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Review

Capillary biomolecular separations

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Abstract

This article summarizes the recent advances in microcolumn separations of biopolymers. Microcolumn liquid chromatography is primarily emphasized for its role as a micropreparative and fractionation tool, whereas high-performance capillary electrophoresis is demonstrated as a highly efficient technique for final analyses. Following a brief discussion of new trends in instrumentation, the recent applications of capillary techniques to proteins, DNA and glycoconjugates are reviewed.

Keywords: Proteins; DNA; Glycoconjugates

Contents

1. Introduction

Capillary separation techniques have had a profound effect on the modern practice of analyzing complex mixtures. While relatively small and lipophilic molecules are now effectively handled by capillary gas chromatography (GC) and microcolumn supercritical fluid chromatography (SFC), microcolumn liquid chromatography (LC) and, in particular, high-performance capillary electrophoresis (HPCE) continue to impact the separation and microanalysis of large biological molecules. The "hybrids" between HPCE and LC, such as micellar electrokinetic capillary chromatography and electrochromatography are also analytically significant in the areas of relatively small organic molecules (pharmaceutical analysis, environmental applications and clinical analysis, for example).

The numerous challenges of modern biology and medicine necessitate the development of analytical methodologies of utmost sensitivity and resolving power. Efforts to miniaturize columns have often been demonstrated to be fundamental to enhanced measurement capabilities of detection techniques. With a continuing quest for characterization of ever larger biomolecular entities, it is imperative that the

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highly sensitive and information-rich devices preserve their analytical value when coupled to high-performance separation systems dealing with large biopolymers. Borrowing from the column technologies and detection methods of other microcolumn techniques, HPCE perhaps now has achieved a greater distinction than any other technique of the past decade. Yet, the sheer pace of instrumental advances and the ever-increasing number of applications appear to suggest that HPCE is far from reaching its full potential. This minireview attempts to summarize the most important recent developments in the biomolecular separations through HPCE and microcolumn LC and point out some promising avenues for further development. After a brief description of recent developments in instrumentation, the three major areas of biochemical interest (protein isolation/characterization, genomic analysis and glycobiology) will be dealt with in relation to the current and future capabilities of the pertinent microcolumn techniques.

2. The evolution of microcolumn separations

Most biochemical researchers are now quite familiar and comfortable with biomacromolecular separations performed chromatographically in conventional (4.6 mm, I.D.) HPLC columns and electrophoretically in flat gel media. While transition to "small-scale" systems may appear revolutionary and far-fetched to them at present, there is an increasing realization that such methods provide qualitatively new avenues to them; the extraordinary resolving power in HPCE, or the unique capability of microcolumns to interface to a mass spectrometer, are good examples. However, transition to the miniaturized technologies is still expected to be a gradual process that must be adequately backed by reliable commercial instrumentation.

Microcolumn separations of biopolymers is the area that has seen a significant degree of cross-fertilization of ideas from other fields in separation science. For example, many important lessons in the miniaturization of separation systems were learnt from as unrelated fields as capillary GC of small molecules (hydrocarbons, petrochemicals or aroma constituents). Some column technologies of both microcolumn LC and HPCE borrow heavily from the surface treatment methods developed in capillary GC, SFC and conventional HPLC. A substantial miniaturization effort in capillary electrophoresis [1,2], which is now widely credited as an important milestone in creating the field of HPCE, utilized the detector miniaturization technologies developed for capillary LC [3–6] and SFC [7]. The polyacrylamide gels of conventional electrophoresis soon found unique utilization in HPCE [8–10] and thus preceded introduction of the now commonly used entangled polymer matrices [11–15] as effective separation media for the separation of polynucleotides, proteins and glycocongugates. The recent advances in polymer research are likely to expand the scope of biocompatible separation media further.

Microcolumn LC and HPCE share certain instrumental characteristics that make them particularly desirable for work with small biological samples. Primarily, these include increased mass sensitivity of concentration-sensitive detectors (devices based on conductivity and optical measurements or on electrochemical principles); easy "hyphenation" and ability to work with small biological objects. Occasionally, these attributes can be combined in increasingly sophisticated applications to measurements in life science (e.g., analysis of single biological cells). With several types of miniaturized detectors, similar interfacing technologies can be utilized for both low-flow microcolumn LC and HPCE.

During the recent period, numerous powerful detection and ancillary techniques have been added to the now standard tools of microcolumn separations: electrochemical detectors for CE [16–18], radiometric detection [19,20], multichannel fluorescence [21] and even inductively coupled plasma mass spectrometry [22] for the benefits of environmental and biochemical elemental speciation. The on-going development of new detection technologies continues to be crucial to the success of capillary separations. Although miniaturized UV detection remains important for the measurement of proteins and peptides, an increasing number of applications are being oriented towards the more sensitive methods of laser-induced fluorescence (LIF) and various techniques of mass spectrometry (MS). With the use of conventional detectors, the typically small size of the sample injections in HPCE limits investigations

to the 10^{-5} *M* concentration range. Certain sample enrichment techniques, such as isotachophoretic preconcentration, sample stacking or electrochromatographic preconcentration, can reduce the detection burden so that (e.g., for trace proteins) picomole to high femtomole levels can be achieved with moderately sensitive detectors. In microcolumn LC, sample preconcentration techniques are relatively easy [23], since the mobile-phase composition can be easily adjusted to cause solute focusing at the column inlet.

A dramatic decrease in the sampling volume requirements that accompanies miniaturization of LC and HPCE (between nanoliters and picoliters) has, several years ago, invited the use of such techniques for investigation of the chemical content of individual biological cells [24,25]. This was the time when the microcolumn techniques joined in the investigations that had been previously limited to the use of microelectrode sensors and microscopic methods whose measurements are confined to either isolated compounds or morphological observations. The capillary techniques provide the opportunity for *multicomponent analyses* and effective removal of interferants prior to ultrasensitive detection. The initial investigations in the area were demonstrated with relatively large single cells, the giant (~100 μm diameter and 1-nl volume) snail neurons [24,25] and tentative characterization of their neurotransmitter-like substances through amperometric detection. More recently, much smaller biological objects, such as adrenomedullary cells [26] and individual red blood cells [27,28] were also analyzed.

An example of determination at the level of single cells is seen in the publication by Cooper et al. [26] who probed single bovine adrenomedullary cells (Fig. 1) using reversed-phase (50 μm, I.D.) packed capillaries and a carbon-fiber electrode detector. The feasibility of quantitative measurements of epinephrine/norepinephrine in different cells was thus established. The electrochemical detection techniques seem to facilitate such analyses at the femtomole to attomole levels, but if suitable fluorescence-tagging procedures are developed for small volumes and solute concentrations, the investigations at the level of zeptomoles (10^{-21} mol) or lower could become feasible.

An interesting twist in the utilization of microscopic techniques in conjunction with microsepara-

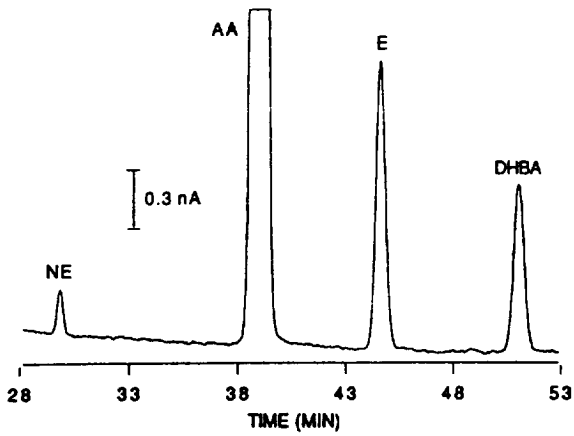

Fig. 1. Chromatogram of a single bovine adrenomedullary cell. Peak identification: NE=norepinephrine; AA=ascorbic acid; E=epinephrine; DHBA=(3,4-dihydroxybenzyl)amine. Reproduced from Ref. [26] with the permission of the American Chemical Society.

tion are the recent publications of Shear et al. [29] and Fishman et al. [30] who employed a single biological cell (Fig. 2) as a detector (biosensor). They have suggested that cellular responses can be tied to highly specific events, such as receptor–ligand recognition, enzymatic activity or transmembrane signaling pathways. For example, such a system could be used for biological screening of receptor agonists and antagonists that are being eluted through a microcolumn [30].

As is often the case in modern analytical separations, the typical sensitivity of new detectors used in microcolumn LC and HPCE runs ahead of the sensitivity of information-rich, ancillary techniques that are needed to confirm identity of a separated biomolecule or to structurally characterize a previously unknown compound. However, the modern techniques of mass spectrometry (MS) seem to be rapidly closing the gap, while their information content is becoming essential in structural elucidation. In particular, electrospray ionization, new designs of mass analyzers and the tandem (MS–MS) techniques are rapidly proving their unique capabilities in the structural elucidation of proteins and glycopeptides and in sequencing efforts. Certain novel interfacing technologies [31,32], using miniaturized electrospray sources and technically advanced mass analyzers, extend the capability of

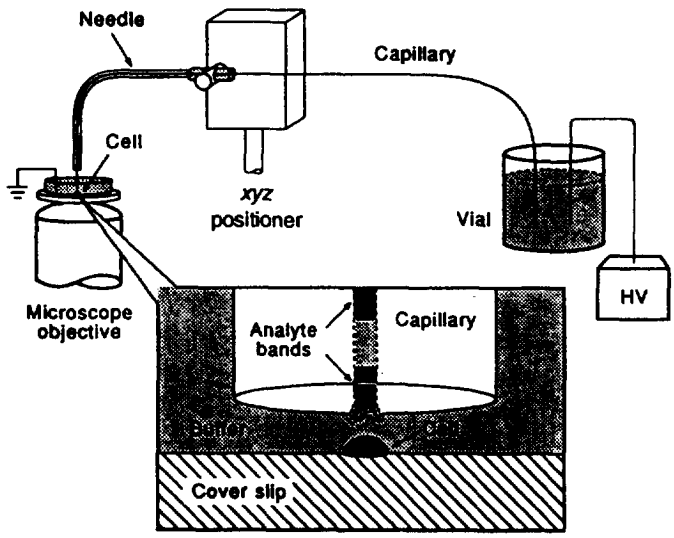

Fig. 2. Single-cell biosensor system for capillary electrophoresis–fluorescence microscopy. Adapted from Ref. [29] with the permission of the American Association for the Advancement of Science.

peptide analysis to below the femtomole level. An example of such instrumental efforts is shown in Fig. 3 [33], with interfacing HPCE through a sheathless (miniaturized) electrospray device to an ion-trap mass spectrometer. Perhaps the leading instrumentation in this area is the combination of HPCE with ion cyclotron resonance (Fourier transform) MS [34] that provides exact molecular mass and isotopic distribution for analytes in complex mixtures.

At present, microcolumn LC appears to match more easily the sample requirements of electrospray MS than those of HPCE. However, significant efforts by both academic researchers and instrument manufacturers are currently directed towards improving the HPCE–MS coupling methods. Because LC and HPCE are orthogonal separation methods, both hyphenated techniques ultimately will be optimized for the benefits of analyzing complex biological mixtures.

Matrix-assisted laser desorption/ionization (MALDI) MS has recently emerged as an extremely popular structural characterization tool for various biomolecules. While its on-line coupling with microcolumn LC and HPCE may well be worth exploring, the current practice involves investigation of the fractions collected after separation [35,36]. The inherent high sensitivity of MALDI MS now seems to allow peptide sequencing [37] at sub-picomole levels and similar techniques can be developed for other biopolymers.

Unfortunately, some highly informative ancillary techniques are inherently limited in their sensitivity. An example is nuclear magnetic resonance (NMR) spectroscopy which, until recently, was not considered seriously for on-line coupling with even conventional HPLC. However, technological advances in NMR instrumentation have gradually enhanced its sensitivity potential. In a series of recent communications, Wu et al. [38,39] and Olson et al. [40] demonstrated the feasibility of miniaturized (microcoil) ^{1}H NMR to be coupled to microcolumn LC and capillary electrophoresis. A section of the separation capillary, provided with a 50 μm copper microcoil, acts as a concentration-sensitive "detector", enhancing mass sensitivity, in nanoliter volumes, by more than 100-fold.

The last decade has certainly seen substantial advances in both HPCE and microcolumn LC. Their increasing utilization in biomolecular analysis now calls for re-assessment of their roles in the overall analysis of complex biological materials. With HPCE clearly taking the lead in ultimate resolving power, speed of analysis and sensitive detection, the roles of chromatography may be most effectively redelegated

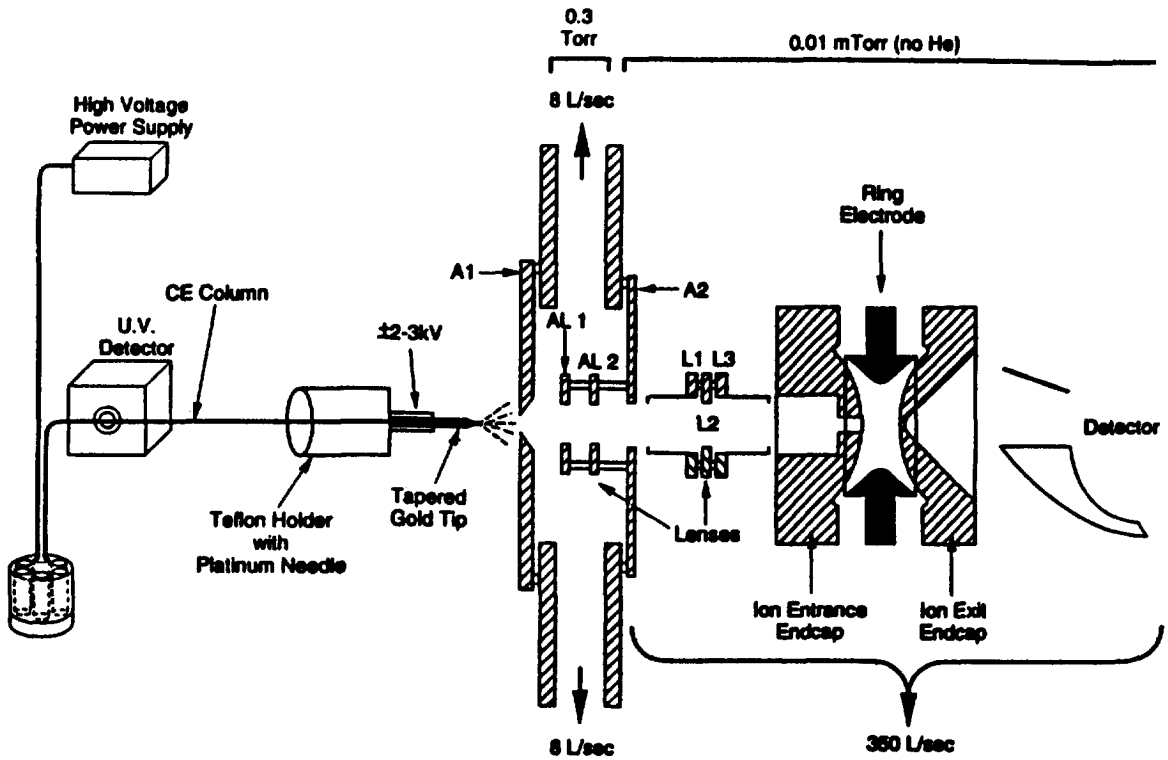

Fig. 3. Schematic diagram of a CE–ion trap mass spectrometer using the sheathless electrospray interface. Reproduced from Ref. [33] with the permission of Wiley and Sons.

to its traditional strengths, i.e., separation selectivity and micropreparative capabilities [41].

Minor exceptions notwithstanding, HPCE is now a clear winner in its various capabilities to perform the final analyses of complex biopolymeric mixtures. A promising recent development in HPCE is the fabrication of separatory channels on a glass microchip [42–47]. Not too long ago, this research area was considered esoteric and highly speculative. However, the precision of current lithographic techniques now allows the formation of adequate separatory geometries (single or parallel) and, in addition, low dead-volume manipulation of buffers on a chip through electroosmosis (without the use of valves or pumps). Importantly, the separation channels can be coupled with precolumn sample treatment or postcolumn fluorescent derivatization [44,45]. An example is shown in Fig. 4 [49]. Thus far, LIF confocal microscopic systems have been used effectively for detection of primary amines and DNA fragments. To analyze multiple samples simultaneously, as needed in modern biopolymer characterization, etching of parallel channels on a microchip is obviously feasible [46,47].

Microcolumn LC is now emerging as a highly complementary rather than a competitive approach to HPCE and, contrary to the popular belief, it has not yet reached its developmental plateau. Initially, a crucial step in microcolumn technology was the development of fused-silica capillaries packed with small particles [48–50]. Miniaturization of such columns below 50 μm I.D. [51,52] has resulted in further substantial gains for their separation performance. Short of some unforeseen developments in separation science, future dramatic improvements in the efficiency of LC microcolumns are not immediately obvious. However, wider use of selectivity in group separations (analyses of complex mixtures) has a very significant rationale. Future design of integrated analytical systems will undoubtedly require the best of chromatographic selectivity (affinity principles, immunoadsorbents, lectins, immobilized

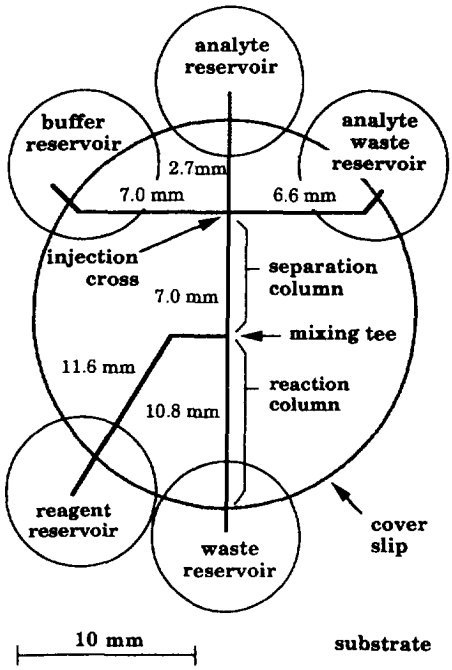

Fig. 4. Schematic diagram of the microchip CE with a postcolumn detection mixer. Reproduced from Ref. [44] with the permission of the American Chemical Society.

enzymes, etc.) in preceding HPCE, or the reversed-phase microcolumn LC, in the final analysis. Conventional packing technologies, or in-capillary polymerization, as described recently by Hjertén et al. [53,54], may be used. Additionally, some room for development may also be available in the future exploration of electrochromatographic principles.

There are numerous reasons for extensive "hyphenation" of microcolumn separation techniques. These have been discussed in some detail by Cortes [55] in his review on multidimensional separation techniques and, in conjunction with other aspects of microcolumn LC, by the author of the present review [56]. In characterization studies with small amounts of proteins, microcolumn LC lends itself uniquely to being combined with additional techniques.

One of the strengths of appropriate two-dimensional micro-LC systems can be the capability of significantly enhancing the peak capacity and resolution (effectively overcoming the fundamental limits of chromatographic efficiency). An example of the complementary use of different phase systems is in the ion-exchange chromatographic analysis of proteins in the first dimension, followed by a size-exclusion run in the second dimension. First, an important condition for effectiveness of such, or similar, "hyphenations" is that the mobile phases between the dimensions are compatible. Second, when the complementary chromatographic techniques operate at roughly the same separation speeds, the "fraction storage" in the valve loops may present some difficulties.

Among the most attractive two-dimensional (2D) techniques are those in which the first run is slower than the second run, so that the eluent of the first column can be continuously sampled by the second column. This condition is fairly easily met in the combination of microcolumn LC and HPCE [57,58]. In peptide-mapping efforts, technical aspects of LC–HPCE are further reinforced by the rationale of combining the separations based on hydrophobicity (reversed-phase LC) and charge-to-mass ratio (HPCE) of the solutes [59,60]. Two-dimensional separations of peptides from larger proteins [61] exemplify the exciting possibilities for rapid characterization in future studies.

3. Protein isolation/characterization and related measurements

In small-scale isolation of proteins, microcolumn LC can be uniquely useful in (a) measuring more easily detectable (compared to conventional HPLC) trace sample components; (b) requiring less dilution during fraction collection and (c) significantly reducing irreversible adsorption due to the drastically decreased volume of the sorption material [62,63]. The benefits of decreasing the column diameter are also being reflected in better compatibility with proteolytic digestion systems [64], microsequencing [65,66] and the analysis of isolated spots from slab gel electrophoresis.

In isolating small quantities of proteins, different LC retention modes can be employed. Using fused-silica packed column technology, our laboratory has reported systems based on the reversed-phase and size-exclusion principles [62,63], immobilized metal affinity [67], and immunoaffinity [68]. An example of a dual-column set-up, combining immunoaffinity

and the reversed-phase microcolumn LC, is shown in Fig. 5 [68]. It has become technologically feasible to use microvalves and miniaturized column connectors at flow-rates of the order of microliters per minute.

Miniaturized UV detectors are quite capable of detecting small amounts of proteins, peptides or nucleotides, but an important question arises concerning the ways of getting some structural information on the collected fractions. Low-picomole quantities of the isolated proteins may still be compatible with the sensitivity of modern methods of amino acid analysis and state-of-the-art gas-phase sequencers and, most certainly, the modern mass spectrometric techniques. It appears that we are not nearly as limited by the availability of sensitive measurement techniques as we are by our capability to carry out chemical reactions (hydrolysis, specific fragmentations, compound tagging, etc.) at low levels of sample.

Miniaturization of proteolytic digests [69–73] combined with high-sensitivity peptide mapping is likely to become significant in future studies on trace proteins, as site-specific fragmentations are among the most informative techniques in protein characterization studies. An example of the sensitivity status of such techniques is shown in Fig. 6 [23], where a peptide map (at subnanogram levels) of a glycoprotein (bovine fetuin) is displayed through UV absorbance detection (above). The series of chromatograms (below) are due to the fluorescence analyses of the discrete peptide fractions for their content of N-acetylneuraminic acid. Both microcolumn LC and HPCE have significant potential in structural characterization studies.

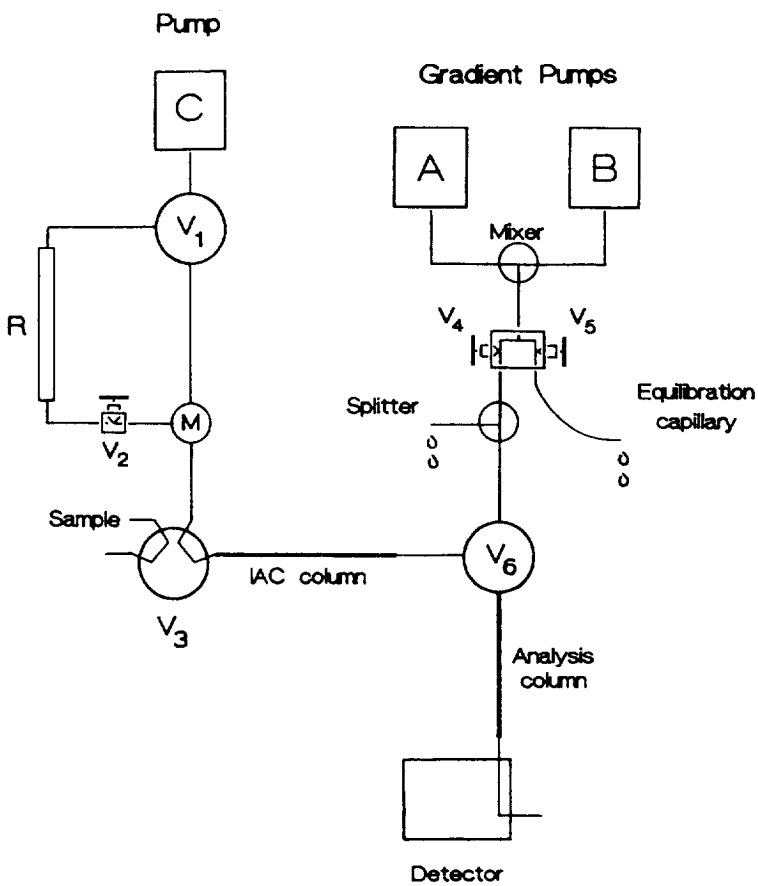

Fig. 5. Schematic diagram of a dual-column immunoaffinity chromatographic system. M=Manifold; V_1–V_6=switchable valves; R=buffer reservoir. Reproduced from Ref. [68] with permission from the American Chemical Society.

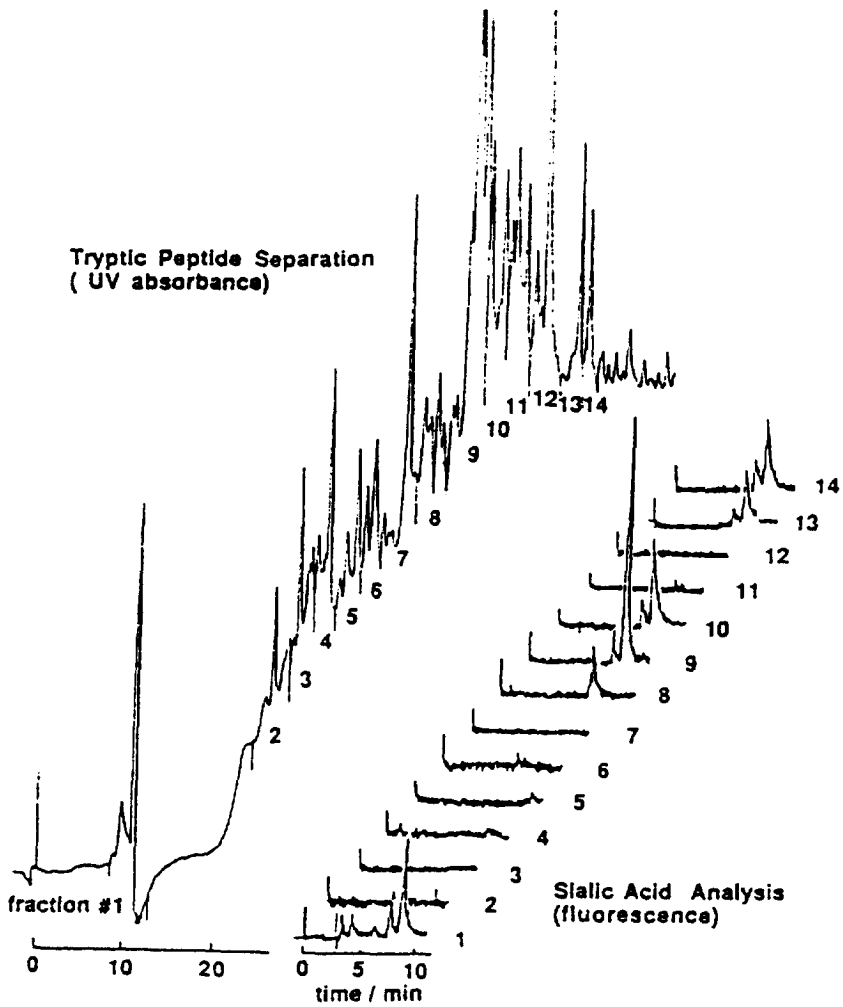

Fig. 6. Tryptic map of bovine fetuin displayed with the chromatograms corresponding to the sialic acid analysis of fractions 1–14. Reproduced from Ref. [23] with permission from Academic Press.

HPCE of peptides and proteins has now progressed from exploratory and optimization studies on model mixtures towards applications that are relevant to the laboratory practice of protein biochemistry. This methodological "maturation" is particularly reflected in the following developments: (a) increased use in quantitative determination of recombinant proteins and their contaminants, where HPCE is increasingly viewed as a complementary technique to the more established HPLC and immunological methods; (b) increased use of HPCE–MS in protein characterization; (c) the development of HPCE-based immunoassays and (d) a rapidly increasing number of ligand–biomolecule binding investigations (the so-called affinity HPCE). The characterization of HPCE-separated proteins requires the best ancillary techniques: on-line electrospray MS; MALDI-MS of recovered fractions and immunodetection. On-line microreactors containing specific enzymes [72,73] can enhance significantly the characterization potential.

Interesting investigations have recently been conducted using HPCE and high-sensitivity detection (typically LIF) to challenge the more established immunoassay techniques. With appropriately labeled antibody, LIF can provide the sensitivity that is

typically needed (10^{-8}–10^{-10} M levels), while HPCE can readily (and rapidly) separate the antigen–antibody complex from other components of the mixture. For example, HPCE-based immunoassays have recently been demonstrated for human growth hormone [74], IgA [75], insulin [76] and digoxin in serum [77].

In 1992, Chu et al. [78] reported that HPCE can be used as a fast and simple method to assess the binding of ligands to biomolecules. They called their technique "affinity capillary electrophoresis" (ACE) and, since then, a number of applications have been identified with this term. In its simplest form, ACE relies on measuring differences in the electrophoretic mobility of the protein–ligand complex and of the uncomplexed protein under differing ligand concentrations, and expressing the data as a Scatchard plot. The method represents an attractive approach for biochemical studies because it consumes small quantities of sample, allows detection of small ligand molecules together with proteins, provides rapid on-line detection, can be coupled to mass spectrometry and works in the absence of radioactive labels. Representative applications of ACE, shown recently, include studies of the interactions between a heat-shock protein and its peptide fragments with various immunosuppressants [79,80], the binding of vancomycin to peptidoglycans [81] and investigations of certain sugar–lectin interactions [82]. ACE has also been utilized to measure the binding constants between albumin and several anti-inflammatory drugs [83]. Some additional directions include the measurement of migration shifts as a consequence of the antigen–antibody interaction [84,85], searching specific interactions of model receptors with constituents of the peptide combinatorial libraries through ACE–MS [86] and determination of binding stoichiometries in protein–ligand interactions [87].

Following the early demonstration [88] that HPCE is a viable method for high-efficiency protein separations, a considerable effort has been directed towards suppressing protein adsorption at the capillary wall (see Ref. [89] for a review of earlier studies). There has been considerable progress to this research area in recent years. Although a "universal" wall treatment may still remain as elusive as ever, dramatic improvements, in terms of reduced protein adsorption and peak symmetry, have been achieved. Recently reported innovations include the use of poly(vinyl alcohol) coatings [90], poly(propylene glycol) treatment [91], polysaccharide-based, hydrolytically stable layers [92,93] and the use of self-assembled alkylsilane monolayers [94]. Researchers are gradually learning that different protein mixtures have different vulnerabilities to the capillary treatment.

4. Genomic analysis

Although liquid chromatography will continue to be important in larger-scale separations of nucleic acid components, gel electrophoresis has become a dominant technique in the analytical separation of DNA molecules. Following the widespread use of slab gel techniques in the area, HPCE using sieving media has become increasingly popular in the analysis of nucleic acids, due to its unprecedented resolution and speed of analysis. Recently, HPCE has been used in a number of novel and exciting applications that range from antisense technology and DNA sequencing tasks to the separation of double-stranded DNA fragments in mutational analysis, restriction fragment mapping and polymerase chain reaction (PCR) product identification in clinical studies (see e.g. Fig. 7) and forensic applications. With the exception of relatively small nucleotides that can be measured through their UV absorbance, almost all recent work uses LIF for detection. Although a search for inexpensive lasers and optimum fluorescent probes is likely to continue, satisfactory results have been reported with the previously employed sequencing fluorescent labels and intercalating dyes. An increasing number of studies stress the need for quantitative information; therapeutic applications of DNA or RNA sequences will soon necessitate the standards of analytical practice that are now common with conventional pharmaceuticals.

The possibilities of fast genomic analysis, in general, and of DNA sequencing, in particular, were initially enhanced by introduction of gel-filled capillaries [8–10], however, the current emphasis has been on the use of entangled polymer solutions. The use of non-crosslinked polymer networks, explained initially by Grossman and Soane [13] using the

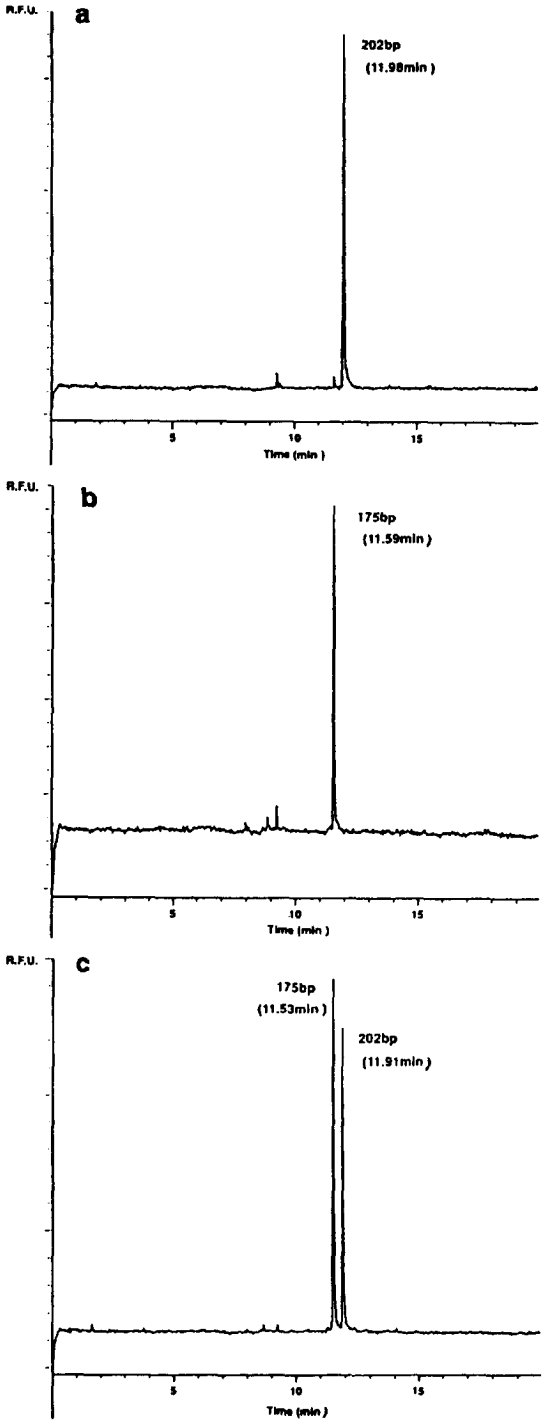

Fig. 7. Electropherograms of DNA fragments obtained by allele specific PCR. (a) Normal subject; (b) MCAD deficiency (homozygote); (c) heterozygous carrier of MCAD deficiency. (MCAD= medium-chain acyl-coenzyme A dehydrogenase deficiency.) Reproduced from Ref. [97].

example of DNA restriction fragments, has led to a great number of applications during recent years. These are particularly evident in the considerations of fast and reliable HPCE in the large-scale genetic analysis programs that have been spurred on by the Human Genome Initiative.

Although a significant gain in speed of analysis over slab gels has been obvious, conventional HPCE permits analysis of only one sample at a time. The problem can be overcome by the use of parallel capillaries and the development of commercial sequencing instrumentation based on multiplexed HPCE is apparently in progress. DNA fragment separations on microfabricated chips with capillary arrays [44–47] may well be the next logical step in this scientifically exciting area.

The use of entangled polymer solutions in HPCE has also impacted the separation of double-stranded DNA fragments. To ensure the reproducibility of migration times that is needed for accurate base-pair assignments, the procedures need optimization [95]. It has been shown that LIF is capable of reliably detecting minute quantities of materials using PCR-amplified DNA fragments. This is particularly important for forensic applications and work with unidentified human remains [96]. The superb resolving power of HPCE now permits genetic studies to be carried out in various organisms using reliable and quantitative DNA fragment analysis. As exemplified by a recent application to the diagnosis of a human dehydrogenase deficiency [97], HPCE may become a key technique in the study of various genetic disorders.

Naturally, the resolution of various DNA fragments deteriorates with increasing molecular mass. This problem is further compounded by the well-known phenomena of molecular stretching and reptation of charged polymers [98] in gels, under the application of an electric field, which leads to a lack of resolution at the level of strands larger than approximately 20 kbp. To counter this disadvantage, Schwartz and Cantor [99] were the first to develop DNA separations (in slab gels) under a pulsed-field regime. Subsequently, this has led to the burgeoning number of applications of the system to molecular biology [100] as well as to numerous investigations aimed at a fundamental understanding of the molecular motions involved [101]. During the last two

years, pulsed-field HPCE has been developed [102–105] as a fast and highly efficient counterpart of the widely practiced [100] slab gel techniques. Different pulsed field regimes have been explored [105] and have been found to cause reliable and reproducible separations in the range up to roughly 100 kbp (see e.g. Fig. 8). However, some difficulties arise in the efforts to expand this methodology to larger strands, presumably due to the undesirable formation of DNA aggregates [106]. The published investigations in pulsed-field HPCE have recently been reviewed [107].

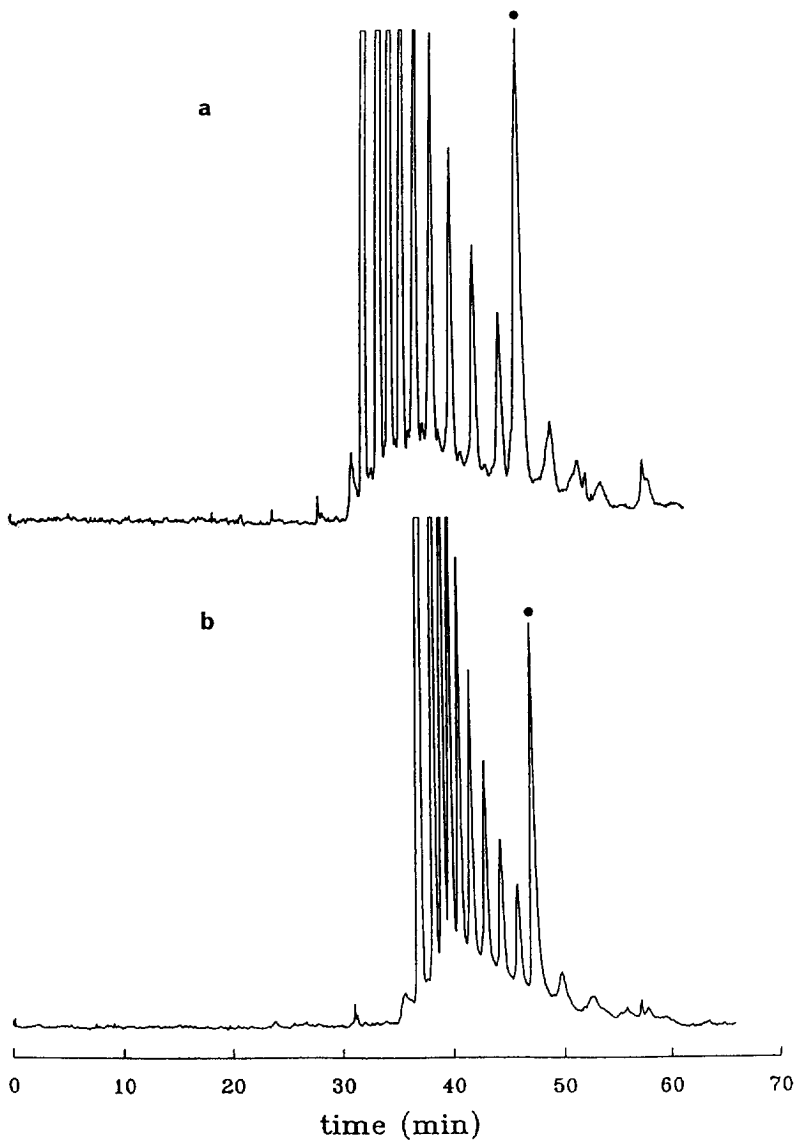

Fig. 8. Pulsed-field CE separation of 5 kb λ DNA concatamers by the square-wave electric field, using a frequency gradient. The dot above the tenth peak indicates a monodisperse λ DNA molecule (48.5 kb) added to the sample. Figures (a) and (b) signify different frequency gradients and matrix concentrations. Reproduced from Ref. [105] with the permission of the Oxford University Press.

5. Glycobiology

Glycoconjugates are being increasingly recognized as important "recognition molecules" whose function is crucial to processes as diverse as cellular adhesion, blood coagulation, antigen–antibody interaction, recognition of toxins or hormones at the level of biological membranes, egg fertilization and targeting aging cells for destruction. Improvements in our knowledge of glycosylation processes is also creating numerous opportunities for the design of new diagnostic techniques and powerful biopharmaceuticals. The fact that very few glycoproteins or proteoglycans have been characterized thus far in sufficient structural detail is largely a reflection of methodological difficulties. During the last decade, numerous analytical advances started to emerge to cope with the exceedingly difficult problems of glycobiology.

Microcolumn LC has significant potential in analytical glycobiology for similar reasons as described in Section 4 on proteins, i.e., microisolation, small-sample handling and selective separations. The limited scope of "biological amplification" for post-translationally produced glycosylated structures places high demands on both the gentle work-up procedures at the nanoscale and high-sensitivity measurements. It must be emphasized that a microisolation procedure (or a sample work-up), if improperly chosen, could become a bottleneck for the overall high-sensitivity analysis of carbohydrates.

Over the last several years, HPCE has made great strides into the ways we analyze glycoconjugates. The recent progress in this area has been reviewed by the present author (Novotny and Sudor [108], Novotny [109,110] and Stefansson and Novotny [111]) and by others [112,113]. The role of HPCE has been primarily emphasized as that of a powerful end method. This is, once again, primarily due to its great resolving power. Concurrently, the combinations of HPCE with LIF detection and mass spectrometry have been emphasized. Although applications in this area are currently less frequent than those in the protein and DNA fields, HPCE is likely to become as indispensable as the more established methods of high-field NMR and HPLC with pulsed amperometric detection. HPCE is expected to carry out the important analytical tasks, varying from display of microheterogeneity in glycoproteins [114], analysis of small saccharides, oligosaccharide mapping, glycopeptide separations, sequencing, etc., to the separation of larger oligosaccharides (as needed in the analyses of glycosaminoglycans or technologically important polysaccharides). The fluorescent tagging of carbohydrates [115–119] has become an important part of the development of HPCE–LIF methodologies.

Investigations of the electromigration mechanisms with various fluorescently tagged oligosaccharides have become essential to recent progress in the area [111,117,120,121]. The buffer composition and secondary equilibria are often crucial to successful resolution of the exceedingly complex oligosaccharide mixtures. The state-of-the-art in oligosaccharide separations in a free-buffer medium is reflected in the component resolution with an enzymatically treated corn amylopectin [122] (Fig. 9). Indeed, HPCE appears to be sufficiently efficient to recognize variously branched isomers as distinct peaks. The analytical use of various debranching enzymes has a significant rationale for structural studies in complex biological mixtures.

Until recently, there has been only a limited success in separating larger polysaccharides, with the exception of polydextran standards migrating in pulsed fields [15] and the water-soluble cellulose derivatives that were charged through addition of suitable buffer additives [120]. Most recently, we (Stefansson et al. [123]) have been successful in demonstrating that high-resolution HPCE of charged polysaccharides (hyaluronic, alginic and polygalacturonic acids) is feasible in appropriately chosen entangled polymer matrices.

The design of suitable fluorescent probes for HPCE–LIF is likely to remain a significant focus for research in high-sensitivity glycoconjugate measurements. The current record for sensitivity in this area has recently been shown [124,125] in the detection limit of 100 analyte molecules (Fig. 10; [125]) with the standard tetramethylrhodamine-labeled oligosaccharides. This result, in itself, might not impress today's analytical community that has become used to the notion of single-molecule detection. However, most importantly, the system described was actually used in the meaningful context of monitoring bio-

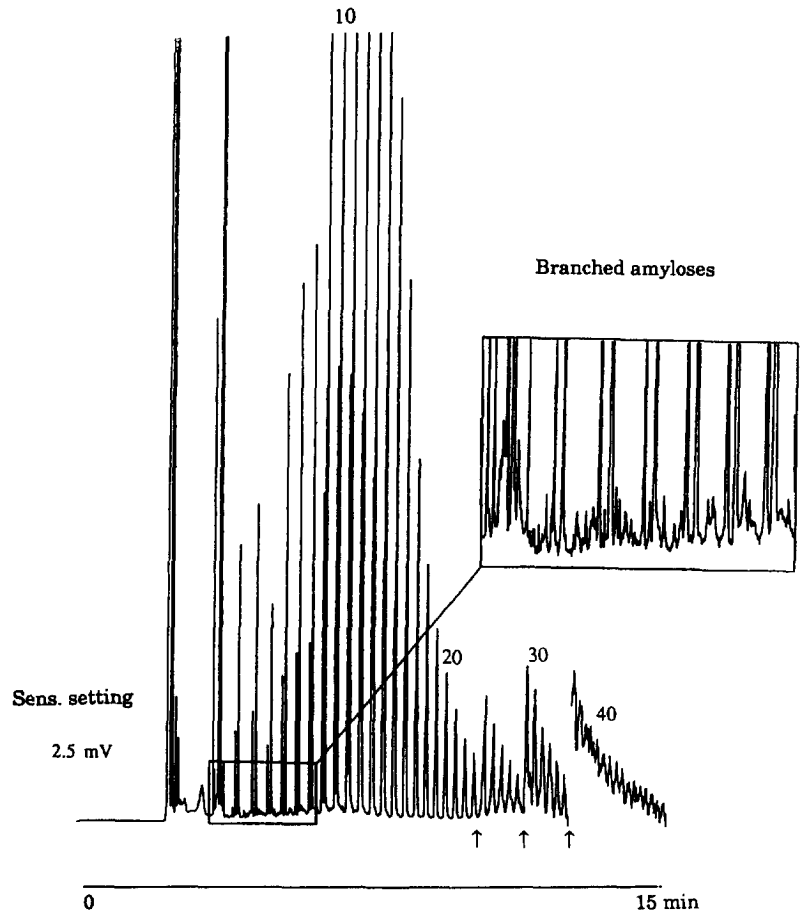

Fig. 9. High-resolution electropherogram of an enzymatically treated corn amylopectin. Adapted from Ref. [122].

synthetic transformation of selected oligosaccharides in cellular preparations.

6. Concluding remarks

Almost a decade has passed since capillary LC was first used in protein analysis and microisolation [62,63] and capillary electrophoresis of proteins [88] and oligonucleotides [9] was first shown at high resolution. While microcolumn LC has continued to gain recognition at a steady pace, HPCE has already revolutionized numerous directions of modern bioanalytical chemistry. The needs of modern biochemical research, molecular biology and biotechnology will continue to promote further methodological advances and conceptual applications.

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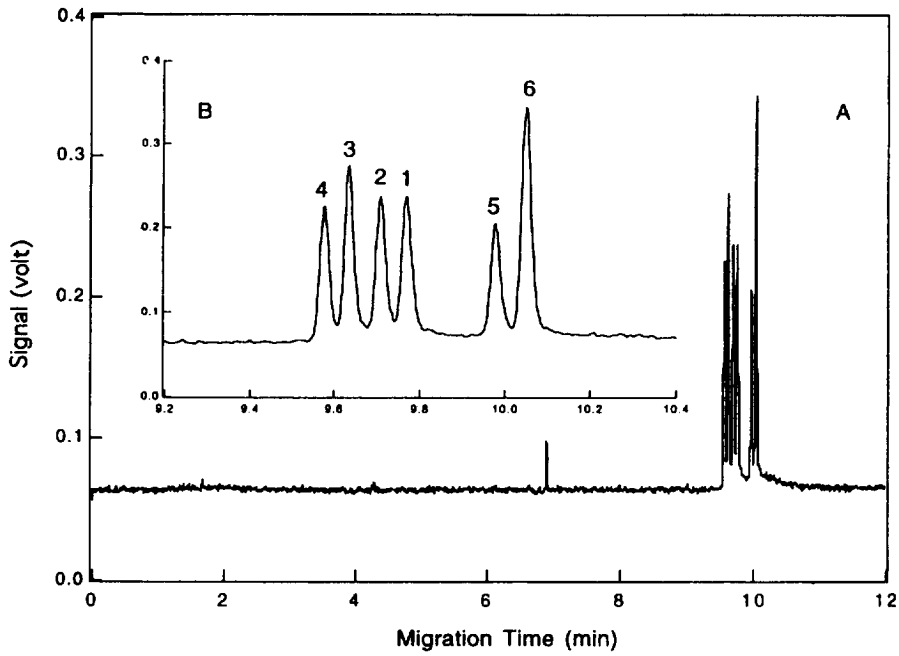

Fig. 10. Capillary electropherogram (with LIF detection) of five standard tetramethylrhodamine-labeled oligosaccharides. A=Entire chromatogram; B=expansion of a narrow migration window. The detection limits correspond to approximately 1200 molecules. Reproduced from Ref. [125] with the permission of Academic Press.

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