Capillary Separation: Micellar Electrokinetic Chromatography

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Key Words

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Abstract

Micellar electrokinetic chromatography (MEKC), a separation mode of capillary electrophoresis (CE), has enabled the separation of electrically neutral analytes. MEKC can be performed by adding an ionic micelle to the running solution of CE without modifying the instrument. Its separation principle is based on the differential migration of the ionic micelles and the bulk running buffer under electrophoresis conditions and on the interaction between the analyte and the micelle. Hence, MEKC's separation principle is similar to that of chromatography. MEKC is a useful technique particularly for the separation of small molecules, both neutral and charged, and yields high-efficiency separation in a short time with minimum amounts of sample and reagents. To improve the concentration sensitivity of detection, several on-line sample preconcentration techniques such as sweeping have been developed.

1. INTRODUCTION

Electrophoresis is a means of separating charged materials under the influence of an electric field. This technique has been employed since the early nineteenth century, but it did not attract much attention until Tiselius (1) invented the moving boundary method in 1930. Thereafter, several electrophoresis techniques have been developed for the separation of proteins, polynucleotides, and other biopolymers.

There is a serious problem in electrophoresis: the thermal convection caused by Joule heating, which deteriorates separation by mixing the separated zones. To prevent this adverse effect, one can use several types of supporting materials, such as gels, films, and papers. Hjertén (2) developed a technique of free-zone electrophoresis using a rotating quartz tube with an inner diameter (i.d.) of 3 mm, an outer diameter (o.d.) of 7.8 mm, and a length of 36 cm. The rotation of the capillary at 40 rpm effectively prevented the solution inside the capillary from convection, but this technique was not widely accepted due to difficult operations. Capillary isotachophoresis (ITP) is another type of free-zone electrophoresis (3) that employs a narrow-bore tube of glass or polytetrafluoroethylene with an i.d. of 0.4 to 0.6 mm. Thermal convection is suppressed inside the narrow-bore capillary, and detection of zone boundaries in ITP is less affected by convection. Capillary ITP is used mainly for the separation of inorganic or small organic ions.

Free-zone electrophoresis in a capillary was developed independently by three research groups (4–6). In particular, Jorgenson & Lukacs (6) attracted much attention among separation analytical chemists by performing a separation with 4,000,000 plates for dansyl-derivatized amino acids in less than 25 min. The authors used a Pyrex™ capillary with a 75-μm i.d., a 550-μm o.d., and a length of 80 to 100 cm, and they applied a voltage of +30 kV. The electroosmotic flow (EOF), which is generated as an electrokinetic phenomenon by the charge on the inside surface and by an applied electric field along the capillary axis, was utilized to carry analytes (including negatively charged ones) toward the cathode. EOF is a pluglike flow, and it helps to shorten analysis time without adversely affecting separation efficiency. However, EOF must be suppressed in capillary ITP and in capillary isoelectric-focusing techniques.

Free-zone electrophoresis performed in a capillary is generally known as capillary zone electrophoresis (CZE). In this technique, analytes migrate toward the cathode due to strong EOF when the capillary surface is negatively charged, which occurs under both neutral and alkaline conditions. The migration velocity is based primarily on the ratio of the charge to the molecular size.

The idea of separating neutral analytes by electrophoresis using ionic micelles was proposed by Nakagawa (7) in 1981, and this approach was successfully demonstrated in 1984 via capillary electrophoresis (CE) (8). The separation principle of this technique is schematically shown in **Figure 1** (9). In this approach the micelle is added to the background solution (BGS) of CZE, and it migrates by electrophoresis under an electric field. The migration velocity of the micelle is different from that of the bulk aqueous phase due to an electrophoretic migration, whereas the bulk solution migrates by EOF. As in CZE, even a negatively charged micelle migrates toward the cathode due to the strong EOF under either neutral or alkaline conditions. The neutral analyte usually migrates at the same velocity as does the bulk solution; hence, no separation occurs. When the micelle is added to the solution, a fraction of the analyte is incorporated into the micelle and migrates at the micelle's velocity. Upon the interaction between the analyte and the micelle, known as micellar solubilization, the reaction quickly reaches an equilibrium state. Therefore, the migration velocity of the neutral analyte depends on the fraction of the analyte incorporated by the micelle.

This method, now known as micellar electrokinetic chromatography (MEKC) (10), has been investigated from the viewpoint of chromatography because the separation principles of the two

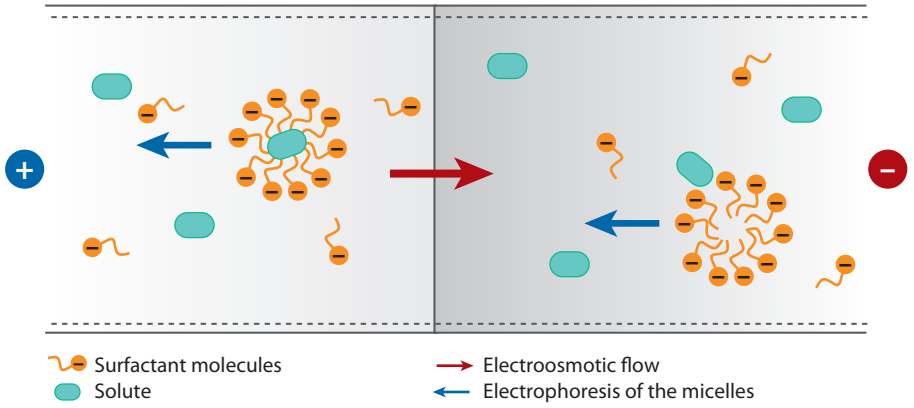

Figure 1

Schematic illustration of the separation principle of micellar electrokinetic chromatography (MEKC). Modified from Otsuka & Terabe (9) with permission. Copyright 1989, Chemical Society of Japan.

techniques are similar. The micelle acts as the stationary phase in chromatography and is here termed the pseudostationary phase (PSP). The surrounding running solution acts as the mobile phase, although the micelle is not stationary inside the capillary but rather migrates. The name electrokinetic chromatography (EKC) refers to the uses of PSP in CE; examples include microemulsions, charged cyclodextrins, polymer ions, and particles other than micelles (10). MEKC is now widely utilized mainly for the separation of small molecules, both charged and neutral. Several books, book chapters, and reviews about MEKC have been published (10–17), as have summaries of recent advances in MEKC (18–20). The fundamental studies of MEKC are relatively mature, and many new applications have been developed. In this review, I survey the fundamental characteristics of MEKC primarily from the viewpoint of recent work. Note that capillary electrochromatography (CEC), which uses EOF to deliver the mobile phase via open tubular or packed capillaries, differs in principle from EKC, although both techniques can separate neutral analytes.

2. FUNDAMENTAL CHARACTERISTICS

2.1. Separation Principle

In chromatography, the ionic micelle functions as the stationary phase, and the surrounding aqueous solution acts as the mobile phase. The micellar solubilization is the partition mechanism. The micelle is the molecular aggregate of the surfactant molecules formed in dynamic equilibrium with single molecules, and it cannot be isolated. The size of the micelle is relatively uniform, and it is distributed homogeneously in the mobile phase. The migration behavior of an imaginary mixture of water, a neutral analyte, and a micelle is schematically shown in **Figure 2** (21). Here, the neutral analyte is assumed to be equally distributed between the micelle and the surrounding aqueous phase. As shown in **Figure 2*a***, water migrates rapidly at the EOF velocity, and the micelle migrates slowly due to the resistance caused by electrophoresis in the opposite direction. The neutral analyte migrates at an average velocity equivalent to those of EOF and the micelle. The three components in the imaginary mixture are assumed to be detectable, resulting in an electropherogram or chromatogram (**Figure 2*b***).

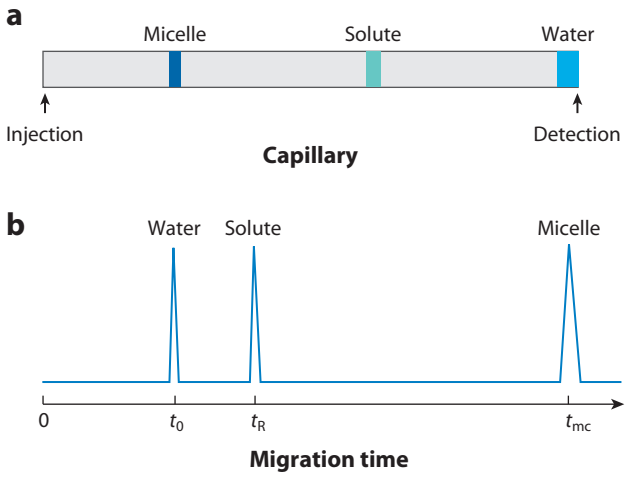

Figure 2

Migration behaviors (*a*) and micellar electrokinetic chromatography (MEKC) electropherogram (*b*) of an imaginary mixture of water, solute and micelle. t_0, t_R, and t_{mc} are the migration times of the water (or electroosmotic flow marker), solute, and micelle, respectively. The solute is assumed to be equally distributed between the micelle and the aqueous phase. Modified from Terabe et al. (21) with permission. Copyright 1985, American Chemical Society.

The retention factor, k, can be defined as

$$k = n_{mc}/n_{aq}, \tag{1}$$

where n_{mc} is the amount of the analyte incorporated into the micelle, and n_{aq} is the amount free of the micelle or the amount in the aqueous phase. Note that in the following discussion, the analyte is assumed to be neutral. The migration time (retention time in chromatography) can be given by (8)

$$t_R = \frac{1+k}{1+(t_0/t_{mc})k} t_0, \tag{2}$$

where t_0 and t_{mc} are the migration times of the EOF marker and the micelle marker, respectively (**Figure 2*b***). Methanol is often used as an EOF marker, and Sudan III or IV is used as a micelle marker. Equation 2 shows that the range of the migration time of the neutral analyte is limited to between t_0 ($k = 0$) and t_{mc} ($k = \infty$). A typical electropherogram of a MEKC separation of neutral analytes is shown in **Figure 3** (8). All of the analytes shown are assumed to be neutral at pH 7.0 from the dissociation constant of each analyte, and they are detected between two marker peaks (methanol and Sudan III). This migration time range is known as the migration time window. The retention factors of these analytes are also given in **Figure 3**. When EOF is completely suppressed, Equation 2 gives

$$t_R = (1 + 1/k)t_{mc}. \tag{3}$$

In the absence of EOF, the micelle migrates through the surrounding aqueous phase, although it corresponds to the stationary phase in conventional chromatography. In this case, we can assume that the micelle is the mobile phase and that the aqueous phase is the stationary phase.

From an electrophoretic point of view, the electrophoretic velocity in MEKC is modified by the micellar additive in BGS (22). Under CE conditions, the ionic micelle migrates via both electrophoresis and EOF. The migration velocity of the micelle v_{mc} differs from that of the bulk flow

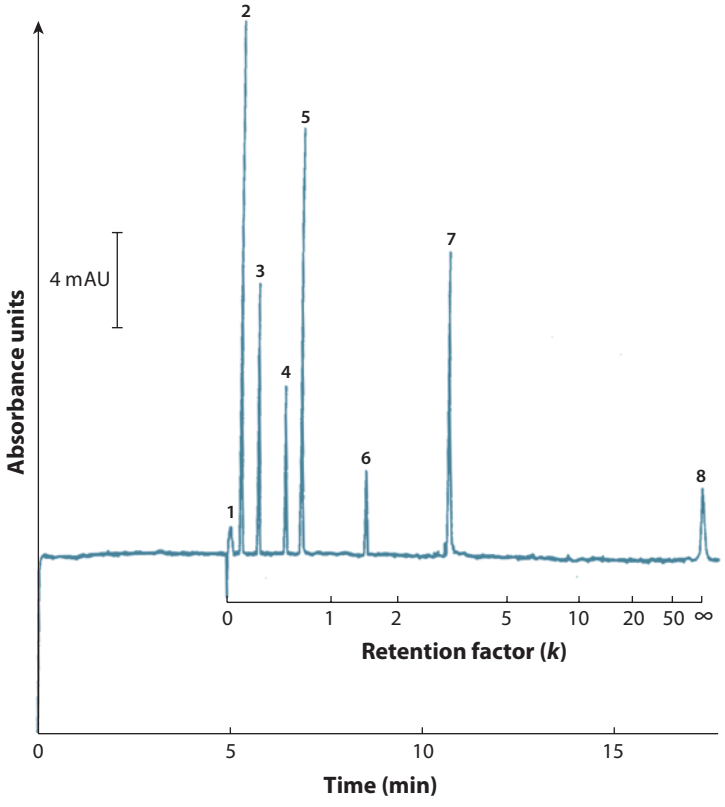

Figure 3

Micellar electrokinetic chromatography (MEKC) separation of standard neutral compounds. 1, methanol; 2, resorcinol; 3, phenol; 4, *p*-nitroaniline; 5, nitrobenzene; 6, toluene; 7, 2-naphthol; 8, Sudan III. Conditions: capillary, 50 μm inner diameter × 65 cm (50 cm to detector); running solution, 30 mM sodium dodecyl sulfate in 50 mM phosphate/100 mM borate buffer (pH 7.0); applied voltage, 15 kV; current, 33 μA; detection, ultraviolet absorbance at 210 nm; temperature, 35° C. Modified from Terabe et al. (21) with permission. Abbreviation: AU, absorbance units. Copyright 1985, American Chemical Society.

or EOF velocity v_{eo} by the electrophoretic velocity of the micelle, $v_{ep}(mc)$. That is, the equation

$$v_{mc} = v_{eo} + v_{ep}(mc) \tag{4}$$

holds. The velocity is a vector quantity and is positive when directed toward the cathode and v_{eo} and $v_{ep}(mc)$ have generally different signs. The migration velocity of the neutral analyte v_a can be modified as

$$v_a = (v_{eo} + v_{mc}k)/(1 + k), \tag{5}$$

where $1/(1 + k)$ and $k/(1 + k)$ are the fraction of the analyte free from the micelle and the fraction incorporated into the micelle, respectively. The velocity of the analyte is limited between v_{eo} ($k = 0$) and v_{mc} ($k = \infty$), as shown in Equation 5. The migration time of the analyte can be obtained by dividing l, the effective length of the capillary from the injection end to the detection point, by v_a, which yields Equation 2 because $l/v_{eo} = t_0$ and $l/v_{mc} = t_{mc}$.

2.2. Experimental Aspects

MEKC can be performed with a conventional CE instrument as CZE simply by using a micellar solution as BGS. To prepare the micellar solution, an ionic surfactant is dissolved into a buffer at a concentration higher than the critical micelle concentration (CMC). The CMC is a free surfactant concentration in equilibrium with the micelle; it is equal to the surfactant concentration, above which the surfactant forms the micelle. It can be measured by electrical conductivity, surface tension, light scattering, spectrophotometry, spectrofluorimetry, cyclic voltammetry, nuclear magnetic resonance, and CE (23). The typical CMC values of several surfactants in pure water have been published in various handbooks; however, CMC depends on several experimental parameters such as temperature, solvent, and additive. For example, the CMC of sodium dodecyl sulfate (SDS), the most popular surfactant employed in MEKC, is ~8 mM in pure water but can range from 2.8 to 6.4 mM, depending on buffers and temperature (24). Therefore, experimental conditions must be kept constant to obtain reproducible data. Micelle polymers are surfactants that show micellar characteristics within a single molecule, and their CMCs are zero. Several micelle polymers have been successfully used as PSPs in MEKC (25, 26), but there is no evidence that using a micelle polymer produces more reproducible results than those obtained when conventional surfactants are employed.

MEKC separations are performed inside the capillary. The inside volume of a 50-μm-i.d., 50-cm-long capillary is ~1 μL; thus, the sample volume injected must be less than a few nanoliters. Usually, untreated or bare fused-silica capillaries are employed in MEKC. The inside surface of the capillary is negatively charged due to ionization of the silanol group above pH ~2, and the EOF points toward the cathode. The EOF is stronger than the electrophoretic migration of an anionic micelle such as SDS; therefore, the micelle also migrates toward the cathode under neutral or alkaline conditions. However, if a cationic surfactant micelle is used as a PSP, the EOF points toward the anode because of the positively charged surface, which results from adsorption of a cationic surfactant such as cetyltrimethylammonium bromide (CTAB) (27). The positively charged surface is formed via the double-layer adsorption of the surfactant on the silica surface, first by the electrostatic interaction between the cationic group of the surfactant and the ionized silanol group, and second by the hydrophobic interaction of alkyl chains between the adsorbed and free surfactants in the solution (28). To suppress EOF, capillaries inside-coated with polyacrylamide or polyethylene glycol can be used, but it is difficult to completely suppress EOF with cationic surfactants.

Photometric detection is generally used when the surfactant solution does not significantly absorb ultraviolet light. Because the peak (zone) volume of the separated analyte is very small (e.g., a few nanoliters), the detection must be performed on capillary. The detection sensitivity of a photometric detector is not high in terms of concentration (i.e., above the micromolar range). Laser-induced fluorescence (LIF) detection is very sensitive, and it can detect concentrations down to the nanomolar scale. The micelle can enhance this LIF sensitivity. Electrochemical detection is another sensitive technique that is particularly suitable for narrow-bore capillaries measuring less than 10 μm i.d. Mass spectrometry (MS) is an indispensable detection method for CE, as well as for gas and liquid chromatography. Several interfaces for CE-MS are available, but MS cannot be routinely used in MEKC due to the presence of significantly high concentrations of nonvolatile surfactant molecules. [Although several techniques (29–31) to circumvent the problems have been developed, I do not discuss MEKC-MS herein.] To improve concentration-detection sensitivity, several on-line sample-preconcentration techniques have been developed; below, I discuss the techniques that are applicable to neutral analytes.

2.3. Resolution Equation

The resolution equation in MEKC for neutral analytes can be given by (21)

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_2}\right)\left(\frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})k_1}\right), \quad (6)$$

where R_s is resolution, N is the theoretical plate number, and α is the selectivity factor defined by k_2/k_1 ($k_2 \geq k_1$), where subscripts 2 and 1 refer to the analyte number, respectively. The retention factor can be obtained from experimental migration times with Equation 7 (derived from Equation 2):

$$k = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})}. \quad (7)$$

Equation 6 indicates that resolution is influenced by four parameters: plate number, selectivity factor, retention factor, and migration time window factor. Although Equation 6 is similar to that derived for chromatographic separation, the last parameter in the right-hand side of the equation is superfluous; it arises from the variable length of the micellar zone, where the analyte can interact with the micelle (described in Section 2.1). The parameters affecting resolution are discussed below in more detail.

2.3.1. Plate number. The plate number in CZE is described by Jorgenson & Lukacs (6) as

$$N = \frac{(\mu + \mu_{eo})V}{2D}, \quad (8)$$

where μ is the electrophoretic mobility of the analyte, which can be obtained by dividing the electrophoretic velocity v_a by the electric field strength, $E = V/L$; V is the applied voltage; L is the total length of the capillary; μ_{eo} is the electroosmotic mobility; and D is the diffusion coefficient of the analyte in the running solution. Equation 8 was derived by assuming that the band broadening is solely due to the longitudinal diffusion of the analyte, as other adverse effects causing zone broadening—e.g., Joule heating, adsorption of the analytes on the capillary surface, extra column effects, injection, and detection volumes—can be minimized by choosing appropriate experimental conditions. Equation 8 indicates that a shorter separation time or a higher applied voltage gives a higher N and that a slower diffusing analyte or a higher-molecular-mass analyte generates a higher N.

The experimental dependence of plate height $H (= l/N)$ on EOF velocity is shown in **Figure 4** (32). Note that the results for Sudan III are invalid due to the presence of contaminants, which were partially resolved from Sudan III as v_{eo} increased (33). These dependencies suggest that band broadening in MEKC arises primarily from longitudinal diffusion. The contribution of longitudinal diffusion to band broadening is given by

$$H_l = \frac{2(D_{aq} + kD_{mc})}{1 + (t_0/t_{mc})k} \times \frac{1}{v_{eo}}, \quad (9)$$

where H_l is the plate height due to the longitudinal diffusion and where D_{aq} and D_{mc} are the diffusion coefficients of the analyte and the micelle, respectively. The average diffusion coefficient can be written as

$$D_{av} = \frac{D_{aq}}{1 + k} + \frac{kD_{mc}}{1 + k} \quad (10)$$

because in MEKC the analyte is distributed between the micelle and the surrounding aqueous phase. D_{mc} is generally 10-fold smaller than D_{aq} because the micelle is larger than the small molecules. Equation 9 suggests that MEKC generates higher plate numbers than does CZE, particularly when k is large; however, large k means a longer migration time.

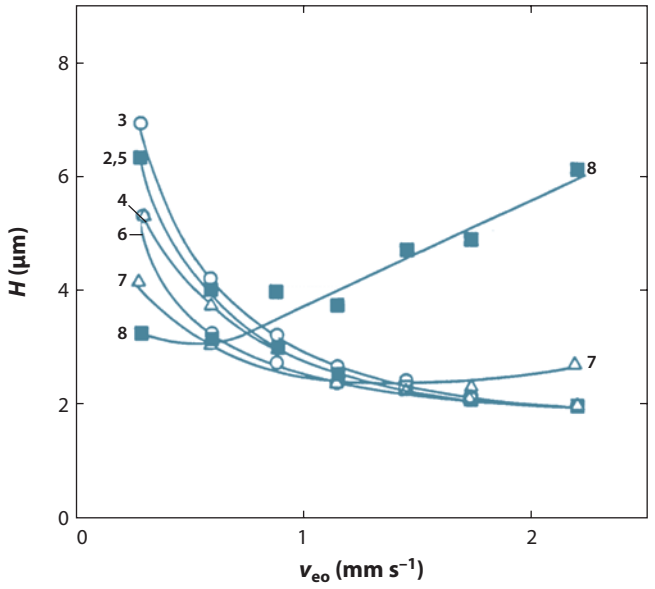

Figure 4

Dependence of plate height (H) on electroosmotic velocity (v_{eo}). 2, resorcinol; 3, phenol; 4, *p*-nitroaniline; 5, nitrobenzene; 6, toluene; 7, 2-naphthol; 8, Sudan III. Conditions: capillary, 50 μm inner diameter × 65 cm (50 cm to detector); running solution, 50 mM sodium dodecyl sulfate in 50 mM phosphate/100 mM borate buffer (pH 7.0); detection, ultraviolet absorbance at 210 nm; temperature, ambient (~25° C). The minimum plate height was approximately 2 μm. The applied voltage was varied to generate different v_{eo}. Modified from Terabe et al. (32) with permission. Copyright 1989, American Chemical Society.

Because MEKC is a chromatographic separation system, we must consider chromatographic factors that may affect band broadening: kinetic parameters on distribution equilibrium and intermicelle mass transfer. Another characteristic parameter of MEKC is the distribution of the micellar size. Joule heating has an effect on band broadening that is different from that of CZE because the radial temperature gradient causes distributions of kinetic parameters in micellar solubilization in addition to the distribution of viscosity. The effect of several parameters on band broadening has been extensively studied by Davis et al. (34, 35).

Longitudinal diffusion is the major contributor to band broadening, and other parameters are not significant under most conventional conditions. Note, however, that parameters for experimental conditions—for instance, injection volume, detection cell volume, and adsorption of the analyte on the capillary wall—should be carefully optimized. When the number of plate numbers is high, injection volume and detection cell volume must be minimized so as not to reduce efficiency, particularly when low detection-volume methods such as LIF and electrochemical detection are employed. The number of plates obtained in conventional MEKC is typically on the order of a few hundred thousand. Some researchers have obtained over a million plates (36), provided that the sample zones were very narrow. Adsorption of analytes on the capillary wall causes poor peak shapes and low plate numbers because MEKC separation does not assume any interactions between the analyte and the capillary wall. However, ionic and hydrophobic interactions between the analyte and the capillary wall may occur. The former can be minimized under low pH, and the latter can be reduced by adding a small amount of organic solvent to the running solution. Using a coated capillary with a hydrophilic polymer can also solve both of these problems.

2.3.2. Selectivity factor. As in chromatography, the selectivity factor α has the greatest impact on resolution. Because the number of plates in MEKC is >10-fold greater than that in high-performance liquid chromatography (HPLC), high resolution can be achieved even if the value of α is less than 1.02 under favorable conditions. The selectivity factor depends mainly on the surfactant structure and on the composition of the aqueous phase. The micelle corresponds to the stationary phase in HPLC, but there are charges on its surface. This results in different selectivity for polar analytes compared with the reversed-phase stationary phase, although hydrophobicity is the major factor affecting selectivity in both MEKC and reversed-phase HPLC. For example, cationic analytes interact with SDS micelles via an ionic interaction, but anionic analytes interact rather repulsively. The relationship between the surfactant structures and the separation selectivity has been studied from the viewpoint of quantitative structure-retention relationship (QSRR) (37) as in HPLC. QSRR studies using experimental descriptors have been successful. In early studies, application of the solvatochromic model revealed several valuable insights into the interaction mechanism (38, 39): Molecular sizes and their hydrogen-bond-accepting basicity of the solutes primarily affect the interaction, and their dipolarity/polarizability and hydrogen-bond-donating acidity play minor roles when anionic surfactant micelles are employed. However, the free-energy-based solvation-parameter model (40) is favored today because solvation parameters are related to energy properties.

Several surfactants are chiral, and the micelles of such chiral surfactants can recognize chirality. Bile salts (41) or amino-acid-derived surfactants (42) have been used in MEKC for enantiomer separation. Techniques to separate enantiomers by CE have also been extensively developed: The use of versatile cyclodextrin (CD) derivatives is particularly popular. MEKC with chiral surfactants is not very widely used because of the these surfactants' limited availability; however, several synthetic chiral surfactants have been shown to be useful for enantiomer separation (43).

2.3.3. Retention factor. The retention factor is the third parameter in the right-hand side of Equation 6. Because the retention factor is also included in the last parameter, its effect on resolution is different from its effect in conventional chromatography; specifically, the larger k does not always produce higher resolution. The optimum k value k_{opt}, which gives the maximum resolution, can be calculated by (44)

$$k_{opt} = \sqrt{t_{mc}/t_0}, \tag{11}$$

assuming $k_1 \approx k_2$ in Equation 6. Under neutral or alkaline conditions, t_{mc}/t_0 is approximately 4; therefore, k_{opt} is approximately 2, whereas the larger k value gives higher resolution at the expense of time in conventional chromatography. The retention factor is related to the phase ratio V_{mc}:V_{aq}, where V_{mc} and V_{aq} are the volume of the surfactant that forms the micelle and the volume of the aqueous phase, respectively (21). Thus

$$k = K\frac{V_{mc}}{V_{aq}} = K\frac{\bar{v}(C_{sf} - \mathrm{CMC})}{1 - \bar{v}(C_{sf} - \mathrm{CMC})}, \tag{12}$$

where K is the distribution coefficient, $\bar{v}$ is the partial specific volume of the surfactant constituting the micelle, and C_{sf} is the surfactant concentration. Because the volume of the micelle is negligibly small compared with the aqueous volume, Equation 12 can be approximated by

$$k = KV_{mc}/V_{aq} \approx K\bar{v}(C_{sf} - \mathrm{CMC}). \tag{13}$$

Equation 13 shows that the retention factor is linearly proportional to the surfactant concentration. This has been experimentally confirmed as shown in **Figure 5** (21), where SDS was employed as the surfactant to form the micelle. All retention factors increase linearly with a rise in SDS concentrations. At an SDS concentration of 10 mM, all retention factors were null, suggesting

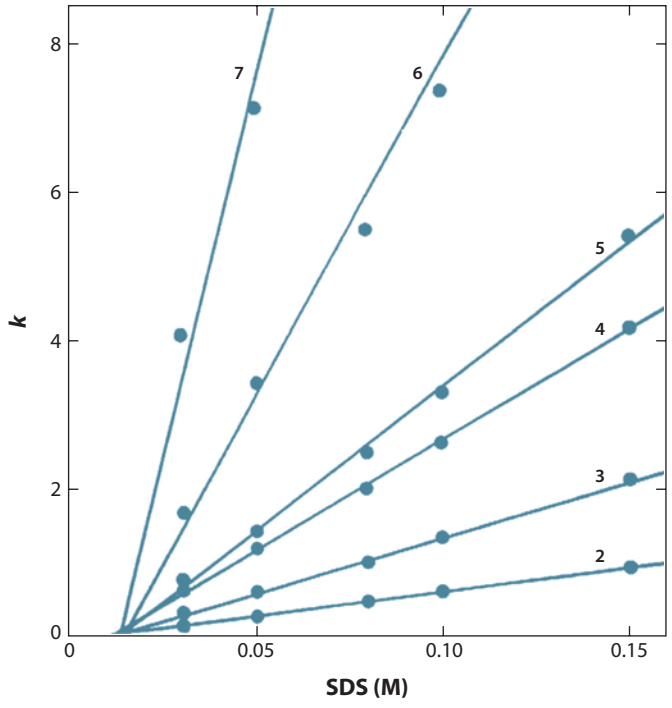

Figure 5

Dependence of the retention factor (k) on the concentration of sodium dodecyl sulfate (SDS). 2, resorcinol; 3, phenol; 4, *p*-nitroaniline; 5, nitrobenzene; 6, toluene; 7, 2-naphthol. Conditions: capillary, 50 μm inner diameter × 65 cm (50 cm to detector); running solution, 30 mM SDS in 50 mM phosphate/100 mM borate buffer (pH 7.0); applied voltage, 15 kV; current, 33 μA; detection, ultraviolet absorbance at 210 nm; temperature, 35° C. Modified from Terabe et al. (21) with permission. Copyright 1985, American Chemical Society.

that the CMC of SDS was ~10 mM under these conditions, although the CMC of 2.6 mM was reported elsewhere (24). The slope of each line gives the distribution coefficient, provided that the partial specific volume is known as predicted from Equation 13. Note that the phase ratio can easily be varied by changing the surfactant concentration; thus, k can easily be adjusted to the desired value if the CMC is known.

2.3.4. Migration time window factor. The last parameter in the right-hand side of Equation 6 is ascribed to the variable length of the micellar solution, where the micelle interacts with the solute. The length corresponds to the column length in conventional HPLC and equals $(v_a - v_{mc})t_R$, where v_a and v_{mc} have the same sign if the micelle and the EOF migrate in the same direction. The available micellar length is lower than the effective capillary length l, which causes loss of resolution. However, if the EOF is weak or if $|v_{eo}| < |v_{mc}|$, the micelle migrates in the direction opposite that of the bulk solution because v_{eo} and v_{mc} have different signs. In this case, the migration direction of the solute depends on k and on the ratio t_0/t_{mc}. The effect of the migration time window on resolution, and its relation to the resolution equation, has been described in detail elsewhere (45, 46). Also, the determination of the migration time window has recently been reviewed (47). Slightly suppressed EOF gives higher resolution but needs a longer separation time. A low pH or a coated capillary can be employed to suppress EOF.

3. PSEUDOSTATIONARY PHASES IN ELECTROKINETIC CHROMATOGRAPHY

3.1. Micelles

SDS is the most widely employed surfactant used to generate the micelle in MEKC because it has several advantages over other surfactants, including its well-characterized properties, high solubilization capability, easy availability, low ultraviolet absorbance, and high solubility to aqueous solutions. Minor disadvantages of SDS are its relatively high CMC (~8 mM in pure water, less in buffer solutions) and its relatively high Krafft point (16° C), which causes precipitation of SDS at low temperatures. Note that potassium dodecyl sulfate has a very high Krafft point (above room temperature; ~40° C) and is therefore unsuitable as a PSP in MEKC; that is, the use of potassium ion as an electrolyte should be avoided when SDS is used.

CTAB is another popular surfactant used in MEKC. CTAB and other cationic surfactants tend to adsorb strongly on the surface of the capillary and to reverse the EOF, as described above. Many different types of surfactants may be used as the PSP in MEKC, and the characteristic parameters of typical surfactants have been described in many review articles (see, e.g., Reference 14). Micelle polymers are also employed occasionally, as discussed above.

Most single-chain surfactant molecules such as SDS and CTAB consist of a single long alkyl group ending in a polar group. Double-chain surfactants such as DBTHX [5,12-bis(dodecyloxymethyl)-4,7,10,13-tetraoxa-1-16-hexadecanedisulfonate] (48) and DOSS (sodium di-2-ethylhexyl sulfosuccinate) (49) yield different separation selectivities compared with SDS and are suitable for separation of highly hydrophobic analytes such as naphthalene derivatives (48) and nonionic aromatic compounds (49). However, one study reported that DOSS did not form a micelle in 40% acetonitrile and that it worked as a solvophobic additive (49). DBTHX and its analogs have low CMCs and provide a wide migration time window compared with SDS (48). Other dimeric surfactants with hydrophilic spacers containing two to six oxygen atoms have been synthesized and characterized as PSPs in MEKC (50).

Two different surfactants can be combined to form a mixed micelle. Mixed micelles consisting of ionic and nonionic surfactants are useful PSPs because they provide a significantly different separation selectivity (51) from that of standard micelles. The change in selectivity can be explained by the alteration of the surface structure of the mixed micelle: The surface of the micelle may be covered by long polyoxyethylene groups of the nonionic surfactant. Esaka et al. (52) successfully added a nonionic surfactant, Tween 20®, to SDS to separate hydrophobic cations such as adrenaline and its precursors. Although hydrophobic cations interact strongly with the SDS micelle, causing poor resolution, adding Tween 20 can reduce the retention factors of such analytes and can provide different selectivity to improve resolution.

3.2. Microemulsions

Microemulsions (oil in water; o/w) consisting of a surfactant, oil, a cosurfactant, and water were first used as a PSP in EKC by Watarai (53) in a technique termed microemulsion electrokinetic chromatography (MEEKC) (54). There are several important differences between MEKC and MEEKC (53, 54). For instance, microemulsions have larger sizes than do micelles, and they are stable if their composition is optimized. Because microemulsions contain additional oil and cosurfactant components, their separation selectivity seems very different from that of MEKC; however, if both methods employ the same surfactant, their separation selectivities do not differ appreciably (53, 54). The component that most affects selectivity in MEEKC is the cosurfactant, as its polar group is located on the surface of the microemulsion. The oil's effect on the microemulsion

is not very significant because most analytes cannot be incorporated into the core oil, but rather remain on the surface.

An advantage of MEEKC over MEKC is that the width of its migration time window can be expanded by changing the surfactant concentration (54). However, the composition of the microemulsion must be kept constant, particularly when the oil is volatile. MEEKC has recently attracted interest with regard to its potential applications to pharmaceutical analysis, and many studies and review articles have been published (55–60). Finally, although MEEKC usually employs o/w microemulsion, water-in-oil (w/o) microemulsion in butanol has also been used with different selectivity (61).

Liposomes, vesicles formed by the aggregation of amphiphilic phospholipid molecules, have also been used as PSPs in EKC (62, 63). The liposome technique is not particularly advantageous compared to EKC using other PSPs, but it is useful to measure the affinity of the solute to the specific liposome.

3.3. Charged Cyclodextrins

Ionic derivatives of β-cyclodextrin, β-CMCD (2-*O*-carboxymethyl-mono-β-cyclodextrin) (64) and CDen [(6-β-aminoethyl-6-deoxy)-β-cyclodextrin] (10), have been used to verify the separation principle of EKC with charged CDs as PSPs, that is, via CD-EKC. Both CD derivatives showed that neutral analytes can be separated via the interaction with the CDs. Because CD is chiral, CDen has been successfully employed to separate enantiomers of dansylated amino acids (10). Unfortunately, CD-EKC is not particularly useful from the viewpoint of practical applications because the characteristic separation selectivity in CD-EKC can be realized by adding a CD derivative to the micellar solution of MEKC (65, 66) in a technique termed CD-modified MEKC (CD-MEKC). Here, the CD derivative to be added to the micellar solution does not need to be charged. Recently, several charged CDs were developed and made commercially available primarily for enantiomer separation in CZE (67). These charged CDs, particularly the highly charged ones, can be useful PSPs (68).

3.4. Charged Polymers

Charged polymers were first used as PSPs for the separation of ions via an ion-exchange mechanism. Although most ions can be separated using conventional CZE, isomeric ions are difficult to separate because their molecular sizes are identical and because the pK_as are close to each other. Cationic polymers such as poly(diallyldimethylammonium chloride) (69, 70), (diethylamino)ethyldextran (69), and polybrene (70) have been employed as PSPs to separate isomeric anions such as naphthalene sulfonic and disulfonic acids (69, 70), as well as several aromatic carboxylic acids (70). This technique is successful in principle, but it has not been widely accepted because the needs for the separation of isomeric ions are not high. For example, separations of isomeric naphthalene monosulfonic and disulfonic acids or of iomeric benzene carboxylic acids are often not required. Adding an ion-pair reagent can also achieve separation of isomeric ions (71).

Micelle polymers, polymerized surfactants of the tail-end geometry (72), are a class of surfactants that have a CMC of zero and that have structures similar to those of conventional micelles. Palmer (72) has synthesized many polymer surfactants for use as PSPs in MEKC and has compared their performance to that of conventional surfactants. A clear advantage of micelle polymers is that they are stable even in the presence of organic solvents (72, 73).

Several charged polymers have also been used as PSPs, including starburst dendrimers (74–76), polyalkylamines (77), polyethyleneimine (78), and polydiallyldimethylammonium (79). These

polymers do not form micelles, and the mechanisms through which they interact with the analytes depend upon their structures. Starburst dendrimers modified with long alkyl chains have selectivities similar to those of micelles (75). Interestingly, a linear solvation model has proven that (*a*) the hydrophobic interaction is insignificant when diallyldimethylammonium is employed and that (*b*) the analyte's interaction with n or π electrons with the polymer is the major mechanism at work (79). When polyethyleneimine is employed, the relevant mechanisms are the ion-dipole interactions between the OH groups of the analyte and the polymer (78). However, if the polymer has long alkyl chains, the hydrophobic interaction is the major mechanism at work (77).

3.5. Proteins

Affinity electrophoresis is widely used to investigate the interactions between proteins and analytes. The analytes may be proteins, peptides, DNA, lipids, carbohydrates, or small molecules. The CE format of affinity electrophoresis provides a high-speed analysis and requires only a small amount of sample to obtain the binding constants (80, 81). The protein bovine serum albumin was first used as a PSP in EKC for the separation of enantiomers of leucovorin (82). Because most proteins tend to adsorb on the capillary wall, one must coat the capillary surface to generate reproducible results in affinity EKC (82, 83). Another problem in affinity EKC is that the presence of the protein in BGS makes photometric detection difficult due to the protein's significantly strong ultraviolet absorption in short-wavelength regions. This detection problem was solved through the use of the partial filling technique (84, 85). In the partial filling technique, the capillary is first filled with a BGS without a PSP. Thereafter, the BGS containing the PSP is introduced into a portion of the capillary from the injection end. While the sample passes through the PSP-containing zone, the components in the sample are separated and reach the detection cell, where each separated component is detected free from PSP. Although affinity EKC is useful for enantiomer separations, it is not particularly popular: Several other techniques for enantiomer separation by CE have been developed, and, the use of CD derivatives as additives in CZE is especially powerful due to the commercial availability of many recently developed CD derivatives.

3.6. Nanoparticles

The use of nanoparticles as PSPs constitutes an intermediate mode between EKC and CEC (unless the nanoparticles are not fixed inside the capillary, in which case the technique used is EKC). Bächmann & Göttlicher (86) modified 500-nm silica particles with 10-(carbomethoxy)decyldimethylchlorosilane and suspended them in BGS as a PSP to separate polycyclic aromatic hydrocarbons and naphthalene derivatives. The particles were charged by ionization of the carboxyl group and migrated by electrophoresis. Nilsson & Nilsson (87) review the uses of several other chemically modified nanoparticles as PSPs. This approach resembles conventional reversed-phase HPLC using silica particles, where the efficiency is relatively low due to the heterogeneity of the particle sizes and the slow kinetics of partition equilibrium.

3.7. Tetraalkylammonium Ions

Walbroehl & Jorgenson (88) used tetraalkylammonium ions added to BGS in CZE to separate hydrophobic neutral analytes by solvophobic interaction. Tetraalkylammonium ions are small molecules that may not be suitable for use as PSPs because the velocity of the analyte–PSP complex is greatly reduced compared with that of the free PSP. This technique, known as hydrophobic-interaction electrokinetic chromatography (91), has been applied to the separation of highly hydrophobic analytes in aqueous organic solvent (89–91).

4. ADDITIVES IN MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Several additives have been developed to improve MEKC separation (16). Most additives primarily modify the aqueous phase, but some are incorporated into and concomitantly modify the micelle. As in HPLC, most popular additives are organic solvents. The addition of an organic solvent such as methanol or acetonitrile reduces the retention factor by increasing the solubility of the analyte into the aqueous phase. Many studies have been published on the effects of the addition of organic solvent to improve the separation of highly hydrophobic analytes; these studies are summarized in a recent review (20). The addition of compounds with both hydrophobic and hydrophilic functional groups (such as alcohols, phenols, and ketones) to the micellar solution significantly affects separation selectivity (92). The effects of adding organic solvents can be illustrated in terms of (*a*) the saturation of the solubilization sites in the Stern layer with the modifiers, (*b*) the specific interaction of the modifiers with the analytes via hydrogen bonding in the micelles, (*c*) the expansion of the core volume with the hydrocarbon bonding in the micelles, and (*d*) the expansion of the core volume with the hydrocarbon moieties of the modifiers (92). Ionic liquids are relatively new additives in MEKC, and their applications are limited (93, 94).

CD derivatives are probably the next most widely used additives. The addition of CD derivatives is useful for the separation of enantiomers, including neutral enantiomers (as mentioned above). CD-MEKC also effectively separates highly hydrophobic analytes and aromatic positional isomers (65, 66). Note that a single surfactant molecule may be incorporated into the cavity of CD; thus, the cavity size available for the guest analyte may differ from its size in the absence of the surfactant. A crown ether (18-crown-6) has been successfully employed for the separation of polyamines and catecholamines by MEKC (95). Several additional additives have been reported: ion-pair regents (96), urea (97), glucose (98), silver (I) (99), polyelectrolyte complex (100), and metal cations (101).

5. ON-LINE SAMPLE PRECONCENTRATION

It is well known that detection sensitivity in CE is low in terms of concentration sensitivity when photometric detectors are used, mostly because of the limited amounts of samples injected and the short light pathlength for the photometric detection. To circumvent this disadvantage, several on-line sample preconcentration techniques have been developed (102, 103). In these approaches, a large volume of the sample solution is injected into the capillary via pressurized injection, then either the analyte is selectively concentrated inside the capillary before separation or it is selectively injected electrokinetically from the sample solution and concentrated at the injection end of the capillary before separation. In the following sections, I discuss preconcentration techniques that are effective in MEKC and that can be applied to neutral analytes.

5.1. Field-Enhanced Sample Stacking

The technique known as field-enhanced sample stacking was originally developed for the concentration of charged analytes (5). When a sample solution is prepared in a dilute electrolyte solution or in a low-conductivity solution, and when BGS is in a high-concentration electrolyte solution or a high-conductivity solution, the analyte ion migrates rapidly in the sample solution but slowly in BGS under electrophoresis because the electrophoretic velocity is proportional to the field strength. This easily implemented technique yields effective sample preconcentration. Several different modes are available; these have been reviewed elsewhere (104). Liu et al. (105) first applied this technique for the preconcentration of neutral analytes in MEKC. Quirino & Terabe (106) extensively developed sample preconcentration techniques for neutral analytes using the field-enhanced technique. Because the sample solution is prepared in a solution with low

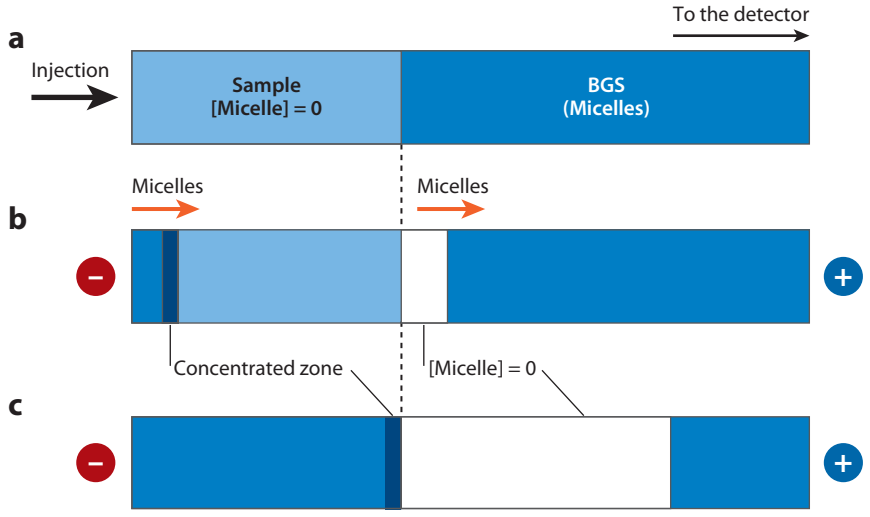

Figure 6

Schematic illustration of the sweeping principle under suppressed electroosmotic flow. (*a*) The sample solution, prepared in a matrix without a micelle but with a conductivity similar to that of the background solution (BGS), is injected as a long plug. (*b*) When voltage of negative polarity is applied, the anionic micelle penetrates the sample solution and accumulates analyte molecules. (*c*) The injected sample zone is completely swept by the micelle, and the analyte is focused into a narrow zone.

conductivity compared with that of BGS, the EOFs of the two solutions differ because of their different electric fields, which adversely affects concentration efficiency. Therefore, the conditions of low EOF are favorable. For neutral analytes, up to a 100-fold enhancement in sensitivity was achieved in MEKC under suppressed EOF compared with conventional MEKC (107, 108).

5.2. Sweeping

Sweeping is a new on-line sample preconcentration method for neutral analytes (109). The principle of sweeping, which differs from that of other electrophoretic techniques, is shown in **Figure 6**. The sample solution is prepared in a matrix without a micelle, and its conductivity is adjusted to be nearly equal to that of the micellar BGS by changing the salt concentration. Thus, no field-enhancement effect occurs during sweeping. The analytes are picked up and concentrated by the micelle, which penetrates the sample zone by electrophoresis. The concentration efficiency can be described as

$$l_{inj}/l_{sweep} = (1 + k),$$

where l_{inj} and l_{sweep} are the injected sample zone length and the swept length, respectively. It is important to select conditions in which the analyte molecules can strongly interact with the micelle during the sweeping process. Specifically, additives to BGS (such as a CD or an organic solvent) can be employed, although such compounds should not be added to the sample matrix. The sweeping phenomenon has been described in detail to illustrate the wide applicability of the technique (110, 111).

Sweeping using other PSPs has been performed by Quirino et al. (68). We have investigated the sweeping process using microchip electrophoresis, wherein the detection point on the separation

channel can be easily moved (112). The narrowing process of the sample zone by sweeping was traced via LIF detector by changing the detection distance from the injection point. The sample zone was gradually swept from the injection side, and then a very sharp zone appeared. The zone width was almost equal to the spot size of the focused laser light at the moment when sweeping finished; zone broadening also occurred as a result of thermal diffusion. The results suggest that sweeping provides an ideal sample injection technique for MEKC separation, yielding an almost infinitely narrow sample zone for analytes that interact strongly with the micelle.

Palmer et al. (113) reported a similar concentration technique termed the high-salt stacking method and claimed that their approach was superior to sweeping. The salt concentration in the sample matrix is higher in the high-salt stacking technique than in the sweeping approach, resulting in stacking (i.e., concentration) of the micelle at the boundary between the sample zone and the BGS. This micelle stacking leads to high efficiency because the concentrated micelle zone penetrates the sample zone. However, there is a possibility of destacking if the concentrated (swept) sample zone broadens upon the stacked sample zone's release from the concentrated micelle zone (114). The pressurized sample injection method is usually employed in sweeping, and the amount of sample volume is limited to less than the volume of the capillary. Electrokinetic injection has been successfully employed in combination with the sweeping technique, enabling injection of a volume greater than that of the capillary (115). The principle of sweeping has been further extended to the concentration of metal ions through in situ complexation reaction and by sweeping with a complexing agent such as ethylenediaminetetraacetic acid (116).

5.3. Combinations of Concentration Techniques

Combining two on-line sample preconcentration techniques efficiently increases detection sensitivity, although the application conditions are rather limited. A combination of sweeping and field-enhanced sample injection (FESI) generated a nearly million-fold increase in sensitivity (117). In one experiment, FESI was used to inject a long concentrated sample zone from a dilute sample solution for a prolonged duration of time; the long concentrated sample zone was further focused by sweeping. The analyte was charged to be compatible with FESI. Although the technique was applied to the concentration of basic compounds, with an SDS micelle as the PSP under acidic conditions, acidic analytes can also be concentrated with a cationic surfactant such as cetyltrimethyl ammonium chloride using a coated capillary to suppress EOF (118).

If the analyte constitutes a mixture of neutral and weakly acidic compounds, it may be possible to concentrate both types of analyte via sweeping under acidic conditions, keeping the analyte neutral. However, a weakly acidic analyte can be successfully concentrated via dynamic pH junction (119). Also, if SDS is added to BGS, a neutral analyte can be simultaneously concentrated by sweeping (120).

5.4. New Techniques

Two new techniques for on-line sample preconcentration were recently developed: transient trapping (121) and analyte focusing by micelle collapse (AFMC) (122). Transient trapping was performed with a short plug of the micelle introduced in the microchannel in microchip electrophoresis. The microchannel was filled with BGS (pH 7.2) without the micelle, then a short plug of the micelle, and finally a long plug of a sample solution. The conductivities of the three solutions were equalized to avoid field-enhanced stacking effects. When the voltage was applied, the analytes were swept by the micelle zone and were concentrated at the end of the micelle zone. Meanwhile, the micelle concentration decreased progressively from the other end of the micelle

zone; the difference in field strength between the micelle zone and BGS generated a gradient concentration. The focused analyte was trapped at the boundary, keeping a narrow zone width until it was released from the boundary in the order of increasing hydrophobicity (*k*) due to the decrease in the micelle concentration. The analytes passed through the micelle zone were released from the micelle when they reached the detection point. A ~400-fold increase in sensitivity was thus obtained (121). Although the transient trapping was performed with the microchip format, this technique is easily transferable to the capillary format. Interestingly, the analytes can be detected when they are free from the micelle, which is promising for MS detection.

In AFMC, the analyte dissolved in a dilute micelle solution (slightly above CMC) can be concentrated at the boundary between the sample zone and a nonmicelle zone (122). Once incorporated into the micelle, the analyte stays there throughout the micelle's gradual collapse; this results in concentration of the analyte. It is important to find good conditions for AFMC. In one study, for example, the capillary was filled with a 100-mM SDS solution in a BGS of 2-mM sodium phosphate (pH 10), and a sample solution prepared in 3 mM SDS in 80-mM sodium phosphate (pH 10) was then injected as a long plug. When a positive voltage was applied at the injection end, the micelle in the sample zone collapsed at the anodic end because of the dilution of the micelle concentration. The analytes stayed in the micelle zone because the migration velocities of the analytes in the nonmicelle zone were higher than that of the sample zone toward the cathode. When all of the micelles in the sample zone collapsed, the analytes were concentrated at the anodic end of the micellar BGS and were then separated. Using this technique, an approximately 200-fold increase in peak height was observed for some corticosteroids (122).

6. CONCLUSIONS

MEKC has been widely accepted as a method of analytical separation, and it is easily performed with commercial instruments in academia. However, MEKC is not very popular in industry, primarily because CE is not widely used as a routine analytical technique. CE, including MEKC, is ready to be used in industry, but it may take more time for CE techniques to gain broad acceptance. Necessary improvements in MEKC include (*a*) increasing the availability of PSP surfactants that have different structures than do SDS, CTAB, and bile salts, particularly micelle polymers, and (*b*) developing more user-friendly CE instruments, both for MEKC and for other CE modes. Interestingly, many on-line sample preconcentration methods have been developed even for neutral analytes based on electrophoretic procedures. Hopefully, in the future MEKC will be able to solve the practical problems in industry.

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Errata

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