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Electrophoretic separation of DNA using a new matrix in uncoated capillaries

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Abstract

A new separation matrix, consisting of polymer poly(*N*-isopropylacrylamide) (PNIPAM) and small molecule additive mannitol, was used for double-stranded (ds) DNA and plasmid DNA separation by capillary electrophoresis. The matrix had a low viscosity, which made it very easy to handle. The additive mannitol dramatically enhanced the sieving performance of PNIPAM in TBE buffer. The optimal mannitol concentration 6% in polymer solution, was determined with the consideration of both speed and resolution. A resolution of 0.95 was achieved on the separation of 271/281 bp in the ΦX174/*Hae*III digest by using 1.5% PNIPAM + 6% mannitol, while the supercoiled, linear and nicked conformers of λ plasmid were separated in 1% PNIPAM + 6% mannitol, demonstrating the potential use of this new matrix for effective DNA separations. The dramatic impact of mannitol on sieving performance of PNIPAM solution was investigated. pH dependent self-coating ability of PNIPAM was revealed. The presence of mannitol in TBE buffer decreased the pH of the buffer, which led to more efficient self-coating ability of PNIPAM probable due to the formation of hydrogen bonds between PNIPAM molecules and silanol groups at the silica wall.

Keywords: Capillary electrophoresis, Dynamic coating, DNA separation, Poly(*N*-isopropylacrylamide), Mannitol

1. Introduction

Capillary electrophoresis (CE) in non-cross-linked polymer solutions is an attractive alternative to slab-gel electrophoresis and becomes a powerful technique for DNA analysis. CE separations are much faster than those in slab-gels and are characterized by high resolution, minimal sample and reagents requirements, high detection sensitivity and ease of automation.

Unlike slab-gel electrophoresis where agarose and cross-linked polyacrylamide were dominant as the DNA separation matrices over 20 years, a number of different hydrophilic polymers have been developed and tested in CE [1], including linear polyacrylamide (LPA) [2,3], polyethylene oxide (PEO) [4,5], polyvinyl pyrrolidone (PVP) [6,7], cellulose and its derivatives [8,9]. Among them, LPA has attained the widely popularity on account of its high sieving capacity in the size range of 100–1000 bp. However, LPA suffers two drawbacks in CE. The polymer solution, like most of the separation matrices used at present, has high viscosity resulting from high molecular mass of its linear chains. The pressure needed to load and refill capillary with high viscosity matrices causes significant problems in the development of capillary-microchannel electrophoresis systems [10]. Another drawback to the use of LPA is that the inner capillary wall has to be coated with LPA [11] or polyvinyl alcohol [12] in order to reduce or eliminate DNA-wall interactions and to suppress electroosmotic flow (EOF). The production of covalently linked polymeric coatings is time-consuming, and the polymerized coatings are difficult to control and optimize. The coatings cannot be regenerated when resolution degrades [13]. Moreover, the in-capillary polymerization step of covalently coatings, which results in the formation of a viscous polymer solution in the capillary lumen that must be expelled under high pressure, limits their practicality for use in small-inner diameter microchannels [14]. The limits of high viscosity and the

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need of wall coatings prompted the work of developing novel promising separation matrices that can circumvent these shortcomings.

Some polymer matrices have been shown to eliminate the need for a covalent wall coating because they can adhere to the capillary inner surface by physical adsorption and form "dynamic" capillary coatings, which reduce EOF to negligible levels [15]. The mechanism of dynamic coating is still a matter of controversy. Either hydrophobic, electrostatic, or hydrogen bonding interactions of the polymer with the wall is thought to be the driving forces for physical adsorption [14–18]. Polymers that have been investigated to have dynamic coating ability include polydimethylacrylamide (PDMA) [19], PEO [5], PVP [6] and some novel acrylamide-based polymers [20–22]. PDMA has the best self-coating ability, which allows good coating ability at rather low concentration. However, the separation performance of PDMA is lower than that of LPA. This reduced effectiveness due to polymer hydrophobicity is also the most significant disadvantage of most presently available dynamic coatings [15,18]. To get both the optimal sieving properties as LPA and the self-coating ability as PDMA, combination of PDMA with LPA or other matrices becomes a focus of attention. Many matrices have been developed by copolymerizing dimethylacrylamide (DMA) with different highly hydrophilic monomers [21,23], or by mixing PDMA with a separation matrix, such as LPA [24,25], PEO [26] and hydroxypropylmethylcellulose (HPMC) [27]. These products can be better alternatives to PDMA used in CE and microfabricated chips, which shows the significance of the above strategy. Another way in the search for homopolymer with hydrophilicity, dynamic coating ability, low viscosity and high sieving ability also results in several novel promising matrices [22].

Poly(*N*-isopropylacrylamide) (PNIPAM) is a well-investigated temperature sensitive polymer applied in biochemistry [28,29]. Since hydropholicity is one of the critical factors for dynamic polymeric wall coating and sieving performance [15,18], and the structures of the monomers indicate that the hydrophilicity of *N*-isopropylacrylamide is between those of acrylamide and DMA, PNIPAM is expected to be a possible ideal separation matrix. In this study, a novel sieving matrix consisting of PNIPAM and small molecular additive mannitol was developed for DNA separation. The effect of mannitol was discussed. This work indicates that PNIPAM might be a useful sieving matrix for DNA separation in CE and microfabricated chips.

2. Experimental

2.1. Chemicals and materials

N-Isopropylacrylamide was obtained from Aldrich (Milwaukee, WI, USA) and was used without further purification. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), ethidium bromide and ammonium persulfate were purchased from Sigma (St. Louis, MO, USA). 2-(*N*-moropholino) ethanesulfonic acid (MES) was from AMRESCO (USA). Tris(hydroxylmethyl)aminoethane (Tris), boric acid, mannitol and EDTA were analytical grade from Shanghai Reagents Co. (Shanghai, China). DNA restriction enzyme digests ΦX174/*Hae*III, and λ plasmid DNA (48502 bp) were from TaKaRa Biotech. Co. (Dalian, China).

2.2. Preparation of polymer

The linear PNIPAM was prepared by free-radical solution polymerization: 6 g of *N*-isopropylacrylamide was dissolved in 100 ml double-distilled water and filtered (0.22 μm). After bubbled with high purity nitrogen for 3 h, the monomer solution was polymerized by addition of 70 μl of 100% (v/v) TEMED and 180 μl of 20% (w/v) ammonium persulfate. The bottle was sealed, and the solution was stirred slowly at room temperature for 2 h. The bottle was then transferred to a refrigerator at 4 °C for 48 h.

The reaction product was extensively dialyzed against water for 2 days, using a 12,000 molecular weight cutoff dialysis membrane tubing (Alpha Biotech. Co., China), lyophilized and then weighed. The yield of the reaction was 81%.

2.3. Measurement of PNIPAM molecular weight

The viscosity-average molecular weight (M_v) was measured from the intrinsic viscosity [η] of the polymer. The kinematic viscosity was determined at 20 °C using an Ubbelohde viscometer (Shanghai, China). The plot of η_{sp}/C (η_{sp}, specific viscosity; C, concentration of polymer) versus the concentration of polymer was extrapolated to zero concentration, producing [η] as the intercept. M_v was calculated according to the Mark–Houwink equation [30] ($[\eta] = KM_v^{\alpha}$ cm^3/g, where $K = 0.112$ and $\alpha = 0.51$). The M_v of PNIPAM was 6.5×10^6 Da.

2.4. Capillary electrophoresis instrumentation and procedures

A laboratory-built CE system with laser-induced fluorescence detection, built in house, was similar to that described in previous work [31]. Briefly, a neodymium-yttrium–aluminium garnet (Nd–YAG) laser with 532 nm output (Quantel, France) was used for excitation. A long wave pass filter at 600 nm was used to block stray light. The signal from the photomultiplier tube (PMT) was fed into a Boxcar averager (M162/165, EG&G, PAR) and then transferred through a 12-bit A/D combining converter and stored in a personal computer.

Capillaries used were uncoated 75 μm i.d. fused silica (Yongnian Optical Fiber Co., Hebei, China), with 32 cm to the detector and a full length of 40 cm. The sieving matrices were 1× TBE buffer (89 mM Tris, 89 mM boric acid and

2 mM EDTA) containing different amounts of PNIPAM in the absence or presence of different amounts of mannitol. As a comparison, another separation matrix was prepared by adding appropriate amount of PNIPAM in MES–NaOH buffer (50 mM MES was adjusted by 1 M NaOH to provide buffers at pH 6.0–7.3). The sieving matrices were loaded into the capillaries under a pressure of 0.3 MPa. DNA samples (10 μg/ml) were introduced into the capillary filled with the sieving buffer containing ethidium bromide (3 μg/ml) by electrokinetic injection at −8 kV for 5 s. The runs were performed under constant voltage at 220 V/cm. After each run, the capillary was rinsed with 1 M NaOH for 5 min and 1 M HCl for 5 min before new sieving medium was pushed through again.

3. Results and discussion

3.1. DNA separation performance

Fig. 1 shows the electropherogram of the ΦX174/*Hae*III digest by using 1.5% PNIPAM in 1× TBE buffer with or without mannitol as the separation medium. When PNIPAM was used alone as the sieving medium, no peak appeared within 50 min. Adding 2% (w/v) mannitol to the polymer solution produced nearly identical results, as shown in Fig. 1a. However, PNIPAM began to show the sieving ability when the concentration of mannitol was 4% (Fig. 1b), but the separation performance was poor. Dramatically improvement was observed after increasing mannitol to 6% (w/v) (Fig. 1c), where all the 11 DNA fragments were baseline separated. No remarkable improvement was observed when the concentration of mannitol was increased up to 10% (Fig. 1d and e). As shown in Fig. 1, the concentration of mannitol showed a great effect on the DNA separation. To clearly understand this effect, the resolution (Rs) was calculated according to the equation $Rs = 2(t_1 - t_2)/(w_1 + w_2)$. Where w_1 and w_2 are the temporal peak widths of DNA fragments 1and 2, respectively, t_1 and t_2 are the migration times of DNA fragments 1 and 2, respectively, and $t_1 > t_2$. The resolutions on separation of 271/281 bp were 0.95, 0.96 and 1.0 by adding 6, 8 and 10% mannitol, respectively, while the migration time of DNA fragments, as shown in Fig. 2, increased significantly when the concentration of mannitol increased from 6 to 10%. As a compromise between resolution and separation speed, 6% mannitol appeared to be the optimal concentration for the 1.5% PNIPAM in 1× TBE solution.

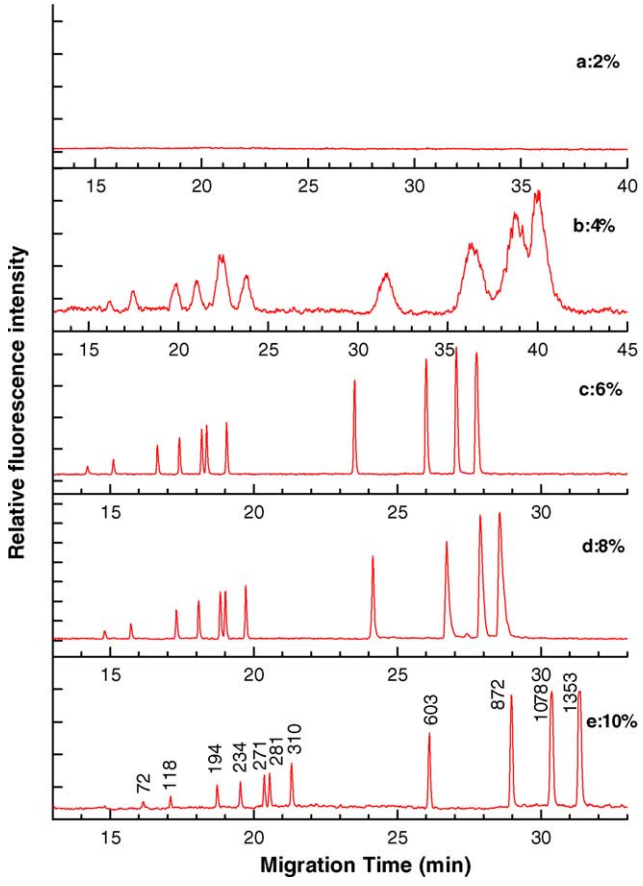

Fig. 1. Separation of ΦX174/*Hae*III DNA by CE with: (a) 1.5% PNIPAM + 2% mannitol; (b) 1.5% PNIPAM + 4% mannitol; (c) 1.5% PNIPAM + 6% mannitol; (d) 1.5% PNIPAM + 8% mannitol; (e) 1.5% PNIPAM + 10% mannitol. Capillary, 75/365 μm i.d./o.d., and 32/40 cm efficient/total length; inject, 5 s at −8 kV; separation electric field strength 220 V/cm.

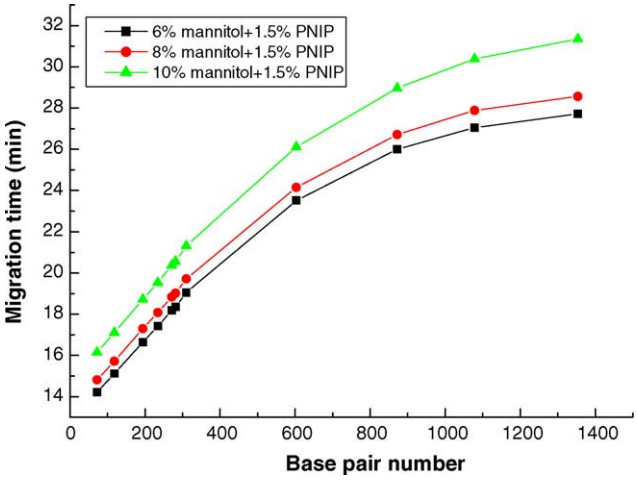

Fig. 2. Plot of migration time versus size of DNA fragment in 1.5% PNIPAM with: (■) 6% mannitol; (●) 8% mannitol; and (▲)10% mannitol. All the data were calculated from the fitting of the electropherogram in Fig. 1.

3.2. Viscosity

The viscosity of the PNIPAM solution over a concentration range of 0.5–5.0% (w/v) with 6% (w/v) mannitol in 1× TBE buffer was measured with a temperature-controlled rotationary viscometer (Shanghai Tongji NDJ-79, China). As shown in Fig. 3, the viscosity of 1.5% (w/v) PNIPAM plus 6% mannitol in 1× TBE buffer was 7 cp at 25 °C, so in the study the polymer solution could be easily pumped into and blown out the capillary under the pressure of 0.3 MPa. Such

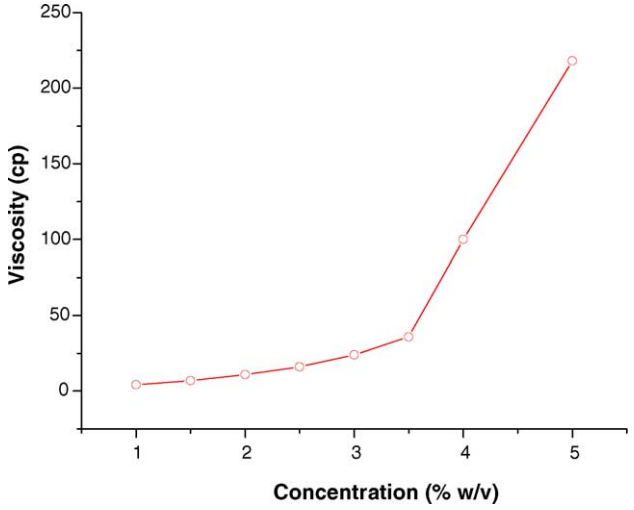

Fig. 3. Viscosity as a function of the concentration of PNIPAM with 6% mannitol.

a polymer solution can also be easily injected or rinsed by a syringe. PNIPAM is a temperature sensitive polymer with a phase transition temperature (also called "lower critical solution temperature", LCST) at ca. 32 °C in aqueous solution [32]. Below the LCST, the polymer chains exist in a random coil conformation, exhibit low viscosity and yield a high sieving ability. Above the LCST, the PNIPAM chains contract and become more hydrophobic, resulting in phase separation and the lost of resolution.

Thermoresponsive matrices developed for DNA separation generally are characterized by switchable viscosities [33]. The thermoresponsive matrix could be loaded into the capillaries or microchannels at a lower temperature with a lower viscosity, while the separation was performed at an increased temperature at which a better separation could be obtained due to an elevated viscosity. Since the PNIPAM here had very low viscosity for loading into the capillary at room temperature, all the separations in this study were performed at 25 °C.

3.3. Impact of mannitol

The results for the DNA separation in PNIPAM with different concentration of mannitol have shown the dramatically impact of mannitol. The mannitol-induced changes in buffer properties, such as conductivity and pH, were investigated. As shown in Table 1, the addition of 6% mannitol to the 1 × TBE buffer reduced the buffer pH from 8.35 to 6.36. This could be explained by the reaction between mannitol and the boric acid in 1× TBE buffer, which is similar to the complex between boric acid and glycerol [34,35]. Complex formation between boric acid and mannitol releases a proton. The released hydrogen ion will increase the ionic strength and decrease the buffer pH.

Table 1
Changes in buffer properties caused by mannitol

Matrix (in 1× TBE)	pH (25 °C)	Current (μA)
1.5% PNIPAM	8.35	4.2
1.5% PNIPAM + 2% mannitol	7.50	11.8
1.5% PNIPAM + 4% mannitol	6.82	13.8
1.5% PNIPAM + 6% mannitol	6.36	14.2
1.5% PNIPAM + 8% mannitol	6.10	13.8
1.5% PNIPAM + 10% mannitol	5.91	12.4

There were some reports about improved effects on DNA separation by incorporating small molecules to the polymer solution, such as the addition of glycerol or mannitol [34,36–38] to the HPMC. These enhancements were attributed to the formation of complexes among polyhydroxylated cellulose polymers, additives and boric acid. The polyhydroxylated additives interacted with the cellulose polymer chains through tetraborate structure, and thus affected the mesh size. Since tetraborate structure cannot be formed between mannitol and PNIPAM, the addition of mannitol should have much weaker interaction with the PNIPAM than cellulose derivatives. Furthermore, if mannitol just affected the mesh size, the increase of mannitol should increase the migration time of DNA fragments, but all the fragments migrated slower in PNIPAM with 4% mannitol than those in PNIPAM with 6% mannitol. Therefore, the effect of mannitol on DNA separation here is different from previous reports.

Theoretically, DNA mobility is affected by the charge over weight ratio of the DNA fragments besides the mesh size. Liang et al. demonstrated that the addition of 25% glycerol in 1× TBE buffer would increase the hydrodynamic radius of DNA and reduce the charge on the DNA fragment [35]. The addition of mannitol may have the same effect on DNA as glycerol and result in the decrease in mobility with increasing concentration of mannitol from 6 to 10%. However, the increase in mobility with mannitol from 4 to 6% should result from other factors.

The addition of mannitol to the PNIPAM solution results in the improvement of separation performance and makes the PNIPAM, which has no self-coating ability at 1× TBE buffer (pH 8.3), become a dynamic coating polymer. The observed change in pH values of the matrices due to the addition of mannitol was assumed to be the critical factor. To verify this assumption, DNA fragments were separated in 1.5% PNIPAM without mannitol by using MES–NaOH instead of TBE as the buffer. As shown in Fig. 4, the separation performance at pH 7.3 and 6.3 were similar with those (Fig. 1) by 1.5% PNIPAM in 1× TBE with 4 and 6% mannitol, respectively. This result also showed the potential use of PNIPAM alone as a self-coating polymer in some buffer with pH at about 6.3.

The achieved self-coating ability of PNIPAM at pH 6.3 was probably due to the hydrogen bonding interactions. Since the hydrogen ion concentration increases at lower pH, the hydrogen bonds between the silanol of the silica surface and the nitrogen of the PNIPAM backbone form easily, which may lead to efficient dynamic coating of the capillary by PNIPAM. From the chemical structure, the electron cloud

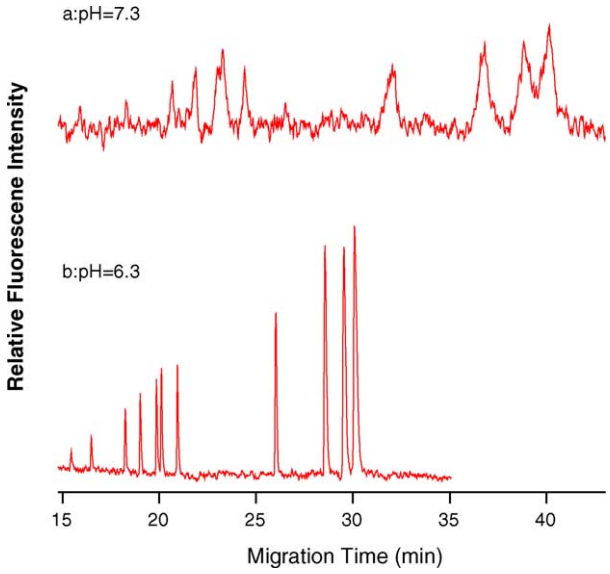

Fig. 4. Electropherogram of ΦX174/*Hae*III digest in 1.5% PNIPAM in 50 mM MES buffer with different pH. (a) pH 7.3; (b) pH 6.3; inject, 6 s at −7 kV; separation electric field strength: 140 V/cm.

density around the nitrogen atoms of PNIPAM is higher due to the electron repulsion of the isopropyl group than that of LPA. This may explain why the PNIPAM has the self-coating ability above pH 6.3. It is expected more *N*-substituted polyacrylamides have the self-coating ability at certain pH. Since in most practical instances DNA is used at pH above 6.0 due to the chemically instable of purine nucleotides at low pH [39], the desirable pH value for polymer achieving self-coating ability is above 6.0.

3.4. Separation of plasmid DNA

There has been increasing interest in CE of plasmid DNA because its importance in cloning and biochemical studies. Plasmid DNA is made up to two covalently closed circular strands of DNA and normally exists in a supercoiled conformation. The polymers that have been reported for capillary electrophoresis of plasmid DNA are much fewer than those for linear ds DNA fragments. Most of the separations had to be performed in covalently coated capillaries. To verify that plasmids could be separated in this matrix in uncoated capillary, λ plasmid sample (48 kbp in size) was tested. As shown in Fig. 5, three peaks were observed for the sample, which were assigned to be the linear, supercoiled and nicked conformers of the plasmid [40]. The peak in the middle was prominent and agreed with that plasmid DNA predominantly existed in a supercoiled conformation in the sample. The nicked conformer and linear conformer are generated by a single-stranded break and double-stranded break in plasmid DNA, respectively. The supercoiled conformer migrated more rapidly than its nicked form but more slowly than the linear form. Our result demonstrates that purity analyses of plasmid preparations are feasible in this matrix in uncoated capillary.

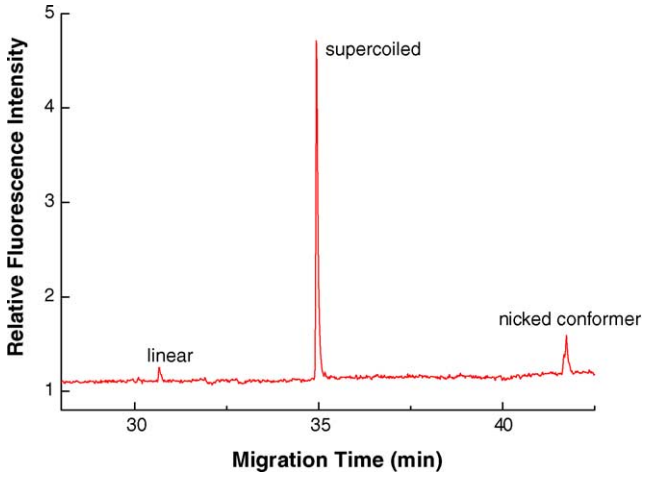

Fig. 5. Electropherogram of λ DNA with 1.0 % PNIPAM + 6% mannitol. Inject, 5 s at −9.8 kV; separation electric field strength 220 V/cm.

4. Conclusions

The work on developing novel homopolymer doesn't only provide novel separation matrices but also allow more information on understanding the mechanisms of self-coating and DNA separation. The separation matrix, consisting of the polymer PNIPAM and small molecular additive mannitol, has shown excellent resolution in the electropherograms of CE for dsDNA and plasmid DNA. The addition of mannitol dramatically enhanced the separation by decreasing the pH of the running buffer, which helped the PNIPAM adhere on the silica wall. This new matrix has very low viscosity, which makes it easy for capillary filling, flushing and refilling. In addition, the pH dependent self-coating ability of PNIPAM is important for reducing the capillary cost and extending its application in fabricated chips. The found of pH dependent self-coating ability allows for useful information on understanding the mechanism of dynamic coating and on developing desiring matrices for biomolecular separation. Further improvement in the separation in performance can be achieved by optimization of variables such as polymer molar mass distribution, separation temperature and novel small molecular substitution for mannitol. On going research is investigating the potential use of this matrix and other N-substituted polyacrylamides for other types of biomolecular separation.

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