INVESTIGATIONS INTO POLYMER AND CARBON NANOMATERIAL SEPARATIONS

DISSERTATION

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By

Cherie Nicole Owens, B.S.

Graduate Program in Chemistry

The Ohio State University

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Dissertation Committee:

Dr. Susan V. Olesik- Advisor

Dr. Prabir K. Dutta

Dr. Anne Co
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ABSTRACT

The work of this thesis follows a common theme of research focused on innovative separation science. Polyhydroxyalkanoates are biodegradable polyesters produced by bacteria that can have a wide distribution in molecular weight and monomer composition. This large distribution often leads to unpredictable physical properties making commercial applications challenging. To improve polymer homogeneity and obtain samples with a clear set of physical characteristics, poly-3-hydroxyvalerate-co-3-hydroxybutyrate copolymers were fractionated using gradient polymer elution chromatography (GPEC) with carefully optimized gradients. The resulting fractions were analyzed using Size Exclusion Chromatography (SEC) and NMR. As the percentage of “good” solvent was increased in the mobile phase, the polymers eluted with decreasing percentage of 3-hydroxyvalerate and increasing molecular weight, which indicates the importance of precipitation/redissolution in the separation. As such, GPEC is an excellent choice to provide polyhydroxyalkanoate samples with a narrower distribution in composition than the original bulk copolymer. Additionally, the critical condition was found for 3-hydroxybutyrate to erase its effects on retention of the copolymer. Copolymer samples were then separated using Liquid Chromatography at the Critical Condition (LCCC) and it was determined that poly(3-hydroxvalerate-co-3-hydroxybutyrate) is a statistically random copolymer.
The second project uses ultra-thin layer chromatography (UTLC) to study the performance and behavior of polyhydroxybutyrate (P3HB) as a chromatographic substrate. One specific polyhydroxyalkanoate, polyhydroxybutyrate, is a liquid crystalline polymer that can be electrospun. Electrospinning involves the formation of nanofibers through the application of an electric potential to a polymer solution. Precisely controlled optimization of electrospinning parameters was conducted to achieve the smallest diameter PHA nanofibers to date to utilize as novel UTLC substrates. Additionally, aligned electrospun UTLC (AE-UTLC) substrates were developed to compare to the randomly oriented electrospun (E-UTLC) devices. The PHB plates were compared to commercially available substrates for the separation of biological samples: nucleotides and steroids. The electrospun substrates show lower band broadening and higher reproducibility in a smaller development distance than commercially available TLC plates, conserving both resources and time. The AE-UTLC plates provided further enhancement of reproducibility and development time compared to E-UTLC plates. Thus, the P3HB E-UTLC phases are an excellent sustainable option for TLC as they are biodegradable and perform better than commercial phases.

A third topic of interest is the study of ordered carbon nanomaterials. The typical amorphous carbon used as a stationary phase in Hypercarb® is known to consist of basal- and edge-plane oriented sites. This heterogeneity of the stationary phase can lead to peak broadening that may be improved by using homogeneous carbon throughout. Amorphous, basal-plane, and edge-plane carbons were produced in-house through membrane template synthesis. Amorphous, basal-plane, and edge-plane carbons were
then used separately as chromatographic phases in capillary electrochromatography (CEC). Differences in chromatographic performance between these species were assessed by modeling retention data for test solutes to determine Linear Solvation Energy Relationships (LSER). The LSER study for the three carbon phases indicates that the main difference is in the polarizability, and hydrogen bonding character of the surface leading to unique solute interactions. These results highlight the possible usefulness of using these phases independently.
DEDICATION

To my family
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I would like to acknowledge all of the individuals who have helped to lead me to my goal of a PhD in chemistry. Firstly, I would like to thank my parents for instilling in me the importance of academics. From kindergarten until today they have convinced me that every day, assignment, and test counts, pushing me to always to my best. Their never-ending support and understanding has also been a safe haven when I felt overwhelmed. Without the foundation that they laid for me, I would certainly not be here today.

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VITA

May 2003 .................................................................McKinley Sr High School
2007.................................................................B.S. Chemistry, University of Akron
2007-2008 .........................................................Graduate Teaching Associate, The Ohio State University
2008 to present ...............................................Graduate Research Associate, Department of Chemistry, The Ohio State University

PUBLICATIONS

Pomeranz, Cherie N.; Olesik, Susan, V. “Separation of poly-3-hydroxyvalerate-co-3-hydroxybutyrate through gradient polymer elution chromatography” J. Chromatogr. A 2011, 1218, 7943-7947.

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Major Field: Chemistry
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CHAPTER 1

BACKGROUND TO PERTINENT SEPARATION TECHNIQUES: HPLC OF POLYMERS, ULTRA-THIN LAYER CHROMATOGRAPHY, CAPILLARY ELECTROCHROMATOGRAPHY

1.1 Introduction to solving problems with polymer commercialization using separation techniques

In the polymer industry, much thought and effort goes into the commercialization of their products. Creating a new polymer of interest can come about in many different ways. Polymer synthesis chemists can formulate new products through new pathways or reactants. There may be a drive toward utilizing wastes from other polymer syntheses or recycling already used products. Or perhaps the polymers are simply discovered as inclusions in organisms. Whichever the pathway toward discovery, much characterization must be done on the new product to be certain of its material properties.
Additionally, depending on the synthetic pathway, most batches of polymers are not homogeneous but instead contain many substituents ranging from leftover reactants to secondary products or even contaminants. Polydispersivity, or the range of polymer characteristics present in a given sample, can be quite problematic for polymers. Frequently, polymers are polydisperse in molecular weight, copolymer composition, end group functionalities, branching distribution, block length, etc based upon the polymer in question.\(^1\) When examining these challenges that face the polymer industry it becomes obvious that separation techniques, which examine specific polymer characteristics, are indispensible toward characterization.

The separation technique that is most commonly used to study a polymer system is size exclusion chromatography (SEC). SEC is a variant of high performance liquid chromatography (HPLC) that is applied to polymers to determine their molecular weights. An HPLC column is packed with porous particles with a surface that is not expected to strongly interact with the samples as they are traversing the column, i.e. no adsorption or partitioning with the stationary phase is desired.\(^2\) Because the packed particles have a range of pore sizes, an intricate flow network is produced as depicted in Figure 1.1. This pore network is available to species with a smaller hydrodynamic volume, or molecular weight, which will cause them to have longer migration times than for larger molecular weight polymers.
Figure 1.1 Diagram of the separation mechanism for size exclusion chromatography. As indicated, larger molecules (higher molecular weight) elute more quickly than smaller molecules that have access to the intricate pore network.
In fact, for all size exclusion columns there is a range of pore sizes present so that a linear region exists where molecular weight correlates inversely with migration time. However, there is of course a cutoff where once a polymer reaches a critical size it will be completely excluded from the pore network. Likewise, there is also a cutoff in the low mass region where all of the pores become accessible once the polymers reach a critical small size. Due to these three size regions, a plot of migration time versus the logarithm of the molecular weight takes on the characteristic S-shape shown in Figure 1.2. The bottom half of Figure 1.2 shows the relationships that these exclusion zones and linear regions represent toward the pore volumes of the packed particles and column. Here, $V_o$ represents the interstitial, or void, volume of the column, which is represented by the volume of the analytes that are fully excluded from the pores of the packing. Conversely, $V_i$ represents the volume of the packed particle pores, and $V_t$ is the total column volume represented by equation 1.1.

$$V_t = V_o + V_i$$  \hspace{1cm} (1.1)
Figure 1.2 Characteristic S-shaped calibration curve of retention volume (mL) versus molecular weights of polystyrene standards subjected to size exclusion chromatography analysis. The dotted lines indicate the information about the pore volumes of the column ($V_o$ interstitial void volume, $V_i$ pore volume of packed particles, $V_t$ total column pore volume) that can be obtained from such an analysis.
The calibration curve for an SEC column is determined by using polymer standards. The samples that will have their molecular weights determined from this curve should lie in the linear region, between $V_0$ and $V_t$, to ensure the lowest error in the calculation. It is best to use low polydisperse polymer standards of the polymers that will have molecular weight determined from this calibration, i.e. if a polymethylmethacrylate (PMMA) polymer will have molecular weight determined, PMMA standards should be used to formulate the calibration curve. This is important since the migration time depends on the hydrodynamic volume of the polymer, which is related to molecular weight for a given homologous series but differs for polymers with different branching and geometries. However, very often this is not possible, and polystyrene standards are used instead to obtain “polystyrene equivalent” molecular weights. These curves are also dependent on the mobile phase composition, operating temperatures, and flow rates so these parameters must also be consistent across calibration and application.

One of the most important factors for size exclusion chromatography is the elimination of enthalpic interactions with the stationary phase. The distribution coefficient, $K$, is a parameter that describes the degree of interaction an analyte has with a stationary phase as it is traversing a column. Under dilute solution conditions, $K$ is related to Gibbs Free Energy ($\Delta G$) by the following equation:

$$\Delta G = -RT \ln (K) \hspace{1cm} (1.2)$$
where \( R \) is the universal gas constant and \( T \) is the temperature of the system. Thus the distribution coefficient is dependent upon enthalpic (\( \Delta H \)) and entropic (\( \Delta S \)) interactions by the relationship with \( \Delta G \) and equation 1.3:

\[
\Delta G = \Delta H - T\Delta S
\]  

Since the SEC mechanism is based upon molecules having access to different pore pathways based upon size, it follows that \( \Delta S \) is the dominant term and that \( \Delta H \) should be minimized. The major contribution to \( \Delta H \) would be adsorption/desorption interactions between functionalities on the stationary phase and the analytes to be separated. As such it is very important to use a stationary phase not expected to strongly interact with the majority of polymer samples. A second contribution to \( \Delta H \) would also be the influence of any precipitation/redissolution phenomena so it is important to use a good solvent for the polymer samples throughout SEC characterization. Keeping the contribution of \( \Delta H \) low is important to have a separation truly based upon size.

Conversely to SEC, separations with a strong influence of \( \Delta H \) compared to \( \Delta S \) show increasing retention times for increasing molecular weights, and this is referred to as interaction chromatography.\(^4\) The larger the polymer, the more functionalities that will be present to interact with the stationary phase and increase retention time. A very
An important separation technique specific to polymer systems that relies on an enthalpic mechanism is gradient polymer elution chromatography (GPEC). In GPEC, a polymer solution is injected onto an HPLC column with a mobile phase that will force adsorption of the sample onto the stationary phase. As previously stated, polymers very often dissolve in only a few solvents, so it is common that this mobile phase may also cause precipitation of the sample at the head of the column. Once the sample is injected, the mobile phase is slowly changed from the adsorbing (or often poorly solvating) mobile phase to a mobile phase that is a good solvent for the polymer. This gradient will cause the polymer sample to partition due to differing solubilities and/or differing degrees of adsorption and interaction with the stationary phase. This procedure is depicted in Figure 1.3. As previously stated, there is often a spread in polymer characteristics, or a polydispersivity, of the sample in polymer applications. This polydispersivity can be in molecular weight, percentage composition, end group composition, branching number, or any other side products of the polymerization reaction. As long as these traits change the polymer solubility or the level of interaction with a stationary phase, GPEC should be able to partition the sample due to composition distribution.
Figure 1.3. Diagram depicting the process of polymer separation in gradient polymer elution chromatography.
If one were to plot the two retention time versus molecular weight relationships for SEC and adsorption/desorption, or interaction, chromatography, it would become obvious that a point could exist between these two modes where retention time is independent of molecular weight, or \( \Delta G = 0 \). At this point the enthalpic adsorption effects balance the entropic size exclusion effects for analytes and retention time for a given polymer is independent of molecular weight.\(^6\) This relationship, referred to as the critical condition, is shown in Figure 1.4.
Figure 1.4. Plot of retention time versus molar mass for differing mobile phases representing the transition between size exclusion chromatography and interaction chromatography modes, with the critical condition shown in the center.
The critical condition is a very useful parameter to have for a polymer to in effect “erase” the contribution of a monomer toward retention. For example, if you have a copolymer composed of A and B block units and operate at the critical condition for monomer A you would achieve retention of the copolymer based upon the monomer B exclusively. Using chromatography at the critical condition would then be a useful way to separate a set of polymers by differing block lengths, branching, end groups, etc.

Achieving the critical condition for a given monomer is a simple approach in theory but can be difficult to achieve in practice. First, it is necessary to find a mobile phase that will minimize adsorption onto the stationary phase and will allow the polymer sample to behave in a size exclusion mechanism. Next, it is necessary to find a mobile phase that will force adsorption of the polymer onto the stationary phase. Due to the limited solubility of most polymers, this very often is a non-solvent for the polymer. Finding the critical condition is then a balance between these two mobile phases, producing the characteristic fan-shaped diagrams as shown in Figure 1.4.

1.2 Introduction to Ultra-Thin Layer Chromatography and Electrospinning

Thin layer chromatography (TLC) involves separation of compounds on a planar substrate and is still indispensable today. TLC is the go-to technique for organic chemists
or pharmaceutical development to observe if a sample has impurities without having to use expensive equipment or a large amount of sample or solvent. TLC involves applying the sample to be separated by capillary near the base of a cut “plate” which it typically 3cm x 7 cm. The plate has a composition normally of silica microparticles, but they can be functionalized to give cyano groups, amide groups, or carbon chains depending on the desired interactions. The stationary phase is attached to a rigid backing for support, typically stainless steel or glass, by use of a binder. Once the sample spot has dried on the plate, one edge is exposed to a developing solution that flows up the plate via capillary action. This developing solution, or mobile phase, carries the sample up the plate a distance depending on how well the sample interacts with the stationary phase, as well as the solvating properties of the mobile phase for the sample.

While this technique is still used fairly often, it does suffer from pitfalls, which if corrected could make this a much more attractive technique. The most important pitfall is the low efficiency of the separations. In chromatography, efficiency (N) is a term that is related to the broadening of a sample band through the separation process. Samples that greatly broaden during a separation have lower resolutions (a parameter related to the degree of separation between the analytes), and lower limits of detection than samples that stay in a tight band. Efficiencies in TLC have been improved using many of the techniques that improved efficiency for liquid chromatography and gas chromatography.
Decreasing the particle sizes of the substrate leads to higher efficiencies. This can be realized by analysis of the Knox equation discussed in Chapter 3.

One way to obtain smaller particle sizes for the substrate is to produce a nanofiber woven mat through the process of electrospinning. Electrospinning is a technique in which a voltage is applied to a polymeric solution causing the surface to eject nanofibers to be collected at a grounded substrate. A typical apparatus is shown in Figure 1.5. The dissolved polymer solution is placed in a syringe with a conductive tip. The syringe is pumped at a steady flow rate while a voltage is applied. At a critical voltage, the surface tension of the droplet of polymer solution at the tip is overcome by the charge at the surface forming a Taylor cone with a continuous nanofiber splaying from it.
Figure 1.5. Typical apparatus for electrospinning polymer nanofibers onto a flat, grounded collector.
Many parameters must be optimized to obtain nanofibers with good reproducibility and morphology. The concentration of the polymer in solution must have high enough viscosity to allow chain entanglement between the polymers during spinning for a continuous nanofiber to be formed. The polymer molecular weight must be high enough for this entanglement as well.\textsuperscript{12} Additionally the solvent used to dissolve the polymer should also be carefully chosen so the solution has a relatively good dielectric constant to properly conduct the voltage applied. It is possible to alter the solution dielectric constants through additives so long as the polymer stays dissolved in solution. Other parameters that also affect fiber morphology and size are voltage, flow rate, distance between tip and collector, and even ambient humidity.\textsuperscript{12} All of these parameters must be optimized to have a stationary phase which is as homogeneous as possible to reduce band broadening from the multipath flow term, or the A term. Additionally, the parameters should be optimized to have the smallest diameter nanofibers as possible to reduce the effective particle size of the stationary phase as well reducing both the A and C terms.

One of the most attractive attributes of using electrospun polymer stationary phases is the ability to change the substrate chemistry by changing the polymer type. There only exist a few options for commercially available TLC plates, such as silica, cyano, octadecyl polysiloxane (C\textsubscript{18}), and amide to name a few. However, as long as electrospinning conditions can be developed to produce low diameter, low dispersion
nanofibers, nearly any polymer could be used as a chromatographic substrate. This would allow a practitioner to tailor the substrate chemistry to the separation of interest providing more selective separations than would be possible when limited to those that are commercially available.

1.3 Introduction to Capillary Electrochromatography

Capillary electrochromatography (CEC) is a separation technique that is quite similar to a typical capillary electrophoresis (CE) experiment. In CE, a potential is applied to a fused silica capillary that induces electroosmotic flow of the buffer contained within. CE separates analytes due to their mass-to-charge ratio differences in this electric field. CEC follows the same procedure as a CE experiment except analytes are also able to partition with a chromatographic phase during this electrophoretic migration as well. This combines the efficiency gains of capillary electrophoresis, due to the plug flow rather than laminar flow, with the selectivity gains of chromatography. Capillaries can be packed with particles for the chromatographic phase, or the phase can flow through with the electroosmotic flow.

The chromatographic phase that is flowing freely with the electroosmotic flow is referred to as a pseudostationary phase (PSP) because the particles are not stationary.
Using a PSP can be desirable since it does not require packing a column or the use of frits which are known to cause bubble formation in the mobile phase. Additionally, fouling the stationary phase from biological or dirty samples is not an issue since the chromatographic phase is replenished with every run. PSPs can be introduced continuously by including them in the run buffer; however, this often causes problems with analyte detection. Thus a partial filling technique can be more desirable if only a small amount of PSP is needed to separate the samples of interest.

Many types of PSPs have been used in CEC to enhance the separation of a set of analytes. The most common PSP is a micelle, and when this is used the separation is referred to as micellar electrokinetic chromatography (MEKC). Various micelles have been used to separate analytes such as surfactants and phospholipids, but the major disadvantage of using these types of PSPs is that they exist only under specific equilibrium conditions, and as such have a much lower range of buffers and pH values under which they would be stable and useable. Having the ability to use many analytical conditions to enhance a separation is vital, and the specific equilibria under which these micelles self-assemble greatly limits the usefulness of MEKC.

Another type of PSP that has been used consists of nanoparticles. Some of these nanoparticle PSPs have been composed of dendrimers, silica, carbon, and gold to name a few. Nanoparticle PSPs avoid the problem of needing specific conditions for use and also can be more compatible with electrospray ionization (ESI) mass spectrometry.
Nanoparticle PSPs have been used in a wide variety of separations ranging from biological analytes to polyaromatic hydrocarbons, acids, and catechols.\textsuperscript{18}

The Olesik group has specific interest in the carbon PSPs due to our studies with the chromatographic performance of amorphous carbon. A typical amorphous carbon structure, as shown in Figure 1.6, is composed of graphene sheets that stack and form the intricate ribbon structure.\textsuperscript{19} When studied closely, it becomes apparent that the amorphous carbon structure is heterogeneous and contains two sites for analytes to interact with: basal- and edge-oriented graphene sheet sites. Basal-oriented graphene sites arise from only the fronts of the graphene sheets being exposed for analyte interactions, whereas edge-oriented sites arise when only the stacked edges of graphene sheets at the end of a ribbon are exposed. This heterogeneity could lead to band broadening due to interactions with both sites, which ultimately could reduce resolution during a separation. The selectivity of the chromatographic phase can be expected to increase by employing each of these phases independently. In fact, this is a topic in not only this dissertation but also in Joseph Zewe’s dissertation (in press). He found differing solid phase extraction performances for basal, edge, and amorphous carbon sorbents for a set of polar and nonpolar analytes. Most notably he found that edge plane oriented carbon better extracted polar analytes than the basal plane oriented carbon. These results point toward edge plane oriented carbon being a more polar phase than basal plane oriented carbon.
Figure 1.6. Intricate ribbon structure of amorphous (glassy) carbon. (Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Jenkins, G.; Kawamura, M. Nature 1971, 231, 175-176), copyright (1971)).
1.4 Research Focus

The work described in this document details investigations into polyhydroxalkanoate (PHA) batch compositions, marked improvement in TLC separations using PHA substrates, and a chromatographic study of the performance differences of graphitic carbons due to changes in molecular orientation. Biodegradable PHA polymers have had trouble in commercialization due to the large spread in physical characteristics in each sample. Chapter 2 describes the analysis of these samples using GPEC with carefully optimized gradients followed by SEC and NMR to characterize the samples. Additionally the critical point for a PHA monomer was carefully determined and applied to the samples for further characterization. Chapter 3 illustrates the lowest diameter nanofibers for PHA to date by a thorough investigation into the electrospinning parameters and then further use of these PHA nanofibers as a substrate for TLC. This is the first use of PHAs as a chromatographic substrate. Additionally, the PHAs were also used to fabricate the first aligned electrospun PHA TLC substrates for comparison. Chapter 4 describes a more fundamental chromatographic study of how a specific carbon chromatographic phase interacts with analytes depending on the molecular orientation of the graphene sheets. The combination of these works results in a clearer understanding in the composition of biodegradable polymers for a better route to commercialization, a novel biodegradable stationary phase for TLC leading to more sustainable separation
science, and a clearer understanding of ordered carbon nanomaterials behavioral differences as stationary phases.
References


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2.1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable linear copolyesters synthesized by a wide variety of bacteria through fermentation of carbon feedstocks, such as sugars, lipids, alkanes, alkenes, and carboxylic acids. Many types of bacteria, including *Pseudomonas* and *Ralstonia eutropha*, have been shown to store PHAs as a carbon source during situations in which carbon feedstocks are abundant but other nutrients, such as nitrogen, are limiting. These biopolymers are found as cytoplasmic inclusions and can account for as much as 70-80% of the dry weight of the bacteria. The bacteria can then later use the PHAs as a carbon source when feedstocks become limited. This
process can be likened to how mammals store adipose tissue to be used at times when nutrients are limiting.

Due to their inherent biodegradability, PHAs are attractive alternatives to petrochemical plastics, which show low degradation rates and as such continue to add to the growing landfill problem. PHAs are currently used in a broad range of applications including food packaging and coatings for beverage bottles. In addition, PHAs show a low inflammatory response making them viable for use in tissue engineering and wound healing applications.²

While over 150 monomers have been produced from different carbon feedstocks for the production of the large family of PHAs, poly-3-hydroxybutyrate P(3HB) and its copolymers remain the most commonly used PHAs.⁴ The structures of the 3HB and 3-hydroxyvalerate (3HV) monomers are shown in Figure 2.1.
Figure 2.1 Chemical structures of 3HB and 3HV monomers.
The type of PHA produced by the bacteria is dependent on the bacteria chosen and the feedstocks provided. For example, when *Ralstonia eutropha* is supplied with glucose it preferentially stores poly-3-hydroxybutyrate (P(3HB)), but when propionic acid is included in the feedstock a copolymer consisting of P(3HB)-co-(3HV) is manufactured.\textsuperscript{2,3} The ratio of propionic acid to glucose supplied correlates with the ratio of 3HV incorporated into P(3HB) backbone. Because the immediate surrounding environment of the bacteria is slightly changing as the feedstocks are consumed, a range of 3HV percentages of copolymers is synthesized causing a sample with a broad distribution. This spread in composition is a significant problem as the physical characteristics of the P(3HB)-co(3HV) copolymer are highly dependent on the percent 3HV contained. For example, pure P(3HB) is a crystalline polymer that exhibits stiff and brittle physical characteristics.\textsuperscript{4} Incorporating 3HV into the P(3HB) backbone increases the strength of the polymer and also alters other characteristics such as the thermal properties.\textsuperscript{5} Figure 2.2 shows how several physical characteristics of the copolymer change with varying the percentage of 3HV.\textsuperscript{6} The linear change in $T_g$ is due to the increasing segmental mobility for the copolymer with increasing the longer side chain percentage.\textsuperscript{6} The $T_m$ and $\Delta H_f$ have a U shaped relationship that reflects the highest crystalline order for 100% 3HV or 3HB with a minimum crystalline strength when the two are mixed due to the mismatch in monomer sterics.\textsuperscript{6} These relationships in Figure 2.2 highlight how the percentage of 3HV in the copolymer changes its physical properties.
Figure 2.2 Plots of changing physical characteristics (melting temperature, heat of fusion, and glass transition temperature) versus content of second monomer in PHA copolymer’s; ■: P(3HB), ○: P(3HB-co-3HV), ●: P(3HB-co-3HP), ▼: P(3HB-co-3HHx), ◇: P(3HB-co-4HB). (Reprinted by permission from John Wiley and Sons Inc: [Macromolecular Symposia] (Ishida, K.; Asakawa, N.; Inoue, Y. Macromol. Symp. 2005, 224, 47-57.), copyright (2005)).
Because of the synthetic pathway, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters have been shown to be mixtures of random copolyesters with a wide distribution of comonomer composition and molecular weight. This variance in copolymer composition is especially problematic for commercial applications since physical characteristics such as mechanical strength, melting temperature, glass transition temperature, and degradation temperature vary according to the percentage of 3HV in the copolymer. The separation of these diverse batches and subsequent characterization of the PHA copolymers is therefore of great importance for utilizing PHAs in commercial applications.

PHAs are typically characterized by subjecting the polymer to partial degradation followed by analysis with gas chromatography coupled with mass spectrometry, GC/MS. A typical mass spectrum of the degraded copolymer is shown in Figure 2.3. However, this does not provide information about the molecular weight spread or the distribution in copolymer content that may be present in the original polymer sample. Additionally, the degradation process may not provide a true copolymer distribution since degradation may not necessarily occur randomly across the backbone but may be preferentially cleaved at certain sites. Lastly, this technique cannot be scaled up for commercial production purposes, which will be very important for the future applications of PHAs. Accordingly, a separation technique is of utmost importance to provide more homogeneous samples through more finite composition distributions.
Figure 2.3 Typical mass spectrum of partially degraded P(3HB-co3HV) copolymer after GC separation. (Reprinted by permission from John Wiley and Sons Inc: [Macromolecular Symposia] (Adamus, G. Macromol. Symp. 2006, 239, 77-83.), copyright (2006)).
Alternatively, characterization of the intact PHV-co-PHB copolymer has been achieved by solubility differences of the many copolymers present in the bulk sample.\textsuperscript{11} The copolymer was precipitated as a function of percentage of 3HV as well as molecular weight by addition of differing proportions of n-heptane to a solution of the copolymer dissolved in chloroform.\textsuperscript{11} This process was able to provide more homogenous copolymer samples but was a time consuming and laborious technique including multiple titration and centrifugation steps as well as twenty-four hour solution equilibration times. To provide a PHA sample with a narrow range of thermal and tensile strength properties, a separation technique that is able to divide the sample into more homogeneous constituents in a facile and automated fashion is needed.

Gradient Polymer Elution Chromatography (GPEC), originally introduced in 1979 by Teramachi \textit{et al.} for the separation of styrene-methyl acrylate copolymers, is a technique commonly used to separate polymer samples into smaller, more homogenous fractions.\textsuperscript{12} In GPEC, a dissolved polymer is injected onto an HPLC column containing a mobile phase that will force the polymer to adsorb onto the stationary phase. Very often, this can cause the polymer to precipitate at the head of the column since polymers inherently have a small range of solvating conditions. Then the polymer is slowly re-dissolved (and/or desorbed from the stationary phase) and separated on the HPLC column by increasing the proportion of good solvent in the mobile phase until there is no longer any non-solvent present.\textsuperscript{13} By using GPEC, a complex polymer sample can be separated
due to differences in solubility and/or interactions with the stationary phase; this process generates fractions with a lower spread in physical characteristics than in the original bulk sample. For example, Glöckner et al. demonstrated the fractionation of poly(styrene-co-acrylonitrile) copolymers by GPEC showing a separation dependent on both the molecular weight as well as the percentage of acrylonitrile in the copolymer.\textsuperscript{14} Interestingly, the retention order was independent of the stationary phase used, i.e. normal- or reversed-phase columns that indicates that GPEC in this case was dominated by a precipitation/redissolution mechanism.\textsuperscript{14} Other chromatographic conditions may lead to a greater influence of the stationary phase on the separation during GPEC by enhancing the adsorptive interactions with a given stationary phase. Macko and Pasch separated linear polyolefins from isotactic, atactic, and syndiotactic polypropylene through differences in enthalpic interactions with the stationary phase.\textsuperscript{15}

Liquid chromatography at the critical condition (LCCC) is also a useful technique to separate polymer samples. In LCCC the entropic energy from a size exclusion mechanism is balanced by the enthalpic energies associated with adsorption/interaction chromatography so that the portion of the polymer is chromatographically “invisible”.\textsuperscript{16,17} Under the critical conditions, the homopolymer elutes with the total volume of the column regardless of its molecular weight.\textsuperscript{17} This critical point is found by starting with a mobile phase that encourages a size exclusion mechanism for a series of homopolymers of different molecular weights and then adding a non-solvent to the mobile phase until
the samples coelute.\textsuperscript{18,19} Unfortunately, higher molecular weight polymers may experience precipitation before the critical point is reached.\textsuperscript{18,20} One advantage of using LCCC over GPEC is its ability to separate polymer samples isocratically, which removes the burden of column re-equilibration between runs and saves time.\textsuperscript{17}

LCCC has been shown to be quite useful in separating polymer samples in fashions unrelated to molecular weight. Steric exclusion effects for one part of the polymer become excluded so that another part of the polymer is alone evaluated under size-exclusion mode.\textsuperscript{17} This makes it possible to separate complex samples by various attributes. Differing block length of the monomers that are not under critical condition can separate blocky copolymers.\textsuperscript{17,18,21} Also, polymers can be separated by functionality differences if the rest of the polymer is chromatographically invisible.\textsuperscript{18,20} Polymers can even be separated by shape differences, as shown in the separation of linear and star copolymers and star copolymers by differing numbers of arms present although they have similar molar masses and hydrodynamic volumes.\textsuperscript{16,21} In summary, polymers with a distribution in block length, functionalities, and shape can be separated with LCCC provided a critical condition exists for a portion of the polymer and the molecular weight is low enough to avoid precipitation.

This chapter describes the first separation of 3HB-co-3HV copolymers by GPEC as well as LCCC. This was achieved by applying GPEC separately to three copolymer samples: P(3HB-co-8 mol\% 3HV), P(3HB-co-12 mol\% 3HV), and P(3HB-co-21 mol\%
Additionally, the critical condition for 3-hydroxybutyrate was developed and applied to the copolymers as well. The average comonomer percentage and molecular weight of the resulting fractions were studied using Nuclear Magnetic Resonance (NMR) and Size Exclusion Chromatography (SEC), respectively.

2.2. Experimental

2.2.1 Materials & Chemicals

Poly(3-hydroxyvalerate-co-3-hydroxybutyrate) (8 mol%, 12 mol%, and 21 mol% HV) copolymer samples were obtained from Metabolix Incorporated, Cambridge, MA. Chloroform with 1% ethanol stabilizer for liquid chromatography was obtained from OmniSolv. HPLC grade reagent ethanol, acetone ACS grade and 2-Propanol ACS grade were from Fisher Scientific. Chloroform-D 100% in 0.75mL one time use vials was obtained from Cambridge Isotope Laboratories Incorporated. The vials used in the autosampler were 1.5mL crimp top glass vials with 1mm crimp seals purchased from National Scientific. The vials used in the fraction collector were 13 x 100 mm Durex™ borosilicate glass culture tubes purchased through VWR International.
2.2.2 Instrumentation

The HPLC system consists of two LC-20AT pumps, a SIL-20A auto-sampler, and a CTO-20A oven with a CBM-20A controlling module, all obtained from Shimadzu Corporation. The detector was an evaporative light scattering detector (ELSD), model 380-LC, obtained from Varian Incorporated. For all HPLC experiments, the ELSD settings were held constant with a nebulizer temperature of 40°C, an evaporation temperature of 62°C, and a gas flow rate of 3.00 standard liters per minute (SLM). The fraction collector was a model FC204 obtained from Gilson Incorporated. The column used for GPEC was a silica gel C<sub>18</sub>, 150 x 4.6 mm I.D., 100 Å, with particle diameter of 5μm, column from Kromasil; and the column used for size exclusion chromatography (SEC) and critical chromatography was a polydivinylbenzene, 300 x 7.5 mm I.D., 10<sup>5</sup> Å, with particle diameter of 5μm, column from PL Gel with an exclusion limit up to 2,000,000 g/mol. The column used to study the effect of the stationary phase composition on the GPEC separations was a silica gel NH<sub>2</sub>, 150 x 4.6 mm I.D., 100 Å, with particle diameter of 5μm, column from Supelco. All solutions were mixed and degassed using a Branson 2210 Ultrasonic cleaner. All <sup>1</sup>H NMR data were collected using a DPX 400 MHz instrument from Bruker.
2.2.3 Gradient Polymer Elution Chromatography

To prepare the copolymers for injection, 25 mg of a copolymer was dissolved in 1.5 mL of chloroform under ultrasonication. Injection sizes for GPEC runs were between 5 and 10 µL for the different polymers. GPEC was performed by transitioning from highly non-solvating conditions (high proportions of ethanol) to more solvating conditions (high proportions of chloroform) throughout a run while maintaining a flow rate of 1 mL/min. Step gradients were optimized individually for each copolymer sample balancing resolution with time, to obtain three (or more) fractions, while the column was kept at 25°C. After gradients were established, the solution was sent to the fraction collector where the partitioned sample was collected. The mobile phase in each fraction was evaporated at 60°C and then the fractions were re-collected. This sequence was repeated 50 times to attain appreciable concentrations of each fraction for subsequent characterization. The samples were then dissolved in 1.5 mL of chloroform and transferred to auto-sample vials for SEC.

To study the influence of the column on the separation, P(3HB-co-21 mol% 3HV) was separated on a normal phase column using the gradient program developed for the reversed phase column. All instrument parameters were identical to the reversed phase column procedure with the exception of the column choice.
2.2.4 Liquid Chromatography at the Critical Condition

To acquire 3HB homopolymer standards for the determination of the critical point, P3HB was subjected to thermal treatment at 190°C for multiple times: 15, and 30 minutes, and 1, 2, 3, 4, 5, and 6 hours. This causes the P3HB sample to degrade to lower molecular weights that were determined through SEC analysis.

The thermally treated P3HB samples were used to determine the critical condition for 3HB by subjecting each to analysis on the SEC column while using different isocratic mobile phases. Initial isocratic mobile phase conditions included a good solvent for P3HB (100% chloroform). Subsequent mobile phases contained portions of non-solvents to vary the solvent strength. Several non-solvents used included ethanol, ethanol:isopropanol, and acetone until the different molecular weight P3HB samples co-eluted. The critical condition was then applied to the three P(3HV-co-3HB) copolymers.

2.2.5 Analysis of fractions

Aliquots (50 μL) of the fractions were injected onto the SEC column. The mobile phase was chloroform with a flow rate of 1 mL/min. The column was maintained at 55°C throughout the SEC analysis. Detection was performed with the ELSD. A
calibration curve using polystyrene standards was used to determine the polystyrene equivalent molecular weights. This calibration curve was constructed by plotting the retention times of ten polystyrene standards with molecular weights of 5,120; 18,700; 29,300; 44,100; 63,000; 114,200; 212,400; 382,100; 1,000,000; 2,316,000 against the log of their molecular weights. Each of these data points was obtained in triplicate. The extrapolated equation from the best fit line of the linear region is what was used to determine the molecular weights of the poly(3HB-co-3HV) samples and fractions by using their retention times on the same SEC column, also conducted in triplicate. The remaining portion of each fraction was then transferred to NMR tubes, the solvent was evaporated at 60°C, and the samples were dissolved in deuterated chloroform for ¹H NMR.

2.2.6 Calibration of ELSD Response

To ensure that the ELSD is operating under linear conditions for response related to polymer concentration, a series of runs to form calibration curves were performed. 21 %3HV-co-3HB was prepared at 3, 5, 10, and 20 mg/mL. The reversed phase column used for GPEC was utilized at three different mobile phase conditions, 100% chloroform, 80:20 chloroform:ethanol, and 60:40 chloroform:ethanol, to replicate the mobile phase
variance experienced during a GPEC run. Integrated peak area was then plotted as a function of both polymer concentration as well as mobile phase composition to observe the linearity of the ELSD response.

2.3. Results & Discussion

The copolymer samples were fractionated by GPEC. Gradients were chosen by first conducting a linear gradient from 10:90 chloroform:ethanol to 100 percent chloroform in 15 minutes. Pure ethanol was not chosen as the initial gradient condition as this causes operating pressures far above the range tolerable by the reversed phase HPLC column due to the solid plug of precipitated polymer formed upon injection. The initial steep gradient (from 90% to 60% ethanol in 3 minutes or less) arose from moving from the initial column conditions to a point where polymer elution begins as quickly as possible. This initial fast step in the gradient helps to ensure retention by starting highly nonsolvating, but also avoids the notorious “breakthrough peak” observed by many when initial mobile phase conditions begin strongly non-solvating.\textsuperscript{22,23,24} Next, the gradient was slowed in order to fractionate the single peak obtained from the linear gradient into three or more peaks. Here the gradient was fine tuned in order to maximize resolution between the fractions as well as to reduce fraction width to facilitate collection. Finally, the
gradient was quickly stepped to 100% chloroform after the polymer was eluted to ensure complete sample recovery from the column. The step gradients chosen and the resulting chromatograms are shown in Figure 2.4A-C. These elution patterns for each polymer and gradient are highly reproducible and have been replicated numerous times over a two years span.
Figure 2.4 GPEC chromatograms and the corresponding step gradients (---) for A:
P(3HB-co-8 mol% 3HV), B: P(3HB-co-12 mol% 3HV), C: P(3HB-co-21 mol% 3HV).
Figure 2.4 continued
Figure 2.4 continued
The linearity of the ELSD response to the amount of polymer represented in each peak was studied. Polymer concentrations of 3, 5, 10, and 20 mg/mL were injected in triplicate onto the reversed phase column used for GPEC under three different isocratic conditions (100:0, 80:20, and 60:40 chloroform:ethanol), which should represent the conditions experienced during a GPEC run. Figure 2.5 shows that the dependence of the ELSD response on the concentration varies for more concentrated samples but approaches zero as the concentration is decreased.
Figure 2.5 Illustration of the change in ELSD response for different polymer concentrations as well as different mobile phase compositions. * 20 mg/mL, ■ 10 mg/mL, ▲ 5 mg/mL, ● 3 mg/mL polymer solutions. The polymer used for this study was P(3HB-co-21 mol% 3HV).
Since the polymer concentrations used for actual GPEC were approximately 16 mg/mL, and each sample was fractionated into at least three portions, we can assume a near linear dependence for ELSD response on the concentration of the polymer for the experimental conditions used. In our situation, the ELSD was the most convenient to use since it shows low baseline fluctuation with mobile phase changes (unlike refractive index or light scattering detectors), and is responsive to the polymers studied, which are not UV active. Any deviation from linearity should be negligible towards the results discussed, since no relationships between polymer concentration and signal are mentioned.

To determine if the GPEC separation of the P(3HB-co-3HV) copolymer samples was influenced by molecular weight, SEC was conducted on the fractions. SEC is a technique consisting of an HPLC column packed with porous particles. Molecules are separated based upon differences in hydrodynamic volume, which is related to their molecular weight. Smaller molecules are able to penetrate the pores of the packed particles and as such have more elaborate elution pathways and elute after larger molecules, which are excluded from some of the pores. In SEC, first a calibration curve must be made using polymer standards of low polydispersivity for the column. It is best to use polymer standards that are similar to the polymer of interest, but when these are not available it is customary to use polystyrene standards. The calibration curve developed for the SEC column used in this work is shown in Figure 2.6. The fractions
applied to SEC showed increasing molecular weight for increasing elution order in the GPEC as well as decreasing polydispersivities ($M_W/M_N$) as compared to the original sample as shown in Table 2.1.
2.6 SEC calibration curve developed from polystyrene standards, and the application of the poly(3HB-co-3HV) copolymers to SEC. ◆ Polystyrene standards, ■ poly(3HB-co-3HV) copolymers.
<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_w$</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB-co-8 mol% 3HV)</td>
<td>360,000</td>
<td>1.9</td>
</tr>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 1</td>
<td>160,000</td>
<td>1.1</td>
</tr>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 2</td>
<td>250,000</td>
<td>1.4</td>
</tr>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 3</td>
<td>280,000</td>
<td>1.4</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV)</td>
<td>270,000</td>
<td>1.6</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 1</td>
<td>48,000</td>
<td>1.6</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 2</td>
<td>130,000</td>
<td>1.5</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 3</td>
<td>230,000</td>
<td>1.3</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 4</td>
<td>260,000</td>
<td>1.3</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV)</td>
<td>180,000</td>
<td>2.1</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 1</td>
<td>100,000</td>
<td>1.7</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 2</td>
<td>150,000</td>
<td>1.4</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 3</td>
<td>160,000</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Table 2.1** Apparent molecular weight information determined by SEC for the fractions collected by GPEC for P(3HB-co-3HV) copolymers.
In GPEC, elution order very commonly correlates with, and is strongly influenced by, molecular weight trends of the polymer under analysis.\textsuperscript{14,15,25} This elution order can be easily understood since most larger molecular weight polymers, including PHAs, require better solvating conditions than necessary for lower molecular weight polymers.\textsuperscript{26} This relationship between molecular weight and precipitation is easily understood if one considers that during the dissolution process solvent-polymer interactions must be greater than polymer-polymer interactions. As the molecular weight of the polymer increases, the cohesive energy of the polymer increases and as such a higher molecular weight polymer is more difficult to dissolve than a lower molecular weight polymer, especially in poorer solvents.\textsuperscript{27} Since the mobile phase conditions are moving from poorer to better solvents then it follows that molecular weights will increase during a run, especially if precipitation/redissolution phenomena are prominent. However, chromatography where adsorption/desorption phenomena are the dominating mechanism retention is also commonly correlated with increasing molecular weight. As the molecular weight of a polymer increases, the number of sites for interaction with the stationary phase increase and as such larger molecular weights become correlated with increased retention. These results alone are not indicative of whether precipitation/redissolution or adsorption/desorption mechanisms are dominant.

Proton NMR was conducted on the fractions to determine if the samples separated by GPEC vary in copolymer composition. A typical proton NMR spectrum for 3HB-co-
3HV is shown in Figure 2.7. The doublet with a chemical shift at ~1.3 ppm (a) represents the methyl group extending off the backbone of 3HB, and the triplet with a chemical shift at ~0.9 ppm (b) corresponds to the methyl group extending off the backbone of 3HV.\textsuperscript{26,28}

The average 3HV-co-3HB copolymer ratios can be determined by taking the ratio of the areas of these two multiplets. These ratios were calculated for all of the collected fractions from the GPEC separation and are depicted in Table 2.2. Also, it is worthwhile to note that this is how the percentage of 3HV in the original copolymers was calculated as well.
Figure 2.7 Proton NMR spectra of a P(3HB-co-3HV) copolymer. The ratio of the integral of the peaks at (a), representing the methyl group hydrogens from the side chain of 3HV, to the integral of the peaks at (b), representing the methyl group hydrogens from the side chain of 3HB, gives the percentage of 3HV to 3HB in the copolymer.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage 3HV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 1</td>
<td>9.1</td>
</tr>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 2</td>
<td>7.7</td>
</tr>
<tr>
<td>P(3HB-co-8 mol% 3HV) Fraction 3</td>
<td>7.0</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 1</td>
<td>13.6</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 2</td>
<td>12.4</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 3</td>
<td>10.9</td>
</tr>
<tr>
<td>P(3HB-co-12 mol% 3HV) Fraction 4</td>
<td>9.9</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 1</td>
<td>24.9</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 2</td>
<td>19.6</td>
</tr>
<tr>
<td>P(3HB-co-21 mol% 3HV) Fraction 3</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Table 2.2 Percentage of 3HV in 3HV-co-3HB copolymer fractions obtained by proton NMR of samples from the GPEC separation.
Firstly, the data show that each copolymer sample actually consists of a spread of copolymers that differ in percentage of 3HV contained. This is what was expected due to the nature of how these samples are processed. Although the bacteria are supplied with a feedstock that should produce a given average percentage 3HV copolymer, the feed stock composition immediately surrounding the bacteria is changing as the process occurs resulting in a spread of copolymer percentage 3HV distribution. If you recall Figure 2.1, the percentage of 3HV in the copolymer greatly influences the physicomechanical properties of the polymer. Having a separation technique that is able to yield samples that are more homogeneous in the percentage of 3HV is a great success toward commercialization of these biopolymers.

Secondly, Table 2.2 shows the trend of decreasing percentage of 3HV in the copolymer for increasing elution time. This agrees with trends observed by Yoshie et al. in the bulk solvent fractionation of 3HV-co-3HB copolymers, with larger percentages of non-solvent leading to higher percentages of 3HV in the copolymer fraction obtained.\textsuperscript{11} The trend of decreasing percentage of 3HV in copolymer fractions eluted with more solvating mobile phases can be explained by solubility differences between the 3HB and 3HV monomers. PHA heteropolymers have been shown to possess different physical characteristics than the 3HB homopolymer such as melting temperature, crystallinity, and solubility.\textsuperscript{3} Ishida et al. specifically states, “the solubility of copolymers in a specific solvent is known to be affected by comonomer unit composition and molecular size”.\textsuperscript{6} As
such, it is clear that the solubility of 3HV-co-3HB copolymers is influenced by both the percentage of 3HV present as well as the molecular weight of the copolymer.

The data in this table also indicates minimal interaction of the PHAs with the stationary phase during GPEC. The elution order of the fractions does not correlate with a reversed phase separation mechanism; separation which elutes the most polar compounds first followed by the most non-polar compounds. Instead, the more non-polar distributions, with higher %3HV in the copolymer, elute first. To further prove the lack of influence of the stationary phase, the gradient used for 21%3HV-co-3HB on the reversed phase column was also applied to a normal phase column. The resulting GPEC chromatogram is indicated in Figure 2.8. The chromatograms both show a similar elution pattern. This validates our assumption that the stationary phase is playing a minor role in the elution of the PHAs studied using these mobile phase conditions, and that the separation is mostly due to precipitation/redissolution effects.
Figure 2.8 GPEC chromatograms for reversed phase (C$_{18}$) (bottom trace) and normal phase (NH$_2$) (upper trace) to show the lack of influence the stationary phase composition has on the elution pattern of P(3HB-co-3HV). The polymer used for this study was P(3HB-co-21 mol% 3HV).
Table 2.2 also gives information about the extent of spread in percentage of 3HV for the three copolymers (P(3HB-co-8 mol% 3HV), P(3HB-co-12 mol% 3HV), P(3HB-co-21 mol% 3HV)). The spread of composition is much lower for the copolymers with a lower average percentage of 3HV than for those with higher average percentage 3HV. For example, the spread in 8% 3HV is across around two units (9.1-7.0) but this increases to around four units for 12% 3HV and increases to nine units for 21% 3HV. This is very important information since the spread in 3HV causes problems by having a spread of physical characteristics. For actual application of these biopolymer systems it follows that conducting GPEC is of utmost importance if higher average percent 3HV copolymers are to be used.

Liquid chromatography at the Critical Condition (LCCC) analysis was also applied to this polymer system. This is a technique in which system conditions are developed so that a homopolymer series of different molecular weights co-elute so that copolymers containing this homopolymer can be separated due to the other portions present. Different molecular weights of P3HB homopolymer were acquired by thermally treating the P3HB sample at 190°C for different durations. This temperature was chosen for degradation since it has been previously shown to provide various molecular weight homopolymers for P3HB when held for various time lengths. The P3HB samples used in this work with their time of degradation and corresponding molecular weights are given in Table 2.3.
<table>
<thead>
<tr>
<th>Time at Degradation Temperature</th>
<th>M&lt;sub&gt;w&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>4,700</td>
</tr>
<tr>
<td>5 hours</td>
<td>5,800</td>
</tr>
<tr>
<td>4 hours</td>
<td>8,300</td>
</tr>
<tr>
<td>3 hours</td>
<td>9,700</td>
</tr>
<tr>
<td>2 hours</td>
<td>16,000</td>
</tr>
<tr>
<td>1 hour</td>
<td>71,000</td>
</tr>
<tr>
<td>30 min</td>
<td>170,000</td>
</tr>
<tr>
<td>15 min</td>
<td>350,000</td>
</tr>
</tbody>
</table>

**Table 2.3** Molecular weights of P3HB homopolymers obtained from partial thermal degradation at 190°C.
Since the 3HV-3HB copolymers have already been shown to separate with a mobile phase consisting of ethanol and chloroform in the GPEC studies, this seems like a logical place to begin the critical condition search. Beginning with a mobile phase of 100% chloroform, isocratic runs were performed with the 3HB homopolymers with different amounts of ethanol included in the mobile phase. The retention time shift of the different homopolymers with the addition of ethanol is shown in Figure 2.9. The percentage of ethanol in the mobile phase was increased in increments of 10% up to 70% ethanol, and then smaller jumps were made up to 80.9% ethanol. As Figure 2.9 shows, the retention times for the P3HB homopolymers shifted toward longer times, but did not appear to be moving closer toward one another. At the highest ethanol mobile phase composition, the homopolymer standards began to show peak splitting as shown in Figure 2.10. Analysis of these split peaks shows that they are the same species, and thus precipitation effects will not allow critical condition determination with this mobile phase combination.
Figure 2.9 Change in P3HB homopolymer retention with the addition of ethanol to a chloroform mobile phase. 10% EtOH, 10% EtOH, 20% EtOH, 30% EtOH, 40% EtOH, 50% EtOH, 60% EtOH, 70% EtOH, 75% EtOH, 78% EtOH, 80% EtOH, 80.5% EtOH, 80.8% EtOH, 80.9% EtOH.
Figure 2.10 Demonstration of homopolymer peak splitting during critical condition experiments for mobile phases with ethanol concentrations that are above 80% in chloroform. A is 84% ethanol, and B is 90% ethanol in the mobile phase, both are the homopolymer heated for 5 hours.
In order to approach the critical condition in smaller increments, an ethanol:isopropanol (IPA) solution was used with chloroform instead of ethanol alone since IPA is a weaker nonsolvent than ethanol. By adding IPA to the ethanol smaller steps can be taken to approach the critical condition, which may avoid the split peak phenomenon. As shown in Figure 2.11, the addition of IPA to the ethanol:chloroform system does not provide the critical condition, and the homopolymers still elute in the size exclusion mode. Additionally, the peak splitting also occurred for this mixed system. As such, it was determined that the critical condition could not be achieved for the ethanol:chloroform mobile phase.
Figure 2.11 Influence of retention time for two P3HB homopolymers for addition of isopropyl alcohol (IPA) to the ethanol (EtOH):chloroform mobile phase in the search for the critical condition for 3HB. • 80% EtOH, • 80% EtOH + 10% IPA, • 80% EtOH + 20% IPA, • 80% EtOH + 30% IPA, • 80% EtOH + 40% IPA, • 80% EtOH + 50% IPA, • 80% EtOH + 60% IPA, • 81% EtOH, • 81% EtOH + 10% IPA, • 81% EtOH + 20% IPA, • 81% EtOH + 30% IPA.
A mobile phase composition of acetone: chloroform was also investigated to determine the critical condition for 3HB. Acetone is a weaker nonsolvent than ethanol and was expected to achieve the critical condition for 3HB before precipitation occurs. Figure 2.12 shows the relationship between the retention times of the homopolymers for the amount of acetone in the mobile phase. The P3HB homopolymers were dissolved in the mobile phase to be studied up to 72% acetone, and were kept there for larger percentage of acetone mobile phases due to solubility considerations. As Figure 2.12 shows, the homopolymers co-elute for mobile phase compositions higher than 45% acetone. This indicates that acetone: chloroform provides more of a “critical region” than a “critical point” for the 3HB homopolymer. In applying the critical condition to the copolymers, 60% acetone was chosen to eliminate problems with precipitation at higher acetone concentrations. When the critical condition was applied to the P(3HV-co-3HB) copolymers a single peak is observed indicating the copolymers studied are linear, statistically random copolymers. The approximate molecular weight of the 3HV portion is 120,000; 110,000; and 130,000 for 8%, 12%, and 21% P(3HB-co-3HV). As Bartkowiak et al. showed, the percentage of the comonomer does influence both the ratio of the peak area and the peak height for conditions at SEC and critical point. Figure 2.13 shows increases in 3HV content give a similar increase for the ratios indicating that critical chromatography can discern the different chemical compositions by the trends in peak height and peak volume, but is not able to fractionate the samples in this study.
Figure 2.12 Determination of critical condition for 3HB using mobile phases of acetone:chloroform. NS refers to acetone being a nonsolvent for 3HB. □ 25% NS, □ 45% NS, □ 50% NS, □ 55% NS, □ 60% NS, □ 65% NS, □ 68% NS, □ 70% NS, □ 72% NS, □ 75% NS (Samples in 72% NS), □ 80% NS (Samples in 72% NS).
Figure 2.13 The ratio of peak areas and peak heights for the samples at SEC versus critical condition for changes in the percentage of 3HV Poly(3HV-co-3HB) copolymers.
2.4. Conclusions

The application of GPEC towards the separation of 3HB-co-3HV PHA copolymers proved efficient in providing distinct, more homogeneous fractions from initial samples with wide distributions in composition. The separations show molecular weight dependence, with larger molecular weight portions eluting after smaller molecular weights, as well as compositional dependence with the percentage of 3HV in the copolymer decreasing with elution order. Through examination of these results as well as application of the gradient to a normal phase column, it was determined that precipitation/redissolution is the dominant mechanism for GPEC of these biopolymers under the mobile phase conditions studied. We have shown that GPEC can be used to provide similar distributional information obtained through bulk fractionation using a facile, automated, chromatographic device. As such, GPEC is indispensible for easily obtaining copolymer samples with a smaller range of physical characteristics as compared to a bulk sample. Additionally, the critical condition for 3HB was determined and applied to the copolymers. The lack of a distribution at the critical condition proves that these are linear, statistically random copolymers without a distribution in functionality and no blocky tendency. Although the critical condition is shown to be sensitive to the percentage of comonomer, which could be useful to predict the average composition of a sample.
References


(8) Feng, L.; Yoshie, N.; Asakawa, N.; Inoue, Y. “Comonomer-Unit Compositions, Physical Properties and Biodegradability of Bacterial Copolyhydroxyalkanoates” 


(14) Glöckner, G.; van den Berg, J. “Gradient Elution Chromatography of Polymers on Reversed-Phase Columns with Tetrahydrofuran as an Eluent Component”

*Chromatographia** 1984, 19, 55-61.


(27) Stevens, M. “Polymer Chemistry: An Introduction” Oxford University Press, New York, **1999**.


3.1 Introduction

The polyhydroxyalkanoates (PHAs) discussed in Chapter 2 are highly desirable to use as a chromatographic substrate. Bacteria manufacture PHAs by supplying carbon feedstocks during other nutrient limiting situations. As such, PHAs do not have a negative environmental impact in synthesis by having to use extremely high temperatures, or toxic solvents. Additionally, PHAs are also biodegradable and are broken down into the carbon feedstocks that were used to manufacture them, which is very important since sustainability has become a major focus in the scientific community recently. For example, Proctor & Gamble have reduced their CO₂ emissions, water
usage, and waste disposal by over 50% in the last decade according to their 2011 Sustainability Report. Over 100 different monomers for PHAs exist which also provides a large number of possible stationary phases with differing chemistries to allow for greater selectivity compared to the small number of stationary phases currently available.

One potential option to formulate chromatographic phases from polymers is through electrospinning. Electrospinning is a technique that involves application of a voltage to a polymeric solution, ejecting nanofibers to be collected on a grounded surface. This process is depicted in Figure 1.7. A dissolved polymeric solution is pumped in a syringe equipped with a conductive tip. A voltage is applied to the tip, and at a critical potential the charge at the surface of the solution overcomes the surface tension forming a Taylor Cone. Provided that there is enough polymer chain entanglement, a continuous jet of polymer is propelled from the Taylor cone and travels to a conductive surface to be collected. If polymer chain entanglement is not high enough, due to low concentration of polymer or low polymer molecular weight, electrospraying occurs instead of electrospinning and droplets are ejected from the Taylor cone.

Electrospun substrates have been shown to be quite useful as stationary phases for ultra thin-layer chromatography (UTLC). Clark et al. has shown the advantages of using electrospun substrates, both carbon and polyacrylonitrile, as UTLC substrates. The electrospun mats show enhanced efficiency and faster development times than
commercially available TLC plates. Additionally, electrospun UTLC plates studied thus far do not require the use of a binder to adhere the stationary phase to the solid support and as such prevent any band dispersion due to interaction with a different material.

As discussed in Chapter 1, one of the reasons why efficiency improvements are observed is due to using small electrospun fiber diameters with a low distribution in size. Thus, it is very important to optimize the electrospinning conditions to obtain fibers with small, reproducible diameters. This can be a time consuming task since fiber morphology depends on many different parameters. As stated above, it is important to use polymers with a high enough molecular weight and at a high enough concentration to allow chain entanglement during the electrospinning process yielding a continuous nanofiber to be ejected from the Taylor Cone. Larger molecular weight polymers can use lower concentrations than smaller molecular weight polymers because the degree of chain entanglement necessary for electrospinning is reached.

The solvent used to dissolve the polymeric solution may also influence the fiber morphology. The conductivity of the solution is very important in electrospinning, so using a solvent with a higher dielectric constant is desirable, especially if the spun polymer does not contain sites to accept charge. Using a solvent with a reasonable dielectric constant can be problematic since many polymers show a very limited range of suitable solvents. One way to remedy this issue is by the addition of co-solvents or salts.
to the solution. Performing these additions allows the polymer to remain dissolved while markedly increasing the conductivity of the polymeric solution.

Many other parameters affect fiber morphology in the electrospinning process. It is important to optimize the solution flow rate. Too high a flow rate may result in larger fiber diameters with poor morphology due to too much solution being deposited at once, but too low and the syringe may clog. The applied voltage is very important to optimize as well since the formation of a Taylor Cone is paramount to the process. The distance between the syringe tip and the collector has also been shown to affect fiber morphology. Too close of a distance and the polymer may not have enough time to dry before reaching the plate which can result in large, wet fibers. Too far of a distance and adequate coverage of the plate may become an issue. Unfortunately, many of these parameters are interdependent and as such, optimization can be a tricky and lengthy process.

It is also possible to change the orientation of the nanofibers collected during electrospinning to produce aligned mats. Aligning fibers during electrospinning has been done with two different techniques. The first technique involves electrospinning onto two grounded electrodes with a gap between them oriented as shown in Figure 3.1A. As shown in Figure 3.1A, the fibers align to bridge the gap between the electrodes. However, the dual electrode technique suffers from a few traits that prevent it from producing TLC mats. Firstly, the distance the electrodes can be separated is quite small,
maybe upwards to two centimeters, which would produce TLC plates that are very short in development length.\textsuperscript{3} Secondly, the thickness of the electrospun mat is not able to reach the 25 \( \mu \text{m} \) preciously used with randomly oriented electrospun mats in TLC.\textsuperscript{2} And lastly, the mat cannot be bound to a solid support after spinning without the use of a binder, which is undesirable as indicated above.
Figure 3.1 Diagrams of setups to obtain aligned electrospun fibers. A: Parallel electrode assembly, B: Rotating mandrel apparatus.
The other technique to produce aligned electrospun mats involves using a rotating collector as depicted in Figure 3.1B. The rotating collector aligns the fibers by winding them onto a rapidly rotating collector so they align similarly to a spindle of thread. Using an aligned, nanofibrous mat can be useful for several reasons. The flow of the mobile phase up the plate can be expected to have less of a multi-path component to band dispersion resulting in better fluid flow and less spreading of the analyte plug during migration. The less tortuous flow would be due to the fact that the fibers are aligned in a common direction with a clear path for the mobile phase without the multi-path problem introduced by the randomly oriented fibers. Because the fibers provide a more clear and straightforward path for fluid flow when aligned than chaotic, this also leads to shortened development times due to faster solvent migration. Lastly, an initial study of aligned fibers also illustrated more reproducible results. This could arise from one of two reasons, or a combination of them: the chaotic fiber mat is not as reproducible from mat to mat due to the random orientation of the fibers, or the actual TLC plate cut from the mat is not in exactly the same position from mat to mat in the randomly oriented fibers, whereas for the aligned fibers there is less room for variation since they are aligned on a common axis on a collector that is only 3 cm wide.

This chapter describes the optimization of electrospinning parameters for poly(3-hydroxybutyrate) (P3HB) and the subsequent use of the mats for UTLC of biological analytes. P3HB electrospinning parameters were systematically varied to produce the
lowest fiber diameters for P3HB to date. P3HB was also used to create the first aligned P3HB UTLC plates with similar mat thickness and fiber diameters. Both types of biodegradable plates were studied for use in analysis of biological analytes and compared to previous electrospun UTLC and commercially available TLC plates.

3.2 Experimental

3.2.1 Materials and Equipment

P(3HB) was obtained from Goodfellow Cambridge Ltd, batch number 6T279/06/2410. The chloroform is the same product as described in chapter 2. The electrospinning apparatus consists of a high voltage power supply (CZE100R from Spellman), a syringe pump (Pump 11 elite from Harvard Apparatus), and a digital hygrometer, thermometer, and dew point detector (from VWR). All electrospinning was performed in an enclosed plexiglass container to prevent exposure to the nanomaterials and to enable humidity control. Chloroform was used as the primary electrospinning solvent, although acetone, ethanol, methanol, isopropanol (all from Sigma Aldrich) were used as additives. The salt added to the electrospinning solution was tetrabutylammonium bromide from Acros Organics. The fluorescent indicator added to
the electrospinning solution was Fluorescence Indicator Green 254nm purchased from Sigma Aldrich. Images of electrospun mats were obtained using a Hitachi S-4300 scanning electron microscope (SEM). The analytes studied are steroids: cholesterol, cortisone, and androsterone, and nucleosides and nucleotides: adenosine (A), adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), which were all purchased from Sigma. Solvents used for mobile phase optimization were ACS grade acetonitrile from Mallinckrodt and certified ACS grade acetic acid from Fisher.

### 3.2.2 TLC of Biological Compounds

To evaluate the performance of the P3HB stationary phase, sets of biological compounds were analyzed. The randomly oriented mat was evaluated for the separation of steroids to allow comparisons to previously published electrospun TLC results. The mobile phase used is the same as published by Clark et al. of 30:70 acetone:water. Additionally, a separation of A, AMP, ADP, ATP was performed using the randomly oriented mats as well the aligned mats and commercially available silica plates to provide a comparison of the chromatographic performance between the three. The mobile phase used for this study consists of various combinations of acetonitrile and water with 5%
acetic acid. Visualization of the spots was performed with an ultraviolet lamp set at 254nm, and analysis of the images was performed with Image J software (available for download from Research Services Branch of NIH, http://rsbweb.nih.gov/ij/).

3.3 Results

3.3.1 Optimization of Electrospinning Parameters

For UTLC experiments, it is important to have well defined fibers with diameters in the range of 300-400 nm. Decreasing the fiber size in electrospun substrates can be likened to a decrease in particle size in traditional substrates. A decrease in particle size typically correlates with an increase in chromatographic efficiency. An examination of the Knox equation, shown below, highlights the importance of particle size.

\[ H = Au^{1/3} + \frac{B}{u} + Cu \]  

(3.1)

Where the terms A, B, and C stand for:
where $D_m$ is the diffusion coefficient of the mobile phase, $u$ is the velocity of the mobile phase, and $d_p$ is the diameter of the stationary phase particles. From terms A and C in the Knox equation it is clear that decreasing particle diameter will result in a decrease in $H$, which is the theoretical plate height related to band broadening during a chromatography experiment. Thus using smaller particle sizes, or nanofibers, will result in an increase in chromatographic efficiency.

Additionally it is important to use particle sizes or nanofibers with a low spread in size. Larger spreads in particle size would lead to a more erratic flow pattern for the mobile phase leading to multi-path flow, which would result in higher band broadening as well. As far as electrospun substrates, it is also important to have well defined fibers for better mat homogeneity leading to less band broadening similar to using small spreads.
in particle diameters. Poor fiber morphology may also result in higher apparent fiber morphology if the fibers are still wet upon impact with the mat resulting in coalescence.

Work from the Olesik group has shown that using lower fiber diameters does in fact correlate with higher chromatographic efficiencies. Clark et al. showed 8-500x better efficiencies for electrospun substrates with fiber diameters around 400nm, which is much smaller than the particles of the commercially available silica TLC plate used for comparison (2-10μm). Given the Knox equation discussion and the previous work from the Olesik group, it is clear that electrospinning conditions need to be developed for P3HB to produce well-defined fiber diameters around 400nm with a low spread in size.

The first electrospinning condition examined were the possible solvents for P3HB. P3HB only dissolves in chloroform and dichloromethane. Using dichloromethane as a solvent proved to be impractical. The boiling point for dichloromethane is quite low (39.6°C) resulting in difficulty dissolving P3HB which is facilitated by heating at 55°C. Using chloroform avoids this issue since the boiling point is much higher (61.2°C). However, the initial electrospinning of P3HB in chloroform did not produce fibers that were optimal for electrospinning. Although increasing the voltage led from a regime of beads (or electrospraying) to beaded fibers to fibers, these fibers were very large in diameter with a wide range in size (1700 ±1000 nm). Figure 3.2 shows a SEM image of the P3HB fibers.
Figure 3.2 SEM image of electrospun P3HB. Conditions: 15% P3HB in chloroform, flow rate 50 μL/min, voltage 15 kV, distance 10 cm. (1 ml/hr, 15 kV, 10 cm).
A possible reason why electrospinning of P3HB in chloroform was so poor is due to the low conductivity of the solution. The solution consisting of P3HB and chloroform can be expected to be a poor conductor of electricity since the polymer does not contain sites to readily adsorb charge and chloroform has such a low dielectric constant (4.81). Electrospinning depends on the solution accumulating a charge density at the surface to form a Taylor Cone and break the surface tension to eject nanofibers. One way to improve the charge carrying ability of the solution is to include a salt. A salt in solution has been shown to allow more charge density to accumulate at the surface of the solution and then in the ejected fibers, leading to higher elongation forces present in the fibers in the presence of the electric field. Additionally, using an organosoluble salt in the electrospinning solution for PHAs has been shown to reduce fiber diameters from 1-4 μm to around 1μm with improved fiber morphology.

The effect of salt addition, tetrabutylammonium bromide due to availability, was studied using the P3HB. Figure 3.3 shows the reduced fiber diameters and enhanced morphology resulting from including the salt in the electrospinning solution. When a salt concentration of 1% w/w is used, the mat appearance looks very similar to conditions without salt being present. However, increasing the salt concentration to 2% w/w yields much more linear fibers with an improved diameter of 1200 ± 400 nm, which is similar to previous electrospinning of PHAs with organosoluble salts. Further increasing the salt concentration to 3% w/w results in brittle and broken fibers as shown in Figure 3.3c.
P3HB is a brittle polymer and as such cannot tolerate the higher elongation forces induced by including larger percentages of salt in solution (3% and higher).\textsuperscript{1,10} For the rest of the electrospinning optimization, a salt concentration of 2% w/w was chosen.
**Figure 3.3** SEM images of P3HB with various amounts of tetrabutylammonium bromide included in the electrospinning solution. A: 1% w/w, B: 2% w/w, C: 3% w/w.
Although including salt with the electrospinning solution improved the fiber morphology and decreased the fiber diameter, the fibers are still not small enough (goal is around 400 nm) for the best TLC performance as well as comparisons to previous electrospun work. Another way to decrease the nanofiber diameters by increasing the electrospinning solution conductivity is with the addition of co-solvents. Due to the limited solubility of PHAs, adding co-solvents rather than changing solvents entirely is the best path to increase the conductivity of the solution. Kim et al. showed a marked improvement of the solution conductivity of PHA solutions and resulting fiber diameters with the addition of ethanol, methanol, and isopropanol.\textsuperscript{6} Table 3.1 shows the dielectric constants for several co-solvents whose effects are studied with P3HB electrospinning in this work. Each of these co-solvents was added to the electrospinning solution, along with 2\% salt to observe the changes to fiber morphology from the higher dielectric constants of the solution. A comparison of SEM images for the three co-solvents is depicted in Figure 3.4.
## Table 3.1

Dielectric constants for solvents studied with electrospinning. (From CRC Handbook of Chemistry and Physics, 92nd Edition)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloroform</td>
<td>4.81</td>
</tr>
<tr>
<td>dimethylformamide</td>
<td>36.71</td>
</tr>
<tr>
<td>methanol</td>
<td>32.7</td>
</tr>
<tr>
<td>acetone</td>
<td>20.7</td>
</tr>
</tbody>
</table>
Although the three co-solvents provide similar fiber sizes and distributions (400 ± 120 nm), it is clear from Figure 3.4 that the morphology is different depending on the co-solvent used. Methanol is clearly the best co-solvent to use in the electrospinning solution as it shows the least swelling of fiber intersections, which would lead to lower chromatographic efficiencies. One would expect dimethylformamide to be the best co-solvent since it has the highest dielectric constant out of the group. However, dimethylformamide has a very high boiling point compared to the solvent and the other co-solvents (153, 65, 56, and 61°C for dimethylformamide, methanol, acetone, and chloroform respectively). This higher boiling point for dimethylformamide does not allow for adequate drying of the electrospinning fibers before deposition that results in the wetter mat appearance as compared to using methanol.
Figure 3.4 SEM images of electrospun P3HB with 5% co-solvent addition to the electrospinning solution. A. dimethylformamide B. methanol C. acetone
The amount of methanol included in the electrospinning solution was also varied to determine the conditions that will result in the smallest, most monodisperse nanofibers. Three concentrations of methanol were applied to the electrospinning solutions: 5, 10, and 15% methanol. The SEM images from the trials are shown in Figure 3.5. Again, through the fiber diameters alone, the best conditions are not obvious since the standard deviations are so large, but it is facile to observe that 5% methanol is the best concentration to use in the electrospinning solution from Figure 3.5. Using lower percentages of methanol in the electrospinning solution increases the solution dielectric constant without giving the wetter mats shown in Figure 3.5B and C which result from using higher percentages of methanol. The wetter mats could be due to polymer precipitation since methanol is a non-solvent for P3HB, or possibly due to the higher boiling point of methanol which may not have fully evaporated upon deposition. For subsequent optimization 5% methanol was included in the electrospinning solution.
Figure 3.5 Effect of the amount of methanol included in the electrospinning solution on mat appearance. A: 5% methanol, B: 10% methanol, C: 15% methanol.
The concentration of P3HB in solution was another parameter investigated. At polymer concentrations below 5% P3HB the solution would not electrospin. For concentrations below 10% P3HB electrospinning was possible but beads were present in the mat regardless of the other parameters. On the higher end, concentrations above 15% P3HB would not dissolve. Thus, a small range of 10-15% P3HB was the only area further studied for the influence of concentration on fiber size and morphology. For several concentrations voltage-distance combinations were varied to discern the impact on fiber diameter with changing P3HB concentration as shown in Figure 3.6. It is difficult to make specific claims since the standard deviations of the fiber diameters are so high, but an overall trend of lower concentrations correlating with lower fiber diameters is present. This agrees with previous studies by others that have noticed higher polymer concentrations lead to higher viscosities resulting in larger fiber diameters.³ Because of these results, 10% P3HB was chosen in order to have the smallest fiber diameters without bead formation.
Figure 3.6 Illustration of the effect of changing polymer concentration on fiber diameter. The percentages refer to the concentration of P3HB in solution for the set, and the x-axis refers only to the number of different parameter combinations for this figure. The key shows the electrospinning distance and voltage for each set. $\bigcirc$ 10 kV, 10 cm; $\square$ 15 kV, 10 cm; $\bigcirc$ 10 kV, 15 cm; $\square$ 10 kV, 20 cm; $\square$ 15 kV, 20 cm; $\bigcirc$ 20 kV, 20 cm; $\square$ 25 kV, 20 cm.
The electrospinning voltage was systematically varied as well to observe its effects on fiber morphology. Below a certain voltage, normally around 7 kV, electrospinning would not occur because the charge accumulation at the surface was not strong enough to overcome surface tension. Electrospinning would occur for all voltages applied above this threshold up to the instrument maximum of 30 kV. However, at the lowest spinnable voltage the fibers coalesce into the background mat upon deposition. This phenomenon is due to the electrospinning solvent evaporating less at lower applied voltages. At the highest applied voltages the fibers appear wet and swollen as shown in Figure 3.7. Higher electrospinning voltages have been shown correspond to a shorter fiber migration times and increase the nominal fiber diameter as well as the occurrence of beads. The problems at the lowest voltages may be due to insufficient applied voltage, leading to unstable jet formation and less polymer whipping during migration to the collector. An operating voltage of 10 kV was chosen since it is on the low end of the range, but is stable creating well-defined fibers.
Figure 3.7 Effect of voltage on electrospinning conditions. Top (A) and bottom (B) images correspond to 12 and 11% P3HB respectively. Left (1) images correspond to the lowest spinnable voltage, and right (2) images correspond to 30 kV.
The effect of electrospinning distance between syringe and collector on fiber morphology was also investigated. Due to the large variation of sizes present in electrospun samples, it is difficult to make strong assumptions about the results shown in Figure 3.8. However, because these electrospun samples will be used for UTLC and need to have a mat thickness of around 25 μm to be similar to Clark et al. for comparison and to have enough stationary phase for UTLC, it is generally best to use the lowest electrospinning distance. At longer electrospinning distances the fiber deposition rate is greatly decreased and unreasonably long electrospinning times would need to be used (several hours). At 10 cm, a thickness of 25 μm is achieved in 30 minutes. A side-on image of the electrospun mat after 30 minutes is shown in Figure 3.9.
Figure 3.8 Effect of electrospinning distance on fiber diameter. 11% P3HB, 12% P3HB, 13% P3HB
Figure 3.9 SEM image displaying P3HB mat thickness. Distance indicated by the arrow is 25 μm.
The remaining electrospinning parameter, feed rate, was varied as the other parameters, but the lowest stable rate was chosen for the optimized conditions. It is well known that a certain flow rate is needed to maintain the Taylor Cone, but higher rates lead to increased fiber diameters and the occurrence of beads.\textsuperscript{2,3} This is due to the greater volume of solution that is leaving the tip for a given time with the same amount of time for stretching to the collector. For the optimized conditions, a flow rate of 5 $\mu$L/min is the lowest rate that provides a stable flow of nanofibers.

Changes in humidity have been known to impact electrospinning in the past. High humidity is a similar situation to including water in the electrospinning solution. Fibers can adsorb water during the migration to the collector plate influencing the rate of solvent evaporation and even partial redissolution of the polymer if water is a good solvent. Humidity is not expected to affect the electrospinning process for P3HB for several reasons. Firstly, water is a non-solvent for P3HB, thus water should not contribute to redissolution. Secondly, the electrospinning solvent is primarily chloroform, which is not miscible with water. The chloroform present would not allow water to affect the spinning. Studies from 0-100\% humidity show no significant difference in fiber morphology or diameter. There is a slight increase in fiber diameter and standard deviation for humidity of 75\%: 340 ± 120 nm versus 300 ± 100 nm for 75\% versus 50\% humidity, respectively. Although this difference is not statistically significant, humidity
was recorded for the remainder of the electrospinning work and decreased if it went above 50%.

The resulting parameters chosen yield fibers with an average diameter of 300 ± 90 nm as shown in Figure 3.4B. To date, the lowest fiber diameters obtained for PHAs have been around 1μm so this is a marked improvement in fiber diameter. To visualize nucleosides and nucleotides 2.5% w/w Fluorescence Indicator Green 254nm was included in the electrospinning solution. This caused no discernable change of fiber diameter or morphology.

Parameters were also developed to create the first aligned P3HB UTLC substrates. As mentioned in the introduction, this technique can be achieved by parallel electrode or rotating mandrel methodology. The parallel electrode method produces highly aligned fibers, but the mat thickness is very small and the mats are difficult to adhere to a solid support for TLC without the use of a binder. For this reason, the rotating mandrel method was used. In the optimization of aligned electrospun mats, the previously developed electrospinning conditions were used but the rotational speed was varied. Because stationary collectors provide randomly oriented electrospun mats, it should be expected that increasing the rotational speed of the mandrel should increase the degree of alignment. Three rotational speeds were investigated: 950, 1100, 1250 rpm. SEM images of each speed are depicted in Figure 3.10. As Figure 3.10 shows, all speeds result in mats that are aligned along a common axis. The images were analyzed to determine the spread
in directionality for the different speeds and the results are shown in Table 3.2. Although increasing the rotational speed of the collector does result in an increase in alignment, 1250 rpm begins to show fiber breakage due to the faster speed of the collector as indicated in Figure 3.10C. A collection speed of 1100 rpm was chosen for UTLC analysis to have the highest degree of alignment without the broken fibers given by faster collection speeds.
**Figure 3.10** SEM images of aligned electrospun P3HB with differing collector rotation speeds. A: 950 rpm, B: 1100 rpm, C: 1250 rpm.
<table>
<thead>
<tr>
<th>Collector rpm</th>
<th>% within 10°</th>
<th>% within 20°</th>
<th>% within 30°</th>
</tr>
</thead>
<tbody>
<tr>
<td>950</td>
<td>39</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td>1100</td>
<td>49</td>
<td>73</td>
<td>84</td>
</tr>
<tr>
<td>1250</td>
<td>57</td>
<td>73</td>
<td>85</td>
</tr>
</tbody>
</table>

**Table 3.2** Degree of alignment for the different collector rotational velocities. Values are the percentage of at fibers within 10, 20, and 30° variations of the alignment direction.
3.3.2 Ultra-Thin Layer Chromatography: Comparisons to Previous Work

To study the performance differences of the P3HB electrospun mats compared to previously obtained results using electrospun substrates, separations of steroids were performed. The three steroids used were androsterone, cholesterol, and cortisone with their structures given in Figure 3.11. Clark et al. separated the listed steroids using polyacrylonitrile (PAN) electrospun stationary phases, with the PAN monomer given in Figure 3.12. These steroids were also separated with the P3HB phases with similar fiber diameters and mat thicknesses to provide a comparison of the influence of polymer functional groups on the chromatographic properties of the substrate. Identical solvents for development and analyte solutions were used to the previous study, and 50nL of each analyte was employed for comparison.
**Figure 3.11** Steroids used for UTLC of P3HB. A: cholesterol, B: androsterone, C: cortisone.
Figure 3.12 Polyacrylonitrile (PAN) monomer.
The results of the differing analyte migrations for the P3HB, PAN, and commercially available cyano plates are depicted in Figure 3.13. As Figure 3.13 shows, the retention order for the analytes is conserved across the three substrates; cholesterol is most retained, followed by androsterone, and then by cortisone which is least retained. This retention order points towards a reversed-phase separation mechanism over all of the plates investigated. However, the cholesterol is completely retained on both of the electrospun phases whereas it co-elutes with androsterone on the cyano plate. This change in retention is due to the presence of a carbon backbone in the polymers that provides increased interaction with the more non-polar cholesterol than is available using the commercially available cyano phase. The more polar analytes are more retained on the P3HB plates than on the PAN plates due to P3HB being a more polar monomer than PAN. Lastly, it is important to note in Figure 3.13 that the standard deviations for migration in the electrospun mats are much smaller than for the commercially available phase, which is an advantageous quality of using electrospun UTLC substrates.
Figure 3.13 Comparison of retardation factors for a set of steroids on UTLC plates of various compositions. ◆ cholesterol ◆ androsterone ◆ cortisone
The performance of the electrospun substrates is superior compared to the commercially available cyano plates. Analyte bands do not broaden as much for the electrospun substrates. Table 3.3 lists the average final bandwidth for the steroids using the three different TLC plates. Analyte bands do not broaden more than 2 mm wide for the electrospun substrates while a common final bandwidth for commercially available plates is around 5 mm and can go as high as 9 mm. This lessened band broadening for the electrospun substrates is due to the nanometer diameter electrospun fibers used as the stationary phase as compared to the large highly variable particle sizes used in conventional TLC (upwards to 10 μm).
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Silica Plate Spot width (mm)</th>
<th>PAN Spot width (mm)</th>
<th>P3HB Spot width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>5.5</td>
<td>1.6</td>
<td>0.5 ± 0.003</td>
</tr>
<tr>
<td>androsterone</td>
<td>5.2</td>
<td>0.6</td>
<td>1.8 ± 0.03</td>
</tr>
<tr>
<td>cortisone</td>
<td>9.6</td>
<td>0.4</td>
<td>2.1 ± 0.03</td>
</tr>
</tbody>
</table>

**Table 3.3** Comparison of analyte broadening during TLC development between commercially available silica plate, electrospun PAN, and electrospun P3HB. Silica and PAN (results from Clark *et al.*, 2009)
3.3.3 Thin Layer Chromatography: Separation of Adenosine and Adenosine Mono-, Di-, and Triphosphates

The electrospun P3HB substrates were then applied to a set of biological compounds with a wide range in polarity: a nucleoside and its set of nucleotides. P3HB is expected to perform well in the separation of these analytes due to the high polarity of the P3HB monomer that contains carbonyl and ester oxygens. These functional groups should be available to participate in hydrogen bonding interactions with the analytes and water present in the mobile phase. Additionally, P3HB has high resistance to dissolution in solvents other than chloroform and dichloromethane making it an ideal substrate for polar biological compounds.

Adenosine and its mono-, di-, and triphosphates (A, AMP, ADP, ATP) were separated on the P3HB stationary phases using a range of development solutions consisting of acetonitrile and water with 5% acetic acid. The structures for A, AMP, ADP, and ATP are given in Figure 3.14. The best separation of this series of compounds was obtained using a mobile phase ranging between 40-60% acetonitrile in 5% acetic acid Nanopure water. Acetonitrile:water mobile phases were chosen because of the overwhelming number of works in the literature that show good separations of nucleotides and nucleosides using this mobile phase.\textsuperscript{12,13,14,15} The acetic acid was included in solution to reduce band broadening since it has been shown to assist in this
matter in previous works.\textsuperscript{16,17} The results for A, AMP, ADP, and ATP at 50\% acetonitrile are given in Table 3.4 and 3.5 as well as Figure 3.15. As Table 3.4 shows, the nucleoside and its set of nucleotides follow a normal-phase separation in which the most polar compounds are the most retained.
Figure 3.14 Structure of adenosine, adenosine mono-, di-, and triphosphate.
Figure 3.15 Separation of adenosine and adenosine mono-, di-, and tri-phosphate using the electrospun P3HB stationary phase. Conditions are 50:50 acetonitrile:nanopure water with 5% acetic acid.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>Spot width (mm)</th>
<th>Efficiency (N/L) (m&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.39</td>
<td>4.3</td>
<td>9,300</td>
</tr>
<tr>
<td>AMP</td>
<td>0.27</td>
<td>2.0</td>
<td>27,000</td>
</tr>
<tr>
<td>ADP</td>
<td>0.23</td>
<td>1.4</td>
<td>42,000</td>
</tr>
<tr>
<td>ATP</td>
<td>0.19</td>
<td>1.2</td>
<td>43,000</td>
</tr>
</tbody>
</table>

Table 3.4 Results for separation of adenosine, adenosine mono-, di-, and triphosphate using P3HB stationary phase with mobile phase of 50:50 acetonitrile:water (with 5% acetic acid).
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:AMP</td>
<td>1.5</td>
</tr>
<tr>
<td>A:ADP</td>
<td>2.2</td>
</tr>
<tr>
<td>A:ATP</td>
<td>3.0</td>
</tr>
<tr>
<td>AMP:ADP</td>
<td>0.97</td>
</tr>
<tr>
<td>AMP:ATP</td>
<td>2.2</td>
</tr>
<tr>
<td>ADP:ATP</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Table 3.5** Resolution between A, AMP, ADP, and ATP using P3HB UTLC plates with 50:50 acetonitrile:water (5% acetic acid).
Adenosine and its mono-, di-, and triphosphates also exhibit a normal phase retention mechanism using the commercially available silica plates. Table 3.6 shows the comparison of different retention factors for the P3HB and silica plates. The main detail to note from Table 3.6 is that adenosine and adenosine monophosphate are much more retained on the P3HB plates than on the silica plates. This increase in retention is similar to the increase in retention of the steroids as compared to the commercially available plate and is due to the carbon backbone of the polymer being available for interaction with less polar compounds. The silica phases do not have sites available to interact with less polar compounds which explains the drastic loss in retention as compared to the polymer phases.
Table 3.6 Comparison of retention factors for the separation of adenosine and adenosine mono-, di-, and triphosphate using the electrospun P3HB phase and commercially available silica TLC plates. Standard deviations given in parentheses.
The difference in retention factors between the two phases also points toward needing a longer TLC plate for the silica phase as compared to the P3HB phase. Adenosine and its nucleotides are separated after traveling 3 cm on the P3HB plate, whereas the silica plate requires 4.5 cm. Using smaller TLC plates is advantageous to conserve resources as well as to save time in solvent development to a shorter distance.

Analyte broadening during development was also compared between the P3HB and silica plates. The change in analyte spot width was recorded before and after development and is depicted in Figure 3.16. Figure 3.16 shows much smaller initial spots for the silica phase but a much greater broadening during development than the P3HB plates show. Analyte plugs broaden much more during development of the commercial plates due to their larger, more variable particle sizes as mentioned earlier in the Knox equation discussion. This increase in broadening during the separation with the commercially available devices results in a loss of resolution as shown in Table 3.7.
Figure 3.16 Comparison of initial and final analyte spot sizes for the separation of adenosine and adenosine mono-, di-, and triphosphate using electrospun P3HB and silica TLC plates.  
- Silica initial,  
- Silica final,  
- P3HB initial,  
- P3HB final.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>P3HB Resolution</th>
<th>Silica Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:AMP</td>
<td>1.5</td>
<td>0.19</td>
</tr>
<tr>
<td>A:ADP</td>
<td>2.2</td>
<td>4.0</td>
</tr>
<tr>
<td>A:ATP</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>AMP:ADP</td>
<td>0.97</td>
<td>4.1</td>
</tr>
<tr>
<td>AMP:ATP</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td>ADP:ATP</td>
<td>1.4</td>
<td>0.022</td>
</tr>
</tbody>
</table>

**Table 3.7** Resolutions for the separation of A, AMP, ADP, ATP on both P3HB and commercially available silica plates.
The nucleoside and its nucleotides were also separated using the aligned electrospun P3HB mat to study the effects that alignment would have on the chromatographic separation. Table 3.8 shows the retention factors for the four analytes on the two types of P3HB mats as well as their development times. The randomly oriented electrospun mats yield similar retention factors to the aligned electrospun mats. Additionally, the aligned substrates take half the time to reach the same development distance as the randomly oriented substrates. However, the aligned electrospun mats give a ten to one hundred-fold improved standard deviation of the retention factors, or a much higher reproducibility.

One possibility for the decreased reproducibility of the randomly oriented mats is due to the randomized fiber network, which is different from run to run. Unlike the aligned mats, the randomly oriented electrospun mats produce a circular mat. This circular mat may have additional variability in mat thickness across the mat. When choosing where to partition this mat to obtain the TLC plates there is more variability, and although it is the goal to take from the most uniform parts of the mat this may not always be straightforward. With the aligned phases, the only cut to be made to obtain the TLC plate is at the top and bottom of the plates, leading to a much more reproducible sectioning of the electrospun mat. Additionally, the fibers are oriented along a common axis yielding more reproducible flow channels than for the randomized mats. These aligned flow channels are also the source of the decreased development time. Figure 3.17
assists in the visualizing the increased number of options of where to section the mat for the randomly oriented samples as compared to the aligned electrospun samples.
Table 3.8 Comparison of retention factors for adenosine and adenosine mono-, di-, and triphosphate separation using randomly oriented and aligned electrospun P3HB stationary phases as well as the development time for each phase. Standard deviations in parentheses.
Figure 3.17 Illustration of the extra variability in UTLC plate choice from randomly electrospun mats (left) as compared to aligned electrospun mats (right).
3.4 Conclusions

This chapter describes the optimization of electrospinning parameters to fabricate the smallest diameter P3HB nanofibers to date and its deployment as a UTLC substrate to separate biological compounds. Electrospun fiber diameters of 300 ± 90 nm were obtained by raising the dielectric constant of the electrospinning solution by salt and co-solvent addition, which is a marked improvement from previous works. The traditional electrospinning parameters (feed rate, applied voltage, distance between tip and collector, polymer concentration) were also systematically varied to achieve the low fiber diameters. P3HB was also used to fabricate the first aligned electrospun P3HB UTLC plates by electrospinning onto a rotating mandrel. The mandrel rotation speed was varied to achieve the highest degree of alignment without fiber breakage, which occurs at 1100 rpm.

Both the randomly oriented and aligned electrospun mats were used as chromatographic substrates in UTLC of biological compounds. The randomly oriented phase was compared to a previously published electrospun UTLC work that focused on the separation of a set of steroids. The P3HB phase showed the same elution order as the electrospun polyacrylonitrile phase, but with increased retention for the more polar compounds. This increase in retention is due to the increased polarity of the 3HB monomer with ester and carbonyl oxygens compared to the acrylonitrile monomer with
only a cyano group for interaction. Additionally, both electrospun phases showed higher reproducibility as compared to the commercially available TLC plate showing the advantages of fabricating TLC plates in house from electrospun polymers. More importantly, the biodegradable P3HB plates did equally well at separating the compounds which is an important move toward sustainability in UTLC.

The P3HB electrospun mats were also studied with nucleoside: adenosine and its nucleotides: adenosine mono-, di-, and triphosphate separations. This set of compounds differs widely in polarity and as such is a complicated separation for thin layer chromatography without use of a gradient. This set of compounds was separated using the P3HB and commercially available silica plates. The advantages from using the P3HB plates are a shorter development distance, which helps to conserve materials, as well as a much lower change in analyte bandwidth during development as compared to the silica plates which results in better resolutions for the separation. Use of the aligned P3HB gave similar retention data, but much lower standard deviations for the data proving the aligned phases are much more reproducible experimentally then the randomly oriented phases. These results are further proof of the usefulness of electrospun polymer stationary phases as TLC substrates and how environmental sustainability can be included more often in separation science.
References


(2) Ramakrishna, S. An Introduction to Electrospinning and Nanofibers World Scientific, New Jersey, 2005.


4.1.1 Introduction to Amorphous Carbon

One of the most popular stationary phases in HPLC separations today uses an amorphous glassy carbon phase to interact with and separate compounds. This amorphous carbon phase is composed of ribbons of graphene sheets with a structure first proposed by Jenkins and Kawamura in 1971 and is shown in Figure 1.6. This intricate ribbon-like structure displays the nature of the amorphous carbon to be heterogeneous. The graphene sheets all lie in a ribbon yielding the smooth surfaces of the sheets, or basal-plane oriented sites, exposed for interactions with analytes. These graphene ribbons additionally form sites where only the edges of the sheets, or edge-plane oriented
sites, are exposed for interactions with analytes. This proposed structure has been confirmed many times, as shown in Figure 4.1.\textsuperscript{2} The combination of both edge- and basal-plane oriented sites in the amorphous carbon structure gives rise to a heterogeneous stationary phase.
**Figure 4.1** High resolution electron micrograph of amorphous graphitic carbon.

“Reprinted from Journal of Chromatography A, 352, Knox, J.; Kaur, B.; Millward, G.,

In a separation it is advantageous to have a homogeneous stationary phase. As a sample plug traverses a stationary phase, broadening of the plug occurs due to mass transfer of solutes with the stationary phase. Additional broadening can occur by having differing interaction sites present in the stationary phase, each with their own enthalpic contributions to solute partitioning. Thus, if various interaction sites are present, this broadening can be worsened for a single sample plug leading to a broader elution profile. If a set of analytes is injected during a single run, the broadness of their individual elution plugs leads to decreased resolution. By using a single type of interaction site, extra broadening due to interacting with several sites is absent leading to higher resolutions and more efficient separations.

It is expected that using edge- and basal-plane oriented carbon materials independently would yield a chromatographic phase with better performance than with using both together in an amorphous carbon stage. One way to fabricate carbon materials with a high degree of orientation is through membrane template synthesis as shown by Hurt et al. In this technique a dissolved carbon precursor is introduced into a membrane template via capillary action. This membrane is a commercially available alumina Anodisc with pore sizes typically in the submicron range. The impregnated Anodisc is then heated to 300°C where the carbon precursor, such as AR mesophase pitch, begins to soften into a homogeneous discotic liquid crystalline phase. If the temperature is further increased, following a specific ramp rate, the graphitic carbon contained within the pores
of the Anodisc begins to shift in orientation so as to maximize the degree of $\pi-\pi$ stacking. When the pores of the Anodisc consist of alumina, the high polarity of the pores and surface roughness cause the graphene sheets align in a stack so that only the edges of the sheets are exposed to the surface of the pore.\(^4\) This process forms edge-plane oriented carbon nanorods that are the dimension of the pores of the Anodisc.

To produce basal-plane oriented carbon nanorods, the surface of the template pores must be modified to induce favorable face on orientations. In order to maximize the likelihood of face-on orientation, the surface of the pores must be able to participate in $\pi-\pi$ or at least $p-\pi$ interactions with the graphene sheets and be extremely smooth. Several surfaces have been shown to induce a basal-plane orientation including mica, graphite, platinum, and silver.\(^4\) These smooth substrates minimize the excluded volume at the surface for basal-plane oriented graphene sheets whereas a rougher alumina surface would be entropically unfavorable for such an orientation due to the large amount of inaccessible volume.\(^4\) An illustration of the graphene sheet orientation difference between basal-plane and edge-plane orientation against the Anodisc substrate is given in Figure 4.2.
**Figure 4.2** Illustration of the graphene sheet orientation of edge-plane and basal-plane carbon at the substrate.
4.1.2. Capillary Electrochromatography

To study the chromatographic performance differences between the different ordered carbon species they must first be incorporated into a chromatographic device. One of the most facile techniques to incorporate nanoparticles into a device for chromatography is capillary electrochromatography (CEC). CEC is similar to a typical capillary electrophoresis setup in that a potential is applied across a capillary, which induces electroosmotic flow of a buffered solution. Samples are separated due to differences in their electrophoretic mobilities, which are related to the size and number of ionized sites per analyte. However, CEC also includes a chromatographic phase to interact with the analytes while they are undergoing the typical capillary electrophoretic migration. This chromatographic support can be adhered or packed in the capillary making it a traditional stationary phase. Alternatively, the chromatographic phase can be flowed through the capillary during the experiment, which is then referred to as a pseudostationary phase (PSP) as it is moving in the buffer solution. A diagram of the CEC apparatus employing a PSP is given in Figure 3.3.
Figure 4.3 Diagram of a capillary electrophoresis setup with a pseudostationary plug of carbon nanorods included for analyte partitioning during migration.
Use of a PSP over traditional stationary phases in CEC has many advantages. Firstly, no nanoparticle packing procedure is necessary, which saves time and resources. Secondly, the absence of packing avoids the need of a frit at the end of the capillary, which has been shown to lead to problems such as bubble formation or even solute degradation at the interface.\textsuperscript{5,6} Lastly, the chromatographic phase is replenished during every experiment so no hysteresis effects can be present such as carry-over or degradation.

Many different PSPs have been used in CEC to give an extra degree of selectivity to the separation beyond electrophoretic mobility differences. The most commonly used PSP consists of micelles and is so prevalent that it is referred to as micellar electrokinetic chromatography (MEKC). MEKC has been shown to aid in many separations including peptides, chiral separations, and even hydrophobic compounds which would not be well separated using capillary electrophoresis in aqueous buffer alone.\textsuperscript{7,8,9} However, MEKC has a very important limitation that makes it undesirable as a universal PSP in CEC. Micelles only form and stabilize in a small range of pH and surfactant concentrations and as such may not be well suited to separate a wide variety of analytes.

The use of nanoparticles as PSPs has also been studied quite extensively. Gold nanoparticles have seen widespread use in the separation of biological analytes such as DNA and peptides.\textsuperscript{10} Nilsson reviewed many other nanoparticle PSPs such as silica, polymer, and molecularly imprinted polymers.\textsuperscript{11} There has been interest in carbon PSPs
as well. Moliner-Martinez provided an extensive review of various carbon PSPs such as fullerenes, nanotubes, and chemically modified nanotubes. Addition of carbon nanoparticles to a background electrolyte is expected to provide a surface for analytes to engage in π-π or van der Waals interactions as well as entropic interactions with any pores that may be present. However, to the best of our knowledge, no one has studied the effects of carbon orientation in carbon nanotube PSPs in CEC to this date. As stated above, it can be expected that by using each carbon orientation independently a more selective separation can be obtained than when using the mixture.

4.1.3 Linear Solvation Energy Relationships

A method of quantitatively assessing the important interactions governing the partitioning of solutes with a chromatographic phase is through the calculation of their Linear Solvation Energy Relationships (LSERs). LSER studies first began as solvatochromic parameter studies with Kamlet and Taft in the 1970’s. These solvatochromic parameter studies focused on modeling the most important solute interactions in solvents that resulted in a shift in some behavior such as resonance, infrared signal, solubility, etc. They determined that solute hydrogen bonding affinity, polarizability, and size were the most important factors governing interactions with
solvents and that these parameters could be modeled using several experiments and multiple linear regression of the resultant data.

It was not until 1988 that this solvatochromic parameter work was applied to studies between analytes and chromatographic phases. The same parameters that are important in solute-solvent effects are also important in solute-stationary phase interactions. By using a set of analytes with known values for hydrogen bonding acceptance or donation affinity, polarizability, and size factors it is possible to compare which interactions are most important between different stationary phases by calculating the coefficients for each phase in the following equation:

\[
\log(k) = \log(k_0) + m \frac{V_x}{100} + s\pi_2 + rR + a\alpha_2 + b\beta_2
\]

In equation 4.1, \(\alpha_2^*\) and \(\beta_2\) refer to solutes hydrogen bond acceptance or donation affinity, \(\pi_2\) and R are related to solute polarizability, and \(V_x\) represents molecular size of the solute. The coefficients \(a\), \(b\), \(s\), \(m\), and \(r\) are specific to a given stationary phase and can be used to compare which interactions are most important for solute partitioning.

LSER studies have provided informative comparisons between chromatographic phases in CEC. For example, LSER has been used to study the chromatographic differences between several micellar PSPs, which illustrated that size and hydrogen bond acceptor strength of the solutes were the most important factors governing interactions.
Additionally Schulte examined the effects on changing the alkyl chain length on modified siloxane PSPs and found that the polarizability change from increasing the alkyl chain length was the most important factor governing the change in retention.\textsuperscript{16}

This chapter describes a detailed chromatographic study of the performance differences between amorphous, basal-, and edge-plane oriented carbon nanorods. First, each of these carbon species was prepared and analyzed using tunneling electron microscopy (TEM). Then the carbon nanorods were employed as a PSP in CEC using optimized conditions for a set of benzene derivatives as a test group. The retention data was processed using multiple linear regression to provide LSER coefficients for each of the carbon nanorod types. Then, a comparison of these coefficients is discussed to elucidate the differences in analyte interaction for changing carbon orientation.

4.2 Experimental

4.2.1 Materials and Instrumentation

AR Mesophase Pitch, naphthalene homopolymer, was purchased from Mitsubishi Gas Chemical America. Alumina Anodiscs with pore sizes of 200 nm and disc thickness of 60 \( \mu \text{m} \) were purchased from Whatman. Silver nitrate, L-tartaric acid, and glucose were
purchased from Sigma Aldrich. Forming gas (5% hydrogen, 95% nitrogen, standard grade) was purchased from Praxair. Analytes for the LSER study are 1-naphthol, 2-naphthol, p-cresol, m-cresol, 3-chlorophenol, 4-chlorophenol, 4-fluorophenol, phenol, resorcinol, 4-ethylphenol, and 3,5-dimethyl phenol purchased from Sigma Aldrich. The neutral marker formamide and sodium hydroxide was purchased from Mallinckrodt. Sodium borate was purchased from J.T. Baker Chemical Co. Pyridine was obtained from Sigma Aldrich and acetonitrile ACS grade was obtained from Mallinckrodt. Ammonium hydroxide and nitric acid were purchased from Fisher Scientific. All water used was in-house 18MΩ Nanopure.

A quartz tube furnace from Thermo Electron Corporation, model Lindberg Blue M, was used for the preparation of the carbon nanorods. All CEC experiments were performed on an Agilent G1600 capillary electrophoresis instrument using UV detection at 204 nm. Capillaries were prepared in-house and consisted of polyimide coated fused silica capillary cut at 45 cm length and detection window burned off at 8.3 cm with an internal diameter of 50 μm and an outer diameter of 360 μm purchased from Polymicro Technologies. Multiple linear regression was performed with Microsoft Excel for the LSER studies.
4.2.1 Preparation of Amorphous, Edge-, and Basal-Plane Carbon Nanorods

The preparation of the carbon nanorod species was modeled after the work of Chan *et al.*[^3] A 1% w/w solution of AR mesophase pitch solution was prepared in pyridine. Alumina Anodiscs were infused with this carbon solution via capillary action. The discs were then placed inside of the quartz tube furnace and purged with forming gas for the duration of the heating protocol. The heating protocol for amorphous carbon was a fast ramp to 300°C (in 10 minutes) followed by a 4 hour hold time and then the furnace was turned off and allowed to cool to room temperature. The heating protocol begins the same for edge-plane carbon, except there is a second ramp to 700°C which takes nearly two hours followed by a hold time of 1 hour at this temperature.

Basal-plane oriented nanorods are formed using the same heating protocol as the edge-plane oriented nanorods; however, the alumina Anodiscs are first coated with silver adapted from Zhang *et al.*[^19] This silver coating procedure involves soaking the Anodiscs in a solution consisting of silver nitrate, L-tartaric acid, and glucose adjusted to a pH of 8 with a 30% nitric acid solution. This silver solution is prepared by first making two solutions: one 30 mL with 7.1 mM silver nitrate and the other 20 mL of 60 mM glucose and 6.7 mM L-tartaric acid. The silver nitrate solution is precipitated by the addition of one drop of 4 M sodium hydroxide by pipette. This precipitate is redissolved by drop wise addition of ammonium hydroxide (typically around 4 drops). This silver solution is

[^3]: Chan et al.  
[^19]: Zhang et al.
then filtered through a 20\(\mu\)m filter and the pH is adjusted to between 8 and 9 by drop wise addition of a 30\% nitric acid solution. The filtered and pH adjusted solution is then added to the L-tartaric acid/glucose solution and the pH is again adjusted using 30\% nitric acid and ammonium hydroxide if necessary. The Anodiscs are then submerged in this liquid in plastic microcentrifuge vials and allowed to soak at 40°C for 48 hours in the dark until the Anodiscs turn black and slightly reflective. The solution is then rinsed off with water.

After the carbon nanorods have been prepared, the alumina Anodisc templates are removed. The unmodified Anodiscs are removed by 6M sodium hydroxide etching followed by centrifugation, decantation, and dilution until neutral pH is achieved. The silver coated Anodiscs first had to be etched with 30\% nitric acid to remove the silver plating followed by centrifugation, decantation, and dilution until neutral pH was achieved and then etched with sodium hydroxide. TEM images were obtained for each of the carbon nanorod samples to ensure graphene sheet reorientation at the surface. All nanorods were stored in a solution of deionized water until use.
4.2.3 Capillary Electrochromatography

The run buffer used was 20 mM borate with 10% acetonitrile buffered to pH of 9.45. At this pH all of the analytes are partially ionized to have a migration time that is different from the neutral marker, formamide. These conditions were chosen to optimize long-term reproducibility of the buffer as well as to maximize the change in analyte migration for the test solutes from formamide. Analytes were prepared at 1 mM with 20 mM formamide in the buffer solution. Analytes were injected for 1 second at 50mbar pressure (~2 nL). The applied voltage was 10 kV, which resulted in an operating current around 15 µA.

The amount of carbon to use in solution was determined by using the highest concentration that would stay dispersed for at least 30 minutes. This is more than double the time necessary for a run. The carbon solution was dispersed by sonication for at least one hour prior to every use. The length of the carbon plug to use was determined by maximizing the retention factor difference of the solutes with and without carbon present. An 80 second carbon plug length (40 seconds before and after the analyte plug) was chosen for all subsequent LSER determining experiments. Each analyte was applied to each type of carbon independently, in triplicate or more to obtain a relative standard deviation of 5% or less, and their changed retention factors were recorded.
4.3 Results & Discussion

4.3.1 Fabrication of Amorphous, Basal-, and Edge-plane Oriented Carbon Nanorods

The procedure outlined in the experimental section was followed to obtain the three types of carbon nanorods. On the macroscopic scale it was obvious that the basal-plane carbon nanorods were much more hydrophobic than the edge-plane or amorphous carbon samples. The basal-plane carbon tended to float on the surface of the water solution, whereas the edge-plane and amorphous carbons would settle to the bottom of the solution over time. TEM images of the three carbon types further proved the success of the orientation and are depicted in Figure 4.4A-C. Figure 4.4A shows amorphous carbon with no orientation, whereas Figure 4.4B shows a high degree of orientation throughout the entire nanorod which is all in an edge-on orientation. Figure 4.4C shows basal-plane orientation but only for the first few layers, which is quite common in carbon that has been fabricated to be face-on. The basal-plane orientation is highly unfavorable compared to edge-plane or even amorphous orientations, which preserve the most internal π-π bonds within the sample, and this is the cause of only the first few graphene layers being truly basal-plane.
continued

**Figure 4.4** TEM image of an A: amorphous, B: edge-plane oriented, C: basal-plane oriented carbon nanorod. Black line indicates graphene sheet orientation.
Figure 4.4 continued
Figure 4.4 continued
4.3.1 Development of Conditions for Capillary Electrochromatography

The choice of run buffer was the first parameter optimized. The test analytes all have $pK_a$ values around 9.5 and as such it is important to choose a buffer that will keep the analytes partially ionized to have a different migration time than the neutral marker. This ensures that even small changes in the retention factor due to interaction with the carbon species will be noticeable without the superposition of the neutral marker if the analytes were uncharged. A borate buffer was chosen to work around this pH regime. Additionally, acetonitrile was also included in the run buffer to assist in the dissolution of more hydrophobic analytes. The concentration of sodium borate and the percentage of acetonitrile in the run buffer were optimized to give the best reproducibility and largest retention factors for the test analytes. It was found that a run buffer strength of 20 mM sodium borate with 10% acetonitrile provided the best results.

Pseudostationary phases can be included in the CEC separation by dispersion in the run buffer, or through a plug injection which both have advantages and disadvantages. Dispersion of the PSP in the entire run buffer offers more PSP for interaction during analysis, however this may also hinder UV detection of the analytes due to extra signal imposed by the PSP. Using plug injection of PSP offers less substrate for interaction, but leaves the majority of the baseline unaffected by signal from the
flowing phase. The carbon nanorod PSP yields UV absorption in the range of interest, and as such will be injected as a plug for ease of analyte detection.

The plug length of carbon for the CEC studies was chosen based upon a study of a change in retention factor for runs with and without carbon for the test analyte phenol. Having the largest difference in retention factors for runs with and without carbon ensures conditions in which the analytes are interacting the most with the PSP. The carbon plug is injected before and after the analyte plug, with equal amounts, to ensure the analyte will traverse the PSP plug. The total carbon plug length is then the sum of the plugs before and after the analyte. Increasing the plug length leads to an increase in interaction between the analyte and the carbon as shown in Figure 4.5. Above an 80 second plug length there appears to be no gain in analyte interaction and the graph appears to level off. Additionally, for plug lengths longer than 80 seconds, the signal from the eluting PSP begins to envelop the signal of the analyte. These results indicate that the environment of the analyte is saturated with carbon, giving no cause to use PSP injection plugs longer than 80 seconds. Thus, for the CEC work, PSP lengths of 80 seconds were employed for basal-plane, edge-plane, and amorphous carbon.
Figure 4.5 Depiction of the degree of interaction between phenol and basal plane carbon with increasing plug length.
4.3.2 Linear Solvation Energy Relationship Study

The LSER study, which provides information for how the different ordered carbon PSPs interact with analytes, begins with choosing analytes with tabulated data for the LSER terms. Table 4.1 gives the analytes and their solute descriptors for each of the terms in equation 4.1. The solvatochromic descriptors for the solutes in Table 4.1 were developed as discussed in Abraham et al. R is excess molar refraction and can be obtained through refractive index measurements, $\pi_2$, $\alpha_2^*$, and $\beta_2$ can be determined from gas chromatography experiments, and $V_x$ is McGowan’s characteristic volume which can be calculated from molecular structure. These solute descriptors have been used in several studies probing the chromatographic performance of different stationary phases and PSPs and thus should assist well in the study of ordered carbon PSP performance.
<table>
<thead>
<tr>
<th>Solute</th>
<th>$V_x$</th>
<th>$\pi_2$</th>
<th>$\beta_2$</th>
<th>$\alpha^*$</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>0.775</td>
<td>0.89</td>
<td>0.3</td>
<td>0.6</td>
<td>0.805</td>
</tr>
<tr>
<td>p-cresol</td>
<td>0.916</td>
<td>0.87</td>
<td>0.31</td>
<td>0.57</td>
<td>0.82</td>
</tr>
<tr>
<td>m-cresol</td>
<td>0.916</td>
<td>0.88</td>
<td>0.34</td>
<td>0.57</td>
<td>0.822</td>
</tr>
<tr>
<td>4-fluorophenol</td>
<td>0.793</td>
<td>0.97</td>
<td>0.23</td>
<td>0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>3-chlorophenol</td>
<td>0.898</td>
<td>1.06</td>
<td>0.15</td>
<td>0.69</td>
<td>0.909</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>0.898</td>
<td>1.08</td>
<td>0.2</td>
<td>0.67</td>
<td>0.915</td>
</tr>
<tr>
<td>3,5-dimethylphenol</td>
<td>1.057</td>
<td>0.84</td>
<td>0.36</td>
<td>0.57</td>
<td>0.82</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>0.834</td>
<td>1</td>
<td>0.58</td>
<td>1.1</td>
<td>0.98</td>
</tr>
<tr>
<td>1-naphthol</td>
<td>1.144</td>
<td>1.05</td>
<td>0.37</td>
<td>0.61</td>
<td>1.52</td>
</tr>
<tr>
<td>2-naphthol</td>
<td>1.144</td>
<td>1.08</td>
<td>0.4</td>
<td>0.61</td>
<td>1.52</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td>1.056</td>
<td>0.90</td>
<td>0.36</td>
<td>0.55</td>
<td>0.80</td>
</tr>
</tbody>
</table>

**Table 4.1** LSER descriptors for the solutes used in ordered carbon PSP CEC study. (from Schulte et al.)
A covariance matrix was constructed for this set of analytes to ensure statistical validity of using this test group. It is important that the parameters in this test group are orthogonal so that the multiple linear regression analysis can be considered not to covary. Table 4.2 gives the covariance matrix for the solutes. Table 4.2 shows a very low degree of correlation between most of the descriptors as the majority are all lower than around 0.3 which has been shown to be an acceptable degree of correlation for these terms studied previously.\textsuperscript{22} There is a relatively significant correlation between the $\alpha_2^*$ and $\beta_2$ terms (0.548). Because all of the analytes contain a hydroxyl group, it is expected that hydrogen bond donation and acceptance should be similar for the set, although differences are present due to the variety of functionalities present in the group. Additionally $V_x$ and $R$ as well as $\pi_2$ and $R$ have stronger correlations, 0.713 and 0.624 respectively. It has been shown that covariance in these parameters cannot be avoided since an increase in molecular size often correlates with an increase in polarizability ($V_x$ and $R$), and the dipolarity and polarizability should of course correlate as well ($\pi_2$ and $R$).\textsuperscript{23} However, multiple linear regression data with very high correlation coefficients (all $R^2$ above 0.98) have been shown to be trustworthy even with some covariance in these terms. With a level of covariance similar to previous studies and the high correlation for the coefficients obtained, chemical interpretations of the LSER coefficients can be considered to be justified.
<table>
<thead>
<tr>
<th></th>
<th>$\pi_2$</th>
<th>$\alpha_2^*$</th>
<th>$\beta_2$</th>
<th>$V_x$</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\pi_2$</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_2^*$</td>
<td>0.338</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>-0.139</td>
<td>0.548</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_x$</td>
<td>0.180</td>
<td>-0.355</td>
<td>0.238</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>0.624</td>
<td>0.054</td>
<td>0.327</td>
<td>0.713</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.2 Covariance matrix for solute descriptors.
Each of the solutes was analyzed independently with edge-plane, basal-plane, and amorphous carbon in CEC. In all cases the presence of carbon in the run buffer changed the migration behavior of the analytes. The presence of each of the carbon species in the run buffer shortened the retention factor for the analytes. The carbon PSPs are uncharged and elute with the neutral marker. The solutes studied are all negatively charged at the pH of the run buffer (9.45), which results in longer migration times than for the neutral marker. Interaction with the carbon PSPs will then shorten the migration time of the analytes by causing them to elute more closely to the neutral marker. The solute migration times are highly reproducible (within 5% RSD) and are indicated in Figure 4.6.
Figure 4.6 Retention factors for analytes subjected to CEC with and without ordered carbon PSPs. □ Amorphous, ▲ Basal-Plane, ▼ Edge-Plane
Retention data for the solutes was used to obtain LSER coefficients for each of the ordered carbon species. A matrix was constructed with the logarithm of the retention factor and the solute descriptors for each carbon type. Multiple linear regression of this matrix provides the LSER coefficients given in Table 4.3. The regression coefficients for the multiple linear regression analysis (all above 0.94) indicate a good correlation between the obtained coefficients and the original retention and descriptor data. However, the standard errors for the coefficients are a bit high, and ideally, the regression coefficients should be higher (around 0.98 and up). In order to observe the predicting quality of the model, plots of experimental -log(k) versus predicted -log(k) were constructed. These plots are depicted in Figures 4.7A-C.
Table 4.3 LSER coefficients for amorphous, basal-plane, and edge-plane oriented nanorod PSPs used in CEC. The standard error for each coefficient is given in parentheses. Coefficients given as 0 are statistically zero due to their standard errors.
**Figure 4.7** Experimental -log(k) versus predicted -log(k) using the LSER model for A: amorphous, B: basal-plane oriented, C: edge-plane oriented carbon. The circled data point is for p-cresol.
Figure 4.7 continued

\[ R^2 = 0.944 \]
Figure 4.7 continued

Predicted -log(k) vs. Experimental -log(k) with $R^2 = 0.982$.
The plots of predicted versus experimental retention data show that the model does not fit the data very well. Coefficients of determination are ranging from 0.94 up to 0.98. Figure 4.7B, which is the plot for basal-plane oriented carbon, displays a data point that is quite far from the regression line. This data point corresponds to p-cresol and has been circled in red on Figures 4.7A-C. The LSER coefficients were recalculated with the removal of p-cresol and are given in Table 4.4. Now the model is a much better fit to the data, with $R^2$ values all above 0.99. Additionally the standard error in the measurements has decreased significantly. A visual depiction of the LSER coefficients is given in Figure 4.8. To further highlight the improvement of the model with the removal of p-cresol, new plots of experimental versus predicted retention data were constructed and are given in Figure 4.9A-C. It is clear that the fit is much improved.
<table>
<thead>
<tr>
<th>Carbon Type</th>
<th>$a$</th>
<th>$b$</th>
<th>$s$</th>
<th>$r$</th>
<th>$m$</th>
<th>$\log(k_0)$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorphous</td>
<td>2.90</td>
<td>-3.75</td>
<td>-1.11</td>
<td>1.15</td>
<td>0</td>
<td>-1.58</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>(0.27)</td>
<td>(0.37)</td>
<td>(0.49)</td>
<td>(0.14)</td>
<td>0</td>
<td>(0.34)</td>
<td></td>
</tr>
<tr>
<td>Basal-Plane</td>
<td>2.98</td>
<td>-4.04</td>
<td>-1.44</td>
<td>1.05</td>
<td>0.40</td>
<td>-1.39</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>(0.31)</td>
<td>(0.43)</td>
<td>(0.57)</td>
<td>(0.17)</td>
<td>(0.23)</td>
<td>(0.40)</td>
<td></td>
</tr>
<tr>
<td>Edge-Plane</td>
<td>2.02</td>
<td>-2.96</td>
<td>0</td>
<td>1.06</td>
<td>-0.54</td>
<td>-1.44</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>(0.30)</td>
<td>(0.42)</td>
<td></td>
<td>(0.16)</td>
<td>(0.23)</td>
<td>(0.39)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.4** LSER coefficients for amorphous, basal-plane, and edge-plane oriented nanorod PSPs used in CEC without p-cresol data. The standard error for each coefficient is given in parentheses. Coefficients given as 0 are statistically zero due to their standard errors.
Figure 4.8 LSER coefficients from modeling of retention data. Basal-Plane, Amorphous, Edge-Plane.
Figure 4.9 Experimental $-\log(k)$ versus predicted $-\log(k)$ using the LSER model for A: amorphous, B: basal-plane oriented, C: edge-plane oriented carbon.
Figure 4.9 continued

![Graph showing predicted vs. experimental -log(k) with a linear regression line and R² = 0.991]
Figure 4.9 continued

\[ R^2 = 0.993 \]

continued
The coefficient \( a \) indicates the relative hydrogen bond acceptor strengths between the carbon species.\(^{17}\) Basal-plane and amorphous carbons have significantly larger coefficients than the edge-plane carbon. It is expected that the basal-plane graphene sheet network can participate in \( \pi \)-H bond formation, which can account for the increased acceptor strength for basal-plane and amorphous over the edge-plane species.\(^{4}\) However, it has also been shown that edge-plane carbon often incorporates functionalities due to unsatisfied valencies on the edge such as oxide groups.\(^{4}\) These groups may be the source of the positive nature hydrogen bond acceptor term for the edge plane PSP since few graphene faces are exposed.

The \( b \) coefficient represents the degree of hydrogen bond donating ability of the PSP.\(^{17,18}\) All of the carbon types have a large, negative value for \( b \) indicating that they are much poorer hydrogen bond donators than the mostly aqueous mobile phase. The carbon nanorods would not be expected to be successful hydrogen bond donators as they are composed of graphene sheets. The edge-plane carbon nanorods have a smaller negative value for hydrogen bond donation strength than the basal-plane and amorphous carbon nanorods. This again can be attributed to functionalities incorporated onto the dangling edges to satisfy valency.

The \( s \) coefficient represents the dipolarity of the stationary phase compared to the mobile phase.\(^{17,18}\) Since the mobile phase is mostly water, it is expected that the carbon species will have a negative value for the \( s \) coefficient. The \( s \) values are negative for the
carbon PSPs; however, edge-plane carbon is much less negative than for amorphous and basal-plane carbon. A larger $s$ coefficient for edge-plane carbon implies that it is more dipolar than the other carbon species. The exposed face of the graphene sheet in basal-plane carbon is a very non-polar environment, as a typical exposed π-network. However, when this is turned on its side the edges are exposed which quite possibly have some attached functionalities which would be significantly more polar than the basal-plane faces, but still much less polar than the mostly aqueous mobile phase.

The $r$ coefficient is a polarizability correction term, thus it can also aid in the description of the polar nature of the different phases. A more polarizable stationary phase is reflected by a larger value for $r$. Additionally, a positive value for $r$ indicates that the phase is more polarizable than the mobile phase and a negative value implies the mobile phase is more polarizable. Water is a very polar molecule whose polarizability is much less influenced by the solutes than the carbon PSP phases. Positive values for $r$ were found for each of the carbon species with nearly the same magnitude. This reinforces the belief that the carbon species are more polarizable than the aqueous mobile phase.

Lastly, the coefficient $m$ is related to the influence of the solutes size on the degree of interaction with the stationary phase. The larger a solute is the more that a stationary phase must adjust to create an appropriately sized cavity for interaction with the solute as well as increasing the dispersion interactions between solute and stationary
The carbon nanorods are a highly rigid system, with the edge plane carbon being the most rigid. Graphene sheets aligning in a face-to-face fashion maximizes \( \pi-\pi \) stacking and is less likely to shift in orientation to accommodate the solutes.\(^4\) Thus, the significantly lower \( m \) coefficient for the edge-plane carbon compared to basal-plane and amorphous carbon is due to the increased rigidity of the carbon nanorods but in general, all of the carbon species show a small \( m \) coefficient.

### 4.4 Conclusions

The chromatographic performance differences between basal-plane, edge-plane, and amorphous carbon were probed in this study. This was accomplished first by careful fabrication of the ordered nanomaterials using membrane template synthesis and precise heat treatment protocols of a pitch solution. The carbon produced with the alumina Anodiscs was either amorphous or edge-plane oriented depending on the final temperature reached during the heating program. Basal-plane oriented nanorods were fabricated by using Anodiscs that are coated with silver prior to the heat treatment procedure to facilitate face-on orientation of the graphene sheets with the pores of the membrane. The technique provides carbon nanorods with molecular order with the graphene sheets oriented in a face-on, edge-on, or amorphous fashion.
The chromatographic differences between these carbon species were studied using a comparison of the LSERs calculated for each carbon type. Applying a set of test analytes to interact with the carbon nanorods and monitoring the retention differences that each carbon species provided determined these LSERs. The carbon nanorods were used as a PSP in CEC to provide interaction with the test analytes.

The LSER study highlighted the major differences in interaction influenced by the ordering of the carbon nanorods. Polarity differences are the most striking variance caused by the molecular orientation of the carbon species. The coefficient related to polarity, $s$, is very different for the edge-plane and amorphous and basal-plane oriented carbon nanorods. Edge-plane carbon has a much larger coefficient indicating that it is a much more polar stationary phase than amorphous or edge-plane oriented carbon. This result is not surprising given the non-polar nature expected from basal-plane oriented carbon which would have a surface dominated with $\pi$-networks.

The LSER study also indicates that the hydrogen bonding nature between edge- and basal-plane carbons is very different. The exposed $\pi$-network of the basal plane carbon offers a stronger hydrogen bond accepting environment than the edges of the graphene sheets. Additionally, edge-plane carbon is a slightly better hydrogen bond acceptor due to the known oxygen functionalities that are often present along the edges to satisfy the valency of the graphitic carbon.
These differences in chromatographic performance between the edge-plane and basal-plane carbon are a very important discovery in separation science. When used together in amorphous carbon, most of the edge plane functionality contributions are lost since the majority of the phase is composed of basal-plane sites. However, band broadening due to interactions with both basal- and edge-plane sites can still be an issue when employing amorphous carbon. Using each of these phases independently may not only reduce this resolution and efficiency problem, but it will also provide a more selective stationary phase. Edge-plane oriented carbon is better suited to the separation of polar compounds whereas basal-plane carbon is better suited to non-polar aromatics. Future work in this subject will continue to probe the advantages of using each of these phases independently to provide a better stationary phase than using the common amorphous carbon phases.
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300.
5.1 Use of Aligned UTLC Substrates in Hydrodynamic Chromatography

Hydrodynamic Chromatography (HDC) is a liquid chromatography variant with a separation based upon size. HDC produces an elution order similar to SEC but without the flow of analytes through a porous packing. Instead, HDC separates particles or large macromolecules through a laminar flow profile across a capillary.\textsuperscript{1,2} This laminar flow has a faster velocity at the center of the capillary and slower velocities near the edges. Smaller particles or macromolecules have access across the entire capillary whereas larger particles are sterically hindered from approaching the capillary wall.\textsuperscript{2} This laminar flow causes larger particles to elute more quickly than smaller particles. An illustration of the HDC separation mechanism is given in Figure 5.1.
Figure 5.1 Illustration of the laminar flow in a capillary separating particles with different sizes due to larger particle sampling the central volume of the capillary.
Although SEC is more common, there are many advantages that make investigations into HDC worthwhile. The molecular sieving mechanism employed in SEC is less attractive for larger macromolecules or particles. The large pore sizes necessary to discriminate between larger analytes suffer from poor mass transfer and homogeneity of flow due to the deep pools of stagnant mobile phase in the large pores. Additionally, larger macromolecules are known to degrade more often in SEC due to shear stresses formed by the intricate pore network and must be operated at low flow rates to attempt to avoid this issue. HDC is thus able to separate larger macromolecules much more quickly than SEC since slow mass transfer in particles or shear stresses causing degradation are not present.

Packed, non-porous, columns have been used in HDC but have been replaced by open-tubular designs. Packed columns provide higher flow resistance as well as a multi-path flow, which can be detrimental to separation efficiency. Use of open-tubular designs avoids these problems, leading to improvements in resolution and reduction in sample and solvent consumption for each experiment. However, bundles of capillaries have also been shown to perform well as HDC substrates. Polystyrene beads have been separated using pillar arrays and polystyrene macromolecules have been separated using bundles of glass rods.

The aligned electrospun substrates have morphologies similar to a bundle of polymer rods or fibers. Solid polymeric fibers are arranged along a common axis
providing linear channels for solvent flow. The following equation describes the relationship between migration time of particles and the size of the channels, neglecting solute wall interactions:

\[
\frac{t_p}{t_m} = \frac{1}{1 + 2\lambda - C\lambda^2}
\]  

(5.1)

where \( t_p \) and \( t_m \) are elution times for the particle and a low molecular weight solute respectively, \( \lambda \) is the aspect ratio of the analyte diameter to flow channel diameter, and \( C \) is most often 1 for models based upon Pouiseille flow.\(^7\) By using the migration times for the particles using the aligned electrospun substrates, an estimation of the interfiber channel diameters can be calculated. Another advantage of using electrospun substrates in HDC is that changing the electrospun fiber diameters can change the interfiber channel diameter fairly readily. The electrospun fiber diameter increases by increasing the concentration of the polymer in the electrospinning solution as discussed in Chapter 3. Additionally, the fabrication of electrospun substrates is facile and inexpensive compared to coating capillaries or designing micropillar arrays, and changing the channel diameter is also easier. By using polyhydroxybutyrate the advantage of having a biodegradable phase is possible which further enhances using electrospun substrates in HDC. Thus, it would be worthwhile to conduct a series of HDC experiments using aligned substrates with both particles and macromolecules.

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5.2 Further Ordered Carbon Works

The preliminary work contained in Chapter 4 describes the change in interactions the carbon PSP has with solutes due to variations in molecular orientation. The study showed a clear difference in the dipolar nature of the carbons due to their orientation as well as a significant difference in their hydrogen bonding activity. More work is needed to verify these results. Firstly, more analytes need to be included in the study. The covariance matrix given in Table 4.2 shows a high degree of correlation between $\alpha$ and $\beta$, $\pi$ and R, and V and R. Including the additional analytes shown in Table 5.1 below would decrease these correlations giving the new covariance matrix shown in Table 5.2.\textsuperscript{8,9} As Table 5.2 illustrates, the addition of the extra solutes decreases the correlations of the solute descriptors. The only significantly large correlation exists between $V_x$ and R, which as stated previously, is very difficult to avoid since increases in molar volume correlate with increases in the polarizability of the molecule.
<table>
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<th>Solute</th>
<th>$V_x$</th>
<th>$\pi_2$</th>
<th>$\beta_2$</th>
<th>$a_2^*$</th>
<th>$R$</th>
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<tr>
<td>o-cresol$^a$</td>
<td>0.916</td>
<td>0.86</td>
<td>0.3</td>
<td>0.52</td>
<td>0.84</td>
</tr>
<tr>
<td>2-fluorophenol$^a$</td>
<td>0.793</td>
<td>0.69</td>
<td>0.26</td>
<td>0.61</td>
<td>0.66</td>
</tr>
<tr>
<td>2-chlorophenol$^a$</td>
<td>0.897</td>
<td>0.88</td>
<td>0.31</td>
<td>0.32</td>
<td>0.853</td>
</tr>
<tr>
<td>3-bromophenol$^a$</td>
<td>0.95</td>
<td>1.15</td>
<td>0.16</td>
<td>0.7</td>
<td>1.06</td>
</tr>
<tr>
<td>4-bromophenol$^b$</td>
<td>0.95</td>
<td>1.17</td>
<td>0.2</td>
<td>0.67</td>
<td>1.08</td>
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<tr>
<td>p-nitrophenol$^a$</td>
<td>0.949</td>
<td>1.72</td>
<td>0.26</td>
<td>0.82</td>
<td>1.07</td>
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<tr>
<td>m-nitrophenol$^b$</td>
<td>0.95</td>
<td>1.57</td>
<td>0.23</td>
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<td>o-nitrophenol$^a$</td>
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<td>1.05</td>
<td>0.37</td>
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<td>1.015</td>
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<tr>
<td>4-methoxyphenol$^a$</td>
<td>0.975</td>
<td>1.17</td>
<td>0.48</td>
<td>0.57</td>
<td>0.9</td>
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**Table 5.1** Solute descriptors for additional analytes (a: from Werlich *et al.*, b: from Bui *et al.*)
<table>
<thead>
<tr>
<th></th>
<th>$\pi_2$</th>
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<th>$\beta_2$</th>
<th>$V_x$</th>
<th>$R$</th>
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<tr>
<td>$\alpha_2^*$</td>
<td>0.350</td>
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<tr>
<td>$\beta_2$</td>
<td>-0.193</td>
<td>0.052</td>
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<tr>
<td>$V_x$</td>
<td>0.204</td>
<td>-0.142</td>
<td>0.231</td>
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<tr>
<td>$R$</td>
<td>0.428</td>
<td>0.109</td>
<td>0.167</td>
<td>0.727</td>
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</tr>
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</table>

Table 5.2: Covariance matrix for solutes in Table 5.1 and Table 4.1.
The addition of the extra solutes also would increase the robustness of the model by increasing a larger spread in solute descriptors for the solutes. It is important that each solute descriptor has a range of values to ensure that the model is general and applicable to more situations than the one described herein. There are large gains in spread for the $\pi_2$ (four times larger) and $\alpha_2^*$ (doubled) terms which should improve the quality of the model.

It may also be worthwhile to conduct experiments with different mobile phases for each of the carbon types. Using a single mobile phase across different stationary phases provides LSER coefficients that describe solute interaction changes only with changing stationary phases. However, in reversed-phase liquid chromatography the retention factor of a solute is related to the mobile phase composition according to the following equation

$$\log(k) = \log(k_w) - S\phi$$  \hspace{1cm} (5.1)

where $k$ is the solute retention factor, $k_w$ is the solute retention factor in water only, $\phi$ is the volume fraction of organic solvent, and $S$ is a constant related to the susceptibility of retention to the changes in organic content.\textsuperscript{10} Combining this equation with the typical LSER equation results in a nearly linear relationship between the LSER coefficients and the mobile phase composition.\textsuperscript{10}
Additional analyses can then be conducted since the slopes for plots of LSER coefficients versus $\phi$ should be similar for stationary phases that have similar retention mechanisms, as discussed by Carr et al.\textsuperscript{10} As such, plots such as the one given in Figure 5.2 can be constructed. As shown in Figure 5.2, the slope for the polystyrene divinyl benzene resin is much smaller than for the other stationary phases, which the authors attribute to this phase having a more adorption-like partitioning process than for the other phases.\textsuperscript{10} By using different mobile phases, perhaps comparisons can be made to previous work by Carr et al. using porous graphitic carbon stationary phases that are composed of amorphous carbon.\textsuperscript{11} He describes work with a system that is 36% tetrahydrofuran in water, which should be possible to replicate here. These extra experiments outlined will provide increased robustness of the model as well as comparisons to previous work, which will further validate the results.
Figure 5.2 Plots for changes in LSER coefficients for changing acetonitrile content in the mobile phases for ○ octadecyl polysiloxane, ● polydivinyl benzene-zirconia, □ phenyl siloxane, ■ polystyrene-zirconia, △ polydivinyl benzene resin. (Reprinted with permission from (Zhao, J.; Carr, P. Anal. Chem. 1998, 70, 3619-3628.), copyright (1998) American Chemical Society.)
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