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CAPILLARY ELECTROKINETIC SEPARATIONS WITH OPTICAL DETECTION

TECHNICAL PROGRESS REPORT FOR THE PERIOD

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INTRODUCTION

This multifarious research program is dedicated to the development of capillary electrokinetic separation techniques and associated optical methods of detection. Currently, research is directed at three general objectives. **First**, fundamental studies of pertinent separation and band broadening mechanisms are being conducted, with the emphasis on understanding systems that include *highly-ordered assemblies* as running buffer additives. The additives include cyclodextrins, affinity reagents, and soluble (entangled) polymers and are employed with electrophoretic (i.e., capillary electrophoresis, CE) and/or electrochromatographic (e.g., micellar electrokinetic capillary chromatography, MECC) modes of separation. The utility of molecular modeling techniques for predicting the effects of highly ordered assemblies on the retention behavior of isomeric compounds is under investigation. In addition, the feasibility of performing separations using a non-aqueous solvent/fullerene electrochromatographic system is being explored. **Second**, the analytical methodologies associated with these capillary separation techniques are being advanced through the development of retention programming (with simplex optimization) instrumentation/techniques and new strategies for performing optical detection. With regard to the latter, the advantages of laser fluorimetry are extended through the inclusion of fluorogenic reagents in the running buffer. These reagents include oligonucleotide intercalation reagents for detecting DNA fragments. Chemiluminescence detection using post-capillary reactors/flow cells is also in progress. **Third**, successful development of these separation and detection systems will fill current voids in the capabilities of capillary separation techniques. In particular, it should be possible to perform rapid, highly efficient, and selective separations of hydrophobic compounds; e.g., higher MW polycyclic aromatic hydrocarbons (PAHs), certain optical isomers, DNA fragments, and fullerene mixtures.

This constitutes the first annual technical progress report in the current trimester grant. Substantial research activity has occurred in each of the proposed areas of research and notable progress has been made in several areas. The following Results and Discussion section is organized based on the evolution of the projects during the past year. The accompanying continuation proposal is similarly organized. In order to simplify and shorten the presentation of results, reference to other DOE documents (recent manuscripts) is made whenever possible and appropriate.

RESULTS AND DISCUSSION

I. Highly ordered assemblies as running buffer additives

Cyclodextrins for isomeric separations: correlation with computationally-derived CD-isomer interaction energies. Simple adjustment of system retention is often inadequate at resolving structurally similar compounds (e.g., geometric and optical isomers) in CE/MECC. We recently reported the use of cyclodextrin (CD) running buffer additives to facilitate MECC separations of geometric isomers of PAHs.^{1,2} In our recent work, the physical, geometric requirements of inclusion complex formation, afforded by using CD additives, facilitated the resolution of benzopyrene isomers (see Table I and separations of 1-position-substituted and methyl-substituted isomers in Figures 3 & 4 of Reference 2). Inclusion complex formation inhibits solute association with the electrophoretically-retarded micellar phase and, thereby, reduces retention in MECC. In CE, complexation decreases solute electrophoretic mobility, which can increase or decrease retention depending on the net mobility of the solute. This work demonstrates the usefulness of MECC for efficient separations of environmentally significant, structurally similar, hydrophobic molecules. We have also explored the use of CD additives for separations of optical isomers; focusing initially on binaphthyl isomers³ and, more recently, DNS-amino acids (see Figure 1 in this report).

Theoretical or empirical predictions regarding the effects of running buffer additives on retention can simplify and expedite system optimization in CE/MECC (or other methods of separation). During the past year, we have modified the computational procedures of a commercial molecular modeling system (SYBYL 6.0, Tripos Associates) to calculate CD-isomer interaction energies.² Considerable effort has been spent in interpreting the large amount of interaction energy data created in order to determine if reasonable correlations with observed CE/MECC retention behavior are possible. Using the average of the five best energy values from interaction energy matrices, correct elution order was predicted for most of the benzopyrene isomers studied (see Table II in Reference 2). We are currently applying these computational techniques to DNS-amino acid enantiomer separations (see accompanying proposal).

Entangled polymers for size-selective DNA separations. With regard to DNA analysis, we had proposed focusing our initial efforts on detection studies using on-column labeling with fluorescent intercalating dyes (see Reference 4 and discussion

below). However, we were also able to establish certain critical parameters for efficient size-selective separations of DNA fragments in standard restriction digests (plate counts greater than 10^6 are demonstrated in Figure 1 of Reference 4). Fused silica capillaries that we surface modified in-house (to eliminate electroosmotic flow and adsorption) were found to perform more reproducibly than those available from commercial sources. Conditions for performing very rapid separations are currently under investigation (the separation shown in Figure 1 of Reference 4 has since been accomplished in about 2 minutes). Our preliminary studies indicate that resolution for large DNA fragments at high fields may be somewhat better for entangled polymer systems, compared to the more commonly used rigid gels, due to the dynamic pore character of the former. Future research will focus on fundamental studies of separation mechanisms, particularly with regard to improving separation speed and extending the applicable size range of DNA fragments (see the accompanying proposal).

Separations involving fullerenes. The competing proposal outlined research aimed at (1) separating fullerene compounds by CE and (2) using the fullerenes as a secondary phase for capillary electrochromatography. Research in this area during the prior year has focused on developing CE methods for efficient separations of fullerenes. An experimental survey of CE-based systems (e.g., MECC using various surfactants and mixed aqueous-organic solvents and solvophobic CE⁵) revealed problems with limited solubility of the inert fullerenes in the separation solution and poor detectability. A positive aspect of this work was the identification of organic solvent systems that supported electroosmotic flow. Based on reports of successful (albeit with poor efficiency) separations of fullerenes by reversed phase HPLC,⁶ our attention has been directed toward investigating electrokinetically-driven packed capillary chromatography⁷ for PAH and fullerene separations. The skills needed to slurry pack capillaries (primarily with reversed phases), electrokinetically inject samples onto these capillaries, and detect on-column (& on-packing) were developed during the summer of 1993. We have obtained reasonable (although not reproducible) electroosmotic flow using non-aqueous solvent systems (e.g., acetonitrile modified with more hydrophobic solvents) and achieved separation efficiencies of 10^5 plates/meter for capillaries packed at relatively low (<2000 p.s. i.) pressure (see Figure 2 in this report for an example). Future work will include fundamental investigations of non-aqueous separations using this technique and refinement of fullerene separations (see accompanying proposal). With regard to the latter, we will attempt to directly verify the

chirality of C₇₆ and C₈₄ fullerenes via high efficiency separations employing chiral phases (e.g., CDs).

Immunoaffinity separations. Affinity reagents such as antibodies represent another form of highly-ordered assembly. The analytical development and evaluation of an immunoaffinity CE system for the determination of aflatoxin B₁ was begun during the summer of 1993. This system was chosen because of the commercial availability of antibodies to B₁ and the convenience and sensitivity of performing laser fluorometric detection of the aflatoxin. The general scheme was to add anti-B₁ to the sample (and, if necessary, the running buffer) to selectively bind the aflatoxin and thereby alter its mobility (relative to other neutral sample components). The analytical figures of merit of this assay were to be evaluated and, subsequently, fundamental aspects of the antibody : B₁ binding were to be investigated. Two problems were identified with these early experiments; i.e., (1) band broadening due to antibody heterogeneity (not protein-capillary adsorption) and (2) insufficient antibody electrophoretic mobility to provide the desired separation between free and complexed B₁. Future studies (see accompanying proposal) will first address these problems.

II. Solvent gradients and simplex optimization

In MECC, the distribution of solutes between micellar and mobile phase (hence capacity factors) can be controlled by employing the organic modifiers and solvent gradients that are traditionally used in reversed phase HPLC. However, predictions concerning the effects of solvent gradients on retention are complicated by the fact that flow rate and the micellar phase volume are effected. We have developed instrumentation for generating gradients in MECC and demonstrated improvements in separations when gradient techniques are used.^{8,9} Gradients with unusual shapes are sometimes important in achieving rapid separations by MECC.⁹ Using flow rate and retention data obtained from isocratic runs, it is possible predict solute retention times with fair accuracy. Most recently, we have employed these predictions along with simplex methods to computationally optimize separations based on evaluation of a *chromatographic response factor*.⁹ Tables 3 & 4 and Figure 4 in Reference 9 illustrate the optimization of an acetonitrile gradient for the separation of selected PAHs. More reliable and easily controlled instrumentation (a versatile automated CE sample injection system may work well in this regard) is needed to carry this research future. Future research in this area is not elaborated upon in the accompanying proposal.

However, if the PI's efforts to acquire automated CE instrumentation through other sources are successful, the project will be continued.

III. Detection based on chemiluminescence

Narrow solute bands and diminutive capillaries complicate detection in CE/MECC. As an alternate mode of optical detection, we have constructed a microscale molded post capillary reactor that simplifies the use of chemiluminescence (CL) detection,¹⁰ relative to previous reports of coaxial flow reactor system.^{11,12} We have employed peroxyoxalate CL in largely non-aqueous solvents for the detection of certain PAHs. The effects of reactor design and operating parameters (e.g., absolute and relative reagent flow rates and reagent concentrations) on signal and peak efficiency are presented in Figures 3 - 5 of Reference 10. For the CL detector tested, concentration limits of detection for injections of perylene and anthracene were 1.5×10^{-8} M and 9.3×10^{-7} M, respectively. Future work will emphasize utilizing this *poor man's* alternative to microfabricating CE instrumentation on a chip,^{13,14} to develop electrokinetic flow manifolds at the outlet of the capillary to enhance detection capabilities (see accompanying proposal).

IV. Detection of DNA fragments based on fluorescent intercalation dyes

The analytical figures of merit of laser-excited fluorescence detection of DNA restriction fragments, based on the enhanced fluorescence of dyes upon intercalation, were determined.⁴ We studied two intercalation dyes (ethidium bromide (EB) and POPO-3) that can be excited using the output (543 nm) of a He-Ne "greenie" laser. Calibration plots were linear over two orders of magnitude in DNA concentration and signal levels increased in approximately a linear fashion with fragment size. Limits of detection were in the low-to-mid femtogram injected range for the individual fragments (a substantial improvement over detection based on spectrophotometry or native fluorescence). The EB system was shown to be stable and easy to use. Future fundamental investigations involving size-selective separations using entangled polymer solutions will rely heavily on detection using this methodology (see above and accompanying proposal).

V. Quantitative aspects of lanthanide metal analysis

Our early work with CE separations of metal ions involved the on-column formation of fluorescent metal complexes for purposes of detection.¹⁵ During the past year, we have investigated the feasibility of using CE for determinations of heavy metals. Reports presenting impressive CE separations, using indirect UV absorbance detection, of complex mixtures of metals, e.g., the complete resolution of the lanthanides,^{16,17} can be misleading with regard to the practical value of this methodology for trace environmental and energy analyses. Traditional measures in quantitative analysis (e.g., detectability, dynamic range, and reproducibility) are strongly influenced by the observed peak shape and by sample stacking effects.¹⁷ We are currently investigating the effects of running buffer composition (supporting electrolyte, background electrolyte, complexing agents), sample composition (particularly ionic strength), and injection method (hydrostatic or electromigration) on these quantitative measures. We have observed dynamic ranges for lanthanide metals that are less than two orders of magnitude in concentration. The complex nature of the injection process and the critical dependence of peak shape on sample and mobile phase composition have adverse effects on reproducibility. In our studies, peak height or peak area RSDs are 1.5-3 times larger than we have generally observed for manual injection in CE/MECC. Furthermore, limits of detection are rather poor; only in the low-to-mid ppm range. Slight changes in experimental parameters can also influence resolution and, hence, the accurate determination of peak height or peak area. Figure 3 illustrates the effect of changing the concentration of the weak complexing agent α -Hydroxyisobutyric acid (HIBA) on the resolution of several lanthanides. In this case, complexation with the Hydroxyisobutyrate anion magnifies the small intrinsic differences in lanthanide ion mobilities and improves resolution. The electrophoretic mobility of the *displaceable* background electrolyte is critical to obtain reasonable peak shape (Ethylimidazole functions better in this regard than the more commonly used Imidazole).

These preliminary studies clearly establish the need for improvements in both detectability and reproducibility. We are preparing a paper on this work for the Eighth Annual DOE Symposium on Separation Science & Technology for Energy Applications to be held October, 1993, in Gatlinburg, TN. We have also establish collaborations with scientists at the Oak Ridge Y-12 plant to facilitate further research in this area (see accompanying proposal).

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FIGURES

Figure 1: CE separation of enantiomers (D form elutes first) of the dansyl derivatives of (A) Leucine, (B) Valine, (C) Threonine, (D) Serine (unresolved), (E) Glutamic Acid, and (F) Aspartic Acid. Separation conditions: running buffer - 10 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 6 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 9.2), 5 mM β -CD and 5 mM γ -CD; capillary - 60 cm x 50 μm id; running voltage - 20 kV; detection - UV absorbance

Figure 2: Electrokinetically-driven, packed capillary separation of benzene, anthracene, and pyrene (in order of elution). Separation conditions: mobile phase - acetonitrile; capillary - 28 cm x 50 μm id packed with 3 μm C_{18} (Vydac); injection - electrokinetic (5 kV, 5 sec); running voltage - 20 kV; detection - UV absorbance

Figure 3: CE separations of lanthanides (La, Pr, Eu, Tb, and Dy , in order of elution) using a running buffer containing (A) 1 mM HIBA and (B) 3 mM HIBA (background electrolyte the only other running buffer component). Separation conditions: capillary - 45 cm x 75 μ m id (C_8 surface modified); running voltage 15 kV; detection - indirect UV using 10 mM Ethylimidazole as background electrolyte

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PERSONNEL

Michael J. Capacci, MS, December 1986, Thesis: Biological Sample Injection for Open Tubular Liquid Chromatography.

Charles N. Kettler, Ph.D., March 1987, Dissertation: Photothermal Detection for Capillary Liquid Chromatography.

D. Edward Burton, Ph.D., March 1987, Dissertation: Development and Application of Micellar Electrokinetic Capillary Chromatography.

Anthony T. Balchunas, Ph.D., December 1987, Dissertation: Surfactant Mediated Development of High Resolution Open Capillary Liquid Chromatography.

Suzanne Landry-Baker, MS, August 1988, Thesis: Macromolecular/Particle Separations Using Capillary Electrokinetic Techniques.

James Bowyer, Ph.D. December 1991, Dissertation: Construction, Evaluation, and Use of a Fluoroimmunochemical-Based Fiber Optic Microscale Regenerable Biosensor.

David Swaile, Ph.D. December 1991, Dissertation: Alternate Methods of Laser-Based Fluorometric Detection in Capillary Electrokinetic Separations.

Roderic Cole, Ph.D. December 1991, Dissertation: Applications of Non-Traditional Organized Media in Capillary Electrophoresis.

Susan Finniss, M.S. May 1992, Thesis: Preliminary Studies of Immunoaffinity Capillary Electrophoresis.

Ricky Holland, M.S. August 1992, Thesis: Evaluation and Improvement of the Qualitative Characteristics of MECC.

A. Craig Powell, Ph.D. December 1992, Dissertation: Optimization of MECC with Solvent Gradients.

*Tracey Staller, fifth year graduate student.

*Beth Colburn, fourth year graduate student.

*Christine Copper, third year graduate student.

*Brian Clark, third year graduate student.

*Kysten Whitaker, second year graduate student.

*Miland Nagale, second year graduate student.

*Mike Stebbins, second year graduate student.

Steve Cosgrove, undergraduate student.

Boris McCubbin, undergraduate student.

Andy Porter, undergraduate student.

*Sonja Starnes, undergraduate student.

Dr. Joe Gorse, visiting faculty research participant (Baldwin Wallace College, OH).

Dr. Art Hoyt, visiting faculty research participant (University of Central Arkansas).

Dr. Chris McGowen, visiting faculty research participant (Tennessee Technical University).

*Dr. Ken Morton, visiting faculty research participant (Carson-Newman College, TN).

*Dr. Joe Davis, visiting faculty research participant (Winthrop University, NC).

*supported at least partially during current 12 month grant period.

END

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