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CAPILLARY ELECTROKINETIC SEPARATIONS WITH OPTICAL DETECTION

TECHNICAL PROGRESS REPORT FOR THE PERIOD

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INTRODUCTION AND OVERVIEW

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 achieving rapid separations and understanding separation systems that include *highly-ordered assemblies* as running buffer additives. The additives include cyclodextrins (CDs), 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. Recently, we have begun investigating a unique dual-CD separation system that may compliment the well-established MECC technique for efficient separations of neutral species. In addition to this open capillary work, the feasibility of performing electrokinetic separations of extremely hydrophobic species (e.g., fullerenes) using packed capillaries and non-aqueous mobile phases has been demonstrated. Capillaries packed with chelating resins are also being evaluated as a means to concentrate heavy metals prior to separation by CE. **Second, instrumentation and methodologies** associated with these capillary separation techniques are being advanced. Most notably, computational-based molecular modeling hardware and software is being used to predict and better understand the effects that highly-ordered running buffer additives exert on separation performance. Acrylic post-capillary manifolds are being constructed and used with electrokinetic flow multiplexing with the eventual aim of enhancing detection capabilities. Work involving on-column labeling for detection purposes continues. Low limits of detection have been obtained for DNA fragments by on-column fluorescence labeling using intercalating dyes. More recently, we have used Arsenazo III for on-column spectrometric labeling of UO_2^{2+} . We have also begun fabricating rugged instrumentation that is compatible with short (< 20 cm) capillaries. This instrumentation will be used for extremely rapid separations and/or field applications. **Third, applications** of these separation and detection systems should 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) and fullerenes), certain optical isomers, DNA fragments, and various pollutants including certain heavy metals.

This constitutes the second 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 prior two years. 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

Uses of Cyclodextrins in capillary electrokinetic separations: isomeric separations. Guest-host inclusion complexation of solutes with CDs is highly dependent on the physio-chemical properties of the solute and the CD. Thus, inclusion complexation can be an extremely selective process. However, since many types of native and derivatized CDs are commercially available, the selectivity is somewhat tunable. We have focused much of our attention on exploring the utility of CDs as running buffer additives in both CE and MECC. They function to alter retention in CE by reducing solute mobility and in MECC by hindering solute interactions with the micellar phase. Our last report highlighted our success with using CD-modified MECC to separate a number of hydrophobic molecules (geometric isomers of various polycyclic aromatic hydrocarbons (PAHs)) that are not easily separated by MECC.^{1,2} The efficiency and selectivity we obtained were excellent, particularly in separations of benzopyrene isomers. These compounds are important due to their interactions with DNA.

During the past year we have focused our attention on using CD-modified CE for separations of optical isomers. CDs are chiral and offer the possibility for enantio-selective separations. Our work involved separations of DNS-amino acids³ and binaphthyl compounds (see accompanying proposal). By altering the concentration of the CD we were able to obtain fundamental information (mobilities and complexation constants) for the DNS-amino acids and, using this information, investigate mechanisms for their enantio-selectivity. Each DNS-amino acid had a distinctive optimum CD concentration, but the type of CD was not extremely critical (both β - and γ -

CD were useful). Somewhat unexpectedly, certain amino acids exhibited differences in the mobilities of their fully-complexed enantiomers. In separations of binaphthyl enantiomers, the type of CD employed was found to be extremely critical (see molecular modeling section below and the accompanying proposal).

Uses of cyclodextrins in capillary electrokinetic separations: a charged-CD / neutral-CD mode of separation. We have recently initiated characterization, with regard to efficiency, selectivity, and system retention, of a new chromatographic-based mode of CE. Separations with this technique are based on the differential distribution of solutes between charged and neutral CD running buffer additives. Preliminary data and a further description of the technique appears in the accompanying proposal. However, it appears that this new mode of separation will complement the MECC technique which has been studied extensively in our laboratory over the past several years.

Uses of cyclodextrins in capillary electrokinetic separations: molecular modeling. The trial and error approach to choosing conditions in CD-modified CE/MECC can be time consuming and certainly does not exhibit significant intrinsic scientific merit. Molecular modeling (MM) is a potentially useful adjunct to actual laboratory experiments in validating separation mechanisms and in simplifying and expediting system optimization. We have employed MM techniques in conjunction both CD-modified CE and MECC. Our approach involves systematically and incrementally translating the guest solute with regard to the CD and calculating the resulting complex's interaction energy at each position (i.e., an energy matrix is created). A departmental workstation with SYBYL 6.0 MM software are employed in this work. Correlations between the interaction energy and the effects of the CD on the separation are sought. This is by no means straight forward. Among the questions to be considered are the following: are the energy terms in the energy summation appropriate?; what orientations (approaches) for the solute and CD are important?; what interaction space and increment are appropriate?; should energy "minimization" of the complex be performed (a time consuming process)?; how should the energies within the matrix be weighted?; is it necessary to consider solvation?; how important are processes and interactions other than those involving the CD (e.g., those involving other running buffer components)?; are entropy and stoichiometric considerations important?; can correlations with specific types of interactions (e.g., hydrogen

bonding) be found?; etc. Our work has indicated that each separation system (sample and technique) is unique with regard to the importance of these questions.

The prior report⁴ highlighted our success with correlating the retention behavior of benzopyrene isomers in CD-modified MECC with the average of the five lowest energies in rather large interaction energy matrices.² Considering all logical orientations between solute and CD was found to be important. This past year we have focused on predictions involving the chiral recognition capabilities of CDs. The correct enantiomer retention order was predicted for DNS-amino acids using CD-modified CE.³ In this case, only one orientation was logical (the DNS label inserted into the CD cavity). However, small increments and energy minimization of the complex was found to be very important. A simple partition function approach to weighting the energies in the matrix was also found to be useful.

Two other MM projects have been pursued recently. Literature reports of HPLC separations of fullerenes using CD stationary phases were evaluated. Based on our MM work, it appears that separations of C60 and C70 are not so much based on discrimination by the CD phases employed (as presented in the literature), but rather are largely a matter of fullerene hydrophobicity differences and interactions with the mobile phase.⁵ We are very interested in capillary electrokinetic separations of fullerenes (see below) and are encouraged by MM results which indicate the feasibility of at least partially resolving fullerenes that are expected (but not yet directly verified) to be chiral, providing high efficiency is achieved (see accompanying proposal). Interpretation of the very distinctive results of CD-modified CE separations of binaphthyl compounds (see above) using MM techniques have not yet been accomplished. These interpretations are complicated by the many binaphthyl/CD orientations that are possible. It also appears that solvation of the CD must be considered in that case or energy minimization of the complex results in unrealistic distortions of the CD and/or binaphthyl compound.

Entangled soluble polymers for size-selective DNA separations. Prior work in this area emphasized laser fluorometric detection. By optimizing on-column labeling conditions, we were able to detect low femtogram (low zeptomole) quantities of DNA fragments using traditional and experimental intercalation dyes as running buffer additives.⁶ Research during the past year has focused on understanding separation mechanisms for entangled polymer size-selective CE, with the aim of optimizing conditions for very rapid separations of DNA restriction digests.⁷ Separation speed is a highly desirable analysis feature in both the fingerprinting and the sequencing of

DNA materials. Other research groups are investigating multiplex approaches (i.e., simultaneous separations using capillary arrays) to increase sequencing rates.⁸ Such instrumentally intensive approaches are beyond the resources of our program. However, our investigations of factors that influence separation speed should compliment efforts to develop capillary arrays.

Our experiments support the recently presented constraint-release theory for DNA migration through entangled polymer networks.⁹ Consistent with that theory, large molecular weight (MW) methyl cellulose entangled polymers were found to perform very similar to rigid gels, but without many of the experimental problems associated with gels. By optimizing parameters such as entangled polymer concentration and MW, capillary dimensions, and applied field (see Figures 1 - 4 in Reference 7) we were able to achieve separations of the tested DNA restriction digests in about two minutes (see Figure 6 in that reference). This represents a three- to five-fold decrease in separation time relative to prior reports for size-selective CE separations of restriction digests. Future work will focus on the use of extremely short capillaries and gradient techniques (see accompanying proposal).

Immunoaffinity CE. Theoretical aspects of immunoaffinity CE were presented in detail in our prior competing proposals. While the addition of affinity reagents, such as antibodies, to the running buffer is theoretically enticing (highly selective control over the retention of the ligand should be possible), the experimental obstacles associated with this technique are formidable. Protein adsorption and poor reproducibility in the surface characteristics of commercial or in-house coated capillaries have proven to be very problematic. Some of the reported measures to eliminate adsorption problems (e.g., pH adjustment or the addition of SDS) can degrade the immune function of antibodies and, thus, are not viable options in this work. Furthermore, antibodies were found to be very heterogeneous (yielded broad peaks exclusive of adsorption effects) and exhibited inadequate intrinsic mobility to provide a proper "retention window" (i.e., retention time difference between analyte and analyte-affinity reagent complex). We had hoped that addressing the specific analysis of a natural fluorophor (aflatoxin B1) for which antibodies are available commercially would simplify matters.⁴ It now seems that a much more concerted effort would be required to make adequate progress in this project (currently one graduate student is working part time on the project).

Work this past year centered on attempting to reproducibly produce polyvinyl alcohol and polyacrylamide coated capillaries and extend the retention window via

adjustments in electroosmotic flow (EOF). The results of the former effort met with very marginal success and efforts to adjust EOF when using coated capillaries are described in a later section of this report. Due to experimental difficulties, limited personnel, and encouraging results in other areas, the accompanying proposal does not emphasize this project.

II. Non-aqueous packed-capillary electrokinetic chromatography for separations of large PAHs and fullerenes

A substantial effort has been made in our laboratories to extend the utility of capillary electrokinetic separation techniques (mainly the MECC technique) to include separations of hydrophobic molecules.¹⁰ As the hydrophobicity of the solutes increases, solubility in traditionally aqueous CE/MECC systems decreases dramatically. Moreover, large concentrations of organic solvent are not compatible with the MECC technique. This situation provided the impetus for investigations of capillary electrokinetic chromatography (CEC) using packed capillaries and non-aqueous mobile phases.¹¹ The establishment of EOF in capillaries under non-aqueous conditions has been reported (see citations in Reference 11); however, it is not well understood and has been limited to very few solvents.

Skills needed to slurry pack capillaries¹² were re-established in our laboratory during the past year, and capillaries containing 3 μm ODS particles were prepared. Van Deemter plots were generated for CEC separations of PAHs using these capillaries, yielding efficiencies greater than 150,000 plate/meter.¹¹ In this work, non-aqueous mobile phases composed of acetonitrile (ACN) modified with less polar solvents such as tetrahydrofuran (THF) and methylene chloride (MeCl_2) were used to separate large PAHs and common fullerenes (see Figure 1 in reference 11). The small magnitude (particularly when solvent strength is increased) and poor reproducibility of EOF under these non-aqueous conditions are problems that need to be addressed (see next section of this report). We have plans to attempt separations of chiral fullerenes using CEC with non-aqueous mobile phases and capillaries containing CD packings (see accompanying proposal).

III. Studies of electroosmotic flow under non-traditional CE conditions

Electroosmotic flow mobility μ_{eo} is often given by Equation 1

$$\mu_{eo} = \varepsilon\psi_o/\eta \quad (1)$$

where ε , ψ_o , and η are solution permittivity, capillary surface charge, and solution viscosity, respectively.¹³ The charge density at the surface is considered to decay exponentially with distance from the capillary surface. Macroscale models do not adequately describe many observations concerning EOF in fused silica capillaries. For example, the addition of long chain cationic surfactant (e.g., CTAB) causes (i) a reversal in the normally cathodic EOF in untreated silica capillaries (the model would predict a neutralization of surface charge and reduction, not reversal, of flow), (ii) generates an anodic flow when capillaries contain a hydrophobic coating, and (iii) generates a rapid cathodic flow when capillaries contain a hydrophillic coating and ionic strength is low. Non-aqueous CEC and affinity CE are two non-traditional modes of CE for which control of EOF is an issue. During the past year, we have explored the uses of various running buffer conditions to achieve proper EOF for these techniques. Because of the aforementioned theoretical limitations, our approach has been largely Edisonian in nature.

Rapid EOF is essential to perform timely CEC (note the long fullerene separation time in Figure 1B of reference 11). We have investigated the utility of various organic solvents with large values of ε/η for CEC separations. ACN provides rapid EOF (~ 7 cm/min in packed capillaries) that is diminished upon the addition of organic salts or most other organic solvents (See Figure 2 in reference 11 as example of effects of adding THF or MeCl_2). Overall, in terms of reversed-phase solvent strength and separation speed, ACN modified with butylnitrile (BN) is the best of the solvents that we have tested.

The importance of adjusting CE separation conditions so as to provide suitable system retention can not be overstated. Affinity CE resembles MECC in that system retention is marked by a retention window. As measured in our laboratory, the intrinsic mobility of whole antibody to aflatoxin B1 (see above) is $\sim +4 \times 10^{-5} \text{ cm}^2/\text{V}\cdot\text{s}$ at physiologic pH. In an untreated capillary ($\mu_{eo} \sim -4 \times 10^{-4} \text{ cm}^2/\text{V}\cdot\text{s}$), using normal operating conditions, this produces a retention time difference between B1 and anti-B1 immunocomplex that is much less than one minute (clearly too small to be practical). Moreover, protein adsorption is a big problem for untreated capillaries.¹⁴ We have had some success creating moderately hydrophillic coated capillaries (see above) that exhibit very small cathodic EOF ($\mu_{eo} \sim -2 \times 10^{-5} \text{ cm}^2/\text{V}\cdot\text{s}$). This provides a wide retention window but analysis time is prohibitively long. We have investigated the use of several common surfactants to modify EOF. μ_{eo} values of $\sim -7 \times 10^{-5}$ or $+5 \times$

10^{-5} cm²/V-s would provide a window of ~ 10 minutes and an overall separation time of ~30 minutes. While we have influenced EOF by adding surfactant to the running buffer, these desirable μ_{eo} values have not been achieved. Moreover, the hydrophillic coated capillaries do not respond reproducibly to the addition of surfactant. This illustrates one of the numerous practical problems associated with developing affinity CE (see above).

IV. Investigations of heavy metal separations by CE

The analytical figures of merit of CE with indirect detection for the separation and quantitative determination of rare earth metals was evaluated previously.¹⁵ Various imidazole derivatives were investigated as to their suitability as running buffer (displaceable) detection ions with α -hydroxyisobutyric acid functioning as a weak chelating agent to enhance separations. More recently, we have directed our attention at the development of CE methodologies for the determination of other, more DOE relevant, heavy metal pollutants. The similar oxidation state and complexation chemistry of the rare earths renders their separation by CE relatively straight forward. The task is more formidable for the actinides and other heavy metals. Thus, we have sought to optimize CE conditions for the determination of heavy metals singularly, focusing initially on uranyl. Although, UO_2^{2+} can be sensitively determined based on its native fluorescence, the required instrumentation is rather complex; pulsed dye laser excitation and time resolved detection are involved.¹⁶ Using very simple CE instrumentation, we have achieved mid-ppb-range LOD for uranyl using on column labeling with the ligand Arsenazo III. A bathochromic shift in the absorbance of this ligand is observed when UO_2^{2+} is complexed (molar absorptivity at 650 nm increases from 2,000 to 22,500). This level of detectability also relies on a significant degree of sample stacking.¹⁵ A calibration plot and a typical peak profile is shown in Figure 1.

Analyses of potentially contaminated ground water streams for uranyl requires low-to-sub- ppb detectability. To achieve this level of detectability we propose the use of short, medium-diameter capillaries, packed with chelating resins such as U/TEVA Spec,¹⁷ as a means of concentrating UO_2^{2+} . It should be possible to use these packed capillaries for rapid field sampling, eliminating the need to handle or transport large volumes of sample. Preliminary studies aim at determining the best conditions to load relatively large volume samples onto columns packed with the resin, and elute with relatively small amounts of CE compatible solvents, have been encouraging (see accompanying proposal).

A. **B.**

Figure 1: Calibration plot (A) and peak profile for 500 ppb UO_2^{2+} (B) for CE determination of uranyl with sample stacking and on-column labeling using the spectrophotometric tag Arsenazo III. Conditions: Capillary - 75 μm i.d. x 50 cm (40 cm to detector); Applied Voltage - 15 kV; Running Buffer - 0.1 mM Arsenazo III, 1/0.6 mM phosphate/borate buffer, 10 mM HClO_4 , 10 mM NaCl; Injection - hydrostatic yielding ~ 25 mm injection length (sample does not contain buffer or NaCl); Detection - absorbance at 650 nm

V. Development of post-capillary multiplexers for improved detection

The development of electrokinetically-controlled, post-capillary multiplexers is based upon our previously described chemiluminescence reactor/flow cell.¹⁸ That work yielded the basic molding techniques required for the production of polyimide manifolds; three 25 μm i.d. detection capillaries are mated to the end of a 50 μm i.d. separation capillary using removable, ultra-thin wires to define narrow connecting channels. The ability to divert discrete bands into detection capillaries without band dispersion offers many advantages for the detection of a small number of separated components. Long time constants, radiochemical counting, post separation analyte derivatization, and multiple modes of detection represent some of the detection opportunities afforded by the multiplexer that we are interested in exploring.

Unfortunately, as with the immunoaffinity CE project, this work has been plagued by numerous experimental difficulties. Only about one in three manifolds are successfully prepared. More detrimentally, the successfully prepared manifolds are very susceptible to occlusion due to foreign materials, precipitation of running buffer components, or dissolution or swelling of the polyimide. During the past year we have developed and tested optical systems to perform laser fluorimetry in each of the three detection capillaries and electronic switching circuits to divert flow or eliminate flow in the capillaries. We have also successfully fabricated at least three dozen manifolds; none of which have survived more than a day of experimentation. We are hopeful that the aforementioned advantages of detection multiplexing can still be demonstrated. However, realistically the multiplexer will not be of practical value unless the occlusion problems can be solved. Because of limited personnel and encouraging results in other areas of our program, the accompanying proposal does not emphasize this project.

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