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Review

Micellar electrokinetic chromatography of proteins

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Abstract

Micellar electrokinetic capillary chromatography (MECC) of proteins is a high resolution capillary electrophoretic (CE) analysis method that utilizes the hydrophobic and electrostatic interaction of protein analytes with surfactant micelles present in the buffer medium to facilitate separation. Through the manipulation of the protein–micelle interaction by the adjustment of variables such as surfactant concentration, solution pH, ionic strength, the presence of an organic modifier and the use of coated capillaries, MECC analyses of a wide variety of proteins have been optimized. MECC has been demonstrated to provide resolution of mixtures consisting of proteins with minor structural variations and also has provided the successful quantitative analysis of proteins present in complex matrices. The adoption of protein MECC as a routine analytical technique may be dependent upon the successful interface of MECC with detection methodology, such as mass spectrometry, which can provide analyte characterization information. © 1997 Elsevier Science B.V.

Keywords: Reviews; Proteins

Contents

1. Introduction

Micellar electrokinetic capillary chromatography (MECC) was first introduced by Terabe et al. [1] as a capillary electrophoresis (CE) technique that utilized buffer solutions containing surfactants to effectively separate analytes possessing identical net charges. Surfactants are molecules that exhibit both hydrophobic and hydrophilic character and, in an aqueous environment, they self-aggregate if the surfactant concentration exceeds a specific critical micelle concentration (CMC). Micelles typically consist of 50–100 surfactant molecules that exist in

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an aqueous environment as an association where the hydrocarbon tail portions are oriented towards the center of the aggregate, while the polar head groups point outwards. During an MECC separation, analytes partition into and out of the micelles in a manner based upon their relative hydrophobic characteristics, an occurrence which influences the electrophoretic migration of the analytes through the capillary. MECC has been applied with great success to the analysis of a variety of small molecules and has proven to offer great advantages over CE for the separation of mixtures containing both ionic and neutral species. However, since the size of the micelles is in the range of 3 to 6 nm in diameter, the types of analytes that may physically partition into the core of the micelle are limited to those of roughly less than M_r 5000, thereby excluding macromolecules such as proteins [2]. Although most proteins are too large to partition into the hydrophobic core of micelles, proteins can associate with micelles through hydrophobic, hydrophilic and electrostatic mechanisms. These interactions have been exploited to manipulate protein separation by techniques such as micellar liquid chromatography [3,4] and the extraction of proteins into reversed micelles [5].

Proteins have provided significant challenges for separation by CE in silica capillaries because of their tendency to adsorb to the capillary walls through a variety of mechanisms, but many successful CE protein separations have been facilitated by the presence of both charged and neutral surfactants. Sodium dodecyl sulfate (SDS) is a negatively charged surfactant that binds to and denatures proteins to form rod-shaped complexes which can be separated on the basis of size during electrophoresis through a capillary filled with a gel matrix, in a manner similar to traditional SDS–polyacrylamide gel electrophoresis (SDS–PAGE) [6]. Protein–capillary wall interactions in both bare silica and coated capillaries have been successfully minimized or eliminated through the use of ionic [7,8], zwitterionic [9] or neutral surfactants [10]. However, in none of these reports was the ability of the surfactant micelles to influence protein separation studies. It is only recently that protein–micelle interactions have been investigated for the achievement of high resolution protein separations by CE.

2. Ionic MECC of proteins

2.1. Ionic MECC in the presence of electroosmotic flow

The use of ionic micellar buffer solutions for protein analyses by CE was initially investigated because it was found that these systems could provide both increased recovery and selectivity for the separation of complex samples. MECC protein separations have been based on the fact that the electrophoretic mobilities of the charged micelles were greater than that of any of the proteins or protein–micelle complexes [11], and that differences in the equilibria of the association of the analytes with the micelles can influence the migration of the analytes through the capillary.

The advent of biotechnology has resulted in a great need for high resolution analyses of drug compounds in the presence of complex matrices. In an attempt to develop such a method using MECC, both cationic and anionic micellar separation buffers were employed by Strege and Lagu [12] to successfully recover and separate a mixture of five model proteins (lysozyme, ribonuclease, myoglobin, β-lactoglobulin and bovine serum albumin) using a variety of pH conditions and surfactant concentrations. Complex relationships between protein migration (relative to a neutral marker) and buffer pH were observed in buffers containing 0.1% cetyltrimethylammonium chloride (CTAC) and these were attributed to changes in protein–micelle, protein–surfactant monomer and protein–surfactant monomer–micelle equilibria (see Fig. 1). The use of C_{18}-derivatized capillaries in conjunction with the micellar buffers was also investigated. By adsorbing a layer of the charged surfactant onto the coating, these capillaries provided a relatively strong and pH-independent electroosmotic flow, which resulted in superior injection-to-injection analyte migration time reproducibility. Experimental evidence suggested that concentrations of the surfactants at levels above the CMC were required for complete saturation of the capillary walls. Also, protein migration times were found to increase as surfactant concentrations were increased, an effect attributed to increased protein–micelle association. Separations of a crude *E. coli*

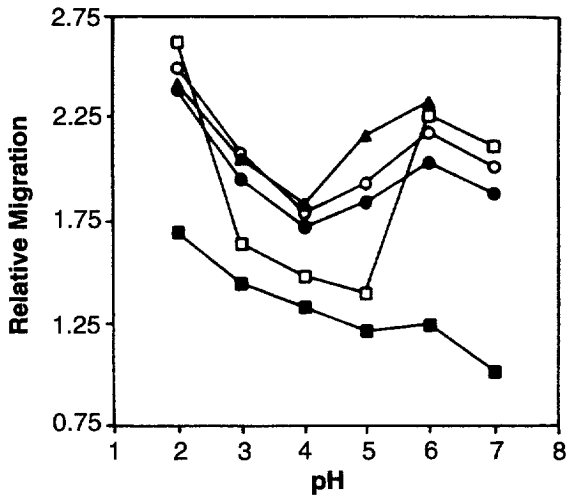

Fig. 1. Dependence of relative protein migration (protein migration time/neutral marker migration time) upon buffer pH, for separations of 2-s injections of 0.5% mesityl oxide and 5-s injections of the protein mixture [1 mg/ml of each protein, (■) ribonuclease; (□) lysozyme; (●) BSA; (○) β-lactoglobulin; (▲) myoglobin] obtained in 50 m*M* sodium phosphate or sodium acetate buffer containing 0.1% CTAC inside a 120-cm C_{18}-derivatized capillary at 30 kV (outlet=anode). Reprinted from Ref. [12] with permission.

fermentation broth preparation were obtained using a 50 m*M* sodium borate buffer, pH 9.0, containing 0.3% SDS (see Fig. 2). Under these conditions, it was observed that a recombinant DNA-derived (rDNA) protein that was present in the broth migrated on the tail-end of a large mass of cellular components (*E. coli* proteins, nucleic acids, etc.), and increased resolution was required for accurate quantitation of the product in this complex matrix. This separation is a good illustration of the unusual selectivities that can be achieved using MECC.

MECC was employed by Eriksen and Holm [13] to separate two forms of rDNA savinase (SAV), a serine protease, which consisted of 269 amino acids and had identical isoelectric points and which differed only in that the SAV and SAV* forms possessed a methionine and serine at position 222 in the primary sequence, respectively. The resolution could not be explained on the basis of the hydrophobicity of the primary sequence of the proteins, since methionine is more hydrophobic than serine, yet SAV migrated more rapidly than did SAV* in the presence of the negatively charged micelles. It was theorized that the separation might have been caused by an opening of the globular protein structure by the SDS and subsequent differential interaction with the micelles.

Peptides differing by one neutrally charged amino acid were also separated via MECC by Yashima et al. [14], who utilized buffers containing SDS or cetyltrimethylammonium bromide (CTAB) to resolve [Leu 13] motilin from [Met 13] motilin, an analysis which could not be achieved in the absence of micelles. Unique to this study was the fact that the investigators also found it necessary to incorporate 5–25% acetonitrile or methanol in the separation buffer to hinder the interaction of the large peptides with the micelles. This approach was also successfully applied to the separation of a mixture of four insulins that differed only by one to three neutrally charged amino acids (see Fig. 3). The analyses were optimized by manipulation of the pH of the buffer, which, in turn, influenced the charge and hydrophobicity of the proteins and the coulombic micelle–insulin repulsion. The nature of the organic modifier also was determined to impact the separations, since acetonitrile was found to be more effective in hindering the protein–micelle interactions than methanol. A study of the MECC resolution of a series of peptides (8–31 amino acid residues) led the investigators to conclude that it would be difficult to separate peptides that consisted of more than twenty amino acid residues without organic modifiers, assuming that the hydrophobicity of the peptides increased with an increase in size.

Glycoproteins represent a class of macromolecules which have demonstrated potent and unique clinical efficacies and, therefore, much effort within the pharmaceutical industry has been devoted to the development of these substances as drugs. A major challenge associated with the development of glycoprotein pharmaceuticals is the analytical chemistry of these molecules, since their structures can be very diverse and complex. MECC represents one separation technique that has shown significant potential for work of this nature. High resolution MECC separations of recombinant human interferon glycoforms were achieved by James et al. [15] who utilized a relatively high ionic strength buffer (400

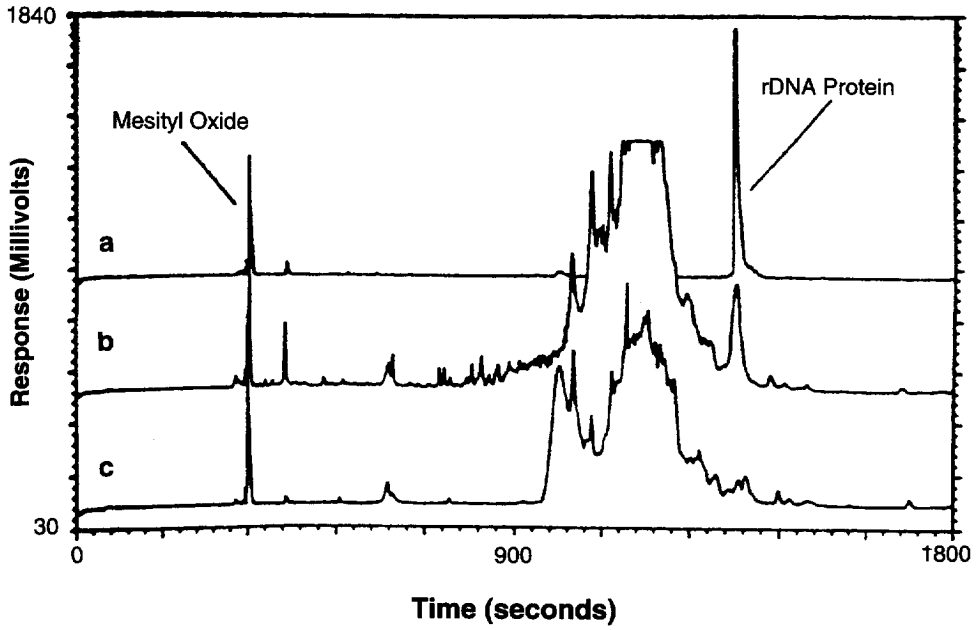

Fig. 2. Separations of a 2-s injection of 0.5% mesityl oxide and a 5-s injection of (a) a 1 mg/ml rDNA protein standard; (b) a fermentation broth sample; (c) a negative control fermentation broth sample obtained in 50 m*M* sodium borate buffer, pH 9.0, containing 0.3% SDS at 20 kV (outlet=cathode) in a 50-cm C_{18} derivatized capillary. Reprinted from Ref. [12] with permission.

m*M* sodium borate buffer, pH 8.5, containing 100 m*M* SDS). Fig. 4 demonstrates the significant increases in resolution that were obtained as the levels of borate and SDS in the buffer were increased. The high separation efficiency in this system was attributed to the synergistic action of both reduced electroosmotic flow and increased surfactant concentration. In the presence of the high concentration of buffer, it was assumed that the glycans present in the glycoprotein structure were extensively complexed with borate, and an additional negative charge on the analytes resulted, which promoted an electrostatic

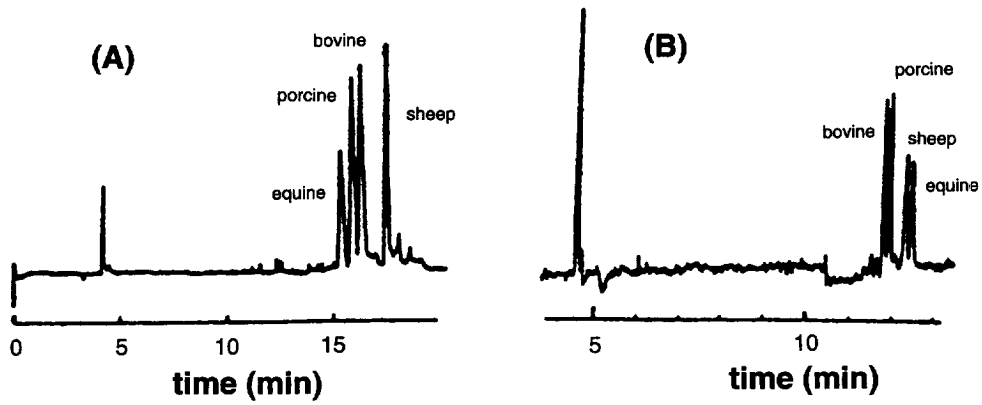

Fig. 3. Separation of insulins by MECC with acetonitrile. Electrophoretic solution: (A) 50 m*M* acetate buffer (pH 3.6) containing 10 m*M* CTAB and 5% acetonitrile; (B) 50 m*M* borate buffer (pH 8.5) containing 50 m*M* SDS and 15% acetonitrile. Reprinted from Ref. [14] with permission.

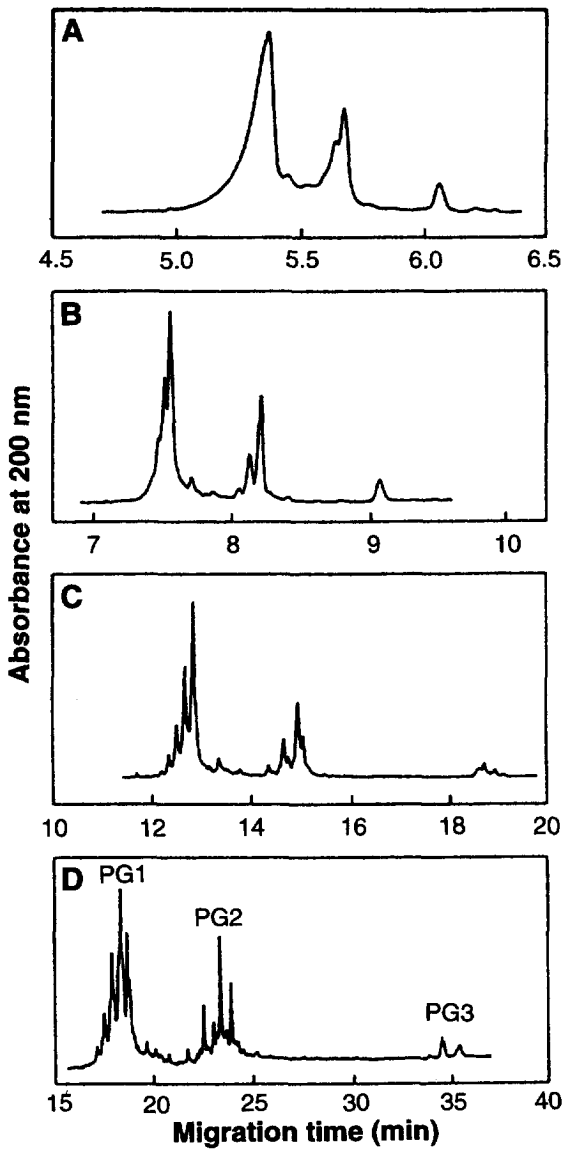

Fig. 4. Optimization of the separation of IFN glycoforms by MECC. Borate–SDS electrophoresis buffer, pH 8.5, was employed at the following concentrations: (A) 40 m*M* borate, 10 m*M* SDS; (B) 40 m*M* borate, 100 m*M* SDS; (C) 400 m*M* borate, 10 m*M* SDS; (D) 400 m*M* borate, 100 m*M* SDS. In each case, IFN, at a concentration of 1 mg/ml in 50 m*M* borate, 50 m*M* SDS, pH 8.5, was injected for 5 s prior to electrophoresis at 22 kV. In (D), the main peak groups are designated PG1, PG2 and PG3 in order of migration. Reprinted from Ref. [15] with permission.

repulsion between the micelles and the glycoprotein. The extent of this electrostatic repulsion was therefore determined by the size of the glycan structure. Since glycoforms that had the greatest interaction with the SDS micelles were expected to be most retarded during migration, it was suggested that those analytes that had the shortest migration times were those associated with the most carbohydrate, i.e. the glycoforms with the largest glycan structures.

Antibodies are glycoproteins whose biological function is to trigger a protective reaction in response to antigenic stress. Kats et al. [16] used MECC to separate four major isoforms of the BR96 antibody following heat-induced reversible interconversions of the molecule in the presence of 25 m*M* SDS in a 12-m*M* sodium borate buffer, pH 9.4 (see Fig. 5), and these analyses were observed to correlate with antibody structural changes detected by circular dichroism. The thermal stability of chimeric BR96 was also examined in detail by Alexander and Hughes [17], who found that MECC could provide the selective separation of antibody-related chains and fragments required for the analysis of degraded samples in a manner superior to traditional electrophoretic and liquid chromatographic methods. Matrix-assisted laser desorption mass spectrometry (MALDI–MS) provided a complimentary impurity profile of the degraded samples from which it was possible to assess the degree of degradation and gain specific molecular mass information on the resulting species formed. Further studies of the MECC of antibodies were performed by Hughes and Richberg [18], who separated the light and heavy antibody chains, the Fab fragment and unconjugated doxorubicin forms of a doxorubicin-linked chimeric antibody.

The analysis of clinical samples also represents an important challenge for separation methods such as MECC. Tadey and Purdy [19] utilized borate buffers, pH 8.3, containing 0.1% SDS for the analysis of plasma apolipoprotein samples isolated from whole blood, and compared their results to those obtained by slab gel SDS–PAGE [19]. Fig. 6 displays the electropherograms of high density lipoproteins (HDL) obtained in the absence and presence of SDS, together with an SDS–PAGE analysis of the same sample. In addition to the benefit of the elimination of protein–protein interaction and aggregation in the presence of surfactant, it was observed that the two

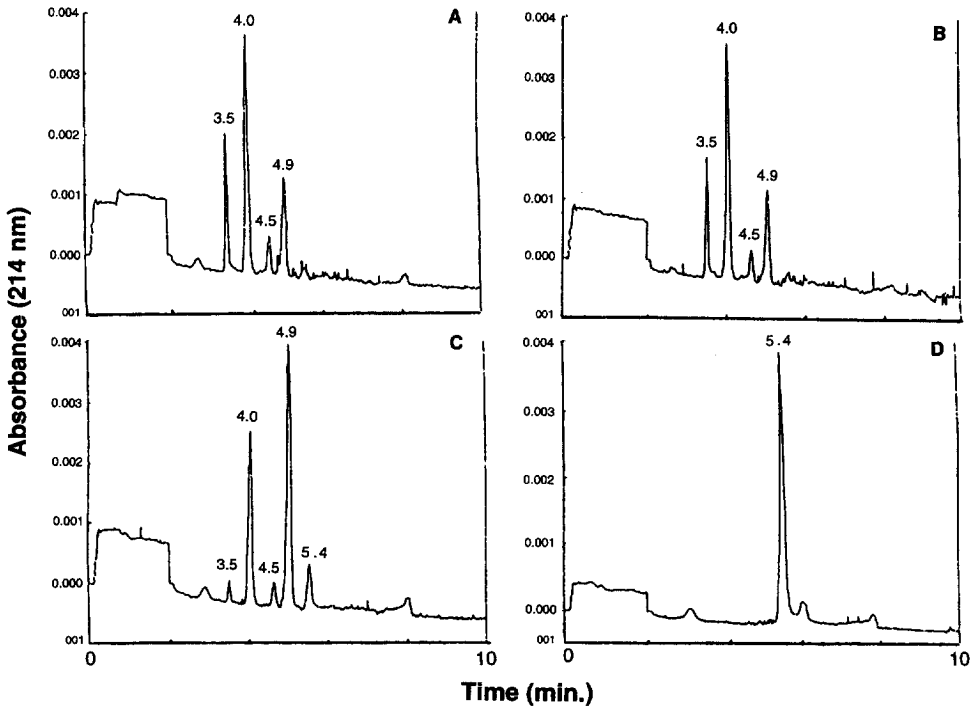

Fig. 5. Electropherograms of the BR96 antibody at (A) 20, (B) 70 and (C) 80°C and (D) incubated at 80°C for 30 min. The separation buffer used was 12 m*M* borate buffer, 25 m*M* SDS, pH 9.4, with detection at 214 nm. Reprinted from Ref. [16] with permission.

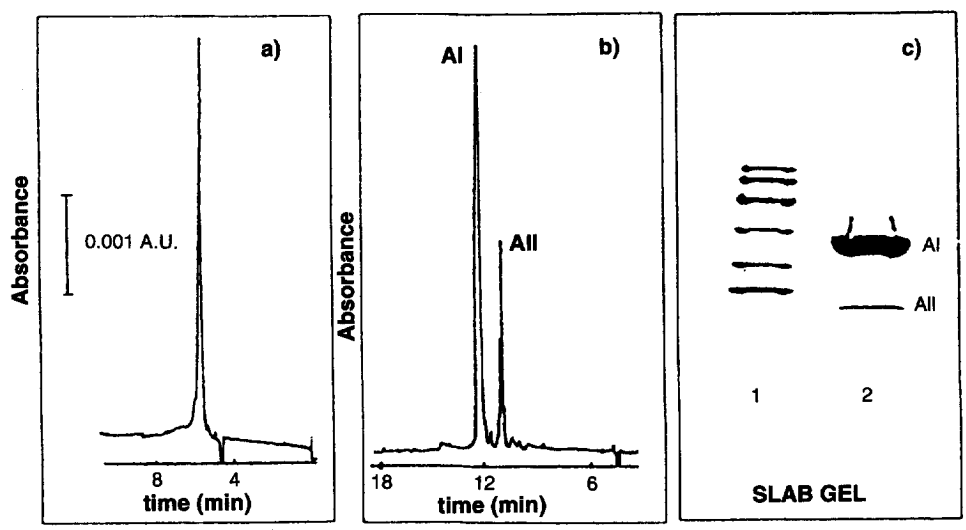

Fig. 6. Electropherograms of HDL apolipoproteins using 30 m*M* borax, pH 8.3, as the running buffer. Conditions: (a) no SDS in the buffer; (b) buffer containing 0.1% SDS; (c) slab-gel SDS–PAGE. Lane 1=molecular-mass standards; lane 2=HDL apolipoproteins. Reprinted from Ref. [19] with permission.

proteins of HDL, apo A-I and apo A-II, were resolved only when SDS was present in the running buffer. Increased resolution was achieved when the separations were performed in polyacrylamide-coated capillaries, where all of the major apolipoproteins were well separated in about 12 min.

Metallothioneins are a family of protein isoforms ($M_r \approx 6500$), found in the liver of mammals, which bind transition metals and have distinctive characteristics through substitution of one–fifteen amino acid residues. Beattie and Richards [20,21] applied SDS MECC to the analysis of both charge-different and charge-similar metallothioneins in tissue extracts. Optimization of the separation conditions facilitated the resolution of up to five and six isoforms from sheep and rabbit sources, respectively. The best separations were obtained when the micelle and protein migration rates were slow (i.e. at 10–20 kV applied potential vs. 30 kV) and, interestingly, additives such as urea or methanol, which modified the analyte's partitioning into the micelles, were found to be deleterious to the separation of isoforms. Borate also appeared to be superior to phosphate as an MECC electrolyte buffer. From these studies, the conclusion was reached that MECC offered great potential for the simultaneous analysis of both proteins and low molecular mass components present in tissue extracts.

The need for powerful high resolution analytical methods also exists within the food science industry. One example of the application of MECC to work of this nature is the study performed by Strickland et al. [22], who utilized buffers containing various concentrations of SDS for the analysis of caseins, peptides and various small molecules present in samples of Cheddar cheese extracts for the purpose of characterizing the biochemical changes associated with cheese maturation. A comparison of the separations achieved using MECC to those obtained via free solution CE (i.e. no surfactant present) revealed that only MECC could provide adequate resolution of the milk proteins and peptides and acceptable durability (the free solution CE system performance degraded after 50 injections, while the MECC system remained robust). MECC profiles of cheese extracts separated in the presence of various concentrations of SDS are displayed in Fig. 7. A major challenge remaining for the investigators following the acquisition of the separations was the identification of the components in the electropherograms.

2.2. Ionic MECC in the absence of electroosmotic flow

As described in Section 2.1, one of the disadvantages of analyzing proteins by ionic MECC in the presence of electroosmotic flow is the relatively long migration time associated with the analytes in these systems. Because the charged micelles possess a high electrophoretic mobility which enables them to resist the electroosmotic flow to a high degree, analytes such as proteins, which associate strongly with the micelles, tend to migrate very slowly through the capillary. However, by eliminating the electroosmotic flow through the use of a polyacrylamide-coated capillary, Strege and Lagu [23] were able to decrease the protein separation time significantly while still preserving the selectivity of the separation of a mixture of five model proteins. In the coated capillary, SDS micelles migrated very rapidly towards the anode, resulting in <8 min separation times for proteins that had previously required 40 min for separation in a bare silica capillary [13]. The selectivity of these analyses could be manipulated by adjusting the surfactant concentration (see Fig. 8) or by employing a cationic surfactant (CTAC) system.

In a subsequent study, MECC in a polyacrylamide-coated capillary was applied to the challenging analysis of a rDNA protein in solubilized *E. coli* fermentation broth [25]. The fermentation analysis was one that had previously been performed in the presence of electroosmotic flow and was found to provide incomplete resolution of the rDNA protein from the other components in the matrix [12]. Successful resolution was achieved in the coated capillary MECC system by incorporation of 25% acetonitrile and 40 m*M* magnesium sulfate into the separation buffer (20 m*M* sodium borate, pH 8.0, 50 m*M* SDS) (see Fig. 9). It was concluded that the presence of both the organic modifier and the additional salt resulted in decreased protein–micelle interaction, which facilitated optimal resolution at the expense of an increase in analysis time (a 30-min separation time was required).

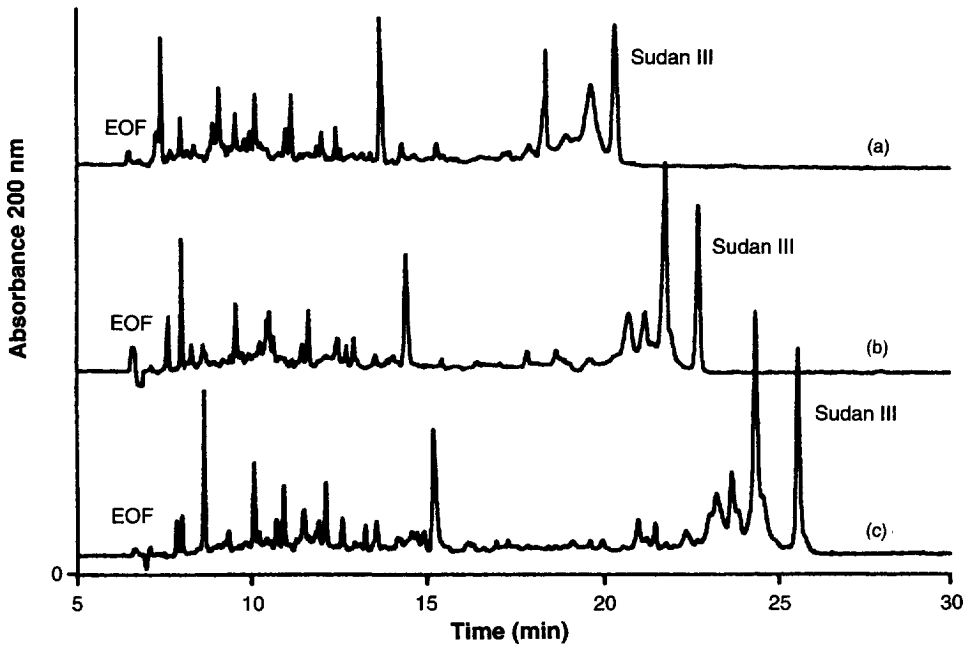

Fig. 7. Micellar electrokinetic capillary chromatography of M_r 30 000 filtrate of the phosphate-soluble Cheddar cheese fraction collected after six months of ripening at 7–10°C. Electrophoresis was performed with 100 m*M* sodium borate buffer (pH 8.5) containing: (a) 20 m*M* SDS; (b) 40 m*M* SDS and (c) 60 m*M* SDS. Reprinted from Ref. [22] with permission.

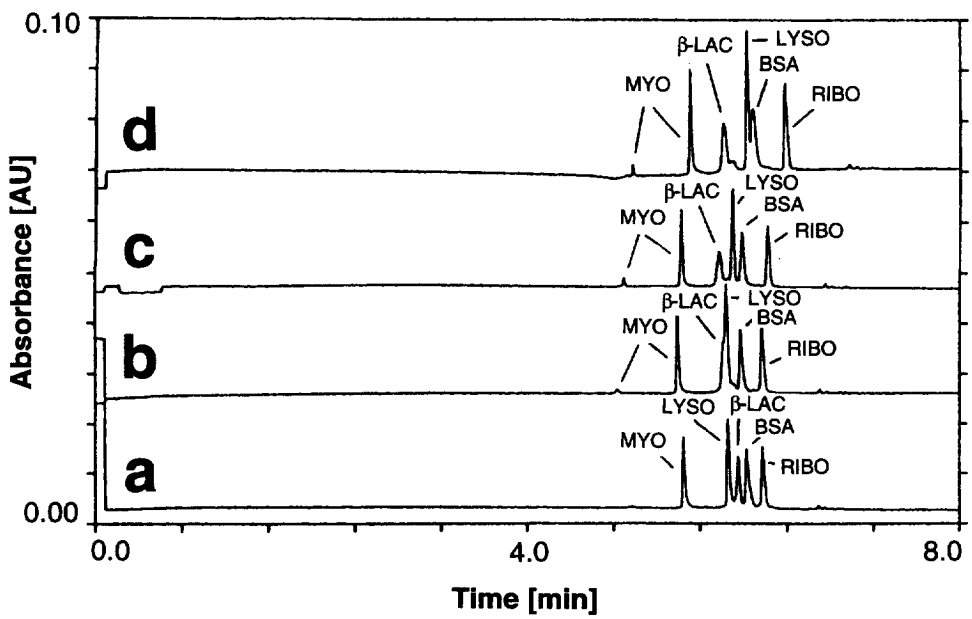

Fig. 8. Separations (outlet=anode) of a 5-s injection of a mixture of myoglobin (MYO), β-lactoglobulin (β-LAC), lysozyme (LYSO), BSA and ribonuclease (RIBO) in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using an applied potential of 30 kV, 214 nm UV detection and 50 m*M* sodium acetate buffers, pH 4.5, containing (a) 0.1% SDS; (b) 0.2% SDS; (c) 0.5% SDS; (d) 1.0% SDS. Reprinted from Ref. [23] with permission.

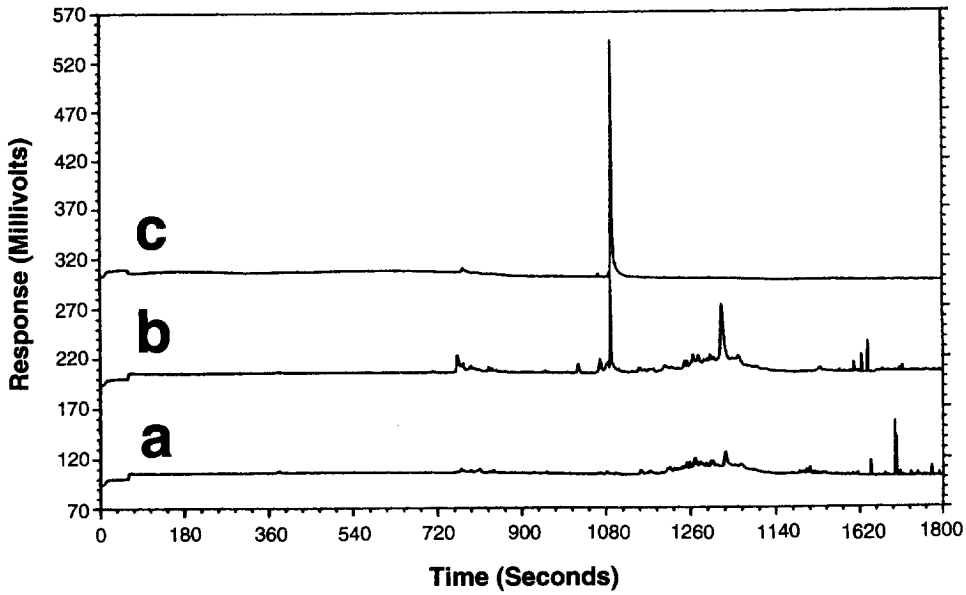

Fig. 9. CE separations of (a) a solubilized negative control *E. coli* fermentation broth sample, (b) a solubilized *E. coli* fermentation broth sample containing approximately 200 μg/ml rDNA protein and (c) a 500-μg/ml rDNA protein standard in 20 m*M* sodium borate buffer, pH 8.0, containing 50 m*M* SDS, 40 m*M* magnesium sulfate, 25% acetonitrile. Reprinted from Ref. [25] with permission.

3. Neutral or zwitterionic MECC of proteins

An attractive alternative to the use of ionic surfactants for the MECC of proteins is the employment of neutral or zwitterionic surfactants. These types of surfactants can be utilized over a wide range of buffer conditions without having significant effects on the properties of the detergent. Since they do not contribute to the solution conductivity, they should not in principle alter the net charge of the analytes to which they are bound, and they should not in general induce protein denaturation. Non-ionic and zwitterionic surfactant micelles have been demonstrated to provide successful MECC separations of hepta- and decapeptides [25,26].

Nashabeh et al. [27] utilized a zwitterionic detergent, N-dodecyl-N,N-dimethyl-3-amino-1-propanesulfonate (DAPS), and a coated capillary which eliminated the effects of electroosmotic flow, to separate rDNA insulin-like growth factor I (IGF-I) variants by MECC. Incorporation of an organic modifier into the separation buffer provided the selectivity required for resolution, as can be observed in Fig. 10, where native IGF-I, methionine-sulfoxide IGF-I and improperly folded IGF-I were separated in a 5-m*M* DAPS, 20 m*M* β-alanine–citric acid buffer, pH 3.8, containing 15% acetonitrile. The migration order of these analytes was identical to that obtained by reversed-phase high-performance liquid chromatography (HPLC) under acidic conditions and, as in reversed-phase chromatography, the choice of or-

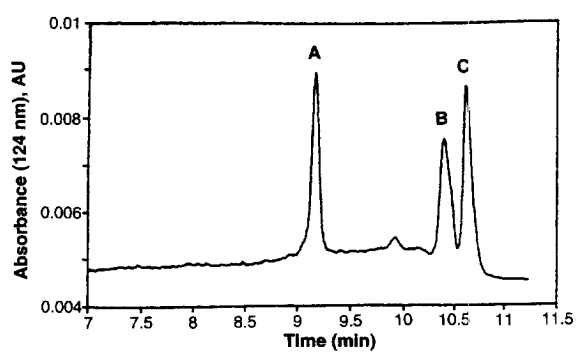

Fig. 10. Separation of native (C), Met-O (B) and improperly folded IGF-I (A) using a siloxanediol linear polyacrylamide-coated capillary and hydrophobic selectivity. Reprinted from Ref. [27] with permission.

ganic solvent added a second level of selectivity. In addition, the employment of coated capillaries (with no electroosmotic flow) together with zwitterionic detergents was compatible with on-line mass spectrometry (see Section 4). The results of this study suggested that operation near the CMC of the zwitterionic detergent in the presence of organic modifiers yielded the greatest selectivity for hydrophobicity-based resolution of protein variants.

As an extension of the study reported by Nashabeh et al. [27], Greve et al. [28] investigated the use of non-ionic surfactants in coated capillaries for the separation of the seventeen amino acid peptides [Leu 15] gastrin and [met 15] gastrin. In contrast to the zwitterionic surfactants, an increase in non-ionic surfactant concentration to levels well above the CMC improved separation. For example, it was found that Tween 20 (CMC=100 μM) required concentrations of 80–250 mM to provide acceptable selectivity.

4. MECC–MS analysis of proteins

Mass spectrometry (MS) has become an extremely valuable technique for the characterization of a wide variety of samples, including proteins and, when coupled to a separation system, can serve as a very powerful on-line detection system. For CE–MS, the choice of buffers to achieve a particular separation has been limited by the sample ionization taking place at the MS interface to buffers that are volatile, such as ammonium acetate and triethylamine. Direct coupling of MECC to MS is especially challenging because of the negative influence of the micelles in the buffer upon the performance of the MS system, resulting in loss of sensitivity and ion source contamination. However, the potential of the combination of this high resolution separation method with the powerful characterization capabilities of MS has generated much interest.

No attempts of the analysis of proteins by ionic MECC–MS have been reported. However, several researchers have had some success with the interface of ionic MECC with electrospray ionization mass spectrometry (ESI–MS) for the analysis of small molecules, and the results of these studies should be directly applicable to the analysis of macromolecules as well. The use of buffers containing the cationic surfactant, CTAC, at concentrations above the CMC for the analysis of peptides (three to thirteen amino acid residues) was investigated by Varghese and Cole [29], who observed a three to four-fold decrease in ESI–MS sensitivity (relative to that achieved in the absence of the surfactant) for the detection of the tripeptides, with a significantly weaker signal generated by the larger peptides. To avoid the problems caused by ionic micelles, Lamoree et al. [30] designed a coupled capillary set-up to provide on-line heartcutting of zones of interest from a capillary containing the micellar separation medium, with subsequent transfer via a second capillary to an electrospray source. Ozaki et al. [31] studied MECC–ESI–MS using a 2% solution of a high molecular mass surfactant, butyl acrylate–butyl methacrylate–methacrylic acid copolymer sodium salt (BBMA) ($M_r \approx 40\,000$, CMC$\approx$0) as the separation buffer. This study found that the phenyltrimethylammonium chloride ion signal intensity in 2% BBMA was ca. 20% of that in the absence of BBMA. An electrospray–chemical ion (ES–CI) interface has been developed by Takada et al. [32] for MECC–MS and preliminary results suggested that the observed ion intensity of aromatic amines was not strongly affected by SDS concentrations up to 50 mM. It was reported that the ES–CI interface could be used over the course of at least ten 20-min analyses, without clogging problems emerging.

A unique MECC–ESI–MS technique was employed by Nashabeh et al. [27], who used coated capillaries and zwitterionic surfactants to obtain protein separations in the absence of electroosmotic flow. Under these conditions, only the charged protein analytes exited the capillary into the ESI interface (i.e. no surfactant ions were observed) and, therefore, the micelles had no effect upon the acquisition of the sample mass spectra. One potential limitation of this system was the fact that, since the analysis was dependent upon the electrophoretic mobility of the analytes, the separation of a complex sample mixture would require the use of an extreme buffer pH to ensure that all of the analytes are either positively or negatively charged and will migrate to the MS interface. Neutral species also would not be detected by this method.

5. Conclusions

Generally applicable rules for the MECC of proteins include the following. Surfactant concentrations in the separation buffer should be at or above the CMC, and the pH of the buffer system should be chosen to provide optimum selectivity and/or solubility of the protein mixture to be analyzed. The choice of an appropriate surfactant will, in turn, be dependent upon the buffer pH, since, in order to prevent protein precipitation, cationic and anionic surfactants are generally useful under acidic and basic conditions, respectively. Neutral surfactants may be employed over a wide pH range. Separation selectivity is achieved by influencing the association of the proteins with the micelles through the introduction of organic into the buffer. When charged micelles are employed, increasing the concentration of salt in the buffer can also inhibit strong analyte–micelle interactions and facilitate increased resolution. For glycoprotein analysis, the presence of borate appears to promote separations based upon the extent of analyte carbohydrate structure.

In comparison to other CE techniques, MECC has demonstrated the potential to provide superior separation selectivity and peak capacity for a wide variety of sample types, including proteins, primarily since it can provide a hydrophobicity-based separation mechanism, in addition to the effects of solvent-accessible net charge that are utilized in traditional free solution CE. For protein analyses, another highly significant benefit of the presence of surfactants in the separation buffer is the elimination of the protein–capillary wall interactions which, historically, have been highly deleterious to the free solution CE of these macromolecules. The successful analysis of complex samples, such as biological extracts, by free solution CE is either not possible or requires a post-separation rinse with base to strip off analytes that have adsorbed to the capillary walls. This capillary cleaning exercise often creates problems with regard to the free silanol equilibration that governs the electroosmotic flow and, in turn, impacts on migration time reproducibility (see Ref. [22] for example). In contrast, the analyses of these types of samples by MECC does not require any special between-injection capillary treatment, resulting in significantly higher analysis precision [12].

The long-term acceptance of protein MECC by the analytical and biochemistry communities requires the consistent demonstration of advantages versus not only traditional slab gel electrophoresis, but especially, reversed-phase HPLC. The similarities of MECC and reversed-phase HPLC (RP-HPLC) separations have been experimentally demonstrated [33,34]. In regard to protein analysis, both techniques have the ability to denature and unravel a protein structure during the separation, exposing the inner core to the solution environment (in the case of MECC) or the chromatographic packing (in the case of RP-HPLC), an effect which then facilitates resolution based on solvent- or packing-accessible hydrophobicity or hydrophilicity. When the investigation of native proteins is desired, MECC has the advantage that it can be non-denaturing through the use of zwitterionic or neutral surfactants. RP-HPLC, however, has been proven to provide rugged protein quantitation capabilities that may be difficult, if not impossible, for MECC to provide, especially in the absence of an internal standard [9,24].

For the optimization of MECC separations of complex samples or proteins of similar structure (especially those differing only by one or more neutral amino acids or chemical modifications), the presence of an organic modifier appears to be necessary to manipulate the interaction between the proteins and micelles. Acetonitrile is perhaps the most commonly used eluent for RP-HPLC and it also appears to be the organic modifier of choice for MECC.

In comparison to bare silica capillaries, coated capillaries that generate no electroosmotic flow have demonstrated the ability to provide increased selectivity and shorter run times, especially for proteins that interact very strongly with ionic micelles. The disadvantages of using coated capillaries include limited integrity of the coating (four days in the presence of SDS at pH 8 [24]) vs. bare silica capillaries, which may be infinitely stable, and the inability to analyze neutral compounds in the presence of zwitterionic or neutral micelles.

Perhaps the biggest challenge hindering the adoption of MECC for routine protein analysis is the difficulty associated with the identification of sample components responsible for generating the peaks in an electrophoretic separation profile, specifically in

the resolution of complex samples. On-line MECC–MS may be the ideal solution to this need, but a successful interface between the two techniques seems to be fraught with many challenges at this point in time and it appears that much development will still be required before the technology can gain widespread acceptance.

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