ELSEVIER

Journal of Chromatography A, 895 (2000) 1–7

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of morphine by capillary electrophoresis immunoassay in thermally reversible hydrogel-modified buffer and laser-induced fluorescence detection

Xin-Xiang Zhang*, Jie Li, Jun Gao, Ling Sun, Wen-Bao Chang

Department of Chemistry, Peking University, Beijing 100871, China

Abstract

In this paper thermally reversible hydrogel used as a replaceable packed material for capillary electrophoresis was examined. A simple and rapid method of detecting morphine was developed, which demonstrated the potential of strong affinity antibodies as a selector for immunologically-based separations in serum by capillary electrophoresis. Polyclonal antibodies were linked to hydrogel and applied to the separation of free fluorescein isothiocyanate (FITC)-labeled antigen and bound FITC antigen. The separation was monitored with laser-induced fluorescence detection. Different separation conditions were studied. The results indicated that poly-*N*-isopropylacrylamide hydrogel (PNIPA) is a kind of steady, replaceable gel. The specific determination of morphine did not require a long incubation time and PNIPA hydrogel-modified antibodies can be stockpiled at 4°C before assay. It can be used to determine morphine with good precision and a detection limit lower than 8.5 ng/ml. Details of the preparation of hydrogel cross-linked polyclonal antibody and of typical separations of bound and free antigen are presented.

Keywords: Capillary electrophoresis immunoassay; Hydrogels; Immunoassays; Morphine

1. Introduction

Screening of urine sample for the presence of drugs in therapeutics and their materials are routinely performed in clinics in different cases for rapidly reversing the function of morphine.

The most common analytical techniques currently used are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) and immunoassay [1,2]. TLC uses low-cost equipment but is labor-intensive, requires high skill and experience and is subject to misinterpretation by the person viewing the color of the sprayed spots. Current commercial immunoassays offer rapid quantitation, high specificity and sensitivity but reagent costs are high because enzyme–antibody (Ab) or enzyme–drug conjugates are employed. The results of HPLC and GC–MS analysis are considered confirmatory but the equipment is expensive and requires great expertise.

Thermal sensitive hydrogel is a kind of polymer that is sensitive to temperature, pH, etc. Its LCST (lower critical solution temperature) can be controlled in synthesis. With its phase-changeable property, hydrogel can be used as capture modifier for collecting samples from a matrix [3]. Different kinds of hydrogels have been applied in bioseparation and drug delivery [4,5]. Makino et al. have investigated the electroosmotic flow (EOF) properties of a hydro-

*Corresponding author. Tel.: +86-10-6275-4174; fax: +86-10-6275-1708.

E-mail address: zxx@chem.pku.edu.cn (X.-X. Zhang).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00590-2

gel surface [6]. It has been also applied as modifier in capillary electrophoresis (CE) [7]. It was also used as an isoelectric focusing matrix [8]. In our previous paper, we have tried to apply it in capillary electrophoresis immunoassay (CEIA) for the analysis of methyltestosterone [9].

The analyte we selected, as an example, is morphine, the major metabolite of heroin and a minor metabolite of codeine that is excreted in the urine either in the free form or conjugated as the glucuronide. Different forms of morphine are analyzed by HPLC–MS or other HPLC-based methods, but, they can be analyzed in total by an immunological reaction. Some reports have dealt with the study of morphine's immunochemical reaction by CE [10,11].

We report here the use of CE with laser-induced fluorescence (LIF) detection as a tool for screening of antibodies cross-linked on hydrogel for binding to a particular antigen (Ag) and for the study of the immunological reaction. We describe the immunochemical analysis of morphine by capillary zone electrophoresis (CZE) and capillary hydrogel electrophoresis. The methods exploit the high affinity and specificity of antibody–antigen binding, the great resolving capability of CE, the sensitivity of LIF and the shift of hydrogel.

2. Experimental

2.1. Apparatus

The experiments were performed using a P/ACE 5000 CE system (Beckman Instruments, Fullerton, CA, USA) equipped with a LIF detector. Fluorescence was excited by argon ion laser at 488 nm. The fluorescence light was detected after passing through a 488-nm cut-off and a 520-nm interference filter.

2.2. Materials and reagents

A fused-silica capillary (75 μm I.D.×375 μm O.D.) was supplied by Beckman Instruments. For the separation studies, a capillary of 57 cm total length and 50 cm to the detection window was employed.

Morphine and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Isopropanol, acrylonitrile, ammonium persulfate (APS), and *N,N,N′N′*-tetramethylethylenediamine (TEMED) were purchased from Beijing Chemicals (Beijing, China). Fluorescein isothiocyanate (FTIC) was supplied by Zhaohui Pharmaceutics (Beijing China). *N*-Isopropylacrylamide was synthesized in our laboratory.

The Tris–borate buffers of different pH and concentration were prepared by mixing 100 mmol/l Tris solution and 100 mmol/l boric acid solution in suitable proportion and diluting to suitable volume. Running buffer containing 100 mmol/l Tris–borate (pH 8.0), 0.5 mol/l sodium carbonate solution (pH 9.4) and other solutions were prepared by dissolving the reagents with pure water supplied by a Milli-Q plus water purification system (Millipore, USA).

The antigen was synthesized by coupling morphine with BSA (M_r=68 000) and the polyclonal antibodies were supplemented by 10% rabbit serum, which was obtained by immunizing rabbit for 4 months (Monoclonal Labs., Department of Biology, Peking University, Beijing, China). The polyclonal antibodies were essentially pure (>95%) as shown by CE. The titer of polyclonal antibody was determined by enzyme-linked immunosorbent assay (ELISA), which is 1:10 000 through the purification steps.

2.3. Preparation of FITC-antigen

A stock solution of 2.6 mg/ml FITC-antigen was made by mixing 0.15 ml of 0.36 mg/ml FITC solution with 1.5 ml of 2.6 mg/ml antigen (dissolved in 0.5 mol/l sodium carbonate buffer at pH 9.5) in a 10-ml beaker [12]. The mixture was vortex-mixed and left in the dark at room temperature for 4–6 h before use.

2.4. Preparation of thermally reversible hydrogel cross-linked antibody [poly(NIPAAm/NAS/Ab)]

The monomer of *N*-isopropylacrylamide (NIPAAm) was synthesized according to the following equation [13]:

$$(CH_3)_2CHOH + CH_2 = CHCN$$
$$\rightarrow (CH_3)_2CHNHCOCH = CH_2$$

A stable opaque gel was formed at 29°C by

mixing 0.4 mg/ml polyclonal antibodies in 0.05 mol/l sodium phosphate, pH 8.0, with 100 mg NIPAAm, 100 μl 0.2% (w/v) *N*-acrylsuccinoamide (NAS) (dissolved in dimethyl sulfoxide, DMSO), 100 μl 1.0% (w/v) APS and 10 μl TEMED were added as free radical initiators.

2.5. Synthesis of different concentrations of PNIPA

The different concentrations PNIPA were formed at 29°C by mixing 50, 100, 150 and 200 mg of NIPAAm in 4 ml, 0.5 mol/l sodium phosphate (pH 8.0). A 100-μl volume of 1.0% (w/v) APS and 10 μl TEMED were added as free radical initiators. The obtained hydrogels were kept at 38°C for 5 min, then centrifuged three times.

2.6. Study of physical qualities of poly(NIPAAm/NAS/Ab)

Since its introduction by Heskin and Guiuet [14] in 1968, thermally reversible hydrogel has been utilized as an important analytical tool in biomedical analysis, drug delivery, biological sensors, biological reactions and separations, etc. It is a kind of special hydrophilic polymer, with infinite swelling in water, which can efficiently absorb water.

The LCST of synthesized poly(NIPAAm/NAS/Ab) was 33.1°C, measured by differential scanning calorimetry (DSC2010, Thermal Analysis, USA). The poly(NIPAAm/NAS/Ab) was centrifuged at 38°C, the supernatant discarded, the deposit dried, then 1 mg of sample was dissolved in 0.5 ml exquisite tetrahydrofuran. The relative molecular mass was determined by gel permeation chromatography (GPC; Waters, USA).

3. Results and discussion

Parameters that affect the overall quality and efficiency of separation were studied, including (i) ionic strength of buffer; (ii) concentration of hydrogel in buffer; (iii) applied voltage; (iv) pH and especially temperature, assuming that other parameters such as column length, are held constant. The optimized separation conditions were used for the study.

3.1. Effect of buffer concentration

The effect of the different buffer ionic strength on resolution was studied by CZE. For a given set of conditions (Fig. 1), the concentrations of buffer have important effects on resolution (R_s) and migration time (t_m). When the buffer concentration increased, R_s and migration time increased for EOF increase. Since the equilibrium constant of Ag–Ab could be affected by ionic strength of buffer to some extent, high salt concentrations must be avoided. And as thermal sensitive hydrogel was added for enhancing resolution, temperature control is very critical. Resolution would be difficult if a high concentration were applied. It is important, therefore for the sake of resolution, time of experiment, and Joule heating, to pay attention to the ionic strength.

3.2. Effect of different pH of buffer

The pH of buffer has an important effect on surface characteristics of the fused-silica capillary and the effective electric charge of the ion. Since the hydrogel polymers swelled in aqueous solution and allowed permeation of different-molecular-mass

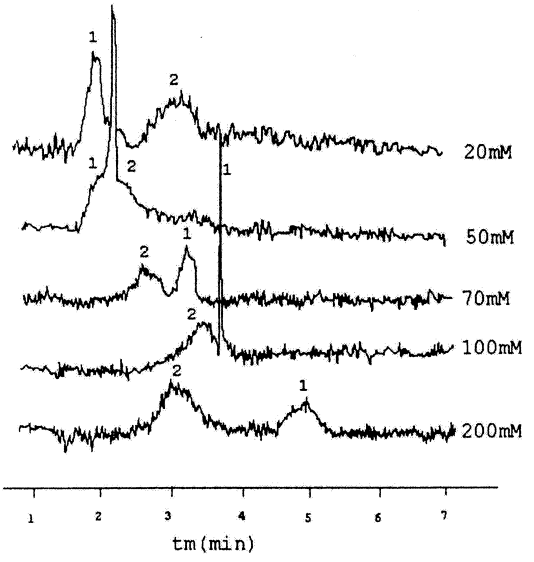

Fig. 1. Effect of different buffer concentrations. 1 = Bound FITC-Ag, 2 = free FITC-Ag. Capillary 50 cm × 75 μm, applied voltage 25 kV, Tris–borate buffers (mixing Tris and boric acid solution), pH 8.0, without hydrogel.

compounds, an equilibrium was attained between the hydrogel polymer layers and the buffer solution, which eliminated the interactions between proteins and the capillary surface and allowed highly efficient and reproducible capillary electrophoretic separations of proteins. But the pH of the buffer has great effect on conformation of antigen and antibody.

The isoelectric point (p*I*) of a solute is critical for the separation. When pH>p*I*, the direction of negative charge of solute is opposite to the EOF, pH<p*I*, which is the same direction as the EOF [15]. Fig. 2A and C shows that the conformation of FITC-Ag and bound FITC-Ag changed greatly and the migration sequences were reversed. According to Terabe et al. [16], the best pH for separation should be lower than the pK_a of the sample. Fig. 2 indicated that the suitable pH value is from 8.0 to 9.0.

3.3. Effect of different hydrogel concentration

Gel concentration and viscosity will affect resolution and analysis time. Fig. 3 indicated that the resolution and migration time increased as the gel concentration increased. The results (Fig. 3A and B) showed that the FITC-Ag and bound FITC-Ag were shifted well by hydrogel uncross-linked Ab and resolution is 2.045 (hydrogel concentration 0.938 mg/ml), but the peak of FITC-Ag was broader. Increasing the concentration of the hydrogel improved the separation. Fig. 3C and D indicated that the binding rate of antibody cross-linked on hydrogel and FITC-antigen also increased without incubation. The results showed that the hydrogel interaction inside the hydrogel which formed a steady hydrogel bond and hydrophobic internal closed space under the high field strength so that there is a good hydrophobic situation for the immunological reaction.

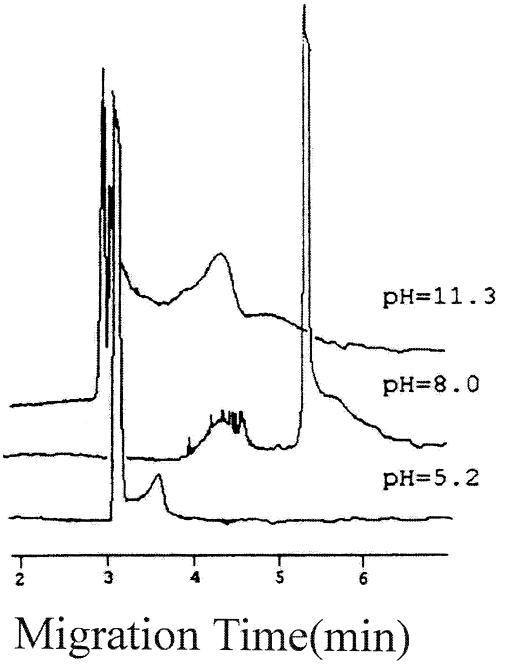

Fig. 2. Separation in different pH. (A) pH 11.3, (B) pH 8.0, (C) pH 5.2. 100 mmol/l Tris–borate buffer containing 0.938 mg/ml hydrogel without cross-linked Ab, other conditions as in Fig. 1.

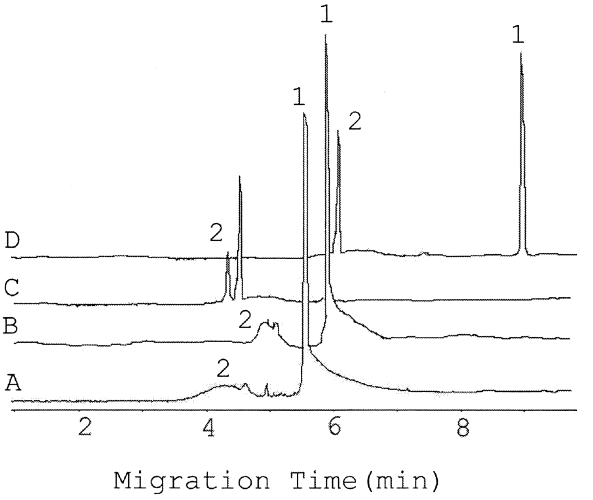

Fig. 3. Effect of different concentrations of hydrogel on separation. (A) 0.313 mg/ml hydrogel without Ab, (B) 0.938 mg/ml hydrogel without Ab, (C) 0.313 mg/ml hydrogel with Ab, (D) 0.938 mg/ml hydrogel with Ab. 100 mmol/l Tris–borate, pH 8.0, other conditions as in Fig. 1.

3.4. Effect of applied voltages

It is known that an increase in applied voltage would lead to an increase in resolution, Joule heating and a decrease in migration time. Since much of the Joule heat in the capillary can be efficiently eliminated by cooling liquid, a relatively high voltage can be applied. A series of electropherograms of bound and free antigen at different field strengths by CZE

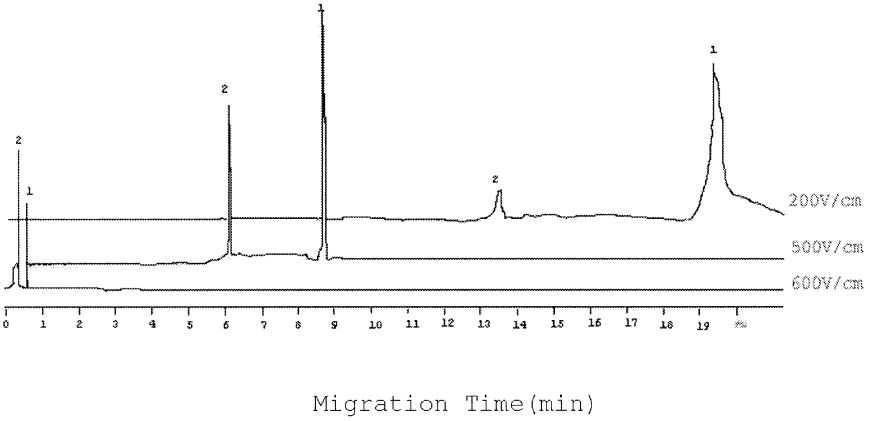

Fig. 4. Migration behaviors in different applied voltages. (A) 200 V/cm, (B) 500 V/cm, (C) 600 V/cm, other conditions as in Fig. 3D.

and by hydrogel matrix cross-linked antibodies are shown in Fig. 4. When the applied voltages were increased from 200 V/cm to 600 V/cm, two phenomena took place: (i) reduction in the time of analysis of free antigen from 19.73 min to 0.50 min, and (ii) improvement of the resolution. With the increase of applied voltage, the migration time of Ab and the Ab-complex in hydrogel containing buffer decreased more rapidly than without hydrogel. This was because the phase change of hydrogel at higher temperature caused by higher applied voltage in microscale and the electroosmotic properties of them have been changed. R_s was a function of the applied voltages and electrophoretic efficient mobility as the length of capillary, temperature were constant. The 600 V/cm applied voltage led to a good result in separating bound and free antigen by hydrogel cross-linked antibodies. The separation was completed in 30 s and the resolution was 3.2. The results indicated that if the concentration of hydrogel was not changed, the higher field strength would lead to good resolution.

3.5. Effect of temperature

The effect of temperature on hydrogel is very important. The temperature range of 25–44°C was studied (Fig. 5) when the concentration of hydrogel was 0.938 mg/ml. At 35°C the bound and free antigen was separated within 4 min with R_s 31.13. And at 37°C they were separated within 5.8 min with R_s 146.84. The result is opposite to the expectation from CZE, where the migration time decreased when temperature increased. The current decreased from 25.43 to 1.7 μA at 43°C. This phenomenon would appear at 41°C when the concentration of hydrogel increased to 1.25 mg/ml. This would partly be because phase transition of hydrogel took place in the capillary and viscosity of the hydrogel increased sharply when the temperature is high. With increasing concentration of hydrogel, the observed phase transition temperature would be decreased.

3.6. Optimization of immunological reactions

Optimization of immunological reactions were performed. In this experiment, a buffer containing 10 μg/ml antibodies (0.938 mg/ml hydrogel) was packed into a capillary and different concentrations of FITC-antigen reacted with Ab, respectively. The results indicated that the best binding rate reached

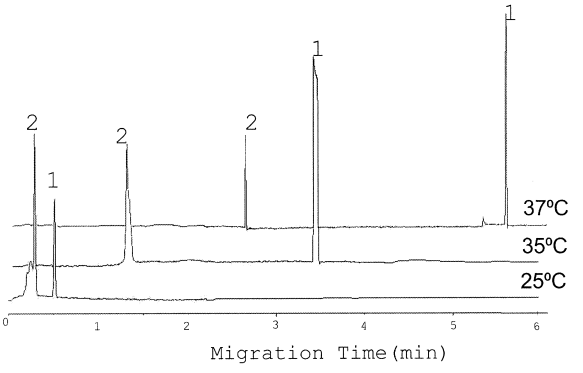

Fig. 5. Effect of capillary temperature on separation. Experimental condition as in Fig. 3D except temperature.

80% with hydrogel at 25°C. The electropherograms have well-shaped peaks with no evidence of dissociation of the complex during separations [17]. For this method, the best concentrations of bound FITC-antigen and free FITC-antigen were determined in solution with 10 μg/ml Ab and 0.536 n*M* FITC-antigen, Fig. 1 showed that the bound FITC-antigen and free FITC-antigen could not be separated completely in a free solution electrophoresis. The observed poor electrophoretic discrimination can be attributed to the following [18]. First, the binding of FITC-antigen to its antibody does not significantly alter the electrophoretic mobility of complex antibody. Second, differences in the amino acid sequence and post-translational modification make both antigen and antibody non-uniform substances. These heterogeneities revealed by the electrophoretic separation lead to very broad and poorly separated peaks. The hydrogel was mixed into buffer, little by little, with a concentration from 0.313 to 1.880 mg/ml and the bound and free antigen were separated when the concentration of the hydrogel is higher than 0.313 mg/ml (Table 1). Obviously, adding hydrogel improved the separation of the immunogenic complex in this mode. The hydrogel is so large a carrier of antibodies that mobility of bound antibody might be adjusted as needed without affecting the mobility of the free form of the labeled antigen. And it governed the mobility of the binding complex but did not influence the mobility of the free labeled antigen, a large mobility shift between the free and bound of FITC-antigen would produce. The general concept was developed by Fuchs et al. [19] in an interesting variant of the well-established sandwich format [20,21]. The peak attributed to free FITC-antigen which can be representative of the actual free FITC-antigen in solution decreased and disappeared when the concentration of gel became higher.

3.7. Immunological reaction

CE–LIF using a replaceable hydrogel has been demonstrated to be a useful technique for performing the separation step required in morphine of immunoassay in our experiments. The type of immunoassay is a competitive assay in which a labeled antigen (FITC-morphine–BSA) and antibody are added to a sample. Analyte, in this case unlabeled antigen (morphine–BSA) with a series of different concentration and the labeled antigen competes to form complexes with the antibody. A CE separation of the bound and free FITC-antigen allows determination of the bound and/or free concentration, which in turn is related to the amount of unlabeled antigen in the samples. After the hydrogel crosslinked antibodies were pressured into capillary, the FITC-antigen mixed with samples was injected for 5 s. The ratios of bound-free FITC-antigen changed according to different morphine concentrations in the sample and the change in area ratios of two peaks was consisted with morphine concentrations. The calibration curve for morphine can be acquired by plotting the relative area over the maximum area ratio (the area ratio of bound to free tracer at zero concentration of morphine) against the morphine concentrations. The relative area ratio was linearly related with log concentration ($r = 0.9523$) within a range of 8.5 to 171.6 ng/ml.

3.8. Analytical application

Since a morphine-positive sample is difficult to obtain, the simulated samples were analyzed by this method. Two samples, one with a low concentration (50 ng/ml) and another with a high concentration (150 ng/ml) of morphine were diluted by a healthy serum from volunteers without taking morphine.

Table 1
Separation of Ag*–Ab and Ag* in different concentrations of hydrogel

Hydrogel concentration (mg/ml)	Migration time (min)		Current (μA)	Resolution
	Ag*–Ab	Ag*		
0.313	4.35	5.55	13.43	2.41
0.625	4.50	5.75	13.38	2.08
0.938	4.50	6.00	12.93	2.04
1.250	4.58	6.54	12.23	2.44

Table 2
Recovery of morphine samples

Sample content (nmol/l)	Detection (nmol/l, $n=5$)	RSD (%, $n=5$)	Recovery (%, $n=5$)
50.0	41.0	3.52	82.0
150.0	130.2	4.01	86.8

Five repetitive experiments were made under the same conditions. Analytical results were listed in Table 2. Results indicated that this method could be applied satisfactorily as a reliable detected means for morphine in serum.

4. Conclusion

These results have demonstrated that a capillary packed with replaceable *N*-isopropylacrylamide hydrogel by use of immobilized crosslinked polyclonal antibodies can be used to rapidly and efficiently separate bound and free antigen. This method can be employed to independently detect morphine in serum at any time as an assistant to GC–MS, HPLC and TLC. Although the method presented in this paper lacks the sophisticated diagnostic capabilities, the technique is more compatible when measurements in high specificity are encountered. In addition, in the future the replaceable hydrogel cross-linking other specific reagent can be used as a convenient and rapid tool in clinic analysis and we believe that temperature reversible polymers represent a new and promising class of electrophoretic media.

Acknowledgements

The authors thank the National Natural Science Foundation of China (29305035, 29775002) and SRFROCS from State Ministry of Education of China for financial support.

References

[1] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop (Eds.), Clarke's Isolation and Identification of Drugs, 2nd ed., Pharmaceutical Press, London, 1986, p. 524.
[2] C.D. Klaassen, M.O. Amdur, J. Doull (Eds.), Casarett and Doull's Toxicology, The Basic Science of Poisons, 3rd ed., MacMillan, New York, 1986, p. 863.
[3] J. Gao, Y.Z. Li, Y.X. Ci, W.B. Chang, Anal. Lett. 32 (1999) 1787.
[4] I.Y. Galaev, B. Mattiasson, Trends Biotechnol. 17 (1999) 335.
[5] T. Miyata, N. Asami, Uragami T, Nature 399 (1999) 766.
[6] K. Makino, K. Suzuki, Y. Sakurai, T. Okano, H. Ohshima, Colliods Surfaces A 103 (1995) 221.
[7] M.X. Huang, M.L. Lee, J. Microcol. Sep. 4 (1992) 491.
[8] T.E. Zewert, M.G. Harrington, Electrophoresis 20 (1999) 1339.
[9] X.X. Zhang, J. Li, J. Gao, L. Sun, W.B. Chang, Electrophoresis 20 (1999) 1998.
[10] R.A. Evangelista, F.-T.A. Chen, J. Chromatogr. A. 680 (1994) 587.
[11] F.-T.A. Chen, R.A. Evangelista, Clin. Chem. 40 (1994) 1819.
[12] N. Monji, A.S. Hoffman, Appl. Biochem. Biotechnol 14 (1987) 107.
[13] M. Kimura, T. Nishigaki, T. Koyama, K. Hanabusa, H. Shirai, React. Polym. 23 (1994) 195.
[14] M. Heskin, J.E. Guiuet, J. Control. Release A2 (1968) 1441.
[15] Y.-Z. Deng, J.-L. He, High-Performance Capillary Electrophoresis, Science Press, Beijing, 1998.
[16] S. Terabe, T. Yashima, N. Tanaka, M. Araki, Anal. Chem. 60 (1988) 1673.
[17] L. Tao, R.T. Kennedy, Electrophoresis 18 (1997) 112.
[18] D. Schmalzing, W. Nashabeh, Electrophoresis 18 (1997) 2184.
[19] M. Fuchs, W. Nashabeh, D. Schmalzing, US Pat. 5 630 924 (1997).
[20] S. Hashida, K. Tanaka, T. Kohno, Anal. Lett. 21 (1998) 1141.
[21] S.D. Lidofsky, W.D. Hinsberg III, R.N. Zare, Proc. Natl. Acad. Sci. USA 78 (1981) 1901.