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Short communication

Single-based resolution for oligodeoxynucleotides and their phosphorothioate modifications by replaceable capillary gel electrophoresis

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Abstract

A replaceable capillary gel electrophoretic (replaceable CGE) method was developed for the separation of two sets of model compounds of single-stranded oligodeoxynucleotide mixtures (18–20 mers), phosphodiester oligodeoxynucleotides (PO-ODNs) and their phosphorothioate modifications (PS-ODNs), with equal sequences differing in a single base. Polyethylene glycol (PEG) 35000 was chosen as the sieving matrix. It was confirmed that PEG polymer solution less influenced resolutions of the PS-ODNs compared with those of the PO-ODNs, while acetonitrile used as an additive in the system improved the separation significantly. It was also noticed that the effect of temperature on separation was much larger than that of denaturant urea.

Keywords: Single-based resolution; Phosphodiester oligodeoxynucleotide; Phosphorothioate modified oligodeoxynucleotide; Replaceable capillary gel electrophoresis

1. Introduction

In recent years, the production of synthetic oligodeoxynucleotides (ODNs) has increased dramatically to meet the demands in the biotechnological markets, including such diverse fields as microbiology, forensics, and medicine. Usually, short segments (generally 12–25 nucleotide units, referred as "*n* mers" in length) of single-stranded and chemically modified ODNs, which are complementary to specific nosogenetic genes are known as antisense oligodeoxynucleotides (ASODNs). ASODNs have recently attracted more attentions as potential drugs for the treatment of cancer and several viral infections [1,2]. In order to gain resistance to exonucleases, chemical modifications of ASODNs, such as the phosphorothioate modification [3] (replacing a non-bridged oxygen of the phosphodiester group by a sulfur atom), have been widely used. The resulting family of ODNs after being synthesized, shortened by one to several nucleotides, may still possess biological activity in vivo [4,5]. A defect in length or sequence is not tolerated especially when they are used as drugs. Hence, a critical question in the evaluation of the synthesized ODNs is their purity check and single-based resolutions (separation of ODNs differing by one nucleotide in length) become necessary.

Since ODN molecules have constant charge/size ratios and their electrophoretic mobilities are almost equal regardless of their chain length, a size-dependent separation of ODN molecules using a properly formulated matrix as a size-sieving matrix is promising. In the past few years, replaceable high molecular polymers solutions filled in capillary electrophoresis, so called replaceable capillary gel electrophoresis (replaceable CGE), have been widely used for the separation of peptides, proteins, and synthetic ODNs [6–8]. The main advantages of replaceable polymers matrices filled in capillaries over the rigid gel are high sieving capacity, low viscosity, chemical resistance, and easy preparation and operation.

For phosphorothioate modified oligodeoxynucleotide (PS-ODN) samples, in addition to the secondary structure, peak

Abbreviations: BGE, background electrolyte; CGE, capillary gel electrophoresis; PAA, polyacrylamide; PEG, polyethylene glycol; PO-ODN, phosphodiester oligodeoxynucleotide; PS-ODN, phosphorothioate modified oligodeoxynucleotide

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broadening due to the phosphorothioate moiety often made replaceable CGE fail to give a content resolution and reproducibility compared with phosphodiester oligodeoxynucleotides (PO-ODNs). Polyethylene glycol (PEG) was often chosen as the sieving matrix [9–13] for its easy and nontoxic preparation procedure. But till now the mechanism of this separation system has not been systematically studied for PO-ODNs and PS-ODNs, especially for their single-based separations.

In this paper, PEG 35000 was used as the sieving matrix to resolve a series of synthetic model single-stranded PO-ODNs and their corresponding phosphorothioate modifications based on a sequence of 20 mer antisense phosphorothioate oligodeoxynucleotide that inhibits telomerase catalytic subunit hTERT [14]. The effects of the concentrations of PEG 35000 and background electrolyte (BGE), column temperature, additive (acetonitrile, ACN), and denaturant (urea) on the migration characteristics of the two series ODN samples were investigated. The goal of this work is to systematically explore parameters influencing single-based separation of PO-ODNs and PS-ODNs.

2. Experimental

2.1. Chemicals and samples

Poly(ethylene glycol), molecular weight 35,000 (PEG 35000) and bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane (Bis–Tris) were purchased from Fluka (Buchs, Switzerland). *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED, 99%) was obtained from Alfa Aesar (Ward Hill, MA, USA) and acrylamide (electrophoresis grade, 99%) was from Acros Organics (Greel, Begium). 3-(Methacryloyloxy) propyltrlmethoxysilane (γ-MAPS, 97%) was obtained from Lancaster Synthesis (Windham, NH, USA). Boric acid and ammonium persulfate (APS, >99%) were all purchased from Amresco (Solon, OH, USA). ACN (HPLC-gradient grade) was from Merck (Darmstadt, Germany). Other reagents were all of analytical grade. Water used for all experiments was de-ionized and double-distilled in-home.

For all measurements the phosphodiester and phosphorothioate modified oligonucleotides (18–20 mers) desalted after purified by denaturing polyacrylamide gel electrophoresis, PAGE-gradient grade, >95%) were synthesized by TaKaRa Biotechnology (Dalian, China) and were used without further purification (Table 1). All samples were dissolved in water, and the concentration of each ODN sample is about 73 μg/ml.

2.2. Instrumentation

CE separations were performed on a HP^{3D} CE capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) using Agilent ChemStation software. Other apparatus were recycling water vacuum pump (SH2-DIII, Gongyi, Henan, China), vortexer (XK96-A, Jiangyan, Jiangsu, China) and ultrasonic bath (SK250LH, Shanghai, China), respectively. Nitrogen (≥99.9%) was used as external pressure gas.

2.3. Replaceable capillary gel electrophoresis

Bare, fused-silica capillaries (Yongnian Optical Fiber Factory, Hebei, China) of dimensions 100 μm i.d. × 375 μm o.d. were permanently coated to prepare with polyacrylamide (PAA) according to the method described by the previous reference using γ-methacryloxypropyltrimethoxysilane and 4% linear polyacrylamide [15]. Unless specified, experiments were performed using a capillary with a total length of 44 cm (35.5 cm effective length). BGEs of all aqueous solutions with different additives were prepared by dissolution of Bis–Tris, boric acid and ACN to the appropriate concentrations. Prior to use, all BGEs were degassed for 10 min in an ultrasonic bath and filtered through a 0.45 μm filter. Replaceable gel was prepared by dissolution of PEG 35000 in the above corresponding BGE solutions to the appropriate concentrations followed by stirred and vacuumized before using. At the start of each analysis day, the PAA-coated capillary was preconditioned by rinsing with the BGE for 5 min. Gel was replaced automatically by external nitrogen connected to the CE apparatus before each run at the pressure of 7–9 bar. Samples were introduced by electrokinetic mode at 10 kV for 5 s. All separations were performed at 25 kV across the capillary using a reversed polarity mode (from negative to positive) and the current through the capillary did not exceed 20 μA. Direct UV detection was at 260 nm with reference wavelength at 350 nm. Exceptions to these settings are stated in Section 3.

3. Results and discussion

As mentioned before, the bandwidth of the PS-ODN was broader than that of unmodified PO-ODN. Therefore, modification and optimization of analytical methods are especially important when the polymer solution was used for the separation of PS-ODNs. Studies in the literature using PEG solution as the sieving matrix [9–13] have revealed that several param-

Table 1
Oligodeoxynucleotides used in this study

No.	Base length (mers)	Base composition	Theoretical mass	Grade	Comments
O-1	18	5′-TCA CTC AGG CCT CAG ACT-3′	5419.610	PAGE	PO-ODN
O-2	19	5′-C TCA CTC AGG CCT CAG ACT-3′	5708.796	PAGE	PO-ODN
O-3	20	5′-AC TCA CTC AGG CCT CAG ACT-3′	6022.008	PAGE	PO-ODN
S-1	18	(S-Oligo) 5′-TCA CTC AGG CCT CAG ACT-3′	5708.772	PAGE	PS-ODN
S-2	19	(S-Oligo) 5′-C TCA CTC AGG CCT CAG ACT-3′	6014.023	PAGE	PS-ODN
S-3	20	(S-Oligo) 5′-AC TCA CTC AGG CCT CAG ACT-3′	6343.300	PAGE	PS-ODN

eters, such as the choice of polymer molecule weight, BGE, and disruption of the secondary structure of ODNs, are critical for the separation of ODNs. In this work, each parameter was systematically varied to find out the optimal condition for the single-based separation of model PO-ODN and PS-ODN samples. Other parameters such as applied voltage and capillary length were used without further optimization.

3.1. Concentration of PEG 35000

Polymers play important roles in replaceable CGE and their concentrations can be adjusted to perform particular separations. Smaller ODN fragments can move more rapidly than the larger ones through this network. However, the solution viscosity increases as the polymer concentration increases. Ideally, one would like to keep a low viscosity of the polymer solution when going to a smaller mesh size. PEG 35000 was shown to be a good sieving matrix used for single base separation of 20–60 mers PO-ODNs with UV detector [12] and for separation of 12–20 mers PS-ODNs coupling with MS detector [13].

In this work, a series of model PO-ODN and PS-ODN samples were separated using different concentrations of PEG 35000 (data were not shown) and the effects of polymer concentrations on separation were also investigated (Fig. 1). For comparison under the same condition, ACN of 30% (v/v) was used as additive in PEG polymer and BGE solution to improve separation, especially for PS-ODNs, for the reasons discussed later in Section 3.3. While the migration time of each ODN sample increased with polymer concentration in the buffer solution, the elution order from small fragments to large for both PO-ODNs and PS-ODNs did not change. Resolutions of PO-ODN samples with different lengths were further improved as the concentration of PEG increased in the concentration range from 10 to 25% (w/v).

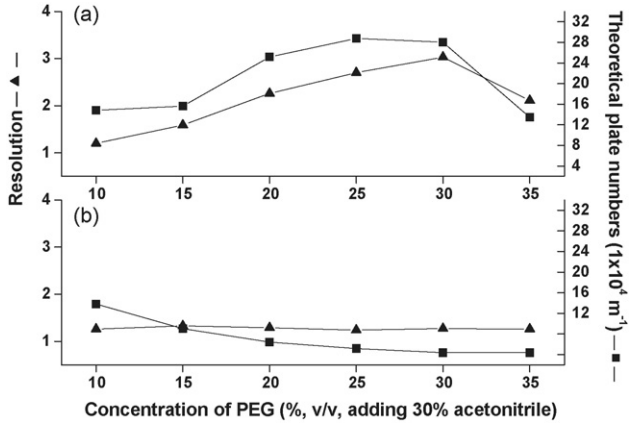

Fig. 1. Effect of polyethylene glycol 35000 (%, w/v, 30%, v/v, acetonitrile as additivity) concentration on separation of (a) phosphodiester oligodeoxynucleotides and (b) their phosphorothioate modified oligodeoxynucleotides (18–20 mers, as listed in Table 1), expressed as means of resolutions (left, ▲) and theoretical plate numbers (right, ■) of the three ODNs. Polyacrylamide (PAA)-coated capillary: 44 cm (35.5 cm effective length), 100 μm i.d. × 375 μm o.d.; electrokinetic injection 10 kV for 5 s; running power: 25 kV, reversed polarity mode (from negative to positive); column temperature: 60 °C; UV detection: 260 nm (reference wavelength 350 nm); BGE solution: 30 mM Bis–Tris, 30 mM boric acid, and 30% (v/v) acetonitrile.

But at the PEG concentration of 30% the efficiencies began to decrease while the resolutions still kept increasing and reached their maximum values under the experimental conditions in this study. Further increasing the polymer concentration resulted in peak broadening and as a result, the resolution dropped quickly. The optimal separation of PO-ODN sample was obtained at the PEG concentration of 25% (Fig. 1a). As for the PS-ODNs, the effects of PEG concentrations on the resolution were not considerable compared with those of the PO-ODN samples. It can be easily seen from Fig. 1b that better separations were performed using the polymer concentration of 15%. Since modified and unmodified ODN molecules have the same base sequence, the cause of band broadening of PS-ODNs in general cannot be solely ascribed to the formation of intramolecular or intermolecular hydrogen bonding. The sulfur substitutions can greatly change the polarity and hydrophobicity of the molecule and as a result, affect the electrophoretic separation. The differences in PEG concentration for the separation of PO-ODN and PS-ODN samples also indicated that for the PS-ODN samples which were usually hard to be fully separated, it is not recommended to vary PEG polymer concentration for improving single-based resolution. Furthermore, the higher the PEG concentration, the longer the analysis time and the more difficult the operation will be due to the increased viscosity.

The parameters to evaluate sieving power in replaceable CGE are usually resolving power R_s, Length-Of-Read (LOR) and Ferguson plot analysis. If the separation mechanism is size-based, Ferguson plots of log (mobility) versus gel composition will yield a straight line (different slope for each sample component) with a common y-intercept, equal to the free-solution mobility [16]. The apparent mobility of each solute in each gel can be calculated by acquiring their migration times, and over the tested range a linear relationship between migration time of ODNs and the PEG concentration was evident in the Ferguson plot. In this work, at the lower PEG concentrations (<30%) the curvatures both for PO-ODNs and PS-ODNs (data were not shown) were close to straight lines, however, at higher concentrations (30–35%) the plots had convex shape. The quite shallow slopes of these plots suggest no efficient sieving characteristics associated with this PEG polymer solution when high PEG concentration was used [17].

3.2. Concentration of BGE solution

Generally speaking, the separation can be greatly improved by increasing the ionic strength of BGE solution in capillary electrophoresis. Although the ionic strength of the sample solution did not appear to affect the sample loading by pressure injection method, sample stacking can be achieved using electrokinetic injection mode, originally proposed by Chien and Burgi [18]. Due to the nature of the stacking process, the method itself is particularly suitable to analyze negatively charged species and, as a consequence, to analyze ODNs [18,19]. The signal response obtained from a BGE solution with the higher ionic strength was greater than that obtained from a lower one at the same concentration of ODN samples. Moreover, the corresponding efficiency of the peak at the higher concentration of BGE, e.g. the last

peak, is $1.6 \times 10^7\ m^{-1}$, which was much higher than that of the lower concentration of BGE, $6.3 \times 10^5\ m^{-1}$. The resolving power did not decrease with higher sample loading because BGE solution with higher concentration and the concentrated narrow sample band compensate for it. Therefore, using electrokinetic injection from low-ionic strength sample solution, sensitivity of ODN analysis can be improved by increasing the ionic strength of BGE solution as well as by changing other accustomed conditions, such as electric power and injection time.

3.3. Additive of ACN

Under the described system the separation was improved by using ACN as an additive in the PEG polymer and BGE solution both for PO-ODN and PS-ODN samples. It is important to note that, without addition of ACN, 18–20 mers PS-ODN samples cannot be fully resolved. A series of experiments (Fig. 2) were carried out to investigate the effect of ACN volume on the migration characters for PO-ODNs as well as for PS-ODNs. Resolutions, especially for PS-ODN samples, were found to change considerably with changes of ACN volume. Additionally, ACN has an obvious effect on the retention time of PO-ODNs. While increasing the ACN volume from 10 to 40% (v/v) slowed down the migration of the three tested PO-ODNs (Fig. 2a), it did not influence the migration of PS-ODN samples (Fig. 2b). Since the two ODN samples have the same base sequence and the electronegativity of phosphodister moiety is much larger than that of phosphorothioate moiety, ACN as the dipolar aprotic solvent may have stronger reaction with phosphodister moiety, and as a result, an decreasing of PO-ODN migration. When ACN volume reached at 50% (v/v), neither for PO-ODNs nor for PS-ODNs had the peak found in the electropherograms. It is estimated that ODN samples are not soluble in the high concentration of ACN due to their large negative phosphate backbone and high polarity.

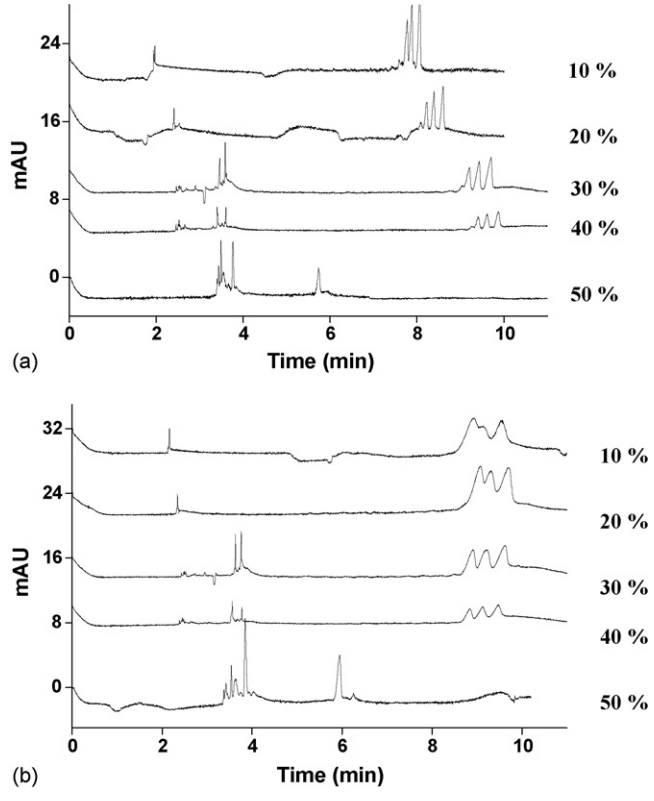

Fig. 2. Effect of acetonitrile concentration (%, v/v) on separation of (a) phosphodiester oligodeoxynucleotides and (b) their phosphorothioate modified oligodeoxynucleotides (18–20 mers, as listed in Table 1). The concentration of PEG was 20% (w/v). PAA-coated capillary was 34 cm (25.5 cm effective length). The acetonitrile composition was as indicated and other conditions were as in Fig. 1.

3.4. Urea

The secondary structure of a single-stranded ODN can be specifically defined as hairpin loops or generally defined as any occurrence of hydrogen bonding between nucleotide pair on the strand, such as A-T and G-C pair. Consequently, the buffer system added in high concentrations of denaturant, such as urea, for the separation of ODNs is often preferred to be capable of suppressing the formation of secondary structure. In this work the sieving systems with and without high concentration of urea (7 M) were compared. However, urea, which is known to be a common denaturant for CGE analysis, did not appear to improve separation of PO-ODN (Fig. 3a) and PS-ODN (Fig. 3b) samples since their sequences have no significant secondary structure. Thus, we conclude that denaturant, urea, is not necessary used in the proposed condition for such ODN analysis.

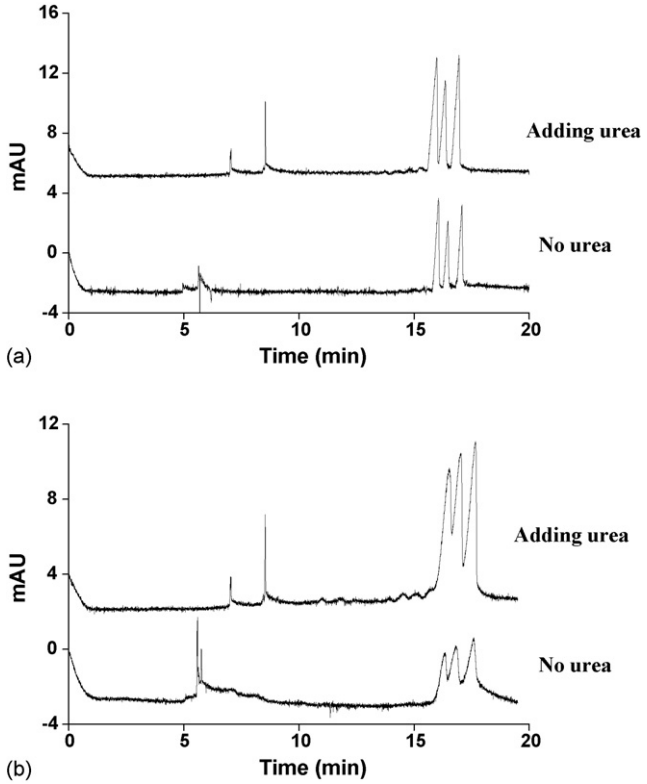

Fig. 3. Effect of 7 M urea as denaturing reagent on separation of (a) phosphodiester oligodeoxynucleotides and (b) their phosphorothioate modified oligodeoxynucleotides (18–20 mers, as listed in Table 1). The concentration of PEG was 20% (w/v) and the acetonitrile composition was 30% (v/v). Adding or no urea was as indicated and other conditions were as in Fig. 1.

3.5. Temperature

In general, an increase of column temperature not only accelerated separation, but also reduced secondary structure and improved resolution [20]. In the present work, the increase of temperature lowered the PEG viscosity and accelerated the ODNs migration in the column resulting in reduced migration time and leading to narrower bands. Either for PO-ODN samples or for PS-ODN samples, the migration orders of the three components did not change under different temperatures, while better resolving powers and efficiencies could be obtained at higher temperature. So the highest temperature of 60 °C was used.

3.6. Endurance of the polyacrylamide-coated capillary

In replaceable CGE, inner-wall coated capillaries are usually used for eliminating electroosmotic flow and avoiding sample adsorption. Such capillaries were prepared by the chemical attachment of linear polyacrylamide to the capillary inner wall described in Ref. [15]. The endurance of the home-made polyacrylamide coated capillary was examined through carefully designed experiments. It was found that the electropherogram showed some degraded performance, i.e. the loss of theoretical plates and increased peak asymmetry after 60 cycle times of injections. It is estimated that high temperature (60 °C) and strong alkalescence (pH 8.5) used in this system, which were usually not recommended, decreased the useful life of this kind of coated capillaries.

4. Conclusion

In this work the parameters that affect the single-based separation of PO-ODN and PS-ODN model samples from their deletion fragments in PEG 35000 replaceable CGE were investigated. Because of the low viscosity of PEG solution, the separation matrix can be easily replaced between runs. Peak broadening due to the phosphorothioate modification often made replaceable CGE fail to give an adequate single-based resolution and reproducibility. It is important that the concentrations of PEG had scarcely influence on the resolving PS-ODNs compared with those on the PO-ODNs, while ACN used as additive in the system improved the separation significantly. The main influence of the concentration of BGE solution was found to accomplish a signal enhancement and efficiency improvement as a result of the sample stacking technique since electrokinetic injection mode was used. Under the experimental conditions in this work, the effect of temperature was much more prominent than that of high concentration of denaturant (urea).

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References

[1] M. Qian, S.H. Chen, E.V. Hofe, J.M. Gallo, J. Pharmacol. Exp. Ther. 282 (1997) 663.
[2] C.F. Bennett, Biochem. Pharmacol. 55 (1998) 9.
[3] C.A. Stein, Y.C. Cheng, Science 261 (1993) 1004.
[4] B.P. Monia, E.A. Lesnik, C. Gonzalez, W.F. Lima, D. McGee, C.J. Guinosso, A.M. Kawasaki, P.D. Cook, S.M. Freier, J. Biol. Chem. 268 (1993) 14514.
[5] S. Akhtar, S. Agrawal, Trends Pharmacol. Sci. 18 (1997) 12.
[6] M.N. Albarghouthi, A.E. Barron, Electrophoresis 21 (2000) 4096.
[7] A. Sartori, V. Barbier, J.L. Viovy, Electrophoresis 24 (2003) 421.
[8] A. Willse, T.M. Straub, S.C. Wunschel, J.A. Small, D.R. Call, D.S. Daly, D.P. Chandler, Nucleic Acids Res. 32 (2004) 1848.
[9] S. Auriola, I. Jääskeläinen, M. Regina, A. Urtti, Anal. Chem. 68 (1996) 3907.
[10] S.H. Chen, R.T. Tzeng, Electrophoresis 20 (1999) 547.
[11] R.T. Pon, S.Y. Yu, Nucleic Acids Res. 33 (2005) 61940.
[12] http://www.agilent.com/chem/supplies, Publication No. 5988-4303EN.
[13] A.V. Brocke, T. Freudemann, E. Bayer, J. Chromatogr. A 991 (2003) 129.
[14] R.X. Lin, C.W. Tuo, Q.J. Lu, W. Zhang, S.Q. Wang, Acta Pharmacol. Sin. 26 (2005) 762.
[15] S. Hjerten, J. Chromatogr. 347 (1985) 191.
[16] C.C. Wang, S.C. Beale, J. Chromatogr. A 756 (1996) 245.
[17] A. Guttman, T. Lengyel, M. Szoke, M. Sasvari-Szekely, J. Chromatogr. A 871 (2000) 289.
[18] R.L. Chien, D.S. Burgi, Anal. Chem. 64 (1992) 1046.
[19] D.M. Osbourn, D.J. Weiss, C.E. Lunte, Electrophoresis 21 (2000) 2768.
[20] J. Zhang, Y. Fang, J.Y. Hou, H.J. Ren, R. Jiang, P. Roos, N.J. Dovichi, Anal. Chem. 67 (1995) 4589.