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A DNA sieving matrix with thermally tunable mesh size

We present a "proof-of-concept" study showing that a blend of thermo-responsive and nonthermo-responsive polymers can be used to create a DNA sieving matrix with a thermally tunable mesh size, or "dynamic porosity". Various blends of two well-studied sieving polymers for CE, including hydroxypropylcellulose (HPC), a thermo-responsive polymer, and hydroxyethylcellulose (HEC), a nonthermo-responsive polymer, were used to separate a double-stranded DNA restriction digest (ΦX174-*Hae*III). HPC exhibits a volume-phase transition in aqueous solution which results in a collapse in polymer coil volume at ~ 39°C. Utilizing a blend of HPC and HEC in a ratio of 1:5 by weight, we investigated the effects of changing mesh size on DNA separation, as controlled by temperature. High-resolution DNA separations were obtained with the blended matrix at temperatures ranging from 25°C to 38°C. We evaluated changes in the selectivity of DNA separation with increasing temperature for certain pairs of small and large fragments. A pure HEC (nonthermo-responsive) matrix was used over the same temperature range as a negative control. In the blended matrix, we observe a maximum in selectivity at ~ 31°C for small DNA, while a significant increase in the selectivity of large-DNA separation occurs at ~ 36°C as the polymer mesh "opens". We also demonstrate, through a temperature ramping experiment, that this matrix can be utilized to obtain high-resolution separation of both small and large DNA fragments simultaneously in a single CE run. Blended polymer matrices with "dynamic porosity" have the potential to provide enhanced genomic analysis by capillary array or microchip electrophoresis in microfluidic devices with advanced temperature control.

Keywords: DNA sieving matrix / Nonthermo-responsive polymers / Thermo-responsive polymers
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1 Introduction

High-throughput, high-resolution, size-dependent separation of both double-stranded (ds) and single-stranded (ss) DNA ranging in size from a few to many thousand bases by capillary electrophoresis (CE) is critical in a number of molecular biology techniques such as DNA sequencing [1–3], restriction mapping of chromosomal DNA [4], polymerase chain reaction (PCR) product sizing [5–7], and forensic analysis [8]. As the electrophoretic mobility of DNA in free solution is size-independent [9–11], fluid sieving polymer solutions, formulated with cellulose derivatives [12–14], polyethylene oxide and its copolymers [15,16], and acrylamide and *N*-substituted acrylamide polymers [17–20], are employed to provide size-dependent DNA fragment separation *via* capillary and chip (microchannel) electrophoresis.

The influences of sieving polymer properties such as average polymer molar mass, matrix composition, and hydrophobicity/hydrophilicity on DNA separation performance have been widely studied [12, 13, 16, 18–21]. It has been shown that large DNA fragments are best separated by CE in less concentrated solutions of high-molar-mass polymers, while low-molar-mass, concentrated polymer solutions tend to provide better resolution of smaller DNA fragments [12, 14, 20, 22–24]. For example, Barron *et al.* [23] observed that low-molar-mass hydroxyethylcellulose (HEC) (M_n 27 000 g/mol) is unable to separate large dsDNA fragments (800–1000 bp) even at low concentration (0.30% w/w), which favors large DNA separation [14]. Meanwhile, high-resolution separations of DNA fragments in this size range were achieved with high-molar-mass HEC (M_n 105 000 g/mol) at concentrations as low as 0.10% w/w in the same study [23].

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Abbreviations: GPC, gel permeation chromatography; **HEC**, hydroxyethylcellulose; **HPC**, hydroxypropylcellulose; **LCST**, lower critical solution temperature; **MALLS**, multiangle laser light scattering; M_n, number-average molar mass; M_w, weight-average molar mass; **TBE**, Tris-borate-EDTA

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As the requirements of separation for small and large DNA fragments by CE in uncross-linked polymer solutions are different, there is a trade-off in choosing a polymer matrix to resolve different size-ranges of DNA fragments in a single run at a given CE condition. To address this, one approach is to use mixtures of low- and high-molar-mass polymers as sieving matrices. It has been observed that the addition of a small amount of a high-molar-mass polymer to a solution of low-molar-mass polymer can provide improved separation of large dsDNA fragments, without any detrimental effect on the resolution of small dsDNA fragments, while the overall viscosity of the solution remains at a practical level [12]. However, this method cannot achieve optimal separation: while an improvement in large-DNA separation is observed when a small amount of high-molar-mass polymer is added to a solution of the low-molar-mass counterpart, the resolution of small DNA fragments is not improved significantly. On the other hand, if the concentration of the low-molar-mass constituent is too high, the positive effect of adding the high-molar-mass polymer on large DNA separation is reduced. Therefore, it can be quite difficult to formulate a CE matrix that offers optimal sieving of both small and large DNA.

Another attractive strategy is to use a varying condition, such as temperature programming [25] or a field strength gradient [26], to create a sieving matrix with "dynamic" properties and/or to change the migration behavior of DNA molecules to resolve both small and large DNA fragments in a single run. The use of thermo-responsive polymers in CE has been investigated [15, 16, 27–29], as reviewed recently by Buchholz *et al.* [30], but the studies carried out so far address only the problem of loading the viscous polymer solutions into the microchannels. The use of optimized "viscosity-adjustable" polymer matrices will enable the development of automated capillary electrophoresis systems in which loading viscous polymer matrices into microchannels can be accomplished rapidly with low applied pressure (*e.g.*, 50 psi). However, to our knowledge, there has been no attempt to explore the thermo-responsive properties of this class of polymers to optimize the sieving performance of the matrices. The application of these thermo-responsive polymer networks in microfluidic devices with advanced spatial and temporal temperature control, such as the one recently demonstrated by Burns *et al.* [31], could lead to substantial advances in the efficiency of high-throughput DNA analysis.

In this study, we investigate the feasibility of changing the mesh properties, and hence the DNA-sieving ability, of a thermo-responsive polymer solution by varying the run temperature. A dsDNA restriction digest (ΦX174-*Hae*III) was separated by CE using a 1:5 blend of hydroxypropylcellulose (HPC) and HEC in Tris-borate-EDTA (TBE) buffer as the sieving matrix. HPC exhibits a volume-phase transition (also called a "lower critical solution temperature" (LCST)) at ~ 39°C in water [32], which results in a collapse in polymer coil volume as the LCST is approached. HPC solutions become cloudy at and above that temperature (the LCST is also called "the cloud point"), disallowing DNA detection at 39°C and above. This phase transition behavior is reversible, as it is under thermodynamic control [27]. We formulated a blended matrix so that the nonthermo-responsive polymer (HEC) acts as a "scaffold" for DNA separation, the "gaps" of which are filled by the thermo-responsive polymer (HPC) at low temperature, and left more open as the thermo-responsive constituent shrinks with increasing temperature. A schematic diagram illustrating the idea of a sieving network with "dynamic porosity" as provided by this type of formulation is shown in Fig. 1. We hypothesized that by raising the electrophoresis run temperature over a certain moderate range, the mesh properties of the matrix could be changed to favor the separation of small DNA fragments at low temperature, and large DNA fragments at high temperature. As our hypothesis turned out to be correct (see below), we then used the results as a guide for the design of a temperature-ramped CE run that provides enhanced selectivity of separation of both small and large DNA fragments simultaneously in a single analysis.

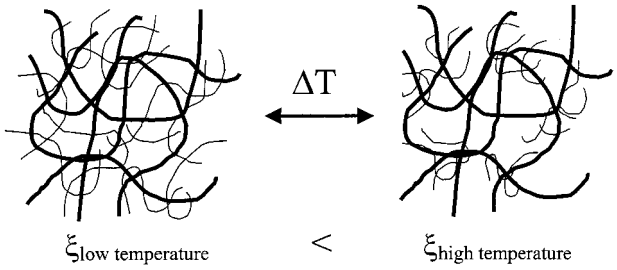

Figure 1. Schematic diagram illustrating the concept of a blended polymer matrix with "dynamic porosity". HEC polymers are shown with thick lines, and HPC with the thin lines; here ξ stands for average mesh size. At low temperature, both polymers adopt a loosely coiled conformation, and form a dense entangled network. As temperature increases, the thermo-responsive constituent shrinks while the nonthermo-responsive constituent remains unchanged, providing an "opening up" of the mesh.

2 Materials and methods

2.1 Materials

A solution of ΦX174-*Hae*III dsDNA restriction fragments (New England BioLabs, Beverly, MA, USA) was diluted to 50 μg/mL in triply deionized water. HPC (Scientific Poly-

mer Products, Ontario, NY, USA, manufacturer-reported number-average molecular weight = 300 000 g/mol) and HEC (Polysciences, Warrington, PA, USA, manufacturer-reported number-average molecular weight = 90 000–105 000 g/mol) were characterized in our laboratory by tandem gel permeation chromatography-multiangle laser light scattering (GPC-MALLS) by methods previously described [30], and the true physical properties determined are reported in Table 1. Reported values represent the average obtained from three analyses. Both polymers were dissolved in water and prepurified before use with a deionizing agent (AG®501-X8 resin, Bio-Rad Laboratories, Hercules, CA, USA). An aqueous buffer consisting of 50 mM Tris, 50 mM boric acid, and 2 mM EDTA (Amresco, Solon, OH, USA and Sigma, St. Louis, MO, USA) with pH 8.3 was used to dissolve the polymer in a pre-determined amount to make up a blended sieving solution of 0.25% w/w HPC and 1.25% w/w HEC. The same solution was used as the running buffer in CE experiments.

Table 1. Physical properties of the HEC and HPC used as determined by tandem GPC-MALLS[a)]

	M_w (g/mol)	M_n (g/mol)	PDI[b)]	R_g (nm)
HPC "300K"	440 000	124 000	3.6	96
HEC "105K"	1 340 000	745 000	1.8	104

a) Data represent the average of three analyses.
b) PDI, polydispersity index

2.2 CE

For CE experiments we employed a single fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 30 cm in length (25 cm to the detector), with 75 μm ID and 360 μm OD, internally coated with a covalently attached layer of linear polyacrylamide (LPA) according to the method of Hjertén [33]. CE was carried out in a BioFocus® Capillary Electrophoresis System (Bio-Rad Laboratories) in reversed-polarity mode. DNA samples were injected electrokinetically at the cathodic end of the capillary, with a field strength of 500 V/cm for 3 s. A run field strength of 265 V/cm was applied. Electrophoresis runs were performed at nine different temperatures (25°C, 27°C, 29°C, 31°C, 33°C, 35°C, 36°C, 37°C, and 38°C), and detection was by UV absorbance at 260 nm.

2.3 Visible spectrophotometry

The turbidity of the polymer matrix as a function of temperature (cloud point transition behavior) was characterized with Cary 500 UV-visible-NIR spectrophotometer with a circulating bath (Varian, Walnut Creek, CA, USA) to control the temperature. The polymer solution was heated and cooled at a rate of 5°C/min, and absorbance data were collected at 500 nm. Data presented were reproducible upon multiple cycles of repeated heating and cooling at this rate.

3 Results and discussion

As shown in Fig. 1, the blended HEC/HPC matrix forms an entangled polymer network, and is hypothesized to undergo a reversible change of mesh size upon temperature change. As temperature increases, the mesh "opens" up as a result of the shrinking of the thermo-reversible constituent (HPC), yet the HEC polymers remain entangled. The physical picture of the blended matrix, *i.e.* the entangled state of HEC polymers with unentangled HPC molecules, can be verified by considering the entanglement threshold concentrations of the polymers. Experimentally, the entanglement threshold concentrations can be determined from a logarithmic plot of specific viscosity (η_s) *vs.* polymer concentration (c) [34]. At low concentrations ($c < c^*$), specific viscosity increases linearly with concentration with slope ≈ 1. At the entanglement threshold ($c = c^*$), the plot deviates from linearity and increases in slope. The entanglement threshold concentrations of the polymers used in this study were determined in previous work [22, 14]. The corresponding c^* values are 0.45% w/w for HPC "300K" [22] and 0.37% w/w for HEC "105K" [14]. Therefore, the HEC polymers form an entangled network, while the unentangled HPC polymers are at a concentration below their c^* value. This lack of HPC-HPC entanglement is important when the volume phase transition occurs, because it should ameliorate the tendency of collapsed HPC polymers to rapidly aggregate with each other and precipitate from solution.

Figure 2 shows representative electropherograms illustrating the CE separation of the ΦX174-*Hae*III restriction digest with the mixed HEC/HPC matrix at temperatures of 25°C, 27°C, 29°C, 31°C, 33°C, and 38°C. We find that this blended polymer solution resolves dsDNA well at both low and high temperatures. The good DNA separation performance of the mixed-formulation matrix suggests that within this range of temperature the two polymers are compatible and fully miscible, and that the presence of thermo-responsive HPC polymers is not detrimental to DNA resolution at temperatures below their cloud point transition temperature of ~39°C.

However, it is definitely true that the useful operating temperature range of the matrix is determined by the volume phase transition behavior of the thermo-responsive con-

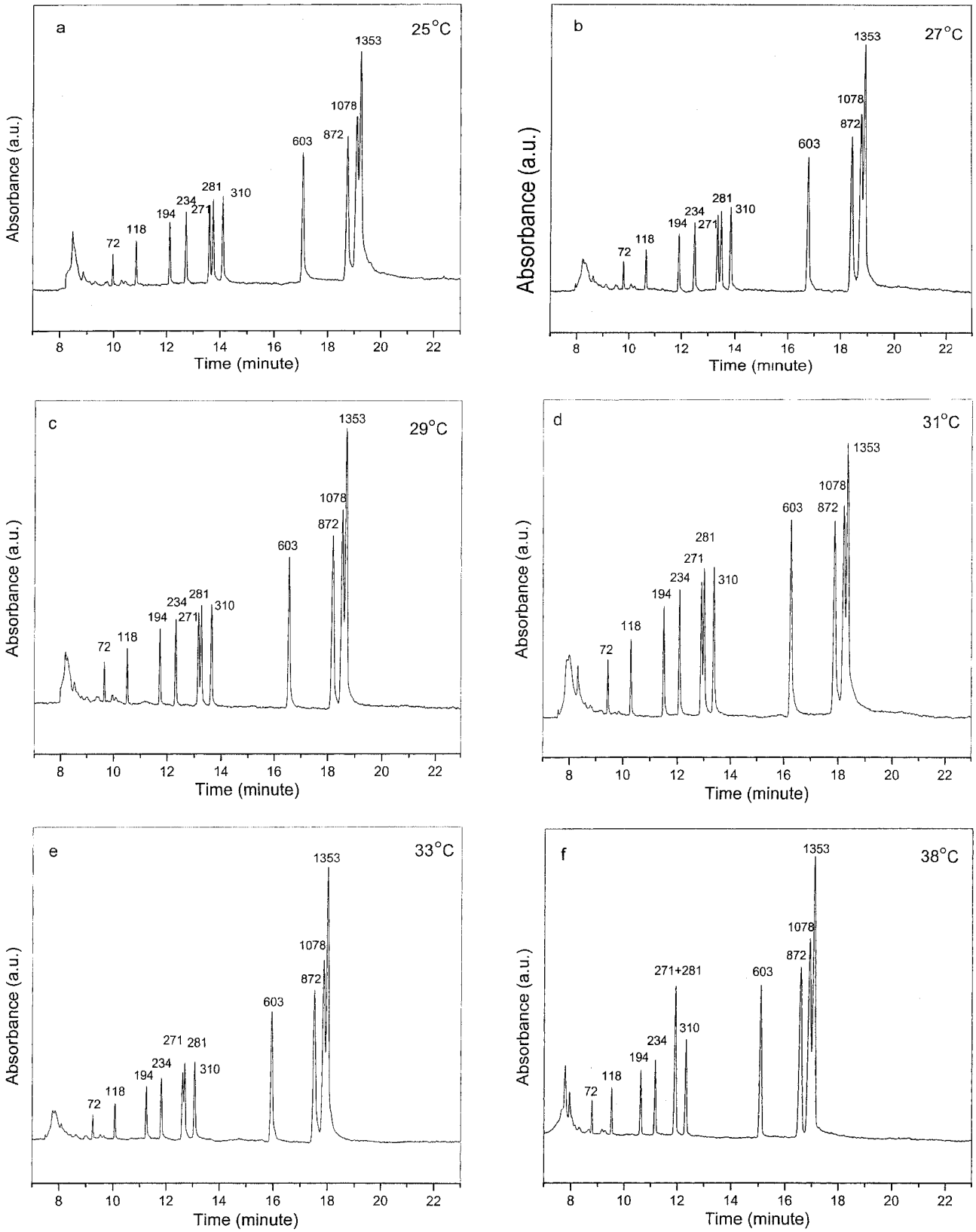

Figure 2. Representative electropherograms of the CE separation of a ΦX174-*Hae*III dsDNA restriction digest at (a) 25°C; (b) 27°C; (c) 29°C; (d) 31°C; (e) 33°C; (f) 38°C. CE conditions: reversed-polarity; electrokinetic injection, 500 V/cm for 3 s; run voltage, 265 V/cm; current at 25°C: 7.2 μA; 27°C: 7.4 μA; 29°C: 7.6 μA; 31°C: 7.8 μA; 33°C: 8.0 μA; 38°C: 8.2 μA.

stituent, HPC. The LCST temperature, or the cloud point temperature, of the blended matrix was determined by temperature-controlled visible spectrophotometry, and the result is shown in Fig. 3a. A sharp volume phase transition is observed, as indicated by an increase in matrix turbidity over a narrow temperature range centered (for the "heating" curve) at ~39°C. There is hysteresis between the heating and cooling curves because it takes some time for collapsed, partially aggregated HPC coils to redissolve upon cooling below the transition. The similarity of the absorbance *vs.* temperature curve of the polymer blend to that obtained with a pure HPC solution with the same total polymer concentration (Fig. 3b) shows that

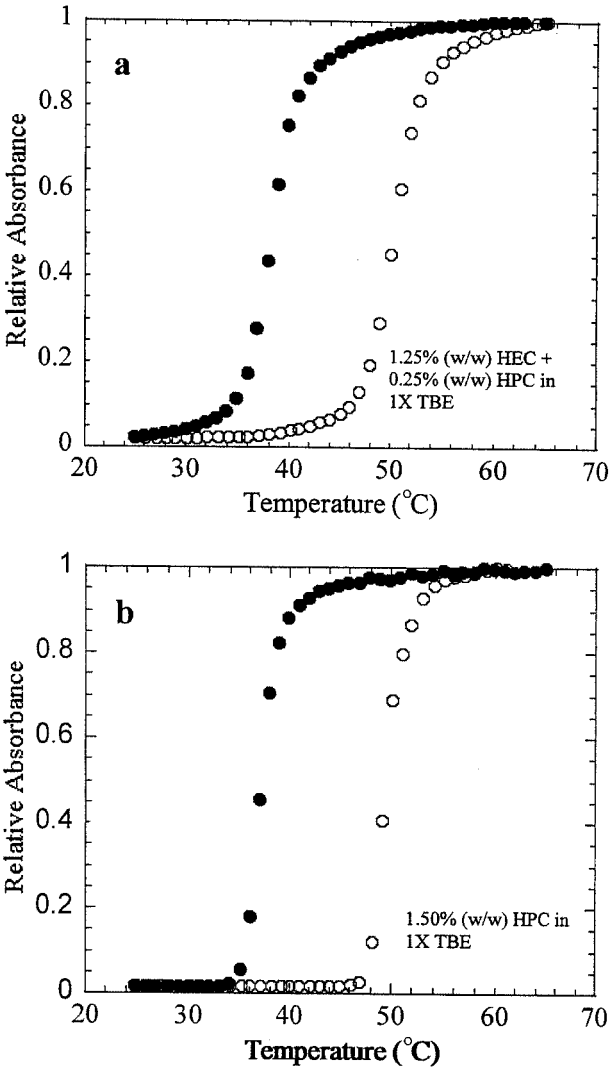

Figure 3. Temperature-dependent absorbance curves for (a) a 1.25% w/w HEC and 0.25% w/w HPC blend in 1 × TBE; (b) 1.5% w/w HPC in 1 × TBE. Open circles depict heating data, and closed circles depict cooling data (5°C/min). The plots represent data obtained in two consecutive cycles of heating and cooling (curves were reversible and reproducible).

the overall volume phase transition behavior is dictated by the thermo-responsive constituent of the matrix. Interestingly, the only major difference in the data presented in Figs. 3a and b is that in the case of the polymer blend containing HEC, the transition is somewhat less sharp, which makes sense since HPC is the minor constituent. No transition in turbidity was observed for a solution of pure HEC at 1.5% w/w over the same temperature range (data not shown). The transition temperature was taken to be the temperature at which the absorbance was equal to 0.5, on a normalized scale of 0 to 1.0, which occurred at ~39°C. Because one cannot detect DNA in a turbid polymer solution, 38°C represents the maximum operating temperature of this matrix. The temperature range investigated for CE experiments (25°C–38°C), shown in Figs. 2a–f, fell in the allowable range.

Whereas the DNA separations depicted in Figs. 2a–f do not appear to be dramatically different, a few interesting trends can be visually observed, and others emerge upon quantitative data analysis. Comparing Figs. 2a–f, we see that at lower temperatures, better resolution is obtained for small DNA (compare in particular the resolution of the 271 bp/281 bp fragments), whereas improved resolution of large DNA (1078 bp/1353 bp fragments) can be observed at high temperatures. The selectivity of DNA separation as a function of temperature for pairs of small (< 300 bp) and large (> 1 kbp) fragments was plotted to illustrate more clearly the temperature-dependence of matrix performance. Selectivity S is defined as:

$$S = (\triangle\mu/\mu_{avg})/\triangle bp \quad (1)$$

where $\triangle\mu$ is the difference in electrophoretic mobility of the two DNA fragments, μ_{avg} is the average of the two mobilities, and $\triangle bp$ is the difference in the DNA sizes in base pairs (bp). (Note that we chose to plot selectivity rather than resolution, so that we could consider peak separation only, and neglect any differences in peak width that might occur from run to run, since peak width can vary with the age of the wall coating as well as with the exact amount of DNA injected.) Figures 4a–c show selectivity as a function of temperature for pairs of DNA fragments including 118 bp-194 bp, 271 bp-281 bp, and 1078 bp-1353 bp, respectively. On each plot, we show the selectivity of separation for a DNA pair for both the blended HEC/HPC matrix (closed triangles) and a pure HEC (nonthermo-responsive) polymer network (open triangles). Clear differences in DNA migration behavior are observed in the two matrices over the temperature range analyzed. We will discuss the behavior of the blended matrix first. In the mixed HEC/HPC solution there is a maximum selectivity at 31°C for the 118 bp-194 bp fragments. On the other hand the selectivity for the 271 bp-281 bp fragments decreases linearly with temperature.

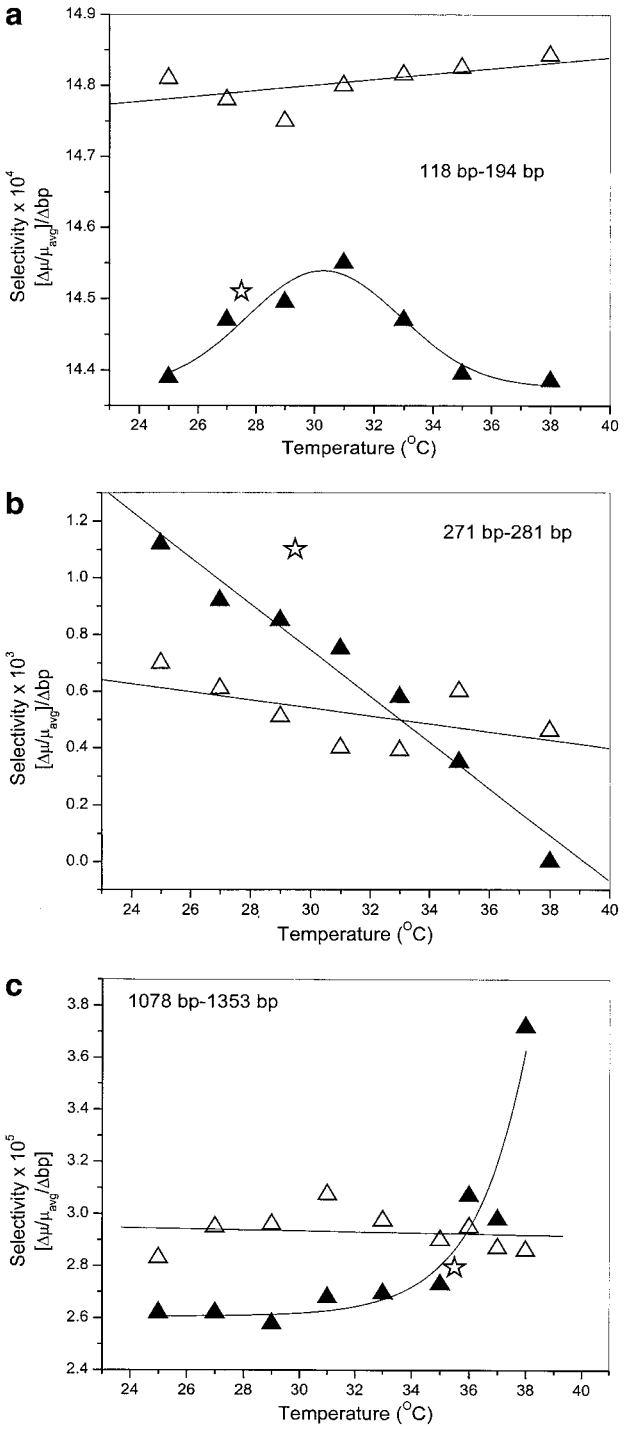

Figure 4. Selectivity as a function of temperature for (a) 118 bp–194 bp; (b) 271 bp–281 bp; (c) 1078 bp–1353 bp. Closed triangles represent the data obtained in 1.25% w/w HEC blended with 0.25% w/w HPC in 1 × TBE. Open triangles represent the data obtained in 1.5% w/w HEC in 1 × TBE (control). Open stars represent the results obtained in the temperature-ramping experiment shown in Figs. 5 and 6. Note that all values plotted represent averages of DNA mobilities for at least three CE runs; typical %RSD in mobilities was around 0.2%.

Significant improvement in the separation of the 1078 bp-1353 bp fragments is observed at higher temperatures (> 35°C).

Based on these data, we believe that a shift of DNA separation mechanism occurs in the blended, thermoresponsive matrix as the mesh size changes with temperature, in particular a shift which is analogous to more typically observed changes in DNA separation performance as polymer concentration is changed [14, 22]. The shrinking of the HPC coils as temperature is increased can be envisioned to create the same effect as a reduction of overall polymer concentration, when the dimension (*e.g.*, R_g) of HPC coils are reduced and the sieving network is "loosened", as if it were a less concentrated solution of HEC polymers only. As more dilute, less entangled polymer solutions are favorable for large DNA fragment separation, the selectivity is improved as the mesh "opens up" at elevated temperature. Previously it has been shown by Heller [35] that the dependences of electrophoretic mobility on mesh size for small and large DNA fragments are different. Generally, the electrophoretic mobility of DNA molecules increases with increasing mesh size. However, there is a stronger dependence of small DNA fragment mobility on mesh size than there is for large ones, as shown by a steeper slope of a logarithmic plot of mobility *vs.* mesh size [35]. Given that the selectivity is determined by the mobility difference, it makes sense that small DNA fragments experience a more dramatic effect than large DNA fragments, as we observe here. At low temperatures (small mesh size), the matrix is a more dense, entangled network. Although the small DNA fragments were baseline-resolved at all CE temperatures (Figs. 2a–f), the selectivity was improved for the 118 bp-194 bp fragments (Fig. 4a) as the mesh size was increased at elevated temperature, up to a certain optimum temperature. This optimal CE temperature presumably corresponds to the most favorable configuration and concentration of the HEC and HPC polymer molecules, corresponding to the polymer network that gives the most effective DNA-polymer interaction for size-dependent separation of this pair. A further increase in temperature, and hence of the matrix mesh size, lowers the resolution of small DNA fragments due to an effect which is analogous to diluting the polymer solution, which is known to be unfavorable for the separation of small DNA fragments. On the other hand, the monotonic decrease of temperature-dependent selectivity that we observed for the 271 bp-281 bp fragments suggests that small DNA fragments, with a very small difference in size, are always best separated in a more constrictive matrix. This is perhaps analogous to the separation of ssDNA sequencing fragments, which always require a highly entangled polymer network for the separation of molecules differing by only one DNA base in length. It is inter-

esting to note that the slope of the decrease in selectivity is much greater for the thermo-responsive polymer blend than for the pure HEC "control" matrix.

Comparing the results obtained for the 118 bp-194 bp and 271 bp-281 bp fragments (Figs. 4a and b), one can see that it is not only the absolute DNA fragment size that determines the optimal choice of sieving matrix, but also that the size difference between the particular DNA pair to be separated may be important. On the other hand, the simple trend that we observe in the selectivity of the matrix for the 1078 bp-1353 bp pair as a function of temperature (Fig. 4c) illustrates the favorable separation of large DNA fragments by a less constrictive matrix, which is quite well known [14, 22, 23], and analogous to the use of a less entangled polymer solution. This finding is consistent with expectation based on the physical picture provided by the transient entanglement coupling model [23], which perhaps best describes the separation of large dsDNA fragments in less entangled polymer solutions.

The control experiments that were done with 1.5% w/w pure HEC allow us to verify that the observed effects are indeed the consequences of "dynamic porosity" of the polymer network, in contrast to simpler effects of electrophoretic mobility change with temperature alone [35]. The results are plotted in Figs. 4a–c as open triangles. The data appear to show a slight temperature-dependence of selectivity, but this is most likely due to the sole effect of increased electrophoretic mobility with temperature. As shown in Eq. (1), the selectivity depends strongly on average electrophoretic mobility, hence a systematic shift in electrophoretic mobility (as seen in Figs. 2a–f) will have a monotonic effect. However, there were much weaker trends with pure HEC than those observed with the thermo-responsive HPC/HEC blend, hence the conformational change of HPC polymers with increasing temperature definitely plays a role. Therefore, the hypothesis that this polymer matrix exhibits a "dynamic porosity" appears to be valid.

One useful way to utilize this matrix, as well as the information gained in this study, is to implement a more sophisticated temperature ramping scheme in a single CE run with the goal of improving the resolution of both small and large DNA fragments simultaneously. The temperature ramp could involve either a spatial or a temporal variation. A spatial temperature ramp could be implemented on an electrophoresis microchip with advanced temperature control [31, 36], while a temporal temperature ramp can be executed in a more traditional CE instrument in a research laboratory, if it has programmable features such as the BioRad® instrument used in this study. Accordingly, a temperature-ramping experiment, with the

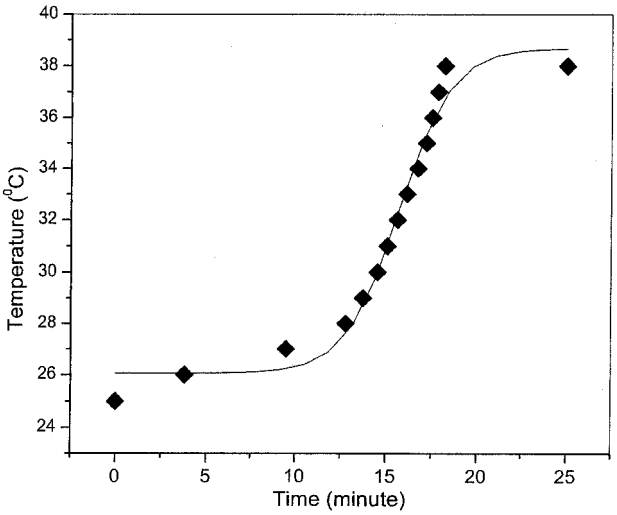

Figure 5. The temporal temperature profile of the temperature-ramping experiment. The profile is obtained by setting up a "timed event" in the BioRad CE instrument software. Temperature was observed as a function of time and plotted.

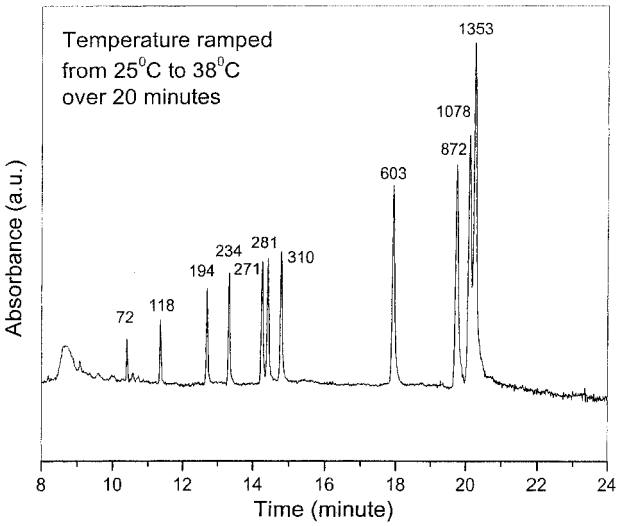

Figure 6. A typical electropherogram obtained in a temperature-ramping experiment with the thermal profile depicted in Fig. 5. CE conditions: reversed-polarity; electrokinetic injection, 500 V/cm for 3 s; run voltage, 265 V/cm; current, ramped from 7.2–8.2 μA.

temperature profile shown in Fig. 5, was performed. A representative electropherogram showing the separation of ΦX174-*Hae*III with the temperature ramp is given in Fig. 6, and the corresponding selectivities determined for different DNA pairs are plotted in Figs. 4a–c (as open stars) for comparison. This temperature-ramping scheme has the advantage of resolving small DNA fragments at low temperature (with almost optimal selectivity) at the beginning of the CE run, while the gradual temperature

ramping, large DNA fragments were well resolved as the run went on. The improved selectivity of the matrix for large DNA separation is not achievable in one CE run with a single temperature. Again, the apparent effect on the resultant electropherogram in comparison with Figs. 2a–f is not dramatic, but we believe that the observed trends in selectivity, shown in Figs. 4a–c, convincingly provide intriguing evidence and a "proof-of-concept" for dynamic porosity that now can be extended to other DNA samples and other blends of thermo-responsive and nonthermo-responsive polymers.

4 Concluding remarks

We have successfully demonstrated the effects of "dynamic porosity" on the resolution of small and large DNA fragments by CE, utilizing a blended matrix of thermo-responsive polymers (HPC) and nonthermo-responsive polymers (HEC), having similar structures to ensure miscibility. The polymer blend provided high-resolution separations of dsDNA at various temperatures ranging from 25°C to 38°C. The selectivity of the matrix for the separation of certain pairs of small and large DNA fragments (118 bp-194 bp, 271 bp-281 bp, 1078 bp-1353 bp) was evaluated between 25°C and 38°C. We found that the variation of matrix mesh size with temperature altered the selectivity of separation for small and large DNA fragments in different ways. The dynamic nature of the matrix mesh size was explored to demonstrate the feasibility of resolving both small and large DNA fragments in a single run through temperature ramping. The proof-of-concept results obtained have important implications for allowing enhanced separations of genomic DNA *via* capillary array or microchip electrophoresis in microfluidic devices with advanced temperature control.

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