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Short communication

Characterization of proteinase activation dynamics by capillary electrophoresis conjugating with fluorescent protein-based probe

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Abstract

In this paper, a novel strategy was reported to characterize dynamics of proteinase activation based on capillary electrophoresis (CE), using caspase-2 as the model system. A fusion protein conjugating an amino acid sequence VDVAD with two fluorescent proteins enhanced cyan fluorescence protein (ECFP) and red fluorescence protein (DsRed), ECFP–VDVAD–DsRed, was specially designed and expressed in HeLa cells as the substrate of proteinase caspase-2. In this substrate, the sequence VDVAD could be specifically recognized and cleaved by caspase-2 as soon as its activation was initiated with treatment of a certain dose of cisplatin to HeLa cells, which led to a break of the substrate into two fragments. Analyses of the cell lysates using CE in a time course of the apoptosis illustrated the dynamics of caspase-2 activation. It showed that the employment of fusion fluorescent protein greatly facilitated both CE separation and identification of the analytes. This result from cell colony by CE was compared with that from single cell achieved by optical imaging.

Keywords: Capillary electrophoresis; Fluorescent protein; Protein activation; Dynamics; Caspase-2

1. Introduction

With completion of human genome project (HGP), to reveal gene function becomes one of central tasks in biological science, which leads to currently fast-growing functional genomics at transcriptomic, proteomic and metabolomic levels. Protein is the product of gene expression. Analyzing protein behavior in regulation network will be of great help to understand gene function. Proteinase is a kind of special enzymes that cleave a large number of different substrates at specific sites of amino acids sequences, for example, caspase, known as a family of 14 death-specific enzymes that are fundamentally vital and play central roles in cell apoptosis or programmed cell death. When apoptosis happens, the so-called "initiator caspases" (e.g., procaspase-8, -9 and -10) are activated at first [1]. The downstream caspases called "effector caspases" (e.g., caspase-3, -6 and -7) are subsequently activated and responsible for cleaving specific cellular proteins in dying cells [2]. Presently, The functions of most of these caspases have been confirmed in apoptotic regulation networks. But for caspase-2, it is not the case. Although caspase-2 is surely involved in apoptotic initiation [3], its activation mechanism is still unclear. Some recent proofs are even contradictory [4–6], which makes it very difficult to confirm its role in signal transduction.

Capillary electrophoresis (CE) is a microscale separation technique of high performance. Owing to its high speed, low sample consumption and automation, CE has been recognized as a mature tool for analyzing bio-molecules of a wide range from macromolecular DNA, protein and polysaccharide [7] to low-mass-molecular metabolite [8]. As a result, CE shows high potential for emerging systems biology that is currently a hot topic and becomes a new challenge of analytical sciences [9]. Recently, using CE to monitor protein activity has been highly focused [10–16], which paves a new way to under-

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stand the underlying molecular events at protein level. Meredith et al. [11] successfully developed a CE based methodology to assay the activation of individual kinases within Xenopus eggs. Recently, Zarrine-Afsar and Krylov [14] demonstrated a unique CE method to monitor the activation of protein kinase A (PKA) in C2C12 cells. To enhance the separation of phosphorylated products from substrate by CE, a novel multiphosphorylatable substrate with a GFP (green fluorescent protein) tag for PKA was generated.

In this communication, we presented a CE-based strategy to characterize the dynamics of proteinase activation using caspase-2 in cell apoptosis as a model system, conjugating with an artificially designed enzymatic substrate. In this unique substrate, a sequence of amino acids VDVAD was sandwiched by two fluorescent proteins ECFP (enhance cyan fluorescent protein) and DsRed (enhanced red fluorescent protein) [17]. While using cisplatin to treat HeLa cells that were transfected with expression construct of this designed substrate, the activated caspase-2 could specifically recognize the VDVAD site and subsequently cleave the fluorescent substrate into two fragments. Analyzing the cleaved and uncleaved substrate in the cell lysates with CE in a time course revealed the activation dynamics of caspase-2 in cell apoptosis. It showed that the employment of fusion fluorescent protein greatly facilitated both CE separation and identification of the analytes. Further, the result from cell colony by CE *in vitro* was validated by optical imaging *in vivo* of single cell.

2. Experimental

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) was obtained from Sigma–Aldrich (MO, USA). Fetal bovine serum was brought from Sijiqing Bioengineering Materials Inc. (Hangzhou, China). Trypsin and Triton X-100 were purchased from Life Inc. (Wuhan, China). All chemicals were of analytical grade. De-ionized and autoclaved water was used for the preparation of all solutions. Electrolyte (25 mM phosphate buffer at pH 8.0) for CE was prepared daily, and then filtered through a 0.22 μm membrane prior to use.

2.2. Construction of ECFP–VDVAD–DsRed probe

Recombinant substrate, ECFP–VDVAD–DsRed, was constructed as reported previously [17] by fusing two fluorescent proteins (ECFP and DsRed) with a linker containing the caspase-2 cleavage sequence (VDVAD). Briefly, to construct CFP–VDVAD–DsRed termed CD2, ECFP–EYFP concatemer from YC2.1 (a gift from A. Miyawaki), was subcloned into PMD18-T (Clontech.) by Hind III and EcoR I. The HindIII/SacI fragment from the PMD18 was replaced with the HindIII/SacI-digested PCR product. With this protocol, the VDVAD sequence was introduced into the N-terminal of ECFP. Then DsRed was amplified from pDsRed2 (Clontech.). The amplified fragment was further digested and ligated into ECFP–VDVAD–EYFP/PMD18-T using SacI and EcoRI, leading to a replacement of YFP with DsRed.

2.3. Cell culture and sample preparation

The CD2-expressing HeLa cells were grown to about 80% confluence in DMEM, supplemented with 10% fetal calf serum, 100 IU/ml penicillin-G and 50 mg/ml streptomycin solutions, in T-25 cm^2 culture flasks at 37 °C in 5% CO_2/95% air. Then the cells were incubated with serum starved DMEM and cisplatin (1 μg/ml) for a given time. After inducement, the cells were washed for three times with cooled PBS and lysed with 0.4 ml 0.5% triton X-100 for 1.5 min. The lysate was then collected and centrifuged at 10,000 rpm for 10 min, and supernatant was collected as cytosolic extract. No purification procedure was further performed.

To obtain DsRed standard sample, DsRed-transfected *E. coli* was cultured for overnight in bacterial growth medium. Selection for positive transformants was carried out in the presence of 50 μg/ml kanamycin (Sigma–Aldrich). Then the bacteria was collected and suspended in lysing buffer, and then subjected to sonication for 5 min. During the sonication, bacteria were kept on ice. After centrifugation at 10,000 rpm for 10 min, supernatant was collected as DsRed standard sample.

2.4. Instruments and procedures

Analyses were performed on a home-made CE system as reported previously [18], consisting of an inverted fluorescence microscope (Olympus IX70, Japan), a cooled charge-coupled device (CCD, Micromax 5 MHz, Roper Scientific) camera as the detector, a high-voltage power supply (Shanghai Nuclear Research Institute, China) and an uncoated fused-silica capillary of 80 cm (60 cm in length to detection window) × 50 μm i.d. and 375 μm o.d. (Yongnian Optic Fiber Inc., China). A 100 mW high-pressure mercury lamp was used as the excitation radiation. Optical imaging was performed on the same fluorescence microscope as described above for CE with identical optical configuration.

The optical pathway in the microscope included an objective (LCAch 20×/N.A. 0.40) and three different excitation cubes, called ECFP, DsRed and FRET channel, respectively. ECFP channel included an excitation filter (425–445 nm HQ), a dichroic mirror (DM450) and a barrier filter (460–510 nm HQ). In the same way, the parameters of other cubes were as follows: DsRed (BP510–550, DM570, BA590), FRET (436/20X, 455DCLP, 620/60 M). The main working preferences of the CCD camera are pixels (582), strips (782), and exposure (280 ms). Data were collected by a computer with Winview32 and Origin7.5 software packages.

A new capillary was pre-treated with 0.1 M HCl, water, 0.1 M NaOH and water for 15 min respectively. Every two injections for electrophoresis, the capillary was washed with 0.1 M NaOH for 10 min, then preconditioned with running buffer for 10 min at room temperature to ensure reproducibility. Samples were hydrodynamically injected into capillary at 10 cm height for 10 s.

Fig. 1. Schematic description of designed fluorescent protein probe CD2. A seventeen-amino-acid peptide (MHDQLTEEVDVADGSEL) was sandwiched by ECFP and DsRed, in which caspase-2 recognition site sequence (VDVAD) was included. Caspse-2 would cleave this artificial substrate at the site as the arrow pointed out.

3. Results and discussion

The purpose of this communication was to establish a CE-based strategy for charactering activation dynamics of proteinase, using caspase-2 in cell apoptosis as the model system. To achieve such a task we designed an artificial substrate as a probe termed CD2 (shown in Fig. 1), in which a specific enzymatic cleavage site VDVAD was sandwiched by two fluorescent proteins ECFP and DsRed [19]. While transfecting this expression construct into HeLa cells, activated caspase-2 would cleave the probe as pointed out by the arrow in Fig. 1. Hence, analyzing the residues of the cleavage reaction (e.g., DsRed or ECFP piece) and the uncleaved substrate CD2 in cell lysates by CE could give a clear description of caspase-2 activation.

3.1. CE separation and identification

Fig. 2 illustrated a separation of the "standard" DsRed and CD2 with a running buffer of 25 mM phosphate (pH 8.0). The so-called standards were obtained from cell lysates of *E. coli* expressing DsRed and HeLa expressing CD2 respectively, as described in Section 2. As shown in Fig. 2, the two analytes were completely separated with a separation resolution of 2.0. The relative standard derivates of the migration time for DsRed and CD2 were 0.72% and 0.94% ($n = 5$), respectively. This good separating quality was partly contributed to the high performance of CE. But another important reason should be also highlighted, that was, the designed molecular structure of the fluorescent probe, in which fluorescent proteins were introduced to each side of the recognizing site VDVAD. Thus, the cleavage of the probe by caspase-2 could cause a big difference in molecular weight between the cleavage products and uncleaved probe. Such strategy was very useful, which greatly facilitated subsequent CE separation. In monitoring the activation of PKA, Zarrine-Afsar and Krylov [14] tried another interesting idea in probe design to enhance CE separation. Eight phosphorylatable sites were simultaneously introduced into the substrate, which could lead to a big difference in molecular charge between the phosphorylated and unphosphorylated probes with activation of PKA.

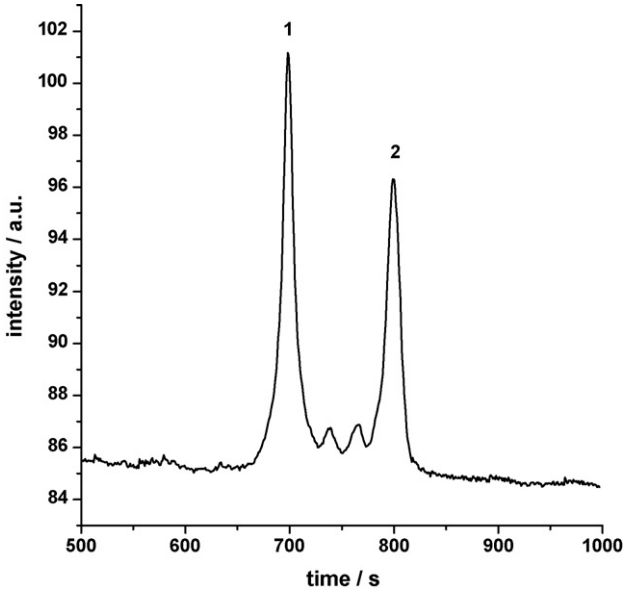

Fig. 2. Electropherogram of mixture of cell lysates serving as "standards" in optimized separation conditions. CE condition: buffer, 25 mM phosphate buffer (pH 8.0); separation voltage, at 16 kV; capillary, 80 cm (total length) × 60 cm (length to detection window) × 50 μm i.d. Detection was performed in DsRed channel. Peak identity: (1) DsRed; (2) CD2.

For peak identification in electropherogram, one kind of fluorescent protein (e.g., DsRed molecular weight (MW): 24 KD, or ECFP, MW: 26 KD) was actually enough for fluorescence detection, by conjugating it to both terminals of the recognizing site such as DsRed–VDVAD–DsRed or ECFP–VDVAD–ECFP. An advantage of employing two different fluorescent proteins was that the cleavage reaction could be monitored in different detection approaches as depicted in Fig. 3, which was definitely meaningful for identification in complex real-world sample like cells lysate. With an incubation of 1 μg/ml cisplatin that was an effective chemotherapeutic reagent for many cancer diseases such as bladder, lung, gullet, stomach and ovarian, HeLa cells were induced to apoptosis. The cell lysates were subjected to CE analyses as shown in Fig. 3A. Two peaks representing DsRed and CD2 were identified in the electropherogram, using DsRed detection channel (excitation and detection filters for DsRed) in microscope. There were to several peaks in the electropherograms, which were believed to be some fluorescent impurities. To validate the result, ECFP detection channel (excitation and detection filters for ECFP) could be also employed in Fig. 3B. Because ECFP was also released from the probe by the cleavage reaction, two peaks appeared in electropherogram, representing ECFP and uncleaved CD2, respectively. Further, the designed probe had a unique feature of fluorescence resonance energy transfer (FRET) [20,21]. While using excitation wavelength of ECFP to irradiate the probe, ECFP would absorb the energy and transfer it to its neighbor DsRed due to inter-molecular dipole–dipole interaction. Consequently it would give out DsRed emission. Because FRET efficiency is very sensitive to the spatial distance between the energy donor and acceptor, it has been widely used to investigate donor-acceptor interactions. In our experiment, FRET channel (excitation filter for ECFP and detection filter for DsRed) could be used for CE detection, as shown in Fig. 3C. The peak of CD2 was undoubtedly detected out. However, a very weak peak belonging to DsRed also occurred because of wide fluorescent excitation spectrum of DsRed.

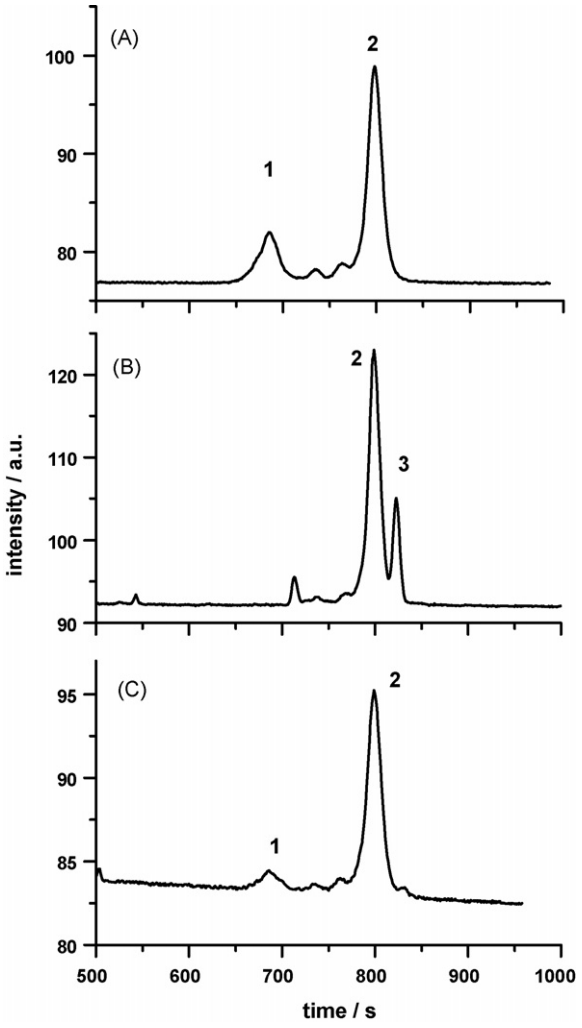

Fig. 3. Electropherograms of lysate of HeLa cells with incubation of cisplatin for 6 h in different detection channels. (A) DsRed channel; (B) ECFP channel; (C) FRET channel. Peak identity: (1) DsRed; (2) CD2; (3) ECFP. The samples were not purified for analyses. Other conditions were the same as in Fig. 2.

3.2. Activation dynamics of caspase-2 in cisplatin induced cell apoptosis

To characterize the activation dynamics of casapse-2, a series of HeLa cell lysate samples were prepared from different incubation time points with cisplatin, following the method as described in experimental section. No procedure for purifying the substrate and its enzymatic cleavage products was carried out because of specificity of fluorescence detection. Fig. 4 showed the results by CE method with DsRed channel as established in above section. The tendency was quite clear. At time point of zero hour, no caspase-2 activation was found. A big peak representing CD2 appeared in the electropherogram. With increase of incubation time, cell apoptosis initiated, and caspase-2 was activated. Thus, the peak representing DsRed appeared, and its height gradually became higher. After 14 h, CD2 was almost digested by caspase-2. Thus, only DsRed could be identified in electropherogram. For a clearer demonstration of this dynamic process, the peak height ratios of DsRed to sum of DsRed and

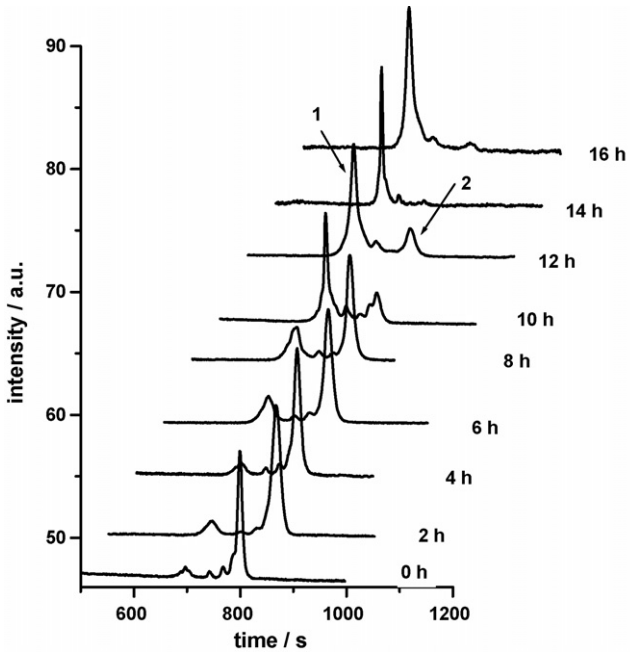

Fig. 4. Electropherograms of lysates of HeLa cells incubated with cisplatin in a time course (waterfall style). Peak identity: (1) DsRed; (2) CD2. Other conditions were the same as in Fig. 2.

CD2 in the time course of apoptosis were calculated. To calibrate the sample-to-sample difference in lysate concentration, all electropherograms were normalized. Fig. 5 gave the plot. It should be pointed out that the strategy as developed above was an *in vitro* method.

3.3. Validation of cell apoptosis using optical imaging

To monitor protein activation *in vivo*, optical molecular imaging has been widely employed that can reveal molecular event at levels from single cell to whole-body of organism [21–24]. For validating above investigations of cisplatin induced cell apoptosis, optical imaging was utilized to monitor the process at single

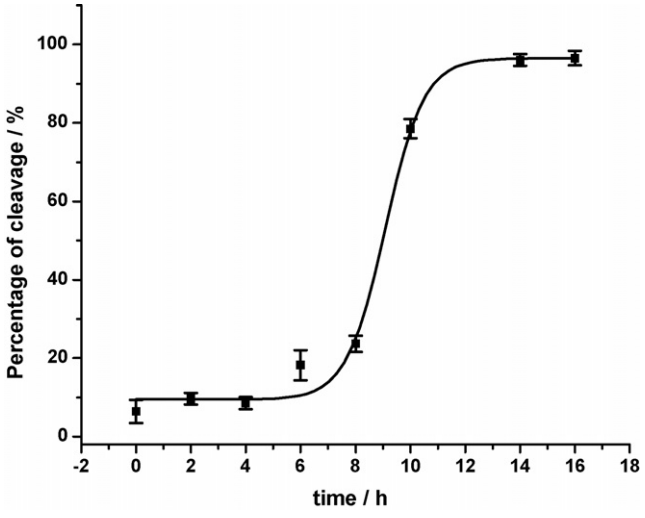

Fig. 5. Dynamics of caspase-2 activation during cisplatin-induced cell apoptosis.

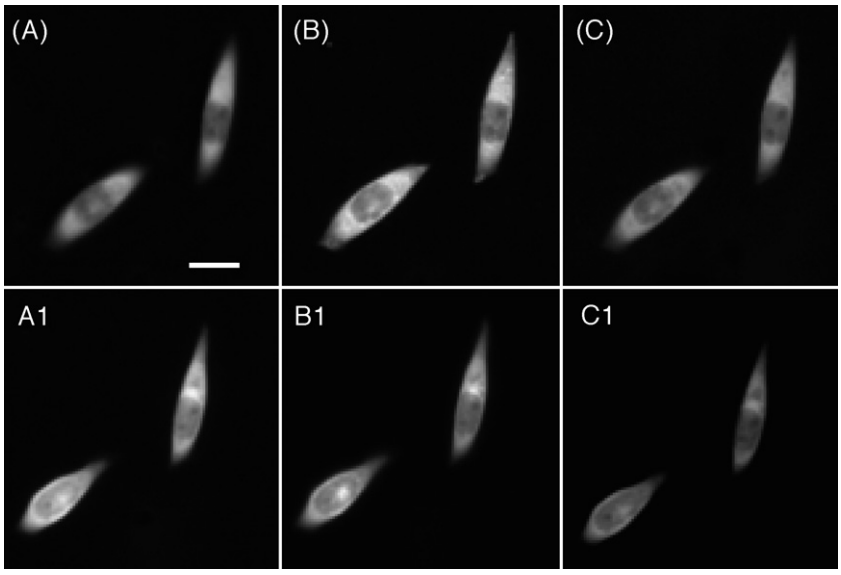

Fig. 6. Micrograph in fluorescence of CD2-expressing HeLa cell apoptosis process by optical imaging. Images of normal HeLa cell captured in ECFP channel (A), DsRed channel (B) and FRET channel (C); images of apoptotic HeLa cell captured in ECFP channel (A1), DsRed channel (B1) and FRET channel (C1) with incubation of cisplatin for 6 h. Scale bar in (A) represents 10 μm.

cell level *in vivo* as shown in Fig. 6. Normal HeLa cell expressing the fluorescent probe CD2 were imaged in different detection channels for ECFP, DsRed and FRET as observed in Fig. 6A, B and C, respectively. With incubation of the given dose of cisplatin, the fluorescence became dim in FRET channel, while brighter in ECFP channel. The activated caspase-2 cleaved the probe CD2, which blocked FRET from CFP to DsRed. It indicated that apoptosis in HeLa cell occurred. Fig. 6A1, B1 and C1 described the cell conditions after cisplatin treatment of 5 h.

4. Concluding remarks

A CE strategy was proposed to characterize the dynamics of caspase-2 activation during cisplatin induced cell apoptosis, conjugating with a fluorescent protein-based probe that contained a VDVAD sequence sandwiched by two different fluorescent tags ECFP and DsRed. The sequence VDVAD could be specifically recognized and cleaved by caspase-2 as soon as it was activated with treatment of a certain dose of cisplatin to HeLa cells. Analyzing the products of cleavage reaction and the uncleaved probe by CE gave an illustration of the dynamics of caspase-2 activation, which was further validated by single cell optical imaging. The established method might be also very useful for similarly characterizing activations of other functional proteins, and also had potential applicability for anti-cancer drug evaluation.

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References

[1] G.S. Salvesen, V.M. Dixit, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10964.
[2] N.A. Thornberry, Y. Lazebnik, Science 281 (1998) 1312.
[3] J.D. Robertson, M. Enoksson, M. Suomela, B. Zhivotovsky, S. Orrenius, J. Biol. Chem. 277 (2002) 29803.
[4] S.H. Read, B.C. Baliga, P.G. Ekert, D.L. Vaux, S. Kumar, J. Cell. Bio. 159 (2002) 739.
[5] Y. Guo, S.M. Srinivasula, A. Druilhe, T. Fernandes-Alnemri, E.S. Alnemri, J. Biol. Chem. 277 (2002) 13430.
[6] J.D. Robertson, V. Gogvadze, A. Kropotov, H. Vakifahmetoglu, B. Zhivotovsky, S. Orrenius, EMBO Rep. 5 (2004) 643.
[7] W.W.C. Quigley, N.J. Dovichi, Anal. Chem. 76 (2004) 4645.
[8] S. Terabe, M.J. Markuszewkksi, N. Inoue, K. Otsuka, T. Nishioka, Pure Appl. Chem. 73 (2001) 1563.
[9] B.-F. Liu, B. Xu, G. Zhang, W. Du, Q. Luo, J. Chromatogr. A 1106 (2006) 19.
[10] S.N. Krylov, Z. Zhang, N.W.C. Chan, E. Arriaga, M.M. Palcic, N.J. Dovichi, Cytometry 37 (1999) 14.
[11] G.D. Meredith, C.E. Sims, J.S. Soughayer, N. Allbritton, Nat. Biotechnol. 18 (2000) 309.
[12] M. Berezovski, W.-P. Li, C.D. Poulter, S.N. Krylov, Electrophoresis 23 (2002) 3398.
[13] S. Yoon, E. Ban, Y.S. Yoo, J. Chromatogr. A 976 (2002) 87.
[14] A. Zarrine-Afsar, S.N. Krylov, Anal. Chem. 75 (2003) 3720.
[15] S. Yoon, K.Y. Han, H.S. Nam, L.V. Nga, Y.S. Yoo, J. Chromatogr. A 1056 (2004) 237.
[16] H.F. Tzeng, H.P. Hung, Electrophoresis 26 (2005) 2225.
[17] J.Q. Lin, Z.-H. Zhang, J. Yang, S.Q. Zeng, B.-F. Liu, Q. Luo, J. Biomed. Opt., in press.
[18] B.-F. Liu, L. Zhang, Y.-T. Lu, J. Chromatogr. A 918 (2001) 401.
[19] J. Lippincott-Schwartz, G.H. Patterson, Science 300 (2003) 88.
[20] E.A. Jares-Erijman, T.M. Jovin, Nat. Biotechnol. 21 (2003) 1387.
[21] W. Du, Y. Wang, Q. Luo, B.-F. Liu, Anal. Bioanal. Chem, in press.
[22] D.J. Stephens, V.J. Allan, Science 300 (2003) 82.
[23] D.L. Farkas, Nat. Biotechnol. 21 (2003) 1269.
[24] Y. Wang, E.L. Botvinick, Y. Zhao, M.W. Berna, S. Usami, R.Y. Tsien, S. Chien, Nature 434 (2005) 1040.