Focusing Review

Chiral Separations Using Avidin as a Chiral Selector and Highly Sensitive Detection Using Thermal Lens Microscopy in Capillary Electrophoresis

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Abstract

A brief review of the author's studies concerning the fundamental characteristics of micellar electrokinetic chromatography (MEKC) and its application along with fundamental and application studies on capillary electrophoresis (CE) as well as fundamental studies on microchip electrophoresis, from the viewpoint of the high performance separation technique, is described. Only limited topics from the authors' investigations, such as chiral separations by MEKC and affinity capillary electrochromatography and highly sensitive detection schemes including on−line sample concentration techniques and the use of thermal lens microscopy in CE and MEKC, are presented.

Keywords: capillary electrophoresis, micellar electrokinetic chromatography, capillary electrochromatography, microchip electrophoresis, chiral separation, on−line sample concentration, thermal lens microscopy.

1. Introduction

During last two decades, capillary electrophoresis (CE) has become popular as a high−resolution separation method. It has been recognized that CE was first introduced by Mikkers et al. [1], Jorgenson and Lukacs [2], and Hjertén [3], as an automated instrumental version of electrophoresis, following the earlier studies [4, 5]. Generally only ionic or charged solutes could be analyzed by CE in principle. However, the development of micellar electrokinetic chromatography (MEKC) [6, 7] has successfully overcome such problems. The principle of MEKC is based upon chromatography using homogeneous solutions containing an ionic "pseudo-stationary phase" or an ionic micelle, in which both neutral and charged analytes can be separated electrophoretically. Several modes of MEKC-like techniques or electrokinetic chromatography (EKC) [8] have been available, where some kinds of pseudo-stationary phases are employed. Among these, however, MEKC is still the most popular technique for the separation of small neutral molecules as well as ions by CE.

The author and co-workers have continuously made fundamental and application studies on EKC as well as CE since the introduction of MEKC in early 1980 s: The key subjects investigated in the early stage, for example, were as follows: (1) derivation of the equations describing the relationship between the migration time and the retention factor and resolution [6, 7]; (2) theoretical and experimental considerations of parameters affecting resolution and separation performance [6, 7, 9−11]; (3) studies on parameters, such as the structure of the surfactants, pH, and temperature, affecting the selectivity [7, 12]; (4) comparison of the structures of the surfactants in terms of the separation characteristics [7, 13]; (5) measurements of the thermodynamic parameters for the micellar solubilization process [7, 14]; (6) effects of the additives to the micellar solutions [15−17]; (7) separations of closely related compounds, *e.g.*, phenylthiohydantoin-amino acids [13], chlorinated phenols [18], and aromatic sulfides [19], (8) quantitation in MEKC [20], (9) chiral separations by CE and EKC [15, 21−25], and by capillary electrochromatography (CEC) [26−29]. Studies on developments of several on-line sample concentration techniques [30−34] and on-line coupling of mass spectrometry (MS) with MEKC (MEKC−MS) and CE (CE−MS) [35−40], and the use of thermal lens microscopy (TLM) as a high-sensitive detection scheme have also been carried out [41]. Recently, the author's interests have been extended to the separation and analysis in smaller fields, or micro and nano scale analytical systems, such as microchip electrophoresis (MCE) and microchip sensing device [42−49].

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This paper briefly overviews recent works of the author's group mainly focused on chiral separations by CEC and novel detection scheme for CE or CE−TLM.

2. Chiral separations

2.1. Chiral separation by MEKC

Chiral separation is one of major objectives of CE as well as MCE and a number of successful studies has been reported [50−54]. As for MEKC, mainly following two modes are employed in chiral separations: (1) MEKC using chiral micelles and (2) cyclodextrin (CD) modified MEKC (CD−MEKC).

In the former system, an ionic chiral micelle is used as a chiral pseudostationary phase. When a pair of enantiomers is injected to the MEKC system, each enantiomer is incorporated into the chiral micelle. However, the equilibrium constant for each enantiomer is expected to be different more or less among the enantiomeric pair; that is, the degree of solubilization of each enantiomer into the chiral micelle would be different each other and hence, the difference in the retention factor would be obtained, resulting a different migration time.

While in the latter system or CD−MEKC, an ionic achiral micelle, *e.g.*, sodium dodecyl sulfate (SDS), and a neutral CD are typically used as a pseudostationary phase and a chiral selector, respectively. At the time of the introduction of a pair of enantiomers into the system, two major distribution equilibria are considered for the enantiomers, namely (1) the equilibrium between the aqueous phase and the micelle, *i.e.*, the micellar solubilization and (2) the equilibrium between the aqueous phase and CD, *i.e.*, the inclusion complex formation. Each enantiomer may have a different equilibrium constant for the inclusion complex formation among the enantiomeric pair due to the enantioselectivity of the CD. Consequently each enantiomer exists in the aqueous phase at a different time among the enantiomeric pair, so that the time spent in the micelle would be varied. In some cases, additionally, an ionic chiral micelle, *e.g.*, a bile salt, is also used as a chiral pseudostationary phase with a CD.

Moreover, cyclodextrin electrokinetic chromatography (CDEKC), where a CD derivative having an ionizable group is used as a chiral pseudostationary phase, has become popular in recent since several commercially available ionic CD derivatives have appeared. Although the CDEKC technique is actually beyond the field of MEKC, it is important method for enantiomer separation by CE.

2.2. Chiral separation by affinity CEC

CEC, the hybrid method of CE and HPLC, is also a useful technique for the chiral separation owing to the high efficiency of capillary electromigration and the high selectivity of chromatography. As well as affinity CE, affinity CEC using proteins as chiral selectors has been employed for enantioseparations. In affinity CEC, mainly following three modes are used: (1) packed CEC (P−CEC) [55,56], (2) monolithic CEC (M−CEC) [29, 57], and (3) open tubular CEC (OT−CEC) [28, 58]. In P−CEC, preparing a frit in the capillary to dam the packing materials is sometimes troublesome, whereas in M−CEC the preparation of capillaries is time−consuming for 2−4 days. In OT−CEC, however, the preparation of the stationary phase of proteins onto the inner wall of the capillary can be achieved by simple modification procedures. Although a low phase ratio in OT−CEC, relatively shorter analysis time compared to P−CEC and M−CEC is expected and thus, higher separation efficiency can be achieved in chiral separations. A short conditioning time, reproducible column preparation, and easy operation are also advantageous in OT−CEC. The immobilization of proteins onto the capillary surface for OT−CEC has been attained by two methods: physical adsorption (dynamic coating) and covalent binding methods. Generally, in the former method the stability of the prepared capillary is not good due to a significant loss of the stationary phase, while in the latter better stability can be expected. Only a few groups, however, have reported the use of the proteins chemically immobilized capillaries for the chiral affinity CEC analysis.

2.2.1. OT−CEC chiral separation

To obtain a stable stationary phase with easy manipulations, avidin, a basic protein, was covalently fixed on the inside wall of the capillary by Schiff base formation reaction [59, 60] and the prepared capillary was applied to the OT−CEC enantioseparation [61]. Under the optimized condition, the successful chiral separation of abscisic acid was attained within 10 min, as shown in Figure 1 [61]. Effects of pH and organic additives on the OT−CEC separation efficiency revealed that both the electrostatic and the hydrophobic interactions between the analytes and avidin play an important roll in the chiral separation of these racemic acidic compounds. The longer lifetime of the capillaries, for 50 days with over 100 runs, in comparison with the physical adsorption method shows that the loss of avidin suppressed by the immobilization through the covalent bond. The prepared capillary was useful for the MS detection because of the stable immobilization of protein or less desorption from the capillary surface. The modification technique can be applied to a microchannel on a quartz chip to achieve a high speed electrochromatographic enantioseparation.

2.2.2. P−CEC chiral separation

The use of magnetic polymer particles (MPs) was investigated to fabricate a capillary for the P−CEC analysis [62]. The MPs could be retained with the magnetic field in the capillary even ap-

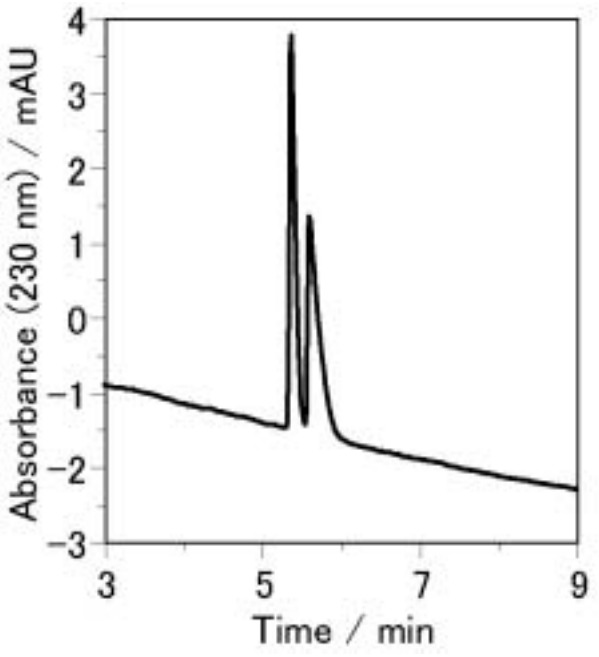

Figure 1. CEC separation of abscisic acid enantiomers using capillaries prepared with avidin concentrations of 2.0 mg/mL. Capillary, 50 cm (effective length, 41.5 cm) × 50 μm I.D.; BGS, 10 mM phosphate buffer (pH 5.0); sample, 0.1 mg/mL abscisic acid; injection, 50 mbar, 3 s; separation, −20 kV; detection, 230 nm; temperature, 25 ℃.

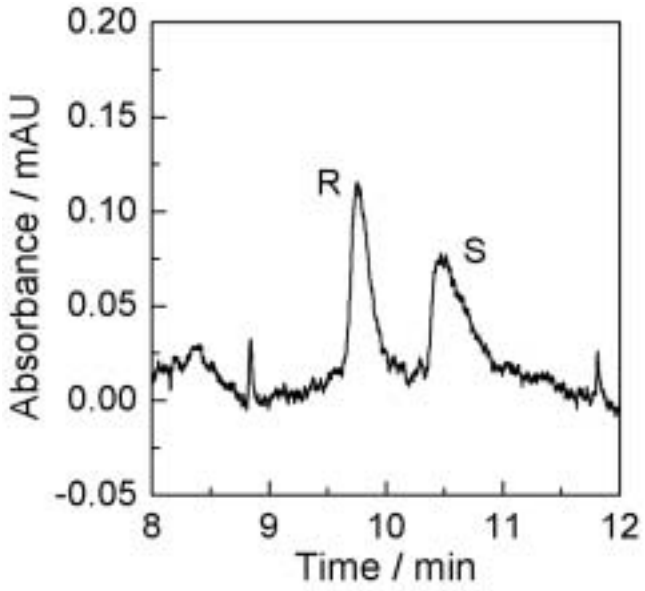

Figure 2. Enantioseparation of ketoprofen by affinity P−CEC with avidin immobilized MPs: Capillary, 52 cm×50 μm I.D. (30 cm to the detector); packed length, 10.0 cm; BGS, 10 mM acetate buffer (pH 5.0); avidin concentration for immobilization, 5.0 mg/mL; detection wavelength, 200 nm; applied voltage, −10 kV; sample, 2.5 μg/mL ketoprofen.

plying high voltage for the CE separation, so that the fritless capillary for P−CEC can be easily prepared and the length of the packed MPs zone also be easily adjusted by changing the magnet length. By employing the fritless avidin coated MPs packed capillary, the chiral separation of ketoprofen was successfully attained with the packing length of 10 cm, as shown in Figure 2 [62]. Effects of the modification condition of avidin, pH of the separation solution, and the packing length on the enantioseparation were investigated. Under the optimal condition the repeatability for the retention time of ketoprofen was better than 1.5% in the relative standard deviation and the capillary-to-capillary reproducibility was also acceptable in the prepared fritless capillaries.

3. Highly sensitive detection schemes

3.1. On−line sample concentration

The low concentration sensitivity in CE is an inherent defect, which is caused by the limited volume of sample that can be injected into the capillary and the narrow optical pathlength for on capillary detection in case of the absorbance detection. By using sample stacking technique, this problem can be successfully reduced in CZE. The sample stacking phenomenon is caused by the different mobilities of the target ions across a boundary, which separates regions of high and low electric fields. Sample stacking in MEKC, however, is not as straightforward as in CZE, since a neutral analyte is not affected by the enhanced electric field. We have achieved several methods for on-line sample concentrations for neutral analytes in MEKC using low conductivity solutions containing no micelles to prepare sample solutions: (1) the hydrodynamic sample injection mode, including normal stacking mode (NSM) [63], reversed electrode polarity stacking mode (REPSM) [64], stacking with reversed migrating micelles (SRMM) [65], and stacking using reverse migrating micelles and a water plug (SRW) [30]; (2) the electrokinetic sample injection mode, including field enhanced sample injection (FESI) [66] and field enhanced sample injection with reverse migrating micelles (FESI−RMM) [67]. It has been found that sample stacking of neutral analytes under acidic conditions, *i.e.*, SRMM, SRW, and FESI−RMM, offers several advantages over those in neutral conditions, *i.e.*, NSM, REPSM, and FESI. By using these concentration techniques, high sensitivity enhancement factors, *e.g.*, 1000-fold increase in detection sensitivity, are easily obtained and optimization schemes are simply encountered.

The development of sweeping has successfully introduced a remarkable enhancement of the detection sensitivity, typically as 5000 to 100000-fold increase in detectability [68, 69]. Fundamental and application studies on sweeping have been extensively reported. The combination of sweeping with stacking or sweeping with the dynamic pH junction technique has been also investigated

to achieve further enhanced sensitivity [70−72].

3.2. Thermal lens microscopy

As one of powerful schemes improving the detection sensitivity in CE, the use of TLM has been investigated as a universal ultra −high−sensitive detector. In the TLM detection scheme, the key to achieve a higher sensitivity is to match the excitation and emission volumes to the separation system, which will provide a low scattering and luminescence background. As well as a confocal microscopic fluorometric detection, the focusing of laser beams on the capillary can facially align in the TLM detection. Although a simple way to make a CE−TLM system possible is the use of the on-capillary method, optical configurations of TLM become complicated since the capillary has a curved surface. The reduce of both the sensitivity and reproducibility will be caused by the complicated refraction, reflection, and scattering of the two laser beams on the capillary surface. To solve the problems, an interface chip (IFChip), which has a flat surface and is connected to the separation capillary for the CE separation, has been introduced [73, 74]. The IFChip was used as a detection window for TLM, so that a sufficient separation efficiency and 100-times higher detection sensitivity than that obtained with CE−UV analysis were achieved since the complicated optical configuration by curved surface of the capillary was improved by using the microchannel with a semicircular shape. An extended study on the CE−IFChip−TLM system has been carried out to make highly sensitive detection scheme possible for the separation of nonfluorescent and neutral analytes by the MEKC mode, in combination with sweeping to obtain further improvement of the concentration sensitivity [41]. The schematic illustration of the CE−IFChip−TLM system is shown in Figure 3, and a typical electrokinetic chromatogram of Sudan R obtained by a sweeping−MEKC condition with the IFChip−TLM system is shown in Figure 4, where dilute sample was introduced with an injection length of 258 mm under an acidic condition (pH 3.0). The value of sensitive enhancement factor (SEF_{height}) was calculated by comparing the peak height obtained in the sweeping condition with that in the conventional MEKC taking into account the dilution factor regardless of the injection volume of the sample solution. The obtained SEF_{height} was ca. 3.9×10^{6} under the acidic condition. Even though the sample amount injected was considered, the concentration efficiency was estimated to be as low as 18000 and hence, an ultra sensitive detection of nonfluorescent compounds was successfully achieved by sweeping−MEKC with the IFChip−TLM system, due to both high spatial resolution of TLM (~μm) and high retention factor of neutral and hydrophobic azo dyes. Further investigations toward the ultrahigh-sensitive detection by using TLM in both CE and MCE are still proceeded in combination with novel separation media and detection fields.

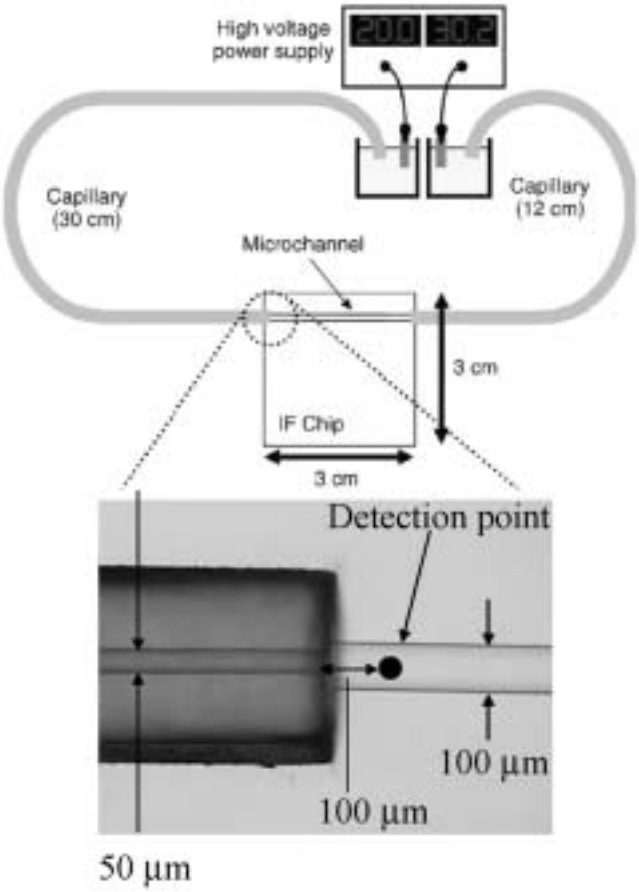

Figure 3. Schematic illustration of the CE−IFChip system for TLM detection.

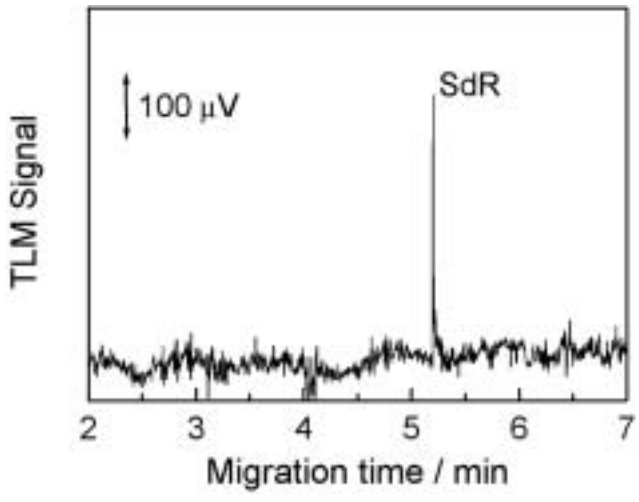

Figure 4. Sweeping−MEKC−IFChip−TLM detection of Sudan R (SdR) at the concentration of 1.8×10^{-12}M under the acidic condition. Data acquisition was started after sample injection. BGS, 1.5×10^{-2}M SDS (pH 3.0); sample matrix, no SDS (pH 3.0) with a conductivity similar to that of the BGS; applied voltage, −20 kV; injection, hydrodynamically 15 cm for 1100 s (injected length, 258 mm).

4. Conclusion

The author has been studying on fundamentals and applications of MEKC since its invention and thinks that MEKC has obviously become one of major modes in CE. As a proof of this fact, Zare found all those articles having the words “Micellar Electrokinetic Chromatography” or “MEKC” in its title or abstract since 1990, as shown in Table 1 [75]. Zare also said, “this selection does not gauge the full impact of MEKC, but it does serve as an interesting measure”. Continuous studies on high performance microscale separation methods not only by MEKC but also by CE, CEC, and MCE have been carried out in the author’s research groups. In this focusing review, however, only his recent typical studies on CE, *i. e.*, chiral separations by affinity CEC techniques using avidin as a chiral selector and highly sensitive detection methods for CE employing on-line sample concentration techniques and TLM detection, are briefly described.

At the present stage, many papers and review articles on MEKC as well as CE and MCE are available, where fundamental characteristics, theoretical treatments, and applications are described from various points of view, so that one can easily refer to those references when detailed information is required.

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Table 1. Number of articles on MEKC published since 1990 in each period [a]

Period	Number of Articles
1990−1991	58
1990−1992	102
1990−1993	179
1990−1994	298
1990−1995	438
1990−1996	591
1990−1997	827
1990−1998	1022
1990−1999	1240
1990−2000	1461
1990−2001	1699
1990−2002	1957
1990−2003	2245
1990−2004	2466
1990−2005	2633

[a] Listed by Zare [75].

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