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Separation of DNA fragments and single strand conformation polymorphism analysis in bare capillaries using poly(acrylamide–dimethylacrylamide) as a separation medium

Jicun Ren*, Zheng-Fa Fang

College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha, 410081, PR China

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Abstract

A short chain poly(acrylamide–dimethylacrylamide) (PADMA) was synthesized in aqueous phase using isopropanol as a chain transfer agent, and was characterized according to the chemical composition and molecular mass. This polymer can form a stable dynamic coating on the inner surface of the capillary, thereby suppressing the electroosmotic flow and DNA–capillary wall interaction. The sieving medium has low viscosity and capillary filling with this medium and medium replacement were conveniently carried out by commercial capillary electrophoresis instruments. The effects of components and concentration of copolymers on the separation of DNA fragments were investigated. Highly efficient separation of DNA fragments, successful single strand conformation polymorphism (SSCP) analysis and good reproducibility of the migration time were obtained in bare capillaries using these copolymers as sieving media. Our preliminary results demonstrate that PADMA will become an alternative matrix for DNA separation by capillary electrophoresis.

Keywords: Single strand conformation polymorphism; DNA; Poly(acrylamide–dimethylacrylamide)

1. Introduction

DNA analysis plays an important role in genetic and medical research, and clinical diagnosis. Most conventional methods are based on slab gel electrophoresis, which is characterized by its time-consuming and labor-intensive nature. Recently, capillary electrophoresis (CE) in polymer solutions has become an attractive alternative to slab gel electrophoresis for DNA analysis [1–7]. This technique has been successfully applied to mutation detection, genotyping, DNA sequencing and gene expression [7–15]. Fused-silica capillaries are widely used to support sieving media in CE. The silanol groups on the capillary dissociate over a wide pH range (>3) and exist as specific sites for Coulombic charge interaction with some biopolymers such as proteins and DNA fragments. In order to perform DNA analysis, the inner surface of the capillary must be modified, usually by covalent bonding of hydrophilic polymers [16]. However, the covalent coating will increase the cost and often causes problems related to capillary deterioration and coating inhomogeneity. Dynamic coating or adsorption of a coating is a promising method to modify the inner surface of the

*Corresponding author. Fax: +86-73-1885-1226.
E-mail address: jicunren@hunnu.edu.cn (J. Ren).

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capillary. Certain water-soluble polymers have been found to form a dynamic coating on the inner surface of the capillary, which suppresses the electroosmotic flow and the capillary wall–DNA interaction. These polymers including poly(ethylene oxide) [17], poly-(vinylprrolidone) [18], polydimethylacrylamide [19] and some copolymers [20,21], have been applied as sieving media for electrophoretic separation of DNA fragments and DNA sequencing in bare capillaries.

Recently, a copolymer-poly(acrylamide–dimethylacrylamide) (PADMA) was synthesized and was used in the concentration gradient capillary electrophoresis of DNA fragments and DNA sequencing as a sieving medium [22–24]. In the present work we report for the first time a simple method to synthesize PADMA, and then demonstrate that the separation of DNA fragments and single strand conformation polymorphism (SSCP) analysis can be successfully performed in bare capillaries using this copolymer as a sieving medium.

2. Materials and methods

2.1. Materials

Acrylamide, pUC 18 Msp I digests (containing 12 DNA fragments) and *N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) were obtained from Sigma (St. Louis, MO, USA). *N*,*N*′-Dimethylacrylamide was from Aldrich-Chemie (Germany). *N*,*N*,*N*′,*N*′-Tetramethylenediamine and ammonium peroxydisulfate, were purchased from Bio-Rad Laboratories (Hercules, CA, USA). SYBR Green I (concentration not given) in dimethyl sulfoxide was purchased from Molecular Probes (Eugene, USA). All other reagents were of analytical grade. The pH of 80 m*M* TAPS was adjusted with 1 *M* NaOH using a pH meter (Lezi Instrument Factory, Shanghai, China). QIAamp blood kits were products of QIAGEN (Hilden, Germany). Fused silica capillaries with 75-μm internal diameter (I.D.) and 365-μm outer diameter (O.D.) were from J&W Scientific (Folsom, CA, USA) and Yongnian Optical Fiber Factory (Yongnian, Hebei, China). 5-Fluorescein-labeled primers were synthesized by Eurogentec (Seraining, Belgium). Double-distilled water was used for preparation of all aqueous solutions.

2.2. Synthesis of PADMA

PADMA was synthesized in water using isopropanol as a chain transfer agent to control the molecular mass of the copolymer. Briefly, 110 ml water and 3.8 ml isopropanol were added to a vessel containing a given amount of dimethylacrylamide and acrylamide (total weight, 10 g). The mixture was degassed with helium for 30 min and heated in a water bath at 50°C for 20 min. Then, 0.625 ml 10% (v/v) *N*,*N*,*N*′,*N*′-tetramethylenediamine and 0.625 ml 10% (w/v) ammonium peroxydisulfate were added, and the polymerization took place for 1.5 h at 50°C.

The reaction product was extensively dialyzed against water for 2 days, using a 12,000 molecular mass cut-off dialysis membrane tubing (Thomas Scientific, Philadephia, PA, USA), and then lyophilized. The sieving medium was prepared by dissolving the copolymer powder into separation buffer.

2.3. Measurement of molecular weight and medium viscosity, and elemental analysis of PADMA

The viscosity average molecular mass (M_v) was measured from the intrinsic viscosity [η] of the polymer. The kinematic viscosity was determined at 25°C using an Ubbelohde viscometer. The plot of η_{sp}/C (η_{sp}, specific viscosity; *C*, concentration of polymer) versus the concentration of the polymer was extrapolated to zero concentration, producing [η] as the intercept. M_v was calculated according to the Mark-Houwink equation ($[\eta]=kM_v^a$ [19,25], where $k=23.2\times10^{-3}$ (ml/g) and $a=0.81$ and the results are shown in Table 1. The viscosity of 4% PADMA solution in 80 m*M* TAPS (pH 7.8) is included in Table 1.

Table 1
Components and molecular mass data of copolymers, and viscosity of media

Ratio	C (%)	H (%)	N (%)	M_v (kDa)	Viscosity (cP)
1:9	41.71	8.41	16.38	39	18
3:7	43.96	8.55	15.95	38	18
1:1	44.50	8.96	15.22	38	18
1:0	51.50	10.88	12.19	38	18

Element analyses were performed on PE 2400 CHN (Perkin-Elmer, Norwalk, USA) and the results are shown in Table 1.

2.4. Measurement of electroosmotic flow (EOF)

EOF measurements were performed on a P/ACE System 5500 with a diode array detector (Beckman Instruments, Fullerton, CA, USA). Briefly, new capillaries with 75-μm I.D. and 27 cm long (20 cm to the detection window) were washed with 0.1 *M* HCl solution for 5 min, then with water and buffer for 1 min each, and finally filled with 0.1% copolymer sieving medium. Running buffer was 80 m*M* TAPS (pH 7.8). An aqueous solution of cytosine (1 mg/ml) was used as the neutral marker and was introduced by pressure injection for 4 s. EOF was measured in positive polarity mode using a field of 296 V/cm and the detection wavelength was 260 nm. EOF (μ_{eof}) values were calculated using the following equation:

$$\mu_{eof} = L_{eff}L_T/VT_0 \quad (1)$$

where L_{eff} and L_T are the effective and total lengths of the capillary, respectively, V is the applied voltage, and T_0 is the migration time of the neutral marker.

2.5. DNA extraction and PCR reactions

DNA was extracted from whole blood using a QIAamp blood kit according to the instructions of the manufacture. The A1298C mutation in MTHFR gene abolishes an MboII restriction site, and the MTHFR genotype was determined by PCR amplification and MboII digestion [26]. The digestion fragments were detected by capillary electrophoresis as described by Ren et al. [27].

The PCR mixture contained 10 m*M* Tris–HCl, pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100, 125 μ*M* of each dNTP, 0.2 μ*M* of each primer, 1 U Taq polymerase (Super Taq, HT Biotechnology, UK) and ~100 ng template DNA in a final volume of 100 μl. PCR reactions were performed on a DNA Thermocycler (Perkin-Elmer, Foster, USA) using a thermocycling profile with 36 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 20 s.

The primers used were 5′-fluorescein-CTTTGGGGAGCTGAAGGACTACTAC (forward) and 5′-fluorescein-CACTTTGTGACCATTCCGGTTTG (reverse). The primers define the PCR product 163 bp in length including a position 1298. The PCR products were precipitated with 70% ethanol according to Ref. [13] and then dissolved in 50 μl diluted buffer (0.1×TBE, pH 8.3). The samples were stored at −20°C until analysis. Before SSCP analysis, the PCR products were heated to 95°C for 5 min, cooled in ice water for 10 min, and then subjected to CE analysis.

2.6. Capillary electrophoresis procedure

The Beckman P/ACE System 5500 was equipped with a laser-induced fluorescence (LIF) detector, in which an argon ion laser provided an excitation light source at 488 nm. A 520-nm bandpass filter was used as an emission cut-off filter. Beckman System Gold software was used in system control, data collection and processing.

A new capillary was first rinsed with 0.1 *M* HCl solution for 10 min, then with water and buffer for 5 min each, and finally filled with copolymer sieving medium. For double strand DNA analysis, SYBR Green I was added to the sieving medium at a dilution of 1:5000. Running buffer was 80 m*M* TAPS (pH 7.8). Samples were introduced by electrokinetic injection and electrophoresis was performed in the reverse polarity mode. Between each run, the capillary was rinsed with the separation buffer for 4 min and then filled with fresh sieving medium.

3. Results and discussion

3.1. Suppression of EOF

Suppression of EOF is an indication of polymer adsorption, and Table 2 shows the EOF values of copolymers. These data illustrated that PADMA could adsorb strongly to the inner surface of the capillary and form a dynamic coating, suppressing the EOF. Some studies [28–32] showed that the adsorption of polymer is associated with its solubility in the solvent from which it was adsorbed. Differing from polyacrylamide, PADMA has a proper hydro-

Table 2
Electroosmotic flow values with and without copolymers

Entry	Dimethylacrylamide to acrylamide ratio	Electroosmotic flow ($\times 10^{-4}$ cm^2/V s)
1	No copolymer	4.10
2	1:9	0.500
3	3:7	0.355
4	1:1	0.356
5	1:0	0.354

phobic nature since it is in part incorporated by dimethylacrylamide. The incorporation of dimethylacrylamide may favor the adsorption of PADMA into the capillary wall and thus improve the stability of the dynamic coating. However, the adsorption mechanism is not completely understood.

3.2. Separation of double strand DNA (dsDNA) fragments

The results above illustrated that PADMA could form a dynamic coating on the inner surface of the capillary and suppress EOF. Next, we examined the separation of DNA fragments in bare capillaries using PADMA sieving medium.

Fig. 1 shows the separation of pUC 18 DNA fragments in a bare capillary using PADMA as sieving media. This system was characterized by high separation efficiency and 12 DNA fragments were well resolved. This is attributed to the fact that the capillary–DNA interaction was limited by the formation of the dynamic coating. Notably, with an increase of the dimethylacrylamide to acrylamide ratio in the copolymer, the resolution between 489 and 501 bp was slightly decreased (as shown in Fig. 2). This may be due to an increase in the hydrophobic nature of the polymer with the dimethylacrylamide to acrylamide ratio.

We also investigated the effects of PADMA concentrations on the separation of DNA fragments (as shown in Fig. 3). The migration times and resolutions increased with the concentration of PADMA, which were similar to some hydrophilic polymers [33,34]. The highest resolutions were obtained at 5% PADMA.

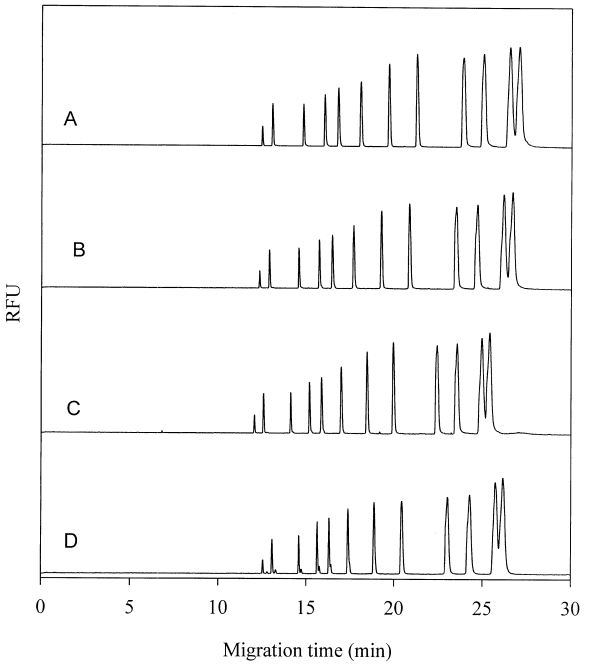

Fig. 1. Separation of dsDNA fragments in PADMA media by CE. A Msp I digest of pUC 18 (2 μg/ml), containing DNA fragments of 26, 34, 67, 89, 110, 147, 190, 242, 353, 404, 489 and 501 bp was separated in a bare capillary (37 cm in length and 75-μm I.D.) filled with 5% PADMA. The dimethylacrylamide to acrylamide ratios in panels A–D were 1:9, 3:7, 1:1 and 1:0, respectively. Electrokinetic injection at −4 kV for 10 s was used, temperature was 25°C and the applied voltage was −8 kV. RFU, relative fluorescence units.

3.3. Reproducibility of migration time of DNA fragments

We tested the reproducibility of migration time of DNA fragments in a bare capillary using PADMA medium. The results obtained (Table 3) demonstrated a high reproducibility (RSD <3%). The data also illustrated that the dynamic coating of PADMA on the capillary was stable.

3.4. SSCP analysis

We also wanted to explore the suitability of PADMA for SSCP analysis as a sieving medium. SSCP analysis is the most popular method to screen unknown mutations and detect known mutations

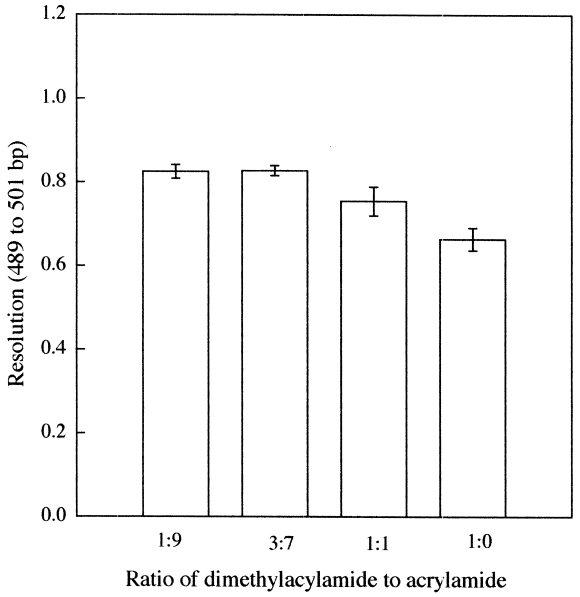

Fig. 2. Resolution between 489 and 501 bp using different dimethylacrylamide to acrylamide ratios. The samples and electrophoretic conditions were as described in Fig. 1 and measurements were performed five times. The resolutions were calculated by Caesar Software (provided by Prince Technologies, The Netherlands).

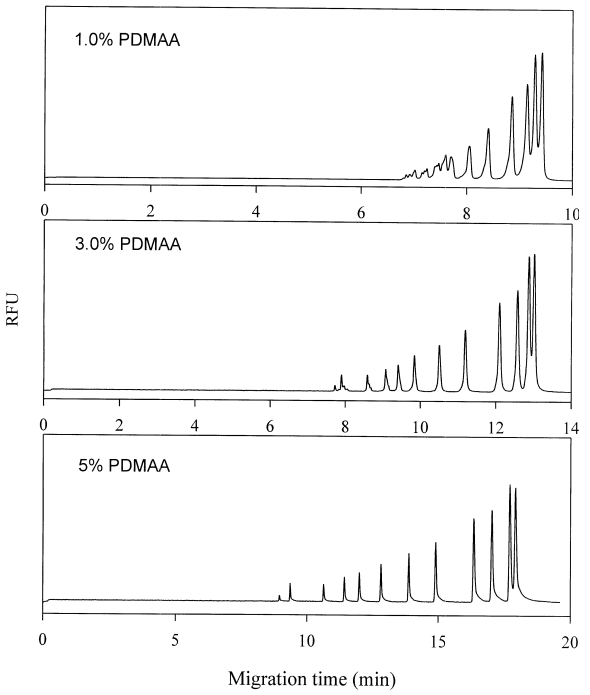

Fig. 3. Effects of the concentration of PADMA on the separation of dsDNA fragments. The dimethylacrylamide to acrylamide ratio of PADMA was 3:7. The samples and electrophoretic conditions were as described in Fig. 1. RFU, relative fluorescence units.

because of its simplicity and low cost [35]. CE has been successfully applied to SSCP analysis instead of slab gel electrophoresis [8,36–41], but in most applications covalently coated capillaries were used to limit the electroosmotic flow and the capillary–DNA interaction. Fig. 4 shows SSCP analysis of the MTHFR gene using PADMA as a sieving medium. Three genotypes of A1298A (− −), A1298C (+ −) and C1298C (+ +) were clearly distinguished by CE, which was in line with that by restriction fragment length polymorphism analysis [26]. These data illustrate that CE using PADMA medium has the high resolution necessary for SSCP analysis.

4. Conclusion

We described a simple method for synthesis of PADMA in an aqueous phase using isopropanol as a chain transfer agent. Our data show that PADMA can form a dynamic coating of the inner surface of the

Table 3
Reproducibility of migration time of DNA fragments ($n=8$)

DNA fragments (bp)	Mean migration time (min)	RSD (%)
26	12.32	1.7
34	12.85	1.7
67	14.45	1.7
89	15.54	1.8
110	16.26	1.8
147	17.41	2.0
190	18.93	2.1
242	20.43	2.2
353	22.97	2.2
404	24.09	2.3
489	25.52	2.3
501	25.96	2.4

The reproducibility experiments were performed on a bare capillary filled with 5% PADMA (dimethylacrylamide to acrylamide ratio, 3:7). Electrophoretic conditions were as described in the legend to Fig. 2.

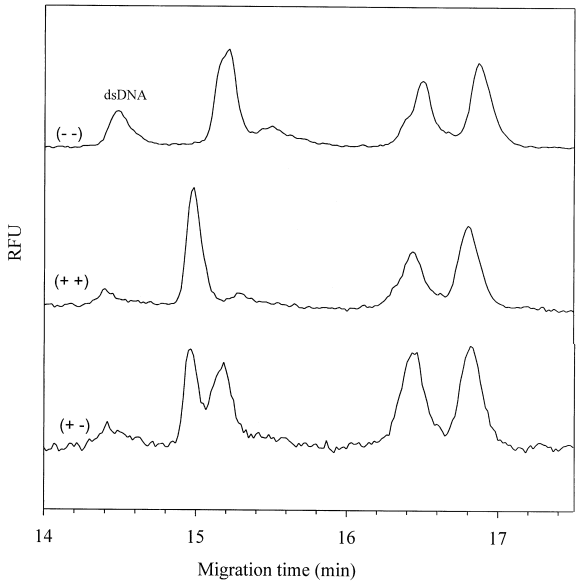

Fig. 4. SSCP analysis by CE. The A1298C mutation of the MTHFR gene was determined by SSCP analysis. The homozygous normal (− −), homozygous mutant (+ +) and heterozygous genotypes are indicated in the panel. The single strand DNA (ssDNA) fragments were separated in 5% PADMA (dimethylacrylamide to acrylamide ratio, 3:7) at 20°C. The other conditions were as described in Fig. 1. RFU, relative fluorescence units.

silica capillary, thereby suppressing EOF and inhibiting the DNA–capillary wall interaction. We also demonstrated highly efficient separations of DNA fragments and successful SSCP analysis in bare capillaries using PADMA as sieving media. The sieving medium has low viscosity and is well suited to DNA analysis by commercial CE instruments. Since acrylamide and dimethylacrylamide are commercially available, this copolymer can be conveniently synthesized by a laboratory and will be an attractive alternative to other non-cross-linked polymers commonly used in CE or chip CE.

5. Nomenclature

CE	Capillary electrophoresis
dsDNA	Double strand DNA
I.D.	Internal diameter
LIF	Laser-induced fluorescence
MTHFR	Methylenetetrahydrofolate reductase
O.D.	Outer diameter
PADMA	Poly(acrylamide–dimethylacrylamide)
SSCP	Single strand conformation polymorphism
ssDNA	Single strand DNA

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