1994

Development of novel separation techniques for biological samples in capillary electrophoresis

Huan-Tsung Chang
Iowa State University

Order Number 9503536

Development of novel separation techniques for biological samples in capillary electrophoresis

Chang, Huan-Tsung, Ph.D.

Iowa State University, 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Development of novel separation techniques for biological samples in capillary electrophoresis

by

Huan-Tsung Chang

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Analytical Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work/

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1994

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

There is no doubt that the challenges in biological separation techniques for analytical chemist in the 1990s have been addressed on two different sides: single cell analysis and DNA sequencing. The importance of these two fields is because human disease always relates to the function of cell; this depends on the variation of the amount and\or the species of chemicals and the damage of DNA in a single cell. Single cell analysis is tedious and somewhat difficult because of complicated chemicals and low amount of interesting chemicals in a single cell. There is pressure to elucidate the sequence of human genome as rapidly and efficiently as possible while minimizing costs, since the accuracy to map the human genome, which contains 3 billion base pairs, is required to diagnose genetic disease. To answer these questions, the analytical chemist has used a novel technique, capillary electrophoresis (CE) for more than one decade. The promise of CE for solving these problems is due to its high speed, excellent resolution, low mass amount and easy automation.

Historical Background

It is not surprising that CE has become one of the most important separation techniques, since its success in biological separation takes the advantages of conventional electrophoresis, miniaturatization, high quality and low cost of fused silica from gas chromatography (GC) and sensitive detection system from high performance liquid chromatography (HPLC).

Based on the Helmholtz's[1] double-layer theory and Kohlrausch's[2] description about the relation between electrophoretic migration of ions and their concentration, modern electrophoresis began in the moving boundary electrophoresis for protein separation by Tiselius[3] during the 1930s. Over the next four decades, research continued on the development of isotachophoresis,[4] tube type zone electrophoresis[5-6] and the introduction of anticonvective media for zone electrophoresis.[7-8] In the 1970s, the use of UV[9] and conductivity as a detector[10] and high voltage applied to the separation in narrow-bore teflon tubes[11] were developed.

The door of modern CE was opened by Jorgenson and Lukacs[12-13] in 1981 who demonstrated high separation efficiency with high electric field strength in narrow (< 100-μm inner diameter) capillaries. When capillaries with small diameters and a low volume-area ratio are used, heat dissipation is significant, and in turn, the band broadening due to the Joule heat effect is minimized. The other

main contribution comes from Terabe[14] who introduced micellar electrokinetic capillary chromatography (MECC or MEKC) which makes it possible to separate neutral molecules via CE.

Basic Theory

In this section we briefly discuss the basic theory of electrophoretic migration in capillary tubes relative to the migration time, electric field strength and physical phenomena that affect the nature of separation. A more comprehensive discussion will be presented in Chapters 2 and 3.

The simple instrumental setup for CE is shown in Figure 1. The basic instrumentation involves a high-voltage power supply (0 to 40 kV), two buffer reservoirs, a capillary and a detector. Generally the flow of the electroosmosis is toward the cathode, hence the detector is put near this end. Sample injection can be performed by hydrodynamic or electromigration injection.

Hydrodynamic injection can be performed simply by raising one end of the capillary to inject sample into the capillary caused by the pressure difference between the two ends. On the other hand, sample injected into the capillary can be down by the flow of analytes when voltage is applied. Generally, hydrodynamic injection is simple but worse in accuracy; electromigration injection is more accurate but with a bias effect because of the difference of mobilities of analytes.

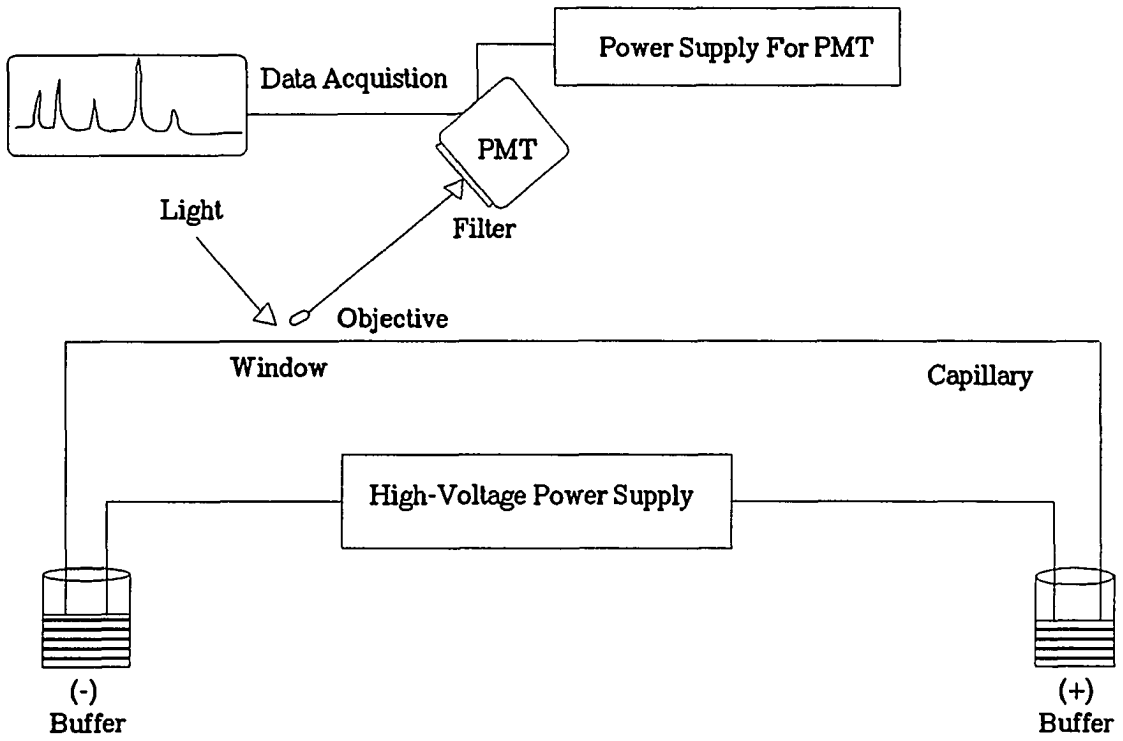


Figure 1. Schematic of CE setup.

Electroosmotic mobility (m_{eo}), which is the flow of solvent in an applied potential field, is one of the flow sources in CE. The flow direction and velocity depend on the net charge of the silica wall surface. Of which pH is a very important factor. For simple zone electrophoresis[15] the silanol functional group dissociates to form negative charge when pH is larger than 4, hence the flow direction is toward the cathode. In addition to the pH effect, temperature, type of buffer and its concentration and viscosity are all important factors which control m_{eo}. Electroosmotic mobility also can be changed by adding modifiers, such as organic solvent or salts into the running buffer. Addition of surfactants[16] and polymers[17] into the running buffer, which can be coated onto the capillary wall, is also suitable to change m_{eo} or the direction of electroosmotic flow.

It is important to note that electroosmosis, which results in a flat flow profile and the lack of stationary phase virtually eliminate any band broadening due to resistance to mass transfer. If heating effects are significantly minimized, the only significant contribution to band broadening is longitudinal diffusion.[18] The comparison of the flow profile between CE and HPLC is shown in Figure 2. Because of the net flow profile in CE, high theoretical plate numbers up to several million in CE are much higher than that in HPLC.

Another flow source in CE, called electrophoretic mobility (m_{ep}) is the natural flow of analyte ions when high voltage is applied. The net migration velocity of analytes in capillaries will be a function of the sum of m_{eo} and m_{ep}. As the analyte

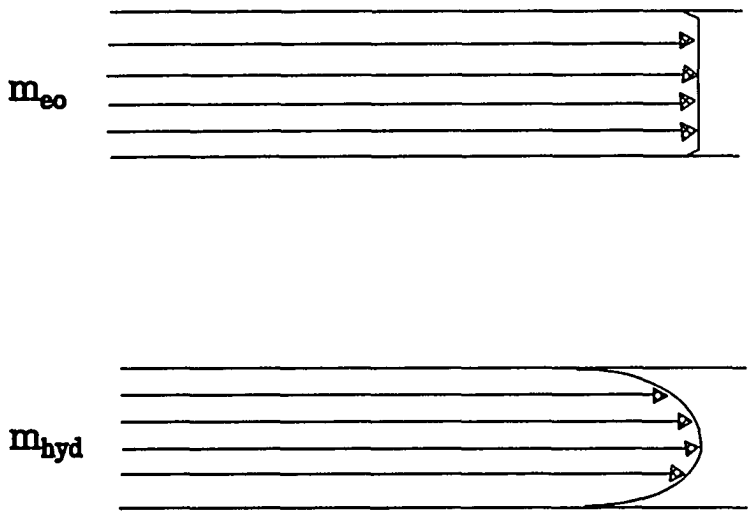


Figure 2. Velocity profiles of liquid flowing in CE and HPLC. m_{eo} is electroosmotic mobility and m_{hyd} is hydrodynamic flow.

moves in a capillary, the migration mobility (m) is given by

$$m = (m_{eo} + m_{ep})\, E = \frac{(m_{eo} + m_{ep})\, V}{L} \qquad (1)$$

where E is the potential field strength, V is the voltage applied across the capillary and L is the capillary length. The migration time (t) for an analyte is expressed by

$$t = \frac{L\, l}{(m_{eo} + m_{ep})\, V} \qquad (2)$$

where l expresses the length of capillary from injection end to the detection point. If only longitudinal diffusion causes band broadening, the variation of the migration zone width (σ^2) can be given by

$$\sigma^2 = \frac{2\, D L\, l}{(m_{eo} + m_{ep})\, V} \qquad (3)$$

where D is the diffusion coefficient of the solute. The maximum separation efficiency (N) is given by

$$N = \frac{L\,l}{\sigma^2} = \frac{(m_{eo} + m_{ep})\,V}{2\,D} \qquad (4)$$

The performance of CE depends on m_{ep}, m_{eo}, D, and V. Hence, pH, ionic strength and viscosity of the buffer which affect these parameters must be well controlled. More detailed discussion will be shown in Chapters 2 to 4.

The following describes the general effects on the separation that result from alternation of the parameters described above.

Applied Voltage

Commercially, most commonly used high voltage power supplies operate well from 0 to 40 kV. Initially, the consideration of the electrode polarity is important. The net flow of solutes should be known to prevent mistake in the decision of the polarity. If there is no any suppression of m_{eo}, and the separation is performed around pH 7, m_{eo} is generally larger than m_{ep}. In this case, the inlet of capillary should be connected to the anode (+), and cathode (-) should be at the outlet. If CE runs at very low pH and the solute has positive charge, the polarity should be reversed.

Although it is much faster when CE runs at high voltage, more specifically, high electric field strength, it is not recommended to use very high voltage to run

CE before the condition is well known. Arcing and producing too much joule heat causing the loss of resolution are common problems if CE is run at high voltage.

Capillary

Most common capillaries are those with 50 and 75 μm i.d.. The use of a capillary with small i.d. has a low volume-to-surface ratio, so it leads to significant heat dissipation, which can prevent a temperature gradient across the capillary. However, the sensitivity will suffer if the detector function is dependent on the path length. Theoretically, resolution is not related to the capillary length. However, electrical field length is the ratio of applied voltage to capillary length. It is recommended to use a 50 to 70-cm capillary. Capillaries coated with polymers are available except the price is much higher (5-10 times higher) than that of bare capillaries.

Buffer

Three parameters, pH, ionic species and ionic strength must be considered seriously to have the best performance of CE.

pH

Generally, m_{eo} increases rapidly when pH changes from pH 4 to 7 and then increases slowly as pH increases from pH 7 to 9. pH also can affect m_{ep} by

changing the dissociation degrees of analytes. Buffer at high and low pH ranges always have high ionic strengths, due to the high mobilities of hydrogen and hydroxyl ions. Usually, it is not a good idea to run CE in those ranges. In the case of difficulty in separation, using buffer pH around the dissociation constants of the solutes can give better resolution. For protein separation, high or very low pH is needed to avoid the adsorption of protein onto the capillary wall. Remember, that pH also can be altered by changing temperature and/or adding organic additives. It is possible, therefore, to generate a temperature induced pH gradient as a way to improve resolution.

Ionic Species

To get better performance in CE, buffer species have to be considered. In order to have a constant buffer system, a species with a high buffer capacity at a certain pH range is important. Since the peak shape of the solute depends on the difference between the mobilities of solute and electrolyte, it is necessary to choose a buffer with mobility close to that of solute. Phosphate, borate, THAM (Tris), citric acid and formic acid are commonly used buffers. Borate forms complexes with diol groups, and is a useful buffer for carbohydrate separations and biological samples. Phosphate can coat onto the capillary to reduce the interaction between analytes and the capillary wall, in turn, to improve performance for CE. Also it has a wide range of buffer capacities which has lead it to become one of

the most popular buffers in CE. Tris-borate buffer works well for DNA separation. The type of the buffer anion or cation is also an important factor since they can affect the ionic strength of the buffer system or may form complexes or interact with the solute.

Ionic Strength

A buffer to solute concentration ratio from 100 to 1000 is normal. Usually, 10 to 20 mM buffer is used in CE, since high ionic strength generates more joule heat. This increases the temperature, and the additional heat causes band broadening. Sometimes, however, it is useful to run CE with high ionic strength conditions. For example, in protein separations, the use of high ionic strength can minilize the interaction between protein and the capillary wall. The running condition can be 0.1 to 0.2 M phosphate at pH 11. Addition of 50 mM NaCl to a running buffer containing 89 mM Tris-borate-EDTA for DNA separation is quite useful. In indirect methods, low ionic strength is necessary to optimize the sensitivity performance.

Detection Modes

Capillaries with small dimension used in CE have the benefits of significant heat dissipation, advanced rapidity, and high resolution. However, high sensitive detectors are necessary due to the small amount of analytes injected and the short

optical path length (OPL). To avoid the loss of resolution in CE from any band broadening resulting from the joints, fittings and connectors that are used in conventional off-column detection system, an on-column detection system is required. Among the used detection systems in CE, absorption, fluorescence, electrochemical and mass spectrometric detectors are most commonly used.

Absorption Detectors

The most popular detection system for CE is UV absorption because of its universal nature.[19-21] Generally, we like to choose a buffer with low or even no absorbance at the wavelength at which solutes have maximum absorbance to get the best sensitivity. For example, absorbance of most proteins increases as the wavelength decreases from 280 to 190 nm. In general, the ratio of absorbance of protein at 218 vs. 280 nm is in the range of 20 to 30. In this case, we prefer to choose a buffer modifier like acetonitrile (urea has a high absorbance and is not suitable) with low background in the deep UV range (e.g. 200 nm).

UV-absorption suffers from low sensitivity due to the short OPL. For instance, we only can detect μM level if the compounds has larger molar aborptivities (e.g. 2×10^{-4} L mol^{-1} cm^{-1}). As a matter of fact, it is usually not this good, since all the light does not pass exactly through the center of the capillary and some kind of electronic noise resulting in increase in background noise. The lack of high sensitivity somewhat limits the use of UV-absorption detector in CE for samples

with low concentration. Researchers have used several different methods to enhance the performance of UV-absorption in CE. Increase in OPL has been developed by using Z-shaped,[22] and bubble-shape flow cells,[23] rectangular capillary,[24] axial beam,[25] and multireflection cell.[26] The sensitivity enhancement factor and comments about these techniques are show in Table 1. Subtraction of the background by using double-beam absorption also has been used to decrease the detection limit by Jorgenson's[27] and Yeung's group.[28]

Table 1. Sensitivity enhancement techniques

Method	ESF	RL	Comments
Z-shape	14	very little	commercial possibility
Bubble-shape	4	problem	commercial possibility
Rectangular	10	very little	commercial possibility
Multireflection	40	no	poor reproducibility
Axial beam	15	no	nonaqueous buffer, data as step function

ESF: enhancement sensitivity factor

RL: resolution loss

Indirect absorption methods are suitable when analytes are not chromophores. To get the best sensitivity by an indirect method, the light source should be stable, displacement process must be efficient and the mobile-phase additive concentration should be kept as low as possible. Limit of detection (LOD) by the indirect method can be given as

$$C_{LOD} = \frac{C_M}{TR \times DR} \qquad (5)$$

where C_M is the concentration of the mobile-phase additive, TR is the transfer ratio, which is usually defined as the number of molecule of the mobile-phase additive displaced by each analyte molecule, and DR is dynamic reserve which is a measure of the baseline stability. Due to the lack of UV-absorption of inorganic ions, indirect UV-absorption is commonly used for the detection of inorganic ions.[29-30]

For a complicated sample which contains interesting chemicals with a wide range of absorption maxima, a fast scan detection system is useful to improve the sensitivity for a certain chemical detected at a certain wavelength. Increase in the selectivity of UV-absorption by using a photo diode array has been introduced in CE.[31]

On-column concentration is another approach for UV-absorption used to

improve detection in CE.[32] Field amplification (or sample stacking)[33,34] by the difference of ionic strength, sample focusing[35,36] by the difference of pH and isotachophoretic sample enrichment[37,38] are common methods used for preconcentration. 100-1000 fold of sensitivity enhancement is obtained reasonably. LOD down to 10^{-8} to 10^{-7} M is possible by combining preconcentration techniques with commercial instrumentation.

Fluorescence Detectors

Unlike the limitation of UV-absorption by the OPL, fluorescence does not depend entirely on the OPL, since its intensity also depends on the intensity of light source, excitation coefficient and quantum efficiency of the fluorophores. The use of a laser is quite suitable since it can provide a high-intensity, coherent and monochromatic source.

The successful use of sensitive fluorescence detection with a laser as an excitation source in HPLC has been well documented. To get a better performance from the fluorescence detector in CE, several factors need to be considered and the problems need to be solved. Stray light from the optical components and the capillary wall causes an increase in the noise level. Also, stray light from the buffer and contamination from solution can result in high background. Good focusing of the light into a capillary and collection of the fluorescence image is necessary. Another drawback is that the maintenance of a

high quality of laser is not easy, especially in the UV range.

Zare is the first one who introduced the laser induced fluorescence (LIF) technique to CE by using a He-Cd laser at 325 nm.[39] Deep-UV LIF with an argon-ion laser at 275 nm has been demonstrated for the detection of proteins without tagging by Lee and Yeung.[40] The detection limit can be lowered down to 0.1 nM. A frequency-doubled argon ion laser is able to generate a stable excitation source with 10-100 mW power at several UV wavelengths (229, 238, 244, 248, 257 and 264 nm). This has been used recently to detect polycyclic aromatic hydrocarbons with detection limits around 6-30 x 10^{-10} M by Zare and co-workers.[41] Minimization of stray light by using sheath flow cuvette has been reported by Dovichi and coworkers.[42] They were able to decrease the detection limit of FITC-arginine to zeptomole levels. Confocal LIF as a CE detector offering sensitivity levels comparable to those with the sheath flow cuvette is used by Guzman and coworkers.[43] Although a sheath flow provides the lowest reported LOD, since the rejection of stray light is essentially perfect, the control of the sheath flow is critical and the sample collection after CE is not easy.

The properties and some general applications of lasers commonly used in CE are shown in Table 2. Although fluorescence is sensitive, this technique is less versatile than UV-absorption because many solutes of interest do not exhibit native fluorescence. Except for certain proteins, porphyrins, drugs, very few interesting chemicals are naturally fluorescent. In this case, the use of a reliable fluorophor

with high fluorescent character to tag the compound of interest is needed. Dansyl chloride,[44] fluorescamine,[45] 4-clair-7-nitrobenzofuran (NBD),[46] o-phthaladehyde (OPA),[47] fluorescein isothiocyanate (FITC),[48] and naphthalenedialdehyde (NDA)[49] are most commonly used.

Table 2. Lasers commonly used for LIF in CE

Wavelength (nm)	Type	Applications	Comments
543.6	He-Ne	DNA	cheap, stable, low power
488 and/or 514	Ar	DNA	stable, ILIF
442	He-Cd	NBD-AA, NBD-CA	short lifetime
363 and/or 334	Ar	DNS-AA, OPA-AA	stable, ILIF
325	He-Cd	HMMC-CAB	short lifetime
305 and/or 275	Ar	protein, PAH, catecholamine	expensive, less stable
248	KrF	protein, DNA	pulsed

ILIF: indirect LIF; AA: amino acid; CA: carbohydrate; CAB: carboxyl compounds; NBD: 4-clair-7-nitrobenzo-2-oxa-1,3-diazole halide; DNS: dansyl halide; OPA: o-phthaldehyde; HMCC: 4-hydroxymethyl-7-methoxycoumarin; PAH; polycyclic aromatic hydrocarbon.

Recently, an on-column reaction based on the zone mixing due to different mobilities of analytes and derivative reagents has been addressed by Reginer's[50] and Yeung's groups.[51] This technique has been applied to study single cell analysis by Yeung and coworkers[52-54] and enzyme microassay by Regnier and coworkers.[55]

An attractive alternative is the use of indirect LIF (ILIF) for compounds which are not naturally fluorescent. Kuhr and Yeung[56] used ILIF to improve the detection limit of amino acids to femtomole levels. A laser must be very stable to work well for ILIF. This detection scheme has been applied to detect amino acids,[57] nucleotides,[58] amines,[59] tryptic digest,[60] and metal ions.[61] More recently, ILIF has been demonstrated successfully to detect the amount of sodium and potassium[62] and lactate, pyruvate in single cells.[63]

Interest in the applications of LIF has been reflected in several different fields such as DNA sequencing, single cell analysis, trace peptide and protein monitoring in clinical analysis, toxic chemicals determinations in environmental analysis and so on. The use of UV diode laser which is much cheaper will be the future. Use of the more sensitive fluorophores for DNA and antigen labelling will keep growing in CE.

Electrochemical Detectors

In contrast to the optical detector, the sensitivity of the electrochemical detector (ECD) is not limited by OPL. However, two principal difficulties must be overcome in order for the significant potential of ECD to be realized in practice: the need to isolate the high voltage applied in CE from the small electrochemical potential used to control analyte oxidation and reduction and the need to supply microsize working electrode. These problems can be solved by replacing a small fracture near the end (or downstream) of the capillary and covering with a porous glass,[64] nafion[65] or cellulose acetate-coated porous polymer[66] and using wires or fibers with 5-50 mm in diameter.[67]

Conductivity detectors for charged species has been used to detect metal ions and amino acids[68] and anions.[69] The problems in conductivity detection are the baseline drift and relatively high detection limits for most species. Detection limits for most ions is 1-10 μM. So far, the lowest detection limit that has been reported is 10^{-6} M for Li^+ (Reference 68).

Amperometric detection, which is more selective and sensitive compared to conductivity detection, has become more popular. It is easy to get detection limits of 10^{-7} to 10^{-8} M. The performance of the amperometric detector can be optimized by miniaturizing both the separation column and the amperometric detector since ECD is not OPL dependent. The applications of amperometric detection in catecholamines,[70,71] carbohydrates,[72,73] and thiols[74] have been published. Due to

its sensitivity, Ewing and coworkers have used 2 and 5-μm diameter carbon fiber electrodes to determine catecholamines in single neuron cells.[75,76]

Resulting from the universality of indirect methods, indirect ECD is also possible to be a good detector in CE. An inditrect amperometric method to detect femtomole levels of amino acids has been reported.[77] In this method, a high and constant background is generated by continuous oxidation of 3,4-dihydroxybenzylamine in the running buffer.

Because of the difficulties in making microsize working electrode and the need for insulation of the high electric field across capillary, ECD is not easy to commercialize. However, the universal capability of ECD still has promised in CE. Indirect methods may extend the application range of ECD in CE. Also the reasonably low cost of ECD is suitable for the applications involving the use of multiple capillaries, if the ease of alignment and insertion of the microelectrodes into the capillaries can be obtained. The use of ECD for neuron cell analysis is predicable.

Mass Spectrometric Detectors

The success in the use of the mass spectrometric detector (MS) for complex mixtures has been well established in GC-MS and HPLC-MS systems. It is not surprising that the combination of MS with CE (with flow rates below 1 μl/min) can work well for the identification of analytes from biological and environmental

samples.

The key for MS as a detector for CE is to have a good interface between them. Based on the electrospray ionization technique, Smith and coworkers[78] developed an interface for CE-MS. So far, the most popular interfaces for CE-MS include sheath flow,[79] liquid junction,[80] and sheathless interface.[81] Problems with these designs are that they all need an additional flow of liquid to incorporate charge-carrying species and invariably degrade detection limit. Hence, in the future the development of a more reliable design without additional flow seems to be necessary. Continuous fast atom bombardment,[82] atmospheric pressure ionization[83] and matrix assisted-laser desorption ionization[84] are other ionization techniques used for CE-MS.

Sensitivity is always considered seriously by analytical chemist, especially when only a small amount of sample is available for the separation in CE. Sensitivity in MS is mass response, unlike that in absorption which is concentration response. Capillaries with small diameters are advantageous if sample volume is limited and greater sensitivity is desired. Another approach to reach high sensitivity is by increasing the overall ion sampling and transmission efficiency. The use of orthogonal time-of-flight (TOF)[85] or ion-trapping[86] MS seems to be the answer.

CE-MS has been applied successfully to quite a wide range of important biological compounds such as peptides and proteins,[84] tryptic digests,[87] metabolites

of flurazepam,[88] sulfonates and amino acids[89] and amines and ammonium salts.[81] The advantages of MS as a detector in CE include its universality and selectivity. That means we can get more information from mass spectra for analytes with masses ranging from several tens of Da (like small organic molecules) to over 100 kDa (like proteins). The sensitivity must be improved to at least attomole or subattomole levels for the detection of biological samples such as the peptide in a single cell. In the future, the use of CE-MS-MS for the structure elucidation of interesting chemicals in complicated samples should become popular.

Separation Methodology

When considering the performance of a certain kind of separation technique, resolution, speed and cost are most common concerns. These requirements have resulted in the emergence of several operation modes which can be performed using CE apparatus. Isoelectric focusing, isotachophoresis, gel and free CE and MEKC are the most commonly used modes. The choice of the separation mode depends on the properties of analytes, the property of the sample and the amount or concentration of interesting analytes in the sample. For instance, MEKC for protein separation is not the best mode if denaturation of protein is not allowable. After the choice of the separation mode, type of buffer, ionic strength and pH of the

buffer, voltage applied, and column diameter must be considered seriously in order to get the best efficiency and resolution.

Capillary isoelectric focusing is a separation technique based on the difference of isoelectric points (pI) of the analytes. It can be performed in gel matrices and free solution. Typical separation procedures include: fill the capillary with sample and ampholyte mixture, immerse the capillary into two reservoirs containing anolyte (like 10-mM H_3PO_4) and catholyte (like 20-mM NaOH) and run at high voltage. The advantages of this technique are concentration effect and ability to separate analytes with small differences in pIs. For instance, if the sample is a mixture of proteins and the concentrations are low, this method is a good separation choice. This technique has been applied to α-chymotrypsin, phosphorylase b, insulin and ovalbumin,[90] ovalbumin, bovine albumin, β-lactoglobulin B and carbonic anhydrase,[91] hemoglobin,[92] RNase mutant,[93] transferrin,[94] and plasminogen.[95]

Isotachophoresis CE is a technique with the separation based on the different mobilities of solutes. Careful choice of the leading ion and its concentration and pH, counter ion, additive and the terminating ion and its concentration is important. The separation of analytes with low concentrations is well done by using this technique because of its stacking effect.[96-97]

MEKC is suitable for the separation of neutral solutes. Adding detergent, such as SDS, with a concentration above its critical micelle concentration (CMC) into the running buffer is an essential step to perform MEKC. Since SDS has a negative

charge, it can migrate slowly in the opposite direction to m_{eo} in turn, to drag the solute migration. Different partitioning of the solutes between the aqueous and micellar phase is the basic mechanism for the separation of solutes in MEKC. Therefore, the migration time, t_r, of solute in MEKC can be given by

$$t_r = \frac{t_0(1 + k)}{1 + \frac{t_0 k}{t_m}} \qquad (6)$$

where t_0 and t_m is the migration time of the solute which is totally insoluble or soluble in micelles, respectively, and k is the capacity factor. The selectivity for solutes in MEKC can be improved by changing k and the factors affecting m_{eo} and m_{ep}. The capacity factor depends on the ability of a solute to partition within the micelles. A solute with a high value of k means it is more hydrophobic. The change of k can be done by changing the type of detergent and its concentration, adding modifiers such as cyclodextrin (CD), or using two or more different detergents. Also, the separation window is a function of the difference between t_0 and t_r, and this ratio affects the migration time of solutes. Thus, well controlling m_{eo} and m_{ep} by adding organic modifiers and changing pH of the running buffer is also important. Several papers have been published involving the enhancement of the separation efficiency in MEKC.[101-103] MEKC has been well demonstrated as a

powerful technique for neutral solute separation and widely applied to the separation of different kinds of solutes.[104, 105] One problem relating to the application of MEKC is that it is limited to the separation of small molecules which can penetrate within the micelles. For example, amino acids can be well separated by this technique but proteins can't. The success of the separation of small molecules show that MEKC can be applied well to the separation of drugs. Also, the addition of a chiral reagent into MEKC system is quite useful to separate chiral compounds which are important to determine the activity of drug.

Capillary gel electrophoresis (CGE) is the other field which has been widely adapted in the separation of protein and DNA. The separation mechanism is based on the sieving effect, i.e., based on the size of solute and the pore of the sieving medium, not on the mass-to-charge ratio. The sieving medium within the capillaries can be high-viscosity gel or low-viscosity polymer network solution. In CGE, cross-linked or non-cross linked sieving matrices have been employed. The difficulty to prepare cross-linked gel and its irreproducibility, nonreplacement and short life-time somewhat limit its wide application. Most common gel media for CGE include polyacrylamide,[106] agarose,[107] and cellulose derivatives.[108] The use of CGE to separate proteins[109-111] and DNA[112-114] provides high resolution and speed compared to conventional slab gel electrophoresis.

Affinity CE (ACE) is a new trend for the study of the binding constants of protein-ligand, separation of protein mixtures and identification of protein and its

amount. An appropriate affinity ligand can be immobilized into the gel, covalently bonded to the capillary or in the running buffer. Problems of ACE include difficult preparation, long migration times without SDS addition (if m_{eo} is equal to 0) and lack of the fundamental principle. ACE has been applied to protein studies.[115-118] More recently, Karger and Shimura addressed a technique, affinity probe CE (APCE) to detect 5×10^{-12} M standard recombinant human growth hormone (rhGH).[119] In this technique, they simply labeled tetramethylrhylrhodamine into the monothiol Fab' in order to detect rhGH via LIF. They also emphasized that the future in this field will be on-line quantitation, microscale analysis and automatic instrumentation.

Practical Applications

The general applications of CE include many fields such as chiral separation, small organic and inorganic ion analysis, carbohydrate analysis, peptide and protein separation, DNA sequencing, clinical analysis, and single cell analysis. The applications of CE in peptide, protein and DNA separations, single cell analysis will be discussed in Chapters 5 to 9. This section focuses on chiral separation, ion analysis, carbohydrate separation, and clinical analysis in CE.

Chiral Separation

Chiral separation is especially important in the pharmaceutical, medical and biological fields. The addition of chelating chiral reagents or CD into the running buffer in CE or MEKC or immobilization of CD into the gel in CGE is a general way to do chiral separations. The addition of Cu-aspartame into the running buffer has been used successfully to separate dansylated DL-amino acids.[120] The use of sodium N-dodecanoyl-L-valinate, bile salts, digitonin and saponin has been published.[121] The most popular way to do chiral separation is the use of CD. β-CD and γ-CD which have suitable cavities to fit the size of the chiral analytes are useful for the separation of DL-amino acids in MEKC.[122,123] The addition of organic solvent, chiral compounds or derivative CDs can generally enhance the enantioselectivity in CD-MEKC. α_1-Acid glycoprotein or albumin packed into capillaries to form a chiral stationary phase for the separation of chiral compounds has been reported.[124, 125]

Ion Analysis

Due to the high separation efficiency approaching 1 million theoretical plates and high speed, CE has been over ion chromatography (IC) in the separation of small ions. So far over 100 different kind of ions have been successfully studied by CE. The general problems of ion analysis CE (ICE) are that most ions are not chromophores, or low conductivities and the large difference of mobilities of ions in

real samples, which will worsen resolution and cause assymetric peak shapes for analytes which have a large difference of the mobilities from buffer species. Therefore, indirect UV absorption, indirect fluorescence and suppressed conductivity are more useful detectors in ion analyses. Chromate,[126] p-hydroxybenzoic acid[127] and quinine[128] are used most commonly for ion analyses by indirect UV-absorption. A suppressed conductometric method has been used by Dasgupta and Bao to detect anions.[129] For trace levels of ions in a real sample, indirect LIF[130] is a good candidate. Generally, real samples always contain a variety of ions, so improvement of the selectivity is very important. The selectivity of ICE can be improved via formation of complexes with chelating agents,[131] size selection by crownether[132] or modification of m_{eo} by adding organic solvents or polymers or changing the pH of the running buffer.[133]

Carbohydrate Analysis

The determination of carbohydrates is important in order to understand the cell function and diabetes. Carbohydrate is not a chromophore and not a charged species, unless at very high pH, which make the determination of carbohydrates via CE difficult. To overcome the limitations on the separation, formation of anionic complexes with borate, then running CE at high pH has been used.[134] Derivitization of carbohydrate with a suitable chromophore or fluorophore not only can form charge species to increase selectivity, but also form chromophores or

fluorophores to be detected by UV-absorption or LIF. 8-Aminonaphthalene-1,3,6-trisulfonic acid,[135] 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde or 3-benzoyl-2-naphthaldehyde[136] or quinoxaline[137] have been employed for this study. Pulsed amperometric detection[65] and indirect LIF[57] have been applied to detect carbohydrates with μM levels. To meet the requirement to get more information in carbohydrate determinations for biological samples, the need to have more sensitive detectors and suitable CE operation modes with better resolution still encourages researchers. The use of MEKC or CGE with MS as a detector seems to be a good candidate.

Clinical Analysis

In clinical analyses, the determination of interesting chemicals and their amount is important, since certain diseases always relate to the presence or absence of some kind of substances or the increase/decrease in their amount. For some diseases, determination of a certain chemical and its total amount in a biological sample is satisfactory. In some more specific diseases such as those which are cell-related, the chemical and its amount in a single cell is necessary to be well solved. The need to have a technique with high resolution and high speed for clinical analysis is also very important, since so many samples needed to be handled and the samples are always very complicated. CE has been done for the separation of different kinds of biosubstances such as proteins, amino acids,

peptide, small organic and inorganic ions, DNA and carbohydrates.[138-140] The low amounts of interesting chemicals and complicated matrices result in the challenge for CE to be a good technique for clinical analysis. Fortunately, several different separation modes of CE with sensitive detectors have been well developed.[141,142] Careful choice of the right operation modes with suitable detectors can answer the questions for a certain sample. In general, the most common samples to be analyzed include urine, serum, tissue, cells and biological fluids.

The most important and difficult chemical needed to be analyzed is protein. Many different proteins with very low concentration in biological samples is a real challenge for chemist. The use of sensitivity enhancement techniques with high sensitivity detection technique such as LIF and the combination of different separation techniques such as HPLC-CE are needed.[143] However, this technique can only give us the information of the amount and indirect information of solutes by comparing the retention time with a standard chemical. The exact structure information can only be given by MS, which is not as sensitive as LIF. To solve this problem, use of several capillaries for separation, then collection of pure sample for MS detection has been reported.[144] The adsorption of biological substances onto the capillary wall, which affects the migration time of analytes and degrades the performance of CE, is a general problem during the separation of biological samples. To overcome this problem, coating capillaries with less hydrophilic chemicals or polymers is necessary.[145,146]

Microdialysis-CE is a powerful technique for in-vivo study. The study of the blood-brain barrier permeability of phenobarbital has done by Tellez and coworkers.[147] Continuous monitoring of the pharmacokinetics of L-dopa in the rat by electrochemical detection technique has been performed by O'shea et al.[148]

Although CE is still not popular in clinical analysis now, the use of CE in this field should gain acceptance very soon.

Future Prospects

The acceptance of CE as a modern separation technique has been emphasized by chromatographers and the truth reflected by several excellent reviews[149-154] and books[155-160] published over the past five years. Definitely, the prediction of CE in the future is quite positive.

Although CE has been developed successfully, there are still several fields which need to be improved to make CE as one of the most powerful techniques. The use of multidimensional capillaries to get more information from complicated samples, miniaturization of capillaries to improve the separation speed, coated capillaries to enhance CE performance for biological samples and suitable gel components for protein and DNA separation is a trend. In order to reach the requirement of the Human Genome Project, improvements in the sensitivity, speed and throughput for the separation of DNA are still needed. The techniques such

as more sensitive detection methods for interesting chemicals and better separation modes involving single cell analysis will keep growing. It is also important to design more reliable CE-MS systems for structure elucidation, especially for peptides, proteins and even DNA. In the future, CE should be well applied in the fields of clinical analysis, drug monitoring, environmental analysis, forensics, molecular biology and single cell analyses.

Dissertation Organization

This dissertation contains 9 chapters. General review of CE is given in Chapter 1. Chapter 2 to 5 are papers which have been published in different journals. Chapters 2 through 4 discuss the basic theories and the applications of gradient techniques in CE. On-column digestion is discussed in Chapter 5. DNA separations by using CGE are shown in Chapters 6 through 8. Chapter 9 discusses single cell analysis. General summary, literature cited and acknowledgements are organized in the end of this dissertation.

CHAPTER 2

OPTIMIZATION OF SELECTIVITY IN CAPILLARY ZONE ELECTROPHORESIS VIA DYNAMIC pH GRADIENT AND DYNAMIC FLOW GRADIENT

A paper published in the Journal of Chromatography[1]

Huan-Tsung Chang and Edward S. Yeung[2]

Abstract

Two different techniques, dynamic pH gradient and electroosmotic flow gradient, were introduced to control selectivity in capillary zone electrophoresis. These two types of gradients showed dramatic effects on the resolution of organic acids. Dynamic pH gradient from pH 3.0 to 5.2 is readily generated by a high-performance liquid chromatography gradient pump. Electroosmotic flow gradient is produced by changing the reservoirs containing different concentrations of cetylammonium bromide for injection and running. The two gradient techniques are applied to the separation of model anions which are not resolved at constant pH or at constant flow conditions.

---

[1] Reprinted with permission from J. of Chromatography 1992, 608, 65-72.

[2] Author for correspondence.

Introduction

Capillary zone electrophoresis (CZE) has played an important role in separation science because of its high efficiency.[1-3] In CZE, the separation performance depends on the electrophoretic mobilities of the analytes and the electroosmotic flow when high voltage is applied. Conventionally, the same electrolyte is used in the capillary tube, the inlet reservoir and the outlet reservoir. In such cases, it is often difficult or impossible to separate a broad range of analytes which have very similar electrophoretic mobilities. Many approaches have been reported to improve the resolution in CZE. Control of the factors governing the electrophoretic mobilities of analytes and the electroosmotic flow are typically used to improve the separation resolution in CZE.

In principle and in practice, changing the pH of the buffer electrolyte seems to be the easiest way to control the electrophoretic mobilities of analytes. For the separation of ionic species such as weak acids or bases, the selection of pH near their dissociation constants (pK_a or pK_b) can generally provide good results. For complex mixtures, however, complete separation may not be possible at any one pH since the components may span a large range of pK values. In such cases, the use of a pH gradient can be advantageous. So far, several approaches for generating pH gradients in CZE have been reported. Boček et al.[4,5] used a three-pole, two-buffer system to force the migration of varying ratios of two ions into the

capillary during separation. Sustacek et al. dynamically modified the pH of the electrolyte at the inlet of the capillary by a steady addition of a modifying electrolyte.[6] A step change in pH can also be used by switching the buffer electrolyte at the column inlet, with $[H^+]$ controlled either directly[7] or by the use of different co-ions.[8] It is also possible to simply introduce a transient pulse of electrolyte at a different pH to enhance separation.[9]

There are some subtle considerations relevant to the implementation of a pH gradient. It is necessary for the changing pH zone to actually interact with the analytes. H^+ (and OH^-) is a special case because of its very high electrophoretic mobility, allowing it to overtake any positively charged analyte within a reasonable distance into the column, even with a high electroosmotic flow rate. Still, to guarantee that the final pH of the buffer at the inlet end actually contributes to the selectivity, the pH change must be completed well before the elution of the components that are to be manipulated. Direct control of $[H^+]$ is practical only at low pH, where the concentration is high enough to overcome other ionic equilibria. Even for unbuffered electrolytes,[4,5] dissolved CO_2, surface silanol groups, and most importantly the analyte, will have be "titrated" to alter the mobilities significantly even at neutral pH conditions. To overcome this problem, co-ions have been utilized[8], such as the carbonate-oxalate system. Since the co-ions are at much higher concentrations, the pH is easily altered. Naturally, the co-ions must migrate past the analyte ions to produce any effect. So far, only a step gradient has been

generated, although one should be able to alter the ratio of the co-ions to generate a continuous gradient.

To guarantee that each analyte species actually experiences the pH step or gradient, a pH gradient derived from temperature changes has been reported.[10] It is also possible to introduce the pH change from the exit (detector) end of the capillary.[11] Regardless of the signs of the electrophoretic mobilities and the electroosmotic flow coefficient, the net travel of the analytes is then opposite in direction to that of the pH front. One can therefore influence the migration of the early eluting components as well as the late-eluting components. So far, only a step change in an unbuffered system has been demonstrated.[11] To provide accurate and reproducible changes over a wide range of pH, it is best to use a polybasic acid (or base) rather than a monobasic acid (or base) for the buffer electrolyte. The pH of a solution of an acid can be controlled by changing the concentration of a counter ion (e.g. Na^+) for a fixed analytical concentration $[A]_0$ of the acid, i.e. the fraction ionized. For a monobasic acid the ratio goes from 0 to 1 with the two extremes providing unbuffered conditions. It is also difficult to introduce very low concentrations of any counter ion since trace contaminants can dominate the equilibria. For polybasic (H_nA) acids in between pK values, e.g. $pK_1 < pH < pK_2$, the ratio goes from 1 to n-1 and external control is easy over a wide, well-buffered range. The direction of the pH change is also important. For a change from high to low pH, one can introduce H^+ and decrease the counter ion

concentration and expect the front to catch up with the analytes. For a change from low to high pH, one must increase the counter ion concentration and decrease H^+ in the column. The mobility of the counter ion rather than the mobility of H^+ becomes important in establishing the gradient. This further restricts the magnitudes and directions of the electrophoretic mobilities and the electroosmotic flow coefficient if the gradient is generated at the inlet end of the capillary. No such restrictions exist if the pH change is introduced from the outlet end, provided there is a net flow of the counter ion into the capillary.

The factors controlling electroosmotic flow, which is governed by the zeta potential on the inner wall of the capillary, include the nature, concentration and pH of the background electrolyte, origin of the capillary and the applied voltage. Adding surfactant to the background electrolyte is one simple way to enhance selectivity in CZE since the surfactant can effectively suppress or change the direction of the electroosmotic flow.[12,13] Organic solvents such as methanol, which can be used to change electroosmotic flow, are also useful to enhance the separation resolution.[14] External electric field, which can change the direction and the rate of electroosmotic flow by external voltage, is another approach to improve the separation ability in CZE.[15] Coating of inner walls of the capillary with silated organic compounds[16] and adding salts[17] like NaCl are other options to affect the separation resolution. Field-amplified CZE,[18,19] where two different concentrations of buffer electrolytes are used for injection and running, is another powerful method

to control the electroosmotic flow.

In this paper, two methods, altering the electrophoretic mobilities of anions by using a dynamic pH gradient and changing the electroosmotic flow rate by running CZE in two different concentrations of CTAB, were described to demonstrate their utility in the separation of organic weak acids at acidic conditions.

Experimental Section

A commercial electrophoresis instrument (Isco model 3850, Lincoln, NE, USA) was used for all electrophoretic experiments. High voltages applied in the experiments of dynamic pH gradient and dynamic flow gradient were 10 kV and 12 kV, respectively. The wavelength was set at 218 nm to detect anions and minimize the background absorption. The fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was 60 cm x 75 μm ID. At 40 cm from the injection end the polyimide coating was burned off to form the detection window. An integrator (SP model 4600, Spectra-Physics Inc, San Jose, CA, USA) was used to record all of the data. A HPLC gradient pump (two 2150 HPLC pumps and a 2152 HPLC controller, LKB, Gaithersburg, MD, USA) was used to introduce the dynamic pH gradient. The setup is shown in Figure 1. Coupling is accomplished through a teflon tube (1.6 mm ID, 3.0 mm OD) with the buffer at the exit end of the capillary constantly modified by the HPLC pump.

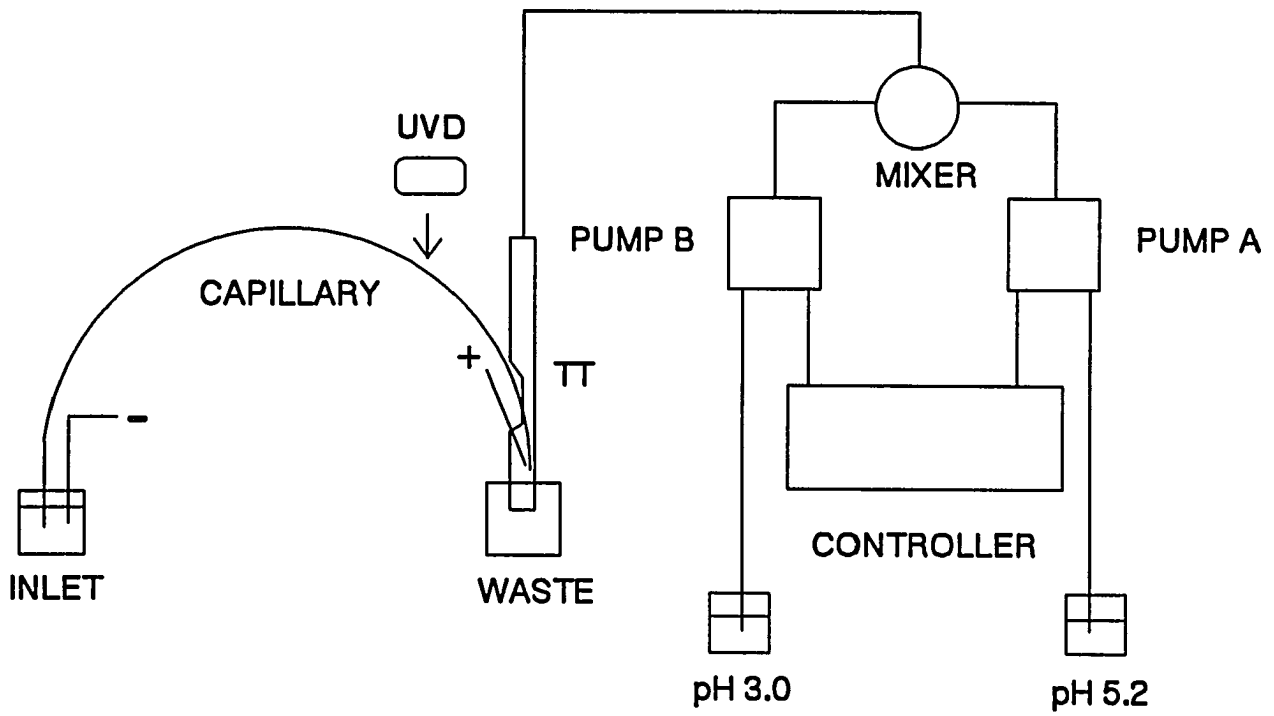


Fig. 1. Schematic of the electrophoresis equipment for generating a dynamic pH gradient. UVD: ultraviolet detector, and TT: teflon tube, in which a small groove was cut in the middle to insert the electrode and the capillary.

All chemicals were of reagent grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), except that phosphoric acid, sodium phosphate, and sodium hydroxide were from Fisher (Fair Lawn, NJ, USA). Buffer solutions of phosphate were prepared from NaH_2PO_4 by adding NaOH or H_3PO_4 to adjust its pH to 5.2, or 4.1 and 3.0, respectively. Acetate buffer solution (pH 6.5) was made by adding NaOH to acetic acid. Cetylammonium bromide (CTAB) was added to buffer solutions to suppress electroosmotic flow. α-Naphthol (a neutral molecule at these pH values) was used to measure the electroosmotic flow. The sample solutions were injected hydrostatically. The buffer vial at the cathodic end of the capillary was raised to 20 cm high for 4 s to introduce samples into the capillary tube.

The capillary was equilibrated for 20 min between each run. Before injecting sample solutions, the capillary was flushed with 0.05 mL of 0.01 M NaOH solution, then 0.5 mL of buffer solution to improve reproducibility. A step change in CTAB was created by injecting sample solutions into the capillary containing a low concentration of CTAB, then running with buffer electrolyte containing a high concentration of CTAB at the exit (anodic) end. The capillary was treated as above before each run.

Results and Discussion

In CZE, the resolution between a pair of adjacent analytes can be calculated from the following equation[20]:

$$R = 0.177\ (m_{eff1} - m_{eff2})\ [V / D\ (m_{av} + m_{eo})]^{1/2} \qquad (1)$$

where R is the resolution, V is the applied voltage, D is the diffusion coefficient, m_{av} is the average mobility of the two analytes and m_{eff1}, m_{eff2} and m_{eo} are the effective mobilities of the two analytes and the electroosmotic flow coefficient, respectively. As equation 1 shows, the resolution can be enhanced by increasing the difference between the effective electrophoretic mobilities of the analytes and (or) decreasing the sum of electroosmotic flow and the average electrophoretic mobilities of two analytes.

Control of effective electrophoretic mobilities with dynamic pH gradient

Effective electrophoretic mobilities of ions are proportional to the fraction of free ion according to the Tiselius equation[21]:

$$m_{eff} = \sum \alpha_i\ m_i \qquad (2)$$

where α_i is the degree of dissociation and m_i is the absolute mobility of the ith ionic form of a molecule. The degree of dissociation again can be calculated from the following equations:

For divalent anions:

$$\alpha_1 = K_{a1}\,[H^+]/([H^+]^2 + K_{a1}\,[H^+] + K_{a1}\,K_{a2}) \qquad (3)$$

$$\alpha_2 = K_{a1}\,K_{a2}/([H^+]^2 + K_{a1}\,[H^+] + K_{a1}\,K_{a2}) \qquad (4)$$

For monovalent anions:

$$\alpha = K_a/([H^+] + K_a \qquad (5)$$

where K_{ai} is the ith dissociation constant of the acid. The amount of negative charge on the inner wall of the capillary and the analyte ions should be a function of pH of the buffer electrolyte as indicated by equations 3-5. For a bare silica column at these pH, electroosmotic flow is generally towards the cathode (injection end). For separation of these anions by migration towards the anode, we need to reverse the electroosmotic flow by adding a cationic surfactant, CTAB. At 0.35 mM CTAB, the amount of the positive charge on the inner wall of the capillary caused by the adsorption of CTAB increases as pH decreases since the degree of

dissociation of the SiOH groups decreases as pH decreases. That is, electroosmotic flow toward the anodic direction increases when pH decreases. Our results show that m_{eo} = -3.15 x 10^{-4}, -1.8 x 10^{-4} and -1.31 x 10^{-4} cm^2 v^{-1} s^{-1} at pH 3.0, 4.1 and 5.2, respectively. The experiments here thus take advantage of the combined results of the changes in electrophoretic mobilities and in electroosmotic flow. However, since m_{eo} is negative (same direction as m_{eff}), the numerator in Eq. 1 dominates in determining the resolution. We note that m_{eo} is smaller than m_{eff} for Na^+ (= 5.0 x 10^{-4} cm^2 v^{-1} s^{-1}) so that the pH change can be introduced from the outlet end.

Figure 2 shows the separation of the model anions at pH 3.0, 4.1 and 5.2, respectively. The calculated electrophoretic mobilities are listed in Table I. The separation of o-nitrobenzoate from fumarate, and o-toluate from m-nitrobenzoate are impossible at pH 3.0. At pH 4.1, there is overlap between the peaks of citraconate and maleate. Serious tailing on the late eluting peaks and the separation of o-toluate from o-nitrobenzoate are problems at pH 5.2. It is worth noting that the electrophoretic mobilities of m-isomers are lower than those of o-isomers. A likely reason is steric effects in the o-isomers which decrease the interaction between the anions and CTAB. The tailing problem is more serious for later eluting peaks at higher pH. Two factors that contribute to this are the stronger interaction between anions and CTAB due to increased dissociations of anions at higher pH and the larger difference of mobilities between

Fig. 2. Effect of change in pH on the migration rates of different anions. Capillary: 75 μm ID, 60 cm total length, 40 cm to detector; Applied voltage = 10 kV; Wavelength = 218 nm, [CTAB] = 0.35 mM, [Phosphate] = 10 mM, [Anion] = 0.2 mM, 1 = Citraconate, 2 = Maleate, 3 = Fumarate, 4 = o-Nitrobenzoate, 5 = o-Toluate, 6 = Benzoate, 7 = m-Nitrobenzoate and 8 = m-Toluate.

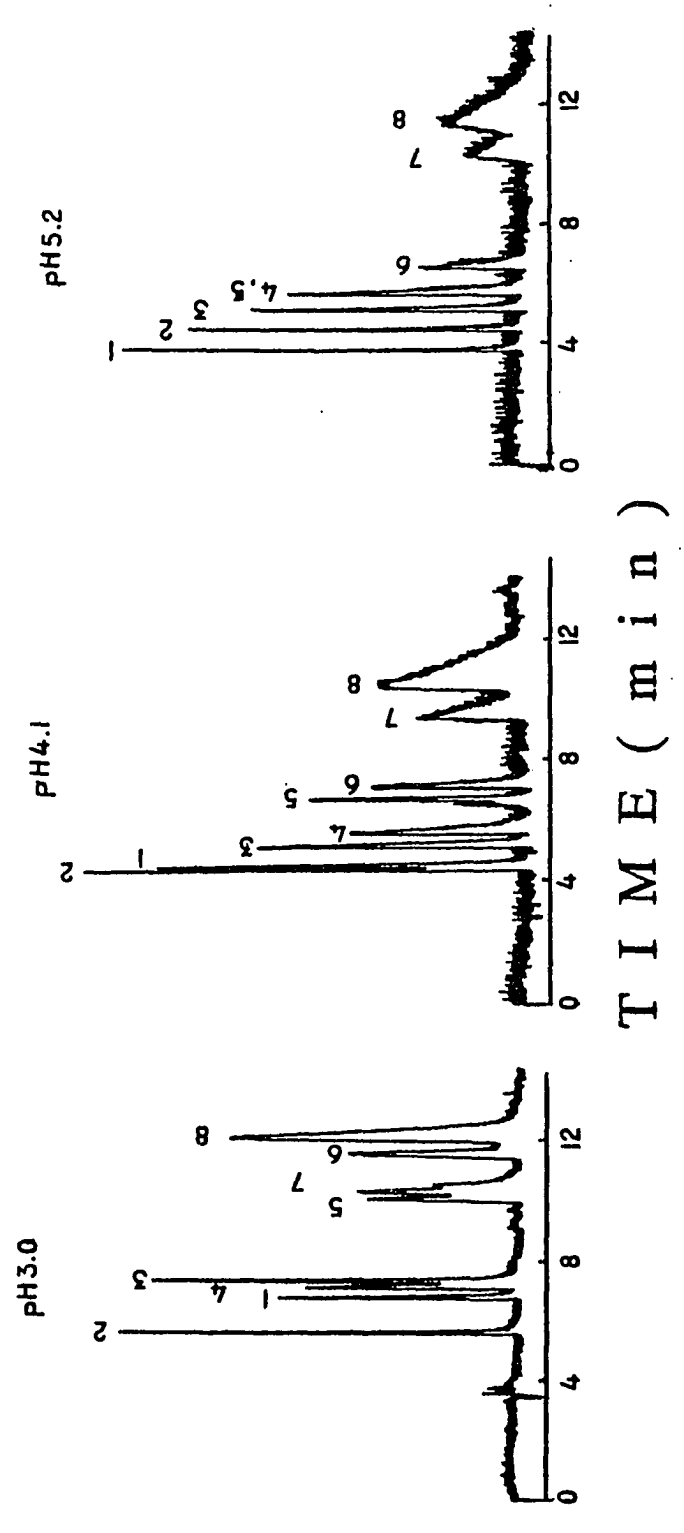

pH3.0
pH4.1
pH5.2
TIME (min)

Table I. Observed electrophoretic mobilities (m_{ep}) of anions

[Anions] = 0.2 mM	$-m_{ep}$ ($x10^{-4}$ cm^2 v^{-1} s^{-1})		
	pH 3.0	pH 4.1	pH 5.2
Citraconate	3.1	7.0	8.2
Maleate	4.1	7.1	7.4
Fumarate	2.7	5.7	6.0
o-Nitrobenzoate	2.8	5.0	5.3
o-Toluate	0.8	4.1	5.3
Benzoate	0.3	3.6	4.4
m-Nitrobenzoate	0.7	2.3	2.4
m-Toluate	0.1	1.9	2.3

Electroosmotic flow coefficient (m_{eo}; x 10^{-4} cm^2 v^{-1} s^{-1}) is negative as electroosmotic flow is toward the anode. m_{eo} = -3.15 at pH 3.0, -1.80 at pH 4.1 and -1.31 at pH 5.2.

the buffer ions and the anions at higher pH.[22,23] Based on literature values[24,25] of dissociation constants and mobilities we have calculated the effective mobilities of this set of anions at these pH. The trends are comparable to those in Table I but the absolute magnitudes are different. In all cases except for fumarate, the calculated values are larger than the experimental values. We note that the literature values are for systems at infinite dilution and for 20 or 25°C. Differences are expected in the presence of the buffer ions and CTAB. Further, since our column temperature is higher than ambient during electrophoresis, the viscosity of water decreases and the effective mobilities should be higher as observed.

As Fig. 2 shows, it is impossible to separate all of the model anions with an isocratic buffer electrolyte. The problem can be overcome by introducing a dynamic pH gradient from pH 3.0 to 5.2 as Figure 3 shows. The resolution was enhanced, the separation took less than 11 mins, and the tailing problem was also reduced by this method. Even though the gradient starts immediately after injection, the components do not meet the moving front until they migrate further down the column. However, every component is guaranteed to meet the moving front in this mode of operation.

Control of the electroosmotic flow by step change in CTAB

To reduce the problem of tailing caused by the difference of mobilities between the buffer ion and the model anions, acetate buffer was used instead of

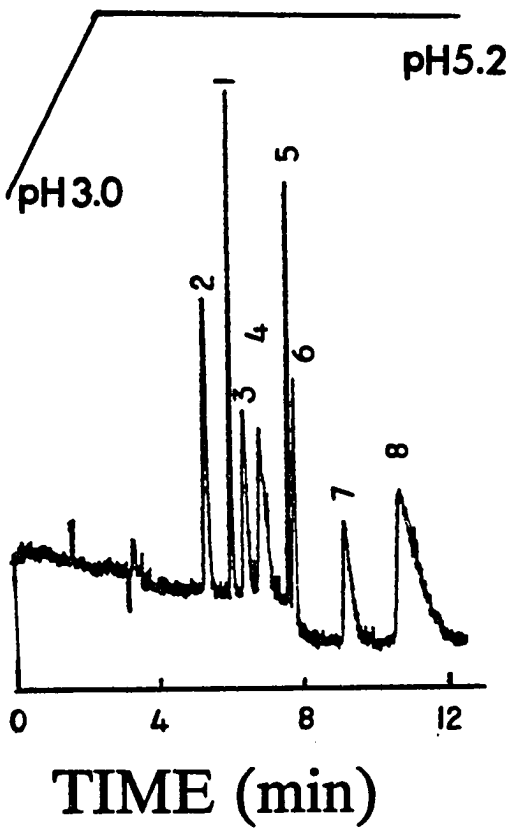


Figure 3. Influence of a pH gradient on the separation of organic anions. Conditions are the same as those in Fig. 2 except a pH gradient is introduced through the outlet end of the capillary as indicated by the top plot in this figure.

phosphate buffer. The use of pH 6.5 was based on the following two reasons. First, to minimize the effect of variations in pH on electrophoretic mobilities the pH of the buffer solution is chosen to be much higher than most of the pK_a. Second, the effect of the surfactant on the electroosmotic flow is larger at higher pH since the dissociation of SiOH on the inner wall of capillary increases as pH increases.

Table II shows that CTAB not only can suppress electroosmotic flow, it can also change its direction at higher concentrations of CTAB, consistent with published reports.[12,13]

Table II. Electroosmotic flow coefficient (m_{eo}) at different concentrations of CTAB

[CTAB] (μM)	m_{eo} (x 10^{-4} cm^2 v^{-1} s^{-1})*
4.0	5.7
6.0	2.9
10.0	0.4
14.0	0.03
40.0	-0.8

*m_{eo} is negative for electroosmotic flow toward the anode.

Table III shows that the electrophoretic mobilities of the model anions are almost constant when the concentration of CTAB changes. Slight variations on electrophoretic mobilities of the anions may be due to the formation of an ion-pair between the anions and CTAB at higher concentration of CTAB. This is evident for the larger anions, naphtholate and coumarate. So, the dominant term in Eq. 1 is the denominator when the CTAB concentration is varied. Figure 4 shows all anions except phthalate and maleate can be separated in less than 18 min at 6 μM CTAB buffer electrolyte. As CTAB increased to 10 μM, α-naphtholate and coumarate cannot be separated and there is little separation between the peaks of phthalate and maleate. The resolution between o-nitrobenzoate and benzoate decreases dramatically as CTAB changed to 40 μM where electroosmotic flow is in the same direction as the electrophoretic mobilities of anions. The increase in m_{ep} + m_{eo} results in the poor resolution of these two anions because the difference between their electrophoretic mobilities is very small. However, the resolution between coumarate and α-naphtholate increases and the elution order changes. This may have resulted from the complexation of CTAB with α-naphtholate and with coumarate. So, Figure 4 shows it is impossible to separate all the model anions under isocratic condition.

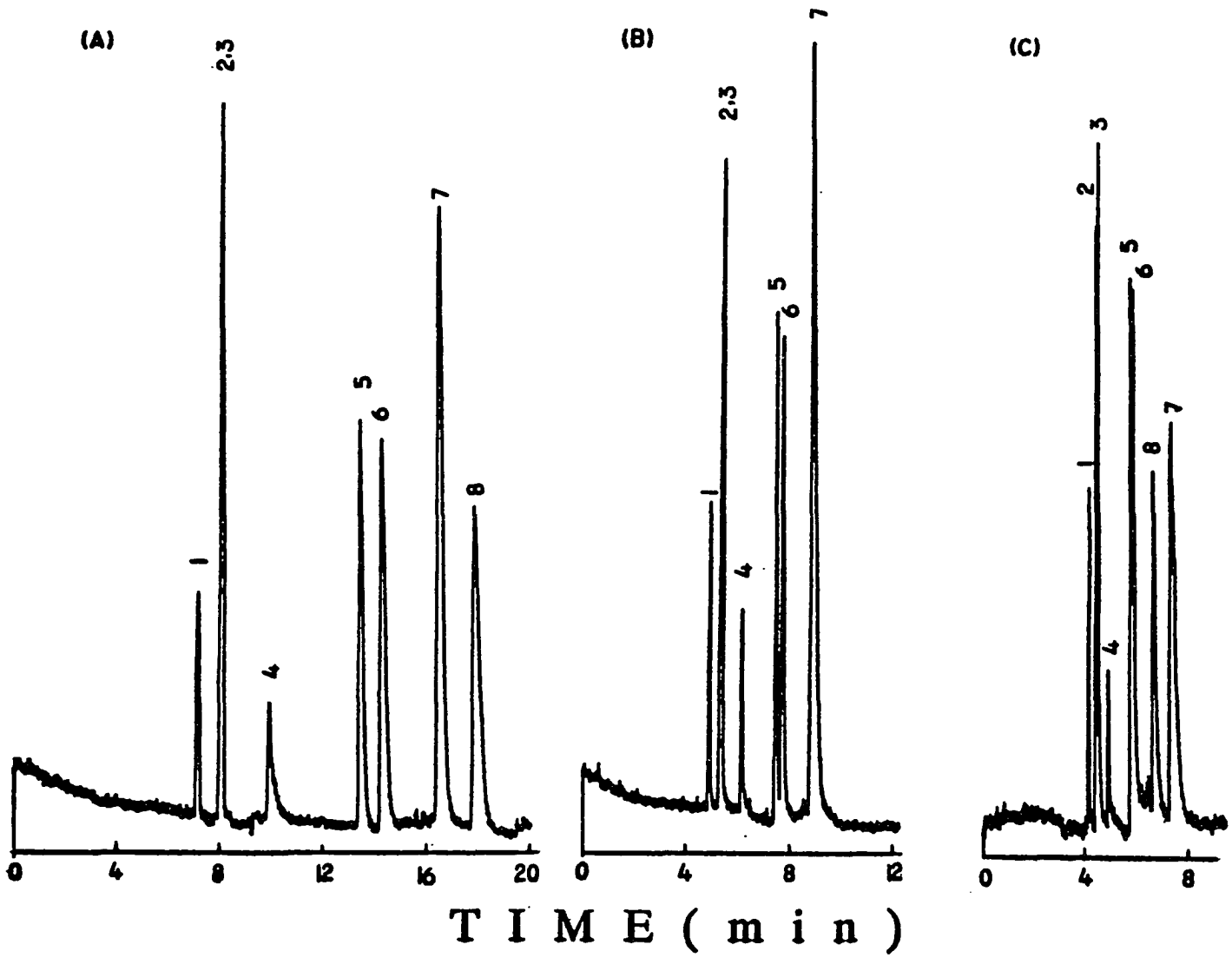
Helmer et al.[18] mentioned that the relationship between the bulk velocity, m_b, and the local electroosmotic velocity, m_l, in the two buffer regions can be expressed by the following equation:

Table III. Electrophoretic mobilities (m_{ep}) of anions* at different concentrations of CTAB

[CTAB] (μM)	6	10	14	40
		$-m_{ep}$ (x 10^{-4} cm^2 v^{-1} s^{-1})		
Citraconate	7.6	7.4	7.4	7.4
Maleate	7.0	6.8	6.7	6.9
Phthalate	7.0	6.8	6.7	6.8
Pyruvate	6.2	6.1	5.8	5.9
o-Nitrobenzoate	5.3	5.1	5.0	4.9
Benzoate	5.1	4.9	4.8	4.8
α-Naphtholate	4.8	4.3	4.2	3.6
Coumarate	4.7	4.3	4.0	4.1

*[Anion] = 0.2 mM, except [pyruvate] = 0.5 mM and [α-naphtholate] = 50 μM.

Fig. 4. Effect of changes in concentration of CTAB on the migration rates of different organic anions. A: [CTAB] = 6 μM; B: [CTAB] = 10 μM ; C: [CTAB] = 40 μM; Capillary: 75 μm ID, 60 cm total length, 40 cm to detector; Applied voltage = 12 kV; Wavelength = 218 nm; [Acetate] = 5 mM ; [Anion] = 0.2 mM except [Pyruvate] = 0.5 mM and [α-Naphtholate] = 50 μM; 1 = Citraconate, 2 = Maleate, 3 = Phthalate, 4 = Pyruvate, 5 = o-Nitrobenzoate; 6 = Benzoate, 7 = α-Naphtholate, 8 = Coumarate.

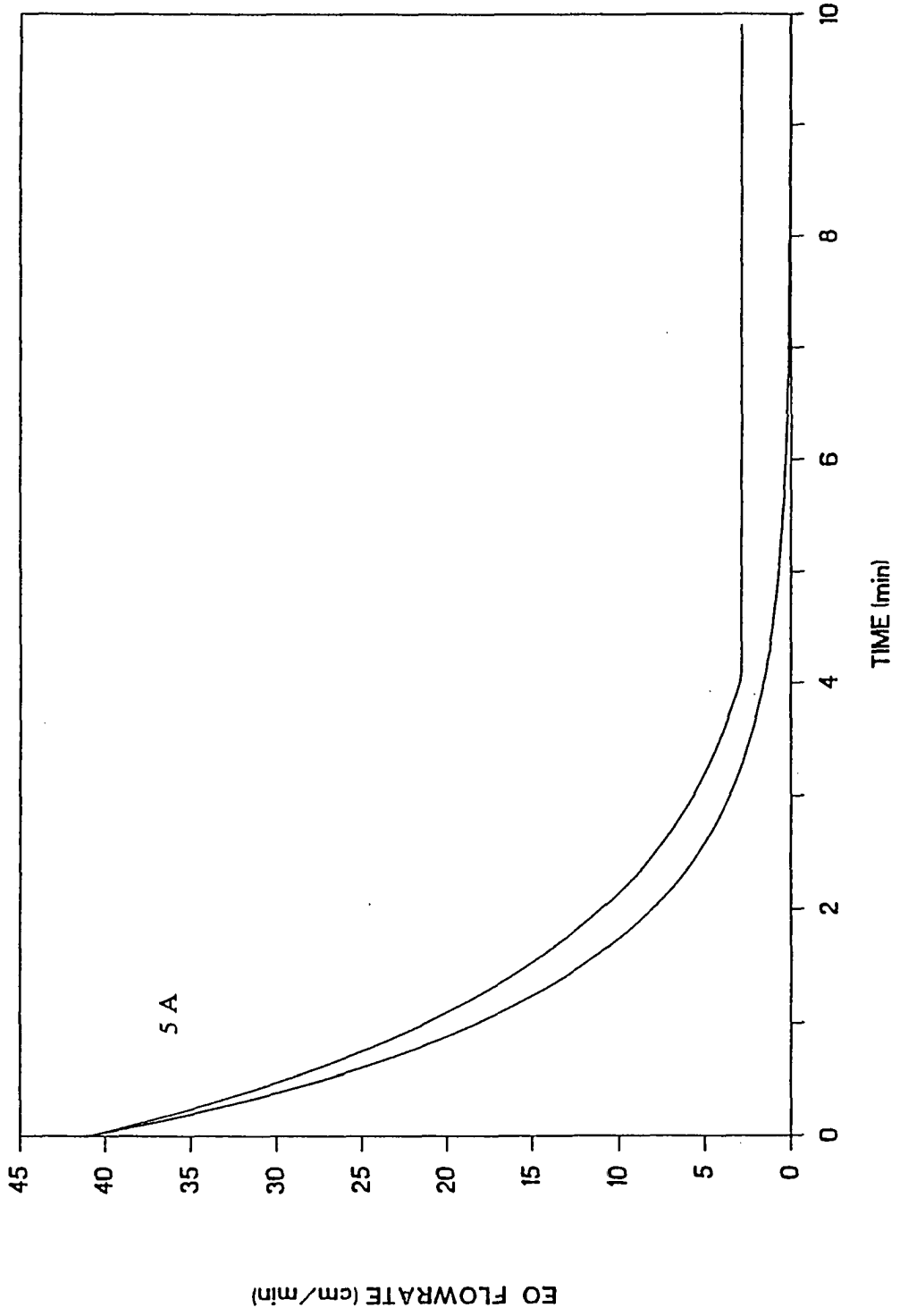

(A)
(B)
(C)
2,3
1
4
5
6
7
8
0
4
8
12
16
20
TIME(min)

$$m_b = x\, m_{l1} + (1 - x)\, m_{l2} \qquad (6)$$

where x is the fraction of length filled with buffer 1 and m_{l1} and m_{l2} are the local electroosmotic flow rates when buffer 1 and 2 are used individually, respectively. This means that if we create a step change of two buffers containing different concentrations of CTAB it is possible to monotonically change the electroosmotic flow. Figure 5 shows a computer simulation of the electroosmotic flow rateresulting from a step change in [CTAB], in agreement with the above discussion. The change is indeed monotonic, but not linear. The rate of change can be controlled by the magnitude of the buffer step. It is interesting to note that the electroosmotic flow can never be reversed in this mode. It is also irrelevant which direction the analytes are moving relative to the flow, since there is only one flow to consider.[18] All analytes experience the same flow gradient regardless of where the front is. Figure 6 shows the effect on the enhancement of the separation of model anions by a step change in the concentration of CTAB. Comparing the results of Figure 6A and Figure 6B, it is obvious that the bulk electroosmotic flow gradient can be controlled by stepping to different concentrations of CTAB. Improvements in resolution and reduction in separation time are simultaneously achieved by the method of step change in [CTAB].

The control of electroosmotic flow rate and electrophoretic mobilities of analytes

Fig. 5. Computer simulation of electroosmotic flow gradient for a step change in [CTAB]. The data in Table II were used. Initial [CTAB] is 4 μM; final [CTAB] is 10 μM for top trace and 40μM for bottom trace. A: flow rate as a function of time; B: location of moving boundary from the exit end of a 60-cm capillary as a function of time. Applied voltage = 12 kV; column length = 60 cm (40 cm to detector).


5 A
EO FLOWRATE (cm/min)
TIME (min)
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10

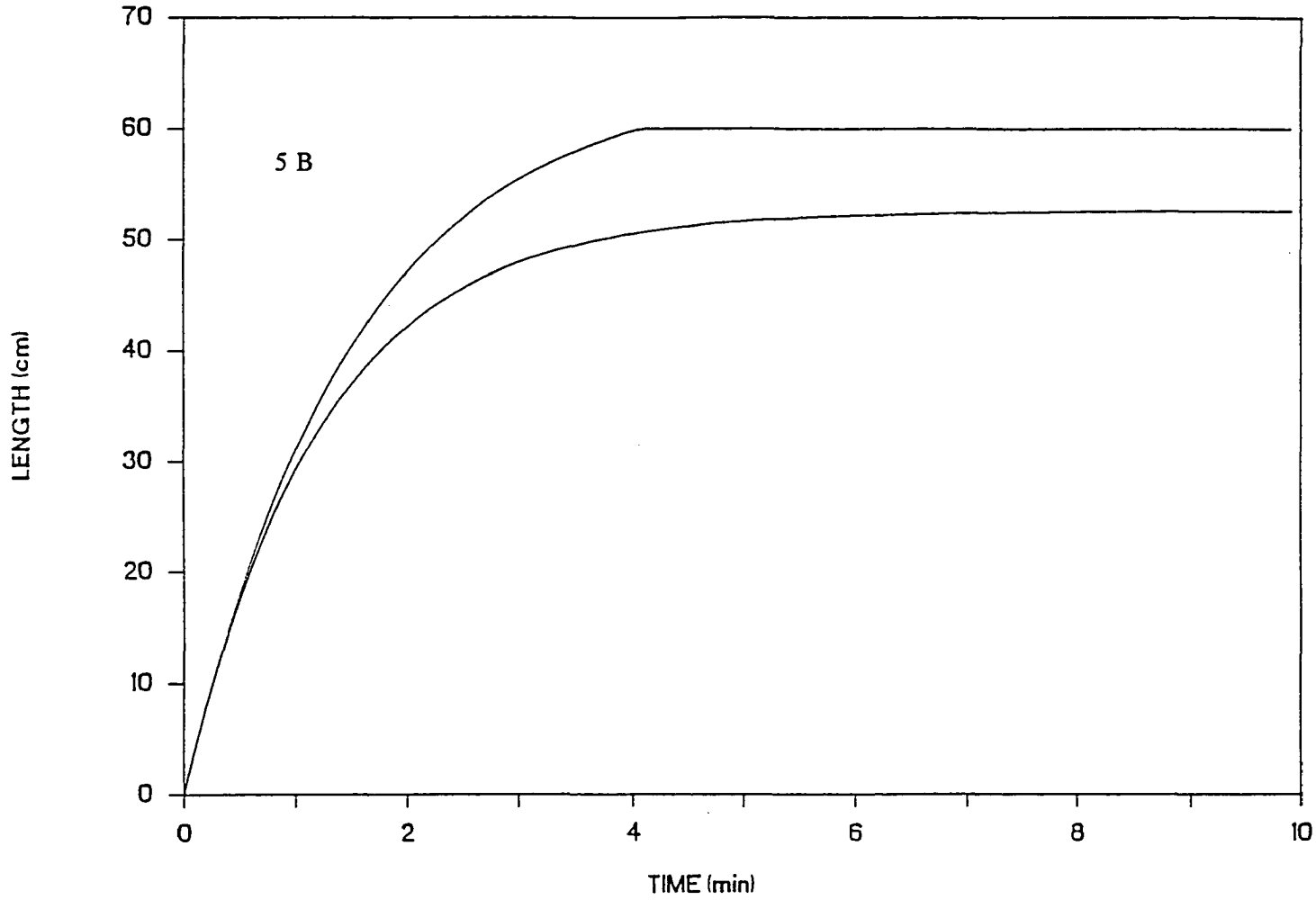


Figure 5. (continued)

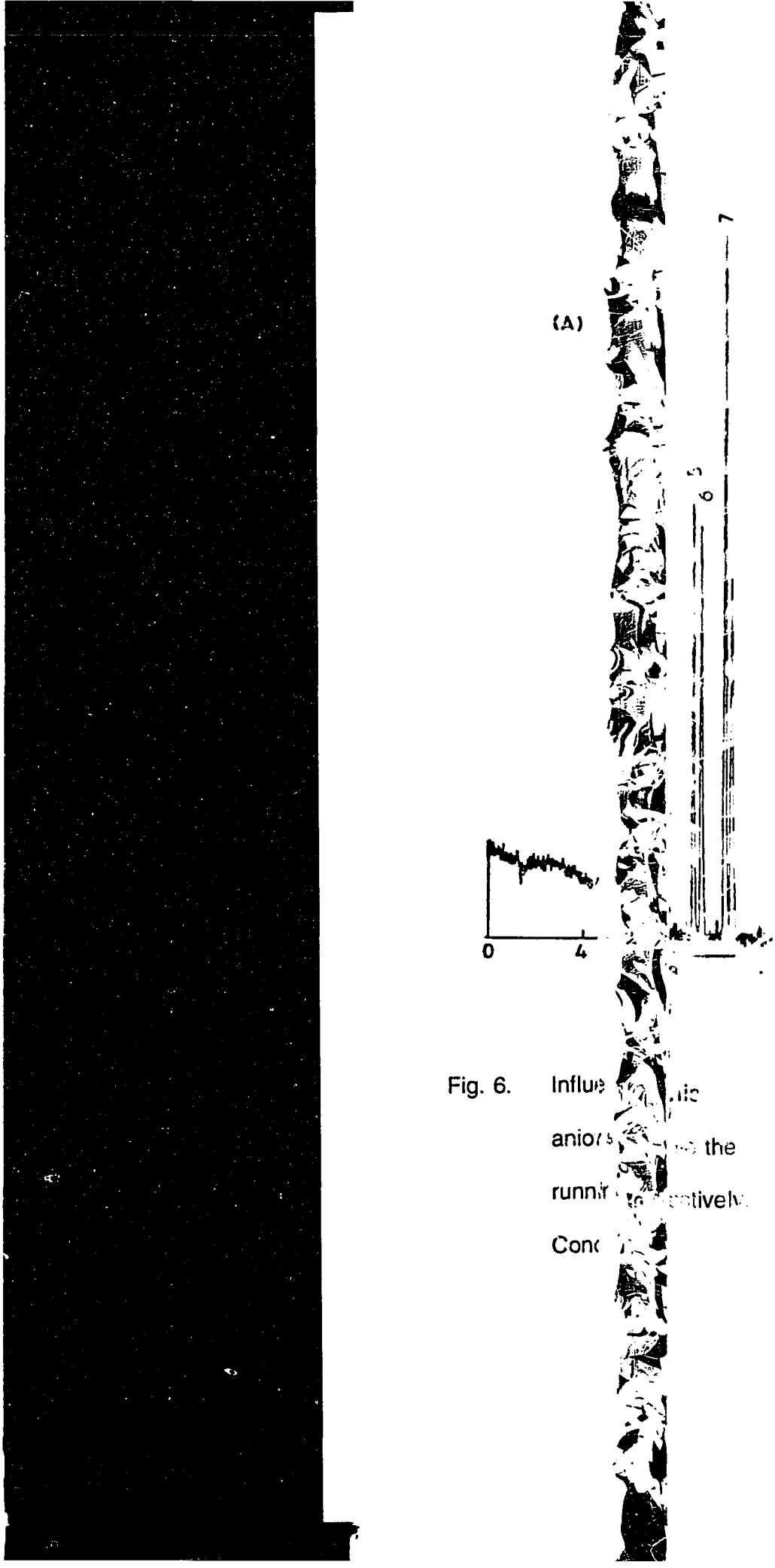


Fig. 6. Influe[illegible] anio[illegible] the runnir[illegible]tively. Conc[illegible]

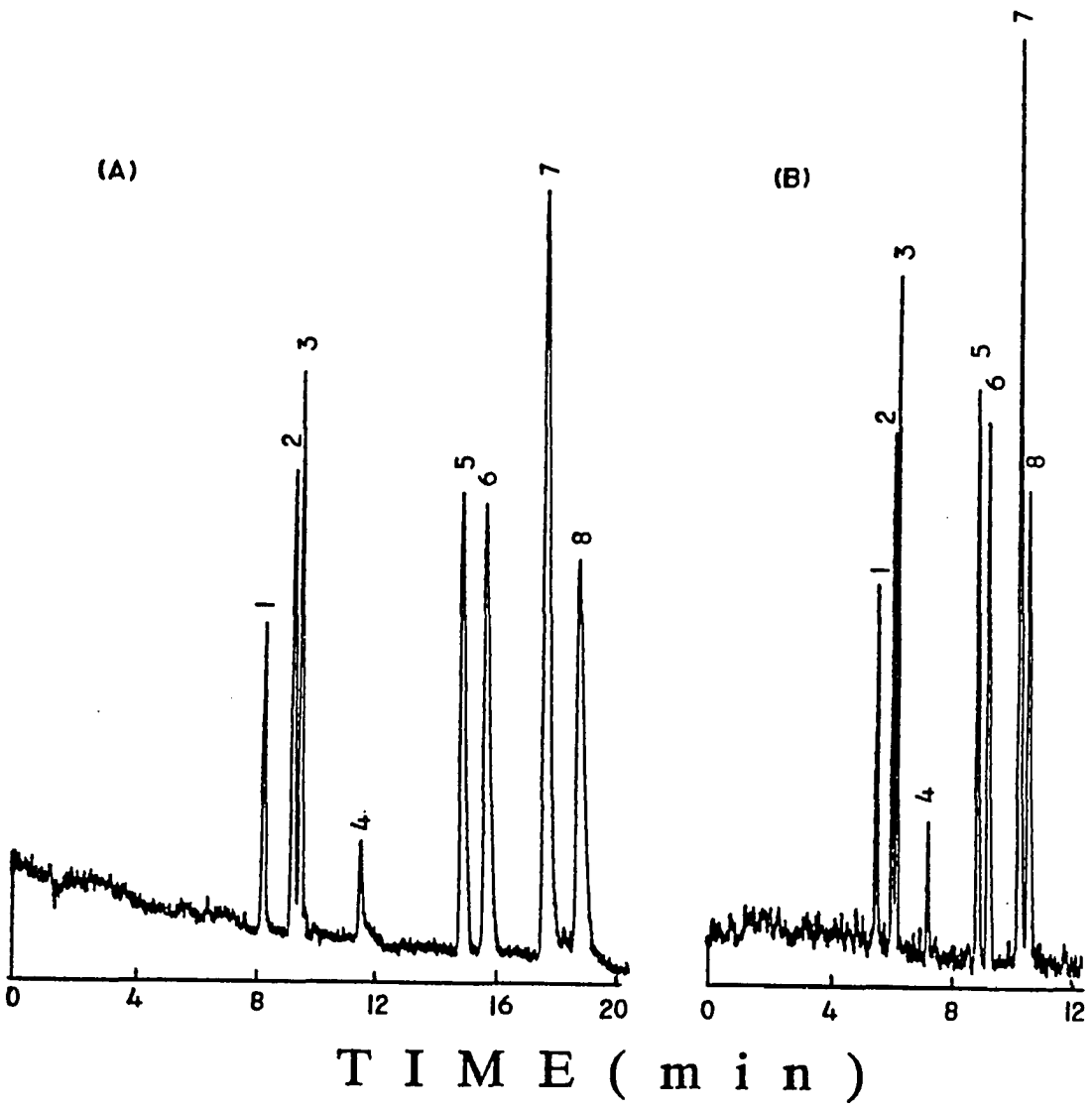


Fig. 6. Influence of a step change in [CTAB] on the separation of organic anions. Before injection, the concentration of CTAB is 4 μM while the running concentrations are 10 μM for A and 40 μM for B, respectively. Conditions are the same as those in Fig. 4.

are important to improve the resolution and reduce the separation time in CZE. Many approaches have already been used to control the electrophoretic mobilities of analytes in order to enhance the separation resolution. In this work, we demonstrated the effect of a dynamic gradient in pH on the effective electrophoretic mobilities of several model anions by introducing the change at the outlet end of the capillary. Altering the electroosmotic flow is another known method for increasing the resolution. In this paper, a simple gradient approach, which is based on the combination of the effect of a surfactant on electroosmotic flow and the idea of field-amplified electroosmosis, is outlined. Eight organic acids are successfully separated by this method in less than 11 min.

References

1. J. W. Jorgenson and K. D. Lukacs, Science (Washington, DC), 222 (1983) 266.
2. R. A. Wallingford and A. G. Ewing, Advanced in Chromatogr., 29 (1989) 1.
3. W. G. Kuhr, Anal. Chem., 62 (1990) 403R.
4. P. Boček, M. Deml, J. Pospíchal and J. Sudor, J. Chromatogr., 470 (1989) 309.
5. J. Pospíchal, M. Deml, P. Gebauer and P. Boček, J. Chromatogr., 470 (1989) 43.

6. V. Sustacek, F. Foret and P. Boček, J. Chromatogr., 480 (1989) 271.

7. F. Foret, S. Fanali and P. Boček, J. Chromatogr., 516 (1990) 219.

8. J. Sudor, J. Pospíchal, M. Deml and P. Boček, J. Chromatogr., 545 (1991) 331.

9. P. Boček, M. Deml and J. Pospíchal, J. Chromatogr., 500 (1990) 673.

10. C. W. Whang and E. S. Yeung, Anal. Chem., 64 (1992) in press.

11. J. Sudor, Z. Stransky, J. Pospíchal, M. Deml and P. Boček, Electrophoresis, 10 (1989) 802.

12. T. Kaneta, S. Tanaka and H. Yoshida, J. Chromatogr., 538 (1991) 385.

13. X. Huang, J. A. Luckey, M. J. Gordon and R. N. Zare, Anal. Chem., 61 (1989) 766.

14. C. Schwer and E. Kenndler, Anal. Chem., 63 (1991) 1801.

15. C. S. Lee, D. McManigill, C. T. Wu and B. Patel, Anal. Chem., 63 (1991) 1519.

16. W. G. Kuhr and E. S. Yeung, Anal. Chem., 60 (1988) 2642.

17. S. Fujiwara and S. Honda, Anal. Chem., 58 (1986) 1811.

18. J. C. Helmer and R. L. Chien, Anal. Chem., 63 (1991) 1354.

19. J. I. Ohms and X. Huang, J. Chromatogr., 516 (1990) 233.

20. J. W. Jorgenson and K. D. Lukacs, Anal. Chem., 53 (1981) 1298.

21. F. M. Everaerts, J. L. Beckers and T. P. Verheggen, Isotachoresis, Elsevier, New York, 1976; pp 27-40.

22. G. D. Roberts, P. H. Rhodes and R. S. Snyder, J. Chromatogr., 480 (1989) 35.

23. S. Hjerten, Electrophoresis, 11 (1990) 665.

24. M. J. Astle, W. H. Beyer and R. C. Weast, CRC Handbook of Chemistry and Physics, 56th ed., CRC Press, Florida; 1984; D 165-166.

25. T. Hirokawa, M. Nishino, N. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi and J. Akiyama, J. Chromatogr., 271 (1983) D1.

CHAPTER 3

VOLTAGE PROGRAMMING IN CAPILLARY ZONE ELECTROPHORESIS

A paper published in the Journal of Chromatography[1]

Huan-Tsung Chang and Edward S. Yeung[2]

Abstract

Temperature is an important factor in capillary zone electrophoresis since it affects the viscosity and the pH of the buffer solution. In this study, a capillary tube with a large radius (130 μm i.d.) and filled with buffer at high ionic strength is used to generate substantial joule heat within the capillary tube to force a significant increase in temperature, in turn to decrease the viscosity and to change the pH of the buffer solution. From a study of the degree of dissociation of analyte at different voltages, we show that voltage-induced pH change is significant in 0.1 M tris(hydroxyamino)methane (THAM) but not in 0.025 M hydrogencarbonate

---

[1] Reprinted with permission from J. of Chromatography 1993, 632, 149-155.

[2] Author for correspondence

buffer system. A step change in voltage from 15 to 25 kV is implemented to generate a pH gradient in the THAM buffer solution. The results show that the method is useful for separating phenols which cannot be separated at a fixed voltage.

Introduction

Temperature is an important parameter in CZE since it affects not only the flow through convection, but also ionization of the analyte and the capillary surface, the viscosity and the pH of the buffer solution.[1,2] The effects are smaller than 0.5% per degree for all these terms except for viscosity which has a 2% per degree change. In CZE, temperature control is often used to provide efficient heat removal.[3-4] Manipulation of chemical equilibria such as metal chelation and micelle partitioning[5-6] within the capillary tube through temperature control has also been proposed.

pH is also an important factor to determine selectivity in CZE since it will affect the dissociation of analytes and ionization of the capillary surface, which in turn changes the electrophoretic mobility of charged analyte and the electroosmotic flow coefficient.[7-8] pH gradients have been used to improve the separation process. Boček et al. used a two buffer system to force the migration of varying ratios of two ions into the capillary during separation.[9,10] Although pH change as a function of

temperature is insignificant for most buffers, it is possible to generate a substantial pH change if a buffer system with a large temperature coefficient (dpH/dT) is used. Whang and Yeung demonstrated the effect of temperature-induced pH change on the separation of dyes.[11] In their study, THAM buffer which has a large temperature coefficient is used to generate a pH gradient via controlling the temperature of the column.

Joule heat is evolved when electrical current passes through the capillary tube. This is usually not desirable because it will distort the zone distribution. However, it is possible to increase the temperature significantly by using a high concentration of buffer, running at a high voltage and using a capillary tube with a large radius.[12] One can then dramatically decrease the viscosity and change the pH of the buffer. As viscosity decreases, the electrophoretic mobility and electroosmotic flow coefficient will increase. Thus, shorter separation time and better resolution may be achieved. Altering the pH further modifies the selectivity for components such as weak acids and bases.

In this paper, the effect of joule heat on CZE is examined. THAM (with large dpH/dT) and bicarbonate (with small dpH/dT) are used to study the effect of voltage-induced pH change on the separation of phenols. We also show the implementation of a step change in voltage from 15 to 25 kV to generate a pH gradient for separating phenols in 0.1 M THAM buffer system.

Theory

Separation in CZE is dependent on the electroosmotic flow coefficient and the electrophoretic mobility. Careful consideration of the factors that affect these parameters is important to obtaining good separations. Equation (1)[13] can be used to relate the decrease in electromomostic flow coefficient, m_{eo}, to the increase in viscosity

$$m_{eo} = \epsilon \, \xi_c / 4 \, \pi \, \eta \qquad (1)$$

where ϵ is the dielectric constant, ξ_c is the zeta potential at the slipping plane and η is the bulk viscosity. Electrophoretic mobility, m_{ep}, can be given by[14]

$$m_{ep} = \epsilon \, \xi_a / 6 \, \pi \, \eta \qquad (2)$$

where ξ_a is the zeta potential of the analyte. Equations (1) and (2) show that both the electroosmotic flow coefficient and the electrophoretic mobility increase when the viscosity of the buffer decreases. In order to force fast flow, low viscosity is

needed. Temperature is an important factor in determining the viscosity of the buffer solution. They are related by

$$1/\eta = A\, e^{-B/T} \qquad (3)$$

where A and B are constants related to the medium. From equation (3), fast flow will be achieved at high temperatures.

Since the effective electrophoretic mobility, m_{eff}, is proportional to the fraction of free ion of the analyte, Tiselius[15] derived the equation

$$m_{eff} = \sum \alpha_i\, m_{ep} \qquad (4)$$

where α_i is the degree of dissociation and m_{ep} is the absolute mobility of the ith ionic form of a molecule. To relate the effective electrophoretic mobility, degree of dissociation for a monovalent ion and viscosity of the bulk solution, equation (4) can be combined with equation (2) to give

$$m_{eff} = \alpha_i\, \epsilon\, \xi_a / 4\, \pi\, \eta \qquad (5)$$

In order to simplify the problem for estimating the changing mobilities of an analyte,

ξ_a is assumed to be constant at different voltages. The ratio of mobilities at different conditions i and j is then

$$(m_{effi}/m_{effj}) = (\alpha_i/\alpha_j)(\eta_j/\eta_i) \quad (6)$$

In this study, η_i/η_j can be calculated from the m_{ep} of benzoic acid at different voltages since it is completely dissociated over the range of pH used.

Since the temperature of the capillary tube is an important factor in establishing the viscosity, pH of the buffer, and the degree of dissociation of the analyte, it is important to know the temperature of capillary tube. It can be estimated by equation (7)[16]

$$T = 1820/[\ln(m_{eo1}) - \ln(m_{eo2}) + 6.11] \quad (7)$$

where m_{eo1} and m_{eo2} are the electroosmotic flow coefficient at 298 K and T K. Therefore, T can be determined by measuring m_{eo1} at a low voltage where we assume little heat is generated, and then measuring m_{eo2} at a high voltage. As soon as the temperature of capillary tube is determined, the pH of the buffer can be calculated from the temperature coefficient of the buffer. Then, α can be estimated from equation (8)

$$\alpha = Ka/(Ka + [H^+]) \quad (8)$$

where Ka is the dissociation constant of the analyte.

Experimental Section

A commercial electrophoresis instrument (Isco model 3850, Lincoln, NE, USA) was used for all electrophoretic experiments. The fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was 55 cm × 130 μm ID. At 35 cm from the injection end, the polyimide coating was burned off to form the detection window. A digitizer (Data Translation model DT 2802, Palo Alto, CA, USA) and a computer (PC/AT, IBM, Boca Raton, FL, USA) were used to collect and store all of the data.

All chemicals were of reagent grade and were obtained from Aldrich (Milwaukee, WI, USA), except that THAM and sodium bicarbonate were from Fisher (Fair Lawn, NJ, USA). Buffer solutions were adjusted by HCl and NaOH to pH 8.5. The injected concentration of each analyte is 2×10^{-4} M, except that mesityl oxide is present at 2×10^{-3} M. Mesityl oxide was used to measure the electroosmotic flow coefficient. Benzoic acid was used as the other marker to estimate the change of electrophoretic mobility as a function of the change in viscosity. The sample solution was raised to 10 cm high for hydrodynamic injection from the anodic end of the capillary for 8 sec.

Results and Discussion

In order to demonstrate the effect of voltage-induced pH change within the capillary tube on the electrophoretic mobilities of analytes, two electrophoresis buffers which have different temperature coefficients are chosen. THAM with a large temperature coefficient and bicarbonate with a small temperature coefficient are suitable buffers in our study. From Table 1, which lists actual experimental measurements in our laboratory, the pH of THAM buffer at different temperatures can be estimated from the following equations

$$pH_2 = pH_1 - 0.023\ (T_2 - T_1) \qquad 40 - 50°C \qquad (9)$$

$$pH_2 = pH_1 - 0.007\ (T_2 - T_1) \qquad 70 - 80°C \qquad (10)$$

$$pH_2 = pH_1 - 0.015\ (T_2 - T_1) \qquad 30 - 40 \text{ and } 50 - 70°C \qquad (11)$$

On the other hand, pH of the bicarbonate buffer increases as temperature increases. The pH of bicarbonate buffer at different temperatures can be calculated from equations (12) and (13)

$$pH_2 = pH_1 + 0.004\ (T_2 - T_1) \qquad 30 - 50°C \qquad (12)$$

$$pH_2 = pH_1 + 0.007\ (T_2 - T_1) \qquad 50 - 80°C \qquad (13)$$

Table 1. The effect of temperature on pH of 0.1 M THAM and 0.025 M bicarbonate buffer solutions

	pH	
Temp. (°C)	THAM	HCO_3^-
30	8.44	8.56
40	8.29	8.60
50	8.06	8.64
60	7.92	8.73
70	7.77	8.79
80	7.70	8.87

The decrease in the viscosity of the buffer is significant as can be seen from the dramatic change in electroosmotic flow coefficient in both systems as the voltage changes from 10 to 25 kV. From Tables 2 and 3, the fractional changes in electroosmotic flow coefficient and the electrophoretic mobility between 10 and 25 kV based on the two markers, mesityl oxide (neutral) and benzoic acid

Table 2. Electroosmotic flow coefficient (m_{eo}) and the electrophoretic mobilities (m_{ep}) of analytes in 0.1 M THAM buffer solution at different voltages

	m_{eo}	m_{ep}								
Voltage	1	2	3	4	5	6	7	8	9	10
10 kV	-4.5	0.4	0.8	1.7	1.8	2.2	2.5	2.8	2.8	2.9
15 kV	-5.2	0.3	0.7	1.7	1.8	2.4	2.9	3.3	3.3	3.4
20 kV	-6.7	0.3	0.7	1.7	2.0	2.8	3.6	4.1	4.1	4.4
25 kV	-9.5	0.2	0.7	1.3	1.8	2.7	4.4	5.1	5.3	6.3

1. mesity oxide, 2. 4-chlorophenol, 3. 3-chlorophenol, 4. 2-chlorophenol, 5. 3-nitrophenol, 6. 2,4-dichlorophenol, 7. 3-methyl-4-nitrophenol, 8. 4-nitrophenol, 9. 2-nitrophenol, 10. benzoic acid.

(completely dissociated), are 2.1 and 2.2 for THAM and 2.0 and 2.0 for bicarbonate buffers, respectively. The results show that our markers are well suited for predicting the change of m_{eo} and m_{ep} due to the change in viscosity of the buffer solution. Also this shows that the dissociation effect of these two markers can be

Table 3. Electroosmotic flow coefficient (m_{eo}) and electrophoretic mobilities (m_{ep}) of analytes in 0.025 M bicarbonate buffer solution at different voltages.

	m_{eo}	m_{ep}								
Voltage	1	2	3	4	5	6	7	8	9	10
10 kV	-5.0	0.8	1.3	2.2	2.3	2.5	2.7	3.0	3.0	3.0
15 kV	-6.0	1.0	1.6	2.6	2.7	2.9	3.2	3.5	3.5	3.5
20 kV	-7.7	1.3	1.6	2.6	2.7	2.9	3.2	3.5	3.5	3.5
25 kV	-9.9	1.8	3.1	4.5	4.8	5.0	5.5	6.0	6.0	6.0

1-10 represent the same analytes as those in Table 2.

neglected in the range of voltage-induced pH change in this work. Based on the m_{eo} values obtained (mesityl oxide), one can calculate the temperature of the liquid at the various operating voltages by using equation (7). This is shown in Table 4.

Since the electrophoretic mobility of an analyte depends on the fraction of its free ion, the degree of dissociation should be determined. It is a function of pH as

Table 4. The calculated temperature and pH of 0.1 M THAM and 0.025 M bicarbonate buffer solutions at different voltages

	THAM		HCO3	
Voltage	Temp. (°C)	pH	Temp. (°C)	pH
10 kV	32	8.41	28	8.54
15 kV	40	8.29	37	8.59
20 kV	54	8.00	51	8.65
25 kV	76	7.73	66	8.77

expressed in equation (8). Hence, in order to illustrate the effect of voltage-induced pH change, it is important to know the degree of dissociation of the analytes at different voltages. Based on the results shown in Tables 2 and 3, the ratio of the degree of dissociation of analytes at different voltages can be calculated from equation (6). The results are shown in Tables 5 and 6. From Table 5, it is obvious that the pH of THAM buffer decreases when the voltage increases since the ratio decreases. The results of observed and calculated values agree each other, which means that the trend of pH change we

Table 5. Comparison of the observed and calculated ratios of the dissociation constant (α) of analytes in 0.1 M THAM buffer solution at different voltages

	Obs.			Cal.		
Analytes	1	2	3	1	2	3
2-nitrophenol	1.01	0.97	0.87	1.01	0.95	0.86
4-nitrophenol	1.01	0.97	0.84	1.01	0.95	0.85
3-methyl-4-nitrophenol	0.99	0.95	0.81	1.01	0.95	0.85
3,4-dichlorophenol	0.93	0.84	0.56	1.03	0.81	0.59
3-nitrophenol	0.85	0.73	0.46	1.06	0.72	0.46
2-chlorophenol	1.12	0.86	0.46	1.07	0.68	0.41
3-chlorophenol	1.19	0.92	0.46	1.10	0.61	0.34
4-chlorophenol	0.64	0.49	0.23	1.11	0.59	0.32

1. α_{15}/α_{10}, 2. α_{20}/α_{10}, 3. α_{25}/α_{10}. The subscript refers to kV operating voltage.

Table 6. Comparison of the observed and calculated ratios of the dissociation constant (α) of analytes in 0.025M bicarbonate buffer solution at different voltages.

	Obs.			Cal.		
Analytes	1	2	3	1	2	3
2-nitrophenol	1.00	0.96	1.00	1.00	1.01	1.02
4-nitrophenol	1.00	0.96	1.00	1.00	1.01	1.02
3-methyl-4-nitrophenol	1.02	0.97	1.02	1.00	1.01	1.02
3,4-dichlorophenol	0.99	0.94	1.00	1.02	1.04	1.08
3-nitrophenol	1.01	0.97	1.02	1.04	1.09	1.17
2-chlorophenol	1.01	0.96	1.02	1.05	1.12	1.24
3-chlorophenol	1.05	1.03	1.19	1.09	1.20	1.45
4-chlorophenoL	1.07	1.04	1.13	1.11	1.24	1.56

1. α_{15}/α_{10}, 2. α_{20}/α_{10}, 3. α_{25}/α_{10}. The subscript refers to kV operating voltage.

estimated is correct. The ratios decrease in the THAM system while they increase a little in the bicarbonate system as the voltage increases. It is worth noting that the ratio changes significantly in the THAM buffer, implying that large voltage-induced pH changes can be obtained in the THAM buffer.

A group of phenolic compounds which have pK_a ranging from 7 to 9.5 are selected to demonstrate the separation improvement due to voltage-induced pH change. The effect of voltage-induced pH change on the separation of phenols can be shown in Fig. 1 and 2 for constant voltage operation. In the THAM system, 2-nitrophenol (pK_a = 7.15) and 4-nitrophenol (pK_a = 7.17) cannot be separated at the lower voltages while they can be separated at 25 kV. The result also agrees with our estimation of the pH of the buffer system in Table 4 because the change in the degree of dissociation of an analyte is more significant when the pH is near its pK_a. The other advantage is that electroosmotic flow increases as viscosity decreases, speeding up the separation. However, resolution among the first three analytes became worse at the high voltage.

In the bicarbonate system, benzoic acid, 2-nitrophenol and 4-nitrophenol, which all have low pK_a, cannot be completely separated. This is due to the insignificant increase in pH in the bicarbonate buffer as voltage increases. Comparison of the result of separation ability in these two buffer systems again supports our conclusion that there is a higher voltage-induced pH change in the THAM buffer than in the bicarbonate buffer.

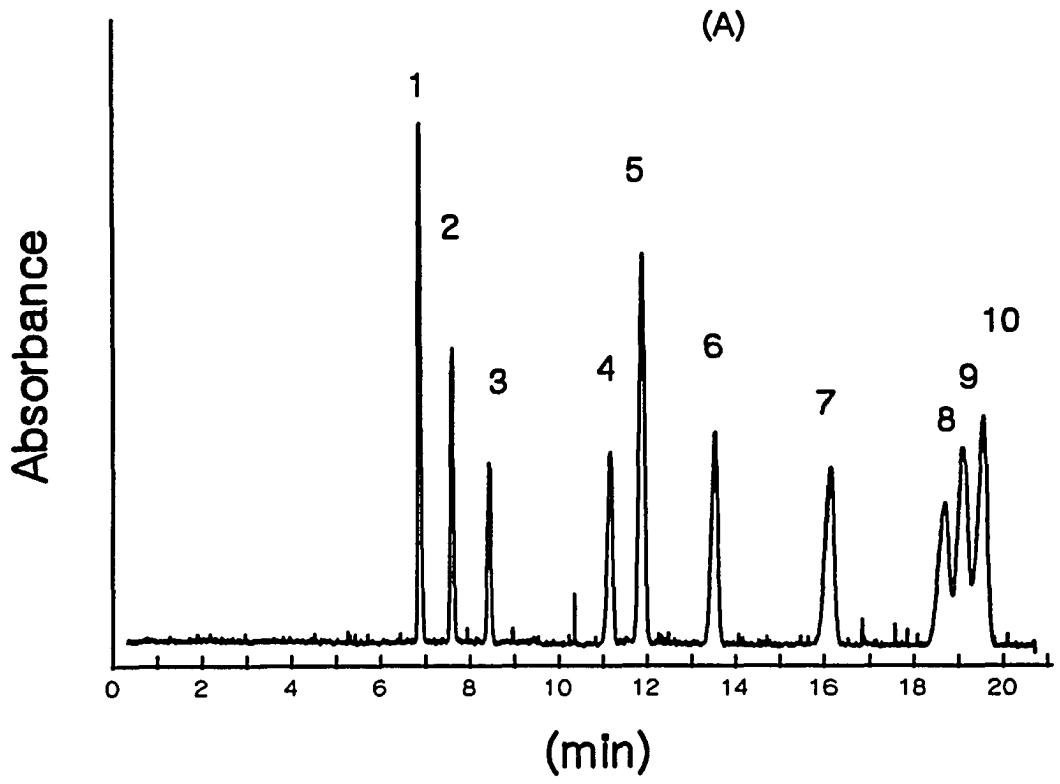


Figure 1. Separation of analytes in pH 8.5, 0.1 M THAM buffer solution at different voltages. Column: 130 μm ID × 360 μm OD. 55 cm total length, 35 cm effective length. Detection wavelength = 218 nm. A: 10 kV, B: 15 kV, C: 20 kV, D: 25 kV. Peak identities:
1. mesityl oxide, 2. 4-chlorophenol, 3. 3-chlorophenol,
4. 2-chlorophenol, 5. 3-nitrophenol, 6. 3,4-dichlorophenol,
7. 3-methyl-4-nitrophenol, 8. 4-nitrophenol, 9. 2-nitrophenol,
10. benzoic acid.

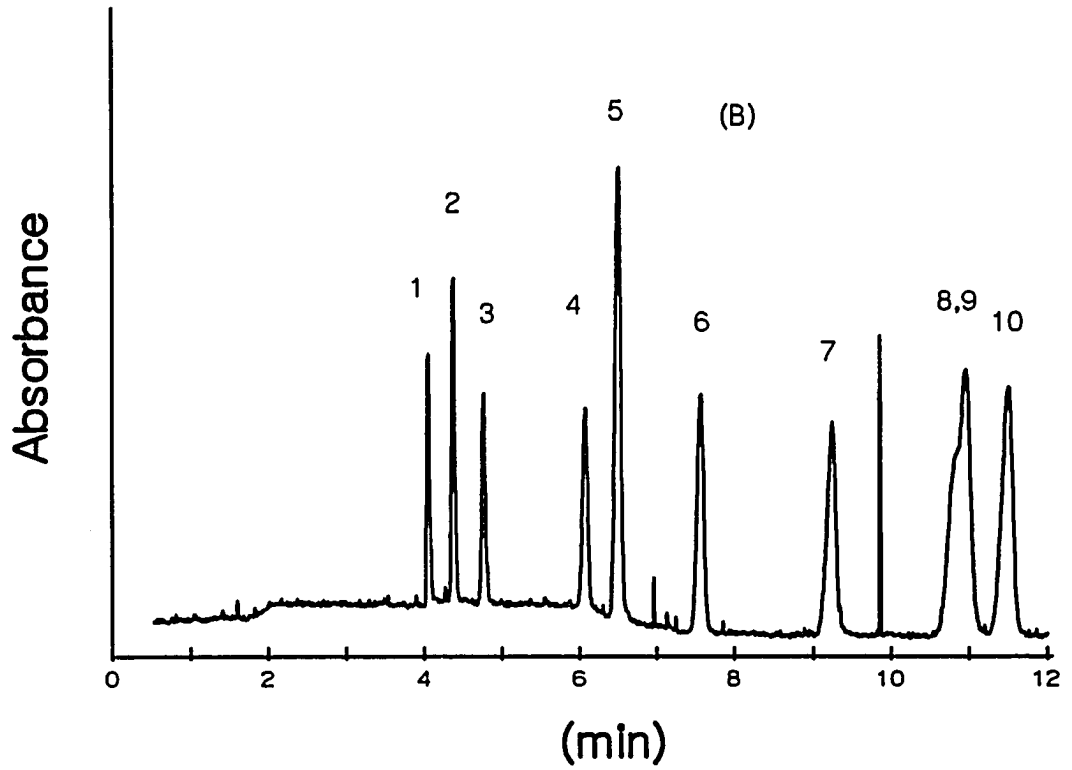


Figure 1. (continued)

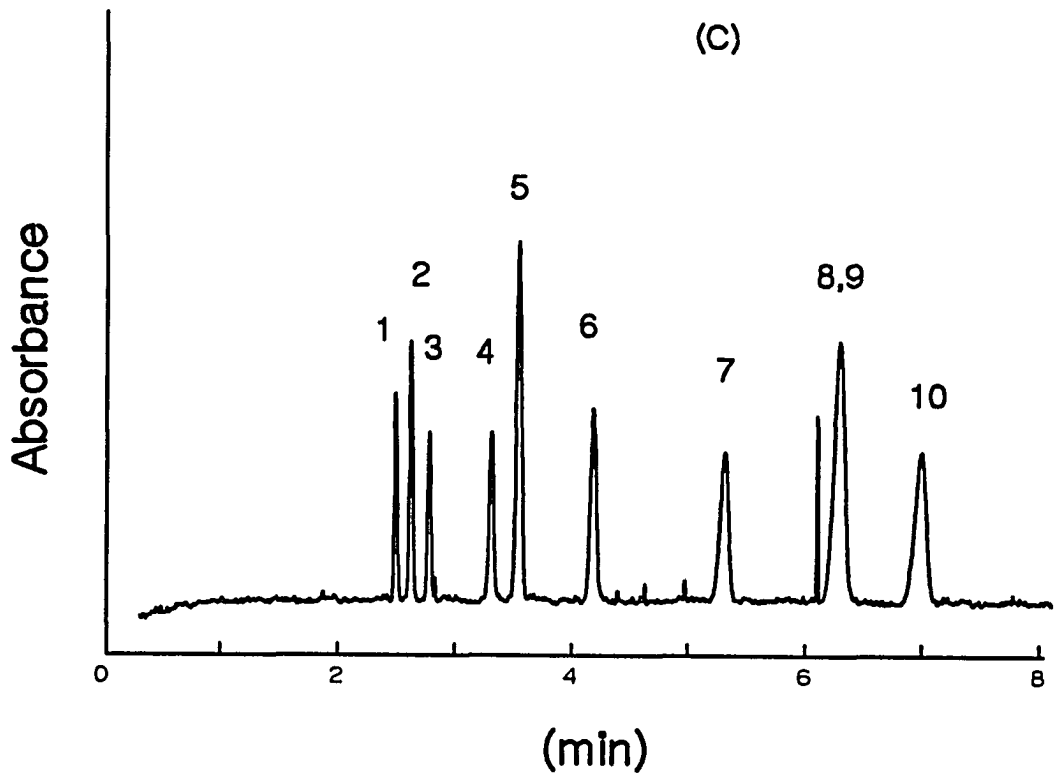


Figure 1. (continued)

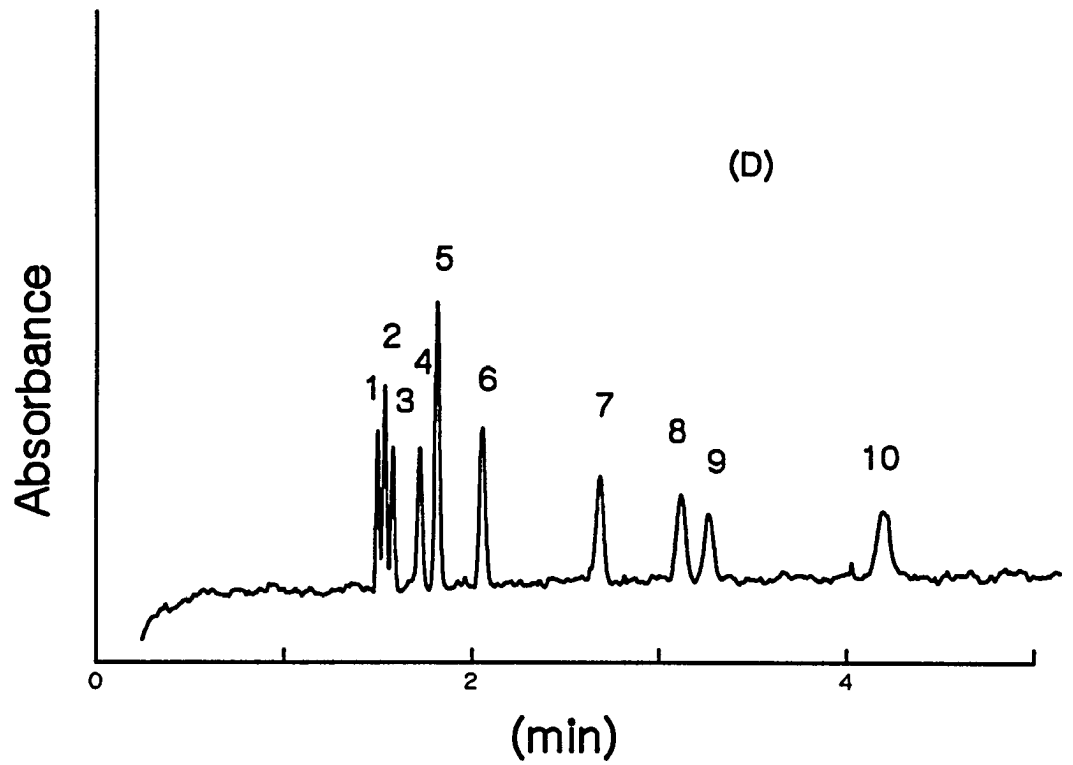


Figure 1. (continued)

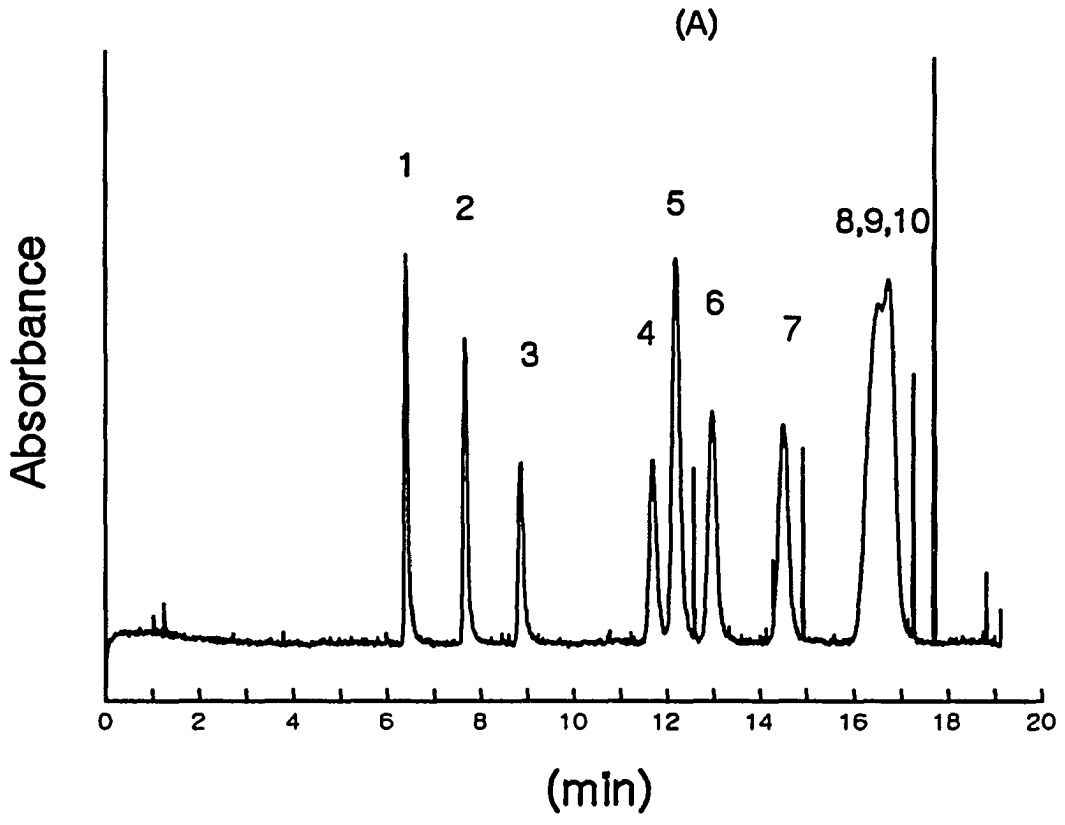


Figure 2. Separation of analytes in pH 8.5, 0.025 M bicarbonate buffer solution at different voltages. Other conditions are the same as those in Fig. 1.

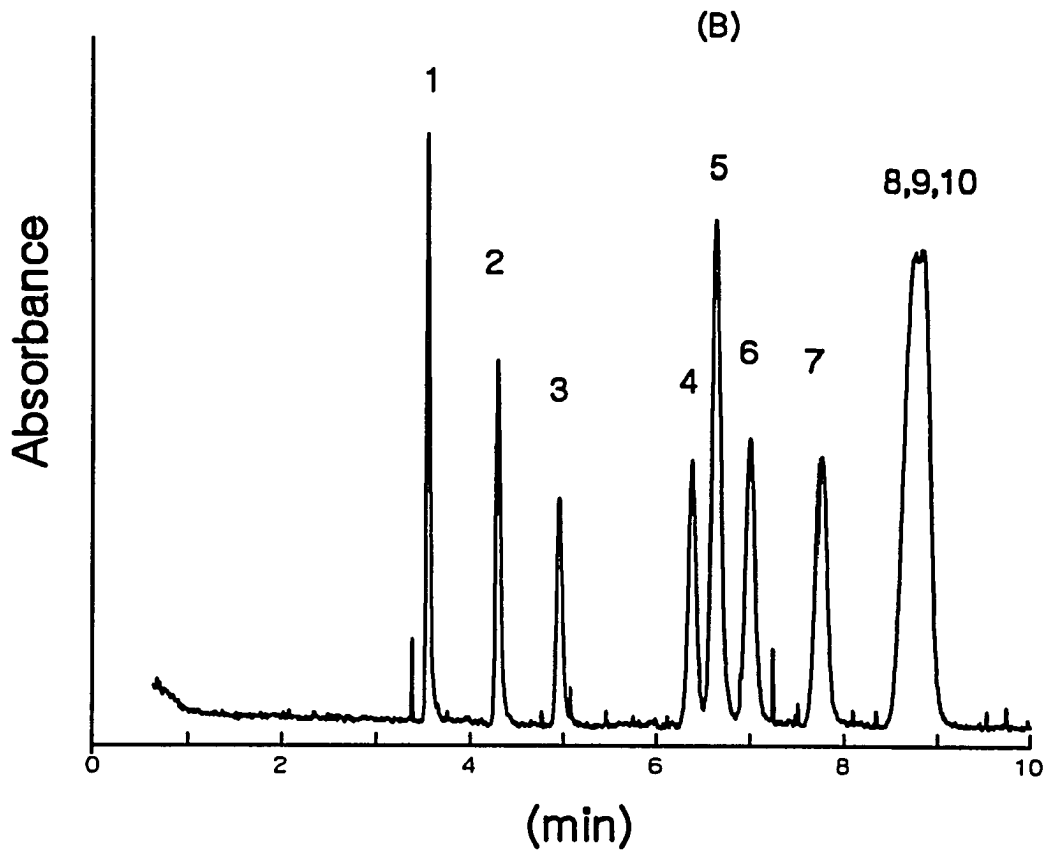


Figure 2. (continued)

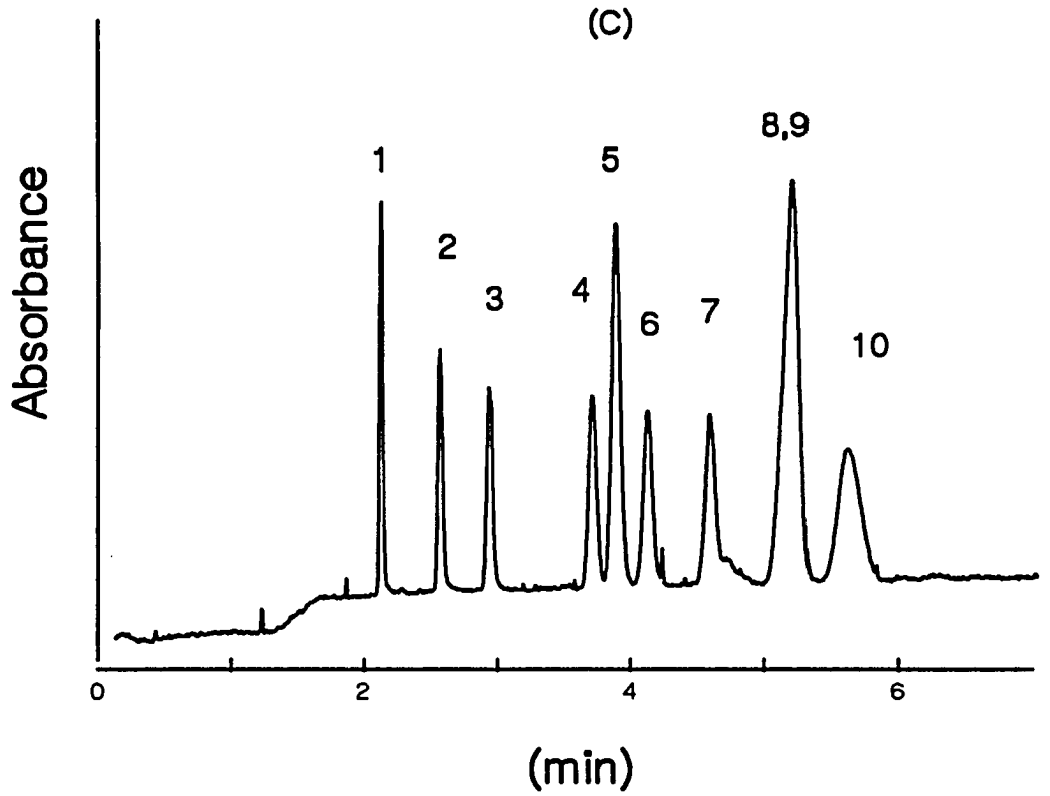


Figure 2. (continued)

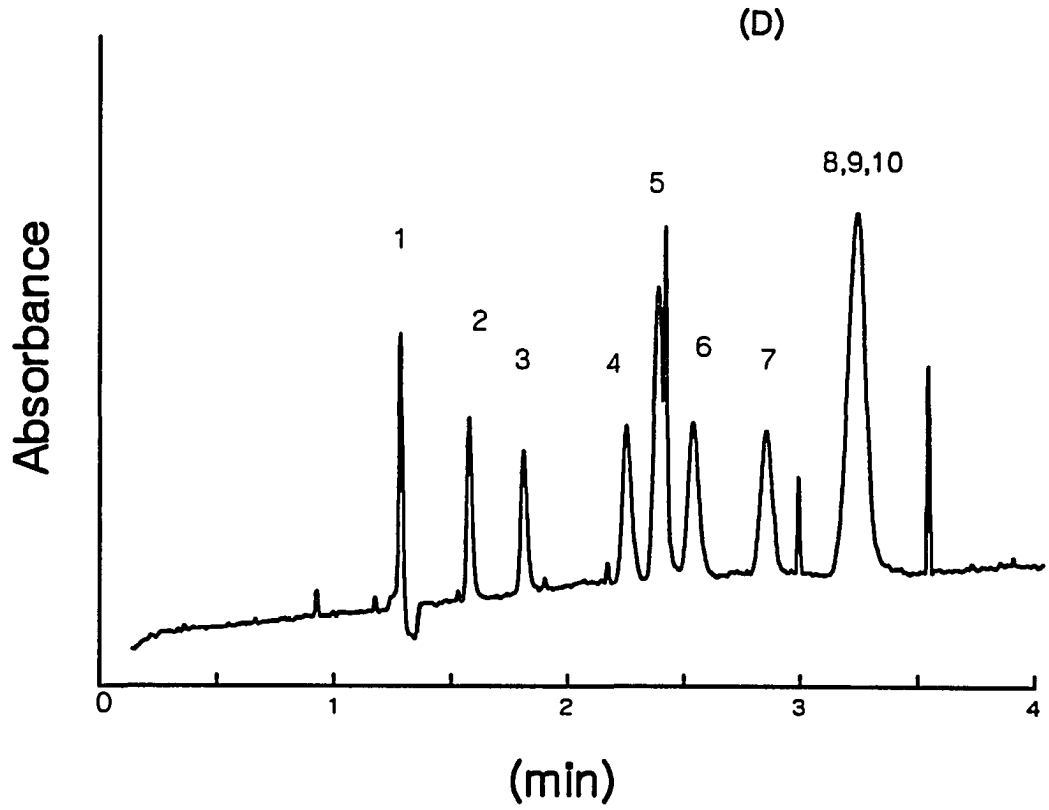


Figure 2. (continued)

As discussed in the introduction, pH gradient is a well known method to improve the separation ability in CZE. To demonstrate the possibility of pH gradient generated via a step change in voltage, we use Fig. 3 as an example. Voltage programming starts from 15 kV for 3 min, then jumps to 25 kV for the remainder of the run. All analytes can be separated in 6 min with little overlap. There is a baseline shift due to the temperature change, as has been reported earlier.[11] From this result, we can conclude that voltage programming is advantageous for certain CE separations. As in the case of pH programming, [9-11] groups of analytes that cannot be separated at any single pH can thus be resolved.

In summary, we have demonstrated the use of joule heat to increase the temperature of the buffer during CZE separation. The resulting increase in result temperature is able to generate a noticeable voltage-induced pH change and a change of the viscosity of 0.1 M THAM buffer to alter selectivity. Implementation is best in large diameter capillaries, with high operating voltage, and at high buffer concentrations. In fact, efficient cooling of the capillary, e.g. in certain commercial CE instruments, is counterproductive in this mode of operation. There is the possibility that high joule heating can reduce the efficiency of the separation. However, for the systems studied here, degradation in efficiency was not observed. the use of voltage programming is in general simpler than the use of temperature programming.[11] The recycle time is also shorter since only the capillary and not

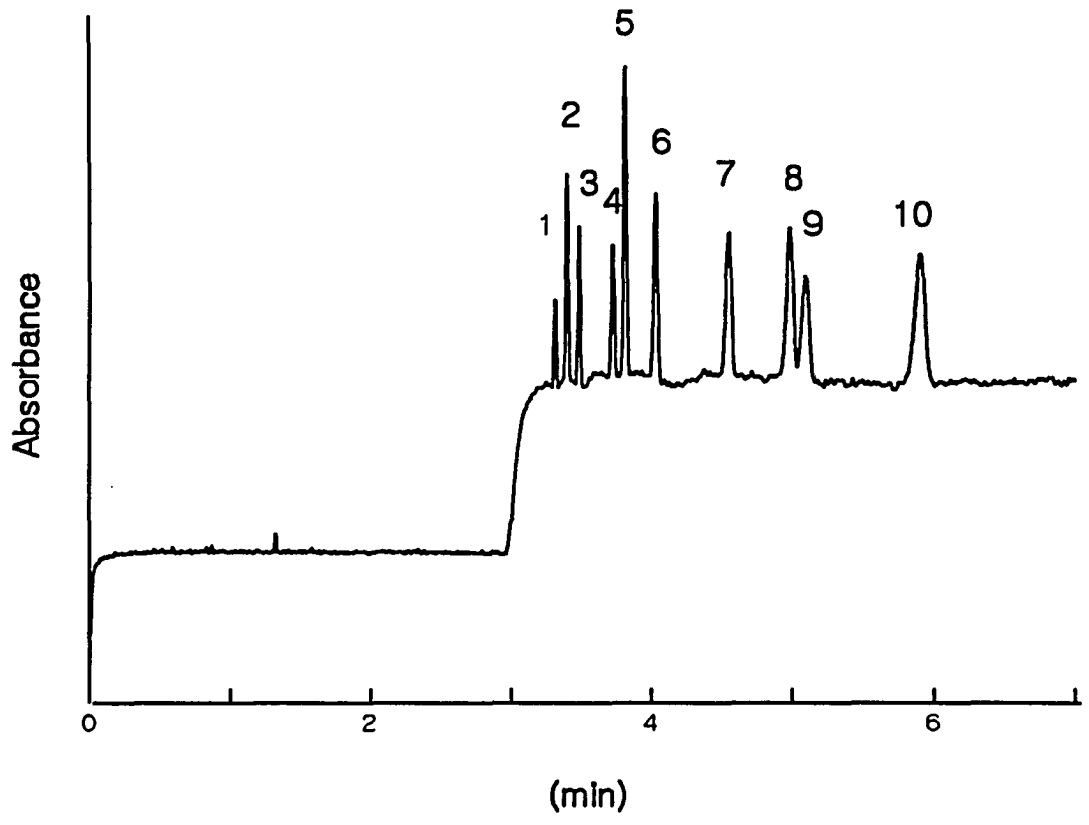


Figure 3. The effect of voltage-induced pH program on the separation of phenols in pH 8.5, 0.1 M THAM solution.

the coolant needs to be returned to the initial temperature before the start of the next run.

References

1. S. Hjerten, Electrophoresis, 11 (1990) 665.
2. P. H. Rhoders, R. S. Synder and G. O. Roberts, J. Chromatogr., 480 (1989) 35.
3. E. Grushka, R. M. McCormick and J. J. Kirkland, Anal. Chem., 61 (1989) 241.
4. Y. Kurosu, K. Hibi, T. Sasaki and M. Saito, J. High Resol. Chromatogr., 14 (1991) 200.
5. S. Terabe, K. Otsuka and T. Ando, Anal. Chem. 61 (1989) 251.
6. A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, Anal. Chem., 59 (1987) 1021.
7. D. L. Middleton and W. J. Lambert, Anal. Chem., 62 (1990) 1585.
8. S. Wren, J. Microcol. Sep., 3 (1991) 147.
9. P. Gebaur, M. Deml, J. Pospichal and P. Boček, Electrophoresis, 11 (1990) 724.
10. M. Deml, J. Pospichal, J. Sudor and P. Boček, J. Chromatogr., 470 (1989) 43.
11. C. W. Whang and E. S. Yeung, Anal. Chem., 64 (1992) 502.
12. S. Hjerten, Chromatogr. Rev., 9 (1967) 122.

13. C. L. Rice, R. Whitehead, J. Phys. Chem., 69 (1965) 4017.

14. J. Th. G. Overbeck and P. H. Wiersema, in "Electrophoresis, Theory, Methods and Application" Vol. 2, M. Bier, ed., Academic Press, New York, 1967, p. 9.

15. F. M. Everaerts, J. L. Beckers and T. P. Verheggen, Isotachoresis, Elsevier, New York, 1967; pp. 27-40.

16. K. Salmon, R. L. Chien and D. Burgi, J. Liq. Chromatogr., 14 (1991) 847.

CHAPTER 4

SELF-REGULATING DYNAMIC CONTROL OF ELECTROOSMOTIC FLOW IN CAPILLARY ELECTROPHESIS

A paper published in the Analytical Chemistry[1]

Huan-tsung Chang and Edward S. Yeung[2]

Introduction

Capillary electrophoresis (CE), primarily because of the impressive separation power and the compatibility with large biomolecules, has established itself as an important separation tool over the past several years.[1,2] One of the major barriers limiting the broad, routine application of the technique is the validation of the reliability of migration times and the quantitative accuracy when dealing with real samples. In particular, sample matrices can alter the nature of the surface of the inner wall of the capillary, leading to irreproducible results. Even when the temperature is constant and when adsorption of the analytes onto the capillary wall

---

[1] Reprinted with permission from Analytical Chemistry 1993, 65, 650-652.

[2] Author for correspondence.

can be neglected, which is the case of pure zone electrophoresis (CZE), the zeta potential of the wall can still be altered by the injected ions.[3] This produces a change in the electroosmotic flow rate[4-6] of the bulk solution, which in turn affects the resolution between analytes.[7] Solution flow rate naturally influences the migration times, but it also contributes to changes in the measured peak areas utilized for quantitation.

Many different approaches have been suggested for controlling electroosmotic flow in CZE. These include the addition of surfactants,[4,5] the use of organic solvents,[6] coating of the capillary walls,[8,9] adding salts,[10] and the application of an external electric field.[11-13] In fact, it has been shown that one can easily program the bulk flow rate during the course of the separation to enhance the resolution.[14] Unfortunately, so far none of these approaches have been successfully applied to improve the reproducibility of migration times of the analytes. The reason is that some sort of interactive control is needed. However, well controlled the initial electroosmotic flow rate is, perturbation of the wall zeta potential by the sample matrix is generally unpredictable. Even in cases where the electroosmotic flow rate has been reduced to zero by the above approaches, adsorption of matrix ions onto the surface will once again cause deviations from the steady-state flow rate. The electroosmotic flow rate must therefore be monitored in real time to allow instantaneous control of the zeta potential to correct for any changes. The use of a neutral marker[15] to measure the electroosmotic flow rate during a particular run

can correct for changes in between runs. It has been shown however that the flow rate (e.g. due to temperature changes) can actually change during a run, and will affect different analytes within the same run differently.[16] The monitoring of the running current[17] can in principle be used to derive the instantaneous electroosmotic flow rate. So far interactive control based on this approach has not been demonstrated, probably because the signal-to-noise ratio for current measurement is typically insufficient unless one can use a high concentration of running buffer. This has the undesirable effect of possibly altering the running temperature[18] and even the electrophoretic mobilities of the analytes.[19]

During our investigation of flow programming to control resolution in CZE,[14] our computer simulations revealed a subtle feature in the changing electroosmotic flow rates. At the start of the run, a low or zero concentration of a cationic surfactant, cetylammonium bromide (CTAB), was present in the buffer vials. For a normal fused-silica capillary at neutral to alkaline pH, this will produce typical electroosmotic flow (from anode to cathode), the magnitude of which will depend on the original CTAB concentration.[5] To start the flow gradient, the buffer vial at the anodic end of the capillary was changed to one containing a high CTAB concentration, but below the critical micellar concentration.[14] Both its electrophoretic mobility and the electroosmotic flow drive the CTAB front into the capillary tube. As higher CTAB concentration builds up along the length of the tube, the electroosmotic flow rate will slow down.[5] Eventually, a condition will exist

whereby the net electroosmotic flow[20,21] will become zero. There is still a net movement of the CTAB front, however, as a result of its small but finite electrophoretic mobility. This eventually builds up more surface charge to create electroosmotic flow in the opposite (cathode to anode) direction, such that the net flow of the CTAB front becomes stationary. This then creates a steady state in the electroosmotic flow. If a sample is injected (at either end) at this stage and if the zeta potential of the capillary wall is affected, the system should react to counter such a change. For example, if the wall becomes more positive, there will be a tendency for the electroosmotic flow to reverse (i.e. cathode to anode), pulling in buffer (at low CTAB concentration) from the cathodic end. Following the concept of field-amplified electrophoresis,[20,21] since the fraction of the capillary containing a low CTAB concentration is then larger, the CTAB front will once again be gradually slowed down to zero. If the wall becomes more negative due to the injected matrix, more CTAB at high concentration will be pulled in from the anodic end to counter the induced flow. Therefore, the CTAB concentration front will always be stationary, except for minor shifts to exactly counteract the change in surface charge due to the matrix. In other words, the electroosmotic flow rate in the column will always be from cathode to anode, at exactly the same magnitude as the electrophoretic velocity of CTAB. In this report, we will demonstrate the utility of such a concept for maintaining a constant electroosmotic flow rate for sample ions in CZE, independent of the changing sample matrix composition. Such an

interactive control of electroosmotic flow has the advantage that the instantaneous flow rate need not be monitored. Once the stationary front is established, no further external intervention is needed.

Experimental Section

A commercial electrophoresis instrument (Isco, model 3140, Lincoln, NE) was used for all electrophoretic components. The fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was 60 cm × 75 μm ID. At 40 cm from the injection end, the polyimide coating was burned off to form the detection window. Electrophoretic runs were performed at 30 kV. The wavelength of the detector was set at 218 nm.

Benzoic acid, naphthoic acid and cetylammonium bromide (CTAB) were purchased from Sigma Chemical Company (St. Louis, MO). Mesityl oxide used to determine the electroosmotic flow coefficient was from Aldrich (Milwaukee, WI). Sodium bicarbonate and hydrochloric acid used to prepare the buffer solutions were obtained from Fisher Scientific (Fair Lawn, NJ). The injected concentrations of acids and mesityl oxide were 3×10^{-4} M and 2×10^{-3} M, respectively. The sample solution was introduced by vacuum injection at level 1 for 3 sec. When different concentrations of CTAB were used, the column was allowed to equilibrate for 2 hr. In the experiment of dynamic control of electroosmotic flow, the column

was flushed with the high CTAB concentration (300 μM) buffer and then filled with 40 μl (ca. 4 times the total volume of the column) of buffer solution without CTAB before running. The period between injections was 8 min in the study of the effect of different sample matrices on the electroosmotic flow.

Results and Discussion

To illustrate the concept of self-regulating dynamic control of electroosmotic flow, two markers are chosen. Mesityl oxide is a neutral compound that has been widely used as a marker for electroosmotic flow because it is a small molecule that exhibits little adsorption on bare silica columns. It is unlikely to be ionized over a broad range of pH conditions and is unlikely to exhibit ion-pairing behavior. Electroosmotic flow can be monitored based on a current change,[17] but that method requires a large change in ionic strength for adequate signal-to-noise ratios. Benzoic acid is a similarly well-behaved small anion that is completely dissociated under our experimental conditions. Dynamic modification of the electroosmotic flow is implemented by controlling the concentration of the cationic surfactant, CTAB.[4,5] Bicarbonate buffer is used throughout to avoid possible complexation with the other ions.

In a static system, the effect of CTAB on the electroosmotic flow and the mobility of the small anion is as expected and is illustrated in Table I. The entries

Table 1. Effect of CTAB concentration on the migration† of a neutral and an ionic analyte.

CTAB (μM)	mesityl oxide m	benzoate m'
pH 6 10 mM bicarbonate buffer		
0	4.4	-3.4
5	-0.7	-3.3
50	-2.9	-3.4
100	-5.0	-2.7
300	-6.6	-2.0
A	-0.2	-3.3
B	-2.4	-3.3
C	-2.1	-3.1
pH 9 10 mM bicarbonate buffer		
0	6.3	-3.4
5	3.8	-3.1
10	*	-3.3
37.5	-0.6	-3.2
75	-1.0	-3.1
150	-1.9	-2.7
300	-5.5	-2.2
A	-0.5	-3.3
B	-2.5	-3.5

†mobilities are in units of 10^{-4} cm^2 V^{-1} s^{-1}; * not detected at either polarity, implying t= ∞ and m = 0; A: column flushed with 300 μM CTAB, filled with 0 μM CTAB, and then run with 0 μM CTAB at both ends. B: column flushed with 300 μM CTAB, filled with 0 μM CTAB, and then prerun with 300 μM CTAB at the anode and 0 μM CTAB at the cathode before injection. C: column flushed with 150 μM CTAB, filled with 0 μM CTAB, and then prerun with 150 μM CTAB at the anode and 0 μM CTAB at the cathode before injection.

can be positive (migration towards the cathode) or negative (migration towards the anode), and are measured by injection at the anodic and cathodic ends, respectively. The mobility of the anion (m') is calculated from the observed migration velocity after correcting for the electroosmotic flow rate (m). The effect of buffer pH is consistent with the increase in ionization of the surface silanol groups at higher pH. Entry A represents pretreatment of the column surface with CTAB but a normal buffer solution was used during electrophoresis. The surface retains some of the surface-adsorbed CTAB that is not easily washed out by an aqueous buffer. Consequently, the mobility of mesityl oxide there is different from that observed for an untreated column. Dynamic control is achieved by using different CTAB concentrations in the anodic (high concentration) and cathodic (low concentration) buffer vials, as depicted in entries B and C. This basically forces two stationary CTAB concentration zones inside the capillary, with the ratio of the zone lengths determined (via self-regulation) by the electrophoretic mobility of CTAB. We note that entries B and C for m are not zero, as predicted above. The fact that all these m values are nearly identical despite the difference in pH and in CTAB concentration confirms that the sole regulating factor is the electrophoretic mobility of CTAB. We are unable to find a literature value for m' for CTAB (monomers) or to experimentally measure it, since CTAB even at low concentrations affects the electroosmotic flow rate. Our results show that m'(CTAB) = + 2.4 $\times$ 10^{-4} cm^2 V^{-1} s^{-1} (to balance the observed m for mesityl oxide), which is a reasonable

value.

The observed mobility m' for benzoic acid is relatively invariant over the entire range of conditions, except when the CTAB concentration is the highest (300 μM). This justifies the choice of benzoate as an ionic marker. At high CTAB concentrations, ion-pairing between CTAB and benzoate will slow down the migration of the latter, as depicted in Table I. The main point is that for conditions B and C, there is no noticeable influence on the mobility of benzoate. This of course is a necessary condition for the present scheme to be of practical utility.

A subtle feature in the establishment of the step concentration gradient inside the column for self-regulated control of electroosmotic flow is the need for pretreatment of the column surface with CTAB. Table I shows a clear difference between an untreated column (0 μM CTAB) and a treated column (condition A). We found that without pretreatment, the steady-state concentration front is difficult to achieve, as evidenced by the irreproducible migration times of both mesityl oxide and benzoic acid. Since CTAB both migrates and is adsorbed, the movement of the concentration boundary is complex in a bare column and may require unacceptably long equilibration times to reach a steady state. Pretreatment essentially first covers up all readily accessible adsorption sites to achieve a steady state in adsorption-desorption. The concentration step can then become self-regulating solely by electroosmotic and electrophoretic forces.

The advantages of the self-regulating control scheme are illustrated in Table II.

Table 2. Matrix effect on the migration times (s) of benzoate and naphthoate in dynamic and static systems

CTAB (μM)	A			B		
benzoate	i	ii	iii	i	ii	iii
0	195	234	234	208	210	212
50	195	156	155	219	223	231
300	153	147	147	222	229	224
800	149	142	142	232	233	230
5000	142	133	130	230	236	236
naphthoate	i	ii	iii	i	ii	iii
0	230	268	277	240	242	246
50	228	181	179	246	255	264
300	175	170	170	257	268	261
800	165	164	165	270	272	266
5000	142	133	130	276	278	279

A: static system; B: dynamic system; i, ii, iii, consecutive injection;
buffer: 10 mM bicarbonate at pH 6; see Table 1 for exact conditions.

Two analyte ions, benzoate and naphthoate, are used in this study. These are dissolved in "sample" solutions with different concentrations of CTAB to simulate different sample matrices. For the static buffer system, A, the migration times change significantly during each series of triplicate injections. This is because the sample matrix (CTAB added) affects the net charge of the column surface and in turn the electroosmotic flow rate. For the sample solutions with no added CTAB, the pre-adsorbed surface positive charge decreases (partially washed away) to promote bulk flow from anode to cathode. This retards the migration of the analyte ions towards the anode in subsequent runs. When CTAB is present at > 50 μM, the additional adsorption increases the positive charge on the column walls to promote bulk flow from cathode to anode. The migration times of the two analytes therefore decreased. The change is most obvious between the first and second runs, since the column walls eventually saturate due to local charge repulsion.[22] Most noteworthy is the fact that the different sample matrices caused different migration times, both for the first injection and for subsequent injections. The decrease in migration time for high CTAB concentrations is again consistent with an increase in surface positive charge on the column.

Once the column is equilibrated in the dynamic control mode (B in Table II), the migration times become remarkably constant over a series of injections. The migration times also change only slightly for sample matrices containing a wide range of CTAB concentrations. There is a slow increase in migration times

towards the highest CTAB concentration samples. The increase is more pronounced for naphthoate than for benzoate. This can be explained by minor adsorption and/or ion pairing between CTAB and the anions, reducing the net electrophoretic mobility. Naphthoate, being more hydrophobic, shows a larger effect. This observation is identical to the change in m' reported in Table I for high CTAB concentrations.

The studies here clearly show that the electroosmotic flow rate can be dynamically controlled in a self-regulating mode. The scheme presented is easy to implement. The final electroosmotic flow rate is not zero, but is equal and opposite to the electrophoretic mobility of the controlling ion (CTAB here). Applications are restricted to analyte ions that do not adsorb or ion pair with the matrix ions, or otherwise change in their fractional charge in different matrices, because the present scheme controls the electroosmotic but not the electrophoretic effects of the sample matrix. Implementation depends on having two buffer solutions that by themselves cause electroosmotic flow in opposite directions. The controlling ion needs to be at low enough concentrations to avoid significant complexation or ion pairing with the analyte ions, but at high enough concentrations to overwhelm the amount of surface charge generated by the sample matrix. If a surfactant is used, the concentration of the controlling ion must be below the critical micellar concentration to achieve steady state. If possible, the controlling ion should be of the same charge as the analyte to minimize complexation or ion pairing. However,

the controlling ion is limited to cations for the case of fused silica capillaries in order to compensate for the inherent negative surface charge.

It is interesting that the CTAB concentration front here is stationary even though typical concentration fronts accompanying a change in electrolytes[17] move at exactly the electroosmotic flow rate. The concentration of CTAB here is negligible compared to the concentration of the main ionic components in the buffer solutions (10 mM bicarbonate), so that the electric field strength is essentially uniform across the column. If however the CTAB concentration contributes to the local field strength (i.e. if present at high concentrations), or if adsorption of CTAB is not at steady state, a more complex behavior will ensue. Naturally, in the event that the matrix material substantially modifies the column surface, the CTAB front may not be able to adequately compensate for the change, and self-regulation will cease. Further studies will be needed to define the useful working range for such a system. Finally, it has been suggested that mixing can occur when different zones with separate electroosmotic flow coefficients exist along a column, since eddy currents are needed to maintain a single bulk flow.[20] In this and our earlier studies,[23] no broadening was observed. This is probably because the maximum theoretical plates achieved is around 100,000. Studies in systems exhibiting much higher plate numbers are needed to evaluate the zone broadening contributions in the present scheme.

References

1. Jorgenson, J. W.; Lukacs, K. D. Science 1983, 222, 266-272.
2. Kuhr, W. G. Anal. Chem. 1990, 62, 403R-414R.
3. Dose, E. V.; Guiochon, G. A. Anal. Chem. 1991, 63, 1154-1158.
4. Kaneta, T.; Tanaka, S.; Yoshida, H. J. Chromatogr. 1991, 538, 385-391.
5. Huang, X.; Luckey, J. A.; Gordon, M. J.; Zare, R. N. Anal. Chem. 1989, 61, 766-770.
6. Schwer, C.; Kenndler, E. Anal. Chem. 1991, 63, 1801-1807.
7. Jorgenson, J. W.; Lukacs, K. D. Anal. Chem. 1981, 53, 1298-1302.
8. Kuhr, W. G.; Yeung, E. S. Anal. Chem. 1988, 60, 2642-2646.
9. Hjerten, S. J. Chromatogr. 1985, 347, 191-198.
10. Fujiwara, S.; Honda, S. Anal. Chem. 1986, 58, 1811-1814.
11. Lee, C. S.; Blanchard, W. C.; Wu, C. T. Anal. Chem. 1990, 62, 1550-1552.
12. Lee, C. S.; McManigill, D., Wu, C. T.; Patel, B. Anal. Chem. 1991, 63, 1519-1523.
13. Wu, C. T.; Led, C. S.; Miller, C. J. Anal. Chem. 1992, 64, 2310-2311
14. Chang, H. T.; Yeung, E. S. J. Chromatogr. 1992, 608, 65-72.
15. Lee, T. T.; Yeung, E. S. Anal. Chem. 1991, 63, 2842-2848.
16. Terabe, S.; Otsuka, K.; Ando, T. Anal. Chem. 1985, 57, 834-841.
17. Huang, X.; Gordon, M. J.; Zare, R. N. Anal. Chem. 1988, 60, 1837-1838.

18. Hjerten, S. Chromatogr. Rev. 1967, 9, 122-129.

19. Tsuda, T. J. Liq. Chromatogr. 1989, 12 (3), 2501-2514.

20. Helmer, J. C.; Chien, R. L. Anal. Chem. 1991, 63, 1354-1361.

21. Chien, R.; Burgi, D. S. Anal. Chem. 1992, 64, 489A-496A.

22. Pfeffer, W. D.; Yeung, E. S. Anal. Chem. 1990, 62, 2178-2182.

23. Whang, C. W.; Yeung, E. S. Anal. Chem. 1992, 64, 502-506.

CHAPTER 5

ON-COLUMN DIGESTION OF PROTEIN FOR PEPTIDE MAPPING BY CAPILLARY ZONE ELECTROPHORESIS WITH LASER-INDUCED NATIVE FLUORESCENCE DETECTION

A paper published in the Analytical Chemistry[1]

Huan-Tsung Chang and Edward S. Yeung[2]

Introduction

The development of improved analytical methods for protein identification and characterization still plays an important role in the field of biochemical and physiological investigation. Peptide mapping is one of the most powerful methods for the structural determination of proteins. In peptide mapping, the sample protein can be selectively cleaved by enzymes (e.g. trypsin[1,2] or pepsin[3,4]) or chemical reagents (e.g. cyanogen bromide[5] or 5-nitro-5-thiocyanobenzoic acid[6]). There are two sets of typical conditions, namely homogeneous and heterogeneous reactions, that digestion by using enzymes can be performed. In the homogeneous environment, generally 1 part of enzyme digests 20 parts of protein in solution at 37° C for

---

[1] Reprinted with permission from Analytical Chemistry1993, 65, 2947-2951.

[2] Author for correspondence.

several hours. In the case of heterogenous reaction, the protein passes through a column on which the enzyme is immobilized. The advantages of heterogeneous reaction over homogeneous reaction are that digestion is faster, the enzyme is more stable and has a longer lifetime, and the enzyme can be used repeatedly. The problems of digestion are the restrictions placed upon the amount of protein and enzyme to be used, poor reproducibility, incomplete digestion, and over-digestion. Hence, one needs optimum control of the pH, temperature, protein: enzyme ratio, and reaction time in order to get an efficient digest and a reproducible peptide map.

After digestion, a separation method has to be used to characterize the fragments. A wide variety of analytical separation techniques have been used for the separation of peptide fragments from digests of proteins. High performance liquid chromatography (HPLC),[7-9] CZE,[10-13] slab gel electrophoresis[14] and thin layer chromatography[15] are the most popular approaches in this field. Among these techniques only microcolumn HPLC and CZE have the requisite efficiency and sensitivity for the analysis of pmole amounts of protein hydrolysate. CZE is gaining acceptance in recent years because of its high speed, friendly environment for biomolecules, simplicity and high efficiency.

Laser-induced fluorescence[16] (LIF) has been used for detection in CZE to offer high sensitivity and selectivity. Cobb and Novotny[17] used LIF to detect arginine-containing peptides and tyrosine-containing peptides after derivatization with 4-

methoxy-1,2-phenylenediamine. Amankwa and Kuhr[18] combined a trypsin-modified fused-silica capillary microreactor, CZE, and LIF to obtain the peptide map of the 2,3-naphthalenedialdehyde-derivatized β-casein. Yeung and coworkers[19,20] used indirect fluorescence to detect the native peptide fragments of β-casein and bovine serum albumen. Recently, native fluorescence (LINF) has been applied to detect amol levels of the tryptic digest of conalbumin and rFIII.[21] Although these methods are quite sensitive for detecting peptide fragments, the total amount of protein used for digestion is still in the microgram to high nanogram range, which still does not quite meet the requirements for the analysis of trace amounts of biological sample. Also, there are inherent problems with methods involving derivatization reactions, such as extra handling, contamination, incomplete tagging, multiple tagging, and sample dilution.

To overcome these shortcomings, we have developed a novel technique to separate and detect peptide fragments which are digested on the same column. In this procedure, pepsin is used to digest low fmol amounts of β-lactoglobulin on the column. Then, CZE and LINF are applied to separate and detect the peptide fragments. The advantages of this method are its simplicity, high sensitivity, high selectivity, efficient operation, and high speed.

Experimental Section

CZE instrument and data collection

The experimental setup is similar to that described in Ref. 16. Briefly, a high-voltage power supply (Glassman High Voltage, Inc., Whitehorse Station, NJ; EH series 0-40 kV) was used to drive the electrophoresis. A 50 μm i.d. or 16 μm i.d. fused-silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) was used. The total length is 80 cm and the effective length is 60 cm. The entire electrophoresis and detection system is enclosed in a sheet-metal box with HV interlocks.

The 275.4 nm line from an argon-ion laser (Spectra Physics, Inc., Mountain View, CA; Model 2045) was isolated from other lines with a prism and focused with a 1 cm focal length lens into the detection region on the window of the capillary tubing where the polyimide coating had been burned off. A UG-1 absorption filter (Schott Glass Technologies, Inc., Duryea, PA) was applied to prevent scattered light from reaching the photomultiplier tube. The signal from LINF was amplified by a current amplifier (Keithley, Inc., Taunton, MA; Model 427) and the data was collected via a 24-bit A/D interface at 5 Hz (Justice Innovation, Palo Alto, CA; Model DT 2802) and stored on a computer (IBM, Boca Raton, FL; Model PC/AT 286).

Sample injection and on-column digestion

Before sample injection, the capillary tubing was equilibrated for 1 hr at 20 kV in the pH 1.9 buffer solution containing 5% (v/v) formic acid and 0.02% (w/v) Tween 20. β-Lactoglobulin and pepsin were prepared with pH 1.9 buffer solution to 5×10^{-5} M and 2.5×10^{-6} M, respectively. Hydrodynamic injection was performed to inject sample protein and the enzyme at 30-cm height. For the 50-μm capillary, pepsin was injected for 60 s, then β-lactoglobulin was introduced for 5 s. For the 16-μm capillary, injection times were 360 s and 30 s, respectively. Even though the sample vial contains a few μL of protein, the injection process only takes up a few nL of material each time. The capillary was put back into the running buffer and electrophoresis started at 10 kV for 20 s (10 s for 16-μm capillary) to allow good overlap between the zones containing the sample protein and the enzyme. Then, the capillary was put into another buffer vial in a water bath at 37° C for 1.5 hr to digest. Finally, the capillary was moved back to the original buffer vial to start the separation.

Materials

β-Lactoglobulin (Bovine milk, MW 36,500) and pepsin (porcine stomach mucosa) were purchased from Sigma Chemical CO. (St. Louis, MO). 88% formic acid was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Tween 20 was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Results and Discussion

Mixing between β-lactoglobulin and pepsin on column

According to the Poiseuille equation,[22] the linear flow rate (V_{hd}) of an analyte in the column can be calculated by using equation 1.

$$V_{hd} = r\, g\, r^2\, \Delta h/8\, h\, L \qquad (1)$$

where r is the solution density, g is the gravitational force constant, r is the internal radius of the capillary, Δh is the difference in height between the two ends of the capillary, h is the solution viscosity and L is the capillary length. In turn, the length (l) of a plug injected into the capillary for time (t) can be calculated from equation 2.

$$l = V_{hd} \times t \qquad (2)$$

The lengths of the plugs of pepsin and β-lactoglobulin injected into column can be obtained for a 50-μm capillary and the results are 1.9 cm and 0.16 cm, respectively, when the hydrodynamic injection times at 30-cm height are 60 s and 5 s, respectively. These zones initially do not mix to any significant degree. The movements of these two plugs when high voltage is applied to the capillary are

dependent on the electroosmotic flow and electrophoretic mobilities of the analytes. At pH 1.9 , β- lactoglobulin (pI = 5.3) has partial positive charge and pepsin (pI = 1) is almost neutral. The net flow of β-lactoglobulin will be the sum of electrophoretic flow and electroosmotic flow, which are in the same direction, and the flow of pepsin will be depend on the electroosmotic flow only. The flow of β-lactoglobulin should be fast enough to drive itself to catch up with the plug of the pepsin because the electroosmotic flow is very small compared to the net flow of β-lactoglobulin at such low pH. By injecting the two proteins separately, we find that the mobilities of β-lactoglobulin and pepsin are 3.43×10^{-4} and 1.70×10^{-4} cm^2/V s, respectively. After running at 10 kV for 20 s , the movement of the plugs can be calculated as 0.86 cm and 0.34 cm for β-lactoglobulin and pepsin, respectively. Here we assume there is no change in the length of the plug during the initial electrophoresis of 20 s at high voltage. The mixing between two plugs as a function of time is shown in Fig. 1. The reason for using a longer plug for pepsin and running for 20 s is to make sure there is a good overlap between the plugs to get an efficient digestion. After all, the electroosmotic flow rate may change depending on the column surface and the predicted locations of the plugs after 20 s are subject to errors.

On-column digestion

The use of pepsin to cleave proteins containing two adjacent hydrophobic

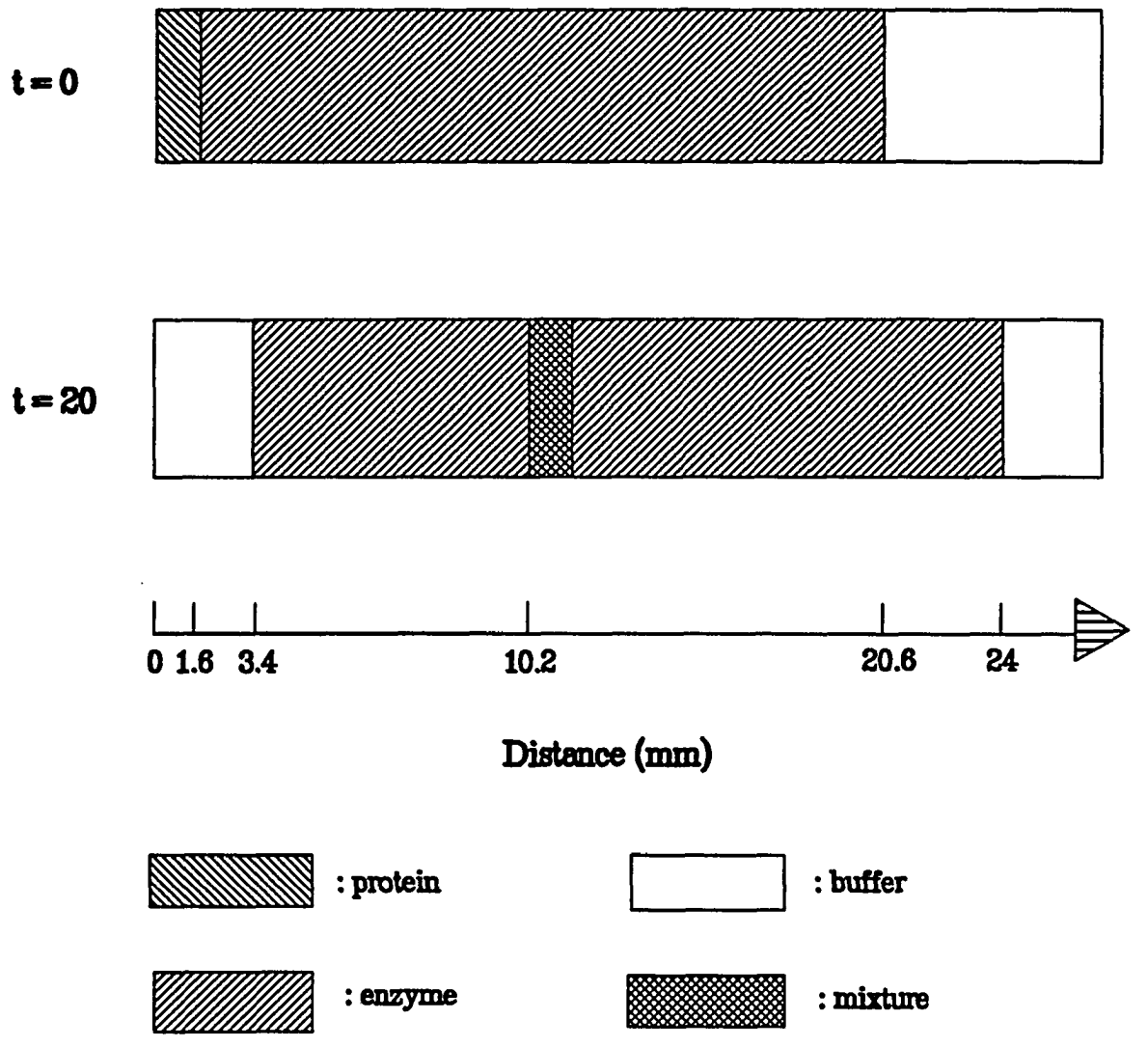


Figure 1. The mixing zone of β-lactoglobulin and pepsin on column as a function of running time t (s).

residues (especially phenylalanine) provides two distinct advantages on digestion: the enzyme is active at low pH, so that prior denaturation of the substrate is rarely required, and disulfide exchange reactions are negligible. It also provides advantages for on-column digestion. The solution used for digestion is well suited as the running buffer in CZE separation because the electroosmotic flow rate is very small and the problem of coating of proteins on the capillary wall is not serious at such low pH. There is no need to add denaturing reagents which may compromise the separation performance. The slow movement and lower native fluorescence of pepsin compared to the peptide fragments give a very clean background as show in Fig. 2A, in turn minimizing interference with the peptide map. Also, the reaction time can be as short as 1.5 hr, so that peak broadening resulting from diffusion can be minimized. The disadvantages of pepsin digestion are that it is very sensitive to reaction time and to the ratio of sample protein to enzyme. Another restriction of on-column digestion by using pepsin at such low pH is that most likely acidic proteins are not suitable because of the difficulty in mixing the protein and enzyme zones (i.e. when they are both neutral) in the scheme shown in Fig. 1.

Native fluorescence

The native fluorescence of proteins is a well-known behavior. Especially, proteins containing tryptophan (W), tyrosine (Y) and phenylalanine (F) have useful levels of fluorescence when they are excited at 275 nm. Lee and Yeung[23] showed

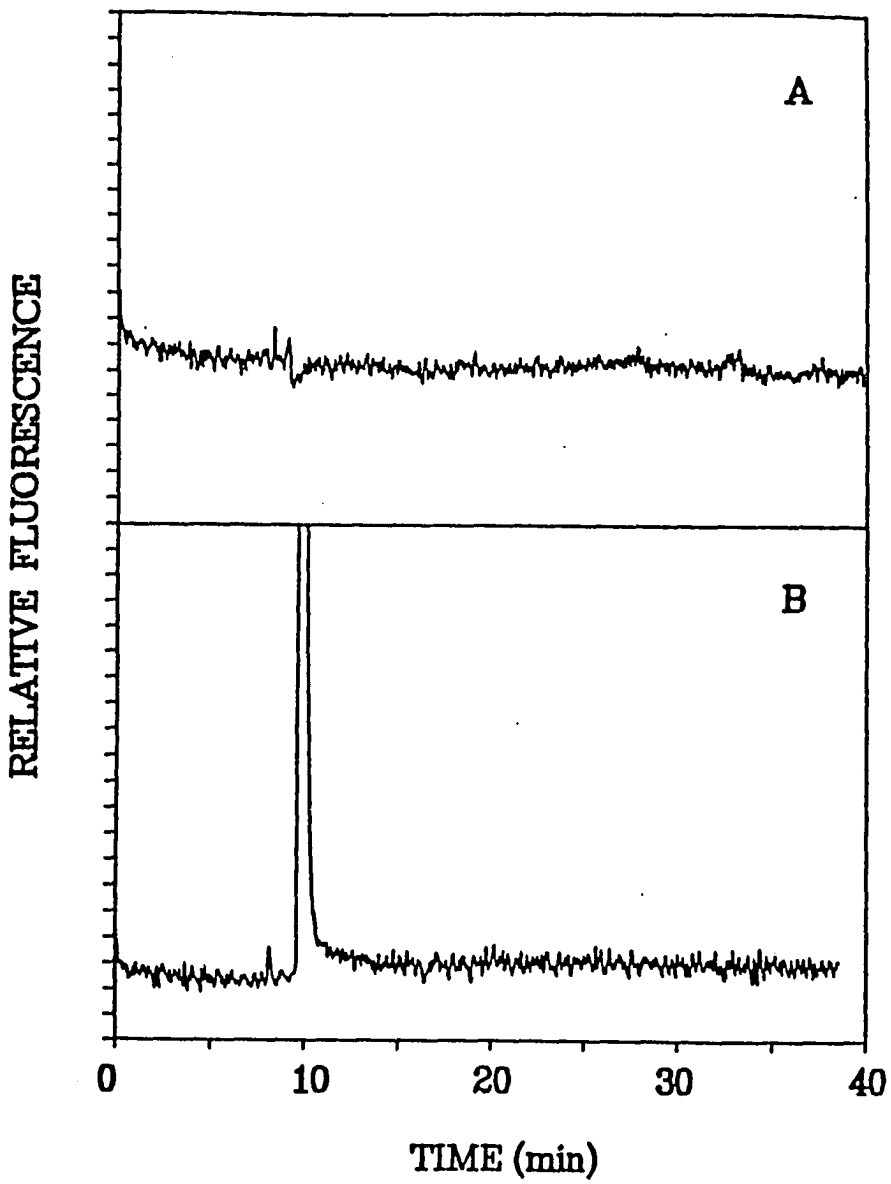


Figure 2. Electropherogram of (A) 2.5 x 10^{-6} M pepsin and (B) 5 x 10^{-5} M β-lactoglobulin after staying on the 50-μm column for 1.5 hr. Running buffer: 5% formic acid and 0.02% Tween 20; pH 1.9; total length and effective length of capillary: 80 cm and 60 cm; applied voltage: 20 kV; excitation wavelength: 275.4 nm.

that the detection limit of some proteins can be below 10^{-9} M in alkali condition. Our model protein, β-lactoglobulin, contains 4 F, 4 Y and 2 W and should have intensive fluorescence. Although acid and base quenching[24] of the fluorescence of W, Y, and F are well known phenomena, there is still adequate fluorescence intensity under our experimental conditions if a UV laser source is used to excite the protein. The result is shown in Fig. 2B. It is obvious that LINF should be a good tool to detect the peptide fragments. Hence the application of LINF for on-column digestion and CZE separation of the peptide fragments should offer the advantages of simplicity, sensitivity and selectivity. While other detection methods, such as UV and fluorescence derivatization, have been used for many years in peptide mapping, UV shows poor sensitivity and selectivity and fluorescence derivatization suffers from the more complicated fingerprint because of side reactions and contamination from the derivatization reagent. It is also not obvious how these other methods can be applied to such small samples.

CZE for peptide mapping

Highly efficient separation of proteins,[25] nucleotides,[26] peptides[27] and amino acids[28] make CZE a powerful tool for the study of biomolecules. Adsorption of the proteins on the capillary wall is a serious problem. This can lead to variable retention times, and band broadening and tailing, especially for separations at the intermediate pH range. Several approaches havd been proposed to reduce the

interaction between the protein and the capillary wall, such as running at low pH,[29] high pH[30] and very high ionic strength,[31] and coating the column.[32] The low pH buffer used for digestion in this work provides highly efficient separations because of low electroosmotic flow rate and weak interaction between the peptide fragments and the capillary wall. The separation of the peptide fragments after on-column digestion is shown in Fig. 3A and 3B, which are quite similar to those from off-column digestion shown in Fig. 4A and 4B. These electropherograms are plotted on an expanded scale to uncover the small fragment peaks. The first peak in those figures represents the undigested protein. From comparison of the relative intensities of the first peak, off-column digestion seems to be more complete. The bands are also sharper for off-column digest, showing a small amount of broadening during the 1.5 hr reaction time. This is most obvious for the peak structures starting around 12 min in Figures 3 and 4. The observed number of theoretical plates are around 40,000 and 100,000 for on-column and off-column digests, respectively, for the fragment at 15 min in Fig. 3A. Even though the resolution is lower in Fig. 3, one can still identify the partially overlapped features with excellent match to those in Fig. 4. In order to confirm that these are true peptide maps, we note that the two blanks, pepsin and β-lactoglobulin on column, shown in Fig. 2, are entirely free of features in the same region as the peptide fragments. The protein peaks in Fig. 3 tell us that 1.5 hours is needed for the reaction but the digests still contain α-lactoglobulin. The percentages of digestion are all around

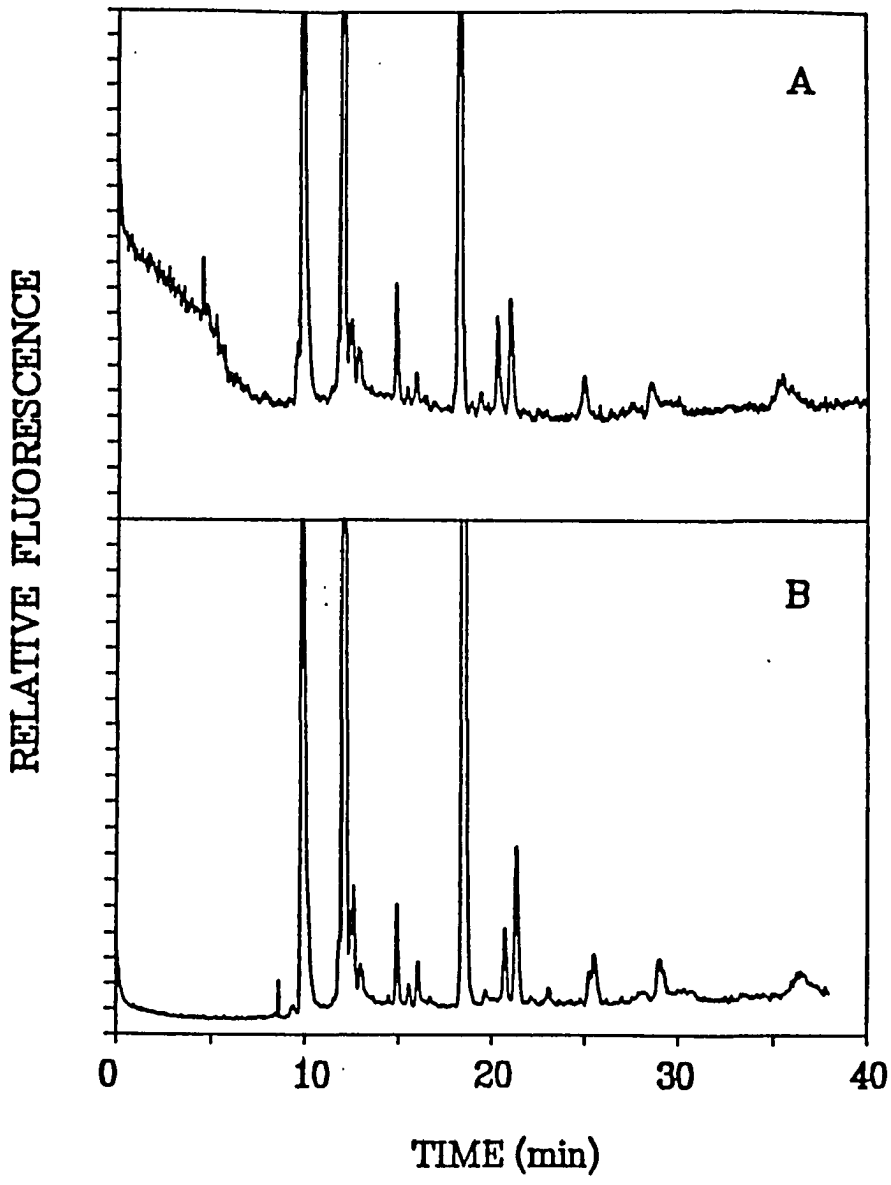


Figure 3. Replicate peptide mapping by on-column digestion of β-lactoglobulin with pepsin in a 50-μm capillary. Conditions are the same as those in Fig. 2. Reaction times: (A) 1 hour, (B) 1.5 hours. The off-scale features at 12 min are split peaks when plotted on scale.

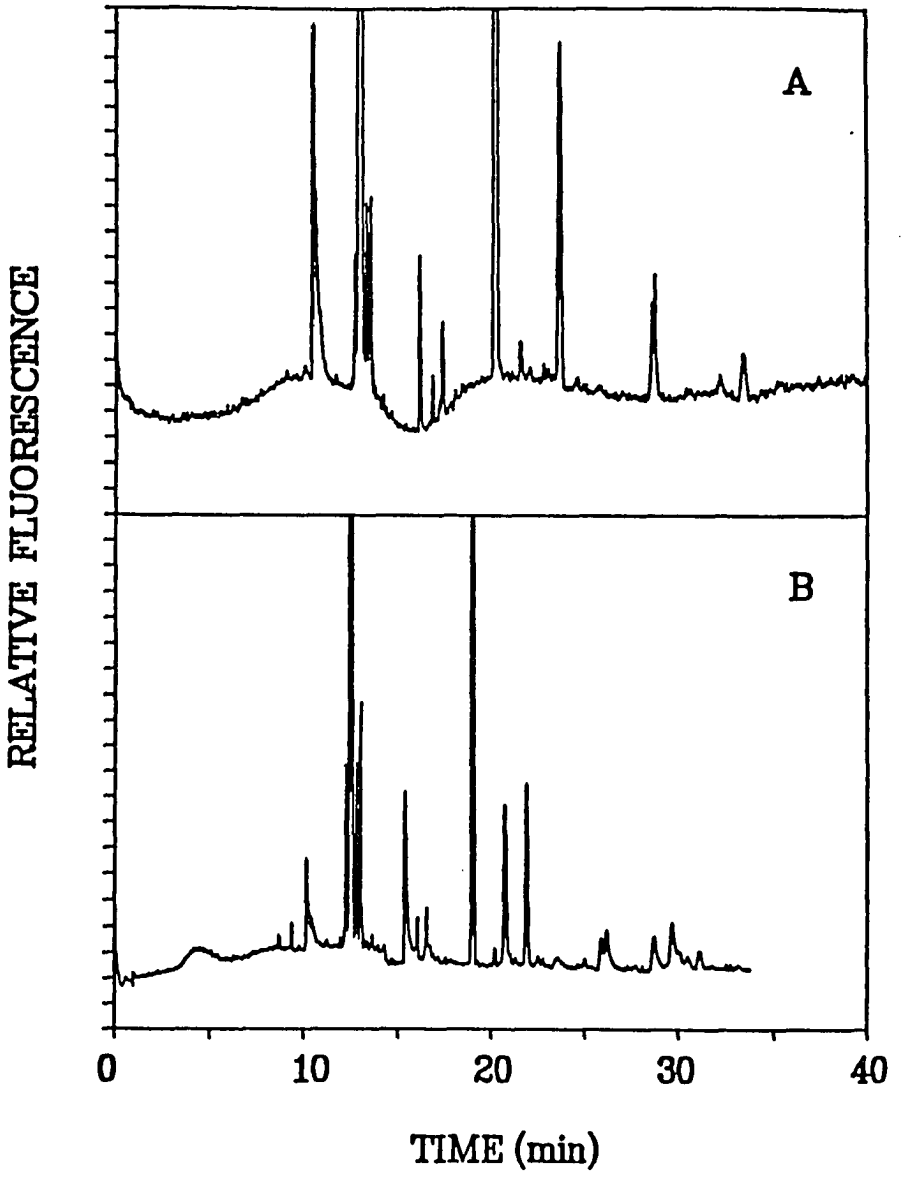


Figure 4. Replicate peptide mapping by off-column digestion of β-lactoglobulin with pepsin in a 50-μm capillary. Conditions are the same as those in Fig. 2, except that 1.5 hours was used in both cases. The off-scale features at 12 min are split peaks when plotted on scale. Migration times in B are shorter due to a change in the electroosmotic flow rate.

70-80% if we simply compare the intensity of the first peak to the peptide peaks. The rough estimation is close to the result (67%) given in Simpson's work.[4] The stronger peaks should correspond to those fragments containing F, Y and W which will have more intense fluorescence. In comparison with the electropherograms of Simpson's work where SDS-PAGE and UV (λ_{max} = 215 nm) are used to separate and detect peptide fragments, our results are obtained in less time, with higher resolution, and are more selective. Our results, as listed in Table I, also show that on-column peptide mapping is very reproducible and produces nearly identical fragments as off-column digests. Variations in migration times are mainly due to changes in electroosmotic flow rates in different columns. In Table I, we have simply normalized the migration times with respect to the protein peak at 9.8 min in Fig. 3A. The normalized migration times are reproducible to 0.3 min, or 1.4%. The identification of these peaks by e.g. mass spectrometry is however beyond the scope of our work.

To illustrate the applicability of this procedure to ultrasmall samples, we repeated the experiments using a 16-μm capillary. The separation of an off-column digest is almost identical to before, as shown in Figures 5A and B. There is an increase in migration times due to a change in electroosmotic flow rate. A well-defined on-column peptide map is shown in Fig. 6A and B, where only 10 fmol of protein was used. There is a direct correlation between the features in Fig. 6 and those in Fig. 3, despite the change in electroosmotic flow rates. Again,

Table 1. Reproducibility of normalized migration times (min) in peptide maps

50-μm capillary				16-μm Capillary			
a	b	c	d	e	f	g	h
9.8	9.8	9.8	9.8	13.8	13.8	13.8	13.8
11.7	11.8	11.9	11.8	15.1	14.9	15.7	15.4
11.9	12.0	12.0	11.9	15.4	15.2	16.0	15.7
12.1	12.2	12.2	12.1	15.6	15.4	16.3	15.9
12.3	12.4	12.3	12.2	15.8	15.6	16.4	16.1
12.5	12.6	12.4	12.3	16.1	15.9	16.8	16.4
12.8	12.9	12.7	12.5	19.3	19.0	20.1	19.7
14.9	15.1	15.1	14.8	24.1	23.9	25.1	24.5
15.4	15.6	15.8	15.4	28.0	27.7	29.2	28.4
15.8	16.0	16.2	15.9	**	**	34.8	33.7
18.3	18.5	18.9	18.3	**	**	36.4	25.6
20.3	20.8	20.2	20.2	**	**	38.7	37.9
21.0	21.4	22.1	21.1	**	**	40.4	39.4
25.0	25.3	26.7	24.7				
27.6	28.2	26.8	24.9				
28.4	29.0	29.9	27.6				
30.0	30.7	31.2	28.5				
35.7	36.0	*	*				

* not recorded

** at detector noise level

Peptide maps are displayed in (a) Fig. 3A, (b) Fig. 3 B, (c) Fig. 4A, (d) Fig. 4B, (e) Fig. 5A, (f) Fig. 5B, (g) Fig. 6A, and (h) Fig 6B. The average standard deviation for a given peptide peak is 0.3 and 0.4 min for all runs for the 50-μm and 16-μm capillary, respectively.

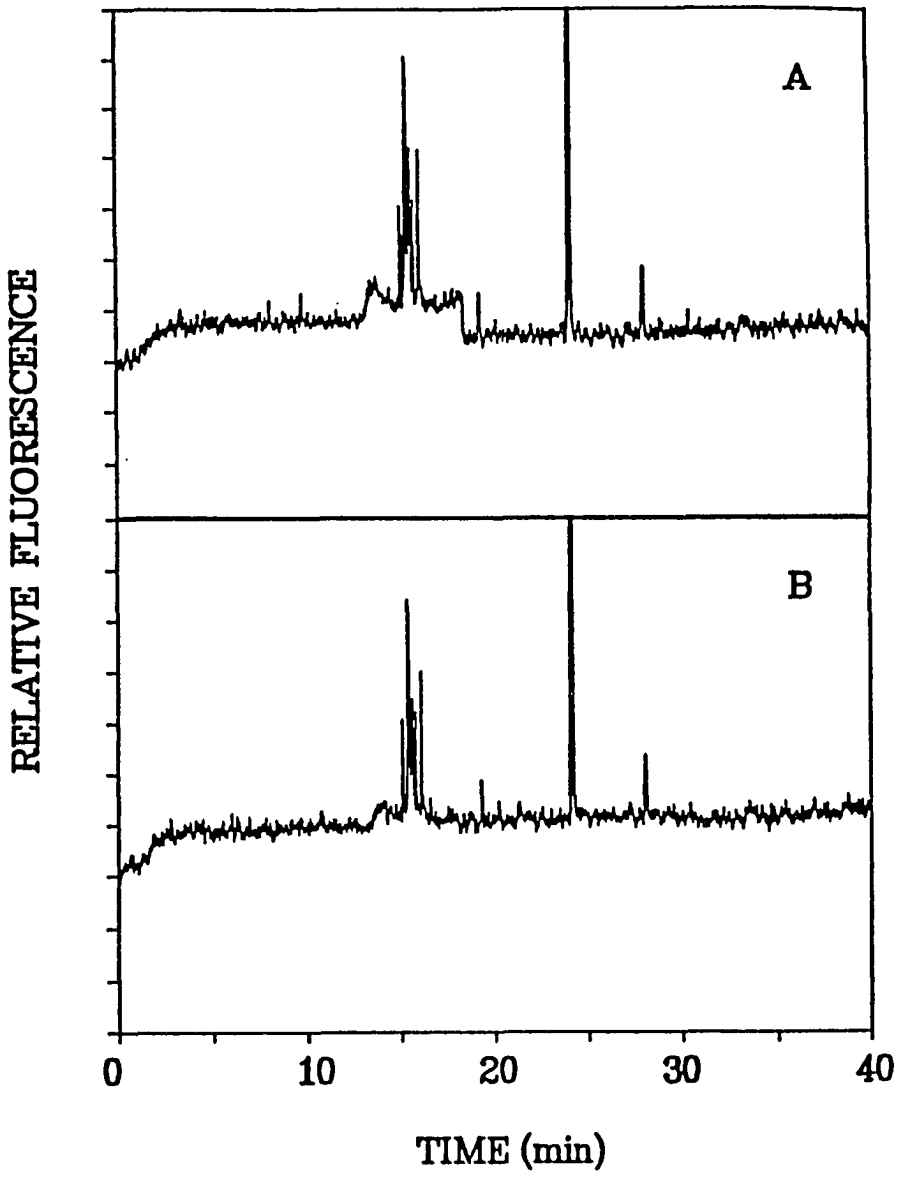


Figure 5. Replicate peptide mapping by off-column digestion of β-lactoglobulin with pepsin in a 16-μm capillary. Conditions are the same as those in Fig. 4.

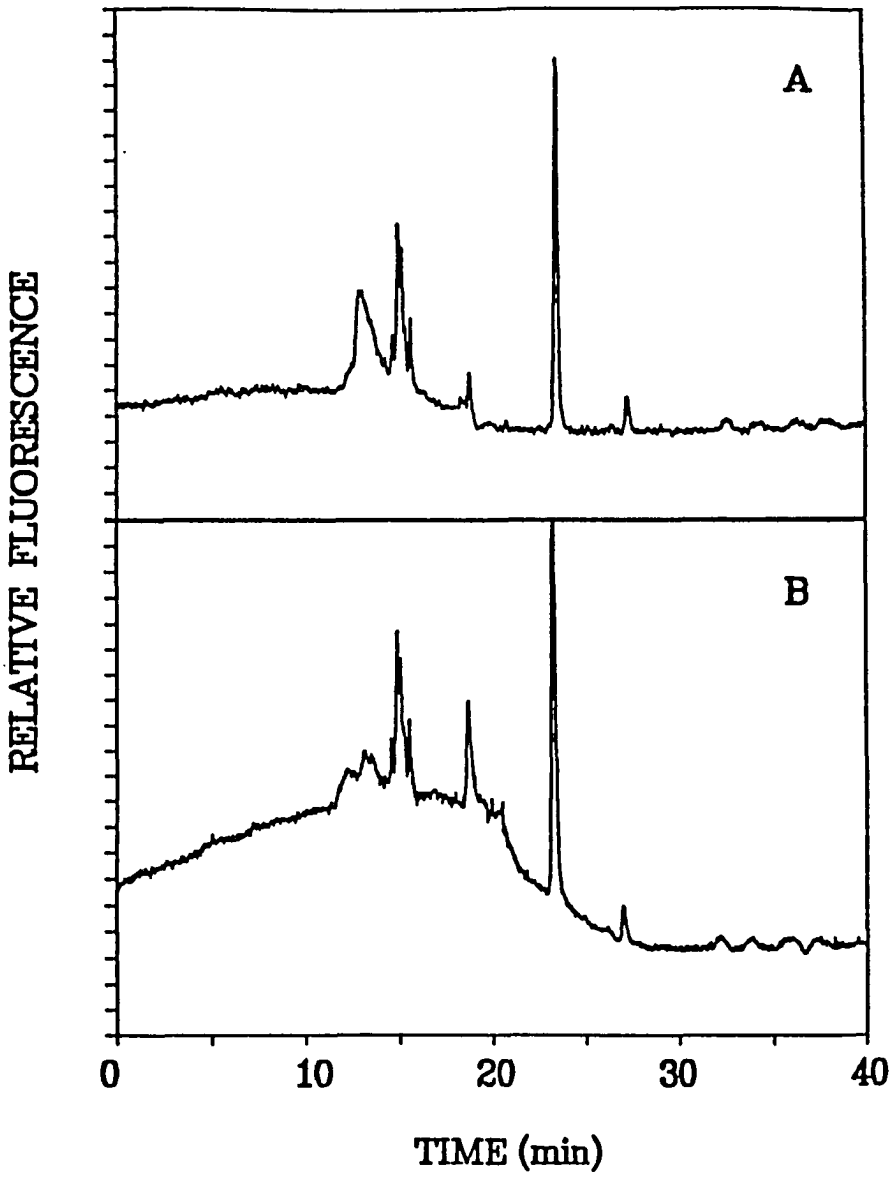


Figure 6. Replicate peptide mapping by on-column digestion of β-lactoglobulin with pepsin in a 16-μm capillary. Conditions are the same as those in Fig. 4.

reproducibility of the migration times are good after normalization to the protein peak at 13.8 min in Fig. 5A. To directly correlate with the fragment peaks obtained in 50-μm capillaries, one will need a more sophisticated normalization scheme[33] to account for changes in both electroosmotic and electrophoretic contributions.

We note that the injected plug length in Fig. 5 is around 0.61 that in Fig. 4 and the amount injected is 1/16 less. Despite the longer injection times, Eq. 1 and 2 actually predict a narrower zone for the protein. The number of theoretical plates in Fig. 5 is around 200,000. The observed peak widths are comparable for Fig. 6 and Fig. 3, showing that diffusion during incubation is the main source for broadening. We also tried similar experiments with trypsin on column. The results are less impressive because the digestion time has to be 15-20 hours, causing broad peptide peaks that are not completely resolved. We note that the digestion times of 1.5 and 15 hours for pepsin and trypsin, respectively, are limited by enzyme kinetics and not by diffusion of the proteins towards each other. This implies that one can introduce viscous polymers such as agarose, polyacrylamide or methylcellulose[34] with the enzyme plug to prevent serious band broadening during digestion. Indeed, agarose-immobilized trypsin is commercially available and is functionally similar to free trypsin. Trypsin also works at conditions that are not ideal for CZE of peptides, complicating the single-column approach.

The lowest amount of β-lactoglobulin used for digestion is 10 fmole (0.37 ng). Table II shows the comparison of the total amount of protein used for digestion in

Table 2. Comparison of the total amount of protein used for digestion

Digestion	Fluorescence	Amount of protein used (μg)	Reference
heterogeneous	derivative	0.05	10
heterogeneous	derivative	10	17
heterogeneous	derivative	60	18
on-column (50 μm)	native	6 x 10-3	this work
on-column (16 μm)	native	3.7 x 10-4	this work

this work with the other published results. It is obvious that the amount of protein used for on-column digestion is much lower here than other methods. Although Amankwa and Kuhr[18] showed that heterogenous digestion of protein by passing it through immobilized enzyme can offer advantages such as stability, longer lifetime and multiple use of enzyme, it still suffers from the inconvenience in preparing the immobilized trypsin column, large actual volume and amount of sample to be digested, and the need to derivatize the peptides for detection afterwards. Fluorescence derivatization may also create chemical noise due to multiple tagging sites and impurities. A practical consideration is that digestion can be performed

without tying up the CE instrument and thus lengthening the cycle time. For example, one can use 3 separate capillaries and start the cycle depicted in Figure 1 every 45 minutes. While one separation is proceeding on the CE instrument, 2 other samples are being digested in the other capillaries in the water bath. Differences in migration times among capillaries can be compensated for as discussed previously[33] and as implemented in Table I.

As is clearly indicated in the experimental section, the concentrations of protein used here are similar to previous work but the amounts are much lower. There are quite a few situations where this feature is important. First, when the available sample is limited, such as in the case of single-cell analysis,[35,36] a one-step scheme is absolutely essential. This also applies when one wants to conserve the sample (e.g. valuable or rare preparations) while allowing a few nL to be withdrawn for characterization. For single-cell studies,[36] it may be eventually possible to directly couple the initially separated proteins into the digestion capillary to provide peptide maps. Second, enzyme kinetics requires that digestion be performed at moderate concentrations such as those used here. At low concentrations, reaction times will be unacceptably long and additional fragmentation of the peptides may occur. When one has e.g. a 1-μL sample at a low concentration, there is a critical difference between first concentrating the protein on the column and applying our scheme versus performing digestion on the original sample, i.e. off column, and then concentrating the products on column to enhance detection. Third, our

scheme is ready for interfacing with capillary isotachophoresis[37] and capillary isoelectric focusing[38], where the sample volumes are in the nL range. Collection of those analyte zones for off-column peptide mapping will involve substantial dilution.

Finally, we need to recognize that Fig. 6 represents a useful map, since most of the peptide fragments identified when using larger samples (Fig. 3) are observed. In particular, the set of 4 peaks between 31 to 39 min in Fig. 6 are reproducible features. Naturally, the detection of a particularly favorable fragment (e.g. the one near 24 min) is possible at much lower levels. We have already shown that sub-attomole levels of tryptophan[23] or proteins[21] can be detected by LINF. Future work may allow 1-10 amol quantities of proteins to be mapped. On the other hand, LINF only reveals those peptide fragments that contain aromatic residues. A complete peptide map is not always recorded.

References

1. Tarr, G. E.; Crabb, J. W. Anal. Biochem. 1983, 131, 99-107.
2. Dong, M. W., Advances in Chromatography, Marcel Dekker, Inc., New York, Vol. 32, 1992, pp. 22-51.
3. Geisow, M. J.; Beaven, G. H. Biochem. J. 1977, 161, 619-625.
4. Ward, L. D.; Ried, G.; Moritz, R. L.; Simpson, R. J. J. Chromatogr. 1990, 519, 199-216.

5. Gross, E. Methods Enzymol. 1967, 11, 238-255.

6. Degani, Y.; Patchornik, A. Biochmistry 1974, 13, 1-11.

7. Simpson, R. J.; Moritz, R. L.; Begg, G. S.; Rubia, M. R.; Nice, E. C. Anal. Biochem. 1989, 177, 221-236.

8. Leadbeater, L.; Ward, F. B. J. Chromatogr. 1987, 397, 435-443.

9. Nice, E. C.; Lloyd, C. J.; Burgess, A. W. J. Chromatogr. 1984, 296, 153-170.

10. Cobb, K. A.; Novotny, M. V. Anal. Chem. 1989, 61, 2226-2231.

11. Grossman, P. D.; Colburn, J. C.; Lauer, H. H.; Nielsen, R. G.; Riggin, R. M.; Sittampalam, G. S.; Rickard, E. C. Anal. Chem. 1989, 61, 1186-1194.

12. Aebersold, R.; Morrison, H. D. J. Chromatogr. 1990, 516, 79-88.

13. Nashabeh, W.; Rassi, Z. E. J. Chromatogr. 1991, 536, 31-42.

14. Lu, H. S.; Lai, P. H. J. Chromatogr. 1986, 368, 215-231.

15. Stephens, R. E. Anal. Biochem. 1978, 84, 116-126.

16. Wang, P.; Li, W.; Giese, R. W.; Yeung, E. S. J. Chromatogr. 1992, 608, 73-77.

17. Cobb, K. A.; Novotny, M. V. Anal. Biochem. 1992, 200, 149-155.

18. Amankwa, L. N.; Kuhr, W. G. Anal. Chem. 1992, 64, 1610-1613.

19. Gross, L.; Yeung, E. S. Anal. Chem. 1990, 62, 427-431.

20. Hogan, B. L.; Yeung, E. S. J. Chromatogr. Science 1990, 28, 15-18.

21. Lee, T. T.; Lillard, S. J.; Yeung, E. S. Electrophoresis 1993, 14, 429-438.

22. Lamb, H., Hydrodynamics, Dover, Inc., New York, 1932, pp. 585-586.

23. Lee, T. T.; Yeung, E. S. J. Chromatogr. 1992, 595, 319-325.

24. White, A. Biochem. J. 1959, 71, 217-220.

25. Tsuji, K. J. Chromatogr. 1991, 550, 823-831.

26. Ng, M.; Balaschke, T. F.; Arias, A. A.; Zare, R. N. Anal. Chem. 1992, 64, 1682-1684.

27. Bongers, J.; Lambros, T.; Felix, A. M.; Heimer, E. P. J. Liq. Chromatogr. 1992, 15, 1115-1128.

28. Gassmann, E.; Kuo, J. E.; Zare, R. N. Science (Washington, DC), 1985, 230, 813-814.

29. Bolger, C. A.; Zhu, M.; Rodriguez, R.; Wehr, T. J. Liq. Chromatogr. 1991, 14, 895-906.

30. Zhu, M.; Rodiguez, R.; Hansen, D.; Wehr, T. J. Chromatogr. 1990, 516, 123-131.

31. Chen, F. A.; Kelly, L.; Palmieri, R.; Biehiler, R.; Schwartz, H. J. Liq. Chromatogr. 1992, 15, 1143-1161.

32. McCormick, R. M. Anal. Chem. 1988, 60, 2322-2328.

33. Lee, T. T.; Yeung, E. S. Anal. Chem. 1991, 63, 2842-2848.

34. Zhu, M.; Hansen, D. L.; Burd, S.; Gannon, F. J. Chromatogr. 1989, 480, 311-319.

35. Hogan, B. L.; Yeung, E. S. Anal. Chem. 1992, 64, 2841-2845.

36. Lee, T. T.; Yeung, E. S. Anal. Chem. 1992, 64, 3045-3051.

37. Foret, F.; Szoko, E.; Karger, B. L. J. Chromatogr. 1992, 608, 3-12.

38. Kilar, F.; Hjerten, S. J. Chromatogr. 1989, 480, 351-357.

CHAPTER 6

POLY(ETHYLENE OXIDE) FOR DNA SEPARATION WITH HIGH RESOLUTION AND SPEED IN CAPILLARY GEL ELECTROPHORESIS

Introduction

The need to increase speed and accuracy for the determination of DNA sequences has been driven by the Human Genome Project (HGP).[1-3] The goal of the HGP is to completely understand the role and origins of all genomic components. With current techniques, it is impossible to sequence the entire genome without substantial time and cost. To answer these questions, several problems involving analytical fields must be overcome by chemists. The speed and efficiency of separation need to be enhanced dramatically, since there are 3×10^9 base pairs in the human genome and 50,000 to 100,000 genes needed to be sequenced. With present technology this sequencing would take several years, the capability of data treatment must be improved significantly. Although the polymerase chain reaction (PCR)[4] can yield more than 10 million copies to allow the analysis of DNA from individual cells, a sensitive detection technique, such as laser induced fluorescence (LIF), still needs to be developed. This technique should minimize the volume of sample used, reduce the effect of the dye on the separation performance, enhance the possibility for multiplexed-operation system

(MOS),[5-7] increase the sequencing acquisition rate of raw sequence data, and sequence for longer fragments of DNA in a single run.

So far, the most promising method to break the barrier of the separation speed for DNA sequencing is the use of capillary gel electrophoresis (CGE) with MOS. With low current and efficient heat dissipation during runs, high electric fields up to 500 V/cm can be applied to DNA separations in CGE. On the contrary, such high fields are impossible in the traditional slab gel system. Since the first reports on the separation of DNA mixtures by CGE,[8] this method has become well known as a technique with high speed and efficiency and has been demonstrated successfully in DNA sequencing[9-11] and separations of restriction fragments[12,13] and PCR products.[14] Traditionally, crosslinked polymers such as polyacrylamide were popular for use as gel matrices in CGE because of their high resolution abilities for the separation of proteins and DNA. The separation performance is controlled by the pore structure of gel matrices, which is based on the amount and characteristics of the monomer and crosslinking agents . However, due to the instabilities, irreproducibilities, nonreplacements and short life time of capillaries filled with crosslinked gel, they are not quite suitable for DNA sequencing, especially, in MOS.

Alternatively, low- to moderate-viscosity entangled polymers have been used to overcome the above problems.[15-25] Unlike crosslinked gels, they are replaceable and more stable for use at higher temperatures and electric field strengths. In

addition, more uniform separation performance and reasonable separation efficiency can be achieved. Thus, entangled polymers are better than crosslinked polymers as gel matrices for CGE-MOS. Linear polyacrylamide gel (0% T) has been used for the separation of DNA or proteins.[9,15-17] In addition, methyl cellulose,[18,19] hydroxyalkyl cellulose,[20-22] polyhydroxy and poly(ethyleneglycol-(meth)acrylate),[23] and polyvinylalcohol[24,25] also have been demonstrated in DNA separation. Entangled polymers are also useful for the separation of biological samples in capillary zone electrophoresis, since they can coat onto the capillary wall to suppress the electroosmotic flow and minimize the interaction between the capillary wall and solutes.[19]

One general problem in DNA separation by using CGE is the interaction between DNA and the wall of capillary which results in poorer separation performance. To minimize this problem, coating of the capillary wall with more hydrohobic polymers is necessary.[26,27]

Although current CGE technology, which uses entangled polymers as gels, works well for DNA sequencing, in terms of speed, cost and sensitivity, it is still not good enough to obtain entire genome information. One approach to increase speed and throughput for DNA sequencing with CGE is to find suitable gel matrices for CGE. To meet the requirements, new gel matrices must provide the following advantages: high separation efficiency, high speed, low background signal in absorbance and fluorescence, ease in preparation, good reproducibility

and high tolerance when run in high electric fields and at high temperatures. In this chapter, we discuss the use of an entangled polymer, poly(ethylene oxide), PEO, for the separation of DNA restriction fragments. Factors such as molecular weight (MW), concentration of polymers and electric field strength are demonstrated to affect the separation performance .

Experimental Section

Materials

ϕX174RF DNA-Hae III digest from United States Biochemical (Cleveland, OH, USA) was diluted to 250 μg/ml. Tris(hydroxymethyl)aminomethane (THAM), boric acid, and ethylenediaminetetraacetic acid (EDTA) used as buffer solution are all from Sigma Chemical Co. (St. Louis, MO, USA). The polymers used for sieving matrices were from Aldrich Chemical Co. (Milwaukee, WI, USA).

Apparatus

50 μm i.d., 360 μm o.d. DB-1 coated GC Capillaries (J & W Scientific, Folsom, CA, USA) with 0.2 μm coating thickness were used without any further modification. The capillary length was 50 cm total, 32 cm effective length. A commercial electrophoresis instrument (Model 3850 ISCO; Lincoln, NE, USA) was

used for all electrophoretic experiments. Electromigration injection was performed throughout for 2 s at the running voltage (-10 to -30 kV). The detection wavelength was set at 260 nm to monitor nucleic acids.

Methods

The buffer solution (TBE) contained equimolar amounts of THAM and boric acid, both being the free base and acid respectively, with EDTA as a chelating reagent for divalent cations to prevent the activation of DNAases. The pH of the 1X TBE buffer was 8.2 without any further adjustment. Gel was prepared with a certain amount of PEO and TBE buffer solution to the desired concentration. Briefly, PEO was gradually added into the TBE buffer in a beaker sitting in a water bath at 85 to 90 °C. During addition of PEO, stirring with a magnetic stirring rod is kept as high as possible to have a well homogenous gel solution. After addition was complete, gel solution was stirring for at least an additional 15 minutes.

Initially, the capillary was flushed with water, methanol, and water for at least 2 cycles. Then, the capillary was filled with very low-viscosity gel (e.g. 0.5% PEO) and run at -10kV for 10 minutes. Finally, the capillary was refilled with separation gel and equilibrated at the running voltage for 15 minutes before any sample injection. It is useful to mention here that it is quite easy to use air pressure to fill the capillary with gel and the total operation time is no more than 5 min. From our experience, the performance of CGE following this treatment is much better, and

the capillary can be used for over 2 weeks and more than 50 runs without any degradation.

Results and Discussion

Separation performance

To obtain the best separation efficiency in CGE, it is important to have gel matrices with the right mesh size to obtain a suitable sieving effect for solutes. Also, suppression of the interaction between the capillary wall and solutes is quite useful to improve separation efficiency. Increase in capillary life time is another consideration in CGE. In this study, we found that it is very easy to have a high-quality capillary, if it is pretreated with very low-viscosity entangled solution. The reason is that it is very difficult to remove bubbles from high-viscosity gel matrices if a bubble is sticking on the capillary wall at the beginning. Pretreatment of capillaries in this way can minimize bubbles sticking onto the wall and make it possible to retreat the capillaries if they are found not work well at the beginning. Loss in separation performance and change of retention times are common phenomena when solutes adsorb onto the capillary wall in capillary electrophoresis. Since DNA fragments form multiple negative charges under the running condition, they can move at a reasonable speed, even though they are large, in the absence of electroosmotic flow. This makes it possible to coat the capillary to minimize any

interaction between the capillary wall and solutes. To minimize DNA fragments adsorbing onto the wall, we use DB-1 capillaries, which have a very thick coating of hydrophobic polymer inside the capillary wall.

Primarily, the separation of proteins or DNA in CGE is based on the sieving mechanism, but also slightly on the mobilities of solutes, since the differences in mobilities alone are too small to have good separations. Gel is a good sieving medium for these solutes. To form a gel, the concentration of polymers has to be higher than a certain value called overlap threshold. As long as the concentration of polymer is higher than the overlap threshold, polymer chains interact with one another to form an entangled solution. The average mesh size (ξ) of a gel can be expressed as[28,29]

$$\xi(\Phi) \approx A\ \Phi^{-0.75} \qquad (1)$$

where Φ is the polymer volume fraction and A is a proportional constant. Since Φ equals $S^{-0.8}$, where S is the size of polymer chain, Eq. (1) can be rewritten as

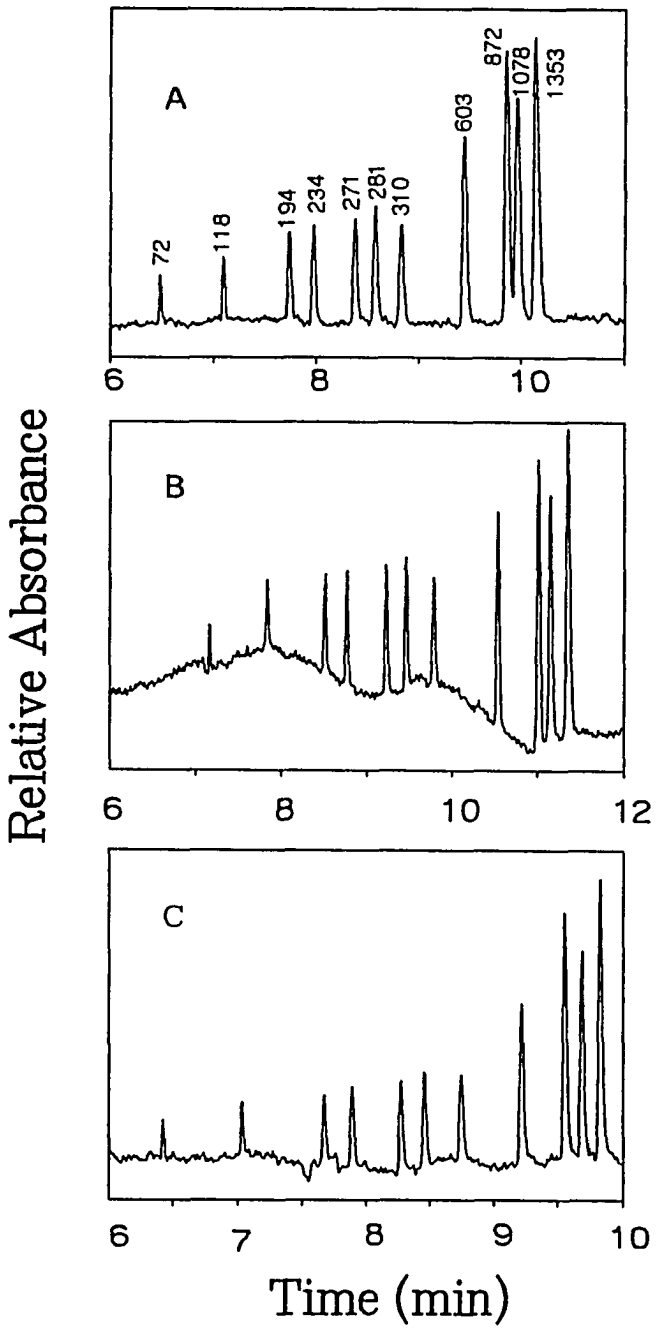
$$\xi(\Phi) \approx A\ S^{0.6} \qquad (2)$$

From Eq. 2, it is easy to estimate optimal mesh size of gel matrices from S. For better separation ability in CGE, in order to fill the capillary easily with gel while

keeping the optimal mesh size for better separation ability, it is nice to have gel matrices with as low viscosity as possible. Hence, in order to create a smaller mesh while minimizing the viscosity of the solution, one wants to use a polymer with short chains and to create a larger mesh one wants to use a polymer with longer chains. In practice, it is not easy to have a gel with the right size for the separation of DNA if the fragments have a wide range of sizes. The separation of DNA fragments in gel matrices prepared from PEO with MW from 300,000 to 5,000,000 at different concentrations are shown in Fig. 1. For a comparable separation performance, concentrations of certain polymers with small chains in solution are always higher than those with long chains. In all conditions, 11 DNA fragments are well separated in a short time period, less than 11 minutes, at -26 kV. It is worth mentioning that the resolution between 271 and 281 pairs of fragments with PEO gel matrices is much better than the use of cellulose-type gel matrices. Also, separation performed in PEO gel matrices provides highly reproducible results for at least 10 runs. The reproducibility among different capillaries and gel batches is also excellent. Overall, PEO gel matrices provide several advantages, including easy preparation, better reproducibility, and longer life time as compared with cellulose type gel matrices. Based on our experience, Table 1 shows a general comparison among three common gel matrices.

Migration times of DNA fragments are different in different gel matrices due to the different mesh size in different entangled solution. Based on the Ogston

Figure 1. Separation of ϕX174RF DNA-Hae III fragments at various PEO gel matrices; (A) 3%, MW 300,000, (B) 3.3%, MW 300,000, (C) 2.75%, MW 1,000,000, (D) 2%, MW 2,000,000, (E) 3%, MW 2,000,000, (F) 2%, MW 5,000,000. Conditions: 1X TBE buffer, 50 cm total length, 32 cm effective length, 50 μm i.d. DB-1coated capillary, wavelength: 260 nm, running voltage: -26 kV.


A
72
118
194
234
271
281
310
603
872
1078
1353
6
8
10
B
6
8
10
12
C
6
7
8
9
10
Relative Absorbance
Time (min)

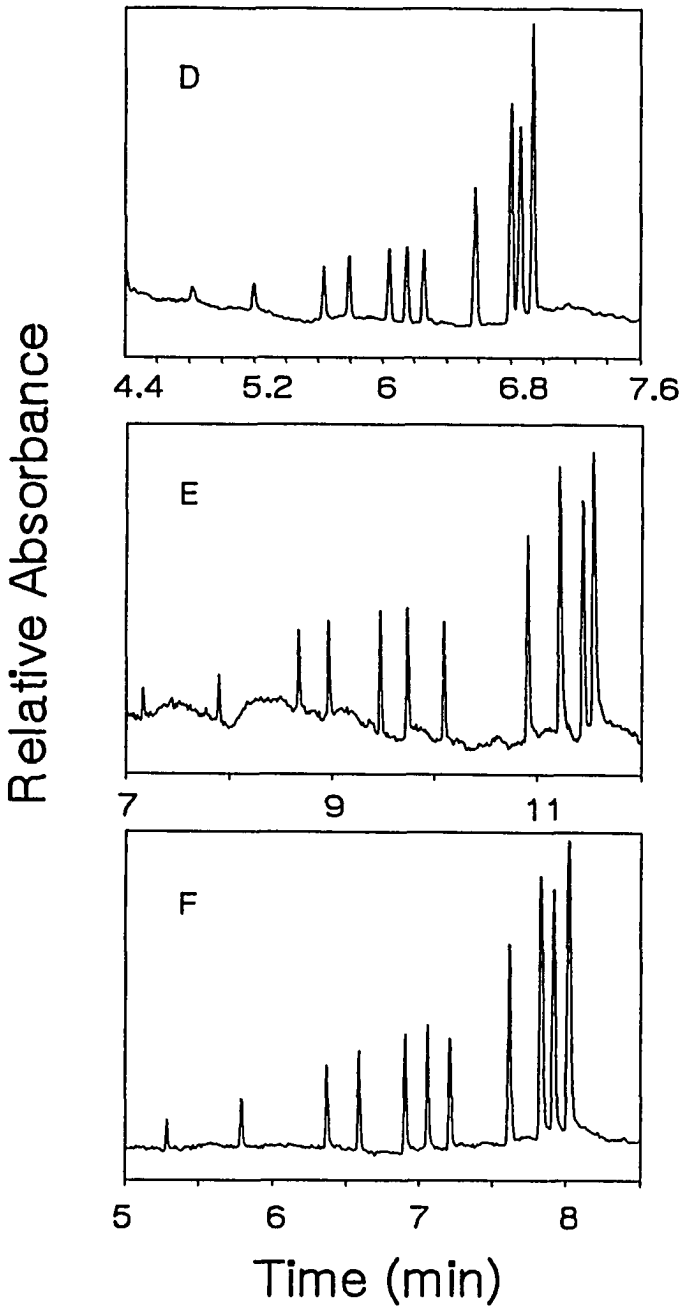


Figure 1. (continued)

Table 1. General comparision of three gel matrices for DNA separation

Gel	Resolution	Sequencing	Preparation	Life time	Stability*	Reproducibility
PEO	high	possible**	easy	long	excellent	excellent
MC	reasonable	bad	difficult	reasonable	good	good
PA	high	excellent	very critical	short	poor	good

MC: methyl cellulose; PA: polyacrylamide, worker must polymerize acrylamide to have polymer with different range of molecular weight, commercial product only has MW range from 5,000,000 to 6,000,000; *: at high electric field strength; **: not shown at this point.

model,[30] mobility (μ) of solute in gel matrices can be given

$$\mu = \mu_0 P \qquad (3)$$

where μ_o is the mobility of the solute in free solution and P is the possibility that a given pore has a radius greater than or equal to the radius of the solute. Keeping P higher while maintaining a good separation performance is important to have a fast separation speed. Obviously, from this prediction, a short migration time for a

certain solute means the solute migrates in gel matrices with bigger mesh size. From our results, migration times for all fragments are shorter in gel matrices prepared from 2 % MW 5,000,000 and 2,000,000 of PEO, which means that these gel matrices should have larger mesh sizes in this serious study. This is reasonable as predicted by Eq. (2). For a given polymer, the mesh size of gel matrices always decreases as the concentration, i. e. viscosity, of polymers in solution increases. Our results also show this trend in Fig. 1A and 1B, and 1D and 1E.

Resolution

In this report, all resolution (R) results are calculated as

$$R = \frac{(2 \ln 2)^{1/2} \Delta t_R}{HW_1 + HW_2} \qquad (4)$$

where Δt_R is the difference in retention time between the two adjacent peaks, and HW is the full width at half maximum. Fig. 2 shows the change of resolution over the difference of number of base pairs as the change in gel matrices. Gel matrices prepared from PEO with MW 2,000,000 at 3% provide the best resolution for DNA fragments. However, it is too viscous to be filled into capillaries easily. Overall, the best performance should be obtained from PEO with MW 5,000,000

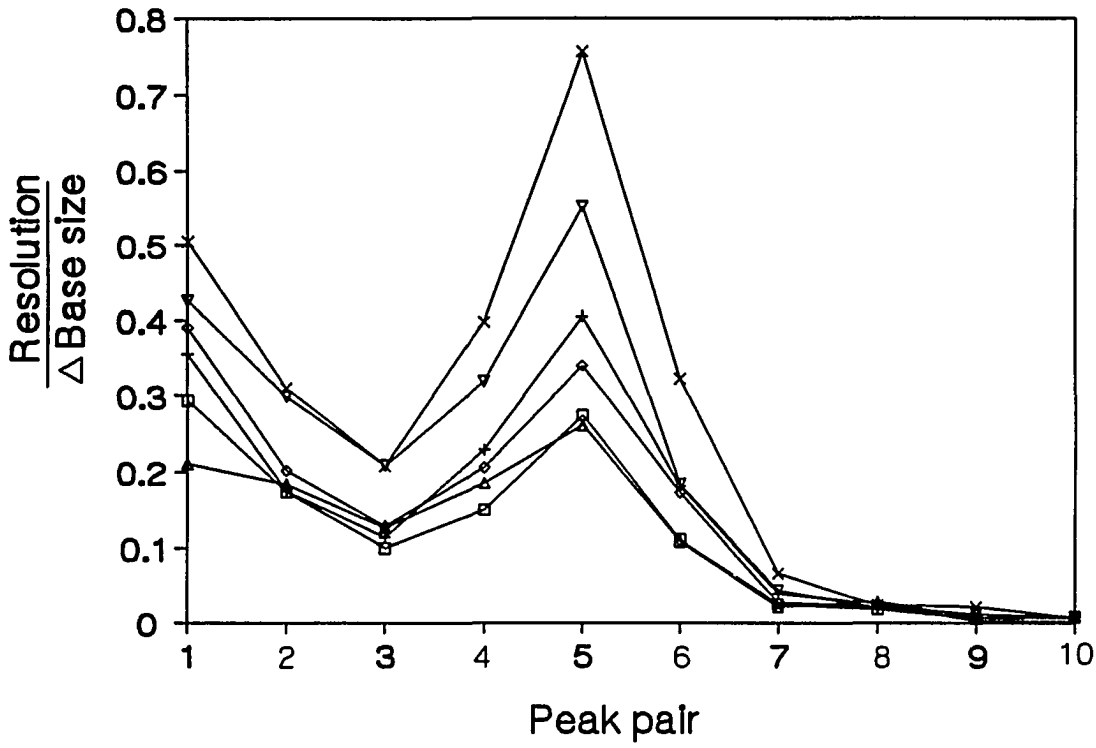


Figure 2. Resolution of DNA fragments in different gel matrices. Data are taken from Fig. 1. Symbols; □: as (A), +: as (B), ◇: as (C), △: as (D), ×: as (E), ▽: as (F). Number of peak pair represents as the order of two adjacent peaks.

and the concentration is a little higher than 2%. It is shown clearly that the maximum resolution for two peaks with a difference of a single base pair is in the range 250 to 350 base pairs, these are the most common lengths for DNA sequencing currently and the resolution degrades as DNA fragments become larger than 600 base pairs. To explain this trend, the following equation is useful[31,32]

$$R = \frac{L\Delta\mu}{2[\,\mu_2(\sigma_{T1}^2)^{1/2} + \mu_1(\sigma_{T2}^2)^{1/2}]} \qquad (5)$$

where L is the capillary length to the detector, μ_1 and μ_2 are the mobilities of solutes corresponding to the adjacent peaks, $\Delta\mu$ is the difference in mobilities between two adjacent peaks, and σ_{T1}^2 and σ_{T2}^2 are the total peak variances. Higher resolution is possible if the difference of the mobilities between the adjacent solutes is larger. On the other hand, smaller total peak variance is necessary to get high resolution. Total peak variances mainly comes from injection and diffusion during the run. So, shorter injection time at lower electric field and high speed separation are important to have a high resolution. Luckey and Smith recently addressed that diffusion variance comes from two terms, longitudinal diffusion and transverse diffusion.[32] In our case, the last term is not as important as first term, since the running current is only 13 μA, thus, a low joule heating effect since the

temperature gradient generated across the capillary is not serious. Low sieving effect (i.e. smaller difference in mobilities), larger peak variance due to longitudinal diffusion and a larger difference in mobilities between solutes and buffer ionic species cause poor resolution for larger fragments. To minimize the longitudinal diffusion, the use of gel matrices with high viscosity and high speed separation are useful. The trends for the increase in the observed resolution are the same as the increase in viscosities in different gel matrices. Corresponding to this observation, it is obvious that longitudinal diffusion is an important factor affecting resolution in this study. Another factor affecting resolution is due to injection variance since a large amount of DNA has to be injected for separation because of poor detection with UV absorption which must be considered seriously in our study. Better resolution can be achieved if the injection variance is decreased by low amount injection through shorter time injection at lower electric field strength. More sensitive detection method such as LIF is a promise for low amount injection. Another benefit to resolution by using LIF comes from the use of capillaries with smaller i. d. when run at higher electric field strength. In this case, smaller longitudinal diffusion due to shorter migration times and smaller transverse diffusion resulting in lower heat generated can be achieved.

Electric field strength is also an important factor affecting separation performance in CGE since the shape of DNA and the network of polymers may change and heat generated differs as the electric field strength changes. In terms

of speed, we like to run CGE at as high an electric field strength as possible to have a shorter separation time. But it has a greater risk to decrease resolution. Fig. 3 and 4 show the effects of electric field strength on resolution. It is interesting to note that the effect of electric field strength on resolution is different in two different gel matrices. For lower-viscosity gel matrices (PEO with MW 300,000 at 3.15%), the decrease in resolution for fragments less than 600 base pairs relates to the electric field strength following the order

$$320 > 420 > 220 > 520 > 600 \text{ V/cm}$$

However, for higher-viscosity gel matrices (PEO with 5,000,000 at 2%), the order is

$$520 > 320 \approx 420 \approx 220 > 600 \text{ V/cm}$$

For both cases, the worst resolution happens when CGE runs at 600 V/cm. Possible reasons include unstable gel matrices, relative transverse effect and large changes both in the shape of DNA and mesh size of gel matrices at extremely high electric field strength. There is no doubt that longitudinal diffusion plays an important factor in both cases. In the first case, the change of DNA from random coil to rod-like shape at high electric field strength[33,34] and the larger change in the orientation of the flexible network in low-viscosity gel matrices[35] may explain this observation. This is because DNA fragments need to snake through the gel matrices with smaller mesh size, in turn, the larger loss in sieving effect causes worse resolution. While in the second case, the effect of electric field strength on resolution is not that clear and significant. More rigidity and larger mesh size of

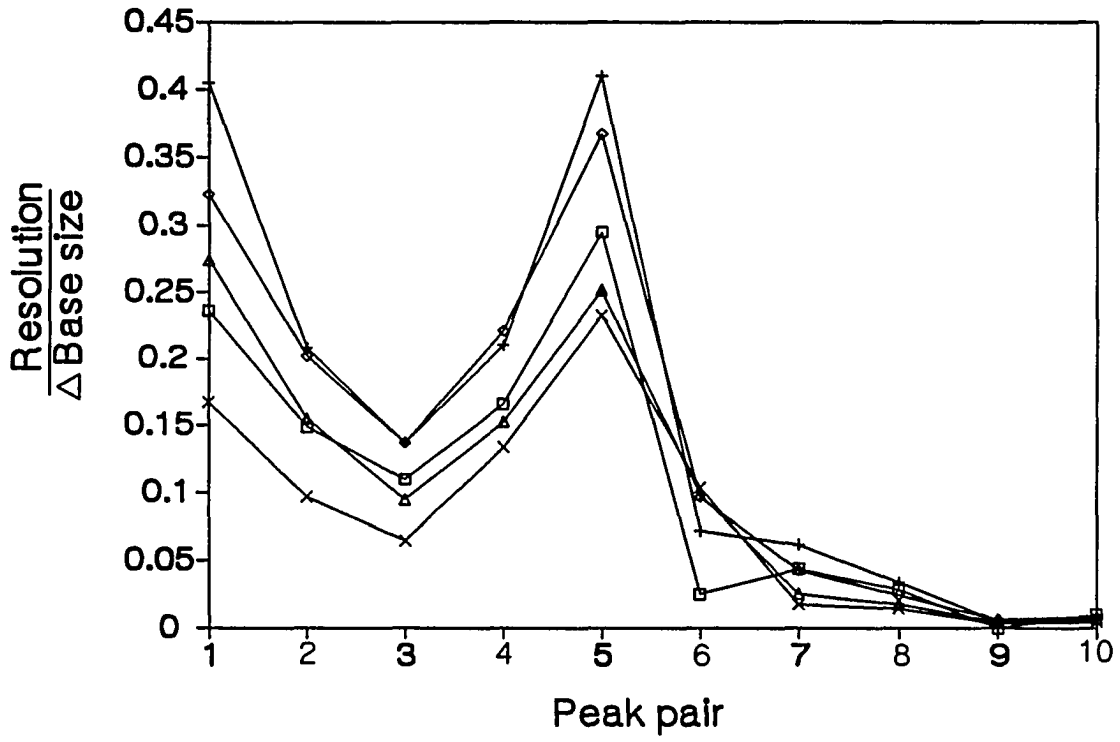


Figure 3. The effect of electric field strength on resolution of DNA fragments in PEO (3.15%, MW 300,000) gel matrices. Conditions are as in Fig. 1 except that □: 220 V/cm, +: 320 V/cm, ◇: 420 V/cm, △: 520 V/cm, x: 600 V/cm.

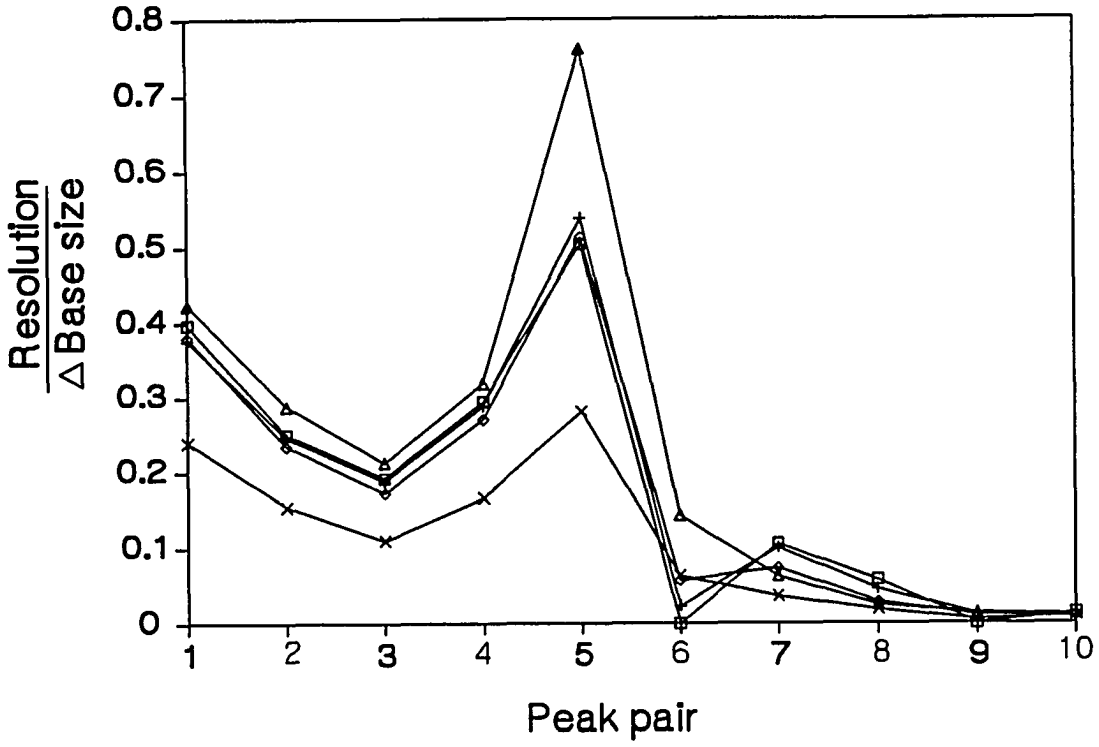


Figure 4. The effect of electric field strength on resolution of DNA fragments in PEO (2%, MW 5,000,000) gel matrices. Conditions are as in Fig. 3.

the gel matrices may be the reasons because the change in shape of DNA fragments is not so significant when the difference in mesh size of gel matrices and DNA fragments is larger. Overall, the effect of electric field strength on resolution is quite different from the use of cellulose-type gel matrices which have the maximum resolution between 200 to 300 V/cm. Also, higher electric field strength can be performed in gel matrices prepared from polymer with higher MW, which provides the benefit of high-speed separation.

Mobility

As Eq. 3 expresses, DNA fragments move faster as the difference between the size of DNA and mesh size of gel matrices is larger. From the comparison with mobilities of DNA fragments in different gel matrices at constant electric field strength, it is easy to estimate the order of the size of gel matrices if heat well dissipates. Fig. 5 shows the mobilities of DNA fragments in different gel matrices. Results show mesh size of gel matrices increases as polymer chain increases or concentration decreases, which is as predicted by Eq. 2. Gel prepared from PEO with MW 2,000,000 at 3 % is not as predicted, because its viscosity is much higher than others and it is impossible to simply predict and compare the mesh size with others only based on the comparison with polymer chain. Fig. 6 and 7 show the change in mobilities of DNA fragments in different gel matrices as the change in electric field strength. In Fig. 6, small increases in mobilities of smaller DNA

fragments due to the decrease in viscosity as electric field strength increases. This reflects the truth that the temperature generated is insignificant. However, results for smaller DNA fragments shown in Fig. 7 are not predictable. For DNA fragments larger than 600 bases, all data are out of the linear range. This means the sieving effect is insignificant for those fragments. In this case, reptation and biased reptation model, instead of the Ogston model, needs to be used to describe the observations. Lumpkin[36] used the following equation to express the relation among mobilities, fragment size (N) and electric field strength (E)

$$\mu \approx K\left(\frac{1}{N} + bE^2\right) \qquad (6)$$

where K is a constant and b is a function of the mesh size of the polymer network as well as the charge and persitence length of the migrating solute. Therefore, if N and/or E become(s) very large, the second term in Eq (6) dominates to affect mobility. Then, prediction of mobility from fragment size is not easy from the plot of ln μ v. s. ln (1/N). Theoretically, the slopes of these plots are close to 1 as DNA fragments migrate through gel matrices without any change in shape. However, this is not the case in our study. Our results show reptation and change in temperature occurs during running. Slopes at lower electric field strength are closer to 1, which means that reptation occurs more significantly at higher electric

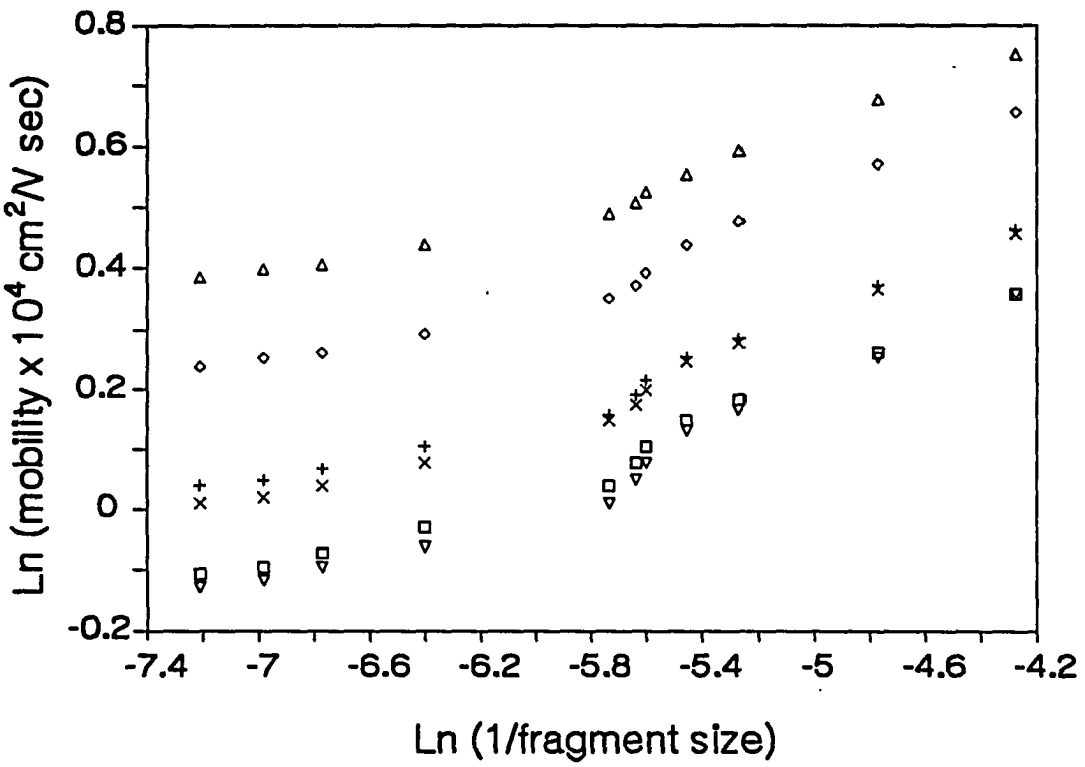


Figure 5. Mobilities of DNA fragments in different gel matrices. Data are taken from Fig. 1. Symbols; ×: as (A), □: as (B), +: as (C), △: as (D), ▽: as (E), ◇: as (F).

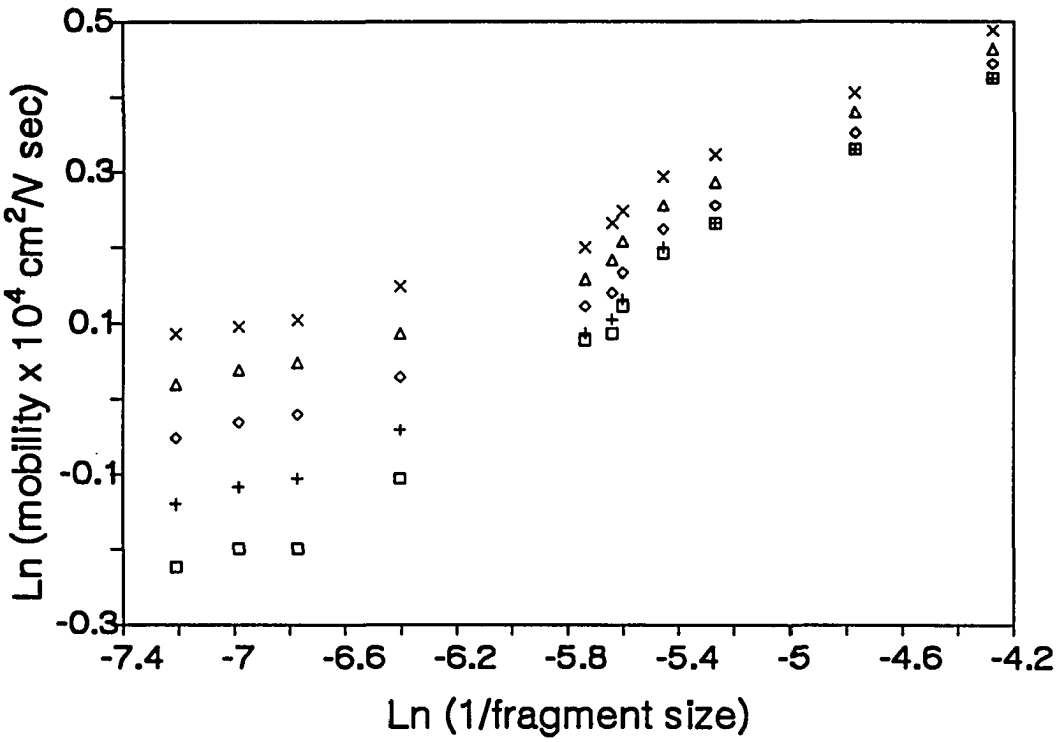


Figure 6. The effect of electric field strength on mobilities of DNA fragments in PEO (3.15%, MW 300,000) gel matrices. Conditions are as in Fig.3.

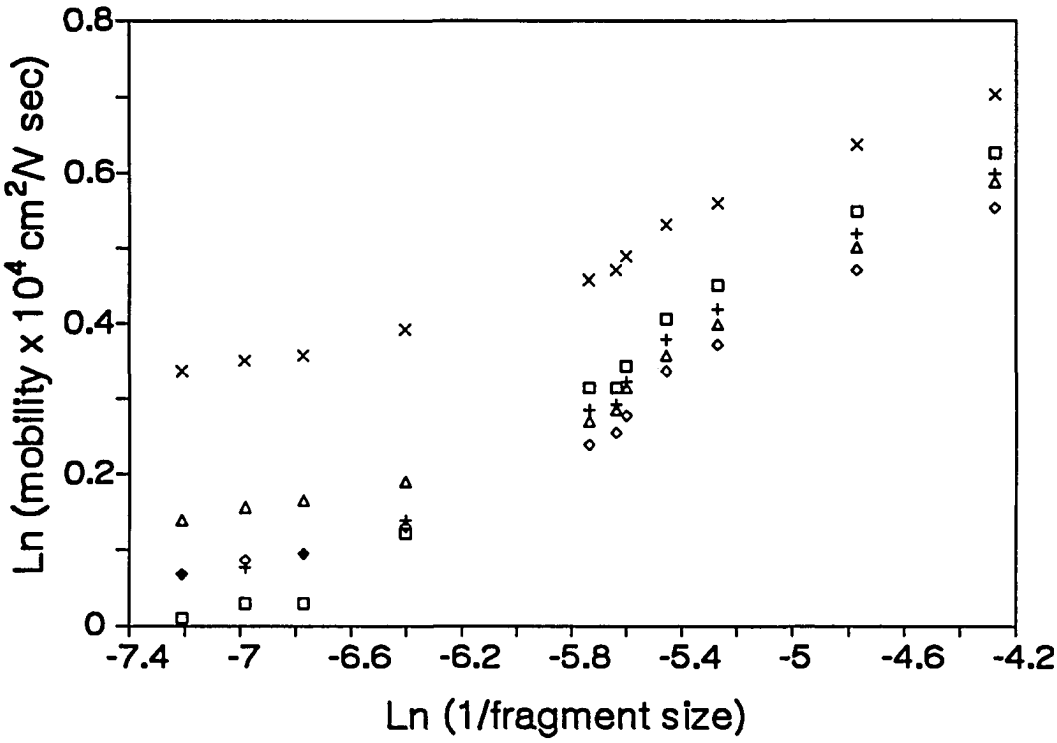


Figure 7. The effect of electric field strength on mobilities of DNA fragment in PEO (2%, MW 5,000,000) gel matrices. Conditions are as in Fig. 4.

field strength. Also, larger DNA fragments must change their shapes from random coil to rod-like when they migrate through gel matrices, which is reflected by the observation of slope almost close to 0. This behavior has been observed for DNA separation through both polyacrylamide gels[37] and agarose.[38]

Conclusion

The need to have gel matrices to provide a good separation ability and high speed performance is always important for DNA sequencing. In this study, we introduce PEO as new gel matrices for the separation of DNA fragments. These gel matrices provide the characteristics of higher reproducible results and long life times. The separation with short migration times and high efficiencies for DNA fragments have been demonstrated. Results show that these new gel matrices should be suitable for DNA sequencing. We also found that the size of the gel matrices as well as the viscosity play an important role in resolution.

Some observations in this work are difficult to explain either by current theory or simple prediction. In the future, more complicated theory and more detailed experiments need to be done to understand the effects of the change of polymer network, shape of DNA and viscosity due to the change of electric field strength and temperature on resolution. It is also important to understand the relationship among gel viscosity, polymer chain and the concentration of entangled polymers

for the choice of right condition for certain samples.

References

1. Ollins, F., Galas, D. Sciences 1993, 262, 43-46.
2. Trainor, G. L. Anal. Chem. 1990, 62, 418-426.
3. Watson, J. D. Science 1990, 248, 44-48.
4. Reynolds, R., Sensabaugh, G., Blake, E. Anal. Chem. 1991, 63, 2-15.
5. Swerdlow, H., Zhang, J. Z., Chen, D. Y., Harke, H. R., Grey, R., Wu, S., Dovichi, N. J., Fuller, C. Anal. Chem 1991, 63, 2835-2841.
6. Huang, X. C., Quesada, M. A., Mathies, R. A. Anal. Chem 1992, 64, 2149-2154.
7. Ueno, K., Yeung, E. S. Anal. Chem. 1994, 66,1424-1431.
8. Cohen, A. S., Najarian, D. R., Paulus, A., Guttman, A., Smith, J. A., Karger, B. L. Proc. Natl. Acad. Sci, U. S. A. 1988, 85, 9660-9663.
9. Ruiz-Martinez, M. C., berka, J., Belenkii, A., Foret, F., Miller, A.W., Karger, B. L. Anal. Chem. 1993, 65, 2851-2856.
10. Heiger, D. N., Carson, S. M., Cohen, A.S., Karger, B. L. Anal. Chem. 1992, 64, 192-199.
11. Konard, K. D., Pentoney, Jr, S. L. Electrophoresis, 1993, 14, 502-508.
12. Guttman, A., Wanders, B., Cooke, N. Anal. Chem. 1992, 64, 2348-2351.

13. Baba, Y., Mitsutomo, T. Trends in Anal. Chem. 1992, 11, 280-287.

14. Schwartz, H. E., Ulfelder, K. J. Anal. Chem. 1992, 64, 1737-1740.

15. Cohen, A. S., Najarian, D. R., Karger, B. L. J. Chromatogr. 1990, 516, 49-60.

16. Ganzler, K., Greve, K. S., Cohen, A. S., Karger, B. L., Guttman, A., Cooke, N. C. Anal. Chem. 1992, 64, 2665-2671.

17. Carson, S., Cohen, A. S., Belenkii, A., Ruiz-Martinez, M. C., Berka, J., Karger, B. L. Anal. Chem. 1993, 65, 3219-3226.

18. Crehan, W. A. M., Rasmussen, H. T., Northrop, D. M. J. Liq. Chroatogr. 1992, 15, 1063-1080.

19. Zhu, M., Hansen, D. L., Burd, S., Gannon, F. J. Chromatogr. 1989, 480, 311-319.

20. Nathakarnkitkool, S., Oefner, P. J., Bartsch, G., Chin, M. A., Bonn, G. K. Electrophoresis 1992, 13, 18-31.

21. Ulfelder, K. J., Schwartz, H. E., Hall, J. M., Sunzeri, F. Anal. Biochem. 1992, 200, 260-267.

22. Grossman, P. D., Soane, D. S. J. Chromatogr. 1991, 559, 257-266.

23. Zewert, T., Harrington, M. Electrophoresis, 1993, 13, 817-824.

24. Kleemib, M. H., Gilges, M., Schomburg, G. Electrophoresis 1993, 14, 515-522.

25. Chrambach, A., Aldroubi, A. Electrophoresis, 1993, 14, 18-22.

26. Hjerten, S. J. Chromatogr. 1985, 347, 191-198.

27. Strege, M., Lagu, A. Anal. Chem. 1991, 63, 1233-1236.

28. de Gennes, P. G. J. Chem. Phys. 1971, 55, 572-579.

29. Grossman, P. D., Soane, D. S. Polymers 1991, 31, 1221-1228.

30. Ogston, A. G. Trans Faraday Soc. 1958, 54, 1754-1757.

31. Luckey, J. A., Norris, T. S., Smith, L. M. J. Phys. Chem. 1993, 97, 3067-3075.

32. Luckey, J. A., Smith, L. M. Anal. Chem. 1993, 65, 2841-2850.

33. Slater, G. W., Noolandi, J. Biopolymers, 1986, 25, 431-454.

34. Doi, M., Edwards, S. F. JCS Faraday Trans. II 1978, 79, 1789-1818.

35. Luckey, J. A., Smith, L. M. Electrophoresis 1993, 14, 492-501.

36. Lumkin, O. J., Dejardin, P, Zimm, B. H. Biopolymers 1985, 24, 1573-1593.

37. Grossman, P. D., Menchen, S., Hershey, D. GATA, 1992, 9, 9-16.

38. Hervet, H., Bean, C. P. Biopolymers, 1987, 26, 727-742.

CHAPTER 7

OPTIMIZATION OF DNA SEPARATION BY CAPILLARY GEL ELECTROPHORESIS WITH ULTRASENSITIVITY

Introduction

One of the most exciting fields in modern science is the development of novel techniques to sequence the human genome. Scanning tunneling microscopy,[1] a flow system with single molecule detection,[2] and capillary gel electrophoresis (CGE)[3-7] have been demonstrated well for DNA sequencing and sizing. Among these methods, CGE seems to dominate, since it provides excellent resolution and reasonable sensitivity, which in turn gives more accurate and complete information.

Conventional 3 D crosslinking gel matrices, which suffer from poor reproducibility and instability, have been replaced recently by linear polymer matrices (LPM). The other advantage of LPM used in CGE is that higher electric field strengths can be used. Therefore, high-speed separations with reasonable resolution can be achieved easily. Common LPMs including polyacrylamide,[8,9] cellulose,[10-13] polyvinylachol,[14] and poly(ethylene oxide)[15] have been used successfully to determine DNA restriction fragments, polymerase chain reaction (PCR) products and proteins. In CGE, the separation mechanism depends on the pore size of the gel and slightly on the mobility of DNA. Important factors affecting

the performance of separation include the molecular weight and the nature of polymers, viscosity of gel matrices, temperature and electric field strength of the running system.

The most universal and inexpensive detection system for DNA is the ultraviolet spectrometer at 260 nm. However, it is not sensitive enough for samples with low concentration and DNA sequencing. One answer is the use of highly sensitive laser-induced fluorescence.[16-18] Unfortunately, the native fluorescence of DNA is weak, although Yeung and Milofsky used an argon-ion laser at 275 nm to detect adenosine monophosphate at 10^{-8} M level and DNA restriction fragments.[19] One alternative in taking advantage of LIF is the use of an inexpensive laser in the visible range to detect DNA tagged with a fluorophore. Common illumination light sources with relatively low cost include green He-Ne lasers (543.6 nm), air-cooled argon-ion lasers (488 and 514.5 nm) and diode-pumped Nd-YAG lasers (532 nm).

To keep reasonable separation performance while achieving high sensitivity, it is critical to choose a suitable dye for intercalating DNA. Optimum dyes should be able to provide high fluorescence intensities for DNA complexes without significant changes in the mobilities of DNA. Dyes with high binding constants for DNA and relatively low fluorescence intensities(compared to the fluorescence intensities of DNA complexes) are more interesting. Monomeric intercalating dyes such as ethidium bromide (EthD),[20, 21] and dimeric intercalators such as thiazole orange homodimer (TOTO), and oxazole yellow homodimer (YOYO)[22-24] are all good

candidates. Monomeric intercalators form complexes with DNA by intercalation and some weak electrostatic binding between the cationic dyes and the anionic phosphate ions of nucleic acids. On the other hand, dimeric intercalators form relatively high-binding complexes with DNA by intercalation, noncooperative binding between the nonintercalated ring and the anionic phosphate ions of DNA and cooperative binding involving nonintercalated rings. Relatively more complicated complexes formed between DNA and dimeric intercalators seem to have more complicated electropherograms. At this point, a more careful choice of dimeric intercalators and the molar ratio between dyes and DNA must be considered seriously to highlight their advantages over monomeric intercalators in term of higher sensitivity.

High-speed and high-sensitivity DNA sequencing has been possible since conventional slab gel and LIF with four different dyes was used for DNA sequencing by Smith and coworkers[25] and Prober and coworkers.[26] The advantages of fluorescence sequencing over radioactive sequencing includes time saving and no limitations in sequencing ordering imposed by the requirement of combining data from four separate lanes on the polyacrylamide gel. However, radioactive sequencing can provide better signal-to-noise and a wide range in the amount of template needed for sequencing.

CE-LIF with LPM has been successful, and this technique shows promise for DNA sequencing. But several problems remain there for scientists to solve.

Improvement in sensitivity is very important since it promises the possibility to read large numbers of base pairs. Gel matrices with high resolution for the range from several base pairs up to 1000 base pairs or even higher still need to be addressed. More reproducible separation performance needs to be improved in order to take advantage of high throughput with multiple capillaries, which is one of the promising ways to enhance the ability of CE for DNA sequencing.

In this chapter, we try to address some of these problems by using quite new gel matrices prepared from poly(ethylene oxide), PEO. Several factors have been studied such as the concentration and type of gel matrices, length of capillaries, electric field strength, type and concentration of tagging dyes, with different lasers as light sources.

Experimental Section

CGE instrument and data collection.

The experimental setup is similar to that described in ref. 27. Briefly, a high-voltage power supply (Glassman High Voltage, Inc, Whitehorse Station, NJ) was used to drive the electrophoresis. The entire electrophoresis and detection system were enclosed in a sheet-metal box with a HV interlock.

An argon-ion laser with 488 nm output from Uniphase (San Jose, CA) and 1-mW He-Ne laser with 543.6 nm output from Melles Griot (Irvine, CA) were used

for the study. For the study of DNA-TOTO, one 535-nm interference filter was used to block scattered light and allow the emission light to reach the PMT when the argon-ion laser was used. For DNA-EthD (for both types of lasers) and DNA-TOTO (with He-Ne laser), one RG 610 cutoff filter and one 630-nm interference filter were used. For PGEM/U sample, two 515-nm cutoff filters were used with the argon-ion laser, and one RG 610 cutoff filter and one 630-nm interference filter were used if the He-Ne laser was used. The signal from LIF was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 4 Hz (Justice Innovation, Palo Alto, CA; Model DT 2802) and stored on a computer (IBM, Roca Raton, FL; Model PC/AT 286).

Capillary and reagents

Capillaries (Polymicro Technologies, Inc, Phoenix, AZ) with 75 μm i.d., 365 μm o.d. were used for coating with γ-methacryloxypropyltrimethoxysilane and polyacrylamide by Hjerten's method.[28] The preparation of gel matrices from PEO was as described in ref. 15.

All chemicals for preparing buffer, gel and coating for capillaries were purchased from ICN Biochemicals, Inc (Irvine, CA), except that acrylamide and foramide were from Sigma Chemical Co. (St. Louis, MO, USA) and poly(ethylene oxide) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The running buffer solution was 1X TBE. EthD was purchased from Sigma. TOTO was

obtained from Molecular Probes, Inc (Eugene, OR). The concentrations of dyes in the running buffer were 1 μg/ml. ϕX 174 RF DNA-Hae digest was purchased from United States Biochemical (Cleveland, OH). PGEM/U was obtained from the Facility of Nucleic Acid Center of Iowa State University (Ames, IA).

Results and Discussion

One of the advantages in DNA separation is that LIF can be used to detect very low amounts of DNA tagged with fluorophores. In order to keep reasonable separation efficiency, it is critical to choose a suitable dye with the right ratio to DNA to minimize the effect of the change in mobilities of DNA. Figure 1 shows the separation of 11 DNA fragments intercalated with a bisintercalator, TOTO for at least 20 min before the separation. Figure 2 and 3 show the separation of DNA fragments intercalated with EthD (a monointercalator) and TOTO during the separation period. The separation ability and sensitivity decreased every time as dyes were in the running buffer. Therefore, it is important that new gel matrices need to be refilled into the capillaries for each run. The degradation was much more serious if TOTO was in the running buffer. Figure 3 shows the irreproducible results for different runs even though we refilled the gel matrices between each run. Possible reasons for this phenomenon are that strong interactions between the dyes and polymer matrices and the capillary wall exist. Dye molecules can be

Figure 1. Influence of voltage on the separation of 0.9 μg/ml of ϕX 174 RF DNA-Hae digest intercalated with 9.5 ng/ml of TOTO before the separation: capillary total length: 60cm and effective length: 52 cm; light source: argon-ion laser at 488 nm. (A): -21 kV; (B): -12 kV; (C): -6 kV.

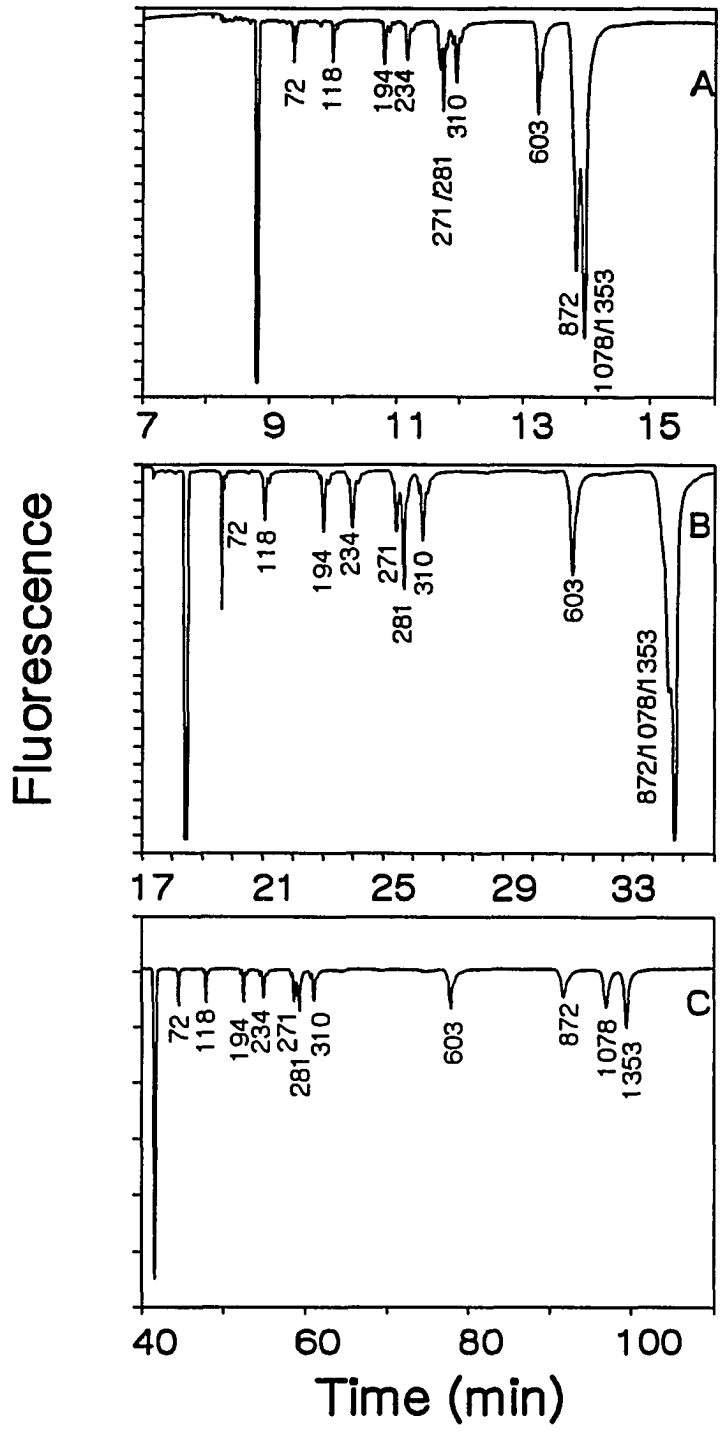

Fluorescence
A
72
118
194
234
271/281
310
603
872
1078/1353
7
9
11
13
15
B
72
118
194
234
271
281
310
603
872/1078/1353
17
21
25
29
33
C
72
118
194
234
271
281
310
603
872
1078
1353
40
60
80
100
Time (min)

Figure 2. Influence of voltage on the separation of 0.9 μg/ml ϕX 174 RF DNA-Hae digest intercalated with 1μg/ml of EthD in the running buffer. Conditions are as in Fig.1.

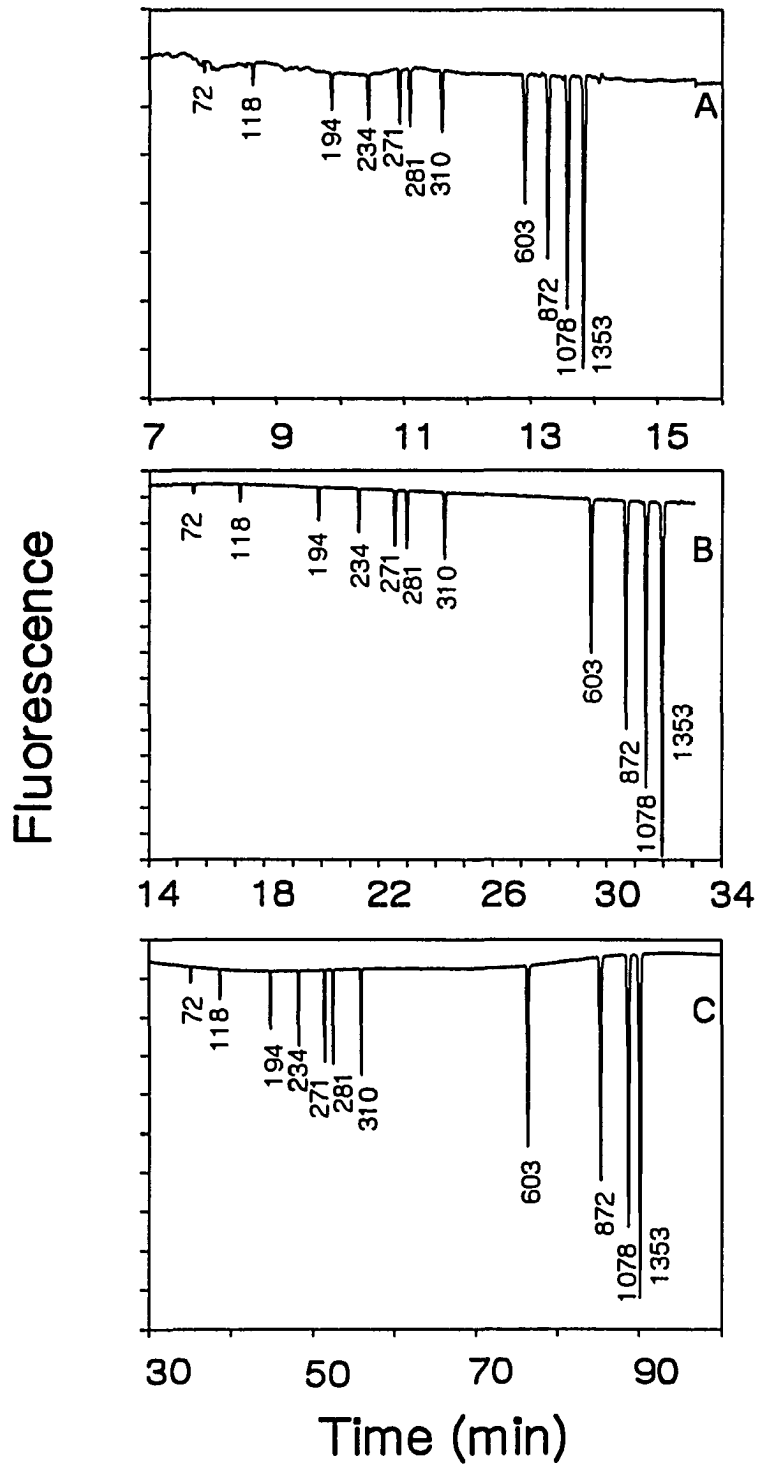
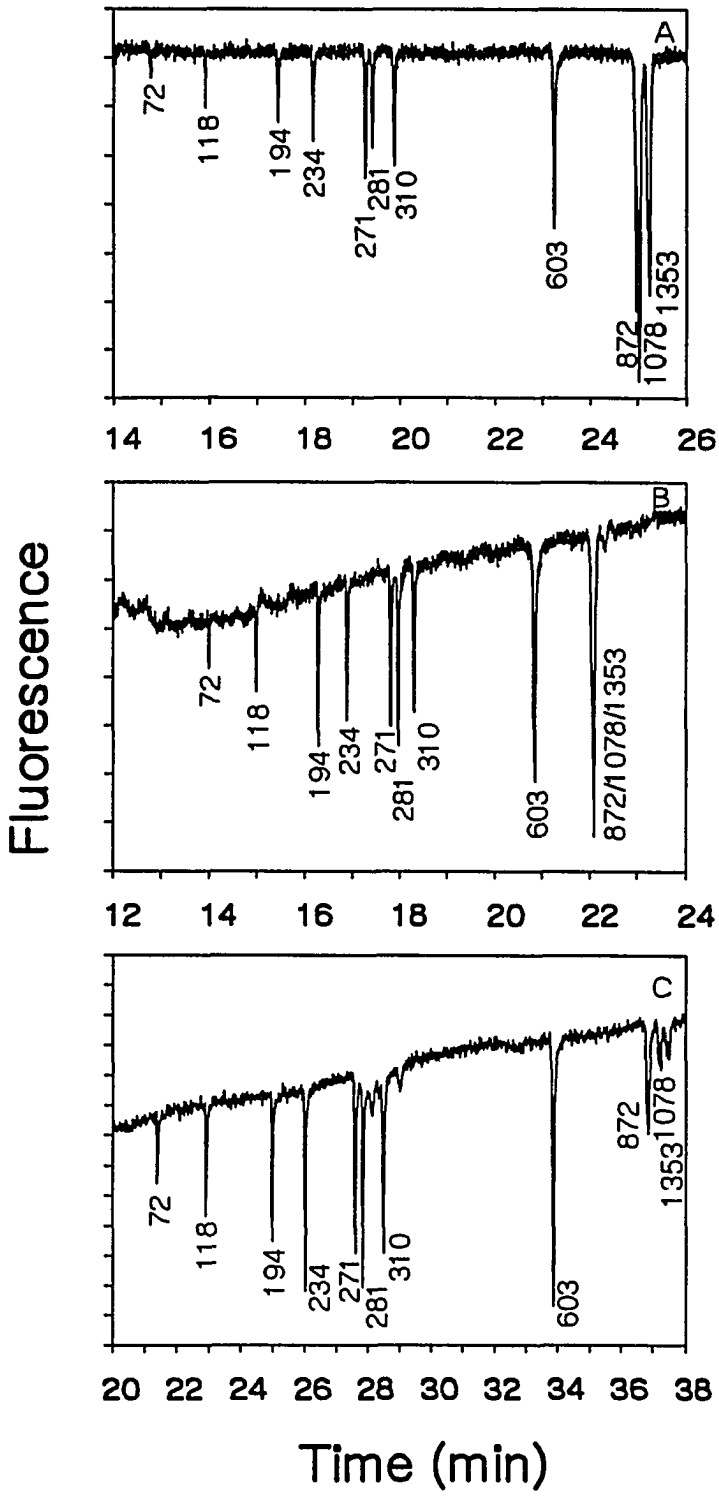
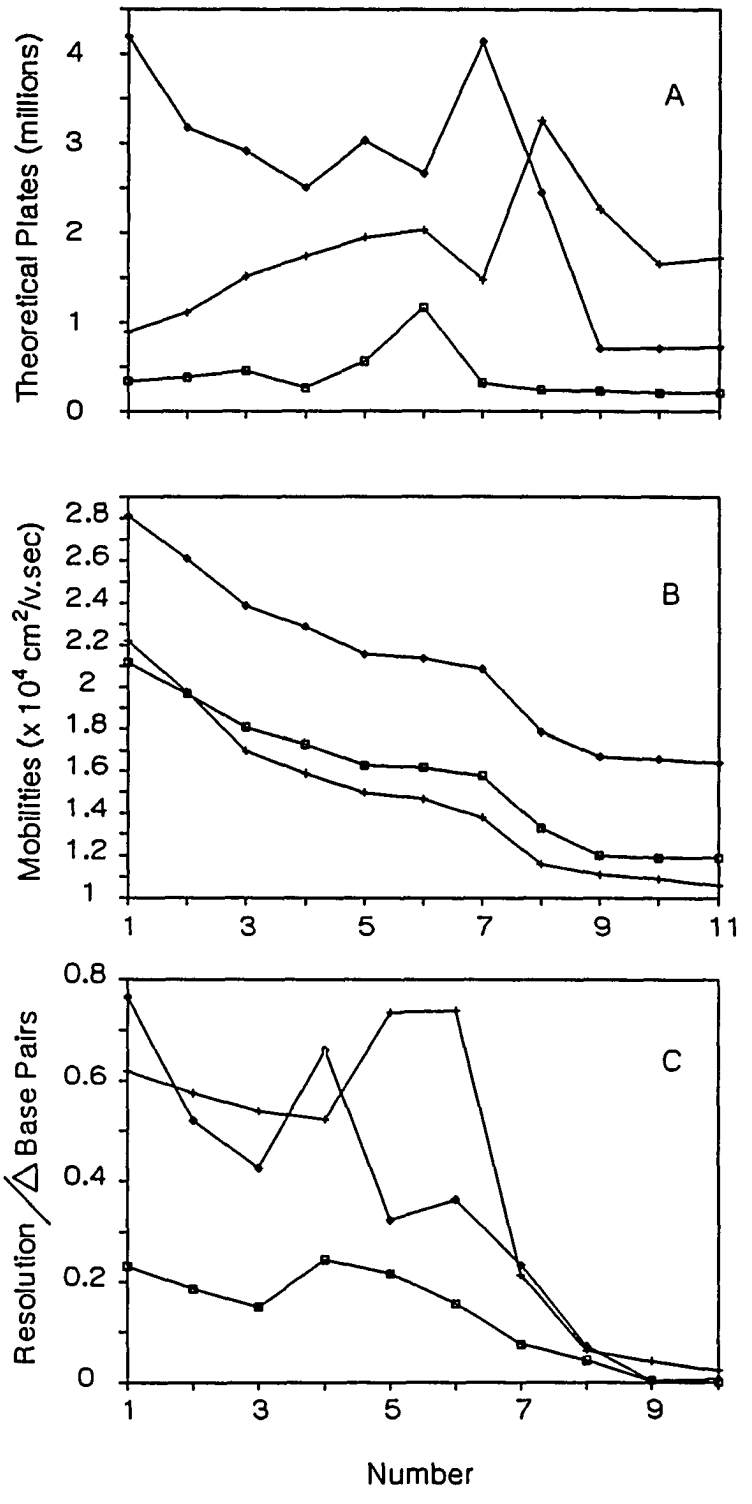

Fluorescence
A
B
C
72
118
194
234
271
281
310
603
872
1078
1353
7
9
11
13
15
14
18
22
26
30
34
30
50
70
90
Time (min)

Figure 3. Electrophoretic separation of 0.9 μg/ml ϕX 174 RF DNA-Hae digest intercalated with 1μg/ml of TOTO in the running buffer at -12 kv. Conditions are as in Fig. 2 except that light source: 1-mW He-Ne laser and(A): First run, (B): second run with new gel matrices, and (C): consecutive run without the change in gel matrices.


A
72
118
194
234
271
281
310
603
872
1078
1353
14
16
18
20
22
24
26
B
72
118
194
234
271
281
310
603
872/1078/1353
12
14
16
18
20
22
24
C
72
118
194
234
271
281
310
603
872
1078
1353
20
22
24
26
28
30
32
34
36
38
Fluorescence
Time (min)

physically entrapped or covalently bonded with polymers by H-bonding, van der Waals force and covalent bonding. As long as the interaction occurs, the morphology of gel matrices changes, and in turn, the sieving effect changes. Also, the dielectric constant of the system was changed as dye was added into the running buffer. This would change the swelling of polymer network. Comparison between the results of Fig 1 and 3 shows that several small peaks are eluted after each main peak, and can be resolved if DNA is intercalated with TOTO for a long period. A probable cause of these small peaks is that DNA complexes forming different conformations, since TOTO is a flexible type of intercalator (numbers of methyl groups between aromatic rings is larger than 8) and it has two different aromatic rings on each sides which can intercalate with the bases of DNA. Therefore, there are at least three different conformations for each fragment. Figure 4 shows the comparison between two dyes in the separation of DNA fragments at -12 kV. Surprisingly, very high theoretical plates, up to several millions, can be achieved if DNA fragments are intercalated with TOTO in the running buffer. However, it cannot provide as a high resolution as EthD, especially for large fragments. A possible reason is that the capillary wall may have stronger interactions with TOTO (MW 1351 g/mol, four positive charges and more flexible) than with EthD (MW 394 g/mol, one positive charge), since we can see that the separation ability decreases each run, even though we refill gel matrices every time when TOTO was in the running buffer. Also, for fragments larger than 800

Figure 4. Comparison the results of Fig 1 to 3 at -12 kV: (A): Theoretical Plates; (B): Mobilities, (C): Calulated Single-Base Resolution. Numbers 1 to 11 corresponds to the order of the peaks in Fig. 1 to 3 for (A) and (B). In (C), numbers 1 to 10 corresponds to the order of two adjacent peak pairs. Symbols: □ = Fig. 1, + = Fig. 2, ◊ = Fig. 3.


Theoretical Plates (millions)
0
1
2
3
4
A
Mobilities (x 10⁴ cm²/v.sec)
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
B
1
3
5
7
9
11
Resolution / Δ Base Pairs
0
0.2
0.4
0.6
0.8
C
1
3
5
7
9
Number

base pairs, the resolution and amount of these fragments eluted decreased dramatically. This phenomenon is not seen in TOTO intercalated with DNA outside the column. Again, these results suggest that there are interactions between the dye and the polymers, and the dye and the capillary wall. It is worth noting that DNA intercalated with EthD provides better resolution, while the mobilities of DNA fragments intercalated with TOTO are less affected than with EthD, if dyes were in the running buffer. Therefore, the change of the characteristics of DNA with TOTO is less than with EthD.[29] However, the change in the polymer network and capillary are much more significant by TOTO. Changes in viscosity of gel matrices and size or shape of DNA fragments are also the possible reasons that DNA intercalated with TOTO move faster. In addition to the stronger interaction of TOTO with the capillary wall, more complexes formed with TOTO is one reason to cause worse resolution. Relatively larger molecules of TOTO than EthD may cause the sieving mechanism for DNA complexes to be worse. The phenomena are more significant for the larger DNA fragments, which cannot be separated well in the TOTO system, since more TOTO has to be intercalated with the bases of DNA.

It is well known that a bisintercalator can provide higher sensitivity and higher affinity to DNA than a monointercalator does. Our studies show that both the use of different concentrations of TOTO and EthD can provide reasonable signal when an argon-ion laser at 488 nm or a He-Ne laser at 543.6 nm is used as the light source. In each case, we were able to get comparable signal when 0.9 μg/ml

DNA is detected as shown in Fig. 1 through 3. Amount injected for analysis is 1.5-fg DNA with 72 base pairs. Figure 5 shows the electropherogram of 0.028 μg/ml of DNA intercalated with EthD when a 1-mW He-Ne laser was used as the light source.

Electric field strength, which can affect the shape of DNA, structure and viscosity of gel matrices, sample injection amount, longitudinal diffusion and transverse diffusion, is one of the major factors in determining the separation efficiency of DNA in CGE. Again, Figures 1 and 2 show the effect of voltage on the separation of DNA fragments. DNA intercalated with TOTO can be well resolved only with very low electric field strength. More detailed comparisons are shown in Figure 6. Greater change in mobilities of the larger fragments is predictable as the shape of DNA changes as high electric field strength is applied. Resolution always increases as electric field strength decreases. For small fragments which have higher diffusion coefficients, resolution is higher at 200 V/cm than at 100 V/cm, since longitudinal diffusion dominates peak variance if running time is too long. Lower resolution at very high electric field strength is due to the large effect of the thermal gradient since more Joule heat is produced and viscosity of the gel decreases significantly. Also the changes in DNA shape and the conformation of polymer network at high electric field may cause worse separation performance. It is interesting to note that the resolutions of DNA-EthD complexes decrease closely to those of DNA-TOTO in high electric field strength. Figure 7

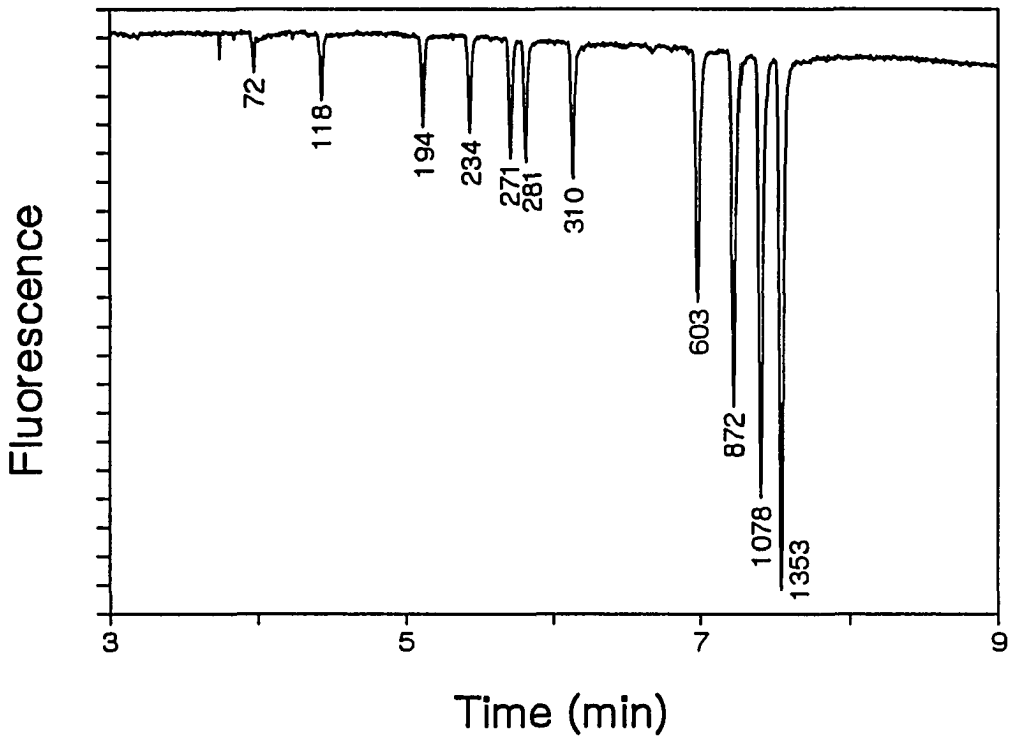


Figure 5. Electrophoretic separation of 0.028 μg/ml of ϕX 174 RF DNA-Hae digest intercalated with 1μg/ml of EthD in the running buffer. Conditions are as in Fig. 3 except that running voltage was -5 kV and capillary total length and effective length was 25 and 17 cm, respectively.

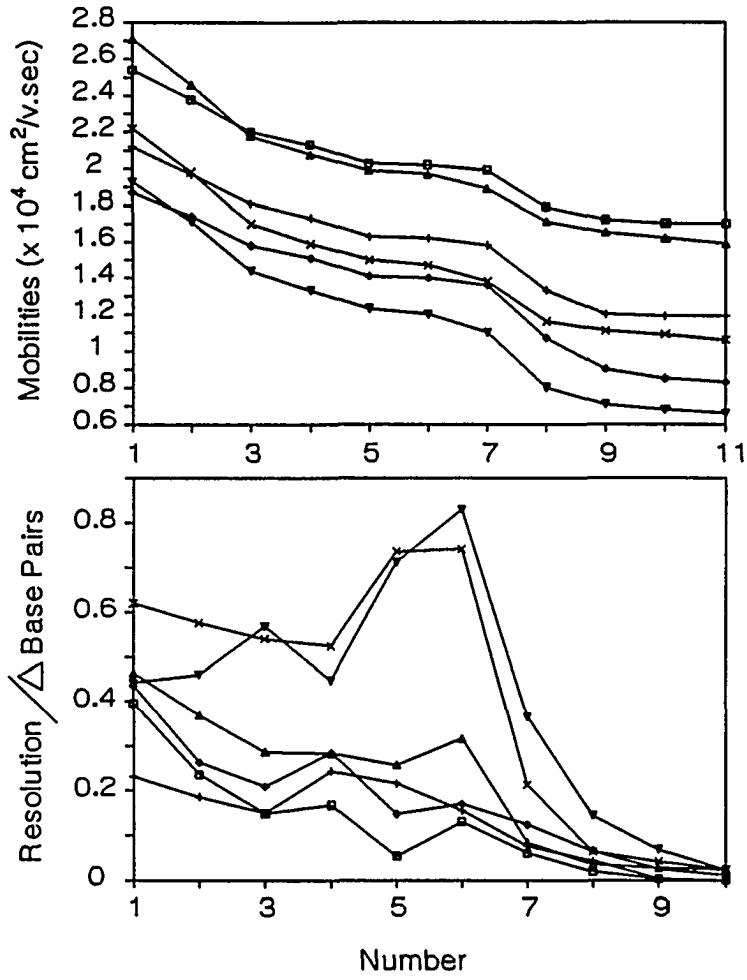
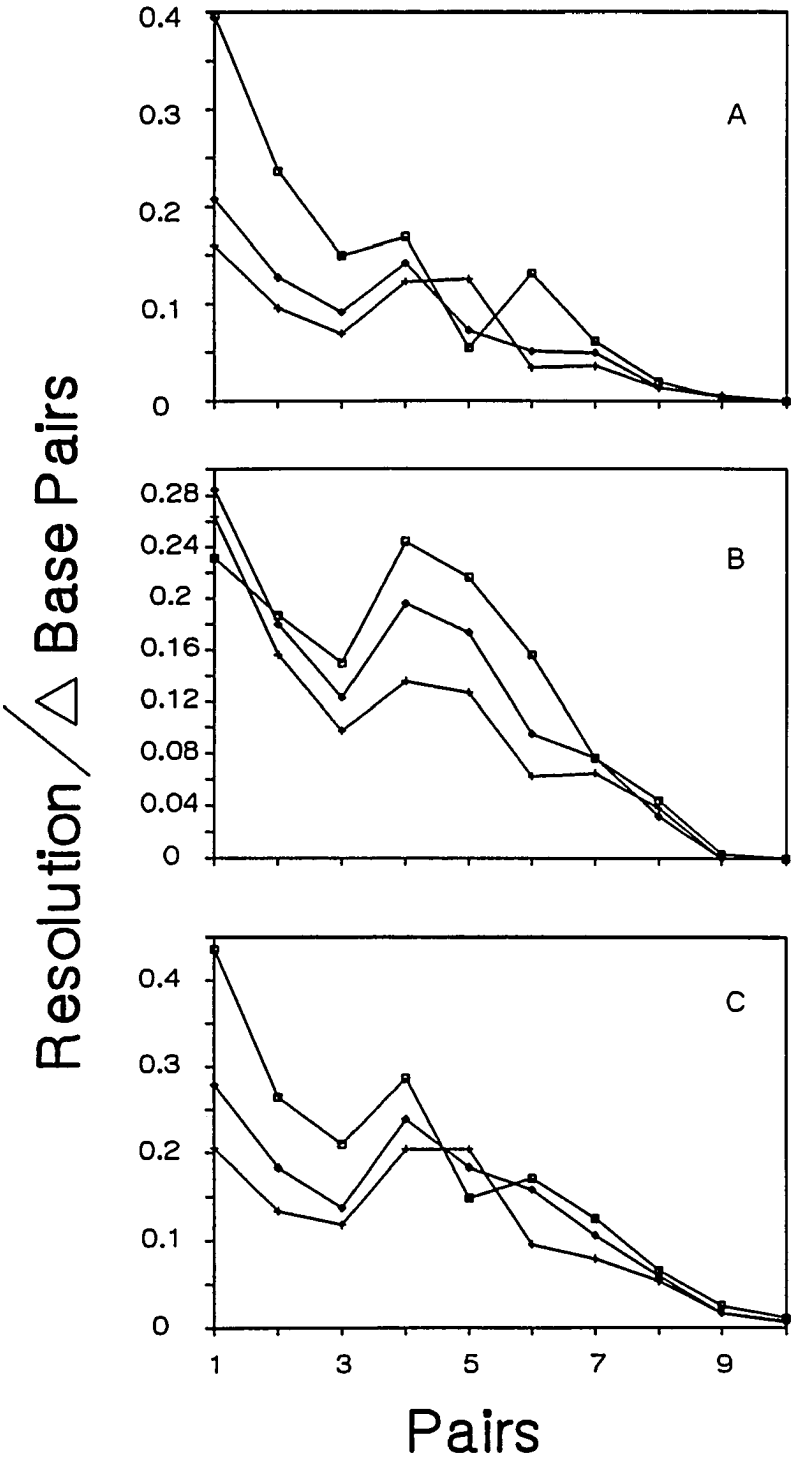


Figure 6. Comparison the effect of voltage on the separation of ϕX 174 RF DNA-Hae digest: results are taken from Fig. 1and 2; (A) Mobilities, □, +,◇ correspods to the results of Fig 1 A, B, and C, respectively. △, ×,▽ corresponds to the results of Fig. 2 A, B, and C, respectively. (B) Calculated single base resolution. Symbols represent as in (A).

Figure 7. Voltage effect on the separation of ϕX 174 RF DNA-Hae digest in different concentration of gel matrices: (A) -21 kV, (B) -12 kV and (C) -6 kV. □, +, and ◊ corresponds to 2.5 , 3 and 3.5 % PEO, respectively.


Resolution / △ Base Pairs
0.4
0.3
0.2
0.1
0
A
0.28
0.24
0.2
0.16
0.12
0.08
0.04
0
B
0.4
0.3
0.2
0.1
0
C
1
3
5
7
9
Pairs

shows the effect of voltage on the resolution of DNA fragments at different concentrations of gel matrices. The trend that better resolution can be obtained with a low electric field strength is still observed. In a suitable range, higher concentration of the gel can provide better resolution. In this study, we found that the resolution cannot become better if the concentration of gel is higher than 2.5%. At higher concentrations, gel cannot provide better resolution, which means that longitudinal diffusion is not a significant factor affecting resolution since the variance due to the fact that the traveling time (longer for small pores) and the diffusion variance (larger for low viscosity gel) compensates each other. In addition, sieving effect and the stability of gel matrice are important. At very low electric field strength, resolution is more comparable in gel matrices prepared from three different concentrations which may be useful to explain that a high concentration gel is less stable at high electric field strengths.

Some authors have mentioned that the capillary length cannot be a factor of resolution in capillary zone electrophoresis.[30] We believe it is another important factor in the performance of CGE, since the sieving effect and the variance of diffusion are proportional to the traveling distance of analytes through gel matrices.[31] Also, it is important to have the right length to keep a reasonable electric field strength at low voltage in order to minimize arcing, which will deteriorate capillary performance. Figure 8 shows the comparisons of theoretical plates, mobilities and resolution in different lengths. Ideally, theoretical plates are

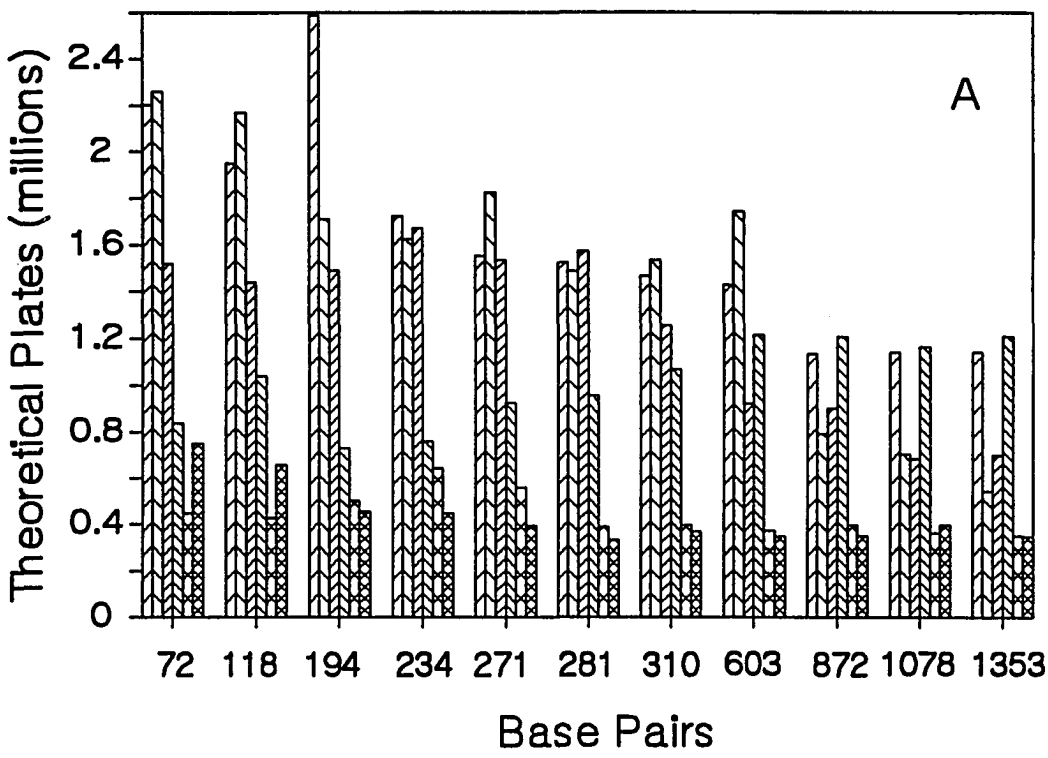


Figure 8. Length effect on the separation of ϕX 174 RF DNA-Hae digest at 200 V/cm: (A): Theoretical Plates, (B) Mobilities and (C) Calculated single base resolution. Symbols; light-left-shaded bar: L = 50 cm, light-right-shaded bar: L = 40 cm, dense-left-shaded bar: L = 35 cm, dense-right-shaded bar; L = 30 cm, light-cross bar: L = 25 cm, dense-cross bar: L = 20 cm.

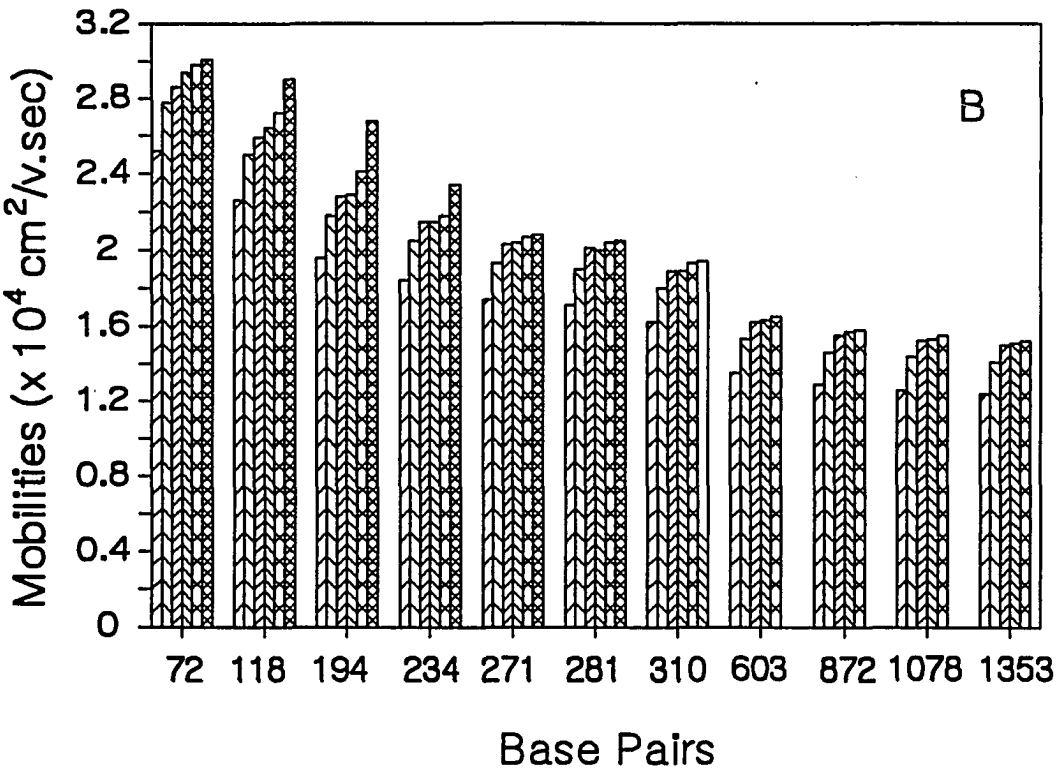


Figure 8. (continued)

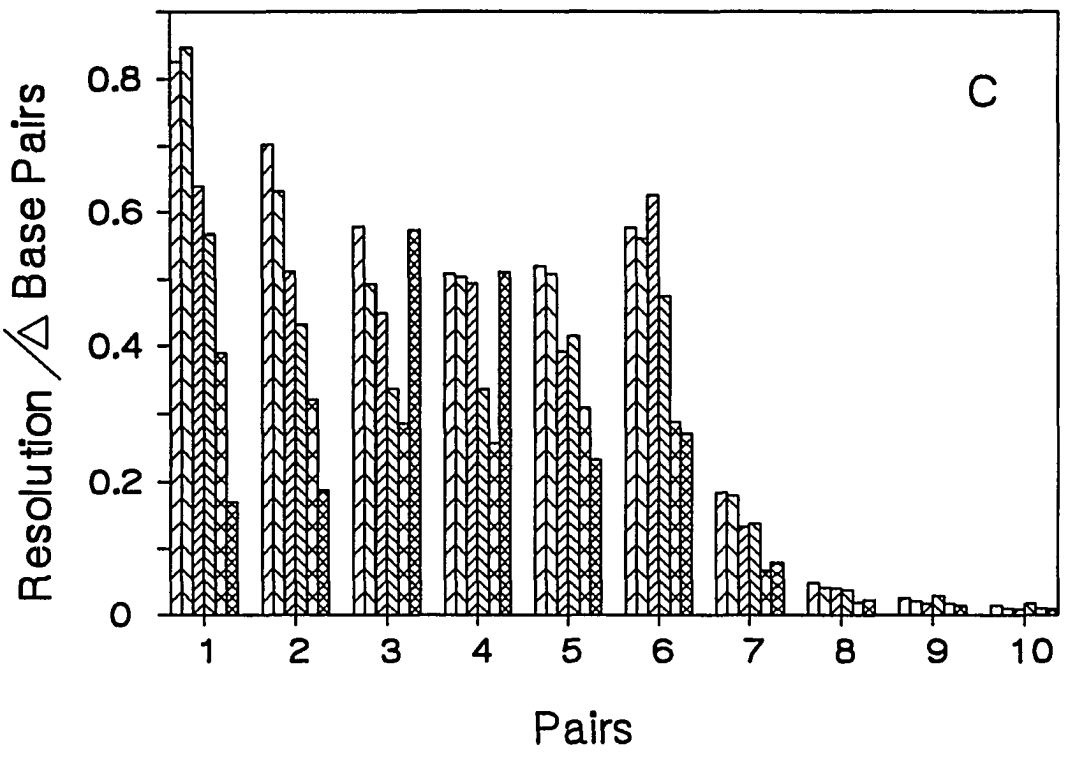
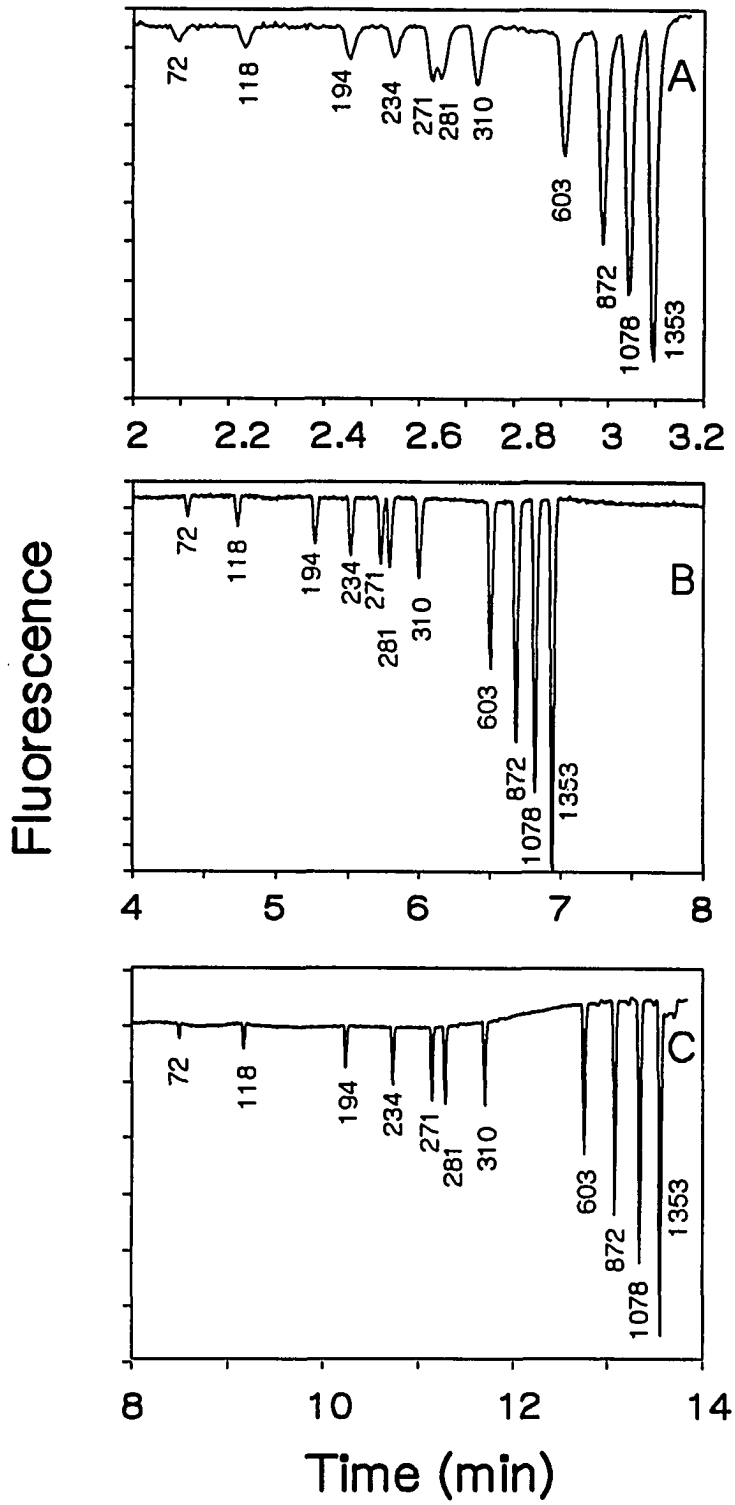


Figure 8. (continued)

directly proportional to the square of capillary length and inversely proportioal to the square of peak variance. Generally, for longer lengths, and the peaks correspond to longer retention times, total peak variance will be dominated by diffusion variance. On the other hand, if a short capillary is used or analytes elute faster, injection variance plays a more important role on the determination of resolution. For small fragments, it is significant that theoretical plates decrease as length decreases, which is predictable as N is proportional to L^2. For larger fragments, the variation of the trend is large and results in poor sieving effect and large peak variances among injections because of a bias effect in electromigration injection. Mobilities of DNA fragments always increase as length decreases. This is because of a small sieving effect and lower fraction of complexes formed between DNA and EthD, which has lower mobilities than DNA with free form, in short capillaries. As length decreases, resolution decreases since there is a smaller sieving effect. This trend is more significant for small fragments and indicates that the effect of length on the resolution is not significant for very large DNA fragments, but very significant for small DNA fragments.

High speed separation with high resolution is one of the features of CGE over conventional high performance liquid chromatography. Figure 9 shows the separations of 11 DNA fragments intercalated with EthD at different electric field strengths and different capillary lengths. Figure 9 A shows that the separation with a reasonable resolution can be achieved in less that 3 min with a very high

Figure 9. High-speed separation of ϕX 174 RF DNA-Hae digest at different length and electric field strength (E): (A) L = 40 cm, E = 525 V/cm, (B): L = 50 cm, E = 420 V/cm, and (C): L = 60 cm, E = 350 V/cm.


Fluorescence
72
118
194
234
271
281
310
603
872
1078
1353
A
B
C
2
2.2
2.4
2.6
2.8
3
3.2
4
5
6
7
8
10
12
14
Time (min)

electrical field, 525 V/cm and short capillaries, 40 cm. This is an important requirement in the study of DNA sequencing, since high speeds with reasonable resolution is necessary to sequence the 3×10^9 base pairs in the human genome.

Figures 10, 11 and 12 show the feature of PEO gel matrices in DNA sequencing. Fig 10 and 11 are the results of PGEM/U sequencing with different capillary lengths, where an argon-ion laser at 488 nm and two 515-nm cutoff filters were used. In this case, peaks with high intensities should respond to A and G. Figure 12 is the result of PGEM/U sequencing that He-Ne laser at 543.6 nm and one 610-nm cutoff filter and one 630-nm interference filter were used. Larger peaks should correspond to A and T. The results show that it is possible to use two lasers and two windows to sequence DNA up to several hundred base pairs. Also, a more sensitive technique seems to be needed in order to get more information from larger fragments.

Conclusion

Several important factors affecting the separation performance of DNA including electrical field strength, nature of dye, length of capillary, and concentration of gel have been well demonstrated in this work. Results show that the separation performance in CGE will be affected by dyes, not only due to the interaction between DNA and dye molecules, but also due to the interaction

Figure10. PGEM/U sequencing: L = 50 cm, E = 200 V/cm, light source: argon-ion laser at 488 nm.

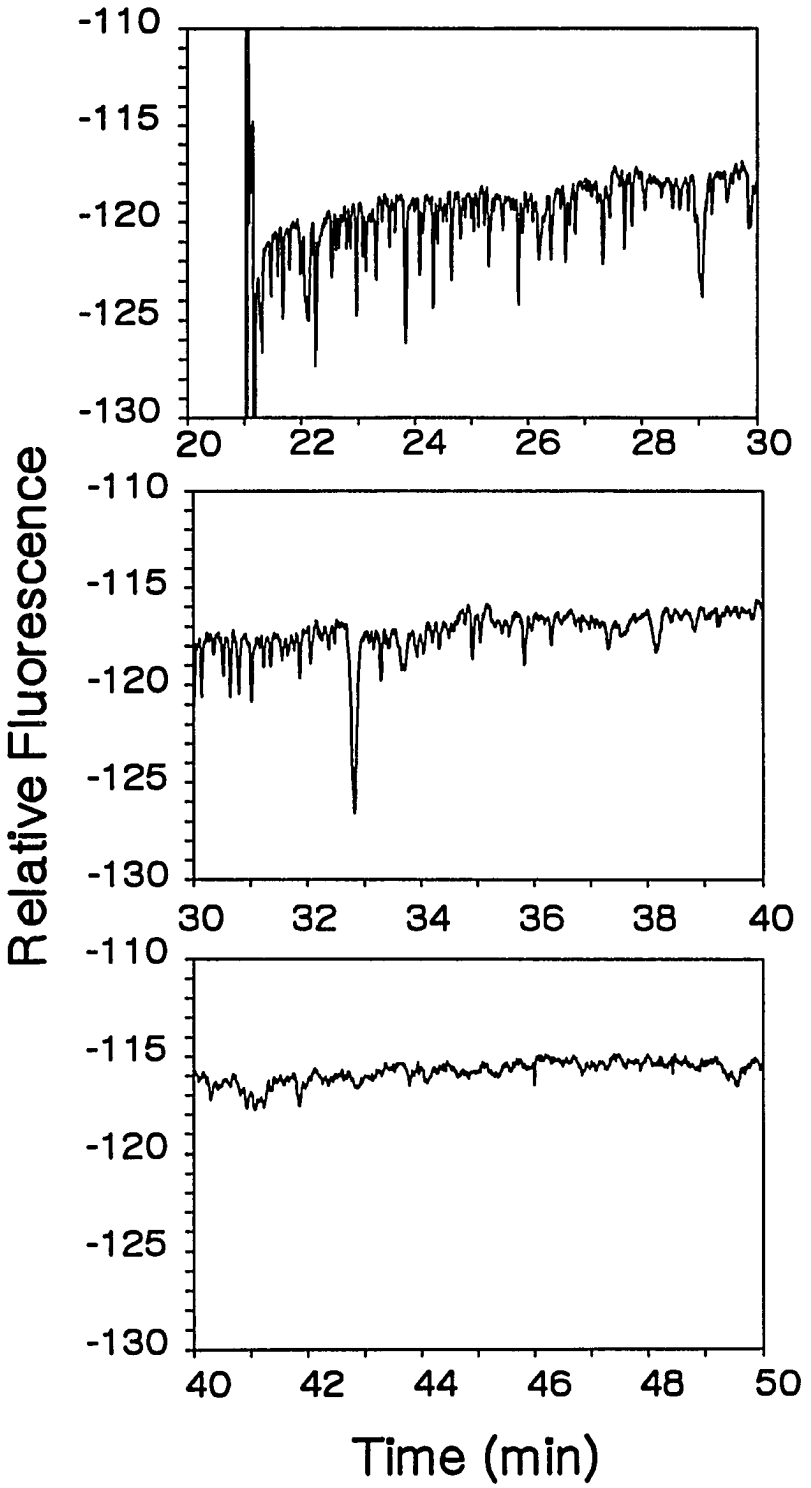

-110
-115
-120
-125
-130
20
22
24
26
28
30
-110
-115
-120
-125
-130
30
32
34
36
38
40
-110
-115
-120
-125
-130
40
42
44
46
48
50
Relative Fluorescence
Time (min)

Figure 11. PGEM/U sequencing. Conditions are as in Fig. 10 except L = 30 cm.

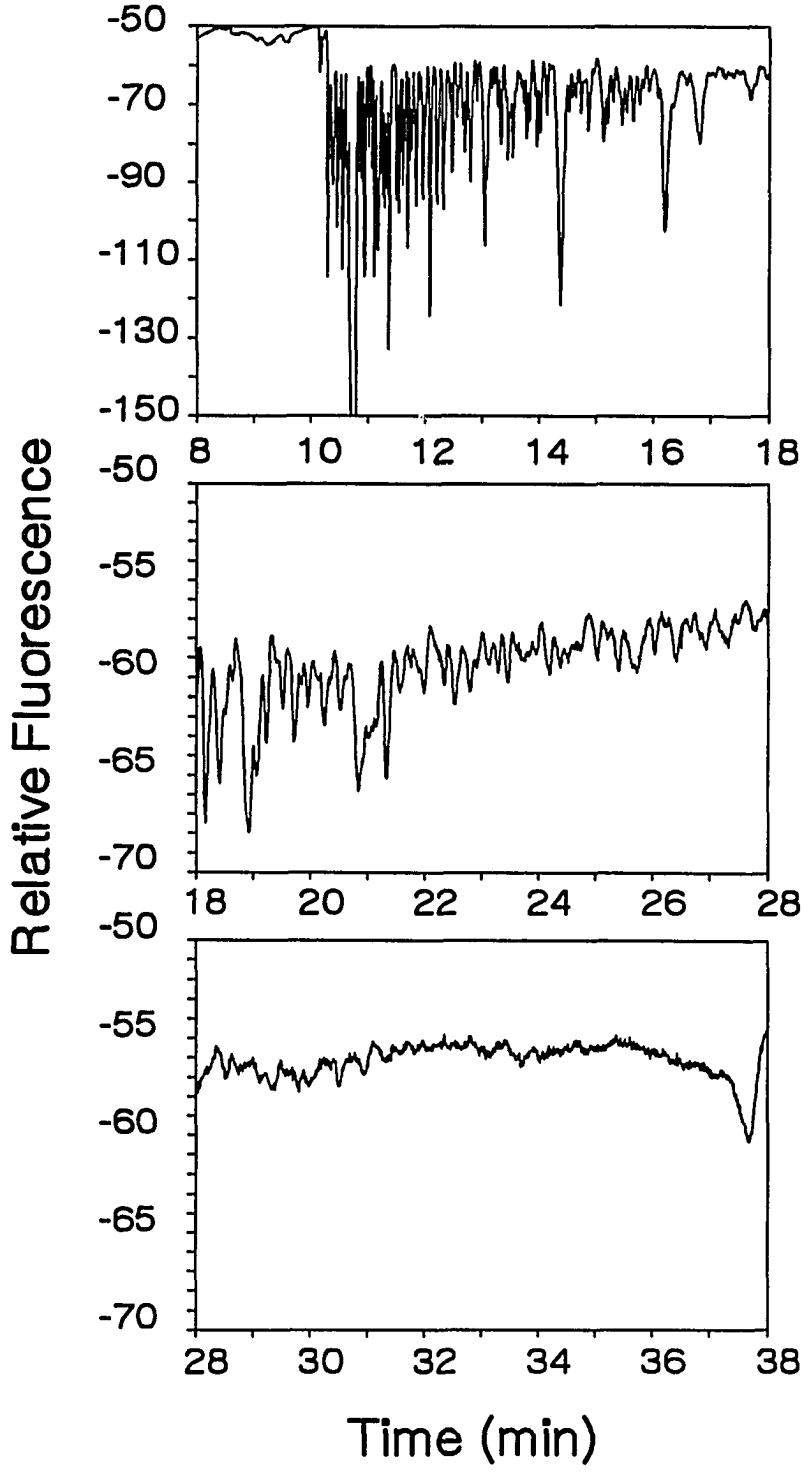
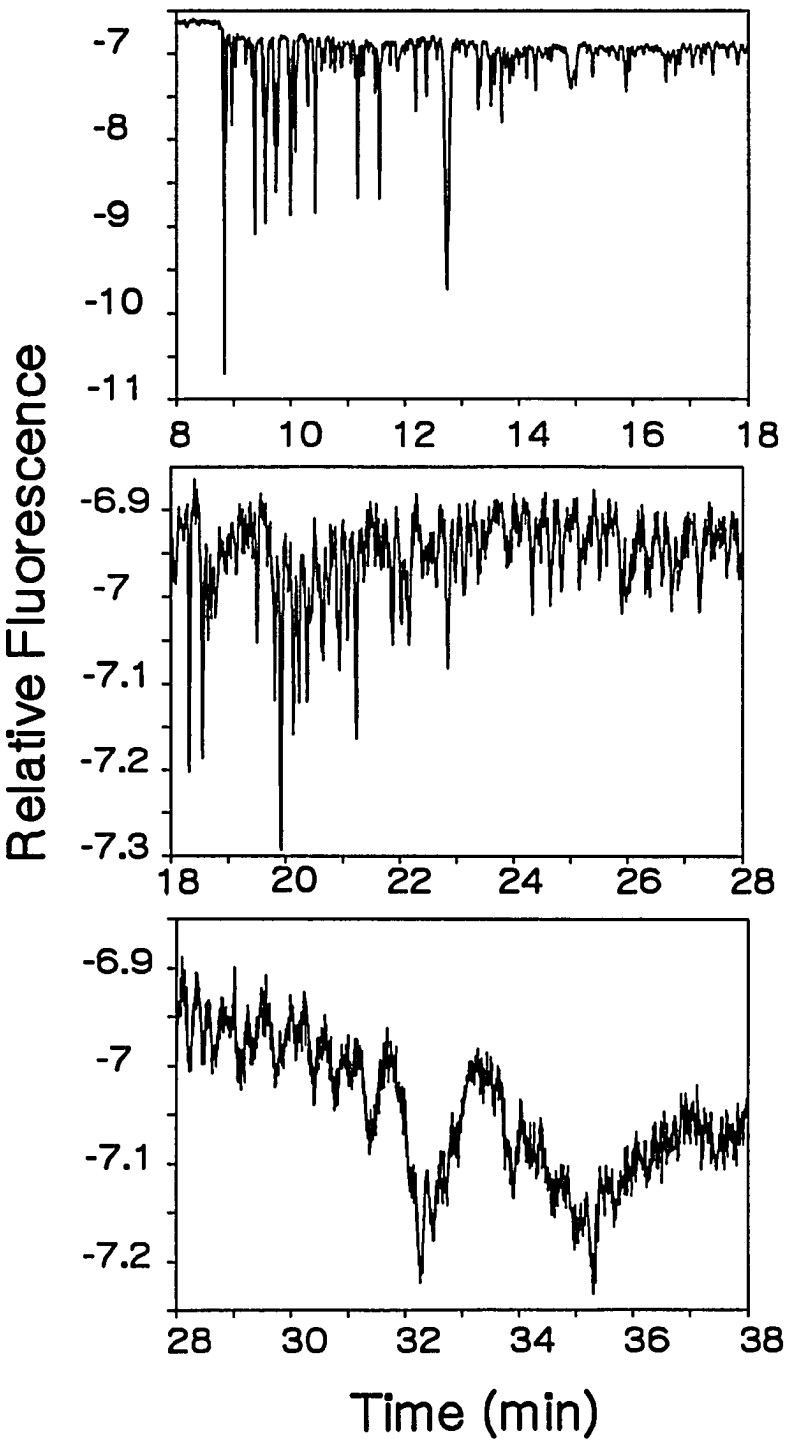

Relative Fluorescence
-50
-70
-90
-110
-130
-150
8
10
12
14
16
18
-50
-55
-60
-65
-70
18
20
22
24
26
28
-50
-55
-60
-65
-70
28
30
32
34
36
38
Time (min)

Figure 12. PGEM/U sequencing. Conditions are as in Fig. 11 except light source: He-Ne laser at 543.6 nm.


Relative Fluorescence
-7
-8
-9
-10
-11
8
10
12
14
16
18
-6.9
-7
-7.1
-7.2
-7.3
18
20
22
24
26
28
-6.9
-7
-7.1
-7.2
28
30
32
34
36
38
Time (min)

between dye with polymer and the capillary wall. High efficient separations (several million plates) by using very low amount (femtogram) of DNA samples in less than 10 min) have been shown in this experiment described herein. Also, preliminary DNA sequencing has been done by using 2.5 % PEO gel matrices with two different lasers. Since a technique with high speed, high resolution and high sensitivity has been demonstrated in this work, it encourages us to use this system to study a genetic disease. For example, the identification of mutations and polymorphisms in human genes by this technique with PCR is helpful to understand the molecular nature of disease and diagnosis of diseases. Although several features of this technique have been developed, there is still a need to develop a better system to have single-base resolution up to 1000 base pairs and better sensitivity like single molecule detection. As long as this requirement is reached, the black box of several kinds of genetic diseases such as cancer can be easily opened.

References

1. Lindsay, S. M., Philipp, M. GATA, 1991, 8, 8-13.
2. Davis, L. M., Fairfield, F.R., Harger, C. A., Jett, J. H., Keller, R. A., Hahn, J. H., Krakowski, L. A., Marrone, B. L., Martin, J. C., Nutter, H. L., Ratliff, R. L., Shera, E. B., Simpson, D. J., Soper, S. A. GATA, 1991, 8, 1-7.

3. Ruiz-Martinez, M. C.; Berka, J., Belenkii, A., Foret, F., Miller, A. W., Karger, B. L. Anal. Chem. 1993, 65, 2851-2856.

4. Heiger, D. N., Carson, S. M., Cohen, A. S., Karger, B. L. Anal. Chem. 1992, 64, 192-194.

5. Swerdlow, H., Zhang, J. Z., Chen, D. Y., Harke, H. R., Grey, R., Wu, S., Dovichi, N. J., Fuller, C. Anal. Chem. 1991, 63, 2835-2841.

6. Huang, X. C., Quesada, M. A., Mathies, R. A. Anal. Chem. 1992, 64, 2149-2154.

7. Swerdlow, H., Wu, S., Harke, H., Dovichi, N. J. J. Chromatogr. 1990, 516, 61-67.

8. Cohen, A. S, Najarian, D. R., Karger, B. L. J. Chromatogr. 1990, 516, 49-60.

9. Carson, S., Cohen, A. S., Belenkii, A., Ruiz-martinez, M. C., Berka, J., Karger, B. L. Anal. Chem. 1993, 65, 3219-3226.

10. Zewert, T., Harrington, M. Electrophoresis, 1993, 13, 817-824.

11. Zhu, M., Hansen, D. L., Burd, S., Gannon, F. J. Chromatogr. 1989, 430, 311-319.

12. Nathakarnkitkool, S., Oefner, P. J., Bartsch, G., Chin, M. A., Bonn, G. K. Electrophoresis 1992, 13, 18-31.

13. Chrambach, A., Aldroubi, A. Electrophoresis 14, 1993, 18-22.

14. Kleemib, M. H., Gilges, M., Schomburg, G. Electrophoresis 1993, 14, 515-522.

15. Chang, H. T. this Dissertation, Ch. 6, pp. 129-156.

16. Takahashi, S., Murakami, K., Anazawa, T., Kambara, H. Anal. Chem. 1994, 66, 1021-1026.

17. Ueno, K., Yeung, E. S. Anal. Chem. 1994, 66, 1424-1431.

18. Navin, M. J., Rapp, T. L., Morris, M. D. Anal. Chem. 1994, 66, 1179-1182.

19. Milofsky, R. E., Yeung, E. S. Anal. Chem. 1993, 65, 153-157.

20. Rye, H. S., Quesada, M. A., Peck, K., Mathesis, R. A., Glazer, A. N. Nucl. Acids Res. 1991, 19, 327-333.

21. Glazer, A. N., Peck, K., Mathesis, R. A. Proc. Natl. Acad. Sci. USA 1990, 87, 3851-3855.

22. Kim, Y., Morris, M. D. Anal. Chem. 1994, 66, 1168-1174.

23. Srinivasan, K., Morris, S. C., Girard, J. E., Kline, M. C., Reeder, D. J. Applied and Theoretical Electrophoresis 1993, 3, 235-239.

24. Schwartz, H. E., Ulfelder, K. J. Anal. Chem. 1992, 64, 1737-1740.

25. Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connel, C. R., Heiner, C., Kent, S. B. H., Hood, L. E. Nature, 1986, 321, 674-679.

26. Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., Baumeister, K. Science, 1987, 238, 336-341.

27. Wang, P., Li, W., Giese, R. W., Yeung, E. S. J. Chromatogr. 1992, 608, 73-77.

28. Hjerten, S. J. Chromatogr. 1985, 347, 191-198.

29. Haugland, R. P., Larison, K. D. Handbook of Fluorescent Probes and Research Chemical, 5th ed.; Molecular Probes, Inc: Eugene, 1992, pp 224-229.

30. Jorgenson, J. W., Lukas, K. D. Science, 1983, 222, 266-272.

31. Luckey, J. A., Noris, T. B., Smith, L. M. J. Phys. Chem. 1993, 97, 3067-3075.

CHAPTER 8

RANDOM-WALK EFFECT ON THE ENHANCEMENT OF DNA SEPARATION IN CAPILLARY GEL ELECTROPHORESIS

Introduction

Capillary electrophoresis (CE), with high resolution and high speed, has become one of the most important separation techniques, especially, for biological samples such as protein and DNA for several decades.[1-5] Although CE has been well demonstrated in the separation of different kinds of samples, in isocratic condition, it is not good enough to be used to separate complicated samples which contain analytes with similar mobilities, and sizes. In this case, CE with gradient modes is necessary.

In capillary zone electrophoresis (CZE), pH and temperature gradients are critically important to enhance the resolution of separation. Since electroosmotic flow and electrophoretic mobilities of analytes both depend on pH, pH gradient has been demonstrated successfully in enhancing the separation ability of organic anions.[6-8] The temperature gradient, which can be generated by the flow of water at different temperature through the outside of the capillary or by voltage programming, has been demonstrated also by Yeung and coworkers.[9-10] In addition to pH and temperature gradients, dynamic flow gradient performed by the control

of the concentration of surfactant, which can adsorb onto the capillary wall to change the electroosmotic flow and its direction, was also used to improve the efficiency of the separation of organic acids by these authors.[11] The radial potential gradient generated by an external electric field driver also has been shown to be an effective method to enhance the efficiency of separation.[12, 13]

Capillary gel electrophoresis (CGE) is a powerful technique to separate macromolecules such as proteins and DNA via a sieving mechanism.[14, 15] At present, the importance of the CGE in the separation of DNA has been driven by the Human Genome Project (HGP).[16] The goal of the HGP is to create dense and cross-referenced genetic, physical and nucleotide-sequence maps of selected genomes in turn to understand the evolution of cells and solve genetic problems such as cancer. Hence, it is important to have a technique that can provide single-base resolution for a wide range of base pairs with high speed. Recently, polyacrylamide gels with relative low viscosity have been successfully demonstrated for single-base separation, up to 300 base pairs, by Karger's group.[17] Field strength gradient has been used to improve the separation of DNA fragments in a relatively short time.[18, 19] Recently, Morris and coworkers showed that variable frequency modulation in DNA separation can provide better resolution than a dc only field for the fragments with 603 to 1353 base pair.[20] In addition, several different gradient methods have been developed to enhance the resolution with different gel matrices in a slab gel system. Denaturing gradient gel electrophoresis

(DGGE) employs a gradient denaturing solvent in a polyacrylamide gel and has been demonstrated for the separation of two DNA fragments with a difference of one base pair.[21, 22] Temperature-gradient gel electrophoresis (TGGE) has also been used to enhance the separation performance by taking the advantage of the abnormal temperature dependence of mobilities of some DNA fragments.[23-25] Chrambach and coworkers have developed transverse agarose pore gradient gel electrophoresis to separate DNA fragments with a large range of sizes.[26, 27] However, in the slab gel system, it suffers from the relatively long separation time.

In this experiment described herein, mixed gel matrices prepared from poly(ethylene oxide), PEO, with different molecular weights were used to enhance the separation performance of DNA fragments with a large range of sizes. This method has been developed to provide several features including simpli speed and better resolution for DNA fragments with a larger range of sizes than the above methods.

Experimental Section

The experimental setup has been described in detail in reference 28. Briefly, a 1-mW He-Ne laser at 543.6 nm from Melles Griot (Irvine, CA) was used to illuminate the analytes to induce fluorescence. One RG 610-nm cutoff filter and one 630-nm interference filter were used to block scattered light from entering the

photomultiplier tube. The fluorescence signal was transferred through a 10-kΩ resistor to a 24-bit A/D interference at 4 Hz (Justice Innovation, Palo Alto, CA; Model DT2802) and stored on a computer (IBM, Boca Raton, FL; Model PC/AT 286).

Capillaries with 75 μm i.d. and 365 μm o.d. purchased from Polymicro Technologies, Inc. (Phoenix, AZ) were coated by the method described by Hjerten.[29] The total length of capillary used for the separation was 50 cm and effective length was 42 cm. A high-voltage power supply (Glassman High Voltage, Inc., Whitehorse Station, NJ, P/SMJ30P0400-11) was operated at +10 kV. The entire electrophoresis and detection system were enclosed in a sheet-metal box with HV interlocks.

The running buffer was 1X TBE with 1 μg/ml of ethidium bromide. All chemicals used to prepare the running buffer were obtained from Sigma Chemical Co. (St. Louis, MO). Gel matrices were prepared from PEO with different molecular weights that were purchased from Aldrich Chemical Co. (Milwaukee, WI). pBR 322 DNA-Hae III , pBR 328 DNA-Bgl I + pBR 328 DNA-Hinf I were purchased from Boehringer Mannhein Biochemicals (Indianapolis, IN). The injection concentration of DNA for the separation was 0.83 μg/ml, and the injection was performed at 6 kV for 3 sec. Between each run, the old gel matrices were flushed out from the capillary, then the capillary was filled with new gel matrices. Before the injection of the analytes, the capillary equilibriated at 10 kV for 10 min.

Results and Discussion

In CGE, pore size of gel matrices and the running electric field strength are the two most important factors affecting the separation performance. In this study, several factors affecting the pore size of gel matrices, including molecular weight of polymer, concentration of gel and the mixture of polymers with different molecular weights, will be discussed in detail. The effect of electric field strength on the resolution of DNA fragments has been discussed in detail in a previous study.[30] The pore size of gel matrices depends on the concentration of gel and the molecular weight of polymer. Equation 1 shows the relationship between the pore size of gel matrices and concentration of gel.[31]

$$\xi = k\, C^{-b} \qquad (1)$$

where ξ is the pore size of gel matrices, C is the concentration of gel, and k and b are experimental constants. As the concentration increases, the pore size will decrease. In terms of separation, the smaller pore size can provide better resolution for small DNA fragments. On the other hand, larger DNA fragments can be separated well in relatively larger pore media. Hence, it is important to choose an optimum concentration of gel to provide an optimum sieving mechanism to obtain better separation performance. The molecular weight of polymer is another

important factor to be considered seriously in CGE since the viscosity and pore size of gel matrices both depend on it. The relationship between viscosity of gel matrices and polymer molecular weight can be described by the Mark-Houwink-Sakurada equation,[32]

$$[\eta] = K\,(MW)^{p} \qquad (2)$$

where $[\eta]$ is the intrinsic viscosity, MW is the polymer molecular weight, K and p are constant characteristics of a given polymer/solvent system at a given temperature. Equation 3 expresses the pore size dependence on the polymer chain length (S).[31]

$$\xi = A\,S^{0.6} \qquad (3)$$

where A is an experimental constant. From Eq. 2 and 3, for a gel prepared from a certain polymer at a certain concentration, it is obvious that as the polymer molecular weight increases, the viscosity and pore size of gel matrices will increase. Again, it is important to prepare gel matrices from polymers with well-chosen molecular weights for better separation performance. From Eq. 1 and 3, pore size of gel matrices depends both on the polymer molecular weight and the concentration of gel. As a rule of thumb,it is essential to use polymers with low

molecular weight to create small pore sizes and to use polymers with longer chains to create larger pore sizes while minimizing viscosity.

Figures 1 through 6 show the separations of DNA fragments in gel matrices prepared from isocratic polymers. In order to have a reasonable separation, gel matrices prepared from polymers with low molecular weight always need higher content of polymers. Figure 1 shows that it is impossible to separate DNA fragments, less than 400 base pairs, in gel matrices prepared from polymers with MW 300,000 even though a high concentration, up to 15 %, was used (results not shown here). This is because the pore size of the gel matrices is too small to have a sieving effect for those DNA fragments. The retention time of DNA fragments in gel matrices prepared from polymer with short chains is longer than that with long chains. This corresponds to the inference from Eq. 3 that DNA fragments move faster through larger pores. The general trend is that the resolution is better as the gel matrices become more viscous and are prepared from polymers with higher molecular weights. The best comparison among the sieving mechanism of different gel matrices is the result of the resolution between base pairs 123 and 124, which clearly shows that gel matrices prepared from PEO with MW 8,000,000 can provide the best resolution. The variation of this trend is due to the different pore sizes of gel matrices. In addition, the difference of the viscosities among different gel matrices is also a possible reason since diffusion variance is an important factor on the determination of resolution. This can be

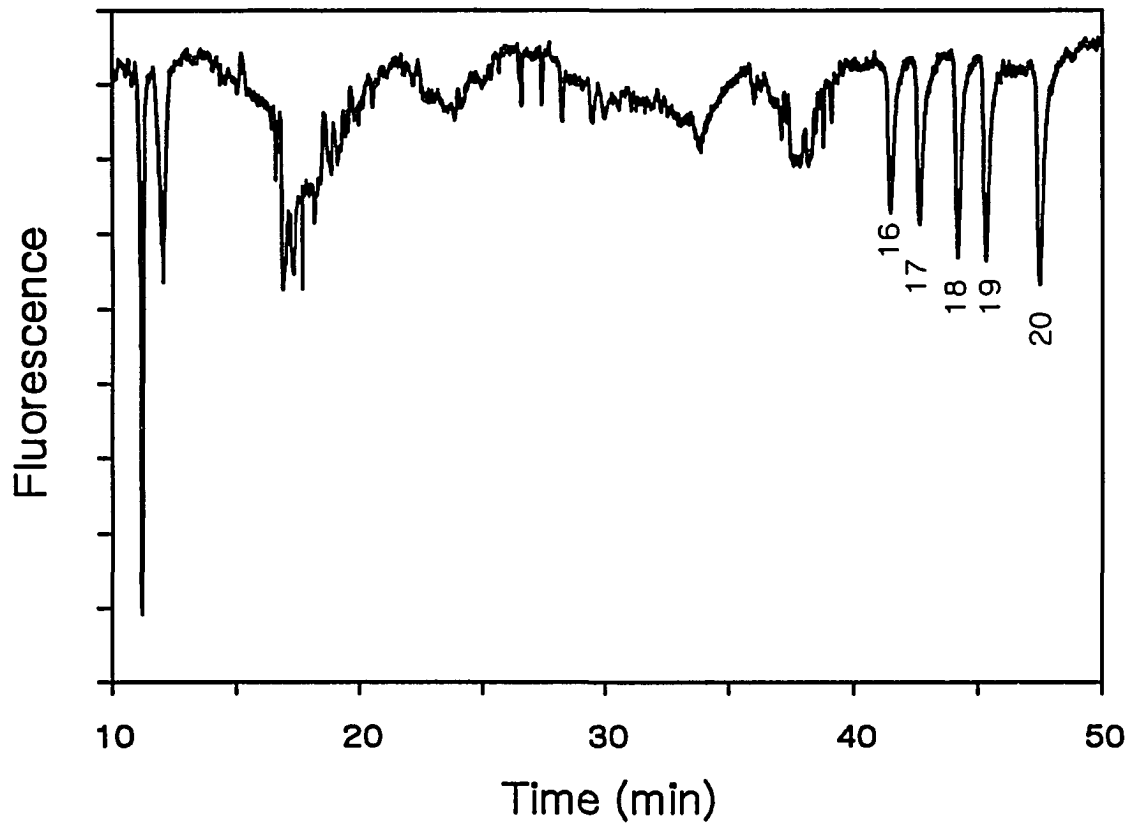


Figure 1. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 9 % PEO, MW 300,000. Other conditions are described in text. Peak assignments: 16 = 434 base pairs (BP), 17 = 458 BP, 18 = 504 BP, 19 = 540 BP, 20 = 587 BP.

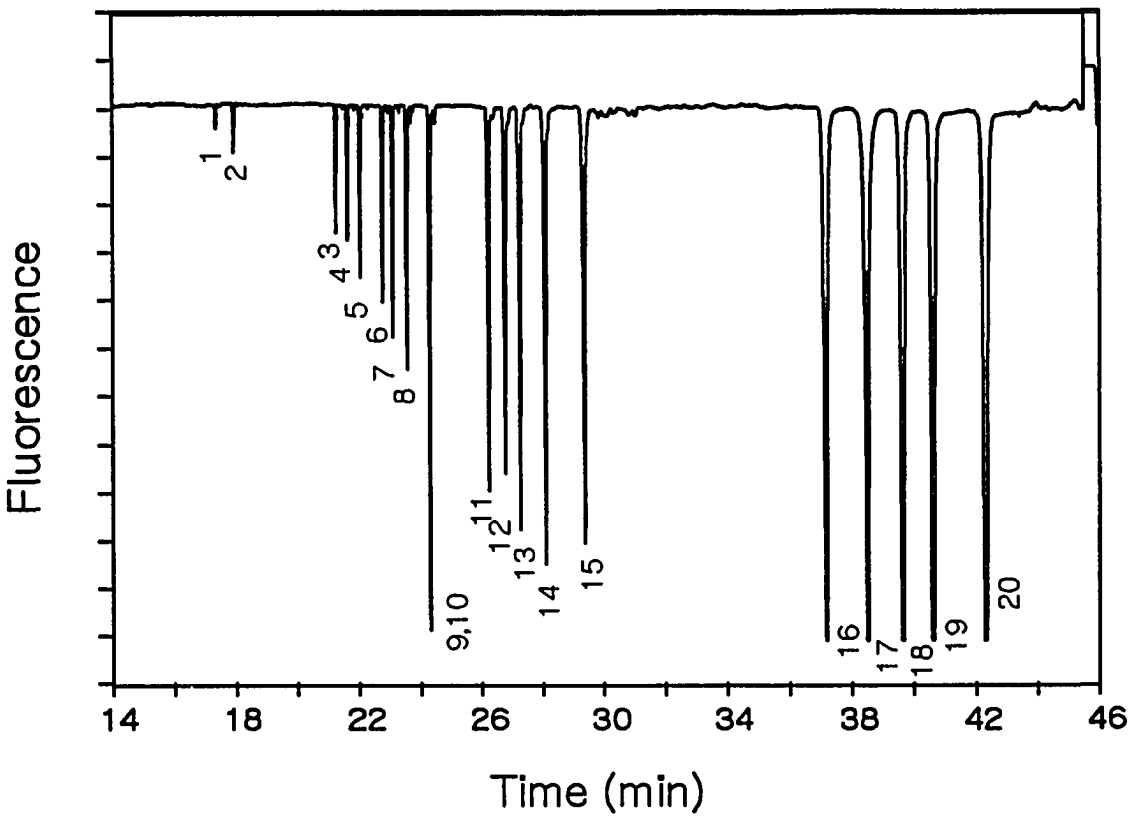


Figure 2. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 6 % PEO, MW 600,000. Other conditions are as in Fig. 1. Peak assignments: 1 = 18 BP, 2 = 28 BP, 3 = 51 BP, 4 = 57 BP, 5 = 64 BP, 6 = 80 BP, 7 = 89 BP, 8 = 104 BP, 9 = 123 BP, 10 = 124 BP, 11 = 184 BP, 12 = 192 BP, 13 = 213 BP, 14 = 234 BP, 15 = 267 BP. 16 to 20 are as in Fig. 1.

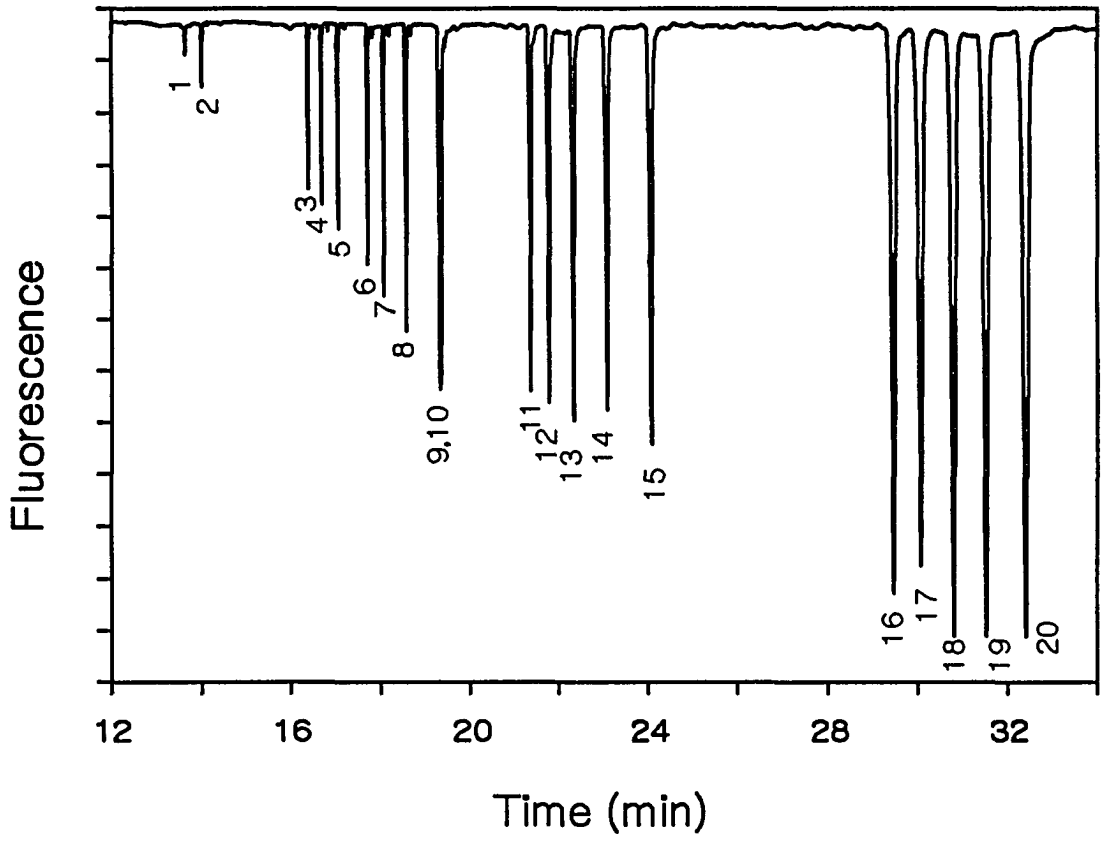


Figure 3. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 3.5 % PEO, MW 2,000,000. Other conditions and peak assignments are as in Fig. 2.

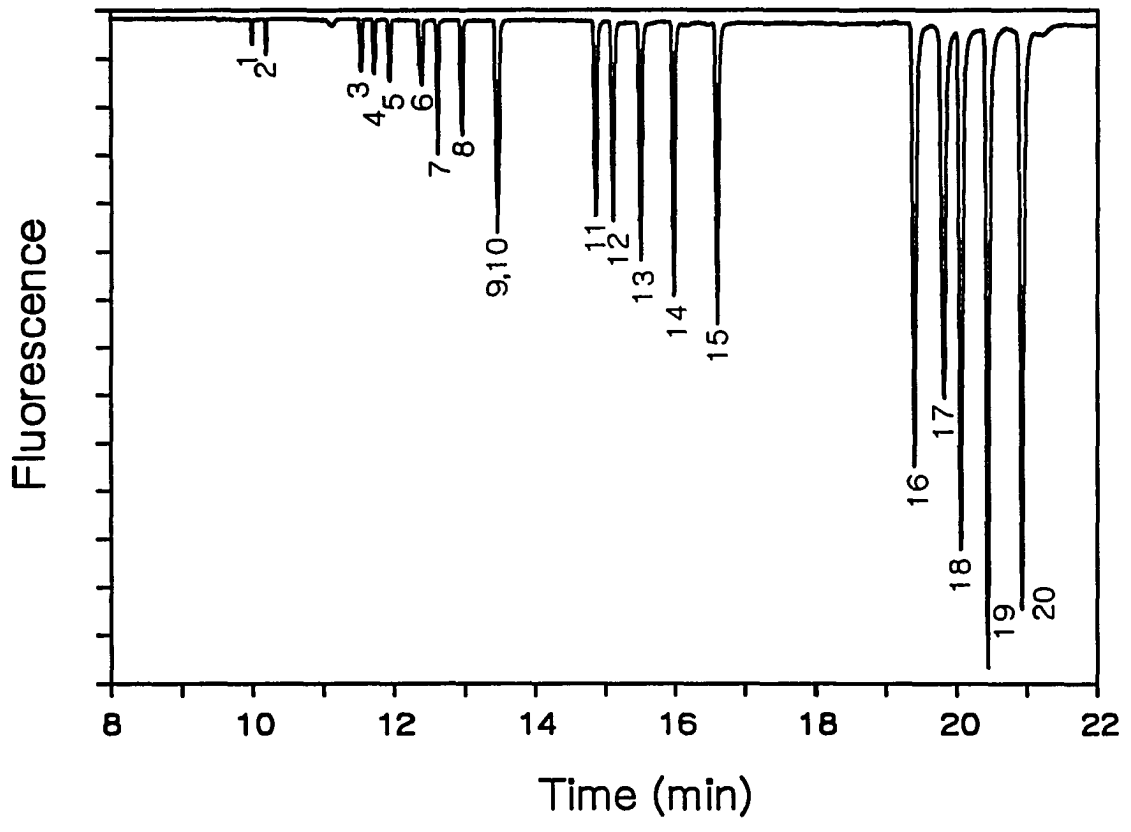


Figure 4. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 2.5 % PEO, MW 5,000,000. Other conditions and peak assignments are as in Fig. 2.

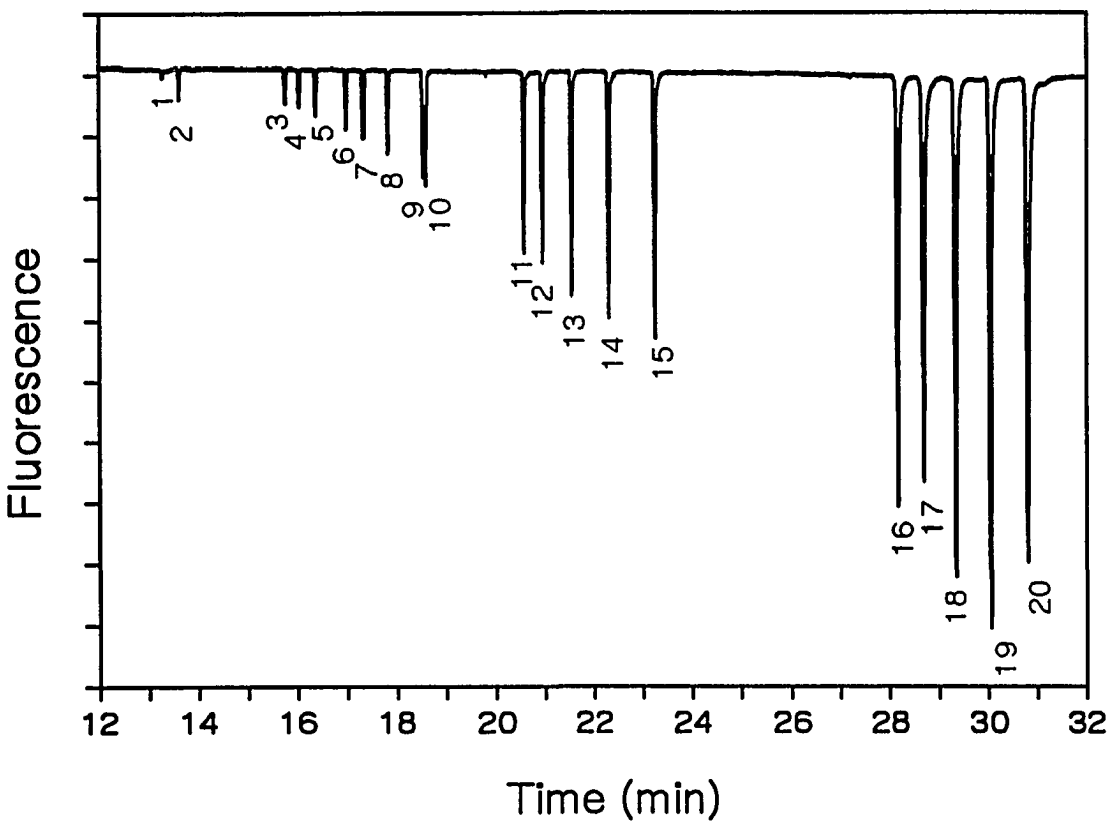


Figure 5. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 2 % PEO, MW 8,000,000. Other conditions and peak assignments are as in Fig. 2.

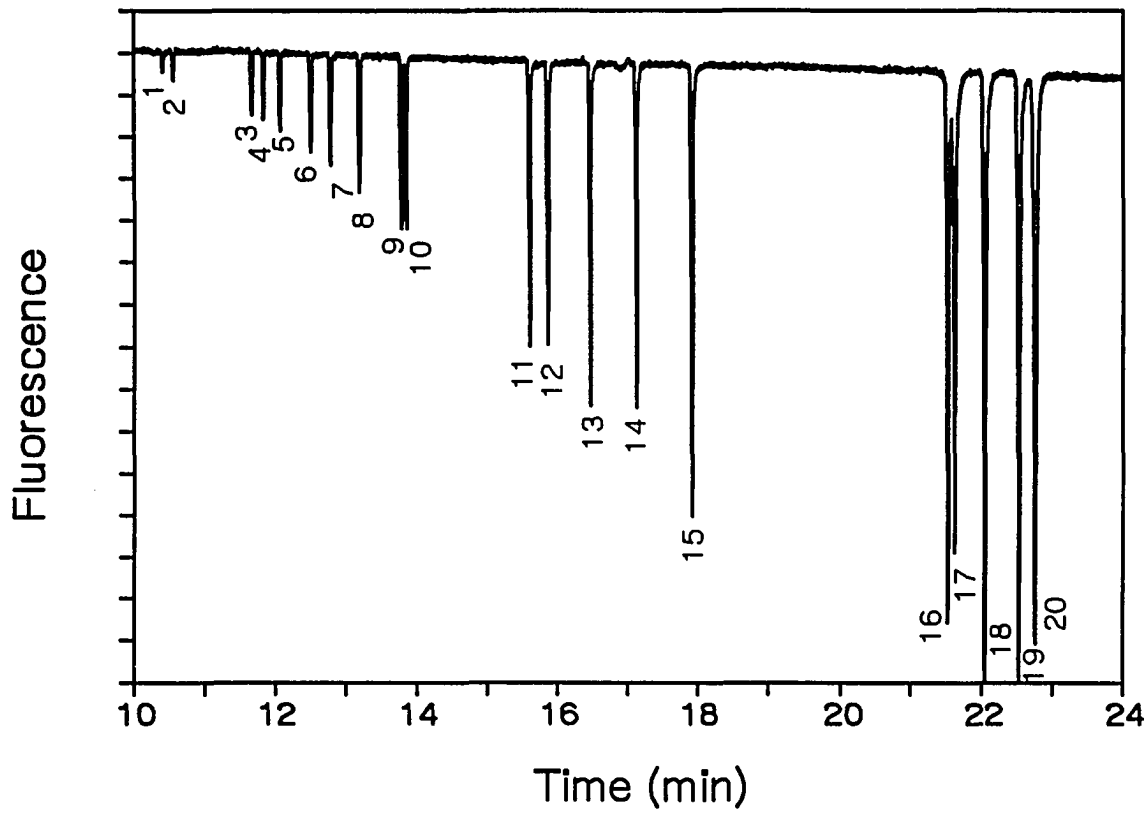


Figure 6. Electrophoretic separation of pBR 322 DNA-Hae III digest: gel matrices were 2.5 % PEO, MW 8,000,000. Other conditions and peak assignments are as in Fig. 2.

proven from the result in the use of the gel matrices made from 6 % PEO (MW 600,000) which has the highest viscosity among these gels. The calculated resolution is high due to the small diffusion variance, but the peaks between base pairs 123 and 124 cannot be separated. These results show that gel matrices prepared from higher concentration of PEO (MW 8,000,000) can provide better resolution, which indicates that the diffusion variance also plays an important role in the determination of resolution. Again, a more detailed comparison from Fig. 7 A and 8 A can prove that the sieving mechanism and viscosity of gel matrices are important to determine the resolution. Clearly, the results show that the smaller fragments can be well separated in gel matrices prepared from a polymer with a MW of less than 2,000,000. For the DNA fragments from 80 to 400 base pairs, better resolution seems to be achieved in gel matrices prepared from polymers with high MW, larger than 5,000,000. For fragments larger than 400 base pairs, the sieving effect of these gel matrices significantly decreases. Figure 9 A shows that the separation of DNA fragments are very efficient. Very high efficiencies can be obtained for large fragments in gel matrices prepared from polymers with larger MW, which shows that these gel matrices can provide larger pore sizes to allow larger fragments to move easily. For small fragments, the distribution between diffusion variance and sieving mechanism can be compensative, since the efficiency is higher while the resolution is lower in the gel matrices prepared from the PEO with smaller MW. All results shown here clearly suggest that it is possible

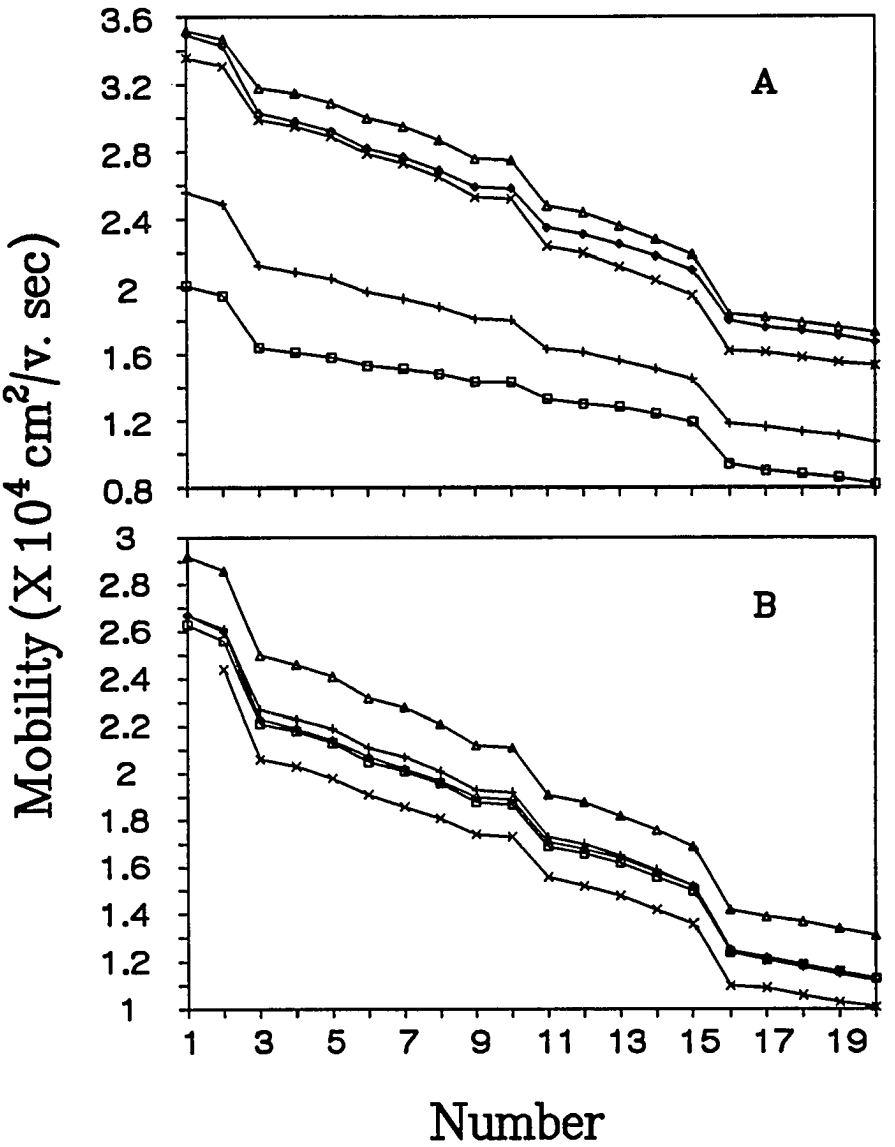


Figure 7. Comparison of the mobilities from the results of Fig. 2 to 6 and 10 to 14: in (A); □: Fig. 2, +: Fig. 3, ◇: Fig. 4, △: Fig. 5, ×: Fig. 6; in (B), □: Fig. 10, +: Fig. 11, ◇: Fig. 12, △: Fig. 13, ×: Fig. 14.

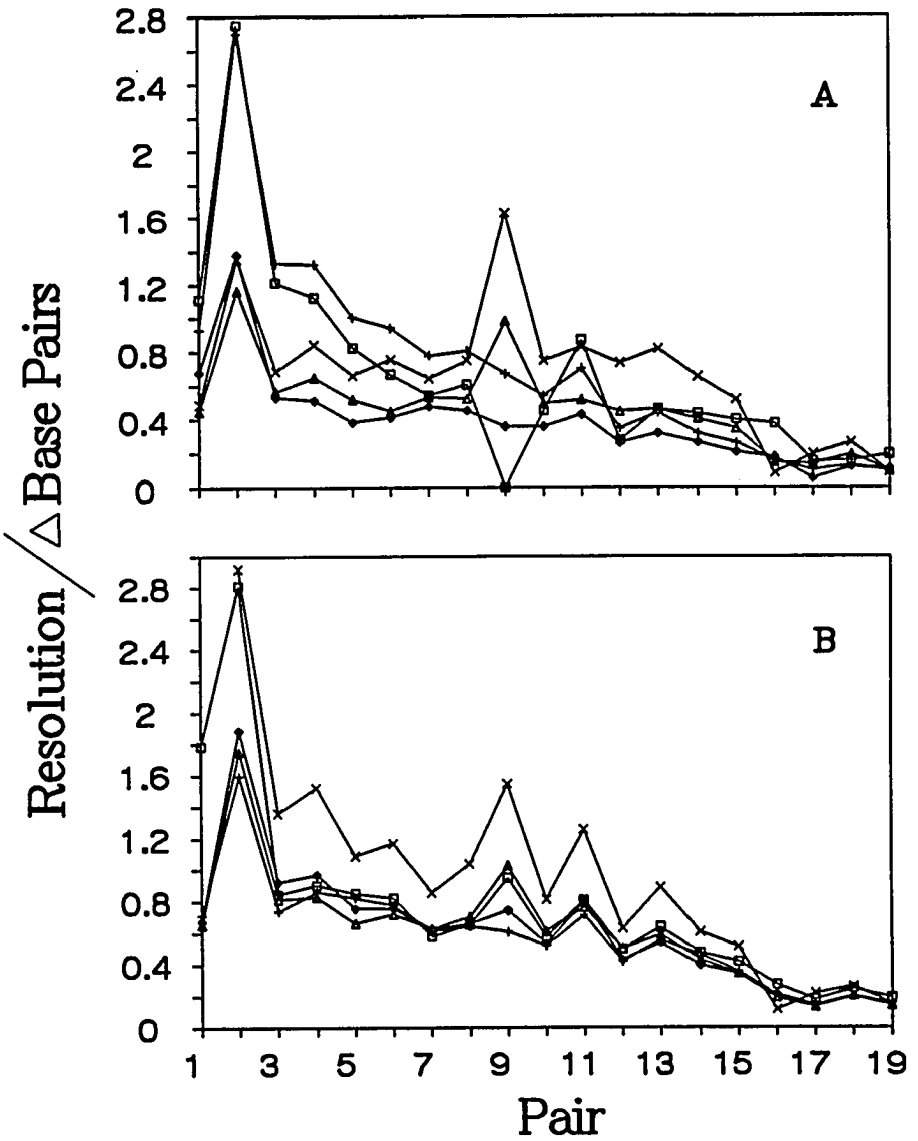
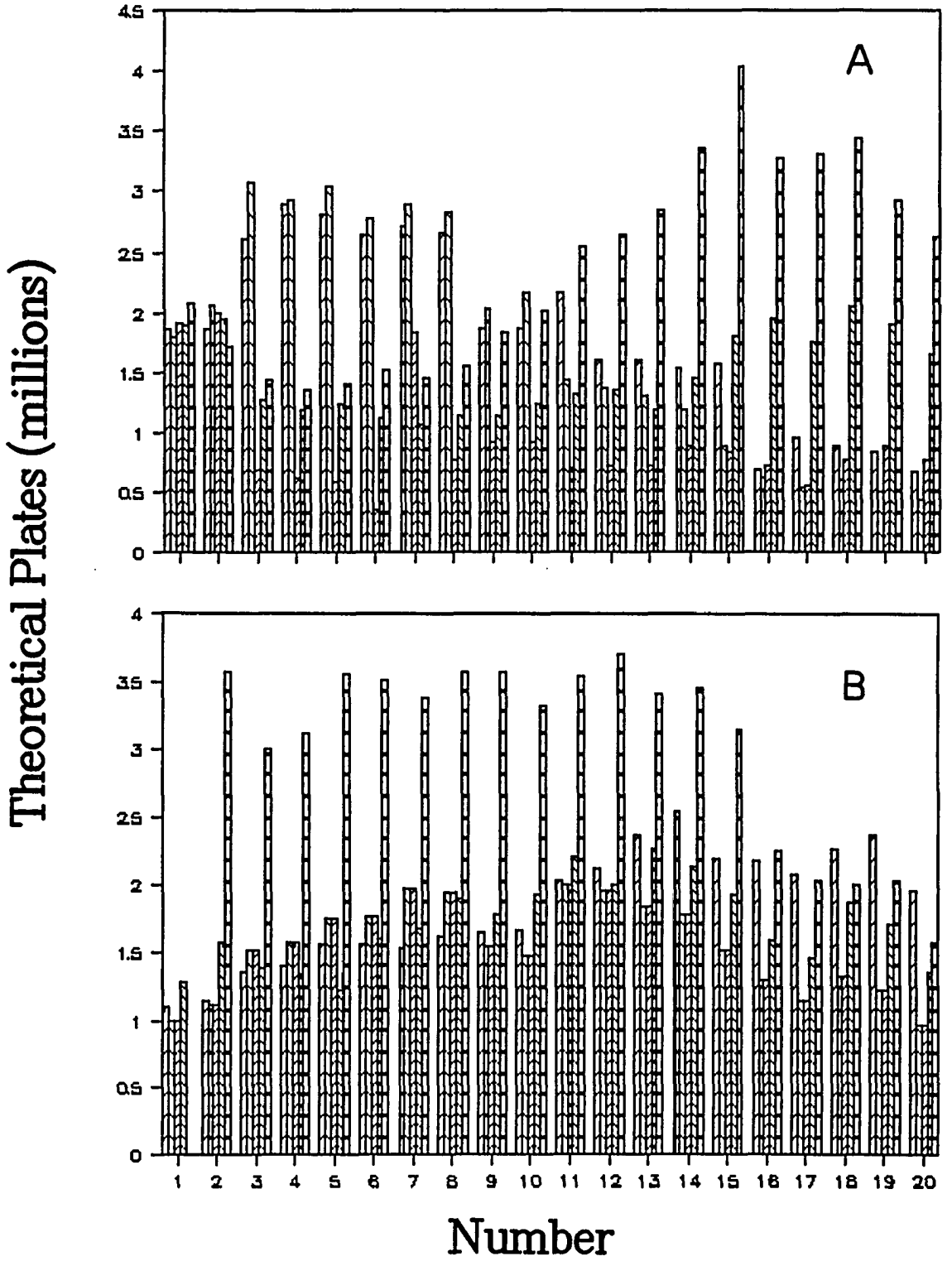


Figure 8. Comparison of the calculated single-base resolution from the results of Fig. 2 to 6 and 10 to 14: symbols are as in Fig. 7.

Figure 9. Comparison of the theoretical plates from the results of Fig 2 to 6 and 10 to 14: in (A); light-left-shaded bar: Fig. 2, light-right-shaded bar: Fig. 3, dense-left-shaded bar: Fig 4, dense-right-shaded bar: Fig. 5, and light-cross bar: Fig. 6. in (B); light-left-shaded bar: Fig. 10, light-right-shaded bar: Fig. 11, dense-left-shaded bar: Fig 12, dense-right-shaded bar: Fig. 13, and light-cross bar: Fig. 14.


Theoretical Plates (millions)
A
B
Number
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

to get better separation performance for all of the range of DNA fragments, if gel matrices can provide different pores through which DNA can move.

In the mixed gel matrices, there are two distributions of pore size. One is the homogeneous pore size distribution as very strong interactions exist among all the polymer chains. The other is random distribution as the interactions among the polymer chains are weak. The former can happen only if the polymer can be well miscible, which is possible if the solution can dissolve the polymers completely. In our study, the latter case should be more statistically possible in aqueous solutions since the very large range of molecular weight distribution among the mixtures and the limit of swelling characteristic of these polymers in aqueous solution. Therefore, a random polymer network with random pores is formed in mixed gel matrices through which DNA fragments move. Of course, the average pore size of gel matrices should be totally different from that in isocratic gel matrices in both cases. Figures 10 through 14 show the results of the separation of DNA fragments in the mixed gel matrices prepared from the mixture of polymers with different molecular weights. At first glance, it is nice to have single-base resolution between 123 and 124 in all mixed gel matrices. It is also obvious that the separation performance is different from that in isocratic gel matrices. This is because the mixed gel matrices provide different pore sizes that allow DNA fragments to move through the gel matrices. From comparison of the results of mobilities of DNA fragments from Fig 7 A and B, the average pore size of the mixed gel matrices is

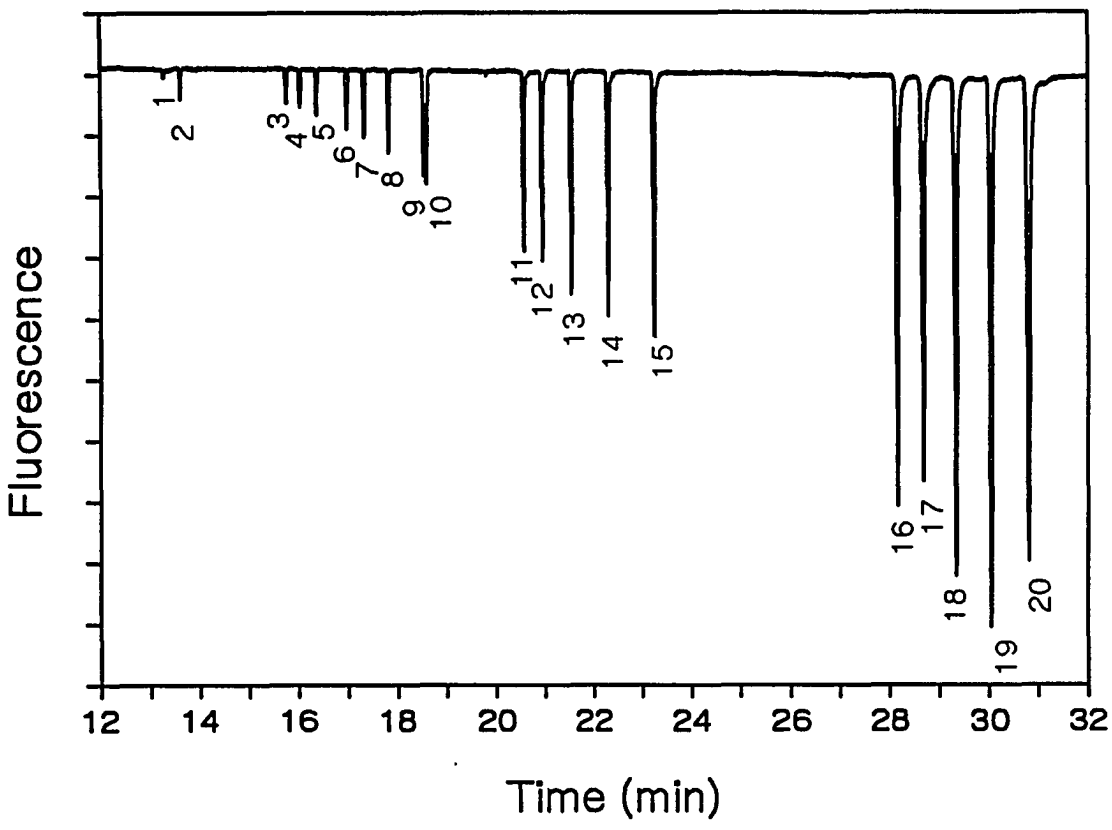


Figure 10. Electrophoretic separation of pBR 322 DNA-Hae III digest: mixed gel matrices were 1.5 % PEO, MW 300,000, 1.8 % PEO, MW 2,000,000, 0.7 % PEO, MW 5,000,000 and 0.7 % PEO, MW 8,000,000. Other conditions and peak assignments are as in Fig. 2.

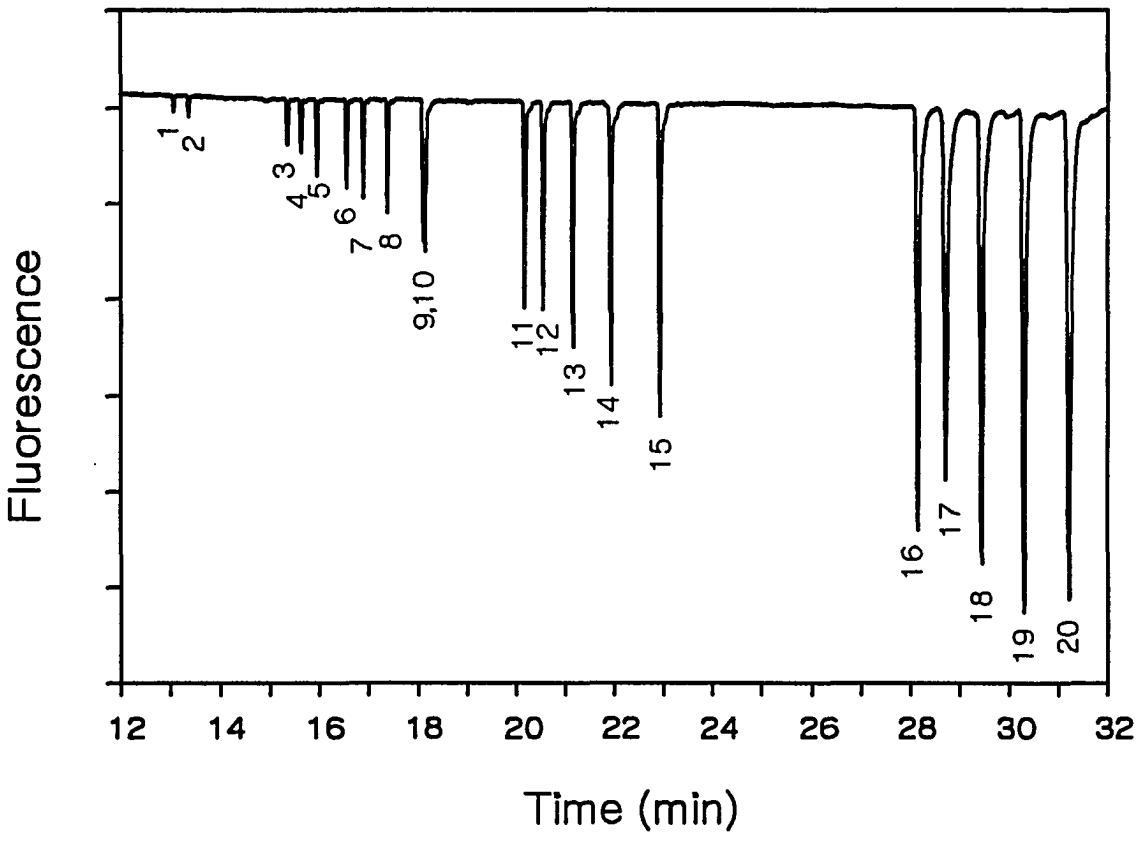


Figure 11. Electrophoretic separation of pBR 322 DNA-Hae III digest: mixed gel matrices were 3.0 % PEO, MW 1,000,000 and 1.3 % PEO, MW 8,000,000. Other conditions and peak assignments are as in Fig. 2.

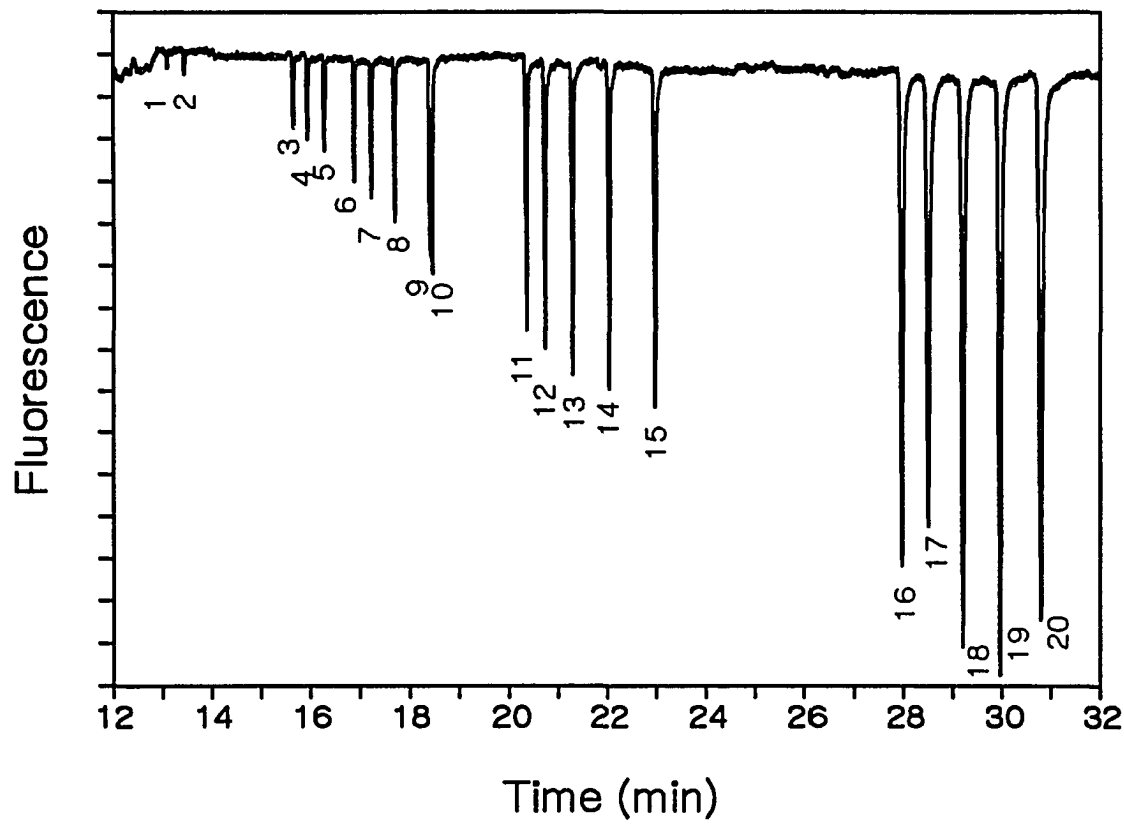


Figure 12. Electrophoretic separation of pBR 322 DNA-Hae III digest: mixed gel matrices were 1.5 % PEO, MW 600,000, 1.0 % PEO, MW 1,000,000 and 1.5 % PEO, MW 5,000, 000. Other conditions and peak assignments are as in Fig. 2.

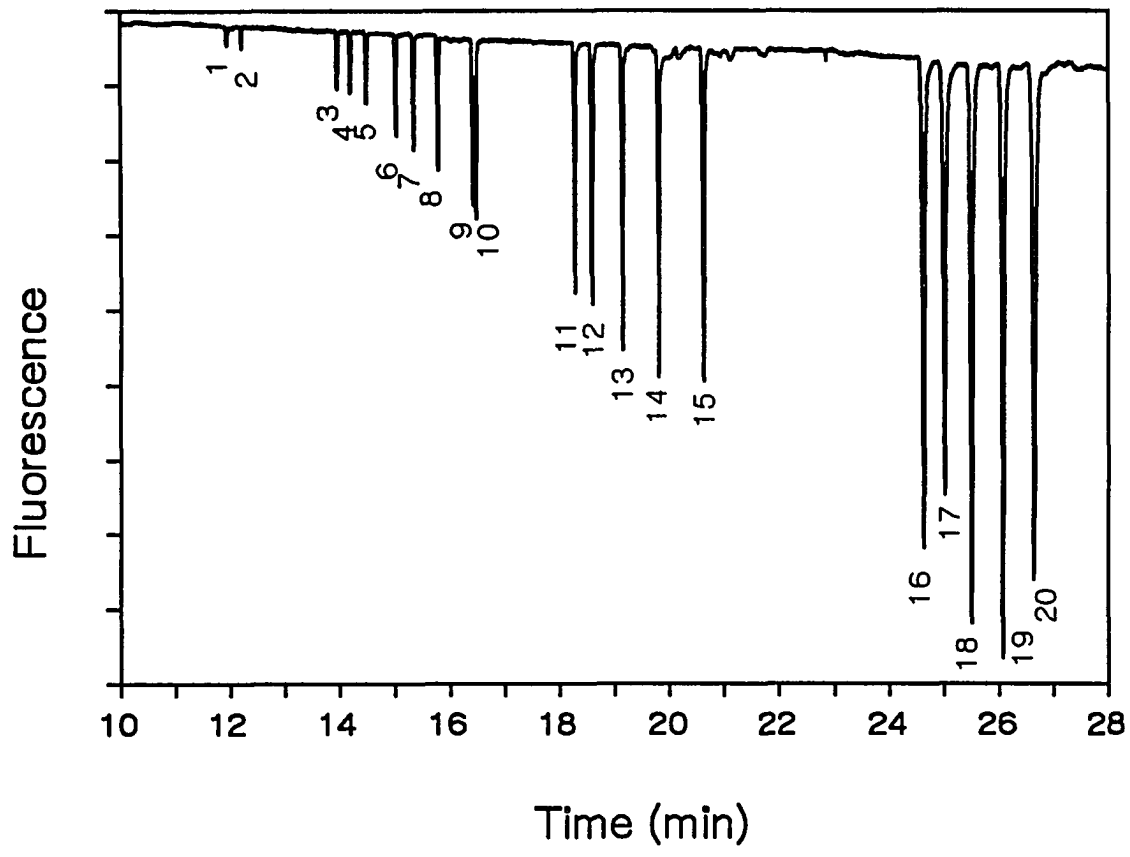


Figure 13. Electrophoretic separation of pBR 322 DNA-Hae III digest: mixed gel matrices were 0.6 % PEO, MW 300,000, 600,000, 1000,000, 2,000,000. 5,000,000 and 8,000,000, respectively. Other conditions and peak assignments are as in Fig. 2.

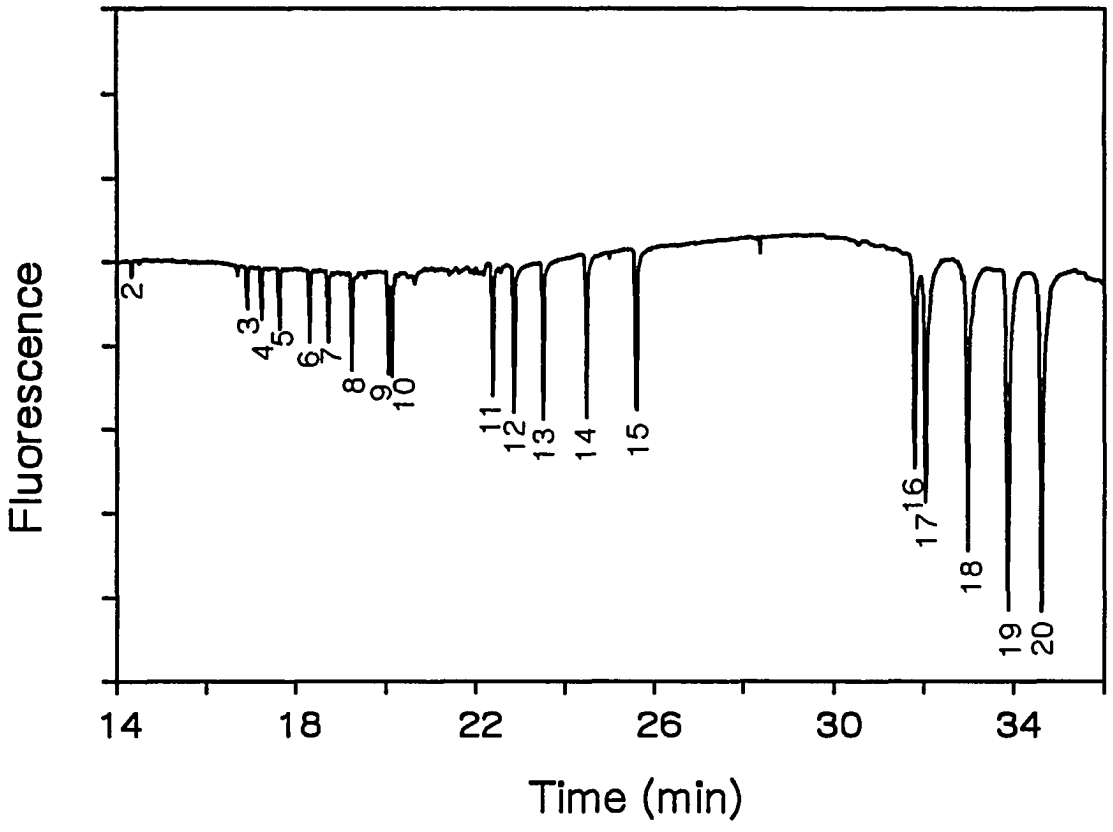


Figure 14. Electrophoretic separation of pBR 322 DNA-Hae III digest: mixed gel matrices were 0.7 % PEO, MW 300,000, 600,000, 1000,000, 2,000,000. 5,000,000 and 8,000,000, respectively. Other conditions and peak assignments are as in Fig. 2.

between that made from 2.5 % PEO (MW 8,000,000) and 3.5 %(MW 2,000,000). While for the larger fragments the resolution is better in mixed gel matrices, the resolution of small fragments in mixed gel matrices is lower than in gel matrices from PEO (MW 2,000,000). On the other hand, gel matrices prepared from PEO (2.5 %, MW 8,000,000) provide slightly higher resolution for DNA fragments from 80 to 400 base pairs, but worse resolution for small fragments than mixed gel matrices do. For DNA fragments larger than 400 base pairs, relatively better resolution can be obtained in mixed gel matrices. Therefore, DNA fragments travel different pathways through mixed gel matrices than isocratic gel matrices.

Random-walk effect is given for the enhancement of the separation of DNA fragments when they move through mixed gel matrices in this study. Figure 8 B also shows that mixed gel matrices prepared from higher content of polymer with shorter chains can provide better resolution for small DNA fragments, but higher content of polymer with longer chains can provide a better sieving mechanism for larger fragments. Again, the different viscosity among different gel matrices caused the variation of resolution. Overall, It is worth noting that mixed gel matrices made from equal amounts of polymers (0.6%) with different molecular weights provide comparable resolution, while keeping much lower viscosity, compared to all gel matrices used in this study. Higher resolution was obtained easily when mixed gel matrices are prepared the same way and from higher amount of polymers (0.7%). Figure 9 B shows that these gel matrices provide

more consistent efficiency for all range of DNA fragments. These phenomena may explain that these gel matrices provide all kinds of pores that allow DNA fragments to walk through the shortest path. Results shown here are important demonstrations in CGE, while the transverse pore gradient, which is not performed as easily in CGE as in slab gel systems, has been performed in slab gel systems for the same purposes. Obviously, mixed gel matrices prepared from different contents of polymers with different molecular weights can provide the same effect as that from gradient techniques, such as voltage programming and frequency modulation, to enhance the separation performance for very small and large DNA fragments. It is important to note here that this technique provides the features of simplicity, high speed, single-base resolution, and relatively high resolution up to 500 base pairs. In order to further show the separation ability of this technique, relatively low viscous gel matrices prepared from equal amounts (0.6% each) of polymers with different molecular weights were used for the separation of more complicated samples. Figure 15 shows the result of the separation of DNA fragments in the mixture of molecular weight markers V and VI which contain fragment sizes from base pairs 8 to 2176. All of the fragments can be separated well in these gel matrices in less than 30 min. The results show that mixed gel matrices can be used to separate the normal DNA sample from the mutated sample, since fragments with 234 base pairs from two different samples can be well separated. Also, this figure highlights the excellent separation ability among

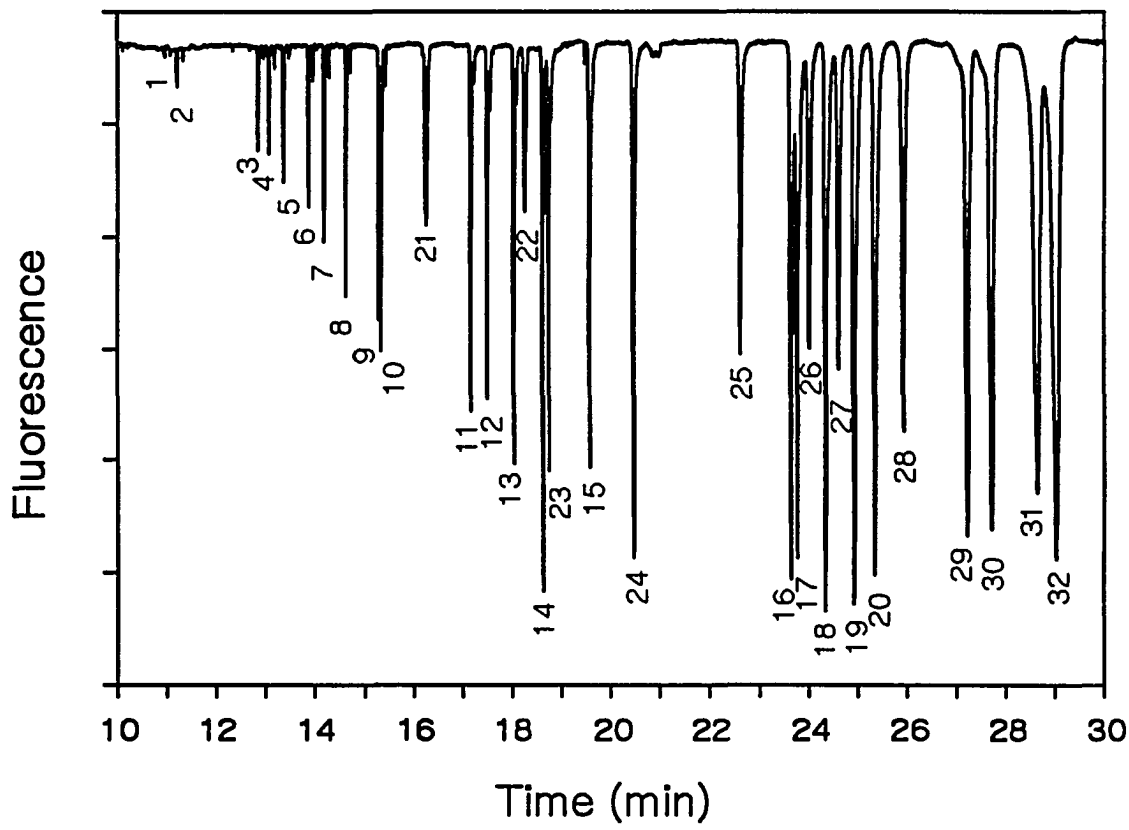


Figure 15. Electrophoretic separation of the mixture of pBR 322 DNA-Hae III, pBR 328 DNA-Bgl I, and pBR 328 DNA-Hinf I. Conditions are described in text. Peak assignments: 1 to 20 are as in Fig. 2, 21 = 154 BP, 22 = 220 BP, 23 = 234 BP (from pBR 328), 24 = 298 BP, 25 = 394 BP, 26 = 453 BP, 27 = 517 BP, 28 = 653 BP, 29 = 1033 BP, 30 = 1230 BP, 31 = 1766 BP, 32 = 2176 BP.

fragments with 434, 453 and 458 base pairs. From this electropherogram, it is guaranteed that these gel matrices can be used to sequence DNA, up to at least 500 base pairs, in a very short period.

Conclusion

To reach the goal of the HGP, it is critically important to have a technique which can sequence the DNA with single-base resolution up to 1000 base pairs in a very short time. CGE with polyacrylamide gels have definitively demonstrated the abilities of single-base resolution. However, it is not advantageous to use polyacrylamide as gel matrices, because of the poor reproducibility and difficulty of polymerization of this polymer. Alternatively, PEO with a large range of molecular weight ranging from 300,000 to 8,000,000 can be commercially purchased. In addition, it is easy to prepare homogeneous gel matrices to provide highly reproducible separation performance with sufficient resolution. Single-base resolution between 123 and 124 base pairs can be achieved easily by the use of gel matrices prepared from PEO (MW 8,000,000) or mixed gel matrices. Also, we have demonstrated that mixed gel matrices can be used to provide better resolution for DNA fragments from 8 to 2176 base pairs. It is also quite easy to change the content and total amount of polymers to achieve higher resolution for different samples with different ranges of fragments. Another advantage of mixed

gel matrices is that relatively high resolution can be obtained while keeping the relatively low viscosity (lower than 4% T of polyacrylamide in 30% foramide and 3.5 M urea) which makes it easier to fill gel matrices into the capillary. The ease to fill the capillary with the gel matrices, high reproducibility of the separation results, high speed, and single-base resolution are quite important for DNA sequencing using a multiplexed capillary array system. In the near future, this group will demonstrate DNA sequencing with 100 capillaries filled with PEO gel matrices.

References

1. Deyl, Z., Struzinsky, R. J. Chromatogr. 1991, 569, 63-122.
2. Landers, J. P. Trends in Biochem. 1993, 18, 409-414.
3. Pedersen, J., Pedersen, M. J. Chromatogr. 1993, 645, 351-361.
4. Stefansson, M., Novotny, M. Anal. Chem. 1994, 66, 1134-1140.
5. Wu, J., Pawllszyn, J. Anal. Chem. 1994, 66, 867-873.
6. Deml, M., Pospichal, J., Sudor, J., Boček, P. J. Chromatogr. 1989, 470, 309-312.
7. Giaffreda, E., Tonani, C., Righetti, P. G. J. Chromatogr. 1993, 630, 313-327.
8. Vinther, A., Søeberg, H. J. Chromatogr. 1992, 589, 315-319.
9. Whang, C. W., Yeung, E. S. Anal. Chem. 1992, 64, 502-506.
10. Chang, H. T., Yeung, E. S. J. Chromatogr. 1993,632, 149-155.

11. Chang, H. T., Yeung, E. S. J. Chromatogr. 1992, 608, 65-72.

12. Wu, C. T., Lee, C. S. Anal. Chem. 1992, 64, 2310-2311.

13. Wu, C. T., Lopes, T., Patel, B., Lee, C. S. Anal. Chem. 1992, 64, 886-891.

14. Goux, A., Athias, A., Persegol, L., Lagrost, L, Gambert, P., Lallemant, C. Anal. Biochem. 1994, 218, 320-324.

15. Baba, Y., Tsuhako, M. Trends in Anal. Chem. 1992, 11, 280-287.

16. Yager, T. D., Zewert, T. E., Hood, L. E. Acc. Chem. Res. 1994, 27, 94-100.

17. Ruiz-Martinez, M. C., Berka, J., Belenkii, A., Foret, F., Miller, A. W., Karger, B. L. Anal. Chem. 1993, 65, 2851-2858.

18. Guttman, A., Wanders, B., Cooke, N. Anal. Chem. 1992, 64, 2348-2351.

19. Luckey, J. A., Smith, L. M. Anal. Chem. 1993, 65, 2841-2850.

20. Navin, M. J., Rapp, T. L., Morris, M. D. Anal. Chem. 1994, 66, 1179-1182.

21. Fischer, S. G., Lerman, L. S. Proc. Natl. Acad. Sci. (USA) 1983, 80, 1579-1583.

22. Yoon, K. L., Modica-Napolitano, J. S., Ernst, S. G., Aprille, J. R. Anal. Biochem. 1991, 196, 427-432.

23. Wartell, R. M., Hosseini, S. H., Moran Jr, C. P. Nucleic Acids Res. 1990, 18, 2699-2705.

24. Theißen, G., Schönborn, J., Wagner, R. Nucleic Acids Res. 1993, 19, 4643-4644.

25. Ke, S. H., Wartel, R. M., Nucleic Acids Res. 1993, 21, 5137-5143.

26. Fawcett, J. S., Wheeler, D., Chrambach, A. J. Biochem. Biophys. Methods 1992, 24, 181-194.

27. Wheeler, D., Chrambach, A. Electrophoresis 1993,14, 570-572.

28. Wang, P. L., Li, W., Giese, R. W., Yeung, E. S. J. Chromatogr. 1992, 608, 73-77.

29. Hjerten, S. J. Chromatogr. 1985, 347, 191-198.

30. Chang, H. T. this dissertation, Ch. 6 and 7, pp. 129-195.

31. Grossman, P. D., Soane, D. S. Biopolymers, 1991, 31, 1221-1228.

32. Brandrup, J., Immergut, E. H. Polymer Handbook, 1989, 3rd ed., John wiley & Sons, New York.

CHAPTER 9

SINGLE ADRENOMEDULLARY CELL ANALYSIS BY CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED NATIVE FLUORESCENCE

Introduction

Interest in the development of techniques to diagnose diseases is growing rapidly in biological, chemical and medical sciences. Single cell analysis is one of the most important techniques which may be able to detect abnormal cells in the earlier stages of disease. In order to get a better understanding about cellular differentiation, the physiological effect of external stimuli such as drug, cell-cell communication and neuron transmission, a single cell analysis should be able to detect the chemical composition and amounts in individual cells, Therefore, the abilities to handle fast responses to the change in components and very small volume of samples, to simultaneously separate and/or detect various compounds with very low amount are necessary for the analysis of single cells.

Thus far, optical and electron microscopic techniques,[1] immunoassay,[2] enzymatic radiolabelling,[3] fluorescence microscopy,[4] and voltametric microelectrodes,[5-6] have been used most commonly for the studies of single cells. However, they suffer from either inadequate sensitivity, poor quantitative capability, the need to perform labelling with fluorescence dyes or an inability to determine

multicomponents. Microcolumn liquid chromatography, with electrochemical or laser-induced fluorescence detection, provides the advantages of excellent separation and low-detection-limit capabilities and small volume of sample needed is appropriate for cell analysis.[7-12] Recently, capillary electrophoresis (CE) in narrow bore (2 to 75-μm I.D.) capillaries has become more popular for biological separation since it provides high-efficiency separation, low mass detection limit, and advanced speed.[13-17] In addition, with the ease for on-line cell injection, CE is ideally suitable for single cell analysis.

Laser induced fluorescence (LIF) with its high sensitivity is a suitable detection technique for CE.[18,19] Unlike electrochemical detection (ECD), which suffers from the difficulty to make micro-size working electrodes and electrode-fouling due to contamination with high-molecule-weight molecules, especially, protein, LIF is easy and suitable for protein detection. A disadvantage of LIF is relatively higher cost than ECD, in terms of instrumentation.

Hemoglobin and carbonic anhydrase in individual human erythrocytes have been determined by using CE-laser-induced native fluorescence (CE-LINF) with an argon-ion laser at 274 nm.[20] Derivatization[21] and an indirect method[22] have been used to overcome the limitation of selectivity of LIF. Recently, these techniques have been well demonstrated for the determination of Na^+ and K^+ and pyruvate and lactate in individual red blood cells.[23-25] More recently, Yeung and Xue have taken advantage of enzyme assay performed by on-column reaction[26] and LIF to detect

lactate dehydrogenase in individual red blood cells via monitoring the fluorescence of NADH at 340 nm.[27] Particle counting based on the on-column immunoassay between glucose phosphate dehydrogenase (G6PDH) and glucose was demonstrated by Yeung and Rosenzweig to detect zeptomole levels of G6PDH in single red blood cells.[28]

The determination of the interesting compounds from thousands of chemicals in a cell makes the requirement to develope novel techniques . Jorgenson and coworkers used 15 to 20-μm open tubular liquid chromatography with ECD to determine quantitatively the neurotransmitter and free amino acid pools in single neuron cells of the land snail Helix Aspersia.[29,30] In addition, CE-ECD has been well demonstrated by Ewing and coworkers for the determination of catecholamine and serotonin in a neuronal cell.[31,32] Those compounds play roles as neurotransmitters in the neuron system.[33]

It is well known that chromaffin cells in the adrenal medulla synthesize and store the catecholamine hormones, epinephrine (E) and norepinephrine (NE), and secrete these hormones in response to stimulation by secretagogue.[34,35] This chapter discusses the use of CE-LINF with an argon-ion laser at 274 nm to determine quantitatively the amount of E and NE in single bovine adrenomedullary cells. Also, the effects of pH, and matrices on the fluorescence intensities of several important amine compounds are shown. High-speed separations with reasonable resolution and high sensitivity for amine compounds were also

demonstrated.

Experimental Section

CZE instrumentation and data collection

The experimental setup is similar to that described in ref 27. Briefly, a high-voltage power supply (Glassman High Voltage, Inc., Whitehorse Station, NJ; EH series 0-40 kV) was used to drive the electrophoresis. A 50-μm or 16-μm-i.d. fused-silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) was used for the separation in different purpose. The total length was 60 cm and the effective length was 40 cm. The entire electrophoresis and detection system were enclosed in a sheet-metal box with HV interlocks.

The 275.4-nm line from an argon-ion laser (Spectra Physics, Inc., Mountain View, CA; Model 2045) was isolated from other lines with a prism and focused with a 1-cm focal-length lens into the detection region of the capillary tubing. Two UG-1 absorption filters (Schott Glass Technologies, Inc., Duryea, PA) were used to prevent scattered light from reaching the photomultiplier tube. The signal from LINF was either amplified by a current amplifier (Keithley, Inc., Taunton, MA; Model 427) or directly passed through a 10-KΩ resistor. The data were collected via a 24-bit A/D interface at 5 Hz (Justice Innovation, Palo Alto, CA,; Model DT 2802) and stored on a computer (IBM, Boca Raton, FL; Model PC/AT 286).

Methods

Cell Preparation and Injection: Chromaffin cells were isolated from bovine adrenal medullae by the method of Livett and coworkers.[36] After purification, the cells were stored at 4°C before use. Before analysis, cells were washed five times with 10-mL balance salt solution. To prevent significant secretion of catecholamine from cells, cells need to be analyzed less than 30 min after cells are washed with balance salt solution. Just before the injection of the cell into the capillary, 5-μL of cell solution is added to 200 μL water solution in the slide. To minimize the secretion of catecholamine from the cells and the adsorption of cells onto the slide wall, injection of a single cell into the capillary should be performed in less than 3 min. To reach this requirement, the injection end of the capillary was etched with HF to form a cone shape. The injection method for a single cell was similar to that described in ref. 23. The separations were performed at 30 kV.

Reagents

All standard amines, polyethylene, boric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Collagenase was obtained from Sigma Chemical Co. (St. Louis, MO). Citric acid, sodium hydroxide, phosphoric acid, sodium phosphate, isopropanol and chemicals for preparation of balance salt solution were purchased from Fisher (Fair Lawn, NJ).

A balance salt solution was composed of 150 mM NaCl, 4.2 mM KCl, 1.0 mM

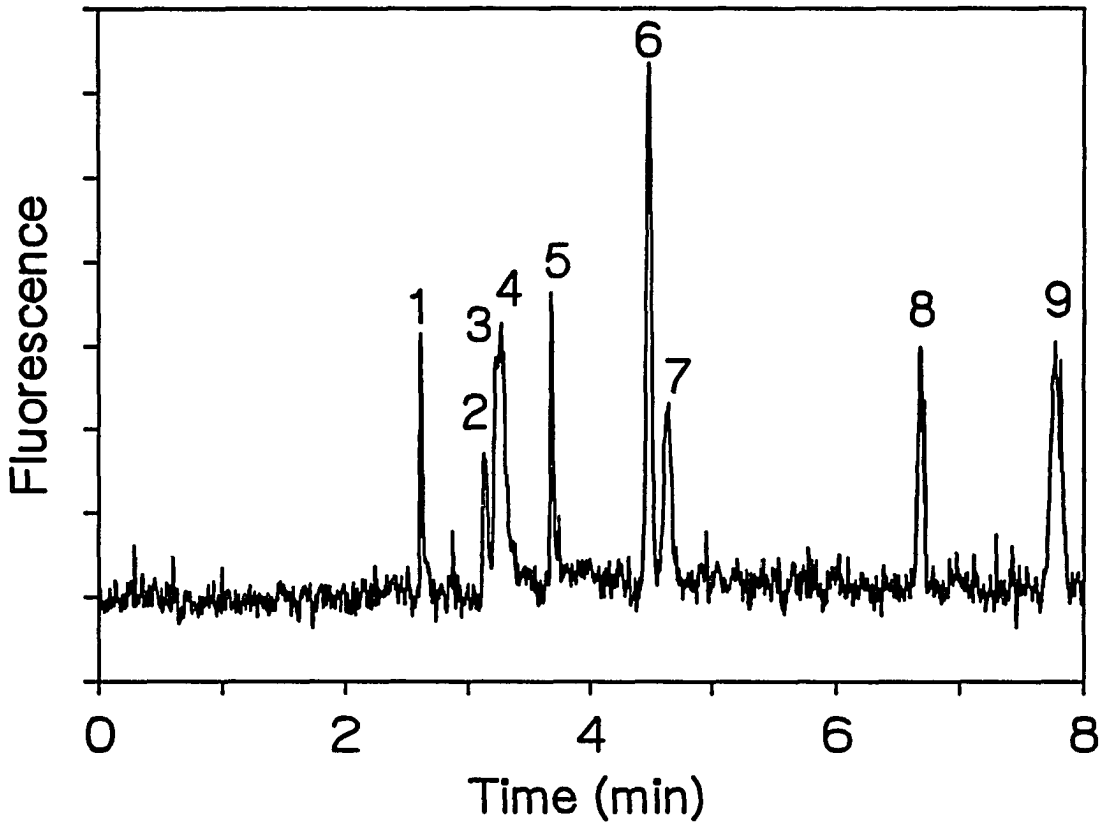
NaH_2PO_4, 11.2 mM glucose, 0.7 mM $MgCl_2$, 2.0 mM $CaCl_2$, and 10.0 mM HEPES and adjusted to pH 7.4 with NaOH. The pH of the running buffer for CE separation was adjusted by sodium hydroxide or phosphoric acid to the desired value.

Results and Discussion

Separation

The separation of catechol and catecholamine is important since they play critical roles as neurotransmitters in the central and peripheral autonomic system and as hormones exerting endocrine and exocrine effects. Figure 1 shows the separation of nine different amines at pH 5.4. High-speed separation in less than 8 minutes is achieved easily due to the same direction of electroosmotic flow and electrophoretic mobilities of analytes. The addition of isopropanol and polyethylene into the running buffer decreases the electroosmotic flow, and in turn provides enough resolution for all amine analytes. The other reason to have polyethylene in the running buffer is to minimize the interaction between the amines and the capillary wall, since protonated amines with positive charges can easily interact with the silanol groups of the capillary wall, which has negative charge at pH higher than 4. One of the features of this separation system is that it is faster and more reproducible than that from the use of micellar electrokinetic capillary chromatography (data are not shown). A possible reason is that ionic amines form

Figure 1. Electrophoretic separation of amines at pH 5.4: capillary; total length = 60 cm, effective length = 40 cm and i.d = 50 μm; sample injection = 30 kV for 3 sec; analyte concentrations = 6 μM for all analytes except that the concentration of serotonin = 0.3 μM, tryptophan = 10 nM, homovanillic acid = 3 μM and 3,4-dihydroxy phenylacetic acid = 10 μM. Other conditions are described in text. Peak assignments: 1 = serotonin, 2 = dopamine, 3 = dopa, 4 = NE, 5 = E, 6 = tryptophan, 7 = catechol, 8 = homovanillic acid, 9 = 3,4-dihydroxyphenylacetic acid.


Fluorescence
1
2
3
4
5
6
7
8
9
0
2
4
6
8
Time (min)

ion pairs with anionic micelles which results in the decrease in the electrophoretic mobilities. Another possible reason is that the complexes may have some interaction with the capillary wall which causes poor reproducibility. It is also possible to separate amines only based on the flow of electrophoretic mobility at very low pH. Figure 2 shows the separation of four amines which are partially or totally protonated at pH 2.8. The separation in this condition provides at least two advantages. First, the resolution is better than that at higher pH such as the result shown in Figure 1. Second, less interferences from acidic compounds and their metabolites can be achieved since only amines can elute at such low pH values.

Detection

This is the first demonstration (to our knowledge) by using laser-induced native fluorescence to detect amines in CE. Traditionally, ECD[37-39] and fluorescence detection with derivatization,[40] which provide enough sensitivity for the determination of biological specimens, are more common. Although some amines with an indole group have natural fluorescence, it is not common to use fluorescence to detect them since traditional light sources are too weak.[41] The use of an argon-ion laser at 275 nm has been well demonstrated as a good light source for the determination of proteins.[20,26] Since the maximum excitation wavelength of these amines is around 280 nm, except serotonin at 298 nm, the excitation at 275 nm with an argon-ion laser is suitable. Two UG-1 filters are

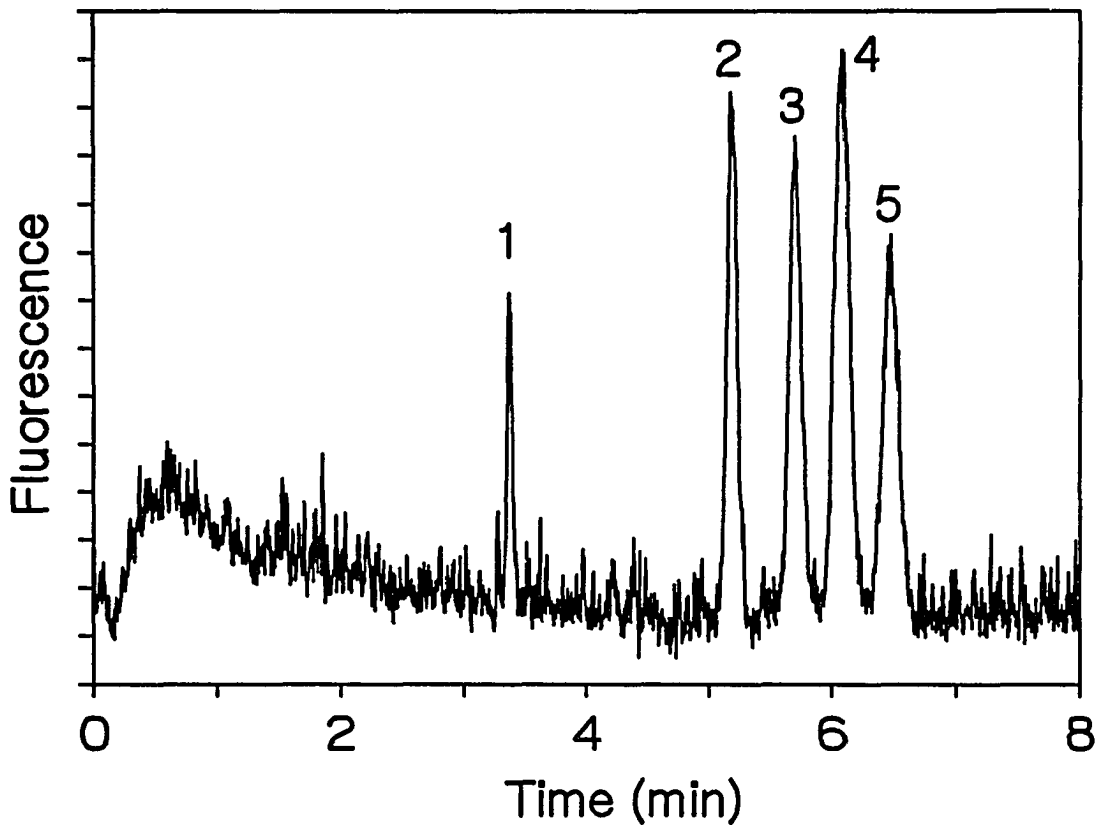


Figure 2. Electrophoretic separation of amines at pH 2.8: concentration were 0.5 μM for NE and E, 10 nM for serotonin and 5 nM for tryptamine; injection was performed at 30 kV for 4 sec. Conditions are as in Fig. 1. Peak assignments: 1 = impurities, 2 = tryptamine, 3 = serotonin, 4 = NE, 5 = E.

useful to block scattered light and allow the emission light (320-350 nm) to reach the PMT. Figure 3 shows the relative fluorescence from pH 2 to 9. The results show that the fluorescence intensity is higher at low pH. It is similar to compounds with an indole nucleus which has higher fluorescence intensity as they are in protonated form. Also, it is worth noting that at this condition amines are more stable. Generally, amines prepared at low pH and stored in the refrigerator can stay for at least one day without significant loss of fluorescence intensity. On the other hand, catecholamine is very unstable at high pH. For example, a yellow color can be found from E and NE solutions after the solution has been prepared for several hours. Since the determination of amines in biological samples generally need pretreatments with organic solvent before analysis, it is important to know the effect of some common solvents on the fluorescence intensity. Figure 4. shows the effect of different matrices on the fluorescence intensity of NE. The fluorescence intensity of NE dissolved in organic solvent such as dimethyl sulfoxide (DMSO) increases. On the other hand, salts with high ionic strength or a strong oxidative acid, such as $HClO_4$ quench fluorescence significantly. The quenching effect may result from the oxidation of amines by strong acids or ion-pair complexes between protonated amines with inorganic ions such as chloride or phosphate from the salt. Table 1 shows the detection limit of amines at two different pH conditions. Since the injection volume of these analytes is in the nL levels, the absolute detectable amount is in the low fmol to low amol levels for

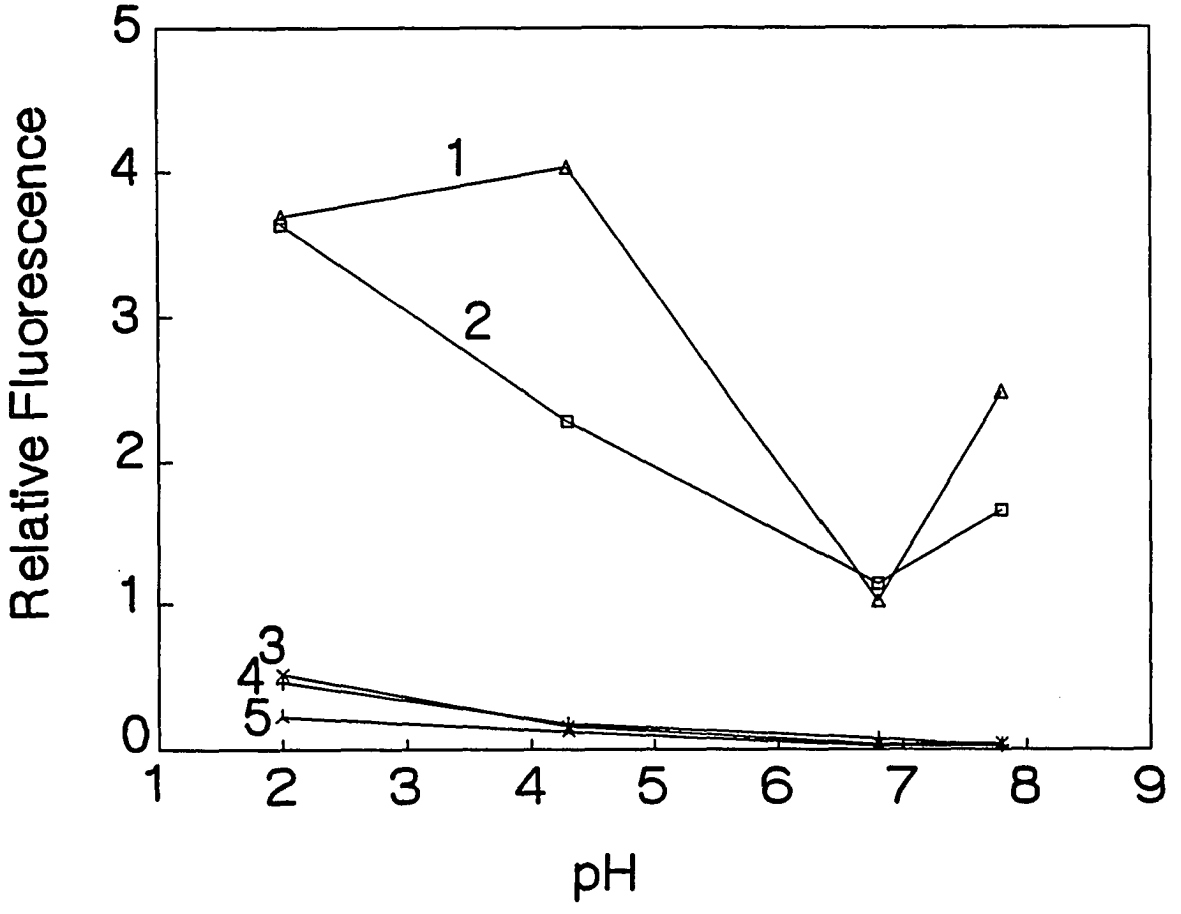


Figure 3. pH effect on the fluorescence intensities of amines. Conditions are described in text. Curve assignments: 1 = tryptamine, 2 = serotonin, 3 = E, 4 = NE and 5 = dopamine.

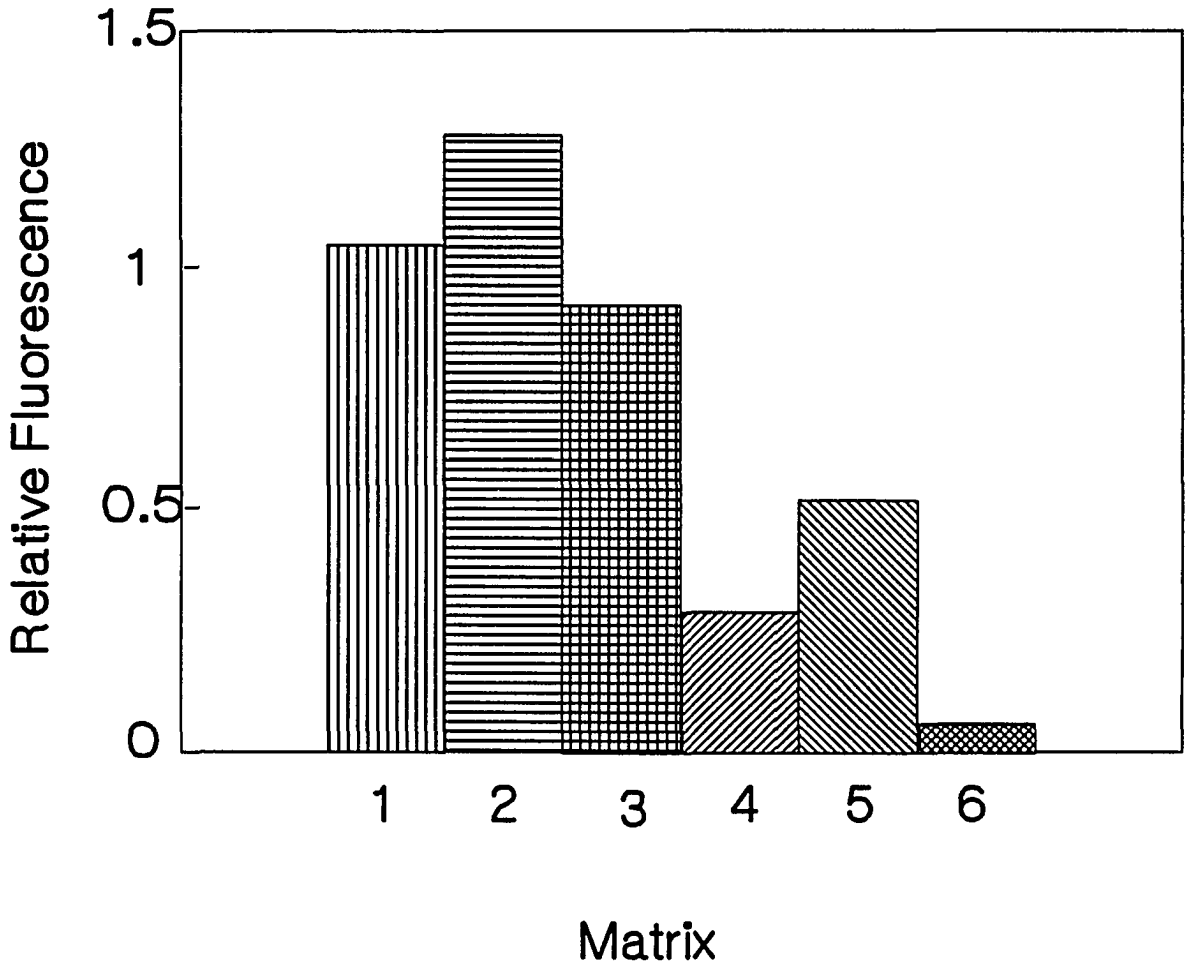


Figure 4. Matrix effect on the fluorescence intensity of amines in 0.1 M citric acid at pH 2.8. Conditions are described in text. Bar assignments: 1 = 6 % glucose, 2 = 1 % DMSO, 3 = 0.6 % Glucose, 4 = balance salt solution, 5 = 0.1 M HCl, and 6 = 0.8 M $HClO_4$.

Table 1. Detection limits of amine compounds

Amines	Concentration (mol/L)	
	pH 2.8[a] (nM)	pH 5.7[b] (nM)
serotonin	1.3	2.5
tryptophan	ND*	0.7
tryptamine	0.5	0.9
epinephrine	65	100
norepinephrine	60	90
dopa	ND	80
dopamine	40	80
metanephrine	ND	25
normetanephrine	ND	5
catechol	ND	200
homovanillic acid	ND	90
3,4-OH phenyl acetic acid	ND	8

a: buffer was 3 mM citric acid; b: buffer was 15% 2-propanol, 1% polyethylene and 0.1M boric acid and pH adjusted by 0.1 M NaOH. ND*: not eluted, ND: didn't measure.

different amines. The results also show that this detection system provides better sensitivity than traditional derivitization methods, which can only detect pmol to fmol levels. Obviously, LINF can be used to determine all kinds of important biological amines and their metabolites because of its high sensitive characteristic, at least 10^{-8} M level. Also, the sensitivity is comparable to that from ECD.[37] The advantages of LINF in this study over ECD are: (1) possible to run separation at very low pH, (2) no need to build up a microelectrode, (3) no fouling effect from large molecules such as proteins on the electrode, (4) more selective and low interference from amino acids and proteins, and (5) possible to determine various amines at a single wavelength. It is obvious that the method we have developed is suitable for the analysis of amines in very complicated biological samples, such as central nervous fluid and blood.

Single Cell Analysis

So far, our results show that the better separation performance and higher sensitivity for the analysis of amines can be performed at lower pH. Also, at pH lower than 4, much less interference can be obtained since only analytes with positive charges can elute because of the lack of electroosmotic flow. In this case, only some amino acids and proteins with electrophoretic mobilities may still cause interference for the determination of amines. Fortunately, by using LINF, among those amino acids, only tryptophan, which has relatively strong fluorescence

intensity, may be detected. However, we didn't see a standard sample elute out at such low pH in 20 mins. The interference from proteins is also almost impossible because of its low fluorescence intensity at such low pH, very low amount of protein (less than amol for certain protein) in a single neuron cell and large difference of retention time from those of amines. The lack of electroosmotic flow also provides a very clean background and almost no spikes. There is still another benefit for choosing such low pH for single cell analysis since cell are easily lysed and NE and E are secreted very quickly from cells by the running buffer. These properties make the single cell analysis much easier.

It is well known that adrenomedullary cells store large amounts, up to several hundred fmols, of catecholamine, NE and E. Hence, it is a good test for our method to apply to the analysis of biological samples. Figure 5 shows that NE and E from a standard sample are well separated at pH 2.3. Since the data are directly transferred from PMT through a 10-kΩ resistor to a 24-bit A/D interface, data showed negative response. From the comparison of the results with and without a current amplifier, there is no significant difference in the signal response.

Figure 6 shows the electropherogram of lysed cells (0.5 μl cells/150 μl buffer solution) from gland 1. If the average size of a cell is 20 μm, cell number corresponding to 0.5 μl is around 1.2×10^8. Based on this assumption and the volume of analytes injected into capillaries, the number of cells injected into capillaries at 30 kV for 3 s is around 14. From the result that the total amount of

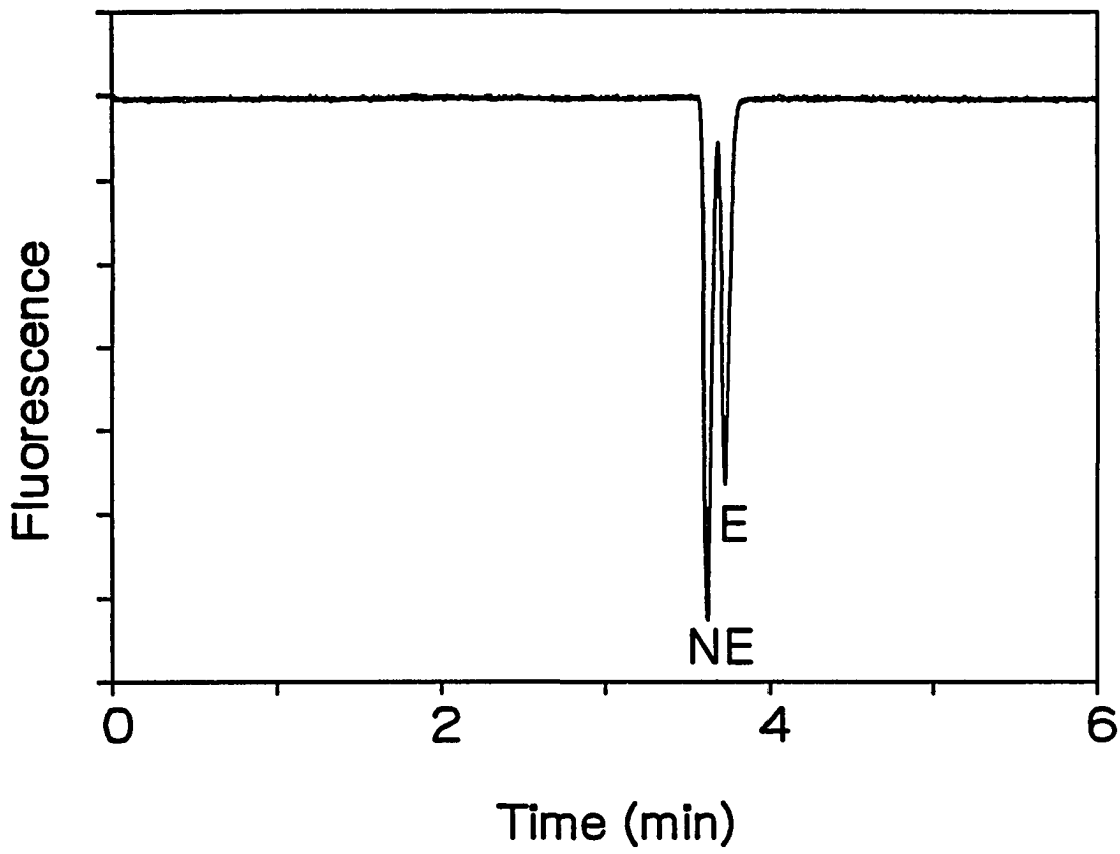


Figure 5. Electrophoretic separation of NE and E in standard solution at 0.1 M citric acid at pH 2.3: capillary; i.d. = 16 μm, total length = 65 cm and effective length = 45 cm; concentration = 20 μM. Other conditions are described in text.

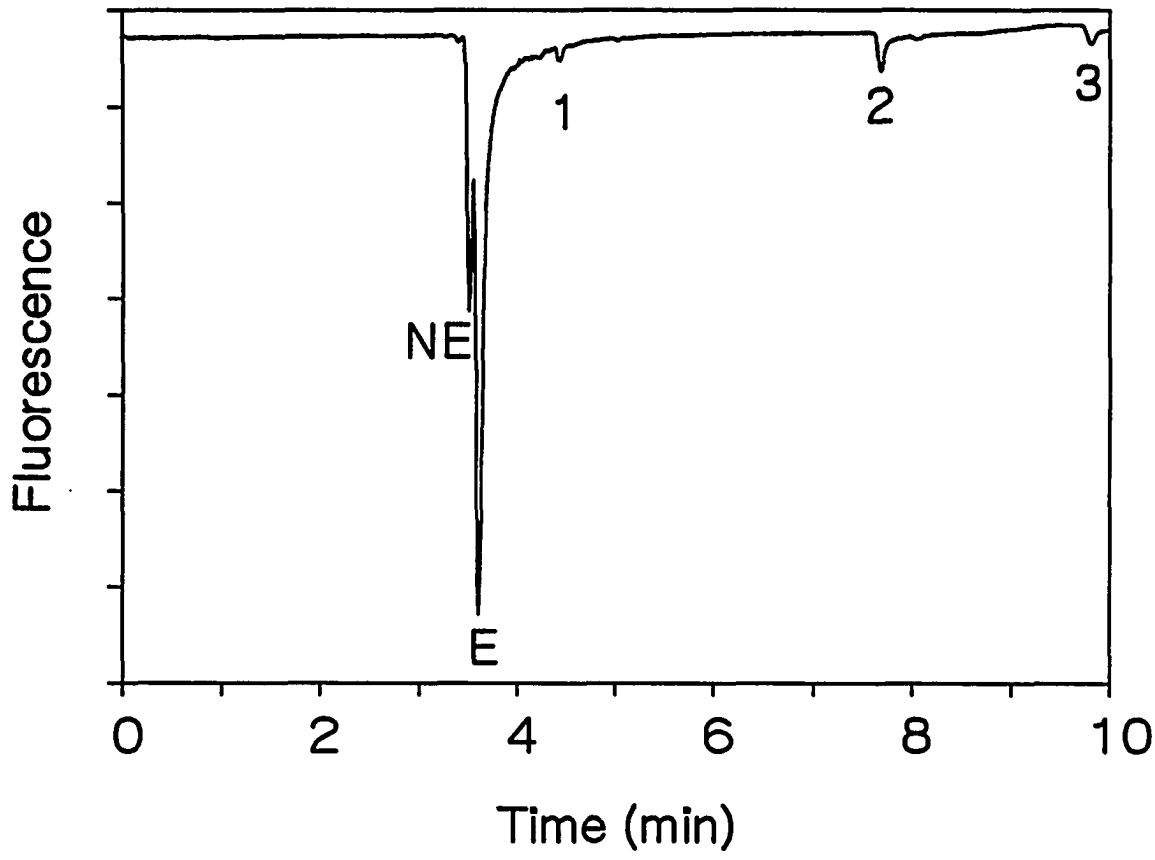


Figure 6. Electrophoretic separation of lysed cells. Conditions are as in Fig. 5. Peaks 1 to 3 are unknown.

NE and E injected into capillary is 220 and 550 fmol, respectively, the average amount of NE and E in individual cells can be estimated as 15 and 39 fmol, respectively. Figure 7 shows the electropherogram of a single cell analysis. Two major peaks correspond to NE and E with a good match of retention time with the standards. In addition, like Fig. 6, there are still several small peaks which may be due to other unknown components such as proteins or other kinds of amines and their metabolites. It is important to identify those components to get more information about a single cell from one run. This may be helpful to further understand more specific functions of a cell. However, it is beyond the goal of this study and more specific experiments are needed.

After several runs, retention time changed due to the adsorption of cell membrane onto the capillary wall. Fortunately, it can be easily overcome by flushing the capillary with running buffer then equilibrating for 5 minutes. Since catecholamine is known to secrete from cells, even though without any chemical or physical stimulation, a blank test after each run is necessary to guarantee there is no or insignificant amount of catecholamine from the balance salt. This was performed by injecting a certain amount of balanced salt solution into capillaries for analysis. Results show that after cells stay in the balance salt solution for 30 min, the average amount of NE and E in blank test which may be injected into capillary with a single cell is less than 1.2 and 3.2 fmol, respectively. In order to minimize any interference from balance salt solution we always pushed out the solution

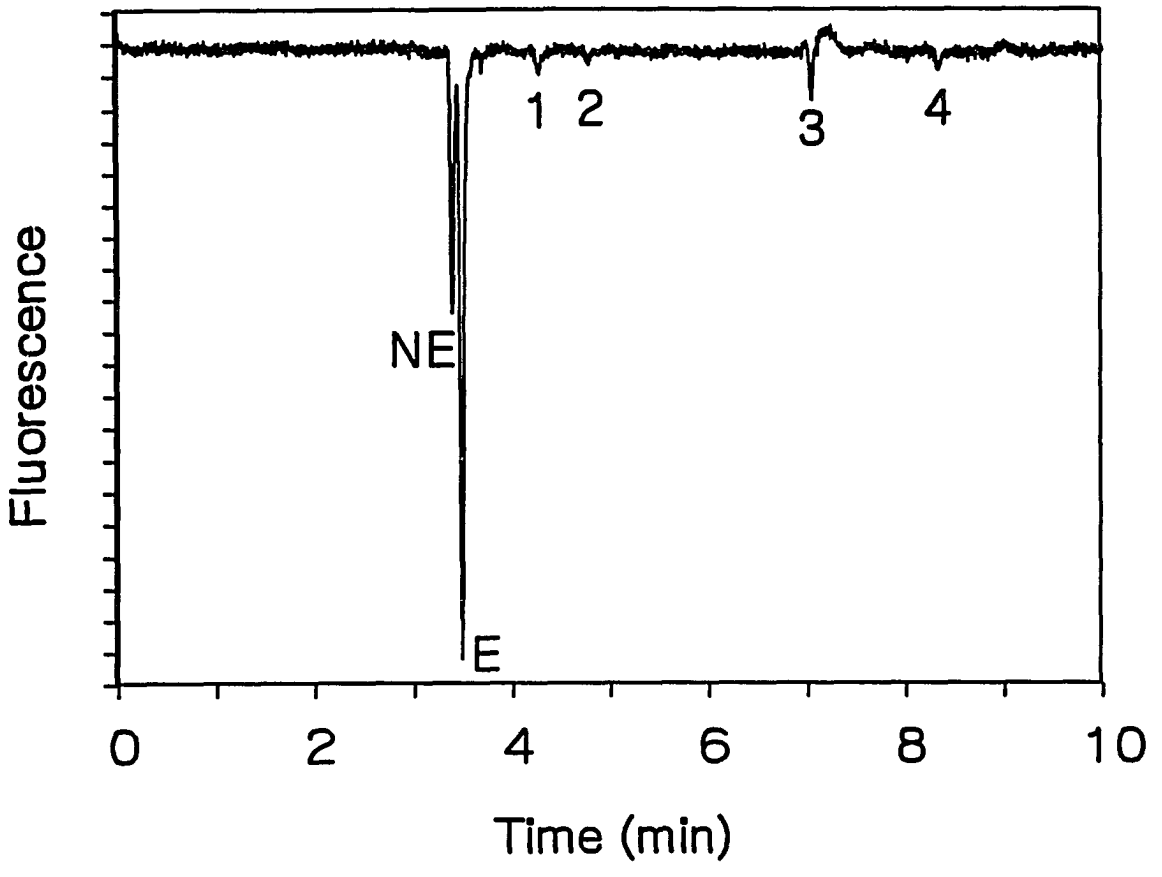


Figure 7. Electrophoretic separation of a single adrenomedullary cell. Conditions are as in Fig. 5. Peaks 1 to 4 are unknown.

injected with a single cell after cell has been injected into capillaries. It is easy to perform since as long as the cell injected into capillaries cell sticks very well onto the capillary wall. Actually the injected amount of NE and E in a single cell analysis from blank is much less than the result from the blank test since the volume injected into capillary with a single cell is much less than that used for a blank test and the cells are analyzed always in less than 30 min in the balance salt solution.

One of the most important features of this method is that the separation is much faster (less than 5 min) than that from HPLC (longer than 50 min).[30] Table 2 shows the amount of NE and E in a single adrenal medulla cell from two different glands. The amounts of catecholamine are significantly different for two different glands from two bovines which is possible. The results for NE and E are 19 ± 9 and 55 ± 30 fmol from 11 different runs for gland 1 and 72 ± 30 and 380 ± 150 fmol from 6 different runs for gland 2. The values we measured are quite reasonable to those measurements by using ECD.[11,30,45] Cell to cell differences in amounts of NE and E are obvious, which means that variation exists among cells. The variation may be natural or due to diseases or aging of the cell. The results from single cell analysis agree with those from lysed cells which further supports our measurements. The ratio of NE to E from two different glands is 2.7 and 5.6 which are close to the results found in some literature.[42,43] However, it is a little surprising that all the results show that the amounts of E are higher than those of

Table 2. Amounts of norepinephrine and epinephrine in individual bovine adrenomedullary cells

	measured amount, fmol		
	NE	E	E/NE
Gland 1			
cell no.			
1	13	40	3.3
2	22	62	2.8
3	25	78	3.1
4	5.1	14	2.7
5	25	71	2.8
6	18	50	2.8
7	4.0	11	2.8
8	22	57	2.6
9	33	120	3.6
10	28	73	2.6
11	11	29	2.6
mean ± sd	19 ± 9	55 ± 30	2.7 ± 0.3
Gland 2			
1	107	524	4.9
2	81	514	6.3
3	91	373	4.1
4	86	481	5.6
5	48	279	5.8
6	17	112	6.6
mean ± sd	72 ± 30	380 ± 150	5.6 ± 0.8

sd is standard deviation

NE in a single cell which is somewhat different from literature results. Some people believe there are two kinds of cells in adrenomedullary cells: E-rich and NE rich, however, our result agrees with Hochman's result.[44] In that paper, he addressed that E content accounts for more than 95% of catecholamine. Faster release of E than NE and the ease of NE converting to E are the reason that E content is higher than NE.[45, 46] Another possible reason that we always obtained a higher value of E than NE in a single cell analysis is that more that 70 % of adrenomedullary cells are E-rich cells. It is reasonable that only E-rich cells were injected into capillaries for the analysis. Finally, the results may be possible that only E-rich cells exist in fresh samples because of the damage of cell due to the sampling.

Conclusion

The present study has demonstrated that CE-LINF provides the features of high sensitivity (down to nM) and high resolution, high speed and low interference from proteins and other amino acids for the analysis of amine compounds at low pH. Also, this method has been applied successfully for the measurement of the amounts of E and NE in individual adrenomedullary cells. Study of stimulus-secretion coupling is an important and exciting subject to understand the function of amines in the role of neurotransmitter which can be performed easily by this method. Application of this method to detect trace amount of amines in biological

samples such as blood and urine is also quite promising. For example, because of its relatively high sensitivity for dopa and dopamine, this method is quite useful for the study of some kinds of diseases, such as Parkinson's disease, due to the deficiency of dopamine. More and more single cell analyses by using CE-LINF for the study of different kinds of neuron cells is also predictable. Overall, this method should join ECD soon as a powerful technique for the study of neurochemistry.

References

1. Betzig, E., Trauyman, J. H., Harris, T. D., Weiner, J. S., Kostelak, R. L. Science, 1991, 251, 1468-1470.
2. Ishikawa, E., Hashida, S., Kohno, T., Hirota, K. Clin. Chim. Acta 1990, 194, 51-72.
3. McCaman, R. M., Weinrich, D., Borys, H. J. Neurochem. 1973, 21, 473-476.
4. Tank, D. W., Sugimori, M., Connor, J, Llinas, R. R. Science, 1988, 242, 773-777.
5. Jankowski, J. A., Schroeder, T. J., Holz, R. W., Wightman, R. M. J. Biol. Chem. 1992, 267, 18329-18335.
6. Lau, Y. Y., Takayuki, A., Ewing, A. G. Anal. Chem. 1992, 64, 1702-1705.
7. Kennedy, R. T, Oates, M. D., Cooper, B. N., Jorgenson, J. W. Science, 1989, 246, 57-63.

8. Leszczszsyn, D. J., Jankowski, J. A., Viveros, O. H., Diliberto, E. J., Near, J. A., Wightman, R. M. J. Neurochem. 1991, 56, 1855-1863.

9. Kennedy, R. T., St. Clair, R. L., White, J. G., Jorgenson, J. W. Mikrochim. Acta 1987, 2, 37-46.

10. Kennedy, R. T., Jorgenson, N. W. J. Anal. Chem. 1989, 61, 436-441.

11. Moro, M. A., Lpez, M. G., Micheleena, L. G., García, A. G. Anal. Biochem. 1990, 185, 243-248.

12. Oates, M. D., Jorgenson, J. W. Anal. Chem. 1990, 62, 1577-1580.

13. Landers, A. P., Oda, R. P., Madden, B. J., Spelsberg, T. C. Anal. Biochem. 1992, 205, 115-124.

14. Stefansson, M., Westerlund D. J. Chromatogr. 1993, 632, 195-200.

15. Ong, C. P., Pang, S. F., Low, H. K., Li, S. F. Y. J. Chromatogr. 1991, 559, 529-536.

16. Watson, E., Yao, F. Anal. Biochem. 1993, 210, 389-393.

17. Nashabeh, W., Rassi, Z. E. J. Chromatogr. 1991, 536, 31-42.

18. Lee, T. T., Lillard, S. J., Yeung, E. S. Electrophoresis, 1993, 14, 429-438.

19. Wu, S., Dovichi, N. J. J. Chromatogr. 1989, 480,141-155.

20. Lee, T. T., Yeung, E. S. Anal. Chem. 1992, 64, 3045-3051.

21. Zhao, J. Y., Waldron, K. C., Miller, J., Zhang, J. Z., Harke, H., Dovichi, N,. J. J. Chromatogr. 1992, 608, 239-242.

22. Yeung, E. S., Kuhr, W. G. Anal. Chem. 63, 1991, 275A-230A.

23. Hogan, B. L., Yeung, E. S. Anal. Chem. 1992, 64, 2841-2845.

24. Lee, Q., Yeung, E. S. J. Capillary Electrophoresis, in press.

25. Xue, Q., Yeung, E. S. J. Chromatogr. 1994, 661, 287-295.

26. Chang, H. T., Yeung, E. S. Anal. Chem. 1993, 65, 2947-2951.

27. Xue, Q., Yeung, E. S. Anal. Chem. 1994, 66, 1175-1178.

28. Rosenzweig, Z., Yeung, E. S. Anal. Chem. 1994, 66, 1771-1776.

29. Oates, M. D., Cooper, B. R., Jorgenson, J. W. Anal. Chem. 1990, 62, 1573-1577.

30. Cooper, B. R., Jankowski, J. A., Leszczyszyn, D. J., Wightman, R. M., Jorgenson, J. W. Anal. Chem. 1992, 64, 691-694.

31. Olefirowicz, T. M., Ewing, A. G., Chimia, 1991, 45, 106-108.

32. Olefirowicz, T. M., Ewing, A. G., Anal. Chem. 1990, 62, 1872-1876.

33. Usdin, E., Synder, S. L., Eds, Frontiers in Catecholamine Research; Pergamon, N. Y., 1973.

34. Moro, M. A., Langley, O. K. J. Neurochem. 1991, 57, 363-369.

35. Vijayaraghavan, S., Schmid, H. A., Mapp, K. S. J. Neurochem.1993, 61, 325-331.

36. Livett, B. G., Kozuousek, V., Dean, D. M. Nature 1979, 278, 256-257.

37. Wallingford, R. A., Ewing, A. G. Anal. Chem. 1989, 61, 98-100.

38. Keating, J., Dratcu, L., Lader, M., Sherwood, R. A. J. Chromatogr. 1993, 615, 237-242.

39. Olefirowicz, T. M., Ewing, A. G. J. Neuroscience Methods 1993, 34, 11-15.

40. Todoriki, H., Hayashi, T., Naruse, H., Hirakawa, A. Y. J. Chromatogr. 1983, 276, 45-54.

41. Peat, M. A., Gibb, J. W. Anal. Biochem. 1983, 128, 275-280.

42. Kong, J. Y., Thureson-Klvin A., Klein, R. L. Neuroscience, 1989, 28, 765-775.

43. MÜller, T. H., Unsicker, K. J. Neuroscience Methods, 1981, 4, 39-52.

44. Hochman, J., Perlman, R. L. Biochim. Biophys. Acta 1976, 421, 168-175.

45. Kilpatrick, D. L., Ledbetter, F. H., Carson, K. A., Kinser, A. G., Sleptis, R., Kinshner, N. J. Neurochem. 1980, 35, 679-692.

46. Greenberg, A., Zinder, O. Cell Tissue Res. 1982, 226, 655-665.

GENERAL SUMMARY

CE-LIF with the features of high speed, high efficiency, high sensitivity, easy automation, and ability to handle very small amount of sample has been well accepted as a powerfui technique for almost any kinds of samples for several decades. The applications for the separation of biological samples such as proteins, peptides, amines and DNA are especially important.

In this dissertation, we covered the basic theories of CE, the applications of pH, temperature and flow gradient on the enhancement of the separation of small organic ions. In addition, CE-LINF has been shown as a powerful technique for the study of peptide mapping and the detection of amines from individual cells. CGE-LIF was also used to study the DNA separation. Technique with high speed, less than 30 min, single base resolution between 123/124 and high sensitivity, 28 ng/ml of DNA has been demonstrated. A new idea to improve the separation performance with mixed gel matrices was developed to function.

The importance to sequence DNA, detect trace amount of important compounds from complicated samples, and determine the components from single cells will make more challenge for analyst in the future. More and more powerful techniques such as CE-MS, microchip electrophoresis, and multicapillary systems will mature soon.

LITERATURE CITED

1. (a) Bard, A. J., Faulkner, L. R., Electrochemical methods: Fundamentals and Applications, John Willey & Sons, New York, 1980, pp. 500-511.
   (b) Helmholtz, H. V. Ann. Phisik 1877, 7, 337.
2. Kohlrausch, F. Ann. Phisik 1897, 62, 209-239.
3. Tiselius, A. Nova Acta Regiae GOC. Sci. Ups. Ser. IV 1930, Vol. 17, No. 4, pp. 1-107.
4. Svensson, H. Acta Chem. Scand. 1961, 15, 325-341.
5. Hjeten, S. Chromatogr. Rev. 1967, 9, 122-219.
6. Virtanen, R. Acta Polytech. Scand. Chem. 1974, 123, 1-63.
7. Laemmli, U. K. Nature 1970, 227, 680-685.
8. Martin, A. J. P., Everaerts, F. M. Anal. Chim. Acta. 1967, 38, 233-237.
9. Alinger, L.; Routs, R. J. Sci. Tools 1970, 17, 21.
10. Verheggen, Th. P. E. M., Ballegooijen, E. C. V., Massen, C. H., Everaerts, F. M. J. Chromatogr. 1972, 64, 185-189.
11. Mikkers, F. E. P., Everaerts, F. M., Verheggen, Th. P. E. M. J. Chromatogr. 1979, 169, 11-20.
12. Jorgenson, J. W., Lukacs, K. D. Anal. Chem. 1981, 53, 1298-1302.
13. Jorgenson, J. W., Lukacs, K. D. J. Chromatogr. 1981, 218, 209-216.
14. Terabe, S., Otsuka, K., Ichikkawa,K., Tsuchiya, A., Ando, T. Anal. Chem.

1984, 56, 111-113.

15. Rice, C. L., Whitehead, R. J. Phys. Chem. 1965, 69, 4017-4024.
16. Altria, K., Simpson, C. Anal. Proc. 1986, 23, 453-454.
17. Foret, F., Fanali, S., Ossicini L., Bocek, P. J. Chromatogr, 1989, 470, 299-308.
18. Giddings, J. J. Chromatogr. 1989, 480, 21-30.
19. Schlabach, T., Powers, J. Am. Lab. 1991, 23, 24-25.
20. Pedersen, J., Pedersen, M. J. Chromatogr. 1993, 645, 353-361.
21. Stefansson, M., Westerlund, D. J Chromatogr, 1993, 632, 195-200.
22. Chervet, J. P. United States Patent 1991, Oct. 15, 5,057,216.
23. Gordon, G. B. United States Patent 1991, Oct. 29, 5,061,361.
24. Tsuda, T., Sweedler, J. V., Zare, R. N. Anal. Chem. 1990, 62, 2149-2152.
25. Taylor, J. A., Yeung, E. S. J. Chroatogr. 1991, 550, 831-837.
26. Wang, T., Aiken, J. H., Huie, C. W., Hatrtwick, R. A. Anal. Chem. 1991, 63, 1372-1376.
27. Walbroehl, Y., Jorgenson, J. W. J. Chromatogr. 1982, 315,135-143.
28. Xue, Y., Yeung, E. S. Anal Chem 1993, 65, 1988-1993.
29. Nielen, M. J. Chromatogr. 1991, 558, 321-326.
30. Xue, Y., Yeung, E. S. Anal. Chem. 1993, 65, 2923-2927.
31. Gebaur, P., Thormann, W. J. Chromatogr. 1991, 545,299-305.
32. Albin, M., Grossman, P. D., Moring, S. E. Anal. Chem. 1993, 65, 489A-497A.
33. Chien, R. L., Burgi, D. S. J. Chromatogr. 1991, 559, 153-161.

34. Chien, R. L., Burgi, D. S. J. Microcolumn Sep. 1991, 3, 199-202.

35. Vinther, A., Everaerts, F. M., Soeberg, H. J. High Resolut, Chromatogr. 1990, 13, 639-642.

36. Aebersold, R., Morrison, H. D. J. Chromatogr. 1990, 516, 79-88.

37. Foret, F., Szoko, E., Karger, B. L. J. Chromatogr. 1992, 608, 3-12.

38. Schwer, C., Lottspeich, F. J Chromatogr. 1992, 623, 345-355.

39. Gassmann, E., Gozel, P., Zare, R. N. Science 1985, 230, 813-814.

40. Lee, T. T., Yeung, E. S. Anal. Chem. 1992, 64, 3045-3051.

41. Nie, S., Dadoo, R., Zare, R. N. Anal. Chem. 1993, 65, 3571-3575.

42. Wu, S., Dovichi, N. J. Chromatogr. 1989, 480, 141-156.

43. Hernandez, L., Escalona, J., Joshi, N., Guzman, N. J. Chromatogr. 1991, 559, 183-196.

44. Green, J. S., Jorgenson, J. W. J. Chromatogr. 1986, 352, 337-343.

45 Walingford, R. A., Ewing, A. G. Anal Chem. 1987, 59, 678-681.

46. Balchunas, A. T., Sepaniak, M. J. Anal. Chem. 1988, 60, 617-621.

47. Wrigth, B. W., Ross, G. A., Smith, R. D. J. Microcol. Sep. 1989, 1, 85-89.

48. Cheng, Y. F., Dovichi, N. J. Science 1989, 242, 562-564.

49. Nickerson, B., Jorgenson, J. W. J. Chromatogr. 1989, 480, 157-168.

50. Harmon, B. J., Patterson, D. H., Regnier, F. E. Anal. Chem. 1993, 56, 2655-2662.

51. Chang, H. T., Yeung, E. S. Anal. Chem. 1993, 65, 2947-2951.

52. Xue, Q.; Yeung, E. S. Anal. Chem. 1994, 66, 1175-1178.

53. Rosenzweig, Z., Yeung, E. S. Anal. Chem. 1994, 66, 1771-1776.

54. Wong, K. S., Yeung, E. S. in preparation.

55. Wu, D.; Regnier, F. E. Anal. Chem. 1993, 65, 2029-2035.

56. Khur. W. G., Yeung, E. S. Anal. Chem. 1988, 60, 1832-1834.

57. Garner, T. W., Yeung, E. S. J. Chromatogr. 1990, 515, 639-644.

58. Khur, W. G., Yeung, E. S. Anal. Chem. 1988, 60, 2642-2646.

59. Gross, L., Yeung, E. S. Anal. Chem.1990, 62, 427-431.

60. Hogan, B. L., Yeung, E. S. J. Chromatogr. Science 1990, 28, 15-18.

61, Bachmann, K., Boden, J., Haumann, I. J. Chromatogr. 1992, 626, 259-265.

62. Hogan, B. L., Yeung, E. S. Anal. Chem. 1992, 64, 2841-2845.

63. Xue, Q., Yeung, E. S. J. Chromatogr. 1994,661, 287-295.

64. Engstrom-silverman, C. E., Ewing, A. G. J. Micrcolumn Sep. 1991, 3, 141-145.

65. O'shea, T. J., Lunte, S. M., LaCourse, W. R. Anal. chem. 1993, 65, 948-951.

66. Chen, I. C., Whang, C. W. Anal. Chem. 1992, 64, 2461-2464.

67. Colon, L. A., Dadoo, R., Zare, R. N. Anal. Chem. 1993, 65, 476-481.

68. Huang, X., Pang, T. K., Gordon, M. J., Zare, R. N. Anal. Chem. 1987, 59, 2747-2749.

69. Foret, F., Demyl, M., Kahle, V., Boček, P. Electrophoresis 1986, 7, 430-432.

70. (a) Wallingford, R. A., Ewing, A. G. Anal. Chem. 1989, 61, 98-100.

(b) Sloss, S., Ewing, A. G. Anal. Chem. 1993, 65, 577-581.

71. Sahin, Y, Kok, W. T. Anal. Chem. 1993, 65, 2497-2501.

72. Ye, J., Baldwin, R. P. Anal. Chem. 1993, 65, 3525-3527.

73. Lu, W., Gassidy, R. M. Anal. Chem. 1993, 65, 2878-2881.

74. O'shea, T. J., Lunte, S. M. Anal. Chem. 1993, 65, 247-250.

75. (a) Olefirowicz, T. M., Ewing, A. G. Anal. Chem. 1990, 62, 1872-1876.
(b) Olefirowicz, T. M., Ewing, A. G. Chimia, 1991, 45, 106-108.

76. Chen, T. K., Lau, Y. Y., Wong, D. K. Y., Ewing, A. G. Anal. Chem. 1992, 64, 1264-1268.

77. Olefirowicz, T. M., Ewing, A. G. J. Chromatogr, 1990, 499, 713-719.

78. Olivares, J. A., Nguyen, N. T., Yonker, C. R., Smith, R. D. Anal. Chem. 1988, 60, 436-441.

79. Smith, R. D., Barinaga,C. J., Udseth, H. R. Anal. Chem. 1988, 60, 1948-1952.

80. Lee, E. D, Muck, W., Henion, J. D.,. Covey, T. R. J. Chromatogr. 1988, 458, 313-321.

81. Smith, R. A., Olivares, J. A., Nguyen, N. T., Udseth, H. R. Anal. Chem. 1988, 60, 436-441.

82. Moseley, M. A., Detrding, L. J., Tomer. K. B., Jorgenson, J. W. Rapid Commun. Mass Spectrom. 1989, 3, 87-93.

83. Johansson, I. M., Pavelka, R., Henion, J. D. J. Chromatogr. 1991, 539,

515-528.

84. Castoro, J. A., Chiu, R. W., Monning, C. A., Wilkins, C. L. J. Am. Chem. Soc. 1992, 114, 7571-7572

85. Sin, C. H., Lee, E.D., Lee, M. L. Anal. Chem. 1991,63, 2897-2900.

86. Ramsey, R. S., Goeringer, D.E., Mcluckey, S. A. Anal. Chem. 1993, 65, 3521-3524.

87. Deterding, L. J., Parker, C. E., Perkins, J. R., Moseley, M. A., Jorgenson, J. W. J. Chromatogr. 1991, 554, 329-338.

88. Wahl, J. L., Gale, D. C, Smith, R. D. J. Chromatogr. 1994, 659, 217-222.

89. Garcia, F., Henion, J. D. Anal. Chem. 1992, 64, 985-990.

90 Wu, J. , Pawliszyn, J. Anal. Chem. 1992, 64, 219-224.

91. Wang, T., Hartwick, R. A. Anal. Chem. 1992, 64, 1745-1747.

92. Wu, J., Pawliszyn, J. Anal. Chem. 1992, 64, 2934-2941.

93. Chen, S. M., Wiktorowicz, J. E. Anal. Biochem. 1992, 206, 84-90.

94. Kilar, F. J. Chromatogr. 1991, 545, 403-406.

95. Yim, K. W. J. Chromatogr. 1991, 559, 401-410.

96. Wanders, B. J., Lemmens, A. A. G., Everaerts, F. M. J. Chromatogr. 1989, 470, 79-88.

97. Widhalm, A., Kenndler, E. Anal. Chem. 1991, 63, 645-648.

98. Kaneta, T., Tanaka, S., Taga, M., Yoshida, H. Anal. Chem. 1992, 64, 798-801.

99. Tsai, P., Patel, B., Lee, C. S. Anal. Chem. 1993, 65, 1439-1442.

100. Balchunas, A. T., Sepaniak, M. J. Anal. Chem. 1987, 59, 1466-1470.

101. Terabe, S. Trends in Anal. Chem. 1989, 8, 129-134.

102. Janine, G. M., Issaq, H. J. J. Liq. Chromatogr. 1992, 15, 927-960.

103. Tanaka, M., Ishida, T., Araki, T., Masuyama, A., Nakatsuji, Y, Okahara, M. J. Chromatogr. 1993, 648, 469-473.

104. Otsuka, K., Terabe, S. J. Liq. Chromatogr. 1993, 16, 945-953.

105. Nishi, H., Terabe, S. Electrophoresis 1990, 11, 691-701.

106. Cohen, A. S., Karger, B. L. J. Chromatogr. 1987, 397, 409- 417.

107. Motsch, S. R., Kleemiss, M. H. J. High Resolut. Chromatogr. 1991, 14, 629-632.

108. Grossman, P. D., Soane, D. S. J. Chromatogr. 1991, 559, 257-266.

109. Dolnik, V., Novotny, M. V. Anal. Chem. 1993, 65, 563-567.

110. Hjerten, S. J. Chromatogr. 1983, 270, 1-6.

111. Guttman, A., Horvath, J., Cooke, N. Anal. Chem. 1993, 65, 199-203.

112. Hegenbrock, K., Schugerl, K., Freitag, R. Electrophoresis 1993, 14, 753-758.

113. Figeys, D., Dovichi, N. J. J. Chromatogr. 1993, 645, 311-317.

114 Gerstile, J. T., Fried, M. G. Electrophoresis, 1993, 14, 725-731.

115. Chu, Y. H., Chen, J. K., Whitesides, G. M. Anal. Chem. 1993, 65, 1314-1322.

116. Honda, S.,Taga, A., Suzuki, S., Kakehi, K. J. Chromatogr.1992, 597, 377-382.

117. Chu, Y. H., Avila, L. Z., Biebuyck, H. A., Whitesides, G. M. J. Med. Chem. 1992, 35, 2915-2917.

118. Sun, P., Wu, N., Barker, G., Hartwick, R. A. J. Chromatogr. 1993, 648, 475-480.

119. Shimura, K., Karger, B. L. Anal. Chem. 1994, 66, 9-15.

120. Gozel, P., Gassmann, E., Michelsen,H.; Zare, R. N. Anal. Chem. 1987, 59, 44-49.

121. Otsuka, K., Terabe, S. Trends in Anal. Chem. 1993, 12, 125-130.

122. Nishi, H., Fukuyama, T., Terabe, S. J. Chromatogr. 1991, 553, 503-516.

123. Shibukawa, A., Lloyd, D. K., Wainer, I. W. Chromatographia 1993, 35, 419-429.

124. Li, S., Lloyd, D. K. Anal. Chem. 1993, 65, 3684-3690.

125. Vespalec, R., Sustacek, V, Boček, P. J. Chromatogr. 1993, 638, 255-261.

126. Romano, J., Jandik, P., Jones, W.R., Jackson, P. E. J. Chromatogr. 1991, 546, 411-421.

127. Weston, A., Brown, P. R., Jandik, P., Heckenberg, A. L., Jones, W. R. J. Chromatogr. 1992, 608, 395-402.

128. Ma, Y., Zhang, R., Cooper, C. L. J. Chromatogr. 1992, 93-96.

129. Dasgupta, P. K., Bao, L. Anal. Chem. 1993, 65, 1003-1011.

130. Yeung, E. S., Kuhr, W. G. Anal. Chem. 1991, 63, 275A-281A.

131. Shi, Y., Fritz, J. Int. Ion Chromatogr. Symp., Linz, Austria Oral Lecture 33, September 21-24, 1992.

132. Bachmann, K., Boden, J., Haumann, I. J. Chromatogr. 1992, 626, 259-265.

133. Jackson, P. E., Haddad, P. R. Trends in Anal. Chem. 1993, 12, 231-238.

134. Stefansson, M., Westerlund, D. J. Chromatogr. 1993, 632, 195-200.

135. Chiesa, C., Horvath, C. J. Chromatogr. 1993, 645, 337-352.

136. Liu, J., Shirota, O., Novotny, M. J. Chromatogr. 1991, 559, 223-235.

137. Miksik, I., Gabriel, J., Deyl, Z. J. Chromatogr. 1993, 638, 343-348.

138. Ling, B. L., Baeyenes, W. R. G., Dewaele, C. Anal. Chim. Acta 1991, 255, 283-288.

139. Tomita, M., Okuyama, T., Nigo, Y. Biomed. Chromatogr. 1992, 6, 91-94.

140. Wildman, B. J., Jackson, P. E., Jones, W. R., Alden, P. G. J. Chromatogr. 1991, 546, 459-466.

141. Nashabeh, W., El Rassi, Z. J. Chromatogr. 1992, 600, 279-287.

142. Tsuda, T., Kolayashi, Y., Hori, A., Matsumoto, T., Suzuki, O. J. Microcolumn Sep. 1990, 2, 21-25.

143. Larmann Jr., J. P., Lemmo, A. V., Moore Jr., A. W., Jorgenson, J. W. Electrophoresis, 1993, 14, 439-447.

144. Guzman, N. A., Trebilock, M. A., Advis, J. P. J. Liq. Chromatogr. 1991,

14, 997-1015.

145. Smith, J. T., Rassi, Z. E. Electrophoresis 1993, 14, 396-406.

146. Schomburg, G. Trends in Anal. Chem. 1991, 10, 163-169.

147. Tellez, S., Forges, N., Roussin, A., Hernandez, L. Proc. Int. Conf. In Vivo Methods, 5th, Rollema, H., Westernik, B. H. C., Drijthout, W. J., Eds: Univ. Cent. Pharm.:Groningen, The Netherlands, 1991; pp 122-124.

148. O'Shea, T. J., Telting-Diaz, M. W., Lunte, S. M., Lunte, C. E., Smyth, M.R. Electroanalysis 1992, 4, 463-468.

149. Khur, W. G. Anal. Chem. 1990, 62, 403R-414R.

150. Khur, W. G., Monnig, C. A. Anal. Chem. 1992, 64, 389R-407R

151. Schoneich, C, Kwork, S. K., Wilson, G. S., Rabel, S. R., Stobaugh, J. F., Willams, T. D., Vander Velde, D. G. Anal. Chem. 1993, 65, 75R-78R.

152. Xu, Y. Anal. Chem. 1993, 65, 425R-433R.

153. Yik, Y. F., Li, S. F. Y. Trends in Anal. Chem. 1992, 11, 325-332.

154. Gareil, P. Analysis 1990, 18, 221-241.

155. Landers J. P , Ed. Handbook of Capillary Electrophoresis; CRC press: Boca Raton, 1993.

156. Guzman, N. A., Ed. Capillary Electrophoresis Technology; Marcel Dekker: New York, 1993.

157. Camilleri, P., Ed., Capillary Electrophoresis: Theory and Practice; CRC Press, Boca Raton, 1993.

158. Jandik, P., Bonn, G. Capillary Electrophoresis of Small Molecules and Ions; VCH Publisher,Inc, New York 1993.

159. Grossman, P. D., Golburn, J. C., Eds, Capillary Electrophoresis: Theory and Practice; Academic Press, San Diego, 1992.

160. Horvath, C., Nikelly, J., Eds, Analytical Biotechnology: Capillary Electrophoresis and Chromatography; Am. Chem. Soc., Washington, 1990

ACKNOWLEDGEMENTS

This work was performed at Ames Laboratory under Contract No. W-7405-eng-82 with U.S. Department of Energy. The United States government has assigned the DOE Report number IS-T 1710 to this thesis.

To say thanks to some special people just like to choose a good topic to do is somewhat difficult since there are too many choices for me. The best and the easiest way is to say thank to all my friends I have met during my life. However, there are some special people who I have to mention here, since I would not have had the chance to learn such many novel techniques and enjoy doing research without their encouragement and support.

One person who I especially have to thank is Dr. Yeung. For me, he is not only the one who creates such many excellent ideas for me to indulge in learning novel separation techniques, but also guides me to learn how to think, face and solve all the problems I have.

Dr. Chuen-Ying Liu, my former advisor for master degree in Taiwan, is the other great person in my life who I must thank for her guidance me to analytical chemistry. Without her encouragement and support during the past years, I probably would have no chance to present my research results here.

It is difficult for me to stay in Ames without true friendship. I would like to give my special thanks to my colleagues, Yu-Chen Chang and Qifeng Xue and his wife,

Mancho Chao. They are always there whenever I need a friend. Also, I must thank Sherri Lillard for her help in English.

Lastly, I thank my family for their support for me in coming to the USA to improve myself. Especially, my two sweet sisters, Shu-Hua and Shu-Jane and my sister in-law, Li-Na, always send me truly love from my families whenever I feel I need it. Also, I like to thank my aunt, Hong, who encouraged me so much to stay here to finish my degree.