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The effects of polymer properties on DNA separations by capillary electrophoresis in uncross-linked polymer solutions

Low-viscosity, aqueous solutions of hydrophilic linear polymers have been shown to be useful for the separation of DNA restriction fragments by capillary electrophoresis (CE). However, the choice of polymer type, size, and concentration remains largely empirical, because the mechanism of high-field electrophoretic DNA separations in polymer solutions is not well understood. To assist in elucidating the mechanism of DNA separation, we experimentally investigated the effects of polymer properties such as stiffness (persistence length), average molecular mass, polydispersity, and hydrophilicity on the separation of DNA ranging from 72 bp to 23 kbp. This was accomplished by comparing the results of DNA separations obtained by counter-migration CE in dilute and semidilute solutions of linear polyacrylamide (PAA), hydroxyethylcellulose (HEC), and hydroxypropylcellulose (HPC) polymers of several different average molecular masses.

1 Introduction

Because crosslinked polyacrylamide gel-filled capillaries are unstable under the high electric fields which are typically employed in CE, uncross-linked polymer solutions are rapidly becoming the CE separation medium of choice for both single-stranded and double-stranded DNA fragments. For example, DNA sequencing requires the single-base resolution of small, single-stranded DNA sequencing fragments, and this can be accomplished by CE in denaturing solutions of long-chain, linear polyacrylamide (9 % T) [1, 2]. Other ubiquitous techniques in molecular biology, such as the restriction mapping of cloning vectors and their inserts [3], and the analysis of polymerase chain reaction (PCR) products [4], routinely require the size separation of double-stranded DNA fragments which range in size from 50 bp to 25 kbp. The CE separation of double-stranded DNA molecules in this size range is best accomplished in polymer solutions less concentrated than those which are necessary to resolve DNA sequencing fragments. Several different types of polymers are useful for such separations. To date, researchers have used at least twelve different water-soluble polymers as DNA separation media for CE, including methyl cellulose [5–8], hydroxyethylcellulose (HEC) [9–13], hydroxypropylcellulose (HPC) [14], hydroxypropylmethylcellulose [14–18], polyethylene glycol [15, 19], galactomannan [19], and polyvinyl alcohol [16, 19, 20], as well as liquified agarose [21], linear polyacryloylaminoethoxyethanol [22], and linear polyacrylamide (PAA) [18, 20, 23–27].

Researchers have attempted electrophoretic DNA separations in such a wide variety of polymers simply because the relative importance of polymer properties such as persistence length, hydrophilicity, molecular mass, and polydispersity, and their impact on DNA separation, are not well understood. Even less well understood is the mechanism of electrophoretic DNA separation in uncross-linked polymer solutions. A recent study by Shi et al. [28] has shed some light on DNA conformational dynamics during electrophoresis in HEC solutions. In this work, very large, fluorescently dyed DNA molecules (225 kbp-1.9 Mbp) were visualized by epifluorescence videomicroscopy while undergoing electrophoresis in HEC solutions. Over the entire concentration range of very dilute to highly entangled HEC solutions, the DNA molecules are seen to transiently adopt U- and V-shaped conformations, as if they were retarded by interaction with one or more uncharged HEC chains, with one primary point of association on the DNA chain. The lifetime and degree of extension of these U- and V-shaped conformations increases with increasing HEC concentration. In less concentrated HEC solutions, the apex of the U or V shape is seen to move rapidly in the field direction. This type of conformational behavior is not consistent with pore-based models of DNA gel electrophoresis, which postulate predominantly end-on motion of the DNA chains.

Our research [11, 29] has shown that the assumption that DNA separation can occur only in entangled polymer solutions [30] by DNA "sieving" through "dynamic pores" [14] is incorrect. Instead, DNA separation will occur even in very dilute, unentangled polymer solutions, a concentration regime for which pore-based models are discarded as inapplicable [29]. For DNA separation in dilute polymer solutions, we postulated a separation mechanism based on DNA entanglement and subsequent dragging of the uncharged HEC chains during electrophoresis of DNA [11, 29], a physical picture which was

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later supported by the videomicroscopy experiments of Shi *et al.* [28] and quantitatively modeled by Hubert and Slater [31].

Although the studies of Shi *et al.* [28] have afforded us a glimpse of the conformational dynamics of DNA molecules during electrophoresis in uncross-linked polymer solutions, systematic experimental data on DNA electrophoretic mobility in various polymer solutions remain an important source of information on the separation mechanism. A few recent studies have compared the DNA separations which are obtained with polymers of different types and/or molecular masses, including those of Barron *et al.* [11, 29], Kleemiß *et al.* [20], Chrambach *et al.* [16], Baba *et al.* [14], and Figeys *et al.* [18]. However, the effects of polymer persistence length (*i.e.*, chain stiffness) and sample polydispersity for the resolution of dsDNA, especially in ultradilute polymer solutions, have not been explicitly investigated. In this work, we address these questions, with a special interest in using these data to make inferences about the mechanism of DNA separation in polymer solutions. In previous work, we described the dependence of DNA electrophoretic mobility on DNA size and polymer concentration for solutions of short-chain and long-chain HEC [29]. Here, we quantitate DNA mobility as a function of DNA size and polymer concentration for three different samples of HPC and two different samples of linear PAA, for comparison to data we obtained in past work with HEC [29]. For these HPC and PAA samples, we report the entanglement threshold concentrations that we have determined from viscosity *vs.* concentration data, and compare them to the minimum polymer concentration required for DNA separation. We give further consideration to the importance of polymer type and concentration for the separation of DNA between 72 bp to 23 kbp , a typical size range for several important molecular biology applications.

2 Materials and methods

2.1 Instrumentation

The CE apparatus employed in these studies employs a single fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 50 cm in length (35 cm to the detector), with 51 μm ID and 360 μm OD, and without internal coating. The capillary connects the anodic reservoir with the electrically grounded cathodic reservoir. A high-voltage power supply with a 30000 V capacity (Gamma High Voltage Research, Ormand Beach, CA, USA) is used to drive electrophoresis. Current is measured over a 1 kΩ resistor in the return circuit of the power supply, using a digital multimeter (Model 3465B, Hewlett-Packard, Palo Alto, CA, USA). On-column detection is by UV absorbance at 260 nm, using a modified variable-wavelength detector (Model 783, Applied Biosystems, Foster City, CA, USA). Data are collected using an integrator (Model 3390, Hewlett-Packard, Palo Alto, CA, USA).

2.2 Chemicals and polymers

A nonstoichiometric mixture of λ-*Hind*III and ΦX174. *Hae*III restriction fragments (λ-*Hind*III fragments are

present at a lower concentration, so that peaks of the larger DNA fragments in this digest will not dwarf those of the ΦX174-*Hae*III digest) was obtained from Pharmacia LKB Biotechnology (Alameda, CA, USA) at a total concentration of 500 μg/mL. Mesityl oxide (Aldrich Chemical Co., Milwaukee, WI, USA) was used as a neutral marker to quantitate the average electroosmotic flow velocity. The buffer used in all experiments was 89 mM Tris, 89 mM borate, and 5 mM EDTA (1× TBE) with a pH of 8.15 (all buffer reagents purchased from Sigma Molecular Biology, St. Louis, MO, USA). Measured amounts of the various polymers were added to prefiltered buffer solutions, which were vigorously shaken by hand, and then mixed for 24 h by tumbling (mechanical stirring sometimes led to incomplete dissolution). Successive dilution was used to make extremely dilute solutions. All concentrations given are on a % w/w basis. Three different (number-average) molecular mass fractions of HEC were used. An HEC sample with a manufacturer-determined number-average molecular mass of $M_n \cong$ 90 000–105 000 g/mol was obtained from Polysciences, Inc. (Warrington, PA, USA). The weight-average molecular mass of this sample was determined in our laboratory by low-angle laser light-scattering to be $M_w \cong$ 1 315 000 g/mol. Another HEC sample with a number-average molecular mass of $M_n \cong$ 438 800 g/mol was donated by the Aqualon Co. (Wilmington, DE, USA) and was designated Natrosol 250H by the manufacturer. The number-average molecular mass of this Natrosol product was previously determined by Sperry [32]. We determined the weight-average molecular mass of this sample to be $M_w \cong$ 1 760 000 g/mol, using low-angle laser light scattering. Three different HPC samples were obtained from Scientific Polymer Products (Ontario, NY, USA), having manufacturer-specified weight-average molecular masses of $M_w \cong$ 100 000 g/mol, $M_w \cong$ 300 000 g/mol, and $M_w \cong$ 1 000 000 g/mol. Two different linear PAA samples were obtained from Polysciences, Inc. One sample was unfractionated with respect to molecular mass, and had a manufacturer-specified weight-average molecular mass in the range $M_w \cong$ 700 000–1 000 000 g/mol. The other polyacrylamide sample was fractionated and well-characterized with respect to molecular mass, and was certified to have a weight-average molecular mass of $M_w \cong$ 1 140 000 g/mol and a number-average molecular mass $M_n \cong$ 456 000 g/mol.

2.3 Counter-migration CE

The technique used in this work is called "counter-migration CE" because DNA is injected at the anodic end of an uncoated capillary, and during electrophoresis is pulled towards the detector by electroosmotic flow in the cathodic direction [33]. To clean the fused silica surface of the capillaries and ensure strong, constant electroosmotic flow to drive the analytes past the detector, each new, uncoated capillary was treated with 1 M NaOH for several hours and then rinsed with deionized water before its first use. Thereafter, before each refilling of the capillary with a given polymer solution, the uncoated inner capillary wall was conditioned first with 1 M NaOH for 10 min, followed by 0.1 M NaOH for 10 min, doubly-distilled deionized water for 10 min, and finally with the

electrophoresis buffer (containing dissolved polymer) for 15 min. (After this treatment, an unlimited number of consecutive runs could be accomplished in such polymer-solution filled capillaries without contamination, loss of DNA resolution, or loss of reproducibility of measured DNA mobilities.) Samples were introduced to the anodic end of the capillary by applying a vacuum of 1–3 in Hg (3386–10159 Pa) for a specific time which depended on the buffer viscosity, to introduce approximately 3 nL (3 × 10^{-6} cm^3) of sample for each run. After the sample was drawn into the capillary, the anodic end of the capillary was replaced in the electrophoresis buffer, together with the anodic electrode, and the voltage of 265 V/cm was applied. The uncoated capillary was enclosed in a plexiglass box and surrounded by convected air at a temperature of 30.0 ± 0.1°C during all experiments. DNA samples containing a mixture of λ-*Hin*dIII and ΦX174-*Hae*III restriction fragments were preheated for 5 min at 65°C and then stored on ice (λ-*Hin*dIII restriction fragments of 4361 bp and 23130 bp have cohesive termini, or "sticky ends"). All DNA samples were pre-mixed with 0.5 μL of a 1:1000 mesityl oxide/TBE solution, and injected without dilution. The average electroosmotic velocity was measured for each electrophoresis experiment, using the time it took the neutral species, mesityl oxide, to move from the entrance of the capillary to the detection window. Absolute electrophoretic mobilities were calculated by subtracting the electroosmotic mobility from the apparent electrophoretic mobility of the DNA band, since the electroosmotic flow and the electrophoresing DNA molecules move in opposite directions [33, 34]. In the electropherograms, peaks were identified by integration of peak areas. A representative plot of peak area as a function of the number of DNA base pairs for the ΦX174-*Hae*III digest can be found in [11]. For the mixed DNA sample that we used, the peaks for the λ-*Hin*dIII digest and the ΦX174-*Hae*III digest were integrated separately, because the former restriction digest is present at a lower concentration than the latter.

2.4 Viscometry

An automated Ubbelohde-type capillary viscometer (Schott Geräte, Hofheim, Germany), controlled by a desk-top PC and thermostated at 25°C in a water bath (Model H-1 High Temperature Bath, Cannon Instrument Co., State College, PA), was used to measure the viscosities of the polymer solutions.

3 Results and discussion

3.1 Establishment of polymer molecular mass distributions and experimental determination of the entanglement thresholds for HEC, HPC, and PAA samples

To provide a baseline for examining the counter-migration CE results we obtained in the various polymer solutions, we begin by establishing the entanglement threshold concentration of each of the seven different polymer samples which we used as DNA separation media. The
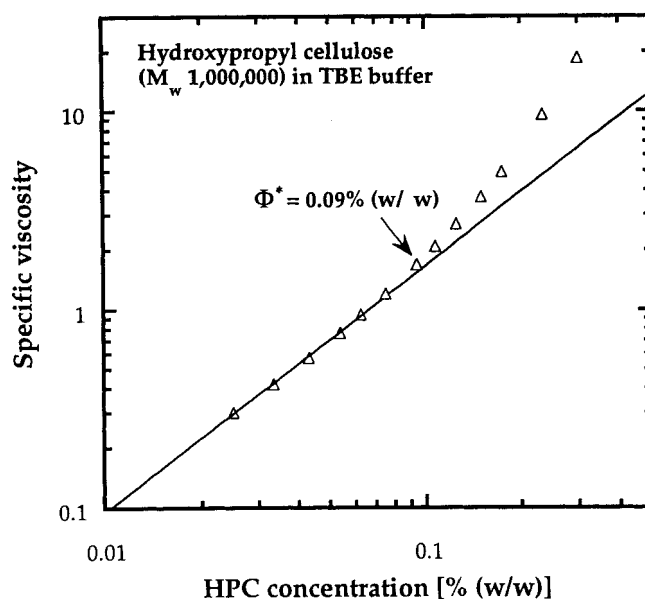

Figure 1. A log-log plot of the specific viscosity of an aqueous polymer solution as a function of polymer concentration. The point of departure of the data from linearity is the approximate concentration at which an entangled polymer network is formed within the solution. In this case, the polymer is HPC, with a weight-average molecular mass of about 1 000 000 g/mol, dissolved in an 89 mM Tris – 89 mM borate – 5 mM EDTA buffer, pH 8.15. The entanglement threshold concentration, Φ*, is approximately 0.09% w/w HPC. Viscosity measurements were taken at 25°C with an automated Ubbelohde viscometer (see Section 2.4).

entanglement threshold concentration of a polymer solution (Φ*), is the approximate concentration above which interaction between polymer chains begins to affect bulk solution properties such as viscosity. Experimentally, Φ* can be estimated from a log-log plot of solution viscosity *vs.* polymer concentration [35]. In a dilute solution there is no strong interaction between solvated polymer molecules, and physical properties change in direct proportion to polymer concentration. Hence, the slope of the log viscosity *vs.* log concentration plot is constant at approximately 1.0. If the polymer chains are of sufficient length, inter-chain entanglements can occur at higher concentrations. The formation of an incipient entangled polymer network in solution [35] is evidenced by a significant increase in the slope of the log viscosity *vs.* log concentration curve. The entanglement threshold concentration is a unique property of a given polymer/ solvent system, which is strongly dependent on the average molecular mass of the polymer sample. The length dependence of Φ* may range from $N^{-0.8}$ for extremely flexible polymers [36] to $N^{-1.2}$ for stiff, hydrophilic cellulose derivatives (where N is the number of monomers in the polymer chain) [11]. Figure 1 shows a log-log plot of solution viscosity *vs.* polymer concentration for one of the HPC samples used, for which $M_w \approx$ 1 000 000 (hereafter referred to as HPC 1M). Qualitatively similar plots were obtained for the other six samples. The molecular mass distributions (to the extent that they are known) and the entanglement threshold concentrations of the polymer samples used in this study are summarized in Table 1. Each polymer sample is referred to in this paper by its abbreviated name, as shown in Table 1. One of these polymer samples, the

Table 1. Molecular mass distributions and entanglement threshold concentrations of commercial polymer samples used for DNA separations

Polymer type and abbreviation	Weight-average molecular mass M_w (g/mol)	Number-average molecular mass M_n (g/mol)	Polydispersity M_w/M_n	Entanglement threshold concentration % w/w
HEC 139K[a]	138 581[a,b]	24 000–27 000[c]	5.1	1.80
HEC 1.32M[a]	1 315 000[a,b]	90 000–105 000[c]	12.4	0.37
HEC 1.76M	1 760 000[b]	438 800[d]	4.0	0.09
HPC 100K	100 000[c]	Unknown	High[e]	0.65
HPC 300K	300 000[c]	Unknown	High	0.45
HPC 1M	1 000 000[c]	Unknown	High	0.09
PAA 1M	700 000–1 000 000[c]	Unknown	High	0.23
PAA 1.14M	1 140 000[c]	456 000[c]	2.5	0.18

a) In previous studies [11, 29, 34], we identified these HEC samples by their number-average molecular masses (refering to them as HEC M_n–27000 and HEC M_n–105000). In this paper, all polymer samples are identified by their weight-average molecular masses for clarity of comparison; hence the change of abbreviation.

b) Determined in our laboratory by low-angle laser light scattering.

c) Specified by the manufacturer (see Section 2.2).

d) Determined by Sperry *et al.* [32].

e) Samples for which the polydispersities are unknown (because M_n is unknown) are unfractionated with respect to molecular weight, and thus are expected to have relatively high polydispersities ($M_w/M_n \geq 4.0$).
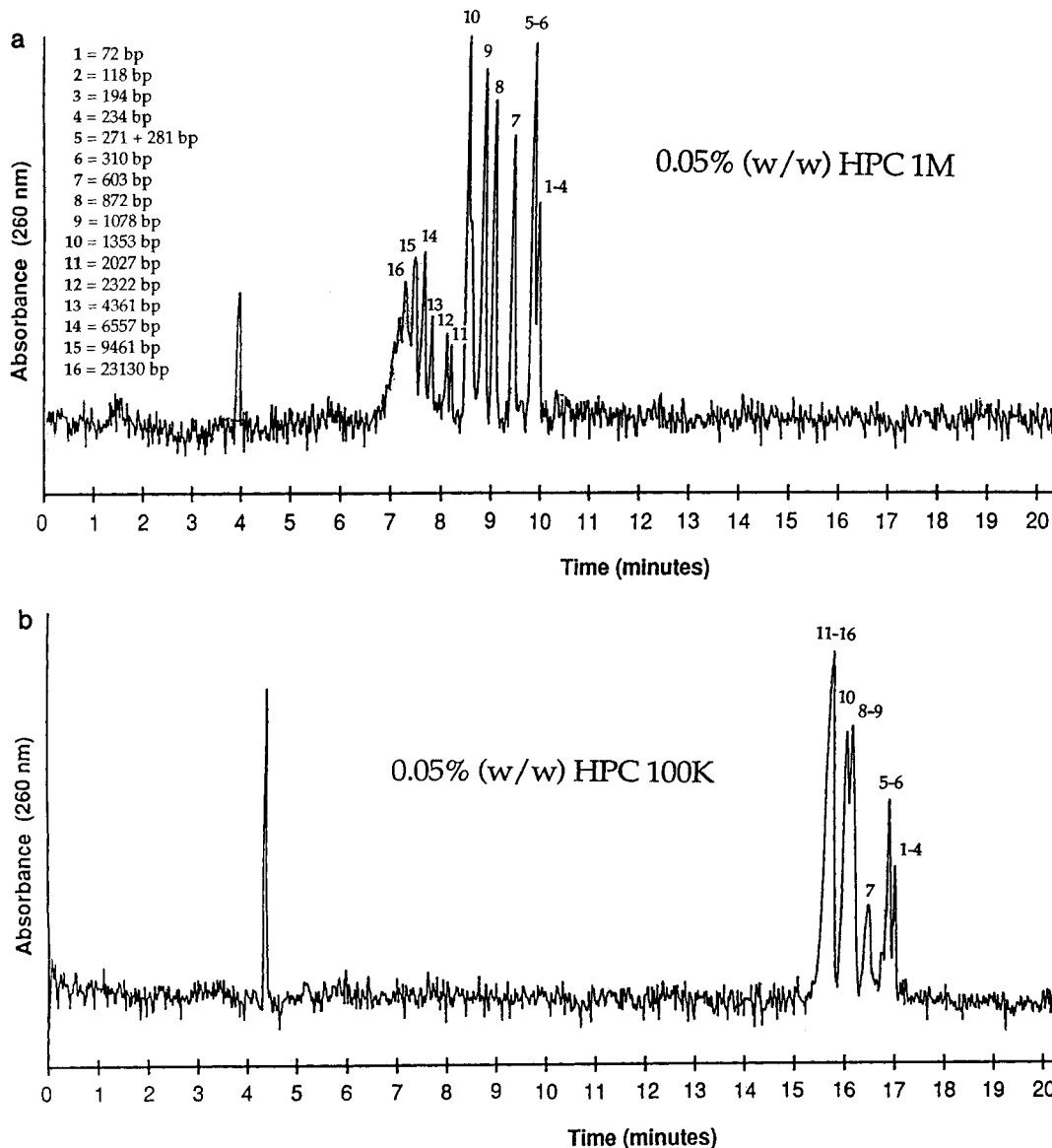
Figure 2. CE of a mixture of λ-*Hind*III and ΦX174–*Hae*III restriction fragments (in non-stoichiometric mixture): (a) in 0.05% w/w HPC 1M; (b) in 0.05% w/w HPC 100K. The far-left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. DNA peaks are identified in part (a). λ-*Hind*III restriction fragments of 125 bp and 564 bp are present at such small concentrations that they are too faint to be seen. Buffer: 1× TBE, pH 8.15. Capillary: 51 μm ID, 50 cm total length (35 cm to detector); temperature, 30 ± 0.1°C. Detection was by UV absorbance at 260 nm. Injection was hydrodynamic. Electrophoresis conditions: Field strength 265 V/cm; current was (a) 7.1 μA, (b) 7.8 μA; RSD of absolute electrophoretic mobilities: (a) 0.78%, n=4; (b) 0.14%, n=4.

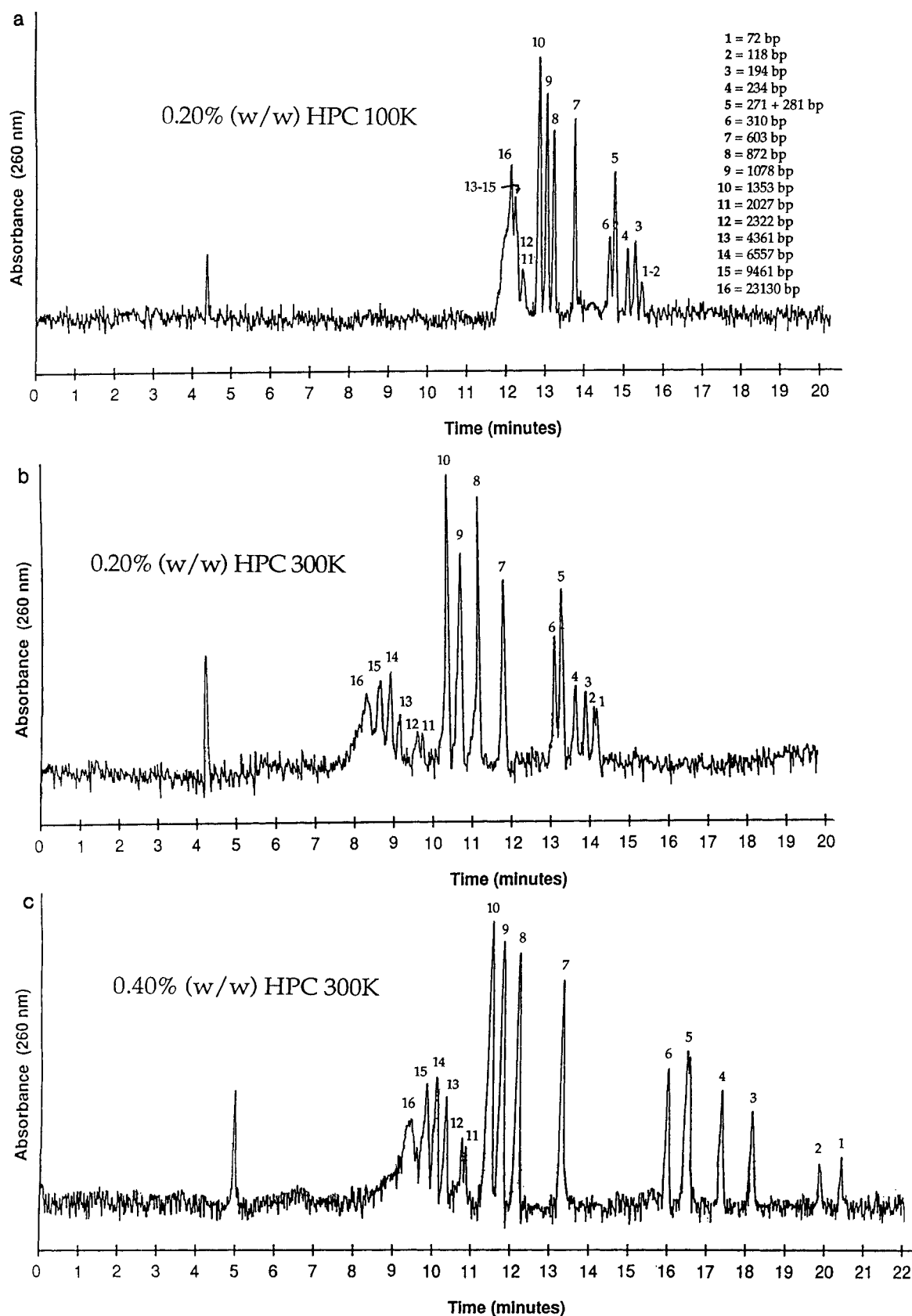*Figure 3.* CE of a mixture of λ-*Hin*dIII and ΦX174-*Hae*III restriction fragments (in nonstoichiometric mixture): (a) in 0.20% w/w HPC 100K; (b) in 0.20% w/w HPC 300K; and (c) 0.40% w/w HPC 300K. The far-left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. DNA peaks are identified in part (a). Electrophoresis conditions: Field strength 265 V/cm, current was (a) 7.8 μA, (b) 7.1 μA, (c) 7.1 μA. RSD of absolute electrophoretic mobilities: (a) 0.16%, n=4; (b) 0.36%, n=6; (c) 0.31%, n=5. All other conditions and specifications as in Fig. 2.

HEC 1.39K, was not used for electrophoresis in this study, but was used in previous studies [11, 29] and is included for the sake of completeness. As one would expect, for a given type of polymer, the entanglement threshold concentration decreases with increasing average molecular mass.

3.2 The importance of polymer chain length: DNA separations in solutions of HPC having different molecular masses

In previous work [11], we have found that the average HEC molecular mass, as well as the HEC concentration in the buffer, has a significant impact on the size range of DNA which can be separated by counter-migration CE [11, 29]. As one would expect, the same general trends that are observed for DNA separations in HEC solutions are also seen when HPC solutions are used; as we will show, however, there are significant differences in the useful concentration ranges of these two types of polymers, resulting from differences in their hydrophilicity and chain stiffness. Figure 2 shows the counter-migration CE separation of a mixture of ΦX174-*Hae*III and λ-*Hin*dIII DNA restriction fragments in (a) 0.05% HPC 1M and (b) 0.05% HPC 100K. Note that because of the opposing electroosmotic flow, the true "effective length" of the capillary used for this separation is 94 cm, although the length from injection to detection is only 35 cm. Thus if a polyacrylamide-coated capillary were used, on would require a 94 cm effective length to observe the same separation shown in Fig. 2. At 25°C, the viscosities of these 0.05% HPC/TBE solutions are 1.58 cP and 0.99 cP, respectively. Both solutions are well below their measured entanglement threshold concentrations; Φ* is about 0.09% for HPC 1M and about 0.65% for HPC 100K (see Table 1). As we have observed for HEC solutions [29], dilute solutions of high-molecular mass cellulosic polymers such as the HPC 1M will separate DNA fragments ranging in size from 600 bp to 23 kbp by counter-migration CE. However, the separating power of the polymer solution decreases rapidly as DNA size increases above 600 bp. Unlike the HPC 1M sample, the low-molecular-mass HPC 100K sample does not provide any significant resolution of DNA larger than 600 bp. Furthermore, at the low concentration of 0.05%, we achieve virtually no resolution of DNA smaller than 600 bp in either HPC sample.

Figure 3a depicts the separation of the ΦX174-*Hae*III/λ-*Hin*dIII mixture in a 0.20% HPC 100K solution (viscosity: 1.23 cP). Virtually no separation of DNA larger than 1.3 kbp is achieved in HPC 100K solutions, neither at 0.20% as shown in Fig. 3a, nor at any other concentration. However, if an HPC sample with triple the average molecular mass of the HPC 100K is used (HPC 300K), much better separation of large DNA is achieved at 0.20% (Fig. 3b); better separation of smaller DNA is achieved as well. Figure 3c shows the further improved resolution that is obtained by doubling the HPC 300K concentration to 0.40%. The greatly improved DNA resolution seen in Fig. 3b, as compared to Fig. 3a, is dramatic testimony to the importance of the average polymer length in the DNA/polymer interactions which lead to DNA separation. We cannot attribute this
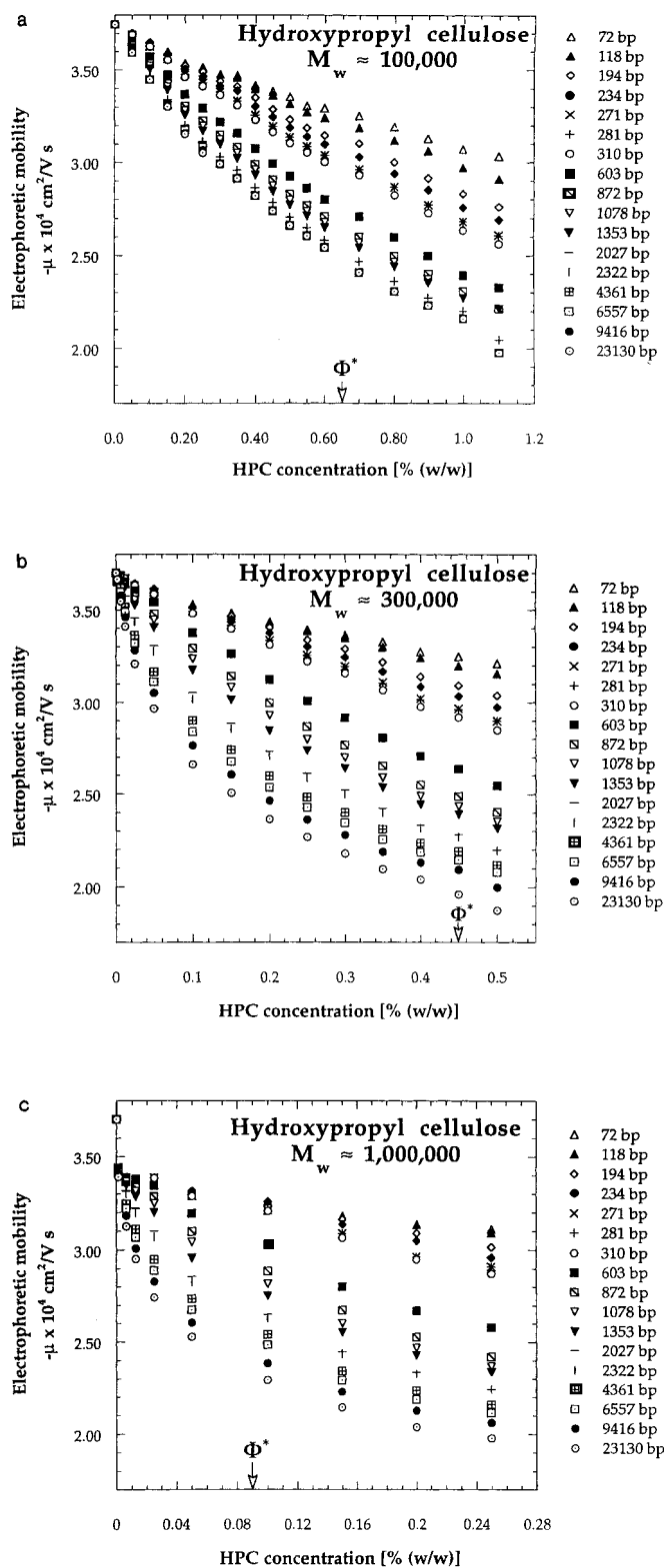

Figure 4. Plots of DNA electrophoretic mobility *vs.* HPC concentration for DNA restriction fragments ranging from 72 bp to 23 130 bp in length, for three different HPC samples having weight-average molecular masses of: (a) 100 000 g/mol, (b) 300 000 g/mol, and (c) 1 000 000 g/mol. The entanglement threshold concentration, Φ*, is indicated on the concentration axis with an arrow. Data points at each HPC concentration are the average of 3–5 individual determinations. Average run-to-run variation in calculated electrophoretic mobilities: (a) ± 0.30%, (b) ± 0.31%, (c) ± 0.47%. The electrophoresis buffer, other conditions and specifications, and DNA sample are as in Fig. 2.

improvement simply to a viscosity difference, or to a difference in the state of entanglement of the HPC polymers in solution. The viscosity of a 0.20% HPC 300K solution is 1.89 cP, not very different from the 1.23-cP viscosity of the HPC 100K solution. Both solutions are well below their entanglement threshold concentrations, which are 0.45% for the HPC 300K and 0.65% for the HPC 100K solution. And, as we have demonstrated for HEC solutions [11, 29], we find once again for the case of HPC that the formation of an entangled polymer network is not a prerequisite for DNA separation.

Figures 4a, 4b and 4c plots of DNA electrophoretic mobility *vs.* polymer concentration, as a function of DNA fragment size, for solutions of HPC 100K, HPC 300K, and HPC 1 M in TBE buffer. The range of electrophoretic mobilities plotted on the *y*-axis is identical in all three figures, so that the DNA separation in the three HPC samples can be compared. However, since the range of HPC concentrations that is useful for DNA separation is highly dependent on molecular mass, the range of concentrations that is plotted on the *x*-axis differs in the three figures.

One can make several observations upon examination of Fig. 4. First, the DNA exhibit the same, size-independent free-solution electrophoretic mobility (about 3.7 $\times$ 10^{-4} cm²/V s). In all cases the separation of small DNA (< 310 bp) continually improves as HPC concentration is increased. For DNA fragments that are 603 bp and greater in length, separation first improves as HPC concentration is increased, and then gradually degrades above a certain concentration. In all three figures, one sees that the entanglement threshold concentration is not a relevant parameter to predict a useful HPC concentration range for DNA separation. Therefore, at these low polymer concentrations, DNA separation does not occur as a result of DNA migration through an entangled network or through "dynamic pores" in such a network, as has been suggested [14, 30]. Instead, these data lend further support to our hypothesis that DNA separation occurs by physical DNA/polymer entanglement interactions, specifically by size-dependent frictional drag, which results from the catching and dragging of the neutral polymer chains by the electrophoresing DNA molecules [11, 29, 34]. If the HPC chains are too small, they do not cause sufficient drag to provide good separation by this "transient entanglement coupling" mechanism.

There is a question as to why the resolution of DNA larger than 603 bp is seen to degrade somewhat at higher HPC concentrations. This trend has also been observed by Mitnik *et al.* [41], and can be interpreted in terms of a shift in the mechanism of DNA separation as polymer concentration is increased and a highly entangled polymer network is formed [29]. At lower polymer concentrations, separation probably occurs by a transient entanglement coupling mechanism, as we have discussed in previous work. At higher concentrations, there is likely a transition to a mode of migration involving elements of reptation with constraint release, as modeled by Viovy and Duke [42]. When DNA reptates in a field-oriented conformation, there is a decrease in the
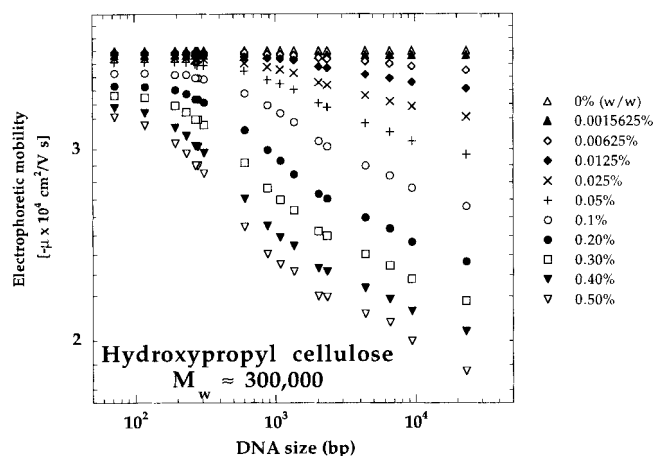

Figure 5. A log-log plot of electrophoretic mobility *vs.* DNA size for DNA separations in HPC 300K solutions. Field strength: 265 V/cm. Electrophoretic buffer, DNA sample, and other conditions and specifications are the same as given in Fig. 4b and Fig. 2.

dependence of DNA mobility on size, particularly for larger DNA.

In semidilute polymer solutions there will be a "gray area" of transition in which the mechanism is neither purely transient entanglement coupling, nor purely reptation with constraint release, but a mixture of both mechanisms. If one plots electrophoretic mobility *vs.* DNA size (log-log), *e.g.* for the HPC 300K solutions (Fig. 5), one observes a gradual transition, as polymer concentration is increased, to a sigmoidal curve similar to that typically seen for DNA gel electrophoresis. The difference is that the dependence of mobility on size is not as strong in uncross-linked polymer solutions as that exhibited in a true gel. Figure 5 looks qualitatively identical to mobility *vs.* size plots for HEC, which we have discussed in detail in a previous paper [29]. We would like to remark that one interesting feature of Fig. 5 is that even at a field strength of 265 V/cm, the mobility of larger DNA does not level off and become size-independent, as it does in agarose slab gels for larger DNA even under very low electric fields (*e.g.*, 5–10 V/cm). This underscores the fact that, in uncross-linked polymer solutions, the mechanism of DNA separation differs from that observed in true gels.

Using the HPC 100K, 300K, and 1M samples, data was obtained for concentrations up to 1.10%, 0.50%, and 0.25%, respectively. In all three cases these upper limits represent the concentrations at which we began to observe significant degradation of resolution of DNA larger than 603 bp. This upper limit is the highest (1.10%) for the HPC 100K, and at this relatively high concentration, we achieve better overall resolution of small DNA fragments (72–310 bp) than we do at the upper-limit concentrations of HPC 300K and 1M (0.50% and 0.25%, respectively). However, we were not able to observe separation of the 271/281 bp fragments using HPC 100K solutions, even at concentrations as high as 1.10%. In fact, we only achieved separation of the 271/281 doublet when we used the HPC 300K sample, in the narrow concentration range of 0.30%–0.45%. The HPC 1M sample did not provide resolution of the 271/281 bp fragments at any of the concentrations we used; how-
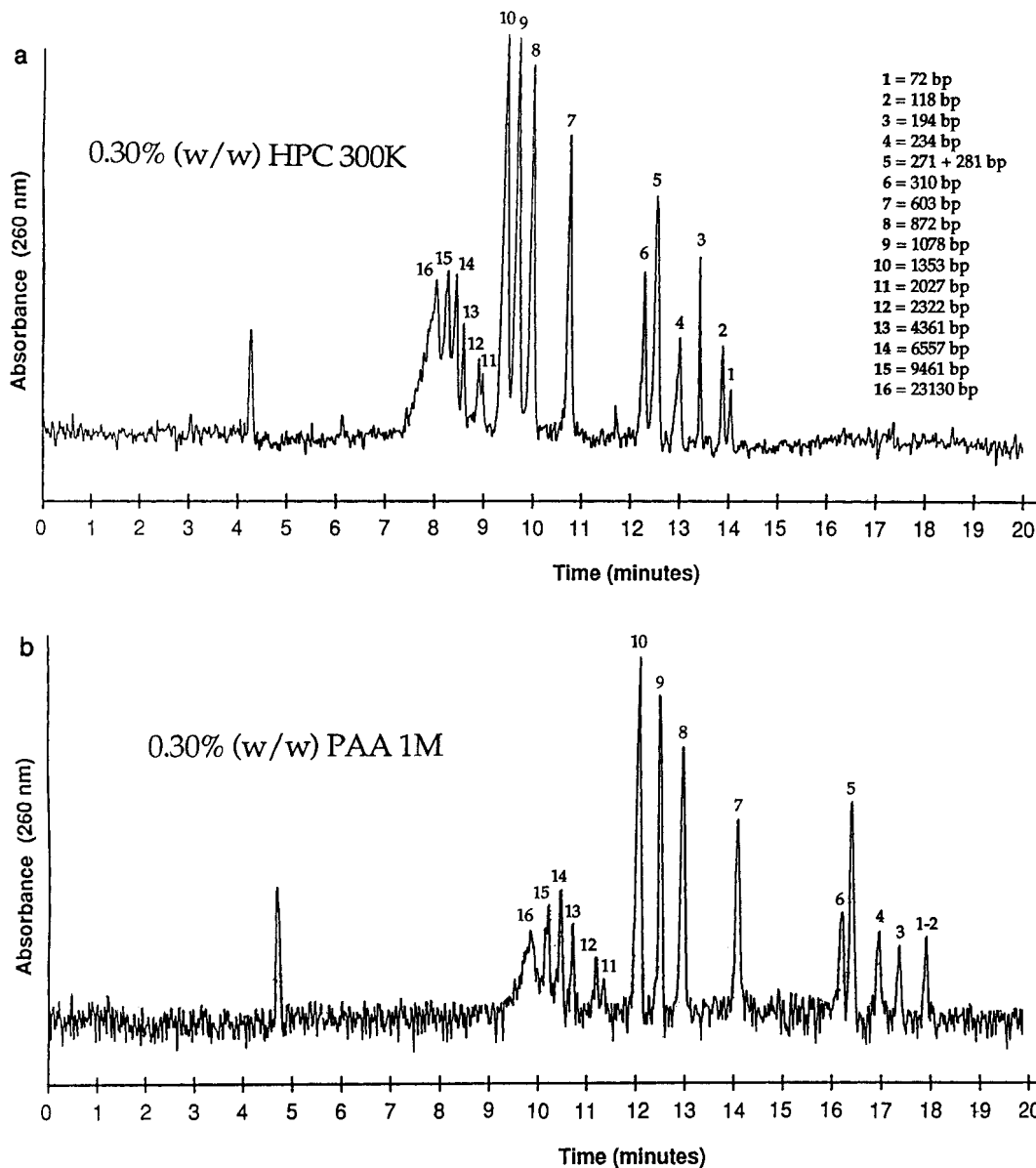
Figure 6. CE of a mixture of λ-*Hin*dIII and ΦX174-*Hae*III restriction fragments (in non-stoichiometric mixture): (a) in 0.30% w/w HPC 300K, (b) in 0.30% w/w PAA 1M. The far-left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. DNA peaks are identified in part (a). Electrophoresis conditions: Field strength 265 V/cm, current was (a) 7.4 μA, (b) 7.2 μA. %RSD of absolute electrophoretic mobilities: (a) 0.20%, *n*=4; (b) 0.24%, *n*=4. All other conditions and specifications as in Fig. 2.

ever, it is likely that we might have observed this finer resolution of small DNA at concentrations higher than 0.25%.

In comparison to HPC 300K and HPC 1M polymers, the HPC 100K sample gives much poorer separation of DNA fragments larger than 603 bp over the entire concentration range studied. The HPC 300K and 1M samples both give good resolution of DNA fragments in the size range of 603–23130 bp. However, for DNA larger than 1 kbp, the separating power of solutions of high-molecular-mass HPC does decrease with increasing DNA size, with 23 kbp being near the upper limit of DNA that can be separated with useful resolution, and with the peak for the 23 kbp fragment being badly tailed. We have observed this same decrease in separating power for high-molecular-mass HEC solutions [29]. In the concentration ranges which were studied, the HPC 300K provides better separation of small DNA fragments than the HPC 1M. This is simply because we tested a larger concentration range for the HPC 300K than for the HPC 1M.

These data imply that the separation of small DNA fragments (< 310 bp) will always improve with increasing HPC concentration, while large DNA fragments are better separated in relatively dilute HPC solutions, with resolution going through an optimum at some low concentration and worsening at higher concentrations.

In general, then, for HPC samples, we find that polymers that are 300000 g/mol and greater in weight-average molecular mass will provide good separation of DNA larger than 1 kbp. If the concentration of these high-molecular-mass HPC solutions is raised sufficiently (though not necessarily above the entanglement threshold), small DNA will also be well resolved, though perhaps at the price of poorer resolution of DNA larger than 600 bp. By this token the intermediate molecular-mass HPC sample, with a weight-average molecular mass of 300000 g/mol, gives the best balance between good resolution of small (72–310 bp) and larger (603–23130 bp) DNA fragments. On the other hand, we see that HPC with a weight-average molecular mass of 100000 g/mol and
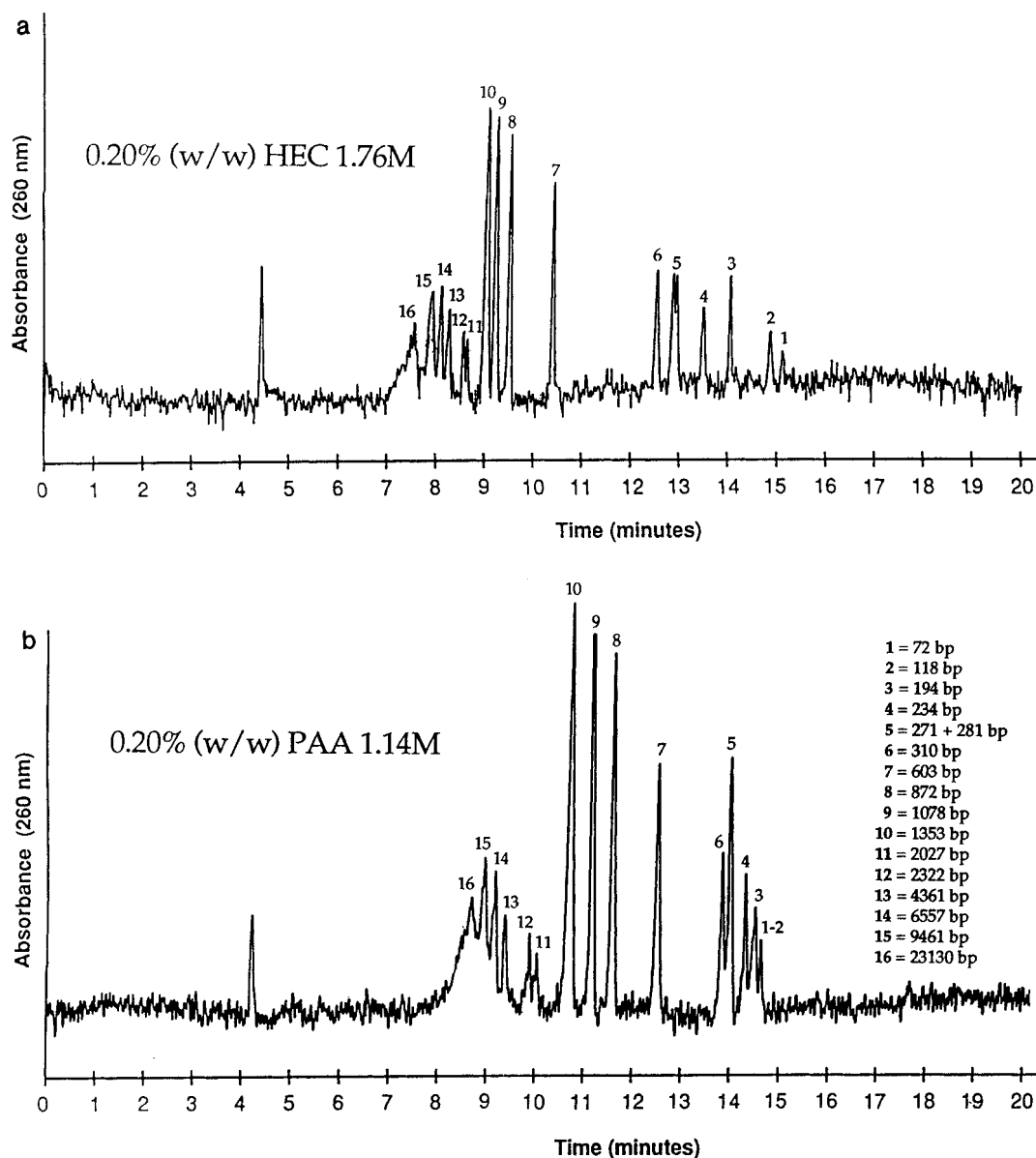
Figure 7. CE of a mixture of λ-*Hind*III and ΦX174-*Hae*III restriction fragments (in non-stoichiometric mixture): (a) in 0.20% w/w HEC 1.76M, (b) in 0.20% w/w PAA 1.14M. The far-left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. DNA peaks are identified in part (b). Electrophoresis conditions: Field strength 265 V/cm; current was (a) 8.2 μA, (b) 7.3 μA. RSD of absolute electrophoretic mobilities: (a) 0.50%, *n*=7; (b) 0.25%, *n*=4. All other conditions and specifications as in Fig. 2.

lower cannot significantly resolve DNA larger than 1 kbp, and does not give 10 bp resolution of the 271/281 bp fragments, although coarser resolution of small DNA is good at semidilute HPC 100K concentrations.

We observed the same general effects of average molecular mass on DNA separation in previous CE experiments in which we used HEC solutions to separate the ΦX174-*Hae*III/λ*Hind*III mixture of DNA restriction fragments [29]. From Fig. 4b and 4c, and from previous experiments with HEC [11], we conclude that above a certain threshold in polymer molecular mass only incremental gains in resolution of large DNA are made as the polymer size is increased beyond minimum (*e.g.*, M_w about 300000 g/mol, for HPC). In other words, HPC 300K and HPC 1M give large-DNA separations that are quantitatively very similar, as do HEC 1.32M and HEC 1.76M (previously designated, in [11] and [29], by their number-average molecular masses of HEC 105000 and HEC 439000). Nonetheless, there is a significant difference in the performance of HEC and HPC solutions as

DNA separation media. In general, at the same concentration, high-molecular-mass HEC polymers give significantly better DNA resolution than do high-molecular-mass HPC polymers. Instead, the concentration of high-molecular-mass HPC must be raised to a higher concentration than that of high-molecular-mass HEC to give similar DNA resolution. For example, we have shown in previous papers [29, 34], that a 0.15% HEC 1.32M solution will provide excellent separation of all DNA fragments in the ΦX174-*Hae*III/λ-*Hind*III mixture. Yet a 0.20% HPC 300K solution gives a poorer separation of these DNA restriction digests (Fig. 3b), and gives no resolution of the 271/281 bp fragments nor of the 72/118 bp fragments.

However, Fig. 3c shows that resolution similar to that achieved with a 0.20% HEC 1.32M solution can be obtained with a 0.40% HPC 300K solution. We attribute this necessity for higher HPC concentrations to the fact that HPC polymers are less hydrophilic than HEC, and hence assume more compact conformations in solution,
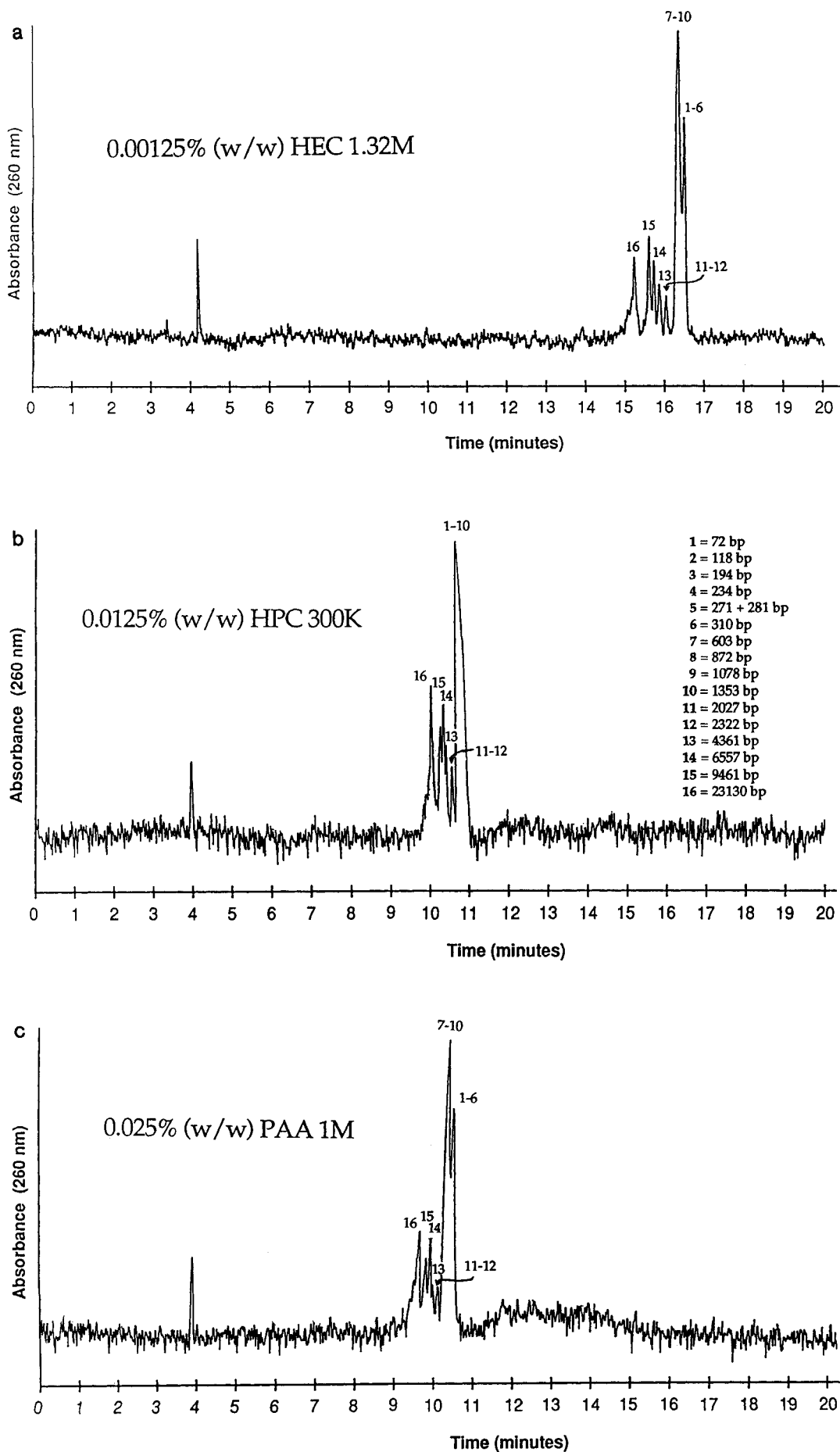
Figure 8. CE of a mixture of λ-*Hin*dIII and ΦX174-*Hae*III restriction fragments (in non-stoichiometric mixture): (a) in 0.00125% w/w HEC 1.32M, (b) in 0.0125% w/w HPC 300 K, and (c) 0.025% w/w PAA 1M. The far left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. DNA peaks are identified in part (b). Electrophoresis conditions: Field strength 265 V/cm; current was (a) 7.2 μA, (b) 7.2 μA, and (c) 7.4 μA. RSD of absolute electrophoretic mobilities: (a) 0.28%, *n*=3; (b) 0.16%, *n*=3; (c) 0.23%, *n*=4. All other conditions and specifications as in Fig. 2.

i.e., the chain is somewhat less stiff and more self-seeking. HEC polymers are known to adopt extended conformations in aqueous solution: the chain is more solvent-seeking than HPC. Stiffer, more extended HEC chains have larger radii of gyration in solution and occupy a greater coil volume on average than do HPC chains. It seems logical that the smaller coil size of HPC chains, due to their greater hydrophobicity than HEC, translates to a decreased probability of DNA encounter and entanglement with these chains, and therefore a larger HPC concentration is required to provide the same resolution. The data which we present in Section 3.4 further substantiates this hypothesis.

### 3.3 The importance of polymer type: Comparison of DNA separations in semidilute HPC, HEC, and PAA solutions

In separating DNA restriction fragments, cellulosic polymer solutions typically are employed at concentrations of 0.20%–1.0%, while linear PAA has been used at much higher concentrations (3.0%–10.0%) to produce comparable resolution of DNA restriction fragments. For example, Strege and Lagu [5] describe the separation in 18 min of a 1 kbp DNA ladder (a size standard useful for the restriction mapping of plasmids) in a 0.5% methyl cellulose solution, while Chiari *et al.* [37] chose a concentration of 4.5% linear PAA to provide the same separation, with similar results but a much longer analysis time (120 min). Single-base resolution of DNA sequencing fragments may be achieved with 6% concentrations of linear PAA [1], or with 2% HEC solutions [38]. It has not yet been clearly explained in the literature why much greater percentage of PAA than of HEC have been employed to gain comparable resolution of DNA restriction fragments. A practical limitation has been that it is difficult to dissolve high-molecular-mass HEC samples at concentrations higher than 2–3%, but long-chain polyacrylamide can be dissolved at concentrations of up to 10%. Furthermore, even though PAA is very hydrophilic, due to its chemical structure it is also a very flexible polymer; HEC polymers are typically quite stiff in comparison [11, 29]. Since, as it appears, DNA separation is achieved by entanglement interactions of DNA with polymer chains, it follows that stiffer polymers like HEC occupy a greater coil volume and also provide stronger entanglement interactions; hence stiff polymers (HEC) can be used at lower concentrations than flexible polymers (PAA, HPC) to provide the same level of DNA separation.

We can now compare the separation of DNA restriction fragments in solutions of HPC, HEC, and PAA polymers. We find that like HEC and HPC, linear PAA also provides good resolution of the ΦX174-*Hae*III/λ-*Hin*dIII fragments at relatively low concentrations. PAA has rarely been used for DNA separation at concentrations of less than 1%; but we find that DNA resolution occurs at concentrations which are ten times more dilute than that. In fact, the DNA resolution achieved in semidilute PAA solutions is quite similar to that obtained in solutions of high-molecular-mass HPC, but not as good as that achieved in high-molecular-mass HEC solutions. We

illustrate the first point with Fig. 6a and 6b, depicting comparable separation of ΦX174-*Hae*III/λ-*Hin*dIII fragments in 0.30% HPC 300K and in 0.30% PAA 1M, respectively. The viscosities of these two solutions are 2.6 cP and 1.9 cP, respectively. The superior DNA resolution that is achieved in semidilute HEC solutions, as compared to PAA solutions, is demonstrated in Fig. 7a and 7b, which show separation in 0.20% HEC 1.76M (7.1 cP) and 0.20% PAA 1.14M (1.7 cP), respectively. In the HEC solution, much better resolution of DNA smaller than 600 bp is achieved, while the separation of larger DNA is comparable. PAA can be used at a higher concentration (*e.g.*, 0.40%) to give better separation of the small DNA fragments. Hence its properties are quite similar to what we observed with HPC in Section 3.2: higher PAA concentrations are required to give the same resolution that is achieved using HEC, a much stiffer polymer.

3.4 The importance of polymer stiffness: DNA separations in ultradilute solutions of HEC, HPC, and PAA

The effect of fundamental differences in properties between hydrophilic, stiff polymers such as HEC, and more flexible polymers like HPC and PAA, is dramatically illustrated by the performance of ultradilute solutions for the separation of large DNA (2.0–23 kbp). First, we should note that ultradilute solutions of long-chain HPC and PAA will provide separation of large DNA, just as we demonstrated in previous work using using ultradilute HEC 1.32M solutions [34]. However, more flexible HPC and PAA polymers must be used at order-of-magnitude higher concentrations to provide the same separation seen in extremely dilute solutions of long-chain HEC. This is shown in Fig. 8a, 8b, and 8c, showing the electrophoresis of ΦX174-*Hae*III/λ-*Hin*dIII fragments in 0.00125% HEC 1.32M solution, 0.0125% HPC 300K solution, and 0.025% PAA 1M solution. The difference in the bulk viscosities of these three ultradilute solutions is negligible: they are 0.94 cP, 0.96 cP, and 1.0 cP, respectively. The electroosmotic flow velocity, as measured by the elution time of a neutral marker, is similar in the three solutions but slightly slower for the HEC 1.32M solution, which at least partially accounts for the later DNA elution time and better apparent resolution. (Recall that a slower electroosmotic flow translates to a longer DNA residence time in the capillary, and hence a longer "effective length.") The somewhat slower electroosmotic velocity seen in Fig. 8a probably does not result from a difference in polymer properties, but from a difference in capillary surface properties – the electropherogram shown in Fig. 8a employed a different fused silica capillary. There are small capillary-to-capillary variations in the electroosmotic flow velocities, which are obtained with polymer/TBE solutions. The salient point which is illustrated by the three electropherograms shown in Fig. 8 is that more flexible polymers like HPC and PAA must be used at higher *concentrations* to give the same separation achieved with stiff, extended HEC polymers. This substantiates our hypothesis that DNA separation in uncross-linked polymer solutions is provided by transient entanglement coupling. Figure 8 also
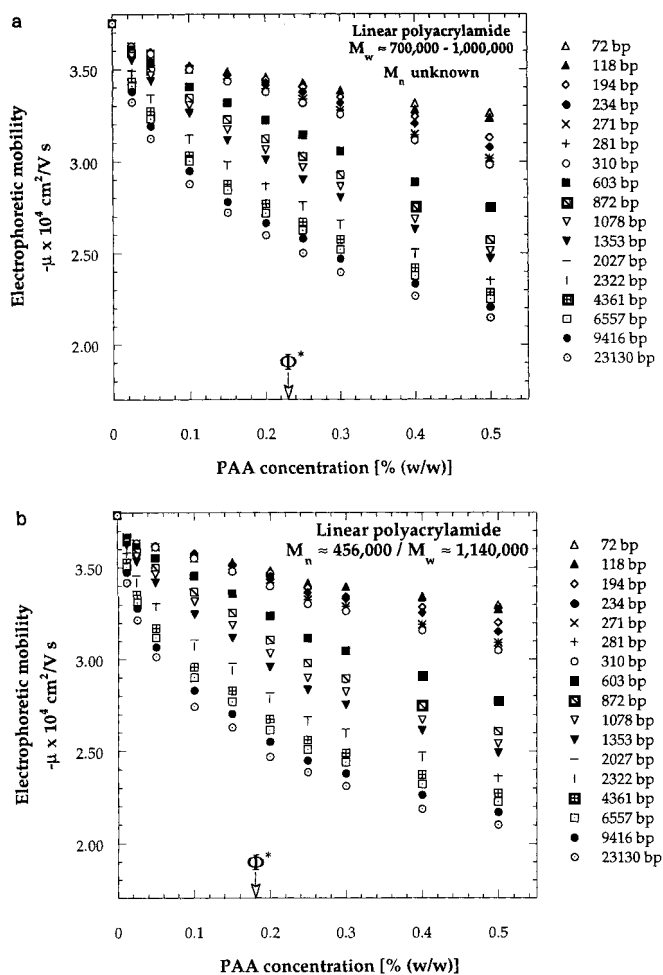
Figure 9. Plots of DNA electrophoretic mobility *vs.* HPC concentration for DNA restriction fragments ranging from 72 bp to 23 130 bp in length, for two different PAA samples: (a) $M_w \approx$ 700 000–1 000 000 g/mol, unfractionated with respect to molecular mass (M_w/M_n unknown); and (b) $M_n \approx$ 456 000 g/mol/$M_w \approx$ 1 140 000 g/mol ($M_w/M_n = 2.5$). The entanglement threshold concentration, ΦX^*, is indicated on the concentration axis with an arrow. The less polydisperse sample, used in part (b), will have a greater number of long PAA chains present in the sample. Data points at each PAA concentration are the average of 3–5 individual determinations. Average run-to-run variation in calculated electrophoretic mobilities: (a) ± 0.33%, (b) ± 0.31%. The electrophoresis buffer, all conditions and specifications, and DNA sample, as in Fig. 2.

shows that this separation mechanism generally applies for DNA separation in linear, hydrophilic polymers, and is therefore not likely to be due to specific chemical interactions but to general, physical DNA/polymer interactions.

3.5 The effect of sample polydispersity: DNA separation in two PAA samples having similar average molecular masses but different molecular mass distributions

One advantageous property of polyacrylamide and related acrylamide-based polymers like polyacryloylaminoethoxyethanol (pAAEE) is that their polymerization can be controlled to give a desired range of molecular mass. Ruiz-Martinez *et al.* [1], Heller and Viovy [39], and Gelfi *et al.* [27] have shown that the molecular mass of linear polyacrylamide may be optimized to provide good

resolution at a reasonably low viscosity, so that the solution can be pushed in and out the capillary easily using a syringe. Although cellulose derivatives are also available in a range of molecular masses, they are not synthetically polymerized, but instead are derived from various natural sources. For example, HEC and HPC are made by the high-temperature, high-pressure reaction of alkali cellulose with ethylene oxide and propylene oxide, respectively. The product is purified, to varying degrees, but rarely fractionated with respect to molecular mass. Hence, derivatized celluloses tend to be quite polydisperse (*i.e.*, $M_w/M_n \gg 1$), with the polydispersity varying quite a bit from sample to sample (see Table 1). For a systematic study of the dependence of DNA separating ability on polymer molecular mass, it might be desirable to use a monodisperse polymer sample; but there has been no indication that polydispersity has any deleterious effects on DNA resolution. In fact, in a recent study Chan *et al.* [40] used solutions of mixed-molecular-mass polyethylene oxide (PEO) to show that controlled polydispersity has advantages for the separation of single-stranded DNA sequencing fragments; by mixing long and short PEO chains they were able to strike a balance between two desirable properties: high DNA resolving power and low solution viscosity.

To study the effect of polydispersity on DNA separation, we took advantage of the availability of relatively monodisperse linear PAA samples. We compared the DNA separation performance of two PAA samples that both have weight-average molecular masses of about 1 million but have very different polydispersities. One of the PAA samples we used, which we will refer to as "PAA 1M", is unfractionated with respect to molecular mass, with $M_w \approx$ 700 000–1 000 000 g/mol. This unfractionated sample is quite polydisperse, with an $M_w/M_n \geq 4$. The other PAA sample, which we refer to as "PAA 1.14M", was fractionated and characterized with respect to its molecular mass distribution, and is certified to have $M_n \approx$ 456 000 and $M_w \approx$ 1 140 000 g/mol, giving a polydispersity index of $M_w/M_n = 2.5$. The difference in the size distribution of these two samples, which both have an $M_w \approx$ 1 million, is that the less polydisperse PAA 1.14M sample contains a greater fraction of long PAA chains, which are nearer to the weight-average molecular mass, in its size distribution. The PAA 1M sample, on the other hand, will have a wider distribution of chain sizes, with a greater number of short PAA chains. Our measurements of the entanglement threshold concentrations of these two samples show this to be the case (see Table 1): $\Phi^* \approx$ 0.23% for the PAA 1M, while $\Phi^* \approx$ 0.18% for the PAA 1.14M. The lower entanglement threshold concentration which is measured for the PAA 1.14M sample indicates that on average, more long chains are present, which can become entangled at a lower concentration than shorter chains.

Figures 9a and 9b show the dependence of DNA electrophoretic mobility on polymer concentration, for the PAA 1M and PAA 1.14M samples. The trends seen are the same as those observed for DNA electrophoresis in HEC and HPC solutions. First, in both PAA 1M and PAA 1.14M solutions, DNA separation occurs at concentrations which are up to two orders of magnitude below

the entanglement threshold concentrations of the PAA samples. Second, DNA which is 310 bp and smaller shows continually improving resolution as the polymer concentration is increased. Third, DNA that is 603 bp and larger is separated in dilute PAA solutions, experiences optimum resolution at some concentration, and then is less well separated as polymer concentration is increased.

From our experiments with HEC and HPC, we expect that the more monodisperse PAA 1.14M sample, which contains a greater fraction of long chains, will provide better separation of large DNA (603 bp–23130 bp) than the more polydisperse sample (PAA 1M). This is indeed the case, although the difference is relatively subtle and not easy to see from a side-by-side comparison of mobility plots. In Fig. 10 we have plotted the difference in electrophoretic mobilities for the large (603 bp–23130 bp) and the small (72 bp–310 bp) DNA fragments. This difference gives an indication of the "envelope" of separation for large and small DNA. Examining Fig. 10, we see that the more monodisperse PAA 1.14M sample, which contains a greater fraction of long chains with M_w near 1000000, gives significantly better resolution of large DNA than the PAA 1M sample, which contains more short PAA chains. However, there is virtually no difference in the ability of these two PAA samples to separate small DNA. These results make it very clear that it is the *long* chains in a polymer sample that are responsible for the separation of large DNA. Further, it shows that small DNA fragments are relatively indifferent to the presence of longer polymer chains in the polymer solution. We can therefore conclude that sample polydispersity has very little effect on the separation of small DNA fragments; instead, overall polymer concentration is the important parameter. However, the separation of large DNA fragments is significantly improved by the presence of a larger fraction of long polymer chains in the solution.

4 Concluding remarks

In this paper we have compared the performance of HEC, HPC, and linear PAA for the separation of DNA restriction fragments ranging in size from 72 bp to 23130 bp, examining the effects of polymer properties such as molecular mass, hydrophilicity, and polydispersity. Overall, we find an overwhelming similarity in results obtained with the three different types of polymers, indicating that the DNA separation mechanism in dilute and semidilute uncross-linked polymer solutions is primarily based on physical entanglement interactions rather than chemical interactions such as hydrogen-bonding between DNA and polymer chains. In all cases, we have found that good DNA separation is achieved at concentrations that are well below the entanglement threshold concentration of the respective polymer samples, with the minimum polymer concentration to separate large DNA (2–23 kbp) being two orders of magnitude below Φ* for high-molecular-mass HEC, HPC, and PAA samples. These results lend further support to the hypothesis that separation of DNA in uncross-linked polymer solutions occurs by a transient entanglement coupling mech-
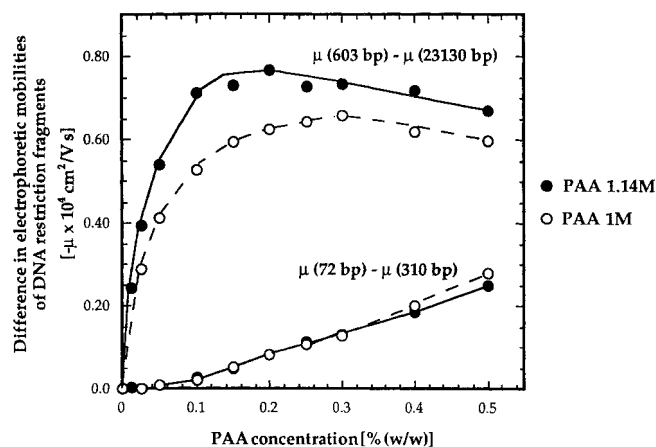

Figure 10. A plot of the absolute difference in DNA electrophoretic mobilities for large DNA (603 bp–23130 bp) and small DNA (72 bp–310 bp), in solutions of two PAA samples having similar weight-average molecular masses but different polydispersities. We differentiated between large and small DNA based on their electrophoretic behavior (see Fig. 9). By subtracting the mobility of the 23130 bp fragment from that of the 603 bp fragment, we obtain the size of the "separation envelope" for larger DNA; and by subtracting the mobility of the 310 bp fragment from that of the 72 bp fragment, a "separation envelope" for smaller DNA. One sees that the PAA 1.14M sample, which is less polydisperse and has a greater fraction of longer PAA chains in its molecular mass distribution, gives superior separation of large DNA in comparison to the PAA 1M sample. Small DNA separation is little affected by the PAA sample polydispersity.

anism, in which electrophoresing DNA catches and drags uncharged polymer chains, creating a size-dependent drag force which is not present when DNA migrates through free solution.

In general, for HPC polymers, we find that a minimum weight-average molecular mass of about 300000 g/mol is required for decent separation of DNA larger than 1 kbp. HPC with an M_w of 100000 g/mol and lower cannot significantly resolve DNA larger than 1 kbp, and furthermore does not provide 10 bp resolution of the 271/281 bp fragments, although coarser resolution of DNA smaller than 600 bp can be obtained at semidilute HPC 100K concentrations. We have found that at a given polymer concentration, the resolution achieved in semidilute PAA solutions is quite similar to that obtained in solutions of high-molecular-mass HPC, but not as good as that achieved in high-molecular-mass HEC solutions. Instead, we have seen that more flexible polymers like HPC and PAA must be used at higher concentrations to give the same separation achieved with stiff, extended HEC polymers. The superior DNA resolution that is achieved in semidilute HEC solutions at a given concentration, as compared to PAA and HPC solutions, can be explained by the fact that HEC is more hydrophilic than these other two polymers, and hence adopts a stiffer, more extended conformation in solution, presenting a larger cross-sectional area to electrophoresing DNA and entangling more strongly than flexible polymers.

The counter-migration CE technique works well whether one uses HEC, HPC, or PAA solutions to provide DNA separations. The type of polymer that is used, and the polymer concentration (as long as one is in the semidi-

lute concentration regime), do not have a significant effect on the velocity of electroosmotic flow in fused silica capillaries. This is discussed in more detail in one of our recent papers [34]. Furthermore, our study of the effect of polydispersity on DNA resolution, we have shown that long polymer chains provide the separation of large DNA restriction fragments in the 603 bp to 23130 bp range. The separation of small DNA fragments, on the other hand, is dependent on the total polymer concentration and is not significantly affected by the presence of long polymer chains in the polymer solution. We conclude that molecular mass distribution in a polymer sample has very little effect on the separation of DNA fragments smaller than 310 bp. However, the separation of large DNA fragments will be significantly improved by the presence of a larger fraction of long polymer chains.

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