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Review

Ultrasensitive chemiluminescence detection in capillary electrophoresis

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Abstract

Capillary electrophoresis techniques offer high plate numbers and are highly suited for the efficient separations of a wide variety of chemical components in diverse matrices. Because of the small capillary and detection cell dimensions, together with the minute volumes of samples to be injected, sensitive detection schemes based on different physicochemical principles are being developed. One logical approach to increased sensitivity in capillary electrophoresis detection has been the development of chemiluminescence-based detectors. The development of on-line ultrasensitive chemiluminescence detection (referred to the concentration detection limit of n*M* order of magnitude or mass detection limit of amol order of magnitude) in capillary electrophoresis system is reviewed. The applications and limitations of the current detection methodology are briefly considered and future prospects for the development are discussed.

Keywords: Reviews; Chemiluminescence detection; Detection, electrophoresis; Luminol; Proteins; Metal cations; Peroxyoxalate

Contents

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1. Introduction

In the past two decades, high-performance capillary electrophoresis (HPCE or CE) has been developed rapidly. CE is an instrumental approach to electrophoresis in which sample components placed between two buffer solutions are separated in an open-tubular capillary with an inside diameter ranging from 2 to 200 μm and a length usually between 10 and 100 cm. The separation is based on the electrophoretic mobilities of the analyte species induced by the large potential (10–30 kV) applied across the capillary. The high efficiency afforded by CE leads to many of the characteristics (high resolving power, large peak capacity, short analysis time) which make this technique to be one of the most powerful tools in the analytical laboratory. The versatility of this technique makes it suitable for chemical, biochemical, biomedical and environmental research, protein analysis, DNA sequencing, chiral analysis, etc.

The extremely small internal diameter of the capillary in CE offers some advantages, such as very low mass detection limits and small sample volume which allows the analysis of biological sample (e.g., single cell assay). On the other hand, the small detection volume often results in a poor concentration detection sensitivity. Moreover, it makes it more difficult to develop methodology for improving sensitivity in CE, which is still a major challenge. The sensitivity limitations of the method are still evident obstacles to progress [1]. Consequently, the development of techniques that can improve detection has been a principal area of CE research.

To date, the majority of commercially available CE instruments employ UV–Vis absorbance detectors because of their simplicity and versatility. The concentration detection limits are only 10^{-5}–10^{-6} M limited by the short path length (2–100 μm) through the column. Various attempts have been made to improve the sensitivity of UV–Vis detectors by increasing the optical path length, including the use of Z-shaped [2] and multireflection detection cells [3], but with some sacrifice in resolution and design simplicity. Continuing efforts have also been made to improve the performance of various CE detectors. Electrochemical detectors are simple, however, the detection limits are 10^{-7}–10^{-8} M [4]. Laser-induced fluorescence (LIF) detection has been shown to be perfectly suited for CE with high sensitivity, and it has been used for detection of single molecules [4]. However, apart from the cost of LIF equipment, additional efforts are also required to introduce fluorophores to nonfluorescent analytes through pre- or post-column derivatization. In addition to LIF, mass spectrometry, Raman and nuclear magnetic resonance (NMR) have been coupled to CE, but the concentration detection limits of these are comparative to that of the absorbance method [4]. Additionally, these detection schemes are either expensive (e.g., lasers, mass spectrometers, NMR spectrometers) or require complex instrumentation.

Chemiluminescence (CL) is an alternative detection scheme. Chemiluminescence can be defined as emission of light (ultraviolet, visible or infrared) from a molecule or atom in an electronically excited state, which occurs by a chemical reaction. Chemiluminescence is highly sensitive and selective, and becomes essential in liquid chromatography, immunoassay and hybridization assay [5]. To date, CL is one of the less developed detection systems for CE, yet there are several features in this method which brings it close to a sensitive detection system. CL detection system is characterized by a simple, cheap optical system requiring no light sources, avoiding the efforts of stray light and the instability of the light source, and thus providing low background noise with excellent sensitivity comparable to that of LIF. Combination of the high separation ability of CE with the high sensitivity of CL is an attractive research line.

There are a number of challenges for the CL detection coupled to CE. One or more reagents should be added to initiate the CL reaction. A CL reagent may yield significant emission not just for one unique analyte, i.e., a lack of selectivity may occur. Moreover, CL emission intensities are sensitive to a variety of environmental factors such as temperature, solvent, ionic strength, pH, and other species present in the system. As a result, separation conditions in CE may not always match the optimum CL emission conditions. The emission intensity from a CL reaction varies with time (light flash composed of a signal increase after reagent mixing, passing through a maximum, then decreasing back to the baseline); therefore, attention must be paid to detect

the signal during strictly defined periods. Because only a small portion of CL emission is measured as the mixture of analyte and CL reagents passes through the detection cell, nonlinear calibration curves are sometimes obtained for reaction with complex kinetics. In addition, the CL emission versus time profile differs widely from one compound to another.

However, the large increase in related publications recently may illustrate the increasing interest and efforts made in this direction. The trends in the various phases of development of this field were well-documented in several dedicated reviews since 1994. Baeyens et al. [6] were the first to review the nature, possible applications and limitations of CL as a detection technique for CE. Later, Staller and Sepaniak [7], Garcia-Campana et al. [8], Sanders et al. [9], Zhang et al. [10], Zhu and Kok [11] and Huang and Fang [12] comprehensively reviewed the principles, instruments and applications of CL detection in CE. More recently, Kuyper and Milofsky [13] gave a review on the recent developments in chemiluminescence and photochemical reaction detection for capillary electrophoresis, they highlighted the developments in the design and configuration of instrumentation used for CL detection coupled to CE. Most recently, Yamaguchi et al. [5] reviewed the principles of chemiluminescence of luminol-type compounds and its analytical application in liquid chromatography and capillary electrophoresis as derivation reagents. In this report, the progress made in combining CE with CL is summarized, and the applications and limitations of the current detection methodology, specially for the ultrasensitive detection (referred to the concentration detection limit of n*M* order of magnitude or mass detection limit of amol order of magnitude), are reviewed.

2. Ultrasensitive chemiluminescence detection systems

Chemiluminescence, although very sensitive, lacks selectivity. However, when CL is combined with a separation method, it can offer excellent analytical sensitivity and selectivity. In the past decade, research in combining CE with CL detection has increased significantly. To date, several CL systems including luminol, peroxyoxalate, acridinium esters and firefly luciferase have been developed for the ultrasensitive detection in CE. Various chemical substances have been detected including metal ions, metal-porphyrins, enzymes, proteins, peptides and ATP, etc.

2.1. Luminol

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and related CL systems are based on the reaction of luminol, or a luminol derivative, with hydrogen peroxide or other oxidants in the presence of a catalyst. The excited state 3-aminophthalate molecules that are produced emit light in the 425–435-nm region of the spectrum. Hitherto, reactions involving luminol are used in CE–CL systems most frequently. In the luminol reaction, CL intensity is proportional to the concentration of luminol, oxidant, and catalyst. Therefore, the system can be applied to the determination of luminol, hydrogen peroxide, or catalysts such as enzymes and metal ions.

At an early stage of the development of CE–CL, Dadoo et al. [14] reported for the first time the use of a luminol-based detection method for CE. The electrophoretic apparatus is composed of an electrophoretic capillary, a reagent capillary, and a reaction capillary held in place by a tee connector (Fig. 1). A 3–4-cm section at the end of the electrophoretic capillary is etched by hydrofluoric acid and then inserted into the reaction capillary. The detection window, which is made on the reaction capillary by burning off the polyimide coating, is placed at the focal point of a parabolic mirror to collimate the emitted light and subsequently is focused on a photomultiplier tube (PMT) connected to a photon counting system. This interface designed is one of the most commonly used in CE–CL so far. The detection limits of 3×10^{-9} *M* and 7×10^{-9}*M* were achieved for luminol and ABEI, respectively. This is an improvement of two to three orders of magnitude over UV absorbance detection.

Hashimoto et al. [15] designed a flow-type CL detection cell using optical fiber for CE. Luminol CL was adapted for use with this detection cell. The detection limit ($S/N=3$) for luminol was 7.6×10^{-9} *M* with theoretical plate numbers of 1.3×10^{5}–1.6×10^{5} and a relative standard deviation of 3.3% for the

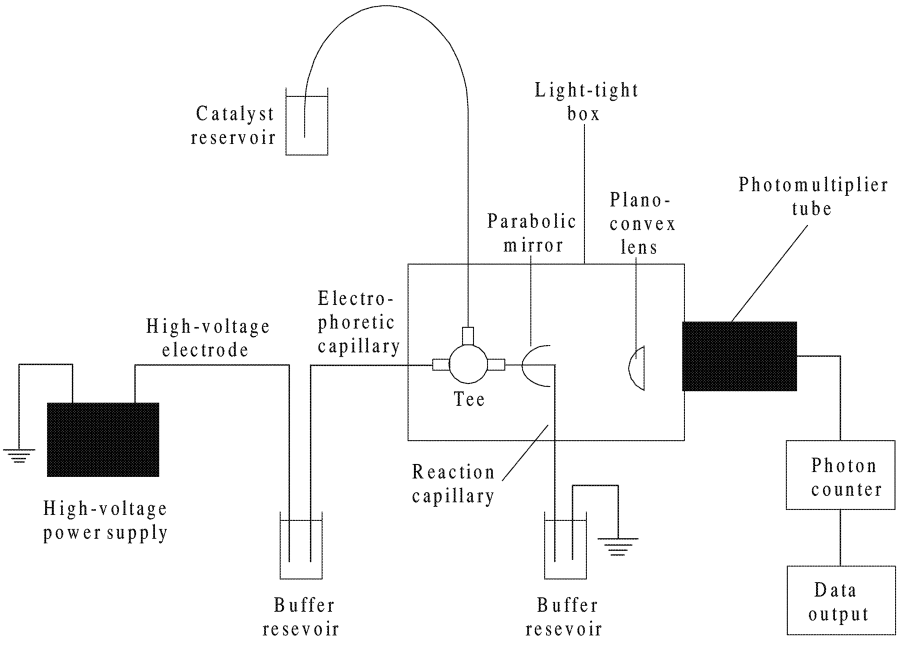

Fig. 1. Schematic of CE–CL detection using luminol. Reproduced from Ref. [14] with permission.

peak height ($n=10$). In a subsequent report from the same laboratory, they [16] used a new and simple CL detection cell and obtained the detection limit ($S/N=3$) of 5.0×10^{-10} *M* for luminol, which was the most sensitive result reported so far.

2.1.1. Detection of metal ions

Metal ion analysis is very important in a variety of research and industrial areas. Macka and Haddad [17] presented an excellent review on the determination of metal ions by CE. As illustrated in that article, interest in this area has steadily increased. However, two main difficulties were encountered in CE [18]. First, lack of sensitivity represents a formidable challenge in this technique mainly due to two reasons: (i) most of metal ions are transparent in UV–Vis region; (ii) inorganic cations are seldom intrinsically fluorescent in the use of LIF detection, and therefore derivatization procedure should be carried out. The second difficulty is the selectivity. It seems very difficult to discriminate these free cations only based on their migration behavior, because inorganic cations have similar or identical ionic equivalent conductances which directly determine the electrophoretic mobilities of ions. To date, two main detection modes, direct detection and indirect detection, have been employed.

A variety of metal ions may be used to catalyze the luminol–hydrogen peroxide CL reaction. To improve the detection sensitivity of metal ions in CE–CL, our group has made continuing efforts based on three aspects as follows [19–26]. First, the performance of apparatus and CL system has been improved and the signal-to-noise ratio has been enhanced. Second, new catalysts with high catalytic activity have been exploited. Third, on-capillary sample stacking technique has been utilized.

To improve the signal-to-noise ratio and prevent the loss of light signal resulting from the mixing of luminol and hydrogen peroxide in advance in the conventional flow injection analysis or ion chromatography–chemiluminescence detection, our group [20,21] have used a novel mixing mode of the reagents (luminol as one of the electrophoretic components and H_2O_2 solution as the only post-capillary reagent to be introduced) in CE–CL. Because luminol, hydrogen peroxide, and the metal ions reach the detector window simultaneously, the fast kinetic rate will produce a light response detected at the same time. Signal to noise ratio of the

system has been improved evidently using the novel mixing mode of the reagents, the detection limit of 5×10^{-13} *M* for Co(II) was achieved.

In CE–CL instrumental configuration, the on-line introduction of one or two reagents into the system is always required for initiating the CL reaction. This feature is reflected in the design of all CE–CL interfaces, where the mixing mode of the analyte and the CL reagent, is one of the crucial factors for achieving good separation efficiencies and sensitivities [12]. Conventional interfaces of CL detection are post-column [8,14], which is similar to the detector originally developed by Rose and Jorgenson [27]. However, this kind of apparatus could still face the following problem. One of the widely used oxide reagents, hydrogen peroxide, in the luminol CL reaction, is easily broken down into bubbles in basic medium. These bubbles can cause an unstable current through the electrophoresis process and even perturb the detection seriously [28,29]. This problem can be solved to a great extent by putting the grounding electrode before the reaction capillary, not in the eluate reservoir, as shown in Fig. 2 [22]. The transfer of the grounding electrode from a point downstream of the detection point to the upstream point significantly improved the performance [12]. The detection limit of 8.1×10^{-9} *M* for 5, 10, 15, 20 - tetrakis(4 - sulfonatophenyl) - 21*H*, 23*H* - porphine manganese(III) based on luminol-H_2O_2 CL reaction was achieved with a theoretical plate number of 3.6×10^{5} using the improved apparatus.

As mentioned earlier, improving the detection sensitivity is a principal area of CE research. One of the most commonly used methods is on-capillary

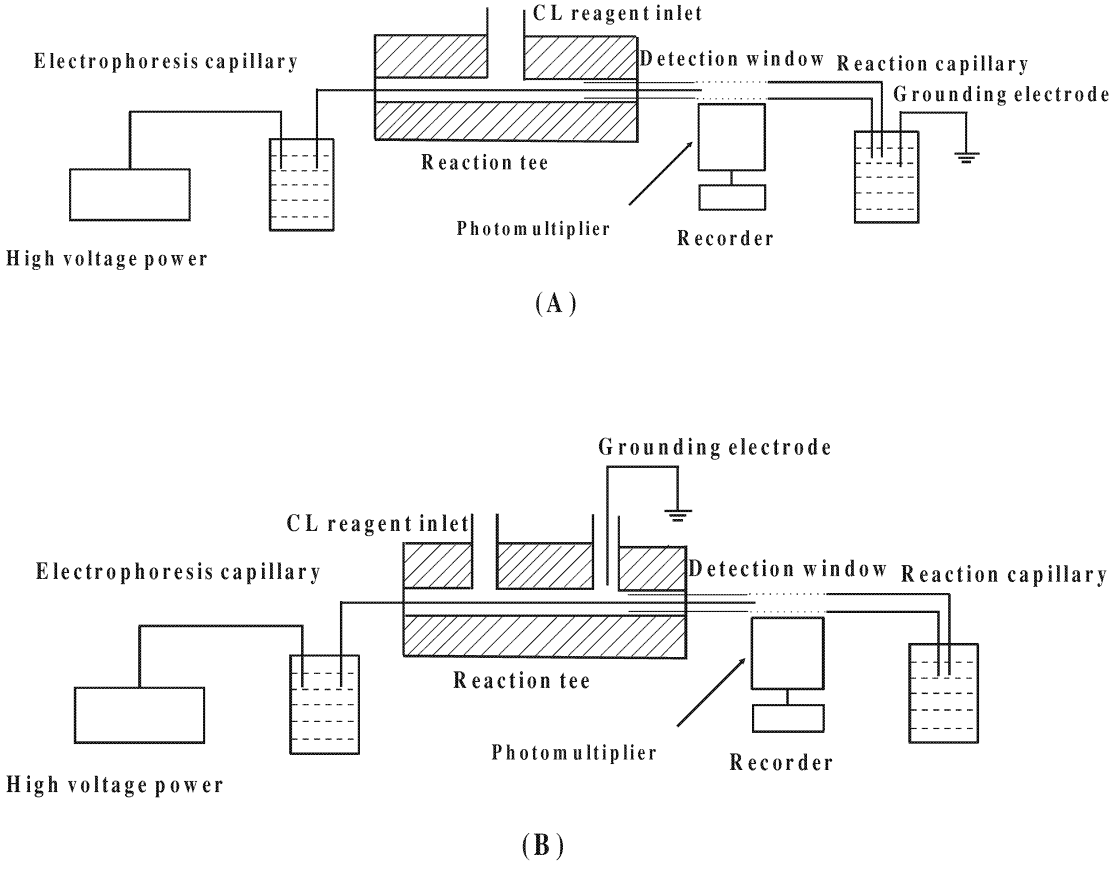

Fig. 2. Schematic diagram of the previous (A) and improved (B) post-column CL detector for CE. Reproduced from Ref. [22] with permission.

sample stacking. More recently, using our newly developed capillary electrophoresis with the chemiluminescence detection system and novel mixing mode of the reagents, coupled with the sample stacking technique, ultrasensitive detection of several metal ions has been performed by our group. Capillary electrophoresis separation and chemiluminescence detection of niobium(V) and tantalum(V) were carried out with 10 m*M* sodium dihydrogenphosphate buffer (pH 2.5) [23]. Signal enhancement was achieved by sample stacking injection in the electrophoresis process. The detection limits for niobium(V) and tantalum(V) ($S/N=3$) are 826 zmol (4.13×10^{-10} *M*) and 640 a*M* (3.2×10^{-8} *M*), respectively.

Interestingly, after investigating the catalytic activity of some metal ions by CE–CL, it is found that the catalytic activity of those metal ions is related to their electronic configuration [24]. According to Hund's rule, when the outer electronic shell is completely occupied (d^{10}), vacant (d^0) or half-occupied (d^5), the corresponding metal ions are more stable. Our experimental results for the detection sensitivity (catalytic activity) of metal ions are listed below: $Zn^{2+}(3d^{10})<V^{5+}(3d^0)<Mn^{2+}(3d^5)<Ni^{2+}(3d^8)<Cu^{2+}(3d^9)<Cr^{3+}(3d^3)$, $Co^{2+}(3d^7)$ and $V^{4+}(3d^1)$. It can be seen from the above results that the catalytic activity of the metal ions is basically consistent with Hund's rule. More recently, it was found that V(IV) has excellent catalytic behavior for the reaction of luminol and hydrogen peroxide. Ultrasensitive CL detection of V(IV) in CE has been achieved [24]. The detection limit ($S/N=3$) for V(IV) is 2.4×10^{-17} *M* (24 a*M*). Up to now, this could be the highest sensitivity for metal ions analysis. In addition, the separation of V(IV) and V(V) has been performed successfully (as shown in Fig. 3). A method of on-line ultrasensitive chemiluminescence detection with capillary electrophoresis for Co(II) was reported [25]. The sub-f*M* level (1.3×10^{-16} *M*, 1.6×10^{-24} mol) detection of cobalt ions with a theoretical plate number of 1.9×10^5 in ultradilute solution was performed. According to the concentration of Co(II) and the apparent volume of injection (12.6 nl), it can be calculated to be one Co(II) molecule in 12.6 nl. The results obtained could be one of the highest sensitivity for metal ions analysis so for. The catalytic behavior of cobalt(II) ion for the chemiluminescence reaction of luminol and hydrogen peroxide and the reaction conditions, such as the concentration of luminol, hydrogen peroxide, and pH of chemiluminescence reagent were investigated. Using the weak complexing agent α-hydroxyisobutyric acid (HIBA) to enhance the differences between the electrophoretic mobilities of metal ions, the separation of f*M* level Co(II) and trace amounts of Ni(II) was performed successfully (Fig. 4). The effects of field-amplified sample injection on the detection limits of Co(II) and other metal ions were also studied in detail (Fig. 5), the detection limits for Cr(III), Cu(II) and Ni(II) reached 10^{-14}, 10^{-11} and 10^{-10} *M*, respectively. By injection of a short plug of water before sample introduction, the sensitivity of metal ion can be further enhanced [26]. The reasons for achieving ultrasensitive detection might be considered as follows. First, the field-amplified sample injection for enhancing sensitivity was utilized. Second, some metal ions have excellent catalytic behavior for the CL reaction of luminol and H_2O_2. Third, the CE–CL detection system including the improved apparatus, novel mixing mode of the reagents, and sensitive detector,

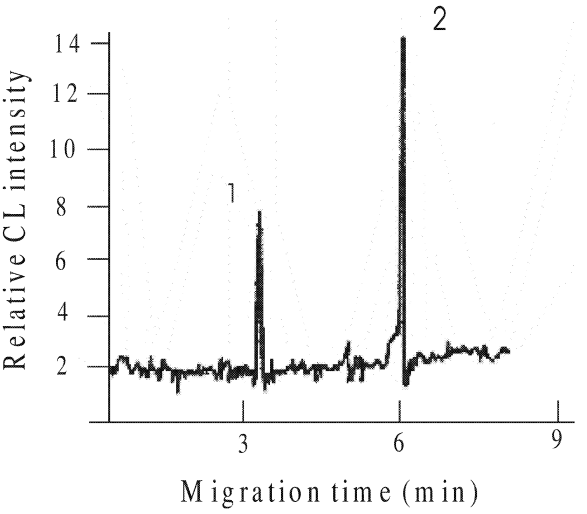

Fig. 3. Separation of 1.3×10^{-16} *M* V(IV) and 6.7×10^{-5} *M* V(V) by capillary electrophoresis with CL detection. Curves: (1) V(IV); (2) V(V). Conditions: 45 cm×50 μm I.D., fused-silica capillary. Electrophoretic electrolyte, 20 m*M* acetate buffer with 1 m*M* luminol at pH 4.5; postcapillary reagent, 50 m*M* H_2O_2 with 25 m*M* acetate buffer at pH 11.5; separation voltage, 20 kV. Reproduced from Ref. [24].

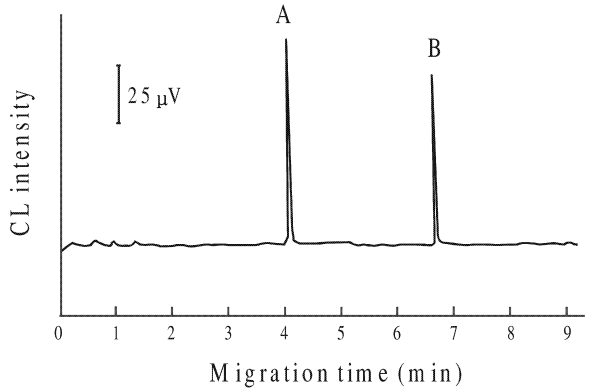

Fig. 4. Separation of 5×10^{-15} *M* Co(II) and 5×10^{-7} *M* Ni(II). Peaks: (A) Co(II); (B) Ni(II). Electrophoretic electrolyte, 1×10^{-3} *M* luminol + 8×10^{-3} *M* HIBA + 4×10^{-2} *M* HAc-NaAc buffer, pH 4.75; CL reagent, 2×10^{-2} *M* H_2O_2 + 2×10^{-2} *M* Na_2CO_3, pH 11.5; sample injection, 5 s at 10 kV; separation voltage, 20 kV. Reproduced from Ref. [25].

etc., has excellent performance. No doubt, CE–CL would provide a new approach for ultrasensitive detection of some metal ions. However, a more systematic study, especially a deep understanding of the field-amplified sample stacking of metal ions and the mechanism of CL reaction in capillary electrophoresis would be needed.

Indirect CL detection, commonly used in liquid chromatography and in flow injection analysis and recently adapted to CE separations, is based on the detection of a nonchemiluminescent analyte that produces interference or suppression of a given CL reaction; the analyte is detected indirectly as an inverted peak [30]. More recently, Ren and Huang [31] first presented a highly sensitive and universal indirect CL detection of cations in CE. The detection interface and mixing mode of the reagents used in the CE–CL system is similar to that reported by our group [21,22]. This method is based on use of the cobalt(II) as a probe ion in the running buffer. A strong and stable background chemiluminescent signal can be generated by the luminol–hydrogen peroxide reaction catalyzed by the cobalt(II) ion. Displacement of the cobalt(II) probe ion in the running buffer by a migrating sample cation results in a quantifiable decrease in the background signal. Under the optimal conditions, the detection limits for manganese(II), cadmium(II), nickel(II), lead(II), and 14 lanthanides are $(3.0–6.0)\times10^{-9}$ *M* ($S/N=3$), which is ~3 orders of magnitude better than indirect UV detection and two orders of magnitude better than indirect LIF detection. To our knowledge, the results obtained could be the highest sensitivity for indirect detection of cations in CE separation. In sodium acetate–lactic acid buffer, a mixture of 18 metal ions including 14 lanthanides was separated rapidly and efficiently within 3.5 min except the

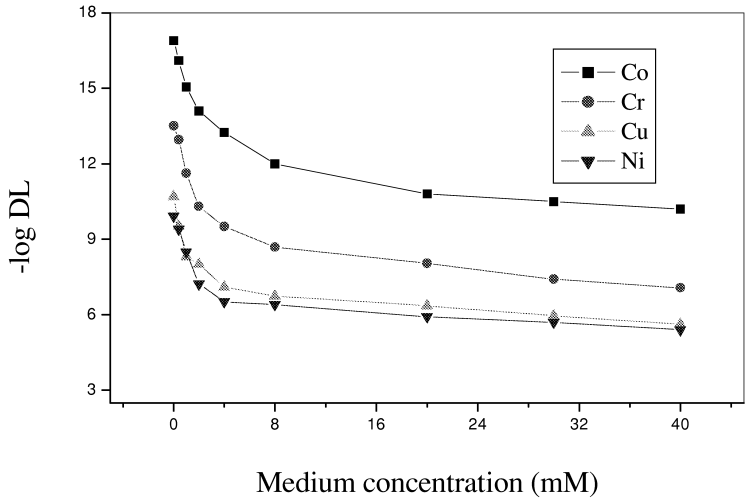

Fig. 5. Effect of concentration of HAc-NaAc in sample on detection limits of metal ions. Conditions: electrophoretic electrolyte, 1 m*M* luminol + 40 m*M* HAc-NaAc buffer, pH 5.10; CL reagent, 20 m*M* H_2O_2 + 20 m*M*Na_2CO_3, pH 11.5; sample injection, 5 s at 10 kV; separation voltage, 20 kV.

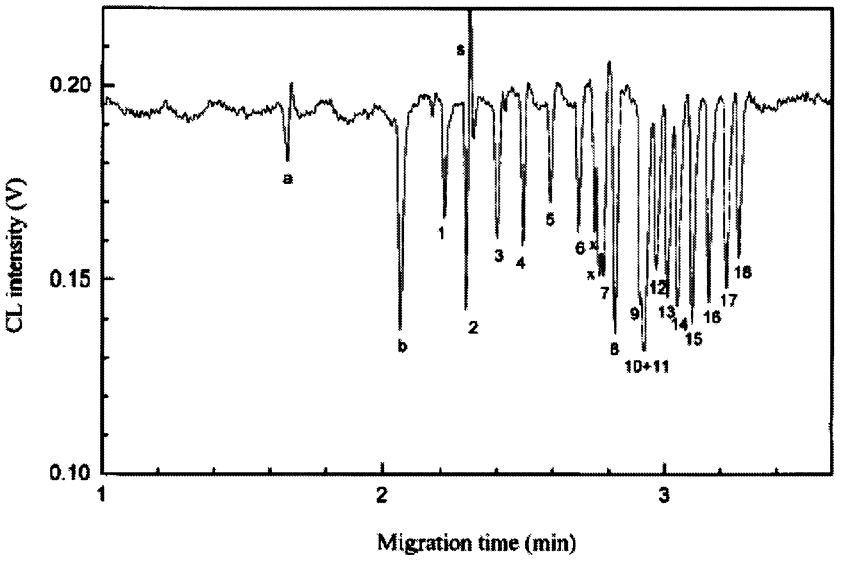

Fig. 6. Indirect CL detection for separation of 18 metal ions. The solution of 1.0×10^{-2} *M* sodium acetate–lactic acid containing 3.0×10^{-4} *M* luminol and 5.0×10^{-7} *M* cobalt(II) ion (pH 4.40) was used as the electrophoretic buffer, and 2.0×10^{-2} *M* sodium acetate solution covering 2.0×10^{-3} *M* of hydrogen peroxide was used as the reaction solution (pH 11.8). The concentration of each metal ion was 5×10^{-8} *M*. Electrokinetic injections at 15 kV for 3 s were used, the temperature was ~21 °C (ambient), and the applied voltage was 20 kV. Peak identification: (1) Mn^{2+}, (2) Cd^{2+}, (3) Ni^{2+}, (4) Pb^{2+}, (5) La^{3+}, (6) Ce^{3+}, (7) Pr^{3+}, (8) Nd^{3+}, (9) Sm^{3+}, (10) Gd^{3+}, (11) Eu^{3+}, (12) Tb^{3+}, (13) Dy^{3+}, (14) Ho^{3+}, (15) Er^{3+}, (16) Tm^{3+}, (17) Yb^{3+}, and (18) Lu^{3+}. The peak denoted s was a system peak; the peaks denoted a, b, and x expressed K^{+}, Ca^{2+}, and unknown ions, respectively. Reproduced from Ref. [31] with permission.

coelution of Sm(III), Eu(III), and Gd(III) ions (Fig. 6). The theoretical plate numbers reached $(3.1–6.5)\times10^{5}$. The high separation efficiency mainly contributes to the fast kinetic nature of the CL reaction and the partial complexing of the lactate to the metal ions. The authors prepared the samples in pure water and used the field-amplified sample stacking in order to enhance the sensitivity. Indirect detection approach expands largely the application of CE–CL. Generally, the limitations of indirect detection method include more interference and relatively low sensitivity compared to direct detection method.

2.1.2. Detection of enzymes

Recent applications of CE–CL to the biochemical analysis have been developed. Using the technique of the electrophoretically mediated microanalysis (EMMA), it is possible to detect the enzyme. It only needs nanoliter volumes of biological sample and has the characteristics of fast speed and simplicity as compared to a conventional colorimetric method. A CL detection system for enzymes using EMMA was presented by Regehr and Regnier [32], the best detection limit of 15 zmol (9300 molecules) was obtained for catalase with injection of 100 p*M* catalase with incubation for 6 min and a separation buffer of 0.1 m*M* H_2O_2 in 25 m*M* phosphate of pH 8. Also, the detection limits of 7.7 amol and 120 zmol were achieved for galactose oxidase and glucose oxidase, respectively.

Recently, CL detection based on the reaction of luminol with peroxide catalyzed by horseradish peroxidase (HRP) was investigated. It is a post-separation detection scheme for microchip-based capillary electrophoresis [33]. The sample was driven by the high voltage source via electrodes inserted in the reservoirs. Luminol was contained in the separation buffer while peroxide was merged to the separation channel. Increasing the amount of sample injected and placing an Al mirror integrated onto the backside of the detection zone to enhance collection efficiency, can enhance the detection sensitivity. If the channel etched to 10 μm deep, the

detection limit was 35 n*M* for 1 nl injected sample plugs of fluorescein conjugate of HRP (HRP-Fl). If the channel etched to 40 μm deep, 8 nl sample plugs gave a detection limit of 7 n*M*. The 7–35 n*M* detection limits for HRP-Fl reported for on-chip CL are about 50–100-fold lower than could be achieved on-chip with absorbance detection. Separation and CL detection of the products of an immunological reaction of a $F(ab')_2$ fragment of the HRP conjugate of goat anti-mouse immunoglobulin G (IgG) with mouse IgG was performed on-chip. Zhang [34] coupled the HRP–luminol–H_2O_2 CL reaction to CE with 4-iodophenol as an enhancer, obtained the detection limits of 1.0 and 2.0 amol for HRP and human IgG, respectively. The IgG in human serum was detected. Therefore, CE–CL immunoassay could be developed a fast, simple and sensitive detection method.

2.1.3. Detection of proteins

In 1997, Tsukagoshi et al. [35] reported for the first time the high-sensitive analysis of heme proteins separated by capillary electrophoresis with on-line chemiluminescence detection using a luminol and hydrogen peroxide system. In general, the adsorption of protein onto the inner wall of the capillary is a serious problem and results in lower separation efficiency and lower detection sensitivity. However, considerable sharp and symmetrical peaks of a mixture sample of hemoglobin and cytochrome *c* were observed in this study. The range of the calibration curve of hemoglobin is 10^{-10}–10^{-6} *M* with a very small detection limit of 10^{-10} *M* ($S/N=2$), which was about 10^4 times as sensitive as the conventional CE-absorption detection. The reasons for the satisfactory results might be as follows. First, since all protein samples were migrated at pH 10, which was higher than or equal to the isoelectric points of the proteins, the interaction between protein surfaces and negative charges due to silanol groups on an inner wall capillary must be either very small or negligible. Second, the concentration of protein samples, which was much lower than that used for ordinary spectrophotometric and fluorometric detection, was subjected to the CE–CL detection method. In fact, using basic medium not only inhibits effectively the adsorption of proteins but also matches the conditions of luminol and hydrogen peroxide CL reaction, and therefore this would be an effective approach for separation and ultrasensitive detection of proteins in CE–CL.

More recently, the same group [36] reported CL detection of heme proteins separated by capillary isoelectric focusing. Hydroxypropylmethylcellulose was added to the sample solution in order to effectively reduce the interactions of protein with the capillary wall as well as the electroendoosmotic flow. A luminol–hydrogen peroxide CL system was utilized, and heme proteins such as cytochrome *c*, myoglobin and peroxidase were satisfactorily separated and cytochrome *c* was detected with a detection limit of 6×10^{-9} *M*. However, the retention time (ca. 30 min) seems to be unsatisfactory for rapid measurements.

2.2. Peroxyoxalate

Peroxyoxalate CL (POCL) reactions are based on the hydrogen peroxide oxidation an aryl oxalate ester which produces a high energy intermediate (1,2-dioxetane-3,4-dione). In the presence of a fluorophore, the intermediate forms a charge transfer complex that dissociates to yield an excited-state fluorophore, which then emits a photon. This type of CL reaction may be applied not only to the determination of hydrogen peroxide and many native fluorescent analytes, but also the analytes which can be labeled with fluorescent tags, as well as indirectly, nonfluorescent analytes which quench the reaction. Several groups have utilized POCL detection in CE. Because of the low solubility and instability of oxalate derivatives in aqueous solution, organic solvents are required which would make several problems when they are used in CE. The organic solvents can influence the migration behavior of the analytes and their mobility in the aqueous electrophoretic buffer, and the high separation voltage can affect the stability of the peroxyoxalate reagents. However, the POCL reaction is still one of the most popular reactions in CE–CL system, and several applications have been demonstrated successfully.

The use of the peroxyoxalate reaction for CE detection was first reported in 1991 by Hara et al. [37] for the determination of the dye Eosine Y. Hydrogen peroxide and bis(2,4,6-trichlorophenyl)oxalate (TCPO) in acetonitrile was used as CL reagent,

and introduced post-column. In a subsequent report from the same laboratory [38], the sensitivity of the system was improved by using rhodamine B isothiocyanate (RITC) as a dye, a detection limit of 5×10^{-8} *M* for bovine serum albumin (BSA) was achieved. Hara's group has continued to focus on improving protein determination, using CE combined with on-line POCL detection. By replacing RITC with tetramethylrhodamine isothiocyanate isomer R (TRITC) dye, they obtained a detection limit of 1×10^{-8} *M* for BSA. The detection limit was further improved to 6×10^{-9} *M* with sample stacking [39]. Tsukagoshi et al. [40] developed a simple and convenient batch-type detection cell for CE–CL using peroxyoxalate reagent. A mixture of dansyl-amino acids was satisfactorily separated. Dansyl-Trp was detected with the detection limit of 10 n*M*.

Besides proteins, Tsukagoshi et al. [41] carried out the CE–CL determination of liposomes labeled with Eosine Y and other dyestuffs using the TCPO–H_2O_2–dyestuff system, the detection limit of 8×10^{-8} *M* was achieved. The authors found that the CL method was about 10 times as sensitive as the fluorescent one. Later on, the same group [42] prepared and analyzed various type of dyestuff-containing liposomes by the TCPO–H_2O_2 CL system. Migration behavior of dyestuff-containing liposomes in CE with CL detection was investigated; however, no detection limits for liposomes were reported.

Despite the impressive limits of detection and inherent selectivity afforded by POCL detection, efficient coupling of POCL to CE remains limited by the relatively slow kinetics of the reactions that drive imidazole-catalyzed chemiluminescence. Moreover, oxalate esters, used in POCL, are sparingly soluble in polar solvents and hydrolyze rapidly, presenting an additional challenge with respect to detection following aqueous phase separations. More recently, Kuyper et al. [43] presented a novel method for coupling an ultra-fast POCL reaction to CE. Post separation electrokinetic delivery of the POCL reagent TCPO was accomplished using a commercially available micro tee (Fig. 7). Electrokinetic addition of TCPO allowed for precise control of the ratio of TCPO to the CL reagents 1,2,2,6,6-pentamethylpiperidine (PMP) and 1,2,4-triazole, spiked into the running buffer. This novel method for CL reagent delivery avoided the problems and costs

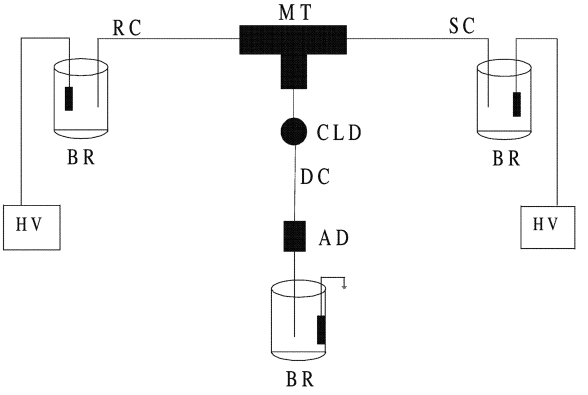

Fig. 7. CE–CL using a micro tee and electrokinetic reagent delivery. HV, high voltage power supply; BR, buffer reservoir; RC, fused-silica reagent capillary; MT, micro tee; SC, fused-silica separation capillary; CLD, chemiluminescence detector; DC, detection capillary; and AD, absorbance detector. Reproduced from Ref. [43] with permission.

associated with using pressure or mechanical pumps to delivery reagents post separation. Use of this dual-component system (PMP and triazole) resulted in intense CL with half-lives of less than 2 s. Optimum conditions for CE–POCL detection were investigated using stopped-flow kinetics. Fig. 8 shows the rapid separation of three model compounds. The detection limit for 3-aminofluoranthene was 9.39×10^{-10} *M*, one of the lowest reported to date. However, a disadvantage for the system is the dead volume of the micro tee (0.029 μl) which leads to peak broadening (Fig. 8).

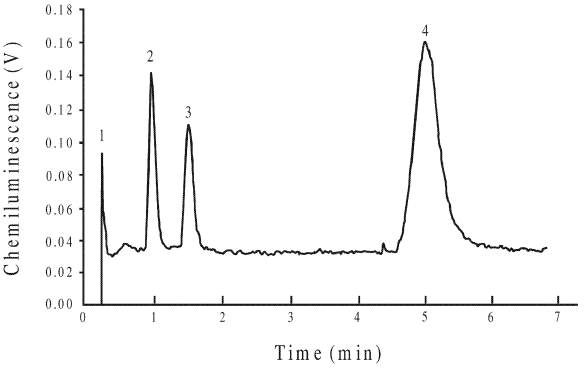

Fig. 8. Electropherogram of three model compounds obtained using CL detection. Reproduced from Ref. [43] with permission.

2.3. Other chemiluminescence reagents

Besides luminol and peroxyoxalate, acridinium esters and firefly luciferase have been also developed for the ultrasensitive CL detection in CE.

Ruberto and Grayeski [28] used the acridinium CL reaction for CE detection, which is based on the oxidation of an acridinium ester by hydrogen peroxide in alkaline medium. A separation of several related acridinium esters has been performed. The detection limit was found to be in the low fmol to upper amol range for acridiniums. Later on, they successfully separated six acridinium-labeled peptides and achieved detection limits in the amol range [44]. Acridinium esters can easily be modified to provide good derivatizing agents for labeling biomolecules, but are easily hydrolyzed above pH 3.

The firefly luciferase reaction is a very efficient bioluminescent reaction in which luciferin reacts with adenosine 5′-triphosphate (ATP) to form adenylluciferin, which subsequently oxidizes to oxyluciferin and emits light. This reaction can be used to determine ATP either directly or coupled with other enzymatic systems. Zare's group [45] demonstrated an end-column CL detector for CE (Fig. 9). The outlet end of the separation capillary was positioned in a buffer reservoir, which contained the CL reagents. Analytes exiting in the capillary initiated a CL reaction. Emission was collected with a fiber optic placed perpendicular to the outlet of the column and detected with a PMT. This system is simple, robust and easily implemented. However, the solution in the post-column reservoir often needs to be renewed to ensure the constant concentration of the CL reagent, and the relatively large detection volume may reduce the separation efficiency. The authors reported a detection limit of 5×10^{-9} M ($S/N=3$) for ATP, which is a factor of 1000–10 000 better than that typically obtained by UV–Vis absorption. The signal is linear over three orders of magnitude in concentration.

3. Conclusion

The combination of CE separation, which is versatile and robust, and CL-based reactions, which are extremely sensitive, is promising for numerous applications in recent years. Obviously, compared with other detection methods widely incorporated in

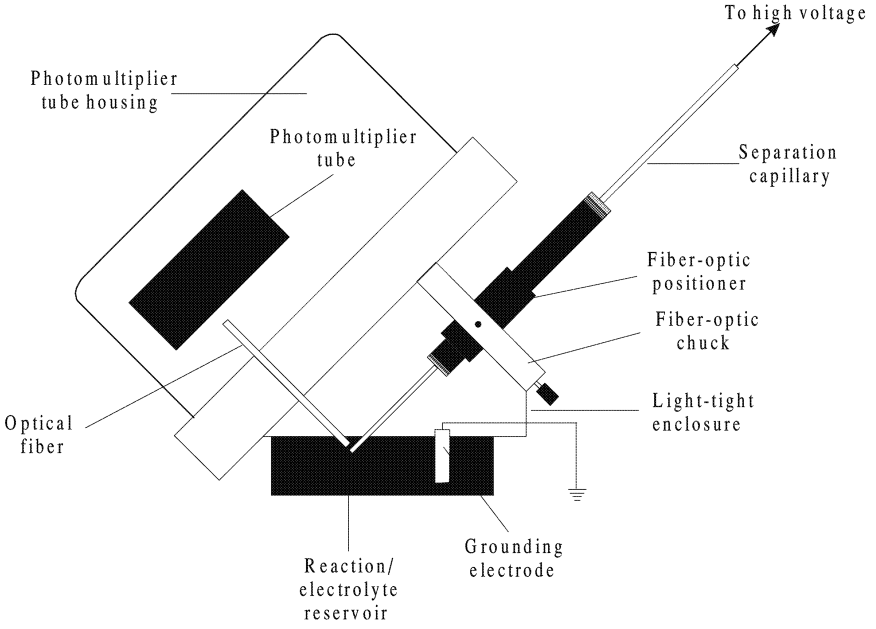

Fig. 9. Schematic of the end-column CL detector for CE. Reproduced from Ref. [45] with permission.

CE, CL detection is an evolving technique. Despite the significant advantages, there are a number of limitations and challenges in CE–CL. So far the number of CL reagents used in CE is still limited, the complicated chemistry involved makes application of these reagents in CE even more difficult. Although many types of reactors (interfaces) have been developed; however, most of them are still in the optimization phase and mainly suited to the fast kinetic reaction. In addition, analysis of real samples in CE–CL is unsatisfactory probably due to matrix interference.

Over the past few years, the micro total analysis system (μ-TAS) has received much attention. As compared with an ordinary CE system, the microchip CE system has advantages as follows: highly analytical speed; low sample and reagent consumption; reduced bench space and potential for the development of portable analytical system. In this respect, Mangru and Harrison [33] have shown the feasibility of performing CL detection on a chip-based system. Tsukagoshi's group has made continuing efforts in microchip CE with CL detection [46–49]. More recently, a flow injection-CE system with CL detection on a chip platform has been developed for the first time by Huang et al. [50]. The performance of the system was illustrated by the separation of Co(II) and Cu(II) using the luminol–hydrogen peroxide CL reaction, achieving baseline separation in 60 s. The detection limits ($S/N=3$) were 1.25×10^{-8} M and 2.3×10^{-6} M for Co(II) and Cu(II), respectively. To date, the concentration sensitivities in the microchip CE detection system were generally lower than that in the conventional CE detection system. However, the chip-based CE–CL systems deserve further exploitation and could become an important research field in the near future.

Future advances in CE–CL should focus on several aspects as follows: improving the CE–CL interface design and signal-to-noise ratio; exploiting new chemiluminescence and bioluminescence reaction with high sensitivity; studying the on-capillary sample stacking techniques and real samples analysis. Field-amplified sample injection (FASI) is a practical and useful on-capillary sample stacking techniques. In order to obtain higher stacking efficiency by FASI, however, the sample should be prepared in a low-conductivity matrix. Therefore, removing the matrix eletrolyte in the sample or convenient preparing the sample in a low-conductivity matrix in real samples analysis is one of research directions. To concentrate the analytes effectively and remove salt and other substances in real sample (e.g., river water), solid-phase extraction (SPE) with C_{18} cartridges prior to separation phenoxy acid herbicides by capillary zone electrophoresis were used recently by Zhu and Lee [51]. Combined with SPE and FASI, the detection limits for the phenoxy acid herbicides as low as 0.01 ng/ml could be achieved by means of a spectrophotometric method. No doubt, this work illustrated an interesting stacking method for ultrasensitive detection in capillary electrophoresis.

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References

[1] A.R. Timerbaev, W. Buchberger, J. Chromatogr. A 834 (1999) 117.
[2] S.E. Moring, R.T. Reel, R.E.J. van Soest, Anal. Chem. 65 (1993) 3454.
[3] T. Wang, J.H. Aiken, C.W. Huie, R.A. Hartwick, Anal. Chem. 63 (1991) 1372.
[4] K. Swinney, D. Bornhop, Crit. Rev. Anal. Chem. 30 (2000) 1.
[5] M. Yamaguchi, H. Yoshida, H. Nohta, J. Chromatogr. A 950 (2002) 1.
[6] W.R.G. Baeyens, B.L. Ling, K. Imai, A.C. Calokerinos, S.G. Schulman, J. Microcol. Sep. 6 (1994) 195.
[7] T.D. Staller, M.J. Sepaniak, Electrophoresis 18 (1997) 2291.
[8] A.M. Garcia-Campana, W.R.G. Baeyens, Y. Zhao, Anal. Chem. 69 (1997) 83A.
[9] M.G. Sanders, K.N. Andrew, P.J. Worsfold, Anal. Commun. 34 (1997) 13H.
[10] Y. Zhang, B. Huang, J.K. Cheng, J. Instrum. Anal. (in Chinese) 17 (1998) 81.
[11] R.H. Zhu, W.T. Kok, J. Pharm. Biomed. Anal. 17 (1998) 985.
[12] X.-J. Huang, Z.-L. Fang, Anal. Chim. Acta 414 (2000) 1.
[13] C. Kuyper, R. Milofsky, Trends Anal. Chem. 20 (2001) 232.
[14] R. Dadoo, L.A. Colon, R.N. Zare, J. High Resolut. Chromatogr. 18 (1992) 133.
[15] M. Hashimoto, T. Nakamura, K. Tsukagoshi, R. Nakajima, K. Kondo, Bull. Chem. Soc. Jpn. 72 (1999) 2673.

[16] M. Hashimoto, K. Tsukagoshi, R. Nakajima, K. Kondo, J. Chromatogr. A 832 (1999) 191.
[17] M. Macka, P.R. Haddad, Electrophoresis 18 (1997) 2482.
[18] B.F. Liu, L.B. Liu, J.K. Cheng, J. Chromatogr. A 834 (1999) 277.
[19] B. Huang, J.J. Li, J.K. Cheng, Chem. J. Chin. Univ. 17 (1996) 528.
[20] B. Huang, J.J. Li, J.K. Cheng, Chin. J. Chromatogr. 13 (1995) 430.
[21] B. Huang, J.J. Li, L. Zhang, J.K. Cheng, Anal. Chem. 68 (1996) 2366.
[22] Y. Zhang, Z.L. Gong, H. Zhang, J.K. Cheng, Anal. Commun. 35 (1998) 293.
[23] E.B. Liu, Y.M. Liu, J.K. Cheng, Anal. Chim. Acta 443 (2001) 101.
[24] E.B. Liu, Y.M. Liu, J.K. Cheng, Anal. Chim. Acta (in press).
[25] Y.M. Liu, E.B. Liu, J.K. Cheng, J. Chromatogr. A 939 (2001) 91.
[26] Y.M. Liu, J.K. Cheng, Electrophoresis 23 (2002) 556.
[27] D.J. Rose, J.W. Jorgenson, J. Chromatogr. 447 (1988) 117.
[28] M.A. Ruberto, M.L. Grayeski, Anal. Chem. 64 (1992) 2758.
[29] J. Zhao, J. Labble, N.J. Dovichi, J. Microcol. Sep. 5 (1993) 331.
[30] Y. Zhang, B. Huang, J.K. Cheng, 363 (1998) 157.
[31] J.C. Ren, X.Y. Huang, Anal. Chem. 73 (2001) 2663.
[32] M.F. Regehr, F.E. Regnier, J. Cap. Electrophoresis 3 (1996) 117.
[33] S.D. Mangru, D.J. Harrison, Electrophoresis 19 (1998) 2301.
[34] Y. Zhang, Ph.D. Thesis, University of Wuhan, Wuhan, 1999.
[35] K. Tsukagoshi, S. Fujimura, R. Nakajima, Anal. Sci. 13 (1997) 279.
[36] M. Hashimoto, K. Tsukagoshi, R. Nakajima, K. Kondo, J. Chromatogr. A 852 (1999) 597.
[37] T. Hara, S. Okamura, S. Kato, J. Yokogi, R. Nakajima, Anal. Sci. 7 (Suppl.) (1991) 261.
[38] T. Hara, H. Nishida, S. Kayama, R. Nakajima, Bull. Chem. Soc. Jpn. 67 (1994) 1193.
[39] T. Hara, H. Nishida, S. Kayama, R. Nakajima, Bull. Chem. Soc. Jpn. 67 (1994) 1193.
[40] K. Tsukagoshi, Y. Okumura, H. Akasaka, R. Nakajima, T. Hara, Anal. Sci. 12 (1996) 869.
[41] K. Tsukagoshi, M. Otsuka, M. Hashimoto, R. Nakajima, H. Kimoto, Chem. Lett. (2000) 98.
[42] K. Tsukagoshi, Y. Okumura, R. Nakajima, J. Chromatogr. A 813 (1998) 402.
[43] C. Kuyper, K. Denham, J. Dickson, J. Murray, R. Milofsky, Chromatographia 53 (2001) 173.
[44] M.A. Ruberto, M.L. Grayeski, J. Microcol. Sep. 6 (1994) 545.
[45] R. Dadoo, A.G. Seto, L.A. Colon, R.N. Zare, Anal. Chem. 66 (1994) 303.
[46] M. Hashimoto, K. Tsukagoshi, R. Nakajima, K. Kondo, A. Arai, Chem. Lett. (1999) 781.
[47] K. Tsukagoshi, M. Hashimoto, R. Nakajima, A. Arai, Anal. Sci. 16 (2000) 1111.
[48] M. Hashimoto, K. Tsukagoshi, R. Nakajima, K. Kondo, A. Arai, J. Chromatogr. A 867 (2000) 271.
[49] K. Tsukagoshi, M. Hashimoto, T. Suzuki, R. Nakajima, A. Arai, Anal. Sci. 17 (2001) 1129.
[50] X.J. Huang, Q.S. Pu, Z.L. Fang, Analyst 126 (2001) 281.
[51] L.Y. Zhu, H.K. Lee, Anal. Chem. 73 (2001) 3065.