Published in final edited form as:

Electrophoresis. 2009 July ; 30(14): 2488–2498. doi:10.1002/elps.200800773.

Reduced Matrix Viscosity in DNA Sequencing by Capillary Electrophoresis and Microchip Electrophoresis Using a Novel Thermo-responsive Copolymer

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Abstract

High viscosity is an inherent problem of sequencing matrices made of hydrophilic polymers. This problem is amplified in separations using microchips where the channels are even smaller. A novel thermal associating graft copolymer, using linear polyacrylamide (LPA) as the backbone and poly(propylene oxide) (PPO) as the graft side chain was synthesized. The injection problem could be resolved by introducing PPO side chains that can be assembled by using temperature changes but without an obvious detrimental effect on the sieving ability of the LPA. Viscosity measurement showed that these LPA-g-PPO copolymers had a transition temperature of ~ 40 °C, above which a significant increase in viscosity was observed. The sequencing performance depended on the thermal association properties of PPO and related parameters. Without optimization, a read length of 1000 bases with a single base resolution of 0.3 was achieved within an hour on an ABI 310 analyzer, using 1.8 wt% LPA-g-PPO (1.8 MDa, PPO, 0.2%). This novel thermal reversible copolymer solution can be a promising candidate as a viable matrix for DNA sequencing in capillary electrophoresis, and even more so in microchip electrophoresis.

Keywords

DNA sequencing; long read length; Thermal-responsive; viscosity switchable

1. Introduction

Thermo-responsive polymers have drawn increasing attention in academic and industrial research. Investigation has been focused mostly in the biological and medical fields, such as drug delivery [1-7], tissue engineering [8], biotechnological devices [9,10], immobilization of enzymes [11] and DNA separation or sequencing [12-19]. These thermally sensitive polymers exhibit a “switchable viscosity” that is driven by volume-phase transition responding to small variations in temperature. In general, there are two kinds of thermo-associating polymers: thermo-thinning and thermo-thickening. Thermo-thinning refers to the phenomenon in which polymer chains entangle at lower temperatures and collapse to a lower viscous state, while thermo-thickening refers to the gel-like state and dramatic increase in viscosity that a polymer solution with a lower viscosity at low temperatures will exhibit at higher temperatures. In particular, when these thermo-responsive polymers are applied as DNA separation matrices, it is possible, if the polymer matrices are well-

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designed, to couple the capillary loading and separation performance by operating at two different temperatures.

The high viscosity of a separation medium often causes much difficulty in injecting the sequencing solution into the capillary. As a result, a fairly high applied pressure (≥ 1000 psi) is often necessary to load the sequencing matrix into the capillary. This type of applied pressure can become one of the critical issues in designing micro-fabricated chips because most microchips cannot tolerate more than ~ 200 psi external pressure before destroying itself, and some plastic chips can only withstand ~ 50 psi [20]. The criteria for a sieving matrix using microchips is basically the same as that in conventional capillary electrophoresis (CE), in terms of sieving ability, dynamic coating ability and viscosity, except that the viscosity problem is amplified in a micro total analysis system (μ TAS).

Some homopolymers, such as poly(*N*-isopropylacrylamide) (PNIPAM) or poly(propylene oxide) (PPO), are able to induce a sol-gel transition at relatively low temperatures (32°C and 5°C , respectively) [21-25], and low concentrations (a few percent), but become turbid after gelation. The turbidity suggests microphase separation and contributes to a detrimental effect in special applications, such as DNA sequencing. It should be noted that a useful separation matrix for DNA sequencing analysis requires a homogeneous network. Microphase separation, as shown by the turbidity appearance, indicates the inhomogeneous nature of the gel-like network. A strategy to solve this problem is to form a graft copolymer by using a hydrophilic polymer such as linear polyacrylamide (LPA) as the backbone and small thermo-sensitive 'triggers' as the side chains. With such a design, the side chains will maintain their thermal gelation properties and induce aggregation at elevated temperatures. The interaction between the polymer backbone and the solvent will make the polymer chain water-soluble in order to maintain the homogeneous nature of the polymer network. On the other hand, the ability of the side chains to change the mesh size and polymer chain disentanglement dynamics remains intact. For example, the LPA-*g*-PNIPAM was reported to be a promising DNA sieving matrix in comparison with commercial matrices. It resulted in a read length of 800 bases in less than one hour [12,26]. It should be particularly important to note that the increase in hydrophobicity caused by the side chains did produce a negative effect on the CE performance [27,28]. Therefore, the optimization of composition and architecture of these polymers becomes very subtle. A proper balance between sufficient positive thermo-thickening aggregation and a negative increase in hydrophobicity (or even worse with an induction to microphase separation) is needed in designing this kind of copolymer for a specific application.

The primary goal of this article is to introduce a new family of thermo-associating graft copolymers as a potential sieving matrix for DNA sequencing. These copolymers try to incorporate the following advantages: 1. a relatively low viscosity at room temperature for easy operation, including solution preparation and gel injection; 2. good sieving ability at typical CE running temperatures (e.g., 60°C) by increasing intermolecular interaction that can be induced thermally to form a more robust network; and 3. lack of (micro-) phase separation in the temperature range from ambient temperature to operating temperature in the obtained matrix.

Considering the above advantages, LPA-*g*-PPO was designed and synthesized as a promising DNA sequencing matrix with LPA as the backbone and PPO as the grafting side chain to form a unique graft copolymer. This was intended to combine the excellent separation performance of LPA and the thermal associative property of PPO. Although copolymers containing PPO, such as F127 ($\text{PEO}_{99}\text{PPO}_{69}\text{PEO}_{99}$) (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)) and F87 ($\text{PEO}_{61}\text{PPO}_{40}\text{PEO}_{61}$) or their mixtures, are well known for their thermo-reversible properties and are used in DNA

separation [17,29-39], for the first time, PPO side chains are being grafted onto LPA so as to increase the performance in DNA sequencing at the desired temperature. As both LPA and PPO are soluble in buffer solution at low temperatures, no aggregation takes place. A relatively low viscosity for the polymer solution is exhibited. When the temperature is increased, the PPO chains become insoluble and start to aggregate. An increase in the apparent polymer molecular weight and solution viscosity results, along with a decrease in mesh size. This in turn causes the formation of a robust network for the separation of DNA fragments, especially the larger ones, which can be attributed to more stable (or slower) polymer chain disentanglement dynamics in the presence of a fraction of aggregated PPO chains acting as cross-linking points. LPA-g-PNIPAM was also synthesized both to determine the effects of different side chains and to serve as a comparison.

2. Materials and Methods

2.1 Chemicals

Methoxy-poly(propylene oxide) acrylate (PPO, Avg. $M_n \sim 475$), 2-amino ethanethiol hydrochloride (AET·HCl), potassium hydroxide (KOH), sodium chloride, dicyclohexylcarbodiimide (DCC), acrylic acid, methanol, diethyl ether, acetonitrile and dichloromethane (CH_2Cl_2), *N*-isopropylacrylamide (NIPAM), ammonium persulfate (APS), ethylenediaminetetraacetic acid (EDTA) and *N*-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS), together with tris(hydroxymethyl)aminomethane (Tris), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used directly without further purification. Acrylamide (AA) was also purchased from Sigma-Aldrich and purified by recrystallization twice in chloroform. *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased from Fisher Scientific (Pittsburgh, PA, USA). *N,N*-dimethylacrylamide (DMA) was purchased from Polysciences (Warrington, PA, USA) and distilled under vacuum (80 °C/20 mmHg) before use.

PDMA ($M_w = 470$ KDa) was synthesized by Song et al as stated in Ref [40], and used as a dynamic coating agent.

The standard DNA samples of BigDye Terminator v3.1 Sequencing Standard, and the Template Suppression Reagent (TSR) were purchased from Applied Biosystems Co. (Foster City, CA, USA). Urea was purchased from sigma-Aldrich and used directly.

The $1 \times$ TTE buffer solution was composed of 50 mM TAPS, 50 mM Tris and 2.0 mM EDTA. The water used in all reactions and solutions was de-ionized to $18.2 \text{ M}\Omega$ with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 Synthesis of LPA-g-PPO & LPA-g-PNIPAM

Both copolymers were synthesized by classical radical polymerization as described in Scheme 1.

LPA-g-PPO—Acrylamide (AA) was first dissolved in a mixed solvent of water/acetonitrile (72 wt% and 21 wt%, respectively), while PPO was dissolved in pure acetonitrile. Both solutions were purged with nitrogen for 30 minutes. The diluted PPO solution was delivered into a Pyrex reactor through the nitrogen inlet/outlet controlled by a program syringe pump (Harvard Apparatus, MA), within which AA solution was present. Total weight percentage of acetonitrile was fixed at ~ 30 wt% of the total amount of solvent. Redox initiator pair APS/TEMED was then added to start the polymerization reaction. The reaction was carried out at room temperature for 15 hours with magnet stirring during the whole process. The

final products were obtained as a white solid by precipitation in excess of acetone and dried under vacuum at ambient temperature.

LPA-g-PNIPAM—The synthesis followed a three-step protocol, described in detail in ref. 20. The first step was to prepare amino-ended PNIPAM. A ratio of AET·HCl/NIPAM to 0.06 was chosen here to obtain the desired chain length of PNIPAM. The estimated molecular weight of the PNIPAM was ~5,000 Da with a yield of ~ 70 %. The second step was to prepare the PNIPAM macro-monomer by coupling it with acrylic acid in the presence of DCC, as seen in Scheme 2.

Finally, AA and PNIPAM macromonomers were dissolved in Mill-Q water and purged with nitrogen. The polymerization was initiated by APS/TEMED according to Scheme 1 and completed in three hours. The white solid copolymer was obtained by precipitation in acetone.

2.3 Laser Light Scattering (LLS)

Static light scattering (SLS) and dynamic light scattering (DLS) measurements were performed by using a laboratory—built laser light scattering spectrometer operating at a wavelength of 532 nm with an output power of 600 mW at 25 °C. The intensity angular dependence was determined between 20° and 136° while the intensity auto-correction function was carried out at the scattering angles of 30°, 60°, 75° and 90°. A Brookhaven Instruments BI-9000 digital correlator was used to detect the intensity autocorrelation function. Benzene was used as a reference for calculating the excess Rayleigh ratio (R_{ex}).

Measurement on dn/dc —A differential refractometer (Phoenix Precision Instruments, NY) was used to measure the dn/dc value of LPA in buffer solution at 25 °C. The instrument was calibrated in advance by using aqueous potassium chloride solutions. At a wavelength of 532 nm, the measured dn/dc of LPA in aqueous solution exhibited a value of 0.17 ml/g. The value of dn/dc of copolymer was estimated to be the weight fraction of each component times their individual value. As the PPO amount was less than 1% in weight, we used the dn/dc of LPA to calculate the molecular weight of LPA-g-PPO.

2.4 Viscosity measurements

Viscosity measurements of LPA-g-PPO were performed using a Brookfield digital viscometer (Brookfield Engineering Laboratories, Inc, MA) with cone/plate geometry. The polymer solutions prepared at room temperature were composed of 7 M urea and 0.1% (w/v) poly(*N,N*-dimethylacrylamide) (PDMA) (470 kDa) in 1 × TTE buffer; the same composition as that used in DNA sequencing matrices.

A Physica MCR301 (Anton Paar, VA) rheometer was used to carry out the viscosity experiments. All were processed using identical procedures in order to make a clear comparison of viscosity change as a function of temperature for different polymers. Initially, polymers were dissolved in Mill-Q water with a concentration of 5% (w/v) and kept in a refrigerator before use. The shear rate was 0.5 s^{-1} , with a rotation speed of 0.383 rpm. The viscosity was measured from 5 °C to 80 °C at a rate of 1 °C/point with a 40 s measuring point rest time. Parallel Plates with a 0.5 mm gap were used to perform the measurements and the solutions sealed with high-boiling-point silicone oil to avoid solvent evaporation.

2.5 DNA sequencing

The BigDye Terminator v3.1 Sequencing Standard was pre-treated with TSR before use according to the protocol as suggested by Applied Biosystems Company. DNA sequencing was carried out on an ABI PRISM 310 Genetic Analyzer, Applied Biosystems Co. (Foster

City, CA, USA) with the control module: 10KV5mpr6mdely6hz510fillIE (Instrument running parameters, which means 5 min pre run at a voltage of 10 KV, 6 min delay before data recording, and data are recorded at 6 points per second; capillary gel filling was performed for 510 seconds.). The effective length (from cathode to detection window) was 50 cm (standard) or 33 cm (for a faster separation).

3. Results and Discussions

3.1 Laser light scattering and Viscosity behavior

The molecular weight of LPA-g-PPO (PPO = 0.2%) was measured to be 1.8 MDa with R_g equal to 60 nm in $1 \times$ TTE buffer. The conditions of synthesis were identical except for the variations in the PPO amount. It was expected that the molecular weight of obtained copolymers was the same, since we measured the molecular weight at 25 °C, below its aggregation temperature. The LPA used as a control in viscosity measurements was measured to be 6.7 M Da, as synthesized by the frozen method [41].

The viscosity of 2.5 wt % LPA-g-PPO in the sequencing buffer was measured at a low shear rate (0.6 s^{-1}) to denote approximately the zero-shear viscosity with a temperature increasing gradient of 1.0 °C. The curve of viscosity as a function of temperature between 25 °C to 45 °C is displayed in Figure 1. It can be clearly seen that the viscosity decreased slightly at the beginning, due to the change of molecular kinetics. When the temperature was increased up to ~ 40 °C, a significant and sharp increase in viscosity took place, with a double increment in a range of 2 °C gap. It implied that the thermal association of PPO side chains was initiated and the entanglement of copolymers was increased (Scheme 3). Note that only 0.2% of PPO in the copolymer was sufficient to induce this thermo-thickening phenomenon. Variation in polymer concentration and shear rate did not change the onset of the apparent increase in viscosity. The transition was completed at ~ 60 °C, a typical running temperature for CE, in agreement with the literature [42].

The evolution of viscosity with temperature for both LPA-g-PPO and LPA-g-PNIPAM can be divided into three stages. This is shown in Figure 2. The first stage was from 5 °C to the starting point of transition, which was ~ 40 °C for LPA-g-PPO (PPO feed ratio 0.2 % w/w) and ~ 50 °C for LPA-g-PNIPAM (PNIPAM feed ratio 10 % w/w). In this stage, the viscosity decreased linearly with increasing temperature, the same tendency as that of LPA used as a control. The LPA-g-PNIPAM showed the lowest viscosity at the first stage, followed by LPA-g-PPO. LPA was highest, probably due to the difference in molecular weight as well as in molecular architecture. The second stage was the process of thermal induced association. This was completed in a range of 15 °C for LPA-g-PPO and 20 °C for LPA-g-PNIPAM, and led to a sharp increase in solution viscosity. At the ending portion of this stage, the viscosity of these two graft copolymers increased up to 10 times than that at first stage, (even exceeded the LPA control), indicating a strong thermo-viscosity increase effect. Note that the amount of PPO was much less than that of PNIPAM to introduce the thermal association. In that respect, the PPO in LPA-g-PPO will likely cause fewer effects on DNA sequencing performance, when compared with that of LPA-g-PNIPAM, as less “hydrophobes” were used. As we know, the hydrophobic groups would interact with DNA-labeling dyes. Finally, the curves at the third stage were almost flat, showing a slight decrease with further increase in temperature.

Interestingly, when compared with the viscosity curve of LPA-g-PPO within the TTE buffer plus 7 M urea (Figure 1), the same onset temperature of the thermal transition (~ 38 °C) was displayed. This was despite the difference of concentration of graft copolymer and the composition of the buffer (ionic strength, urea, and LMW PDMA). It has been reported that the presence of salts or/and urea influences the self-assembling behavior of PPO-containing

block copolymers (PEOPPOPEO) in aqueous solutions, with sodium chloride used to decrease the critical micellar temperature (CMT) and urea to increase the CMT [43-47]. The effect of urea was ascribed to an indirect mechanism in which urea interacts with hydrated PEO block and water molecules by breaking the hydrogen bonds between water and PEO. It does not interact with PPO directly, as was determined by the results of ^1H NMR [43]. In our case, LPA is more hydrophilic than PEO. So it is unlikely to replace the hydrogen bonds between LPA and water molecules, and will exert limited influence on the aggregation of graft copolymers. Therefore, we believe that a similar phenomenon could be observed in a sequencing buffer.

3.2 Sequencing performance

As sequencing matrix plays a critical role in DNA sequencing, different sequencing matrices have specific running conditions in order to achieve optimal desired separation. The relationship between the polymer properties and the separation performance has been assessed systematically, as discussed in the review [14,48,49]. Briefly, polymers with higher average molecular weights and lower concentrations favor separation of larger DNA fragments if their concentrations are still above the overlap concentration. Higher overall concentration is better for smaller DNA fragments. At the same time, more hydrophilicity results in a more effective separation. However, in order to achieve the thermally responsive self-assembly behavior, it is unavoidable to introduce a certain moiety of more hydrophobic components (PPO). So, the amount of PPO is critical. The increase of the portion of side chains will increase the cross-linking points at an elevated temperature, and in turn increase the apparent molecular weight and lead to a more stabilized network. On the other hand, it will also increase the hydrophobicity of the matrix and deteriorate the resolution partially due to the fact that the hydrophobic portion of the copolymer will also interact with the labeling dye. Thus, only a limited amount of thermal “trigger (PPO side chains)” is applied to alleviate its adverse impact on DNA separation efficiency, but the “amount” should be just beyond the threshold where a significant thermal “viscosity switch” is achieved. Hereby, the PPO chain we used was very short ($M_n \sim 475$) and at a low weight percentage ($\sim 0.1\%$ to 0.8%).

One-base resolution was calculated in order to make a comparison of separation performance among different matrices. Several peaks were selected and calculated using the formula below [40,50,51]:

$$R = \frac{2(t_{1+n} - t_1)}{1.699n(w_{1+n} + w_1)}$$

where t and w are the migration time and peak width at half maximum, respectively. The subscripts, 1 and 1+n, denote two peaks with n base difference. A ‘Peakfit’ program (SPSS Inc) was used to calculate w , the peak width at half height (FWHM), and t , the center value of the peak (1 or 1+n).

Temperature—The thermal associating behavior of graft copolymer adds new importance to CE operating temperature, in that polymer architecture changes over different temperature ranges. DNA sequencing was performed from 45 °C to 70 °C, a temperature range covering the whole three stages, with a 5 °C increment in a 33-cm-long capillary. This was intended to illustrate the influence of temperature on sieving behavior of the matrix. The plot of resolution versus base number of electrophoresis at 45 °C (within transition state) and 60 °C (after transition) are shown in Figure 3. Obviously, the sieving performance at 60 °C is significantly enhanced in sequencing of those fragments larger than 500 bases, when compared with that at 45 °C, while the smaller region is similar. Generally speaking, the

temperature used was as high as possible, so as to pursue a quicker separation without an obvious drop in resolution and to improve throughput. In other words, the resolution would not be improved substantially by simply increasing the temperature for a normal sieving system. Hence, the dramatic enhancement of resolution as shown in Figure 3 should be at least partially, if not wholly, attributed to the fact that it has induced the thermal association when the temperature has gone beyond the transition temperature. This association has increased the apparent molecular weight and strengthened the transient network, thus favoring the sequencing of larger DNA molecules but not affecting the smaller ones. This observation conforms to the results in viscosity measurements. Note that the migration time for base 909 is 40 min at 60 °C and 42 min at 45 °C, only a 2-min difference. The effects of temperature on DNA migration were cancelled by the viscosity change.

Concentration—Polymer overall concentration is another important parameter that should be assessed. It impacts the thermal thickening behavior of the graft copolymer under consideration, in addition to the common polymer matrices. As shown in Figure 4, a 1.8% (g/ml) solution of copolymer (PPO, 0.2 % w/w) exhibits a better sieving ability for base numbers larger than 400 bases than that of 2.3 % (g/ml) polymer solution, as indicated by the resolution. The peaks in an electropherogram can be described as readable peaks and unreadable peaks. Readable peaks mean those having adequate resolution and signal-to-noise ratio, which can be identified by current available base-calling software or other methods. Unreadable ones have low resolution or signal-to-noise ratio, and usually occur at the beginning and the ending portion of the electropherogram. Those occurring after the readable peaks, but before the final large mixed finishing peak, give a hint of the possible separation limit of the longest DNA fragments. And they provide another viewpoint to compare the sieving performance for long DNA fragments, though they are not able to be read by available technologies. On the other hand, these peaks may be identified with the development and modification of reading skills, including DNA preparation protocols, instrument detection technology, and base-calling software. In fact, Karger *et al* substantially extended the read length up to 1300 bases by optimizing the base-calling software and doubling the G-terminal DNA fragments. In our case, the low signal-to-noise ratio beyond 1000 bases was caused partially by the low signal from G-terminated fragments using the standard reaction protocol [52].

Figure 5 illustrates the results of DNA sequencing using the matrices composed of 1.8 % (g/ml) and 2.3 % (g/ml) copolymer at different temperatures (selected whole electropherograms were listed in Appendix A). Obviously, all the electropherograms can be read up to 1000 bases at the detected column temperatures and polymer concentrations. However, the total number of visible peaks at the tail portion increases with the temperature increasing from 50 °C to 60 °C, and further to 70 °C for both concentrations. At the same temperature (60 °C or 70 °C), more peaks occur on the electropherograms obtained from the matrix made of 1.8 % (g/ml) copolymer than that composed of 2.3 % copolymer, indicating that 1.8 % (g/ml) has a higher separation ability for DNA fragments larger than 1000 bases at given conditions. Therefore, a higher temperature (70 °C) and lower concentration (1.8 % g/ml) is preferred for separating larger DNA fragments, due possibly to the thermal association of PPO, which has increased the apparent molecular weight of the graft copolymer. The increase in the apparent molecular weight of the graft copolymer, in turn, requires less copolymer when forming a robust network. Moreover, the migration time is about 13 minutes shorter for a 1.8 % (g/ml) solution than that for a 2.3 % (g/ml) solution under identical conditions. Notwithstanding the overall polymer concentration, only a 2 minute time change results with an increment of 10 °C in column temperature.

A similar length existed in the region representing the DNA fragments with more than 1000 bases on Figure 5b and Figure 5c. Figure 5c was obtained at a sieving temperature of 70 °C,

using the matrix with 2.3 % (g/ml) LPA-g-PPO (0.2 % PPO/(LPA+PPO), wt%) and Figure 5b was acquired at 60 °C, using the matrix composing of 1.8 % (g/ml) LPA-g-PPO. The results suggested that separation temperature and polymer concentration had the compensating effects on triggering the self-aggregation of PPO side chains on LPA-g-PPO under a limited concentration and temperature span. Correspondingly, these two parameters can be balanced in order to choose a more effective and convenient way to achieve comparable resolution. It is worthwhile to note that it took only about 50 minutes to sequence up to 1000 bases at 60 °C with the concentration of 1.8 % (g/ml) in a 50-cm long capillary (effective separation length). Therefore, with regard to efficiency, a 1.8 % (g/ml) of polymer concentration was employed as the CE condition for most other experiments.

Polymer composition—The thermal associating property of graft copolymers can add a significant impact on several critical aspects of DNA sequencing performance including resolution, read length and separation time. However, the physical property itself is determined by the chemical composition of the copolymer, i.e., main-chain molecular weight (LPA), side-chain molecular weight (PPO) and grafting density of the side chains (PPO). At a fixed ratio of side chain to main chain, the viscosity at of the graft copolymer at elevated temperature was independent of graft size and the dependence of resolution on graft size was rather weak with a better resolution at shorter graft size, as examined by Viovy *et al* [26]. This is because the interaction becomes stronger with increasing side-chain molecular weight. On the contrary, the number of linking points decreases correspondingly. These two factors are therefore complementary in inducing thermal assembly effects, as long as there are enough grafting points per main chain. Based on the above knowledge, in our premier experiments, dependence of DNA sieving ability upon the graft copolymer was tested relative to the weight percentage of PPO in the copolymer (PPO/LPA ratio), rather than that of graft density and chain length, which would be optimized later on.

The single base resolution of the electropherogram obtained at 60 °C in a capillary with a 50-cm effective length, using 1.8% (g/ml) LPA-g-PPO at a different PPO/LPA ratio is plotted in Figure 6. The graft copolymer with a 0.3 % (w/w) of PPO showed the highest resolution, especially in the larger bases region. That of 0.2 % exhibited a slight decrease, but was still second highest. This was followed by 0.4 %, and finally, that of 0.8 % (w/w) PPO. That was the poorest resolution with an apparent decline in the separation range beyond 600 bases. It implies that a concentration of about 0.3 % (w/w) of PPO in the polymer is the desired composition. 0.8 % (w/w) is far above the amount needed under current running conditions. It showed that the amount of PPO was critical in forming a robust network with a suitable mesh size.

3.3 Comparison with LPA-g-PNIPAM

Based on the discussion above, the performance of LPA-g-PPO as a sieving matrix for DNA sequencing can be manipulated. The chemical structure of the copolymer, the composition of the solution, as well as external conditions, such as separation temperature and applied voltage, can all be utilized for this purpose. The quantitative relationship between these parameters and sieving performance becomes rather complicated when the thermal-assembling effects of the matrix, including polymer composition, polymer concentration and solution temperature are considered.

Considering the long read length and separation time (efficiency), the following conditions were chosen as preferred by using LPA-g-PPO (of the current composition) as the sieving matrix for DNA sequencing. The matrix was set at 1.8 % (g/ml) LPA-g-PPO with the weight percentage of PPO equal to 0.3 % in 1 × TTE buffer + 7 M urea. 0.23 % PDMA ($M_w = 470$ KDa) was added to be the dynamic coating agent. The running temperature was constant at

60 °C. The applied voltage was 200 V/cm. DNA was injected for 40 seconds under a voltage of 39 V/cm. The experiment was carried out in a capillary with an inner diameter of 50 μm and an effective length of 50 cm. The data obtained are shown in Figure 7.

Viovy *et al* [26] reported that a read length of 800 bases was obtained on a DNA ladder in less than one hour with a resolution of ~ 0.5 on an ABI 310 instrument without optimization, using LPA-g-PNIPAM as the separation media. Compared with the published data of LPA-g-PNIPAM matrix, LPA-g-PPO samples have demonstrated a better sequencing performance. The new matrix can achieve a read length of 1000 bases routinely, as shown in Figure 5 and Figure 7, on the same commercial ABI 310 analyzer. The numerical comparison is illustrated in Table 1. It can be seen that LPA-g-PPO distinguishes itself from LPA-g-PNIPAM as a sieving matrix by using a lower graft density, less overall polymer concentration, and higher resolution in sequencing large DNA fragments. To be more certain of this finding, we synthesized LPA-g-PNIPAM with a similar main chain length and ran CE at corresponding conditions. Results are compared with those of LPA-g-PPO in terms of single base resolution. As shown in Figure 8, the resolution curve for LPA-g-PPO is above that for LPA-g-PNIPAM, based on current data, which agrees with the previous comparison in Table 1. The resolution is lower than that stated in Table 1, as we used a shorter column (33 cm effective length) for a shorter experimental run time. It should be realized that the DNA standard we used was BigDye Terminator v3.1 Sequencing Standard kits, which is more difficult to separate than the DNA ladder used by Viovy *et al* [26].

3.4 Comparison with current available matrices

We tried to improve the sequencing performance of the matrices in terms of separation rate, read length, and cost reduction, along with easy handling associated with media preparation and injection. It is recognized that our innovative thermal switchable copolymer has shown a viscosity transition at around 40 °C. Gel preparation and injection was performed at room temperature, at a much lower temperature, as shown in previous sections. DNA sequencing was carried out at 60 °C, at which temperature the thermal transition had been completed. In the present case, the viscosity problem associated with the HMW LPA [41] was resolved, at least partially. The low viscosity separation matrix, should become even more important as a sieving matrix in microchip applications.

To demonstrate LPA-g-PPO as a promising separation medium for sequencing long DNA fragments, we compared its performance with commercially available matrices promoted for long read length. Our findings are listed in Table 2. It can be seen that the matrices, including the long read matrix from GE Healthcare (Amersham Bioscience) and the CEQ separation gel LPA-1 from Beckman Coulter, all suffer from high viscosity. POP6 and POP7 from Applied Biosystems show fairly low viscosity, but exhibit a longer separation time with a fairly good read length. Note that a shorter read length will produce more gaps, requiring more redundancy in genome assembling. This will result in a substantially higher final cost, especially for complicated genomes such as those of human beings [53]. In order to compete with the POP series in viscosity, we developed this “viscosity switchable” copolymer LPA-g-PPO. As discussed previously, we have achieved a read length of 1000 bases within one hour; and it surpasses both POP6 and POP7 in terms of read length and separation time.

We understand that for a better evaluation, the read length and accuracy from the base-calling software should be presented. However, we were not able to adjust the base mobility shift and other matrix-specific parameters to process the data analysis by using the ABI software. On the other hand, the results obtained so far showed that the LPA-g-PPO matrix is a promising thermal associating medium for DNA sequencing in the region of middle and larger size DNA fragments. An improvement of data analysis software (base mobility

correction and resolution reading limit), as well as in enhancement of signal-to-noise ratio (i.e. modification on DNA preparation protocols), would sufficiently extend the read length, as there are visible peaks after base 1007. At the same time, the CE parameters, including the composition of the matrix, polymer concentrations, and the architecture of the copolymer, (such as length of PPO chains, grafting density, and length of LPA backbones,) may all need to be further manipulated in pursuit of the best result.

4. Concluding Remarks

A new thermo-gelling graft copolymer LPA-g-PPO was successfully synthesized in the mixture solvent of water/acetonitrile. Viscosity tests (in the absence of urea) showed that the copolymer had a transition temperature of about 40 °C. It exhibited a low viscosity below this temperature, and experienced a sharp increase in the following 10 °C. A much more viscous state at about 50 °C was found due to the increase in hydrophobicity of PPO at increasing temperatures and the aggregation of PPO side chains.

The copolymer was tested as a sieving matrix for DNA sequencing. Data showed that a read length of 1000 bases can be achieved routinely in one hour under limited optimized conditions. The sieving performance could be particularly influenced by the separation temperature, polymer overall concentration, and graft density. These parameters affected thermal associating behavior of the graft copolymer, and could be considered as a group when determining the copolymer self-assembly behavior, along with the hydrogen bond breaking buffer. Based on available results both from our lab and literature [26], we have concluded that LPA-g-PPO is a promising candidate to be a separation matrix in DNA separations by capillary electrophoresis, especially in the microchannel format. Our next step is to form an IPN with our novel graft copolymer with PDMA so as to enhance the sequencing performance. At the present time, although a more detailed study is required to optimize the composition and the fine structure of LPA-g-PPO, the current experiments have clearly shown that a better thermo-reversible matrix for DNA sequencing has been developed. And it may find its position as a potential matrix for the microchip format.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

BC gratefully acknowledges the support of this research by the National Science Foundation, Polymers Program (DMR 0454887), and by the National Human Genome Research Institute (NIH 2R01HG01386-09).

Abbreviations

AA	acrylamide
APS	ammonium persulfate
DMA	<i>N,N</i> -dimethylacrylamide
EDTA	ethylenediaminetetraacetic acid
LPA	linear polyacrylamide
NIPAM	<i>N</i> -isopropylacrylamide
NMF	<i>N</i> -methylformamide
PDMA	poly(<i>N,N</i> -dimethylacrylamide)

PEO	Poly(ethylene oxide)
PNIPAM	poly(<i>N</i> -isopropylacrylamide)
PPO	Poly(propylene oxide)
TAPS	<i>N</i> -tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid
μTAS	micro total analysis system
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TSR	Template Suppression Reagent

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Fig. 1. Plot of viscosity vs. temperature of LPA-g-PPO (PPO, wt% = 0.2 %) with a polymer concentration of 2.5 % (g/ml) in 1 $\times$ TTE + 7 M urea. The shear rate is 0.6 s $^{-1}$.

Fig. 2.

Plot of viscosity vs. temperature of LPA, LPA-g-PPO (PPO, wt% = 0.2 %) and LPA-g-PNIPAM (PNIPAM, wt% = 6 %) in Mill-Q water with a polymer concentration of 5 % (g/ml). The shear rate is 0.5 s^{-1} .

Fig. 3.

Plot of resolution vs. base number in DNA sequencing using LPA-g-PPO as the separation medium by CE operated at different column temperatures. The sequencing matrix was composed of 2.3 % (w/v) LPA-g-PPO (PPO, wt% = 0.2 %) + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 20 seconds at 41 V/cm and measurements were carried out at 200 V/cm. Total capillary length = 44 cm with an effective length of 33 cm and ID = 50 μ m.

Fig. 4.

Plot of resolution vs. base number in DNA sequencing using LPA-g-PPO as the separation medium by CE operated at different polymer concentrations. The sequencing matrix was composed of LPA-g-PPO (PPO, wt% = 0.2 %) + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 20 seconds at 41 V/cm and measurements were carried out at 200 V/cm and 60 °C. Total capillary length = 61 cm with an effective length of 50 cm and ID = 50 μ m.

Fig. 5.

Sequencing of four-color DNA at different temperatures and polymer concentrations with only data larger than 665 bases and base G being shown in the plot. The sequencing matrix was composed of LPA-g-PPO (PPO, wt% = 0.2 %) + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 20 seconds at 41 V/cm and measurements were carried out at 200 V/cm. Total capillary length = 61 cm with an effective length of 50 cm and inner diameter of 50 μ m. (The complete data are presented in the appendix)

Fig. 6.

Plot of resolution vs. base number in DNA sequencing using LPA-g-PPO as the separation medium by CE operated at different PPO percentage. The sequencing matrix was composed of 1.8 % LPA-g-PPO + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 20 seconds at 41 V/cm and measurements were carried out at 200 V/cm and 60 °C. Total capillary length = 61 cm with an effective length of 50 cm and ID = 50 μm.

Fig. 7.

Sequencing of four-color DNA with only data representing base G being shown in the plot. The sequencing matrix was composed of 1.8% LPA-*g*-PPO (PPO, wt% = 0.3 %) + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 30 seconds at 41 V/cm and measurements were carried out at 200 V/cm and 60 °C. Total capillary length = 61 cm with an effective length of 50 cm and ID = 50 μm.

Fig. 8.

Plot of resolution vs. base number in DNA sequencing using 1.8 % of LPA-g-PPO (PPO, wt % = 0.3 %) or 2.3 % LPA-g-PNIPAM (PNIPAM, wt% = 6 %) as the separation medium by CE operated at different PPO percentage. The sequencing matrix was composed of copolymer + 0.23 % (w/v) PDMA (470 KDa) in 1×TTE + 7 M urea. DNA was injected for 20 seconds at 41 V/cm and measurements were carried out at 200 V/cm and 60 °C. Total capillary length = 44 cm with an effective length of 33 cm and ID = 50 μm.

Scheme 1.
Synthesis of graft copolymers

Scheme 2.
Synthesis of PNIPAM macromonomer

Scheme 3.

Simplified scheme representing thermally reversible association of LPA-g-PPO

Table 1

Comparison of LPA-*g*-PPO and LPA-*g*-PNIPAM (P(Am1500PNIPAM10/10%)) as DNA separation media. Copolymers were dissolved in 1× TTE + 7 M urea, consisting of 0.23 % (g/m l) PDMA (470 KDa). Measurements were carried out at 200 V/cm and 60 °C; total capillary length = 55 cm with an effective capillary length of 50 cm and ID = 50 μm.

Sample	Graft chains	Concentration of media	DNA	Resolution (1000 bases)
LPA- <i>g</i> -PPO (0.2%)	PPO, $M_n = 475$, 0.2 wt%	2.0 % & 2.5 %	BigDye 3.1 Standard	0.4
P(Am1500PNIPAM10/10%) [12,25]	PNIPAM, $M_w = 10000$, 10 wt%	3.0 % & 5.0 %	ssDNA "ladder"	<0.3

Table 2

Comparison of our matrices with commercial products.

Matrix	Characteristics (Advantages/disadvantages)	Sequencing performance
LPA-g-PPO ⁽¹⁾	“Viscosity switch”; long read length with short separation time	1000 bases in 48 min with additional visible peaks
UHMW LPA ⁽¹⁾	Long read length; high viscosity	1000 bases in 1 hour or 1200 bases in 114 min with an accuracy of 95 % read by “ BASE” before optimization
Quasi-IPN	Long read length with short separation time high viscosity	750 bases of contiguous sequence in 37 min with 98.0 % accuracy or 1000 bases in 39 min, or 1200 bases in 60 min [53]
POP6 ⁽²⁾	Acceptable read length with long separation time	600-700 bases in 150 min [54]
POP7 ⁽²⁾	Long read length with long separation time	850-900 bases in 125 min [54]
MegaBACE Long Read Matrix (LPA) ⁽³⁾	Long Read length with long separation time; high viscosity	up to 1000 bases in 3 hours [55]
CEQ separation gel LPA-1 ⁽⁴⁾	Acceptable read length high viscosity	700 bases at better than 98% accuracy in about 100 min [56]

¹ 0.23 % of LMW PDMA was added to the solution to be the dynamic coating agent

² Applied Biosystems

³ GE Healthcare (Amersham Biosciences)

⁴ Beckman Coulter