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# Microchannel DNA Sequencing Matrices with a Thermally Controlled "Viscosity Switch"

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**Polymers and hydrogels that swell or shrink in response to environmental stimuli such as changes in temperature, pH, or ionic strength are of interest as switchable materials for applications in biotechnology. In this paper, we show that thermoresponsive polymers offer some particular advantages as entangled matrices for DNA sequencing by capillary and microchip electrophoresis. Matrices based on conventional water-soluble polymers demand a compromise in their design for microchannel electrophoresis: whereas highly entangled solutions of high molar mass polymers provide optimal sequencing performance, their highly viscous solutions require application of high pressures to be loaded into electrophoresis microchannels. Here, we demonstrate the reproducible synthesis, precise characterization, and excellent DNA sequencing performance of high molar mass, thermoresponsive polymer matrices that exhibit a reversible, temperature-controlled "viscosity switch" from high-viscosity solutions at 25 °C to low-viscosity, microphase-separated colloidal dispersions at a chosen, elevated temperature. The viscosity switch decouples matrix loading and sieving properties, enabling acceleration of microchannel flow by 3 orders of magnitude. DNA sequencing separations yielding read lengths of 463 bases of contiguous sequence in 78 min with 97% base-calling accuracy can be achieved in these matrices. Switchable matrices will be particularly applicable to microfluidic devices with dynamic temperature control, which are likely to provide the next major leap in the efficiency of high-throughput DNA analysis.**

Although a "working draft" of the human genome has been completed, DNA sequencing throughput still must be doubled if an accurate version of the genome is to be completed by 2003, the target set by the Human Genome Project (HGP).[1] Moreover, in addition to the HGP, over 345 genome projects have been launched for other organisms.[2] These massive sequencing projects and their many applications in medicine, forensics, molecular biology, and biotechnology will continue to demand rapid evolution of high-throughput DNA sequencing technologies toward increased speed and reduced cost.

The industrial implementation of capillary array electrophoresis (CAE) has revolutionized the operation of genome centers.[3,4] Commercial CAE instruments, including the MegaBACE 1000 DNA sequencing system from Molecular Dynamics and the ABI PRISM 3700 DNA analyzer from Applied Biosystems, have decreased sample and reagent requirements and increased sequencing throughput by providing increased run automation.[5] Integrated instruments that enable start-to-finish automation of sequence generation, including sample preparation, cleanup, and analysis by CAE, have been investigated[6,7] but are not yet commercially available.

Currently, DNA separation matrices based on viscous solutions of entangled, water-soluble polymers are used in CAE (as recently reviewed[8]) and are replaced from capillary arrays before each sequencing run by the application of high pressure (1000 psi or greater). In particular, linear polyacrylamide,[9] poly(dimethylacrylamide),[10,11] and poly(ethylene oxide)[12] are utilized as separation matrices for DNA sequencing by CAE and provide practical read lengths of 550–600 bases/capillary with 2–4 h of electrophoresis. In both of these CAE systems, fused-silica capillary arrays typically are used for at least 150 consecutive runs, with pressurized polymer matrix replacement before each run (a fresh gel is required for each sequencing separation). Capillary arrays themselves are eventually replaced due to a gradual drop in performance that is generally associated with either fouling or degradation of the polymeric wall coating. The ability to use capillary arrays for more than 150 runs brings down the cost per DNA base considerably, in comparison to the cost of single-use, crosslinked gel-filled capillaries, which are not practical for high-throughput sequencing. With highly optimized polyacrylamide matrix formulation, base-calling algorithms, and near-complete removal of salt and template from DNA sequencing samples, CAE has shown the potential to provide up to 1300 bases of contiguous sequence per capillary in less than 2 h, in poly(vinyl alcohol)-

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coated capillaries that in this case could be used for more than 300 consecutive runs with matrix replacement before each run.[9]

Microfluidic devices ("electrophoresis chips") offer the possibility of a 5–10-fold increase in DNA sequencing rate over CAE instruments,[13,14] primarily because of the narrow sample zone and highly controlled injections that are attainable with these devices. With narrow sample zones, even relatively short separation channels on glass microfluidic devices (e.g., 11 cm) can deliver reasonably long DNA sequencing reads (e.g., 550 bases) in under 27 min.[15–18] However, no group has yet demonstrated the ability to carry out practical, automated matrix replacement on microfluidic chips between sequencing runs. Hence, in their present embodiment, glass sequencing chips would serve as single-use, parallel sequencing devices, i.e., single-use monolithic capillary arrays.

Electrophoresis chips can be fabricated not only from glass[19] but also from plastic[20,21] in a variety of channel configurations. Prototype chip devices with integrated reagent measuring and mixing as well as spatial temperature control have been demonstrated,[22] innovations that will facilitate on-chip implementation of integrated genetic sample preparation steps including PCR[23] and sample cleanup.[24] Chips with advanced temperature control will also enable optimal use of the thermoreversible polymer matrices that we describe here.

Certainly for some applications it would be convenient if microfluidic chips could serve as single-use, disposable genetic analysis devices. However, present DNA sequencing chips, which are fabricated from glass and lack the possibility of automated matrix replacement between runs, may be impractical as single-use, throwaway devices because of the relatively high cost and effort associated with producing individual glass chips by serial photolithography and chemical wet etching. On the other hand, plastic microfluidic devices, which have significantly lower production costs, often suffer from lower detection sensitivity than glass chips owing to autofluorescence of the plastic substrate.[25–28] There is not yet any published work indicating that disposable plastic chips can be used for high-performance, long-read DNA sequencing. Hence, there is a present need for easily replaceable DNA separation matrices that would enable glass microfluidic chips to serve as multiuse devices, just as fused-silica capillary arrays are presently reused for 150–200 sequencing runs. The ability to reuse silica-based sequencing devices would drive down the cost per DNA base substantially, making this platform more attractive as an alternative to CAE.

DNA separation matrices that are appropriate for use in CAE instruments are not necessarily the most advantageous for use in electrophoresis chips. Conventional matrices, which are highly entangled solutions of high molar mass linear polymers such as polyacrylamide, have high zero-shear viscosities (e.g., 120 000 cP)[29] and hence require the application of high pressure (e.g., 1000 psi) to initiate microchannel flow and fill capillary arrays within a reasonable time. For this reason, matrices based on conventional water-soluble polymers may be difficult to implement for automated replacement in glass chips, because of the inherent difficulty of applying positive pressure to chip microchannels. Furthermore, chip devices cannot physically withstand high delivery pressures (e.g., some polymeric devices have a pressure limitation of 50 psi.[21])

Automated replacement of separation matrices in electrophoresis chips is predicted to be a necessary technological breakthrough for the development of high-throughput DNA sequencing in multiuse microfluidic devices.[18] To date, separation matrices that have been used in electrophoresis chips have been either polymerized in situ or, in more recent work, prepared externally and hand-loaded into chips with a syringe or pipet.[15,23,30] The properties of polymers produced by in situ reaction within chip microchannels are difficult to control; moreover, these matrices will contain unpolymerized, reactive monomer that can cause irreproducible sequencing results. On the other hand, manual or even automated loading of viscous separation matrices into chip microchannels through application of high pressures is a less attractive technological approach than automated matrix loading under low applied pressures. It seems that unless it is possible to replace separation matrices from glass chips and hence to use them for as many runs as one uses a fused-silica capillary array, it is questionable whether a glass chip format will actually provide a significant cost and throughput advantage over CAE for high-throughput DNA sequencing.

It seems clear, then, that practical introduction of microfluidic chips into a high-throughput sequencing environment could be facilitated by development of a new class of DNA separation matrices that provide good sequencing performance and that also can be loaded rapidly into chip microchannels under low applied pressure (e.g., 50 psi). Here, we show that the thermodynamic volume phase transitions of thermoresponsive, *N,N*-dialkylacrylamide-based copolymers in aqueous 7 M urea solution can be exploited to dynamically control matrix viscosity, fulfilling this requirement for rapid and easy microchannel loading, as well as


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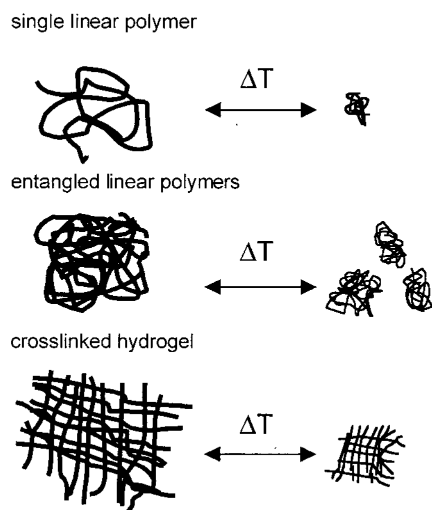


**Figure 1.** Schematic representation of LCST-exhibiting polymer and hydrogel systems as they undergo a reversible volume phase transition. As the temperature is increased, a single (dilute) linear polymer and entangled linear polymers undergo the collapse transition either as singular or as entangled chains that can then coalesce into separate polymer microdomains that reach colloidal dimensions, clouding the solution. On the other hand, cross-linked hydrogels undergo the volume phase transition as single monolithic entities.

rapid and efficient DNA sequencing analyses by microchannel electrophoresis.

As schematically shown in Figure 1, individual thermosensitive polymer chains that are not part of a cross-linked network undergo a thermodynamically driven volume phase transition over a narrow range of temperature, in which they abruptly collapse from a solvated, coiled chain conformation to an insoluble, globular state.[31] This sudden change in polymer solubility is triggered by a shift in the relative strengths of enthalpically favorable hydrogen bonding and entropically unfavorable hydrophobic hydration interactions between the polymer segments and the aqueous solvent.[32−35] In semidilute or entangled polymer solutions, polymer globules aggregate after collapse through kinetically controlled hydrophobic association to form dispersed, insoluble droplets of colloidal dimensions, which turn the solution cloudy.[31,36] Given sufficient time, these polymer globule microphase droplets will coalesce and precipitate to form a second, immiscible, polymer-rich liquid phase. Hence, thermally driven collapse transitions of un-cross-linked thermoresponsive polymer solutions present a physical scenario different from the more typically studied volume phase transitions exhibited by cross-linked, semisolid hydrogels,[36] in that a cloudy, low-viscosity dispersion is formed upon polymer collapse and aggregation (Figure 1). The temperature at which this phase transition occurs is termed the lower critical solution temperature (LCST) or the "cloud point" of the solution.

Solution viscosity decreases precipitously when a thermoresponsive polymer solution is switched into its microphase-separated state by raising the temperature above the cloud point of the solution, as a result of the dramatic collapse in polymer coil volume. It has been noted previously that this property could be exploited to facilitate easy capillary loading of DNA separation matrices.[37,38] Numerous polymers of the poly(*N*-alkylacrylamide) family,[39] including poly(*N,N*-diethylacrylamide)[35] and poly(*N*-isopropylacrylamide),[40−43] display volume phase transitions in aqueous solution at elevated temperatures. Preliminary tests of thermoresponsive polymers as separation matrices for double-stranded DNA, including poly(*N*-isopropylacrylamide)-*g*-poly(ethylene oxide) [44] and poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) triblock copolymers,[45−48] have indicated that viscosity-adjustable separation matrices can be used for the separation of double-stranded DNA fragments, but none of these matrices has been demonstrated to provide the single-base resolution required for DNA sequencing.

In work more relevant to the present study, entangled matrices composed of poly(*N,N*-diethylacrylamide)-*co*-poly(*N,N*-dimethylacrylamide) (DEA/DMA) were tested for DNA separation by CE with single-color fluorescence detection and were shown to provide limited single-base resolution of small, single-stranded DNA fragments (<130 bases),[37] which indicated that thermoresponsive matrices of this class might be useful for DNA sequencing. The challenge in developing these copolymers to serve as useful DNA sequencing matrices was to create polymers having both the desired thermoresponsive behavior and viscosity transitions *and* the appropriate chemical and physical properties for high-resolution separations of DNA sequencing fragments. Toward this end, we have developed new polymer synthetic methods and separation matrix formulations specifically for this purpose. As described below, we have succeeded in creating optimized thermoresponsive polymers that offer all of the desired chemical and physical attributes to serve as DNA sequencing matrices with a dramatic, thermally controlled "viscosity switch".

## EXPERIMENTAL SECTION

**Polymer Synthesis.** Ultrapure *N,N*-diethylacrylamide and *N,N*-dimethylacrylamide (Monomer-Polymer and Dajac Labs, Inc., Feasterville, PA) were copolymerized (53 wt % DEA/47 wt % DMA) in an aqueous solution (7.0% (w/v) total monomer concentration), thermostated at 47 °C, and degassed with high-purity helium prior to initiation. Initiator V-50 (2,2′-azobis(2-amidinopropane) dihydrochloride, Wako Chemical USA, Inc.,

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Richmond, VA) was dissolved in water and injected into the reaction flask. After 16 h, the resulting mixture was allowed to come to room temperature, poured into 1000 molecular weight cutoff cellulose ester membranes (Fisher Scientific, Pittsburgh, PA), and dialyzed against deionized, distilled water for 10 days with frequent water changes. The polymer solution was then frozen and lyophilized using a freeze−dry system (Labconco, Kansas City, MO), resulting in a white, stiff, foamlike polymer mass that was then redissolved in aqueous buffer by slow rotation overnight (Roto-Torque, Cole-Parmer Instrument Co., Inc., Vernon Hills, IL).

**Polymer Characterization.** To characterize the molar mass distributions of the DEA/DMA copolymers that were synthesized, samples were fractionated by gel permeation chromatography (GPC) prior to analysis by on-line multiangle laser light scattering (MALLS) detection, using a Waters 2690 Alliance Separations Module (Milford, MA) with Shodex (New York, NY) OHpak columns SB-806 HQ, SB-804 HQ, and SB-802.5 HQ connected in series. In this tandem GPC-MALLS mode, the effluent from the GPC system flows into the DAWN DSP laser photometer and Optilab DSP interferometric refractometer (both, Wyatt Technology, Santa Barbara, CA). Sample aliquots of 100 $\mu$L (sample concentration, 0.5 mg/mL) were injected into the system (mobile phase 0.1 M NaCl, 50 mM $NaH_2PO_4$, and 200 ppm $NaN_3$; flow rate 0.35 mL/min). The tandem GPC-MALLS data was processed using ASTRA for Windows software from Wyatt Technology.

A rotational Bohlin VOR rheometer (Cranbury, NJ) was used to determine the steady-shear viscosity of the DNA separation matrices as a function of temperature. At each given, fixed temperature (held with a circulating water bath), the viscosity was measured at a range of shear rates. To measure solution turbidity as a function of temperature and detect the cloud point transition of the polymer solutions, visible spectroscopy was performed using a Cary 500 UV−visible−NIR spectrophotometer outfitted with a circulating water bath (Varian, Walnut Creek, CA). Matrices were heated and cooled at 5 °C/min, and absorbance data were collected at 500 nm. Capillary loading times were measured using a laboratory-built, aluminum device pressurized with a nitrogen gas cylinder. Both the loading device and the capillary (excluding the open tip) were fully immersed in a temperature-controlled water bath.

**Copolymer Composition.** $^1$H NMR spectroscopy was performed with a Varian Inova 500 (Walnut Creek, CA) to determine the composition of the DMA/DEA copolymers and the actual proportions of each monomer incorporated into the copolymers. $^1$H NMR (500 MHz, $D_2O$) $\delta$ (ppm): 1.12 ($2CH_3$ of DEA, br s), 1.43−1.73 ($CH_3\mathit{CH_2}$, m), 2.66 ($\mathit{CH}CH_2$, m), 2.95 ($2CH_3$ of DMA, br s), 3.32 ($2CH_2$ of DEA, m). Integration of NMR signals at 1.12 and 2.95 ppm was used to calculate the relative molar proportions of DEA and DMA monomers, respectively, incorporated into the copolymer.

**DNA Sequencing.** DNA sequencing was performed on a MegaBACE 1000 DNA sequencing system (Molecular Dynamics, Sunnyvale, CA) CAE instrument equipped with 96 fused-silica capillaries (75-$\mu$m inner diameter, 64-cm total length, 40-cm effective length) covalently coated with linear polyacrylamide. A buffer consisting of 50 mM tris(hydroxymethyl)aminomethane (Tris), 50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid (TAPS), and 2 mM ethylenediaminetetraacetic acid (EDTA) (Amresco, Solon, OH, and, Sigma, St. Louis, MO) containing 7 M urea (Amresco) was used to dissolve the 53% (w/w) DEA/47% (w/w) DMA copolymer to a concentration of 7.35% (w/v). MegaBACE sequencing standards (Amersham Pharmacia Biotech, Piscataway, NJ) consisting of M13 DNA sequencing reaction products were used without further purification. As purchased, sequencing reaction products were purified by the manufacturer by standard ethanol precipitation. Sequencing matrix was loaded into the capillaries under a pressure of 1000 psi for 200 s, followed by a relaxation time of 20 min and a prerun electrophoresis for 5 min at 140 V/cm. After electrokinetic sample injection (93.75 V/cm, 30 s), the DNA was electrophoresed at 140 V/cm and 44 °C. Laser-induced fluorescence data were collected, analyzed, and translated into DNA sequence using the MegaBACE 1000 DNA sequencing software version 2.0.

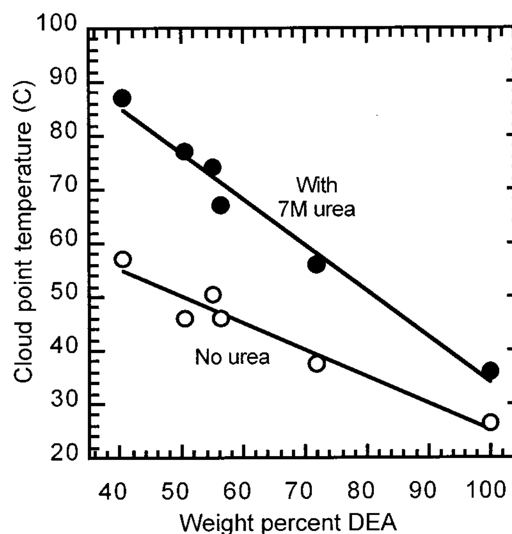


**Figure 2.** Dependence of cloud point on polymer composition for random, linear thermosensitive copolymers formulated from different proportions of DEA and DMA. Cloud points were determined by light absorbance measurements at 500 nm while samples were heated and subsequently cooled at 5 °C/min using a Cary 500 UV−visible spectrophotometer. Filled points: 7.35 (w/v)% copolymer in 50 mM Tris/50 mM TAPS/2 mM EDTA buffer with 7 M urea. Open points: 7.355 (w/v) copolymer in 50 mM Tris/50 mM TAPS/2 mM EDTA buffer.

## RESULTS AND DISCUSSION

Random copolymers were formulated from a mixture of two different monomers: DEA, which produces a thermosensitive homopolymer with a cloud point of ~30−35 °C in aqueous solution, and DMA, which is soluble in water up to 100 °C and is known to be useful for DNA sequencing as a homopolymer.[10] Through copolymerization of these two monomers, the cloud point of the resultant thermoresponsive polymers in water can be tailored between 30 and 100 °C by adjustment of the comonomer composition (Figure 2).[49] With the addition of 7 M urea, as necessary to create denaturing conditions for DNA sequencing, cloud points are higher as a result of the change in solvent quality but remain linearly dependent on copolymer composition. Extension of the abscissa of Figure 2 to copolymer compositions below

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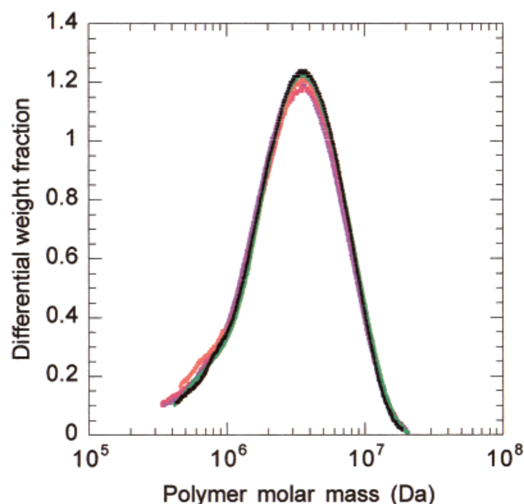


**Figure 3.** Tandem GPC-MALLS molar mass characterization of five different batches of thermoresponsive copolymers synthesized by random free-radical copolymerization of 53 wt % DEA and 47 wt % DMA. Results from each batch of copolymer are shown in a different color. Polymerization conditions are given in the Experimental Section.

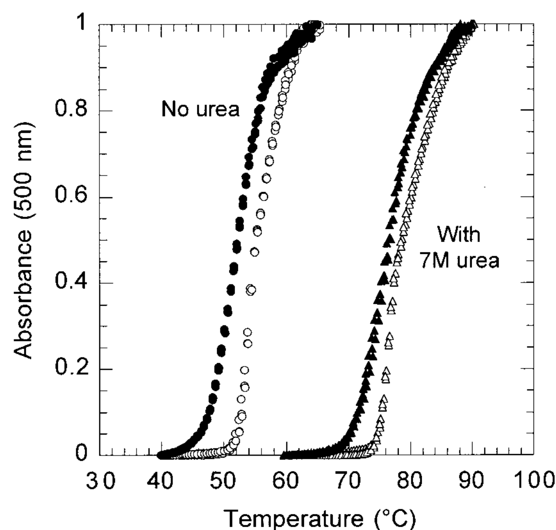


**Figure 4.** Transition behavior of thermoresponsive 53 wt % DEA/47 wt % DMA copolymers as measured by visible light absorbance at 500 nm, using a Cary 500 temperature-controlled UV−visible spectrophotometer. Left (circles): 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA. Right (triangles): 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA with 7 M urea. Both: Open points depict heating data; filled points depict cooling data. Samples were heated and cooled at 5 °C/min. Data depicted represent the results of three consecutive cycles of heating and cooling at this rate.

40% (w/w) DEA is not possible, as the cloud point temperatures of these matrix formulations in 7 M urea solution exceed 100 °C and cannot be measured without boiling the solvent. In any case, these formulations are not of interest for the study as such high transition temperatures are inaccessible for dynamic matrix viscosity switching.

The two predominant commercial CAE sequencing instruments, the MegaBACE 1000 DNA sequencing system and the ABI PRISM 3700 DNA analyzer, are run at maximum electrophoresis temperatures of 44 and 60 °C, respectively.[50,51] Hence, for DNA sequencing purposes we chose a copolymer formulation that simultaneously provides an accessible transition temperature (75 °C in a sequencing buffer containing 7 M urea) well above the 44 °C maximum run temperature of the MegaBACE 1000 DNA sequencing system in our laboratory and excellent DNA separation performance at 44 °C. At 44 °C, the copolymer matrix properties are essentially those of a normal, entangled polymer solution in which the polymers have some degree of hydrophobic character.[52]

Since it is known that matrices composed of high molar mass polymers give the longest DNA sequencing read lengths,[9,53] free-radical solution polymerization conditions were adjusted to produce copolymers with the highest achievable molar mass. Under a given set of conditions, we find that solution copolymerization gives highly reproducible results, as indicated by GPC-MALLS analyses of the molar mass distributions of five separate polymer batches (Figure 3). Optimized synthesis conditions consistently produced copolymers with weight-average molar mass ($M_w$) of 4.0 MDa, weight-average radius of gyration of 99 nm, and a polydispersity index of 1.70. $^1$H NMR spectroscopy confirmed that incorporation of DEA and DMA monomers into the copolymer is consistent with the composition of the initial monomer stock. We expect that higher molar masses could potentially be achieved by inverse emulsion polymerization.[29]

The phase transition behavior of denaturing and nondenaturing matrices composed of 7.35% (w/v) copolymer were characterized in detail by temperature-controlled visible spectrophotometry and steady-shear rheometry. Both denaturing and nondenaturing DNA separation matrices undergo a sharp volume phase transition, as indicated by their rapid increase in turbidity over a narrow temperature range (Figure 4). As noted previously, the addition of urea to the matrices raises the cloud point temperature by more than 20 °C, owing to the change in solvent quality. Both denaturing and nondenaturing polymer matrices exhibit some hysteresis in absorbance between heating and cooling cycles, as a result of intrachain interactions that are formed in the aggregated state and that take some time to dissipate.[42] However, we were able to identify thermal cycling conditions under which the volume phase transitions for both matrices are fully reversible, even over three consecutive cycles of relatively rapid heating and cooling (5 °C/min), as shown in the figure. Despite the existence of some polymer dissolution hysteresis, the data shown in Figure 4 demonstrate that the matrices can be heated and cooled rapidly, multiple times, for microchannel loading and sequencing steps, respectively, with no precipitation of thermosensitive polymers from solution.

Panels a and b of Figure 5 show the dependence of matrix viscosity and capillary loading time on temperature, respectively. Within the temperature range over which the phase transition occurs, the steady-shear viscosities of thermoresponsive copolymer solutions precipitously decrease, dropping by at least 1 order of magnitude. This occurs for both nondenaturing and denaturing matrices, with the viscosity transition appearing to be somewhat more dramatic for the nondenaturing matrix. These steep reductions in matrix viscosity provide an even greater relative

(50) www.megabace.com.
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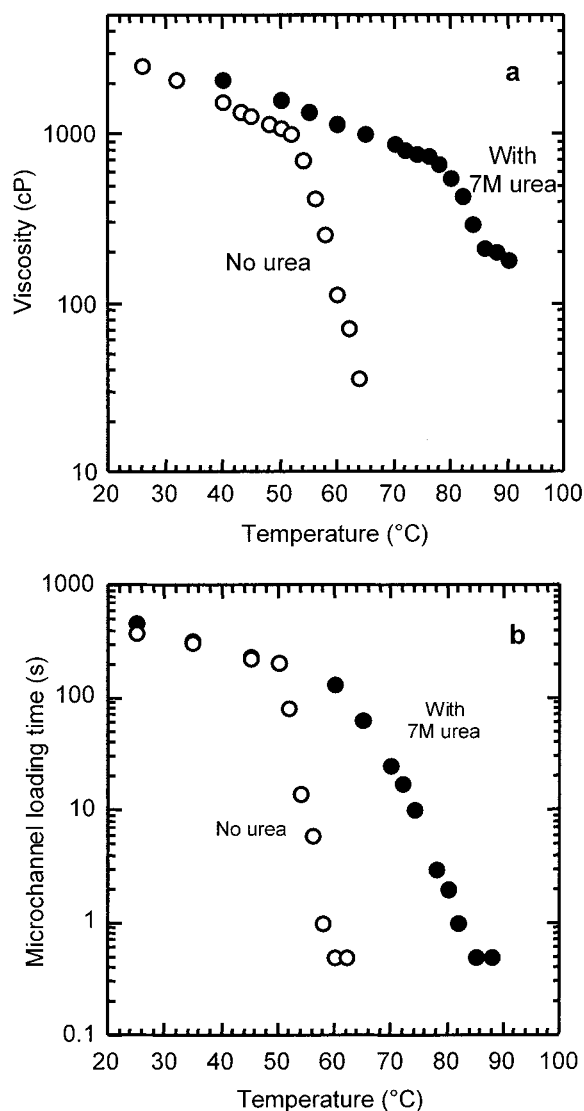


**Figure 5.** (Panel a) Rheological characterization of thermoresponsive 53 wt % DEA/47 wt % DMA copolymer solutions as a function of temperature. Experiments were run on a Bohlin rheometer with a Couette cell. The shear rate for both samples was 11.6 $s^{-1}$. Filled circles: 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA with 7 M urea. Open circles: 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA. (Panel b) Pressurized loading times of 53 wt % DEA/47 wt % DMA copolymer solutions into a 10 cm × 50 $\mu$m capillary microchannel under a constant applied pressure of 50 psi at varying temperatures. Filled circles: 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA with 7 M urea. Open circles: 7.35% (w/v) in 50 mM Tris/50 mM TAPS/2 mM EDTA.

decrease in microchannel loading time, as seen in Figure 5b. Both matrices, when heated above their cloud point, can be rapidly loaded into a 10-cm capillary with a 50-$\mu$m inner diameter, under a very low applied pressure of 50 psi. Here, capillary dimensions and maximum loading pressure were chosen to mimic those of microchannels in a typical microfluidic DNA sequencing device. The results are dramatic: whereas room-temperature microchannel loading of the matrices requires ~8 min at 50 psi, in their low-viscosity supercritical state, the matrices can be loaded within 500 ms. This represents a reduction of 3 orders of magnitude in microchannel loading time with the use of the viscosity switch.

Analysis of ssM13mp18 DNA sequencing fragments in the same thermoresponsive polymer solution was performed on a MegaBACE 1000 DNA sequencing system (Figure 6). The denaturing DNA sequencing matrix, loaded at 44 °C, yields a DNA sequencing read length of 421 bases in 75 min with 98.5% base-calling accuracy and a read length of 463 bases in 78 min at 97.0% accuracy. The read lengths that we report here are quite conservative, as it is usually best to "train" a base-caller for a given class of matrices; the commercial base-caller we used is optimized for linear polyacrylamide matrices. With the use of slightly hydrophobic, thermosensitive polymers, base-calling accuracy could likely be improved substantially with custom mobility shift corrections for the four different, base-specific dyes.

We also wished to simulate the use of the viscosity switch prior to sequencing, in which thermoresponsive matrices would be rapidly heated (at rates of >5 °C/min), loaded into chip microchannels under 50 psi pressure in under 1 s, and then rapidly cooled to the run temperature for use as sequencing matrix. To accomplish this, we heated the matrix from room temperature to a temperature above its volume phase transition for a few minutes using a water bath, cooled it to room temperature for a few minutes, and then immediately used it for sequencing. After this process of heating and cooling, the same matrix discussed above yielded a somewhat shortened read length of 380 bases in 69 min with 98.5% accuracy and 432 bases in 74 min with 97% accuracy. Reduction in read length upon matrix heating and cooling may indicate that interchain hydrophobic interactions, formed when the polymer solution is heated above its volume phase transition and held there for some time, persist transiently after the matrix is cooled. We believe that this effect, which here caused a 10% reduction in read length, can be averted in actual chip devices through the implementation of a Peltier thermal control device that will allow rapid (e.g., >10 °C/min) in-chip heating and cooling and prevent the occurrence of extensive kinetically controlled polymer aggregation at temperatures above the cloud point.

While the 420-base read length with 98.5% accuracy that we report here is shorter than those typically provided by optimized commercial matrices for CAE based on conventional water-soluble polymers such as linear polyacrylamide and polyDMA (550–600 bases in 2–4 h), we note that our results are achieved in the significantly shorter time of 75 min and that most likely they could be extended by at least 50 bases through appropriate training of base-called software.

Throughout the course of this study, we noticed that read lengths in DEA/DMA copolymer matrices are quite sensitive to copolymer formulation and that more hydrophobic polymers (with lower transition temperatures) generally deliver shorter reads. In a companion study to this one, we have investigated the impact of polymer chemical and physical properties on matrix viscosity and DNA sequencing separations.[52] Careful characterization and comparison of various DMA/DEA copolymer matrices confirmed that, at a fixed average molar mass, the weight-average root-mean-square radius of gyration of the polymer chains, matrix viscosity, extent of chain entanglement, and DNA sequencing read length all decrease monotonically with increasing DEA content in the copolymer.[52] On the basis of the results of that study and this work, it is possible to choose a copolymer formulation that gives the desired viscosity switching temperature, with full knowledge

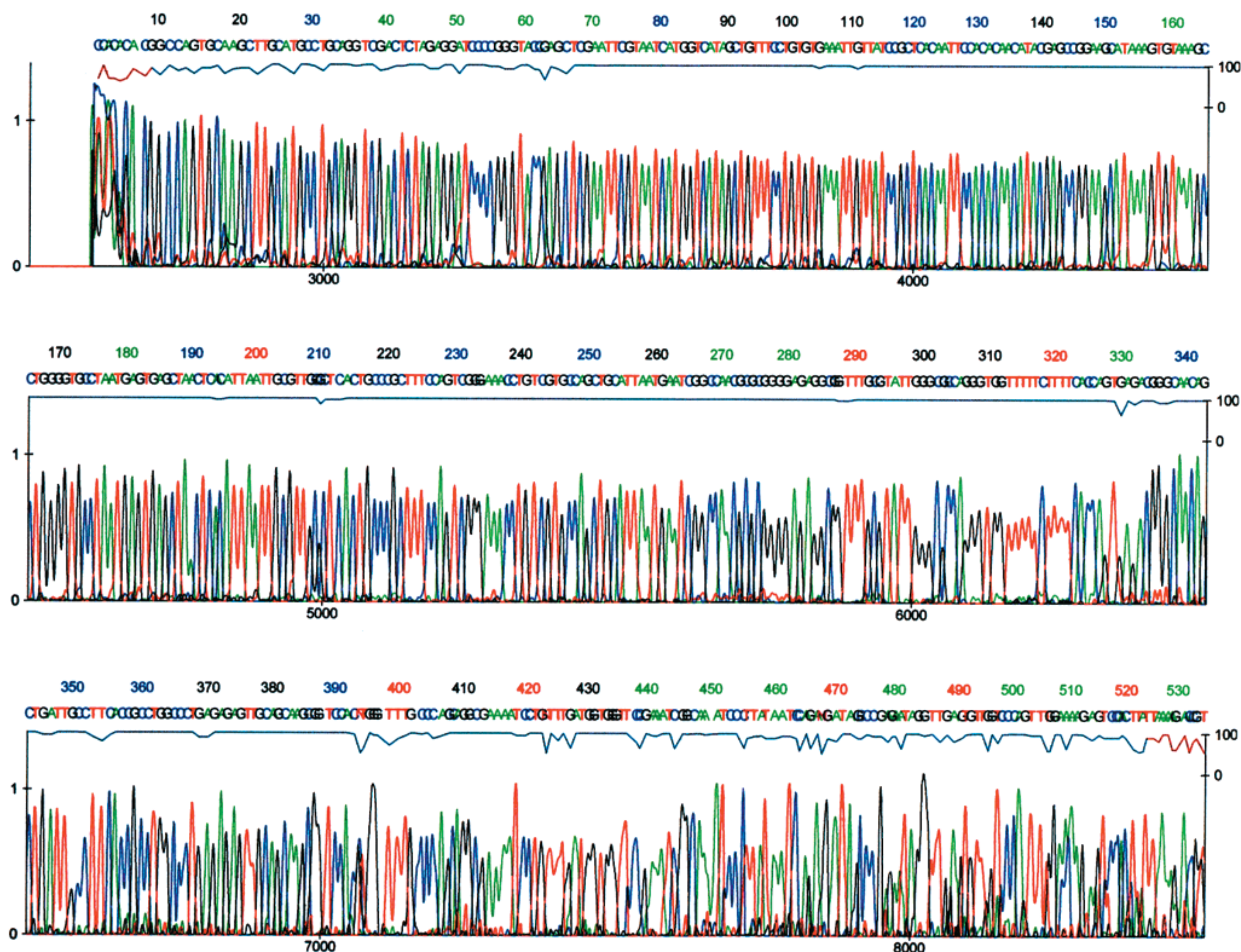


**Figure 6.** Electrophoretic separation of ssM13mp18 MegaBACE DNA sequencing standards labeled with fluorescent DYEnamic ET primers, performed on a MegaBACE 1000 CAE instrument, using a 53 wt % DEA/47 wt % DMA thermoresponsive copolymer solution as the separation matrix. Separation was achieved in 7.35% (w/v) copolymer in 50 mM Tris/50 mM TAPS/2 mM EDTA buffer with 7 M urea, at 140 V/cm and 44 °C.

of the expected read length and matrix viscosity. DEA/DMA copolymers comprising 10% less DEA monomer than those demonstrated here (e.g., 43% DEA and 57% DMA) provide ~50 bases more contiguous DNA sequence (yielding at least 515 base reads) and will exhibit a transition temperature in 7 M urea buffer of ~85 °C (Figure 2). This temperature should be accessible on a chip with a Peltier thermal control device. Since the matrix would only have to be held at this temperature for 1−2 s to accomplish channel loading (Figure 5b), thermal degradation of buffer components such as urea is not expected to be a problem.

Depending upon the requirements of the DNA sequencing application, the achievement of long reads (>400 bases) may or may not be necessary. Although long reads will most likely remain desirable for high-throughput sequencing efforts, for many clinical applications of DNA sequencing, shorter, faster reads such as those demonstrated here may be optimal. Thermoresponsive matrices should also be useful for other types of DNA separations including double-stranded DNA analysis for PCR fragment sizing and other types of genetic analyses used for the discovery of SNPs or the detection of genetic mutations. For example, single-strand conformation polymorphism analysis (SSCP) is a sensitive technique for the detection of single nucleotide changes in DNA fragments of typically ~300 base pairs or less that has traditionally been carried out in dense polyacrylamide slab gels.[55] However, CE-SSCP with entangled polymer matrices has recently been used to detect sequence variations in DNA fragments up to 600 base pairs long; this type of mutation detection analysis could also be carried out in thermoresponsive polymer matrices, perhaps allowing more concentrated solutions to be used to improve the separation of the SSCP fragments, which have identical or very similar chain lengths (depending on the mutation) but different secondary structure.[56]

While investigating alternate copolymer formulations for this study, we observed at times that hydrophobic interactions between polymer side chains and some classes of fluorescently labeled DNA molecules could be detrimental to the sequencing results.[52] All results presented in this paper were obtained using DNA sequencing fragments labeled with DYEnamic ET dyes (Amersham-Pharmacia), for which we did not observe any deleterious

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hydrophobic interactions. In the ET dye system, donor and acceptor dyes are linked by a few bases of DNA, which increases the water solubility and hydrophilicity of the ET dye complex. In addition to working with ET dye-labeled sequencing samples, we also carried out limited tests of our thermoreversible matrices with DNA sequencing fragments labeled with ABI PRISM BigDye terminators (Applied Biosystems Inc., Foster City, CA), another common fluorescent labeling kit for DNA cycle sequencing reaction products. Donor and acceptor dyes in the BigDye complexes are linked by chemical moieties that are organic and hydrophobic in nature; hence, they are substantially more hydrophobic than the ET dyes. These hydrophobic BigDye complexes, particularly the T-terminator dye complex, appeared to have substantial hydrophobic interactions with thermoreversible copolymers that comprised more than 40% DEA. These interactions altered the mobility of shorter DNA BigDye fragments and also led to band broadening and band shifting that made calling of accurate DNA sequence difficult when BigDyes were used. Hence, use of BigDye-labeled DNA fragments resulted in a dramatic reduction in read length and resolution compared to the MegaBACE sequencing standards labeled with fluorescent ET primers, for which we did not observe hydrophobic interaction.

Finally, we note that DNA sequencing experiments presented in this work were performed in fused-silica capillaries that were covalently coated with linear polyacrylamide (arrays that are available from Molecular Dynamics for use in the MegaBACE). Research ongoing in our laboratory indicates that DEA/DMA copolymers have excellent self-coating properties in fused-silica capillaries, which may eliminate the need for a covalent wall coating for capillaries and for glass-based microfluidic devices (data not shown).

## CONCLUSIONS

We have shown that thermoresponsive DNA separation matrices in a supercritical, phase-separated state can be loaded quickly into microchannels under low pressure (50 psi), yet provide highly effective DNA sequencing separations upon cooling below the cloud point. Exploitation of this thermodynamic phenomenon will allow for well-designed polymer matrices with switchable viscosities to be implemented on temperature-controlled microfluidic electrophoresis chips, which have high potential to enable integrated, totally automated genomic analyses including both sample preparation and analysis steps. With further optimization of copolymer formulation, molar mass, and matrix composition to extend read lengths, separation matrices comprising thermoresponsive copolymers will facilitate the implementation of chip-based DNA analysis systems in a high-throughput sequencing or clinical environment. As a typical DNA sequencing separation on a chip requires less than 25 min,[13] reduction of matrix loading time from 8 min to 500 ms, as we showed here, will greatly decrease sample turnaround time on chips with replaceable matrices. Both denaturing and nondenaturing matrices should prove to be useful for a variety of different types of nucleic acid-based analyses. Moreover, aqueous polymer matrices with a thermally triggered viscosity switch that can be chemically tailored for triggering at any temperature between 30 and 100 °C may be useful for a variety of other, unforeseen applications in biotechnology.

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