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Ultra-thin-layer agarose gel electrophoresis II. Separation of DNA fragments on composite agarose–linear polymer matrices

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Abstract

The effect of hydrophilic linear polymer additives (non-cross-linked polyacrylamide, hydroxyethyl cellulose and polyethylene oxide) on the migration behavior of double stranded DNA molecules, ranging from 200–1000 base pairs, were studied in ultra-thin-layer agarose gel electrophoresis. The detection sensitivity was found to be less than 0.1 ng/band using To-Pro-3 fluorophore labeling and fiber optic bundle-based scanning detection system with a 640 nm red diode laser. Among the various polymers investigated, addition of linear polyacrylamide resulted in the best separation performance (steepest Ferguson plots), while composite gels with hydroxyethylcellulose and polyethylene oxide still exhibited adequate resolving power. Using the composite matrices of 1% agarose–linear polyacrylamide (0.5–3%), 1% agarose–hydroxyethylcellulose (0.2–1%) and 1% agarose–polyethylene oxide (0.2–1%), the mechanism of the separation was found to be in the Ogston sieving regime. Activation energy curves were also plotted based on the slopes of the Arrhenius plots of the various composite matrices, and exhibited decreasing characteristics for the agarose–linear polyacrylamide composite matrix and increasing characteristics for the agarose–hydroxyethylcellulose and agarose–polyethylene oxide composite matrices.

Keywords: Agarose–polymer matrices; Polymer–agarose matrices; DNA

1. Introduction

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 dalton agarbiose polymer units [1]. Its high mechanical strength, even at low gel concentrations, biological inertness and stability in the pH range of 4–9 made agarose a popular separation matrix. Agarose gel electrophoresis based analysis of DNA fragments has been practiced for decades [2]. After the separation process, the resolved bands are usually visualized with some kind of a staining process, such as silver staining or high sensitivity fluorescent dye staining (e.g., ethidium bromide, Sybr Green or Sybr Gold) [3]. These latter ones can even be part of the separation matrix, i.e., gel–buffer system, assuring extremely high detection sensitivity for the separated bands by illuminating the gel with the appropriate light source emitting in the 480–530 nm wavelength range. More recently, dyes in the red excitation regime were also introduced and exhibited excellent signal to noise ratios due to lower background (e.g., very little auto-fluorescence of the glass

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plates). Our group have previously reported on the separation and detection of Cy5-labeled DNA fragments, as well as ''in-migratio'' labeling of dsDNA molecules using the fluorophore complexing agent To-Pro-3 [4]. This latter technique proved to be useful in both providing a better detection limit and also in enhancing the separation selectivity. In order to increase mechanical strength and/or resolving power of agarose based separation matrices, composite gels, containing agarose mixed with other polymers such as cross-linked polyacrylamide have been introduced [5]. More recently, non-cross-linked polyacrylamide [6] and other hydrophilic linear polymers, such as nonionic polysaccharides [7] were successfully employed as composite additives for agarose gels.

Ultra-thin-layer agarose gel electrophoresis is a novel combination of agarose slab gel electrophoresis and capillary agarose gel electrophoresis [8]. The relatively large temperature difference between the gelling and melting temperatures of the agarose matrix (35–40°C vs. 70–90°C) makes possible its use as an effective separation medium even at higher electric field strengths of >40 V/cm, under ultra-thin-layer separation conditions, where extra Joule heat dissipation is efficient [9]. In this paper, we investigated the effects of several linear polymer additives, such as linear polyacrylamide (LPA), hydroxyethylcellulose (HEC) and polyethylene oxide (PEO), on the separation properties of low electroendosmosis (EEO) agarose gels in ultra-thin-layer electrophoresis of double-stranded DNA (dsDNA) ladder standards. The detection sensitivity and the influence of the linear polymer additives on the Ferguson, reptation and Arrhennius plots were evaluated using To-Pro-3 intercalator dye for ''in migratio'' visualization.

2. Materials and methods

2.1. Chemicals

In all the experiments 1% low EEO agarose gel ($-m_r=0.1$) (Sigma, St. Louis, MO, USA) was dissolved in 45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA·Na_2 buffer, pH 8.3 (0.5× TBE). Tris, boric acid and EDTA·Na_2 were obtained from ICN (Costa Mesa, CA, USA), all of 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), polyethylene oxide (PEO) (M_r 600 000) was from Aldrich (Milwaukee, WI, USA). The To-Pro-3 fluorescent dye was purchased from Molecular Probes (Eugene, OR, USA). During the electrophoresis separation experiments, the 100 base pair (bp) DNA ladder (Life Technologies, Gaithersburg, MD, USA) was used, diluted with double deionized water (18 MΩ) to the working concentration of 5–40 ng/μl and stored at −20°C until use.

2.2. Instrumentation

The automated ultra-thin-layer agarose gel electrophoresis system equipped with real time laser induced fluorescent detection was described earlier [10]. This particular system employed a fiber optic bundle-based scanning illumination/detection unit using a 640 nm red diode laser excitation source and avalanche photodiode detection (APD) with a 682±22 nm wide band interference filter. A lens set scanned across the multilane separation platform at 6 cm from the injection site by means of a translation stage and collected the emitted fluorescent light. The APD analog signal was digitized in a micro-controller and 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 plastic buffer reservoirs at both ends. The 100 bp DNA ladder standard was injected by previously discussed membrane mediated loading technology from the cathode side of the platform [11]. The injection side of the ultra-thin separation slab gel had a straight edge with no individual wells. Small amounts of sample (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 higher viscosity and low ionic strength well solution was used to interface between the membrane and the separation matrix [12], 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 100 bp dsDNA ladder standard molecules were fluorescently labeled during the separation process by ''in migratio'' intercalation with To-Pro-3. The intercalator dye was dissolved in the separation gel–buffer system in 2 n*M* concentration.

2.3. Procedures

The appropriate amount of agarose powder was suspended in 0.5× 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 0.5–3.0% with 0.5% increments for LPA and 0.2–1% with 0.2% increments for HEC and PEO, respectively. The composite matrices were kept at 60°C for 10 min before the appropriate amount of staining dye (2 n*M* To-Pro-3) was added. For ultra-thin-layer agarose gel electrophoresis, the preheated separation cassettes (45–50°C) were filled with the melted agarose–additive mixture and after several minutes of cooling/solidification the gel filled cassette was ready. The used gels were replaced in the separation cassette by simply pumping through fresh melted composite matrices after each run. Preheating of the separation cassette helped to prevent premature solidification of the freshly poured gel during the replacement process.

The effective separation length of the agarose gel filled ultra-thin-layer cassette was 6 cm. 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

As we have derived previously [13], the electrophoretic mobility of the migrating dsDNA molecules in ultra-thin-layer agarose gel electrophoresis can be described as:

$$\mu = \text{constant} \cdot e^{-K_R P_{\%}} n^k e^{-E_a/RT} \quad (1)$$

where μ is the apparent electrophoretic mobility, K_R is the retardation coefficient at $P_{\%}$ sieving matrix concentration [14], n is the number of base pairs comprising the DNA molecule, the exponent k represents information about the apparent shape of the DNA molecule during its migration by the influence of the applied electric field [15], E_a is the activation energy of the actual conformation of the viscous separation media, R is the universal gas constant and T is the absolute temperature.

When a complexing agent is present in the gel–buffer system during the separation, e.g., ''in-migratio'' visualization with intercalating dyes for real time detection, an affinity term should be added to Eq. (1):

$$\mu = \text{constant} \cdot e^{-K_R P_{\%}} n^k e^{-E_a/RT} \cdot \frac{1}{1 + K_c[L^{2+}]^m} \quad (2)$$

where K_c is the complex formation constant, L is the equilibration concentration of the ligand (To-Pro-3) and m is the number of the ligand molecules in the DNA/To-Pro-3 complex. $e^{-K_R P_{\%}}$ (term I), n^k (term II), $e^{-E_a/RT}$ (term III) and $1/(1+K_c[L^{2+}]^m)$ (term IV) are referred to as Ferguson, reptation, Arrhenius, and affinity terms, respectively.

Fig. 1 depicts a typical ultra-thin-layer agarose gel electrophoresis separation of the 100 bp DNA ladder using ''in migratio'' fluorophore labeling with To-Pro-3. The amounts of total DNA injected onto the ultra-thin-layer platform ranged from 2.5 to 20 ng. The separation matrix in this instance was a composition of 1% agarose and 1.5% linear polyacrylamide (M_r 700 000–1 000 000) in 0.5× TBE buffer containing 2 m*M* To-Pro-3 dye. As Fig. 1 exhibits, the detection limit in this case was better than 2.5 ng DNA per lane, corresponding to less than 0.1 ng/band. It should be noted, however, that the To-Pro-3 concentration (2 n*M*) in the gel–buffer system was significantly less (50–500-fold) than that of was found earlier to be necessary for appropriate visualization of dsDNA molecules in capillary gel electrophoresis separations, reported by Rampal et al. [16]. One of the most important implications of the ability of using such a low concentration visualization agent, is its very minor effect on the mobilities of the migrating DNA fragments. Over the con-

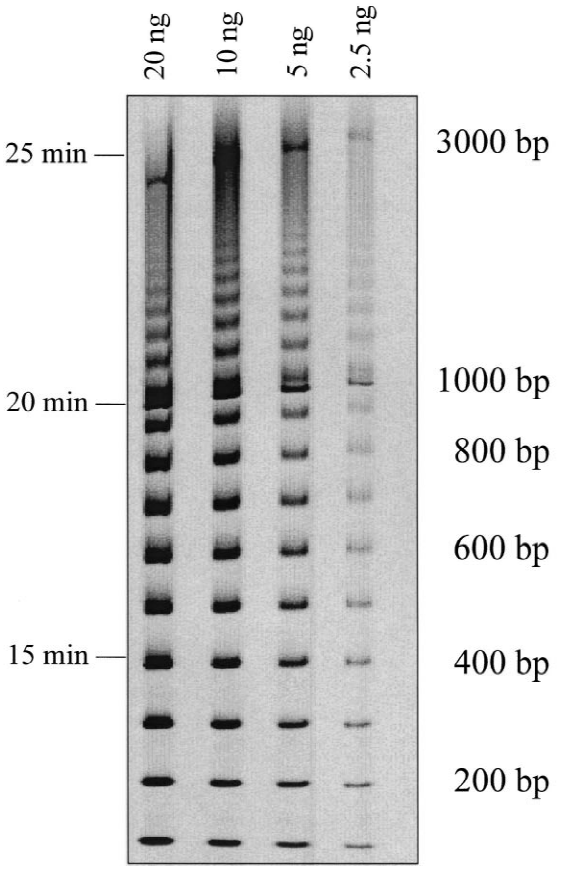

Fig. 1. Detection sensitivity of the ultra-thin-layer agarose gel electrophoresis–laser-induced fluorescent scanning detection system using To-Pro-3 ''in migratio'' labeling. Lanes correspond to the injected amount of the 100 bp DNA sizing ladder: 20; 10; 5 and 2.5 ng/lane. Separation conditions: gel: 1% agarose, 1.5% LPA (M_r 700 000–1 000 000) in 0.5× TBE (pH 8.3), 2 n*M* To-Pro-3; separation buffer: 0.5× TBE (pH 8.3); separation voltage: 750 V; effective separation length: 6 cm; gel thickness: 190 μm; temperature: ambient; sample loading: membrane mediated: 0.5 μl/tab.

centration range of 0.5–25 n*M* To-Pro-3, dissolved in the gel–buffer system, we observed no alteration on the migration times of the separated bands (data not shown). Please note that even in the lane where the smallest amount of DNA was injected (2.5 ng), the DNA/dye ratio was still only 150 bp/dye-molecule. This represents significantly less dye molecules in the complex than that was defined earlier as having a noticeable influence on the apparent mobility of the migrating DNA molecules (40 bp/dye) [17]. The slight increase in DNA mobilities in the lanes where higher concentration samples were injected (20 ng and 10 ng DNA/lane) were due to the common overloading problem described earlier by Johnson et al. [18].

3.1. Effect of the linear polymer additives on the electrophoretic mobility of dsDNA fragments

To evaluate the effect of hydrophilic linear polymer additives on the electrophoretic mobility of dsDNA molecules, increasing amounts of linear polymers were added to 1% low EEO ($-m_r=0.1$) agarose gels. Fig. 2 exhibits the Ferguson plots for the composite gels of agarose–linear polyacrylamide (Fig. 2a), agarose–hydroxyethylcellulose (Fig. 2b) and agarose–polyethylene oxide (Fig. 2c). Please note that the observed electrophoretic mobilities were corrected with the individual electroosmotic mobilities of the corresponding agarose–linear polymer additive compositions, defined earlier [19]. For better comparability, we used the same range of ln μ at the *y*-axis in Fig. 2a–c. As Fig. 2a depicts, when 0.5 to 3.0% (in 0.5% increments) linear polyacrylamide (M_r 700 000–1 000 000) were added to 1% agarose in ultra-thin-layer electrophoresis, nonlinear convex plots were attained, similar to that obtained earlier with regular slab [20] and ultra-thin-layer [13] agarose gels. A somewhat more interesting situation can be observed in Fig. 2b, where hydroxyethylcellulose (M_r 90 000–105 000) was used as composite additive in 1% agarose, ranging from 0.2 to 1.0% in 0.2% increments. It should be noted, that higher than 1% HEC additive concentration increased the viscosity of the composite matrix beyond the ease of filling and replacing the gel in the separation cassette. As one can see, at higher additive concentrations (>0.4%) the plots have similar convex shapes as the plots in Fig. 2a, however, at lower additive concentrations (<0.4%) the curvature is somewhat disturbed. We do not have an adequate explanation of this phenomenon yet, but it is under further investigation in our laboratory.

When polyethylene oxide (M_r 600 000) was used as composite additive to 1% agarose gel (0.2 to 1.0% in 0.2% increments), the resulting Ferguson plots exhibited almost regular linear behavior (Fig. 2c).

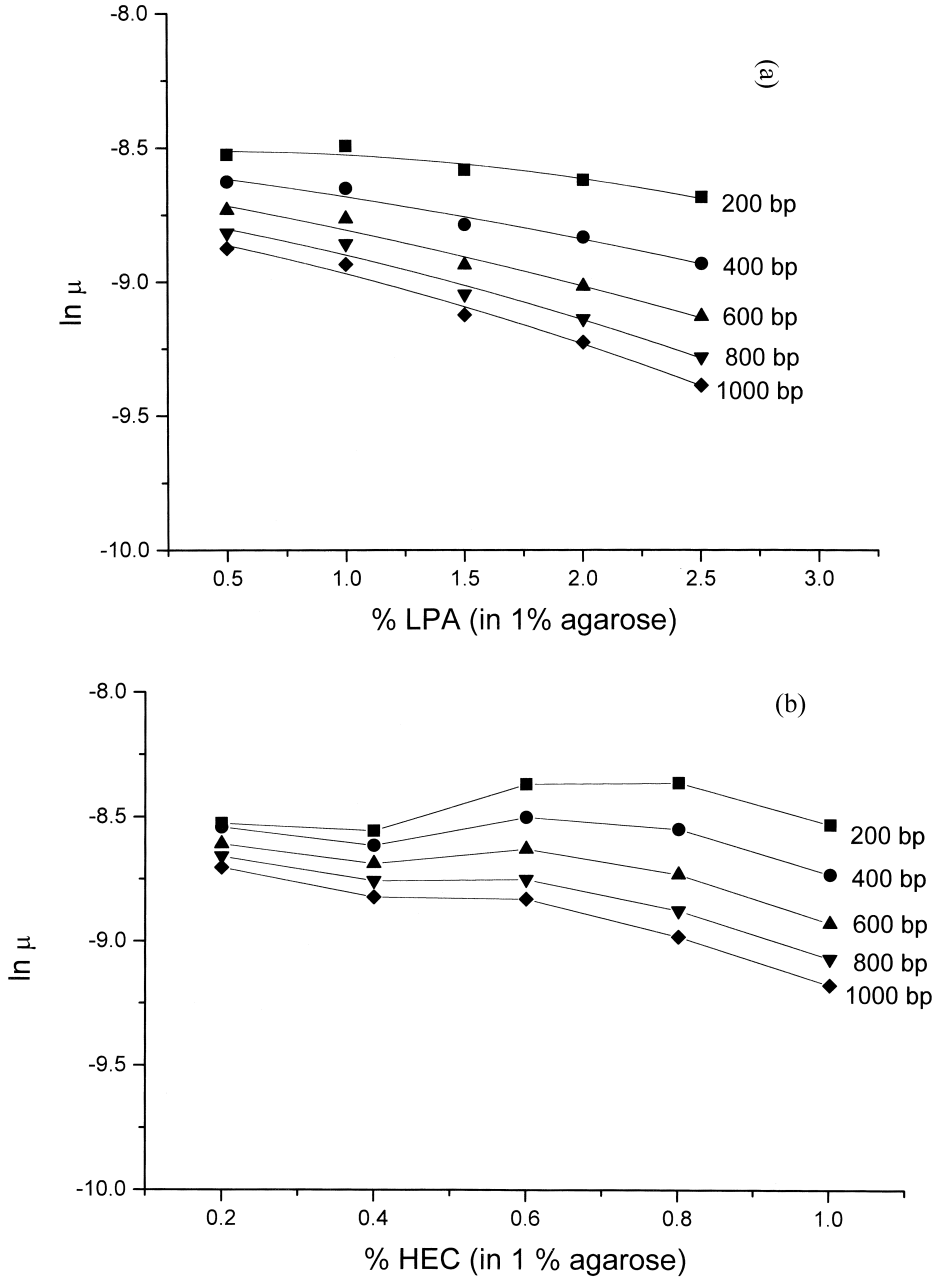

Fig. 2. Ferguson plots of the 100 bp DNA sizing ladder fragments in ultra-thin-layer agarose gel electrophoresis using LPA (a), HEC (b) and PEO (c) composite additives. The observed mobility values were corrected with the EOF values of the individual composite formulations. Numbers on the plots correspond to the chain length of the individual DNA molecules.

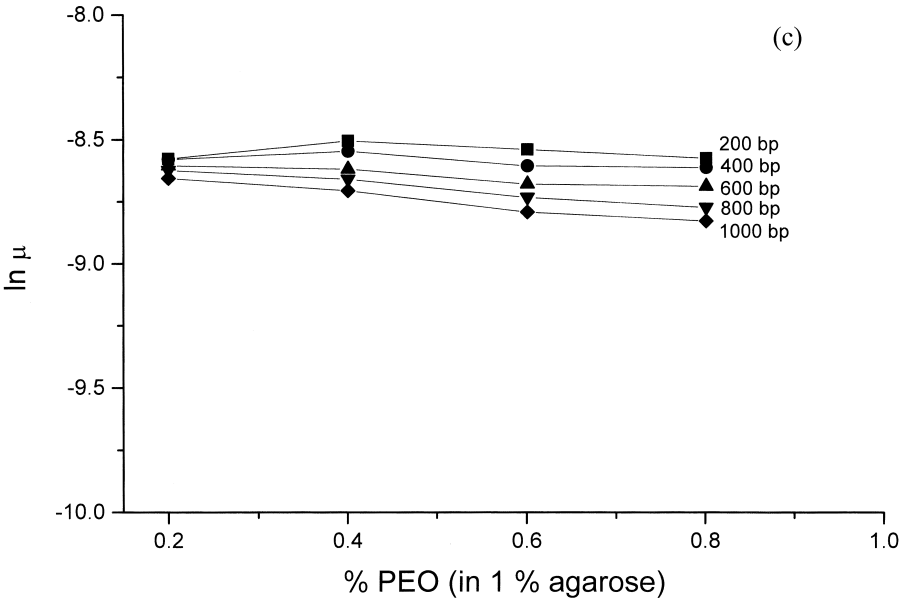

Fig. 2 (*continued*).

The slopes of these plots were quite shallow, suggesting no efficient sieving characteristics associated with this polymer as composite additive material at the concentration range used. When PEO was added in higher than 1% concentration, the solidification process of the composite agarose matrix was not sufficient, probably due to the increasing effect of the PEO on the hydrogen bridge formation, that is necessary to result in a solid agarose structure. Considering the ease of gel pouring, mechanical stability and the overall sieving efficiencies of the composite gels discussed above, linear polyacrylamide was found to be the most favorable additive.

Fig. 3 depicts the so-called ''reptation'' plots of the natural logarithms of relative mobility values vs. the natural logarithms of the chain lengths of the solute molecules [21]. The agarose–linear polymer composite matrices tested in this experiment contained 1.5% LPA, 0.6% HEC and 0.4% PEO, respectively. The free solution mobility values (μ_0) were obtained by extrapolation of the corresponding plots in Fig. 2 to zero gel concentration. The average slope values of the individual plots (k in term II, Eq. (2)) were found to be -0.339, -0.251 and -0.125 for the LPA, HEC and PEO containing composite gels, respectively. The fact, that these values were significantly less than unity, suggests that the migration of DNA fragments in the 200–1000 bp range using ultra-thin-layer agarose–linear polymer (LPA, HEC and PEO) composite gels can be appropriately characterized by the Ogston sieving theory [22].

3.2. Effect of the separation temperature

The Arrhenius plots of natural logarithm of mobility vs. reciprocal absolute temperature for the various composite matrices are shown in Fig. 4. The effect of the separation temperature on the mobility values of the DNA ladder standard fragments was examined between 20 and 50°C for the agarose–LPA and agarose–HEC composite gels. In the instance of agarose–PEO matrices, due to mechanical stability issues discussed above, the temperature range between 20–40°C was evaluated. The observed mobility values were corrected with the temperature influenced viscosity change of the solvent (1.1%/K) [23] and with the temperature dependent EOF changes (20%/10 K) [24]. Fig. 4a depicts the Arrhenius plots when 1.5% LPA was used as composite additive to 1% agarose. As expected, the mobility values of the DNA fragments increased with

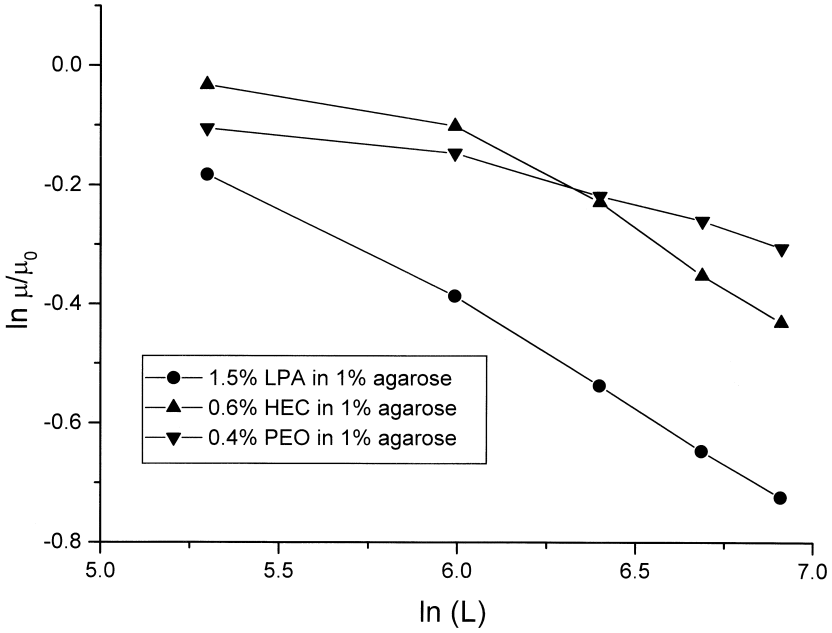

Fig. 3. Reptation plot of the natural logarithm of relative electrophoretic mobility vs. the natural logarithm of chain length of the DNA fragments using 1.5% LPA (●), 0.6% HEC (▲) and 0.4% PEO (▼) containing composite agarose gels. The observed mobility values were corrected with the EOF values of the individual composite formulations.

elevated temperatures. Similar plots were obtained when 0.6% HEC and 0.4% PEO additives were added to 1% agarose, as shown in Fig. 4b and c, respectively. It has been reported that the migration of DNA fragments during gel electrophoresis is an activated process [25]. The activation energy of the actual separations (E_a, term III, Eq. (2)) were calculated from the slopes of the Arrhenius plots in Fig. 4, as we discussed in our previous paper [13]. The activation energy values were plotted as a function of the chain length of the separated DNA fragments in Fig. 5 for the LPA, HEC and PEO containing agarose matrices, respectively. The agarose–LPA composite separation matrix exhibited a decreasing function with increasing DNA fragment length, similar to that of Lu et al. observed in capillary electrophoresis of DNA sequencing fragments using crosslinked polyacrylamide gels [26]. On the other hand, when HEC or PEO was added to 1% agarose gel, the activation energy values increased with increasing fragment length, similar to that was observed earlier by the same group in capillary electrophoresis using non-crosslinked acrylamide gels [25], and by our group in ultra-thin-layer gel electrophoresis using plain agarose gels [13].

4. Conclusion

This paper discussed the effects of hydrophilic linear polymer additives on the migration properties of dsDNA molecules (200–1000 bp) in ultra-thin-layer agarose gel electrophoresis using To-Pro-3 fluorophore dye for ''in migratio'' visualization. The detection sensitivity was found to be less than 0.1 ng/band for the fluorophore labeled fragments, which value was not apparently influenced by the various linear polymer additives used in the experiments. It is important to note, that only 2 n*M* To-Pro-3 was used in the separation gel–buffer system, which was more than two-orders of magnitude less that was suggested earlier in conventional slab gel and capillary gel electrophoresis based separation methods. The separation mechanism with the composite matrices of agarose–LPA, agarose–HEC and agarose–PEO were all found to follow the

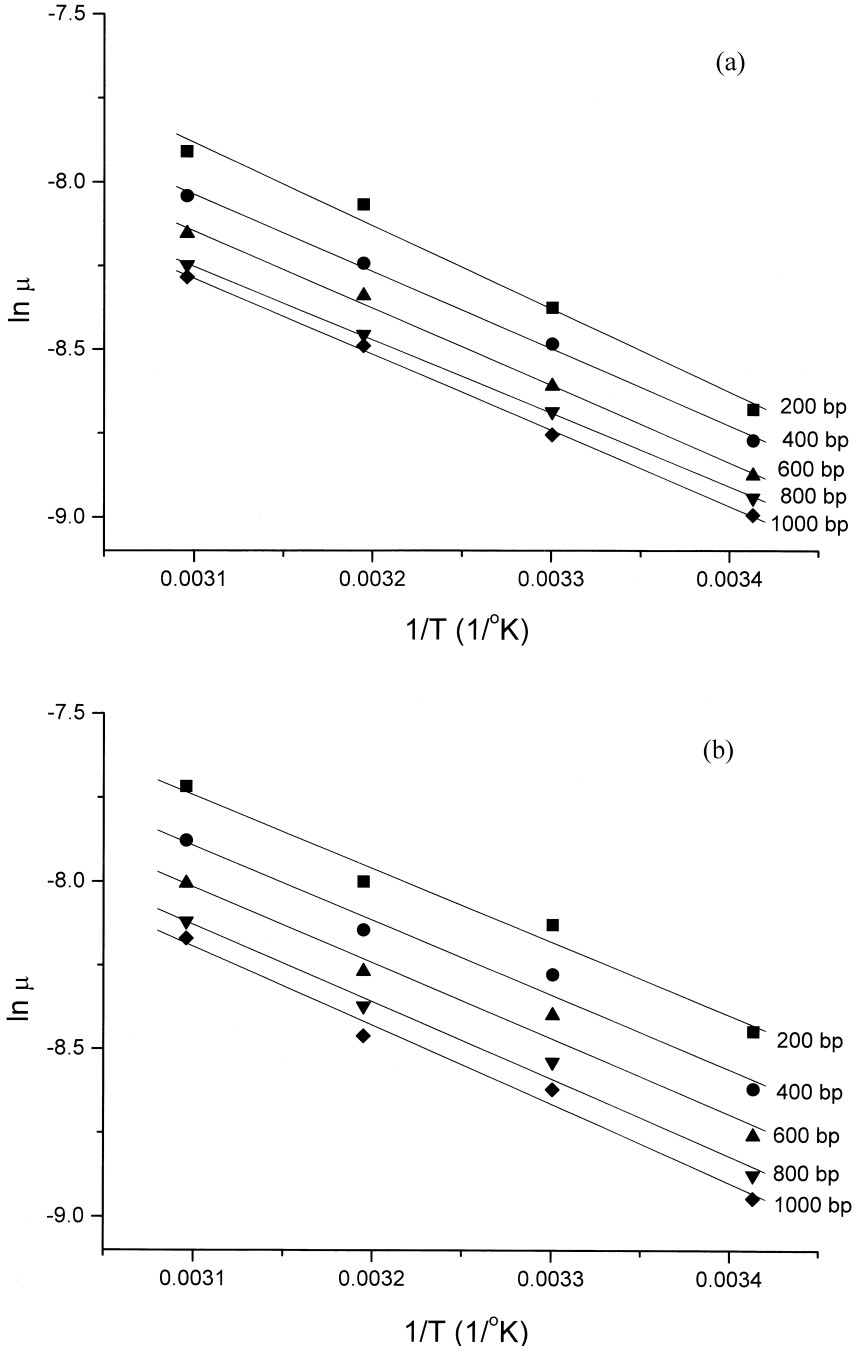

Fig. 4. Arrhenius plots of the logarithmic mobility and the reciprocal absolute temperature for the various size DNA fragments in ultra-thin-layer agarose gel electrophoresis using 1.5% LPA (a), 0.6% HEC (b) and 0.4% PEO (c) additives. The observed mobility values were corrected for temperature-mediated viscosity and EOF changes. Numbers on the plots correspond to the chain length of the individual DNA molecules.

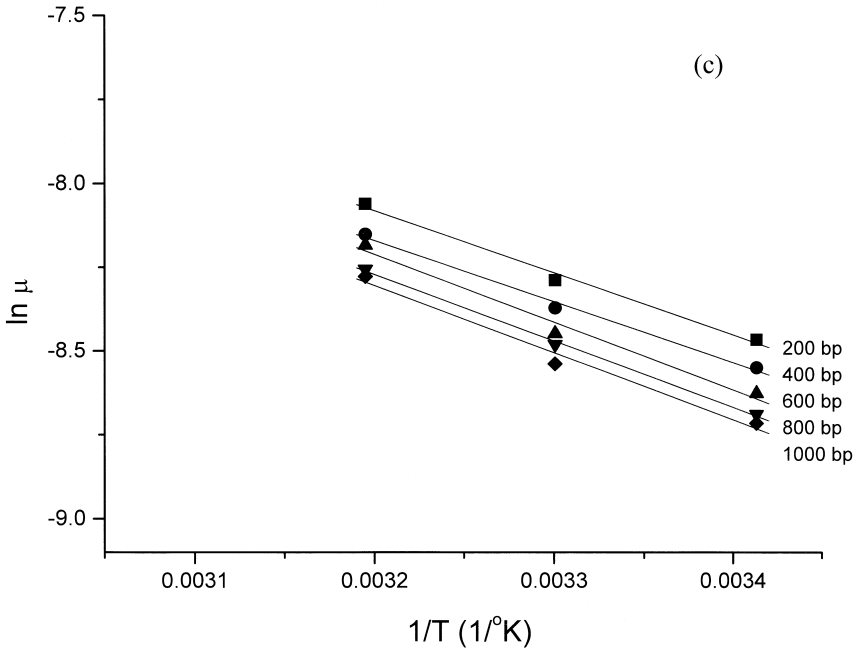

Fig. 4 (*continued*).

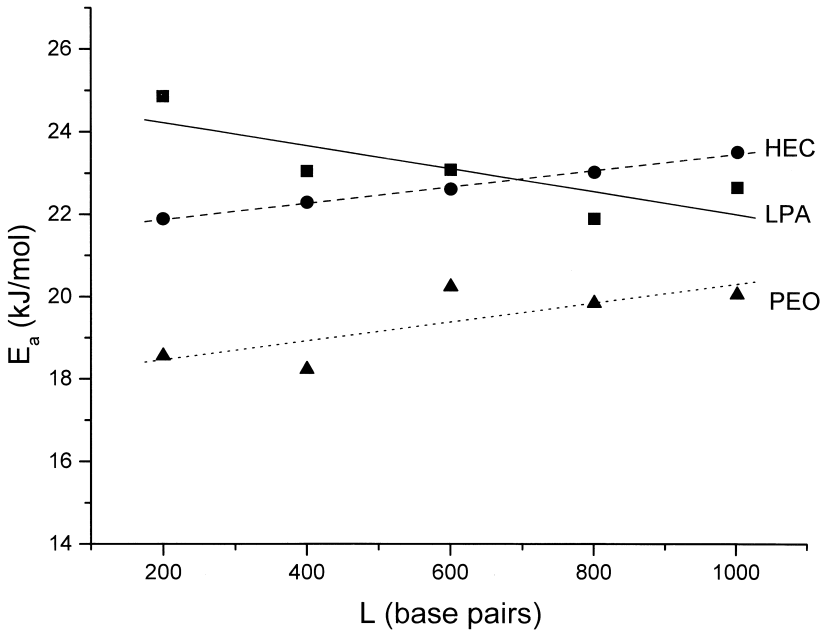

Fig. 5. Activation energy of the sieving matrix as a function of the fragment length of the solute for 1.5% LPA (■) (r^2=0.81), 0.6% HEC (●) (r^2=0.99) and 0.4% PEO (▲) (r^2=0.79) composite agarose gels.

Ogston sieving model in the molecular mass range of the DNA fragments investigated. Using LPA as composite additive resulted in a decreasing slope of the activation energy vs. fragment length plot, very similar to that reported earlier for non-crosslinked linear polyacrylamide, suggesting distortion in the polymer fibers in allowing the passage of the DNA fragments. In the case of using HEC and PEO as composite additives, the activation energy increased with increasing fragment length, thus, exhibiting strong correlation to that which was observed earlier with regular agarose and crosslinked polyacrylamide gels, i.e., suggesting higher activation energy requirement for the separation of longer DNA fragments.

Acknowledgements

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