Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 68 (2006) 940–944
Talanta
www.elsevier.com/locate/talanta

DNA mutation analysis based on capillary electrochromatography using colloidal poly(*N*-isopropylacrylamide) particles as pseudostationary phase

Joon Myong Song [a,*], Amit Asthana [b], Dong Pyo Kim [b,**]

[a] *College of Pharmacy, Seoul National University, Seoul 151-742, South Korea*
[b] *Department of Fine Chemical Engineering and Chemistry, Chungnam National University, Daejon 305-764, South Korea*

Received 8 April 2005; received in revised form 15 June 2005; accepted 15 June 2005
Available online 10 August 2005

Abstract

Poly(*N*-isopropylacrylamide) (PNIPAM) is an interesting class of temperature sensitive, water soluble polymer that has a lower critical solution temperature (LCST) of 32 °C. Above the LCST, PNIPAM gets phase-separated and precipitates out from water. The fascinating temperature-sensitive property of PNIPAM has led to a growing interest in diverse fields of applications. Recently, capillary electrochromatography (CEC) has gained attention due to the wide range of applications based on the use of open tubular capillaries. In this paper, the use of phase-separated PNIPAM as a pseudostationary phase for CEC is demonstrated for the detection of single nucleotide polymorphisms (SNPs). Owing to the dynamic coating, the phase-separated PNIPAM particles did not require any immobilization technique and could exist as a mobile stationary phase in the open tubular capillary. The heteroduplex analyses of mutation samples could be successfully performed based on the phase-separated PNIPAM particles in the constructed CEC system. The CEC system, based on PNIPAM particles capable of having a narrow size distribution, shows great potential as an alternative to conventional DNA mutation systems.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Poly(*N*-isopropylacrylamide); Pseudostationary phase; Open tubular capillary electrochromatography; SNP analysis

1. Introduction

Single nucleotide polymorphisms (SNPs), which are the most common form of genetic variation in the human genome, have attracted much attention. Single-base substitutions, insertions, and deletions in the genome can be efficiently used as genetic markers for tracking disease genes and population history. It has been reported that 2.1 million SNPs in the human genome occur at a frequency of 1/1.25 kb of sequence [1]. Many different methods have been developed for the identification of SNPs. DNA sequencing [2], single-stranded conformational polymorphism analysis (SSCP) [3], temperature gradient gel electrophoresis (TGGE) [4], denaturing gradient gel electrophoresis (DGGE) [5], DNA chip [6], and mass spectrometry [7] are representative tools that identify SNPs. Another powerful tool used in the detection of SNPs is denaturing high performance liquid chromatography (DHPLC). DHPLC performs separations of homoduplexes and heteroduplexes under partially denaturing conditions using ion-pair reversed-phase (IP-RP) HPLC [8]. The separation of homoduplexes and heteroduplexes is based on electrostatic interactions between negatively charged DNAs and positively charged alkylated particles employed as a stationary phase. The ion-pairing agent such as amphiphilic triethylammonium ion binds to the alkylated particle and renders the surface of the particle positively charged. Heteroduplexes can form partially unwound DNA fragments at melting temperature that does not allow the partial denaturation of homoduplexes. This is because heteroduplexes have

* Corresponding author. Tel.: +82 2 880 7857; fax: +82 2 874 8928.
** Corresponding author. Fax: +82 42 821 6695.
E-mail addresses: jmsong@cnu.ac.kr (J.M. Song), dpkim@cnu.ac.kr (D.P. Kim).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.06.052

lower melting temperature than homoduplexes. As a result, heteroduplexes mediate less interaction with the alkylated particles and have faster migration times than homoduplexes. DHPLC that uses alkylated nonporous particles as a stationary phase does not require regeneration of the separation medium at every run, unlike gel-based separation techniques such as SSCP, TGGE, and DGGE. The advantages of DHPLC include speedy analysis, the use of automated instruments, and high reproducibility.

Capillary electrochromatography (CEC) performs separations based on a chromatographic separation mechanism between a stationary phase (typically 1.5 μm C_{18}-silica) filled with the capillary column and a mobile phase electroosmotically driven through the capillary. An open tubular chromatographic column is a capillary where a stationary phase is coated on the capillary inner wall. Open tubular capillaries, which have no end frit, have several advantages over packed capillaries, for example, utilization of surface modification, higher compatibility with columns of smaller internal diameter, and higher detection sensitivity [9]. Several separation modes, such as ion exchange, molecular, or chiral recognition, are available in open tubular CEC due to the diverse immobilized stationary phases. CEC provides higher separation resolution compared with HPLC for the same particle size and column length, mainly due to the flat flow profile induced by electroosmosis.

Aqueous poly(*N*-isopropylacrylamide) (PNIPAM) solution is very sensitive to thermal conditions. PNIPAM is readily soluble in water below the lower critical solution temperature (LCST) of 32 °C. Above the LCST, PNIPAM is dehydrated and precipitated [10]. This is mainly due to the conformation changes of the polymer chain arising from hydration changes of isopropyl side groups. Accordingly, heating a solution of linear PNIPAM above the LCST can be utilized as an effective tool that produces PNIPAM particles. In this work, the use of phase-separated PNIPAM particles as a pseudostationary phase is demonstrated in a CEC system for DNA mutation analysis. Obviously, open tubular capillaries that do not need end frit formation allow much easier preparation and provide important advantages compared with packed columns. However, open tubular capillaries in CEC consist of an immobilized stationary phase obtained through surface chemistry or synthetic processes inside the capillaries. Although these processes significantly improve the selectivity and the separation toward a specific analyte, they are laborious, time-consuming, and quite complex. Compared with the immobilized stationary phase, the PNIPAM particles as a pseudostationary phase can be easily prepared without using immobilization chemistry by the temperature-controlled phase transition. The PNIPAM particles are heterogeneous particles precipitated in aqueous solution above the LCST and freely mobile inside the capillaries. The precipitated PNIPAM particles can be considered as a kind of packed material playing a role that interacts with DNA molecules in the capillaries. Accordingly, the mobile PNIPAM particles can be described as a pseudostationary phase. The packing of PNIPAM particles can be easily performed by pretreatment of the capillary inner wall with poly(vinylpyrrolidone) (PVP) gel that efficiently suppresses the electroosmotic flow [11,12], followed by dynamic coating and heating of the aqueous PNIPAM solution. Some materials as pseudostationary phase have been applied to CEC [13–17]. Polymeric nanoparticles have been prepared by utilizing a precipitation polymerization technique and were used in CEC with electrospray ionization mass spectrometry detection [13]. Polymeric surfactants have proven to improve the CEC separation of a mixture of cholesterol and 12 ester derivatives [14]. In addition, ion-exchange particles, polymer additive, and dextran sulfate have been used as pseudostationary phase for CEC. The combination of temperature-controlled PNIPAM particles with open tubular capillaries provides advantages to CEC for heteroduplex analysis. First, the precipitated PNIPAM particles readily form around the temperatures used to provide the partially denaturing condition for the separation of homoduplexes and heteroduplexes. In addition, PNIPAM particles as a pseudostationary phase are readily adaptable to the open tubular capillaries by dynamic coating. As a result, column preparation can be easily achieved without using any immobilization chemistry. In this condition, neither end frit formation nor a series of steps for immobilization of the stationary phase are necessary. Compared with the conventional open tubular CEC, the CEC system using the precipitated PNIPAM particles can reduce the cost and time needed for the immobilization process of the stationary phase. In this work, we present a DNA mutation analysis using a CEC system based on the temperature-controlled phase transition of PNIPAM.

2. Experimental

2.1. Reagents and sample preparation

Capillaries of 50 μm i.d. and 365 μm o.d. were obtained from polymicro Technologies (Phoenix, AZ). Linear poly(*N*-isopropylacrylamide) (M_n 20,000–25,000) and poly(vinylpyrrolidone) (PVP) were obtained from Aldrich (Milwaukee, WI). Ethidium bromide was purchased from Molecular Probes (Eugene, OR). 1× Tris–borate–EDTA (1× TBE) buffer solution was prepared by dissolving the premixed powder that yields 89 mM Tris, 89 mM borate, and 2 mM EDTA in deionized water according to the method specified by the supplier (Amresco, Solon, OH). The commercially available DNA sample for the heteroduplex analysis was purchased from Transgenomic, Inc. (San Jose, CA). The commercial DNA samples that consist of 209 bp fragments from the human Y chromosome have an A to G mutation at position 168 [8]. The commercial DNA samples were labeled by the blue fluorescent dye carboxyfluorescein (FAM). Other types of DNA samples were prepared by polymerase chain reaction (PCR). The PCR was performed with 0.02 units/μL of AmpliTaq DNA poly-

merase and 100 ng template. Forward primer GCACTG-GCGTTCATCATCT and reverse primer ATGTTCATTATG-GTTCAGGAGG were used at 0.2 μM final concentration each. The PCR was performed with the following protocol: 34 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. The size of the PCR product was 388 bp and the mutation was at position 243. The mutation type was A to G. The PCR-amplified DNAs without label were detected using ethidium bromide [12]. Ethidium bromide powder (0.5 mg) was dissolved in 1000 mL of 1× TBE buffer so that the final concentration was 0.5 μg/mL.

2.2. Fluorescence detection system

Fig. 1 represents a schematic diagram of capillary electrochromatography using PNIPAM particles. A 488 and 514.5 nm beam from an Ar-ion laser (American Laser Corporation, Salt Lake City, UT) were used to excite the FAM-labeled DNAs and ethidium bromide-stacked DNAs, respectively. A planoconvex lens of 40 mm focal length was used to focus the laser beam onto the capillary. The polyimide coating of the capillary was removed to produce a detection window at the position of laser irradiation. A temperature controller (Global Lab., Model #: GLTC-D) was used to heat the capillary. The capillary was wrapped by the heating element of the temperature controller so that the temperature of the capillary was controlled to a 1 °C interval and measured using a thermocouple. The length of the capillary wrapped by the heating element was 30 cm. A 20× microscope objective (Nikon, 0.40 NA) that was set perpendicular to the incident laser beam was used to collect the fluorescence from the detection window and focused onto a photomultiplier tube (PMT) (Hamamatsu Corporation, Model #: R595). The PMT was operated at −900 V using a digital photometer (Model 124, Pacific Instruments, Inc., Concord, CA). A bandpass optical filter (center wavelength: 520 nm, FWHM: 10 nm) was positioned in front of the PMT to remove the 488 nm laser beam scattering. A long-pass optical filter (cut-off position: 590-nm, Edmund Industrial Optics) was used to eliminate the laser scattering when the 514.5 nm laser beam was used.

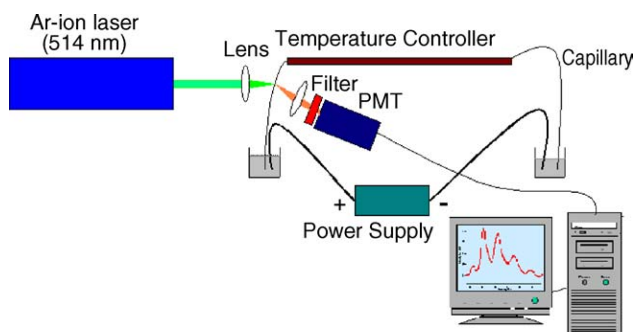

Fig. 1. A schematic diagram of the experimental apparatus.

The PMT signal was read out and saved by a computer. A custom written software interface constructed with Labview was used for the data acquisition process.

2.3. Capillary electrophoresis

Two percent of PVP (M_r, 130,000) solution was prepared by dissolving the PVP powder in 1× TBE buffer solution and then shaken softly for 10 min. Two percent PNIPAM prepared in 1× TBE buffer was used as a separation medium. A 100 μL syringe was used to load the polymeric solutions. Before the 2% PNIPAM solution was loaded onto the capillary for the DNA separation, the capillary was treated with the prepared PVP solution so that the electroosmotic flow was suppressed. When the temperature of the capillary is above 32 °C, the loaded PNIPAM precipitates. The electric field was generated by a high-voltage power supply (ConverTech, Inc., Model #: SHV 200, 40 kV/5 mA). The total and effective length of the capillary was 45 and 34 cm, respectively. Electrokinetic injections of DNA sample solutions were performed at 6 kV. An electric field gradient of 150 V/cm was applied to the capillary for the separation of homoduplexes and heteroduplexes. After each run, the separation medium in the capillary was flushed out with water using a 100 μL syringe.

3. Results and discussion

Fig. 2 represents electropherograms of the commercially available heteroduplexes and homoduplexes obtained as a

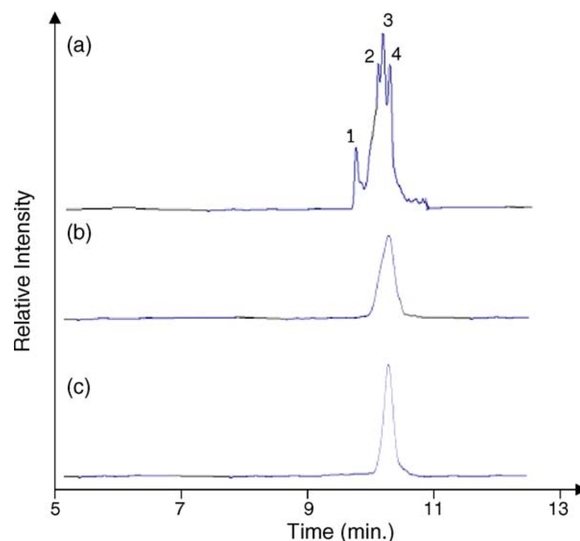

Fig. 2. Separation of homoduplexes and heteroduplexes as a function of temperature using the colloidal PNIPAM particles. The DNA samples were 209 bp fragments from the human Y chromosome. The separation conditions were 2% PNIPAM and 150 V/cm electric field gradient. The temperature applied to the capillary were: (a) 58 °C, (b) 45 °C, and (c) room temperature. Wild-type and mutant have (A,T) and (G,C) base pair at position 168, respectively. Peak legend in (a): (1) homoduplex (G,C), (2) homoduplex (A,T), (3) heteroduplex (A,C), and (4) heteroduplex (G,T).

function of temperature using a 2% linear PNIPAM sieving matrix. The operating temperatures in Fig. 2(a–c) were 58 °C, 45 °C, and room temperature, respectively. At room temperature, PNIPAM exists as a homogeneous gel and forms a dynamic coating in the inner wall of the capillary. At 45 and 58 °C, which are above the LCST, the PNIPAM is precipitated by the phase transition and subsequently produces heterogeneous particles in the capillary. The electropherogram obtained at room temperature does not show any separation of homoduplexes from heteroduplexes. Fig. 2(b) shows the same result as Fig. 2(c). Although the phase transition occurs at 45 °C, the separation of homoduplexes from heteroduplexes was not achieved in the PNIPAM particles. This means that the temperature elevation from room temperature was not sufficient to allow the melting of DNA molecules. At 58 °C, the electropherogram clearly shows four peaks that correspond to homoduplexes and heteroduplexes. The interactions of the precipitated PNIPAM particles with homoduplexes and heteroduplexes in the electric field could lead to the successful separation of homoduplexes from heteroduplexes. These results are in agreement with the previous result obtained using DHPLC [8]. The above results show that colloidal PNIPAM particles as a separation medium in CEC can provide the separation resolution for SNP detection. As the analysis is performed around the melting temperatures of both homoduplexes and heteroduplexes, the thermally stable colloidal dispersion of PNIPAM can be considered as an appropriate medium for SNP detection. The colloidal PNIPAM particles can be described as a pseudostationary phase in CEC because the precipitated PNIPAM particles are not immobilized on the inner capillary wall; they are mobile and interact with DNAs. Unlike DHPLC, a bulkier DNA fragment has a lower electrophoretic mobility in the present CEC system. Accordingly, migration times of heteroduplexes that have lower melting temperature are slower than those of homoduplexes.

One of the important properties required for packing materials in DHPLC is uniform size distribution. This is because high separation resolution can be expected from packing materials with narrow size distributions. The phase-separated PNIPAM particles as a separation medium for CEC fulfill the above requirement. Pelton et al. reported that PNIPAM can produce remarkably uniform particles [10]. The size of phase-separated PNIPAM particles is capable of being variable as a function of PNIPAM concentration and ionic strength. However, the size distributions are very narrow. Pelton et al. reported that the maximum polydispersity of 0.14 and the maximum standard deviation of the mean diameter of 0.5 nm were obtained when the size of PNIPAM with weight-average molecular weight of 547,000 Da was measured at 40 °C by dynamic light scattering [18]. Fig. 3 shows an electropherogram of the DNA sample by using 2% PVP as a sieving matrix by keeping the temperature of the capillary as 58 °C. The 2% PVP provides an unresolved peak. Compared with the conventional PVP gel, the phase-separated PNIPAM particles show the successful separation of homoduplexes from heteroduplexes under identical conditions.

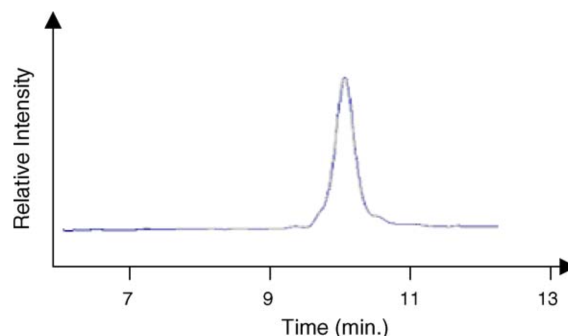

Fig. 3. Electropherogram of the DNA sample using 2% PVP as a separation medium. The DNA samples were 209 bp fragments from the human Y chromosome. The separations were performed at 58 °C and 150 V/cm electric field gradient.

The uniform size distribution of PNIPAM particles is thought to contribute greatly to the separation capability of the CEC system. This result demonstrates the suitability of the colloidal PNIPAM particles as a separation medium in CEC for SNP analysis with high resolution.

An analysis of the PCR products was performed to further explore the potential of precipitated PNIPAM particles as stationary phase for CEC for SNP analysis. Fig. 4 represents the electropherograms of the PCR-amplified products obtained at 62 °C using 2% PVP and 2% PNIPAM. As expected, Fig. 4(a) clearly shows four well-resolved peaks of the homoduplexes and heteroduplexes. On the other hand, 2% PVP shows an unresolved broad peak. The present study clearly demonstrates the feasibility of CEC based on the colloidal PNIPAM

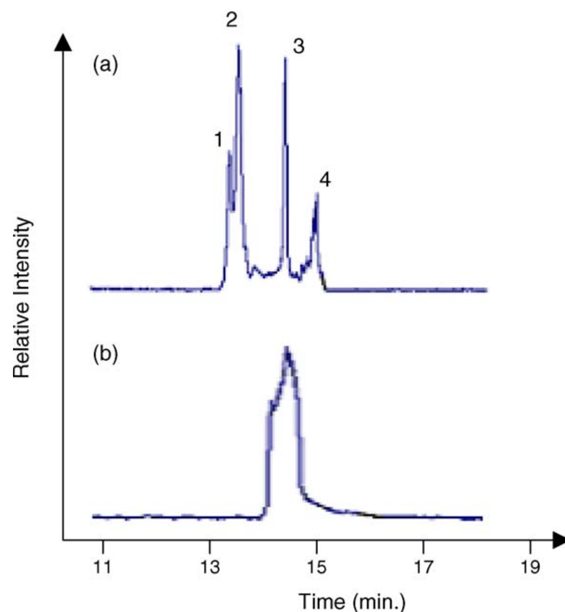

Fig. 4. Detection of SNPs for the PCR products using 2% PVP and 2% PNIPAM. The applied temperature and electric field gradient were 62 °C and 150 V/cm, respectively. (a) 2% PNIPAM and (b) 2% PVP. Peak legend in (a): (1) homoduplex (G,C), (2) homoduplex (A,T), (3) heteroduplex (A,C), and (4) heteroduplex (G,T).

particles as an alternative to conventional DNA mutation analyses.

Acknowledgement

This work was supported by Korea Research Foundation Grant (KRF-2004-042-103115).

References

[1] J.C. Venter, et al., Science 291 (2001) 1304.
[2] V.B. Rao, Anal. Biochem. 216 (1994) 1.
[3] M. Orita, H. Iwahana, H. Kanazawa, K. Hayashi, T. Sekiya, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 2766.
[4] D. Riesner, G. Steger, R. Zimmat, R.A. Owens, M. Wagenhoder, W. Hillen, S. Vollbach, K. Henco, Electrophoresis 10 (1989) 377.
[5] S.G. Fischer, L.S. Lerman, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 1579.
[6] J.Y. Deng, X.E. Zhang, Y. Mang, Z.P. Zhang, Y.F. Zhou, Q. Liu, H.B. Lu, Z.J. Fu, Biosens. Bioelectron. 19 (2004) 1277.
[7] R.W. Giese, Chem. Res. Toxicol. 10 (1997) 255.
[8] K.H. Hecker, P.D. Taylor, D.T. Gjerde, Anal. Biochem. 272 (1999) 156.
[9] E. Guihen, J.D. Glennon, J. Chromatogr. A 1044 (2004) 67.
[10] R. Pelton, Adv. Colloid Interface Sci. 85 (2000) 1.
[11] J.M. Song, E.S. Yeung, Electrophoresis 21 (2000) 807.
[12] J.M. Song, J. Mobley, T. Vo-Dinh, J. Chromatogr. B 783 (2003) 501.
[13] P. Viberg, M. Jornten-Karlsson, P. Petersson, P. Spegel, S. Nilsson, Anal. Chem. 74 (2002) 4595.
[14] S. Thiam, S.A. Shamsi, C.W. Henry III, J.W. Robinson, I.M. Warner, Anal. Chem. 72 (2000) 2541.
[15] M.C. Breadmore, M. Macka, P.R. Haddad, Electrophoresis 20 (1999) 1987.
[16] M. Gilar, A. Belenky, A.S. Cohen, Electrophoresis 21 (2000) 2999.
[17] Y. Chen, X. Lu, Z. Han, L. Qi, M.X. Wang, X. Yu, G. Yang, L. Mao, H. Ma, Electrophoresis 26 (2005) 833.
[18] K. Chan, R. Pelton, J. Zhang, Langmuir 15 (1999) 4018.