Preparation and Characterization of Temperature-Responsive Chromatographic Column Containing Poly(*N*-isopropylacrylamide) and Poly([2-(methacryloyloxy)-ethyl]trimetylammonium chloride)

Rongji Dai,[1] Lei Chen,[1] Zongjian Liu,[1] Haihui Wang,[1] Dingyu Hu,[1,2] Yulin Deng[1]

[1]*School of Life Science, Beijing Institute of Technology, Beijing 100081, China*
[2]*The Chinese People's Armed Police Force Academy, Langfang, Hebei 065000, China*

Received 22 April 2010; accepted 23 November 2010
DOI 10.1002/app.33830
Published online 16 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: This article described the preparation of temperature-responsive chromatographic column containing poly(*N*-isopropylacrylamide) and poly quaternary ammonium salt. Poly (*N*-isopropylacrylamide) and poly ([2-(methacryloyloxy)ethyl]trimetylammonium chloride) were synthesized and grafted to amino modified silica gel. The temperature responsive polymer grafted silica gel was characterized using Fourier transform infrared spectroscopy, thermoweight loss, and elemental analysis. The temperature responsive chromatographic column was used to analyze lactic acid and creatine phosphate disodium salt by controlling of the column temperature from 10 to 50°C. The mixture of lactic acid and creatine phosphate disodium salt was baseline separated at pH 6.93. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 2233–2238, 2011

Key words: temperature-responsive polymer; separation; poly(*N*-isopropylacrylamide); chromatography; poly ([2-(methacryloyloxy)ethyl]trimetyl-ammonium chloride)

INTRODUCTION

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a temperature-responsive material with a lower critical solution temperature (LCST) of 32°C.[1–3] The polymer chain of PNIPAAm is hydrated and is water soluble below its LCST in aqueous solution, and it was dehydrated to form an insoluble conformation above LCST. The properties of the PNIPAAm grafted solid surfaces have been investigated.[4,5] The surface shows hydrophilic to hydrophobic property alteration with temperature. The thermally responsible stationary phases[6–21] were applied for the separation and analysis of phenyl thiohydantoin amino acids[9–11,13] and hydrophobic steroid separations.[2,8,10,12,14] Thermoresponsive modifiers on silica surfaces effectively modulate separation efficiencies in these chromatography systems in purely aqueous milieu and the surface property of the stationary phase is controlled by external temperature. Reverse-phase liquid chromatography (RPLC) is the most widely used HPLC technique in the separation, purification, and study of peptides.

Temperature-responsive chromatography is performed with an aqueous mobile phase without using an organic solvent, such as methanol or acetonitrile that are used in RPLC. On HPLC columns packed with thermoresponsive polymer-modified silica, effects similar to gradient elution can be achieved with a single-mobile phase by controlling external temperature.[6–21] In conventional RPLC, column temperature can be used to modulate the chromatography, although the influence of temperature is less significant than organic solvent compositions in mobile phase.

Lactic acid is a metabolite under hypoxia condition. The levels of lactic acid increase significantly under the serious hypoxia environment (such as 6–8% O_2), while the levels of creatine phosphate decrease sharply to produce more ATP, which can be used to make more energy. Analyses and separation of lactic acid and creatine phosphate, as the two important indexes, have significance on the measurement of the mechanism in cell energy metabolism. In recent research on the analyses and separation of lactic acid and creatine phosphate, enzymology, spectrophotometric method,[22] hydrophilic interaction chromatography (HILIC),[23] ion

Correspondence to: R. Dai (dairongji@bit.edu.cn); Y. Deng (Deng@bit.edu.cn).

Contract grant sponsor: National Sciences Foundation of China; contract grant number: 20675008.
Contract grant sponsor: National Basic Research Program of China (973 Program); contract grant number: 2007CB914103.
Contract grant sponsor: National Key Technology R and D Program of China; contract grant number: 2006BAK03A07.

chromatograph[24] are used universally. Now, a new method for the analyses and separation of lactic acid and creatine phosphate was designed. In this study, a novel stationary phase with PNIPAAm and polymers carrying a residue of [2-(methacryloyloxy)-ethyl]trimetylammonium chloride was used to separate the mixture of lactic acid and creatine phosphate disodium salt. [2-(methacryloyloxy)ethyl]-trimetylammonium chloride was introduced to the stationary phase as a cationic charged monomer to improve separation performance of the stationary phase by electrostatic interactions with the analytes.

EXPERIMENTAL

Apparatus

The LCST of PNIPAAm was performed on Shimazu UV-2500 ultraviolet–visible spectrophotometer. Gel-permeation chromatography was finished on Waters 1515 Isocratic HPLC equipped with Waters 2414 Refractive Index Detector. Spectrum BX FT-IR system of PerkinElmer was used to determine the infrared spectrum of polymers. Thermogravimetric analysis of modified silica gel was performed on TGA 2950 of Thermogravimetric Analyzer.

Chemicals

N-isopropylacrylamide (NIPAAm) and 3-mercaptopropionic acid (MPA) were purchased from Aldrich. Amino-modified silica gel with diameter 5 μm and aperture 100 Å was got from Tianjin Borui Chromatography. 2-(methacryloyloxy)ethyl] trimetylammonium chloride was obtained from Alfa Aesar (Tianjin) Chemical. Other chemicals were all analytical or chemical grade.

Synthesis of PNIPAAM containing carboxyl group[25]

NIPAAm (2.67 g, 0.0235 mol), AIBN (69.42 mg, 4.23×10^{-4} mol), and MPA (66.75 μL, 6.20×10^{-7} mol) were dissolved in 8 mL of DMF. The reaction mixture was stirred under vacuum for 30 min at ambient temperature to remove the oxygen in the mixture. The polymerization was carried out at 70°C for 12 h. After the reaction was finished, the polymer solution was concentrated by evaporator. Then the reaction mixture was poured into 50 mL of diethyl ether to precipitate the polymers. The polymer was further purified by repeated precipitation from 10 mL of acetone into 100 mL of diethyl ether. 2.24 g of yellowish powder was obtained, yield 74.67%. Molecular weight was determined using acid-basic titration, M_n = 4030. LCST: 32°C. IR (KBr, cm^{-1}): 3299 (γ_{N-H}), 1650 ($\gamma_{C=O}$) (CONH). ^{1}H-NMR(δ, ppm, DMSO-d_6): 1.1 [6H, $CH(CH_3)_2$], 1.7 [2H, $S(CH_2CH)_n$], 2.1 [1H, $S(CH_2CH)_n$], 4.0 [1H, $CH(CH_3)_2$], 7.1 (COOH).

Synthesis of poly[2-(methacryloyloxy)ethyl] trimetylammonium chloride containing carboxyl group

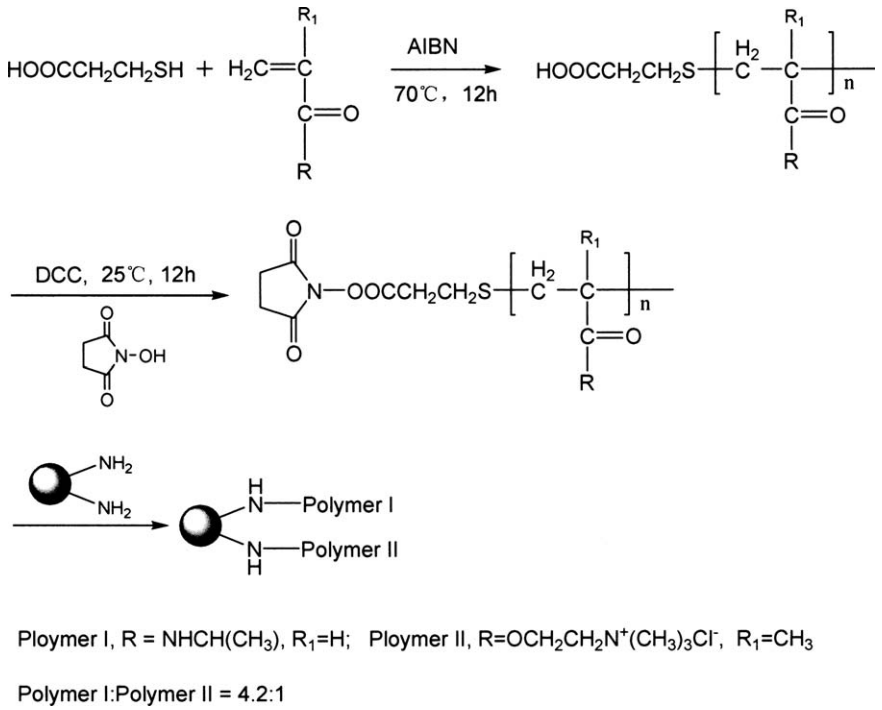
Acetone was added into aqueous solution of 2-(methacryloyloxy)ethyl trimetylammonium chloride to get the pure 2-(methacryloyloxy)ethyl trimetylammonium chloride. The precipitate was washed using acetone three times to remove polymerization inhibitor. Then 2-(methacryloyloxy)ethyl trimetylammonium chloride (2 g, 9.63×10^{-3} mol), AIBN (28.29 mg, 1.72×10^{-4} mol), and MPA (65.83 μL, 6.20×10^{-7}mol) were dissolved in 8 mL of DMF. The polymerization was carried out at 70°C for 12 h. After the reaction was finished, the product was precipitated directly. The polymer was further purified by repeated precipitation from 10 mL of ethanol into 100 mL of acetone. Yield: 93.33%, M_n = 8333, IR (KBr, cm^{-1}): 1750 ($\gamma_{C=O}$) (COOC). ^{1}HNMR (δ, ppm): 0.9–1.2 [1.58H, $S(CH_2CH)_n$], 2.0 [0.79H, $S(CH_2CH)_n$], 3.2 [4.16H, $N(CH_3)_3$], 3.7 [1H, $N(CH_3)_3CH_2$], 4.4 (1H, CH_2OCO), 7.8 (0.04H, COOH).

Preparation of PNIPAAM containing *N*-succinimidehydroxyl ester

PNIPAAm containing carboxyl group (2.24 g, 5.56×10^{-6} mol) was reacted with *N*-hydroxysuccinimide (0.28 g, 2.43×10^{-3} mol) in the presence of dicyclohexylcarbodiimide (DCC) (0.5 g, 2.42×10^{-3} mol) in anhydrous ethyl acetate (20 mL) at 0°C for 2 h, then at 25°C for 12 h. After the precipitated dicyclohexylurea (DCU) was filtrated, the mixture was concentrated by evaporation. The activated polymers were purified by precipitation from dry diethyl ether. A yellowish material was obtained, yield 66.42%. FTIR (KBr, cm^{-1}): 3297 (γ_{N-H}), 1650 ($\gamma_{C=O}$) (CONH), 1738 ($\gamma_{C=O}$) [$(CH_2CO)_2NOCO$]. The presence of the succinimidyl group at the end of the polymer was confirmed by the appearance of a new peak in the IR spectrum at 1738 cm^{-1}, corresponding to the C=O stretching, and an ultraviolet absorption at 260 nm in NH_4OH, corresponding to the succinimidylanion.

Preparation of poly([2-(methacryloyloxy)ethyl]-trimetylammonium chloride) containing *N*-succinimide hydroxyl ester

Poly([2-(methacryloyloxy)ethyl] trimetylammonium chloride) containing *N*-succinimide hydroxyl ester was synthesized using the same method in Section "Preparation of PNIPAAm containing *N*-succinimidehydroxyl ester." Carboxyl groups terminating Poly ([2-(methacryloyloxy)ethyl] trimetylammonium chloride): yield: 51.39%. FTIR (KBr, cm^{-1}): 1750 ($\gamma_{C=O}$) (COOC), 1780($\gamma_{C=O}$)[$(CH_2CO)_2NOCO$].



Scheme 1 Preparation of polymer-grafted silica gel.

Preparation of polymer-grafted silica gel[26]

Three grams of amino group modified-silica gel was added to a solution of 2.79 g of activated PNIPAAm and 1.365 g activated poly([2-(methacryloyloxy)ethyl]-trimetylammonium chloride) in 25 mL of dioxane. The reaction was held at 25°C for 12 h. The modified-silica was washed with 100 mL of ethanol and 500 mL water three times, and dried under vacuum at ambient for 12 h. The ratio of polymer linked to the silica gel was calculated by elemental analyses and thermogravimetric analysis.

Elemental analyses (%): (1) aminopropyl silica, C, 2.94; H, 0.83; N, 1.06. (2) Polymer-modified silica (%): C, 11.71; H, 2.09; N, 2.53.

Measurement of LCST

The LCST of PNIPAAm was determined by measuring the optical transmittance of polymer aqueous solutions. The optical transmittance of polymer solutions (5 mg/mL) was measured at 500 nm at various temperatures using a UV–vis spectrophotometer. The LCST values for each polymer were defined as the temperature where 50% optical transmittance of polymer aqueous solutions was observed.

Temperature-responsive chromatography

A polymer-grafted silica gel support was packed into a stainless steel column (length: 150 mm × 4.6 mm I.D.) on Altech 1000 column packing equipment. The column was connected to an HPLC system (SY-8100 pump; SY-8200 UV-monitor). The column oven was controlled by a HCT-360 HPLC column heater. Milli-Q grade water was used as the mobile phase. The elution behaviors of the samples were recorded at a flow rate of 1 mL/min at various temperatures.

RESULTS AND DISCUSSION

The preparation of PNIPAAm and poly([2-(methacryloyloxy)ethyl]-trimetylammonium chloride)-grafted silica gel was shown in Scheme 1. The ratio of PNIPAAm to poly([2-(methacryloyloxy) ethyl]-trimetylammonium chloride) is about 4.2 : 1.

Characterization of temperature responsive polymers

LCST of PNIPAAm and its derivatives are 32°C in water. The LCST of PNIPAAm was changed in acetonitrile as in Figure 1. The LCST of PNIPAAm was changed from 32 to 4.9°C when the concentration of acetonitrile was from 0 to 20%. When the concentration of acetonitrile was in the range of 20–48%, the PNIPAAm solution is always epinephelos, and no LCST was determined. When the concentration of acetonitrile was in the range of 48–50.5%, the LCST of PNIPAAm was in the range of 18.2–41.2°C. When the concentration of acetonitrile is more than 50.5%, the PNIPAAm solution becomes clarified.

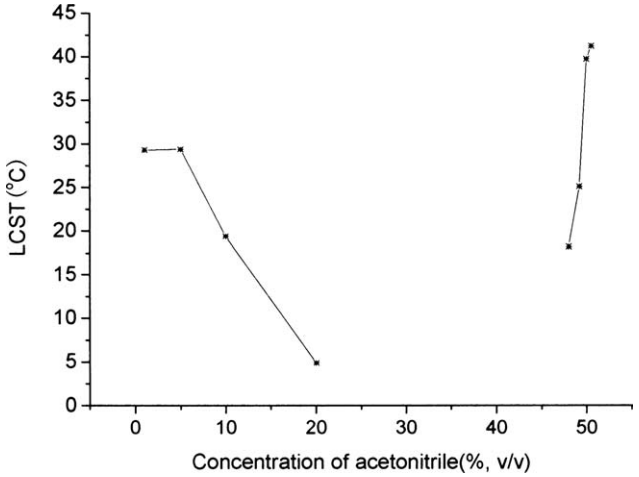


Figure 1 LCST of PIPAAm in acetonitrile aqueous solution.

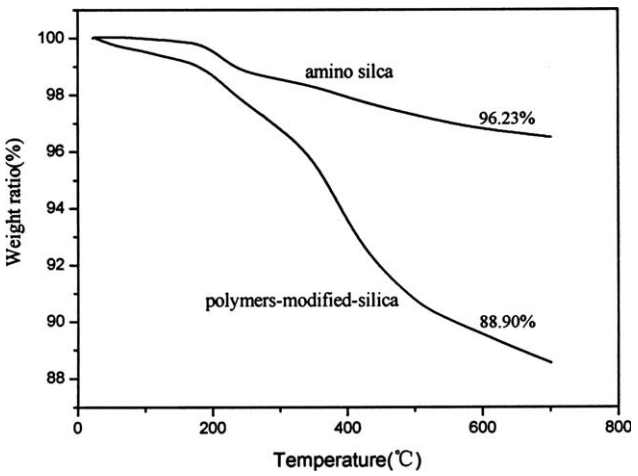


Figure 3 TGA curves of amino silica and polymers-modified silica.

Characterization of polymer-grafted silica gel

The presence of the succinimidyl group at the end of the polymer was confirmed by the appearance of a new peak in the IR spectrum at 1738 cm^{-1}, corresponding to the C=O stretching, and an ultraviolet absorption at 260 nm, corresponding to the succinimidylanion. The FTIR of four polymers are shown in Figure 2. The grafted silica gel of PIPAAm was characterized using elemental analyses and TGA. The results of elemental analyze showed that PIPAAm was successfully grafted on silica gel. In aminopropyl silica, carbon is 2.94%, hydrogen is 0.83%, and nitrogen is 1.06%. In polymer-modified-silica gel, carbon is 11.71%, hydrogen is 2.09%, and nitrogen is 2.53%. The TGA of polymer-modified-silica gel was shown in Figure 3. The weight loss of amino silca gel is 3.77%, and the weight loss of polymer-modified-silica gel is 11.10%. The ratio of PIPAAm grafted on silica gel is 7.33%. Figure 4 shows SEM images of the polymers-grafted silica and unmodified silica. Polymers-grafted silica showed large amounts of polymers were covered on the silica surface compared to unmodified silica.

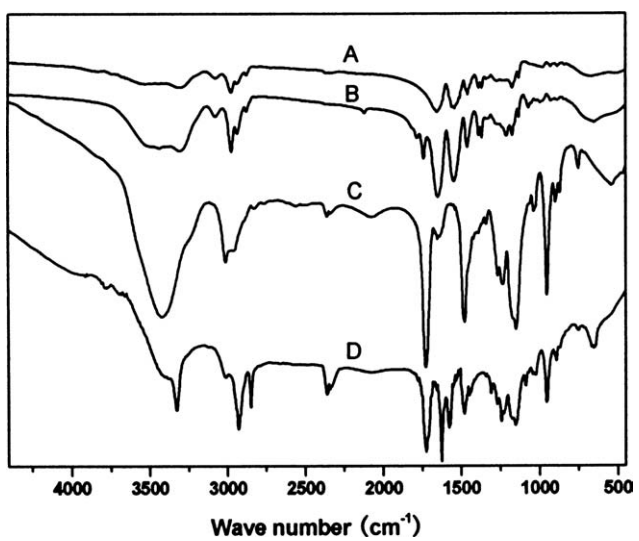


Figure 2 The FTIR of four polymers. (A) PNIPAAm containing carboxyl group. (B) PNIPAAm containing N-succinimidehydroxyl ester. (C) poly[2-(methacryloyloxy)ethyl] trimetylammonium chloride containing carboxyl group. (D) poly([2-(methacryloyloxy)ethyl]ethyl]-trimetylammonium chloride) containing N-succinimidehydroxyl ester.

Separation of lactic acid and creatine phosphate disodium salt

The PIPAAm-grafted silica gel was used as stationary phase to separation lactic acid and creatine phosphate disodium salt. The results showed that both the buffer pH and column temperature affected the separation results, as shown in Figures 5–7. At pH 6.0, the two compounds were separated partly; the resolutions are less than 0.2, as shown in Figure 5. The best resolution was obtained at pH 6.93 and 40°C, as shown in Figure 6. The buffer pH is the key factor on the separation.

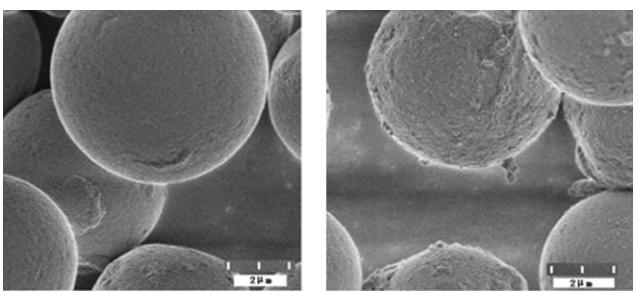


Figure 4 SEM images of amino silica and polymers-modified silica. (A) Amino silica. (B) Polymers-modified silica.

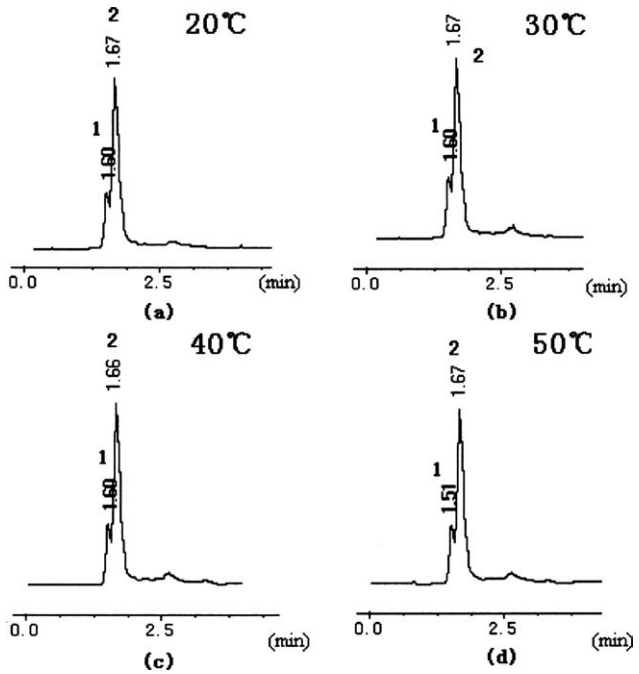


Figure 5 The chromatograms of lactic acid and creatine phosphate disodium salt on PIPAAm stationary phase at pH 6.0. Mobile phase: 26% acetonitrile, 74% ammonium acetate pH 6.0; flow rate, 1.0 mL/min; detection wavelength, 210 nm. (A) Column temperature, 20°C, $R = 0.17$; (b) column temperature, 30°C, $R = 0.17$; (c) column temperature, 40°C, $R = 0.16$; (d) column temperature, 50°C, $R = 0.17$. Peak 1, lactic acid; Peak 2, creatine phosphate disodium salt.

The impact of acetonitrile concentration and column temperature was further investigated to find the best separation results. At pH 6.93, the influences of acetonitrile concentration and column temperature on separation results were shown in Table I

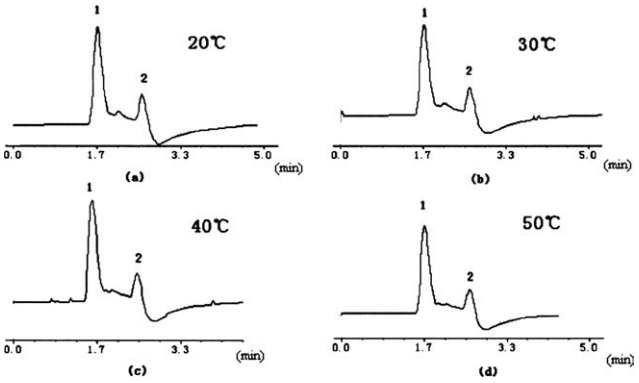


Figure 6 The chromatograms of lactic acid and creatine phosphate disodium salt on PIPAAm stationary phase at pH 6.93. Mobile phase: 26% acetonitrile, 74% ammonium acetate pH 6.93; flow rate, 1.0 mL/min; detection wavelength, 210 nm. (a) Column temperature, 20°C, $R = 1.75$; (b) column temperature, 30°C, $R = 1.96$; (c) column temperature, 40°C, $R = 2.22$; (d) column temperature, 50°C, $R = 2.12$. Peak 1, lactic acid; Peak 2, creatine phosphate disodium salt.

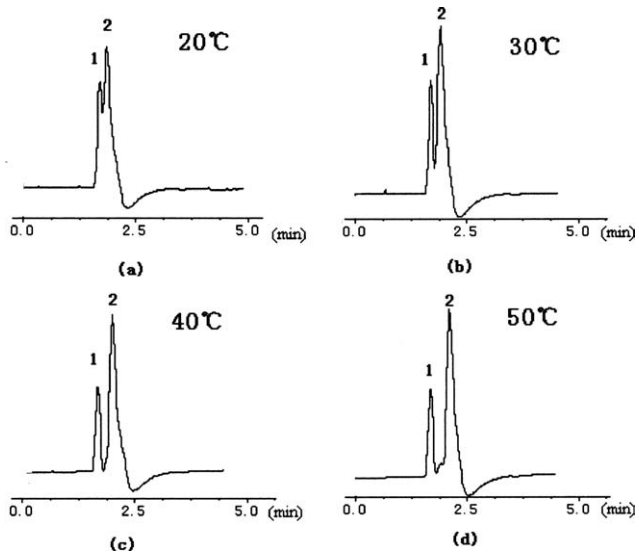


Figure 7 The chromatograms of lactic acid and creatine phosphate disodium salt on PIPAAm stationary phase at pH 8.0. Mobile phase: 26% acetonitrile, 74% ammonium acetate pH 8.0; flow rate, 1.0 mL/min; detection wavelength, 210 nm. (a) Column temperature, 20°C, $R = 0.52$; (b) column temperature, 30°C, $R = 0.74$; (c) column temperature, 40°C, $R = 1.11$; (d) column temperature, 50°C, $R = 1.45$. Peak 1, lactic acid; Peak 2, creatine phosphate disodium salt.

and Figure 5. The concentrations of acetonitrile were 0, 10, 20, 26, 40, and 60%. The column temperatures were 15, 20, 30, 40, and 50°C. From Figure 5, the best resolution temperature can be performed at any acetonitrile concentration. The best temperature and acetonitrile concentration were 15°C and 40%, respectively. Using the same mobile phase, there will be a column temperature to get the best separation. When the acetonitrile concentration is 0%, the two compounds were not separated at any temperature. When the acetonitrile concentration is 10 and 20%, the optimal column temperature is 30°C. When the acetonitrile concentration is 26%, the optimal column temperature is 40°C. When the acetonitrile concentration is 40%, the optimal column temperature is 15°C. When the acetonitrile concentration is 60%, the optimal column temperature is 50°C. The best resolution temperature changed with

TABLE I
The Resolution at Various Acetonitrile Concentration and Column Temperature

Acetonitrile concentration	15°C	20°C	30°C	40°C	50°C
0	0	0	0	0	0
10	0.93	0.98	1.06	0.99	0.86
20	1.21	1.68	1.84	1.76	1.41
26	1.00	1.75	1.96	2.22	2.12
40	2.61	2.28	2.18	2.14	2.40
60	2.34	1.98	2.20	2.12	2.58

acetonitrile concentrations; the effects of acetonitrile concentration on LCST of PNIPAAm grafted silica contributed to this. After acetonitrile was added to the water, LCST of PNIPAAm decreased. From Figure 1, when acetonitrile concentrations are 10 and 20%, LCSTS of PNIPAAm decrease to 19.4°C and 4.9°C, respectively. So under these conditions the best resolution temperature is a little low. When acetonitrile concentration increased to 40%, the best resolution temperature, i.e. 15°C is much lower because LCST of PNIPAAm is much lower. Even though column temperature exceeds it, the resolution did not change better. But when acetonitrile concentration gets 26%, the best resolution temperature is 40°C which is much higher anomalously. Maybe this attributes to another factor effecting on the resolution. Interaction between sample and mobile solvent can be influenced by temperature. In this condition, this factor impacted resolution, so only after temperature is 40°C, the best resolution can be accepted. When acetonitrile concentration is 60%, PNIPAAm loses temperature-responsive property and dissolves in solvents; the best resolution has to get at 50°C.

CONCLUSIONS

We prepared silica stationary-phase surfaces modified with PNIPAAm and poly ([2-(methacryloyloxy) ethyl] trimetylammonium chloride) to separate the mixture of lactic acid and creatine phosphate disodium salt. We investigate the dependence of the resolution on the column temperature, buffer pH, and acetonitrile concentration in the mobile phase. Finally, we found that this stationary phase is very effective to separate the mixture of lactic acid and creatine phosphate disodium salt and we found the optimal separation condition to get the maximal resolution of 2.61.

References

1. Heskins, M.; Guillet, J. E.; James, E. J Macromol Sci Chem A 1968, 2, 1441.
2. Miron, T.; Wilchek, M. Anal Chem 1982, 126, 433.
3. Yamada, N.; Okano, T.; Sakai, H.; Karikusa, F.; Sawasaki, Y.; Sakurai, Y. Makromol Chem Rapid Commun 1990, 11, 571.
4. Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Sakurai, Y.; Okano, T. Macromolecules 1994, 27, 6163.
5. Yakushiji, T.; Sakai, K.; Kikuchi, A.; Aoyagi, T.; Sakurai, Y.; Okano, T. Langmuir 1998, 14, 4657.
6. Hosoya, K.; Kimata, K.; Araki, T.; Tanaka, N. Anal Chem 1995, 67, 1907.
7. Kanazawa, H.; Yamamoto, K.; Matsushima, Y.; Takai, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. Anal Chem 1996, 68, 100.
8. Kanazawa, H.; Kashiwase, Y.; Yamamoto, K.; Matsushima, Y.; Kikuchi, A.; Sakurai, Y.; Okano, T. Anal Chem 1997, 69, 823.
9. Kanazawa, H.; Sunamoto, T.; Matsushima, Y.; Kikuchi, A.; Okano, T. Anal Chem 2000, 72, 5961.
10. Kanazawa, H.; Sunamoto, T.; Ayano, E.; Matsushima, Y.; Kikuchi, A.; Okano, T. Anal Sci 2002, 18, 45.
11. Sakamoto, C.; Okada, Y.; Kanazawa, H.; Ayano, E.; Nishimura, T.; Ando, M.; Kikuchi, A.; Okano, T. J Chromatogr A 2004, 1030, 247.
12. Seino, M.; Yokomachi, K.; Hayakawa, T.; Kikuch, R.; Kakimoto, M.; Horiuchi, S. Polymer 2006, 47, 1946.
13. Kanazawa, H.; Ayano, E.; Sakamoto, C.; Yoda, R.; Kikuchi, A.; Okano, T. J Chromatogr A 2006, 1106, 152.
14. Nagase, K.; Kobayashi, J.; Kikuchi, A.; Akiyama, Y.; Kanazawa, H.; Okano, T. Langmuir 2007, 23, 9409.
15. Lynenl, F.; Heijl, J. M. D.; Du Prez, F. E.; Brown, R.; Szucs, R.; Sandra, P. Chromatographia 2007, 66, 143.
16. Yi, Z.; Caihua, N.; Dan, S.; Xiang, J. Acta Polym Sinica 2007, 8, 765.
17. Nagase, K.; Kobayashi, J.; Kikuchi, A.; Akiyama, Y.; Annaka, M.; Kanazawa, H.; Okano, T. Langmuir, 2007, 23, 9409.
18. Roohi, F.; Antonietti, M.; Titirici, M. M. J Chromatogr A 2008, 1203, 160.
19. Song, Y. X.; Wang, Q.; Su, Z. X.; Chen, D. Y. Chromatographia 2001, 54, 208.
20. Nagase, K.; Kobayashi, J.; Kikuchi, A.; Akiyama, Y.; Kanazawa, H.; Okano, T. Biomacromolecules 2008, 9, 1340.
21. Nagase, K.; Kobayashi, J.; Kikuchi, A.; Akiyama, Y.; Kanazawa, H.; Annaka, M.; Okano, T. Biomacromolecules 2010, 11, 215.
22. Van Der Laarse, A.; Dijkshoorn, N. J.; Hollaar, L.; Caspers, T. Clin Chim Acta 1980, 104, 381.
23. Mora, L.; Sentandreu, M. Á.; Toldrá, F. Meat Sci 2008, 79, 709.
24. Ikeda, S.; Morino, H.; Motonaka, J.; Mishima, Y. Anal Chim Acta 1999, 384, 45.
25. Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. Bioconjugate Chem 1993, 4, 42.
26. Bückmann, A. F.; Morr, M.; Johansson, G. Makromol Chem 1981, 182, 1379.