Engineering polyanhydride microspheres for the stabilization and controlled release of proteins

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Engineering polyanhydride microspheres for the stabilization and controlled release of proteins

by

Amy Sywassink Determan

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

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Ames, Iowa
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For the Major Program
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ABSTRACT

This work investigated the use of polyanhydride microspheres as drug delivery carriers for therapeutic proteins. The specific polyanhydrides investigated were poly(sebacic anhydride) and copolymers of poly[1,6-bis(\(\rho\)-carboxyphenoxy)hexane] (poly(CPH)) and poly(SA), 20:80 (CPH:SA), 50:50 (CPH:SA), and 80:20 (CPH:SA). The model protein bovine serum albumin (BSA) was encapsulated in poly(SA), 20:80 (CPH:SA), 50:50 (CPH:SA), and 80:20 (CPH:SA) microspheres to determine the feasibility of using polyanhydrides as protein carriers. Poly(SA) and 20:80 (CPH:SA) microspheres were found to stabilize the encapsulated BSA and were used for all future studies. The compatibility of three proteins (ovalbumin, lysozyme, and tetanus toxoid) with polyanhydride or polyester degradation products was investigated. This work provided a rational approach for selecting compatible protein/polymer systems prior to encapsulating a protein in a polymeric microspheres. The role of the microsphere fabrication process on protein stability was studied by encapsulating ovalbumin in microspheres fabricated by four different fabrication techniques (water-oil-water, water-oil-oil, soli-oil-oil, and a cryogenic atomization method). The in vitro release kinetics, encapsulation efficiencies, and structural stability of the encapsulated and released ovalbumin were investigated. The cryogenic atomization method was used in future applications. The therapeutic protein, uterocalin, was encapsulated in polyanhydride microspheres and its biological activity upon release in vitro was measured. Uterocalin released from poly(SA) and 20:80 (CPH:SA) microspheres was biologically active. This work demonstrates that polyanhydrides microspheres are suitable drug delivery devices for therapeutic proteins.
CHAPTER 1
INTRODUCTION

1.1 Introduction

Between 1980 and 2001 the food and drug administration (FDA) approved 554 new therapeutic drugs [1]. Small molecular weight drugs made up the majority of the new therapeutics approved (504), followed by recombinant proteins (40), and monoclonal antibodies (10). As recombinant DNA practices have become more routine, the number of recombinant DNA products entering the market has increased. With the increasing number of therapeutic proteins being marketed in United States each year more research is being directed towards developing superior methods of delivering proteins. Unlike low molecular weight drugs, proteins are complex three-dimensional molecules, whose functionality depends on their higher-order structure [2]. Proteins are prone to chemical (e.g., deamidation, oxidation) and physical (aggregation, precipitation, and adsorption) alterations [2-5]. The mechanisms by which proteins undergo structural alterations are protein specific; but, there are known factors that decrease the stability of proteins such as elevated temperature and moisture [3, 4, 6, 7]. Parenteral administration remains the most common method of delivering proteins; however, this method often provides no method of stabilizing the shelf life of the protein or increasing the half-life of the protein in vivo [8].

Controlled delivery devices are more advantageous than traditional methods of administering drugs. Traditional drug delivery requires repeat administration to maintain therapeutic levels. Repeat administration results in poor patient compliance and peak concentrations of the drug. If administered too frequently, fatal concentrations of the drug
may occur, while infrequent administration may result in the inability to maintain therapeutic levels of the drug. Figure 1.1 illustrates the *in vivo* drug concentration during traditional drug administration. Controlled delivery devices that initially elevate the drug concentration in the serum to therapeutic values and maintain that concentration for a prolonged period (ideally the length of the ailment) are desirable.

![Graph showing drug concentration over time](image)

**Figure 1.1.** Schematic demonstrating the *in vivo* concentration of a drug administered by traditional methods.

A vehicle that can be delivered via parenteral administration and is capable of preserving the function of native proteins while simultaneously providing controlled release *in vivo* is needed. The ability of biodegradable polymers to meet these requirements is being investigated. The biodegradable polymer that has received the most attention as a protein delivery vehicle is poly(D,L-lactide-co-glycolide) (PLGA). PLGA has been used to encapsulate and release numerous model and therapeutic proteins, such as bovine serum albumin (BSA) [9-16], lysozyme [15, 17, 18], recombinant human growth hormone (rhGH) [19-22], and recombinant human insulin like growth factor-1 (rhIGF-I) [23-25]. Because
PLGA is a bulk-eroding polymer, as soon as it is exposed to an aqueous environment the encapsulated protein is exposed to elevated moisture content. This increase in moisture can cause covalent aggregation of the encapsulated protein [6, 7]. The addition of excipients (e.g., trehalose or dextran) is needed to help prevent covalent aggregation of the encapsulated protein [12, 13, 26]. As PLGA degrades, the pH within the polymeric device drops significantly, providing a less than ideal microclimate for the protein [27]. Co-encapsulating basic moieties, such as antacids, are needed to stabilize the encapsulated protein [9].

Polyanhydrides are biodegradable polymers that have also been investigated as protein carriers [28]. Polyanhydrides are more hydrophobic than PLGA. Polyanhydrides are surface eroding polymers as a result of their increased hydrophobicity. By eroding from the surface inward the moisture level that an encapsulated protein is exposed to is minimized, reducing moisture induced protein aggregation [29-32]. As polyanhydrides degrade, the pH surrounding the degrading device does not drop as severely as in PLGA, providing a more suitable microclimate for encapsulated and released proteins [31].

For parenteral formulations, microspheres are the most commonly used controlled delivery vehicle to encapsulated proteins or low molecular weight drugs. Microspheres can be fabricated by three different procedures: hot melt, solvent removal, and spray drying [33-40]. The hot melt procedure is not advantageous when encapsulating proteins, because the elevated temperatures needed to melt the polymer can denature proteins. Solvent removal, either oil-in-oil (O/O) or water-in-oil-in-water (W/O/W) (also known as the double emulsion methods), and spray drying can be performed at or below room temperature. While spray drying requires the use of an atomizer the solvent removal techniques require no special equipment. Care must be taken when encapsulating proteins using a solvent removal
technique because the presence of a water/oil interface can cause protein inactivation (e.g., denaturation) [41].

When encapsulating a protein in a polymer microsphere, there are three stages in which the protein must maintain its stability: microsphere fabrication, storage, and release of the protein from the eroding microspheres. A detailed understanding of each of these considerations is needed before any polymer can be used clinically for the delivery of proteins. Maintaining the functionality of the protein during the encapsulation process requires an understanding of how a specific protein denatures, and one must avoid those conditions during the microsphere fabrication process. Once the protein has been successfully encapsulated within a polymer matrix, it is necessary to characterize the interactions between the polymer matrix and the protein. By characterizing the interactions, it is possible to determine if the function of the protein has been altered (e.g., through non-covalent interactions) and if the shelf life of the protein has been extended. Finally, it is important to be able to control the release of the protein from the device by understanding: how the release of the protein will be influenced by the protein distributed within the microsphere, how the protein interacts with the degradation products of the polymer, and how the microenvironment surrounding the microsphere affects the stability and diffusion of the released protein.

The overall objective of this research is to demonstrate that polyanhydrides can be used to stabilize and provide a controlled release of proteins. Rational strategies that can be generalized and applied to several different protein/polymer systems will be developed. This will be accomplished by meeting the following specific goals:
1. Demonstrate the feasibility of using polyanhydrides microspheres as protein carriers.
2. Discern the compatibility of proteins with biodegradable polymer degradation products.
3. Study the role of microsphere fabrication methods on protein stability.
4. Apply the insights gained from specific goals 1, 2, and 3 to design uterocalin-loaded polyanhydride microspheres to expedite cell migration for applications for applications in wound healing.

The polymers to be used in this work are poly(sebacic anhydride) (poly(SA)) and copolymers of poly[1,6-bis-\(p\)(carboxyphenoxy)hexane] (poly((CPH)) and poly(SA). The repeat units of poly(SA) and poly(CPH) are shown in Figure 1.2.

a.

\[
\left[\frac{\text{O}}{\text{C}} - \left(\text{CH}_2\right)_6 \text{C} - \text{O}\right]_n
\]

b.

\[
\left[\frac{\text{O}}{\text{C}} - \text{O} - \left(\text{CH}_2\right)_6 \text{O} - \text{C} - \text{O}\right]_n
\]

Figure 1.2. The chemical structures of (a) poly(sebacic anhydride) (poly(SA)) and (b) poly[1,6-bis-\(p\)(carboxyphenoxy)hexane] (poly(CPH)).

1.2 References


CHAPTER 2
ENGINEERING SURFACE-ERODIBLE POLYANHYDRIDES WITH TAILORED MICROSTRUCTURE FOR CONTROLLED DELIVERY


2.1 Introduction

This chapter summarizes the literature concerning polyanhydride synthesis, characterization, and degradation. A section describing mechanisms of protein inactivation is included, as well as a review of protein-loaded microsphere systems including fabrication and characterization techniques, followed by a section discussing release of proteins from polymeric microspheres.

2.2 Polymer Synthesis

Butcher and Slade first synthesized polyanhydrides in 1909 [1]. Twenty years later, Hill and Carothers studied aliphatic polyanhydrides for use in textiles. They soon discovered that polyanhydrides are hydrolytically unstable (e.g., water) making them a poor choice for textiles [2, 3]. It was not until the 1980’s when Langer suggested using polyanhydrides as drug delivery devices that a practical use for polyanhydrides was identified [4]. Since the

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\textsuperscript{2} Department of Chemical and Biological Engineering, Iowa State University
\textsuperscript{3} Major professor; corresponding author
1980's, two polyanhydrides, poly(sebacic anhydride) (poly(SA)) and poly(1,3-bis(p-carboxyphenoxy) propane) (poly(CPP)), have been approved for human use by the U.S. food and drug administration (FDA) [5]. Table 2.1 shows the monomer structure of poly(SA) and poly(CPP) and other prevalent polyanhydrides used in medial applications.

Table 2.1: Common anhydride monomers used for drug delivery systems

<table>
<thead>
<tr>
<th>Structure</th>
<th>Chemical Name (Abbreviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure 1" /></td>
<td>n = 4 adipic anhydride (AA)</td>
</tr>
<tr>
<td><img src="image2" alt="Structure 2" /></td>
<td>n = 5 azelaic anhydride</td>
</tr>
<tr>
<td><img src="image3" alt="Structure 3" /></td>
<td>n = 6 suburic anhydride</td>
</tr>
<tr>
<td><img src="image4" alt="Structure 4" /></td>
<td>n = 8 sebacic anhydride (SA)</td>
</tr>
<tr>
<td><img src="image5" alt="Structure 5" /></td>
<td>n = 10 docecandioic anhydride (DD)</td>
</tr>
<tr>
<td><img src="image6" alt="Structure 6" /></td>
<td>n = 1 p-carboxyphenoxy methane (CPM)</td>
</tr>
<tr>
<td><img src="image7" alt="Structure 7" /></td>
<td>n = 3 p-carboxyphenoxy propane (CPP)</td>
</tr>
<tr>
<td><img src="image8" alt="Structure 8" /></td>
<td>n = 4 p-carboxyphenoxy valeric anhydride (CPV)</td>
</tr>
<tr>
<td><img src="image9" alt="Structure 9" /></td>
<td>n = 1 p-carboxyphenoxy acetic anhydride (CPA)</td>
</tr>
<tr>
<td><img src="image10" alt="Structure 10" /></td>
<td>n = 4 p-carboxyphenoxy valeric anhydride (CPV)</td>
</tr>
<tr>
<td><img src="image11" alt="Structure 11" /></td>
<td>n = 8 p-carboxyphenoxy octanoic acid (CPO)</td>
</tr>
<tr>
<td><img src="image12" alt="Structure 12" /></td>
<td>n = 1 p-carboxyphenoxy acetic anhydride (CPA)</td>
</tr>
<tr>
<td><img src="image13" alt="Structure 13" /></td>
<td>n = 4 p-carboxyphenoxy valeric anhydride (CPV)</td>
</tr>
<tr>
<td><img src="image14" alt="Structure 14" /></td>
<td>n = 8 p-carboxyphenoxy octanoic acid (CPO)</td>
</tr>
<tr>
<td><img src="image15" alt="Structure 15" /></td>
<td>Phenylendipropionic anhydride (PDP)</td>
</tr>
</tbody>
</table>
Table 2.1: Common anhydride monomers used for drug delivery systems (continued)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Chemical Name (Abbreviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure" /></td>
<td>Fumaric Acid (FA)</td>
</tr>
<tr>
<td><img src="image2" alt="Structure" /></td>
<td>1, 4'-cyclohexanedicarboxylic anhydride (CHDA)</td>
</tr>
<tr>
<td><img src="image3" alt="Structure" /></td>
<td>Trimellitylimido-glycine (TMA-GLY)</td>
</tr>
<tr>
<td><img src="image4" alt="Structure" /></td>
<td>Trimellitylimido-alanine (PMA-ALA)</td>
</tr>
<tr>
<td><img src="image5" alt="Structure" /></td>
<td>Trimellitylimido-tyrosine (TMA-TYR)</td>
</tr>
</tbody>
</table>

2.2.1 Melt polycondensation

Melt polycondensation reactions can be broken up into two separate steps. In the first step, dicarboxylic acids are refluxed with an excess amount of acetic anhydride resulting in prepolymer with a degree of polymerization ranging from 1 to 20. In the second step, the prepolymer is polymerized in a dry environment, under vacuum, and at elevated temperatures, typically 150-200 °C, Figure 2.1 [5].
When synthesizing poly(SA) and poly 1,6-bis(p-carboxyphenoxy)hexane (poly(CPH)), the diacids (e.g., sebacic acid and 1,6-bis(p-carboxyphenoxy)hexane), are allowed to reflux with acetic anhydride for 30 and 60 min, respectively. To purify the prepolymer, they are dissolved in chloroform and filtered. Sebacic acid is commercially available, however, CPH diacid is not. The chemistry outlining the synthesis of CPH diacid is similar to the procedure used by Conix for making CPP diacid [7].

Briefly, CPH diacid is synthesized by adding sodium hydroxide, \( p \)-hydroxybenzoic acid, and water to a reaction vessel. The mixture is stirred and heated. Once it has started to reflux, dibromohexane is added dropwise to the vessel. The contents are then allowed to reflux overnight. The white precipitate is then filtered and dissolved in warm water and fuming sulfuric acid is added. This acidifies the precipitate and the dibasic acid is collected by filtration and dried under heat and vacuum.

The second step of the melt polycondensation reaction is to polymerize the prepolymer. This is done under heat and vacuum. The polymerization of poly(SA) and poly(CPH) is done at 180 °C and at \( 3 \times 10^{-1} \) torr. The polymerization of both poly(SA) and poly(CPH) proceeds in a stepwise fashion. The molecular mass of the polymers is highly dependent on the strength of the vacuum and the purity of the diacids used in the formation of the prepolymer [5].
There are four variables that affect the molecular weight of a polymer made by melt polycondensation: the monomer purity, the temperature of the reaction, the length of the reaction, and the adequate removal of the condensation product [8]. By taking advantage of these variables, pure prepolymerized at 180 °C under a vacuum of $10^{-4}$ mm Hg for 90 minutes, Domb and Langer [8] obtained polyanhydrides with weight-average molecular weights ($M_w$) above 100,000. By over heating or extending the length of time of the reaction, the polymers became susceptible to depolymerization, resulting in a lower molecular mass product. The removal of the acetic anhydride condensation products was essential to obtain high conversions.

When catalysts such as cadmium acetate, calcium carbonate, earth metal oxides, and ZnEt$_2$-H$_2$O were used to synthesize polyanhydrides by melt condensation, higher molecular weight polymers were obtained in a shorter length of time [8]. Because all of the catalysts except calcium carbonate are toxic, catalysts are rarely employed when synthesizing polyanhydrides for biomedical use.

2.2.2 Solution polymerization

One drawback of melt polycondensation reactions is that the polymerization must take place at elevated temperatures. Some monomers are heat sensitive (e.g., dipeptides and therapeutically active diacids) and others have such a high melting temperature that the product may be charred [9], thus, precluding the use of melt condensation reactions. An alternative to melt condensation reactions is solution polymerization in which the dicarboxylic acid is polymerized in one step at room temperature. Schotten-Baumann
condensation and dehydrative coupling are two common mechanisms for solution polymerization [9-12].

Schotten-Baumann condensation, shown in Figure 2.2, is essentially the dehydrochlorination between a diacid chloride and a dicarboxylic acid. The diacid and the base, such as triethylamine or tri-n-propylamine, are dissolved in an organic solvent. To this solution, the diacid chloride is added dropwise and allowed to stir at room temperature until the reaction is completed (~1 h). To precipitate the polymer petroleum ether is added. Polymers with number average molecular weight ($M_n$) in the range of 2000 to 5000 have been reported using Schotten-Baumann condensation [9].

![Figure 2.2. The Schotten-Baumann mechanism used to polymerize polyanhydrides via a solution polymerization. Reprinted with permission from ref. [6].](image)

Polyanhydrides can also be polymerized in solution by using dehydrative coupling agents at room temperature. Mestres et al. [10] and Cabre-Castellvi et al. [11] reported the formation of anhydrides by reacting $N,N$-bis[2-oxo-3-oxazolidinyl]phosphorodiamidic chloride with carboxylic acids. Leong et al. [9] later used bis[2-oxo-3-oxazolidinyl]phosphinic chloride, $N$-phenylphosphoroamidochloridate, and diphenyl chlorophosphate to synthesize polymeric anhydrides from dicarboxylic acids such as sebacic acid and 1,4-pheylhylenedipropionic acid. Bis[2-oxo-3-oxazolidinyl]phosphinic chloride and $N$-phenylphosphoroamidochloridate were more effective coupling agents, resulting in polymers with $M_n$ of 3000, while diphenyl chlorophosphate had a lower coupling activity.
leading to discolored lower molecular weight polymers. One drawback of using coupling agents is the need of an additional separation step needed to remove the catalyst derivatives that form during the reaction. This procedure often leads to the hydrolysis of the polymer [9].

Phosgene and disphosgene have also been used as coupling agents in the synthesis of polyanhydrides with the use of poly(4-vinylpyridine) (PVP) and K₂CO₃ as proton acceptors [12]. High molecular weight poly(SA) \( (M_n = 16000) \) was obtained when a heterogeneous acid acceptor (e.g., PVP) was used, as opposed to the lower molecular weight polymer that was obtained when the non-amine heterogeneous base K₂CO₃ was used.

2.2.3 Ring opening reactions

In the 1930's, Hill and Carothers studied aliphatic polyanhydrides formed using the diacids HOOC-(CO₂)ₙ-(COOH), \( (n \text{ ranged from 4 to 12}) \) and the different transformations to which these polymers were susceptible [2, 3]. When the diacids were reacted with acetic anhydride linear low molecular weight polymers resulted (denoted \( \alpha \)-anhydride). To increase the molecular weight of the \( \alpha \)-anhydride, it was subjected to a molecular distillation. During this process a higher molecular weight polymer formed (\( \omega \)-anhydride) and cyclic monomers and dimers were distilled off (\( \beta \)-anhydride). The \( \beta \)-anhydride was converted to a higher molecular weight polymer on standing (Figure 2.3). Using an aniline reaction, Hill and Carothers [3] were able to determine if the products were cyclic monomers, dimers, or aliphatic polymers. When aniline was reacted with a monomer only one product resulted, acid monoanilide, however, when aniline was reacted with a dimer or a polymer there were three possible products: dibasic acid, acid monoanilide, and acid dianilide [2, 3].
Albertsson and Lundmark [13] synthesized poly(adipic anhydride) (poly(AA)) by first preparing oxepane-2,7-dione and depolymerizing it using two different ring opening mechanisms. In the first, the oxepane-2,7-dione prepared by reacting adipic acid with acetic anhydride was dissolved in methylene chloride and allowed to react with ZnCl₂ (1% w) for 6 h at room temperature. The resulting poly(AA) had a \( M_n \) of 1700 [14]. The second mechanism that they employed was to catalytically depolymerize oxepane-2,7-dione under vacuum. The concentration of the catalyst (stannous 2-ethylhexanoate), the reaction time, and temperature were all found to influence the depolymerization. The resulting poly(AA) had a \( M_n \) no greater than 5000. By studying the stannous 2-ethylhexanoate reaction with \(^1\)H-NMR and infrared spectroscopy (IR), it was deduced that a non-ionic insertion polymerization mechanism was occurring initially in the reaction. However, after 2 h at 80°C the anhydride exchange was the dominating reaction [13].

![Figure 2.3. Mechanism of polyanhydride formation using ring opening polymerization. Reprinted with permission from ref. [6].](image)

More recently, poly(AA) has been synthesized by ring opening polymerization initiated by dibutylmagnesium, potassium poly(ethylene glycol)ate, and aluminum triisopropoxide respectively [15-17]. The highest molecular weight was achieved using aluminum triisopropoxide as the initiator, and a \( M_n \) of 58,000 was obtained [16]. The high
molecular weight polymer was polymerized in bulk at 80°C in the presence of the initiator and nicotine (nicotine acts as a Lewis base).

2.3 Polymer Characterization

In order to optimize the performance of polyanhydrides as biomaterials, their chemical composition, molecular weight, degree of crystallinity, and thermal properties need to be characterized. These are discussed below.

2.3.1 Chemical composition

When copolymerizing aliphatic and aromatic monomers there are three different covalent bonds that could result in the polymer backbone: aliphatic-aliphatic, aliphatic-aromatic (or aromatic-aliphatic), and aromatic-aromatic [18, 19]. These different bonds are each represented in the $^1$H-NMR spectra of a copolymer, thus, making $^1$H-NMR a useful analytical technique for characterizing the chemical composition. In the model system poly(CPP:SA), the copolymer has a doublet at 8.1 and 8.0 ppm ($J=8.7$ Hz) and two triplets at 2.6 and 2.4 ppm ($J=7.4$ Hz), while the SA monomer has one triplet at 2.4 ppm and the CPP monomer has one doublet at 8.1 ppm [19]. Figure 2.4 shows how the $^1$H-NMR spectra of poly(CPP:SA) changes as the ratio CPP to SA is varied. The additional peaks in the copolymer are attributed to long-range deshielding and shielding effects of the additional bonds found in the copolymer. The peak at 2.6 ppm is a result of the aliphatic-aromatic bond between CPP-SA in the copolymer that has undergone an upward shift, while the peak at 8.0 ppm is a result of the aliphatic-aromatic bond between CPP-SA that undergoes a downward shift. Using the frequency of specific bonds between aliphatic and aromatic monomers the
reactivity ratio of aliphatic and aromatic monomers, can be determined. A reactivity ratio of one for SA monomers with CPP and CPH has been reported [19, 20].

$^{1}$H-NMR can also be used to determine the number average sequence length by integrating the area under peaks specific to each particular monomer. Thus, $^{1}$H-NMR can be used to: verify the percent conversion of the polymerization [21], determine the exact composition of copolymers [19, 22, 23], monitor the rate of degradation [24], and determine the degree of randomness of the copolymer (e.g. if it has a block like or random sequence distribution). The sequence distribution is dependent on the ratio of monomers in the copolymer. In the CPP:SA and CPH:SA copolymer systems, the dominating monomer has a longer number average sequence length and the copolymer displays a block like sequence distribution [19, 23]. When no monomer dominates (50:50 (CPP:SA) or (CPH:SA)) the distribution of the monomers mimics an alternating copolymer.

Fourier transform infrared (FTIR) and Raman spectroscopy have also been used to characterize homopolymers [9-12, 18, 21, 25-29], determine monomer content in copolymers [18, 20, 27, 30-32], study reactions between the anhydride bond and amines [33, 34], and study the degradation process of polyanhydrides [31, 35-38]. Aliphatic polyanhydrides display an anhydride doublet at 1740 and 1810 cm$^{-1}$, while aromatic polyanhydrides display an anhydride doublet at 1720 and 1780 cm$^{-1}$, as shown in Figure 2.5. When aliphatic and aromatic polyanhydrides are copolymerized, two distinct peaks are observed at 1810 cm$^{-1}$ and 1780 cm$^{-1}$, and a third distinct peak is observed between 1720-1740 cm$^{-1}$. As the polyanhydrides undergo hydrolysis the anhydride doublet disappears and is replaced by a peak at 1700 cm$^{-1}$ corresponding to carboxylic acid [39].
Figure 2.4. 1 H NMR spectra of poly(CPP:SA) copolymers, from ref [19].
FTIR was used to study the degradation properties of poly(fumaric anhydride:sebacic anhydride) poly(FA:SA) copolymers in three different buffers (0.1M citrate pH 4.2, phosphate pH 7.4, and tris pH 8.8 buffers) [36]. In this study, the ratio of the areas under the anhydride (1860-1775 cm\(^{-1}\)) and carboxylic acid (1770-1675 cm\(^{-1}\)) peaks were plotted as a function of time. From this information, the amount of free acid formation was also calculated. This study helped support the theory that crystalline regions degrade slower than amorphous regions and that polyanhydrides degrade faster in alkaline solutions [36].

![FTIR spectra of aliphatic polyanhydride (SA) and aromatic polyanhydride (CPH).](image)

Figure 2.5. FTIR spectra of aliphatic polyanhydride (SA) and aromatic polyanhydride (CPH).

2.3.2 Molecular mass

Gel permeation chromatography (GPC) and viscosity measurements have been used to determine the molecular weight of polyanhydrides [19]. The weight average molecular weight (M\(_w\)) ranges from 2,000 to 300,000 and the intrinsic viscosity (in chloroform) ranges from 0.13 to 1.25 dL/g. The viscosity of the polymers was measured using an Ubbelohde
viscometer. The Mark-Houwink relationship for poly(CPP:SA) was determined from the viscosity data and the $M_w$ values determined by using polystyrene standards.

$$ [\eta]_{CHCl_3}^{23^\circ C} = 3.88 \times 10^{-7} M_w^{0.658} \tag{1} $$

From the Mark-Houwink relationship of polystyrene and poly(CPP:SA), a universal calibration curve was obtained that allows for direct determination of $M_w$ of polyanhydrides without determining the viscosity of the polymer. All the experimental $M_w$ data collected were found to fit equation 1 [19].

2.3.3 Crystallinity and thermal properties

Differential scanning calorimetry (DSC) has been used to report the thermal properties of polyanhydrides. The glass transition temperature ($T_g$), the melting temperature ($T_m$), and the heat of fusion of the copolymers (CPH:SA), (CPP:SA), (FA:SA), trimellitylimidoglycine:CPH (TMA-gly:CPH), (TMA-gyl:SA), pyromellitylimidoalanine:SA (PMA-ala:SA), and (PMA-ala:CPH) and the individual homopolymers have been reported [22, 36, 40-42]. A typical trend of the $T_g$ is that as the chain length increases, the $T_g$ decreases; this is a result of the increased molecular motion due to the increased flexibility of the polymer chain. It is essential to know the $T_g$ and the $T_m$ values for polyanhydrides because they are important parameters for fabricating drug delivery devices. For example, $T_g$ is the minimum temperature needed for compression molding while $T_m$ is the minimum temperature needed for injection molding or melt pressing [35]. The $T_g$ and $T_m$ for poly(SA) are $62^\circ C$ and $79^\circ C$, respectively [40]. The $T_g$ and $T_m$ for poly(CPH) are $32^\circ C$ and $140^\circ C$, 
respectively [8]. The $T_g$ and $T_m$ of copolymers of poly(CPH) and poly(SA) fall between the values of the homopolymers [40].

The degree of crystallinity of polyanhydrides is an important factor in the rate of polymer erosion. The degree of crystallinity of both homopolymers and copolymers of polyanhydrides has been determined using X-ray diffraction [26, 36, 43, 44], differential scanning calorimetry (DSC) [26, 36, 44-46], small angle X-ray scattering (SAXS) [44, 47], and $^1$H-NMR [26]. X-ray diffraction can be used to determine the crystallinity of a polymer only when a clear separation between the amorphous halo and the crystalline pattern exists. When such a clear separation is not available, DSC and $^1$H-NMR methods are usually employed.

The degree of crystallinity for the homopolymers poly(SA), poly(CPP), and poly(CPH) are 67%, 61%, and 20% respectively [26]. When these monomers are copolymerized, the more prevalent monomer in the copolymer determines the crystal structure, and when a non-crystalline monomer is polymerized with a crystalline monomer, a decrease in the crystallinity results [26, 40]. When an equal monomer ratio of SA and CPP were copolymerized, the resulting copolymer was amorphous; this was a direct result of the random presence of both monomer units in the polymer chain. However, when an equal monomer ratio of CPH and SA were copolymerized the resulting polymer had a degree of crystallinity between poly(SA) and poly(CPH); this was to be expected because poly(CPH) has a lower degree of crystallinity than poly(SA). Kipper et al. [48] used time-resolved SAXS to study the isothermal crystallization kinetics of homopolymers and copolymers of poly(CPH) and poly(SA). This work provided new insights on the crystal morphology via measurements of lamellar dimensions.
2.3.4 Stability

The stability of aliphatic and aromatic polyanhydrides was examined in solution and solid state [49]. Aromatic polyanhydrides such as poly(CPH) and poly(CPP) maintained their original molecular weight both as a solid (under dry argon and under vacuum) and in solution for over a year. Aliphatic polyanhydrides, poly(SA), underwent molecular weight loss both in solution and in the solid state. Aliphatic-aromatic copolymers were found to undergo molecular weight loss according to their monomer content, the more aromatic monomer present the more stable the copolymer. The depolymerization of the aliphatic polyanhydrides followed first-order kinetics, with an activation energy of 7.5 kcal/mol. The depolymerization of the polymers was found not to be a result of hydrolysis but rather an internal anhydride exchange. This result was justified by the repolymerization of the depolymerized polymer, by heating to 180ºC for 20 min. From ¹H NMR data it was confirmed that the depolymerized polymer repolymerized. If it had undergone hydrolysis this would not have been possible [49].

Polyanhydrides that are to be used as drug delivery devices must be sterilized before they can be used in vivo. It is important that the sterilization process not alter the physical properties of the polymer, thus altering the polymers’ degradation profile. γ-irradiation and ethylene oxide are two methods that can be used to sterilize polyanhydrides [38, 50]. γ-irradiation at a dose of 2.5 Mrad was found not to affect the molecular weight of aliphatic polyanhydrides; however, the molecular weight of polymers containing an anhydride conjugated to unsaturated bonds did show molecular weight loss after undergoing γ-irradiation [50]. The decrease in molecular weight was due to the conversion of a fully unsaturated anhydride bond to a more stable half-saturated anhydride bond. This internal
anhydride exchange lead to the formation of low molecular weight polymer rings that hydrolyzed faster than the original polymer.

Leach et al. [38] used ethylene oxide to sterilize double-walled microspheres of poly(CPP:SA) and poly(lactic acid). The sterilization caused some degradation of the polyanhydride core, as detected by an increase in oligomers and carboxylic acid groups by DSC and FTIR respectively. Prior to sterilization, 93% of the sample had a $M_w$ greater than 2000 and after the sterilization only 52% of the sample had a $M_w$ greater than 2000. The sterilization was performed under humid conditions; thus, the polyanhydrides probably underwent hydrolysis due to the content of the medium and not the ethylene oxide [38].

2.3.5. Biocompatibility

Polyanhydrides have been extensively studied as a potential biomaterial for site directed drug delivery. However, before polyanhydrides could be used clinically, their biological inertness had to be verified. The biocompatibility of polyanhydrides has been studied in the brains of rats, monkeys, and rabbits, subcutaneously in rats, and in the corneas of rabbits [33, 51-60]. In all studies, the localized inflammatory response was minimal. The inflammatory response to the polyanhydride implants was comparable to other surgical implants, even when large doses (40 to 120 times greater than would be used in humans) were used [51, 55, 56].

Because the brain is protected from foreign substances or toxins by the blood brain barrier, it is exceptionally difficult to administer drugs to ailments located within the brain [61]. Due to the limited effectiveness of drugs on ailments such as brain cancer by traditional drug delivery methods, the use of site directed drug delivery in the brain using
polyanhydrides was investigated. After undergoing phase I, II, and III human clinical trials, the copolymer 20:80 poly(CPP:SA) became the first surface eroding polymer to be approved by the FDA for use in humans [62]. The polyanhydride implant is a wafer composed of 20:80 poly(CPP:SA) encapsulating 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), a chemotherapeutic agent. It is marketed under the name Gliadel™ and is used for the treatment of glioblastoma multiforme, a fatal form of brain cancer [63].

Domb et al. [64] studied the in vivo elimination of a 20:80 poly(CPP:SA) implant in rat brains by radiolabelling each of the monomers separately. The majority of poly(SA), 40%, exited the rat as CO₂, with minimal excretion of polymer in urine (10%) and feces (2%). At the completion of the study (7 days), 10% of the radiolabelled polymer remained at the site of the implant with the remainder of the radiolabelled polymer in the process of being metabolized. Poly(CPP) was not expired as CO₂. The main route of elimination for CPP in seven days was in urine (~1%) and feces (~3%).

2.4 Structures

The term “polyanhydride” describes a polymer containing an anhydride bond in each “mer” of its backbone, yet the term does not provide any additional information about the backbone. Polyanhydrides of varying structures, molecular weights, degrees of crystallinity, hydrophobicity, and mechanical strength have been synthesized. Each of these characteristics is a function of the polymer backbone. Several different polyanhydride structures, and the properties of these particular polymers will be discussed.
2.4.1 Aliphatic

Poly(SA) is one of the most commonly studied polyanhydrides. It was first synthesized in 1932 by Hill and Carothers [3] and has since been approved by the FDA for use as a biomaterial. However, poly(SA) is only one of many saturated aliphatic polyanhydrides all of which have similar properties. Saturated aliphatic polyanhydrides are crystalline and rigid. The length of the diacid monomer influences the polymer's melting temperature, solubility in common organics, and the degradation time of the polymer. As the length of the diacid increases, the melting temperature of the polymer increases (the melting temperatures for aliphatic polyanhydrides are usually below 100°C). As the length of the diacid monomer increases, the solubility of the polymer in common organic solvents and the rate of degradation decreases [57]. Domb et al. [57] reported that enzymes do not affect the rate of degradation of aliphatic polyanhydrides; instead the rate was solely dependent on the rate of hydrolysis. Aliphatic polymers are degraded and eliminated from the body within weeks. Aliphatic polyanhydrides showed a decrease in molecular weight over a one year period when stored in either solid state or in an organic solution [18].

2.4.2 Aromatic

Aromatic polyanhydrides are more hydrophobic than aliphatic polyanhydrides. The increase in hydrophobicity increases the polymer stability. Aromatic polyanhydrides studied for a year showed no molecular weight loss in either solid state or in an organic solvent [18]. The increase in hydrophobicity decreased the degradation rate of the polymers. The degradation rate of aromatic polymers was on the time scale of years [65]. Aromatic polyanhydrides were less soluble in organic solutions and have melting temperatures
significantly higher than aliphatic polymers. Aromatic homopolymers are rarely used due to their poor solubility in organics, lengthy degradation time, and their high melting temperatures [45]. The solubility of aromatic polyanhydrides can be improved by altering the ring substitution pattern from para to ortho [66-68].

Aromatic diacids separated by branched aliphatic spacers were synthesized and copolymerized with SA [69]. The new polymers fabricated were: poly(1,2-bis(4-carboxyphenoxy)propane-co-SA), poly(1,3-bis(4-carboxyphenoxy)-2-methyl propane-co-SA), and poly(1,3-bis(4-carboxyphenoxy)-2,2-dimethly propane-co-SA). The polymers had a low solubility in organic solvents. A fluorinated aromatic polyanhydride was synthesized by melt condensation of 4,4'-(hexafluoroisopropylidene)bis benzoic acid [70]. The fluorinated polymer had a high solubility in organic solvents and had $M_w$ in the range of 15,000-18,000.

2.4.3 Unsaturated

Fumaric acid (FA), acetylenedicarboxylic acid (ACDA), and 1, 4'-stilbendicarboxylic acid (STDA) were polymerized as unsaturated polyanhydrides by either melt or solution polymerization [29]. The structure of the polymers produced was $\text{-[-(CO-CH=CH-COO-)-]_x-(-CO-R-COO-)-]_y-\text{n}}$. Infrared spectroscopy (IR) and NMR confirmed the existence of the double bond after the polymerization. The unsaturated homopolymers were insoluble in common organic solvents and water. The polymers were also highly crystalline. Copolymers of fumaric acid and aliphatic diacids resulted in less crystalline polymers that were soluble in chlorinated hydrocarbons. The $M_w$ of all polymers studied ranged from 10,000 to 30,000 [29].
2.4.4 Aliphatic-aromatic homopolymers

Homopolymers based on aliphatic-aromatic diacids (5-(p-carboxyphenoxy)valeric acid (CPV), 8-(p-carboxyphenoxy)octanoic acid (CPO), and 2-(p-carboxyphenoxy)acetic acid (CPA)) were synthesized by melt and solution polymerization residue [18]. An aromatic head and an aliphatic tail characterized the diacids, as shown in Table 1. The maximum $M_w$ obtained was 44,600 and the polymers had low melting temperatures. The stability of the polymers was studied both in solid state and in chloroform. The polymers did not undergo any molecular weight loss in solid state during a 6-month period, however, they did undergo molecular weight loss when stored in chloroform for the same amount of time. The degradation rate of the homopolymers was dependent on the length of the alkaloid acid.

2.4.5 Amino acid-based

Polyanhydrides have poor mechanical properties, preventing them from being used as sutures or as orthopedic devices. To overcome this shortcoming, amide and imide bonds were incorporated into the backbone of polyanhydrides. The first step in the polymerization of amino acid based polyanhydrides was to convert the amino acid (either naturally occurring or $\omega$-amino acids) into a diacid by condensation with trimellitic anhydride [32, 42]. The diacid was then polymerized forming mixed anhydrides and prepolymer, and was further polymerized by either solution or melt polycondensation reactions [22, 41, 42, 71, 72].

Poly(anhydride-co-imides) based on natural occurring amino acids, $\omega$-amino acids, sebacic acid, and 1,6-bis(p-carboxyphenoxy)hexane were studied [22, 32, 41, 42], see Table 2.1. By incorporating an imide into the backbone of either poly(CPH) or poly(SA), the mechanical properties of the polymer were enhanced. Poly(anhydride-co-imides) containing
a high aromatic or imide content were only soluble in polar solvents, while polymers with a high aliphatic or low imide content were more soluble in common organic solvents. Increasing the molar percent of imide in a copolymer caused a decrease in the molecular weight of the polymer [22]. In polymers containing aliphatic anhydrides the incorporation of imides increased the polymer hydrophobicity, however, little to no change was seen when the imide was incorporated into an aromatic backbone.

Glycine, β-alanine, γ-aminobutyric acid, L-leucine, and L-tyrosine have all been incorporated into N-trimellitylimido acid polymers [32]. The incorporation of an imide in the backbone of N-trimellitylimido acid polymers produced brittle polymers with a $M_w$ of less than 10,000. Incorporating an aliphatic spacer, sebacic acid, produced higher molecular weight polymers. As the content of the aliphatic spacer increased the polymer’s solubility in common organic solvents increased, and the tensile strength of the polymer decreased.

The *in vivo* degradation and biocompatibility of compression molded disks[TMA-gly:CPH (10:90, 30:70, and 50:50) and PMA-ala:CPH (10:90 and 30:70)] implanted subcutaneously in rat tissues were examined [58, 73]. PMA-ala:CPH caused minimal inflammation and fibrosis. TMA-gly:CPH (10:90 and 30:70) caused mild inflammation while the 50:50 composition induced a moderate inflammatory response. The difference in the histological response was a result of the increased degradation rate of 50:50 (14 days) compared to 10:90 and 30:70 (60 days). The PMA-ala:CPH and TMA-gly:CPH (10:90 and 30:70) disks maintained their shape throughout the two month study [73].
2.4.6 Fatty acid-based

Fatty acids are naturally occurring hydrophobic materials that if incorporated into polyanhydrides would increase the hydrophobicity and degradation time of the polymers. Fatty acids have been incorporated into the backbone of polyanhydrides in two ways: 1) by converting the monofunctional fatty acid into a diacid fat based monomer and 2) by using the monofunctional fatty acid as a chain terminator [50, 74-79].

Using the first approach, two unsaturated fatty acids, oleic acid and erucic acid, were made into dimers [79]. The fatty acid dimers contained two carboxylic acids that were further polymerized by either melt of solution polymerization [78-81]. The dimers themselves formed liquid oil, and homopolymers of the fatty acids are viscous liquids with a $M_w$ of 133,000 [82]. When copolymerized with sebacic acid, the $M_w$ was increased to 235,000 [77].

Linear fatty acids (C8-C18) have been added to the terminal position of poly(SA) [50, 83]. The resulting fatty acid terminated polymer had a waxy off-white appearance [50, 83] with $M_w$ in the range of 5,000 to 9,000 (for 30% and 10% w/w fatty acid respectively). The higher the fatty acid content in the polymer the lower the molecular weight, due to the shortening of the polymer chain. The addition of the fatty acid at the terminal position increased the hydrophobicity of the polymer and increased the storage stability of the polymer. Fatty acid terminated poly(SA) showed no molecular weight loss when stored at 17°C or 4°C for over a year, as compared to non-terminated poly(SA) that showed significant molecular weight loss during the same period [83]. A comprehensive review of fatty acids and polyanhydrides by Kumar et al. [76] is available.
2.4.7 Poly(ester-anhydrides) and poly(ether-anhydrides)

Both random and block copolymers of poly(SA-co-ethylene glycol) have been synthesized by melt polycondensation [25, 35, 84]. The $M_w$ of both the block and random copolymers decreased as the molar percent of poly(ethylene glycol) (PEG) was increased. The presence of the PEG in the copolymer backbones increased the flexibility of the sebacic acid, which increased the crystallinity of the poly(SA) component. However, the presence of the poly(SA) in the backbone decreased the flexibility of the PEG, preventing the formation of PEG crystals. As the content of PEG in the backbone was increased from 0 to 30%, the density of the polymer decreased [84]. The mechanical strength of the random copolymer was superior to either of the homopolymers, supporting the idea that the random copolymer can prevent energy accumulation by absorbing external forces, which is particularly important for biomaterials that would be subjected to large impacts [30]. The degradation rate of the block copolymer was found to be faster than the degradation rate of poly(SA) [20]. In addition, PEG coated poly(SA) nanospheres were fabricated by the use of the diblock PEG-SA polymers [82].

Vogel and Mallapragada [85] first synthesized aromatic anhydride monomers containing ethylene glycol. Torres et al. [86] later modified the synthesis mechanism to obtain polymers that could more easily be fabricated into controlled delivery devices. These novel polymers 1,8-bis($p$-carboxyphenoxy)-6-dioxaoctane (CPTEG) and 1,14-bis($p$-carboxyphenoxy)-3,6,9,12-tertaoxatetradecane (CPOEG-5) combined the hydrophobic surface eroding properties of polyanhydrides with the more hydrophilic bulk eroding properties of ethylene glycol. The aim of incorporating PEG into an aromatic polyanhydride was to maintain a controlled release for an extended time while improving protein-polymer
interactions. By copolymerizing CPTEG with CPH, the mechanism of polymer degradation could be varied from bulk to surface erosion.

A salicylic acid-derived poly(ester-anhydride) was synthesized by a melt polycondensation reaction [28]. The backbone of the polymer was composed of alkyl chains connected to the aromatic salicylic acid groups by ester bonds (Figure 2.6). As the polymer underwent hydrolysis, the biologically active salicylic acid was released and induced new bone growth [87].

\[
\text{Scheme 2.6. The chemical structure of salicylic acid-derived poly(anhydride-esters). Reprinted with permission from ref. [6].}
\]

Triblock poly(lactic acid) (PLA) terminated poly(SA) chains have been synthesized by melt polycondensation [88]. The molecular weights of the polymers ranged from 3000 to 9000 as the content of PLA increased the molecular weight decreased. The incorporation of the PLA into the polymer backbone increased the degradation time of the polymer as compared to poly(SA). Poly(SA) completely degraded in 10 days while the triblock copolymer took 3 weeks to completely degrade.

A poly(ester anhydride) was formed by incorporating ricinoleic acid, a natural fatty acid, into the backbone of poly(SA) [89]. The hydroxyl group of ricinoleic acid reacted with the anhydride group of poly(SA) via a transesterification reaction. As the ricinoleic acid content was increased, the $T_m$ and the crystallinity decreased.
2.4.8 Crosslinking

The need for a high strength biodegradable material that can be used in orthopedic applications has lead researchers to study the mechanical properties and the degradation rates of crosslinked polyanhydrides. Crosslinking polyanhydrides resulted in high strength three-dimensional networks. Crosslinked polyanhydrides were prepared by chemical and photo crosslinking [21, 29, 90, 91]. In the case of chemical crosslinking unsaturated monomers (e.g. divenyl benzene (DVB)), and a catalyst (e.g. benzoyl peroxide (BzP)) were added to unsaturated polymers (e.g. poly(FA:SA)) and allowed to react either in solution or in bulk. The resulting crosslinked polymer was insoluble in organic solutions and decomposed at temperatures over 200°C [29].

Photopolymerization is an attractive way of crosslinking polyanhydrides because the monomers can be injected into a fracture sight and then polymerized, allowing them to take the shape of a complicated fracture or break. The anhydride monomers of SA, CPP, CPH, tricarballylic acid (TCA), and PMA-ala have been capped with methacrylates (MSA, MCPP, MTCA, and MPMA-ala) and photopolymerized to form crosslinked polyanhydrides [90, 91]. The crosslinking of the homopolymer MSA showed a two fold decrease in the degradation time over the linear homopolymer poly(SA). By copolymerizing MSA and MCPH the degradation rate of the crosslinked copolymer was varied from 2 days to over one year. MSA was also copolymerized with linear CPP, CPH, poly(methyl methacrylate) (PMMA), and 50:50 (CPP:CPH) to study the degradation rate of semi-interpenetrating polymer networks (IPNs) [90, 92]. The copolymers resulted in crosslinked MSA that had chemically independent yet physically entangled the linear polymers. The degradation rate of the IPN was significantly reduced as compared to the crosslinked homopolymer MSA. Burkoth et al.
copolymers MSA with methacrylated steric acid (MStA) and methacrylated cholesterol (MC). The rate of mass loss of the copolymers MSA:MStA and MSA:MC was significantly slower than the homopolymer MSA. The mechanical properties of all photopolymerized polyanhydrides were greatly increased as compared to linear polyanhydrides. The tensile modulus of poly(MSA) and poly(MCPP) was 40 ± 120 MPa and 640 ± 80 MPa respectively. These values were between the reported tensile modulus of the cortical and trabecular, 17-20 GPa and 50-100 Mpa, respectively [90].

Poshusta et al. [93] examined the histological effects of IPNs (MSA copolymerized with linear CPP and CPP:CPH) implanted subcutaneously in rats, and the in situ photopolymerization of MSA:MC in the rat tibia. The IPN formulation MSA:CPP invoked a heightened inflammatory response while MSA:CPP:CPH invoked a minimal response and encouraged a cellular response. The in situ photopolymerization of MSA:MC also encouraged a cellular response with no adverse effects from the reaction.

2.4.9 Branched

Random and graft branched poly(SA) were synthesized by polymerizing sebacic acid with 1,3,5 benzenetricarboxylic acid and poly(acrylic acid), as shown in Figure 2.7 [94]. The branched polymers had similar thermal and crystalline properties as linear poly(SA). The only morphological differences between the linear and branched polymers were that the branched polymers had a higher molecular weight and lower specific viscosity. The degradation rate of the two branched polymers were compared to the degradation rate of linear poly(SA), the two branched polymers degraded at relatively the same rate yet faster than the linear polymer [94].
2.4.10 Blends

When two or more polymers are physically mixed the resulting blend may display microscopic or macroscopic heterogeneity depending if the blended polymers are miscible (totally or partially) or immiscible, respectively. If the mixed polymers are miscible the polymer blend will take on new properties distinct from the individual components of the blend. Domb [77] reported that aliphatic, aromatic, and copolymer polyanhydrides were miscible and the blends had lower \( T_m \) and crystallinity than the starting polymers. The polymers were miscible independent of the molecular weight of the initial polymers. Polyanhydrides are partially miscible with poly(orthoesters), poly(hydroxybutyric acids), and low molecular weight (< 2000) polyesters. These partially miscible polymers formed uniform mixtures with polyanhydrides, yet displayed two melting temperatures, one for each of the blended polymers. Domb et al. [75, 83] also reported that polyanhydrides are immiscible with polycaprolactone or lactide-glycolide copolymers.

Blends of poly(trimethylene carbonate) poly(TMC) and poly(AA) have been studied both \textit{in vitro} and \textit{in vivo} as a potential biomaterial [31, 60]. The partially miscible blend was found to be biocompatible and contained two \( T_g \)’s corresponding to the two starting polymers. By blending poly(TMC) and poly(AA), the new polymer blend had a more acceptable degradation rate than either of the starting polymers. The poly(AA) in the
polymer blend degraded first leaving behind a porous polymer matrix, enhancing the
degradation rate of the poly(TMC).

The effects of blending poly[bis(glycine ethyl) phosphazene] (PGP) with poly(SA-co-
TMA-GLY)-block-PEG) poly(STP) (30:50:20) was investigated [95]. PGP was partially
miscible with poly(STP) as a result of hydrogen bonding. The blend had two distinct \( T_g \)'s.
As the poly(STP) content was increased, the \( T_g \) associated with PGP increased, as the content
of PGP was increased the \( T_g \) associated with poly(STP) decreased. The \textit{in vitro} degradation
of the blend was investigated, increasing the amount of poly(STP) in the blend increased the
erosion rate of the blend.

The effects of blending PEG and poly(SA) were studied using DSC and FTIR [96].
Blends with less than 10% PEG were miscible with poly(SA) while blends containing more
than 10% PEG were not miscible. In miscible blends of poly(SA) and PEG, the crystallinity
of poly(SA) was increased due to a decrease in the \( T_g \) of the poly(SA) component as a result
of the PEG.

2.5 Characterization of Polymer Erosion

The distinction between the terms “erosion” and “degradation” is important.
Degradation of a polymer is defined as the cleavage of polymer bonds either by hydrolysis or
enzymatic reactions, leading to the formation of oligomers and monomers. Degradation is
the most important step of the erosion process. As oligomers and monomers are formed by
chain scission, they dissolve in the water and eventually diffuse away from the polymer
system. Erosion is the sum of all of the above steps. To better understand and predict the
erosion process for polyanhydrides, several models have been developed. Kipper \textit{et al.} [97]
developed a model to predict the release kinetics of drugs from the model systems poly(CPH:SA) and poly(CPP:SA). The model took into account the microphase separation of the copolymer and assumed that degradation was the controlling step of the erosion process. The model did not take into account the degree of crystallinity of the copolymer, the local pH, or the polymer molecular weight. Kipper and Narasimhan [47] later improved the model by including differences in degradation rates between micro-phase separated domains and by including the preferential partitioning of drugs into domains. The erosion process of polyanhydrides is complex, and no model has been developed that takes all variables into consideration. In the following sections, the complexity of the modeling problem will become more apparent as the different variables that affect polyanhydride degradation and erosion are discussed. There are several review articles available that discuss the complexity of polymer erosion and modeling of this phenomenon [92, 98-100].

2.5.1 Polymer degradation

There are certain factors that are known to influence the rate of degradation of polyanhydrides, even though the degradation mechanism of every polymer is unique. These factors include the pH of the system, the degree of crystallinity of the polymer, the mobility of the water in the polymer, and the type of bonds that are present between monomer units. The degradation rate of copolymers is determined by the most prevalent monomer. Polyanhydrides were found to degrade much faster under alkaline conditions than under neutral or acidic conditions [25, 36]. The amorphous regions of polyanhydrides degrade faster than crystalline regions [24]. However, the most influential determinant of how fast a polymer will degrade is the type of bonds that connects the monomers in the polymer.
backbone. Polyanhydrides, poly(ortho esters), poly(esters), and poly(amides), four common polymers used in biomedical applications, have half lives of 0.1 h, 4 h, 3.3 yr, and 83,000 yr, respectively [101, 102]. The degradation rate for polyanhydrides is very fast in the presence of water, as compared to other biomaterials.

2.5.2 Polymer erosion

Polymers can be classified as either bulk (homogenous) or surface eroding (heterogeneous) materials, as shown in Figure 2.8. In the case of bulk eroding materials, the polymer allows water to penetrate the material. As this occurs, the polymer undergoes degradation and erosion throughout the device [92]. Bulk eroding devices maintain their initial shape until the entire matrix has been eroded. Poly(D,L-lactide-co-glycolide) (PLGA) is an example of a bulk-eroding polymer. In the case of surface eroding materials degradation occurs faster than the water can penetrate the system, limiting degradation and erosion to the surface of the device. Polyanhydrides are considered surface eroding materials even though eroding polyanhydrides have properties of both surface and bulk eroding polymers [92]. Göpferich et al. [103] showed that the there are three different layers to an eroding polyanhydride: two outer layers where the buffer has eroded the polymer and an inner layer where erosion has not begun. The outer zones have a higher ratio of degraded carboxyl groups to undegraded anhydride bonds as compared to the inner regions of the polymer matrix [104].
2.5.2.1 Morphology. The erosion of poly(CPP:SA) disks was studied by fabricating disks of various thickness, while preserving the total surface area [104]. Initially the erosion kinetics of the different disks with different thicknesses were identical, proving that the erosion of the polymer was occurring from the surface inward. As the erosion front reached the center of the thinner films, the erosion kinetics differed depending on the thickness of the film. As the erosion front moved towards the center of the disk, the polymer changed from a non-porous material to a highly porous material, and all of the poly(SA) in the “erosion zone” was depleted, leaving only a porous poly(CPP) shell. The resulting pores were classified as either micropores (diameter of 0.1 μm) or macropores (diameter of 100 μm). Micropores were the result of the faster erosion of the amorphous regions as compared to the crystalline regions, while macropores were a result of surface cracking that occurs soon after the polymer begins to erode [92, 99]. The disks maintained their original shape for a long time, even after the erosion front reached the center of the matrix.

2.5.2.2 pH. The bioactivity of encapsulated proteins and drugs is dependent on the pH of the environment into which they are released. The rate of diffusion of oligomers and
monomers that form as a result of hydrolysis is also dependent on the pH, thus studies have
been conducted to determine the pH of the microclimate inside and surrounding degrading
polyanhydrides. Scanning electron microscopy (SEM) was used to measure the pH profile
surrounding the degrading polymer, 20:80 poly(CPP:SA), by relating the gray levels from
channels 1 (540 nm) and 2 (600 nm) to the pH [103]. The pH surrounding the copolymer
was plotted as a function of distance from the copolymer surface. From this data it was
apparent that the pH directly surrounding the polymer matrix was lower than the pH of the
bulk fluid. Using spectral spatial electron paramagnetic resonance imaging (e.p.r.i.) the
internal pH of the degrading copolymer was determined [105]. During the early stages of
degradation the copolymer disk had an internal pH of 4.7. As the copolymer eroded the pH
slowly increased to the pH of the buffer. For comparison, the pH within eroding PLGA was
reported to be as low as 1.5 [106].

2.5.2.3 Crystallinity. McCann et al. [24] studied the changes in crystallinity of
copolymers of poly(CPP:SA) using liquid-state and solid-state $^1$N NMR. Amorphous regions
of copolymers were found to undergo erosion faster than crystalline regions. As the
amorphous regions of the copolymer eroded there was an increase in crystallinity of the
polymer. This finding contradicted the result reported by Göpferich and Langer [103] that as
the copolymer eroded there was a decrease in observed crystallinity by using DSC and X-ray
diffraction. This discrepancy arises due to lack of accounting for the changes in
experimental enthalpies due to changes in mass of the eroding polymer [24].
2.6 Mechanisms of Protein Denaturation

As recombinant DNA techniques become more routine, the number of commercially marketed pharmaceutical proteins continues to increase [107]. With this new wave of therapies come new challenges. Unlike conventional low molecular weight drugs, proteins are large globular molecules whose function is related to their structure. The amino acid sequence (the primary structure) that encodes each protein contains reactive moieties and chemically labile bonds. Any alteration in the amino acid sequence can be detrimental to the pharmaceutical potential of the protein. Amino acid sequences interact with themselves to form structures such as α-helixes and β-sheets. These structures interact with each other to form a globular protein molecule. In many instances the globular structure that a protein exhibits is essential for it to carry out its biological function. For example, lipocalins are a family of proteins that are composed of eight anti-parallel β-sheets that form a β-barrel [108]. The calyx of the barrel is where the protein binds low molecular weight ligands. If the structural hierarchy of the protein is altered and the protein no longer forms a β-barrel, then the molecule may be inactive (denatured). Before a therapeutic protein can be used, it must be stabilized in order to prevent an immunogenic response. A protein's stability is defined by its resistance to changes in its molecular weight or biological function when exposed to denaturants (e.g. heat or acidity). The FDA defines a stable pharmaceutical as one that deteriorates less than 10% in two years [109]. Pharmaceutical proteins typically have to be lyophilized to achieve this level of stability. However, lyophilization can induce unwanted stress on the protein [110, 111]. In order to stabilize a protein for therapeutic use, it is important to understand and prevent the mechanism of denaturation of the protein. The most common methods of protein
denaturation are protein aggregation (e.g., the formation of inter-molecular disulfide bonds), deamidation, and oxidation [112]. Each will be discussed below in more detail.

2.6.1 Disulfide formation & aggregation

Disulfide bonds are covalent cross-links, responsible for maintaining the structural and biological integrity of many proteins. Disulfide bonds are the de novo bonds synthesized in globular proteins. The formation or breakage of a disulfide bond does not always result in loss of activity for the protein. α-Interferon has two disulfide bonds between residues 1 and 98 and then between residues 29 and 138. If the bond is broken between residues 1 and 98, the protein undergoes almost no activity loss, whereas, if the bond between 29 and 138 is broken, the protein loses most of its biological function [113].

Disulfide scrambling can also be a problem with proteins. Lyophilized insulin has three disulfide bonds and no free Cys residues. However, the protein is prone to disulfide scrambling which results in aggregates of the lyophilized protein [109].

Bovine serum albumin (BSA) also undergoes covalent aggregation as a result of either thiol-thiol group oxidation or thiol-disulfide interchanges [114-116]. (Ovalbumin also forms covalent aggregates via thiol-disulfide interchanges but to a lesser extent than BSA [115].) A thiol-thiol group oxidation occurs when the sulfhydryl group of two BSA molecules react, eliminating free thiol groups. The thiol-thiol group oxidation produces a non-reactive aggregate. A propagation reaction occurs when a thiol of one BSA molecule attacks (nucleophilic) a disulfide bond of another BSA molecule. The result is an intermolecular disulfide interchange and the conservation of a free reactive thiol.
2.6.2 **Deamidation**

Deamidation is a hydrolysis reaction, in which the amide group is removed from either asparagine (Asn) or glutamine (Gln) forming aspartic acid (Asp) or glutamic acid (Glu), respectively [112]. Deamidation can occur under basic or acidic conditions, and at high temperatures. The rate at which deamidation occurs is influenced by the local sequence and structure of the polypeptide. For instance polar residues attached to the N-terminus of Asn or Gln increase the rate of deamidation while large bulky hydrophobic groups attached to the C-terminus side decrease the rate of deamidation.

Deamidation has varying effects on the biological activity of a protein. Deamidation does not affect interleukin-1α, insulin, or aminotransferase, however, it does reduce the activity of cytochrome C, lysozyme, and calmodulin. One way in which deamidation can disrupt the activity of a protein is by increasing the net negative charge on the protein surface, which can lead to conformational changes [112].

2.6.3 **Oxidation**

In order for a species to undergo oxidation, the oxidation state of the species must be altered. Trace amounts of metal ions, oxidants (e.g. singlet oxygen, hydrogen peroxide, ozone), and exposure to light are known catalysts for oxidation. The amino acids that are most susceptible to oxidation are methionine (Met), cysteine (Cys), tryptophan (Trp), tyrosine (Tyr), and histidine (His) [112].

Oxidation almost always results in some biological activity loss of a protein, but the extent is usually less than if the protein underwent deamidation or hydrolysis. One exception is human insulin-like growth factor 1 (hIGF-1) which has three possible routes of
degradation: oxidation at Met$^{59}$, deamidation of Asn$^{26}$ and Gln$^{15}$, and the reduction of three different disulfide bridges (Cys$^{6}$-Cys$^{48}$, Cys$^{47}$-Cys$^{52}$, and Cys$^{18}$-Cys$^{48}$). The oxidation at the Met$^{59}$ is the controlling route of degradation for insulin like growth factor 1 (IGF-1) [113].

2.6.4 Degradation by proteases

Proteins are susceptible to biological degradation by proteases, natural occurring enzymatic proteins. Proteases are found in both extracellular and intracellular pathways. Trypsin and chymotrypsin are two examples of digestive proteases that break down ingested proteins into smaller fragments. These small peptide fragments can then be transferred to the bloodstream [117].

2.7 Microsphere Fabrication

Due to the biological instability of proteins, they cannot be administered orally [113]. Instead, they need to be delivered via an injection. The in vivo half-life of therapeutic proteins is short and the therapeutic value of a single injection is limited. Therefore, the medical community is seeking a delivery device that is capable of encapsulating, stabilizing, and providing a controlled release of proteins. Polymer microspheres are small enough to be administered via injection and have demonstrated the ability to encapsulate, stabilize, and provide a controlled release of proteins [6]. Thus, microsphere fabrication techniques have received much attention. Microspheres have been fabricated by hot-melt microencapsulation, solvent removal, and spray drying. Polyanhydride microspheres of varying diameters and polymer compositions have been studied. The results from these studies are discussed below.
2.7.1 *Hot-melt microencapsulation*

Acid orange (AO), *p*-nitroaniline (PNA), insulin, and myoglobin have been encapsulated in (21:79) poly(CPP:SA) microspheres by hot-melt encapsulation [118]. By heating the copolymer to 81°C, 5°C above the T_m of poly(SA), and suspending a fine powder of a low molecular weight drug or protein in the viscous polymer, encapsulation of the molecules was possible. The melted polymer/drug slurry was then added to either silicon or olive oil and stirred at 81°C until the emulsion was stabilized. Once the emulsion was stabilized the suspension was cooled, while being stirred. The microspheres were then washed with petroleum ether to obtain a free flowing powder.

The size of the microspheres could be controlled by the rate of stirring. The newly formed microspheres had a smooth external surface with few pores as determined by SEM. SEM was also used to examine the microspheres after 15 h, 24 h, and 10 days of degradation. From this study it was shown that the microspheres underwent surface degradation. As long as the same molecular weight polymer was used reproducible yields, size, and loading distributions were obtained with an error of less than 5% [118].

The main drawback of this fabrication technique is the need to melt the polymer and expose the drug/protein to elevated temperatures. By using high temperatures drug/polymer interactions are more prevalent and loss of biological activity becomes a concern.

2.7.2 *Solvent removal microencapsulation*

Solvent removal techniques are used most frequently to fabricate polymeric microspheres. The general idea behind this fabrication method is that an emulsion is formed between a dissolved polymer and a non-solvent. The polymer solvent is then allowed to
evaporate leaving solid polymer microspheres that are suspended in a non-solvent. Different solvent removal techniques have been employed including: oil-in-oil (o/o), water-oil-oil (w/o/o), or water-in-oil-in-water (w/o/w) emulsions. Solvent extraction techniques are relatively straightforward and do not require any specialized equipment. Figure 2.9 outlines the steps required when fabricating microspheres using a solvent removal technique.

![Figure 2.9. Schematic of the double emulsion technique used to fabricate polymer microspheres by the double emulsion technique. (a) An aqueous protein solution is added to a larger volume of dissolved polymer these phases are emulsified to form the primary emulsion. (b) The primary emulsion is then emulsified in a larger volume of an organic solvent (W/O/O) or aqueous solution (W/O/W) containing a surfactant forming microspheres.](image)

The oil/oil solvent removal technique has been employed to fabricate polyester (e.g. PLGA) and polyanhydride (e.g. SA, CPH, CPP) microspheres [119-122]. Using an oil/oil emulsion the polymer and the drug/protein were co-dissolved or suspended in an organic solvent, typically methylene chloride. This solution/suspension was then added dropwise to a second organic phase, silicone oil, which contained a surfactant. The microspheres were stirred for an hour before petroleum ether was added to the suspension. The addition of
petroleum ether and the continued stirring caused the microspheres to harden. The microspheres were collected by centrifugation, washed with petroleum ether, and dried overnight. The surface morphology of the resulting microspheres was dependent on the polymer/copolymer used to make the microsphere. In the case of poly(SA) microspheres the surface was covered with crevices. The surface roughness of poly(CPP:SA) and poly(CPH:SA) copolymers was dependent on the molar ratio of CPP or CPH, as the CPH or CPP ratio was increased the surface of the spheres became smoother. The difference in surface morphology was attributed to be a function of the degree of crystallinity of the polymer and the rate at which it precipitates [119].

Water-soluble molecules have also been encapsulated using the o/o method in solution (e.g., dissolved in water) by emulsifying the aqueous phase with the organic polymer phase forming an inner emulsion. This emulsion is then added to a larger organic solution, in which the polymer is not soluble and emulsified again. This method is called the w/o/o method [6].

An advantage of using the o/o technique to fabricate microspheres is that the polymers do not undergo degradation during the fabrication process. On the contrary the polymers are exposed to several organic solutions that need to be completely removed from the final product, due to their toxicity. Protein loading efficiencies greater than 90% have been reported for both polyesters and polyanhydride microspheres fabricated via o/o [120, 123].

The second type of solvent encapsulation is the w/o/w technique. The w/o/w fabrication technique has been used the most to fabricate protein loaded polyanhydride and
polyester microspheres, as shown in Figure 2.9. In this method proteins are dissolved in an aqueous phase, either with or without a surfactant [e.g. poly(vinyl alcohol) (PVA)]. The protein solution is then added to a polymer solution and emulsified. The protein/polymer emulsion is then added to a larger water phase with a surfactant, typically PVA, and stirred for several hours. The microspheres are washed, collected by centrifugation, and freeze-dried to obtain a free flowing powder. Yang et al. [124] used PVA as a surfactant in both the inner and outer water emulsions. A higher PVA content in the inner emulsion led to higher encapsulation efficiency, more even distribution of the protein, and a smaller initial burst. The concentration of PVA in the outer emulsion influenced the diameter of the microspheres; higher concentrations led to smaller diameter microspheres, as a result of preventing the polymer/protein droplets from coalescing during the second emulsion [124, 125]. The benefit of this method over o/o is that this method reduces the number of toxic organics that the microspheres are exposed to, making the procedure more suitable for in vivo applications. There are three drawbacks to the w/o/w method. The first is that the proteins are exposed to an organic/aqueous interface, which is known to alter the bioactivity of proteins [126, 127]. The second is that the polymer begins to undergo hydrolysis during the several hours it is suspended in water [128]. The third is that high protein loading efficiencies are rarely obtained [124, 125].

2.7.3 Spray drying microencapsulation

An alternative to the hot melt or solvent removal microsphere fabrication methods is spray drying. Mathiowitz et al. [129] reported the fabrication of poly(SA), poly(CPP:SA), poly(CPH:SA), and poly(FA:SA) microspheres by spray drying. The drug/protein was first
co-dissolved or suspended in an organic solution with the polymer. The solution/suspension was then spray dried, as the particles fell from the bottom of the spray dryer they were dried by an upward flow of nitrogen. The microspheres fabricated with 50:50 poly(CPP:SA) and 50:50 poly(CPH:SA) were prone to fusing together before they were dried, it was hypothesized that the low $T_g$ of these polymers was responsible for this phenomenon. To prevent the microspheres from fusing together lower polymer concentrations were used.

Using SEM it was revealed that poly(SA) microspheres had a surface that was covered with crevices. Copolymers made with SA and the aromatic monomers CPP and CPH displayed smoother surfaces as the molar ratio of the aromatic component was increased. Spray drying is an advantageous procedure for fabricating microspheres because it can be easily scaled up, it is highly reproducible, it can be done at or below room temperature, and it eliminates the creation of a water/oil interface.

Berkland et al. [130] reported the fabrication of monodisperse PLG microspheres and later the fabrication of drug loaded polyanhydride microspheres [131] using a modified version of spray drying. The drug-loaded polyanhydride microspheres (poly(SA)) were fabricated by first dissolving the polymer (20% w/v) and drug (rhodamine B (RhBB) 3% w/w, PNA 10% w/w, or piroxicam 10% w/w) in methylene chloride. The solution was then pumped through a small-gauge hypodermic needle at various velocities. Uniform droplets of the polymer/drug solution were obtained by exciting the solution using an ultrasonic transducer controlled by a frequency generator. The diameter of the droplets was controlled by a carrier stream of 1% w/v PVA in deionized water that engulfed the polymer/drug stream. The particles were collected in a solution of 1% PVA and allowed to stir for 3 h.
The resulting poly(SA) microspheres were monodisperse, and 90% of the microspheres had a diameter within 1.5 μm of the average diameter.

Cryogenic atomization has also been employed to fabricate protein-loaded PLGA microspheres [132-135]. When using this method the drug/protein was first dissolved in an appropriate solution and spray dried to obtain particles with diameters between 1-5 microns. The polymer was then dissolved in a solvent (e.g. methylene chloride) and the solid protein particles were suspended in the polymer solution by mechanical mixing (e.g. sonication). The suspension was then pumped to an ultrasonic atomizing nozzle. As the newly formed microspheres left the nozzle they fell into a layer of liquid nitrogen overlaying a frozen layer of ethanol, see Figure 2.10. The liquid nitrogen immediately froze the microspheres. The vessel was then stored at -80 °C for three days. During this time the ethanol slowly thawed and the solvent was slowly extracted from the polymer, leaving solid microspheres. The microspheres were then collected by filtration and lyophilized. Lam et al. [135] reported 100% loading efficiency of IGF-I in PLGA microspheres using this method.
2.8 Protein-Loaded Microsphere Characterization

When encapsulating proteins in polymer microspheres, the protein is exposed to conditions that are less than ideal (e.g. organic solvent, mechanical stresses, and hydrophobic interactions with the polymer) [37, 126, 136-138]. These conditions could lead to the deactivation of the protein. In previous work little attention has been given to studying the stability of proteins encapsulated in polymer microsphere. Once administered to a patient the protein-loaded microsphere begin to degrade. As it degrades the water content within the microsphere increases affecting the stability of the protein. It is therefore essential to test the stability of the encapsulated as well as the released protein. As mentioned previously in this chapter pharmaceutical proteins must have a stable shelf life (deteriorate less than 10% in 2 years) in order for the FDA to approve their use. In the following section, methods of
determining the protein distribution within the microspheres as well as testing the structural stability of encapsulated and released proteins are discussed.

2.8.1 Confocal laser scanning microscopy

Confocal microscopy has been used to study protein distribution within polymeric microspheres [124, 125, 139]. Sun et al. [125] used confocal laser scanning microscopy to study the distribution of BSA-FITC in PLGA microspheres made using the W/O/W technique. As the amount of BSA-FITC initially used to fabricate the microspheres was increased the protein distribution changed from uniform distribution to pockets of protein aggregation.

2.8.2 Analysis of secondary structure

By mixing protein-loaded microspheres with an excess of potassium bromide, transmission Fourier transform infrared spectroscopy (FTIR) has been used to study the secondary structure of proteins encapsulated in polyester microspheres [123, 127, 140-145]. The absorbance spectrum of blank microspheres (microspheres containing no protein) was subtracted from the spectra of protein-loaded microspheres to obtain spectra of the protein. In order to quantify the structural components of the protein within the amide I region (1700-1600 cm\(^{-1}\)), Gaussian curves were fit to the corrected spectra. The corrected spectra were obtained by either performing a Fourier self-deconvolution or by taking the second derivative of the protein spectra. Fu et al. [144] encapsulated BSA in PLGA microspheres via the w/o/w fabrication technique and reported an \(\alpha\)-helix content of 18% as compared to a 37%
and 27% α-helix content of the protein when in an aqueous solution or lyophilized, respectively.

FTIR has also been used to study the secondary structure of proteins in aqueous solutions [146-152]. Aqueous protein solutions can be analyzed using either calcium fluoride windows (transmission) or by using a zinc selenide attenuated total reflectance (ATR) accessory. Unlike circular dichroism (CD) or fluorescence, studies that require high protein concentrations, concentrations as low as 10 nM provide adequate signal-to-noise ratios for structural analysis using FTIR [152].

CD and fluorescence have also been used to study the secondary and tertiary structures of proteins released from polymer microspheres into aqueous solutions [142, 153, 154]. Carrasquillo et al. [142] used CD to report a decrease in α-helix content of BSA when encapsulated in PLGA microspheres via the o/o fabrication technique. Zhu et al. [153] used a combination of CD and fluorescence to confirm that BSA released from PLGA microspheres was still intact.

2.8.3 Analysis of primary structure

Size exclusion chromatography-high pressure liquid chromatography (SEC-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been used to determine the molecular weight of released proteins from polymer microspheres [141, 142, 144, 153, 155-157]. Park et al. [157] used reducing and non-reducing SDS-PAGE to show that lysozyme extracted from PLGA microspheres was still intact, (e.g., no cleavage or aggregation). Castellanos et al. [141] used SEC-HPLC to determine if BSA released from PLGA microspheres had undergone aggregation via thiol-disulfide interchange.
2.9 In Vitro Release

Prior to administering therapeutic protein-loaded microspheres to animals or human patients the release kinetics of the protein need to be understood in vitro. This includes establishing the initial conditions of the release (is there a burst) and the length of time it will take for the encapsulated protein to be released.

2.9.1 In vitro release kinetics of protein-loaded microspheres

Crotts et al. [155] reported that the release profile of BSA was dependent on the medium used for the experiment. BSA was released in 5 mM PBS/0.01% sodium azide and 5 mM SDS/PBS/0.01% sodium azide solutions. Ten-20% more BSA was released from the polymer microspheres when released in the buffer containing surfactant (SDS), supporting the idea that BSA does absorb to the degrading polymer.

Zhu et al. [153] reported that only 20% of the BSA loaded into PLGA microspheres via the w/o/w technique was released, and it was released as protein dimers, trimers, and as peptide fragments as a result of the acidic microclimate. The remaining 80% of the protein was never released from the polymer matrix due to the formation of insoluble aggregates. By co-encapsulating Mg(OH)$_2$, a basic salt, over 80% of the encapsulated BSA was released as monomeric protein, hence eliminating aggregation due to the microclimate.

Castellanos et al. [141] reported that the solvent and the use of excipient influenced morphology, initial burst, and length of protein release from PLGA microspheres made via the s/o/w technique. When BSA and trehalose were co-encapsulated the initial burst was reduced to only 16%, as compared to over 20% when BSA was encapsulated alone and acetate was used as the solvent. Castellanos et al. [141] reported a loading efficiency of
85% for BSA when co-encapsulated with trehalose, and the protein was continuously released for a little over 600 h.

Tabata et al. [137] encapsulated chicken egg lysozyme, bovine pancreatic trypsin, heparinase, chicken egg albumin, BSA, and bovine immunoglobin into copolymers of poly(FAD:SA). The enzymatic activity of the released enzymes and the release profile of all of the proteins encapsulated in microspheres made by w/o/w procedure were reported. The encapsulation efficiency, irrespective of the protein used, was 85% and more than 90% of the microspheres fabricated had diameters in the range of 50 to 125 μm. There was no initial burst and all of the proteins have similar release profiles. The activity of released trypsin and heparinase after 2 days was compared to the activity of the proteins left in solution for 2 days. The activity of the encapsulated proteins was higher than that of the non-encapsulated proteins; trypsin in solution lost 80% of its activity while trypsin released from microspheres lost only 10% of its activity [137]. The release profiles were found to be independent of the initial molecular weight of the copolymer. Tabata et al. [137] used SEM to determine how the morphology of the degrading polymer microspheres changed. Initially the microspheres were smooth with no traces of BSA on the exterior surface. The microspheres were also sectioned prior to any degradation in order to view the interior of the microspheres. The interiors were dense, and after 44 h, only the surface of the spheres had undergone degradation.

Chiba et al. [158] and Hanes et al. [159] used poly(anhydride-co-imide) and poly[trimellitylimido-L-tyrosine-co-sebacic acid-co-1,3-bis(carboxyphenoxy)propane] (poly(TMA-Try:SA:CPP)) microspheres to release BSA. The release of the BSA was independent of the initial molecular weight of the polymer and closely followed the erosion of the
microspheres, which was dependent on the pH of the system [158]. The more acidic the release buffer, the slower the polymer degraded [158]. As the amount of CPP in the backbone increased, the hydrophobicity of the copolymer increased, and the release of BSA was significantly slowed.

The effect of loading protein into poly(FA:SA) microspheres on the erosion profile was studied [160]. Blank poly(FA:SA) microspheres and raw polymer eroded slower than microspheres loaded with BSA. As the BSA on the surface of the microspheres was released, more surface area of the microsphere was exposed. This increase in surface area lead to an enhanced erosion profile.

2.9.2 In vitro effect of polymer degradation products on protein stability

Johansen et al. [161] incubated tetanus toxoid in the presence of lactic and glycolic acid to study the effect of the degradation products on the stability of the protein. This study was motivated by the need to understand how the degradation products of biodegradable polymers affect released proteins. It was reported that the temperature was more detrimental to the stability of the protein than the low pH. Xing et al. [154] performed a similar study using free tetanus toxoid to determine the effect that pH and temperature had on the stability of the protein. During the first few days of the experiment, tetanus toxoid began to unfold under the warm acidic conditions (37°C and pH 2.5).

2.10 Summary

In the last two decades polyanhydrides have emerged as a very promising biomaterial. Much effort has gone into synthesizing and characterizing polyanhydrides.
There is a plethora of literature discussing microsphere fabrication techniques and extensive studies have shown that polyanhydrides of varying compositions are biologically inert and have minimal interactions with many drugs/proteins studied. Yet the FDA has approved only one drug delivery device for human use, and this device requires surgical implantation in the brain [62]. In order for polyanhydrides to be used for routine delivery of proteins a more detailed understanding of how polyanhydrides and proteins interact is necessary.

2.11 References


3.1 Research Objective

Chapter 1 demonstrated the importance of developing controlled delivery devices for proteins. The idea of delivering proteins via a controlled delivery device is not novel, and much attention has been given to this topic, as was discussed in Chapter 2. However, no two proteins behave exactly the same. Therefore, no one material is capable of stabilizing all of the recombinant proteins that are being marketed today. The majority of the current research in this field is done with poly(\(D,L\)-lactide-co-glycolide) (PLGA) and little of the available literature has focused on the importance of determining how proteins interact with the polymers in which they are encapsulated. The research described in the following chapters focuses on the polyanhydrides, poly(sebacic acid) (poly(SA) and copolymers of poly(SA) and poly[1,6-bis-p(carboxyphenoxy)hexane] (poly(CPH)) with regards to their ability to provide a means of stabilizing proteins while providing an extended controlled release. The overall goal of this research is to demonstrate that polyanhydrides can be used to stabilize and to provide a controlled release of therapeutic proteins. The four specific goals of this work were:

1. Demonstrate the feasibility of using polyanhydrides microspheres as protein carriers.
2. Discern the compatibility of proteins with biodegradable polymer degradation products.
3. Study the role of microsphere fabrication methods on protein stability.

4. Apply the insights gained from specific goals 1, 2, and 3 to design uterocalin-loaded polyanhydride microspheres to expedite cell migration for applications in wound healing.

Specific goal 1 will provide evidence to support the hypothesis that polyanhydrides can stabilize proteins and provide a controlled release profile. Bovine serum albumin (BSA) has been chosen as the model protein. The in vitro data obtained by encapsulating and releasing BSA from polyanhydride microspheres will lay the foundation for further studies with the therapeutic protein, uterocalin. After completing specific goal 1, a comprehensive understanding of the experimental techniques needed, including microsphere fabrication, and an in depth analysis of the encapsulated and released protein(s).

It is important to understand how proteins released from degrading polymer devices interact with polymer degradation products. Specific goal 2 will demonstrate the importance of such interactions on the structural and enzymatic or antigenic activity of lysozyme, ovalbumin, and tetanus toxoid. These experiments will demonstrate rational methods for selecting compatible polymer/protein systems prior to protein encapsulation. The stability of three proteins (tetanus toxoid, lysozyme, ovalbumin, and uterocalin) in the presence of various polymer degradation products will be evaluated. The data will aid in expediting the development of protein-loaded drug delivery devices by establishing methods of discerning incompatible systems, reducing time and resources spent studying incompatible systems.

The data collection upon successful completion of Specific goal 3 will provide information on how different microsphere fabrication processes affect protein stability and
release kinetics. These experiments will be designed to compare the stability of ovalbumin encapsulated in microspheres fabricated using four different fabrication techniques (water-oil-water, water-oil-oil, solid-oil-oil, and a cryogenic atomization technique). By directly comparing the different fabrication methods used to encapsulate the same protein, the superior fabrication method will be identified and used for future studies.

The knowledge gained by completing specific goals 1, 2, and 3 will be used to develop and test uterocalin-loaded microspheres. The stability of the encapsulated uterocalin will be assessed as will the bio-activity of the released protein by performing cell culture studies.

3.2 Dissertation Organization

Each of the following four chapters discusses the work that was performed to complete each specific goals of this project, starting with chapter 4 addressing specific goal 1 and ending with chapter 7 addressing specific goal 4.

The overall conclusions of this work are discussed in chapter 8 along with the future applications for using polyanhydrides as controlled delivery devices.
4.1 Abstract

In order to determine the efficacy of using polyanhydrides as a carrier for therapeutic proteins, the model protein bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA) was encapsulated in microspheres of poly sebacic anhydride (poly(SA)), and random copolymers of poly(SA) and poly(1,6-bis-p-carboxyphenoxy)hexane (poly(CPH)). The microspheres were fabricated via the double emulsion (water/oil/water) technique and were characterized using scanning electron microscopy, gel permeation chromatography, confocal microscopy, and particle size determination. The effect of protein loading, protein distribution, and change in polymer composition was examined in an in vitro release study. The secondary structure of the encapsulated FITC-BSA was determined with Fourier transform infrared spectroscopy. The primary structure of the released protein was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Poly(SA) and 20:80
(CPH:SA) microspheres were found to conserve both the primary structure of the released protein and the secondary structure of the encapsulated and released protein, and showed a sustained delivery for approximately 15 and 30 days, respectively. As the CPH content in the copolymer increased, the secondary structure of FITC-BSA was not conserved, as indicated by the steep decrease in the α-helix content.

4.2 Introduction

Between 1980 and 2001 the U.S. Food and Drug Administration (FDA) approved 554 new therapeutic drugs [1]. Small molecular mass drugs made up the majority of the new therapeutics approved (504), followed by recombinant proteins (40), and monoclonal antibodies (10). As recombinant DNA practices have become routine, the number of protein products entering the market has increased. With the increasing number of therapeutic proteins being marketed in the United States each year more research is being directed towards developing superior methods of delivering proteins. Unlike small molecular mass drugs, proteins are complex three-dimensional molecules, whose functionality depends on their higher-order structure [2]. Proteins are prone to chemical (e.g., deamidation, oxidation) and physical (aggregation, precipitation, and adsorption) alterations [2-5]. The mechanisms by which proteins undergo structural alterations is protein specific, however, there are known factors that decrease the stability of proteins such as elevated temperature and moisture [3, 4, 6, 7]. Parenteral administration remains the most common method of delivering proteins, however, it provides no stabilization of the shelf life of the protein or increase of the half-life of the protein in vivo [8].
A vehicle capable of encapsulating proteins, minimizing the mechanisms of degradation, maximizing the \textit{in vivo} activity, and providing controlled release that can be delivered via parental administration would be a valuable asset for the effective delivery of various drugs or vaccines. The polymer that has received the most attention as a protein delivery vehicle is poly(D,L-lactide-co-glycolide) (PLGA). PLGA has been used to encapsulate and release numerous model and recombinant proteins, such as bovine serum albumin (BSA) \cite{9-16}, lysozyme \cite{15, 17, 18}, recombinant human growth hormone (rhGH) \cite{19-22}, and recombinant human insulin like growth factor-1 (rhIGF-I) \cite{23-25}. Because PLGA is a bulk-eroding polymer in an aqueous environment, the encapsulated protein is quickly exposed to elevated moisture content. This increase in moisture has been shown cause aggregation of the encapsulated protein \cite{6, 7}. The addition of excipients (e.g. trehalose or dextran) was shown to prevent covalent aggregation of the encapsulated protein \cite{12, 13, 26}. Another potential negative issue with PLGA is that, as it degrades, the pH within the polymeric device drops significantly \cite{27}, which can be detrimental to the protein. This problem can be overcome by the co-encapsulation of basic compounds, which have proven to help stabilize the encapsulated protein \cite{9}.

Polyanhydrides are biodegradable polymers that have also been shown to stabilize and provide sustained release of proteins \cite{28}. Unlike PLGA, polyanhydrides are surface eroding polymers, an advantage that minimizes the moisture level to which an encapsulated protein is exposed, thus reducing protein aggregation due to moisture \cite{29-32}. Another benefit of polyanhydrides is that the pH of the degrading polymeric material does not drop as severely as PLGA, thus providing a more suitable (or less hostile) microclimate for encapsulated (and released) protein molecules \cite{31}. 
In parenteral formulations, microspheres are the most commonly used vehicle to encapsulate proteins or small molecular mass drugs. Microspheres can be fabricated by three different procedures: hot melt, solvent removal, or spray drying [33-40]. The hot melt procedure is not advantageous when encapsulating proteins because the elevated temperatures needed to melt the polymer can denature the protein. Solvent removal, either oil-in-oil (O/O) or water-in-oil-in-water (W/O/W) (also known as the double emulsion method), and spray drying can both be performed at room temperature. While spray drying requires the use of an atomizer, the solvent removal technique requires no special equipment; however, care must be taken when encapsulating proteins via the solvent removal technique because the presence of a water/oil interface can cause protein inactivation [41].

The polymers used in this study are poly(sebacic anhydride) (poly(SA)) and random copolymers of poly(SA) and poly(1,6-bis-p-carboxyphenoxy)hexane (poly(CPH)) (see Figure 4.1). These polymers are of interest because they have vastly different degradation rates. Poly(CPH) degrades on a time scale of years, while poly(SA) degrades on a time scale of weeks [42]. Random copolymers of SA and CPH have degradation times that fall in between the degradation time of the homopolymers [43]. By varying the polymer chemistry a suitable degradation time can be achieved to meet delivery needs.

![Figure 4.1. Structure of repeating units of polymers (a) poly(CPH) (b) poly(SA).](image-url)
The release of \( p \)-nitroaniline (PNA), a small molecular mass model drug, from microspheres of homopolymers and copolymers of poly(CPH) and poly(SA) has been previously studied [44]. It was shown that the release profile of PNA is a function of polymer erosion rate and the interaction of the drug with the polymer. The hypothesis of this research is that the release of proteins from polyanhydrides is also a function of polymer erosion rate and the interaction of the protein with the polymer.

The focus of this work is to determine the effectiveness of polyanhydride carriers such as poly(SA) and copolymers of poly(CPH) and poly(SA) with respect to the stabilization and release of bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA). The double emulsion technique was employed to fabricate the microspheres. The protein-loaded microspheres were then characterized by various techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the primary structure of FITC-BSA after it was released from the eroding polyanhydride microspheres. The secondary structure of FITC-BSA encapsulated in the microspheres was assessed using Fourier transform infrared spectroscopy (FTIR).

### 4.3 Materials and Methods

#### 4.3.1 Materials

Sebacic acid (99%), \( p \)-carboxy benzoic acid (99+%), and 1-methyl-2-pyrrolidinone anhydrous (99+%), and KBr (FTIR grade) were purchased from Aldrich (Milwaukee, WI). 1,6-dibromohexane (98+) and poly(vinyl alcohol) (99+) (PVA) were purchased from Acros (Fairlawn, NJ). Fluorescein isothiocyanate bovine serum albumin (FITC-BSA) was purchased from Sigma (St. Louis, MO). The BCA assay kit was purchased from Pierce
Chemicals (Rockford, IL). 40% polyacrylamide/bis 19:1, the SDS-PAGE silver staining kit, and molecular mass standards for SDS-PAGE were purchased from BioRad (Hercules, CA). Petroleum ether (hexane, 55% n-hexane) was purchased from Fisher Scientific (Pittsburgh, PA) and dried and distilled over sodium and benzophenone (Fisher) before use. All the other chemicals were of analytical grade and were purchased from Fisher Scientific (Pittsburgh, PA).

4.3.2 Polymer synthesis

The prepolymers of SA and CPH were synthesized by the methods described by Shen et al. [45], and for the CPH prepolymer, the method was similar to the one described by Conix [46] for 1,3-bis(p-carboxyphenoxy)propane. The polymers were synthesized by melt polycondensation as described by Kipper et al. [44].

4.3.3 Polymer characterization

Neat polymers were characterized by the procedures described by Kipper et al. [44]. The chemical structure of the polymers was characterized with $^1$H NMR. Samples were dissolved in deuterated chloroform (99.8% atom-d) and the chloroform peak was used to calibrate the chemical shift of the polymer. Polymer molecular mass was determined using gel permeation chromatography (GPC). A PL Gel column from Polymer Laboratories (Amherst, MA) and a Waters GPC system (Milford, MA) were used to separate the samples dissolved in chloroform. The elution times of the samples were compared to poly(methyl methacrylate) standards from Fluka (Milwaukee, WI). Using differential scanning
calorimetry (DSC) (DSC7, Perkin Elmer, Shelton, CT), the melting point of the semicrystalline polymers was compared to that reported by Shen et al. [45].

4.3.4 *Microsphere fabrication*

A double emulsion technique was employed to encapsulate FITC-BSA in microspheres of varying polymer composition. The following polymers were used: poly(SA), 20:80 (CPH:SA), 50:50 (CPH:SA), and 80:20 (CPH:SA). The fabrication technique used was similar to the method reported by Tabata and Langer [28]. Briefly, FITC-BSA (5 mg) was dissolved in de-ionized water (200 µl) and was added to polymer (100 mg) dissolved in methylene chloride (2 ml). The two phases were emulsified with a homogenizer at 10,000 rpm for 30 seconds (Tissue-Tearer™, Biospec Products, Inc., Bartlesville, OK) forming the inner emulsion. Four ml of 1% PVA saturated with methylene chloride (80 µl) was immediately added to the inner emulsion and homogenized for 30 sec at 10,000 rpm to form the double emulsion. The microspheres were then dispersed in 1% PVA (100 ml) and stirred for 2 h at 300 rpm using an overhead stirrer with a 3-in impeller (Wiarton, Ontario). The microspheres were collected by centrifugation for 10 min at 1500 g using an Eppendorf Centrifuge 5403 (Westbury, NY). After the first centrifugation the supernatant was collected and then replaced with de-ionized water to wash the microspheres. The microspheres were washed two additional times to ensure all of the free protein and PVA had been removed. The microspheres were then suspended in 4 ml of de-ionized water, flash frozen, and dried under vacuum overnight.
4.3.5 Microsphere characterization

The mass of microspheres recovered divided by the initial mass of polymer and protein was used to calculate the yield. To determine the size distribution the microspheres were dispersed by sonication in Coulter® Balanced Electrolyte Solution prior to using a Beckman Model Multisizer™ Three Coulter Counter (Fullerton, CA). Scanning electron microscopy (SEM) (Hitachi S-2460N) was used to study the surface morphology of the microspheres. Confocal microscopy (Nikon Eclipse TE200 microscope and Chiu Technical Corporation 100W mercury source) was used to analyze the FITC-BSA distribution within the microspheres. The molecular mass loss during the fabrication process was determined using GPC by comparing the molecular mass of blank microspheres to that of neat polymer.

4.3.6 Protein loading

The amount of FITC-BSA encapsulated in the microspheres was indirectly determined using a procedure similar to the one described by Bouillot and co-workers [11]. During the fabrication process the microspheres were collected by centrifugation, and the supernatant was collected. The BCA protein assay was utilized to determine the concentration of FITC-BSA present in the supernatant, thus providing the mass of protein not encapsulated. A mass balance was performed to determine the amount of FITC-BSA that was loaded into the microspheres. The protein that was lost while washing the microspheres during the fabrication process was not accounted for when calculating the loading. Hence the loading and loading efficiency are slightly overestimated. The encapsulation efficiency of the protein was determined by dividing the mass of the loaded protein by the initial mass of protein.
4.3.7 *In vitro protein release*

Fifteen mg of microspheres were suspended in 1 ml of water containing 3% (w/w) sodium dodecyl sulfate (SDS). The samples were placed in an incubator at 37°C and continuously agitated at 100 rpm. To determine the mass fraction of FITC-BSA released the supernatant from the samples was collected at predetermined times and fresh release media was added to maintain perfect sink conditions. The concentration of protein in each sample was determined using the BCA protein assay upon removal. The amount of protein released was normalized by the amount of protein initially loaded into the microspheres. The experiments were done in triplicate. The Bonferroni multiple comparison adjustment was used to determine that the p-values for all the multiple comparisons.

4.3.8 *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was used to determine the primary structure of the FITC-BSA released from microspheres *in vitro*. Microspheres were suspended in water containing 3% (w/w) SDS under the same conditions as the microspheres used for the *in vitro* release study. The microspheres were allowed to degrade until the concentration of released BSA reached at least 30 μg/ml in the aqueous phase. The samples were mixed with an equal volume of a SDS (1% w/v), Tris-HCl (pH 6.8, 0.06 mM), glycerol (3 mM), bromophenol blue (0.01% w/v) solution with and without β-mercaptoethanol (0.05% v/v) for staining with silver. β-mercaptoethanol is a reducing agent that breaks covalent disulfide bonds [47], thus by comparing the samples with and without β-mercaptoethanol, it was possible to determine if the released FITC-BSA had formed inter-protein disulfide bonds. The samples were resolved through a 10% polyacrylamide gel with a 5% polyacrylamide stacker.
Electrophoresis was performed using a BioRad Mini-Protean II electrophoresis setup at a constant voltage (120 volts) as described by the manufacturer. The resolved proteins were stained using a Silver Stain kit (BioRad). The apparent molecular mass of the detected bands was compared to standards. FITC-BSA samples were also run on the gel to. The silver stained gels were immediately photographed and dried overnight.

4.3.9 *Fourier transform infrared spectroscopy (FTIR)*

FTIR spectroscopy was performed using a Nicolet Nexus 470 (Madison, WI), equipped with a cooled MCT/A detector and an Ever-Glo source. Omnic 5.2 software was used to collect the data and to perform initial data analysis. Dry air was purged through the optical bench throughout data acquisition in order to reduce IR absorbance due to water. Samples were prepared by mixing microspheres (either BSA-loaded or blank) with KBr (4% w/w). In order to compare the native structure of the protein with that of the encapsulated protein, FITC-BSA samples were also mixed with KBr (4% w/w). Prior to collecting FTIR spectra all samples were stored at 50 °C under vacuum overnight to eliminate any residual moisture. The dry powder was then placed in a 7 mm dye and a pellet was formed with a hand press (Spectra-Tech Inc., Sheldon, CT).

Each spectrum was collected by running a total of 256 scans at a resolution of 4 cm⁻¹. The spectra of the encapsulated FITC-BSA were obtained by subtracting the spectra of the blank microspheres from the spectra of the FITC-BSA loaded microspheres. A subtraction was considered successful if the resulting spectra had a straight baseline in the region of 1800-2500 cm⁻¹ [48]. Fourier self-deconvolution was then performed on the amide I region of the subtracted spectra using the Omnic 5.2 software with an enhancement factor of 1.2 and
a bandwidth of 20 kHz [49]. Gaussian curves were fit to the Fourier self-deconvoluted spectra [15, 49]. The same procedure was also carried out on the spectra of the native protein. The assignment of secondary structure either: \(\alpha\)-helix, \(\beta\)-sheet, or unordered was done using the assignment of peaks for BSA listed by Fu et al. [15]. The area under each peak in the amide I region was calculated and used to determine the secondary structure of the protein using procedures described elsewhere [12-15, 22, 48-51]. A Student t-test was preformed to determine if the secondary structure of the encapsulated ovalbumin differed significantly from that of the native protein. Treatments that different significantly from the native BSA were noted.

4.4 Results and Discussion

4.4.1 Microsphere characterization

The yield for the microspheres was 56-67%, with the centrifugation step being the most pivotal in the overall recovery of microspheres, see Table 4.1. The yield was calculated from no less than five batches of microspheres of all compositions. The size distribution of all compositions of microspheres was found to be uni-modal. As an example, the size distribution of poly(SA) is shown in Figure 4.2. The maximum volume percent of all the microspheres used in this study was found to occur at a particle diameter of ca. 20 \(\mu\)m.
Table 4.1. Characteristics of microsphere yields and loading efficiency of FITC-BSA.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Yield %</th>
<th>Loading Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>68 ± 11</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>20:80</td>
<td>56 ± 9</td>
<td>68 ± 13</td>
</tr>
<tr>
<td>50:50</td>
<td>67 ± 8</td>
<td>48 ± 16</td>
</tr>
<tr>
<td>80:20</td>
<td>57 ± 10</td>
<td>54 ± 8</td>
</tr>
</tbody>
</table>

*± values are standard deviation values calculated from a minimum of five batches*

The anhydride bond is labile in an aqueous environment and the fabrication of the polyanhydride microspheres was done in the presence of water, therefore, the amount of degradation that the polymers underwent during the fabrication process was quantified by GPC (Table 4.2). Poly(SA) showed the greatest $M_n$ loss while 80:20 CPH:SA showed the least. With increasing amounts of CPH in the copolymers, the number of CPH:CPH bonds increases. It is known that CPH:CPH bonds are not as labile as CPH:SA or SA:SA bonds [52, 53], therefore, these chemical properties control the decrease in the $M_n$ associated with copolymers containing increasing amounts of CPH. These results are similar to those reported by Kipper et al. [44]. Figure 4.3 shows the surface morphology of the microspheres obtained by using SEM. The surface morphology of the microspheres varies with the polymer composition such that the more hydrophobic the polymer (e.g., the higher the CPH content), the smoother the surface of the microspheres. As the content of SA increases, the surface appears to get rougher and dark circles below the surface become apparent. The rough surface morphology of the SA-rich compositions is a consequence of the fast degradation of SA:SA bonds [54-57]. The dark circles that are present in microspheres containing higher contents of SA are attributed to the inner emulsion breaking near the surface. Figure 4.4 shows the distribution of FITC-BSA within the microspheres obtained by
confocal microscopy. The protein is uniformly distributed in the poly(SA) microspheres. As the CPH content increases, the BSA-FTIC is distributed less evenly throughout the microspheres, as is apparent by both the protein rich (identified by the intense fluorescence) and protein void regions (identified by the lack of fluorescence). The uneven distribution of FITC-BSA in the more hydrophobic polymers is attributed to unfavorable protein-polymer interactions.

Figure 4.2. Size distribution of the polyanhydride microspheres based on volume percent of microspheres as a function of particle diameter for poly(sebacic acid).

Table 4.2. Molecular mass characteristics of polymer microspheres as determined by GPC.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mₙ/Mₚ of neat polymer</th>
<th>Mₙ/Mₚ of microspheres</th>
<th>Mₚ loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SA)</td>
<td>74000/15000</td>
<td>37000/11000</td>
<td>25</td>
</tr>
<tr>
<td>CPH:SA (20:80)</td>
<td>21000/10000</td>
<td>18000/9000</td>
<td>11</td>
</tr>
<tr>
<td>CPH:SA (50:50)</td>
<td>15000/7000</td>
<td>14000/7000</td>
<td>0</td>
</tr>
<tr>
<td>CPH:SA (80:20)</td>
<td>20000/10000</td>
<td>20000/10000</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4.3. Scanning electron micrographs of polyanhydride microsphere surface morphologies (a) poly(SA) (b) 20:80 (CPH:SA) (c) 50:50 (CPH:SA) (d) 80:20 (CPH:SA).

Figure 4.4. Fluorescence photomicrographs depicting the cross-sectional distribution of FITC-BSA in polyanhydride microspheres (a) poly(SA) (b) 20:80 (CPH:SA) (c) 50:50 (CPH:SA) (d) 80:20 (CPH:SA).
4.4.2 Protein loading

The encapsulation efficiency of FITC-BSA was approximately 50%, and is similar to previous reports that use the W/O/W method, see Table 4.1 [58, 59]. Because of the variation in loading efficiency from batch to batch, the protein content of each batch of microspheres was determined \textit{a priori} in order to perform the normalization.

4.4.3 In vitro protein release

The \textit{in vitro} release was conducted in an aqueous solution containing 3\% SDS. The SDS acted as a surfactant to ensure that the (sticky) protein did not bind to the reaction vessel, the sampling equipment, or the degrading microspheres [10]. Because SDS denatures proteins, the secondary structure of the released protein could not be assessed. The addition of SDS to the release media did slightly increase the amount of FITC-BSA released (data not shown) and aided in more accurate BCA readings and resulted in protein concentrations detectable by SDS-PAGE.

The release profile of FITC-BSA loaded microspheres was a function of the polymer chemistry as shown in Figure 4.5. The Bonferroni multiple comparison adjustment was used to determine that the p-values for all the multiple comparisons were less than 0.001 indicating that release profiles of each polymer composition are statistically different [60]. The release of FITC-BSA from poly(SA) and 20:80 (CPH:SA) shows a small initial burst followed by a period of zero order release. The initial burst seen from the SA-rich microspheres was due to the accelerated surface degradation that occurred during the fabrication. The release of FITC-BSA from 50:50 (CPH:SA) and 80:20 (CPH:SA) did not show an initial burst. The lack of an initial burst was attributed to the fact that each
microsphere had a slightly different distribution of FITC-BSA as seen with the confocal microscopy studies. It is hypothesized that the varying distribution of FITC-BSA within the microspheres counteracted any fluctuations that may have resulted from the protein rich regions or protein void spaces near the surface, as seen in Figure 4.4. As the hydrophobicity of the polymer increased, the duration of release increased. After 10 and 20 days, respectively, the majority of the protein encapsulated in poly(SA) and 20:80 (CPH:SA) was released. After 50 days, only 50% of the encapsulated protein was released from 50:50 (CPH:SA) and only 10% was released from 80:20 (CPH:SA). This data suggested that by altering the copolymer composition both the release rate of FITC-BSA as well as the shape of the release profile. It is possible that some of the FITC-BSA was released as insoluble aggregates undetected by the BCA assay.

Figure 4.5. *In vitro* release of FITC-BSA from polyanhydride microspheres incubated in 1 ml of a 3% SDS solution at 37°C. Aliquots of 0.75 ml were collected and 0.75 ml of fresh solution was added to the sample to maintain perfect sink conditions. Results are representative of three replicates. Errors bars represent the standard deviation of the samples.
4.4.4 SDS-PAGE analysis

Figure 4.6 shows the SDS-PAGE analysis of the released FITC-BSA and native FITC-BSA under reducing and non-reducing conditions. Any protein aggregates present in the FITC-BSA would be conserved when analyzed without β-mercaptoethanol. Because BSA was known to form inter-protein multimers via disulfide bonds [61], β-mercaptoethanol was used in order to break any disulfide bonds providing insight on the possible formation of protein multimers. Samples with and without β-mercaptoethanol were compared. A small percentage of the commercially available FITC-BSA contained multimers, as indicated by the higher molecular mass bands seen in lane 2 of Figure 4.6a. These same bands were seen in lanes 3-6 of Figure 4.6a, the protein released from the polyanhydride microspheres. In the absence of β-mercaptoethanol the released BSA showed no additional indications of inter-protein multimers as compared to the unencapsulated protein, which would have been represented by a larger percentage of higher molecular mass bands. With the addition of β-mercaptoethanol, the disulfide bonds that were present in the unencapsulated BSA were eliminated as were the higher molecular mass bands seen in the BSA released from the polyanhydride microsphere. This data is shown in Figure 4.6b.
Native bovine serum albumin has a molecular mass of 66,400 Da [61] and was represented in lane one of Figure 4.6. If the released protein was still intact, it should have a band corresponding to 66,400 Da. As shown in Figure 4.6, bands corresponding to 66,400 Da were present in all the lanes. However, faint low molecular mass bands were also observed. These low molecular mass bands indicated that a small portion of the protein underwent hydrolysis due to the experimental conditions. The pH of the release medium drops slightly as the polyanhydride microspheres degrade especially in the absence of a buffering solution. Because protein concentrations high enough to be detected by silver staining can only be obtained by allowing the microspheres to degrade for several days, the released protein was exposed for a long time to a high temperature and mildly acidic conditions which lead to hydrolysis [62]. This observation was consistent with the data.
reported by Igartua et al. [16] indicating that BSA incubated at 37°C for one day remained intact, BSA incubated for several days was hydrolyzed [16]. To verify this, the native protein was incubated with 15 mg of polymer in a 3% SDS solution. After several days the media was sampled and run on a gel. The protein did undergo cleavage as a result of the acidic conditions and the incubating temperature (data not shown). Because the darkest bands (indicating the highest protein content) correspond to 66,400 Da for all polymer compositions, it was concluded that the primary structure of FITC-BSA encapsulated within polyanhydride microspheres was generally conserved.

4.4.5 FTIR analysis

The secondary structure of FITC-BSA encapsulated within polyanhydride microspheres was determined to assess what effects the microsphere fabrication process and the presence of the polymer had on the storage stability of the protein. The two typical signatures of protein secondary structure are α-helices and β-sheets [63]. Lyophilized proteins have a higher content of β-sheets than proteins in aqueous solutions due to intermolecular interactions [49]. Because the β-sheet content of lyophilized powder was not always indicative of the integrity of protein structure, only the α-helix content was used to determine the secondary structure of FITC-BSA [49]. Three sets of spectra were collected: blank microspheres, FITC-BSA loaded microspheres, and the native protein. The spectra of the encapsulated protein were obtained by subtracting the spectra of the blank microspheres from that of the BSA-loaded microspheres. This spectrum was then compared to the spectra of the native protein.
The α-helix content of the lyophilized FITC-BSA was 45% (Figure 4.7), which was comparable to the values reported previously for unlabeled BSA [12, 13, 15, 49, 50]. To determine if the fabrication process or the presence of the polymer altered secondary structure of FITC-BSA, the spectrum of the encapsulated protein was compared to the native protein. The FITC-BSA encapsulated in poly(SA) and 20:80 CPH:SA microspheres showed little or no deviation in the α-helix content when compared to the native protein (see Figure 4.7). This indicated that neither the polymer nor the microsphere fabrication process had an effect on the protein secondary structure. Thus, FITC-BSA can be stabilized in poly(SA) and 20:80 CPH:SA and released continuously for three weeks. However, as the CPH content in the microspheres was increased to 50% and beyond, the % α-helix content decreased steadily, to a point at which no α-helices were detected for the protein encapsulated in 80:20 (CPH:SA). This decrease in the α-helix content was attributed to the polymer hydrophobicity, and not to the microsphere fabrication process, since no change in α-helix content was observed in the poly(SA) microspheres, which were fabricated by the same process. To further verify this contention, lyophilized protein was physically mixed with each polymer (poly(SA), 20:80 (CPH:SA), 50:50 (CPH:SA), and 80:20 (CPH:SA)) and analyzed as before. The spectrum of the protein was obtained by subtracting the spectrum of the polymer from the spectrum of the polymer/protein blend. Fourier self-deconvolution was then performed on the amide I region and Gaussian curves were fit to the spectra. The secondary structure of protein mixed with polymer was found to match the secondary structure of the encapsulated protein (data not shown). To verify that this phenomenon was not an artifact of the polymer peaks interfering with the subtraction process, protein was
added to a polymer pellet without physically mixing the two and analyzed as before. The subtracted spectra of the polymer from the protein/polymer spectra (when not physically mixed) showed α-helices, indicating that the polymer was not interfering with the data analysis. Hence, the change in protein secondary structure due to increased polymer hydrophobicity was a result of the polymer-protein interactions.

Figure 4.7 also shows the β-sheet content of the encapsulated FITC-BSA in each of the polymer microspheres. Native FITC-BSA had a β-sheet content of 23%. Once encapsulated, the β-sheet content increased, indicating that the encapsulated protein aggregated and forms pockets of protein-rich regions in the microspheres, trying to minimize exposure to the polymer. These observations were consistent with the confocal microscopy images in Figure 4.4.
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Figure 4.7. Histogram showing the secondary structure (as determined by FTIR) of native FITC-BSA vs. FITC-BSA encapsulated in polyanhydride microspheres. The results are representative of a minimum of three experiments (± indicates the standard deviation). * Represents p-value < 0.05 and ** represents p-value < 0.001 as determined by a Student t test.

4.5 Conclusions

It has been shown that polyanhydrides are capable of releasing FITC-BSA for an extended time period, and by varying the copolymer composition the release profile of the protein can be altered. Polyanhydride microspheres rich in SA fabricated by the double emulsion technique were successful in preserving the primary structure of the encapsulated FITC-BSA without the addition of excipients or lyoprotectants. The hydrophobic polyanhydride microspheres also prevented the FITC-BSA from undergoing covalent inter-protein multimers via the formation of disulfide bonds. The primary structure of the released protein was intact and underwent hydrolysis only after it was allowed to incubate at 37 °C in mildly acidic media for several days. Though the protein has been shown to be stable while encapsulated and capable of being released at its native molecular mass, the secondary
structure of the protein could be altered upon encapsulation into or release from the microspheres. Because BSA has no measurable biological activity and the secondary structure could not be determined in these studies, further work needs to be performed to further support the conclusion that poly(SA) and 20:80 (CPH:SA) microspheres can deliver biologically active therapeutic proteins.

Though polyanhydride microspheres offer a sanctuary against the formation of covalent disulfide bonds and provide a method of prolonged delivery, not all polyanhydrides are suitable vehicles for protein stabilization. From this study, it was evident that the increased hydrophobicity of CPH provided a harsh climate for the FITC-BSA. The secondary structure of the protein, quantified by the α-helix content, underwent significant perturbations. The increased degradation time (~few months) of the CPH-rich copolymers also limits the usefulness of such copolymers for medical applications. The data indicates that polyanhydrides such as poly(SA) and 20:80 (CPH:SA) are well suited for protein stabilization and delivery.

4.6 Acknowledgments

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50. Castellanos JJ, Cuadrado WL, and Griebenow K. Prevention of structural perturbations and aggregation upon encapsulation of bovine serum albumin into
CHAPTER 5
PROTEIN STABILITY IN THE PRESENCE OF POLYMER DEGRADATION PRODUCTS: CONSEQUENCES FOR CONTROLLED RELEASE FORMULATIONS

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5.1 Abstract

When encapsulating proteins in polymer microspheres for sustained drug delivery there are three stages during which the stability of the protein must be maintained: (1) the fabrication of the microspheres, (2) the storage of the microspheres, and (3) the release of the encapsulated protein. This study focuses on how proteins would be affected by the polymer degradation products during the release of a protein from degrading microspheres. Tetanus toxoid, ovalbumin, and lysozyme were incubated for 0 or 20 days in the presence of ester (lactic acid and glycolic acid) and anhydride (sebacic acid and 1,6-bis(p-carboxyphenoxy)hexane) monomers. The structure and antigenicity or enzymatic activity of each protein in the presence of each monomer was quantified. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, circular dichroism, and fluorescence spectroscopy were used to assess/evaluate the primary, secondary, and tertiary structures of the proteins,

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respectively. Enzyme-linked-immunosorbent-assay was used to measure changes in the antigenicity of tetanus toxoid and ovalbumin, and a fluorescence-based assay was used to determine the enzymatic activity of lysozyme. Tetanus toxoid was found to be the most stable in the presence of anhydride monomers, while ovalbumin was most stable in the presence of sebacic acid, and lysozyme was stable when incubated in the presence of all of the monomers studied.

5.2 Introduction

As the number of therapeutic proteins being marketed in the U.S. each year continues to increase, new challenges are arising [1]. Proteins have shorter half-lives and are more prone to chemical and physical mechanisms of deactivation in vivo in comparison to small molecular weight drugs [2-7]. The delicate nature of proteins limits the feasibility of oral administration of protein-based therapeutics. Traditional delivery techniques often require high doses and multiple administrations of vaccines or pharmacological mediators, placing a high burden upon patient compliance (e.g., self administration of the next dose, repeat office visit) for the treatment regimen to be effective [2]. Thus, an injectable biodegradable controlled delivery device that is capable of encapsulating and providing a sustained release of biologically active proteins is desirable.

Encapsulating proteins into polymer microspheres is the most common method of fabricating an injectable controlled delivery device. Numerous biodegradable polymers have received attention as protein carriers, including polyesters and polyanhydrides [8-11]. Polyesters and polyanhydrides have different erosion mechanisms. Polyesters are bulk-eroding materials with elevated moisture levels within the degrading device, that results in
moisture-induced aggregation of some proteins [12, 13]. In contrast, polyanhydrides are surface-eroding materials that significantly reduce the moisture level within the degrading device [12, 14]. Another difference between polyanhydrides and polyesters is the strength of acidity generated by the degradation products. As polyesters erode, their degradation products are more soluble in water than anhydride monomers and create a microenvironment with a pH that is lower than that created by eroding polyanhydrides [15-17]. The acidic environment produced by either degrading polymer could denature some proteins, with those induced by polyesters likely to be more destructive to protein integrity. Polyesters are used as drug delivery vehicles more frequently than polyanhydrides, though both are approved by the U.S. Food and Drug Administration (FDA) for human use. One reason that polyesters are used more frequently is because they are less hydrophobic than polyanhydrides reducing the risk of non-covalent interactions with the encapsulated and released protein [18].

Before a protein can be encapsulated in a polymeric microsphere, care must be taken to select a polymer that can stabilize the protein of interest. The FDA defines a stable pharmaceutical as one that deteriorates less than 10% in two years [2]. Every protein has varying levels of stability and each has a different mechanism of denaturation [2-7, 19]. Thus, it is important to understand the conditions that can be detrimental for a protein and try to prevent the exposure of the protein to those conditions. When encapsulating proteins in polymeric microspheres, there are three stages in which the protein must maintain its stability: microsphere fabrication, storage, and the release of the protein from the eroding microsphere. The work presented here investigates the changes in structure and activity of three proteins (tetanus toxoid, ovalbumin, and lysozyme) after being incubated with the degradation products (monomers) of both polyesters and polyanhydrides. The goal of this
work was to study the effect of ester or anhydride monomers on the stability of different proteins. These experiments simulated the conditions that the protein would be exposed to as it is released from a degrading microsphere. The esters (in the form of polymer degradation products) used for this study were lactic acid (LA) and glycolic acid (GA). The anhydrides (in the form of polymer degradation products) used for this study were sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH). The primary, secondary, and tertiary structures, and the antigenicity or enzymatic activity of each protein were determined after incubating at 37°C for 0 and 20 days with each of the monomers. For these studies, tetanus toxoid, ovalbumin, and lysozyme were chosen as model therapeutic proteins to give a broad perspective on how different polymer degradation products affected the stability of different proteins.

5.3 Materials and Methods

5.3.1 Materials

Sebacic acid (99%), p-carboxy benzoic acid (99+%), 1-methyl-2-pyrrolidinone anhydrous (99+%), DL-lactic acid (85% solution in water), and glycolic acid (99%) were purchased from Aldrich (Milwaukee, WI). 1,6-dibromohexane (98+%) was purchased from Acros (Fairlawn, NJ). Chicken egg white ovalbumin, hen egg white lysozyme, monoclonal anti-chicken egg albumin (clone ova-14 mouse ascites fluid), anti-chicken egg albumin developed in rabbit, anti-rabbit IgG alkaline phosphatase conjugate developed in goat, fetal calf serum (FCS), Coomassie R-250, Sigma 104 phosphatase substrate, and p-nitrophenyl phosphatase (pNPP) liquid substrate system were purchased from Sigma (St. Louis, MO). Mini gradient gels (4-20%, Tris-Glycine) were purchased from Gradipore (Australia).
Protein molecular mass standards were purchased from BioRad (Hercules, CA). The fluorescent-based assay, EnzCheck®, used to determine the enzymatic activity of lysozyme, was purchased from Molecular Probes (Eugene, OR). Tetanus toxoid (TT) was purchased from University of Massachusetts Biologic Laboratories (Jamaica Plain, MA) at a purity of 1.5 mg/ml and 490Lf/ml. Tetanus antitoxin was purchased from Fort Dodge Laboratories, Inc (Fort Dodge, IA). The IgG fraction was purified from tetanus antitoxin using the T-gel purification kit with immobilized protein A from Pierce (Rockford, IL) following the manufacturer's instructions. Biotin-conjugated goat anti-horse IgG (H&L) was purchased from KPL, (Gaithersburg, MD). Alkaline phosphatase-conjugated streptavidin was purchased from Southern Biotechnology Associates (Birmingham, AL). All the other chemicals were of analytical grade and were used as purchased from Fisher Scientific (Pittsburgh, PA).

5.3.2 In vitro incubation

The stability of tetanus toxoid (TT), ovalbumin, and lysozyme when incubated for 0 and 20 days with different ester monomers, anhydride monomers, or phosphate buffered saline (PBS) pH 7.4, was examined. The 20-day samples were incubated at 37°C and agitated at 100 rpm. The LA and GA solutions were prepared by dissolving the acids in de-ionized water (5 mM) and filtering (0.22 μm). CPH diacid was synthesized as described previously [20]. The CPH and SA diacid solutions were obtained by incubating an excess of each diacid in de-ionized water at 37°C (100 rpm) overnight. The diacid solutions were then centrifuged and filtered (0.22 μm). The final concentration of the CPH and SA diacid solutions were 1 mM and 5 mM, respectively. Mixtures of LA/GA and CPH/SA were
obtained by mixing equal volumes of each solution (prepared separately) together. Table 5.1 shows the pH and the concentration of each of the solutions used. Stock solutions of tetanus toxoid, ovalbumin, and lysozyme (1.5 mg/ml) were made in a saline/thimerosal solution (8.5 g/L NaCl, 0.003% thimerosal). The protein solution was added to the monomer solution at a volume ratio of 20%. The final protein concentration of all samples was 300 µg/ml, unless otherwise stated. Experiments were repeated a minimum of three times.

Table 5.1: Polymer degradation products used, and the pH of the solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
<th>Type of Polymer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH</td>
<td>1 mM (saturated)</td>
<td>Polyanhydride</td>
<td>5.5</td>
</tr>
<tr>
<td>SA</td>
<td>5 mM</td>
<td>Polyanhydride</td>
<td>4.2</td>
</tr>
<tr>
<td>CPH/SA</td>
<td>0.5/2.5 mM</td>
<td>Polyanhydride</td>
<td>5.0</td>
</tr>
<tr>
<td>LA</td>
<td>5 mM</td>
<td>Polyester</td>
<td>3.5</td>
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<tr>
<td>GA</td>
<td>5 mM</td>
<td>Polyester</td>
<td>3.6</td>
</tr>
<tr>
<td>LA/GA</td>
<td>2.5/2.5 mM</td>
<td>Polyester</td>
<td>3.5</td>
</tr>
</tbody>
</table>

5.3.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess changes in the primary structure of the proteins after being incubated with the polymer degradation products for 0 and 20 days. All the gels were run under reducing conditions. Each sample was mixed with an equal volume of a solution containing SDS (1% w/v), Tris-HCl (pH 6.8, 0.06 mM), glycerol (3 mM), bromophenol blue (0.01% w/v), and β-mercaptoethanol (0.05% v/v). The samples were boiled for 10 min and allowed to cool to room temperature before loading onto the gel. The samples (7.5 µg/lane of ovalbumin and lysozyme and 18.75 µg/lane of TT) were then resolved through 4-20% precast gradient
polyacrylamide gels (Gradipore). Electrophoresis was performed using a BioRad Mini-
Protean II electrophoresis setup at a constant voltage (100 V) as described by the
manufacturer. Prestained BioRad low-range standards were used to estimate the molecular
mass of each protein sample. The gels were stained either with GelCode Blue (Pierce)
following manufacturer directions or with Coomassie Blue (methanol 10% v/v, acetic acid
10% v/v, and Coomassie Blue R-250 0.1% w/v) overnight and then destained with a
methanol (30% v/v) solution. The gels were then photographed and dried. The molecular
weight of the detected bands was compared to that of standards.

The TT samples used for the SDS-PAGE experiments were incubated at a
concentration of 150 µg/ml. To increase the visibility of the TT bands on the gel, the
samples were concentrated prior to loading onto the gel. The TT samples were concentrated
using Vivaspin centrifugation (Vivascience, Hannover, Germany) (3000 MW cutoff)
following the manufacturer’s instructions. Samples were diluted in PBS and concentrated to
remove the monomers. Equal quantities of each TT sample were loaded per lane (18.75 µg
protein based on original concentrations).

5.3.4 Circular dichroism (CD)

Far UV circular dichroism (CD) (190-250 nm) was used to monitor changes in the
secondary structure of the proteins after 0 and 20 days of incubation with the polymer
degradation products. Due to the high absorbance of the PBS solution (without any protein)
water was used as a control for all CD experiments. To ensure that the ellipticity of TT and
lysozyme was within a workable range for these experiments, the protein concentration was
reduced from 300 µg/ml to 150 µg/ml. All CD spectra were collected on a Jasco J-710
(Easton, MD) instrument with a 1 cm quartz cell. Using the Jasco software, the background solution (polymer degradation product in de-ionized water with saline/thimerosal solution added) was subtracted from each protein spectrum. A minimum of three protein spectra were collected per sample and averaged.

5.3.5 Fluorescence spectroscopy

To determine if the protein underwent any changes in tertiary structure while in the presence of the polymer degradation products, fluorimetry was performed (Varian Cary Eclipse Fluorescence Spectrometer, Australia). The emission spectrum (300-500 nm) of each sample was collected at an excitation wavelength of 280 nm. When excited at 280 nm, the emission spectrum is a result of contributions from both tryptophan and tyrosine residues in the protein [21]. Each protein spectrum was corrected by subtracting the spectrum of the appropriate blank solution (no protein) to eliminate spectral features from the solvent.

5.3.6 TT-specific enzyme-linked-immunosorbent-assay (ELISA)

Ninety-six well microtiter plates (Costar high protein binding) were coated overnight at room temperature with 100 μl PBS (pH 7.4) containing different dilutions of the incubated TT samples, in duplicate. By measuring different dilutions of TT, the concentration at which the antigenicity of TT was no longer detectable was determined. To remove unbound TT, plates were washed with PBS containing 0.05% Tween 20 (PBST) and then blocked for 2 h at room temperature with PBST containing 2% gelatin. Anti-TT antibodies (100 μl/well) (IgG fraction purified from Tetanus Antitoxin, 1 mg/ml diluted 1:20,000) were diluted in PBST supplemented with 1% FCS (PBST-FCS). Following an overnight incubation (18 h at
4°C) the plates were washed three times with PBST. A volume of 100 µl of PBST-FCS containing biotin-conjugated goat anti-horse IgG (H&L) (0.5 mg/ml diluted 1:2000) was then added to each well. After a 4 h incubation, the plates were washed three times with PBST and 100 µl alkaline phosphatase-conjugated Streptavidin (diluted 1:1000) was added. After a 2 h incubation, the plates were washed three times with PBST and 100 µl of sodium carbonate buffer (pH 9.3) containing phosphatase substrate (1 mg/ml) was added and allowed to react for 1 h at room temperature. The optical density (OD) of the reaction was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale, CA).

5.3.7 Ovalbumin-specific enzyme-linked-immunosorbent-assay (ELISA)

High binding Costar ninety-six well plates were coated overnight at 4°C with 100 µl of mouse monoclonal anti-chicken egg albumin diluted 1:2000 in PBST. The plates were then washed three times with PBST and blocked for 2 h at room temperature with a 2% BSA solution in PBST. The blocking solution was removed and the plates were washed three times with PBST. One ovalbumin sample incubated with each monomer or PBS was then diluted in PBST to a final concentration of 150 µg/ml and 100 µl was added to each well (in triplicate). Following an overnight incubation (18 h at 4°C), the plates were washed three times with PBST. A volume of 100 µl of anti-ova developed in rabbit (2 µg/ml) was then added to each well and allowed to incubate at room temperature for 2 h. The plates were then washed three times with PBST. After washing, 100 µl of anti-rabbit IgG alkaline phosphatase developed in goat diluted in PBST (1:30,000) was added to each well. After incubating for 2 h at room temperature the plates were washed three times with PBST. A volume of 100 µl of pNPP liquid substrate was then added to each well and allowed to react.
for 1 to 2 h. The OD of the reaction was measured using a Bio-TEK EL 340 microtiter plate reader (Winooski, VT) at 405 nm. All the values were normalized by the initial protein and reported as relative epitope availability. A Student t test was used to determine the statistical differences between the ovalbumin incubated in PBS and each of the monomers investigated. Treatments showing a statistical difference were noted.

5.3.8 **Lysozyme activity test**

The activity of lysozyme was quantified using the fluorescent-based kit EnzChek® as described by the manufacturer. Briefly, one sample recovered from each monomer or PBS incubation was diluted with 1X reaction buffer (0.1M sodium phosphate, 0.1 M NaCl, 2 mM sodium azide, pH 7.5) and 50 μl per well was added to a 96 well plate. Fifty μl of lysozyme substrate (*Micrococcus lysodeikiticus* labeled with fluorescein diluted in 1X reaction buffer) was added to each sample. The samples were then allowed to react in the dark for 30 min at 37°C. The fluorescence intensity was then measured using a Varian Cary Eclipse Fluorescence Spectrometer by exciting the samples at 494 nm and measuring the emission at 518 nm. All samples were compared to standards of known activity. A Student t test was used to determine the statistical differences between the ovalbumin incubated in PBS and each of the monomers investigated. Treatments showing a statistical difference were noted.
5.4 Results

5.4.1 Stability of tetanus toxoid

5.4.1.1 SDS-PAGE analysis. Tetanus toxoid (TT) is a 150 kDa heterodimer known to lose its immunogenicity when exposed to high moisture environments [22]. TT was included in the study because both polyesters and polyanhydrides have been used to encapsulate TT with the intent to develop a single dose vaccine [9, 23, 24]. Mature TT is composed of 1,351 amino acid residues comprising two polypeptide chains (N-terminal light chain, 52 kDa and the C-terminal heavy chain, 98 kDa) that are linked by a disulfide bridge [25]. In order to address changes in the primary structure of TT, changes in the molecular mass were investigated using SDS-PAGE after 0 and 20 days of incubation in the presence of ester and anhydride monomers (Figure 5.1). Three bands were visible by SDS-PAGE analysis when the TT was suspended in PBS and incubated for 0 days. The highest intensity band corresponded to intact TT (i.e 150 kDa) and the two smaller bands corresponded to the heavy and light chains of TT, respectively. Because TT is prepared as a formalin-fixed solution, the majority of the TT supplied by the manufacturer was present as a heterodimer with a molecular size of 150 kDa; however, a smaller fraction of the TT preparation was present as separate light and heavy chains. The TT that was dissolved in solutions containing polyester or polyanhydride degradation products and immediately analyzed by SDS-PAGE showed similar banding patterns, indicating that the primary structure of TT was not affected by these solutions. After 20 days of incubation in solutions containing the different monomers (Table 1), changes in the primary structure of TT were investigated. Following incubation in the indicated solution, an equal amount of total TT was loaded into individual lanes of the gel. The staining intensity of the bands corresponding to 150, 98, and 52 kDa for
TT samples incubated in the presence of the polyester monomers was much lower than that of the protein incubated with PBS or the polyanhydride monomers. The disappearance of the TT bands is a consequence of protein aggregation. Because TT is a large protein (150 kDa) its aggregated form is difficult to resolve on a gel. Johansen et al. [26] also observed that TT became undetectable by SDS-PAGE after the samples had been incubated in 100 mM of either LA or GA as a consequence of aggregation. The results of this experiment demonstrated that incubation of TT in the presence of polyester degradation products lead to protein aggregation, while the primary structure of TT incubated with anhydride monomers was unaffected.

![SDS-PAGE analysis](image)

**Figure 5.1.** SDS-PAGE analysis of tetanus toxoid samples (18.75 μg/lane) incubated in the presence of the indicated ester and anhydride monomers for (a) 0 days or (b) 20 days. Lane 1: molecular mass ladder; lane 2: 1 mM 1,6-bis(p-carboxyphenoxy)hexane (CPH); lane 3: 5 mM sebacic acid (SA); lane 4: 0.1/2.5 mM CPH/SA; lane 5: 5 mM lactic acid (LA); lane 6: 5 mM glycolic acid (GA); lane 7: 2.5/2.5 mM LA/GA; lane 8: phosphate buffer saline (PBS).

5.4.1.2 Circular dichroism analysis. The secondary structure of TT incubated with polyester and polyanhydride degradation products was monitored using far UV CD (190-250 nm). The spectra of TT incubated in water for 0 and 20 days were identical. In water, the TT spectrum had two minima at 208 and 222 nm, in agreement with previous work, as shown
in Figure 5.2 [27]. The two minima at 208 and 222 nm are signatures of α-helices and α-helices + β-sheets [28, 29]. After 0 days of incubation with either the ester or anhydride monomers, there was no change in the spectra of TT. After the TT was incubated for 20 days with LA, GA, and LA/GA, the minimum at 222 nm shifted to 217 nm indicating an increase in the β-sheet content of the protein [28]. The spectra of TT incubated with the polyanhydride degradation products for 20 days showed no deviation from those of TT incubated in water for the same length of time. This experiment showed that TT underwent conformational alterations in the presence of polyester degradation products, but not in the presence of polyanhydride degradation products.

![Graph](image)

Figure 5.2. Circular dichroism spectral analysis of tetanus toxoid (150 μg/ml) after 20 days of incubation in the presence of water (closed circle), 5 mM sebacic acid (SA)(open square), or 5 mM glycolic acid (GA) (open triangle). Results are representative of three replicate experiments.

5.4.1.3.1 Fluorescence spectroscopy. The fluorescence spectra of TT incubated with each of the polymer degradation products for 0 and 20 days are shown in Figure 5.3. The TT samples dissolved in PBS and analyzed immediately generated one large peak with a
maximum absorbance \( (\lambda_{\text{max}}) \) at 330 nm and a much smaller peak with a \( \lambda_{\text{max}} \) at 445 nm. Similar results were observed by Xing et al. [30]. The smaller peak at 445 nm corresponded to a tryptophan oxidation product [30]. The fluorescence spectra obtained immediately after solubilizing TT in solutions containing the ester or anhydride monomers were similar to the spectrum obtained when TT was solubilized in water. After a 20-day incubation in solutions containing the polymer degradation products, the intensity of the 330 nm peak decreased while the intensity of the 445 nm peak increased, as shown in Figure 5.3b. Following incubation of TT in solutions containing the polyester degradation products, the fluorescence spectra displayed the highest intensity at the 445 nm peak at day 0 and 20, indicating an alteration of the tryptophan residues [30].

5.4.1.4 Antigenicity. Tetanus toxoid has multiple immunologic epitopes to which serum antibodies specific of TT can bind. The overall antigenicity of a protein will be defined by the total sum of the antibody molecules bound to the individual epitopes. If protein denaturation destroys an antibody-binding site (e.g., epitope), the overall antigenicity
of the resultant peptide mixture would be reduced. However, if a protein was degraded and
the epitopes remain intact, the overall antigenicity of the peptide mixture would remain
unchanged. In order to assess the effects of the polyester and polyanhydride degradation
products on the antigenicity of TT, an ELISA was performed using polyclonal equine
antisera to detect the antigenic integrity of TT. The ELISA results analyzing TT samples
incubated in the indicated solutions for either 0 or 20 days are shown in Figure 5.4. The
antigenic reactivity of TT samples was unchanged when analyzed immediately after
preparation of the TT-containing ester or anhydride solutions. After 20 days of incubation,
the antigenicity of TT was unaffected for samples incubated in the presence of PBS or the
anhydride monomers. However, incubation of TT for 20 days in solutions of the ester
monomers resulted in a significant decrease in the antigenicity of the protein. The loss of
antigenicity was most evident when the lowest detectable concentration of total protein was
determined. TT incubated in the presence of SA or SA/CPH monomers lost about 50% of its
antigenicity while TT incubated with the ester monomers was four times less antigenic than
TT incubated in PBS. Similar to previous results reported by Xing et al. [32], these results
suggested that the loss of antigenicity was due to protein degradation associated with the
more acidic environment created by the ester monomers in comparison to the solutions
containing the anhydride monomers.
Figure 5.4. The antigenicity of tetanus toxoid measured by ELISA using a standard dilution of equine anti-tetanus toxoid. The antigenic stability of tetanus toxoid was evaluated as described in Materials and Methods after being incubated with ester and anhydride monomers for (a) 0 days or (b) 20 days and was compared to tetanus toxoid incubated in phosphate buffered saline for the same time periods. Results are representative of three replicate experiments.

5.4.2 Stability of ovalbumin

5.4.2.1 SDS-PAGE analysis. Ovalbumin (48 kDa) is an abundant protein that has previously been used as a model antigen for microencapsulation [31-34]. SDS-PAGE was used to monitor changes in the primary structure of ovalbumin after being incubated in the presence of polyanhydride and polyester degradation products for 0 or 20 days. The SDS-PAGE profiles for ovalbumin incubated in the presence of the ester or anhydride monomers for 0 or 20 days were identical, hence only the gel depicting the samples incubated for 20 days is shown in Figure 5.5. Under these experimental conditions, the primary structure of ovalbumin was not altered by any of the monomer-containing solutions. There was no evidence that ovalbumin underwent aggregation or hydrolysis in these solutions. Because ovalbumin is known to form covalent aggregates via disulfide bonding [35], the samples
were also run under non-reducing conditions to verify the absence of aggregates. The results (not shown) confirmed that ovalbumin did not form covalent aggregates in any of the treatment solutions. This experiment demonstrated that ovalbumin resisted changes in its primary structure when incubated in the presence of either ester or anhydride monomers.

![Figure 5.5. SDS-PAGE analysis of ovalbumin samples (7.5 μg/per lane) after being incubated for 20 days. Lane 1: molecular mass ladder; lane 2: native ovalbumin; lane 3: 1 mM 1,6-bis(p-carboxyphenoxy)hexane (CPH); lane 4: 5 mM sebacic acid (SA); lane 5: .5/2.5 mM CPH/SA; lane 6: 5 mM lactic acid (LA); lane 7: 5 mM glycolic acid (GA); lane 8: 2.5/2.5 mM LA/GA; lane 9: phosphate buffer saline (PBS).]

5.4.2.2 Circular dichroism. Far UV CD (190-250 nm) was used to monitor the secondary structure of ovalbumin incubated with polyester and polyanhydride degradation products and water. Koseki *et al.* [36] reported that incubating ovalbumin in an acidic environment alone does not denature the protein, instead the protein takes on a molten globular form in which the flexibility of the side chains is increased, decreasing the stability of ovalbumin. The spectra of ovalbumin incubated for 0 and 20 days in water were identical, indicating that ovalbumin did not undergo any conformational changes. Each spectrum had two minima at 208 and 222 nm (Figure 5.6), consistent with previous studies [36]. The
ovalbumin incubated with the ester and anhydride monomers for 0 days also had two minima at 208 and 222 nm. After a 20-day incubation with LA, the spectrum of ovalbumin was unchanged. When incubated with GA and LA/GA for 20 days, the spectrum of ovalbumin was altered, and the minimum at 222 nm shifted to 218 nm indicating an increase in the β-sheet content of the protein. The spectra of ovalbumin incubated with the polyanhydride degradation products for 0 days was identical to the spectrum of ovalbumin in water. After 20 days of incubation, the ovalbumin incubated with the CPH and the SA showed no changes, while the spectrum of ovalbumin incubated for 20 days with CPH/SA showed deviation. The spectrum of ovalbumin after incubation in CPH/SA for 20 days still contained minima at 208 and 222 nm; however, the ratio of $[\theta]^{222}/[\theta]^{208}$ changed from 1.32 (water) to 0.85 (CPH/SA) indicating an increase in the α-helical content [21]. Zemser et al. [21] and Takeda and Moriyama [37] reported similar findings when SDS was added to ovalbumin. The anionic detergent, SDS, interacts with the hydrophobic side chains of ovalbumin disrupting the hydrophobic bonds within the protein. The disruption of the hydrophobic bonds favors the formation of α-helices. Because the CPH monomer contains hydrophobic regions, it is anticipated that the monomer interacts with the hydrophobic side chains of the ovalbumin in the same way that SDS does.
Figure 5.6. Circular dichroism spectral analysis of ovalbumin (300 μg/ml) after 20 days of incubation in the presence of water (closed circle), 5 mM sebacic acid (SA) (open square), or 5 mM glycolic acid (GA) (open triangle). Results are representative of three replicate experiments.

5.4.2.3 Fluorescence spectroscopy. When ovalbumin was excited at 280 nm, the resulting fluorescence spectrum had a single peak with a λ<sub>max</sub> between 331 and 342 nm, corresponding to the intense emission of tryptophan [21]. Ovalbumin contains three tryptophan residues that are located in the interior of the molecule. As the protein unfolds, or undergoes changes in its tertiary structure, the location of the internal tryptophan residues change and the resulting fluorescence spectrum is altered [21].

The fluorescence spectra of ovalbumin incubated for 0 and 20 days are compared in Table 5.2. The λ<sub>max</sub> of all protein spectra at day 0 were within the range of 331 to 342 nm. Following a 20-day incubation, the ovalbumin incubated with PBS, the anhydride monomers, and LA had a λ<sub>max</sub> within the normal range of 331-342 nm. Though the λ<sub>max</sub> of the ovalbumin incubated with CPH and CPH/SA was within the acceptable range, there was a shift in the λ<sub>max</sub> to longer wavelengths. A similar shift of the λ<sub>max</sub> was seen when 0.1 M and
0.01 M SDS was added to ovalbumin indicating an increase in α-helix formation [21]. This is consistent with the CD results of ovalbumin incubated with CPH/SA for 20 days.

When ovalbumin was incubated with GA and LA/GA for 20 days, the $\lambda_{\text{max}}$ of the resulting spectra fell outside of the expected range of 331 to 342 nm (see Table 5.2 for $\lambda_{\text{max}}$ values at days 0 and 20), indicating that the protein was unfolding [21]. This finding is consistent with the work of Koseki et al. [36] where the side chains of ovalbumin were found to be more flexible and susceptible to denaturation when kept at acidic pH for an extended period of time. This change in the tertiary structure is also consistent with the changes in the secondary structure of ovalbumin incubated with GA and LA/GA as observed with CD. The change in the tertiary structure of ovalbumin is attributed to the glycolic acid interfering with the hydrogen-bonding pattern within the polar regions of the protein as seen when ovalbumin is incubated with guanidine [21].

Table 5.2. Wavelength maxima ($\lambda_{\text{max}}$) of ovalbumin incubated in the indicated monomers for 0 and 20 days.

<table>
<thead>
<tr>
<th>Polymer Degradation Products</th>
<th>$\lambda_{\text{max}}$ Day 0 (nm)</th>
<th>$\lambda_{\text{max}}$ Day 20 (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH</td>
<td>339</td>
<td>332</td>
</tr>
<tr>
<td>SA</td>
<td>336</td>
<td>339</td>
</tr>
<tr>
<td>CPH/SA</td>
<td>337</td>
<td>335</td>
</tr>
<tr>
<td>LA</td>
<td>340</td>
<td>340</td>
</tr>
<tr>
<td>GA</td>
<td>336</td>
<td>344</td>
</tr>
<tr>
<td>LA/GA</td>
<td>340</td>
<td>346</td>
</tr>
<tr>
<td>PBS</td>
<td>339</td>
<td>337</td>
</tr>
</tbody>
</table>

Data were obtained by fluorescence spectroscopy. *Italicized* values represent $\lambda_{\text{max}}$ values that fall outside of the 331-342 nm range.

5.4.2.3 Antigenicity. A capture ELISA was used to monitor the changes in the antigenicity of ovalbumin incubated in the presence of polyester or polyanhydride
degradation products for 0 or 20 days. The results are shown in Figure 5.7. The ELISA values represent a relative epitope availability of ovalbumin after the values had been normalized by the epitope availability of native ovalbumin (e.g., incubated in PBS). If no structural alterations occurred, the ratio of the relative epitope availability would be 1, as observed for the ovalbumin solubilized in PBS or anhydride monomers and assayed immediately. These results are consistent with previous reports that the antigenicity of ovalbumin was retained when the pH was >3.0 [38]. The increase in the relative epitope availability exhibited by ovalbumin solubilized in the polyester monomers was a result of protein unfolding and exposing more epitopes that were being recognized by the polyclonal antibody used in the assay. Because the solutions containing the ester monomers were more acidic (Table 1), the flexibility of the side chains was increased.

Figure 5.7. The antigenicity of ovalbumin (150 µg/ml) measured using ELISA, after being incubated with ester and anhydride monomers for (a) 0 days or (b) 20 days (± indicates standard deviation). * represents p-value < 0.05 and ** represents p-value < 0.001 as determined by a Student t test.
Ovalbumin incubated for 20 days in PBS, CPH, or CPH/SA containing solutions showed no change in ovalbumin antigenicity. Ovalbumin incubated in solutions containing SA, LA, GA, or LA/GA showed a large increase in the fractional antigenicity of ovalbumin. The mechanism underlying this increase in antigenicity was likely associated with the unfolding of this protein that resulted in exposing more linear epitopes that are being recognized by the polyclonal antibody used in these studies. On a weight comparison, the apparent increase in antigenicity of ovalbumin incubated in the presence of select monomers is likely attributable to the excessive unfolding of ovalbumin as a consequence of the higher acidity of these monomers. Though no previous experiment in this work provided evidence of ovalbumin undergoing conformational changes in the presence of SA, this solution was still more acidic than the PBS, CPH, or CPH/SA solutions due to the higher water solubility of SA [15]. The data suggested that this increase in acidity affected the overall stability or conformation of the protein.

5.4.3 Stability of lysozyme

5.4.3.1 SDS-PAGE analysis. Lysozyme (14 kDa) is a model protein that is widely available and has been used for microsphere encapsulation. SDS-PAGE was used to monitor changes in the primary structure of lysozyme after being incubated with polymer degradation products. The results from days 0 and 20 were identical. The 20-day incubation data are shown in Figure 5.8 and indicate that the protein did not undergo any changes in its primary structure after being incubated with either ester or anhydride monomers. These results indicate that all of the degradation products provided a suitable environment for maintaining
the primary structure of lysozyme. These results are consistent with previous observations that lysozyme is stable under acidic conditions [39].

Figure 5.8. SDS-PAGE analysis of lysozyme samples (7.5 μg/per lane) after being incubated for 20 days in the presence of the indicated monomers. Lane 1: 1 mM 1,6-bis(p-carboxyphenoxy)hexane (CPH); lane 2: 5 mM sebacic acid (SA); lane 3: .05/2.5 mM CPH/SA; lane 4: 5 mM lactic acid (LA); lane 5: 5 mM glycolic acid (GA); lane 6: 2.5/2.5 mM LA/GA; lane 7: phosphate buffer saline (PBS); lane 8: native lysozyme; lane 9: molecular mass ladder.

5.4.3.2 Circular dichroism. Far UV CD was used to monitor the stability of lysozyme in the presence of water, polyester, and polyanhydride degradation products. There was no change in the spectra of lysozyme incubated in water for 0 and 20 days. The spectra of lysozyme dissolved in water displayed two minima at 208 and 222 nm, as previously reported [40]. The spectra of lysozyme incubated in either polyester or polyanhydride degradation products for 0 days were identical to that of lysozyme incubated with water. Incubation with LA or LA/GA for 20 days were the only treatments that were found to alter the secondary structure of lysozyme. Instead of having two minima, the lysozyme incubated with either LA or LA/GA had a single minimum at 208 nm, and representative examples are shown in Figure 5.9. This corresponds to an increase in the α-helix content, and is consistent with a previous study in which lysozyme was incubated in methanol [41].
5.4.3.3 Fluorescence spectroscopy. The fluorescence spectrum of lysozyme, like ovalbumin, had one peak with a $\lambda_{\text{max}}$ between 330-345 nm [30]. Unlike ovalbumin, lysozyme is more stable under acidic conditions, and thus more resistant to unfolding. To determine if lysozyme unfolded in the presence of any of the polymer degradation products after 20 days, fluorescence spectroscopy was used to monitor the tertiary structure of the protein. The $\lambda_{\text{max}}$ of all of the lysozyme samples from days 0 and 20 fell within the 330-345 nm range, indicating the tertiary structure of the protein had not been detectably altered, as shown in Figure 5.10. These results suggest that lysozyme would be stable when released from either polyester or polyanhydride microspheres.
Figure 5.10. Fluorescence spectral analysis of lysozyme (300 µg/ml) incubated with ester and anhydride monomers for (a) 0 days (b) 20 days. Results are representative of three replicate experiments.

5.4.3.4 Activity. In order to denature lysozyme, denaturants are needed (e.g., to denature 50% of native lysozyme 1.1 M of guanidinium thiocyanate, 2.8 M guanidinium hydrochloride, or 7.4 M urea are required) [39]. Thus, it was anticipated that lysozyme would lose little of its activity after being incubated with polyanhydride and polyester degradation products. Figure 5.11 shows the measured activity of lysozyme after being incubated with polyanhydride and polyester degradation products for 0 and 20 days. The enzymatic activity of lysozyme incubated with the CPH and CPH/SA monomers for 20 days was statistically reduced from lysozyme incubated in PBS for 20 days (~20% activity loss). This loss in activity was attributed to hydrophobic interactions between the protein and the CPH monomer. There was little enzymatic activity lost by lysozyme after being incubated with SA or polyester monomers for as long as 20 days. This indicates that any of the polymers (studied in the form of monomers) would provide a suitable environment for lysozyme and could be chosen as a delivery vehicle for similar acid-stabilized enzymes.
5.5 Conclusions

To effectively administer drugs or vaccines in fabricated delivery vehicles, it will be essential that the released compounds be functionally active or retain immunogenicity. Recently, Kipper et al. [9] described the use of polyanhydride microspheres as vaccine delivery vehicles. The current studies complement those studies by evaluating protein stability following exposure of test proteins to solutions containing anhydride or ester monomers. The experiments described above were performed using the same concentration of both ester and anhydride monomers. Yet, the concentrations represent a best and worse case scenario for the polyesters and polyanhydrides, respectively. Poly(LA) and poly(GA) are bulk-eroding polymers, thus the entire bulk of the microsphere degrades simultaneously. Under these conditions, a protein encapsulated in a polyester microsphere would be exposed to concentrations of LA or GA near saturation with an even lower pH than the solutions used in these experiments. Poly(CPH) and poly(SA) are surface-eroding materials. As they

Figure 5.11. The enzymatic activity of lysozyme (300 μg/ml) was measured using a fluorescence-based assay, after being incubated with ester and anhydride monomers for (a) 0 days or (b) 20 days (± indicates standard deviation). Results are representative of two/three replicate experiments. ** represents p-value < 0.001 as determined by a student t test.
degrade, the polymer degradation products would be at the surface of the microsphere and would quickly diffuse away. It is unlikely that proteins released from polyanhydride microspheres would be exposed to a saturated solution of CPH or SA diacids for a prolonged period [15]; therefore, these experiments represented a worse case scenario for the anhydride monomers.

This work has demonstrated that TT, ovalbumin, and lysozyme behaved differently when incubated for extended periods of time with different polymer degradation products. In the presence of polyester degradation products, TT showed evidence of aggregation and structural unfolding resulting in a larger decrease in antigenicity, which is consistent with previous work [26]. The structural and antigenic changes that occurred in the presence of the ester monomers could result in the induction of poor to undetectable immune responses if the polyesters were used as the delivery vehicle for TT. The results of this study have shown that the structure and antigenicity of TT would be better maintained following encapsulation into polyanhydrides in comparison to polyesters. Thus, polyanhydrides are viable candidates for use as a drug delivery vehicle for proteins that are susceptible moisture induced aggregation.

The consequences of encapsulating a protein known to take on a molten globular structure in an acidic environment (e.g., ovalbumin) such as that formed in polyester microspheres could also lead to the induction of suboptimal immune responses as a result of a denatured protein being released from the microspheres. Ovalbumin in the presence of the ester monomers showed a decrease in stability as evidenced by the CD, fluorimetry, and ELISA experiments. CPH should be used with caution to encapsulate and release proteins similar to ovalbumin. Though no change was exhibited in the antigenicity of the protein
when incubated in the presence of the CPH, the secondary and tertiary structure of the protein was altered indicating a hydrophobic interaction between the protein and CPH. Sebacic acid provided the most stabilizing environment for ovalbumin and these results indicated that polymers containing SA should be further investigated as a delivery vehicle for proteins that take on a molten globular structure under acidic conditions.

Minimal changes in structural integrity and enzymatic activity were detected following the incubation of lysozyme in solutions containing either polyester or polyanhydride degradation products. These experiments demonstrated that there was good potential for polymers based on either esters or anhydrides to successfully deliver acid-stabilized proteins.

These experiments have demonstrated for the first time how anhydride and ester monomers affected the stability of tetanus toxoid, lysozyme, and ovalbumin. These studies demonstrated that proteins behave differently in the presence of the anhydride or ester monomers and provided a “blueprint” for the analyses that should be performed prior to encapsulating proteins in microspheres that are intended for in vivo use. Finally, these studies demonstrated that a rational approach can be employed for selecting compatible polymer/protein systems prior to encapsulation of the protein.

### 5.6 Acknowledgments

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References


CHAPTER 6
THE ROLE OF MICROSPHERE FABRICATION METHODS ON THE STABILITY
AND RELEASE KINETICS OF OVALBUMIN ENCAPSULATED IN
POLYANHYDRIDE MICROSPHERES

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6.1 Abstract

The effect of microsphere fabrication methods on the stability and release kinetics of ovalbumin encapsulated and released from polyanhydride microspheres was investigated. The polyanhydrides used were poly(sebacic anhydride) (poly(SA)) and a 20:80 random copolymer of poly[(1,6-bis-p-carboxyphenoxy)hexane] (poly(CPH)) and poly(SA).

Microspheres were fabricated using three double emulsion methods (water/oil/water (w/o/w), water/oil/oil (w/o/wo), and solid/oil/oil (s/o/o)) and a cryogenic atomization technique. The encapsulation efficiency was the highest for the cryogenic atomization technique and lowest when the w/o/w technique was used. Microspheres fabricated by the s/o/o method had the largest initial burst of released protein. All the fabrication methods resulted in zero-order release of the protein after the burst. The release of ovalbumin from poly(SA) and 20:80 (CPH:SA) microspheres was complete at ~3 and ~6 weeks, respectively. For all fabrication methods the primary structure of released ovalbumin was conserved as determined by gel

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electrophoresis. The secondary structure of ovalbumin encapsulated in 20:80 (CPH:SA) w/o/w microspheres was not conserved.

6.2 Introduction

The use of biodegradable microspheres is among the most commonly used parenteral delivery regimens [1, 2]. Advances in microencapsulation technology have produced numerous successful microsphere-based controlled release devices [3]. pharmaceutically acceptable encapsulation technologies for proteins using biodegradable polymers include spray drying and solvent extraction (e.g. double emulsion) techniques. However, major challenges remain in the area of encapsulation and release of biologically active proteins. While the complex physicochemical nature and the three-dimensional structural intricacies of proteins enhances their value as therapeutic drugs, it also makes them fragile under pharmaceutically relevant processing conditions. When a protein is encapsulated within a polymer microsphere, the diverse functional groups of the protein are prone to a plethora of deleterious processes under conditions relevant to their formulation, storage, and delivery [4]. In addition to conserving the hierarchical structure of the protein and its activity, it is also important to sustain the release of the protein over the desired time interval to maintain optimal drug concentration in serum and to improve patient compliance by reducing the frequency of administration.

Surface-erodible polyanhydrides have been studied as potential drug delivery carriers for about two decades [5]. Studies have focused on the synthesis and characterization of random copolymers, their physicochemical properties, and biocompatibility [6]. This class of water-insoluble polymers degrades (by backbone chain scission across the anhydride bond)
into water-soluble monomers that can be absorbed by the body. The microstructural characterization and phase behavior of random copolymer polyanhydrides has been carried out using differential scanning calorimetry (DSC), small angle X-ray scattering (SAXS), wide angle X-ray diffraction (WAXD), and atomic force microscopy (AFM) [7-10]. The "block-like" microstructure of some random copolymer polyanhydrides has been confirmed by liquid state $^1$H NMR spectroscopy, solid state NMR, and SAXS [11]. It has also been shown that drug release profiles do not match polymer erosion profiles over the entire duration of release [8, 12]. In fact, drug release profiles match individual monomer release profiles depending on their "affinity" to the monomer phase, pointing to the existence of microphase separation. The use of polyanhydride microspheres as carriers for proteins has been demonstrated [13]. In these experiments, fluorescein isothiocyanate labeled bovine serum albumin (BSA-FITC) was encapsulated in polyanhydride copolymer microspheres. It was found that increasing the hydrophobicity of the copolymer led to a decrease in the stability of the protein (via loss of secondary structure). These results are consistent with studies by Langer and co-workers [2, 14] on the release of polypeptides from polyanhydrides indicating that the water penetration into the core of the polyanhydride was reduced, thus, helping prevent covalent aggregation.

The most commonly used methods for microsphere fabrication include solvent extraction (e.g. double emulsion) methods such as water-in-oil-in-water (w/o/w), water-in-oil-in-oil (w/o/o), solid-in-oil-in-oil (s/o/o), and a cryogenic atomization procedure. Microspheres are most commonly fabricated using double emulsion techniques such as w/o/w and w/o/o [15]. These methods are attractive because they do not require any special equipment and are relatively straightforward. However, these methods have drawbacks since
they result in variable loading efficiencies and the creation of a water/oil interface [16-22]. This interface causes proteins to migrate and act as surfactants. Once at the interface, the protein may begin to unfold. Ovalbumin is one of many proteins vulnerable to unfolding at the water/oil interface. It is known that after a 0.5 mg/ml solution of ovalbumin is emulsified with methylene chloride only 37.8% of the initial protein is recovered from the aqueous-phase when no surfactant is added [21].

The s/o/o technique and the cryogenic atomization method offer viable alternatives to the aqueous double emulsion techniques by eliminating the water/oil interface and by increasing the loading efficiency [23, 24]. In order to encapsulate a protein using either of these methods, the protein must first be lyophilized. The lyophilized protein is then suspended in the organic polymer phase. Achieving a uniform distribution of a protein within microspheres fabricated with these methods is challenging. Large bursts (14 - 70% burst) of initial release are typically seen when lyophilized protein is encapsulated in microspheres [23-27]. Ultimately, the fabrication method needs to be capable of achieving the highest loading efficiency, providing reproducible and desired release kinetics, and stabilizing the encapsulated protein and releasing a functional protein.

The objectives of this study are to evaluate the role of the fabrication method on the stability and the release kinetics of proteins encapsulated in polymer microspheres. The polyanhydrides used in this work are poly(sebacic anhydride) (poly(SA)) and a copolymer of poly(SA) and poly[1,6-bis-p-carboxyphenoxy)hexane] (poly(CPH)), 20:80 (CPH:SA). These polymers are of interest because they are biodegradable and varying the composition of SA and CPH monomers can control the rate of degradation of the copolymer. Poly(CPH) is more hydrophobic than poly(SA), hence the copolymer 20:80 (CPH:SA) is more
hydrophobic than poly(SA) and will take longer to degrade [28]. These two polymers are also of interest because previous work has shown that BSA-FITC can be stabilized within and released from microspheres based on these two polyanhydrides [13].

6.3 Material and Methods

6.3.1 Materials

Sebacic acid (99%), 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone anhydrous 99.5%, 1,6-dibromohexane, potassium bromide (FTIR grade), Hanks’ balanced salt solution (without phenol red), poly(vinyl alcohol) (PVA), and ovalbumin from chicken egg white (grade V minimum 98% gel electrophoresis) were purchased from Sigma-Aldrich (Milwaukee, WI). The BCA kit used to quantify the release of ovalbumin was purchased from Pierce Biotechnology Inc. (Rockford, IL). 40% acrylamide, low molecular massstandards, and the silver stain plus kit were purchased from BioRad (Hercules, CA). Dow corning fluid 550 and 200 proof ethanol were purchased from in-house bulk supplies. All other chemicals were purchased from Fischer Scientific (Pittsburgh, PA) and used as received.

6.3.2 Polymer synthesis & characterization

The synthesis of SA and CPH prepolymers used has been previously described [7, 12]. The synthesis of CPH prepolymer was performed by modifying the synthesis of 1,3-bis(p-carboxyphenoxy)propane as described by Conix [29]. The SA and CPH prepolymers were polymerized via a melt polycondensation reaction under vacuum (3 x 10^{-1} torr) for 90 min at 180 °C [7, 12]. The polymers were characterized using gel permeation
chromatography (GPC), proton nuclear magnetic resonance (\(^1\)H NMR), and Fourier transform infrared spectroscopy (FTIR).

6.3.3 Ovalbumin lyophilization

To obtain solid ovalbumin with a small particle size (<1 μm) the ovalbumin was constituted in a 50 mM ammonia bicarbonate solution. The protein solution (5 mg/ml) was then placed in a gas tight syringe and pumped (3 ml/min) to an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY). The protein solution was atomized at an output of 2.5 W into 400 ml of liquid nitrogen. The liquid nitrogen was allowed to boil off and the frozen atomized protein was dried under vacuum overnight. To characterize the lyophilized protein some of the ovalbumin particles were coated with 200 Å of gold and viewed with JOEL 840A scanning electron microscope (SEM).

6.3.4 W/O/W microsphere fabrication method

The w/o/w solvent removal technique employed to fabricated ova-loaded microspheres has been previously described [13]. Briefly, an ovalbumin solution (5 mg of ovalbumin dissolved in 200 μl of de-ionized water) was added to a polymer solution (100 mg polymer dissolved in 2 ml methylene chloride). The two solutions were emulsified, forming the inner emulsion, with a Tissue-Tearor™ (Biospec Products, Bartlesville, OK) at 30 000 rpm for 30 sec. A 1% PVA (4 ml) solution saturated with methylene chloride (80 μl) was then added to the polymer/protein emulsion and further emulsified at 10 000 rpm for 30 sec to form the double emulsion. The newly formed microspheres were added to a stirring 1% PVA (100 ml) solution. The dispersion was stirred (300 rpm) with an overhead stirrer with a
3 in impeller (Wiarton, Ontario) on ice for 2 h. The dispersion was then centrifuged for 10 min at 1500 g (Eppendorf Centrifuge 5403, Westbury, NY) to collect the microspheres. To remove any unencapsulated protein and PVA from the surface of the microspheres the supernatant was removed and replaced by fresh de-ionized water. The microspheres were centrifuged and washed a total of three times. The washed microspheres were then suspended in 4 ml of de-ionized water and flash frozen and dried overnight under vacuum.

6.3.5 W/O/O microsphere fabrication method

The w/o/o procedure used for this study was similar to previously reported methods [30, 31]. An ovalbumin solution (5 mg dissolved in 200 μl of de-ionized water) was emulsified with a polymer solution (100 mg polymer dissolved in 2 ml methylene chloride) using a Tissue-Tearor™ at 30 000 rpm for 30 sec, forming an inner emulsion. Dow Corning oil 550 (3 ml) saturated with methylene chloride (4 ml) was added to the protein/polymer emulsion and homogenized at 30 000 rpm for 30 sec forming a double emulsion. To extract the solvents the newly formed microspheres were stirred (300 rpm) in 200 ml of heptane on ice for 2 h. The hardened microspheres were then collected by filtration and dried under vacuum overnight.

6.3.6 S/O/O microsphere fabrication method

The s/o/o fabrication technique used was modified from the procedure described by Carrasquillo et al. [27]. Microspheres were constructed by adding atomized ovalbumin (5 mg) to polymer (100 mg) dissolved in methylene chloride (2 ml). The protein was suspended in the organic phase by mechanically mixing using a Tissue-Tearor™ at 30 000 rpm for 1
min. Dow Corning oil 550 (3 ml) saturated with methylene chloride (4 ml) was then added to the polymer/protein suspension and emulsified at 20 000 rpm for 1 min. The resulting microspheres were added to 200 ml of heptane stirring at 300 rpm (overhead stirrer) on ice. After stirring for 2 h the microspheres were filtered and dried under vacuum overnight.

6.3.7 Cryogenic atomization method for microsphere fabrication

The cryogenic atomization method used in this work has been previously described [24, 32]. Atomized ovalbumin (5 mg) was suspended in a polymer solution (100 mg of poly(SA) in 3 ml of methylene chloride or 100 mg of 20:80 (CPH:SA) in 1 ml of methylene chloride) using a Tissue-Tearor™ at 10 000 rpm for 30 sec. The suspension was transferred to a gas tight syringe and pumped (3 ml/min) to an ultrasonic nozzle operating at 2.5 W or 1.5 W for SA and 20:80, respectively. The suspension was atomized into 200 ml of liquid nitrogen overlaying 300 ml of frozen ethanol. The frozen microspheres and ethanol were stored at –80°C for three days, during which time the ethanol thawed and the methylene chloride was extracted from the polymer, leaving behind solid microspheres. The microspheres were then collected by filtration and dried overnight under vacuum.

6.3.8 Microsphere characterization

The yield of each batch of microspheres was calculated by dividing the mass of microspheres recovered by the initial mass of polymer and protein used. The surface morphology of the microspheres was examined by coating the samples with 200 Å of gold and examining them using SEM. To determine the molecular massloss that occurred during the fabrication process, the molecular massof blank microspheres was compared to the
molecular mass of neat polymer using GPC. The GPC columns were purchased from Polymer Laboratories (Amherst, MA) and polystyrene standards were used for calibration.

6.3.9 Encapsulation efficiency

The amount of protein loaded into each batch of microspheres was determined by extraction as previously reported by Lam et al. [24]. Ovalbumin-loaded microspheres (~10 mg) were suspended by sonication (Sonics & Materials Inc., Newton, CT) in 1 ml of 17 mM NaOH. The suspension was then loaded into a syringe. To ensure all microspheres were recovered, the test tube was rinsed twice with 1 ml of 17 mM NaOH. All 3 ml were then loaded into 3 ml Slide-A-Lyzer® (Pierce Biotechnology Inc., Rockford, IL) and suspended in 600 ml of 17 mM NaOH. After 4 days at room temperature under constant agitation (100 rpm), the NaOH solution was removed from the dialysis cassette and analyzed using the BCA assay to determine the amount of ovalbumin that had been loaded into the microspheres. The encapsulation efficiency was calculated by dividing the amount of ovalbumin obtained from the extraction study by the amount of ovalbumin used at the start of the microsphere fabrication process.

6.3.10 In vitro release studies

Microspheres made by each of the fabrication methods described above were suspended (15 mg) separately in 1.0 ml Hanks' balanced salt solution. Sodium azide (0.01% w/w) was added to the salt solution to prevent microbial contamination. The microsphere suspensions were incubated at 37°C under constant agitation at 100 rpm. At predetermined times the supernatant was sampled (750 µl) and then replaced with fresh buffer (750 µl) to
maintain sink conditions. The sampled supernatant was stored at 4°C until the protein concentration was quantified using the BCA assay. Prior to using the BCA assay the supernatants were centrifuged (10 000 rpm, 10 min, at 4°C). The in vitro release data was normalized by the cumulative amount of ovalbumin released. Release studies were done in triplicate for each microsphere fabrication method and for both polymer compositions.

6.3.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The conservation of primary structure of the protein following its encapsulation and in vitro release was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The microspheres were incubated in Hanks' balanced salt solution under the conditions described above. After 2 h of incubation, the supernatant was removed and replaced with an equal volume of fresh salt solution. After 46 h of additional incubation the supernatant was removed and analyzed using SDS-PAGE. This ensured that any un-encapsulated protein was removed prior to the release of the encapsulated protein, and that any bands on the developed gels were a result of ovalbumin released from the microspheres.

An equal volume of the sampled supernatant from each experiment was mixed with SDS-loading dye (SDS 1% w/v, 0.06 mM Tris-HCl pH 6.8, 3.0 mM glycerol, bromophenol blue 0.01% w/v, and β-mercaptoethanol) and loaded (10.0 μl) into polyacrylamide gels for electrophoresis (10% acrylamide running gel and 5% acrylamide stacking gel). Electrophoresis was also performed on ovalbumin samples (0.125 mg/ml) in Hank's balanced salt solution for comparison with the released samples. All the samples were compared to standards.
The BioRad Mini-Protean II electrophoresis setup was used to perform these experiments, with a constant voltage of 120V for 70 min. The gels were silver stained using the BioRad silver stain kit. The gels were photographed and dried.

6.3.12 Fourier transform infrared spectroscopy (FTIR)

Preservation of secondary structure of encapsulated ovalbumin was examined using Fourier transform infrared (FTIR) spectroscopy. Samples were prepared by mixing protein-loaded microspheres, protein-free microspheres, or native ovalbumin with 99+% FT-IR (5% w/w) grade potassium bromide. Pellets were pressed under vacuum using a Carver press (Wabash, IN) at 2000 lb.

Spectroscopy was performed with a Nicolet Nexus 470 (Madison, WI) utilizing a cooled MCT/A detector and an Ever-Glo source beam. The data collection software used was Omnic 7.2. Each spectrum consisted of 256 scans at a resolution of 4 cm\(^{-1}\). Background spectra were collected every 100 minutes. Following spectra collection, the spectra of the encapsulated protein were obtained by subtracting the spectra of the blank microspheres from the spectra of protein-loaded microspheres. The resulting spectrum was used only if a flat baseline in the region of 1800-2500 cm\(^{-1}\) was obtained from the subtraction [33]. Fourier self-deconvolution was preformed on the amide I region of the encapsulated protein spectra using a bandwidth of ~20 and an enhancement factor of 1.5 [34]. Gaussian curves were fit to the deconvoluted spectra and the area of each deconvoluted peak was used to calculate the secondary structure of the encapsulated ovalbumin [35]. Each peak represented a secondary structure: \(\alpha\)-helix, \(\beta\)-sheet, \(\beta\)-turn, or random coil and was assigned according to the designation made by Dong et al. [36]. A Student t-test was preformed to determine if the
secondary structure of the encapsulated ovalbumin differed significantly from that of the 
native protein. Treatments showing a statistical difference were noted.

6.4 Results and Discussion

6.4.1 Ovalbumin atomization

Ammonium bicarbonate is a volatile salt when dried under vacuum. Hence, it was 
chosen as the salt for the solution in the cryogenic atomization of ovalbumin. Using this 
volatile salt ensured that only ovalbumin (and not salts) was loaded into microspheres (e.g., 
s/o/o and cryogenic atomization methods). After the ovalbumin was atomized and dried, the 
size of the resulting protein particles was inspected visually using SEM. The atomized 
particles were smaller than the initial ovalbumin (data not shown) flakes. The small protein 
particles took on a fibrous form with <1 μm diameter and lengths ranging from <1 μm to 5 
μm. Upon mechanical mixing (or further scraping of the protein on the SEM stub), the size 
of the proteins was further reduced, enabling encapsulation in microspheres.

6.4.2 Microsphere characterization

A minimum of three batches were used to calculate the yield of poly(SA) and 20:80 
(CPH:SA) microspheres made by each of the four fabrication methods studied. As expected, 
the percent yield was independent of the type of polymer used as demonstrated by the little 
variation between poly(SA) and 20:80 (CPH:SA) microspheres fabricated by the same 
method. The results for poly(SA) microspheres are shown in Table 6.1. Microspheres 
fabricated using the w/o/w, w/o/o, or s/o/o methods resulted in yields of ~75%. When 
microspheres were fabricated using the cryogenic atomization procedure, the percent yield
was lower (~ 60%). This lower yield was a result of some of the atomized microspheres not falling into the liquid nitrogen overlaying the frozen ethanol. As the polymer/protein suspension is atomized a fine mist falls from the nozzle into the liquid nitrogen. As this occurs, the liquid nitrogen begins to boil making it difficult to ensure a higher of the atomized microspheres.

Table 6.1. Yields and loading efficiencies for poly(SA) microspheres.

<table>
<thead>
<tr>
<th>Microsphere Fabrication Method</th>
<th>Yield (%)</th>
<th>Encapsulation Efficiency (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/O/W</td>
<td>72 ± 14</td>
<td>60 ± 23</td>
</tr>
<tr>
<td>W/O/O</td>
<td>78 ± 9</td>
<td>85 ± 20</td>
</tr>
<tr>
<td>S/O/O</td>
<td>73 ± 6</td>
<td>85 ± 20</td>
</tr>
<tr>
<td>Cryogenic Atomization</td>
<td>51 ± 9</td>
<td>100 ± 2</td>
</tr>
</tbody>
</table>

^a ± values are standard deviation values calculated from a minimum of three batches

To address the question of how much polymer degradation occurred during the microsphere fabrication process, the molecular mass of neat polymer was compared to the molecular mass of protein-free microspheres fabricated by each of the four methods using GPC. The results are shown in Table 6.2. The amount of molecular massloss was proportional to the amount of water used in the fabrication process. Neither the s/o/o nor the cryogenic atomization methods required any water. However, in the case of poly(SA) microspheres fabricated via the s/o/o method the polymer did undergo a 13% reduction in \( M_n \). This reduction in molecular mass may be due to the water from the atmosphere degrading the polymer during the handling of the microspheres and by the water in the
atmosphere dissolved in the non-solvent (heptane) during the solvent extraction process. For all fabrication methods where molecular mass loss was detected, the largest molecular mass loss occurred in the poly(SA) microspheres. This result is expected because poly(SA) is more hydrolytically labile than 20:80 (CPH:SA).

The surface morphology of the microspheres was analyzed using SEM. The photomicrographs of poly(SA) and 20:80 (CPH:SA) microspheres are shown in Figures 6.1 and 6.2, respectively. The amount of polymer degradation that occurs during the fabrication process and the rate of solvent extraction influence the surface morphology of the microspheres. The rate of solvent extraction is influenced by temperature, the type of polymer, the non-solvent, and the stability of the inner emulsion present in the newly formed microspheres. As the volume of water used to fabricate the poly(SA) microspheres and the number of emulsions used to create the microspheres decreased, the surface of the microspheres got smoother. The surface of the poly(SA) microspheres fabricated using the w/o/w method was the roughest because this technique required the largest volume of water (leading to more polymer degradation). This method also required an inner emulsion that increased the rate of solvent extraction, leading to a rough surface morphology. The surface morphology of the poly(SA) microspheres fabricated using the w/o/o and s/o/o processes looked similar as a result of using the same non-solvent (e.g., heptane) in the outer emulsion and the inner aqueous phase (w/o/o method), thus having little effect on the rate of solvent extraction. The surface morphology of poly(SA) microspheres fabricated using the cryogenic atomization technique had the slowest rate of solvent extraction leading to the smoothest surface. The cryogenic atomization technique required the solvent extraction to occur at -80°C and ethanol was used as the non-solvent.
Table 6.2. Molecular mass of poly(SA) and 20:80 (CPH:SA) microspheres compared to neat polymer as determined by GPC

<table>
<thead>
<tr>
<th>Microsphere Fabrication Method</th>
<th>Poly(SA) $M_n$ Loss (%)</th>
<th>20:80 (CPH:SA) $M_n$ Loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/O/W</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>W/O/O</td>
<td>19</td>
<td>Not detected</td>
</tr>
<tr>
<td>S/O/O</td>
<td>13</td>
<td>Not detected</td>
</tr>
<tr>
<td>Cryogenic Atomization</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Figure 6.1. SEM photomicrographs depicting the surface morphology of ovalbumin-loaded poly(SA) microspheres fabricated via (a) w/o/w; (b) w/o/o; (c) s/o/o; (d) cryogenic atomization
The surface morphology of 20:80 (CPH:SA) microspheres was independent of the microsphere fabrication process used. This was a result of the solvent being extracted from the 20:80 (CPH:SA) polymer at similar rates even when different non-solvents were used. Because the cryogenic atomization method produced extremely smooth poly(SA) microspheres, 20:80 (CPH:SA) was atomized into different non-solvents to try to obtain similar results. The surface roughness of ovalbumin-loaded 20:80 (CPH:SA) atomized microspheres remained the same when either isopropanol or heptane were used as non-solvents. However, when protein-free 20:80 (CPH:SA) microspheres were atomized into isopropanol, the surface morphology was smooth and similar to that of the poly(SA)
atomized microspheres (data not shown) indicating that ovalbumin increased the rate of solvent extraction, leading to microspheres with a rougher surface topography.

6.4.3 Encapsulation efficiency

The degradation for polyanhydrides is base catalyzed [5, 37, 38]. Thus, a 17 mM NaOH solution was used to accelerate the degradation of ovalbumin-loaded microspheres in order to quantify the encapsulation efficiency of ovalbumin. The molarity of the NaOH used was chosen by determining the highest NaOH concentration in which ovalbumin was soluble. By placing the microsphere suspension in a dialysis cassette, the released ovalbumin was retained in the cassette while the soluble polymer degradation products were removed.

Table 6.1 shows the encapsulation efficiencies of ovalbumin-loaded poly(SA) microspheres made by the various fabrication methods. The results for the 20:80 (CPH:SA) microspheres were similar. The highest encapsulation efficiency was obtained when microspheres were fabricated using the cryogenic atomization method; almost 100% of the protein was loaded into the microspheres. Microspheres fabricated using the w/o/o and s/o/o techniques had similar loadings, as a result of using the same non-solvent. The lowest encapsulation efficiency was observed when using the w/o/w method. This method not only contains a poorly stabilized inner emulsion but also an exterior aqueous phase, which increases the probability that the protein within the forming microsphere would partition to the exterior aqueous phase reducing the encapsulation efficiency. If a surfactant had been used to stabilize the inner emulsion of either the w/o/w or w/o/o methods, it would have been co-encapsulated in the microspheres with the ovalbumin. No surfactant was used to prevent alterations of the release profiles and to allow comparison of the protein interacted with the
polymer after being fabricated by each of the four methods. However, by not using a surfactant in the inner emulsion, the emulsions were less stable and the variability of the encapsulation efficiency increased.

6.4.4 In vitro release of ovalbumin

The in vitro release studies were conducted in Hanks’ balanced salt solution to mimic the physiological conditions. Sodium azide was added to the medium to inhibit microbial contamination.

The in vitro release of ovalbumin from poly(SA) and 20:80 (CPH:SA) microspheres is shown in Figure 6.3. The largest bursts occurred when the s/o/o method was used to fabricate either the poly(SA) or 20:80 (CPH:SA) microspheres. For the poly(SA) microspheres, the second largest burst (~24%) was seen by microspheres fabricated by the cryogenic atomization technique. The 20:80 (CPH:SA) microspheres fabricated by this technique had a similar burst of ~30%. In general, the larger bursts were associated with the protein being encapsulated as a solid. In these methods, it was difficult to maintain a uniform distribution of a solid protein during the microsphere fabrication process. This was consistent with previously reported data [23-27]. The w/o/o fabrication technique had varying effects on the release kinetics depending on the polymer used to fabricate the microspheres. The poly(SA) microspheres fabricated using the w/o/o technique had a 14% burst while the 20:80 (CPH:SA) microspheres had a 32% burst. This difference was attributed to the 20:80 (CPH:SA) polymer being more hydrophobic and decreasing the protein compatibility with the more hydrophobic polymer. The microspheres fabricated by the w/o/w techniques (both poly(SA) and 20:80 (CPH:SA)) showed the smallest initial
bursts. This was attributed to the protein maintaining a uniform distribution within the microspheres throughout the fabrication process. Similar results were observed when BSA-FITC was encapsulated in these polymers and the protein distribution viewed with confocal microscopy [13].

![Graphs showing in vitro release of ovalbumin from poly(SA) microspheres and 20:80 (CPH:SA) fabricated by various methods.](image)

Figure 6.3. *In vitro* release of ovalbumin from (a) poly(SA) microspheres (b) 20:80 (CPH:SA) fabricated by various methods. Studies were performed using Hanks’ balanced salt solution with 0.01% sodium azide. Results are representative of three replicates.

After the initial burst the release of ovalbumin from the microspheres, a period of zero order release followed for all the polymers and the fabrication methods studied. After 10 days, all of the protein was released from poly(SA) microspheres fabricated by the s/o/o and w/o/o methods, providing the shortest release profile. The ovalbumin encapsulated in poly(SA) microspheres fabricated by the w/o/w and cryogenic atomization methods showed a more prolonged release lasting ~3 weeks. Thus, depending on the release kinetics desired, the appropriate fabrication method could be selected. If a large burst followed by a release of protein for ~1 week is needed, the s/o/o fabrication method could be employed. If a smaller burst was needed over a seven day period, the w/o/o method could be employed. For longer release times, either the w/o/w or cryogenic atomization method could be employed.
depending on the desired initial burst. The release of ovalbumin from 20:80 (CPH:SA) microspheres continued for ~6 weeks and was independent of the fabrication method used.

6.4.5 SDS-PAGE analysis

The molecular mass of the ovalbumin released from poly(SA) and 20:80 (CPH:SA) microspheres fabricated using the w/o/w, w/o/o, s/o/o, and cryogenic atomization was investigated using SDS-PAGE. The results are shown in Figure 6.4. The ovalbumin released from all of the microsphere formulations showed only one band corresponding to a molecular mass of 45 KDa. No additional bands were present, indicating the released protein was neither aggregated nor fragmented. Similar findings for ovalbumin encapsulated and released from PLGA microspheres fabricated via w/o/w and w/o/o methods have been previously reported [39]. These results also agree with the previous work in which BSA-FITC was released from poly(SA) and 20:80 (CPH:SA) microspheres [13]. Similar to BSA-FITC, ovalbumin was known to form moisture-induced covalent aggregates [40]. Because there are no additional bands of higher molecular weight, it can be concluded that polyanhydrides microspheres prevented the covalent aggregation of ovalbumin.
6.4.6 FTIR analysis

FTIR was used to determine what effect, if any, the different microsphere fabrication methods had on the secondary structure of the encapsulated ovalbumin. Typical signatures of the secondary structure of ovalbumin are α-helices, β-sheets, β-turns, and random coils [36]. It is known that the β-sheet and random coil content of a protein vary as a protein is transferred between a solid and liquid state [34]. A lyophilized protein will have a higher β-sheet content than when dissolved in an aqueous solution. Hence, only the α-helical content of the encapsulated ovalbumin was used to determine if the fabrication processes altered the secondary structure.

The α-helical content of native lyophilized ovalbumin was determined to be 24% (Figure 6.5a) and was consistent with the value determined by crystallography [41]. The spectrum of the encapsulated protein was compared to that of the native protein to determine if the fabrication process affected the secondary structure of the ovalbumin. The results of the spectral analysis are shown in Figure 6.5. Ovalbumin encapsulated in poly(SA)
microspheres showed no statistical difference in α-helical content when compared to the native protein.

The secondary structure of the ovalbumin encapsulated in 20:80 (CPH:SA) microspheres was maintained when the microspheres were fabricated using the w/o/o, s/o/o, or cryogenic atomization techniques. Ovalbumin encapsulated in 20:80 (CPH:SA) microspheres fabricated using the w/o/w technique showed a small, but statistically significant loss in α-helical content indicating that the fabrication process lead to the destabilization of the protein. When fabricating microspheres using the w/o/w method, two water/oil interfaces were created increasing the possibility of protein destabilization. This loss in stability was not observed when poly(SA) was used to fabricate w/o/w microspheres. This difference in stability of encapsulated ovalbumin is attributed to the different interactions that occur between the protein and the polymers. As the hydrophobicity of the encapsulating polymer was increased, the stability of the protein was jeopardized [13].

Figure 6.5. The secondary structure of native ovalbumin vs. ovalbumin encapsulated in (a) poly(SA) microspheres (b) 20:80 (CPH:SA) microspheres. The * indicates statistical differences (p-value < 0.1) when compared to the secondary structure of native protein as determined by a Student t-test.
6.5 Conclusions

This study has shown how microsphere fabrication processes affect the stability and release kinetics of ovalbumin from polyanhydride microspheres. Proteins encapsulated in an aqueous form (w/o/w and w/o/o) typically result in lower initial bursts and lower encapsulation efficiencies. As the hydrophobicity of the polymer increases, caution should be used when encapsulating a protein in an aqueous form due to the increased possibility of the protein being destabilized. This was observed by the reduction of α-helices in ovalbumin encapsulated in 20:80 (CPH:SA) microspheres fabricated by w/o/w method. By encapsulating a protein as a solid, the likelihood of the protein undergoing conformational alterations was reduced. The drawback to using solid phase proteins was that it was difficult to obtain uniform distribution within a polymer solution when fabricating microspheres resulting in larger bursts of protein release.

The poly(SA) microspheres fabricated using the s/o/o and w/o/o methods had the least desirable release kinetics due to the extremely fast release of ovalbumin (completely released in 10 days). The release of ovalbumin from the 20:80 (CPH:SA) from all the batches of microspheres lasted ~6 weeks, and no significant differences were observed in the size of the initial burst.

From these studies, the cryogenic atomization method maintained the stability of the encapsulated protein and provided reasonable bursts for all the polymers used. The highest encapsulation efficiency was obtained when microspheres were fabricated using the cryogenic atomization method, which is an important consideration when working with therapeutic proteins. This method also prevented alterations in the secondary structure of the encapsulated ovalbumin.
6.6 Acknowledgements

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6.7 References


CHAPTER 7
UTEROCALIN-LOADED POLYANHYDRIDE MICROSPHERES EXPEDITE CELL MIGRATION FOR WOUND HEALING APPLICATIONS

A paper to be submitted to the Journal Biomedical Materials Research
Amy S. Determan\textsuperscript{1,2}, Marit Nilsen-Hamilton\textsuperscript{3}, Balaji Narasimhan\textsuperscript{2,4}

7.1 Abstract
A cryogenic atomization method was used to fabricate uterocalin (24p3, SIP24, Lcn2)-loaded polyanhydride microspheres. The polymers used for this work were poly(sebacic anhydride) (poly(SA)) and a 20:80 copolymer composed of 1,6-bis(p-carboxyphenoxy)hexane] (CPH) and SA. The \textit{in vitro} release kinetics of uterocalin from the polyanhydride microspheres was zero order after an initial burst of 22\% of the encapsulated protein. Uterocalin released from the polyanhydride microspheres maintained its primary structure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The \textit{in vitro} biological activity of uterocalin released from polyanhydride microspheres was investigated using a cell migration assay based on human colon epithelial cells (HT29). Uterocalin released from either poly(SA) or 20:80 (CPH:SA) microspheres maintained its biological activity as demonstrated by the increased rate of HT29 cell migration. This work demonstrates the therapeutic use of polyanhydride microspheres as protein carriers.

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7.2 Introduction

For chronic diseases such as diabetes or rheumatoid arthritis, traditional therapeutic strategies require patients to undergo frequent drug administration. In the last two decades, the concept of using biodegradable polymers for sustained drug delivery, which reduces the frequency of drug administrations, has received much attention. In particular, polymeric microspheres have been extensively studied for use in sustained drug delivery because they are injectable and do not require surgical removal [1].

Poly(D,L-lactide-co-glycolide) (PLGA) is one of the most commonly investigated polymers for use in sustained drug and protein delivery [1]. Research has demonstrated that proteins can be stably encapsulated and released from PLGA microspheres. Nutropin Depot®, which encapsulates recombinant human growth hormone, is one example of a sustained drug delivery system approved for use in humans by the U.S. Food and Drug Administration (FDA) [2]. However, PLGA microspheres are not suitable delivery vehicles for many therapeutic proteins. Proteins are complex three-dimensional molecules that are usually biologically inactive or can cause adverse reactions if administered in a denatured form. One reason PLGA is not a suitable carrier for many proteins is because PLGA is a bulk-eroding polymer, and allows water to penetrate the entire volume of the degrading polymer microsphere. This increases the possibility of moisture-induced protein aggregation [3-5]. A second concern when using PLGA to encapsulate proteins is that the microenvironment in and around a degrading PLGA microsphere can become very acidic, with the pH reaching as low as 1.5 [6]. This acidic microenvironment denatures many proteins [7].

Polyanhydrides have been investigated as an alternative to using PLGA as a vehicle for therapeutic proteins [8-11]. Unlike PLGA, polyanhydrides are surface-eroding polymers,
and, as such, they reduce the risks of moisture-induced aggregation of proteins [3, 4, 12]. Additionally, the degradation products of polyanhydrides are less soluble in water than the degradation products of PLGA. As a result, the microenvironment surrounding a degrading polyanhydride device is not as acidic as that produced by PLGA reducing any unwanted stress on an encapsulated protein [13].

Previous work has shown that the polyanhydrides microspheres based on poly(sebacic anhydride) (poly(SA)) and the 20:80 copolymer of 1,6-bis(p-carboxyphenoxy)hexane (CPH) and SA are capable of stabilizing the structure of encapsulated proteins [10, 11]. Little work to date has examined the ability of proteins encapsulated and released from microspheres made of either poly(SA) or 20:80 (CPH:SA) to maintain their biological activity [14]. The goal of this work is to determine that polyanhydrides based on SA and CPH can not only stabilize the structure of an encapsulated protein but also remain biologically active upon release. To this end, uterocalin (SIP24, 24p3, Lcn2) was encapsulated and released from poly(SA) and 20:80 (CPH:SA) microspheres. Uterocalin was encapsulated in the polyanhydride microspheres using a cryogenic atomization technique [10, 15, 16]. This technique was used because it has been shown to minimize protein loss during the fabrication process, it eliminates water/oil interfaces, and reduces protein denaturation [17].

Uterocalin is an acute phase protein encoded in the mouse genome by the gene Lcn2, and it is a member of the lipocalin protein family [18]. The human homolog of uterocalin is neutrophil gelatinase-associated lipocalin (NGAL). Uterocalin is a 24 kDa protein composed of a β-barrel [19]. It is hypothesized that uterocalin binds ligands in the calyx of the protein. It is also hypothesized that uterocalin would no longer be biologically active if the structure
of the protein was altered [20]. Though the in vivo function of uterocalin is still being investigated, two examples of in vitro functions of uterocalin have been identified. These include siderophore binding by uterocalin and the ability of uterocalin to increase cell migration of wounded epithelial cells [21, 22]. In this paper, we are interested in the wound healing application.

One of the first responses the body has after being wounded is the secretion of acute phase proteins into the blood stream [23]. This is followed by the migration of epithelial cells. Because uterocalin is an acute phase protein that can increase the rate of epithelial cell migration in vitro, it is hypothesized that it plays a role in the wounding healing process in vivo. This work examines the ability of uterocalin to increase the rate of human colon epithelial (HT29) cell migration after being released from polyanhydride microspheres.

It is the hypothesis of this work that uterocalin encapsulated in either poly(SA) or 20:80 (CPH:SA) microspheres will be stabilized and will be released as a biologically active protein, capable of stimulating and sustaining epithelial cell migration in vitro. This work lays the groundwork for future therapeutic applications of uterocalin and NGAL while demonstrating the usefulness of polyanhydrides as vehicles for protein delivery.

7.3 Materials and Methods

7.3.1 Materials

The following chemicals were purchased from Sigma-Aldrich (Milwaukee, WI): sebacic acid (99%), 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone anhydrous 99.5%, 1,6-dibromohexane, bovine serum albumin (BSA), and Hanks’ balanced salt solution (without phenol red). The BCA kit, dialysis cassettes (3500 MW cutoff), and mini dialysis cassettes
(3500 MWCO) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Precast 12% polyacrylamide gels (Tris-HCl), low molecular mass standards, 2-mercaptoethanol, and a silver stain plus kit were purchased from BioRad (Hercules, CA). Bacto™ yeast extract, tryptone, and agarose were purchased from Becton, Dickinson, and Company (Sparks, MD). Ampicillin (sodium salt) and isopropyl-β-D-thiogalactopyranoside (IPTG, dioxane free) were purchased from Research Products International Corporation (Mt. Prospect, IL). Ni-NTA was purchased from Qiagen (Valencia, CA). Human epithelial growth factor (EGF) was purchased from R & D Systems (Minneapolis, MN) and reconstituted in 10 mM acetic acid and 0.1% BSA. Ethanol (200 proof) was purchased from in-house bulk supplies. All other chemicals were purchased from Fischer Scientific (Pittsburgh, PA) and used as received.

7.3.2 Polymer synthesis & characterization

Sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH) prepolymers were used to synthesize poly(SA) and the copolymer 20:80 (CPH:SA) by a melt polycondensation reaction. The synthesis of the SA and CPH prepolymers has been reported previously by Shen et al. [24]. The synthesis of poly(SA) and the copolymer (20:80 CPH:SA) has been previously described [25]. The newly synthesized polymers were characterized by Fourier transform infrared spectroscopy (FTIR) and gel permeation chromatography (GPC).

7.3.3 Protein purification

The plasmid pTrcHisUtc2, previously constructed by Ryon et al. [26], containing the full-length uterocalin cDNA and a 6X histidine tag, was amplified in XL1 Blue E. coli. The cells were first inoculated in a 10 ml culture (100 g/L tryptone, 5 g/L yeast extract, 5 mM
NaCl, 143 mM ampicillin) and grown at 37°C, 200 rpm for 16 h. The culture was then added to 2 L of superbroth (32 g/L tryptone, 5 g/L yeast extract, 170 mM NaCl, 5 mM NaOH, 143 mM ampicillin) and allowed to grow under the same conditions until the optical density (OD) was between 0.7 and 0.9. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture to induce the production of the uterocalin. The cells were allowed to grow an additional 5 h before harvesting by centrifugation. The harvested cells were lysed (lysis solution 50 mM NaPi, 300 mM NaCl, pH 8.0), flash frozen (using liquid nitrogen), and stored at −80°C until purification.

Ni-NTA resin was equilibrated with the lysed cells for 1 h at 4°C. The resin and cell solution was then loaded into a narrow bore column (Bio-Rad, Hercules, CA). The resin was allowed to settle and the solution was passed through the column. After all of the lysed cell solution had run through the column, the resin was washed with additional lysis solution until the effluent had an OD < 0.01 at 280 nm. The next step of the purification process was to wash the resin with a more acidic wash buffer (50 mM NaPi, 300 mM NaCl, pH 6.0). The resin was washed with the wash buffer until the effluent had an OD < 0.01 at 280 nm. The uterocalin was then eluted from the Ni-NTA resin in the column using an elution buffer (50 mM NaPi, 300 mM NaCl, pH 4.5). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were used to verify that the purified protein was uterocalin. The purified uterocalin was then dialyzed at 4°C against a 5 mM ammonia bicarbonate solution for a minimum of 4 h. The dialysate was exchanged a minimum of two times before the uterocalin sample was removed for further use.
7.3.4 Protein lyophilization

The purified uterocalin, dissolved in 5 mM ammonia bicarbonate solution was atomized, as previously reported, to obtain a fine lyophilized powder [10]. The dissolved protein was transferred to a 10 ml gas tight syringe from where it was pumped at 3 ml/min to an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY) operating at 2.5 W. The atomized protein was collected in 400 ml of liquid nitrogen. The liquid nitrogen was allowed to boil off at room temperature and the frozen protein particles were dried under vacuum overnight. A JOEL scanning electron microscope (SEM) was used to determine the size of the solid protein particles.

7.3.5 Microsphere fabrication

Blank and uterocalin-loaded microspheres were fabricated using a cryogenic atomization technique [10, 15, 16]. The polymers (100 mg) were first dissolved in methylene chloride (3 ml for poly(SA) or 1 ml for 20:80 (CPH:SA)). To fabricate uterocalin-loaded microspheres, the atomized uterocalin was suspended by mechanical mixing (Tissue-Tearor™, Biospec Products, Bartlesville, OK) at 10,000 rpm for 30 s in the polymer solution. The protein/polymer suspension (uterocalin-loaded microspheres) or polymer solution (blank microspheres) was then transferred to a 10 ml gas tight syringe and pumped at 3 ml/min through an ultrasonic atomizing nozzle operating at 1.5 W. From the atomizing nozzle, the newly formed microspheres were collected in a layer of liquid nitrogen (200 ml) overlaying a frozen layer of ethanol (300 ml). The frozen microspheres and ethanol were stored at −80°C for three days to allow the ethanol to slowly thaw, extracting the methylene chloride from the
microspheres. The microspheres were collected by filtration and dried under vacuum overnight.

7.3.6 Microsphere characterization

The percent yield of each batch of uterocalin-loaded microspheres was quantified by dividing the mass of recovered microspheres by the initial mass of polymer and protein. Scanning electron microscopy (SEM) was used to study the external morphology of the uterocalin-loaded microspheres. The samples were coated with 200 Å of gold and then examined using a JOEL SEM.

The loading efficiency of uterocalin (the percent of initial uterocalin loaded into the microspheres) was quantified by measuring the amount of protein extracted from the uterocalin-loaded microspheres. Polyanhydrides degrade faster under alkaline conditions; hence, sodium hydroxide was used to expedite the polymer degradation, as previously reported [10, 16]. Briefly, the protein-loaded microspheres (15 mg) were suspended by sonication (Sonics & Materials Inc., Newton, CT) in 1 ml of 17 mM NaOH and loaded into a syringe. The test tube was then rinsed with 2 ml of 17 mM NaOH to ensure all microspheres were collected. The 3 ml microsphere suspension was then injected into a 3500 molecular weight cut off (MWCO) dialysis cassette (Pierce). The cassette was suspended in 600 ml of 17 mM NaOH for 4 days at room temperature. The 3500 MWCO dialysis cassette was used to retain the released uterocalin while allowing the polymer degradation products (monomers and oligomers) to be removed from the cassette. The concentration of the uterocalin in the dialysis cassette after 4 days in the alkaline solution was determined using the BCA assay.
7.3.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Uterocalin-loaded microspheres (15 mg) were suspended in 1 ml of Hanks’ balanced salt solution, 0.01% sodium azide and incubated at 37°C and mixed at 100 rpm. After 2 h of incubation, the supernatant was removed and replaced with fresh solution. The microspheres were incubated an additional 46 h at which time the supernatant was again sampled and the molecular mass of the released protein was analyzed using SDS-PAGE. Each protein sample was mixed (1:2) with a loading buffer (1% w/v SDS, 0.006 mM Tris-HCl pH 6.8, 3 mM glycerol, 0.01% w/v bromophenal blue, and 0.05% v/v β-mercaptoethanol) and heated to 96°C for 10 min. The samples were then resolved through a 12% polyacrylamide precast gel (Tris-HCl; Bio-Rad) using a constant voltage (100 V). The gels were silver stained, photographed, and dried.

7.3.8 In vitro release

To ascertain the rate of uterocalin release from the poly(SA) and 20:80 (CPH:SA) microspheres in vitro release experiments were conducted. The uterocalin-loaded microspheres (15 mg) were suspended in 1 ml of Hanks’ balanced salt solution containing 0.01% sodium azide. The microsphere suspension was incubated at 37°C and 100 rpm for the entire duration of the experiment. At predetermined times, 0.75 ml of the supernatant was removed and fresh solution was added to maintain constant sink conditions. The concentration of uterocalin in the sampled supernatant was quantified using the BCA assay. All experiments were performed in triplicate. The cumulative amount of uterocalin released at each time point was normalized by the total amount of uterocalin released from the microspheres to obtain the mass fraction of uterocalin released as a function of time.
7.3.9 Incubation of microspheres for cell migration assay

Playford et al. [22] reported that uterocalin increased the rate of cell migration of HT29 cells in a dose dependent manner up to a concentration 15 μg/ml, and that above this concentration there was no increase in the rate of cell migration. The results from this work demonstrate that uterocalin administered at a concentration of 5 μg/ml increases the rate of cell migration. After the formation of a wound (i.e., scraping away part of the cell monolayer \textit{in vitro}), the cells migrate for \textasciitilde24 h before proliferation begins. In the first 24 h, only 35% and 24% of the encapsulated uterocalin was released from the poly(SA) and 20:80 (CPH:SA) microspheres, respectively (see results of \textit{in vitro} release experiment below). When using 2 mg of microspheres this small percentage of released uterocalin would not be enough to raise the concentration of uterocalin in the medium to 5 μg/ml. To ensure that enough uterocalin was released from the polyanhydride microspheres to reach a concentration of 5 μg/ml, the microspheres were incubated in phosphate buffer saline (PBS) prior to being added to the wounded cells. Approximately 2 mg of microspheres (either blank or uterocalin-loaded) were suspended in 100 μl of PBS and allowed to degrade at room temperature for 5 days. After five days, the microspheres were then removed by sterile filtration using a Spin-X 0.22 μm centrifuge tube filter (Costar, Cambridge, MA) and the sterilized solution was then used in the cell migration assay. To test the affect blank microspheres and free uterocalin had on the rate of cell migration the blank microspheres were allowed to degrade for 5 days and were then mixed with the free uterocalin (15 μg/ml) and then the suspension was sterile filtered.
7.3.10 Cell migration assay

HT29 (human colonic epithelial cells) cells were provided by Dr. Diane Birt at Iowa State University. The cells were grown at 37°C in 10% CO₂ until confluent in McCoy’s 5A medium (Gibco-Life Technologies, Grand Island, N.Y.) containing 10% calf serum and 10 units/ml of penicillin and streptomycin. The cells were grown on 35 x 10 mm polystyrene culture dishes with an external 2 mm grid on the bottom of the dish. Once the cells were confluent, the medium was removed and replaced with McCoy’s 5A media (0.2% calf serum, 10 units/ml of penicillin and streptomycin). The cells were then incubated for 24 h in the reduced serum medium before wounding the cells. The cells were wounded by removing ¾ of the cells on the plate (leaving the cells in the middle of the plate) using a cell lifter (Corning Incorporated, Corning, N.Y.). The culture dishes were then washed three times with Tris-HCl buffer saline (TBS) to remove non-adherent cells. A volume of 2 ml of fresh McCoy’s 5A media (0.2% calf serum, 10 units/ml of penicillin and streptomycin) was then added to the cells. At this time, the treatments were added to the culture dishes. The following treatments were tested: epidermal growth factor (EGF) (used as positive control), buffer, free uterocalin (15 μg/ml and 5 μg/ml), the sterilized release medium in which blank poly(SA) microspheres were degraded in (5 days), uterocalin released from poly(SA) microspheres (final uterocalin concentration 5 μg/ml), the sterilized release medium in which blank poly(SA) microspheres were degraded in (5 days) plus free uterocalin (15 μg/ml), the sterilized release medium in which blank 20:80 (CPH:SA) microspheres were degraded in (5 days), uterocalin released from 20:80 (CPH:SA) microspheres (final uterocalin concentration 5 μg/ml), the sterilized release medium in which blank 20:80 (CPH:SA) microspheres were degraded in (5 days) and free uterocalin (μg/ml). The final concentration of EGF used was
10 nM, a concentration previously shown to increase the rate of cell migration [27]. Table 7.1 shows the concentrations and volumes used in each treatment.

The wounded cells were imaged immediately after the treatments were added and the same region of the wound (determined by the exterior grid on the bottom of the petri dish) was imaged 24 h later. The rate of cell migration was determined by quantifying how far from the initial wound edge the cells had migrated after 24 h. The cells were magnified 10-fold with a Nikon Eclipse TE200 microscope and imaged with a Hamamatsu digital camera. During the imaging period, the cells were incubated at 37°C in 10% CO2. A minimum of five images was taken of each wound at day 0 and day 1.

Table 7.1. Experimental treatments tested for their ability to increase the rate of HT29 cell migration after being wounded.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterocalin</th>
<th>Microspheres</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EGF</td>
<td>-</td>
<td>-</td>
<td>10 nM</td>
</tr>
<tr>
<td>Uterocalin</td>
<td>15 &amp; 5 μg/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poly(SA) blank microspheres</td>
<td>-</td>
<td>2 mg</td>
<td>-</td>
</tr>
<tr>
<td>Uterocalin-loaded poly(SA) microspheres</td>
<td>-</td>
<td>2 mg</td>
<td>-</td>
</tr>
<tr>
<td>Poly(SA) blank microsphere and free uterocalin</td>
<td>15 μg/ml</td>
<td>2 mg</td>
<td>-</td>
</tr>
<tr>
<td>20:80 (CHF:SA) blank microspheres</td>
<td>-</td>
<td>2 mg</td>
<td>-</td>
</tr>
<tr>
<td>Uterocalin-loaded 20:80 (CHF:SA) microspheres</td>
<td>-</td>
<td>2 mg</td>
<td>-</td>
</tr>
<tr>
<td>20:80 (CHF:SA) blank microspheres &amp; free uterocalin</td>
<td>15 μg/ml</td>
<td>2 mg</td>
<td>-</td>
</tr>
</tbody>
</table>

*a47.5 μl of sterile PBS was added to all cells, either with or without uterocalin. *b100 μl of PBS was added to all cells with or without suspended microspheres. *c6 μl of 10 mM acetic acid and 0.1% BSA with or without EGF was added to all cells.
7.3.11 Data analysis

Adobe Photoshop 9.0 was used to label the wound edge on the cell images from day 0. The images from day 1 were superimposed and aligned on the day 0 images. The original wound edge from day 0 was applied to the day 1 image. The distance that the cells had moved from the initial wound edge in 24 h was measured in ten different locations on each picture using the Image J software (NIH).

The average distance of cell migration for each individual plate was calculated from the five pictures per wound (10 points per picture). All treatments were then compared to the negative control (buffer) using a linear multiple regression model that accounted for the unequal variance between treatments by taking the log of the response. The treatments that were significantly different from the negative control were noted. The differences in treatment responses were not correlated when the experiment was performed, and hence the variance due to different days was not included in the statistical model. The variances between treatments were found to be unequal, hence the log of the responses were used in the linear multiple regression statistical model.

7.4 Results

7.4.1 Uterocalin lyophilization

In order to obtain salt-free lyophilized uterocalin, ammonium bicarbonate (a volatile solution under vacuum) was used as the solvent for the lyophilization process. After the uterocalin solution had been atomized into liquid nitrogen and dried under vacuum, SEM was used to verify that the solid protein particles had diameters < 1 μm. It was essential that the protein particles be smaller than the polymer microspheres (average diameter ~ 18 μm) in
order to ensure that the protein could be encapsulated in the polymer microspheres and to
increase the encapsulation efficiency. In all cases, the uterocalin lyophilized by the above
procedure fit this criterion.

7.4.2 Microsphere characterization

The average yield of seven batches of either the poly(SA) or 20:80 (CPH:SA) uterocalin-loaded microspheres was 69%, as shown in Table 7.2.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Yield (%)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SA)</td>
<td>69 ± 7</td>
<td>51 ± 16</td>
</tr>
<tr>
<td>20:80 (CPH:SA)</td>
<td>69 ± 9</td>
<td>77 ± 19</td>
</tr>
</tbody>
</table>

Table 7.2. Yields and loading efficiencies of uterocalin-loaded poly(SA) and 20:80 (CPH:SA) microspheres.

All numbers are calculated from a minimum of seven different microsphere batches. The ± values represent a 95% confidence interval.

The surface morphology of the uterocalin-loaded microspheres was analyzed using SEM. The microspheres were first viewed at low magnifications to ensure that the particles were spherical. Higher magnifications were then used to view in more detail the surface morphology of individual microspheres. Figure 7.1 shows two magnifications of uterocalin-loaded poly(SA) and 20:80 (CPH:SA) microspheres. The poly(SA) microspheres had a smoother surface than the 20:80 (CPH:SA) microspheres as previously reported [10]. The difference in the surface morphology of the poly(SA) and 20:80 (CPH:SA) microspheres is attributed to the two polymers having different rates of precipitation in the non-solvent, ethanol. Poly(SA) had a smoother external morphology because it precipitated slower, while 20:80 (CPH:SA) precipitated more rapidly resulting in a rougher external surface.
The encapsulation efficiency of uterocalin loaded into the poly(SA) and 20:80 (CPH:SA) microspheres was 51% and 77%, respectively, as shown in Table 7.2. These values were in agreement with the total mass fraction of uterocalin released from the poly(SA) and 20:80 (CPH:SA) microspheres in the in vitro release experiment as discussed below. These encapsulation efficiencies were lower than previously reported encapsulation efficiencies using the same fabrication method [10, 16]. The lower encapsulation efficiencies may be a result of the N-terminal poly-histidine tag of the protein interacting with the polymer degradation products (being more reactive with the SA degradation products) and interfering with the detection assay.

7.4.3 SDS-PAGE analysis

The release of uterocalin from the polyanhydride microspheres for the SDS-PAGE experiments was performed using Hanks' balance salt solution with 0.01% sodium azide. Hanks' balanced salt solution was used to mimic physiological conditions and sodium azide
was added to the solution to inhibit bacterial contamination. In order to determine the primary structure of the encapsulated and released uterocalin, the supernatant was sampled after 2 h of incubation to remove any uterocalin that may not have been encapsulated or that was loaded onto the surface of the microspheres. This ensured that only uterocalin that had been encapsulated and released from the microspheres was studied by electrophoresis.

The uterocalin released from poly(SA) and 20:80 (CPH:SA) microspheres detected on the resolving gel appeared at 24 kDa consistent with its native primary structure, see Figure 7.2. This result indicated that the released uterocalin was intact and no alterations in its primary structure (cleavage or aggregation) occurred during the encapsulation or release of uterocalin from the polyanhydride microspheres.

![Figure 7.2. SDS-PAGE analysis of uterocalin released from polyanhydride microspheres. Lane 1: molecular mass standards, lane 2: uterocalin released from poly(SA) microspheres, lane 3: uterocalin released from 20:80 (CPH:SA) microspheres.](image)
7.4.4 In vitro release kinetics

The in vitro release profile of uterocalin from poly(SA) and 20:80 (CPH:SA) microsphere is shown in Figure 7.3. To obtain the mass fraction of uterocalin released, the total amount of uterocalin released was used to normalize the concentration of uterocalin at each time point in the experiment.

The concentration of released uterocalin was measured 2 h after the start of the experiment to determine the initial burst. The initial burst accounted for ~22% of the total uterocalin released from the microspheres. This is comparable to previous work in which a 30% burst was observed when ovalbumin was encapsulated in poly(SA) microspheres fabricated using the cryogenic atomization method [10]. When solid proteins are encapsulated in polymeric microspheres, larger bursts are typically seen due to the difficulty of evenly distributing the protein throughout the polymer [28]. The release of the uterocalin from either poly(SA) or 20:80 (CPH:SA) microspheres followed a zero-order release after the initial burst. All of the uterocalin was released from the poly(SA) microspheres within 15 days while it took 30 days for all of the uterocalin to be released from the 20:80 (CPH:SA) microspheres. Because 20:80 (CPH:SA) is more hydrophobic than poly(SA), it was anticipated that the uterocalin would be released more slowly from the 20:80 (CPH:SA) microspheres.
Figure 7.3. *In vitro* release of uterocalin from (filled circles) poly(SA) and (open squares) 20:80 (CPH:SA) microspheres. Release of uterocalin from the microspheres was performed in triplicate as described in Materials and Methods.

### 7.4.5 Cell migration assay

Twenty-four hours prior to wounding, the cell monolayer the culture medium was changed from 10% calf serum to 0.2% calf serum. The cell migration assay was then performed under the serum-limiting conditions. The reduced serum concentration was used to lower the concentration of proteins and growth factors present in the serum and secreted by the growing cell population. These proteins and growth factors could influence the rate of cell migration minimizing the observable differences between the treatments.

The results of the cell migration assay are shown in Figure 7.4. All treatments were compared to the negative control (buffer) and any statistical differences between a given treatment and the negative control are noted on the histogram. Differences between treatments were also compared and are noted by brackets on the histogram. EGF (10 nM), used as a positive control, showed the largest increase in the rate of cell migration over a 24 h period. This increase in cell migration due to EGF is well documented in the literature [27].
Playford et al. [22] have previously reported that uterocalin affects the rate of HT29 cells migration in a dose dependent manner up to a concentration of 15 μg/ml. To verify these experimental results, two concentrations (15 μg/ml and 5 μg/ml) of free uterocalin were added to wounded HT29 cells and tested for their ability to increase the rate of cell migration. No difference was found between the rates of cell migration when 15 μg/ml or 5 μg/ml of uterocalin was added to the cells, though both treatments increased the rate of cell migration over the negative control.

Figure 7.4. Histogram showing the average distance (arbitrary units) that wounded HT29 cells migrated in 24 hours after being exposed to each treatment: (1) buffer, (2) EGF (10 nM), (3) uterocalin (15 μg/ml), (4) uterocalin (5 μg/ml), (5) blank poly(SA) microspheres (2 mg), (6) uterocalin-loaded poly(SA) microspheres (2 mg), (7) blank poly(SA) microspheres (2 mg) and free uterocalin (15 μg/ml), (8) blank 20:80 (CPH:SA) microspheres (2 mg), (9) uterocalin-loaded 20:80 (CPH:SA) microspheres (2 mg), (10) blank 20:80 (CPH:SA) microspheres (2 mg) and free uterocalin (15 μg/ml). Error bars represent 95% confidence intervals of treatments. ** represents p-value of < 0.01, * represents p-value of < 0.05 when compared to the negative control (buffer), brackets show statistical difference between treatments.
To assess if blank poly(SA) or 20:80 (CPH:SA) microspheres stimulated or inhibited cell migration, the supernatant in which ~2 mg of blank microspheres had been incubated for 5 days was administered to the 2 ml culture of wounded HT29 cells. The rate of cell migration for the cells treated with buffer and the blank microspheres were identical indicating that the blank microspheres had no effect on the migration of the cells.

When uterocalin released from either poly(SA) or 20:80 (CPH:SA) microspheres (previously incubated for 5 days) was added to the wounded monolayers, a statistically significant increase in the rate of cell migration was observed as compared to the negative control (buffer) and to the blank microspheres added to the wounded cells. There was no statistical difference between the cells treated with the uterocalin-loaded microspheres and cells treated with free uterocalin. This indicated that the uterocalin released from the polyanhydride microspheres was released as a biologically active protein.

Uterocalin mixed with blank microspheres (that had been allowed to degrade for 5 days in PBS) of either poly(SA) or 20:80 (CPH:SA) and added to the wounded cells failed to increase the rate of cell migration. This is attributed to the adsorption of the free protein to the surface of the microspheres prior to the sterile filtration.

7.5 Conclusions

Uterocalin was successfully encapsulated in poly(SA) and 20:80 (CPH:SA) microspheres using the cryogenic atomization fabrication technique. The encapsulation efficiency of uterocalin in polyanhydride microspheres was lower than that reported in previous work using the same polymers and the same microsphere fabrication technique [10]. This result is attributed to the interaction of the N-terminal poly-histidine tag of the protein.
with the degradation products of the polymers (primarily of poly(SA)), thus interfering with the detection assay. Uterocalin is known to bind low molecular weight ligands, though the monomers of CPH would be more hydrophobic than SA monomers there would be more SA monomers present in the supernatant. The strength of the bonds between monomer units is as follows CPH-CPH > CPH-SA and SA-SA, thus it would take less time for a SA-SA bond to be broken than it would for a CPH-CPH bond in the copolymer [29].

This work has demonstrated that polyanhydride microspheres can conserve the structural integrity of an encapsulated protein and that the released protein is biologically active. The uterocalin released from the poly(SA) and 20:80 (CPH:SA) microspheres was released as an intact protein, as demonstrated by SDS-PAGE analysis. The uterocalin released from the polyanhydride microspheres was found to increase the rate of cell migration of HT29 cells supporting previous reports [22] and indicating that the biological activity of the protein was maintained after being encapsulated in poly(SA) and 20:80 (CPH:SA) microspheres.

When blank microspheres (previously incubated for 5 days) were added to the wounded cells that were treated with 15 μg/ml of free uterocalin, no difference in the rate of cell migration was observed. The blank microspheres used in this work were semi-crystalline and their erosion rates are slower than that of the uterocalin-loaded microspheres [30]. This is attributed to the decreased degree of crystallinity of polyanhydrides when they are mixed with small amounts of compatible drugs. As a result, the increased hydrophobic surface area of the blank microspheres causes more of the free uterocalin to adsorb to the polymer microspheres and prevents it from interacting with the cells. Thus, no difference in the rate of cell migration is observed. Uterocalin mixed with poly(SA) microspheres
deviated statistically from the samples treated with uterocalin-loaded poly(SA) microspheres. However, there was only weak evidence of a statistical difference between the cells treated with free uterocalin mixed with 20:80 (CPH:SA) microspheres and cells treated with uterocalin-loaded 20:80 (CPH:SA) microspheres (p-value < 0.1). Because poly(SA) is more crystalline than 20:80 (CPH:SA) [24] there is a larger difference in the rate of degradation between a blank poly(SA) microsphere and a uterocalin-loaded poly(SA) microsphere than there is between a blank 20:80 (CPH:SA) microsphere and a uterocalin-loaded 20:80 (CPH:SA) microsphere.

The potential for using polyanhydride microspheres as protein carriers is enormous. They can stabilize proteins while providing a controlled release that can be altered by varying the polymer chemistry.

7.6 Acknowledgements

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7.7 References


CHAPTER 8
CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this research was to demonstrate that polyanhydrides, specifically poly(sebacic anhydride) (poly(SA)) and copolymers of [1,6-bis-p(carboxyphenoxy)hexane] (CPH) and SA, can be used to stabilize and provide controlled release of proteins. This project was broken up into four specific goals:

1. Demonstrate the feasibility of using polyanhydrides microspheres as protein carriers.
2. Discern the compatibility of proteins with biodegradable polymer degradation products.
3. Study the role of microsphere fabrication methods on protein stability.
4. Apply the insights gained from specific goals 1, 2, and 3 to design uterocalin-loaded polyanhydride microspheres to expedite cell migration for applications in wound healing.

Specific goal 1 was discussed in Chapter 4. Bovine serum albumin (BSA) was encapsulated and released from poly(SA), 20:80 (CPH:SA), 50:50 (CPH:SA), and 80:20 (CPH:SA) microspheres. The microspheres were fabricated using the standard water-oil-water fabrication technique. The BSA released from the polyanhydride microspheres was not aggregated; it was released as an intact polypeptide chain. Each of the polymer compositions used to encapsulate BSA prevented the formation of inter-protein multimers via disulfide
bonds. Cleavage of the protein only occurred when the protein was allowed to incubate for extended periods of time with the acidic polymer degradation products. As the hydrophobicity of the polymer increased (increasing amounts of CPH) the α-helix content of the encapsulated protein decreased steadily to a point where no α-helices were detected in the protein encapsulated within 80:20 (CPH:SA). These results demonstrated that poly(SA) and 20:80 (CPH:SA) were capable of stabilizing BSA. This work also showed for the first time that the increased hydrophobicity of the copolymers 50:50 (CPH:SA) and 80:20 (CPH:SA) was not conducive for stabilizing encapsulated proteins.

Specific goal 2 was addressed in Chapter 5. The changes in structure and activity or antigenicity of ovalbumin, lysozyme, and tetanus toxoid were studied after the proteins were incubated with the monomers (degradation products) of polyanhydrides (poly(SA) and poly(CPH)) and polyesters (poly(lactic acid) and poly(glycolic acid)). The results of this work demonstrated the importance of understanding the effect of the polymer degradation environment on the structure of the protein. Ovalbumin and tetanus toxoid were least stable in the presence of the ester monomers. However, ovalbumin was more stable in the presence of the SA monomer while tetanus toxoid was more stable in the presence of the CPH monomer. Lysozyme, an acid stabilized protein, was found to be stable in the presence of either ester or anhydride monomers. These experiments demonstrated a rational approach for selecting compatible protein/polymer systems prior to encapsulating proteins in polymer microspheres.

Though specific goal 2 answered a lot of questions, it has raised new questions that should be addressed in future investigations. The work demonstrated a protocol for determining protein/monomer compatibility. Future investigations should address how well
the described protocol predicts the stability of proteins in *in vitro* and *in vivo* release experiments. These experiments should first be done with proteins encapsulated in polymer tablets to ensure that any instability of the protein is a result of the polymer/protein interactions and not a result of a fabrication technique (e.g., microsphere fabrication). The work could then be repeated with polyester and polyanhydride microspheres to obtain an in-depth comparison of the two types of polymer delivery systems.

Chapter 6 addressed specific goal 3. Four different microsphere fabrication techniques were used to examine the stability of ovalbumin released from poly(SA) and 20:80 (CPH:SA) microspheres. Poly(SA) and 20:80 (CPH:SA) were used because these were the polyanhydrides that stabilized the encapsulated and released protein in specific goal 1. The methods used to fabricate the poly(SA) and 20:80 (CPH:SA) microspheres were: water-oil-water (w/o/w), water-oil-oil (w/o/o), solid-oil-oil (s/o/o), and a cryogenic atomization procedure. The ovalbumin released from the poly(SA) and 20:80 (CPH:SA) microspheres made by each of the four fabrication methods maintained its primary structure. The only case in which the protein secondary structure deviated (i.e., reduced α-helix content) from that of the native protein was 20:80 (CPH:SA) microspheres fabricated via the water-oil-water method. This decrease was a combination of the increased hydrophobicity of the copolymer and the presence of two water-oil interfaces during the microsphere fabrication process. It is well known that in the presence of a water-oil interface, ovalbumin is more susceptible to aggregation than BSA [1].

By directly comparing the yield, protein loading efficiency, protein stability, and protein release kinetics it was possible to determine the preferred microsphere fabrication method for different delivery applications. When the protein was encapsulated as a
lyophilized powder (s/o/o and cryogenic atomization) the initial burst was larger than when it was encapsulated in an aqueous solution (w/o/w and w/o/o). In all cases, after the initial burst, the release of ovalbumin followed zero order kinetics. The cryogenic atomization method should be used for future applications (in which the delivery application requires a substantial burst followed by a zero order release) because it had the highest loading efficiency (it did have the lowest yield but this was later improved in specific goal 4), eliminated any water-oil interfaces, stabilized the encapsulated protein, and provided a controlled release with a moderate initial burst.

Because each protein is different, future investigations should repeat the above studies with different proteins to determine the preferred fabrication method for each protein (e.g., low molecular weight proteins, or proteins with a free thiol group). Future applications should also test how the different microsphere fabrication methods affect the biological activity of the released protein.

Chapter 7 addressed specific goal 4. In this work, uterocalin was encapsulated in poly(SA) and 20:80 (CPH:SA) microspheres using the cryogenic atomization method. The primary structure of the released protein was conserved and the protein was biologically active as determined by a cell migration assay. The uterocalin released from polyanhydride microspheres was added to a monolayer of wounded human colon epithelial cells (HT29) and its ability to increase the rate of cell migration was studied. The uterocalin released from poly(SA) and 20:80 (CPH:SA) microspheres maintained its biological activity, providing evidence that polyanhydrides are suitable protein carriers.

Future therapeutic applications of protein-loaded polyanhydride microspheres should focus on diseases that require prolonged drug therapy (e.g. cancer, chronic diseases, and
preventative medicines). Once an appropriate alignment is identified further research should focus on identifying the most appropriate delivery route to the body and how the body uptakes the device and the encapsulated drug. One area that should be considered is targeting macrophages with drug-loaded nanoparticles (< 1 µm). Macrophages are excellent targets for drug delivery devices because they not only can remove nanoparticles from the blood within minutes of injection but they also contribute to inflammation (e.g. rheumatoid arthritis) and serve as hosts to infectious diseases (e.g. HIV) [2]. Because human macrophages typically have a lifespan of 1-3 months, similar to the amount of time needed to degrade a polyanhydride, polyanhydride nanoparticles may make excellent drug carriers for macrophage drug targeting. This work would require the fabrication of nanoparticles, which could be obtained by altering the fabrication techniques discussed in this work.

Besides targeting macrophages, polyanhydrides should be investigated as vehicles for targeted drug delivery. By functionalizing a polymer (e.g., by chemically attaching an antibody or peptide chain) it can be made to specifically target specific tissues or organs in the body. Specifically targeting a tissue or organ in the body and localizing the drug delivery there maximizes the drug potential while minimizing the side effects. Polyanhydrides are end-capped with carboxylic acid groups, thus enabling the chemical attachment of antibodies or peptide chains. Previous work done with polyester (PLGA) microspheres/nanospheres has shown that by covalently linking ligands to the carboxylic end groups of the polymer, it is possible to deliver a drug-loaded polymeric particle to a specific site [3, 4].

This work has provided evidence that polyanhydrides stabilize proteins (BSA, ovalbumin, and uterocalin) and that the degradation products of polyanhydrides do not reduce the antigenic response of proteins (e.g. ovalbumin and tetanus toxoid). Previous work
in our research group showed the potential of polyanhydride microspheres loaded with tetanus toxoid to modulate the immune response of mice [5]. This novel trait of polyanhydrides should be further investigated with other antigenic proteins.

In summary, this work has demonstrated the ability of polyanhydride microspheres to stabilize and provide a controlled release of proteins. This work has addressed questions raised in previous research and voids in the literature including the design of a rational approach for selecting compatible polymer/protein systems and the direct comparison of microsphere fabrication methods. This work has also laid the groundwork for future applications of protein-loaded polyanhydride microspheres.

8.1 References