Review

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On-line sample concentration techniques in capillary electrophoresis: Velocity gradient techniques and sample concentration techniques for biomolecules

Methods with a high sensitivity and high separation efficiency are goals in analytical separation techniques. On-line sample concentration techniques in capillary electrophoresis (CE) separations have rapidly grown in popularity over the past few years because they achieve this goal. This review describes the methodology and theory associated with a number of different techniques, including electrokinetic and chromatographic methods. For small molecules, several on-line concentration methods based on velocity gradient techniques are described, in which the electrophoretic velocities of the analyte molecules are manipulated by field amplification, sweeping, and isotachophoretic migration, resulting in the on-line concentration of the analyte zones. In addition, the on-line concentration methods for macromolecules are described, since the techniques used for macromolecules (DNAs and proteins), are different from those for small molecules, with respect to either mechanism or methodology. Recent studies relating to this topic are also discussed, including electrophoretic and chromatographic techniques on capillary or microchip.

Keywords: Capillary electrophoresis / On-line sample concentration techniques / Review / Stacking / Sweeping
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Contents

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Abbreviations: **ASEI-sweeping-MEKC**, anion-selective exhaustive injection-sweeping-micellar electrokinetic chromatography; **BGS**, background solution; **CGE**, capillary gel electrophoresis; **CSEI-sweeping-MEKC**, cation-selective exhaustive injection-sweeping-micellar electrokinetic chromatography; **CTAC**, cetyltrimethylammonium chloride; **EKS**, electrokinetic supercharging; **FASS**, field-amplified sample stacking; **LVSS**, large-volume sample stacking; **PS**, pseudostationary phase; **tr-ITP**, transient isotachophoresis

1 Introduction

Of the efforts that have been made to increase sensitivity in capillary electrophoresis (CE), which include applications of a bubble cell, Z-shaped or multiple-reflection capillaries to extend the detection path-length, seldom have been mentioned, since several order of magnitudes improvement in sensitivity can be obtained when the so called on-

line sample concentration techniques are used. Except for some techniques that involve different mechanisms based on physical means, such as liquid- and solid-phase extraction, most of these techniques were developed to accommodate a large volume for sample injection, since the limit of detection (LOD) is proportional to the amount of sample injected. Unfortunately, an improvement in LOD cannot be achieved by simply increasing the length of the sample solution or the injection time (in the case of electrokinetic injection), because individual electrophoretic parameters, such as buffer conductivities, pH values, the magnitude and direction of the electroosmotic flow (EOF), the concentration of surfactants (if needed) used, the injection length of sample solution, and even the polarity of the electrode, must be optimized. Many on-line sample concentration methods for small molecules have been proposed and no apparent loss in separation efficiency occurs. One of the earlier examples in CE is the capillary isotachophoresis (ITP) method [1, 2]. These early studies led to the later success in sample-stacking techniques, such as field-amplified sample stacking (FASS) [3–5], large-volume sample stacking (LVSS) [6, 7], pH-mediated stacking [8, 9], and stacking in the micellar electrokinetic chromatography (MEKC) mode [10–12]. Shihabi [13], Beckers *et al.* [14], Terabe *et al.* [15], and Lunte *et al.* [16] have all published comprehensive reviews on these topics. More recently, Breadmore *et al.* [17] cited numerous reports on topics relating to determination of inorganic and small organic anions; Sentellas *et al.* [18] also summarized the analysis of biological samples based on on-/off-line preconcentration methods.

On the other hand, for the analysis of neutral and/or charged analytes, the sweeping technique is one of the most frequently used methods. The phenomenon of sweeping in electrokinetic chromatography under a considerably homogeneous electric field was theoretically examined and experimentally verified by Terabe *et al.* [19–21]. Later, methods that dramatically increased sensitivity, the so-called cation- and anion-selective exhaustive injection-sweeping-micellar electrokinetic chromatography (CSEI-, ASEI-sweeping-MEKC) methods were reported [22–25]. In addition, a velocity difference-induced focusing (V-DIF) method, using a dynamic pH junction method, has been established, in which this on-line focusing method is based on the different mobility of cationic analytes between the background solution (BGS) and the sample matrix [26, 27]. Similar to this, but with additional surfactant (such as sodium dodecyl sulfate (SDS)) added to the BGS, the dynamic pH junction-sweeping method can be used to improve the separation efficiency, compared to the dynamic pH junction or sweeping techniques [28]. Terabe *et al.* [29] recently authored a comprehensive review on the topic of on-line preconcentration strategies for the trace analysis of metabolites. Undoubtedly, all of these techniques clearly have the capability to open new fields for investigating low levels of small molecules and have already overcome the drawbacks associated with the short light path of the capillaries.

In this article, the methodology and theory of these on-line sample concentration techniques in CE that are currently in use are reviewed. Their applications, limits of detection, sample concentration efficiency, and reproducibility are also discussed. Furthermore, from the point of view of macromolecules, capillary gel electrophoresis (CGE) plays an important role in the separations of DNAs and proteins. In DNA analysis, CGE is a powerful technique for fragment separation, genotyping, single nucleotide polymorphism, and sequencing. In these applications, trace analysis is essential because of the limited amounts of sample that are often available. On the other hand, protein analysis is more complex since numerous proteins are present at different concentrations in biological tissues and cells. Thus, minor components in a sample must be concentrated by an off-line or on-line method. In particular, CGE is expected to be a powerful separation method in the field of proteomics. Therefore, on-line concentration methods have been extensively investigated in this area by several researchers. On-line sample preconcentration techniques for proteins and peptides have been reviewed by Stroink and co-workers [30]. Recent works on this topic are also discussed.

2 On-line sample concentration techniques for small molecules

2.1 Stacking in CZE

2.1.1 FASS

Figure 1 shows schematic diagrams of the FASS method, which is considered to be the simplest technique for on-line sample concentration. In this method, the sample is prepared in a low-conductivity matrix, which is different from the BGS (a high-conductivity buffer). A convenient sample preparation method uses a 1/100–1/1000 diluted BGS, resulting in a low-conductivity solution, in which the sample is dissolved (sample solution). In the initial step, the capillary is filled with a high-conductivity BGS and the sample solution is then injected into the capillary to a certain length. After completion of the sample injection, upon application of a high positive voltage, a proportionally greater electric field develops across the sample zone causing the ions to migrate more rapidly. Once the ionic analytes reach the boundaries between the sample zone

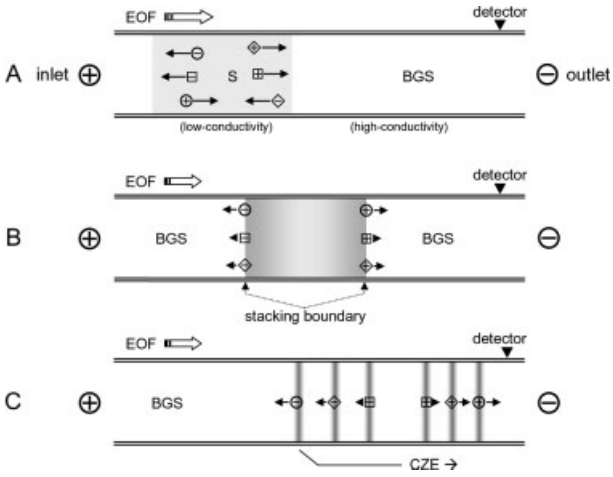

Figure 1. Schematic diagrams of the FASS model. (A) The capillary is conditioned with a BGS (a high conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and a high positive voltage is applied; (B) focusing of the analytes occurs near the boundaries between the sample zone and the BGS because of its mobility changes; (C) stacked analytes migrate and are separated by the CZE mode.

and the BGS, the electric field strength suddenly decreases and migration becomes slower, causing the sample analytes to be focused near the boundaries. Since the mobility of EOF is greater than those of the charged analytes, all analytes will finally move toward the detection window (the cations migrate faster than the anions), while the analytes are separated by the CZE mode. In this method, the sample injection volume (10–30% of capillary length) must be optimized because too large a volume of sample injection will cause the sample focusing to be difficult and an insufficient length is left for the separation. Applications of this method, as listed in Table 1, including the recently reported analysis of opioids [31], alkaloids [32], heroin metabolites [33], and arsenic species [34], with 10- to 10 000-fold enhancements achieved.

2.1.2 LVSS

LVSS can be performed by using a similar buffer system as that used for the FASS method, but the electrode polarity is switched so as to acquire a reversed EOF. Basic schematic diagrams of LVSS are illustrated in Fig. 2. In the initial step, the sample is dissolved either in a low-conductivity buffer or water. When the capillary is filled with a high-conductivity BGS, the sample solution is then injected into the capillary to a certain length and a negative polarity is applied. At this moment, the direction of the

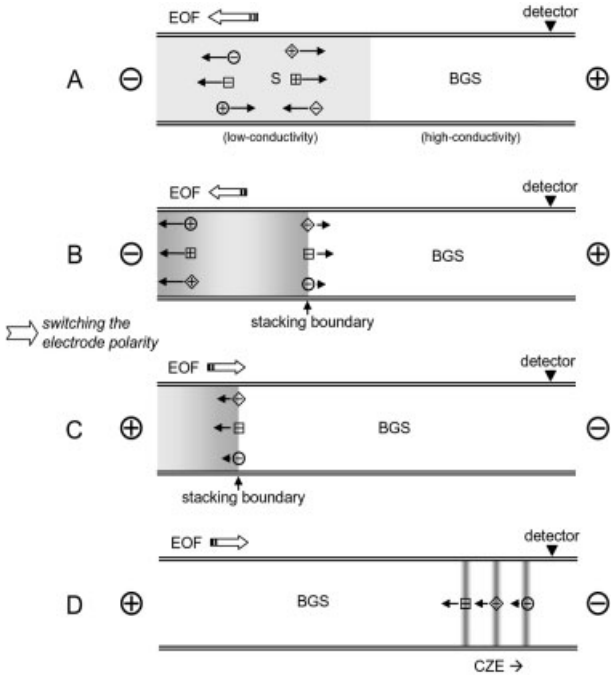

Figure 2. Schematic diagrams of the LVSS model. (A) The capillary is conditioned with a BGS (a high-conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and then a high negative voltage is applied (EOF is toward the inlet); (B) the anionic analytes move toward the detection end (outlet) and stack at one side of the boundary, whereas the cations and neutral species move and exit the capillary at the injection end (inlet); (C) the electrophoretic current is carefully monitored until it reaches approximately 95–99% of its original value, and the polarity is then quickly returned to positive (EOF is reversed); (D) the following separation occurs by CZE mode.

EOF is toward the inlet. The anionic analytes move toward the detection end (outlet) and stack at one side of the boundary between the sample zone and the BGS. Meanwhile, the cations and neutral species move and exit the capillary at the injection end (inlet). The electrophoretic current should be monitored carefully until it reaches approximately 95–99% of its original value, and the polarity is then quickly returned to positive, leading to a reversed EOF. If this is not done, the anionic analytes may be lost. The following separation occurs by the CZE mode. Compared to FASS, this method can provide a much larger sample injection without any significant loss in separation efficiency. For acquiring a good reproducibility, monitoring the current requires some care. Furthermore, it should be noted that this method cannot be used for the simultaneous separation of anions and cations and also is limited to analytes with low mobilities. Applications of this method are listed in Table 1.

Table 1. Examples of different compounds analyzed using different types of stacking methods

Technique	Compound	LOD	Concentration efficiency	Reproducibility RSD (%)	Ref.
FASS	Opioids	1 ppb	1000	1.2–7	[31]
	Alkaloids	0.7–0.9 ppb	1000–10 000	4–6	[32]
	Heroin metabolites	40 ng/mL	~10	1.2–3.6	[33]
	Arsenic species	< 1 μM	400–2100	3.1–5.8	[34]
	Pharmaceutical species	< 10 nM	> 500	10%	[48]
	Pb, Hg, Se complex	< 1 ng/mL	1700		[49]
	Metformin	0.1 μg/mL		15.6%	[50]
LVSS	As, Se species	0.1–7.4 mg/L	2.6–4.5	6.0–8.4	[35]
	Kanamycin	0.1 μg/mL	25	3.4–11.2	[36]
	Naphthalenesulfonate	4 μg/mL	100	0.1–1.0	[37]
	Metallothioneins	0.31 μg/mL	7–13	2	[38]
	Phenolic species	5–25 μg/L	40	2.1–12.6	[39]
	Food colorants	2–26 μg/L	80	0.03–7.69	[40]
	Anions	1.8–6.5 ppb	100	4.54–12.04	[41]
	Peptides	0.3–2 pM	10–15		[42]
	Iron complexes	< 0.1 μM	> 50	5.2–7.8	[43]
	Anions	ppb levels	> 300		[51]
	Bromide ion	15 μg/L		0.14–10%	[52]
pH-mediated	Haloacetic acids	0.5–4.0 μg/L	97–120	1.0–7.0	[44]
	Peptides	1 nM	10		[45]
	Coumarin metabolites	0.1 μM	10–17	6.5–17.0	[46]
	Physiological anions	0.3 μM	66	1.4–1.7	[47]
Stacking-MEKC	Plant hormones	0.3 ng/mL	10–600	0.12–0.28	[57]
	Triazine	3.3–8.5 μg/L	4–12	4.0–6.4	[58]
	Sildenafil citrate	17 ng/mL	11	0.96–8.67	[59]
	Herbicides	0.1–2.7 μg/L	70–1540	2.5–22.8	[60]
	Riboflavin	20 ng/mL	24	1.8–6.2	[61]

2.1.3 pH-mediated stacking

The FASS and LVSS methods described above are performed with the sample dissolved in a low conductivity buffer. However, this is not always convenient because the conductivities of some samples, such as urine or blood analytes which contain salts, are usually too high, leading to difficulties in the application of stacking, unless a pretreatment is used to reduce the conductivity. One of the methods for overcoming this drawback is the use of a pH-mediated stacking method. Schematic diagrams are shown in Fig. 3. In the initial step, the sample is prepared in a high-ionic strength medium and is electrokinetically injected into the capillary. Then, a plug of strong acid is electrophoretically injected and a positive separation voltage is applied. The strong acid titrates the sample solution to create a neutral zone (a high-resistance zone). Thus, a proportionally greater field will develop across the neutral zone, causing the ions to migrate faster. As a result, the analytes are stacked at the boundary between the titrated zone and the BGS. Separation by the CZE mode then occurs. Applications of this method are also listed in Table 1.

2.2 Stacking in MEKC

2.2.1 Normal-stacking MEKC

The stacking techniques described above are performed based on the CZE mode; stacking in the MEKC mode is also convenient. Figure 4 shows schematic diagrams of the normal stacking-MEKC separation when SDS is used as surfactant. In the initial step, the sample is dissolved in a low-conductivity buffer or water; the BGS contains SDS to form the micelles. After the background and sample solutions are injected, respectively, a positive voltage is applied. Meanwhile, the SDS micelles from the inlet enter the sample zone (since the mobility of EOF is greater than

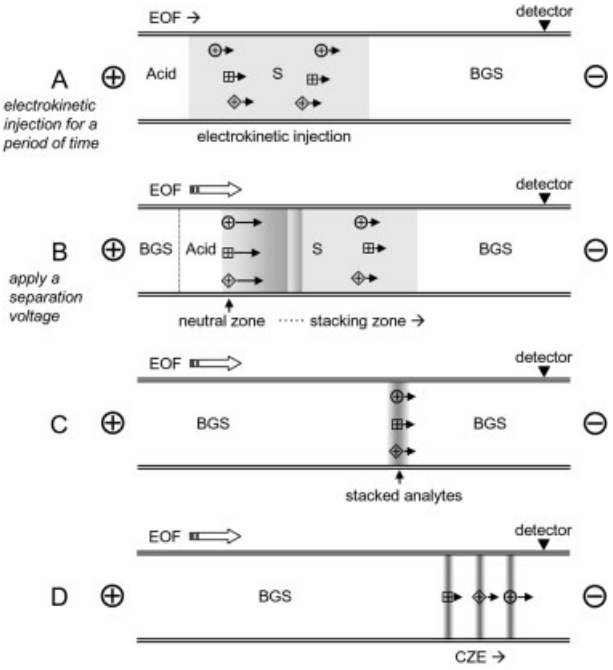

Figure 3. Schematic diagrams of a pH-mediated stacking model. (A) The capillary is conditioned with a high-conductivity BGS, the cationic analytes dissolved in a low-conductivity buffer are electrokinetic injected into the capillary, and then a plug of strong acid is also electrokinetically injected; (B) a positive separation voltage is applied; (C) the strong acid titrates the sample solution to create a neutral zone causing the ions to migrate faster and become stacked; (D) the subsequent separation occurs by the CZE mode.

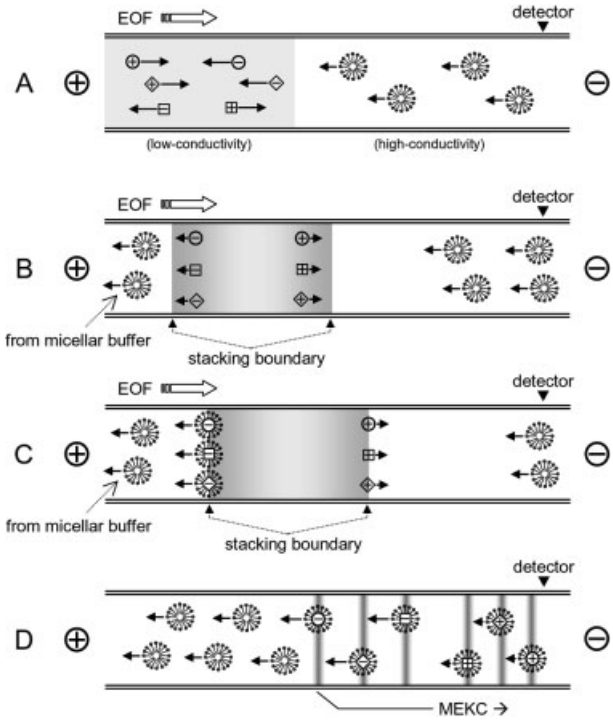

Figure 4. Schematic diagrams of a normal-stacking MEKC model. (A) The sample is dissolved in a low-conductivity buffer, BGS, consisting of SDS to form the micelles; after the background and sample solution are injected, respectively, a positive voltage is applied; (B) the SDS micelles from the inlet enter the sample zone and then permit the analytes to migrate and become stacked; (C) then the SDS-analytes are separated by the MEKC mode.

that of the SDS micelles), and then carry the analytes to migrate. Once the SDS-analytes reach the boundaries between the sample zone and the BGS, sample focusing occurs, as described above. Following this, the SDS-analytes are separated by the MEKC mode. Based on this principle, a cationic surfactant can also be used but the electrode polarity must be switched [70]. These methods can be used to separate either charged or neutral analyte molecules, and several different modes have been proposed. Terabe *et al.* [10] published a comprehensive review of the conditions for various stacking modes in the MEKC mode when SDS micelles are used.

2.2.2 Reversed-stacking MEKC

The schematic diagrams for reversed-stacking MEKC, the so-called reversed electrode polarity stacking mode (REPSM), are shown in Fig. 5. The sample is dissolved in a low-conductivity buffer, whereas a high-conductivity micellar buffer (such as SDS) is used as BGS. When a negative polarity is applied, the EOF moves toward the inlet; the anionic analytes move toward the outlet and stack at one side of the boundary, whereas the cations and neutral species move and exit the capillary at the injection end (inlet). The electrophoretic current reaches approximately 95–99% of its original value, the polarity is quickly returned to positive, leading to the reversal of EOF. The micelles from the BGS will carry and stack the analytes at the stacking boundary and the analytes are separated by the MEKC mode. Instead switching the electrode polarity, an acidic micellar BGS can be used to reduce the EOF [53]. Thus, this method, the so-called stacking with reverse migrating micelles (SRMM), is the only negative polarity needed. As a result, a better reproducibility can be achieved, since a polarity-switching step is no longer necessary. A series of reversed-stacking modes have been reported by Terabe *et al.*, including stacking using reverse-migrating micelles and a water plug (SRW) [54], field-enhanced sample injection (FESI) [55], and field-enhanced sample injection with reverse migrating micelles (FESI-RMM) [56]. Each of these meth-

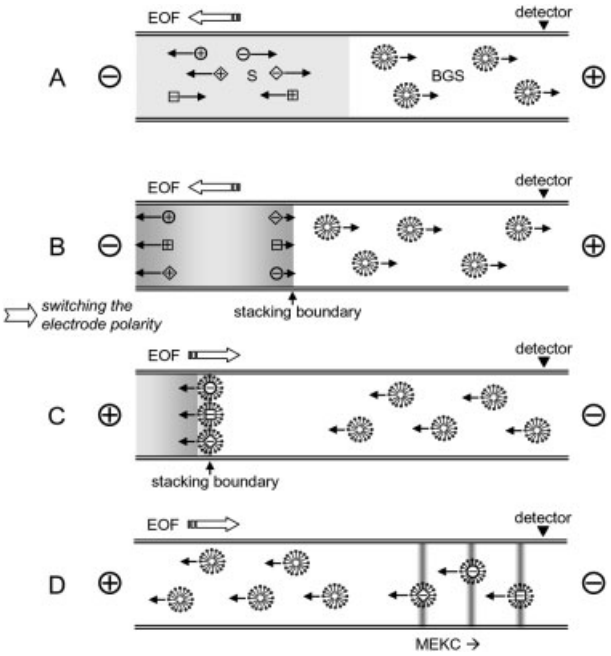

Figure 5. Schematic diagrams of a reversed-stacking MEKC model. (A) The sample and BGS are prepared as described in Fig. 4A but a negative polarity is applied; (B) the EOF moves toward the inlet, the anionic analytes move toward the outlet and stack at one side of the boundary; (C) the electrophoretic current reaches approximately 95–99% of its original value, the polarity is quickly returned to positive, reversing the EOF; (D) then the SDS-analytes are separated by the MEKC mode.

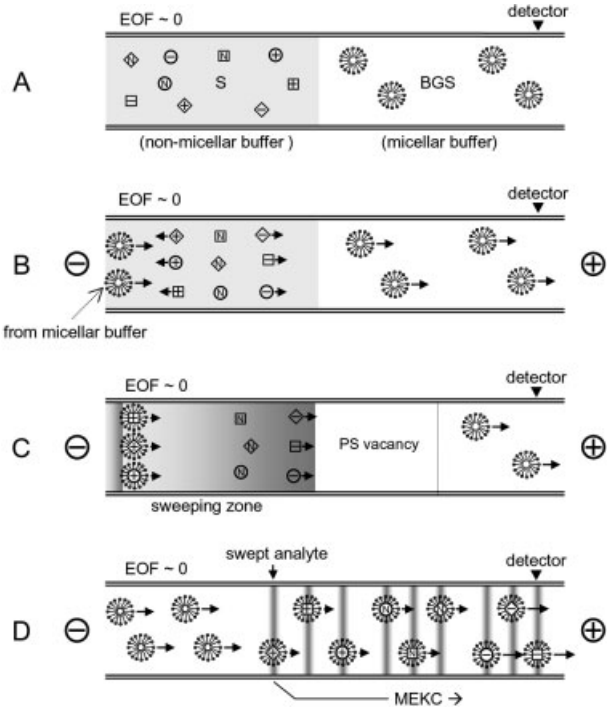

Figure 6. Schematic diagrams of a reversed-sweeping MEKC model. (A) The BGS consists of a surfactant (for example, SDS, a negatively charged surfactant) and electrolytes to form a micellar buffer but the samples are dissolved in a nonmicellar buffer; (B) after the injection of the BGS and the sample solution, a negative polarity is applied to power the CE separation; (C) the cations and anions move toward the inlet and outlet , respectively, and anionic SDS micelles enter the capillary sweeping the analytes; (D) the analytes are completely swept by SDS, the subsequent separation occurs by the MEKC mode.

ods has unique advantages and disadvantages with respect to sensitivity, precision, and simplicity of use. The applications of stacking-MEKC are summarized in Table 1.

2.3 Sweeping

2.3.1 Sweeping-MEKC

Figure 6 shows schematic diagrams of sweeping. This is a simple and convenient on-line sample concentration method for either charged or neutral analytes. The sample concentration effect relies on how the pseudostationary phase (PS) enters the sample solution zone (nonmicelle buffer) and sweeps the analytes. In this method, the BGS consists of a surfactant (for example, SDS, a negatively charged surfactant) and electrolytes to form a micellar buffer; the samples are dissolved in a nonmicelle buffer. The pH of these solutions should be kept at a low value to suppress the EOF, since this method is independent of EOF. When the injection of BGS and the sample solution are completed, a negative polarity is applied to power the CE separation. The cations move toward the inlet, anions move in the reverse direction. As a result, anionic SDS micelles enter the capillary and sweep the analytes. Once the analytes are completely swept by SDS, the followed separation occurs by MEKC mode. In sweeping, the length of the resulting zone after sweeping (l_{sweep}) is given by [20]:

$$l_{sweep} = l_{inj}\frac{1}{1+k}$$

where l_{inj} is the length of the injected sample zone and k is the retention factor of the analyte. The k values in the sample zone when filled with PS are assumed to be equal to those in the separation zone. Compared to a normal injection, a 2–3 order of magnitude enhancement has been reported. Applications of sweeping-MEKC are summarized in Table 2. Furthermore, hyphenation techniques, such as sweeping-cyclodextrin-MEKC [88] and sweeping on a microchip [89], have also been achieved.

Table 2. Examples of compounds analyzed by different kinds of sweeping methods

Mode	PS	Compound	LOD	Concentration efficiency	Ref.
Sweeping					
	SDS	Alkyl phenyl ketones		88–836	[19]
		Naphthalene derivatives		92–259	
		Dialkyl phthalates		272–691	
		Steroids	1.7–9.6 ppb	1541–2564	
		Reserpine, nicardipine, quinine, trimipramine		3312–5044	
	SDS	Proguanil, 4-chlorophenylbiguanide, cycloguanil	10–20 ppb		[62]
	SDS	Estrogens	53–100 nM		[63]
	SDS	Doxorubicin, daunorubicin	1 nM		[64]
	SDS	*trans*-Resveratrol	5 ppb	1500	[73]
	SDS	Corticosterone	3 ppb	2000	[74]
		17-Hydroxycorticosterone	3 ppb	2300	
	SDS	Dopamine-labeled NDA	3.1×10^{-8} M		[75]
		Reserpine	2×10^{-9} M		
	SDS	Lysergic acid diethylamide	16 ppb		[76–78]
		iso-Lysergic acid diethylamide	22 ppb		[76–78]
		Lysergic acid *N*,*N*-methylpropamide	18 ppb		[76–78]
	SDS	Corticosterone	5 ppb		[79]
		17-Hydroxycorticosterone	4 ppb		
	SDS	Bisphenol A and alkylphenols	6.5–21 ppb	41–69	[80]
	(SDS)-SB-12	*p-tert*.-Butylphenol	24 ppb	122	[83]
		2,4,6-Trichlorophenol	19 ppb	360	
	Borate complexation	Pyridine and adenine nucleotides	2×10^{-8} M		[81]
	SDS	Triadimenol		9–12	[85]
	EDTA	Metal ions (Cu^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+})	$1.8–23.4 \times 10^{-8}$ M	60–180	[23]
	TTAB	Bisphenol A and alkylphenols	30–159 ppb	29–67	[84]
	TTAB	*o*-, *m*-, *p*-Nitroanilines		20	[65]
		Aromatic carboxylic acids		1000	
		NSAs	0.47–0.96 ppb	670–760	
	TTAB	Steroids	9–20 ppb	270–370	[66]
	CTAC	2-Naphthoic acid, salicylic acid	0.4–3.1 ppb	590–600	[66]
	TTAB	Triazines	9–15 ppb	30–110	[67]
	Brij 35	4-Chlorophenol, 4-ethylphenol, 3-methylphenol	19–28 ppb	54–100	[68]
	Microemulsion	Steroids		138–278	[69]
	poly SUS	Heptanophenon, quinine		50–230	[70]
	pSAm-28	Quinine	42 ppb	580	
	pSAm-24	Quinine	3–5 ppb	5800–10 000	
	SC	Corticosteroids	50 ppb		[71]
	SC	Estrogens	118 ppb		[72]
CSEI-sweeping-MEKC					
	SDS	Laudanosine, 1-naphthylamine	4.1–8.0 ppt	550 000–900 000	[22]
	SDS	Aromatic amines	0.1 ppt	12 000–146 000	[86]
	SDS	Paraquat, diquat, difenzoquat	0.075–1.0 ppb	3000–51 000	[87]
	SDS	Corticosterone	5 ppb	1200	[74]
		17-Hydroxycorticosterone	4 ppb	1500	

Table 2. Continued

Mode	PS	Compound	LOD	Concentration efficiency	Ref.
	SDS	Reserpine	2×10^{-10} M		[75]
	SDS	Lysergic acid diethylamide	58 pg/mL		[76]
		iso-Lysergic acid diethylamide	68 g/mL		[77,78]
		Lysergic acid *N*,*N*-methylpropamide	80 g/mL		
	EDTA	Metal ions (Cu^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+})	$2.4–25 \times 10^{-11}$ M	140 000	[23]
ASEI-sweeping-MEKC					
	CTAC	Aromatic carboxylic acids		2400–2700	[24]
		Dansyl amino acids	0.8–1.2 ppb	1150–1430	
		Naphthalenedisulfonic acids	60–80 ppt	5800–5900	
	SDS	Phenoxy acidic herbicides	100 ppt	100 000	[82]

Abbreviations: NDA, naphthalene-2,3-dicarboxaldehyde; SB-12, zwitterionic *N*-dodecyl-*N*,*N*-dimethylammonium-3-propane-1-sulfonic acid; TTAB, tetradecyltrimethylammonium bromide; NSAs,naphthalenesulfonic acids; Brij 35, polyoxyethyl-ene (23) lauryl ether; poly SUS, poly(sodium 10-undecenyl sulfate); pSAm-24, -28, poly(2-acrylamido 2-methyl propane sulfonic acid-*co*-stearylacrylamide (-24, -28 indicates the mole percentage of the hydrophobic monomer in the polymer); SC, sodium cholate

2.3.2 CSEI-sweeping-MEKC

The CSEI-sweeping-MEKC model was first reported by Terabe *et al.* [22]. This method provides for a more sensitive detection than sweeping and is sufficiently flexible to offer the potential for achieving an increase in the detection limit of more than 100 000-fold, for positively chargeable analytes [22]. Figure 7 shows schematic diagrams of this method. As the beginning of the runs, the capillary is conditioned with a non-micellar BGS, followed by the injection of a high-conductivity buffer void of organic solvent, and finally by the injection of a short water plug. Using electrokinetic injection (at a positive polarity) the cationic analytes are prepared in a low-conductivity matrix or water. The cationic analytes enter the capillary through the water plug at high velocities, and are then focused or stacked at the interface between the water zone and the high-conductivity buffer. The continued electrokinetic injection provides a high efficiency for sample concentration. However, if this is too long, the separation efficiency will decrease. Once an optimized injection time is determined, the injection is then stopped and the micellar BGSs are replaced at both ends of the capillary. The voltage is then switched to a negative polarity, thus permitting the entry of micelles from the cathodic vial into the capillary to sweep the stacked and introduced analytes to the narrow bands. Finally, the separation can be performed using MEKC in the reversed-migration mode.

2.3.3 ASEI-sweeping-MEKC

The principle of ASEI-sweeping-MEKC is similar to that for CSEI-sweeping-MEKC but a cationic surfactant is used. Under optimized conditions, an approximately 1000 to 6000-fold improvement in LODs can be obtained [24]. Applications of CSEI-sweeping-MEKC and ASEI-sweeping-MEKC methods are listed in Table 2.

2.4 Dynamic pH junction

2.4.1 Dynamic pH junction

A pH junction occurs between the sample zone and the BGS, and this can be used to focus zwitterionic analytes as well as weakly acidic compounds because this junction causes a mobility difference in the analytes [90, 91]. Figure 8 shows schematic diagrams of the dynamic pH junction method. In the initial step, the capillary is filled with the BGS (higher pH value; *e.g.*, borate buffer) and the sample solution (lower pH value; *e.g.*, phosphate buffer) is then injected to a certain length; a pH junction occurs at the interface of the two solutions. In acidic solution, the analytes are neutral species, but they acquire a negative charge if the solution is alkaline. Because of this property of the analytes, when a high positive voltage is applied, a discontinuous electrolyte zone results. OH^- and $B(OH)_4^-$ ions move toward the sample zone and the EOF migrates in the reverse direction (toward the BGS zone). As a result,

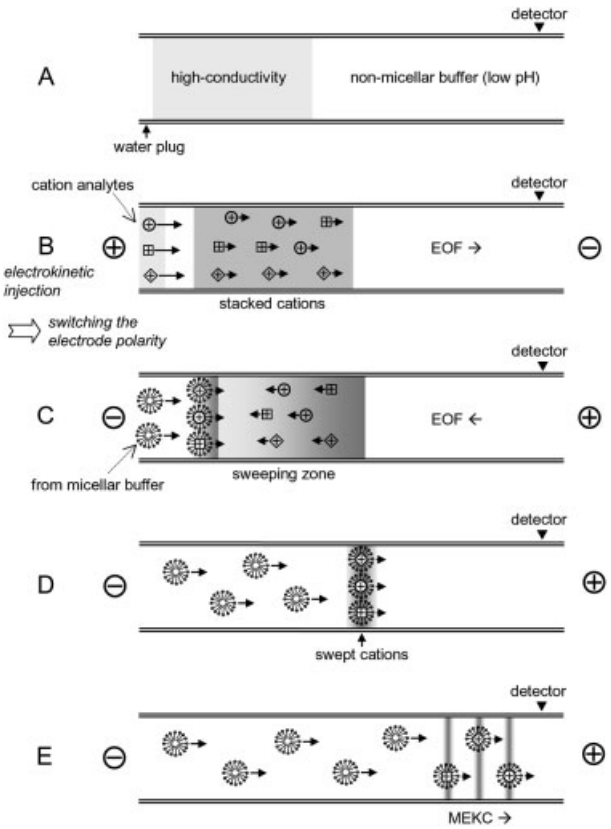

Figure 7. Schematic diagrams of CSEI-sweeping-MEKC model. (A) The capillary is conditioned with a nonmicellar BGS, followed by the injection of a high-conductivity buffer void of organic solvent, lastly, by the injection of a short water plug; (B) the cationic analytes, prepared in a low conductivity matrix or water, are electrokinetically injected for a defined time period; (C) the micellar BGSs are replaced at both ends of the capillary and the voltage is then switched to negative polarity; (D) the micelles from the cathodic vial enter the capillary to sweep the stacked and introduced analytes to narrow bands; (E) the following separation is achieved by the reversed MEKC mode.

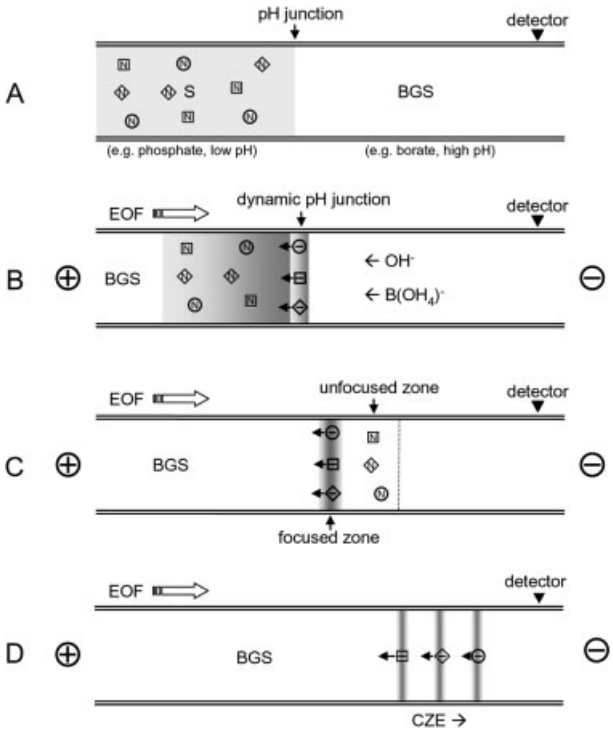

Figure 8. Schematic diagrams of a dynamic pH junction model. (A) The capillary is filled with a high pH-BGS and a section of sample solution (prepared in a lower-pH buffer); (B) a high positive voltage is applied, resulting in a discontinuous electrolyte zone; (C) the anionic analytes are focused on the boundary of the pH junction; (D) separation of the analytes occurs by the CZE mode.

the analytes are focused on the boundary at the pH junction. Subsequently, the analytes are separated by the CZE mode. The focusing effects are dependent on the pH, the concentrations (both of the BGS and the sample matrix), and protonation and deprotonation properties (pK_a values) of zwitterionic analytes. All of these factors determine the changes in the local velocity of the analytes in the two different segments of the capillary. This focusing technique is different from sample stacking since the conductivity of the sample matrix is not of great importance; it can be less than or greater than that of the BGS. This method also can be easily incorporated into conventional CE separations through appropriate changes in the composition of the sample matrix relative to the BGS. However, it cannot be used for the analysis of cationic analytes.

However, a modified method has been reported. By means of a cationic surfactant, namely cetyltrimethylammonium chloride (CTAC), the EOF can be reversed [92]. Figure 9 shows schematic diagrams of this modified method. In the initial step, the BGS, a higher-pH buffer containing CTAC, is used to fill the capillary. The analytes are prepared in a lower-pH matrix which is injected into the capillary to a certain length after the filling of the BGS. Under these conditions, the analytes are positively charged. When a negative voltage is applied, the EOF is reversed (because of the added CTAC) and moves toward the outlet; the interface between the BGS and sample zones move at the same mobility and in the same direction as the EOF. The cationic analytes move toward the inlet with a higher electrophoretic mobility than the EOF and are converted to neutral species by deprotonation at the rear boundary due to the change in pH changing. As a result, sample focusing occurs at the dynamic pH junction; the neutralized and focused analyte zones migrate

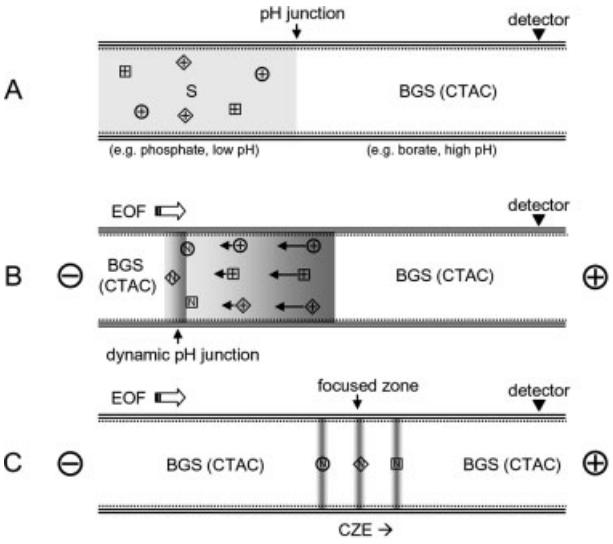

Figure 9. Schematic diagrams of the reversed dynamic pH junction model. (A) The capillary is filled with BGS (prepared in a higher-pH buffer containing CTAC) and sample solution (prepared in a lower-pH matrix); (B) a negative voltage is applied because of the addition of CTAC the EOF moves toward the outlet; the cationic analytes move toward the inlet and change to neutral at the rear boundary due to the change in pH; (C) separation of the analytes occurs by the CZE mode.

as independent zones through the BGS. It should be noted that the analytes must be partially charged to be separated from each other by CZE. An application of this method includes the analysis of anilines with a 100- to 160-fold enhancement in comparison to conventional injection; the LODs for the test anilines were determined at the ppb level by UV detection without any pretreatment procedure [92]. Some applications of dynamic pH junction methods are listed in Table 3.

2.4.2 Dynamic pH junction-sweeping

The dynamic pH junction-sweeping method is similar to the dynamic pH junction mode but additional surfactant (such as SDS) is added to the discontinuous electrolyte system [28]. Schematic diagrams are shown in Fig. 10. The BGS used is a micellar buffer, *e.g.*, a solution containing sodium tetraborate and SDS (high pH). The analytes are prepared in a nonmicellar buffer (low pH). In the initial step, the capillary is filled with BGS and the sample solution is then injected for a certain length into the capillary. When the injection is completed, a positive polarity (if a negatively charged SDS surfactant is used) is applied to power the CE separation. The OH^- ions and anionic SDS micelles enter the capillary. As a result, neutral analytes are converted to anions and are swept by the SDS micelles. Since the mobility of the EOF is greater than that of the SDS micelles, the SDS micelles enter the capillary from the inlet and are used in the subsequent MEKC mode. Once the anionic analytes are completely swept by the SDS micelles, separation occurs by the MEKC mode. This method is very effective for overcoming the often poor band-narrowing efficiency of conventional sweeping (using anionic micelles) and the dynamic pH junction for hydrophilic and neutral analytes, respectively, even if the migration time needed for separation is much longer than that of a conventional dynamic pH junction or sweeping. Applications of this method include the analysis of flavins in plasma and urine with a picomolar detectability by CE-LIF detection, without the need for laborious off-line preconcentration [99].

Table 3. Examples of compounds analyzed by different types of pH junction methods

Technique	Compound	LOD	Concentration efficiency	Reproducibility RSD (%)	Ref.
pH junction	Catecholamines	4×10^{-8} M	250		[26]
	AMP	4×10^{-8} M	50	2.5	[27]
	Myoglobin, lysozyme, α-lactalbumin	25.2–3.1 nM	30–69	2.5–3.4	[90]
	Ethynyl estradiol		100	1.4	[93]
	Xanthine	4×10^{-8} M		2.9	[94]
	Aniline, *m*-anisidine, *p*-bromoaniline	$1.5–3.3 \times 10^{-8}$ M	100–160	0.67–1.1	[92]
	Purine	8×10^{-8} M	50	2.38	[95]
	Serotonin	1×10^{-8} M		1.8	[96]
	8OHdG	20 nM		4.79	[97]
	Pyridine, adenine	2.4×10^{-8} M		10–2.4	[98]
pH junction-sweeping	Flavins	4.0×10^{-12} M	1200	3.8	[99]

Abbreviations: AMP, adenosine monophosphates; 8OHdG, 8-hydroxy-2'-deoxyguanosine

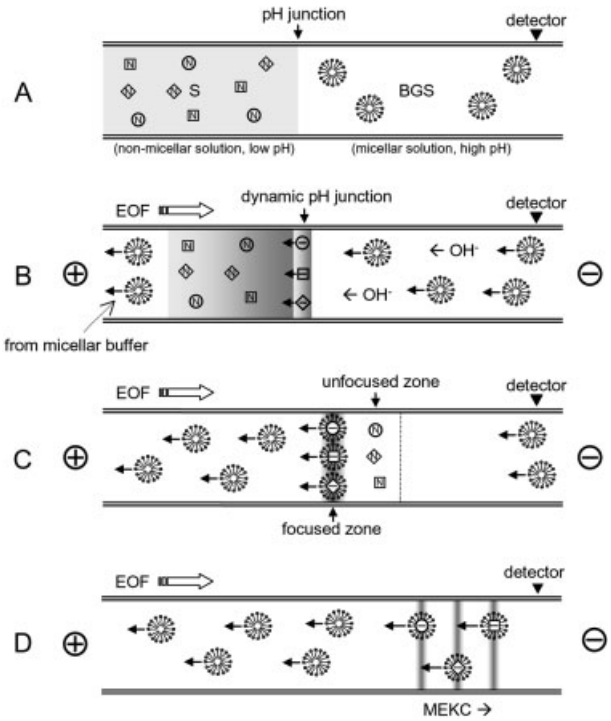

Figure 10. Schematic diagrams of the dynamic pH junction-sweeping model. (A) The micellar (such as SDS) BGS and the sample solution (a nonmicellar buffer) are injected into the capillary, respectively; (B) when the injection is complete, a positive polarity is applied (if a negatively charged SDS surfactant is used) to power the CE separation; (C) the neutral analytes are converted to anions and are swept by the SDS micelles; (D) separation occurs by the MEKC mode.

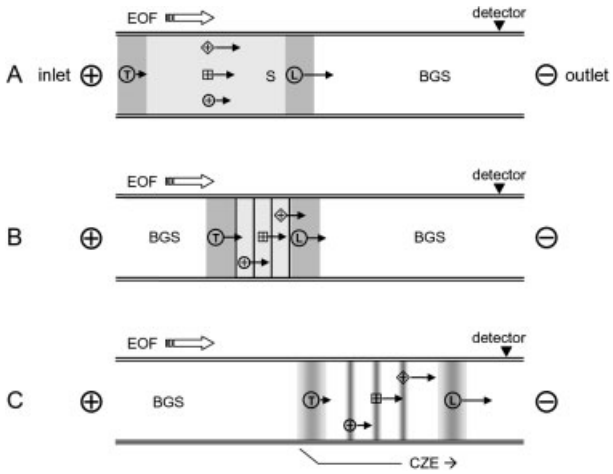

Figure 11. Schematic diagrams of a tr-ITP model for cations. (A) The capillary is conditioned with a BGS, the leading electrolyte, sample solution, and terminating electrolyte are then injected in turn, and a high positive voltage is applied; (B) concentration of the analytes occurs between the leading and the terminating ions during tr-ITP migration; (C) the concentrated analyte zones are separated by the CZE mode.

2.5 Transient ITP and electrokinetic supercharging

ITP was employed as an on-line concentration method in early CE studies [100]. Transient-ITP (tr-ITP) has several advantages beyond FASS, *e.g.*, no dilution of the sample is required and a relatively large sample volume is injected. However, it should be noted that only cations or anions can be concentrated by the tr-ITP method, since analyte ions must be sandwiched between the leading and terminating ions that migrate in the same direction as the analyte ions. Figure 11 shows schematic diagrams of a standard tr-ITP method for cations. For anions, EOF is usually suppressed or reversed by modification of the capillary surface or appropriate additives to the BGS as reversed polarity (cathode is located at the injection side) is employed. A sample solution is injected between the leading and terminating electrolytes which contain faster migrating ions (leading ion) and slower (terminating ion) than the analyte ions, respectively (Fig. 11A). After applying an electric potential, the analyte ions are concentrated between the leading and termination ions during the tr-ITP process (Fig. 11B), and then are separated by normal CZE (Fig. 11C).

In ITP, the concentrations of the analyte ions are regulated by the concentration of the leading ion. Thus, the concentration of the leading ion is adjusted so as to be relatively high (~mM) to obtain high concentration efficiency. Hirokawa and co-workers demonstrated several applications of tr-ITP to the determination of rare-earth ions [101], strontium and lithium ions [102], and iodide ion [103]. They compared FASS with tr-ITP in the concentration of rare-earth ions [101]. In FASS, when the sample volume was increased to more than 5% of the capillary length (effective length, 87.7 cm), the separability deteriorated by the reduction of the separation window and the mismatch of the EOF at the leading boundary between the sample plug and BGS. However, the injection volume could be increased by ~20% in tr-ITP under optimum conditions where the leading and terminating electrolytes were added into the sample before the injection. Furthermore, they also proposed an on-line concentration method referred to as electrokinetic supercharging (EKS) [104]. The method is an electrokinetic injection method with a tr-ITP process. Their studies demonstrated the high concentration efficiency of the method, both theoretically and experimentally [104]. In addition, EKS has been applied to the determination of minor components in rare-earth ore samples, such as erbium, thulium, and ytterbium ions [105]. Both tr-ITP and EKS have advantages and dis-

advantages. For example, tr-ITP is applicable to samples that contain high concentrations of salts, while EKS is not. On the other hand, the amount of the sample that can be injected is limited to ~20% of the length of the capillary in tr-ITP. However, EKS permits the injection of larger amounts than tr-ITP by two concentration steps, electrokinetic injection and tr-ITP, resulting in higher concentration efficiency than tr-ITP.

3 On-line concentration methods for macromolecules

3.1 On-line concentration method for DNA

In general, CGE is a powerful separation technique for DNA separations, including sequencing, genotyping, single nucleotide polymorphism, and fragment analyses. The human genome project has, in fact, been successful as the result of developments in capillary-array electrophoresis systems. In the case of DNA sequencing, the sample is prepared in a mixed solvent of water and formamide, which denatures single-stranded (ss) DNA fragments. Formamide causes a reduction in the electrical conductivity of the sample solution. As a result, ssDNAs are efficiently stacked by electrokinetic injection. For both ssDNA and double-stranded (ds) DNA, polymerase chain reaction (PCR) is frequently utilized for DNA amplification. However, PCR has a disadvantage in quantitative analysis because of poor reproducibility in the reaction yield. Several on-line preconcentration methods have been developed by several researchers. Chang and co-workers [106–108] demonstrated the large-volume injection of a DNA sample by reducing the electrophoretic mobilities induced by a polymer solution containing poly(ethylene oxide) (PEO). In the presence of an EOF, after injecting a large volume of the ds DNA sample in a capillary filled with a buffer solution, the injection end of the capillary was immersed in a PEO solution. When an electric potential is applied, DNA fragments are stacked between the sample zone and the PEO solution. The method is applicable to the on-line concentration of DNA samples prepared in low and high-conductive media. Therefore, no pretreatment is usually required for sample preparation. Park and Swerdlow [109] developed a unique device for concentrating DNA molecules. The device consists of two gap junctions, each of which is covered with a conductive membrane material and built upon a flow channel made of poly ether ether ketone (PEEK) tubing. A sample solution was hydrodynamically introduced from the end of the PEEK tubing. An electric field was applied between the gap junctions, where the negatively charged DNA molecules migrate toward the hydrodynamic flow stream of the sample solution. As a result, DNA fragments are trapped between the junctions. The captured DNA fragments were recovered in a concentrated form by simply turning off the electric field. The recovery of DNA was up to 95%. The effectiveness of the method was demonstrated by an improved sensitivity and separation efficiency in conventional DNA sequencing by CE. The method has also been applied to the concentration of proteins, as described below.

In recent reports, microfluidic channels have been employed for concentrating DNA and proteins. Dai *et al.* [110] have reported on the electrokinetic trapping of DNA molecules in a microfluidic channel. The microfluidic channel consists of a nanoporous polyester membrane sandwiched between two poly(dimethylsiloxane) (PDMS) blocks, each of which contained a single fluidic channel. The design of the microfluidic channel is similar to those reported by Whitesides [111] and Sweedler [112] who employed such a device for investigations of chemical interactions and the control of fluidic transfer. The channels and reservoirs were filled with 1 × Tris-borate-EDTA (TBE) buffer. For the preconcentration of DNA molecules, an electric field was applied between two PDMS blocks across the membrane. The anode and cathode were placed in the waste and source reservoirs, respectively. The buffer solution in the source reservoir was replaced with a buffer solution containing DNA fragments after pretreatment of the channels, which includes the application of an electric potential until the current becomes stabilized. DNA fragments migrate toward the membrane and are then stacked in front of the membrane. It was concluded that the stacking mechanism is not a consequence of physical blocking or size exclusion, but rather the balance of electrophoretic and electroosmotic mobilities. Namely, the electrophoretic mobilities of DNA fragments must be larger than the electroosmotic mobility in the source channel, while the electroosmotic mobility must be larger than the electrophoretic mobilities of DNA fragments in the membrane. When these requirements are satisfied, DNA fragments are concentrated at the surface of the membrane. Enrichment factors exceeding 100 could be achieved by the concentration method.

The on-line concentration of DNA fragments for microchip electrophoresis has been demonstrated by Hirokawa and co-workers [113]. They employed EKS in a single channel microchip. To compare the concentration method with conventional microchip electrophoresis, a cross-column geometry chip and the single-channel chip were employed for conventional electrophoresis and EKS, respectively. Under the EKS mode, the limit of the detectable concentration for dsDNA fragments was improved by more than 10 times. About 2–3 min was required to complete the entire analytical process on the microchip.

3.2 On-line concentration method for proteins

Some of the on-line concentration methods for DNA described above can also be used to concentrate proteins. Astorga-Wells and Swerdlow [114] demonstrated the concentration of proteins, as well as DNA fragments, using a fluidic preconcentrator device. Hirokawa and coworkers [115] applied EKS to the concentration of proteins. A large-volume sample stacking with an electroosmotic flow pump has been demonstrated by Kim *et al.* [116]. The method has previously been applied to small molecules under acidic conditions [117]. Kim *et al.* [116] employed coated capillaries to suppress the EOF. In this method, a sample solution was prepared by dissolving the proteins in water and almost the entire capillary volume was initially filled with sample solution. The cathode was placed at the injection side of the capillary, that is, a reverse polarity was applied. In the stacking process, the proteins were stacked at the boundary between the sample plug and the separation buffer during their migration to the cathodic side by electroosmotic pumping. After replacing the sample plug with the separation buffer, the proteins were stacked at the injection end of the capillary and the proteins then begin to migrate toward the anodic side, resulting in a separation. The magnitude of the electroosmotic mobility is the most important factor in achieving stacking, since the high electroosmotic flow pumps out the proteins before stacking occurs. An enhancement factor of more than 100 times was achieved for proteins with high p*I* values (p$I > 4$) using this method.

A chromatographic method is also applicable to the on-line concentration of peptides as reported by Vizioli and co-workers [118]. A piece of fused-silica capillary (150 μm ID $\times$ 8 mm in length) was filled with the chromatographic packing material, to serve as a concentrator. The beads were held inside the column by two porous frit structures. The concentrator was connected to two longer capillary columns (75 μm ID). Samples were introduced into the capillary for 1 min by hydrostatic injection. Removal was done by flushing with separation buffer. The desorption of bound analytes was carried out introducing an elution buffer containing 75% acetonitrile by hydrostatic injection for 10 s, followed by the introduction of a separation buffer for 5 s. Using this concentrator, the detection sensitivity was enhanced from 200- to 380-fold for synthetic peptides. Isoelectric focusing is potentially useful, not only for separation, but also for preconcentration. Zhang *et al.* [119] demonstrated the on-line hyphenation of capillary isoelectric focusing (CIEF) and CGE by a dialysis interface. The study mainly aimed at the development of an on-line two-dimensional CE system. In this system, two capillaries were connected by inserting them into a short section of hollow dialysis fiber. A protein sample was separated by CIEF in the first capillary, followed the separation by CGE. The method permits a large-volume sample injection in the CIEF step, resulting in preconcentration of the sample.

Wei and Yeung [120] employed an etched porous joint for the on-line concentration of proteins. To construct the etched porous joint, the polyimide coating of a short section of a bare fused-silica capillary (~0.5–1 cm) was removed. The exposed section of the capillary was immersed in 49% HF for 60 min at room temperature. The etching time is critical, since 50 min is insufficient and 70 min was too long. The etched section became a porous membrane that allowed electrical conductivity but prevented the passage of analyte ions. Three buffer vials were used in the CE experiments and high voltages were independently applied between the inlet end and the etched section and between the outlet end and the etched section. When a high voltage was applied to the concentration capillary (between the inlet end and the etched section), proteins and peptides were concentrated at the etched portion. After focusing, the narrow sample zone was introduced into the separation capillary (between the outlet end and the etched section). More than 100-fold stacking could be achieved using this method. A similar setup was employed for the on-line preconcentration of proteins by Lee *et al.* [121]. Instead of constructing an etched capillary, two capillaries were connected by inserting into a short section of a hollow dialysis fiber, similar to that employed for the 2-D system described above [119]. Protein analytes were concentrated by approximately 60- to 200-fold under various conditions.

On-line concentration methods for large molecules, DNA, and proteins have also been described. Several techniques based on electrokinetic phenomena facilitate on-line purification and concentration of DNA and proteins in a solution containing a high concentration of salts and other small molecules. CE is currently one of the most powerful analytical techniques for DNA as demonstrated in genotyping, single nucleotide polymorphism, and sequencing analyses, because it permits rapid and high-throughput performance analyses. A lack of sensitivity can be overcome by a suitable on-line concentration method. The on-line concentration of proteins promises to play important roles in proteome analysis in the future. CE-MS is a promising technique for use in proteomics, although further improvements in sensitivity are necessary at present. Therefore, on-line preconcentration techniques have generated considerable interest in recent years in CE-MS [45, 122–123].

4 Conclusions

CE separation is a rapidly growing separation technique and is now a well-established and widely used analytical method in many fields. The most currently used detection methods are probably UV-absorbance as well as laser-induced fluorescence detection. Improving the detection limits by several orders, on-line sample concentration techniques in CE become beyond doubt popular, important, and have a wide range of applications for both small and macromolecules. Several modes, depending on the various analytes, are now available. Compared to conventional sample concentration methods, such as liquid- and solid-phase extractions, these methods are rather economical, sensitive, rapid, simple, and reproducible. Of very considerable importance in future research on on-line sample concentration techniques in CE is its combination with the other detection methods, so-called hyphenation techniques, such as combinations with MS or microchips. These capabilities will lead to an extension of the utility of CE as useful analytical technique.

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