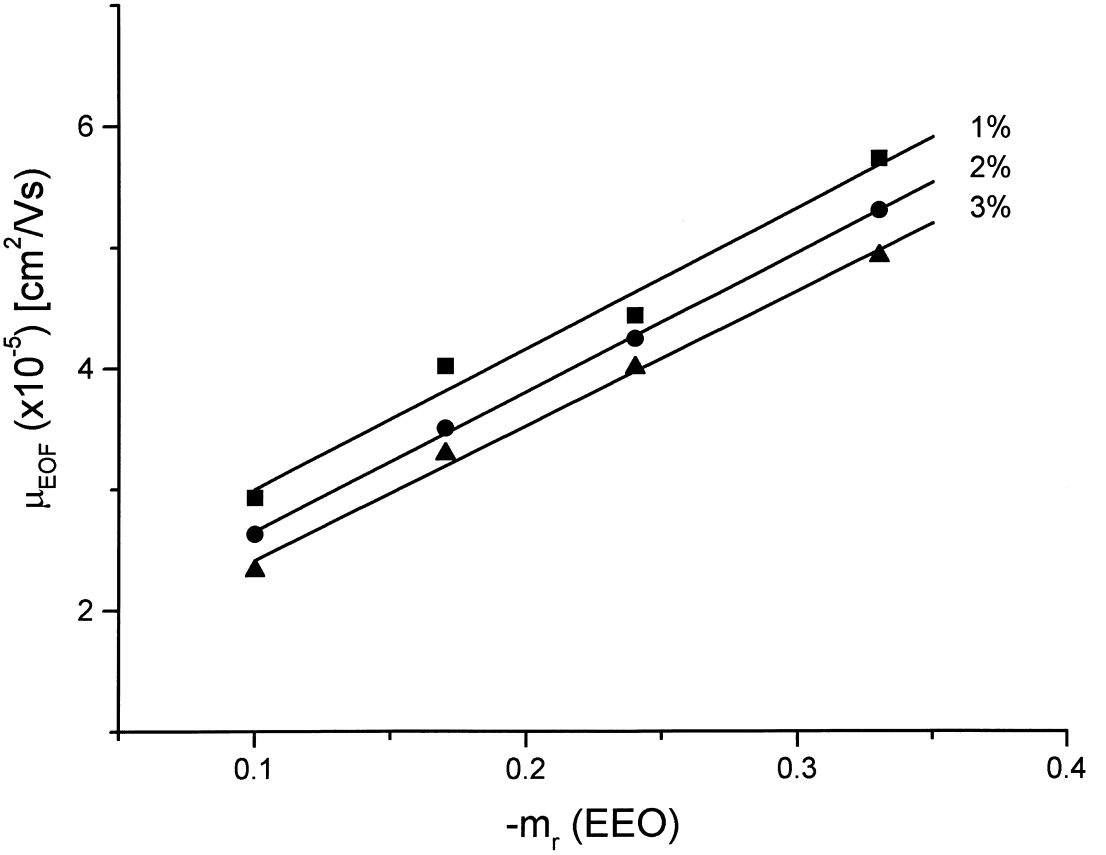

Fig. 1. Correlation of the electroosmotic flow (EOF) mobility measured with various agarose gels in 1, 2 and 3% concentrations in ultrathin layer electrophoresis to the EEO ($-m_r$) values given by the manufacturer. EOF mobility was calculated from the migration time of the neutral, fluorescent marker, Bodipy FL Hydrazide. Conditions: E = 42 V/cm, Injection to read distance (l) = 2 cm, running buffer: 0.5x TBE, gel size: 7.5 cm × 18 cm × 0.19 mm.

tration, suggesting their enhanced effect on EOF suppression. The electroosmotic mobility values were most depressed by the addition of HEC in higher than 0.4% concentration.

3.3. Effect of the electroosmotic flow on the separation of dsDNA fragments

The λ DNA Hind III restriction digest fragments were used as model compounds to evaluate the effect of electroosmotic properties of the various EEO agarose gels during ultrathin layer electrophoresis separation. The sample was injected by membrane mediated loading technology, and the mobilities of the individual DNA fragments were calculated from their respective migration times (l = 6 cm). Again, all mobility values were corrected to the common temperature of 20°C. Fig. 3 shows the double logarithmic plots of the observed mobilities (lower four plots: L, M, H, sH) and the EEO corrected mobility values (upper four plots: l, m, h, sh) of the DNA fragments, as the function of their chain length (base pairs, bp). As one can see, parallel decreasing plots were observed with increasing fragment length for the various EEO agarose gels, exhibiting highest apparent mobilities for the low EEO agarose (L) and lowest apparent mobilities for the super high EEO agarose (sH). This is in agreement with Eq. (1),

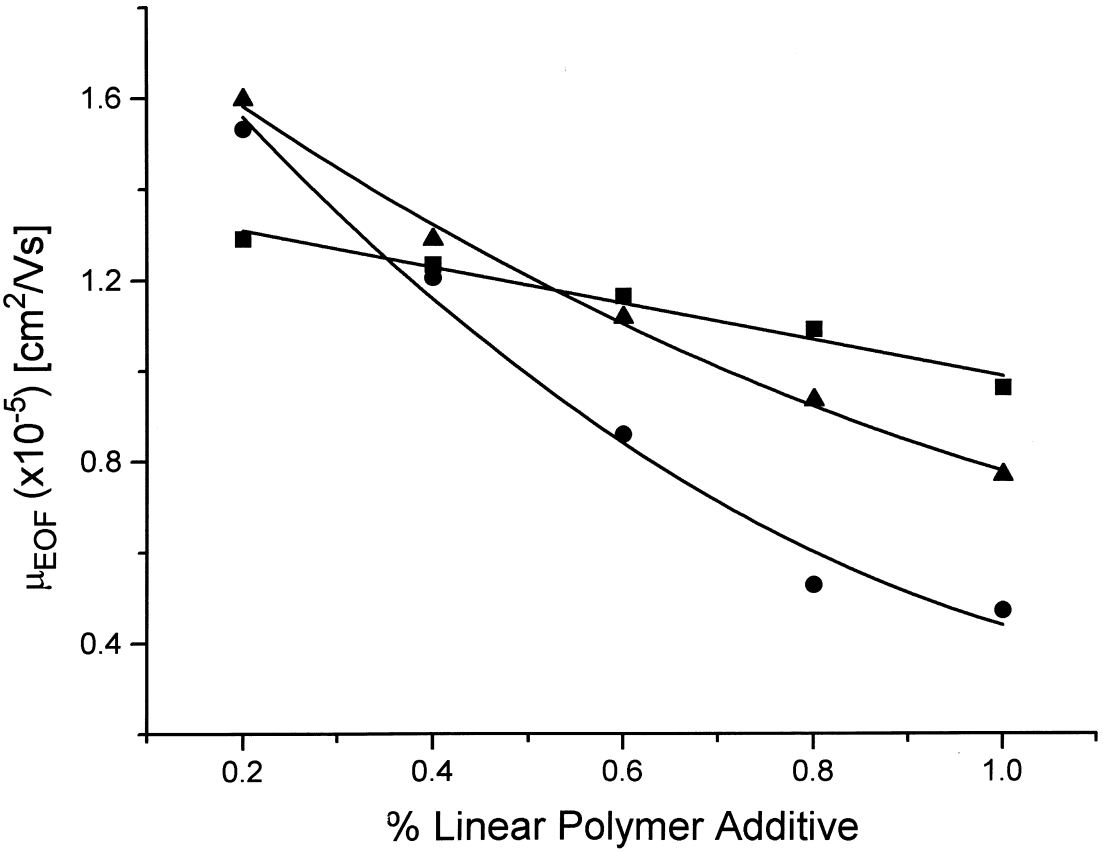

Fig. 2. Effect of the amount of various linear polymer additives on the observed electroosmotic flow mobility in ultrathin-layer electrophoresis. EOF mobility was calculated from the migration time of the neutral, fluorescent marker, Bodipy FL Hydrazide. ■=LPA: linear polyacrylamide (M_r 700 000–1 000 000); ●=HEC: hydroxyethylcellulose (M_r 90 000–105 000); ▲=PEO: polyethylene oxide (M_r 600 000). Conditions are the same as in Fig. 1.

since the super high EEO agarose generates the highest EOF (Table 1), resulting in the slowest apparent mobility of the analyte. When the observed mobility values of the DNA fragments were corrected with the actual EOF values of the respective agarose gels (see values in Table 1), the resulting plots (l, m, h, sh) became very close to, almost overlapping each other. This suggests, that the various EEO gels may have very similar retardation (sieving) charateristics, and probably their respective electroosmotic properties cause the shifts in the apparent mobility values of the solute. In conclusion, our results strongly suggest, that the observed mobilities obtained in ultrathin-layer agarose gel electrophoresis separation of DNA fragments should be corrected with the electroosmotic mobility values of the agarose gels used.

Under ultrathin-layer separation conditions, the electroosmotic properties of the agarose gels may also be responsible for some disruption in the separation performance. This effect is probably enhanced by the high applied electric field strength used in this format (42 V/cm). Fig. 4 compares the separation of the λ DNA Hind III restriction digest fragments using 1% low EEO agarose (A) and the composite matrices of 1% low EEO agarose mixed

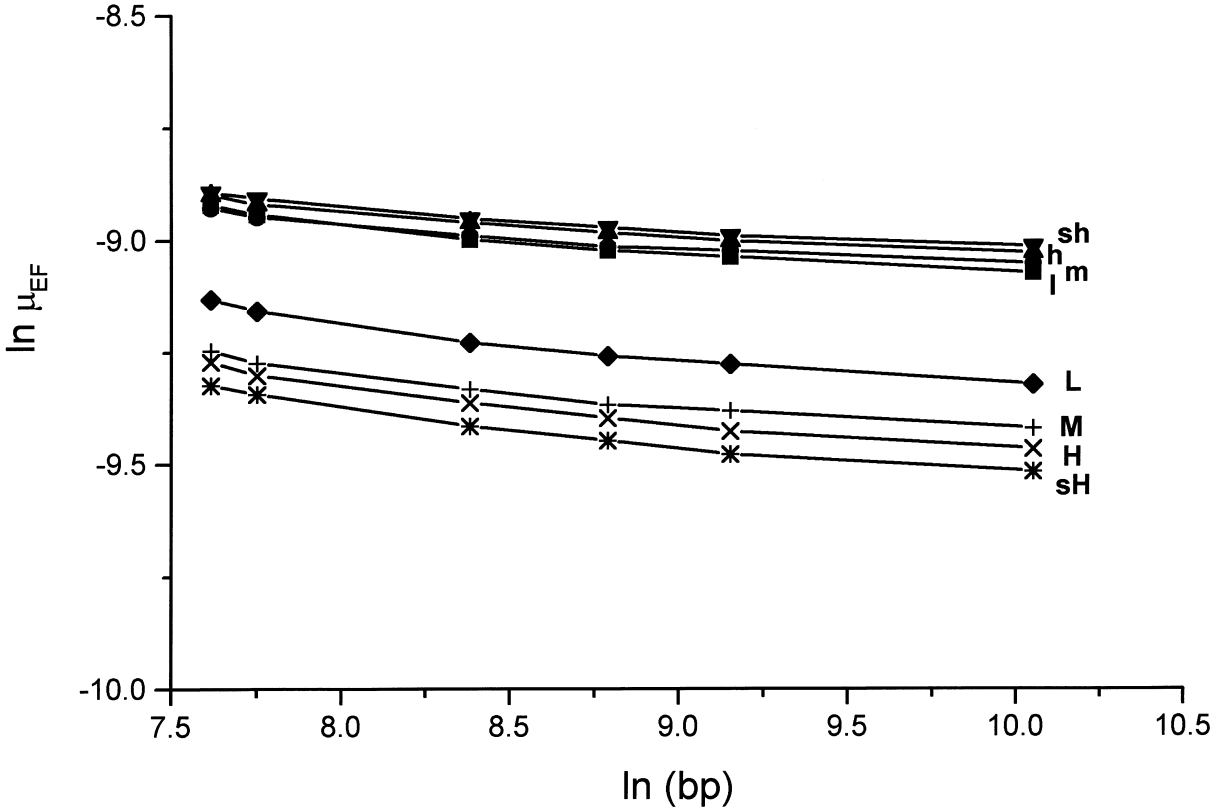

Fig. 3. Double logarithmic plot of the mobility of the λ DNA Hind III restriction fragments as a function of the fragment length, with (plots l, m, h, sh) and without (plots L, M, H, sH) electroosmotic mobility correction with the respective EOF values of the agaroses used. L and l: low EEO, M and m: medium EEO, H and h: high EEO, sH and sh: super high EEO. Conditions: same as in Fig. 1, but the injection to read distance (*l*) was 6 cm and the separation gel contained 50 n*M* ethidium bromide.

with 1% LPA (M_r 700 000–1 000 000) (B); 1% HEC (M_r 90 000–105 000) (C) and 1% PEO (M_r 600 000) (C), respectively. The effect of electroosmotic flow is especially pronounced in panel (A) where the separation of the lower base pair fragments (bands 1, 2 and 3) were apparently disrupted. Interestingly, the higher base pair fragments (bands 4, 5 and 6) were not affected in the same extent. Panels B–D in Fig. 4 clearly depict, that the use of linear polymer additives (LPA, HEC and PEO) can significantly improve the separation performance, probably by reducing the electroosmotic characteristics of the agarose. Please note, that handling of the composite gels is also easier, due to their enhanced rigidity. The overall longer migration times of the DNA fragments in the composite gels are due to the additional retardation contributed by the linear polymer additives. The individual and combined effects of the agarose and linear polymer additives on the mobility and separation performance is subject of further investigation.

Acknowledgements

This work was supported by the US–Hungarian Science and Technology Joint Research Fund, Project JF 654/96 and FKFP 0658/1999. The help of Robert J. Nelson in the preparation of the manuscript is also highly appreciated.

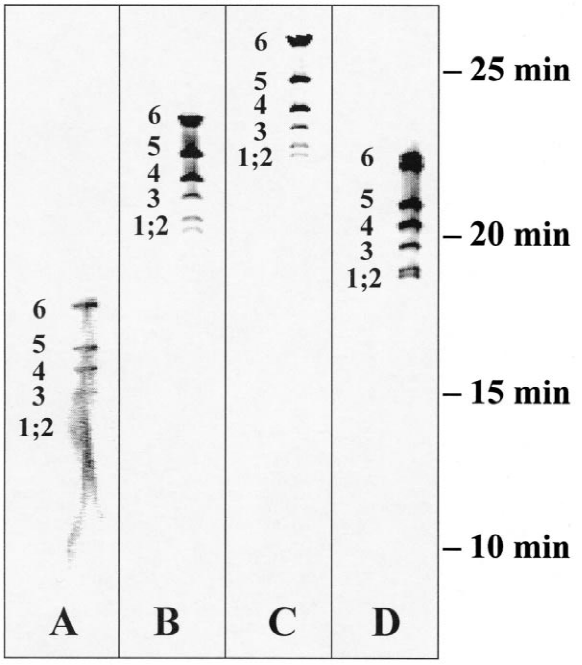

Fig. 4. Separation of λ DNA Hind III restriction digest mixture by ultrathin-layer gel electrophoresis using 1% low EEO agarose (A) and the composite matrices of 1% low EEO agarose mixed with 1% LPA (B), 1% HEC (C) and 1% PEO (D), respectively. Bands: 1=2027; 2=2322; 3=4361; 4=6557; 5=9416; 6=21 130. Conditions: Same as in Fig. 3.

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