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Review

Ionic boundaries in biological capillary electrophoresis

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Abstract

Ion migration in electrophoresis always leads to the formation of ionic boundaries. While some types of the ionic boundaries can be formed intentionally, e.g., to improve separation efficiency or sensitivity of the analysis, other, naturally formed boundaries, may cause unexpected effects during the electrophoretic experiment. Some of the boundaries often go unnoticed in practice; however, many effects of the ionic boundaries formed by the sample matrix and background electrolyte components are frequently observed in capillary electrophoresis (CE). The interpretation of these effects may not be trivial and different explanations of the same phenomena may be found in the literature. This critical article attempts to review some of the important effects of the ionic boundaries observed or utilized during the CE of biologically important samples.

Keywords: Ionic boundaries; DNA

Contents

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1. Ionic boundaries—fundamental features

For the past 60 years, the electrophoretic techniques were the most important tool for analysis of biological samples. At present, the slab gel electrophoresis and modern capillary electrophoresis instrumentation still are the workhorse for the separations of proteins and DNA fragments. While the solid theoretical basis for the explanation of most phenomena related to ion migration has been laid down over hundred years ago, the observation and description of number of the individual manifests of the ion boundaries is still the topic of a number of scientific communications. This is especially true for the development of capillary electrophoresis where many of the effects related to the ion boundaries, unnoticed in the slab gel experiment, can be observed by the on column detectors. Additionally, the intentional creation of the concentration boundaries for sample preconcentration is an evergreen in the CE with generally poor concentration-based sensitivity. While most of the sample preconcentration procedures are based on the same principle, number of different names can be found in the literature in an attempt to stress a specific experimental condition.

When the electric current passes across an initially sharp boundary between two different electrolyte solutions, new boundaries form, which move away from the initial boundary position. This fact has been well known for more than fifty years and in the case of strong electrolytes such moving boundaries have afforded the most precise determination of transference numbers—the moving boundary method [1,2]. A typical example, frequently taught in the basic course of physical chemistry, is the migration of a nicely visible sharp boundary between potassium nitrate and potassium permanganate in a U tube with two electrodes.

In the case of solutions of proteins, the moving boundary method has made possible an important method for the analysis of complex mixtures such as plasma [3] published first by the Nobel Prize winner Arne Tiselius [3]. Since that time the moving boundary method developed into one of the most powerful separation method—electrophoresis.

Theory of the moving boundary systems has been developed extensively, and in case of moving boundary systems formed by strong electrolytes, and with the assumption that relative ion mobilities are constant throughout the system, explicit solutions and predictions have been developed. These give lucid insight into the dynamics of formation and define basic terms and phenomena of the ion boundaries in a simple way [1,4–8]. For recent description of the above-mentioned items one can mention here following monographs on capillary isotachophoresis [9] and capillary zone electrophoresis [10]. Brief description of the problem is discussed here, too, since it will be useful later on in the description of the behavior of actual biological substances.

As an example a system of two electrolyte solutions in a capillary of a constant cross-section can be considered. At the start of the experiment part of the capillary is filled with a solution of a background electrolyte LR, where R is a cation and L the corresponding anion. The remaining section of the capillary contains a solution of a mixture of background electrolyte LR and a sample SR. The situation scheme is in Fig. 1a. Further, the zone formed by pure background electrolyte is denoted as number 1 and the zone containing sample by number 3. The boundary separating these two zones is denoted as 3:1. The concentrations of the electrolytes are shown in Fig. 1b. Obviously, zone 1 is formed by a BGE of a certain concentration and the zone containing the sample is in fact sample dissolved in a diluted BGE.

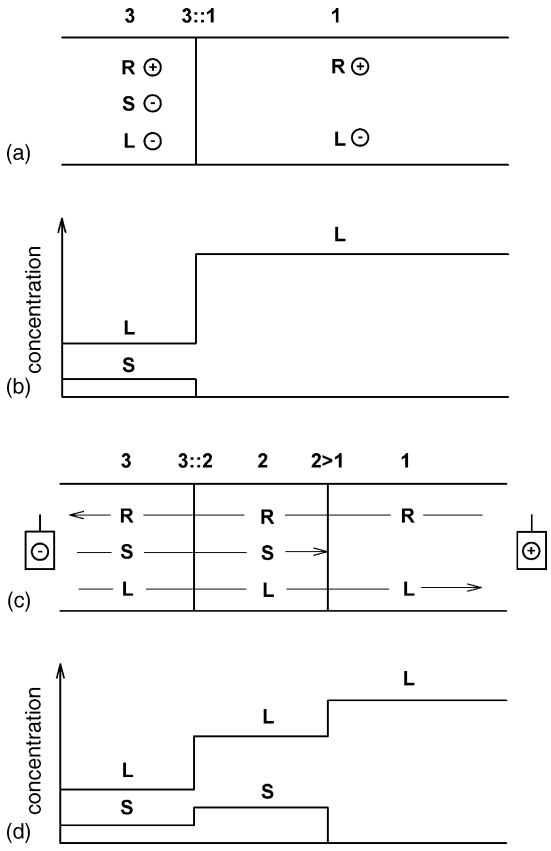

Fig. 1. Scheme of the stationary and moving boundaries. For description and explanation see text.

On passage of the electric current, see Fig. 1c, sample S migrates to the right as depicted by arrows in the figure and, a new moving boundary 2 → 1 is created which migrates with a certain velocity v_{21}. Behind this boundary, a new zone (zone 2) is being formed. Concerning the terminology, the first ionic species, see zone 1, in the direction of migration followed, is named leading and usually denoted by L.

The concentrations of the sample, S, as well as of the leader, L, in the new created zone 2 ($c_{S,2}$, $c_{L,2}$) differ from their concentrations in zone 3 ($c_{S,3}$, $c_{L,3}$), see Fig. 1d. These concentrations are not arbitrary but they are fixed to the values which obey the Kohlrausch regulating function. This function is dependent only on the position along the electromigration path only and does not depend on time—its value is given by the situation prior to the passage of the electric current [4,5].

Besides the name the Kohlrausch function, it is also known under the name omega function.

The value of the Kohlrausch function is proportional to the sum of all concentrations of the ionic species present in the capillary cross-section at a given position [11,12]. In the previous example, the zone 2 is occupying the position of the zone 1 prior to the passage of the electric current. As follows from Fig. 1b, the Kohlrausch function in zone 1 is larger than in zone 3, and, consequently, the newly created zone 2 is more concentrated than zone 3 since it should reach the Kohlrausch function value of zone 1. Concerning the terminology, it is said that the sample is stacked behind boundary 3:2 and its concentration is adjusted to the concentration of the original BGE in zone 1.

At the position of the original zone boundary 3:1, the stationary boundary (also called concentration boundary) remains. It is characteristic for this boundary that the qualitative composition on both sides of it is the same and that the mutual ratios of all the components involved are the same. The ratio of L and S in zone 2 is the same as in the zone 3; however, the overall level of both components is higher in zone 2. The sample becomes more concentrated—stacked. It is worth to note again that the sample stacking occurs at the boundary 3:2, i.e., past the position of the original boundary between the sample and the BGE, in the direction of the electromigration. For recent reviews on stacking see [13–17].

Obviously, the different composition of the zones indicates their different conductivities. Since the density of an electric current is the same in the whole system, different electric field strengths exist in different zones. The migration velocity of the moving boundary $2 \rightarrow 1$, v_{21}, gives a true picture of the mobility of a sample species S, since this species is the only one present on one side of this boundary. This mobility can be expressed as: $u_S = v_{21}/E_2$, where E_2 is the electric field strength on that side of the boundary $2 \rightarrow 1$ which contains the sample (zone 2).

Another important aspect of the migration behavior of the moving boundaries is their dispersive or steady-state character. Referring again to the boundary $2 \rightarrow 1$ in Fig. 1c and d, two cases can be considered with certain simplification (for exact treatment see, e.g., [18]). In the first case the mobility of the sample is less than that of the co-ion, L, of the background electrolyte, and, obviously the conductivity of the zone 2 is less than that of the zone 1. Due to the constant density of the electric current, the electric field strength E_2 is larger than E_1. In this case, the result is the steady-state character of the moving boundary $2 \rightarrow 1$, where the diffusion of the sample species is counteracted by the self-sharpening effect of this boundary. If an ion S enters the zone 1 by diffusion, it experiences lower electric field strength E_1 and its velocity is decreased relative to that of the boundary $2 \rightarrow 1$, resulting in its return to its own zone 2. In this way, a steady-state concentration profile of a sample species S is reached across the moving boundary. The sharpness of the boundary depends on the difference in the mobilities of S and L and on the current density applied [7].

In the second case the mobility of the sample, u_S, is larger than u_L. Obviously, the conductivity of the zone 2 is higher than that of zone 1 and the electric field strength E_1 is larger than E_2. When an ion S enters the zone 1 by diffusion, it is accelerated by the higher electric field strength and moves faster than the boundary $2 \rightarrow 1$. The concentration profile across the boundary becomes flat with time. This dispersion is called electromigration dispersion and its magnitude is proportional to the difference in mobilities of S and L.

Generally for systems of strong electrolytes containing n species, the number of the boundaries formed, their velocities and the composition of the zones created may be theoretically predicted, providing that the compositions of the original solutions as well as the mobilities and the electric current density are known. The resulting description includes differential equations of continuity, the electroneutrality requirement and specifications of mobilities of the species as functions of the composition [12]. As a rule, if the total number of the different ionic species in the system is n, then after passage of an electric current, $n - 1$ boundaries evolve. One boundary is stationary and $n - 2$ boundaries move.

In the system of weak electrolytes, the situation is similar, however, in contrast to strong electrolyte systems, new phenomena are observed because of chemical reactions which take place in the moving boundaries and which reflect abrupt changes in the dissociation of weak acids or protonation of weak bases [19,20]. Obviously, the migrating boundaries in weak electrolyte systems may be characterized not only as the migration boundaries of the substances involved, but also as the moving boundaries of the pH fronts [21,22]. The theoretical predictions are more difficult, and, in most cases, only computer simulations and or numerical solutions are available. Moreover, the self-sharpening effect of the boundaries becomes more complex, since a species in considerations may strongly vary their mobilities when crossing the migrating boundaries. Very thorough and detailed theoretical and experimental studies of these phenomena have been published in connection with the separability of species in capillary isotachophoresis, see [9,16,23].

2. Ionic boundaries generated by the sample

In capillary electrophoresis the initial situation prior to the passage of an electric current, can be characterized as follows. The capillary contains a suitable background electrolyte and, at the injection side there is a sample plug. Both the injection and the detection ends of the separation capillary are dipped into the electrode chambers filled with suitable electrolytes, typically the operational BGE. Thus, at the beginning of the experiment only two original boundaries, front and rear boundary of the sample plug are formed.

After turning on the electric current both these boundaries split into two new subsystems of moving boundaries where the boundaries formed by analytes migrate in the direction to the detection end of the capillary and the boundaries formed

by counterions migrate into the electrode chamber on the injection side of the capillary. Once these subsystems of boundaries have left the original sample spot, they migrate in the opposite direction and do not influence each other. This situation has very important consequences, once the zones of the analytes have left the original sample spot (the original zone represented by the introduced sample plug) and are mutually separated, they migrate in a given BGE independently of each other and their migration order and migration velocities are constant as long as the composition of the BGE (first of all the pH of BGE) is kept constant. This situation facilitates reproducible analyses where the migration order of the analytes does not change, it is fixed, and the migration times of individual zones can strongly aid to the identification of the species analyzed.

An important feature that differs from the general moving boundary systems is stemming from the fact that a sample introduced into the capillary is not a front of infinite length but a zone of a finite length demarcated on both sides by chemical boundaries. The sample zone between these boundaries contains finite amounts of analytes and respective counterions. The overall situation can be described on an example shown in Fig. 2. The BGE consists of 20 mM β-alanine titrated with a solution of KOH to pH 10.7. The sample contains 2 mM peptides A2 and A5, dissolved in 3 mM KOH. The sample plug introduced is 3 mm long and is situated between 5 and 8 mm of the separation capillary measured from the capillary inlet. This situation is depicted in Fig. 2a. The capillary contains the BGE on both sides of the sample. The migration of the sample is anionic or to the right in our scheme.

Upon application of the electric current the anionic sample peptides migrate to the right. The situation after 20 s of migration is in Fig. 2b. It can be seen that the peptides are stacked after they had passed the front stationary boundary (see original sample zone in Fig. 2a) and the front boundaries of their stacked zones migrate directly behind the rear boundary of anionic β-alanine from BGE. Of course, this rear boundary of the β-alanine zone had already left its original position and had moved to the right. From the rear part of BGE, see left of the sample, the original high concentration of β-alanine is decreased after it had passed the stationary boundary and it is adjusted to the lower level of concentrations of the original sample.

The situation after 90 s of migration is in Fig. 2c. Both the sample peptides had migrated out of the original zone; their concentration profiles form typical electrophoretic peaks with a slight tailing. It can be seen that both peptides are now partially separated. The original sample spot, demarcated by two stationary boundaries, is already fully filled with the BGE the concentration of which is decreased. This is a very frequent situation in practice and this original sample spot is frequently a crucial problem since it represents a zone with low conductivity and thus a zone prone to excessive Joule's heating.

The final Fig. 2d represents the situation after 700 s. It can be seen that both peptides are fully separated. Their migration

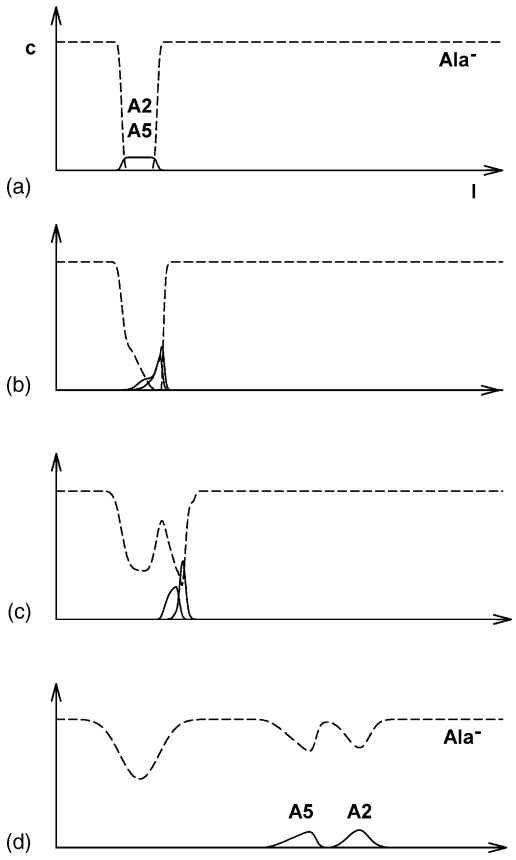

Fig. 2. Simulation of the analysis of two peptides. The composition of the BGE and sample was 20 mM β-alanine + 15 mM K^+ (pH 10.67) and 2 mM A2 + 2 mM A5 + 3 mM K^+, respectively. The simulation was performed for a 3 mm long sample zone at a current density of 200 A m^{-2} using the program Simul [40]. The ionic mobilities of the peptides A2 and A5 were selected as 22.6×10^{-9} and 14.9×10^{-9} m^2 V^{-1} s^{-1}, respectively, and their pK_a 9.8. For explanation see text.

is accompanied by migrating local changes in BGE. In the migrating sample zones, the composition and pH of the BGE used is altered. This phenomenon is of strong importance for practice since in many cases the slight differences between the actual pH in the sample zone and that of original BGE may bring strong changes in the migration behavior of the analytes. The situation at the original sample spot changed slightly due to diffusion, which slowly but continuously equilibrates the concentrations at this point. Hence, the low concentration BGE in the site of the original sample is continuously increased up to the level of the original BGE. The position of this site is, however, fixed, since it does not move electrophoretically. In practice, it can be transported along the separation capillary by residual hydrodynamic flows or by electro-osmosis.

3. Ionic boundaries and transient isotachophoresis

The fact that a sample introduced into the capillary has a form of a discrete zone of a finite length has serious con-

sequence on the migration of sample species during the first stage of their analysis. In many cases, a special mode of electromigration can be formed (often intentionally) for a limited time interval and is called transient isotachophoresis. Isotachophoresis means that a couple of ionic species having mutually different effective electrophoretic mobilities migrate with the same velocity (iso-tacho) in neighboring zones with a common ionic boundary. Another characteristic feature of isotachophoresis is that the coions in isotachophoretic zones are individual pure species.

For an example, referring to the previous section and Fig. 2b it can be seen that the sample species A2, which is faster than A5, crossed the stationary boundary and formed a zone directly behind the zone of β-alanine which is the co-ion of BGE. Obviously, these two zones are in direct contact and the ionic species migrate with the same speed since the electric current would be interrupted upon their separation. This transient isotachophoretic migration survives for such a period of time till the β-alanine from the rear side penetrates through the zones of analytes A5 and A2. Very important feature of transient isotachophoresis is its concentrating power. Due to the adjustment of the concentrations (Kohlrausch regulating function) original diluted sample may be concentrated by orders of magnitude.

4. Ionic boundaries generated by other means

There are very useful arrangements of the operational electrolyte systems in capillary electrophoresis where there are other original boundaries formed at the ends of the capillary used. These arrangements may offer efficient stacking of diluted samples or higher selectivity for difficult separations.

This mode of analysis can be implemented by using electrolytes in one or both electrode chambers different from the BGE used for separation. After turning on the electric current, various pH fronts may be generated at both ends of the capillary and these fronts may effect the migration of analyte zones. In most cases the migration of pH fronts is very fast, usually much faster than the migration of zones of analytes. Hence, the pH fronts formed by co-ions and generated at the injection end of the capillary, though initially situated behind the sample in the direction of their movement, may pass through the zones of the analytes and influence their migration. On the other hand, the pH fronts formed by counterions and generated at the detection end of the capillary migrate in the opposite direction towards the zones of analytes and inevitably pass through them.

A broad spectrum of artificially created migrating boundaries not induced by the sample is covered by the collecting term “dynamic changes” [24–32]. These changes (that are in fact moving discontinuities in concentration, pH and conductivity) comprise (i) a sharp step, (ii) a diffuse gradient, and (iii) a pulse that is usually composed of a sharp step and a diffuse gradient, see Fig. 3. All these changes can be applied either as co-migrating or counter-migrating with respect to

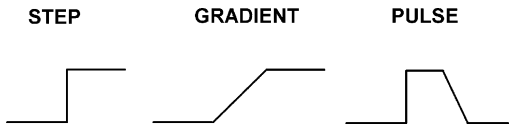

Fig. 3. Typical shapes of dynamic changes in the electrolyte systems.

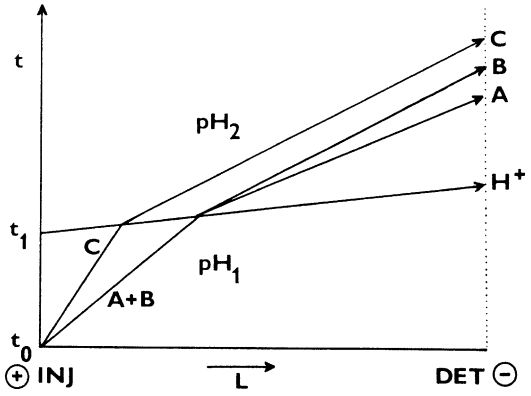

Fig. 4. Migration trajectories of cations A, B and C in a co-migrating pH step. From [29]; with permission.

the migration of the sample zones. The spectrum of possibilities how to generate these changes is quite broad and varies from simple off-line exchange of the content of an electrode reservoir to sophisticated instrumentation with multiple electrode chambers [25,30,32].

Fig. 4 shows an example of the effect of a co-migrating pH step on the separation of cations A, B and C [29]. A and B do not separate at pH_1 and B and C do not separate at pH_2. The trajectories in Fig. 4 show clearly how a co-migrating pH step (from pH_1 to pH_2) can assure complete separation of all three cations. Fig. 5 shows an application of this technique to the separation of a protein mixture [28]. Separation at pH 3.0 (panel A) brings partial resolution only and separation at pH 4.5 (panel B) is accompanied by extensive tailing although the resolution is improved. The application of a co-migrating pH step (4.5–3.0) solves the problem (panel C).

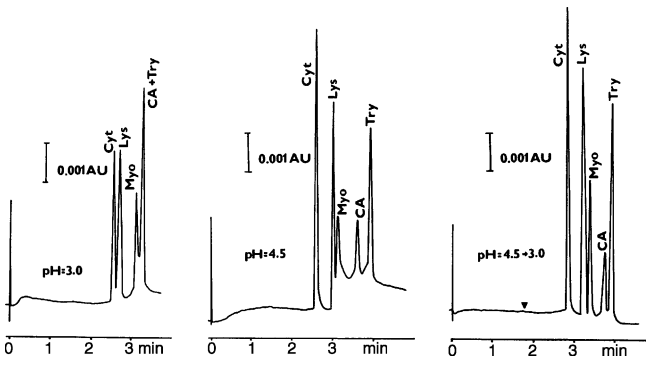

Fig. 5. Cationic separation of proteins: left—at constant pH 3.0 (8 kV, 13 μA); middle—pH 4.5 (8 kV, 12 μA); right—using a dynamic pH step (pH change from 4.5 to 3.0). UV detection at 206 nm. Cyt = cytochrome c; Lys = lysozyme; Myo = myoglobin; Try = trypsinogen; CA = carbonic anhydrase. From [29]; with permission.

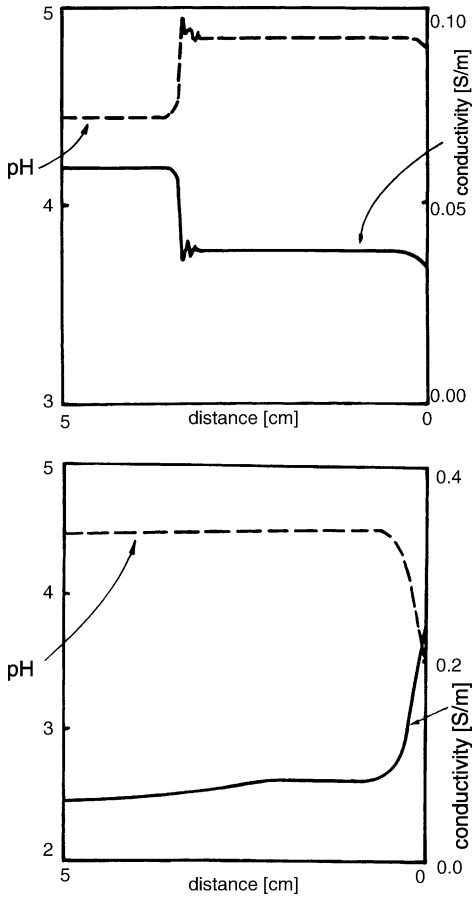

Fig. 6. Computer simulation of the pH and conductivity profiles of the ionic boundary formed during electrophoresis in 20 mM ε-aminocaproic acid/phosphoric acid, pH 4.4, as BGE. Conductivity is in Sm^{-1}. (top) 1% acetic acid as LS. (bottom) 1% formic acid as LS. From [33] with permission.

A practical example of countermigrating pH step is the formation of a moving boundary induced by the liquid sheath in coupling of the capillary electrophoresis with electrospray mass spectrometry (CE-ESI/MS) [33]. Here the BGE and the liquid sheath (LS) may contain different ions, leading to the formation of the moving boundary inside the separation capillary during the electrophoresis. Since this boundary moves opposite to the analyte zones, a change in migration of the separated compounds can be observed due to a different pH, ionic strength, conductivity, and type and extent of ionic interactions. Fig. 6 shows the computer-simulated pH and conductivity profiles of the ionic boundary formed during electrophoresis in 20 mM ε-aminocaproic acid/phosphoric acid, pH 4.4, as BGE, with 1% acetic acid (A, top) and 1% formic acid (B, bottom) as LS [33]. The effective mobility of acetic acid is lower than that of phosphoric acid and therefore a sharp boundary migrates into the separation capillary, accompanied by a considerable conductivity and pH step. In case of formic acid as LS, the mobility of the LS counterion is higher than that of the BGE, and therefore, a diffuse zone migrates into the capillary that does not change pH or conductivity considerably. Fig. 7 shows the effects of the above-mentioned

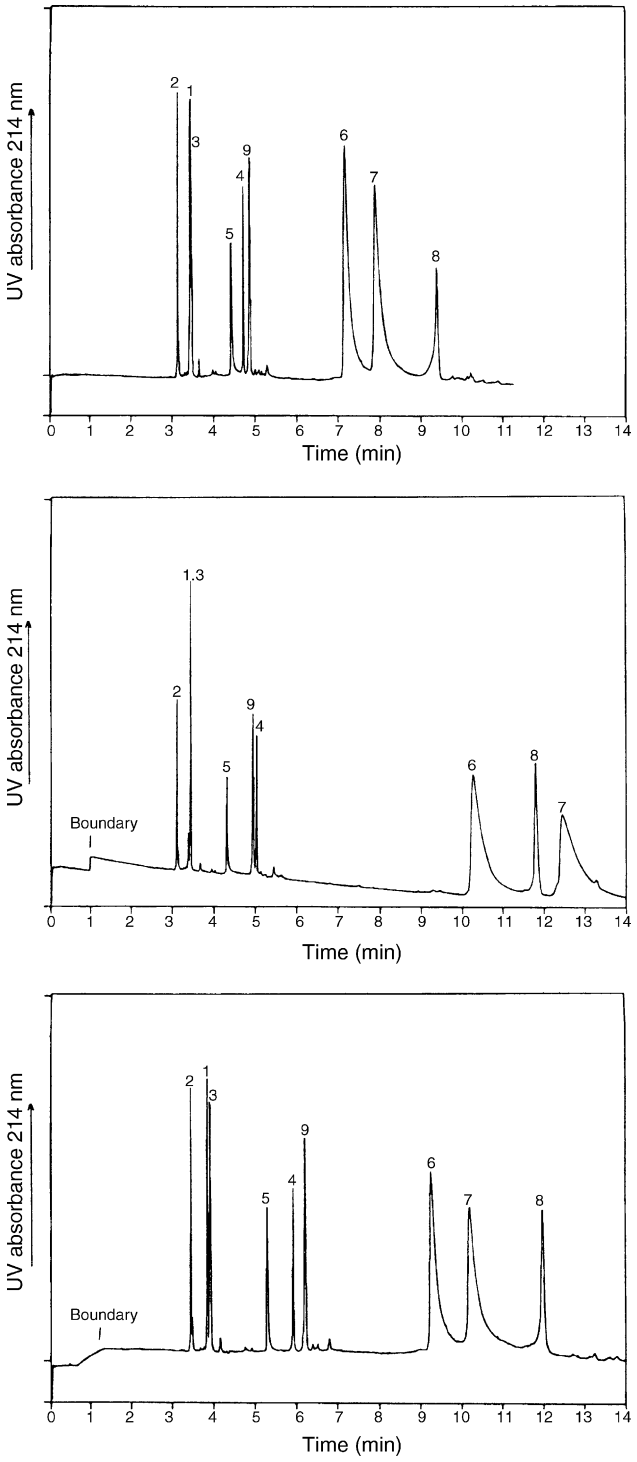

Fig. 7. UV detection of ionic boundaries during the separation of the model protein sample. BGE, 20 mM ε-aminocaproic acid/phosphoric acid, pH 4.4. Capillary, 36 (27) cm × 75 μm i.d. Constant voltage, 25 kV. (top) Reference separation with the BGE in both electrode chambers; stable current, 13 μA. (middle) 1% acetic acid in 50% methanol/water in cathodic reservoir; continuously decreasing current, 13–6 μA. (bottom) 20 mM formic acid in 50% methanol/water in cathodic reservoir; continuously increasing current, 12–18 μA. The peak identification is (1) cytochrome c; (2) lysozyme; (3) aprotinin; (4) myoglobin; (5) RNase A; (6) β-lactoglobulin B; (7, 8) β-lactoglobulin A + carbonic anhydrase; (9) α-chymotrypsinogen A. From [33]; with permission.

boundaries on the separation of a protein mixture. The differences between the reference separation (LS = BGE, panel A) and the separations with LS moving boundaries (LS = acetic acid, panel B; LS = formic acid, panel C) clearly demonstrate the potential of these effects.

From the above results, it is obvious that such a process can result in either an unwanted effect that negatively affect the separation or in a positive change of selectivity during the separation. The counter-migrating boundary can be either diffuse or sharp, depending on whether the effective mobility of the sheath liquid counterion is higher or lower than that of the BGsE, respectively.

5. Prediction of the ionic boundaries by computer simulation

The migrating ionic boundaries are a frequent phenomenon in electrophoretic separations. Although they may significantly affect the separation process and the results of an analysis, they frequently remain undiscovered. A very useful tool to get insight into the behavior of electrophoretic systems is the computer simulation. Computer simulation of electrophoresis in its most frequent form is based on the numerical solution of a one-dimensional approximation of basic transport equations. Using the concentration distributions of all substances along the capillary, their mobilities and pK_a values as input data, the simulation shows how all the concentration profiles change with time and allows monitoring the electrophoretic separation process including all migrating boundaries.

There have been published a number of simulation models differing mainly by the extent of simplifications to achieve the simulation on a given computer platform. The models also differ by the types of supported substances (all models cover univalent small ions). Two simulation models that became widely used as a real tool for theoretical research of electrophoresis will be mentioned here. The first one reported by Mosher and co-workers [34,35] underwent many improvements until recent days including the possibility to simulate proteins [36–38]. It is capable to perform high-resolution simulations of complicated systems at real laboratory conditions in its present form. The second model by Gas et al. [39] is currently available as a shareware program called Simul [40] and became very popular for its simple and user-friendly interface.

Fig. 8 shows the typical computer simulation, which can be used to explain the behavior of a complex system [41]. The simulation corresponds to the system with a stacking zone containing a highly mobile co-ion. This mobile co-ion (ammonium in this case) ensures stacking of the analyte zone (a model peptide) into a sharp zone due to transient isotachophoresis. It is seen how the stacking of the sample starts in Fig. 8b and is completed in Fig. 8c. Fig. 8d then shows the start of the destacking process when the background co-ion moves from the sample.

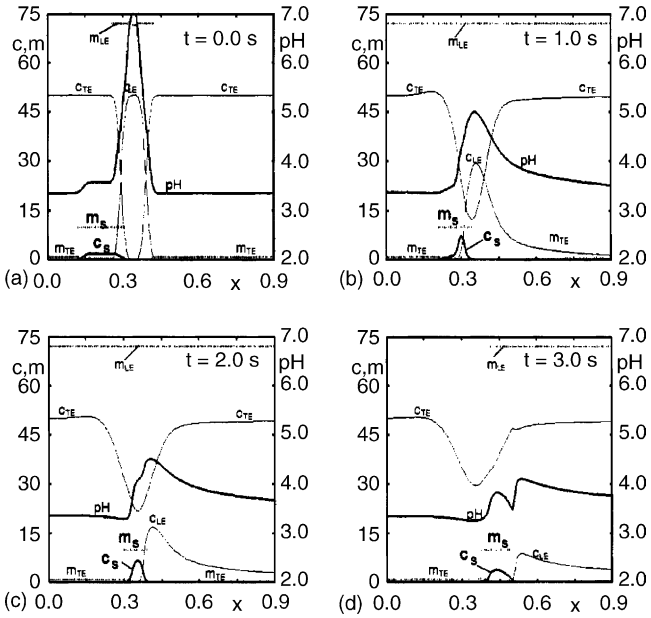

Fig. 8. Computer simulation of the stacking of a peptide in the ammonium/betaine stacking system (BGE: 50 mM betaine acetate, pH = 3.3; stacking zone: 50 mM ammonium acetate). The mobility of the peptide was assumed to be constant, having a value of 10×10^{-9} m^2 V^{-1} s^{-1}. The current density was 1000 A m^{-1}. c_{LE}, c_{TE}, c_S, m_{LE}, m_{TE} and m_S are the concentrations or the effective mobilities, respectively, of the stacking electrolyte, the background electrolyte, and the sample. The concentration c is given in mM, the mobility m in 10^{-9} m^2 V^{-1} s^{-1}, and the length coordinate x is in mm. From [41]; with permission.

6. Selected applications

6.1. Preconcentration of proteins and peptides

Sample preconcentration can help improving both the resolution and detection limit of the analysis. One of the first and still widely used technique for preconcentration of proteins has been developed by Ornstein [42] and Davies [43] and adopted under the term disc electrophoresis. The preconcentration is based on the formation of discontinuities in both the gel concentration and electrolyte composition to form a sharp, concentrated protein zone at the start of the separation gel. Typically, the gel is divided into the stacking and resolving areas. The resolving gel, with the pore dimension suitable for the size of the separated proteins, is prepared in a background electrolyte containing 0.4 M Tris–HCl buffer at pH 8.8. The diluted stacking gel with large pores contains 0.1 M Tris–HCl buffer at pH 6.8. The electrode buffer on the sample application side contains Glycine. After the application of the sample on the stacking gel the proteins electromigrate through the large gel pores and are displaced by the glycine ions. Since the glycine (pI 6.7) has a very low electrophoretic mobility at pH 6.8 the sample is isotachophortically focused between the leading chloride and terminating glycine ions. After migrating through the dilute stacking gel the focused (but unresolved) protein band enters the separation gel and the proteins start separating according to their size through the gel

pores. At the same time the increased pH accelerates the terminating glycine ions, which now migrate faster and form the new background electrolyte for the protein separation. The disc electrophoresis is being used in a number of variations. Besides the attempts to miniaturize the standard slab gel into ultra-thin format with 150 μm gel thickness [44], capillary version of the technique has been tested for protein separation at the cellular level as early as in 1966 [45]. Although successful protein separation has been achieved, further applications were delayed by the lack of a suitable detection system. The development of capillary electrophoresis instrumentation in the 80s and 90s of the last century has brought a renewed interest in preconcentration techniques suitable for proteins and peptides. Although the original disc electrophoresis method could, in principle, be adopted to the capillary format the technical complexity of creating the multiphasic sieving matrix in the capillary [46] directed the development to a different path. After the first successful application of in capillary polymerized cross-linked gels [47] the next development has focused on the main strength of the capillary format—replenishment of the separation matrix after each analysis [48]. At present the peptides and proteins are separated either in free solution, based on differences in the electrophoretic mobilities, or using a replaceable sieving matrix [49]. The most simple sample preconcentration method is based on the injection of sample dissolved in pure water or diluted background electrolyte. The preconcentration is based on the concentration rearrangement when the sample ions move across the stationary concentration boundary sample-BGE [50]. This technique, suitable for creating sharp concentrated starting sample zone using either pressure or electromigration injection is frequently termed "sample stacking" or field amplified sample stacking" [51,52]. The prerequisite of having the sample dissolved in pure water or dilute BGE predetermines this technique mainly for use in quality control of pharmaceuticals. In cases when the sample matrix contains salts or is dissolved in the background electrolyte (e.g., to maintain solubility or prevent protein precipitation) the preconcentration is still possible with the properly selected background electrolyte. It has also been found experimentally, that the addition of organic solvents to the sample can, in some cases lead to the zone sharpening due to the selective changes in the electrophoretic mobilities of the sample and sample matrix ions. For example the addition of acetonitrile at 66% in the sample reversed the deleterious effect of salts and favored the stacking by the electrokinetic injection leading to better separation efficiency [53]. Similarly, adjustment of the sample pH has experimentally led to preconcentration of some of the sample constituents [54] and such an approach has been referred to as pH mediated field amplification on-column preconcentration [55]. Most of such experimental approaches generally lead to the generation of moving boundaries and focusing of the sample components by the isotachophoretic principle. The ITP preconcentration and selective enrichment of extremely large sample volumes (up to 30 μL) can be achieved using a dedicated column coupling instrumentation [56,57]. In this case, the separation proceeds in the ITP mode in the first, larger bore column and only a selected section of the focused sample zones is directed into the second CE separation capillary. The resulting preconcentration of more than 3 orders magnitude allowed protein separations with the limit of detection in the subnanomolar region [58]. Besides the standard two column arrangement the use of longitudinally narrowing channel was also suggested for further increase of the focusing in a continuous pH gradient [59]. Additional advantage of the coupled column separation system is the capability to work with samples containing large amounts of salts such as blood serum [57]; however, the required instrumentation is not widely available. The typical single capillary instrument can be used in a transient isotachophoresis (tITP) mode for preconcentration and separation of sample volumes reaching the total volume of the separation capillary [60]. Two basic modes of the transient sample preconcentration are schematically shown in Fig. 9. In one approach (Fig. 9A) a regular ITP system is used, consisting of a leading (LE) and terminating (TE) electrolyte with the sample injected in between. After turning on the voltage the sample components focus into sharp ITP zone with the concentration close to that of the leading ion. After replacing the terminating electrolyte by a solution of the leading electrolyte the fast leading ions migrate through all the zones formed during the ITP step, resulting in the zone destacking. In the second approach (Fig. 9B) only a single background electrolyte (BGE) is used for both focusing and separation.

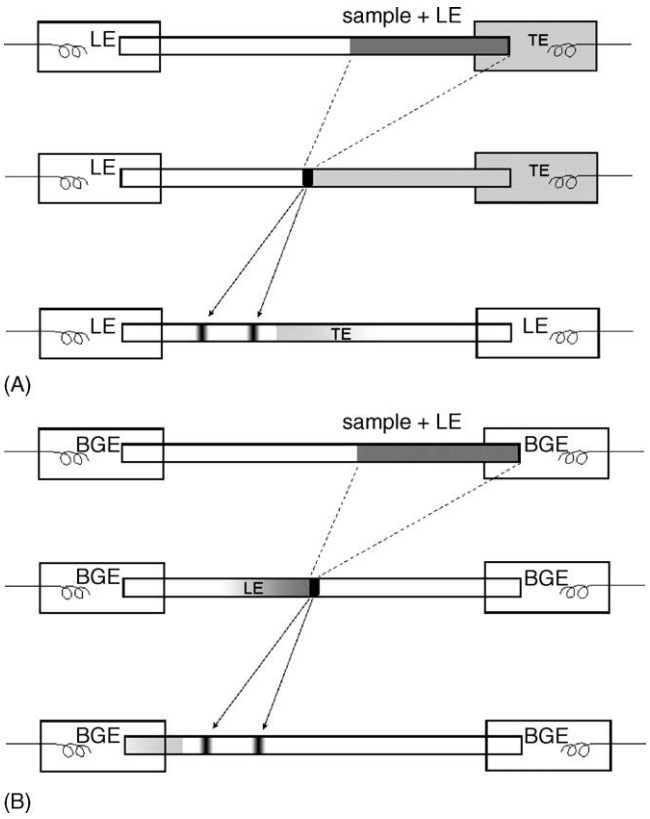

Fig. 9. Schemes of the transient isotachophoretic preconcentration.

The sample is focused behind the zone of fast ions from the sample matrix (sodium, potassium, chloride, sulphate, etc.) forming the transient leading zone. Real samples frequently contain such ions in large quantities. The mobility of the co-ion of the BGE must have sufficiently low mobility to serve as the terminating ion. During migration, the concentration of the asymmetric fronting zone of the leading ions decreases and at certain moment (depending on the original leading ion concentration) the sample bands destack and start migrating independently in the zone electrophoresis mode.

It is worth noting that the zones detected after the tITP may appear much sharper than without the preconcentration; however, the calculation of the separation efficiency is meaningless since the zones migrated in the ITP stack for a significant part of the analysis. Some examples of suitable electrolyte systems are listed in the Table 1. An example of the tITP analysis of a protein mixture in an uncoated capillary is in Fig. 10 where the sample focused behind the zone of the chloride ions. In this example, the electro-osmotic flow transported the whole capillary content to the cathode, allowing the detection of the original injection zone (S).

Table 1
Examples of electrolyte systems for on-column transient ITP preconcentration

Cationic analysis
Method A
Leading electrolyte:
0.01–∼ 0.05 M hydroxides (or suitable salts) of high mobility cations, e.g., Na, K or NH_4 titrated to desired pH by: formic acid, pH range 3.5–4.2 and acetic acid, pH range 4.2–5.2
Terminating electrolyte: 0. 01 M acetic acid
Method B
BGE:
(1) 0.01–0.1 M acetic acid
(2) 0.01–0.1 M β-a1anine + acetic acid, pH 3.54.3
(3) 0.01–0.1 M ε-aminocaproic acid + acetic acid, pH 4.1–4.8
(4) 0.01–0.1 M histidine + acetic acid, pH 5.5–6.3
Sample additive (leading ions) Na, K or NH_4 acetate
Anionic analysis
Method A
Leading electrolyte:
0.01–0.1 M acetic acid titrated to desired pH by: histidine, pH range 5.5 ∼ 6.5; Tris, pH range 7.5–8.5 (For analytes with high electrophoretic mobilities, HCl can be used instead of the acetic acid.)
Terminating electrolyte: 0.01M TAPS(*N*-Tris(hydroxymethyl]methyl 3-aminopropanesulfonic acid)
Method B
BGE:
(1) 0.01–0.1 M MES (2-[*N*-morpholino]ethanesulfonic acid) + histidine (or Tris), pH 5.5–6.5
(2) 0.01–0.1 M TES (*N*-Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid) + Tris, pH 7.0–8.0
(3) 0.01–0.1 M TAPS (*N*-Tris[hydroxymethyl]methyl-3-aminopropane-sulfonic acid) + Tris, pH 8.0–9.0
Sample additive Tris—acetate, sulfate or chloride

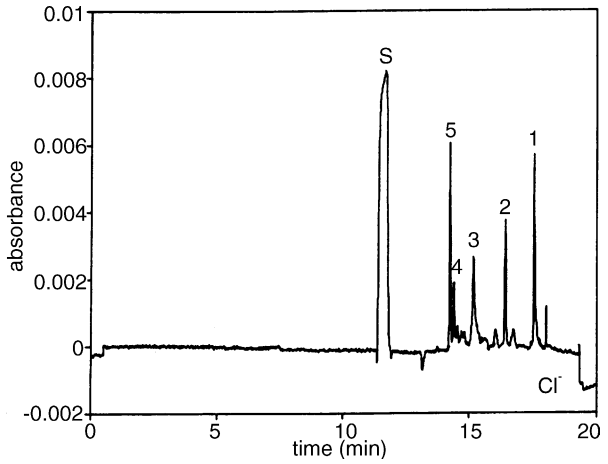

Fig. 10. Anionic tITP analysis of the mixture of acidic proteins in bare fused silica capillary 100 cm × 75 μm i.d. Sample volume 160 nL. BGE: 0.02 M TAPS–Tris, pH 8.3. Sample: (1) glucose-6-phosphate dehydrogenase (2×10^{-8} M); (2) trypsin inhibitor (2.5×10^{-7} M); (3) β-lactoglobulin B (2.5×10^{-7} M); (4) L-asparaginase (2×10^{-8} M); (5) α-lactalbumin (10^{-7} M) dissolved in 10 mM Tris–HCl. Detection at 191 nm. After ref 60; with permission.

The potential of a number of variations of the transient ITP sample preconcentration has been documented on a number of different samples containing peptides and proteins [41,61–67]. As in any separation technique the selection of an optimum electrolyte system for a given sample will always depend on the sample matrix composition and results of the preliminary experiments. The use of the computer simulation as described in the previous section may greatly simplify the experimental work. The simulation of the stacking described in the previous section (see Fig. 8) was performed for the slowest migrating peptide of the of α s1 casein tryptic digest. The practical example of the separation of the digest without and with the stacking is shown in Fig. 11. In this example, a dramatic improvement in both the separation efficiency and signal intensity was achieved in spite of the sample being dissolved in the background electrolyte. Transient ITP stacking was initiated by loading a plug of the leading ammonium acetate electrolyte prior to the sample. The BGE served as the transient terminator during the focusing stage.

It is fair to say that although the electrophoretic sample preconcentration can greatly improve the performance of the CE analysis, there may be cases when additional sample cleanup is required. Besides the standard off-line protocols [68] a number of on-line bead or membrane-based sample preconcentrators have been described [69–71]. In these cases the preconcentrator is typically connected to the injection side and after selective entrapment the sample components are eluted into the separation capillary. Since this process typically does not form a sharp starting zone, additional tITP zone focusing was applied to improve the sep-

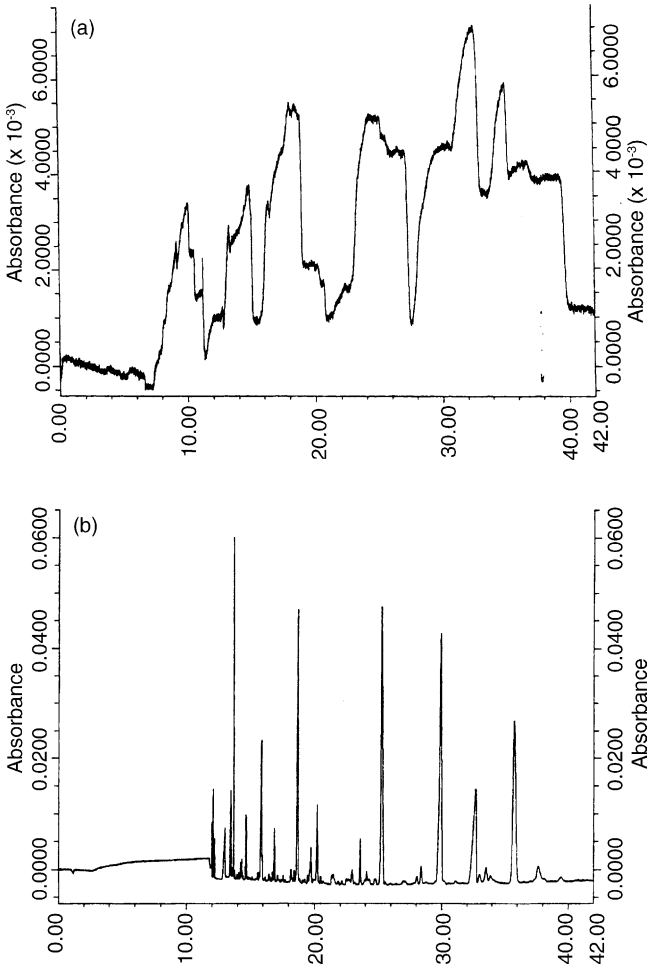

Fig. 11. Electropherogram of a tryptic digest of α s1 casein under nonstacking and tITP conditions. Background electrolyte: 50 mM betaine/50 mM acetate, pH 3.3. Sample (25 s injection): (a) 15 pmol/μL dissolved in the BGE; (b) the same sample injected after 25 s loading of 50 mM ammonium acetate serving as the leading electrolyte zone. From ref. [41]; with permission.

aration. Such an approach was applied to the analysis of major histocompatibility complex (MHC) class I peptides derived from a K-b precipitation of mouse EL-4 cells followed by the tandem mass spectrometry analysis [72]. Additional approaches for selective sample enrichment based on the use of porous joints were also recently described [73–76].

6.2. Ion boundaries in mass spectrometry

Mass spectrometry is the indispensable tool for the analysis of proteins and peptides. The use of the separations (chromatography, electrophoresis) prior to the mass spectrometry analysis can be viewed as an on line sample pretreatment minimizing the ion suppression effects frequently observed when complex mixtures are analyzed directly by the MS [77]. Although interfaces were developed for MS coupling via the matrix assisted laser desorption ionization (MALDI) and electrospray, the most common on-line coupling uses the latter option. Similarly to the standard CE applications sample preconcentration is very important for successful CE–MS analysis. Besides the proper selection of the electrolyte composition with respect to electrophoretic mobilities, one has to keep in mind its compatibility with the ESI process. Thus, the electrolytes containing phosphate, detergents (especially ionic, e.g., SDS) or soluble polymers should be avoided in favor of volatile electrolytes based on acetic and formic acids and ammonia salts. Following the rules described in the previous section the use of transient ITP preconcentration for analysis of proteins was first demonstrated in CE–MS with a triple quadrupole instrument [78]. More than 100-fold improvement in sensitivity was achieved. Discontinuous electrolyte system for ITP preconcentration was applied for the determination of paralytic shellfish poisoning toxins in shellfish tissues using capillary electrophoresis/electrospray mass spectrometry [79]. This preconcentration technique was found to be entirely compatible with electrospray mass spectrometry and permitted the analysis of scallop extracts containing submicromolar levels of PSP toxins. Similar sensitivity enhancement was also observed with tITP sample preconcentration for the simultaneous analysis of endogenous neurotransmitters and neuropeptides in brain tissue [80] and for monitoring of peptidomimetic direct thrombin inhibitor melagatran, endogenous peptides, substance P and calcitonin gene-related peptide [81]. An important aspect of the CE–MS coupling is the ion migration in the ESI interface which provides the transfer if the separated ions from the liquid phase into the gas phase via creation of the fine mist of charged droplets. The interface also closes the electrical circuit for the CE separation current. Currently, there are three basic arrangements of the CE–MS interfaces shown schematically in the Fig. 12. In the coaxial sheath liquid arrangement the separation CE capillary is inserted into a stainless steel needle supplied with a sheath liquid (SL) for maintaining the electric connection of both the CE current and the ESI high voltage (HV). In the liquid junction arrangement, the ESI needle is decoupled from the CE capillary in a reservoir containing the spray liquid and the electrode for the CE and ESI voltage connection. In the last design the spray is generated directly off the CE capillary. The electric connection is

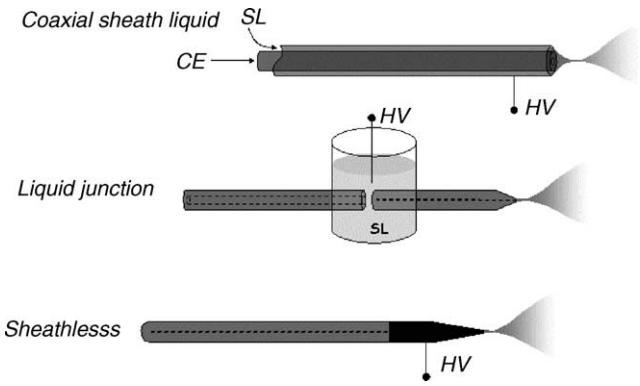

Fig. 12. Schemes of the CE–MS interfacing.

maintained via electrically conductive coating on the sharpened tip. While the detailed description of the ESI interfacing is off the scope of this review it is interesting to note the potential effects of the ion boundaries generated at the CE capillary exit. During the electrophoretic separation the sample zones migrate towards the interface and are sprayed after exiting the separation capillary. At the same time the counterions of the background moving in the opposite direction are replaced by the new counterions from the spray liquid or by the electrolysis products generated at the conductive tip of the sheathless ESI arrangement. This ion substitution creates a moving ion boundary and will occur in all cases when there is no liquid flow in the CE capillary (capillary with a neutral wall coating), is slower than the rate of electromigration or the liquid flows into the capillary (poorly balanced liquid level in the injection electrode reservoir; of opposite to the electromigration). Since the sheathless arrangement typically operates with the flow directed towards the MS analyzer the effects of the counterion migration typically do not appear. Depending on the composition of the BGE and spray liquid the creation of the ion boundaries can effect the migration of the analytes in interfaces based on the sheath liquid and liquid junction. The evolution such boundaries as well as an example of the separations affected by the boundaries were discussed in the Section 4.

7. DNA analysis

In the practice of DNA analysis, various preconcentration techniques were investigated with the aim to increase both sensitivity and resolution. Less attention, however, has been paid to the examination of effects connected with migration of "invisible" zones of sample matrix components, which can be the reason of some unwanted phenomena including variation of the detection signal and peak compressions. In DNA sequencing, for example, the sudden regions with compressed peaks or regions with consecutively decreasing peak heights could be attributed not only to the incompletely denatured guanine and cytosine rich fragments and errors in sequencing reaction, but also to the effect of highly conductive or resistive co-migrating zones.

7.1. Analysis of double-stranded DNA fragments

Since the DNA separations are typically performed in viscous sieving matrices electromigration is the most frequently used injection method. Careful removal of the salts from the sample is typically required to achieve sufficient injected quantity. In principle, electrokinetic injection from relatively large sample volumes (several microliters) typically results in more than 100-fold sample preconcentration [82]. This decreased the minimum detectable concentration of double-stranded DNA intercalated by a fluorescent dye YOYO down to 22 ng mL^{-1} when detected by LIF [83]. In many cases, additional stacking is achieved not only by the concentration adjustments at the sample/matrix boundary, but also behind the zone of chlorides originating from the sample. Chlorides are common components of the environment for DNA polymerase or sequencing reactions (140 mM), physiological solutions (156 mM), electrolytes of DNA standards (50 mM), etc. The zone of chlorides migrating in front of the lower mobility DNA fragments results in the sample preconcentration described in Section 2. The effect of salt concentration and long injection times on migration behavior of double-stranded DNA zones was studied in detail [84] with respect to irreproducible migration times, peak fronting or splitting resulting in the application of transient isotachophoretic preconcentration followed by zone electrophoresis (ITP-CZE) [85]. In this technique, the capillary was filled completely by the solution of a sieving medium with a background electrolyte. Next, plugs of free solution buffer with the same electrolyte composition followed by a sample were introduced hydrodynamically. This procedure assured the same length of a sieving medium and the same course of electric field strength for all consecutive separations. After the sample injection, the inlet and outlet of the capillary were placed in terminating and leading electrolytes, respectively. Key parameter for success is a proper choice of the terminator, which must be slower than DNA in the free solution, but faster in the zone of a sieving medium (linear 3% polyacrylamide) and, therefore, becomes leading behind the free solution/sieving matrix boundary. Thus, the transition from ITP to CZE is achieved by the mobility shift of DNA migrating from free solution to sieving matrix. Butyrate, which meets the mobility criterion, was chosen as the terminator. The method enabled the injection of up to 700 nL volume of a sample [6]. The similar system was used for the preconcentration and clean-up of therapeutically important 20–30 antisense phosphorothioate oligonucleotides. Up to 3 μL sample could be injected into the capillary without significantly disturbing the separation performance. The detection limit of 8×10^{-9} mol L^{-1} for the 20-mer phosphorothioate was obtained with a UV absorbance concentration [86].

The course of the transient ITP can be better controlled, when a terminator is replaced by a leading ion after the preconcentration step. The optimum time needed for an effective preconcentration, must be found empirically. It was shown that up to 20% of the capillary volume can be filled with a solution of short oligonucleotides (19–24 nucleotide pd(A) oligomers) by a pressure injection and effectively stacked. The best results were obtained with a 12% (w/w) solution of poly(ethylenglycol) (molecular mass above 1000 g mol^{-1}) in 100 mM ammonium formate buffer (pH 4.5). Here the formate anions play the role of leading and 100 mM 2-(*N*-morpholino)ethanesulfonic acid titrated to pH 4.5 with 2 M ammonium hydroxide was used as a terminator. The acidic pH was chosen to suppress electro-osmotic flow in bare fused silica capillaries. After the on-column preconcentration, the terminating electrolyte was replaced with back-

ground electrolyte, and separation continued in the zone electrophoretic mode. The reported detection limit of the method was100 ng mL^{-1} for the 16-mer oligonucleotide with UV detection (signal-to-noise ratio 10) [87]. Similar stacking of DNA size standards after hydrodynamic filling half of the capillary volume with the sample was also reported [88].

The use of a concentrated zone of sodium ions was suggested as a means to improve the shape of the fronting DNA bands. In this case, the DNA analysis was performed in Tris–borate buffer and the zone of sodium was launched in the opposite direction against the DNA bands. Thus, formed fronting gradient of sodium ions improved the apparent peak shape of the DNA fragments and the analysis time decreased [89].

Since the electrophoretic migration of the DNA is influenced by the polymeric sieving matrix much stronger than the small buffer ions it was suggested that the boundary free solution/sieving matrix could be used for sharpening and preconcentration. This process, similar to the sweeping preconcentration used in micelar electrokinetic chromatography [90,91], was tested for the analysis of dsDNA fragments. A large volume of a sample was introduced by electro-osmotic flow into an uncoated capillary filled with a free solution BGE. Next, the sample vial was replaced by a reservoir with a polymer solution and electro-osmotic flow pumped the sieving medium into the capillary. The electro-osmotic mobility was higher than the electrophoretic mobility of DNA fragments. Thus, the DNA migrated against the zone of the sieving medium and was separated according to the size; the longest fragments first. Although the stacking principle was not detailed, the authors declared about 66-fold increase in sensitivity when 0.67 μL of sample was injected in the capillary and swept by a 1.5% solution of poly(ethylene oxide) in 100 mM Tris–borate buffer (pH 9) [92]. The supposed advantage of this technique is a possibility to electro-osmotically pump a stepwise concentration gradient of a polymer solution into the capillary [93].

7.2. DNA sequencing

DNA sequencing represents the most demanding electrophoretic separation problem. Single stranded DNA fragments differing by one nucleotide in length must be separated up to the maximum possible fragment length. Under optimized separation conditions sequencing read length over 1500 nucleotides were obtained in less than 2 h [94]. Formation of a sharp starting sample zone is critical for achieving good separation and detection sensitivity. Besides the injection of the desalted sequencing reactions dissolved in water, alternative solvents can also help the sample focusing. For example, pure formamide as a solvent for the preconcentration of DNA sequencing fragments is not only advantageous for denaturing of single stranded sequencing fragments but also, due to the low conductivity, creates a high voltage drop across the sample. Thus, the DNA fragments migrate at a high but size-independent velocity and concentrate at the boundary between the sample and sieving medium at the capillary entrance. In this way, an estimated 75% of the DNA contained within 3 μL of the sample was injected into the sequencing capillary [95]. Similarly, long injection time from deionized formamide was used for the separation and detection of DNA sequencing fragments in narrow, 10 μm i.d., capillaries [96].

Additional way of sharpening the starting DNA zone is the transient isotachophoretic sample preconcentration based on the pH-induced mobility changes. This strategy was applied to concentrate long plug of DNA sequencing fragments with Tris–HCl buffer as a background electrolyte, which upon a titration by OH^- ions produced low conductivity terminating zone. Thus, after a long sample injection (up to 180 s at 50 V cm^{-1}), the end of the capillary was inserted into a vial with 0.1 M solution of NaOH and OH^- ions were let migrating behind the zone of DNA for up to 80s at 160 V cm^{-1}. Within this low conductivity zone of high electric field, DNA molecules moved at higher velocities and were concentrated at its front edge. The main benefit of this technique is a possibility to inject a crude sequencing samples into capillaries without any pretreatment and the resolution is comparable to runs performed using standard injection of purified samples in a conventional buffer system [97]. Similar pH-mediated tITP stacking was also applied for the analysis of multiplexed short tandem repeats. In this application, the same buffer system as described previously [98] was used, but the PCR fragments were injected into the zone already titrated by OH^- ions.

In our previous work [99], the effect of highly alkaline background electrolytes on the denaturing electrophoresis of single-stranded DNA fragments was investigated. It was found that the OH^- ions are not ideal for the migration of a DNA fragment zone due to the large difference in the mobilities. The electromigration dispersion of injected zones at the start of the separation may limit the separation efficiency substantial for a successful separation of sequencing fragments. Such a phenomenon can play a crucial role especially when short capillaries or microfluidic systems are used. To eliminate this source of zone spreading a very low mobility compound, such as monocarboxy poly(ethylene glycol) (PEG^-) can be selected as a temporary terminator for transient isotachophoresis. Additional benefit is the superior denaturing ability of electrolytes with pH 12 and higher for resolving the GC rich fragments prone to band compressions. The importance of the stacking obtained under these conditions is demonstrated in Fig. 13. Here, the separation records of the same sample with and without the stacking are compared. Completely lost resolution due to bad separation efficiency indicates the effect of the electromigration dispersion at the very beginning of the separation.

7.3. DNA analysis in microfluidic systems

The short separation distances on the microfluidic devices require a special attention on the injection of a sharp start-

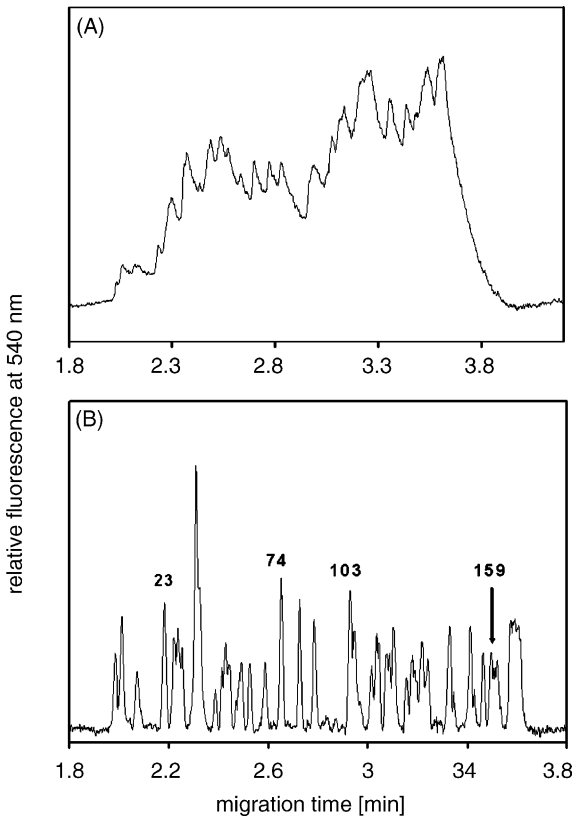

Fig. 13. Comparison of the separation under nonstacking (panel A) and tITP conditions (panel B). Separation records of guanine-terminated DNA sequencing fragments synthesized by cycle-sequencing technology on a PCR product of a size of about 180 bp specifically labeled by dRhodamine dyes. Electrophoresis: in 4% (w/w) solution of LPA (mol. mass: ∼9 MDa) with 0.04 M NaOH; electric field strength: 240 V cm^{-1}. Bare fused silica capillary of effective (total) lengths: 7 (12.5) cm; i.d.: 50 μm; temperature: −30 °C. The ITP stacking was achieved by using monocarboxy (polyethylene glycol) (PEG^-) as transient terminator. Following the sample injection the 2% (w/v) PEG^- solution was introduced electrokinetically for 16 s. Fragment sizes are in number of nucleotides. From ref. [99]; with permission.

ing zone. Although often not applied knowingly the sample focusing methods will be of key importance for successful practical analysis. Typically, the length of a zone introduced into the separation column is determined by the geometry of the microfabricated twin-T injector. While the separation process requires the shortest possible injection plug this also reduces the injected amount and the sensitivity of the analysis. The effect of the stacking in the microfabricated injector for DNA sequencing was investigated with respect to the band broadening and injection bias of fragments with different sizes [100]. It was found that when coupled to an array of separation capillaries the system can combine the advantages of sample purification and stacking with uniform signal intensity profile typical for the microfabricated twin-T injectors [101]. A special microfluidic device for the preconcentration of double-stranded DNA fragments by a transient isotachophoresis was designed recently [102]. A set of 32 separation systems was microfabricated on a plastic card. Each separation element was divided into segments for the sample injection, stacking, and separation. The segments were connected with reservoirs for sample, terminating electrolyte, sample waste, leading electrolyte and outlet. Thus, by applying the voltage consecutively between the couples of reservoirs, the sample was injected, focused and finally separated. A 22 mm long sample plug was preconcentrated into a narrow band by ITP discontinuous buffers and then separated on a 45 mm separation distance. A 40-fold increase in the detection sensitivity was reported. Similar improvements were also reported with the commercial microfluidic system [103]. Microfabrication technology also offers different possibilities for sample preconcentration based on the creation of ion semipermeable junctions capable of selective enrichment of the large molecular mass compounds [104].

7.4. Analysis of DNA adducts

The DNA adducts are formed in the body when the enzymatic repair mechanisms fail to correct these chemical modifications caused by the metabolic processes. It is generally believed that the interaction of cellular DNA with an exogenous chemical or a metabolite is the initial step in the induction of mutations, neoplastic cell transformations and chemical carcinogenesis. Thus, the structural and quantitative analysis of very small amounts of these compounds is of biological and medical importance. Although the DNA itself is typically separated by electrophoresis in a sieving separation media and detected by fluorescence, the identification of the numerous modifications on the nucleotide units requires mass spectrometric analysis. The use of capillary electrophoresis combined with the negative ion electrospray MS has been explored for the identification and quantification of such adducts. Since the sample preparation typically involves extensive extraction steps the samples are available as low ionic strength solutions. Thus, a large sample volume can be injected into the separation capillary and preconcentrated on the concentration boundary with the background electrolyte. The low ionic strength inside the separation capillary induces a strong electro-osmotic flow and, therefore, the analysis starts with the high voltage polarity causing the transport of the sample plug towards the injection end of the capillary. The sample ions are focused at the end of this plug, while a large portion of the capillary filled with water is being removed by the electro-osmotic flow. The water removal is monitored as the current increase and the process can be calibrated such that almost all water is removed prior to the reversal of the polarity and start of the analysis. In principle, the whole separation column can be filled with the sample at the start of the analysis. DNA adducts with several compounds were identified and quantified by this approach at low levels. The analyses of DNA adducts with benzo[a]pyrene and its metabolites [105,106] styrene oxide mono-adducts in di-, tri- and tetranucleotides [107], benzo[*a*]pyrene diol epoxide [108], *N*-acetoxy-*N*-acetyl-2-aminofluorene [107,109] and phenyl glycidyl ethers have been published [110–112].

8. Conclusion

Studying the evolution of the zones and ionic boundaries in electrophoresis can help understanding many of the effects frequently encountered in practice. Proper selection of the operational conditions can minimize the potential adverse phenomena and maximize the desired effects leading to better separation efficiency and sensitivity of the analysis. The aim of this short critical article was to briefly review some of the aspects of the evolution of ionic boundaries important in the analysis of biologically important samples, such as peptides, proteins and DNA. It should be noted that the selection of the discussed applications was arbitrary and the same phenomena are commonly observed in all applications and modifications of electromigration techniques including micellar electrokinetc chromatography and isoelectric focusing. Additionally, the manifestations of the ionic boundaries are of key importance for correct interpretation of the detection signals and system peaks directly related to the ionic boundaries such as in indirect (optical, electrochemical) or conductivity detections.

Acknowledgment

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