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**CAPILLARY ELECTROKINETIC SEPARATIONS
WITH OPTICAL DETECTION**

**TECHNICAL PROGRESS REPORT FOR THE PERIOD
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INTRODUCTION AND OVERVIEW

The seminal work of Jorgenson in 1981 ushered in the modern era of capillary electrophoresis (CE).¹ Since that time, research activities involving capillary electrokinetic methods of separation have grown exponentially. Numerous conferences, symposia, monographs, and dedicated journals attest to the maturing of these techniques. While many of the obvious approaches have been explored, and instrumentation is reasonably well-developed, the full potential of CE has clearly not yet been reached. Moreover, CE techniques are not universally accepted as desirable alternatives to traditional chromatographic and electrophoretic methods of separation. Thus, it is likely that research into various aspects of capillary electrokinetic separations will continue at a torrid pace for at least the remainder of this decade.

Since the mid 1980s, we have conducted DOE-sponsored research aimed at the fundamental understanding and practical development of capillary electrokinetic separation techniques and associated methods of optical detection. Our multifarious approach involves the following elements: (i) Advancing CE Instrumentation with the aim of improving the analytical capabilities of the technique, as well as providing a means to study fundamental aspects of CE separation processes; (ii) Developing New Separation/Detection Strategies to facilitate separations of substances of interest to the DOE (e.g., energy-related, environmental metals and organic toxins, and DNA materials). To this end, we have made extensive use of highly organized media (e.g., micelles cyclodextrins, CDs, and complexing agents) as running buffer additives. Most notably, we extended prior work involving the fundamental development of micellar electrokinetic capillary chromatography (MECC) to include more complex modes of separation using CD and mixed CD-micellar systems; (iii) Performing Fundamental Studies to better understand the factors and phenomena that influence solute band migration and dispersion. In this regard, we have been one of the few groups with a strong interest in

the fundamental aspects of CE separation processes; (iv) Apply capillary electrokinetic techniques to the separations of substances of energy and environmental interest.

The separations research (see Publications and Reports Section) performed by our group, comprised of 8-10 graduate students and an occasional visiting faculty collaborator (see Personnel Section), has been supported in large part by DOE/OBES. The broad scope of our research and approach is well-suited to the varying interests and abilities of these academic group members. Moreover, it has provided the flexibility to respond quickly to DOE interests and, indeed, many of our accomplishments compliment other research programs supported by the Department. This document constitutes the Technical Progress Report covering the 36 month period from February 1, 1993 to January 31, 1996. It is organized around the four elements discussed above. In order to simplify and shorten the presentation of results, numerous references are made to figures, tables, and related discussion in pertinent DOE registered publications (copies enclosed). The outline of this report (i) highlights these recent publications.

RESULTS AND DISCUSSION

Instrumentation

Gradients for MECC and SSCE. When separating the components in complex samples, it is often observed that a single set of operating conditions is not appropriate to resolve all of the components of interest. This "general elution problem" is particularly significant in micellar electrokinetic capillary chromatography (MECC) due to the limited elution range of the technique² and in size-selective capillary electrophoresis (SSCE) separations of complex DNA materials. We have developed and tested instrumentation that permits the generation of linear- and curve-shaped solvent gradients in MECC (see Figs 1 & 2 in Ref 3).³ Using a previously developed retention model⁴ and this instrumentation, a simplex procedure was employed to optimize gradients for

separations of polycyclic aromatic hydrocarbon (PAH) mixtures (see Fig 4 & Tables 3 & 4 in Ref 3). Very recently, we developed a spatial scanning detection scheme for SSCE (see below) that also permits the in-situ generation of size-sieving gradients.⁵ This approach to SSCE separations of DNA digests was shown to reduce analysis time, which is a very important issue in sequencing applications (see Fig 3 in Ref 5).

Spatial scanning LIF detector. The aforementioned laser induced fluorescence (LIF) detector (see apparatus Fig 1 in Ref 5) permits real time or post-separation inspection of nearly the entire length of the separation capillary.^{5,6} The proven or potential advantages of this detection scheme are numerous and include decreased analysis time by tracking or "seeking" bands (see Fig 6 in Ref 6), improved detectability resulting from relaxed time constant constraints (see Fig 7 in Ref 6), and better peak area reproducibility. Fundamental studies of CE phenomena are also possible (see below). Thus, through implementation of this novel instrumentation, we expect to develop a better understanding of SSCE (and other CE) separation processes. This instrumentation will be improved, adapted for field use, and employed extensively in future work (see accompanying grant application).

Flow multiplexing with post capillary junctions. A new method of micro-fabrication was developed and employed in the production of low dead volume, post-capillary junctions.⁷ These devices (see apparatus Figs 1-5 in Ref 7) were used for chemiluminescence detection and to selectively divert separated bands prior to a multiplexed approach to LIF detection (see Fig 11 in Ref 7). Despite some success in this project, complications with fabricating the junctions, and the recent success of "chip" approaches to CE,⁸ have reduced the significance of the project.

ELP Capillaries/LIF. Extended light path (ELP) "bubble" capillaries from Hewlett Packard were shown to offer advantages over conventional capillaries for LIF detection.⁹ In addition to improved sensitivity due to longer pathlengths, the ELP capillaries simplify

optical alignment and spatial rejection of optical background, thereby providing further improvements in detectability (see Fig 4 in Ref 9).

Separations-based fiber optic sensors. We are integrating our expertise with fiberoptic sensing¹⁰ and capillary electrokinetic separations in the development of separations-based fiber optic sensors (SBFOS). High selectivity is a necessary analytical characteristic in sensing work since measurements are generally performed in situ. This selectivity is often provided chemically, as with the immunochemical based sensors we developed earlier.¹⁰ We have begun exploiting the CE attributes of high efficiency, speed, diminutive size, remote control, and exceptional selectivity in the development of SBFOS.¹¹ Frontal electropherograms of laser dyes were obtained and exhibited efficiencies of several thousand plates for a 5 cm long SBFOS separation element (see preliminary manuscript and data presented in Ref 11). We fully expect that numerous important remote sensing problems (e.g., monitoring various inorganic and organic chemicals in ground water, at toxic burial sites, and in waste storage tanks) could be addressed with well-developed SBFOS. (See accompanying grant application for future studies in this area.)

New Separation/Detection Strategies

On-column labeling for LIF and absorbance detection. Optical modes of detection are commonly employed in CE. As a consequence of the diminutive sizes of CE capillaries, absolute limits of detection (LODs) are often very low but concentration LODs can be rather poor. The inherent sensitivity of LIF renders it a highly desirable mode of detection. During the current grant period, we extended prior work^{12,13} with on-column fluorescence labeling as a means to apply LIF to non- or poorly-fluorescing analytes. Hydrophobic probes (the arylaminonaphthalenes ANS & TNS) were used to detect non-chromophoric surfactant/micelle systems and for indirect LIF detection in MECC.¹⁴ We also studied the use of two intercalation dyes (ethidium bromide, EB, and POPO-3) for

on-column labeling of DNA materials.¹⁵ In the latter work, limits of detection were in the low femtogram injected range (see Table II in Ref 14).

To address the detection of heavy metals of interest to the DOE, we have used indirect spectrophotometric methods to detect lanthanides separated by CE.¹⁶ More significantly, we recently developed a unique but simple CE method for the rapid screening of ground waters for uranyl ion.¹⁷ That work involved on-column complexation with a ligand, Arsenazo III, that experienced a red-shift in its absorbance spectrum upon complexation (see Fig 1 in Ref 16). By employing a unique sample stacking procedure, LODs were in the low ppb range (considerably lower than normally achieved for absorbance detection in CE). In response to pressing DOE environmental management needs, the accompanying proposal contains plans to address the separation of other metals of significance to DOE using on-column complexation chemistries (e.g., extensions of our prior work with 8-HQS¹²) and instrumentation designed for field use and sensing.

Non-aqueous capillary electrochromatography (CEC). Separations of hydrophobic molecules (e.g., higher MW PAHs) is not easily accomplished by CE techniques. Modifying MECC running buffers with organic solvent and CDs provides a limited solution to this problem.¹⁸⁻²⁰ Recently we have been the first group to employ a non-aqueous mode of electrochromatography in packed capillaries.²¹ Efficiencies of 160,000 plates/m were achieved in CEC separations of PAHs and common fullerenes. The composition of the non-aqueous mobile phase was shown to dramatically influence electroosmotic flow (EOF) rate (which was quite slow in some cases) and capacity factors (see Figs 2 & 3 in Ref 21).

Rapid size-selective CE of DNA fragments. Speed is an essential characteristic in DNA analyses, especially those associated with sequencing for human genome work. We have studied fundamental aspects of SSCE separations using running buffers that contain entangled polymer for sieving.²² By optimizing conditions, we were able to

separate certain restriction digests in less than 3 minutes. Polymer concentration, size-selective gradients, and manipulation of effective capillary length, were shown to be important factors in reducing separation time.^{5,22} This work compliments multiplex approaches to increase sample throughput.²³ Current and proposed work in this area involve studies of DNA band migration and dispersion using the novel spatial scanning LIF detector mentioned above (see Figs 2, 3, & 6 in Ref 6 & accompanying grant application).

CD-modified CE/MECC. Cyclodextrins are macrocyclic sugar molecules that possess a hydrophobic cavity and a hydrophilic exterior. The most common CDs contain 6 (α -CD), 7 (β -CD), and 8 (γ -CD) sugar molecules. Many organic compounds form inclusion complexes with CDs. Inclusion complexation constants depend on the size, geometry, and physiochemical properties (including stereochemical) of the compound and CD. We have shown the utility of CDs as running buffer additives in CE for enantio-selective separations of DNS-amino acids²⁴ and binaphthyl compounds.²⁵ In these CE separations, the inclusion of the enantiomers with the CD resulted in a reduction in observed mobility (see Fig 2 in Ref 24 and Fig 1 in Ref 25 for typical separations).

Our work with CD-modified MECC is particularly significant to the assessment of the impact of fossil fuels on the environment. Separations of PAHs by conventional MECC is difficult because the hydrophobicity of these analytes causes them to associate completely with the micellar phase and co-elute at the effective retention time of the micelle.¹⁸ Organic modifiers and relatively polar surfactants provide marginal resolution in some cases.¹⁸ The use of CDs to inhibit the association of hydrophobic PAHs with the micellar phase has distinct advantages in these separations. Conditions were optimized for separations of 3-, 4-, and 5-fused ring PAHs¹⁹ and the advantages of CD-modified MECC for the accurate determination of benzo(a)pyrene (BaP) in a shale oil sample was demonstrated (see Fig 6 in Ref 19). The carcinogenic properties of substituted PAHs are often dependent on the position of substitution. We utilized the geometric specificity of

inclusion into the CD cavity to provide extremely efficient ($>10^5$ plates/m) and highly selective separations of substitutional isomers of BaP.²⁰ The type of CD (native and derivatized were used) had a strong influence on these separations (see Table 1 in Ref 20).

CD distribution capillary electrochromatography. Very recently, we have begun evaluating a new capillary electrokinetic approach which we refer to as cyclodextrin distribution capillary electrochromatography (CDCE).^{26,27} With this technique, neutral analytes are separated based on their differential distribution between a neutral CD phase, which migrates with EOF, and an electrophoretically-mediated charged CD phase. Our preliminary investigations have revealed that CDCE offers efficiency that is similar to the more established MECC technique. However, the specificity associated with solute-CD inclusion provides for unique and, more importantly, tunable selectivity (see Figs 1 & 3 in Ref 27). Moreover, the technique is robust with regard to the use of organic solvents in the running buffer and retention can be conveniently manipulated by adjusting CD phase ratio (see Table 1 & Fig 4 in Ref 27). The accompanying grant application contains a description of further studies to characterize and develop this technique.

Fundamental Studies

Band migration and dispersion in SSCE. Factors that influence selectivity and efficiency in DNA separations by SSCE were studied.²² DNA separations are complicated by the tendency of DNA to change conformation under electrophoretic conditions. Adherence to a given migration model is dependent on separation conditions. When using large MW methyl cellulose (MC) as an entangled polymer for sieving of DNA fragments, we observed Ogston model behavior for small fragments and bias reptation model behavior for large fragments. This indicates that large MW entangled polymers operate much like rigid gels in DNA separations (as these models

were developed to explain migration through gels). Plots of selectivity versus MC concentration resulted in a good correlation between calculated mesh size, calculated DNA size, and observed selectivity (see Fig 1 in Ref 22). This was not the case when small MW MC was used for sieving (see Fig 2 in Ref 22). Our results agree with the constraint-release theory of migration through entangled polymers²⁸ and indicate that the concept of entangled polymer mesh size loses its significance when the entangled polymer can rapidly relax around a larger migrating DNA fragment.

Surprisingly, comparisons of separations using large and small MW MC revealed that DNA migration rate does not depend on the macro-viscosity of the sieving medium. Conversely, efficiency was higher in high viscosity media (see Fig 3 in Ref 22). The latter observation could lead to the conclusion that axial diffusion is the principal source of band dispersion. However, static diffusion coefficients determined by using the spatial scanning LIF detector contradict this conclusion in that the values were too low to account for the observed efficiency (see Fig 3 in Ref 6). The issue of efficiency requires further study, including evaluations of dynamic diffusion coefficients. Experiments to investigate efficiency in SSCE and other modes of separation (e.g., metal ion separations using CE), using this novel instrumentation, are described in the accompanying grant application.

Band migration and dispersion in CD-modified CE/MECC. Migration in CD-modified CE is strongly influenced by CD-analyte inclusion (or more generally binding) constants. Numerous models describing the influence of binding on electrophoretic mobility have appeared in the literature. Rundlett and Armstrong discuss these models and point out that in some cases there are redundancies in the models and often they do not consider all the phenomena that impact mobility.²⁹ Nevertheless, we observed a close experimental adherence to one such model based on plots of CD concentration versus DNS-amino acid enantiomer mobility (see Eq 3, Fig 3, and Table 1 in Ref 24) and binaphthyl enantiomer mobility (see Fig 2 and Table 1 in Ref 25). Proper adjustment of

CD type and concentration was important in resolving these enantiomers. (Refer to the next section for molecular modeling of these systems.) Slow mass transfer (inclusion-disassociation) can limit efficiency in CD-modified CE. However, in the applications cited above, inclusion constants were not particularly high (< 1000) and it was anticipated that rate constants for the process would provide relatively rapid mass transfer. This is borne out by the observed high efficiencies (see electropherograms in Refs 24 & 25).

Migration and band dispersion behavior is more complicated for the mixed systems used in CD-modified MECC and CDCE. Analytes can exist in free form in the running buffer, associated with micelle, or associated with CD (and in some systems there are numerous types of CDs). Adsorption to capillary walls, non-inclusion association with the CD, and surfactant interactions with CD or analyte are possible complications as well. However, our BaP isomer separations using a γ -CD/SDS-micelle system were relatively simple. The analytes were largely insoluble in the running buffer and separations were postulated to involve a direct exchange of analyte between the CD cavity and the electrophoretically-mediated micelle. Exceptional selectivity was observed but this was greatly diminished when a derivatized CD was employed (see Table 1 in Ref 20). We originally attributed this to the greater flexibility of the derivatized CD. However, recent work with the CDCE technique indicates that the complexity of commercial derivatized CDs products (degree and position of substitution vary greatly in a given product) may be responsible for the loss of selectivity.

In the separations presented in Reference 20, very high efficiency is observed. Mass transfer of BaP isomers between micelle and CD, which had comparable phase volumes, was apparently rapid. Based on one-step sorption-desorption mass transfer calculations, exchange rates would need to be greater than 100 Hz to account for observed efficiency. The adverse effects of micelle polydispersity also were not seen, as is usually the case when surfactant concentration is well above the critical micelle concentration.² The derivatized CD used in this work was polydisperse, but all forms

migrated with EOF and, as such, did not constitute a possible mass transfer problem (such is not the case for the charged CDs employed in CDCE). Band migration and dispersion phenomena in CDCE are rather complicated. These topics are briefly discussed in Reference 27 and covered in more detail in the accompanying grant application.

Molecular modeling of CE system selectivity. Molecular modeling (MM) is a potentially useful adjunct to actual laboratory separation experiments (e.g., in validating separation mechanisms and in expediting system optimization). In a simple sense, MM utilizes empirical force fields to determine an energy, relative to a hypothetical ideal case, for a chemical system. Significant to our work involving CDs, the "interaction energy" of a analyte-CD complex can also be determined. Thus, we have attempted to correlate interaction energies, computationally-derived using SYBYL 6.0 MM software run on an Evans and Sutherland workstation, with selectivity in CD-modified CE/MECC.^{20,24,25} The implementation of MM techniques in this manner is by no means straight forward and we are relatively early in the learning curve. There are numerous considerations to be made and these are discussed in the accompanying grant application. Below we present a very brief summary of results obtained to date.

In the CD-modified MECC separation of BaP substitutional isomers,²⁰ a systematic survey of BaP-CD interaction space was used to create an interaction energy matrix. It was determined that all reasonable orientations between BaP and the CD must be considered. Because these isomers are relatively rigid, energy "minimization" was not attempted (to save computational time). No energy barriers were observed and the average of the five lowest interaction energy values correlated with elution order with only a couple exceptions (see Table 2 in Ref 20). In the CD-modified CE separation of DNS-amino acid enantiomers,²⁴ there was only one logical orientation between analyte and CD (DNS inclusion as depicted in Fig 1 of Ref 24). Small increments within the interaction space and minimization of each energy value was necessary to probe the

subtleties of enantio-selectivity. A partition function applied to the data provided correct enantiomer elution order (see Table 2 in Ref 24). In the CD-modified CE separation of selected binaphthyl (BN) enantiomers,²⁵ there appeared to be significantly different interaction mechanisms for the structurally diverse BNs. This conclusion is based on a lack of correlation between BN-CD interaction energies and separation performance for different BNs. However, there was a correlation between BN-CD interaction energy for different CDs with a given BN compound, which could prove useful in choosing CD type and separation conditions (see Table 2 in Ref 25).

Applications

PAHs and other environmental toxins. PAHs are widespread pollutants resulting from the incomplete combustion of fossil fuels. Carcinogenic properties of BaP and other PAHs are closely related to substitutional patterns (both as they exist in the environment and as a result of metabolic processes).³⁰ Sensitive and highly selective methods of analyzing environmental and biological samples for these compounds are essential. As discussed above, the high efficiency and exceptional selectivity of CD-modified MECC has made it a valuable analytical tool in the analysis of structural isomers of PAHs,^{19,20} and other environmental pollutants such as highly toxic mycotoxins.³¹ This is evidenced by the efficient separations in these references. The qualitative aspects of mycotoxin analysis and the quantitative aspects of BaP measurement were among the analytical applications highlighted in this work (see Tables I & II in Ref 31 and Fig 6 in Ref 19, respectively). Future work in the area of organic pollutant analysis will involve MM assisted development of the aforementioned CDCE technique (see the accompanying grant application).

DNA restriction digests. Rapid SSCE separations of ϕ X-174-HeaIII,⁵ pBR322,²² and pBR328²² restriction digests were accomplished (see Fig 2 in Ref 5 and Fig 6 in Ref 22 for 2-3 minute separations). This work was performed largely to determine the upper

limits of analysis speed and what experimental factors impose those limits. Information involving the optimization of running buffer conditions (e.g., entangled polymer size and concentration) and instrumental factors (e.g., applied fields, capillary diameter, gradients and detection scanning) in these restriction digest separations should also apply to sequencing. In addition to fundamental studies of SSCE, future studies will include an assessment of CE for performing gel retardation analyses to study protein-DNA interactions (see accompanying grant application).

Heavy metals. Lanthanide metal ion separations based on on-column complexation with α -hydroxyisobutyric acid and indirect detection employing displacable imidazoles were performed.¹⁶ More recently, a CE protocol was developed (see above) that permitted the quick and highly selective determination of 10 ppb - 10 ppm levels of uranyl ion in ground waters.¹⁷ Future studies will include developing CE-based methodologies for other significant metals and developing field-ready instrumentation to perform such analyses.

Chiral compounds. Enantio-selective CD inclusion was used to separate the optical isomers of DNA-amino acids²⁴ and binaphthyl compounds.²⁵ This research could have significance in the development of methods to separate chiral geological biomarkers and chiral PAHs. Moreover, information gained from these studies should be useful in understanding factors that influence the separation and proper molecular modeling of other achiral isomeric separations.

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