Fundamentals of Capillary Electrochromatography: Migration Behavior of Ionized Sample Components

Rong Xiang and Csaba Horváth*

Department of Chemical Engineering, Yale University, New Haven, Connecticut 06520-8286

The mechanism of separating charged species by capillary electrochromatography (CEC) was modeled with the conditions of ideal/linear chromatography by using a simple random walk. The most novel aspect of the work rests with the assumption that in sufficiently high electric field ionized sample components can also migrate in the adsorbed state on the ionized surface of the stationary phase. This feature of CEC leads to the introduction of three dimensionless parameters: α, reduced mobility of a sample component with the electrosmotic mobility as the reference; β, the CEC retention factor; and γ, the ratio of the electrophoretic migration velocity and the velocity of surface electrodiffusion. Since the interplay of retentive and electrophoretic forces determines the overall migration velocity, the separation mechanism in CEC is governed by the relative importance of the above parameters. The model predicts conditions under which the features of the CEC system engender migration behavior that manifests itself in a relatively narrow elution window and in a gradient like elution pattern in the separation of peptides and proteins by using pro forma isocratic CEC. It is believed that such elution patterns, which resemble those obtained by the use of external gradient of the eluent, are brought about by the formation of an internal gradient in the CEC system that gave rise to concomitant peak compression. The peculiarities of CEC are discussed in the three operational modalities of the technique: co-current, countercurrent, and co-counter CEC. The results suggest that CEC, which is often called "liquid chromatography on electrophoretic platform" is an analytical tool with great potential in the separation of peptides and proteins.

Whereas capillary electrochromatography (CEC) has established itself as a promising high-performance analytical separation technique for mixtures of small neutral molecules,[1−3] its potential for the separation of biological macromolecules with ionogenic functions is yet to be demonstrated. Although novel columns have been developed especially for the CEC of proteins and peptides,[4−8]

progress in applying CEC to this area of bioseparations has remained slow. One of the major impediments to enlarge the scope of CEC applications is the lack of our theoretical understanding of the interplay of electrophoretic and chromatographic forces in bringing about the separation of polyelectrolytes with an efficiency greater than with traditional chromatography or electrophoresis alone. In view of the growing significance of proteomics,[9,10] there is an increasing need for high analytical performance that CEC might provide in the field of protein/peptide separations.

The main goal of this paper is to examine the physicochemical underpinning of the separation process for electrically charged biological macromolecules in CEC. So far we have drawn the following conclusions from earlier observations: (i) migration behavior of neutral sample components in CEC obeys the same rules as in HPLC;[11,12] (ii) with electrically charged sample components, the elution window can be significantly narrower than that in traditional HPLC under similar conditions;[5,6,13] (iii) peak capacity can be several times greater with electrosmotic flow (EOF) than with pressure-driven flow in traditional HPLC;[1,14] (iv) chromatograms of charged species obtained with isocratic CEC look very similar to those obtained in traditional HPLC with gradient elution;[8] (v) in contradistinction to HPLC, the dependence of the (logarithmic) retention factor on the organic modifier or salt concentration in CEC is mostly nonlinear.[15]

These features of migration behavior have not yet found a satisfactory explanation despite the theoretical work published so far.[16−19] Therefore, we have developed a model to examine the

* Corredsponding author: (tel) (203) 432-4357; (fax) (203) 432-4360; (e-mail) csaba.horvath@yale.edu.

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mechanism of the separation process in CEC with charged sample components. The results are expected to facilitate the interpretation of CEC data, the design of the chromatographic system, and the optimization of the operating conditions. In this endeavor, we shall not use the velocity frame, which is mostly employed in the treatment of CEC, but we are taking an approach based on the notion of a simple random walk[20] and assume that charged sample components in the adsorbed state on the stationary phase can also migrate under the influence of the electric field.

RANDOM WALK MODEL OF CEC

We shall treat the migration of a sample component in CEC as a random walk that consists of a series of partitioning steps between the mobile and stationary phases.[20] We assume that each step has the same length and the time spent by the sample molecule in the stationary and mobile phases are given by τ_s and τ_m, respectively. If we denote the average number of partitioning steps during the migration time of the sample component across the column by N, the overall migration time is given as

$$t = N(\tau_s + \tau_m) \tag{1}$$

Following the definition of the retention factor in HPLC (as a peak locator), we obtain the migration factor in CEC, k_{cec}, as[19]

$$k_{cec} = (t_{cec} - t_0)/t_0 \tag{2}$$

where t_{cec} is the migration time in CEC. In the following, reference will be made frequently to the use of a given column in the CEC and the HPLC modes, i.e., with and without electric field, under otherwise identical conditions, respectively. For instance, the migration time of a neutral unretained tracer, t_0, that is given by

$$t_0 = N_0\tau_m \tag{3}$$

where N_0 is the value of N in the same column operated in the HPLC mode under otherwise identical separation conditions. In this case, N_0 is the same for all sample components because in HPLC all of them migrate with the mobile-phase velocity. Consequently, the actual migration length of all sample components in the mobile phase is also the same and is given by

$$L_0 = N_0 u_m \tau_m \tag{4}$$

where u_m is the mobile phase velocity. The retention factor in isocratic HPLC mode, k_{lc}, is given by

$$k_{lc} = \frac{t_{lc} - t_0}{t_0} = \frac{N_0(\tau_s + \tau_m) - N_0\tau_m}{N_0\tau_m} = \frac{\tau_s}{\tau_m} \tag{5}$$

As seen from eq 5, the retention factor, k_{lc}, is the ratio of the time the sample component spends in the stationary and mobile phases.

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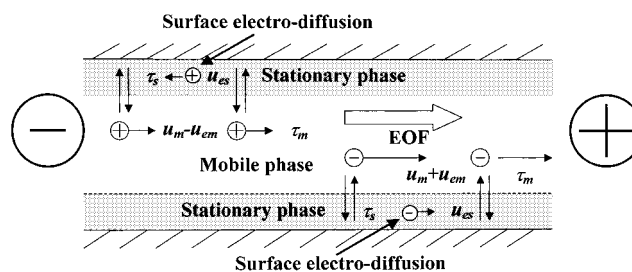
Figure 1. Schematic illustration of a simple random walk by ionized sample components in CEC. Negatively charged sample components migrate codirectionally toward the anode while the positively charged sample components migrating counterdirectionally.

The electrophoretic migration of the sample components in CEC can be in the same direction as that of EOF, called codirectional, or in the direction opposite to that of EOF, called counterdirectional. The overall migration velocity is the sum of the two velocities, and this is shown schematically in Figure 1. It is seen that negatively charged sample components move codirectionally toward the anode, which are faster than those positively charged sample components moving counterdirectionally. For a sample mixture containing both codirectionally and counterdirectionally migrating sample components, the separation process is called co-counterdirectional.

For the sake of simplicity, we shall assume first that the sample components are moving codirectional with the mobile phase. The velocity of the sample molecules migrating in the mobile-phase stream is the sum of u_m and u_{em}, where u_{em} is the electrophoretic velocity in the mobile phase as it is determined by its electrophoretic mobility and the field strength. We assume that when an electrically charged migrant is sorbed by the charged stationary phase, it may not be arrested completely by adsorption on the stationary phase. Rather, while in the stationary phase it will migrate under the influence of the electric field. This phenomenon, which is conveniently called surface electrodiffusion, makes the actual migration length L in the mobile phase appear to be shorter in codirectional mode, as shown in Figure 1. The migration velocity of the "retained" sample component in the stationary phase, u_{es}, is not expected to be higher than u_{em} due to the higher resistance to mass transfer in the stationary phase, although it is possible that, in certain rare cases, u_{es} may be larger than u_{em}. For the sake of simplicity, in this work we assume that u_{es} is lower than u_{em}. It is important to note that it is a unique feature of CEC that both retention and surface electrodiffusion occur simultaneously in or on the stationary phase, in contrast to the conventional view in the chromatographic theory that the sample components are immobilized when they are retained by the stationary phase. Of course, electrophoretic migration of the "retained" sample component only occurs when the migrant is electrically charged.

The number of partitioning steps N is different for each component and given by

$$N = \frac{L_0 - N\tau_s u_{es}}{(u_m + u_{em})\tau_m} \tag{6}$$

Substituting eqs 4 and 5 into eq 6, we obtain

$$N = N_0 u_m / (u_m + u_{em} + k_{lc} u_{es}) \qquad (7)$$

Substitution of eqs 1 and 7 into eq 2 results in the following expression

$$k_{cec} = \frac{u_m}{u_m + u_{em} + k_{lc} u_{es}}(1 + k_{lc}) - 1 \qquad (8)$$

Let us express γ by the ratio of the electrophoretic migration velocities of the migrant at the stationary-phase surface and in the mobile phase as follows

$$\gamma = u_{es}/u_{em} = \mu_{es}/\mu_{em} \qquad (9)$$

Equation 9 shows that γ reflects the strength of the surface electrodiffusion, which is related to the thickness, porosity, tortuosity, and charge properties of the stationary phase.

Another important migration parameter α is expressed as

$$\alpha = u_{em}/u_m = \mu_{em}/\mu_m \qquad (10)$$

where α is conveniently called reduced mobility.[21] In our approach, α plays a pivotal role together with parameters β_{cec} and β_{lc} that are defined by

$$\beta_{cec} = t_{cec}/t_0 = 1 + k_{cec} \qquad (11)$$

and

$$\beta_{lc} = \frac{t_{lc}}{t_0} = \frac{\tau_s + \tau_m}{\tau_m} = 1 + k_{lc} \qquad (12)$$

Substituting eqs 9–12 into eq 8, we obtain

$$\frac{1}{\beta_{cec}} = \frac{1 + \alpha(1 - \gamma)}{\beta_{lc}} + \alpha\gamma \qquad (13)$$

In CEC, β_{cec} is always positive and β_{lc} is always greater than unity. The fact that β_{cec} is always positive overcomes the inconvenience caused by the negative value of k_{cec}.

The factor $1 + \alpha(1 - \gamma)$ in eq 13 could be called reduced relative mobility that reflects the difference in the reduced mobility of the sample component between the mobile phase $(1 + \alpha)$ and the stationary phase $(\alpha\gamma)$. The importance of this factor will be discussed in a subsequent section.

When α is zero, β_{cec} equals β_{lc}. This means that the CEC system behaves as if it would be isocratic HPLC or when all the sample components were neutral. When β_{lc} is unity, as in capillary zone electrophoresis, β_{cec} equals $1/(1 + \alpha)$, which is in agreement with the definition of β_{cec}.

MIGRATION PARAMETERS IN CEC

When γ is unity, the two velocities u_{es} and u_{em} are the same, and eq 13 will yield the following relationship

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$$1/\beta_{cec} = 1/\beta_{lc} + \alpha \qquad (14)$$

In contradistinction, when γ is zero, i.e., in absence of surface electrodiffusion, eq 13 is reduced to

$$1/\beta_{cec} = (1 + \alpha)/\beta_{lc} \qquad (15)$$

Plots of β_{cec} against β_{lc} with γ as the parameter are depicted in Figure 2. As illustrated, in codirectional CEC, β_{cec} is usually smaller than β_{lc}. When γ is zero, β_{cec} depends linearly on β_{lc}.

In most cases in CEC, γ is believed to range from 0 to 1. The particular value depends on the relative magnitude of the migration velocity of the sample component in the stationary phase by surface electrodiffusion. Generally, when γ is increasing, β_{cec} is decreasing so that the increase of u_{es} results in a smaller apparent retention. Following the definition of k_{lc} in HPLC, where the sample components are assumed to migrate with the same velocity in the mobile phase, we define an actual chromatographic retention factor, $k_{lc/cec}$ in CEC. However, unlike k_{cec}, which is defined as a peak locator by considering the differential migration of the sample components in both the mobile phase and stationary phase, $k_{lc/cec}$ does not entail the differential migration of sample components in the mobile phase. Thus, it solely reflects the retention between the stationary phase and mobile phase. Parameter $k_{lc/cec}$ has to be measured with an unretained tracer that has the same electrophoretic migration velocity in the mobile phase of a sample component instead of a neutral unretained tracer.

It can be derived by using the same path and expressed as

$$k_{lc/cec} = \frac{(1 + \alpha - \alpha\gamma)k_{lc}}{1 + \alpha + \alpha\gamma k_{lc}} \qquad (16)$$

Equation 16 shows a rectangular hyperbolic relationship of $k_{lc/cec}$ on k_{lc} which is formally similar to Langmuir adsorption isotherms as shown in Figure 3a. As seen in Figure 3b, when α and γ are constant, plots of $1/k_{lc/cec}$ against $1/k_{lc}$ are linear. Since the maximum value of $k_{lc/cec}$ is determined only by α and γ, it is independent of k_{lc}. It is seen from eq 16 that when γ equals zero, e.g., the sample components to be separated are neutral, $k_{lc/cec}$ equals k_{lc}. When γ is not zero, i.e., surface electrodiffusion takes place, $k_{lc/cec}$ will be smaller than k_{lc}.

It follows from the above considerations that the difference between the separation mechanisms in CEC and HPLC is not merely the difference of the velocities in the mobile phase, which has been considered to be the only difference in previous work.[14] The surface electrodiffusion changes the magnitude of chromatographic retention in the stationary phase that could be explained either by the change of the actual migration length L or by the change in the value of τ_s.

Since neutral compounds are not expected to migrate electrophoretically, the migration behavior and the separation mechanism of neutral sample components in CEC are believed to be the same as in HPLC provided that both the stationary and the mobile phases are unaffected by the electric field. This also can be seen from eqs 13 and 16 by letting α equal to zero.
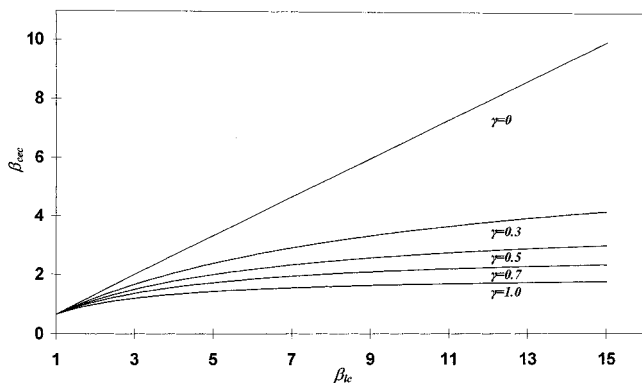
Figure 2. Plot of β_{cec} against β_{lc} with γ as the parameter in codirectional CEC. The value of α is set at 0.5.

$$Slope = (1+\alpha)/(1+\alpha-\alpha\gamma)$$
$$Intercept = \alpha\gamma/(1+\alpha-\alpha\gamma)$$
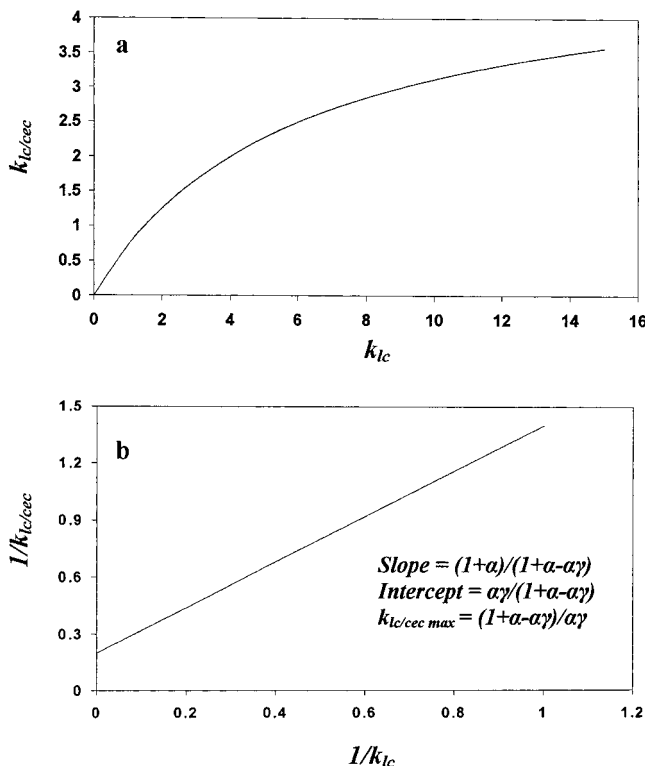$$k_{lc/cec\ max} = (1+\alpha-\alpha\gamma)/\alpha\gamma$$

Figure 3. Plot of $k_{lc/ec}$ against k_{lc} in codirectional CEC. The value of both α and γ is set at 0.5.

For the examination of counterdirectional separation, we follow the same path and get

$$\frac{1}{\beta_{cec}} = \frac{1 - \alpha(1 - \gamma)}{\beta_{lc}} - \alpha\gamma \qquad (17)$$

Alternatively, we can simply combine eqs 13 and 17 to obtain

$$\beta_{cec} = \frac{\beta_{lc}}{1 + \alpha(\gamma\beta_{lc} + (1 - \gamma))} \qquad (18)$$

where α is a signed quantity. Positive and negative α values indicate that the CEC system is codirectional and counterdirectional, respectively.

Experimental data are easy to present graphically by using eqs 13, 17, and 18 to evaluate γ or even α. Parameters β_{cec} and β_{lc} can
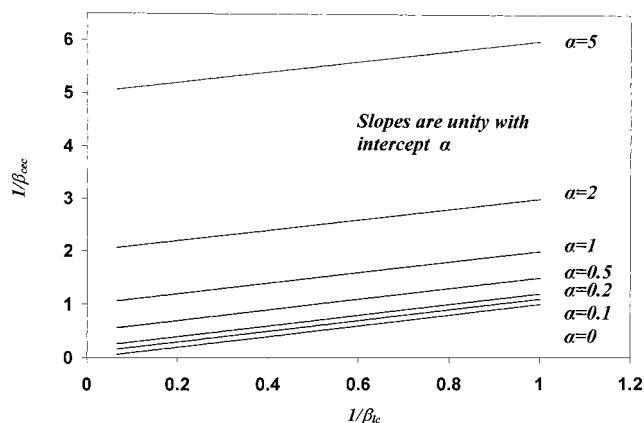

Figure 4. Double-reciprocal plot of $1/\beta_{cec}$ against $1/\beta_{lc}$ with α as the parameter in codirectional CEC. The value of γ is set at 1.0.

be evaluated from experimental runs with a given column in both CEC and HPLC modes under otherwise identical conditions. On the other hand, α can be evaluated from pertinent μ_m and μ_{em} data measured experimentally under both CEC and CZE conditions. Then γ can be calculated by solving eq 18. Under certain specific conditions, e.g., when separating homologues series, α and γ may be approximately taken as constants. In this case, α and γ could be calculated from the slope and intercept of the lines obtained by plotting $1/\beta_{cec}$ against $1/\beta_{lc}$ according to eqs 13 and 17.

In the next section, we will discuss the migration behavior of charged sample components by using these equations for treating the three different separation modes: codirectional, counterdirectional, and co-counterdirectional CEC.

CODIRECTIONAL SEPARATIONS

In codirectional CEC, the charged sample components migrate in the direction of EOF under the influence of the electric field, as illustrated in Figure 1 by the migration of negatively charged sample components. In other words, codirectional separation occurs when the sign of the surface charge of the column packing is opposite to that of the sample component under investigation. In other words, a negatively charged chromatographic surface with positively charged sample components or a positively charged chromatographic surface with negatively charged migrants is used in the separation by CEC. Since the total migration velocity is the sum of the EOF velocity and the electrophoretic velocity, the codirectional mode of separation is preferred in CEC because it facilitates a relatively high speed of separation. In many cases, some or all peaks of the sample components elute before the inert tracer.[7,15]

A graphical illustration of eq 14 is provided in Figure 4 by plots of $1/\beta_{cec}$ against $1/\beta_{lc}$ with α as the parameter in codirectional separation when γ is set at unity. In Figure 4, it is shown that when the reduced mobility α is very large, although the sample component has strong chromatographic retention i.e., large β_{lc}, the value of β_{cec} will become much smaller than that of β_{lc} according to eq 15. The linear plot of $1/\beta_{cec}$ against $1/\beta_{lc}$ can be used to evaluate α graphically from the intercept of the straight line.

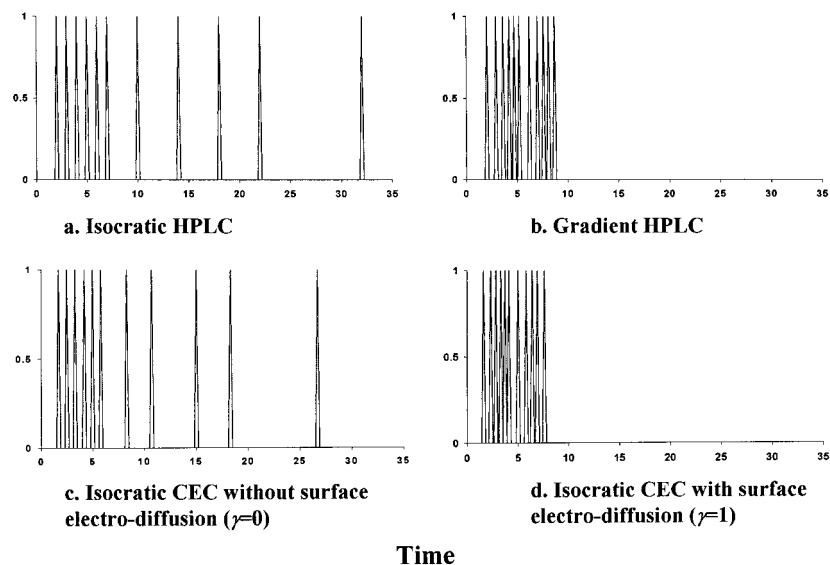As depicted in Figure 5, chromatograms of charged sample components obtained under isocratic conditions by CEC can

a. Isocratic HPLC

b. Gradient HPLC

c. Isocratic CEC without surface electro-diffusion ($\gamma=0$)

d. Isocratic CEC with surface electro-diffusion ($\gamma=1$)

Time

Figure 5. Computer-simulated chromatograms showing the postulated internal gradient effect in codirectional CEC. In gradient HPLC, the gradient steepness parameter b is set at 0.1. In isocratic CEC, the value of α is set at 0.2 and the values of γ are set at 0 without and 1.0 with surface electrodiffusion, respectively.

exhibit a pattern very similar to those obtained with gradient elution in HPLC. We attribute this phenomenon to the combined effect of electrophoretic migration and surface electrodiffusion of the sample components.

To explain this unusual behavior, which has ample experimental support,[7,15,16,22] let us postulate the existence of some internal gradient in the CEC column that affects the separation in a way similar to that of external gradient elution,[23] at least in the separation of charged sample components, e.g., proteins and peptides. From this observation we infer that, in codirectional CEC, isocratic conditions suffice to bring about separations that look as if they would have been made by external gradient elution. This means that the conditions are only pro forma isocratic, i.e., the mobile-phase composition is invariant at the column inlet. Such peak focusing by an internal gradient may be particularly important in the separation of slowly diffusing species, for instance, proteins and peptides, because of the expected relatively high column efficiency.

In HPLC, the retention factor with linear gradient elution in HPLC is usually expressed as[24]

$$1/k = 1/k_{lc} + 2.3b \tag{19}$$

where k_{lc} is the retention factor under isocratic elution conditions, k is the apparent retention factor at the outlet of the column, and b is the gradient steepness parameter given by [24]

$$b = (V_M/Ft_g) \log(k_a/k_b) \tag{20}$$

where t_g is the time of gradient development and k_a and k_b are the isocratic retention factors of the eluite of interest in the neat

eluents A and B, respectively. The total mobile-phase volume in the column is V_m and F is the volumetric flow rate. The value of b ranges from 0.1 to 0.3 in chromatographic practice.

The chromatograms in Figure 5 illustrate the effect of a postulated internal gradient in CEC, in comparison to the pattern obtained by external gradient and isocratic elution in HPLC. The results explain why isocratic elution in some cases may suffice for the separation of charged components, such as peptides and proteins, in codirectional CEC. Nevertheless, external gradient elution still can be effective and in fact needed in the separation of neutral compounds or substances having low molecular mass and charged components in counterdirectional or co-counterdirectional separations.[25-28]

The function of the reduced mobility α in CEC is similar to that of parameter b in HPLC with external gradient elution in determining the elution pattern as can be seen by comparing eqs 14 and 19. Normally, different sample components have different α values, whereas parameter b has the same value for all components eluted by a given linear solvent-strength gradient. As a result, each component may have its own internal gradient with its characteristic steepness that enhances the separation selectivity and peak efficiency. Compared to isocratic elution in traditional HPLC, peak capacities are also higher in CEC, due not only to the higher column efficiency but also to the focusing effect of the internal gradient. As a result, the elution windows also become significantly narrower. The operating range of b is from 0.1 to 0.3, but α has a much wider range in which it may be greater than unity. However, in some cases, it can make the gradient steepness too sharp so that relatively high column efficiency is

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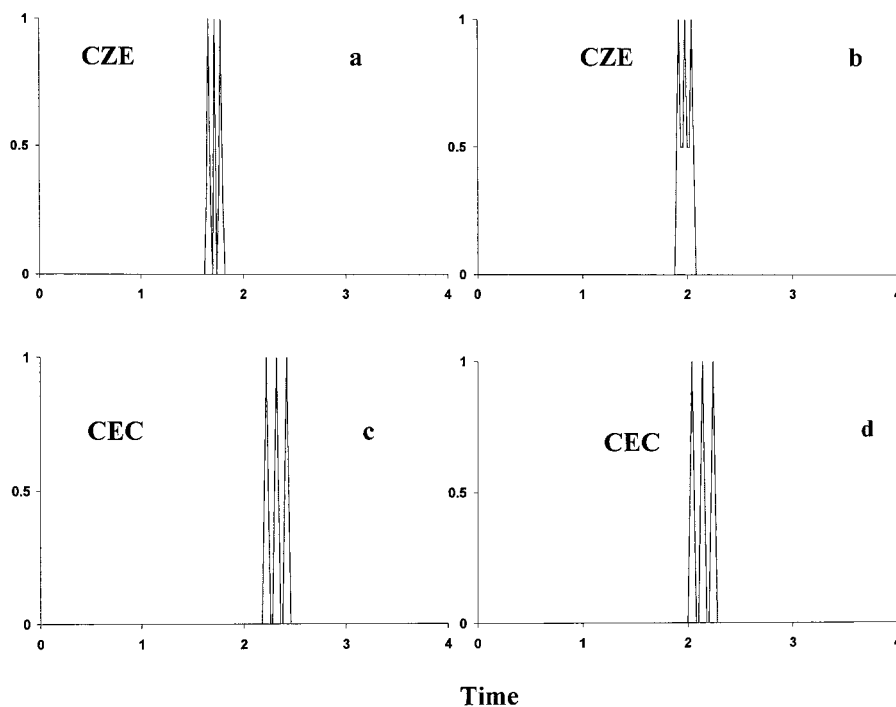(28) Que, A. H.; Kahle, V.; Novotny, M. V. *J. Microcolumn Sep.* **2000**, *12*, 1−5.

Figure 6. Computer-simulated chromatograms showing a peak shift in codirectional CEC and CZE upon changing the value of α. The value of α is initially set at 1.8, 1.9, and 2.0, respectively in (a) and (c) and then reduced by 20% in (b) and (d). The value of γ is set to unity and the value of k_{lc} is assumed to be 4.0 for all three sample components.

required to avoid overlap of the peaks due to the too steep internal gradient.

In certain cases, we found that with a CEC system the optimization of the separation conditions is easier than with a CZE system.[5] This is exemplified by the computer-generated chromatograms depicted in Figure 6 which may help to understand this phenomenon. Within the realm of this investigation, the resolution in CZE becomes worse, whereas in CEC, the resolution changes very little or becomes even better. This is due to the fact that, in CEC, the relative change in elution time by changing the reduced mobility can be magnified by the chromatographic retention, because when $\beta_{lc} > 1$ then the following inequality holds

$$\frac{\beta_{lc}}{1 + \alpha\beta_{lc}} > \frac{1}{1 + \alpha} \qquad (21)$$

This inequality makes the relative change of β_{cec} in CEC larger than that in CZE according to eq 14. It means that, by the appropriate choice of β_{lc}, overlap of neighboring peaks can be controlled easier in CEC than in CZE. Furthermore, changing β_{lc} can also cause a change in β_{cec}. This behavior of CEC imparts to the system relatively high flexibility that makes it easier to find optimal separation conditions.

COUNTERDIRECTIONAL SEPARATION

In counterdirectional CEC, the sample components migrate in the direction opposite to that of the EOF velocity, and as a result, the total or the net migration velocity is given by the difference of the EOF velocity and electrophoretic velocity of the sample component. Although the separation is slower due to the counterdirectional movement of the sample components, it is often used in CEC when electrostatic repulsion can facilitate the
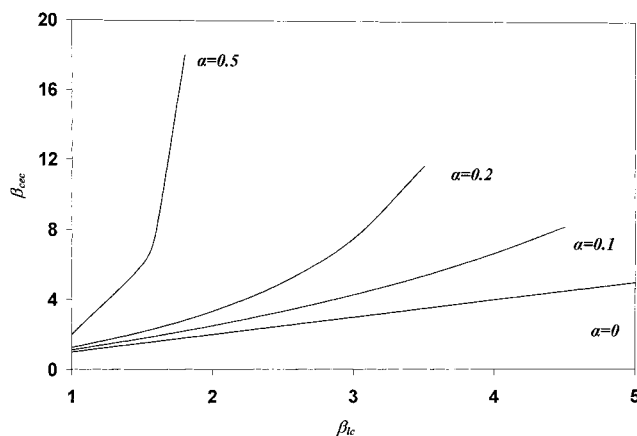

Figure 7. Plot of β_{cec} against β_{lc} with α as the parameter in counterdirectional CEC. The value of γ is set at 1.

separation. Counterdirectional separation occurs when the fixed charges at the chromatographic surface and the charges of the sample components have the same sign, i.e., negatively charged packing surface with negatively charged samples or positively charged packing surface with positively charged samples. Figure 7 illustrates counterdirectional CEC by plots of β_{cec} against β_{lc} with α as the parameter according to eq 17 with γ set to unity. From Figure 7, we can see that the reduced mobility α plays an important role in augmenting the superficial chromatographic retention. Under isocratic elution conditions in CEC, the migration patterns resemble those obtained in chromatographic systems featuring an internal gradient in the mobile phase. However, as discussed above, this internal gradient is different from the external gradient commonly used in HPLC. In fact, the signs of their gradient slopes are opposite, and the reversal of the gradient slopes makes the elution window even wider.
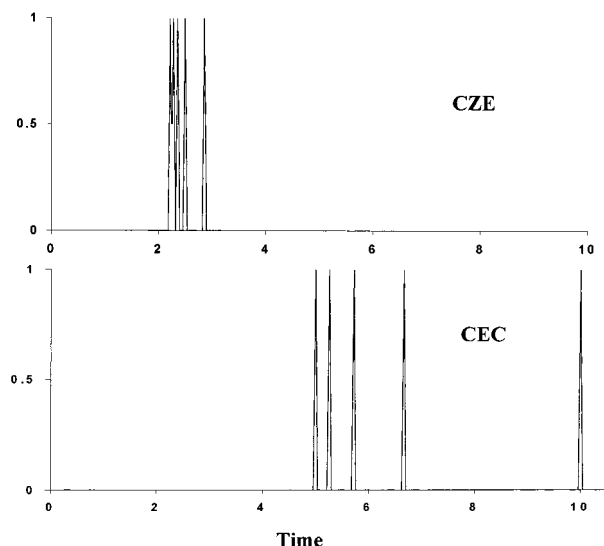
Figure 8. Computer-simulated chromatograms to show protein separation in counterdirectional CEC compared with CZE. The vaue of α is set at 0.1, 0.12, 0.15, 0.2, and 0.3, and the value of γ is set to 1.0. The values of k_{lc} are assumed to be 1.0 for all sample components.

In counterdirectional CEC, elution of the sample components may take a longer time than in HPLC and codirectional CEC under comparable conditions. It is due to the relatively high apparent retention factors enlarged by the relatively highly reduced mobilities in the opposite direction shown in Figure 7. According to eq 18, a sample component may not be eluted, if the following inequality holds

$$1 - \alpha(\gamma\beta_{lc} + (1 - \gamma)) < 0 \qquad (22)$$

According to eq 22, counterdirectional runs should be used when the reduced mobility is not very large, i.e., the electrophoretic velocity is much lower than the EOF velocity, and the chromatographic retention is moderate. This finding offers some guidelines for the design of novel stationary phases in CEC which will be discussed in the conclusions.

Figure 8 shows simulated chromatograms of a protein mixture by counterdirectional CEC. It illustrates the separation of five proteins having much smaller electrophoretic mobility than electrosmotic mobility and low chromatographic retention. The calculated chromatograms show good qualitative agreement with published results.[6,8]

The advantages of using counterdirectional separations rest with the enhancement of the difference between the migration velocities of the sample components and are associated with attenuation of irreversible adsorption by taking advantage of arising electrical repulsion. Nevertheless, if the EOF is very small and the sample components are strongly retained, the separation will take an unduly long time and some of the sample components even cannot elute within the time of the experiment. Consequently, the counterdirectional separation has its limitations. The operating conditions have a major impact on the efficiency of the separation, and optimization may require a significant effort at present. The use of pressure-assisted CEC may alleviate a part of the problem.[29-31] It increases the driving force for the mobile phase

and makes the reduced mobility significantly smaller; thus, it facilitates rapid separations by counterdirectional CEC.

As we mentioned in the section on codirectional separation, the relative change in the elution time in counterdirectional CEC can be controlled by adjusting β_{cec}. It is magnified by changing the reduced mobility because, when $\beta_{lc} > 1$, the following relationship holds

$$\frac{\beta_{lc}}{1 - \alpha\beta_{lc}} > \frac{1}{1 - \alpha} \qquad (23)$$

This inequality makes the relative change of β_{cec} in CEC greater than in CZE when α is negative. It means that, by appropriate choice of β_{lc}, the overlap of neighboring peaks can be more easily controlled in CEC than in CZE. Changing β_{lc} can also cause a corresponding change in β_{cec}. This behavior imparts more flexibility to the chromatographic system and makes it easier to find favorable separation conditions in CEC than in CZE.

CO-COUNTERDIRECTIONAL SEPARATIONS

Co-counterdirectional CEC occurs when the sample mixture contains both codirectionally and counterdirectionally migrating sample components. It usually happens when mixtures of complex positively and negatively charged components have to be separated, e.g., mixture of proteins or peptides having a wide range of isoelectric points or a mixture of acidic and basic compounds. [32-34] In this case, each sample component will migrate in the direction determined by its charge, as well as the composition of the mobile phase and the ζ potential of the stationary phase. Co-counterdirectional separations are hybrids of codirectional and counterdirectional separations. They could manifest the advantages or disadvantages of the two separation modes.

A major problem with co-counterdirectional CEC separation is that, if some components migrate counterdirectionally, part of them may not be eluted from the column or may not even enter the column by electrokinetic injection. Therefore, some peaks of the sample components may be easily lost. This is quite different from reversed-phase liquid chromatography, in which nearly all of the sample components can be eluted by applying a mobile phase having sufficient eluent strength.

For this reason, it is advisable to first separate the sample mixture codirectionally by changing the composition of the mobile phase. The resulting chromatogram is expected to give some hints regarding the migration properties of the sample components. Then co-counterdirectional separation is carried out by further adjusting the composition of the mobile phase, and thus, the separation is optimized by trial and error. Other anisocratic means of elution, e.g., gradient elution, pressure assistance, and temperature programming, may help to get satisfactory separation.

CONCLUSIONS

It has been shown that a simple random walk model can account for the coupled separation mechanism in CEC, which can

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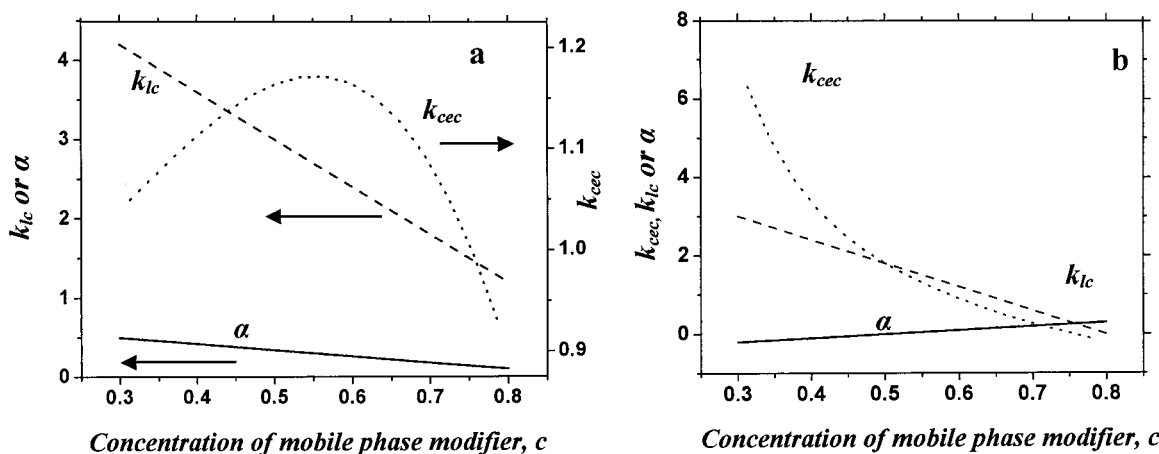(34) Gillott, N. C.; Euerby, M. R.; Johnson, C. M.; Barrett, D. A.; Shaw, P. N. *Anal. Commun.* **1998**, *35*, 217−220.

Figure 9. Computer-simulated plots illustrating the effect of modifier concentration c on k_{cec}, k_{lc}, and α with the value of γ set at 0.5. Both k_{lc} and α are assumed to have linear concentration dependence and, with increasing concentration c, α decreases in (a) and increases in (b).

be considered a hybrid of micro-HPLC and capillary electrophoresis. Isocratic CEC of multicharged sample components such as peptides and proteins has been shown to yield, in certain cases, a separation pattern that has a strong resemblance to that obtained in HPLC with linear gradient elution. The use of the critical migration parameters α, β, and γ introduced in this paper is discussed in the three different separation modes: codirectional, counterdirectional, and co-counterdirectional CEC. The combination of electrophoretic migration in the mobile phase and surface electrodiffusion in the stationary phase in CEC offers a unique analytical technology for separation of charged sample components.

Now the frequently asked question whether a given CEC separation is dominated by chromatographic retention or by electrophoretic migration can be crudely answered as follows. The relative importance of the contributions by each of the two separation mechanisms depends according to eq 18 on the magnitude of the critical migration parameters α, β, and γ. When the sample is neutral, the separation is only dependent on the difference in the chromatographic retention of the components. With an increase of the electrophoretic mobility of the sample components, the apparent retention is attenuated or augmented depending on the reduced mobility. When the reduced mobility is sufficiently large, the effect of the chromatographic retention seems to have a very small contribution to the overall migration.

Figure 9 shows that the apparent retention factor changes significantly by altering the separation conditions, e.g., by modifying the ionic or organic solvent strength of the mobile phase. The dependence of the (logarithmic) retention factor on organic solvent (reversed-phase chromatography) or salt concentration (ion-exchange chromatography) in CEC of charged sample components may be nonlinear. This is in agreement with our experimental results.[35] The migration behavior of sample components in co-counterdirectional CEC is not as easy to predict as that in HPLC. On the other hand, this behavior provides more flexibility to optimize the separation conditions. With the accumulation of further experimental experience and development of theoretical considerations, this may be a potential advantage in separating complex mixtures.

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The stationary phases for use in CEC should be different from the stationary phases being used in HPLC. For example, in codirectional separation, the charge of the sample components is opposite to that of the chromatographic surface. It can be a more serious problem when the sample mixture is composed of complex biological molecules, which are subject to multisite binding and have low diffusivity. The design of new stationary phases for use in CEC must be aimed at eliminating strong binding sites to avoid irreversible adsorption and, at same time, maintain a vigorous EOF. One of the possible ways is to mask strong binding sites at the surface of the stationary phase by coating with a suitable thin hydrogel layer. Such a layer should shield the charges at the stationary-phase surface without too much attenuation of EOF. New stationary phases should also have a high and uniform charge density at the surface of the stationary phase that offers favorable electrokinetic properties through optimal functionalization and topology of the surface. Increase of the ζ potential will raise the EOF velocity and the higher mobile-phase velocity that facilitates fast separations.

Another approach to augment the speed of the separation is based on the use of pressure assistance. The combination of pressure-driven and voltage-driven streams in the column is believed to bring about high mobile-phase velocity without significant loss of column efficiency. Pressure assistance can also be used to adjust selectivity, because it allows a change of the relative mobilities, as follows from eq 18, and thus makes the apparent retention factors in CEC different.[30]

GLOSSARY

b	gradient steepness parameter in external gradient elution in HPLC
c	modifier concentration in the mobile phase
CEC	capillary electrochromatography
CZE	capillary zone electrophoresis

EOF	electrosmotic flow
F	volumetric flow rate
k	apparent retention factor in gradient elution in HPLC
k_a	isocratic retention factors of a given eluite in neat eluent A in gradient HPLC
k_b	isocratic retention factors of a given eluite in neat eluent B in gradient HPLC
k_{cec}	apparent retention factor in CEC in eq 2
k_{lc}	retention factor in isocratic HPLC
$k_{lc/cec}$	actual retention factor in CEC in eq 16
L	actual migration length in CEC
L_0	actual migration length in HPLC
N	number of random walk steps in CEC mode
N_0	value of N in the same column operated in the HPLC mode under otherwise identical conditions
t	migration time of sample component under investigation
t_0	migration time of a neutral unretained tracer
t_g	time of the gradient development in external gradient elution in HPLC
t_{cec}	migration time of sample component in CEC
t_{lc}	migration time of sample component in HPLC
u_{em}	electrophoretic velocity of sample component in the mobile phase
u_{es}	electrophoretic velocity of sample component in the stationary phase
u_m	mobile-phase velocity
V_m	total mobile-phase volume in the column in external gradient elution in HPLC

Greek Letters

α	reduced mobility of sample component in eq 10
β_{cec}	retention factor in CEC in eq 11
β_{lc}	retention factor in HPLC in eq 12
γ	ratio of the electrophoretic migration velocities on the stationary phase and in the mobile phase in eq 9
τ_m	time spent by the sample component in the mobile phase during a partition step
τ_s	time spent by the sample component in the stationary phase during a partition step
μ_{em}	electrophoretic mobility of the sample component in the mobile phase
μ_{es}	electrophoretic mobility of the sample component in the stationary phase
μ_m	electrosmotic mobility