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Characterization of the interaction between phospholipid and protein by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

We report an electrophoretic mobility shift-based method to study the interactions between phospholipids and proteins by capillary electrophoresis with laser-induced fluorescence detection. A fluorogenic dye, 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), was used to label phosphatidylserine (PS). Then the FQ labeled PS (FQ-PS) was used as the fluorescent probe for monitoring the association between PS and bovine serum albumin (BSA). Two conjugates were observed to form between each PS species and BSA, indicating that two interactions exist between these PS species and BSA. We can also detect the competitive association with BSA between labeled PS and unlabeled PS. This method only needs a minute volume of sample. It is highly sensitive and can be used to detect the interaction between phospholipids and nanomolar concentrations of proteins, such as BSA. © 2001 Published by Elsevier Science B.V.

Keywords: Electrophoretic mobility shift assay; Phospholipids; Lipids; Proteins; Phosphatidylserine; Furoylquinoline carboxaldehyde; Albumin

1. Introduction

Biomolecular interactions are of fundamental importance for life. Many metabolic and signaling pathways are controlled by interaction between DNA and protein [1], protein and protein [2], protein and carbohydrate [3], and lipid and protein [4]. Consequently, identifying and characterizing these interactions are very important for our understanding of biological systems.

Analytical methods play a central role in studying these biospecific interactions. An extremely popular technique is spectroscopy, which includes fluorescence spectroscopy, electron spin resonance (ESR), Raman spectroscopy, nuclear magnetic resonance (NMR), infrared spectroscopy, surface plasmon resonance, X-ray spectroscopy, etc. Separation methods are also very important for studying biomolecular interactions. For instance, affinity chromatography is often used to identify the interaction mechanism and to determine the binding constant [5]. Electrophoretic mobility shift assay (EMSA) is widely used to study protein–protein and DNA–protein interactions [6], especially for detection of the sequence-specific interactions between proteins and DNA. Recently, capillary electrophoresis has also been widely used for studying biospecific interactions between proteins and ligands, proteins and drugs, and proteins and carbohydrates [7–10].

Lipid–protein interactions are central to many basic cellular processes. Characterization of these interactions is very crucial for our understanding of

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membrane structure and function, membrane biogenesis, ion transport, cell recognition, lipid metabolism, and lipid transport. For studying lipid–protein association, the widely used methods include ESR, NMR, and fluorescence spectroscopy such as fluorescence polarization, fluorescence quenching, and fluorescence recovery after photobleaching [11]. In this work, we introduce a CE–laser-induced fluorescence (LIF) method for the detection of protein–lipid interaction. CE is a microscale separation technique that requires only a minute volume of biological samples, which is particularly useful when the biological sample is very expensive or not easily obtained. In addition, CE provides high-resolution separation for biomolecules. Combining the high resolution of CE with the high sensitivity of the sheath flow LIF detector developed in this lab, we can detect multiple interactions between phospholipid and protein BSA at a nanomolar level. As far as we know, this is the first CE–LIF method developed for studying protein–lipid interaction.

2. Experimental

2.1. Apparatus

The CE instrument with a sheath flow LIF detector was built in the laboratory and has been described previously [12]. Briefly, high voltage was provided by a 0–30 kV d.c. power supply (CZE 1000, Spellman, Plainview, NY, USA). The excitation source for the sheath flow LIF detector was provided by an argon ion laser (Model 2211-15SL, Uniphase, San Jose, CA, USA) operated at 12 mW. The 488-nm laser line was focused at ~30 μm from the tip of the capillary using a 6.3× objective (Melles Griot, Nepean, Canada). Fluorescence was filtered with a 630DF30 bandpass filter (Omega Optical, Brattleboro, VT, USA), collected with a 60×, 0.7 NA microscope objective (MO 0060LWD, Universe Kokagu, Oyster Bay, NY, USA) and then detected with a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ, USA). Data sampling was accomplished by a 16-bit data acquisition board (NB-MIO16XH-18, National Instruments, Austin, TX, USA) connected to a Macintosh computer.

2.2. Materials and reagents

Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 150 μm o.d. and 20 μm I.D. were used in this work. Before use, the capillary was flushed sequentially with NaOH, water, and running buffer using a laboratory-made pressure device. Typical CE running buffer was 10 m*M* NaH_2PO_4–10 m*M* Na_2HPO_4 (pH 7.4).

Bovine serum albumin (BSA, M_r 66 000) was purchased from Sigma (St Louis, MO, USA). Phospholipids were purchased from Avanti-Polaris (Alabaster, AL, USA). 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) and KCN were obtained from Molecular Probes (Eugene, OR, USA). Stock solution of 25 m*M* KCN was prepared with water. Stock solution of 100 m*M* FQ was prepared with methanol. FQ solution (10 μl) was then placed into a 500-μl microcentrifuge tube and the solvent was vacuumed with a Speed Vac (Savant, Farmingdale, NY, USA). The dried FQ aliquots (100 nmol) were stored at −20°C. These precautions were used to prevent FQ degradation in solutions.

2.3. Procedures

To label the lipids, 8-μl lipid stock solution and 2-μl 25 m*M* KCN were mixed in a vial containing 100 nmol of previously dried FQ. The mixture was incubated for 10 min at 65°C and then diluted with 40 μl running buffer.

For monitoring the interaction between BSA and lipids, 5 μl of FQ-labeled lipid solution was mixed with 5 μl BSA and incubated typically at room temperature for 2.5 min. After incubation, the mixture was injected on to the capillary column at 100 V/cm for 5 s and separated at a high voltage of 400 V/cm. For control experiments, 8-μl running buffer and 2-μl 25 m*M* KCN were mixed in a vial containing 100 nmol of previously dried FQ. The mixture was also incubated for 10 min at 65°C and then diluted with 40 μl running buffer. Then 5 μl of this diluted solution was mixed with 5 μl BSA and incubated at room temperature for 2.5 min. After incubation, the mixture was injected on to the capillary column at 100 V/cm for 5 s and separated at 400 V/cm.

To detect the competition, labeled lipid and un-

labeled lipid were mixed with BSA solution at the same time and incubated at room temperature for 2.5 min. The solution was then injected on to the capillary at 100 V/cm for 5 s and separated at 400 V/cm.

3. Results and discussion

3.1. Labeling phospholipid using FQ

Phospholipids exist in abundance in the cell membranes of animals and plants. It is known that chemical and physical properties of cell membranes are largely dependent on the phospholipid composition. Most phospholipids are esters of glycerol comprising two fatty acyl residues (nonpolar tails) and a single phosphate ester substituent (polar head group). They do not contain fluorophores, so they are often labeled with fluorescent reagents, which are then employed as probes for studying biological membranes or as tracers for studying lipid metabolism and transport. Commercial fluorescent phospholipid probes include 4, 4-difluoro-4-boro-3a,4a-diaza-s-indacene (BODIPY), 4-chloro-7-nitrobenzofurazan (NBD-F), and pyrene derivatives [13]. However, pyrene lipid probes need to be excited at 340 nm by UV laser, while the excitation wavelength for NBD lipid probe does not match with the argon ion laser very well and its fluorescent quantum yield is relatively low.

In this work, we use FQ for labeling phospholipids. We have developed highly sensitive CE–LIF methods based on FQ labeling to analyze the proteins [14,15] and phospholipid classes including phosphatidylethanolamine (PE), phosphatidylserine (PS), lysophosphatidylethanolamine (LysoPE), and lysophosphatidylserine (LysoPS) [16]. FQ is a fluorogenic dye, so high concentration of FQ can be used to label phospholipid and will not cause high background. This ensures the highest derivatization efficiency for labeling reaction, resulting in high detection sensitivity for phospholipids. Moreover, FQ derivatives of phospholipids are very stable. Even in solution, FQ-phospholipid derivatives can be used for over 1 month. In addition, the excitation wavelength for FQ derivatives is compatible with the argon ion laser, which is a very popular and economical excitation source for LIF detection.

We studied various forms of phosphatidylserine that differ in the length of the nonpolar tails. These compounds include the *n*-hexanoic ester ($C_{6:0}$), the *n*-octanoic ester ($C_{8:0}$), the *n*-decanoic ester ($C_{10:0}$), and the *n*-dodecanoic ester ($C_{12:0}$).

3.2. Interaction between phospholipid and BSA

BSA was chosen in this work because serum albumin is a transport protein and often used as a model protein for many physicochemical studies. Fatty acid transport is an important function of albumin [4]. In this work, $C_{6:0}$ PS was first labeled with FQ and used as a fluorescent probe to identify the interaction between PS and BSA. The results for monitoring the conjugate formation between FQ-PS ($C_{6:0}$) and BSA are shown in Fig. 1. Fig. 1A shows the electropherogram obtained for FQ-PS ($C_{6:0}$) without incubation with BSA. Peak 1 corresponds to FQ-PS. Fig. 1B shows the electropherogram obtained for the mixture of FQ-PS and BSA after incubation

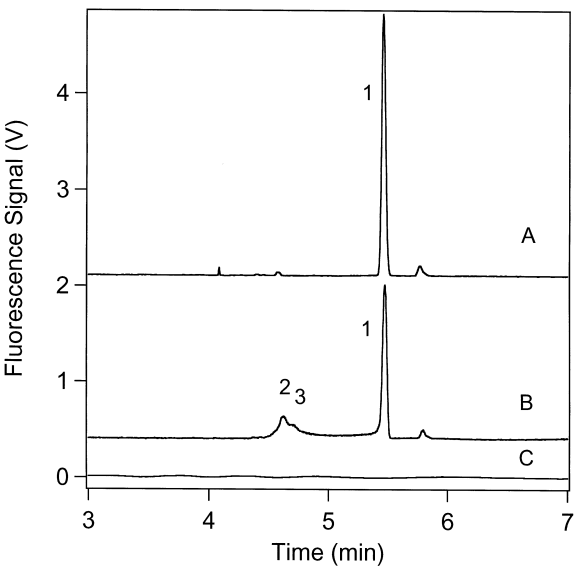

Fig. 1. Electropherograms showing the conjugate formation between FQ-PS ($C_{6:0}$) and BSA. Conditions: separation, 400 V/cm; injection, 100 V/cm for 5 s; Capillary: 20 μm×30 cm; FQ-PS: 18.4 μ*M*; BSA: 200 n*M*. Peaks 1: FQ-PS, 2 and 3: FQ-PS conjugates with BSA. (A) FQ-PS; (B) Conjugates of FQ-PS with BSA.

for 2.5 min, whereas Fig. 1C shows the electropherogram obtained for the control experiment (see Section 2.3). Comparing Fig. 1A with Fig. 1B, it can be seen that the peak height of FQ-PS decreases after incubation with BSA, indicating that some FQ-PS has been consumed. Meanwhile, two new CE peaks (peaks 2 and 3) are generated, demonstrating that conjugates form between FQ-PS and BSA. Although these two peaks were not completely resolved, it is still clear that two different conjugates formed. This result means that two types of interactions may exist between FQ-PS ($C_{6:0}$) and BSA.

Similar results were obtained for other species of PS. Fig. 2 presents the electropherograms of the conjugate formed between $C_{8:0}$ PS and BSA (Fig. 2A), $C_{10:0}$ PS and BSA (Fig. 2B), and $C_{12:0}$ PS and BSA (Fig. 2C). Two new peaks were observed for each PS species after incubation with BSA, indicating that two different interactions exist between each PS species and BSA.

Our results are consistent with previous studies on the interaction of lipid and serum albumin. Many studies have shown that serum albumin has different binding sites for lipids and the specificity of these binding sites is also different [4]. These binding sites are correlated with different subdomains of serum albumin. For example, subdomains 1-C, 2-C and 3-C appear to be the binding sites for long chain fatty acids ($C>12$), while subdomains 2-AB and 3-AB are probably the binding sites for short chain fatty acids ($C\leq 12$) [4]. Our results not only demonstrate that BSA provides two main different binding sites for short chain phospholipid PS, but also identify that one binding sites of BSA always has higher affinity for phospholipid PS species than the other. This can be clearly concluded from Figs. 1 and 2, which show that first conjugate always has higher fluorescence intensity than the second one for each PS species.

The effect of incubation time on the interaction between FQ-PS ($C_{6:0}$) and BSA was also investigated. From our experiments, the incubation time does not have much influence on the conjugate formation. Almost identical electropherograms were obtained when BSA and FQ-PS ($C_{6:0}$) were incubated at different times of 15 s, 30 s, 1 min, 2.5 min, 5 min and 10 min. This indicates that the association of FQ-PS and BSA reaches equilibrium rapidly.

Fig. 3 presents the electropherograms for monitoring the conjugate formation between the same concentration of FQ-PS ($C_{6:0}$) and different concentrations of BSA. As the concentration of BSA increases, the peak heights for the new conjugates (shown as peak 1) increase. In contrast, the peak height of FQ-PS (shown as peak 2) decreases, which means more FQ-PS has combined with BSA. In addition, the resolution of the conjugates degrades when high BSA concentrations were incubated with FQ-PS.

Fig. 4 shows the electropherograms for detection of the interaction when BSA is at nanomolar concentrations. The conjugates (shown as an asterisk in the figure) were also observed even when BSA

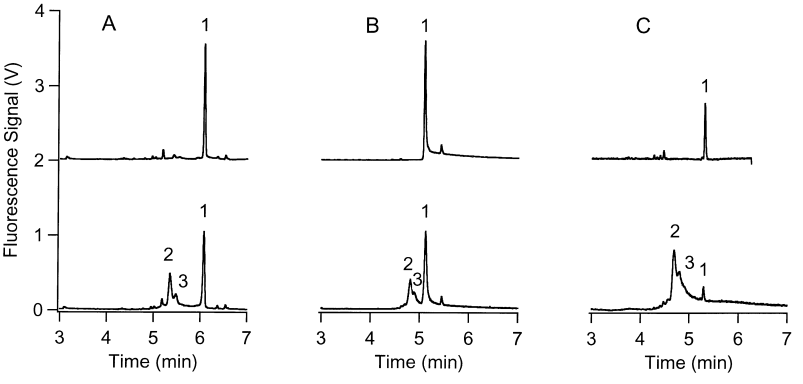

Fig. 2. Electropherograms showing the conjugate formation between FQ labeled PS species and BSA. The top traces are the blank electropherograms generated by injection of the FQ-labeled phosphatidylserine. The bottom traces are the electropherograms obtained by incubating BSA with: (A) $C_{8:0}$ PS: 17.3 μM; (B) $C_{10:0}$ PS: 20 μM; (C) $C_{12:0}$ PS: 12.5 μM. Peak identification and other conditions are as in Fig. 1.

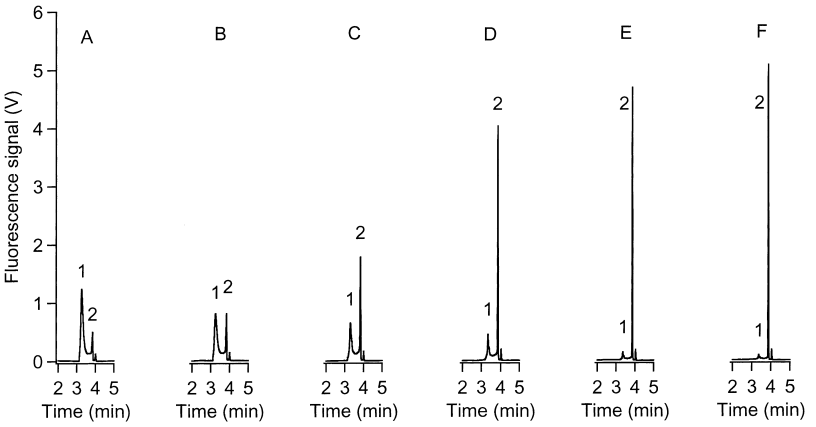

Fig. 3. Comparison of the interaction between different concentrations of BSA and the same concentration of FQ-PS ($C_{6:0}$). Peaks 1: BSA-FQ-PS conjugate, 2: FQ-PS. Capillary: 20 μm×25 cm; FQ-PS: 28.0 μ*M*; BSA concentration: (A) 1.2 μ*M*, (B) 800 n*M*, (C) 400 n*M*, (D) 200 n*M*, (E) 100 n*M*, (F) 40 n*M*. Other conditions as in Fig. 1.

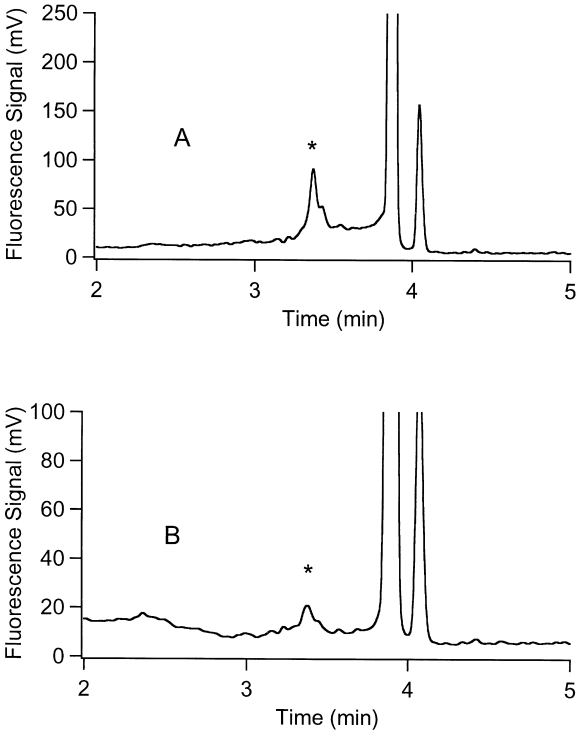

Fig. 4. Electropherograms showing the interaction between FQ-PS ($C_{6:0}$) and nanomolar protein BSA. The conjugate is denoted with an asterisk. BSA concentration: (A) 100 n*M*, (B) 40 n*M*. Other conditions as in Fig. 3.

concentration was as low as 40 n*M* (Fig. 4B). The corresponding injection amount of the conjugate of FQ-PS and BSA is estimated to be a few attomoles or lower, demonstrating that this method is highly sensitive for the detection of biomolecular interactions.

3.3. Competition between labeled phospholipid and unlabeled phospholipid

If FQ-labeled PS ($C_{6:0}$) and unlabeled PS ($C_{6:0}$) were incubated with BSA at the same time, then competitive binding occurs. As presented in Fig. 5, if we increase the concentration of unlabeled PS ($C_{6:0}$) while keeping the same concentrations of BSA and FQ-PS ($C_{6:0}$), the peak area for the conjugates of FQ-PS with BSA (shown as peak 1) was observed to decrease, but the peak area of FQ-PS (shown as peak 2) was observed to increase. This means that some unlabeled PS has replaced FQ-PS to combine with BSA. Similar competitive association with BSA between FQ labeled $C_{8:0}$ PS and unlabeled $C_{8:0}$ PS was also observed. As shown in Fig. 6, the peak area for the conjugates of FQ-PS with BSA (shown as peaks 1 and 2) decreases while the peak area of FQ-PS (shown as peak 3) increases if we increase the concentration of unlabeled PS ($C_{8:0}$) while keeping the same concentrations of BSA and FQ-PS ($C_{8:0}$). This approach may be used in the determination of binding constants for lipids with proteins.

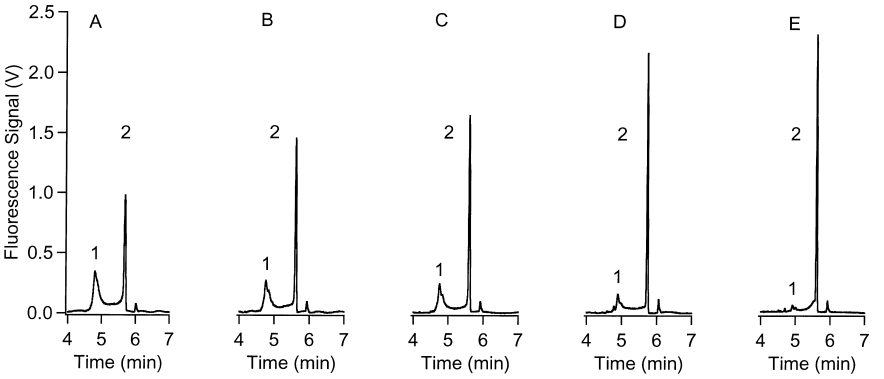

Fig. 5. Electropherograms showing the competitive association with BSA between labeled PS ($C_{6:0}$) and unlabeled PS ($C_{6:0}$). Peaks 1: BSA-FQ-PS conjugate, 2: FQ-PS. FQ-PS: 18.4 μ*M*; BSA: 400 nM; unlabeled PS concentration, (A) 0 μ*M*, (B) 100 μ*M*, (C) 200 μ*M*, (D) 300 μ*M*, (E) 400 μ*M*. Other conditions as in Fig. 1.

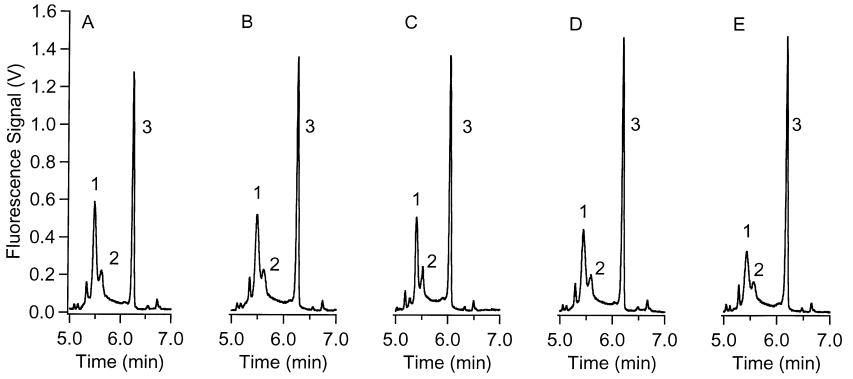

Fig. 6. Electropherograms showing the competitive association with BSA between labeled $C_{8:0}$ PS and unlabeled $C_{8:0}$ PS. Peaks 1 and 2 are the BSA-FQ-PS conjugate. FQ-PS: 17.3 μ*M*; BSA: 250 nM; unlabeled PS: (A) 0 μ*M*, (B) 21.6 μ*M*, (C) 43.2 μ*M*, (D) 108 μ*M*, (E) 216 μ*M*. Other conditions as in Fig. 1.

However, these data are generated by electrokinetic injection, and our estimate of the relative amounts of bound and free FQ-PS must be corrected for the biased injection of the two.

3.4. Conclusion

By employing a model system of phospholipid PS and BSA, we have demonstrated that CE–LIF can be used for study of the interactions between phospholipid and protein. High detection sensitivity, ultrasmall sample volume, and high resolving power are demonstrated by this method. Future work will focus on application of this method to studying the interactions of lipids with lipoproteins or lipid transfer proteins.

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References

[1] W.A. Lim, R.T. Sauer, A.D. Lander, Methods Enzymol. 208 (1991) 196.

[2] A.R. Mendelsohn, R. Brent, Science 284 (1999) 1948.
[3] I.J. Goldstein, Carbohydrate–Protein Interaction (ACS Symposium Series, American Chemical Society, Washington, DC, 1979.
[4] P.C. Jost, O.H. Griffith, Lipid–Protein Interactions, Wiley, New York, 1982.
[5] T. Kline, in: Handbook of Affinity Chromatography, Marcel Dekker, New York, 1993, p. 219.
[6] J.R. Cann, Anal. Biochem. 237 (1996) 1.
[7] J. Gao, M. Mammen, G.M. Whitesides, Science 272 (1996) 535.
[8] G. Rippel, H. Corstjens, H.A.H. Billiet, J. Frank, Electrophoresis 18 (1997) 2175.
[9] M.H.A. Busch, L.B. Carels, H.F.M. Boelens, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[10] F.A. Gomez, L.Z. Avilla, Y. Chu, G.M. Whitesides, Anal. Chem. 66 (1994) 1785.
[11] P.F. Devaux, M. Seigneuret, Biochim. Biophys. Acta 822 (1985) 63.
[12] D.M. Pinto, E.A. Arriaga, D.B. Craig, J. Angelova, N. Sharma, H. Ahmadzadeh, N.J. Dovichi, Anal. Chem. 69 (1997) 3015.
[13] R.P. Haugland, Handbook of Fluorescent Probes and Research Chemicals, 6th ed., Molecular Probes Inc., Eugene Or, 1995, pp. 287–293.
[14] I.H. Lee, D.M. Pinto, E.A. Arriage, Z. Zhang, N.J. Dovichi, Anal. Chem. 70 (1998) 4546.
[15] Z. Zhang, S. Krylov, E.A. Arriaga, R. Polakowski, N.J. Dovichi, Anal. Chem. 72 (2000) 318.
[16] L. Zhang, S. Krylov, S. Hu, N.J. Dovichi, J. Chromatogr. A 895 (2000) 129.