Cross-Linked Hydrogels for the Delivery of Growth Factors in Tissue Engineering

Chad David Brown

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2003

Program Authorized to Offer Degree: Department of Bioengineering

UMI Number: 3102630

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to Proquest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature: Chad W Brown

Date: August 18, 2003

University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Chad David Brown

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Co-Chairs of Supervisory Committee:

Allan S. Hoffman

Patrick S. Stayton

Reading Committee:

Allan S. Hoffman

Patrick S. Stayton

Cecilia M. Giachelli

Wayne R. Gombotz

Date: August 13, 2003

University of Washington

Abstract

Cross-Linked Hydrogels for the Delivery of Growth Factors in Tissue Engineering

Chad David Brown

Co-Chairs of Supervisory Committee:
Professor Allan S. Hoffman
Professor Patrick S. Stayton
Department of Bioengineering

The goal of tissue engineering is the regeneration or replacement of failed or damaged tissues with viable and functional tissues that integrate with the host system. However, this vision has not yet been fully realized. One difficulty in producing large tissue substitutes is maintaining the viability of cells on or within polymer scaffolds. Many factors, several associated with undesirable wound healing, may lead to the demise of cells contained within a tissue-engineering scaffold. These factors include difficulty in the delivery of nutrients or removal of waste products, attack of implanted cells by the host immune system, or a lack of proper cell proliferation and differentiation. The recent increase in the number of available recombinant proteins, including growth factors has allowed for the manipulation of cell function and differentiation. However, the success of these important biomolecules is dependent on the amount delivered to the cell and the timing of their delivery. Important processes such as inflammation, wound healing and associated angiogenesis, immune regulation, and cellular differentiation and proliferation may be mediated when these factors are delivered in an appropriate manner. Many

traditional tissue-engineering scaffolds, such as poly(lactide-co-glycolide), are relatively hydrophobic in nature and require processing conditions that can denature proteins. Further, protein delivery systems fabricated from these polymers result in non-uniform protein distribution and lead to non-ideal release characteristics. Hydrogels provide a more stable environment for proteins; however, they swell rapidly and release entrapped biomolecules quickly. It is hypothesized that proteins can be delivered in a controlled and stable manner by combining appropriate ratios of hydrophobic and hydrophilic polymers in cross-linked networks, thus promoting beneficial biological functions. In this work, synthetic and natural polymers were utilized to develop physically or chemically cross-linked protein delivery systems capable of stable and controlled delivery of growth factors with appropriate individual release kinetics in an effort to promote proper wound healing events such as angiogenesis.

Table of Contents

List of Figures

Figure Number **Page**

List of Tables

Table Number **Page**

Acknowledgements

I would like to express my appreciation to my research advisors, Professors Allan Hoffman and Patrick Stayton, for their support and advice during the tenure of my graduate schooling. I cherish the time I have spent at the University of Washington, and hope to continue to learn from these scholars in the years to come.

I would also like to thank the many collaborators that I have had the opportunity to work with during my graduate school career. Masashi Nakakura and Ninus Caram-Lelham begun the work involving the controlled release of PEG-(GM-CSF) from chitosan scaffolds. Wayne Gombotz, Dean Pettit, and Lotte Kreilgaard, who at the time were at Immunex Corporation, were instrumental collaborators on this project and their expertise and willingness to offer advice were great assets. Ari Karchin, Kelvin Wu, and Joan Sanders provided the compression testing expertise on the photo-crosslinkable hyaluronic acid hydrogel work. Jeff Anderl was an invaluable asset on the development of the Prolease® technique for developing microparticles. His initial groundwork on getting the equipment and technique worked out aided greatly in the production of this work. He has also been a wealth of information for ideas and trouble shooting many of the experiments discussed in this dissertation. Lisa Weister, Janet Cuy, and Cecilia Giachelli collaborated on a chitosan heart valve tissue engineering project not discussed in this work. However, this collaboration helped guide my interest in tissue engineering and provided me with many molecular biology skills.

I must also express my gratitude to the great researchers and friends I have had the pleasure to work with while at the University of Washington. All current and former

members of the Hoffman and Stayton groups have provided me with many interesting scientific discussions and moments of friendship. In particular, I must personally thank those who have had a profound effect on the scientist and person I am today. Kathy Leach was an instrumental part in my decision to pursue the field of controlled release. Her mentorship in my early graduate years helped define who I am as a scientist. I will always welcome the opportunity to travel to the "country cottage" for a meal complete with friendship and surrounded by dogs. Andreas Goessl also played an early role in shaping my research interests as well as opening my eyes to the great adventures available in the Pacific Northwest. He also taught me that any piece of equipment could be made or rebuilt if you look in enough dumpsters. I owe to Niren Murthy all the practical organic chemistry knowledge and skills that I possess today. His advice was invaluable in solving many synthesis problems. His passion for science is contagious. Natalie Winblade, Fiona Black, Volga Bulmus, Monica Woodward, and Jason Hwang were lab mates who provided many interesting scientific and "not-so"-scientific discussions in and outside of lab. I am hard-pressed to choose which was more valuable. The opportunity to work with great scientists who turned out to be great friends is a pleasure I will always cherish.

Of course I would be remiss if I did not acknowledge the friends whose dedication to my well being outside of research kept me going during bleak times and made the joyous times even more stellar. Yak-nam Wang will forever remain "my darling" who is always welcome for an ice cream break. Aaron "Jake" Jacobsen, an aficionado for God, Led Zeppelin, and home brewing, eerily reminds me of myself. Chuck Cheung, who endured

6 years at the University of Washington with me and shared many grand adventures, and in the process became one of my best and most trusted friends. Kelly Halle, who reminded me that life really is funny and meant to be enjoyed, opened my heart to things that I thought impossible. You are the ones that provide my life with laughter and hope for the future. I will hold all of you close to my heart.

In addition, I must thank my family whose support never wavered. Mom and Dad, thank you for always believing in me and loving me, even when I didn't myself. The life long lessons of discipline and perseverance you instilled in me growing up may have been all I needed. Nate, you were the best brother I could have had. I am glad that we have remained close and will always enjoy your laughter and enthusiasm. I look forward to our continued friendship in the future.

Most of all, I would like to express humble and respectful gratitude to my Creator, Lord and Savior from whom all strength comes.

Dedication

To my family and friends:

Mom and Dad, your unconditional love and support
gave me the belief that I could accomplish anything.

Nathan, we grew up together as brothers,
and became best of friends
Thanks for all the laughs.

Friends, you have been angels sent by God to
carry me through the trials,
I hope someday I can return the many blessings.

"For I know the plans I have for you," declares the Lord.
"Plans to prosper you, not to harm you.
Plans to give you hope and a future."

-Jeremiah 29:11

Chapter 1: Background and Introduction

1.1 Importance of Growth Factor Delivery in T issue Engineering

The development of devices for the stable delivery of growth factors is a major area of current research in tissue engineering [1-7]. Growth factors can direct many complex cellular functions such as inflammation, wound healing, angiogenesis, immune regulation, and cellular proliferation and differentiation [3]. Thus, the ability to deliver growth factors in a controlled and stable manner from tissue engineering scaffolds can have a dramatic impact on the ultimate wound healing response and viability of engineered tissues. However, precise control over the kinetics and timing of delivery is needed in order to mimic conditions seen during embryonic development and wound repair. Conventional methods for the delivery of therapeutics are inadequate for the delivery of growth factors in tissue engineering due to the short plasma half-life and the large size of proteins. Further, due to slow tissue penetration of growth factors and rapid diffusion from the site of injection, high concentrations of protein are needed when delivered systemically, leading to potential systemic toxicity [2, 4]. Growth factor delivery from tissue engineering scaffolds would allow for local administration of lower amounts of growth factor while providing the chemotactic gradients necessary for cellular processes such as angiogenesis and innervation [8].

In order to correctly influence the development of tissue by controlled delivery of growth factors several factors must be taken into consideration [3]. First, there must be concern for maintaining the native structure and functionality of the protein. Second, the

kinetics of release must be precisely controlled to mimic those seen *in vivo* in order to maintain correct tissue development and avoid tissue overgrowth. Next, due to the integration of signals from multiple growth factors in the development of a functional tissue, the ability to control the rate of release of multiple proteins from a single matrix is essential. Finally, it may be necessary to assure that the growth factor reaches the correct cell population through the targeted delivery of the protein.

In addition to concerns relevant to the actions and properties of the growth factor, one must also consider the properties of the scaffold being utilized and how they will affect growth factor release [9]. First, it is important to be able to load sufficient quantities of growth factor into the scaffold. Further, the formulation strategy must allow the growth factor to be loaded in a manner that allows for even dispersion throughout the entire scaffold in order to ensure uniform release. Next, the scaffold must allow for the stable encapsulation of the protein by avoiding harsh, denaturing conditions thus allowing growth factors to maintain their full bioactivity. Also, it may be advantageous for the scaffold to be degradable; however, the degradation products must not be detrimental to the stability of the protein. Further, the scaffold must degrade in a manner that allows for the appropriate release kinetics of the entrapped protein. Finally, a growth factor delivery system that is versatile and can be extended to a wide range of tissue engineering applications would be an invaluable tool for the tissue engineer.

1.2 Current Limitations in Growth Factor Delivery

Two main scaffolding strategies have been the primary focus for the development of growth factor delivery devices in tissue engineering. One class is the synthetic, degradable, and relatively more hydrophobic polymers such as poly(lactide) (PLA), poly(glycolide) (PGA), and their copolymers poly(lactide-co-glycolide) (PLGA). These polymers have been extensively utilized due to their apparent biocompatibility and FDA approval. A second class is the natural and synthetic hydrogel polymers such as collagen, chitosan, hyaluronic acid, and poly(ethylene glycol). The popularity of these matrices is owed to a high aqueous environment that is conducive to cell survival and protein stability. Examples of various scaffolds of each type and their utilization as growth factor delivery devices in tissue engineering are shown in Table 1-1.

Table 1-1: Scaffolds and growth factors utilized in tissue engineering applications

Polymer Scaffold	Growth Factor	Tissue	References
I. Synthetic/Hydrophobic			
PLA/PGA/PLGA	EGF, bFGF	Liver	[10-13]
	VEGF/PDGF	Angiogenesis	[14, 15]
	BMP-2, PDGF	Bone	[16-18]
	N/A	Cartilage	[19]
II. Synthetic/Hydrogel			
PEG	RGD, TGF-β	Vascular	[20, 21]
	N/A	Cartilage	[22-24]
PEG-co-PLA	IGF-1, TGF-β	Cartilage	[25]
III. Natural/Hydrogel			
Alginate	VEGF	Angiogenesis	[26]
	N/A	Bone	[27]
Chitosan	ECGF	Angiogenesis	[28]
	PDGF	Bone	[29, 30]
Hyaluronic Acid	BMP-2, bFGF, TGF-β	Bone/Cartilage	[31-33]
Fibrin	β-NGF, bFGF	Nerve	[34, 35]
Collagen	N/A	Cartilage	[36]
Gelatin	BFGF, TGF-β, BMP-2	Vascular/Bone	[37]

1.2.1 Synthetic/Hydrophobic Systems – Poly(lactide)

Poly(lactide) and its copolymers have received the most attention in the tissue engineering and controlled release fields due to their prior FDA approval as delivery devices and sutures. Further, these polymers are biocompatible owing to their degradation to the natural by-products of lactic acid and glycolic acid [38]. Control over the release of bioactive molecules is easily modulated by altering the degradative

properties of the polymer through changes in polymer molecular weight and copolymer ratio. However, these polymers are hydrophobic in nature and usually require phase transitions, high temperatures, and organic solvents such as methylene chloride for scaffold fabrication. These conditions can cause denaturation of entrapped growth factors with an accompanying loss of bioactivity [39]. In addition, the degradation of these polymers follows a bulk degradation process, leading to the generation of acidic local microenvironments within the matrix, which can be detrimental to the bioactivity of the encapsulated growth factor [40, 41]. The decrease in pH of the local microenvironment (as low as pH=1.5) can lead to modification of protein functional groups and peptide bond cleavage with accompanied enzymatic hydrolysis [7, 42]. In addition to the loss of bioactivity, denatured proteins may cause unwanted side effects such as an increase in immunogenicity. Further, many formulation strategies involve organic/aqueous interfaces that lead to unfolding of protein at the interface and leaching of incorporated protein, thus decreasing the efficiency of entrapment of these expensive biomolecules. Finally, due to the relative hydrophobic nature of the polymers, proteins tend to aggregate on the surface and pores of the polymer after fabrication. This leads to release profiles of growth factor that begin with a large quantity of protein released initially as the surface absorbed protein is quickly desorbed, followed by a slower rate of release of entrapped protein as the scaffold slowly swells and degrades.

1.2.2 Hydrogel Systems

In contrast to the synthetic/hydrophobic scaffolds described above, hydrogel scaffolds provide a much more stable environment for proteins largely due to their aqueous processing conditions [43]. Most scaffolds of this type are fabricated from the physical or chemical cross-linking of water-soluble polymers to give a swollen network. The high water content of these scaffolds provides a non-fouling background that allows for greater control over the signals delivered to cells through growth factor release and scaffold modifications [44]. However, these scaffolds swell extensively, generating large pores and allowing for rapid release of entrapped proteins. Thus, extended and controlled release of growth factors is challenging. Further, due to their highly swollen state, hydrogel scaffolds tend to be mechanically weak in comparison to the synthetic/hydrophobic systems described above.

Table 1-2: Advantages and Disadvantages of Hydrophobic/Synthetic and Hydrogel Delivery Systems

	Advantages	**Disadvantages**
Hydrophobic/Synthetic	**Mechanical Strength** **Degradable** **Biocompatible** **Extended Release**	**Denaturing Processing Conditions** **Acidic Degradation Products** **Surface Aggregation of Protein**
Hydrogels	**Aqueous Formulation** **Non-Fouling Surface**	**Rapid Swelling** **Large Pores** **Mechanically Weak** **Rapid Protein Release**

1.2.2.1 Chitosan

Chitosan is a natural hydrogel that has recently been investigated for tissue engineering applications due to its biodegradability and biocompatibility [45, 46]. Chitosan is a natural linear copolymer of β(1→4)-linked glucosamine and N-acetyl-D-glucosamine and is obtained from the base-catalyzed partial N-deacetylation of chitin (Figure 1-1) [47, 48]. Chitin is the second most abundant natural polymer next to cellulose and is the major constituent of the shells of shrimp and crab [49]. The biocompatibility of chitosan is due to its degradation by lysozyme to the natural byproducts of glucosamine and N-acetyl-glucosamine, which are incorporated into glycoproteins or excreted as carbon dioxide [45, 50-52]. Chitosan is insoluble in neutral aqueous solutions; however, chitosan becomes protonated and dissolves in dilute acidic solutions, such as acetic acid [53, 54]. Thus, tissue engineering scaffolds capable of growth factor delivery can be fabricated from chitosan under milder conditions than those for synthetic/hydrophobic systems such as poly(lactide). Due to its positive charge, chitosan has been utilized to deliver negatively charged compounds such as acidic proteins, glycosaminoglycans, and DNA [28, 54-63]. In addition, chitosan has been used as a component in wound healing formulations [54, 58, 59]. The acceleration of wound healing by chitosan is believed to be due to its ability to increase the rate of infiltration of polymorphonuclear cells and the production of collagen by fibroblasts [64]. Further, it is speculated that chitosan may bind growth factors with or without the aid of complexed heparin [58, 59, 65]. This protects growth factors from degradation by proteolysis, and increases their availability in the wound bed. More recently, chitosan has been

investigated as a tissue-engineering scaffold because of its structural resemblance to glycosaminoglycans, an important structural element of the extracellular matrix of many tissues [66-68]. The use of chitosan as a tissue engineering scaffold has been investigated for a variety of tissues including bone, liver, neural tissue, vascular grafts, cartilage, and skin. The tissue engineering applications of chitosan and several of its modifications are shown in Table1-3 [46]. The ability of chitosan to stably deliver growth factors combined with its wound healing effects makes it an interesting material for the fabrication of tissue engineering scaffolds that modulate the wound healing process.

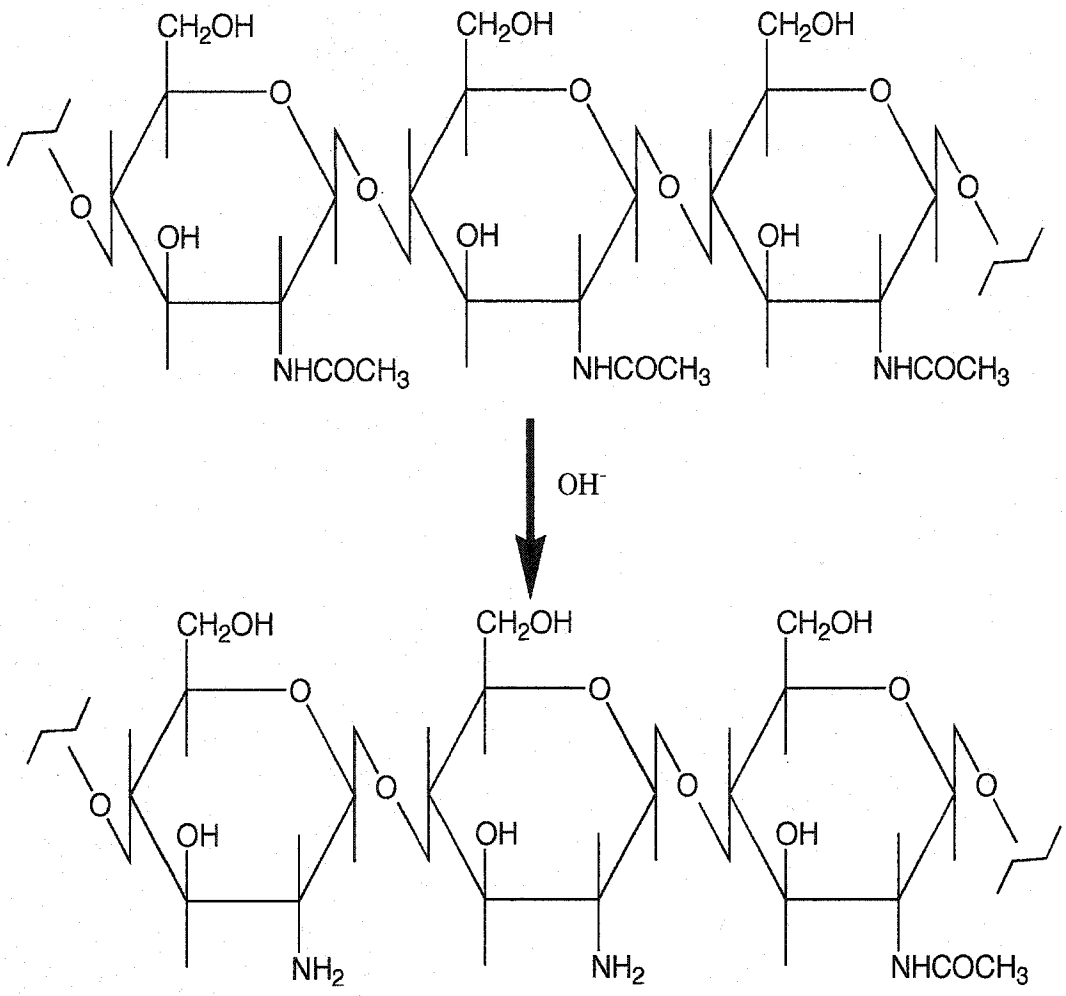


Figure 1-1: Base catalyzed N-deacetylation of chitin to produce chitosan

Table 1-3: Examples of chitosan modifications utilized in tissue engineering

Modification	Tissue	Reference
Carboxymethylated	Bone	[69-71]
Tripolyphosphate cross-linked	Bone	[29]
Glutaraldehyde cross-linked	Liver	[72, 73]
Sugar Conjugation	Liver, Neural	[73, 74]
Protein Blending	Liver, Neural, Skin	[28, 68, 75-77]
Glycosaminoglycans	Cartilage, Skin, Vascular	[58-60, 76, 77]
Hydroxypropylated	Vascular	[78, 79]

1.2.2.2 Hyaluronic Acid

Another polysaccharide that has been widely investigated as a drug delivery and tissue engineering scaffold is hyaluronic acid (HA) [80]. HA is a natural, anionic, high molecular weight copolymer of β (1-4) linked 2-acetamide-2-deoxy-D-glucose and β (1-3) linked D-glucuronic acid (Figure 1-2). Due to its high biocompatibility and degradation *in vivo* by the action of hyaluronidase, HA has been developed into products approved for the treatment of osteoarthritis, ophthalmic surgery, cosmetics, and drug delivery [81]. It is the only nonsulfated glycosaminoglycan (GAG) and is found abundantly in the extracellular matrix. The ability of HA to form highly viscous solutions allows HA its biological role as a provider of tissue hydration and extracellular spacing. This is the result of strong hydrogen bonds that allow HA to entrap up to 1000 times its weight in water [80]. Due to its abundant presence in fetal development, HA has been implicated in scarless fetal wound healing [82]. This has been attributed to the high swollen volume of HA preventing proliferating cells from receiving inhibitory contacts. HA also binds cellular ligands that direct cell adhesion and motility [83] and promote cellular proliferation and differentiation [84]. These cellular interactions, namely CD44, RHAMM, and ICAM-1, play an active role in wound healing events such as the inflammatory response and angiogenesis [80, 85, 86]. High molecular weight HA has been shown to inhibit angiogenesis while degradation products of HA (4-25 disaccharide units) induce endothelial cell proliferation and blood vessel formation [87-89]. This endothelial cell proliferation is due to induction of protein tyrosine kinase activity mediated by CD44 binding of HA oligosaccharides [90]. Further, the angiogenic

effect of HA oligosaccharides has been shown to be synergistic when delivered with VEGF, but not in conjunction with bFGF [91].

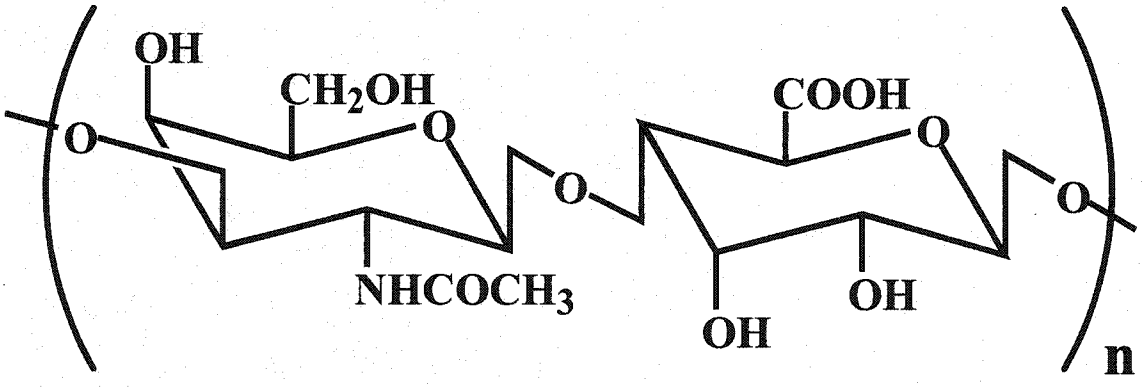


Figure 1-2: Chemical structure of hyaluronic acid, a linear alternating copolymer of β (1-4) linked 2-acetamide-2-deoxy-D-glucose and β (1-3) linked D-glucuronic acid.

The role of HA in the promotion of wound healing and angiogenesis makes it an attractive biomaterial for drug delivery and tissue engineering; however, the mechanical properties of HA are relatively poor. Thus, several chemical modifications of HA have been attempted in order to improve the mechanical properties for use as a tissue engineering and drug delivery scaffold [80, 92]. These modifications take advantage of the hydroxyl and carboxyl reactive groups that are present on native HA. One strategy that provides a water insoluble derivative of HA with improved mechanical properties, while maintaining its biocompatibility and degradability, involves the esterification of the HA carboxyl with benzyl alcohol [93, 94]. These materials are particularly ideal for the tissue engineering of cartilage and have been shown to enhance the repair of articular cartilage defects in rabbits [95, 96]. The benzyl esters of HA have also been investigated

for their ability to deliver several growth factors including basic fibroblast growth factor, bone morphogenic protein-2, nerve growth factor, calcitonin, and insulin [31, 32, 97-99]. Another important modification of HA is the production of hydrogels through the use of various cross-linking chemistries. These include cross-links formed by reaction with divinyl sulfone, glutaraldehyde, and hydrazide chemistries [80, 92, 100, 101]. Hydrogels of HA have also been produced by preparing a photo-crosslinkable derivative of HA using methacrylic anhydride to modify the 6-carbon hydroxyl of the disaccharide [102, 103]. This strategy is of particular interest due to the introduction of pendant methacrylate groups that can be used to photo-crosslink HA and entrap proteins and cells under milder chemical conditions.

1.3 Growth Factors in Wound Healing

Once a tissue-engineering construct has been implanted, the desired result is integration of the construct with the host system. The implant site is essentially a wound bed in which the encouragement of proper wound healing and accompanying angiogenesis is necessary for successful integration. Upon examination of the wound bed, one sees the complex and coordinated interaction of many cell types in the closure and healing of the wound [104]. These cells interact and communicate with one another through the use of potent growth factor signaling proteins. However, in order to produce optimized healing, these growth factors are strictly regulated requiring specific amounts of growth factor at specific times. Further, many cell responses require the establishment of a chemotactic gradient to encourage cell migration toward the wound site. Controlled

release systems are ideal for establishing these types of localized gradients; however, the release system design must also be capable of supporting growth factor delivery that is tunable, allowing for temporal and kinetic control of protein release.

1.3.1 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that has been shown to have stimulatory effects on the growth and maturation of hematopoietic cells. For this reason, it has been used clinically to enhance hematopoietic recovery after bone marrow transplantation and for reducing neutropenia associated with chemotherapy [105]. GM-CSF has also been shown to accelerate wound healing by promoting keratinocyte proliferation, granulation tissue formation, and vascularization [106]. It is a net-negatively charged protein with a molecular weight between 14.5 and 19.5 kD depending on the degree of glycosylation. Local extended release formulations are of interest due to the relatively short half-life of GM-CSF (60 min). Thus, high dosages are required to reach local therapeutic levels, leading to toxic side effects such as pericarditis, fluid retention, and venous thromboses [107]. It has been shown that the plasma half-life of GM-CSF can be extended through PEGylation of the growth factor, decreasing its dosage requirements [108]. The typical mode of administration of GM-CSF is daily intravenous infusion at a dosing rate of 250 $\mu g/m^2$ for 14-21 days [107]. Patient compliance and comfort would be greatly improved by an effective controlled release formulation for GM-CSF.

1.3.2 Vascular Endothelial Growth Factor (VEGF)

The survival of cells transplanted on tissue engineering matrices is largely dependent on the removal of cellular waste products and the delivery of nutrients, especially oxygen. It has been shown that cells positioned more that 100μm from a capillary suffer hypoxic conditions [109]. Thus, the ability of a tissue engineering scaffold to promote angiogenesis and generate its own vascular supply is essential in developing successful tissue engineering constructs.

One growth factor that has demonstrated importance in regulating angiogenesis is vascular endothelial growth factor (VEGF). VEGF, a 42 kD heparin binding growth factor, is a potent stimulator of angiogenesis due to its mitogenic effect on vascular endothelial cells, promotion of cell migration, and regulation of microvessel permeability [110]. Initiation of angiogenesis necessitates the presence of VEGF making it the most critical player in vessel growth [111]. Expression of VEGF leads to the degradation of the extracellular matrix by endothelial cells, followed by endothelial cell migration, and tube formation [112]. In addition, the survival of endothelial cells within these tubes is dependent on VEGF *in vitro*. *In vivo,* VEGF is produced by stimulated endothelial cells, macrophages, fibroblasts, and smooth muscle cells [113]. VEGF is a potent biomolecule requiring concentrations as low as 1 ng/ml for initiation of angiogenic events [114].

The delivery of VEGF must be closely regulated in order to avoid detrimental results on existing vasculature. A high dose of VEGF may lead to leaky and hemorrhagic vessels [111]. Further, the extended release of VEGF can lead to the development of tumors *in vivo*. In addition, the plasma half-life of VEGF is only 50 minutes after

intravenous injection [115]. These temporal and quantitative requirements on VEGF delivery may be most efficiently maintained using a controlled release approach. Richardson, et al, reported an optimal angiogenic release rate of VEGF of 1.7 pmol/day [14]. Further, the vasculature obtained was optimized when VEGF was delivered with platelet derived growth factor (PDGF) (0.1 pmol/day), showing the synergistic effect of these two molecules and demonstrating the importance of versatile release systems with controllable release rates.

1.4 Recent Progress in Growth Factor Delivery

Due to the importance of protein therapeutics in disease management and tissue engineering, a wide variety of approaches have been investigated to stabilize growth factors during the controlled delivery of these important biomolecules [116]. Modification of proteins with PEG is an important breakthrough that provides the ability to increase the plasma half-life of proteins by reducing their clearance and proteolysis. Another technique combines the benefits of synthetic/hydrophobic scaffolds and hydrogel scaffolds through the utilization of block copolymers of PLA and PEG. A third strategy known as SABER™, utilizes a non-aqueous solvent to provide an injectable viscous polymer solution with hydrogel type properties that aids in the dispersion and decreased burst effect of PLA type scaffolds. Finally, a technique for forming protein-loaded microparticles known as Prolease®, has allowed for the miniaturization of protein delivery systems. Each of these systems will be discussed in detail below.

1.4.1 Protein PEGylation

One strategy to increase the bioavailability of important protein therapeutics is modification with poly(ethylene glycol) (PEG). PEG is a neutral synthetic polymer consisting of repeat units of (CH_2CH_2O) with molecular weights of medical interest ranging from a couple hundred Daltons to 20 kD. A full review of the properties and medical applications of PEG can be found in the books by Harris et al [117, 118]. Briefly, PEG molecules are highly mobile with a large exclusion volume in water, are soluble in both aqueous and organic solvents, form strong hydrogen bonds, and show little toxicity. Many applications have been devised to take advantage of the wide range of physical and chemical properties of PEG.

The most widely utilized property of PEG is its ability to provide "stealth" characteristics to surfaces and proteins to which it has been conjugated [119]. This was first demonstrated by Abuchowski et al. who showed that the covalent attachment of PEG to bovine serum albumin reduced the immunogenicity of the protein in a PEG molecular weight dependent manner [120]. In addition to reducing the antigenicity of a protein, PEG conjugation also leads to a decrease in enzymatic proteolysis suggesting that PEGylation may be an effective strategy for the delivery of protein therapeutics via the oral route [121, 122]. The "stealth" properties afforded to proteins by PEGylation in regards to immunogenicity and proteolysis, in addition to a reduction in glomerular filtration and reticuloendothelial clearance leads to an overall increase in plasma circulation half-lives [122, 123]. This allows for less frequent administration and increased patient compliance. In addition to its ability to increase circulation half-lives,

PEG has been shown to promote mucoadhesion, making PEGylation attractive for oral and pulmonary protein delivery strategies [124]. In fact, bioactive PEGylated granulocyte colony stimulating factor has been successfully delivered via the enteral and pulmonary routes [108, 125]. Modification of proteins with PEG also confers thermal and interfacial stability to the protein by significantly decreasing the ability of proteins to aggregate and denature [126]. Although, coupling of PEG to proteins decreases denaturation, decreases in protein bioactivity are almost universally exhibited due to the highly hydrated PEG chain blocking access to the active site of the protein [123]. Gombotz and Pettit have reviewed PEGylation strategies that minimize this loss in bioactivity while maintaining the stealth properties of the conjugate [127]. The lower pKa of the N-terminal amine allows for its selective PEGylation over the more basic lysine and arginine amine groups at slightly acidic pHs [128]. Thus, if the N-terminus is away from the active site of the protein, the conjugated PEG molecule can be placed where it less likely to interfere with biological function. Another strategy is to bind the protein on an affinity column during the PEGylation step in order to protect the active site from undesired conjugations. This method was shown to successfully maintain the activity of etanercept, a fusion protein consisting of the extracellular domain of TNFR and the Fc portion of IgG, over a solution PEGylation technique [127].

The biocompatibility of PEG has been well documented [118, 129, 130]. Working et al. have demonstrated that PEG (MW ~1500) is renally secreted and cleared from the plasma within 3 to 4 days. They also found no observed adverse effect levels of greater than 1500 mg/ml and 600 mg/ml of PEG (MW~1500) in the rat and dog, respectively.

Due to its biocompatibility, PEG has gained FDA approval for gestation and injection and is found as a pharmaceutical excipient in enteral and topical formulations. PEG is also present in many foods, cosmetics, and personal care items. PEG has also found success clinically as many PEGylated growth factors are undergoing advanced clinical trials [117, 119]. Several PEGylated proteins have gained FDA approval including, PEG-asparaginase for the treatment of lymphoma and leukemia [131], PEG-adenosine deaminase [132] for the treatment of SCIDS, PEGASYS (PEG-interferon alpha 2a) for the treatment of hepatitis C [133], and PEG-filgrastim (PEG-granulocyte colony stimulating factor) for the treatment of neutropenia following chemotherapy [134]. Although, PEGylation has demonstrated great success by increasing the availability of many therapeutic proteins, it has been underutilized in controlled release strategies and may be applicable to many oral drug delivery and tissue engineering efforts.

1.4.2 PLA/PEG Protein Delivery

Delivery systems that combine the respective benefits of PLA and PEG have received attention lately due to their ability to entrap and release protein without denaturation in a controlled and extended manner. The balance of hydrophobic and hydrophilic segments in these systems allows for control over mechanical properties, swelling, degradation, and protein release rates. Jiang, et al, studied the effect of using blends of slowly degrading PLA (145 kD) and PEG (10 and 35 kD) on BSA release from microspheres [135]. The microspheres were produced by an oil in oil (o/o) emulsion solvent extraction technique. They found that by incorporating greater than 20% PEG in the formulation that

continuous release could be achieved over 29 days; however, less than 20% PEG led to incomplete release, suggesting BSA aggregation within the polymer. With less than 10% PEG in the formulation, released BSA was found to be significantly denatured. In all cases, the protein release rate was high initially, followed by a period of much slower release. It is speculated that the PEG in these systems acts as a pore former to increase the permeability and hydrophilicity of the delivery device, thus allowing for greater diffusion for the removal of acidic degradation products. Further, the high molecular weight PLA leads to a slower rate of generation of acidic degradation products. Combined, these effects provide a stabilizing environment for BSA with low acid and high water content. An extension of this technique is to form physically cross-linked hydrogels with PLA-PEG-PLA block copolymers using hydrophobic interactions [43, 136]. Kissel, et al, demonstrated an accelerated degradation of the PLA component and an increase in swelling in comparison to PLA devices [137]. In addition, the delivery devices demonstrated molecular weight dependent release characteristic of cross-linked hydrogels. PLA-PEG-PLA block copolymers provide the advantage of increased drug loading, controlled and extended release, and less aggregation of protein in the delivery device. However, the increase in PLA degradation led to a build up of acidic degradation products leading to stability issues during release. Further, fabrication of these hydrogels involves a water in oil in water (w/o/w) double emulsion solvent extraction technique that leads to the leaching of protein and a decrease in entrapment efficiency. Jeong, et al, produced *in situ* thermo-gelling protein delivery systems using PEG-PLA-PEG block copolymers [138]. These systems are formulated in an aqueous solvent allowing for

stable entrapment of protein. Further, the gelling of this system occurs after injection of an aqueous solution of copolymer and protein. The gelation occurs from the outer surface of the gel and proceeds inward. This is advantageous in preventing an initial burst of protein release from the system. In a final example, Anseth, et al, have produced PLA-PEG-PLA block copolymers with vinyl functionalized end groups which can be used to form photo-crosslinked degradable networks with improved physical and mechanical properties for tissue engineering applications [25]. The modulus of these scaffolds decreased exponentially with time while the swelling increased exponentially. These scaffolds were found to support the formation of cartilage when chondrocytes were co-encapsulated with insulin-like growth factor (IGF).

1.4.3 SABER™

A relatively recent advancement in protein delivery is sucrose acetate isobutyrate extended release (SABER™) [139-142]. This biodegradable release system relies on the dissolution of growth factors and other biomolecules in a non-aqueous high viscosity liquid solution of sucrose acetate isobutyrate (SAIB). The formulation is made less viscous for injection by diluting SAIB with benzyl benzoate. Upon injection the benzyl benzoate slowly diffuses from the system leaving an adhesive, biodegradable, and biocompatible release system. This technology has been utilized for the delivery of rhVEGF and rhGH with controlled and extended release of growth factor [139, 140]. Further, the system has been shown to maintain the bioactivity of the delivered growth factor. An extension of this technique is to suspend PLGA microspheres loaded with

growth factor within the SABER™ system [141, 142]. This resulted in a much lower burst of rhGH from microspheres (from 14% after one hour to <2%) and continual release *in vivo* for over 21 days. Another extension of this technique investigated the effect of dissolving VEGF directly into a solution of poly(lactide) in benzyl benzoate and benzyl alcohol for use as an injectable depot [143]. This system was shown to increase angiogenesis in a bone repair model.

The strategies discussed above rely on the use of a biocompatible solvent, benzyl benzoate. Besides these applications, benzyl benzoate has also been utilized as a topical acaricide [144], as a subcutaneous, injectable carrier for hydrophobic drugs such as RU-486 [145], and as a carrier in nanocapsules carrying the anti-inflammatory molecules diclofenac and indomethacin [146-148]. In all cases, the safety and biocompatibility of benzyl benzoate has been established. Thus, SABER™ provides improved control over protein release and stability utilizing a biocompatible solvent system; however, this technique is not readily transferred to tissue engineering applications due to the depot mode of delivery.

1.4.4 Prolease™

Prolease™ is a protein micro-encapsulation technique originally patented by Gombotz et al. involving the entrapment of protein in polymeric microspheres using a cryogenic spray-freeze drying strategy (Figure 1-3) [149]. The strategy is as follows: First, a solution of polymer in solvent is prepared and protein is dispersed throughout the organic phase. This mixture is then atomized using an ultra-sonic nozzle by which the

vibrational energy of the nozzle is transformed into standing waves in the polymer solution on the nozzle tip [150]. At a critical amplitude, below that necessary for cavitations, the waves collapse releasing tiny droplets of polymer solution. This fine spray of polymer solution is then immediately frozen in a frozen non-solvent bath overlayed with liquid nitrogen. The frozen non-solvent is allowed to slowly thaw and the solvent is slowly extracted from the polymer particles. After filtration, polymeric microparticles containing entrapped protein are recovered.

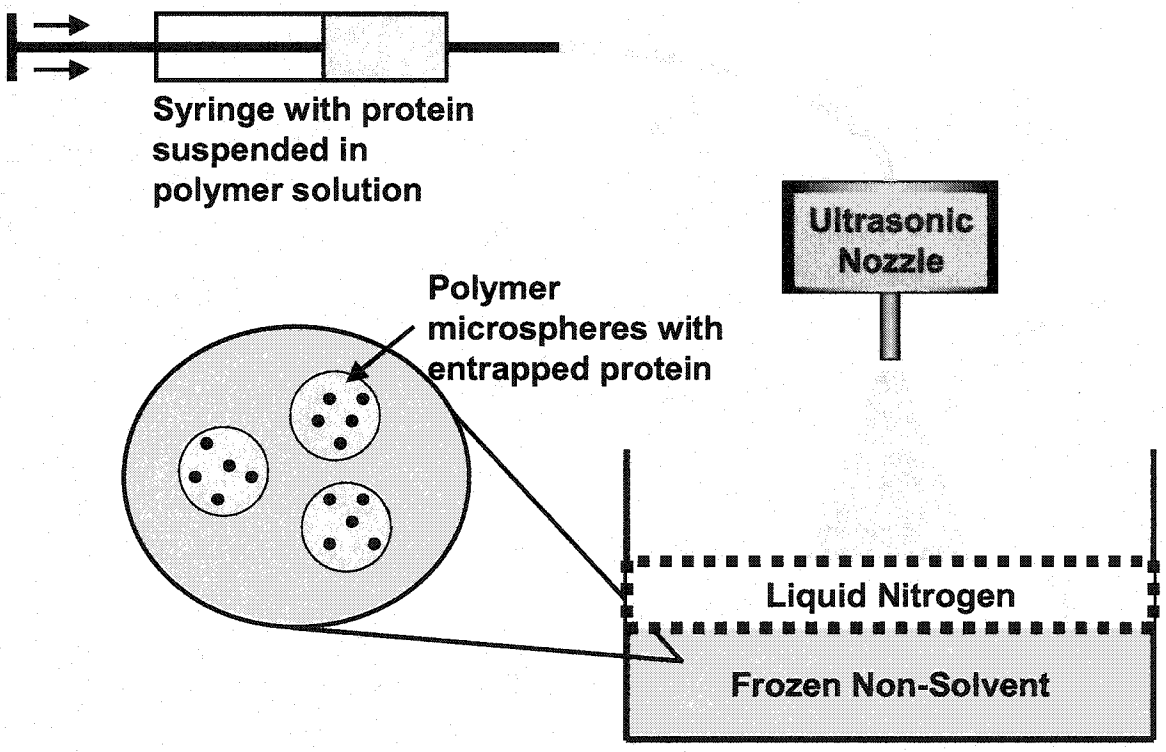


Figure 1-3: Preparation of microparticles using a Prolease® strategy. Atomized polymer/protein solution is frozen in a liquid nitrogen/non-solvent bath cold. Slow increase in temperature results in extraction of solvent leaving behind polymer microspheres with entrapped protein.

The most important variable in controlling the release of protein from microparticles fabricated using this technique is the particle-size of the entrapped protein [151]. Larger protein particles lead to an increase in initial protein release (burst effect). Thus, spray-freeze drying of protein is typically utilized to minimize protein particle size prior to encapsulation in a polymer matrix [152]. Prolease™ has shown promise in its ability to release bioactive protein. This is partly due to the absence of a water/organic interface [116]. Proteins are amphipathic and tend to denature and lose their activity at these interfaces. Many excipients have been investigated for their ability to stabilize the protein during the spray-freeze drying process. The addition of carboxymethyl cellulose, mannitol, trehalose, ammonium sulfate, and zinc complexation has been shown to increase the stability of spray-freeze dried protein [151, 153]. However, trehalose, and ammonium sulfate led to an undesired increase in protein particle size, while carboxymethyl cellulose, mannitol, and ammonium sulfate only partially stabilized the protein.

The Prolease™ strategy has recently been licensed by Alkermes (Cambridge, MA) for the delivery of human growth hormone (hGH) [154]. *In vivo* and clinical success have been achieved using this technique with controlled release of bioactive hGH producing serum levels of hGH and insulin growth factor I greater than those seen with traditional daily injection of equivalent amounts of hGH [155-158]. For this reason, Prolease™ strategies have been investigated to control the release of many therapeutic growth factors including NGF, VEGF, IFN-γ, and IGF-1 [159-164].

1.5 Experimental Outline

It is hypothesized that proteins can be delivered in a controlled and stable manner by combining appropriate ratios of hydrophobic and hydrophilic polymers in cross-linked networks, thus aiding in the successful integration of tissue engineering scaffolds. In this work, synthetic and natural polymers were utilized to develop physically or chemically cross-linked protein delivery hydrogels capable of stable and controlled delivery of growth factors with appropriate individual release kinetics in order to promote proper wound healing events such as angiogenesis.

In Chapter 2, our goal is to develop a delivery system for PEG-(GM-CSF) based on a physically cross-linked chitosan carrier that would allow for the controlled delivery of GM-CSF. Our strategy is to form films of chitosan containing GM-CSF or PEG-(GM-CSF) and varying amounts of glycerol as a physical cross-linker. Glycerol should hydrogen bond with chitosan amine groups allowing for their deprotonation. This should generate a more hydrophobic scaffold with decreased swelling and protein release profiles. To this end, films comprised of various weight ratios of chitosan to glycerol were analyzed for their ability to control the release rate of GM-CSF and PEG-(GM-CSF).

In Chapter 3, the mechanism behind glycerol's ability to slow and reduce the release of PEG-(GM-CSF) and to mechanically stabilize the chitosan films was investigated. Thus, the contribution of hydrogen bonding on the mechanism of release was investigated utilizing gravimetric, pH, and FTIR measurements. In addition, several

molecules were screened for their ability to provide "glycerol-like" stabilizing effects in chitosan formulations.

Next, we modified another naturally occurring hydrogel, hyaluronic acid, using glycidyl methacrylate to produce hyaluronic acid with various degrees of methacrylation as described in chapter 4. Thus, using photochemical techniques, we can form hydrogels of hyaluronic acid with various cross-link densities. These hydrogel scaffolds were characterized using mechanical compression tests to determine their suitability as a cell encapsulating scaffold. Further, the ability of the cross-linked HA hydrogels to control the release of biomacromolecules was examined using FITC-labeled dextrans of various molecular weights, lysozyme, and bovine serum albumin (BSA).

In chapter 5, we have prepared interpenetrating polymer networks (IPNs) comprised of poly(ethylene glycol) dimethacrylate (PEGDMA) and poly(lactide) (PLA). The IPNs were synthesized using photochemical techniques in a mixture of benzyl benzoate/benzyl alcohol (BB/BA), which is a co-solvent for both the polymers and protein. The use of this solvent system for preparing our IPNs allows for uniform protein dispersion throughout the matrix, which may lead to more uniform release kinetics (elimination of initial burst). The hydrophobic/hydrophilic balance of the IPN, and thus the swelling and release rate can be manipulated by varying the polymer molecular weights and the PEG/PLA ratio. The effects of these parameters on IPN swelling and release of a model protein, bovine serum albumin (BSA), were investigated with the goal of providing a tunable protein delivery system with engineering handles to control the rate of protein release.

Finally, in chapter 6 we describe a method for producing microparticles from our PEG/PLA system. The ability to produce microparticles of the PEG/PLA IPNs provides versatility in applying this protein release system to a variety of tissue engineering strategies. With this in mind, we investigated the ability of these microparticle PEG/PLA IPNs to control the release of VEGF, a potent angiogenic growth factor.

Chapter 2: Formulation of Physically Cross-Linked Chitosan/Glycerol Films for the Delivery of PEG-(GM-CSF)

2.1 Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that has been shown to have stimulatory effects on the growth and maturation of hematopoietic cells. For this reason, it has been used clinically to enhance hematopoietic recovery after bone marrow transplantation and for reducing neutropenia associated with chemotherapy [107]. In addition, GM-CSF has been shown to promote proliferation of keratinocytes, formation of granulation tissue, and vascularization of the wound bed [106]. Due to its role in wound healing, GM-CSF may be an interesting mediator in tissue engineering applications. Typically, GM-CSF is administered daily by intravenous infusion at a dosing rate of 250 $\mu g/m^2$ for 14-21 days. However, lower dosages may be required when delivered locally in tissue engineering or wound healing applications. Either way, patient compliance and comfort would be greatly improved by an effective controlled release formulation for GM-CSF.

Modification of proteins with poly(ethylene glycol) (PEG) is an effective way to increase the plasma circulation half-life of the protein by reducing glomerular filtration, reticuloendothelial clearance, and proteolysis [121-123]. These combined effects allow proteins to be delivered at lower doses and less frequently. Furthermore, PEG provides a stealth effect that protects the protein from the host immune system, thus decreasing its antigenic and immunogenic profiles [120, 122]. Interestingly, PEGylation has achieved some success in protecting G-CSF when delivered by the enteral and pulmonary routes

[108, 125]; however, it may be necessary to make the PEG conjugation degradable to achieve optimal bioavailablity via these routes.

Our goal is to develop a delivery system for PEG-(GM-CSF) based on a physically cross-linked chitosan carrier that would allow for the controlled delivery of GM-CSF. Chitosan is insoluble in neutral aqueous solutions; however, in dilute acetic acid solutions (pH<6), the chitosan amino groups become protonated making the charged polymer soluble. The cationic character of chitosan and its mild processing conditions make it an ideal carrier for the delivery of GM-CSF, which is net-negative at physiological conditions. Chitosan has also been utilized in many tissue-engineering applications due to its apparent biocompatibility and structural similarities to glycosaminoglycans [46]. In addition, chitosan has been shown to have an accelerating effect on wound healing [64]. Finally, chitosan has been shown to be mucoadhesive, making it an ideal component for applications requiring oral or mucosal delivery of GM-CSF [45, 54, 66, 165].

Our strategy is to form films of chitosan containing GM-CSF or PEG-(GM-CSF) and varying amounts of glycerol. Glycerol is an FDA approved excipient for topical, ocular, and parenteral administration. Glycerol may be capable of forming hydrogen bonds with both chitosan and PEG, thus providing a physically cross-linked delivery system with resulting control over the rate of protein release. Films comprised of various weight ratios of chitosan to glycerol were thus analyzed for their ability to control the release rate of GM-CSF and PEG-(GM-CSF).

2.2 Materials and Methods

2.2.1 Materials

Chitosan (VNS-579) with an average degree of deacetylation of 84% and a viscosity of 490 cps was obtained from Vanson Corporation (Redmond, WA). Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was provided by Immunex Corporation (Seattle, WA). PEG-(GM-CSF) was synthesized and purified by Immunex Corporation using lysine specific PEG succinimidyl propionate chemistry (M-SPA-PEG: 2 kD, 5 kD, and 20 kD, Shearwater Polymers, Huntsville, AL) and a Q-sepharose ion exchange column from Pharmacia (Uppsala, Sweden) [127]. The various PEG-(GM-CSF) conjugates were a mixture of mono- and di-PEGylated products (2 kD: 25% mono/75% di, 5 kD: 18% mono/82% di, and 20 kD: 25% mono/75% di). Glacial acetic acid (A.C.S. Plus) was obtained from Fisher Scientific (Pittsburgh, PA). Anhydrous glycerol (A.C.S. Reagent) was received from J. T. Baker, Inc (Phillipsburg, NJ). Physiological phosphate buffered saline (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) and HCl were obtained from Sigma (St. Louis, MO). Ninhydrin and Na-S sample diluent buffer were obtained from Beckman (Fullerton, CA).

2.2.2 Preparation of Chitosan/Glycerol Films

Chitosan/Glycerol films with various ratios of chitosan to glycerol were prepared using the following protocol. First, chitosan was dissolved in 0.5 to 1.0% acetic acid at 10 mg/ml and centrifuged and filtered before use to remove any insoluble material. A 10

mg/ml solution of glycerol was prepared by dissolving anhydrous glycerol in distilled and deionized water. Casting solutions of the appropriate ratios of chitosan to glycerol were prepared by mixing the chitosan and glycerol solutions at the desired ratio while maintaining a 0.5% acetic acid concentration and a constant total film weight of chitosan and glycerol. PEG-(GM-CSF), GM-CSF, or physical mixtures of PEG and GM-CSF in 10 mM Tris buffer, pH=7.4 were added to the casting solutions to give a concentration of 1% by weight GM-CSF in the solution. Approximately 5.0 ml of casting solution were cast into a 10 ml Teflon beaker and allowed to evaporate at room temperature and pressure for 7 days. After 7 days, the films were placed under vacuum (>100 kPa vacuum) at room temperature to dry for an additional 7 days. The dry weight of each film was recorded (initial dry weight). Samples were prepared in duplicate for each release condition. Typical compositions of these films are shown in Table 2-1.

Table 2-1: Composition of casting solutions for films containing 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF) with various weight ratios of chitosan (C)/glycerol (G)

C:G (wt)	Glycerol:NH_2 (molar)	C (mg)	G (mg)	GM-CSF (mg)	PEG (mg)
1:1	2.2	23.2	23.2	0.47	1.1
1:0.67	1.45	28.8	19.2	0.47	1.1
1:0.5	1.09	31.0	15.5	0.47	1.1
1:0.25	0.54	38.4	9.6	0.47	1.1
1:0	-	46.4	-	0.47	1.1

2.2.3 Swelling and Release Profiles of Chitosan/Glycerol Films

The swelling and release profiles of chitosan/glycerol films containing PEG-(GM-CSF), GM-CSF, or physical mixtures of PEG and GM-CSF were examined using the following procedure. Approximately 5 ml of release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) were added to a 20 ml glass scintillation vial. The weight of the buffer-containing vial was recorded. A vacuumed-dried chitosan/glycerol film was then added to each vial and the vials were subsequently placed in a shaking water bath at 37 °C. After time periods of 10, 20, 30, 60, 180, and 300 minutes the film was removed from the vial and placed into a new vial with fresh release buffer. At each time point, the weight of the vial along with the remaining release buffer was determined in order to calculate the amount of water absorbed by the film. After completion of the release study, the remaining film was lyophilized and reweighed (final dry film weight). The swelling ratio was calculated by determining the weight of water absorbed by the film and comparing it to the final dry film weight by the following equation:

$$\mathbf{Q = (Ww+Wd)/Wd}$$

Where,

Q = the swelling ratio

Ww = weight of water absorbed by the film

Wd = final dry film weight

The amount of PEG-(GM-CSF) and GM-CSF released at each time point was determined using size exclusion high performance liquid chromatography (HPLC-SEC). A BioSil 125 column (BioRad, Hercules, CA) with an elution buffer of 100 mM sodium phosphate, 150 mM NaCl, pH 6.8 and a flow rate of 1.0 ml/min was utilized. Results were expressed as a percentage of the amount of theoretically loaded protein. Values for duplicate films were averaged at each time point.

The amount of protein loaded into the films was verified using amino acid analysis. Briefly, film samples were incubated anaerobically at 110°C in concentrated HCl for 24 to 72 hours in order to hydrolyze the protein. The HCl was evaporated off and the samples resuspended in Na-S sample diluent buffer. The solution was filtered to remove any insoluble film residue. The amino acids were separated and quantified using ion-exchange chromatography (Beckman sodium column) followed by reaction with ninhydrin reagent and detection at 570 nm.

2.2.4 Bioactivity of Released GM-CSF

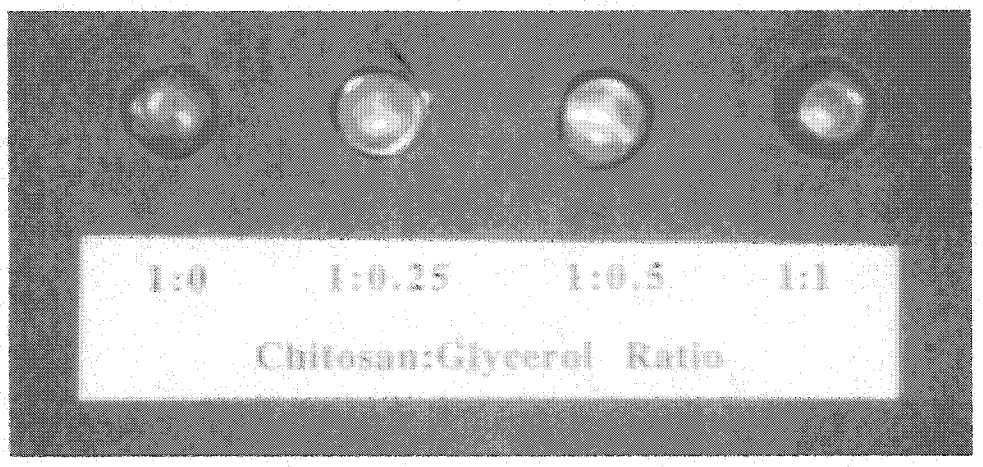
A human erythroleukemia cell line (TF-1) was used to determine the bioactivity of GM-CSF before and after release from the chitosan/glycerol films. The proliferation of this cell line is dose-dependent on GM-CSF [166]. Therefore, the degree of proliferation provides a measure of the bioactivity of the released GM-CSF.

2.3 Results

2.3.1 Release of PEG-(GM-CSF) and GM-CSF from chitosan/glycerol films

Films composed of either a 1:0, 1:0.25, 1:0.67, or 1:1 weight ratio of chitosan to glycerol and containing 20 kD PEG-(GM-CSF) were placed in release buffer and allowed to swell (Figure 2-1). As the glycerol content was increased, the film swelled to a lesser extent, demonstrated increased mechanical integrity, and did not dissolve during the duration of the experiment (Figure 2-2). A decrease in swelling with time is hypothesized to be contributed to the extraction of glycerol, buffer salts, and residual acetic acid resulting in a decrease in driving force for osmotic swelling. The overall result was a dramatic decrease in the percent release of 20 kD PEG-(GM-CSF) with increasing amounts of glycerol in the film (Figure 2-3). Films containing no glycerol swelled rapidly, formed a weak gel, dissolved and released the PEG-(GM-CSF) as a burst. On the other hand, films containing a 1:1 weight ratio of chitosan to glycerol exhibited much slower release with almost complete retardation of 20 kD PEG-(GM-CSF) release from the film

(a)



(b)

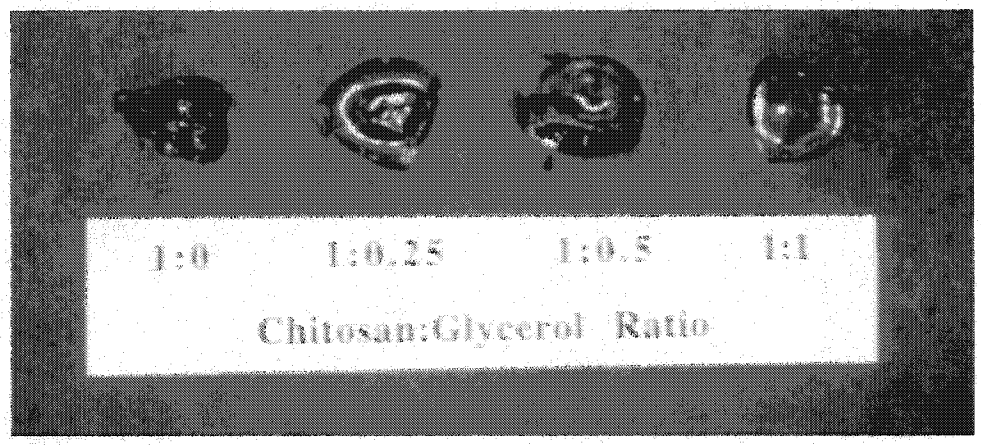


Figure 2-1: Chitosan:Glycerol films (a) before and (b) after submersion in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C for 10 minutes.

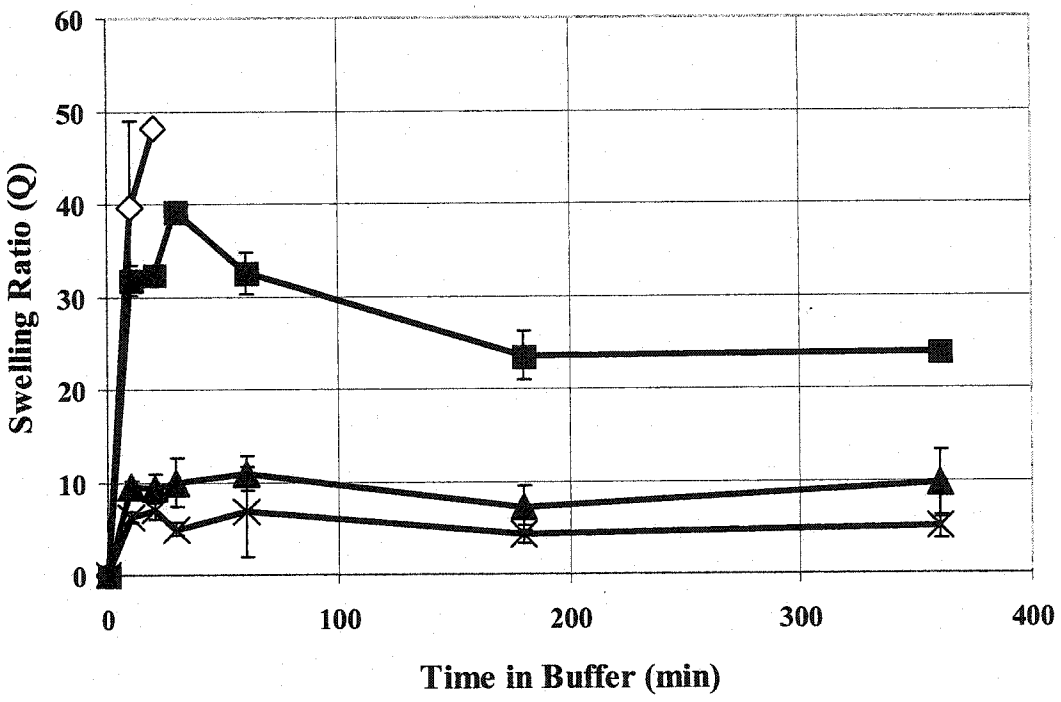


Figure 2-2: Swelling profiles of chitosan/glycerol films containing 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Chitosan to glycerol weight ratios: 1:0 (◊), 1:0.25 (■), 1:0.67 (▲), and 1:1 (X).

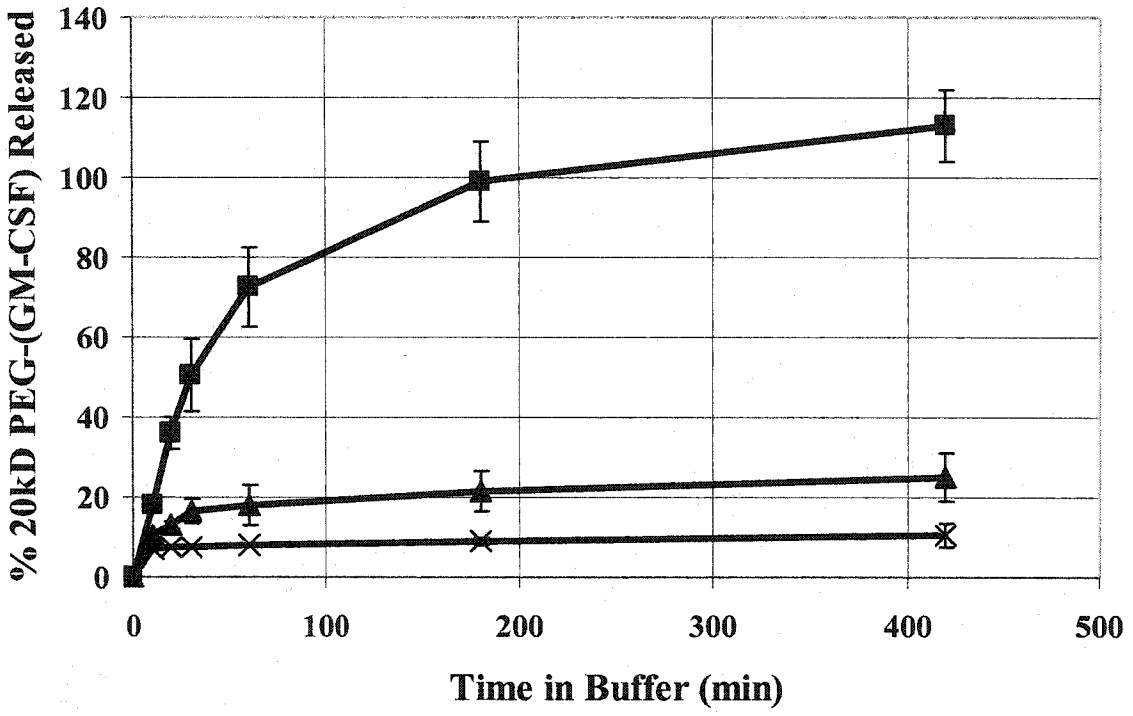


Figure 2-3: Release profiles of 20 kD PEG-(GM-CSF) from chitosan/glycerol films containing 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Chitosan to glycerol weight ratios: 1:0.25 (■), 1:0.67 (▲), and 1:1 (X).

The decrease in the amount of PEG-(GM-CSF) released was found to be dependent on the molecular weight of the PEG conjugated to GM-CSF. Films comprised of a 1:1 weight ratio of chitosan to glycerol and containing PEG-(GM-CSF) with PEG molecular weights of 2 kD, 5 kD, or 20 kD were allowed to swell in release buffer. The amount of PEG-(GM-CSF) released decreased as the molecular weight of the conjugated PEG increased (Figure 2-4a). This decrease in PEG-(GM-CSF) release is most likely due to an increase in steric hindrance or in chain entanglements between PEG and chitosan when PEGs of higher molecular weight are conjugated to GM-CSF. It is also possible that PEG may form hydrogen bonds with chitosan forming a more highly cross-linked

network, which could add diffusional resistance as the molecular weight of PEG is increased. Both of these effects would hinder the protein molecule during its diffusion out of the chitosan film. Controls containing physical mixtures of PEG and GM-CSF did not show this same dependence on PEG molecular weight. Films with a 1:1 weight ratio of chitosan to glycerol containing GM-CSF or physical mixtures of GM-CSF and non-conjugated PEG of molecular weights of 2 kD, 4.6 kD, or 19.6 kD were prepared, maintaining the same ratio of PEG to protein as that used for the PEG conjugated GM-CSF. In all cases, the rate of release of GM-CSF from the chitosan/glycerol film was similar to that with GM-CSF alone (Figure 2-4b). This supports the hypothesis that the decrease in PEG-(GM-CSF) release with increasing PEG molecular weight is due to increased steric hindrance or chain entanglements of the PEGylated molecule and not due to hydrogen bonding effects between PEG and chitosan leading to the formation of a higher cross-linked network. Importantly, it was found that the released GM-CSF and PEG-(GM-CSF) in all studies retained their activity as demonstrated by the TF-1 bioactivity assay (Table 2-2). In addition, amino acid analysis of dried films accounted for 100% of the theoretically loaded GM-CSF in the chitosan/glycerol films.

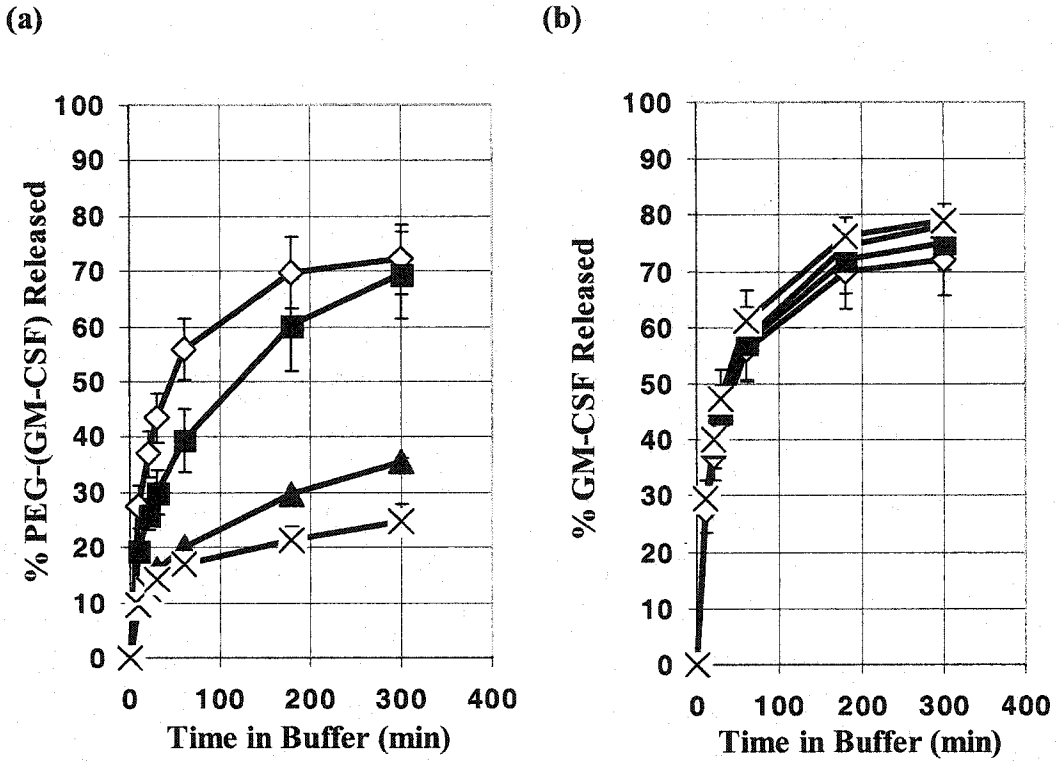


Figure 2-4: Effect of PEG molecular weight on the release profile of PEG-(GM-CSF)/GM-CSF from chitosan/glycerol films consisting of a 1:1 weight ratio of chitosan to glycerol, (a) conjugated with no PEG (◊), 2 kD PEG (■), 5 kD PEG (▲), and 20 kD PEG (X), or (b) physical mixtures with no PEG (◊), 2 kD PEG (■), 4.6 kD PEG (▲), and 19.6 kD PEG (X). Release studies were carried out in 10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 37°C. All films contained 1% (w/w) GM-CSF.

Table 2-2: Specific activity of GM-CSF and PEG-(GM-CSF) before and after 420 minutes of release from a chitosan/glycerol film with a 1:1 weight ratio of chitosan:glycerol. Values may contain greater than 20% error due to error introduced during HPLC detection and inherent error in the bioassay.

	Specific Activity Before Release (units/μg)	Specific Activity After Release (units/μg)
GM-CSF	67050	94950
2 kD PEG-(GM-CSF)	53767	20430
5 kD PEG-(GM-CSF)	43688	19490
20 kD PEG-(GM-CSF)	1590	2353

2.4 Discussion

The effects of PEG molecular weight and glycerol on the swelling and PEG-(GM-CSF) release profiles of chitosan/glycerol films are summarized in Table 2-3. First, it was observed that as the molecular weight of the conjugated PEG increased there was a dramatic effect on the percent release and release rate of PEG-(GM-CSF). In fact, at a 1:1 weight ratio of chitosan to glycerol the release of 20 kD PEG-(GM-CSF) is almost completely prevented. It is hypothesized that this decrease in release rate is due to an increase in steric hindrance or in chain entanglements of the PEGylated protein during its diffusion out of the chitosan film. Further, it was observed that the demonstrated decrease in release rate of PEG-(GM-CSF) with an increase in PEG molecular weight was dependent on the conjugation of PEG to GM-CSF. This suggests that the decrease in the percent of PEG-(GM-CSF) released was not due to hydrogen bonding between chitosan and PEG, which would cause the formation of a more highly cross-linked network and provide a higher diffusional resistance for the PEGylated protein during its diffusion out of the chitosan film. It may prove beneficial in this formulation to PEGylate GM-CSF with degradable PEG linkages such that full release of the PEG-(GM-CSF) will occur. Due to the almost complete retardation of release of the 20 kD PEG-(GM-CSF) from chitosan films with a 1:1 chitosan to glycerol ratio, the rate of release would be governed by the rate of degradation of the degradable PEG linkage rather than the slow biodegradation of the chitosan matrix. Further, oral formulations may benefit from degradable PEG linkages, facilitating the mucosal penetration and transport of the growth factor.

In addition, it was found that as the glycerol content of the chitosan/glycerol film increased, that the swelling of the chitosan film decreased with a corresponding decrease in the percent release and release rate of PEG-(GM-CSF). It is interesting to note that the swelling of the chitosan film decreases progressively and appears to reach a threshold at a 1:0.5 chitosan to glycerol weight ratio. This weight ratio corresponds to approximately a 1:1 glycerol to chitosan amine molar ratio. This suggests that glycerol is in fact acting as a physical cross-linking agent as designed. It is our hypothesis that the decrease in swelling and protein release rate seen with glycerol is due to the ability of glycerol to hydrogen bond with the chitosan amine groups, allowing for the complete deprotonation of the amines, and thus complete removal of residual acetic acid from the formulation during the drying of the film. The deprotonation of chitosan by the addition of glycerol has the overall effect of increasing the hydrophobicity of the matrix. Thus, the hydrophobicity of the matrix can easily be adjusted by varying the amount of the physical cross-linking agent. Any residual acetic acid in the formulation would allow for protonation of the amines when the films are placed in buffer and would lead to rapid swelling and release of entrapped protein. This hypothesis will be investigated further in the following chapter.

Table 2-3: General trends observed in the release of PEG-(GM-CSF) from chitosan/glycerol films.

	Swelling	**PEG-(GM-CSF) Release**
PEG MW Increased	Increases Slightly	Decreases
Glycerol Content Increased	Decreases	Decreases

Chapter 3: Mechanism of PEG-(GM-CSF) Release from Physically Cross-Linked Chitosan/Glycerol Films

3.1 Introduction

It was noted in Chapter 2 that chitosan/glycerol films containing increasing amounts of glycerol exhibited less swelling, produced more stable films, and entrapped PEG-(GM-CSF), whereas films containing no glycerol swelled rapidly, dissolved, and released PEG-(GM-CSF) in a burst fashion. In order to understand the mechanism of glycerol action to slow and reduce the release of PEG-(GM-CSF) and also to mechanically stabilize the chitosan films, the hypothesis that glycerol aids in the removal of residual acetic acid from the films was tested. It is hypothesized that glycerol acts by hydrogen bonding with chitosan amines allowing for their deprotonation and aiding in the complete removal of acetic acid during the drying of the film. This produces a chitosan film that is more hydrophobic due to the deprotonated state of the amines. Any residual acetic acid in the film would allow for the rapid protonation of the chitosan amine groups, which in turn would lead to rapid swelling of the chitosan film as the chitosan began to solubilize. This leads to a controlled release formulation with poor control over the rate of protein delivery. Thus, the contribution of hydrogen bonding on the mechanism of release was investigated utilizing gravimetric, pH, and FTIR measurements. In addition, the chitosan/glycerol formulations were further characterized by examining the rate of glycerol release. Finally, several molecules were screened for their ability to provide "glycerol-like" stabilizing effects in chitosan formulations.

3.2 Materials and Methods

3.2.1 Materials

Chitosan (VNS-579) with an average degree of deacetylation of 84% and a viscosity of 490 cps was obtained from Vanson Corporation (Redmond, WA). Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and PEGylated GM-CSF [PEG-(GM-CSF)] was provided by Immunex Corporation (Seattle, WA). PEG-(GM-CSF) was synthesized, purified and characterized as in Chapter 2. Glacial acetic acid (A.C.S. Plus) was obtained from Fisher Scientific (Pittsburgh, PA). Urea (Reagent), anhydrous glycerol (A.C.S. Reagent), PEG (400 Dalton), and sucrose were received from J. T. Baker, Inc (Phillipsburg, NJ). Physiological phosphate buffered saline (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4), propanediol, glucose, and trehalose were obtained from Sigma (St. Louis, MO). Pentaerythritol, mannitol, sorbitol, and trimethylolpropane were obtained from Aldrich (Milwaukee, WI). Dextran (10 kD) was obtained from Pharmacia (Uppsala, Sweden).

3.2.2 Release of Glycerol from Chitosan/Glycerol Films

Films comprised of either a 1:0, 1:0.5, or 1:1 chitosan to glycerol weight ratio were prepared and allow to swell in release media (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) as described in Chapter 2. These films were weighed dry before and after the release study to determine the extent of removal of glycerol from the films. Further,

careful analysis of the expected and measured initial weights may provide insight into the removal of acetic acid from the films.

The rate of release of glycerol from the chitosan/glycerol films was determined using size exclusion high performance liquid chromatography (HPLC-SEC). A BioSil 125 column (BioRad, Hercules, CA) with an elution buffer of 100 mM sodium phosphate, 150 mM NaCl, pH 6.8 and a flow rate of 1.0 ml/min was utilized. Concentrations were determined from peak areas against glycerol standards of known concentration. Results were expressed as a percentage of the theoretically added amount of anhydrous glycerol.

3.2.3 Acidification of Release Media

To further test the hypothesis that increased glycerol content leads to an increased efficiency of residual acetic acid removal from the drying film, the pH of the release medium was measured at various time points throughout the release study. Films comprised of either a 1:0, 1:0.5, or 1:1 chitosan to glycerol weight ratio were prepared and allow to swell in release media (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) as described in Chapter 2. The release media was collected at various time points and replaced with fresh media. At each time point the pH of the release media was measured using an Accumet 950 pH meter (Fisher Scientific).

3.2.4 Effect of Acidification and H-Bond Disruption on Release of PEG-(GM-CSF)

Films comprised of a 1:1 chitosan to glycerol weight ratio and containing 20 kD PEG-(GM-CSF) were allowed to swell in PBS buffer as described in Chapter 2. After

180 minutes, 25 µl of glacial acetic acid were added to the 5 ml of release media in order to overwhelm the buffering capacity of the media and to verify whether the entrapped PEG-(GM-CSF) would be released upon swelling of the chitosan film.

In addition, in order to investigate possible hydrogen bonding effects of glycerol within chitosan films, 100 µl of an 8 M urea solution were added to the casting solution of films comprised of a 1:1 chitosan to glycerol weight ratio and containing 20 kD PEG-(GM-CSF). Urea is known to disrupt hydrogen bonding and should have an effect on the release rate of PEG-(GM-CSF) if the mechanism of glycerol stabilization involves hydrogen bonding. As a control, films comprised of a 1:1 chitosan to glycerol weight ratio and containing PEG-(GM-CSF) but without the addition of urea to the casting solution were prepared. However, these films were treated with 100 µl of an 8M urea solution after 180 minutes of release in order to determine if the hydrogen bonding effects of glycerol were more important during film formation or during film swelling.

3.2.5 FTIR Analysis of Chitosan/Glycerol Films

The hydrogen bonding effects of glycerol on the proton state of the chitosan amines can be monitored using Fourier transform infrared spectroscopy (FTIR). A peak at approximately 1560 cm^{-1} has been previously attributed to the presence of $NH_3^+\cdots CH_3COO^-$ in chitosan [167]. Another peak at 1152 cm^{-1} is due to the ether bond in chitosan and is unaffected by the proton state of the amino groups [53]. The ratio of the peak height and area at 1560 cm^{-1} to that of the peak at 1152 cm^{-1} was used to provide a measure of the acetate content of the films. Casting solutions containing chitosan and

glycerol in acetic acid were prepared as described in Chapter 2 except without the protein addition step. Films were generated by placing 1.0 ml of solution into a 10 ml Teflon beaker (2.2 cm internal diameter) and drying, thus producing thinner films than those used in the release studies. These thin films were analyzed by placing the film vertically in the laser path of a Perkin Elmer 1720 FTIR spectrophotometer. Spectra of glycerol blanks were obtained by coating a thin film of anhydrous glycerol on a NaCl salt plate. All spectra were an average of 32 scans. A background spectrum containing no sample was subtracted from each spectrum. Peak heights and areas were calculated using a software package supplied by Perkin Elmer. Four films were analyzed at each condition and the results plotted as an average plus or minus one standard deviation.

3.2.6 Screening of Compounds with Glycerol-Like Behavior

In order to analyze the ability of other hydrogen bonding compounds to act as physical cross-linking agents and to enhance the removal of acetic acid from the drying films, the pH of the film was monitored by adding bromothymol blue to the casting solution of various chitosan film formulations. Bromothymol blue is a colorimetric pH indicator that is yellow at pH 5.5 and blue at pH 7.5. Using this technique, the color of the film could be monitored during the drying process and used to indicate which films contained higher amounts of residual acetic acid. Films containing agents that have effects similar to glycerol will exhibit a dark green color after drying (pH~7), while films not containing glycerol-like agents should remain yellow.

3.3 Results

3.3.1 Release of Glycerol from Chitosan/Glycerol Films

It was noted above that glycerol confers mechanical stability, decreased swelling, and a decreased rate of protein release to chitosan films. It is hypothesized that glycerol acts by aiding in the removal of acetic acid from the film through hydrogen bonding, thus providing stability to chitosan films when placed in aqueous media. By comparing the dry weight of chitosan/glycerol films before and after the release study it was found that as the glycerol content increased, the initial dry weight of the film steadily decreased to below the expected value (based on the amount of chitosan and glycerol present in the film, Figure 3-1) suggesting that glycerol has indeed aided in the removal of residual acetic acid from the film. On the other hand, the final dry weight of the film was comparable to the expected value after complete extraction of the glycerol component, indicating the results are not due to a lower than expected amount of chitosan in the film (Table 3-1). The much lower final weight for the 1:0 ratio is due to solubilization of the highly swollen film. Together, these results suggest that glycerol does aid in the removal of bound acetic acid and water from the drying film.

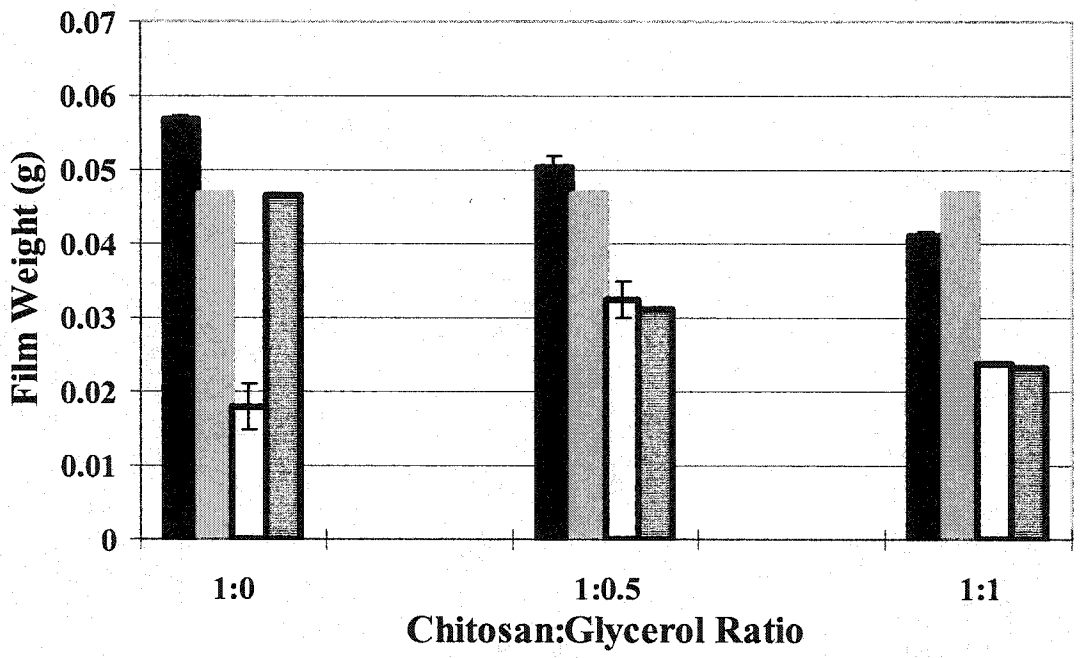


Figure 3-1: Initial and final dry weights of chitosan/glycerol films consisting of various chitosan to glycerol ratios: Initial dry weight (■), expected initial dry weight based on chitosan and glycerol content (▒), final dry weight (□), and expected final dry weight based on complete removal of glycerol component (⊟).

Table 3-1: Mass of chitosan/glycerol film with a 1:1 weight ratio of chitosan:glycerol loaded with 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF) before and after 10 minutes of release.

	C (mg)	G (mg)	GM-CSF (mg)	PEG (mg)	Theoretical/measured (mg)
before	23.2	23.2	0.47	1.1	47.97/41.0
after*	23.2*	0*	0.40*	0.94*	24.54*/23.9

*estimated values based on complete glycerol removal and 15% PEG-(GM-CSF) release.

In order to evaluate how quickly the glycerol component is being removed from the chitosan/glycerol films, the release media was analyzed for glycerol content by SEC-HPLC. It was found that a majority of the theoretically loaded glycerol was released within ten minutes of placement of the chitosan/glycerol film in the release media (Figure 3-2). No further glycerol release was seen at longer time points suggesting incomplete glycerol release. However, this observation is most likely due to inaccuracies in measuring and adding the anhydrous glycerol component to the formulation. Further, it is also likely that the glycerol utilized was not completely anhydrous allowing for the addition of water weight to the film. In either case, it is obvious that the glycerol component is quickly removed from the film and that its role in stabilizing the chitosan/glycerol films is most likely during film formation and not during the swelling process.

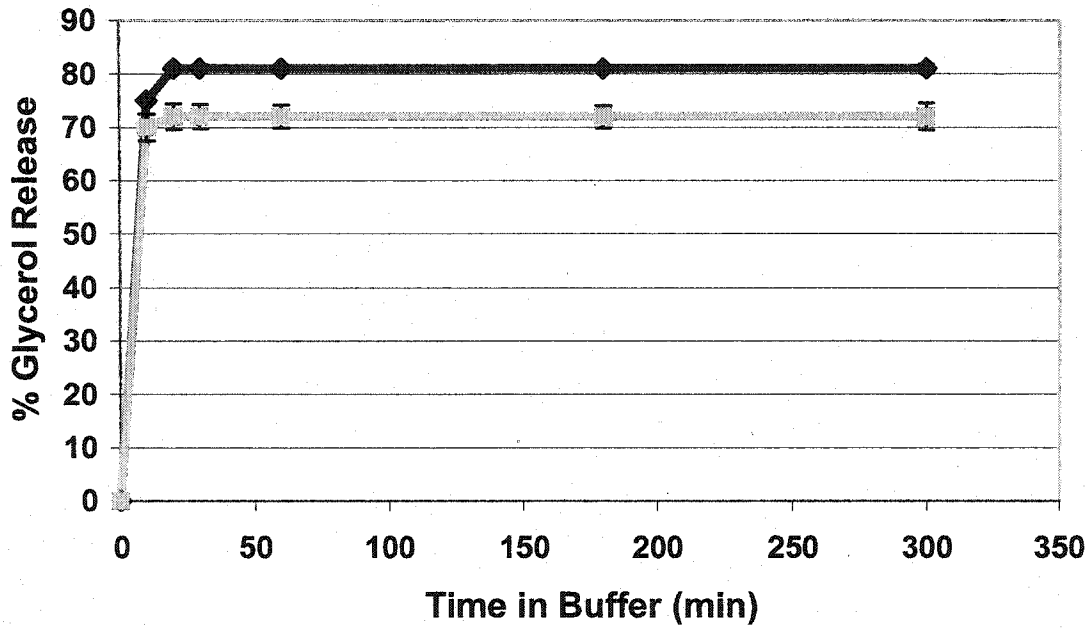


Figure 3-2: Release profiles of glycerol from chitosan/glycerol films with a 1:1 weight ratio of chitosan to glycerol placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. With 18.5 kD PEG (♦) or without PEG( ).

3.3.2 pH-dependent swelling of chitosan/glycerol films

To further test the hypothesis that glycerol aids in the removal of residual acetic acid from the chitosan/glycerol films, the pH of the release medium was measured throughout the release study. It was discovered that the release medium for chitosan films without glycerol became acidic with pH levels reaching those necessary for chitosan solubilization (pH<6, Figure 3-3). On the other hand, films containing increasing amounts of glycerol exhibited higher pH levels in the release media; further supporting the hypothesis that glycerol enhances the evaporation and removal of acetic acid during the drying of the films.

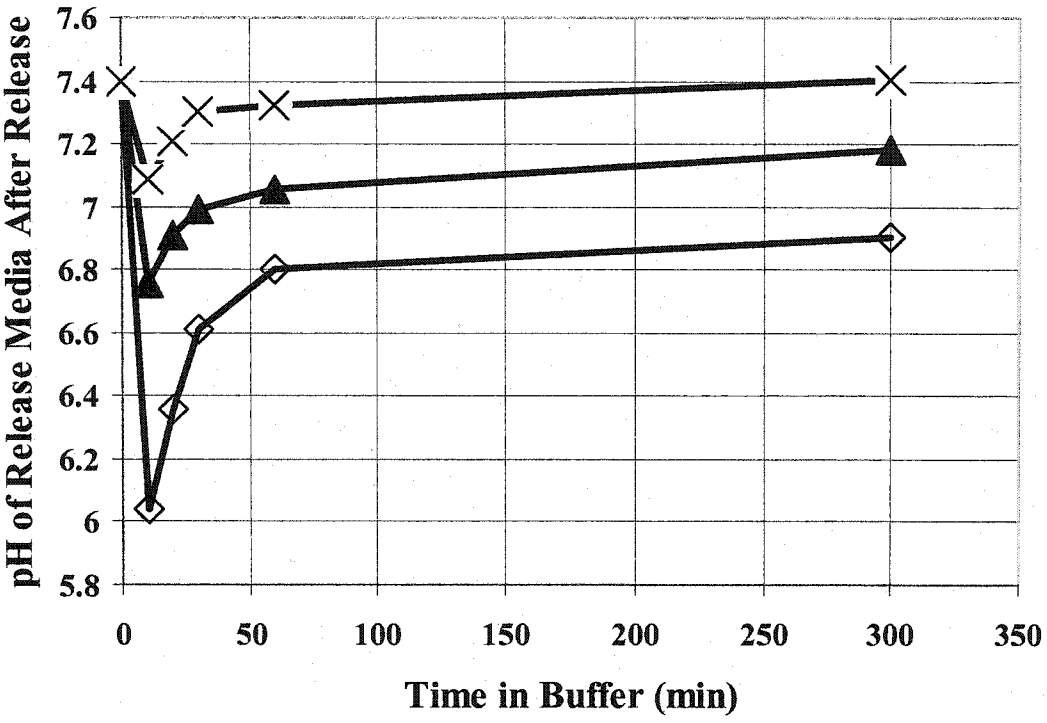


Figure 3-3: pH profile of release media (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 37°C) after release of 20 kD PEG-GM-CSF from chitosan/glycerol films containing 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF). Chitosan to glycerol weight ratios: 1:1 (X), 1:0.5 (▲), and 1:0 (◊).

3.3.3 Effect of Acidification and H-Bond Disruption on Release of PEG-(GM-CSF)

Films comprised of a 1:1 chitosan to glycerol weight ratio and containing 20 kD PEG-(GM-CSF) were allowed to release into release media. After 180 minutes, glacial acetic acid was added. This resulted in dissolution of the film and a burst of 20 kD PEG-(GM-CSF) release at 180 minutes (Figure 3-4). This result demonstrates that the limited release of 20 kD PEG-(GM-CSF) from films with a 1:1 weight ratio of chitosan to glycerol is due to the entrapment of the protein in the film by steric hindrance or ionic interactions with chitosan. When acetic acid is added to the film the chitosan amine

groups become protonated and may undergo ion exchange with acetate counter ions. This allows for film swelling, release of entrapped 20 kD PEG-(GM-CSF), and eventual dissolution of the chitosan film.

Next, in order to investigate possible hydrogen bonding effects of glycerol with chitosan in the films, urea was added to the casting solution of a film with a 1:1 chitosan to glycerol weight ratio containing 20 kD PEG-(GM-CSF). This film exhibited a greater release rate than control films containing no urea or films treated with urea after 180 minutes of release (Figure 3-4). This observation suggests that hydrogen bonding between glycerol, PEG, and chitosan may be the mechanism by which glycerol aids in the stabilization of the chitosan film; however, the presence of urea could also lead to an increased driving force for osmotic swelling and a subsequent increase in release rate.

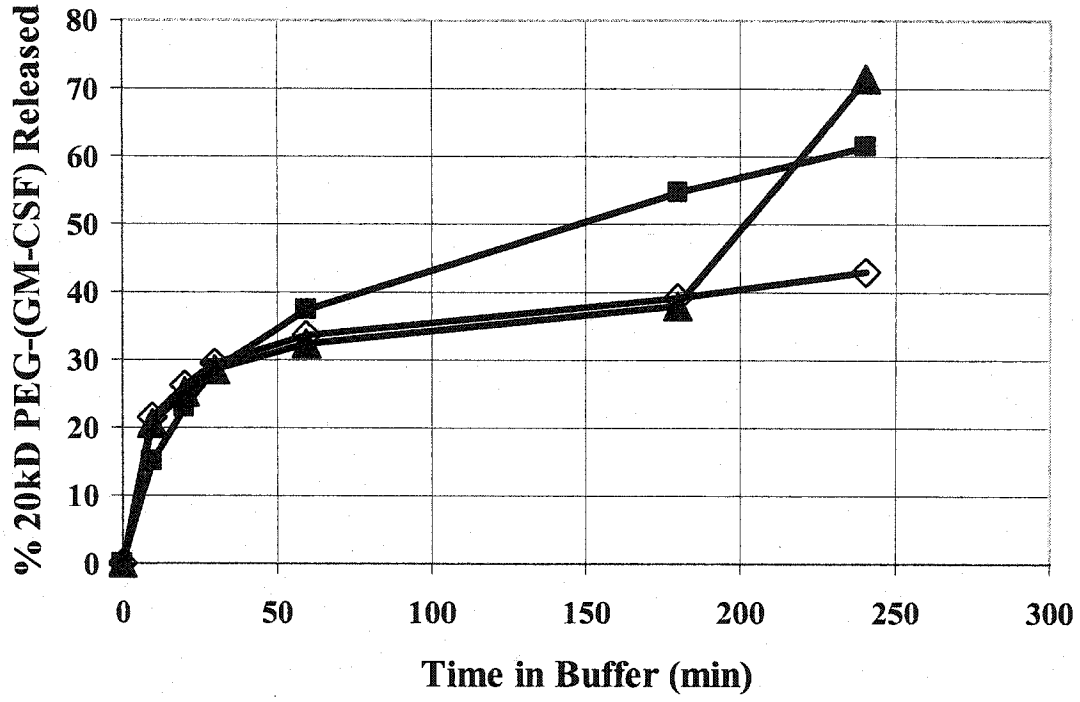


Figure 3-4: Release profiles of 20 kD PEG-(GM-CSF) from chitosan/glycerol films consisting of a 1:1 weight ratio of chitosan to glycerol placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C with urea added in the casting solution (■), glacial acetic acid added after 180 min of release (▲), or urea added after 180 min of release (◊). All films contained 1% (w/w) 20 kD PEG-(GM-CSF) (based on GM-CSF).

3.3.4 FTIR analysis of chitosan/glycerol films

FTIR spectroscopy was utilized to further investigate the effect of glycerol on the displacement of acetic acid from chitosan/glycerol films. A peak at approximately 1560 cm^{-1} has been previously attributed to the presence of $NH_3^+\cdots CH_3COO^-$ in chitosan [167]. Another peak at 1152 cm^{-1} is due to the ether bond in chitosan and is unaffected by the proton state of the amino groups [53]. Thus, a ratio of the peak height and area at 1560 cm^{-1} to that of the peak at 1152 cm^{-1} can be used to provide a measure of the acetate

content of the film. Figure 3-5 provides representative FTIR spectra for films with various chitosan/glycerol ratios, in comparison to the spectrum for glycerol. It is important to note that the spectrum for glycerol does not contain any interfering peaks in the regions of interest (1560 cm^{-1} and 1152 cm^{-1}). (Note: spectrum is given as transmission data, thus peaks are shown as downward deflections). A shift in the peak from 1560 cm^{-1} to 1590 cm^{-1} can be seen as the glycerol content increases, indicating a change from asymmetric NH_3^+ bending vibrations to NH_2 bending vibrations in the chitosan molecules [168]. Further, peak area and height ratios for the two peaks of interest significantly decrease with increasing glycerol content (Figure 3-6), signifying a shift from the protonated chitosan-acetate salt to the deprotonated form of chitosan. This further supports the hypothesis that glycerol displaces bound acetic acid from the chitosan films during drying leading to a more hydrophobic, deprotonated chitosan matrix.

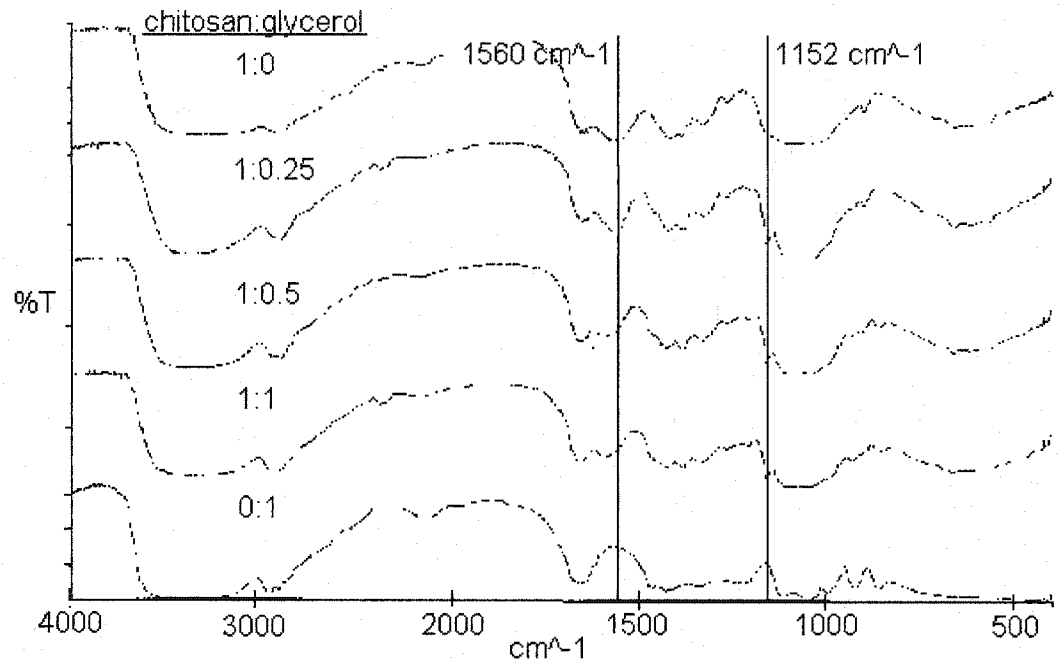


Figure 3-5: Representative FTIR spectra of thin chitosan/glycerol films consisting of various weight ratios of chitosan to glycerol.

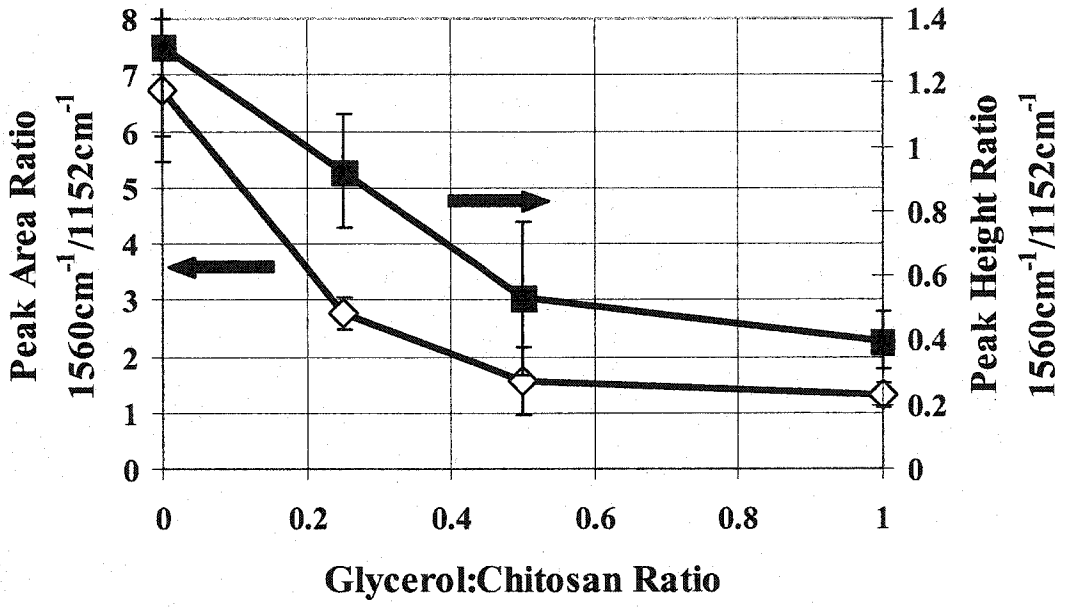


Figure 3-6: Analysis of 1560 cm^{-1} to 1152 cm^{-1} peak height (■) and area (◊) ratios of chitosan films consisting of various weight ratios of chitosan to glycerol.

3.3.5 Screening of Compounds with Glycerol-Like Behavior

Hydrogen bonding strength decreases with an increase in the molecular weight of the compound. Likewise, as the number of hydroxyls on a molecule increases, the hydrogen bond strength is increased. To this end, other hydrogen bonding agents with varying molecular weight and hydroxyl content were investigated for their ability to enhance the removal of acetic acid from the drying films by monitoring the pH of the drying film using the addition of Bromothymol blue (BTB) in the casting solution. Compounds that had effects similar to glycerol on the film pH include: propanediol, trimethylolpropane, and 400 Dalton PEG (Table 3-2). Other compounds that were investigated and found not to have glycerol-like behavior were pentaerythritol, mannitol, sorbitol, glucose, sucrose,

trehalose, and 10 kD dextran. This suggests that the molecular weight of the molecule had a much stronger effect on the ability of the hydrogen bonding agent to bond with chitosan than the number of hydroxyls. Lower molecular weight molecules demonstrated the ability to drive acetic acid from the films while slightly higher molecular weight molecules with greater numbers of hydroxyls did not efficiently remove acid from the system. One reason for this may be the presence of secondary versus primary hydroxyls on many of the saccharide compounds making them less accessible for hydrogen bonding. Poly(ethylene glycol) was also capable of removing residual acetic acid from the drying film despite its higher molecular weight and two primary hydroxyl end groups; however, it is known that the ether oxygens in PEG are strong hydrogen bonders and thus increase the hydrogen bonding potential of this molecule.

Table 3-2: Other hydrogen bonding compounds that aid in the removal of residual acetic acid from chitosan/glycerol films.

Hydrogen Bonding Agent	"Glycerol-Like" Activity	Molecular Weight (g/mol)	Number of Hydroxyls
Propanediol	X	76.10	2
Glycerol	**X**	**92.09**	**3**
Trimethylolpropane	X	134.18	3
Pentaerythritol		136.15	4
Mannitol		183.17	6
Sorbitol		183.17	6
Glucose		183.17	6
Sucrose		342.3	8
Trehalose		378.33	8
400 Da PEG	X	400	2 (9)
10 kD Dextran		10,000	~186

3.4 Discussion

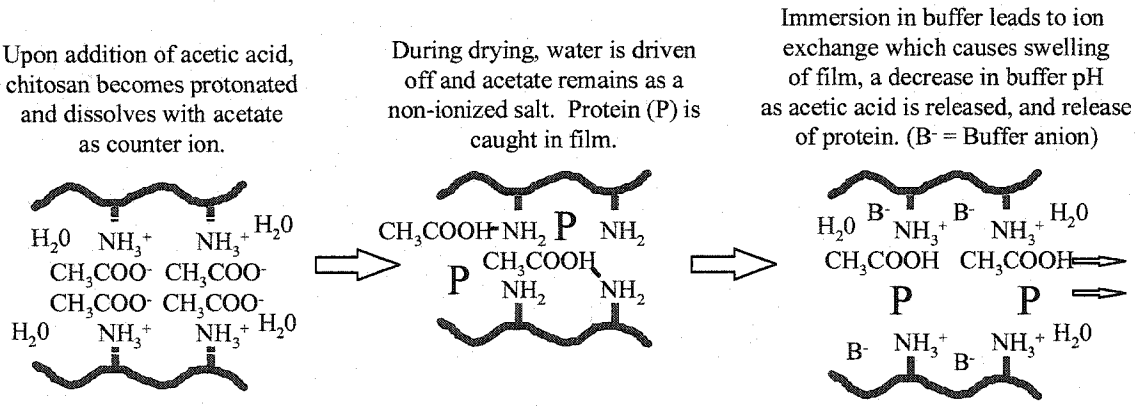
In order to describe the role of glycerol in the chitosan delivery system the mechanism of film formation and protein release will be rationalized with and without the presence of glycerol (Figure 3-7). The first step in film formation is dissolution of the chitosan in acetic acid. In order for this to occur, the chitosan becomes protonated and associates with acetate counter ions. When glycerol is not present in the casting solution, water is driven from the film during the drying procedure, leaving behind acetate as a non-ionized salt. At this point the film is immersed in release buffer, which leads to ion exchange. This causes film swelling, a decrease in buffer pH as acetic acid is released, and release of protein. When glycerol is present in the casting solution it hydrogen bonds with chitosan, leading to amine deprotonation and an increase in matrix hydrophobicity. The acetic acid can then be successfully volatilized and removed during the drying procedure. Immersion of the chitosan/glycerol film into release buffer causes slight swelling of the film and rapid release of glycerol. The pH of the medium is maintained by the buffer and the protein remains entrapped in the film. At this point, addition of glacial acetic acid to the release media causes protonation of the chitosan amines, swelling of the matrix, dissolution of the chitosan film, and release of entrapped protein.

The results from the swelling, pH, FTIR, and protein and glycerol release experiments all support the hypothesis that glycerol displaces bound acetic acid from chitosan films during drying, through hydrogen bonding between glycerol and chitosan amine groups. This provides a physically cross-linked chitosan network with increased hydrophobicity. These films exhibit decreased swelling and an increased stability when

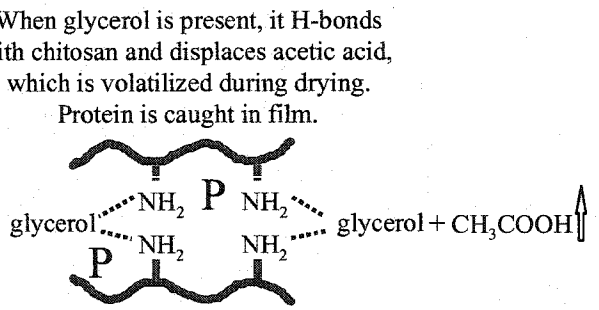
placed in release buffer. This leads to entrapment of PEG-(GM-CSF) in the films when immersed in PBS buffer and a decrease in PEG-(GM-CSF) release rate. Furthermore, due to the decreased swelling of these films, PEG-(GM-CSF) molecules that are PEGylated with increasing molecular weight PEGs experience increased steric hindrance and chain entanglements between PEG and chitosan, resulting in decreased release kinetics in comparison to GM-CSF.

The delivery system described here may hold promise for the delivery of anionic therapeutics (growth factors, DNA, and glycosaminoglycans) from tissue engineered constructs, wound coverings, or oral protein delivery formulations comprised of chitosan. However, the delivery of cationic molecules will require different matrix properties and formulation strategies. Further, it may be beneficial to chemically cross-link versus physically cross-link the delivery system in order to avoid the co-delivery of high amounts of physical cross-linking agent. High release rates of the physical cross-linking agent (glycerol) may prove toxic at local sites. With these criteria in mind, delivery systems comprised of a chemically cross-linked hyaluronic acid were prepared and characterized as described in Chapter 4.

Mechanism Without Glycerol



Mechanism With Glycerol



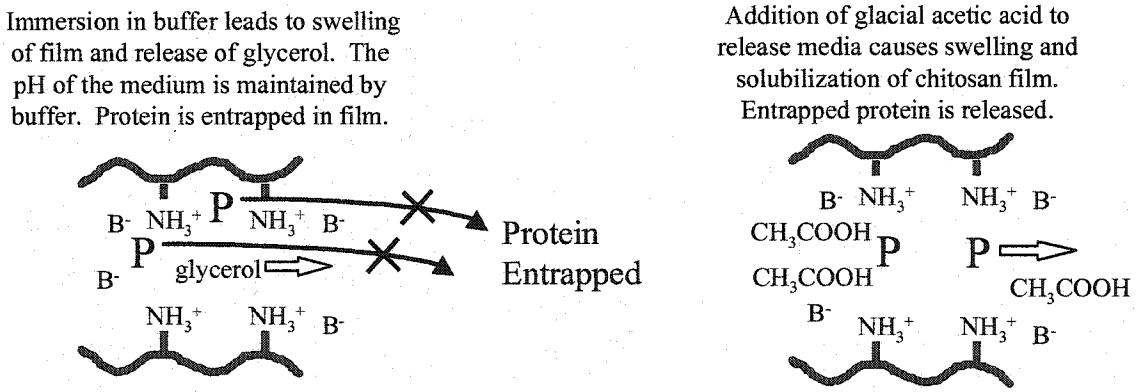


Figure 3-7: Proposed mechanism of the ability of glycerol to hydrogen bond with chitosan, aiding in the removal of residual acetic acid and allowing for film stabilization and protein entrapment.

Chapter 4: Hyaluronic Acid as a Chemically Cross-Linked Delivery System

4.1 Introduction

Hyaluronic acid (HA) is a natural linear copolymer hydrogel consisting of alternating saccharides units of β (1-4) linked 2-acetamide-2-deoxy-D-glucose and β (1-3) linked D-glucuronic acid. Due to its role in wound healing and high biocompatibility, HA has been extensively investigated as a tissue engineering and drug delivery matrix [100, 169, 170]. Each disaccharide unit of HA contains a carboxyl moiety that gives HA its extremely hydrophilic nature and water solubility. This carboxyl group may be used to complex and deliver cationic therapeutics such as basic growth factors, i.e. VEGF and bFGF. In addition, the non-fouling nature of HA should allow for the entrapment of sensitive proteins without denaturation, leading to release of stable and bioactive protein. However, due to the aqueous solubility of HA, a cross-linking strategy must be employed to formulate a solid hydrogel scaffold capable of cell and growth factor encapsulation.

Photo cross-linking has been investigated recently as a formulation strategy for the delivery of cells and therapeutic proteins [21-24, 171, 172]. Our strategy is to modify the hydroxyl group on the 6-carbon of 2-acetamide-2-deoxy-D-glucose using glycidyl methacrylate as shown in Figure 4-1. This introduces methacrylate moieties on the backbone of HA allowing it to be chemically cross-linked using photochemical techniques. In this manner, we can encapsulate cells and proteins by mixing them in an aqueous solution of the glycidyl methacrylate modified HA (HA-GMA), and upon exposure of this mixture to UV radiation, form a cross-linked HA scaffold. A similar

strategy has been employed by Smeds, et al, wherein methacrylate moieties are introduced on the HA backbone using methacrylic anhydride [102, 103]. However, we have found that this technique is difficult to reproduce due to the quick hydrolysis of the anhydride bond in aqueous reaction conditions. Therefore, in this study we have synthesized HA-GMA with varying degrees of glycidyl methacrylate modification (cross-link density) and have formed cross-linked hydrogels using photochemical techniques. These hydrogel scaffolds were characterized using NMR and mechanical compression tests. Further, the ability of the cross-linked HA hydrogels to control the release of biomacromolecules was examined using FITC-labeled dextrans of various molecular weights, lysozyme, and bovine serum albumin (BSA).

4.2 Materials and Methods

4.2.1 Materials

Medical grade hyaluronic acid with a molecular weight of 1.2 MD was donated by Genzyme, Inc (Cambridge, MA). Glycidyl methacrylate (97%), methacrylic anhydride (94%), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (98%, Igracure 2959), methanol, and isopropanol were obtained from Aldrich (Milwaukee, WI). Physiological phosphate buffered saline (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4), bovine serum albumin (BSA, fraction V), lysozyme (from chicken egg white), and fluorescein isothiocyanate-dextrans (FITC, MW 4400 and MW 71600) were obtained from Sigma (St. Louis, MO). Sodium hydroxide (NaOH, 1N) was obtained from VWR Scientific

Products (Plainfield, NJ). Deuterium oxide (d_2O) was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA).

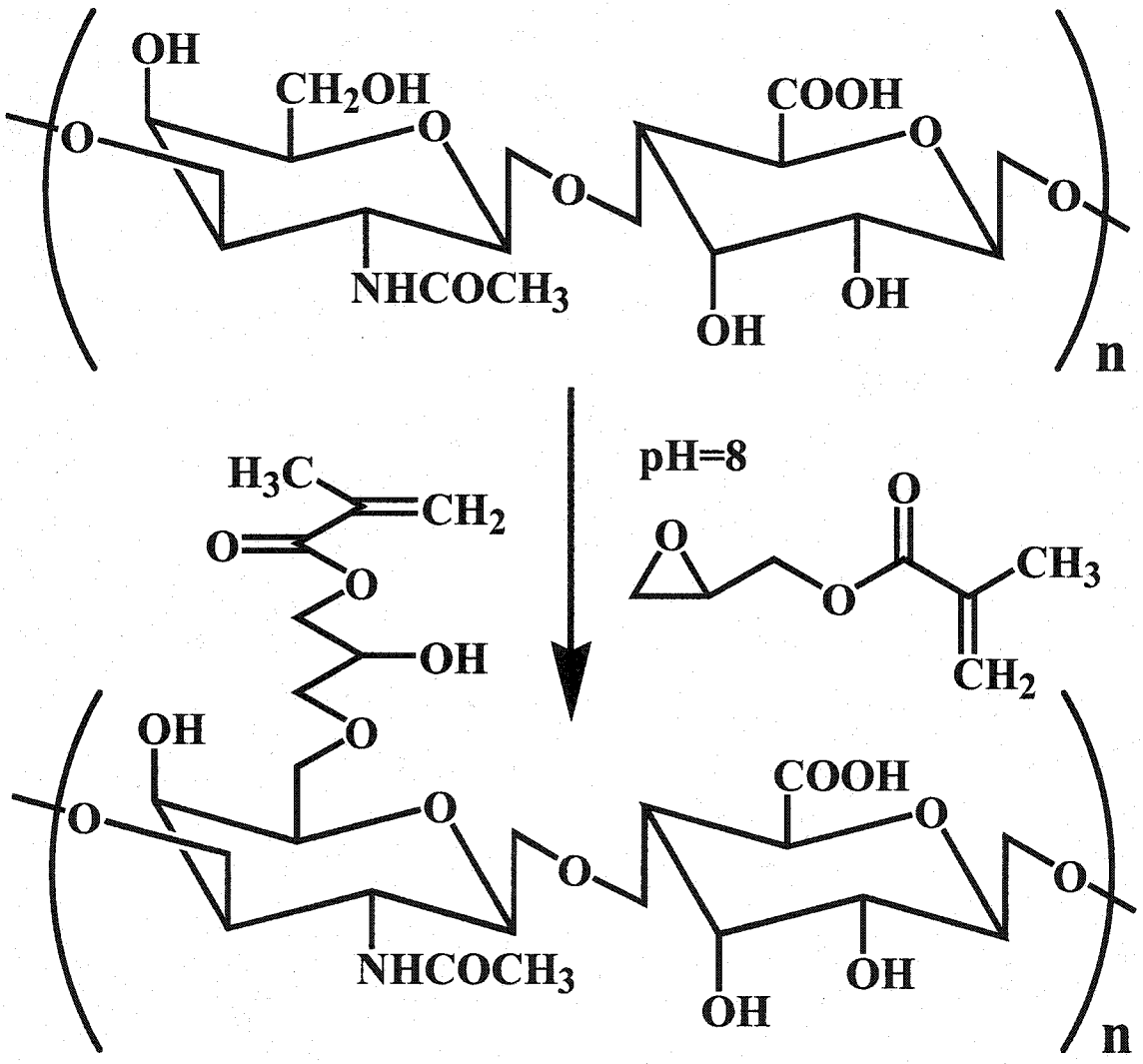


Figure 4-1: Synthesis of glycidyl methacrylate modified hyaluronic acid (HA-GMA).

4.2.2 Synthesis of Hyaluronic Acid-Glycidyl-Methacrylate Hydrogels

Glycidyl methacrylate modified hyaluronic acid (HA-GMA) was synthesized (Figure 4-1) by preparing a 1.5% (w/w) solution of HA in deionized and distilled water. The pH of the HA solution was adjusted to pH = 8 using 1N NaOH. Next, glycidyl methacrylate at a 25X molar excess was slowly added to the HA solution under constant stirring. The primary hydroxyl on the 6-carbon of 2-acetamide-2-deoxy-D-glucose is the more reactive hydroxyl in HA and should be modified selectively over the remaining secondary hydroxyls. The reaction was allowed to proceed at room temperature and pressure for time periods of 1, 3, 5, 7, and 10 days. At a given time point, the HA/glycidyl methacrylate solution was slowly dripped into ice cold isopropanol under constant stirring to precipitate the HA-GMA. Precipitations were carried out three times to fully remove all unreacted glycidyl methacrylate. The HA-GMA was then filtered and dried under vacuum. The extent of methacrylation was determined using ^{1}H-NMR (499MHz) in d_2O at 330 K and comparing the number of methacrylate protons at 6.0-6.5 ppm to the number of saccharide ring protons at 3.5-4.3.

Cross-linked HA-GMA hydrogels were obtained by dissolving HA-GMA in physiological PBS (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) at a concentration of 20 mg/ml. Approximately 225 microliters of this solution was added to a well of a 48-well tissue culture polystyrene plate as a casting mold. In this manner, hydrogels of reproducible size and shape were produced. In order to initiate cross-linking 20 microliters of a 20 mg/ml solution of Igracure 2959 in methanol was added to each well and mixed. The wells were then exposed to UV radiation at 365 nm using a long-

wave ultra violet lamp (Blak-Ray, Model B100AP) for 20 minutes. The gels were allowed to stand overnight before further testing.

4.2.3 Mechanical Testing of Hyaluronic Acid-Glycidyl Methacrylate Hydrogels

Compression testing of cross-linked HA-GMA hydrogels comprised of various methacrylation densities was performed using a parallel plate compression instrument (Figure 4-2) [173]. Data was collected using a Sensotec (Model #31, Columbus, OH) tension/compression load cell with a 10-pound capacity. The signal was filtered using a Vishay signal-conditioning amplifier (Vishay Gain =7000, Malvern, PA). The data was acquired using a National Instruments data acquisition card (PCI-1200, Austin, TX) with 12 bit resolution and 100 kilosamples per second sampling rate on a Power Macintosh 8500/180 MHz computer running Labview 4.1 software and a custom VI. Tests were conducted by inputting a force-controlled signal using a triangle waveform at 0.02 Hz and measuring the resultant displacement data. Discs of HA-GMA hydrogels for compression testing were prepared by casting 1.5 ml of a 20 mg/ml solution of HA-GMA in deionized and distilled water into a 12-well tissue culture polystyrene plate. The gels were cross-linked by the addition of 150 microliters of a 20mg/ml solution of Igracure 2959 in methanol, followed by a 20 minute exposure to UV radiation at 365 nm. Gels were allowed to cross-link overnight prior to testing. Sample diameters and thickness were measured using digital calipers to relate force and displacement data to stress and strain. A linear fit over the 20% strain region was utilized to determine the modulus of the various samples.

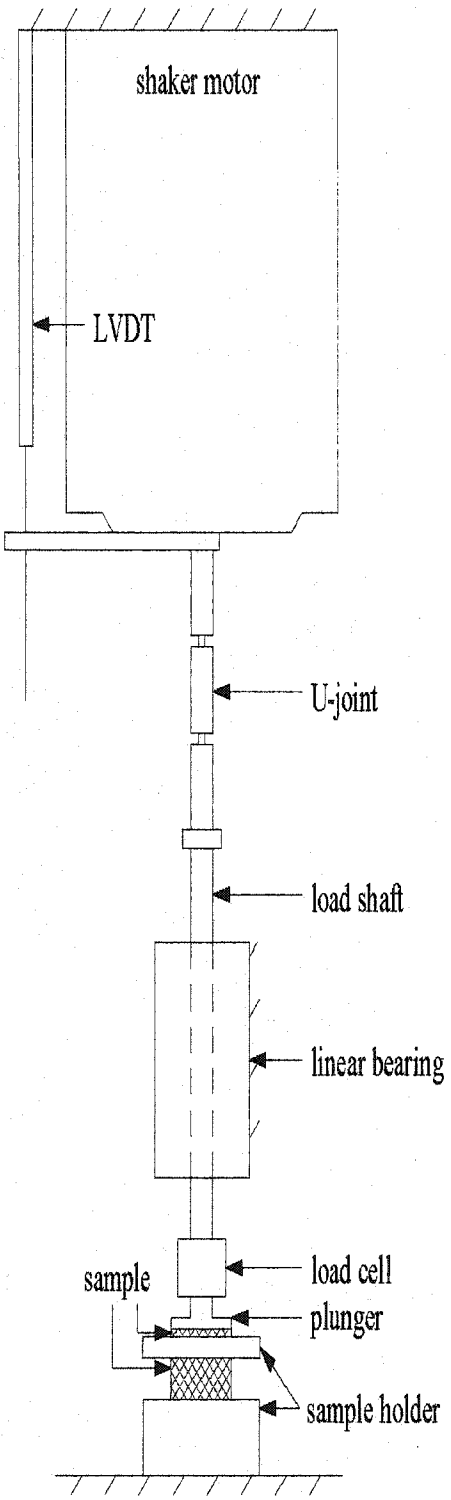


Figure 4-2: Schematic of parallel-plate compression instrument utilized to test mechanical strength of HA-GMA hydrogels.

4.2.4 *Release of Macromolecules from Hyaluronic Acid-Glycidyl Methacrylate Hydrogels*

HA-GMA hydrogels with varying degrees of methacrylation were analyzed for their ability to control the release of macromolecules of different sizes and charge. FITC-dextrans of low (4.4 kD) and high (71.6 kD) molecular weight were loaded into the HA-GMA hydrogels to probe the effective pore size of the gels at different cross-link densities. Hydrogels of HA-GMA were loaded with FITC-dextran by dissolving HA-GMA in physiological PBS (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) at a concentration of 20 mg/ml. Next, FITC-dextran as a solution in PBS was added to the HA-GMA solution to give a 10% loading by weight. Approximately 225 microliters of this solution was added to a well of a 48-well tissue culture polystyrene plate. In order to initiate cross-linking 20 microliters of a 20 mg/ml solution of Igracure 2959 in methanol was added to each well and mixed. The wells were then exposed to UV radiation at 365 nm for 20 minutes. The gels were allowed to cross-link overnight before performing release studies. Each condition was prepared in triplicate.

The release profile of FITC-dextran from the various HA-GMA scaffolds was analyzed by removing the cross-linked hydrogel from the 48-well plate and placing it in one milliliter of release media (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) in a glass scintillation vial. The hydrogels were incubated at 37 °C in a shaking water bath. The samples were kept shielded from ambient light to minimize the effects of photo bleaching on the release results. At time points of 1, 2, 4, 8, 24, and 48 hours the release media was removed and replaced with fresh release media. Release media samples were

stored frozen (-20 °C) until further analysis. The amount of FITC-dextran unreleased from the hydrogel was determined by degrading the HA-GMA gel completely with hyaluronidase to liberate any entrapped FITC-dextran. The percent FITC-dextran released at each time point was then determined by measuring the absorbance at 493 nm and comparing it to a standard curve. Results were expressed as an average of triplicate samples plus or minus one standard deviation.

The ability of HA-GMA hydrogels with varying degrees of methacrylation to control the release of growth factors was examined using two model proteins with different physical properties. Lysozyme, a cationic protein with a molecular weight of 14.3 kD, and BSA, a anionic protein with a molecular weight of 66 KD, were entrapped in HA-GMA gels of varying cross-linking densities using the method described above for the entrapment of FITC-dextran. Briefly, HA-GMA was dissolved in physiological PBS (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) at a concentration of 20 mg/ml. Next, lysozyme or BSA as a solution in PBS was added to the HA-GMA solution to give a 10% loading by weight. Approximately 225 microliters of this solution was added to a well of a 48-well tissue culture polystyrene plate. Cross-linking was initiated by adding 20 microliters of a 20 mg/ml solution of Igracure 2959 in methanol to each well and exposing the wells to UV radiation at 365 nm for 20 minutes. The gels were allowed to cross-link overnight before performing release studies. Control gels were prepared in the same manner without the protein addition step.

The hydrogels were not dried prior to the release study as these materials are to be used in the co-encapsulation of growth factors and viable cells. Although lyophilization

of the hydrogels would lead to improved release kinetics, this processing step would prove detrimental to encapsulated cells. Thus, the release profiles of lysozyme and BSA from HA-GMA scaffolds with varying degrees of cross-linking were analyzed in the swollen state by removing the cross-linked HA-GMA hydrogel from the 48-well plate and placing it in 1.5 ml of release media (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) in a glass scintillation vial. The hydrogels were incubated at 37 °C in a shaking water bath. At time points of 1, 2, 4, 8, 24, and 48 and 132 hours the release media was removed and replaced with fresh release media. Release media samples were stored frozen (-20 °C) until further analysis. The percent of lysozyme or BSA released at each time point was then analyzed using the Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL). The BCA method is a colorimetric assay that utilizes a calibration curve to determine protein concentration. The assay is based on the known reduction of Cu^{2+} to Cu^{1+} by proteins and the sensitivity of BCA to Cu^{1+} [174]. The reaction of BCA with Cu^{1+} produces a purple color that can be measured using absorbance readings at 562 nm. Triplicate samples were measured and compared to blank scaffolds containing no protein. The percent release of loaded protein was expressed as an average plus or minus one standard deviation at each time point.

4.3 Results

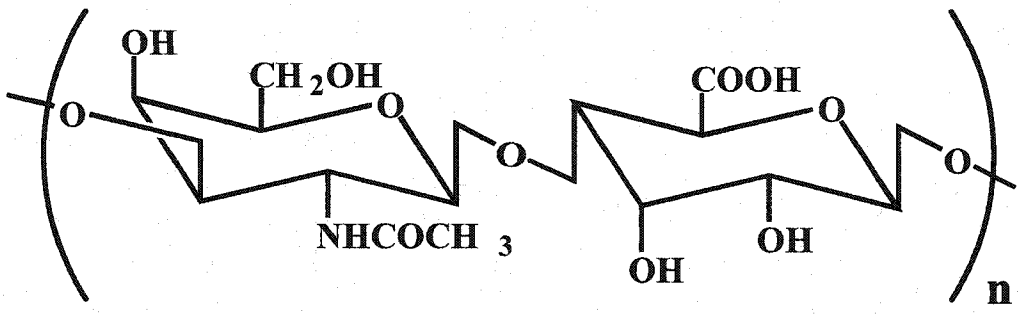
4.3.1 Synthesis of Hyaluronic Acid-Glycidyl Methacrylate Hydrogels

Methacrylation of hyaluronic acid by reaction of the hydroxyl group on the 6-carbon of 2-acetamide-2-deoxy-D-glucose with glycidyl methacrylate produced HA-GMA with

various degrees of methacrylation in a reproducible manner. Analysis by ^{1}H-NMR shows the introduction of the methacrylate moieties to hyaluronic acid with characteristic proton peaks at 6.0-6.4 ppm (Figure 4-3 and Figure 4-4). HA-GMA with percent methacrylations of 1% to 8% was produced after 1 to 10 days of reaction (Table 4-1). The resulting HA-GMA was easily cross-linked using photochemical techniques producing hydrogels that filled the entire volume of their casting solutions. The gels were easily handled and removed from their casting molds allowing for the production of hydrogel films of reproducible size and shape. Qualitatively, the solution viscosity of HA-GMA solutions with higher methacrylation densities was significantly lower. This suggests that the modification with glycidyl methacrylate may have either lead to degradation of the polymer backbone providing HA with a lower molecular weight, or has interfered with hydrogen bonding between HA chains. In either case, the decreased solution viscosity of HA-GMA with a higher percent methacrylation allowed for more thorough mixing during processing steps and may have contributed to the superior properties observed with these materials.

Table 4-1: Time-dependent percent methacrylation of hyaluronic acid as determined by ^{1}H-NMR in d_2O at 330K.

Days of Reaction	Methacrylate Protons	Hexose Ring Protons	% Methacrylation
1	0.01	3.16	<1
3	0.05	3.72	2.7
5	0.06	2.89	4.2
7	0.09	2.48	6.9
10	0.10	2.55	7.8



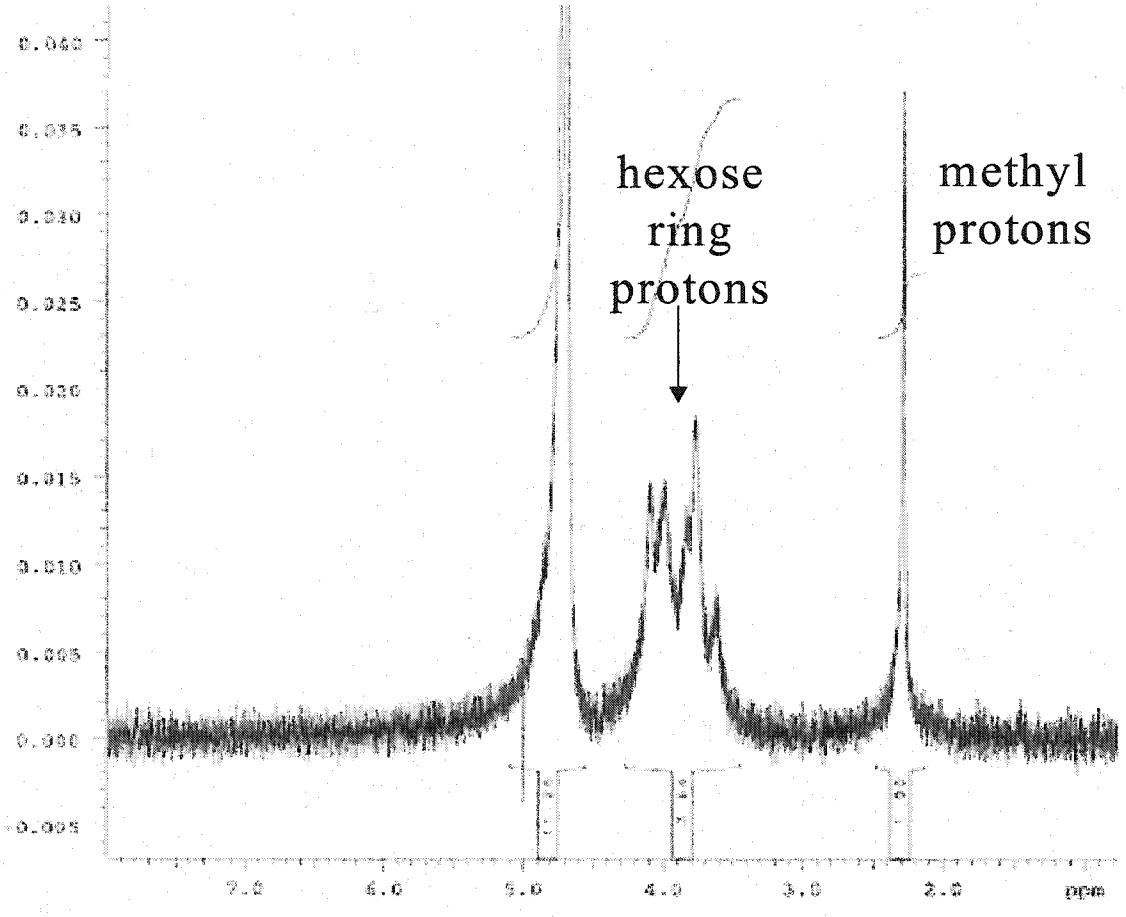


Figure 4-3: ^{1}H-NMR spectrum of hyaluronic acid in d_2O at 330 K.

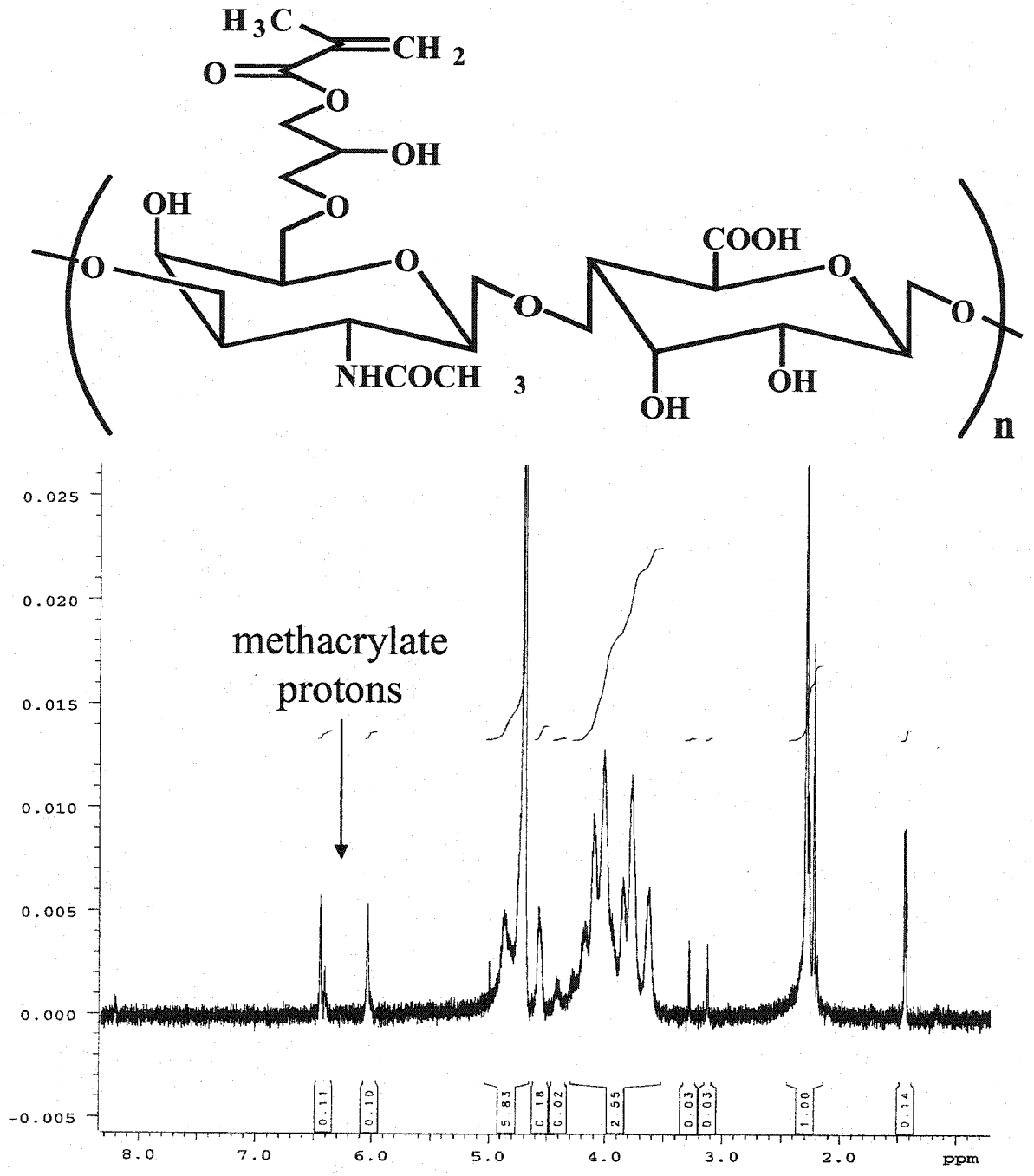


Figure 4-4: ^{1}H-NMR spectrum of hyaluronic acid modified with glycidyl methacrylate (HA-GMA) in d_2O at 330 K.

4.3.2 *Mechanical Testing of Hyaluronic Acid-Glycidyl Methacrylate Hydrogels*

Cross-linked hydrogels of HA-GMA with the lowest (<1%) and highest (7.8%) percent methacrylation were compression tested using a parallel-plate compression instrument (Figure 4-5). Hydrogels with a higher percent methacrylation demonstrated a higher resistance to strain than those of a low percent methacrylation. The simple moduli for the 7.8% and <1% methacrylated HA-GMA gels at low strain (~20%) are 1.2 kPa (+/- 0.3 kPa) and 265 Pa (+/- 31 Pa) respectively. Further, the behavior of the gels diverged rapidly at higher strain, demonstrating the improved mechanical strength of the higher methacrylated product. Thus, HA-GMA gels formulated with a higher percent methacrylation provide a more densely cross-linked and stronger network.

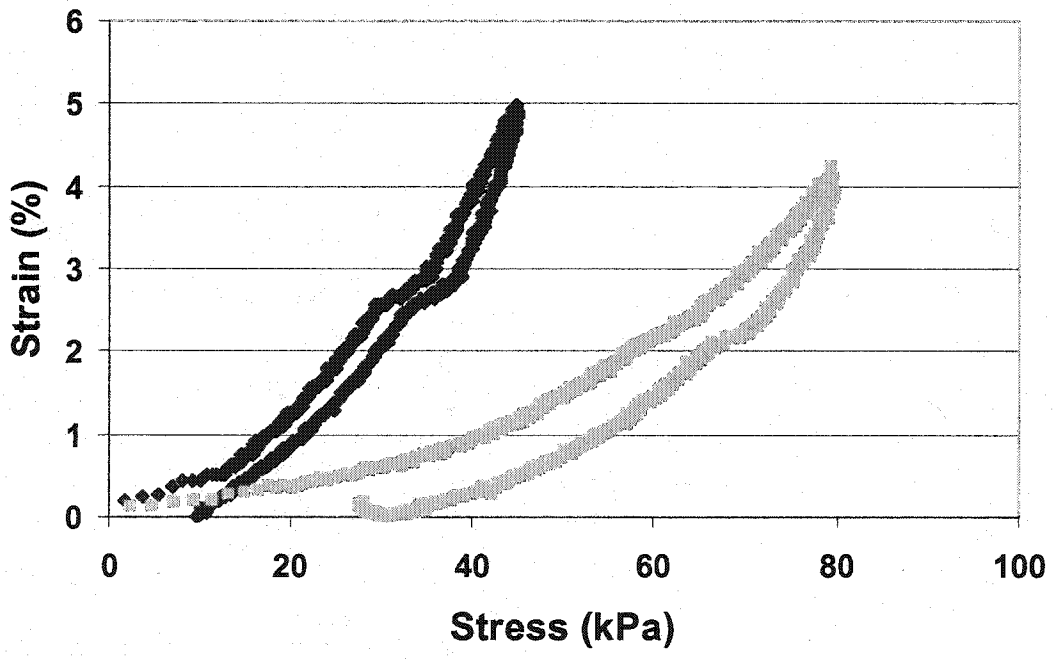


Figure 4-5: Stress versus strain plot of 7.8% methacrylated HA-GMA (■) and <1% methacrylated HA-GMA (■) as determined using a parallel plate compression instrument.

4.3.3 *Release of Macromolecules from Hyaluronic Acid-Glycidyl Methacrylate Hydrogels*

In order to probe the molecular weight dependent diffusional properties of the HA-GMA hydrogels, matrices of various cross-linked densities were loaded with FITC-dextran of either high (71.6 kD) or low (4.4 kD) molecular weight. These gels were placed in physiological PBS release media (pH=7.4) and analyzed for FITC-dextran release. It was found that for both FITC-dextran molecular weights, that the percent release of FITC-dextran decreased as the percent of methacrylation of the HA-GMA hydrogel increased (Figure 4-6 and Figure 4-7). This suggests that the higher methacrylated product leads to a hydrogel scaffold with increased cross-linking and an increased resistance to diffusion (smaller pore size). Similarly, it is seen that at a given percent methacrylation of HA-GMA, that the release of the higher molecular weight FITC-dextran is reduced in comparison to the lower molecular weight FITC-dextran (Figure 4-8). This result is most evident at intermediate methacrylation percentages. At the lowest methacrylation percentage (<1), the pore sizes are large enough in the cross-linked HA-GMA to allow for similar diffusion of FITC-dextran of the two molecular weights. In contrast, at the highest methacrylation percentage (7.8%), the pore sizes are small enough to significantly inhibit the diffusion of both FITC-dextran molecular weights. At the intermediate methacrylation percentages, the cross-linked HA-GMA hydrogels provide a pore structure capable of providing high diffusion rates for low molecular weight FITC-dextran, while providing increased resistance to diffusion for the higher molecular weight FITC-dextran. The effects of photo bleaching on the FITC-

molecules confound this data making it difficult to verify FITC-dextran loadings; however, the release data show trends that are expected from gels with increasing cross-link density. Studies using proteins of different molecular weights were used to further probe the diffusional properties of the HA-GMA hydrogels.

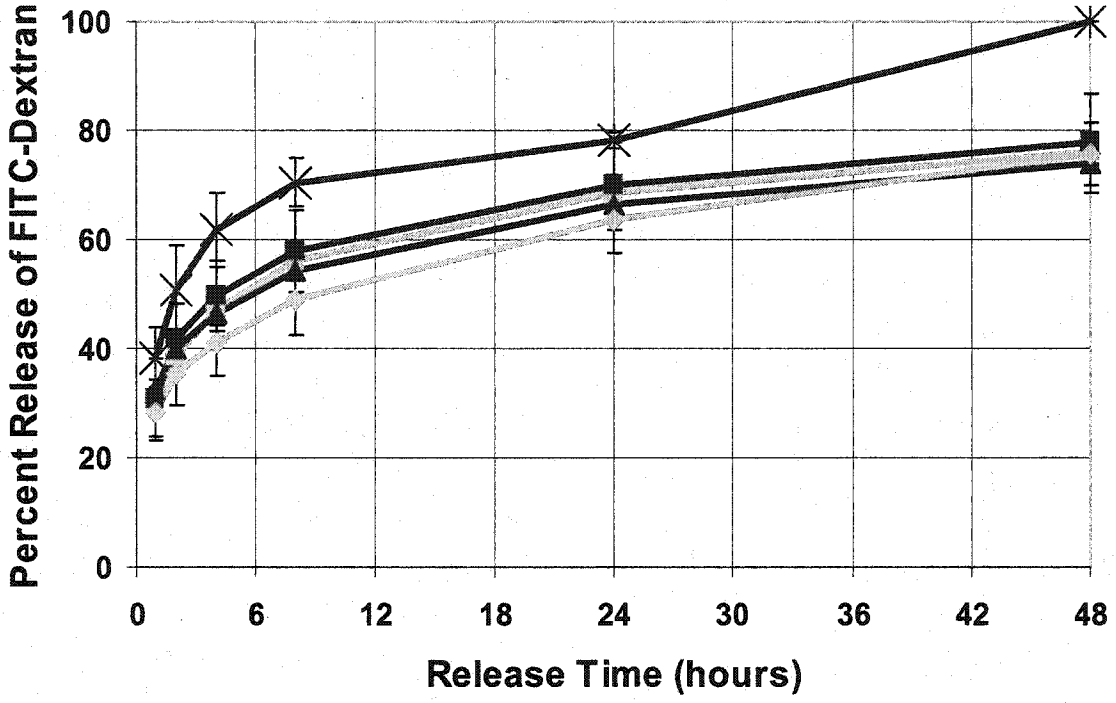


Figure 4-6: Release of 71.6 kD FITC-dextran from HA-GMA hydrogels with various percent methacrylations placed into release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Percent methacrylations of <1% (X), 2.7% (▒), 4.2% (▲), 6.9% (■), and 7.8% (◊).

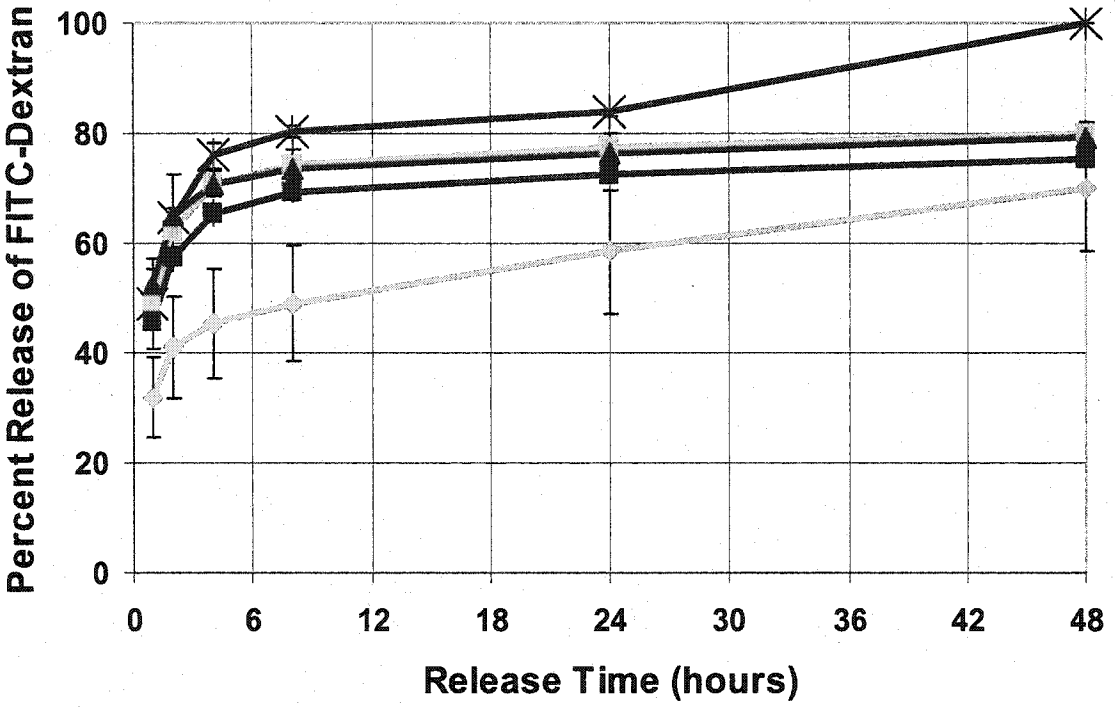


Figure 4-7: Release of 4.4 kD FITC-dextran from HA-GMA hydrogels with various percent methacrylations placed into release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Percent methacrylations of <1% (X), 2.7% (▒), 4.2% (▲), 6.9% (■), and 7.8% (◆).

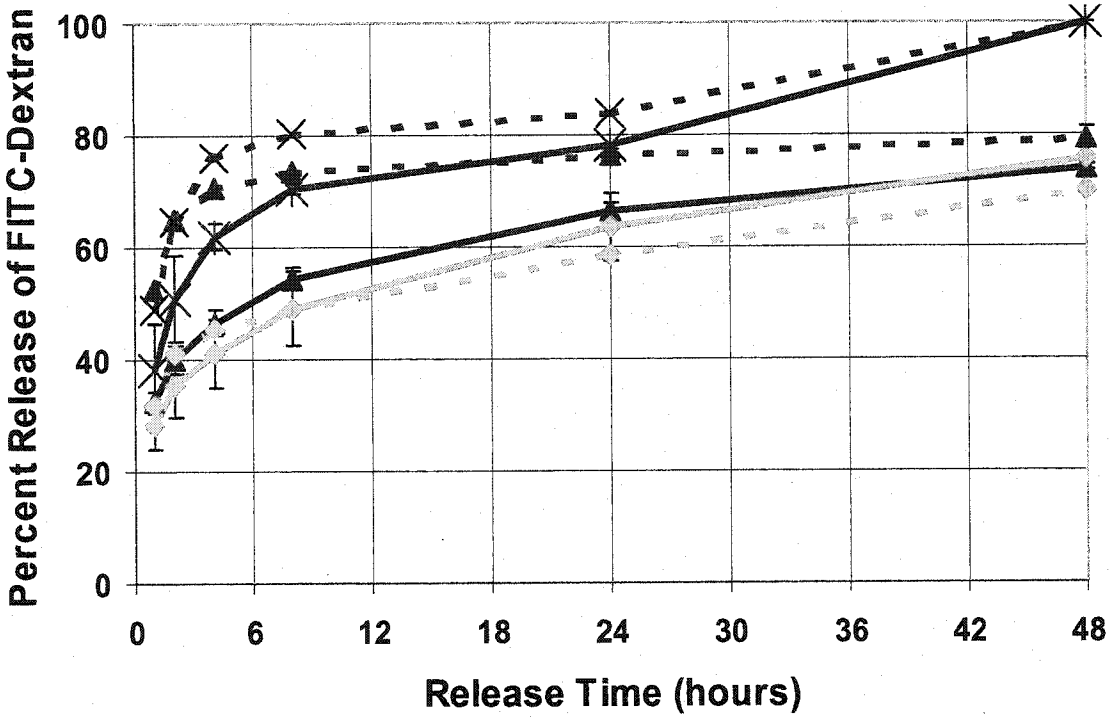


Figure 4-8: Release of 4.4 kD (dashed) and 71.6 kD (solid) FITC-dextran from HA-GMA hydrogels with various percent methacrylations placed into release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Percent methacrylations of <1% (X), 4.2% (▲), and 7.8% (◆).

In order to investigate the ability of the HA-GMA hydrogels to control release rates of proteins of various charges and sizes, matrices with a percent methacrylation of 4.2% were loaded with either lysozyme (14.3 kD, cationic) or BSA (66 kD, anionic). These gels were placed in physiological PBS release media (pH=7.4) and analyzed for protein release using a BCA assay. Similar release profiles were found for both proteins with approximately half of the loaded protein being released within 2 hours followed by a slower release profile over the 5 day period of the study (Figure 4-9). The similar release profiles despite the vastly different physical properties of the proteins may be due to the

cationic charge of the smaller protein ionically hindering its diffusion out of the anionic HA-GMA scaffold, thus providing it with diffusional properties similar to a larger protein. Further, the ability of the HA-GMA scaffold to retain the anionic BSA despite the potential for ionic repulsion may pertain to the ability of BSA to form complexes with 18 disaccharide repeat units of HA (18 carboxyls) [175].

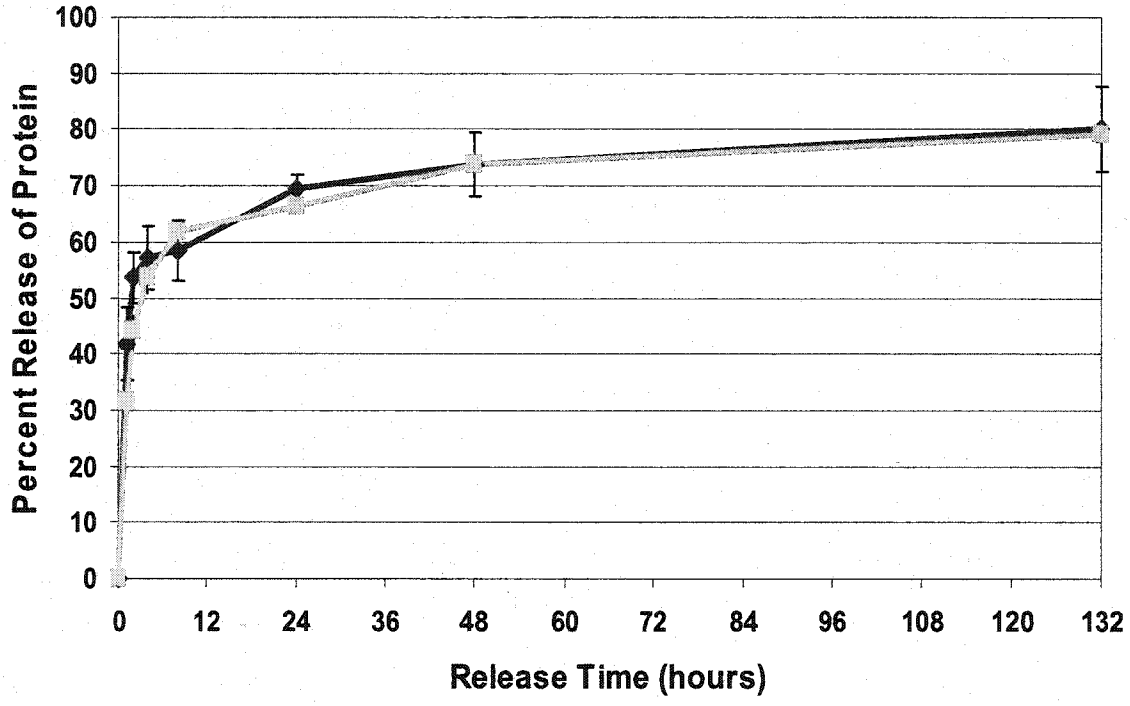


Figure 4-9: Release of BSA (♦) and lysozyme (▫) from HA-GMA hydrogels with 4.2% methacrylation placed into release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C.

To further characterize the ability of HA-GMA hydrogels to control the rate of protein release, BSA was added to HA-GMA gels with varying methacrylation percentages. The release of BSA was then determined by placing the cross-linked hydrogels into physiological PBS release media (pH=7.4) and measuring protein release

using a BCA assay. It was found that with all HA-GMA hydrogels except for those with the highest percent methacrylation, that the BSA was released rapidly and without control over the release rate (Figure 4-10). However, with hydrogels comprised of the 7.8% methacrylated HA-GMA, nearly half of the loaded protein was released after one day of release. This was followed by a slower release rate for the duration of the study (10 days), at which time the gels had fully dissolved. The high initial release rate in these studies is due to the pre-swollen state of the gels, which is necessary to maintain cell friendly encapsulation conditions.

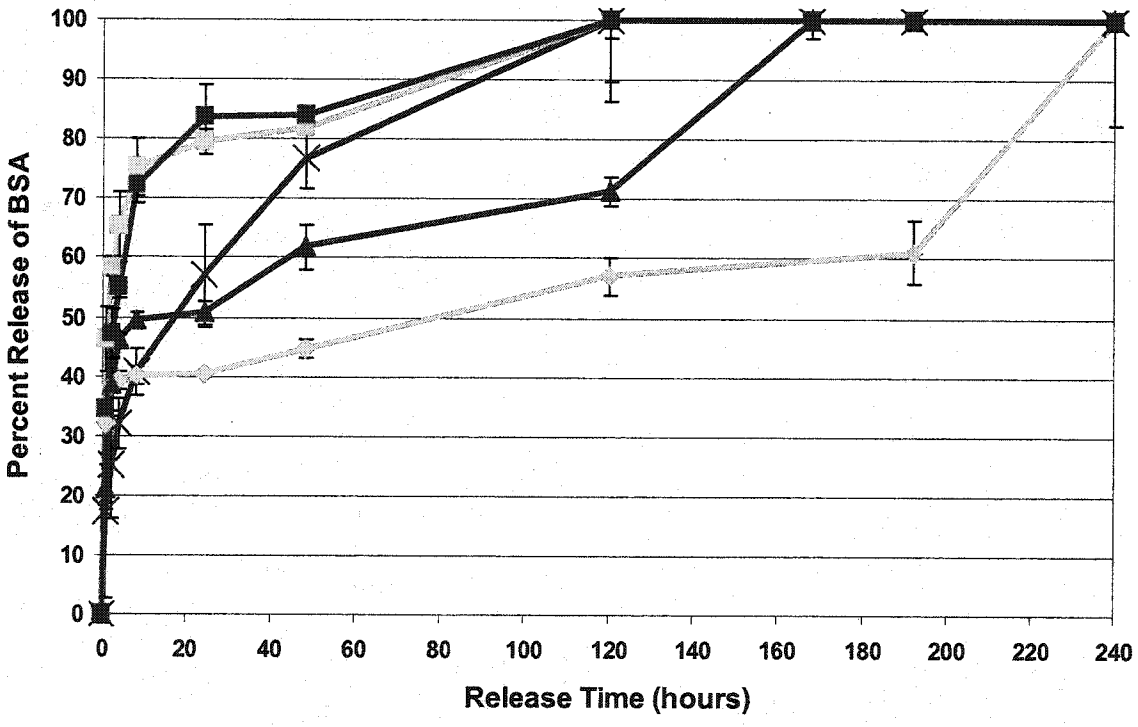


Figure 4-10: Release of BSA from HA-GMA hydrogels with various percent methacrylations placed into release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. Percent methacrylations of <1% (X), 2.7% (▒), 4.2% (▲), 6.9% (■), and 7.8% (◇).

4.4 Discussion

Glycidyl methacrylate modified hyaluronic acid (HA-GMA) of varying methacrylation densities was reproducibly synthesized and cross-linked using photochemical methods. The mechanical properties and protein release profiles of these gels were determined and are summarized in Table 4-2. In general, as the percent methacrylation increased the mechanical strength of the hydrogel increased and the protein release rate decreased. Further, it was noted qualitatively that the solution viscosity of HA-GMA with a higher percent methacrylation was significantly decreased suggesting degradation of the HA backbone or interference with hydrogen bonding between HA chains. However, this decrease in solution viscosity aids in the cross-linking of the HA-GMA gels as processing conditions, namely mixing, are improved.

Table 4-2: General trends observed in HA-GMA hydrogels with an increase in methacrylation percentage

Property of HA-GMA Hydrogel	Effect with Increased Cross-Linking
Solution Viscosity	Decreased
Mechanical Strength	Increased
FITC-Dextran Release	Decreased
Protein Release	Decreased (only at highest density)

The compression testing of HA-GMA hydrogels demonstrated a simple moduli that increased with increased methacrylation density from 265 Pa for the <1% methacrylated HA-GMA to 1.2 kPa for the 7.8% methacrylated HA-GMA. This is a significant increase in mechanical strength and is substantially better than that seen with HA gels produced by the methacrylic anhydride technique of Smeds, et al, who showed a 90 Pa

simple moduli for an 8% cross-linked HA [102]. The improved strength of the HA-GMA hydrogels should provide a strong and protective matrix for encapsulated cells, allowing them to be handled and to endure physiological stresses after implantation. Further, the FITC-dextran release data demonstrates quick diffusion of small and large molecules through the HA-GMA hydrogel suggesting that nutrient delivery and waste product removal would be facilitated, aiding in the survival of encapsulated cells. In the wake of the work produced in this chapter, Leach et al, have produced glycidyl methacrylate modified HA using a technique similar to that described here [176]. This study found decreased swelling and degradation rates of crosslinked HA hydrogels with an increase in crosslink density. Further, they found *in vivo* endothelial cell invasion of the hyaluronic acid hydrogels to be similar to that of fibrin gels. This further suggests that the HA-GMA produced here may be ideal for cell encapsulation strategies.

Although the mechanical and diffusive properties of the HA-GMA hydrogels appear to be ideal for cell encapsulation, the scaffold proves to be too hydrophilic for controlling protein release rates. The decrease in release rate with increasing percent methacrylation that was seen with FITC-dextran was not exhibited in the release of BSA. Only the 7.8% methacrylated HA-GMA provided controlled release of BSA beyond five days of release. HA-GMA hydrogels at all methacrylation percentages demonstrated approximately 50% release of BSA as a burst within the first few hours of release. These non-ideal release characteristics may be improved by the lyophilization of the HA-GMA hydrogel; however, this would be detrimental to tissue engineering strategies involving the co-encapsulation of cells and protein growth factors, as was the goal of this project. The

rapid release of BSA from the HA-GMA hydrogels may also be influenced by the ionic repulsion of the negatively charged BSA and HA which may contribute to the driving force for release. The cross-linked HA-GMA hydrogels produced in these studies may be more ideally suited for the delivery of cationic growth factors, such as VEGF or bFGF, or proteins that contain a HA binding domain. In either case, a growth factor delivery system that is more hydrophobic, to decrease the initial rapid release of protein, yet still provides a stable environment for protein encapsulation without leading to denaturation and loss of bioactivity is needed. Further, the ability to form microparticles of this delivery system would allow for the co-encapsulation of the microparticles and cells in hydrogel scaffolds, such as the HA-GMA described here, and may prove beneficial for tissue engineering strategies. With these criteria in mind, we have designed a micro-particulate system comprised of interpenetrating networks of poly(lactide) (PLA) and poly(ethylene glycol) dimethacrylate (PEGDMA) as described in chapters 5 and 6.

Chapter 5: Formulation of Chemically Cross-Linked Interpenetrating Networks of Poly(Ethylene Glycol) and Poly (Lactide)

5.1 Introduction

Many traditional drug delivery and tissue engineering matrices, including poly(lactide) (PLA), poly(glycolide) (PGA), and their copolymers poly(lactide-co-glycolide) (PLGA), require processing conditions such as organic solvents or extreme temperatures and pressures that can lead to the denaturation of proteins. Further, the hydrophobicity of these polymers leads to surface aggregation of the protein, which causes protein unfolding and non-ideal release profiles (high initial burst). In addition, these polymers typically follow a bulk degradation process leading to acidic local microenvironments within the matrix, which can be detrimental to the bioactivity of the encapsulated growth factor [40, 41]. On the other hand, hydrogel scaffolds are processed under aqueous conditions making them conducive to entrapping proteins in a stable manner [43]. However, these scaffolds swell extensively and release entrapped proteins quickly as seen with the cross-linked hyaluronic acid gels in chapter 4. By combining traditional synthetic/hydrophobic polymers with hydrogels we hypothesize that matrices with properties of both polymer types can be obtained. Thus, the mechanical strength and control over release provided by synthetic/hydrophobic scaffolds can be acquired in a system that allows for the stable and non-denaturing encapsulation of growth factors as seen with hydrogels. Further, the hydrophilic nature afforded by the hydrogel component should lead to increased transport of acidic by-products allowing for better control over degradation and providing an environment that is more conducive to protein stability.

To this end, we have prepared interpenetrating polymer networks (IPNs) comprised of poly(ethylene glycol) dimethacrylate (PEGDMA) and either poly(lactide) (PLA) or a newly synthesized copolymer of PLA with pendant vinyl groups. The IPNs were synthesized using photochemical techniques in a mixture of benzyl benzoate/benzyl alcohol (BB/BA), which is a co-solvent for both the polymers and protein. This solvent has been utilized with protein therapeutics as a carrier for injectable protein delivery depots and has been found to be biocompatible and to have no detrimental effects on the protein [139, 141]. The use of this solvent system for preparing our IPNs allows for uniform protein dispersion throughout the matrix, which may lead to more uniform release kinetics (elimination of initial burst). The hydrophobic/hydrophilic balance of the IPN, and thus the swelling and release rate can be manipulated by varying the polymer molecular weights and the PEG/PLA ratio. Further, the use of a vinyl pendant PLA will allow for a more highly cross-linked and homogeneous hydrogel allowing for further control over the release kinetics. The effects of these parameters on IPN swelling and release of a model protein, bovine serum albumin (BSA), were investigated with the goal of providing a tunable protein delivery system with engineering handles to control the rate of protein release.

5.2 Materials and Methods

5.2.1 Materials

Poly(DL-lactide) with inherent viscosities of 0.36 dL/g (38 kD), 0.49 dL/g (52 kD), and 0.67 dL/g (78 kD) were purchased from Birmingham Polymers, Inc. (BPI,

Birmingham, AL). Poly(DL-lactide) with an inherent viscosity of 0.2 dL/g (30 kD) was purchased from Polysciences, Inc (Warrington, PA). Poly(ethylene glycol) diacrylate MW=3400 Da was donated by Shearwater Corporation (Huntsville, AL). Poly(ethylene glycol) dimethacrylate MW=550 Da, anhydrous benzyl alcohol 99.8%, benzyl benzoate 99+%, 2',4'-dimethoxy-2-phenylacetophenone 98% (Igracure 651), 3,6-dimethyl-1,4-dioxane-2,5-dione (lactide), 1,4-cyclohexanediol 99%, 2-bromoisobutyryl bromide 98%, pyridinium chlorochromate 98%, 3-chloroperoxy benzoic acid 77% max, 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) 98%, 1-dodecanol 98+%, sodium biocarbonate 99%, magnesium sulfate 99%, triethylamine 99.5%, stannous octoate, chloroform (ACS grade), anhydrous ethyl ether (ACS grade), ethyl acetate (HPLC grade), hexanes (HPLC grade), dichloromethane (HPLC grade), tetrahydrafuran (HPLC grade), n-heptane (HPLC grade), and anhydrous toluene 99.8% were all obtained from Aldrich (Milwaukee, WI). Physiological phosphate buffered saline (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) and bovine serum albumin (BSA, fraction V) were purchased from Sigma (St. Louis, MO). Hydrochloric acid (HCl, 1N) was obtained from VWR Scientific Products (Plainfield, NJ). Deuterated chloroform ($CDCl_3$) was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). The Bio-Rad DC protein assay was purchased from Bio-Rad Life Sciences (Hercules, CA).

5.2.2 Synthesis of PEG/PLA IPNs

Poly(ethylene glycol)/poly(lactide) (PEG/PLA) IPNs of various ratios and molecular weights of PEG and PLA were synthesized by dissolving PLA and PEGDMA in a 95/5

mixture of benzyl benzoate/benzyl alcohol (BB/BA) to give a total polymer concentration of 300 mg/ml (Figure 5-1). Various PEG/PLA ratios were prepared by adjusting the respective amounts of each polymer in the solution while maintaining a constant total concentration. BSA was added to each solution at a concentration of 5% by weight (based on total polymer weight). The IPNs were photo cross-linked by adding 350 μl of the polymer solution to a Teflon mold (14 mm in diameter). To each mold 40 μl of 2,2-dimethyl-2-phenylacetophenone (Igracure 651, 100 mg/ml) was added followed by exposure to UV at 365nm for 1 hour (Blakray B100AP). The resulting hydrogel discs were allowed to react overnight before being removed from the Teflon molds and used in the release study. Blank IPNs containing no protein were prepared in the same manner without the protein addition step. Due to the extremely low volatility of BB/BA, the solvent cannot be removed from the hydrogel prior to delivery. Thus, the solvent will be released along with the entrapped protein from our delivery device. However, BB/BA has been used as a biocompatible carrier for injectable depot formulations for the delivery of proteins and the inclusion of the solvent in our delivery device is not of concern.

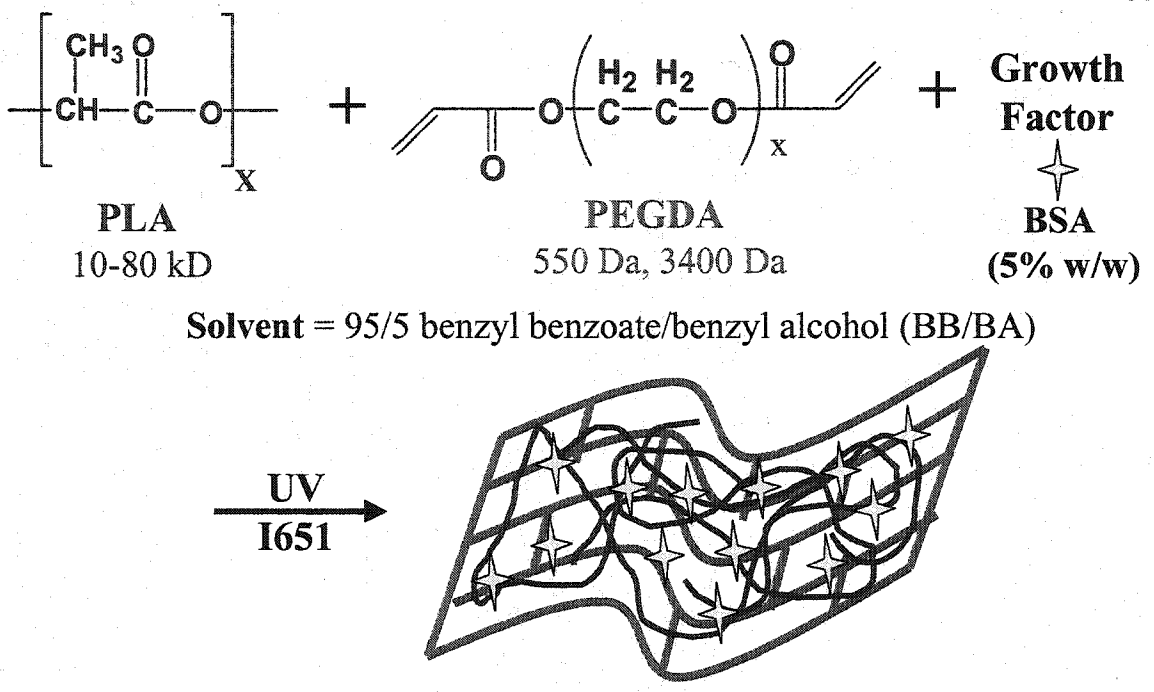


Figure 5-1: Schematic representation of the photo cross-linking of PEGDA/PLA IPNs loaded with BSA in benzyl benzoate/benzyl alcohol

5.2.3 Swelling and Release Profiles of Bovine Serum Albumin from PEG/PLA IPNs

The swelling and BSA release profiles of PEG/PLA IPNs of various ratios and molecular weights of PEG and PLA were examined using the procedure detailed below. Approximately 3 ml of release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH=7.4) was added to a 20 ml glass scintillation vial. PEG/PLA IPNs were then added to each vial and the vials were incubated at 37°C in a shaking water bath. After various time periods the release buffer was removed and replaced with fresh buffer. The release media was centrifuged to remove any particulates and stored at 4 °C until analyzed for protein concentration. After completion of the release study, the swollen PEG/PLA IPN

was weighed (wet weight), lyophilized, and reweighed (dry weight). The equilibrium swelling ratio (Q) was calculated using the following relationship:

$$Q = (\text{wet weight})/(\text{dry weight})$$

Protein loading was verified by degrading the remaining PEG/PLA IPN in 1N NaOH for 24 hours and measuring the resulting protein concentration.

The amount of BSA released at each time point was determined using the BioRad DC protein assay. BioRad DC is a colorimetric assay that utilizes a calibration curve to determine protein concentration. The assay is based on the reaction of protein with copper tartrate followed by reduction of Folin reagent by the copper treated protein [177]. The reduced Folin reagent has a characteristic blue color whose absorbance can be measured at 750 nm. It is a detergent compatible assay and was found to give more reproducible results in the presence of PLA degradation products than other protein assays. Triplicate samples were measured and expressed as an average plus or minus one standard deviation. Results were presented as a percentage of the amount of loaded protein released at each time point compared to blank IPNs containing no protein.

In order to analyze the diffusive properties of the PEG/PLA IPN hydrogels, the release of BSA from the PEG/PLA IPNs was plotted as the amount of BSA released versus the square root of release time. These results were fitted to the Higuchi model, which describes the release of an active agent dispersed in a monolithic device where the agent diffuses from the device rapidly relative to the degradation of the polymer [40]. Our system fits the assumptions of this model due to the hydrogel nature of the PEG/PLA

IPNs, which allows for fairly rapid release in comparison to the slow degradation of the PLA component. The Higuchi model is given by the following relationship:

$$dM_t/dt = (A/2)\,(2PC_o/t)^{1/2}$$

Where,

M_t = the amount of agent released at time t

P = the permeability of the polymer to the agent

A = the surface area of both sides of the disc

C_o = initial concentration of agent in the polymer

Thus, after integration the release is described by:

$$M_t = kt^{1/2}$$

Where,

k = a constant describing the permeability of the polymer and the diffusivity of the agent from the matrix.

This relationship provides a linear plot when the amount released is plotted against the square root of time. The slope was used to give an estimate of the combined effect of permeability and diffusivity and provided a quantitative measure for the comparison of protein release rates from the different PEG/PLA IPN formulations.

5.2.4 *Degradation of PEG/PLA Interpenetrating Networks*

PEG/PLA IPNs consisting of various molecular weights of PLA were prepared in order to analyze the degradation profile of the IPNs. Briefly, IPNs were photo cross-linked in the same manner as described for the release study without protein loading. The cross-linked IPN was placed into physiological PBS (pH=7.4) and incubated at 37 °C with constant shaking. At time points of 1, 5, 10, and 20 days, a PEG/PLA IPN was removed from the release media and lyophilized. Next, the PLA portion of the dry PEG/PLA IPN was extracted into tetrahydrafuran. The extracted sample was analyzed by gel permeation chromatography (GPC) against polystyrene standards in order to track the degradation of the PLA component. Each condition was prepared in triplicate and presented as an average plus or minus one standard deviation.

5.2.5 *Synthesis of Vinyl-Pendant Poly(lactide)*

In order to confer vinyl functionality to PLA, a co-monomer that is capable of under going ring-opening polymerization was synthesized. Our strategy involves synthesizing a monomer with a pendant tertiary bromide, followed by ring-opening copolymerization with PLA, and subsequent elimination of the tertiary bromide to produce a pendant vinyl group. To this end, γ-(2-bromo-2-methyl propionyl)-ε-caprolactone was synthesized as reported in the literature by Mecerreyes, et al, in a 3-step reaction (Figure 5-2) [178]. Briefly, (4-hydroxycycloheyl)-2-bromo-2-methylpropionate was synthesized by dissolving 20 g (173 mmol) of 1,4 cyclohexanediol and 40 ml (347 mmol) of triethylamine in 200 ml of dry tetrahydrafuran (THF). Next, 22 ml (173 mmol) of 2-

bromoisobutyryl bromide was slowly added and allowed to react for 24 hours. The THF was removed by rotoevaporation and 350 ml of methylene chloride was added. The solution was then washed 3 times with 1 N HCl and twice more with ddH_2O. The solution was dried over $MgSO_4$, filtered, and purified using flash chromatography (silica gel using hexane/ethyl acetate gradients of 5, 10, 20, 30, 40, and 50% ethyl acetate). The product was found by thin layer chromatography and rotoevaporated to give an orange liquid that was further purified by distillation (135 °C, 5 mmHg) to give a clear liquid (Yield: 17.1 g, 38%).

(4-ketocyclohexyl) 2-bromo-2-methylpropionate was prepared by oxidizing (4-hydroxycycloheyl)-2-bromo-2-methylpropionate with pyridinium chlorochromate (PCC). Approximately, 17.1 g (65 mmol) of (4-hydroxycycloheyl)-2-bromo-2-methylpropionate and 16.7 g (78mmol) of PCC were dissolved in 140 ml of dichloromethane and allowed to react for 3 hours. Next, 100 ml of anhydrous ether was added and the solution was filtered through silica gel. The yellow solution was rotoevaporated and purified using flash chromatography (silica gel using hexane/ethyl acetate gradients of 5, 10, 20, 30, and 40% ethyl acetate). The product was found by thin layer chromatography and rotoevaporated to give a white crystalline powder (Yield: 13.8 g, 81%).

γ-(2-bromo-2-methyl propionyl)-ε-caprolactone (BMPCL) was produced using a Bayer-Villiger oxidation to transform the ketone into a cyclic ester. Approximately 13.8 g (53mmol) of (4-ketocyclohexyl) 2-bromo-2-methylpropionate was dissolved in 100 ml of chloroform. To this solution, 125 ml of a 240 mg/ml solution of 3-chloroperoxybenzoic acid (m-CPBA, 123 mmol) was added drop-wise. The mixture was

allowed to react for 24 hours, followed by filtration over celite. The yellow solution was washed 2 times with 2 M $NaHCO_3$ and one time with brine. The product solution was rotoevaporated and purified using flash chromatography (silica gel using hexane/ethyl acetate gradients of 5, 10, and 20% ethyl acetate). The monomer was then recrystallized from anhydrous ether to give a white crystalline product (Yield: 8 g, 55%). ^{1}H-NMR analysis ($CDCl_3$, 599MHz) was used to confirm the presence and purity of the BMPCL.

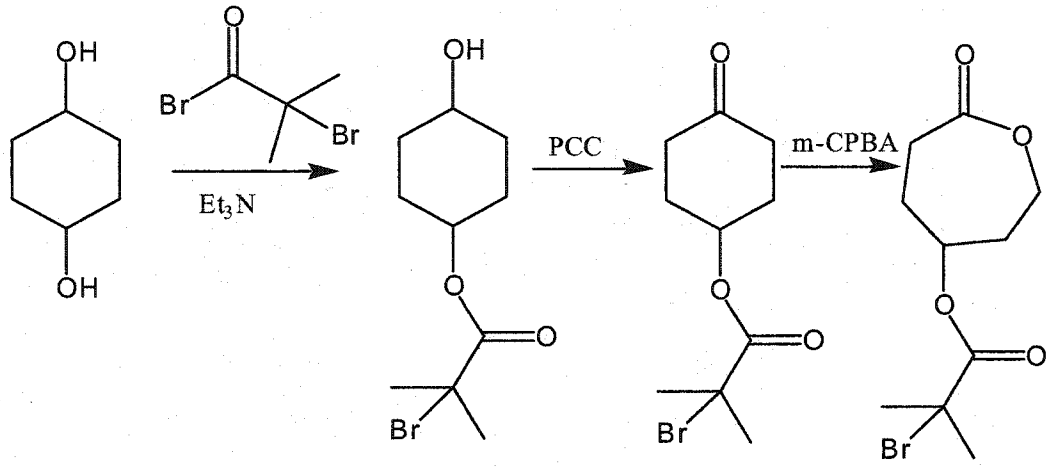


Figure 5-2: Synthetic strategy for the synthesis of γ-(2-bromo-2-methyl propionyl)-ε-caprolactone

BMPCL was copolymerized with 3,6-dimethyl-1,4-dioxane-2,5-dione by ring opening polymerization to produce PLA-co-BMPCL (Figure 5-3). The polymerization was carried out using a technique similar to that of Detrembleur, et al [179]. The procedure for producing a 90:10 random copolymer with a molecular weight of 14 kD is described briefly here. Approximately 900 mg 3,6-dimethyl-1,4-dioxane-2,5-dione and

200 mg of BMPCL were dried under vacuum. The reaction flask was then heated to 135 °C and the monomers were melted. Approximately 148 µl of dodecanol in dry toluene (100mg/ml) was added as an initiator. The polymerization was catalyzed by adding 60 µl of stannous octoate in dry toluene (100mg/ml). The polymerization was allowed to proceed overnight under an N_2 atmosphere and dry toluene was added to adjust the viscosity of the polymerization. The polymers were dissolved in THF, precipitated repeatedly in n-heptane, and dried under vacuum. The polymers were characterized for molecular weight using gel permeation chromatography (GPC) against polystyrene standards in THF and for polyester formation using ^{1}H-NMR ($CDCl_3$, 599MHz).

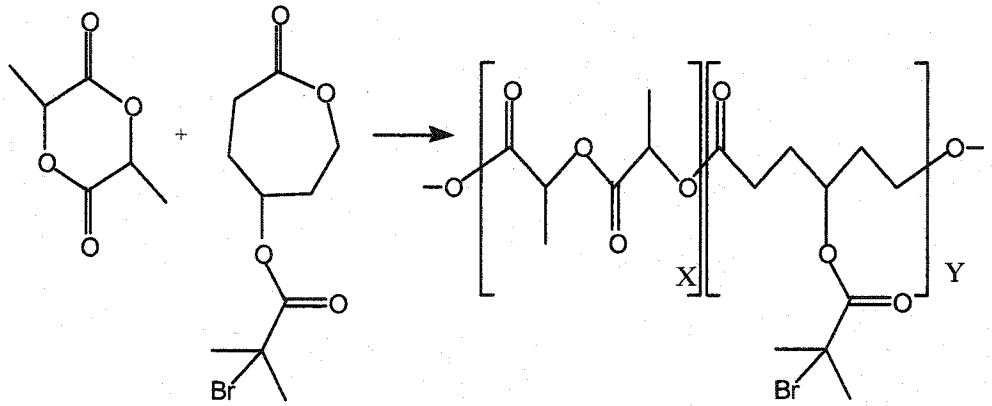


Figure 5-3: Random copolymerization of γ-(2-bromo-2-methyl propionyl)-ε-caprolactone and 3,6-dimethyl-1,4-dioxane-2,5-dione by ring opening polymerization

Vinyl-pendant PLA was produced by the dehalogenation of PLA-co-BMPCL using the technique of Detrembleur, et al (Figure 5-4) [179]. Briefly, 300 mg of PLA-co-BMPCL and 125 µl of 1,5-diazabicyclo[4.3.0]-non-5-ene (DBN) were dissolved in 4 ml

of dry toluene. The reaction was carried out for 10 hours at 80 °C under an N_2 atmosphere. The vinyl-pendant PLA was then repeatedly precipitated in ice cold n-heptane and dried under vacuum. The polymer was characterized for molecular weight using gel permeation chromatography (GPC) against polystyrene standards in tetrahydrafuran and for polyester formation using ^{1}H-NMR ($CDCl_3$, 599MHz).

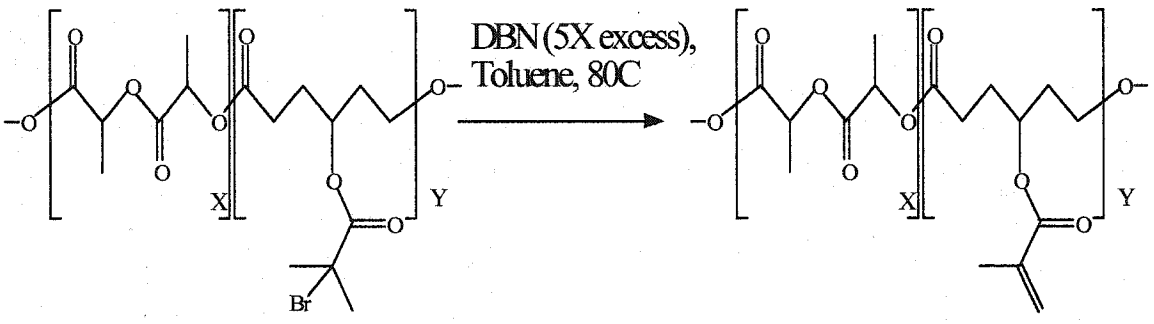


Figure 5-4: Dehalogenation of PLA-co-BMPCL to produce vinyl-pendant PLA

5.3 Results

5.3.1 Swelling and BSA release profiles of PEGDA/PLA IPNs

Interpenetrating networks of PEG and PLA were successfully synthesized using photochemical techniques producing hydrogels that occupied the entire volume of their reaction solvent. The gels were easily handled and removed from their casting molds allowing for the generation of hydrogel discs of reproducible size and shape. IPNs were synthesized at PEG/PLA weight ratios of 100/0, 75/25, and 50/50; however, cross-linking was unsuccessful at a 25/75 ratio due to an insufficient density of vinyl groups in the solution and these samples were not analyzed.

In order to evaluate the effect of PEGDMA molecular weight on the swelling and protein release profiles of the PEG/PLA IPNs, hydrogels consisting of 15 kD PLA and 550 or 3400 Da PEG were allowed to swell in release media and the equilibrium swelling determined. A dependence on PEG molecular weight and PEG/PLA ratio was seen with a higher PEG molecular weight and higher PEG content leading to an increase in the swelling of the IPN (Figure 5-5). This is expected, as PEG is the more hydrophilic polymer and should cause a greater influx of water into the PEG/PLA IPN. Further, the larger PEG molecular weight should allow for a larger pore size in the swollen hydrogel and a greater uptake of water.

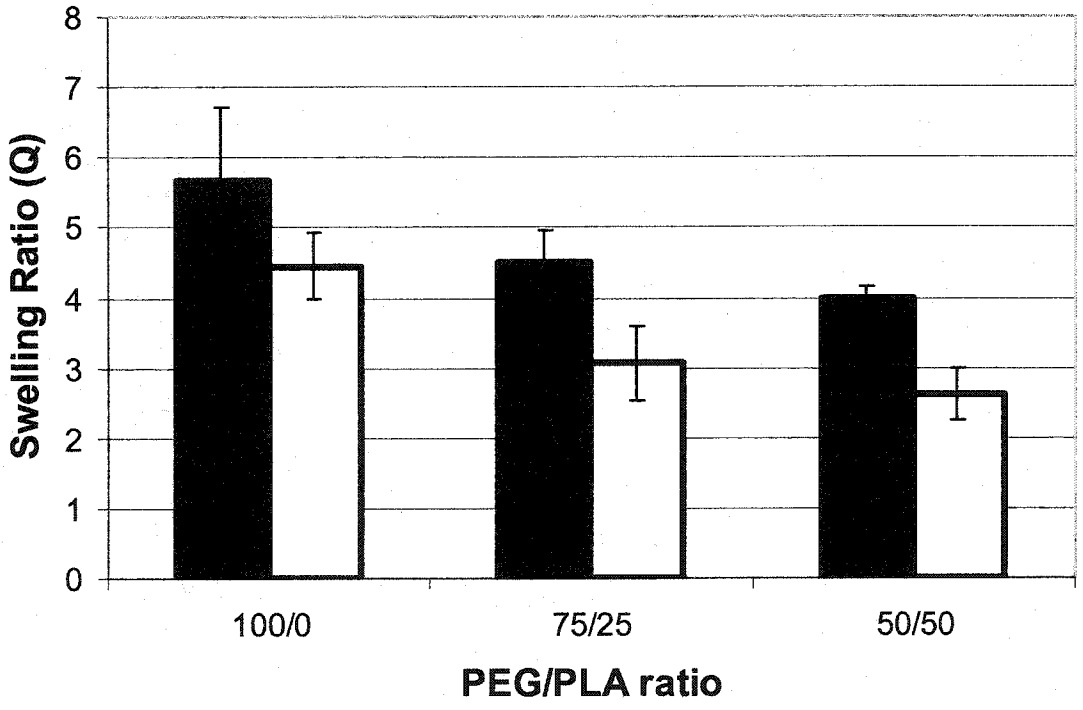


Figure 5-5: Equilibrium swelling of PEG/15 kD PLA IPNs containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PEGDA molecular weights: 3400 (■) and 550 (□).

The ability of the PEG/15 kD PLA IPNs to control the release of a model protein, BSA, was analyzed at both PEG molecular weights and at various PEG/PLA ratios. PEG/PLA IPNs formed with 3400 Da PEGDA released BSA at a similar rate regardless of the PEG/PLA ratio. Approximately 32% of the loaded protein was released as a burst within the first hour and complete release occurred after approximately 8 days (Figure 5-6). On the other hand, IPNs formed with 550 Da PEGDMA demonstrated better control over the rate of protein release (Figure 5-7). IPNs at the lowest PEG550/PLA ratio (50/50) exhibited a decreased initial burst and extended release times. These IPNs released 20% of the loaded BSA within one hour followed by a much slower release with approximately 60% of the loaded BSA released after 10 days. In contrast, a PEG550/PLA ratio of 100/0 demonstrated an initial release of approximately 40% followed by a slower release profile with nearly complete release of the BSA after 10 days. The higher release rate seen with 3400 Da PEG/PLA IPNs may be due to an increase in the pore size of the swollen hydrogel matrix due to the lower cross-link density. On the other hand, the reduction in burst of protein release from the 550 Da PEG/PLA IPNs occurs due the smaller pore size provided by the lower molecular weight PEG leading to a decrease in initial swelling. The gradual degradation of the more hydrophobic PLA component then leads to a slow release of entrapped protein at later time points. Due to the ability of PEG/PLA IPNs formulated with 550 Da PEGDMA to provide control of protein release rate with changes in the PEG/PLA ratio, only IPNs composed of 550 DA PEGDMA were investigated further.

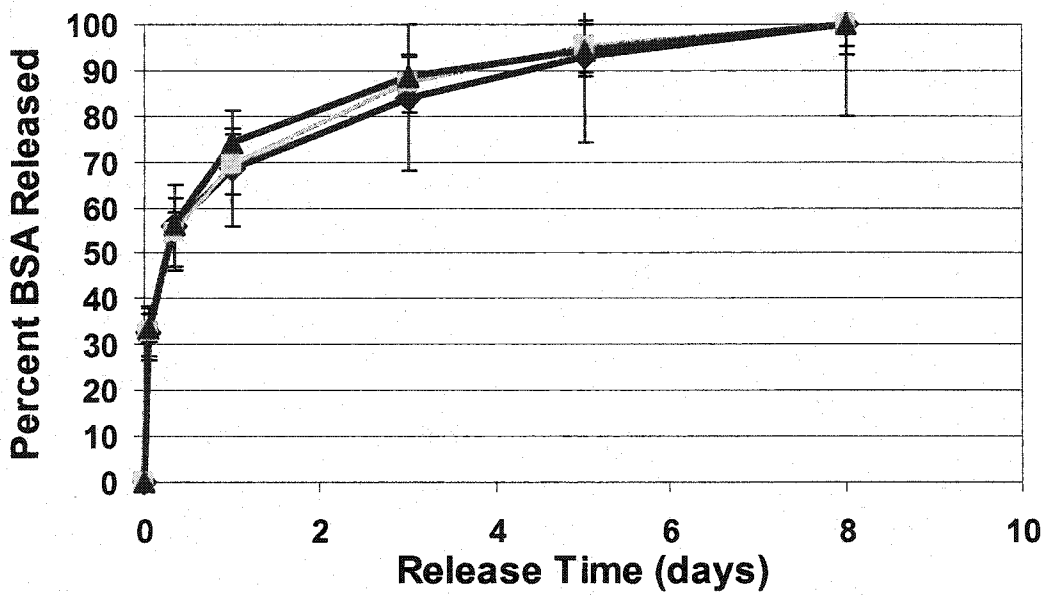


Figure 5-6: Release profiles of BSA from 3400 Da PEG/15 kD PLA IPNs containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PEG/PLA ratios: 100/0 (♦), 75/25 (▒), and 50/50 (▲).

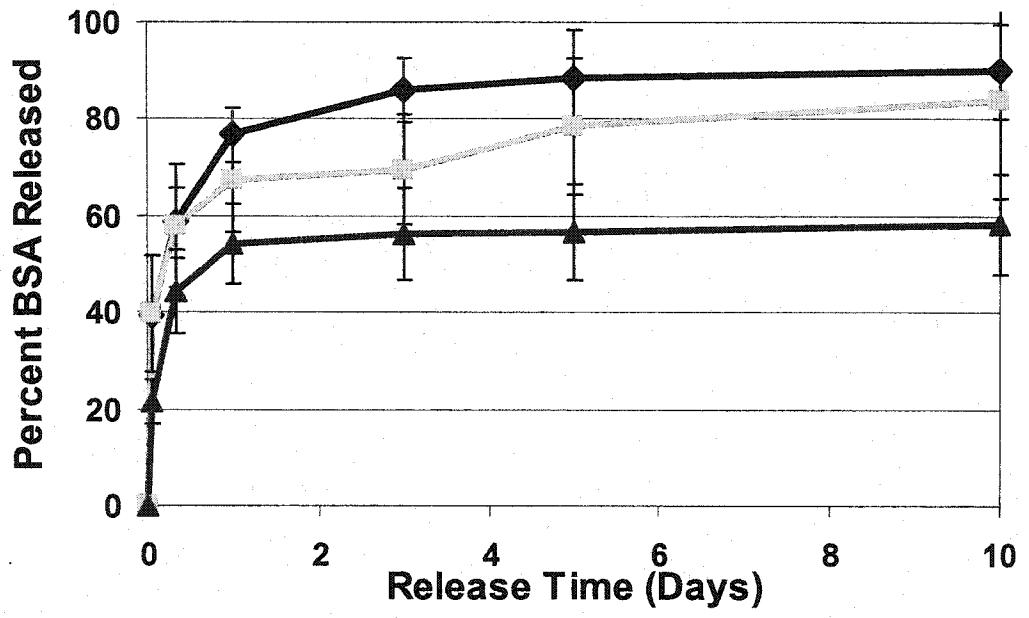


Figure 5-7: Release profiles of BSA from 550 Da PEG/15 kD PLA IPNs containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PEG/PLA ratios: 100/0 (♦), 75/25 (■), and 50/50 (▲).

Next, the effects of PLA molecular weight and polymer concentration on protein release rates from 50:50 PEG/PLA IPNs were investigated. IPNs consisting of 550 Da PEGDMA and various molecular weights of PLA were prepared. Additionally, 50:50 PEG/PLA IPNs with a higher vinyl group density were fabricated by increasing the total polymer concentration from 300 mg/ml to 600 mg/ml. As before, the IPNs were loaded with 5% (w/w) BSA and examined for their protein release rates by allowing the IPN to swell in physiological PBS (pH = 7.4). As the molecular weight of the PLA component was increased, a decrease in equilibrium swelling was observed in accordance with the increased hydrophobicity of the PLA (Figure 5-8). Similarly, at a given PLA molecular weight the equilibrium swelling decreased as the total polymer concentration was

increased from 300 mg/ml to 600 mg/ml. This is due to a higher vinyl group density in the solution leading to a higher crosslink density in the IPN.

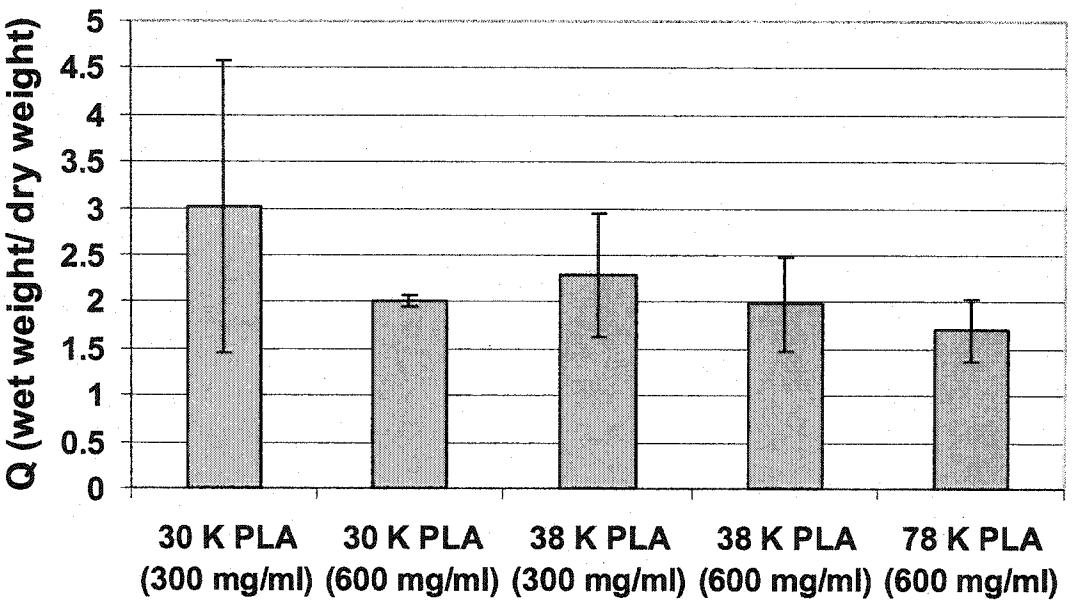


Figure 5-8: Equilibrium swelling of 550 Da PEG/PLA IPNs (50:50 ratio) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C with various PLA molecular weights and polymer concentrations.

The PLA molecular weight and total polymer concentration also had a dramatic effect on the release of BSA from the 550 Da PEG/PLA (50:50 ratio) IPNs (Figure 5-9). As the molecular weight of the PLA was increased, the release rate of BSA decreased significantly. Likewise, at a given PLA molecular weight the release rate of BSA decreased significantly with an increase in total polymer concentration and thus cross-linking density. For the highest PLA molecular weight (78 kD) and highest polymer

concentration (600 mg/ml), a significant decrease in the initial burst was seen followed by a very slow release of BSA with approximately 15% of the loaded protein being released over a 20 day period. However, most formulations exhibited a 50% or greater initial burst followed by slower release rates over the 20 day release period. This is most likely due to the quick extraction of the benzyl benzoate and benzyl alcohol from the IPN when placed in physiological PBS. Since protein is soluble in the BB/BA solvent, the BSA in quickly extracted along with the solvent. A strategy for removing benzyl benzoate and benzyl alcohol will be important for reducing the rapid initial release of protein.

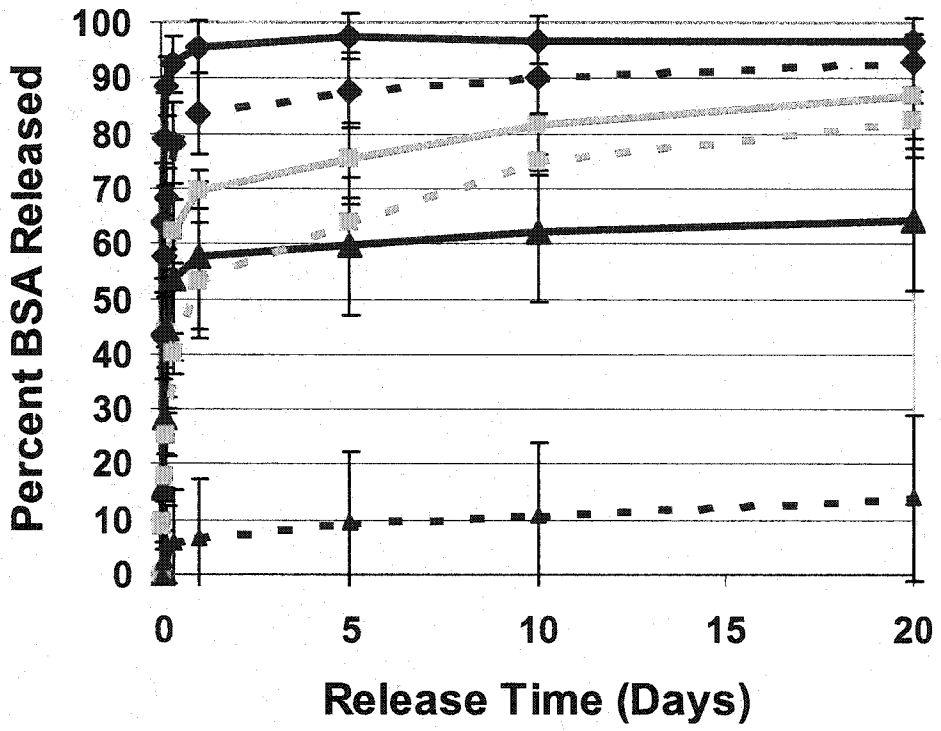


Figure 5-9: PLA molecular weight and polymer concentration dependent release of BSA from 550 Da PEG/PLA IPNs (50:50 ratio) containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PLA molecular weights: 38kD (♦), 52kD (▒), and 78kD (▲). Total polymer concentrations of 300 mg/ml (solid) and 600 mg/ml (dashed).

By fitting the initial release data to the Higuchi model a linear relationship is demonstrated between the percent of BSA released versus the square root of time (Figure 5-10). This suggests the initial rapid release of protein is diffusive in nature. This release regime is then followed by a slower release of BSA that may be diffusive or degradation controlled. Calculating the slope of the Higuchi model fit, a diffusive rate for each formulation can be determined (Table 5-1). This allows comparison of the initial release rates of the different formulations and quantification of the differences seen with an

increase in PLA molecular weight or total polymer concentration (cross-link density). An order of magnitude difference was seen between the slope of formulations containing the lowest molecular weight of PLA (38 kD) and the highest PLA molecular weight (78kD) when utilizing the higher polymer concentration. The deviation from linearity of the IPNs with lower molecular weight PLA is due to the near complete release of protein during the early time points.

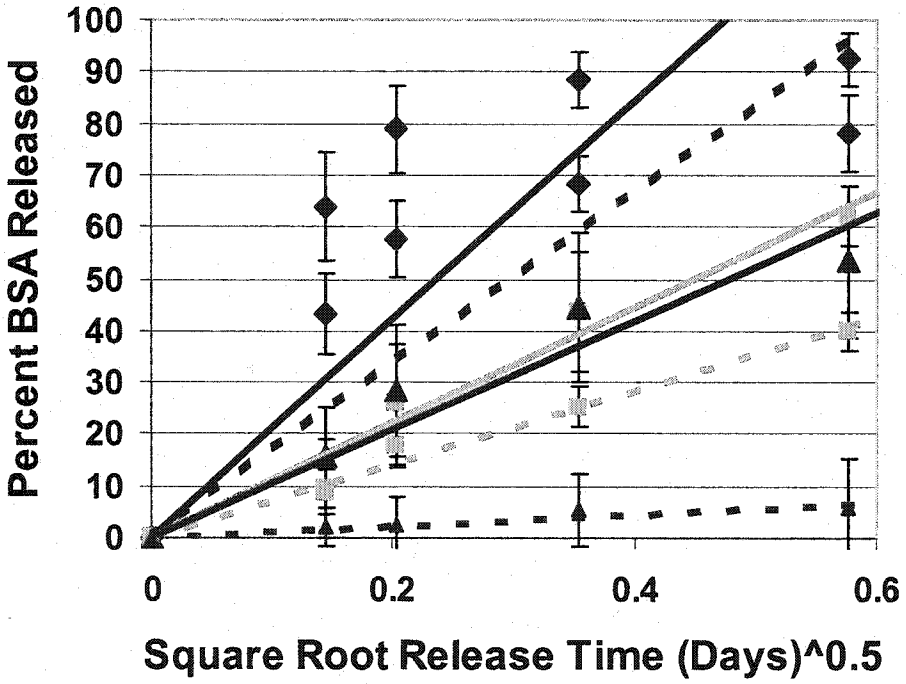


Figure 5-10: Higuchi model fits of initial PLA molecular weight and polymer concentration dependent release of BSA from 550 Da PEG/PLA IPNs (50:50 ratio) containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PLA molecular weights: 38kD (♦), 52kD (■), and 78kD (▲). Total polymer concentrations of 300 mg/ml (solid) and 600 mg/ml (dashed).

Table 5-1: Release rates as determined by the Higuchi model for 50:50 PEG/PLA IPN formulations with various PLA molecular weights and total polymer concentrations

PLA MW/Polymer Conc.	Slope (percent/day$^{0.5}$)	R^2
38 kD/300 mg/ml	211	0.4
38 kD/600 mg/ml	168	0.64
52 kD/300 mg/ml	111	0.97
52 kD/600 mg/ml	71	0.99
78 kD/300 mg/ml	105	0.92
78 kD/600 mg/ml	11	0.89

5.3.2 Degradation of PEG/PLA Interpenetrating Networks

The degradation of 550 Da PEG/PLA (50:50 ratio) IPNs comprised of various molecular weights of PLA and total polymer concentrations (cross-linking densities) was analyzed by extracting the PLA component into tetrahydrafuran after various time periods in physiological PBS (pH = 7.4). The extracted PLA was analyzed by gel permeation chromatography against polystyrene standards in order to determine the percent decrease in PLA molecular weight. With all formulations, there was an initial 10 to 20 percent decrease in PLA molecular weight, followed by very slow degradation over the 20 day period of the study (Figure 5-11). The PLAs used in these studies are hydrophobic and known to degrade slowly over a period of months to years. It was speculated that the increased water uptake of these IPNs due to the hydrophilic PEG component might lead to an increase in degradation rate. However, this does not appear to be the case and the protein release seems to be mostly diffusional in nature. The initial 10 to 20 percent drop in molecular weight may be due to UV scission of the polyester bonds of PLA.

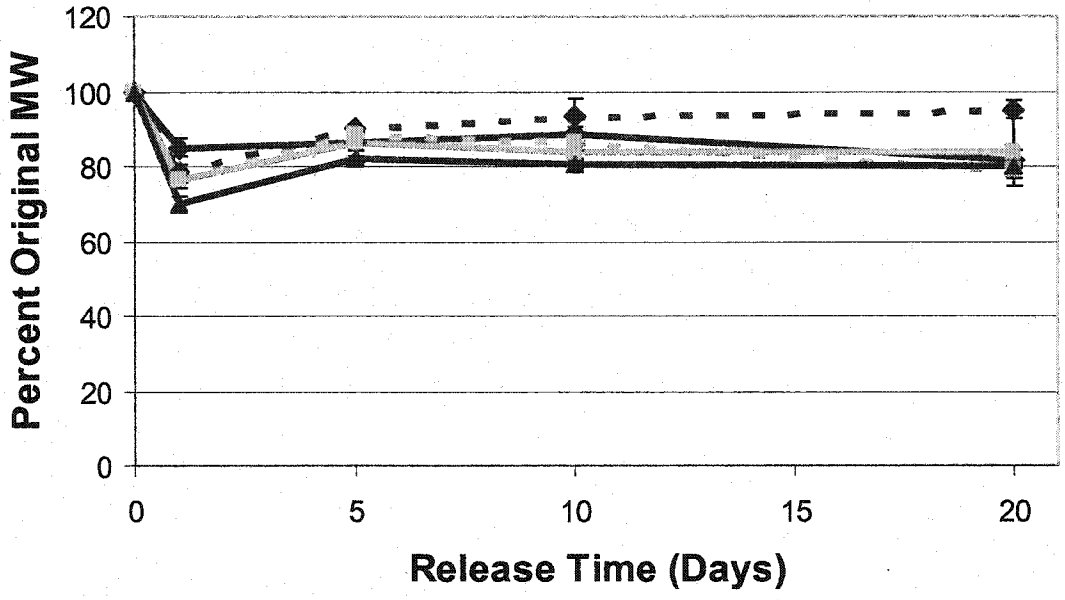


Figure 5-11: Degradation of the PLA component of 550 Da PEG/PLA IPNs (50:50 ratio) containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C. PLA molecular weights: 30kD (♦), 38kD (▒), and 78kD (▲). Total polymer concentrations of 300 mg/ml (solid) and 600 mg/ml (dashed).

5.3.3 Synthesis and characterization of vinyl-pendant PLA

In order to increase the vinyl group density and thus the cross-linking density of the PEG/PLA IPNs, a vinyl-pendant PLA was synthesized. The necessary BMPCL monomer was successfully synthesized and purified as demonstrated in the ^{1}H-NMR spectra below (Figure 5-12). This monomer was capable of undergoing ring-opening polymerizations with 3,6-dimethyl-1,4-dioxane-2,5-dione to form PLA-co-BMPCL at various copolymer ratios and molecular weights. Copolymer ratios of 95/5 to 80/20 PLA/BMPCL were successfully prepared with molecular weights ranging from 10 kD to 25 kD. ^{1}H-NMR analysis confirmed the polymerization and removal of unreacted

monomer (Figure 5-13). Dehalogenation with DBN resulted in a PLA copolymer containing pendant vinyl groups. The presence of the methacrylate moieties (denoted by i) can be clearly seen in the ^{1}H-NMR spectrum of vinyl pendant PLA (Figure 5-14). However, a 50% decrease in molecular weight was seen after the elimination reaction. Further, there was incomplete conversion of the tertiary bromides to vinyl groups. These issues caused difficulty in designing and synthesizing polymers of desired molecular weight and vinyl group density in sufficient amounts for these studies. Thus, an increase in PEGDMA concentration rather than vinyl-pendant PLA was used to increase the vinyl group density in the IPNs as described above.

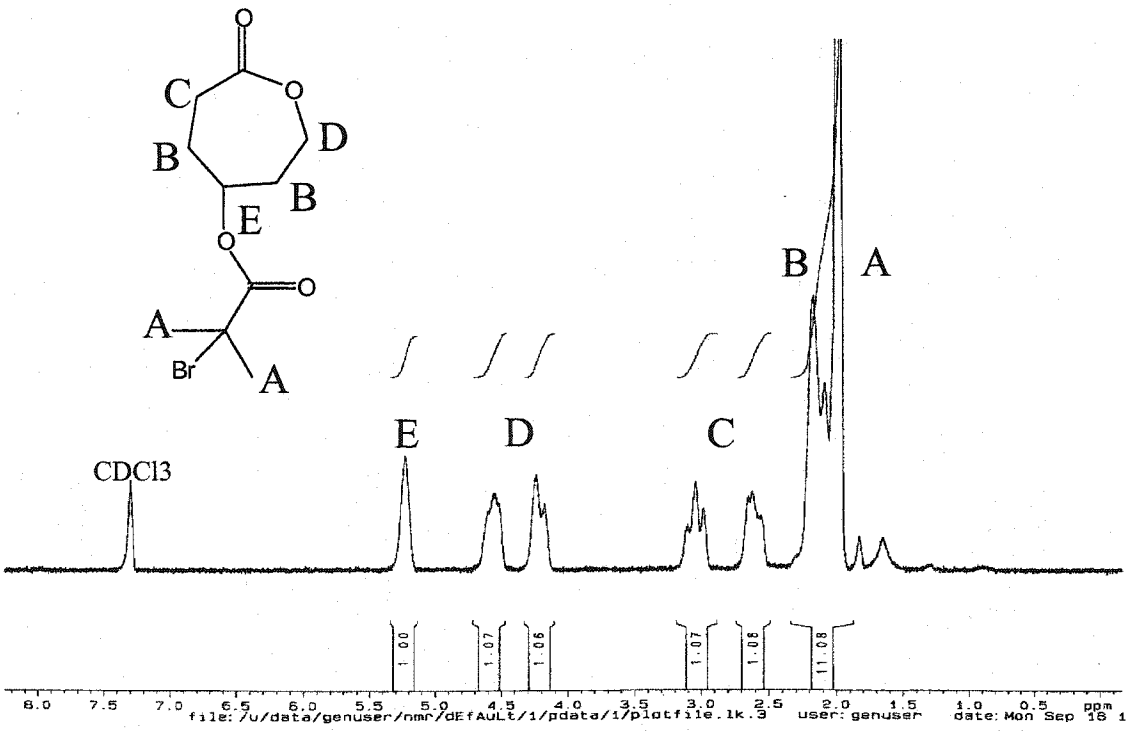


Figure 5-12: ^{1}H-NMR spectrum of γ-(2-bromo-2-methyl propionyl)-ε-caprolactone in $CDCl_3$

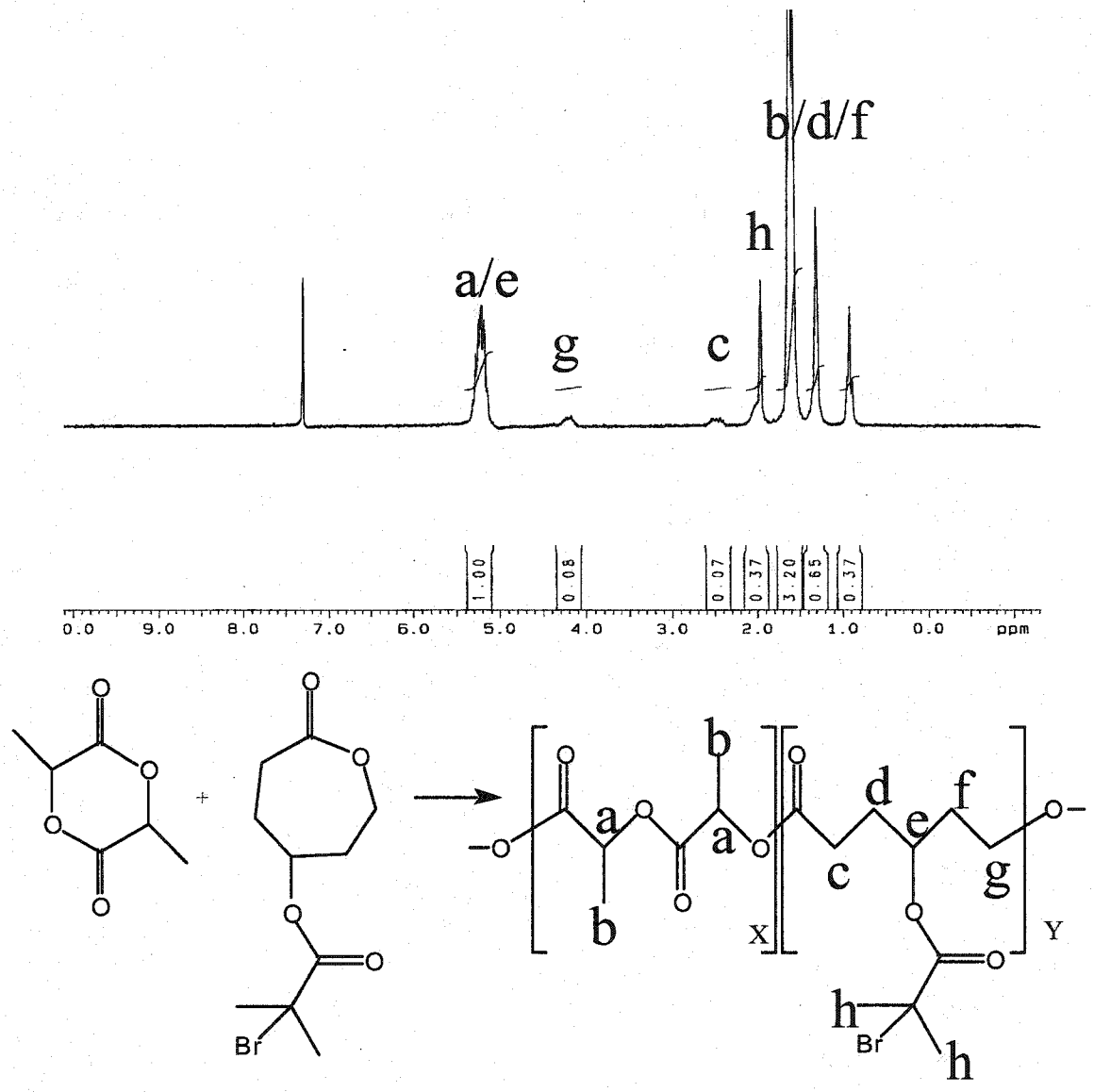


Figure 5-13: ^{1}H-NMR spectrum of 90:10 23kD PLA-BMPCL in $CDCl_3$

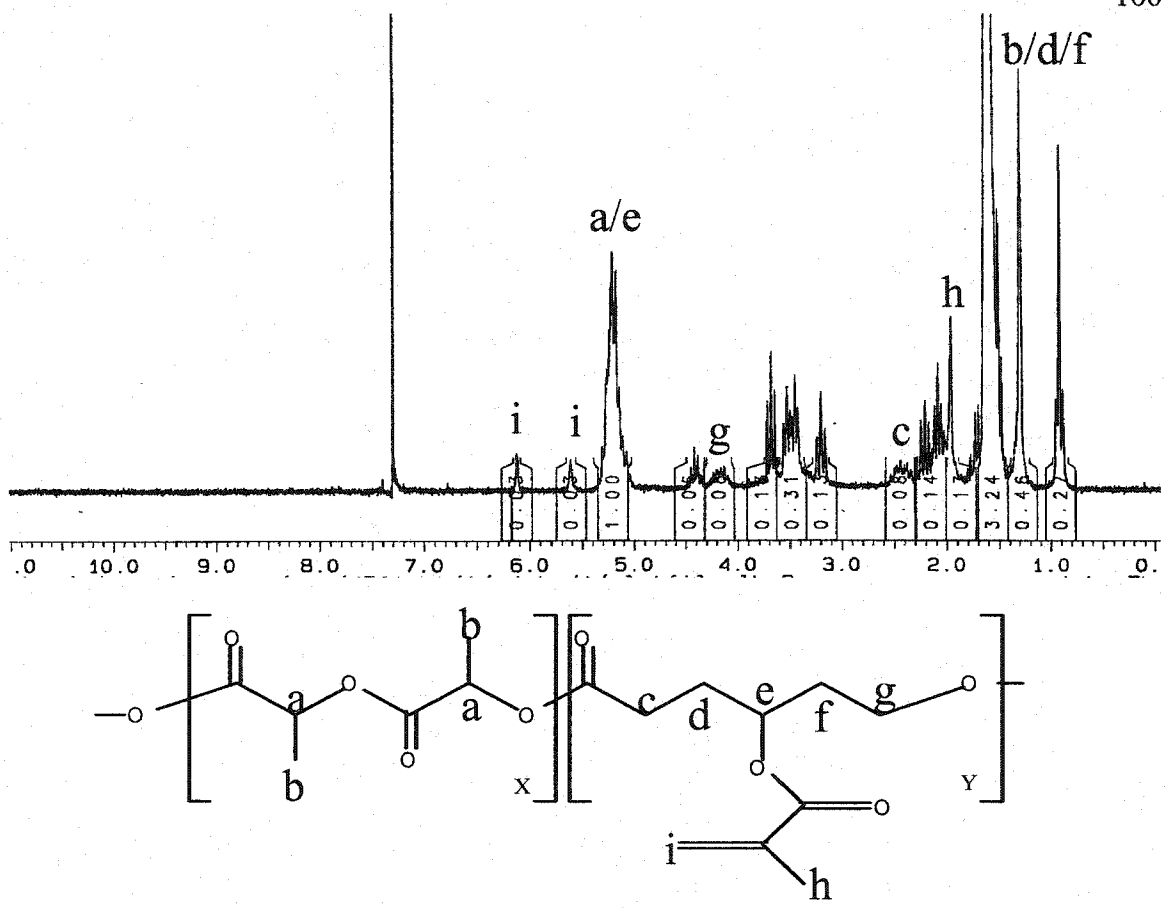


Figure 5-14: ^{1}H-NMR spectrum of 11 kD vinyl-PLA in $CDCl_3$

5.4 Discussion

Interpenetrating networks of poly(ethylene glycol) dimethacrylate and poly(lactide) of varying molecular weights and polymer ratios were reproducibly cross-linked using photochemical methods in a benzyl benzoate and benzyl alcohol solvent system. BSA as a model protein was uniformly dispersed within the IPN using this cross-linking strategy. The effects of polymer molecular weight and polymer ratio on the swelling and protein

release profiles of the IPNs are summarized in Table 5-2. Briefly, as the amount of the more hydrophobic component was increased by increasing the PLA molecular weight, there was a significant decrease in the swelling and protein release rate of the IPN. Further, as the amount of the more hydrophilic component was increased by increasing the PEGDMA molecular weight or the PEG/PLA ratio, the IPNs exhibited a significant increase in equilibrium swelling and protein release rates. These results were expected based on the ability of the more hydrophilic PEG component to aid in the absorption of water by the IPN leading to increased swelling and protein release rates. IPNs comprised of the higher molecular weight PEGDA (3400 Da) showed similar release rates at all PEG/PLA ratios, suggesting that the hydrated pore size of these IPNs is too large to allow for tuning of protein release rates using the PEG/PLA ratio. However, the lower molecular weight PEGDMA (550 Da) allowed for IPNs with a range of protein release rates as the PEG/PLA ratio was varied. This is due to the smaller pore size of the PEGDMA network leading to a decrease in swelling volume and causing an increase in diffusional resistance for the entrapped protein.

Table 5-2: Engineering 'handles' and their effects on the swelling and BSA release rate of PEG/PLA IPNs

Variable	Swelling	BSA Release Rate
PLA MW ↑	Decreased	Decreased
PEG MW ↑	Increased	Increased
PEG:PLA Ratio ↑	Increased	Increased
Vinyl Group Density ↑	Decreased	Decreased

A vinyl-pendant PLA was successfully synthesized to increase the cross-linking density of the IPN, but an inability to produce high enough amounts of the polymer with defined molecular weight and vinyl group density prohibited its use in these studies. Instead, the polymer concentration was increased to afford an increase in vinyl group density in the solution and thus increase the cross-link density of the IPN. IPNs of various PLA molecular weights demonstrated a decrease in swelling and protein release rate with an increase in polymer concentration, suggesting this technique was successful in increasing the cross-link density of the IPN. Indeed, the best control over the rate of protein release as shown by a decrease in the initial burst of release was seen with IPNs comprised of the highest molecular weight of PLA (78 kD) and highest polymer concentration (600 mg/ml). In the future, this variable could be more closely scrutinized by synthesizing 4-(Acryloyloxy)-ε-caprolactone by the method of Mecerreyes, et al. This monomer can be copolymerized with lactide to provide vinyl pendant PLA without the need for the difficult tertiary bromide elimination step [180]. In this manner, the cross-link density of the IPN could be increased independent of polymer concentration.

The release data obtained in this study was fitted to the Higuchi model by plotting the percent of protein released versus the square root of release time. This model fits the data to a linear relationship whose slope defines the permeability and diffusional properties of the IPN. PEG/PLA IPNs exhibited a decrease in Higuchi model slope with an increase in PLA molecular weight and total polymer concentration, demonstrating that the protein release rate can be successfully altered by manipulating the hydrophobic/hydrophilic balance of the IPN using these variables. Further, the early time points of these release

studies exhibit a linear relationship when fitted to the Higuchi model suggesting that the release from these systems is diffusive in nature. Likewise, analysis of the degradation of the PLA component of these IPNs showed little to no degradation of the PLA, further suggesting that the release is due to diffusion from the IPN and not to degradation of the PLA.

All of the PEG/PLA IPN formulations demonstrated similar release profiles independent of their initial release rate. This was characterized by a significant initial burst of protein release in which 40-80% of the entrapped protein was released, followed by a much slower rate of protein release for the duration of the study. It is our hypothesis that the entrapped benzyl benzoate and benzyl alcohol solvent in the IPN is aiding in the quick release of protein from the system. Due to the solubility of the protein in the BB/BA solvent, the protein is quickly removed during the extraction of the solvent from the IPN. Thus, a method that allows for the removal of the BB/BA solvent during processing would aid in decreasing the initial burst of protein release from the IPN and would allow for better control over protein release rates. In chapter 6, we describe a method for producing microparticles from our PEG/PLA system that allows for the extraction of the BB/BA component. Further, the ability to produce microparticles of the PEG/PLA IPNs provides versatility in applying this protein release system to a variety of tissue engineering strategies. With this in mind, we investigated the ability of these microparticle PEG/PLA IPNs to control the release of VEGF, a potent angiogenic growth factor.

Chapter 6: Release of VEGF from Cross-Linked Microparticles of Poly(Ethylene Glycol) and Poly(Lactide)

6.1 Introduction

The need exists for stable and controlled delivery of growth factors in tissue engineering. Further, the ability to deliver multiple growth factors at various rates from the same tissue engineering construct would allow the orchestration of complex tissue development as well as control over wound healing. Our strategy is to produce micro-particles of the PEG/PLA IPNs developed in Chapter 5 for incorporation into existing hydrogel tissue engineering scaffolds. There has recently been much interest in encapsulating cells in photo-crosslinkable hydrogel scaffolds such as hyaluronic acid, alginate, and polyethylene glycol [24, 102, 103, 181]. We envision a micro-particulate system that could be suspended in these scaffolds and entrapped along with cells during the initial cell encapsulation step. Further, a mixture of micro-particles comprised of different polymer molecular weights and PEG/PLA polymer ratios could be utilized to release different growth factors at different rates from the same tissue engineering scaffold. Together, this would lead to a versatile method for the delivery of combinations of growth factors at designed rates from tissue engineering scaffolds. Richardson, et al. have demonstrated the importance of a tunable growth factor delivery system with the dual delivery of VEGF and PDGF from a hydrophobic PLGA matrix [14]. This system involves the fabrication of a porous PLGA scaffold that has VEGF loaded into the pores of the scaffold and PDGF loaded into the polymer bulk. As a result, VEGF is released quickly (1.7 pmol/day) and early after implantation, while PDGF is released later at a

slower rate (0.1 pmol/day). This is ideal for both the initiation of blood vessel formation (VEGF) and for their maturation through stabilization by smooth muscle cells (PDGF), and thus significantly increases the rate of formation of a vascular network.

In this study, we investigated the release of VEGF from micro-particulate IPNs prepared by a modified Prolease® strategy (Figure 6-1). This strategy involves the atomization of a polymer solution through an ultrasonic nozzle followed by rapid freezing of the polymer particulates in cold hexane. During the atomization step, the polymer solution is exposed to UV radiation in order to initiate cross-linking. The cold hexane is a non-solvent for PLA, PEG, and protein, but is miscible with the solvents used to fabricate the microparticles, benzyl benzoate (T_m = 21°C) and benzyl alcohol (T_m = -15°C). Thus, as the temperature of the frozen particles is slowly increased, the benzyl benzoate and benzyl alcohol slowly thaw and are extracted into the hexane phase. By this technique cross-linked PEG/PLA IPNs without benzyl benzoate and benzyl alcohol are prepared, which should provide greater control over the release kinetics, namely a decrease in the initial burst of protein release. Using this technique, microparticulate IPNs comprised of various ratios of PEGDMA and PLA were fabricated and analyzed for their ability to deliver VEGF at different release rates.

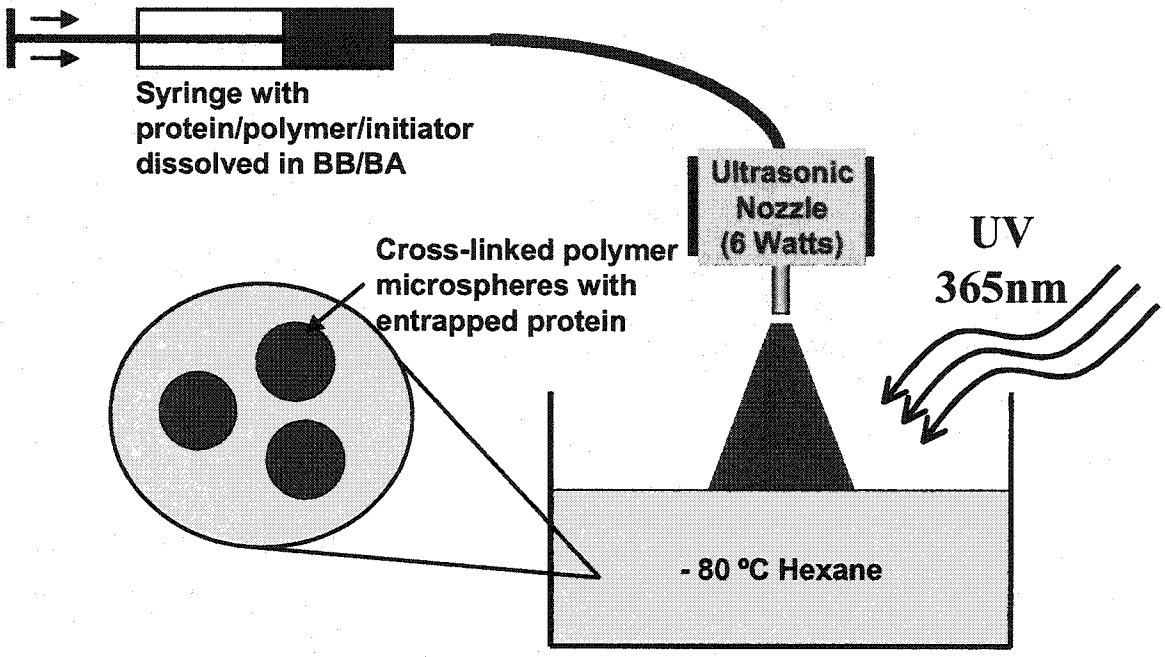


Figure 6-1: Preparation of PEG/PLA IPN microparticles using a modified Prolease® strategy. Atomized polymer/protein/photo-initiator solution is exposed to UV radiation and frozen in cold hexane. Slow increase in hexane temperature results in extraction of benzyl benzoate and benzyl alcohol into hexane leaving behind cross-linked polymer microspheres with entrapped protein.

6.2 Materials and Methods

6.2.1 Materials

Poly(DL-lactic acid) with an inherent viscosity of 0.36 dL/g (38 kD) was purchased from Birmingham Polymers, Inc. (BPI, Birmingham, AL). Poly(ethylene glycol) dimethacrylate MW=550 Da, anhydrous benzyl alcohol 99.8%, benzyl benzoate 99+%, 2',4'-dimethoxy-2-phenylacetophenone 98% (Igracure 651), hexanes (HPLC grade), tetrahydrafuran (HPLC grade), and NaN_3 were all obtained from Aldrich (Milwaukee, WI). Physiological phosphate buffered saline (10 mM PBS, 138 mM NaCl, 2.7 mM KCl,

pH=7.4) and bovine serum albumin (BSA, fraction V) were purchased from Sigma (St. Louis, MO). Sodium hydroxide (NaOH, 1N) was obtained from VWR Scientific Products (Plainfield, NJ). Recombinant human vascular endothelial growth factor (VEGF) expressed in *E. Coli* was purchased from PeproTech Inc (Rocky Hill, NJ). The Bio-Rad DC protein assay was purchased from Bio-Rad Life Sciences (Hercules, CA). The Quantikine® human VEGF immunoassay was purchased from R&D Systems, Inc. (Minneapolis, MN).

6.2.2 Fabrication of Cross-Linked PEG/PLA Micro-Particles

Cross-linked micro-particles of PEGDMA and PLA were prepared using a modified Prolease® strategy (Figure 6-1). PLA (38 kD) and PEGDMA (550 Da) were dissolved at various ratios of PEG/PLA at a total polymer concentration of 300 mg/ml in a 95/5 mixture of BB/BA. To these solutions, Igracure 651 photo-initiator (4% w/w), VEGF (13 ng/mg polymer), and/or BSA as a carrier for VEGF (5% w/w) were added. These solutions were atomized through an ultrasonic nozzle (Sonotek, model #8700-120, Milton, NY) at a volumetric flow rate of 0.8 ml/min and a power of approximately 6.0 Watts. The atomized stream was exposed to UV radiation at 365nm before being immediately frozen in a non-solvent bath containing hexane (approximately 400ml) maintained at -80°C. The microparticles were placed at -80 °C for 1 day to ensure freezing of the microparticles. After one day, the particle suspension was stirred to break up any conglomerates of microparticles and the mixture was placed at –20 °C where the BB/BA solvent began to slowly thaw and was extracted into the hexane non-solvent.

After 5 days the microparticles were placed at 4 °C and stirred continuously to extract any remaining BB/BA for 5 days. On the fifth day, one gram of Igracure 651 was dissolved in the hexane non-solvent and the microparticle suspension was exposed to UV radiation at 365 nm for 30 minutes with stirring. Approximately 100 ml of absolute ethanol was then added to extract any unreacted PEGDMA and spent photo-initiator from the microparticles. After 1 day, the microparticles were filtered in a dry ice box and lyophilized to remove residual solvent. After lyophilization, a free-flowing powder was produced that did not dissolve in aqueous or organic solvent suggesting successful cross-linking of the microparticulates.

6.2.3 Size Characterization of PEG/PLA Micro-Particles

Microparticles of PEG/PLA IPNs were imaged using bright field light microscopy. Microparticles were resuspended in cold physiological PBS, pH = 7.4 containing 0.05% NaN_3 and ultrasonicated using a Branson Sonifier 450 ultrasonic probe (10 pulses, 10% duty, output=1). In order to observe the cross-linked network of the PEG/PLA IPNs, microparticles were resuspended in BB/BA. This allowed for the dissolution and extraction of the PLA component and imaging of the remaining cross-linked PEG network. The particles were imaged using an inverted Nikon TE200 microscope with a Nicon 10X objective. The images were captured using a Hamamatsu digital camera (model# C4742-98) and Metamorph 4.6r3 software.

6.2.4 *Release of protein from Cross-Linked PEG/PLA Micro-Particles*

Release of protein from the PEG/PLA IPN microparticles was investigated by suspending 10 mg of microparticles in 1 ml of physiological PBS, pH = 7.4 containing 0.05% NaN_3 as an antibacterial and 0.25% BSA as a stabilizer for released VEGF (omitted when BSA added as a carrier for VEGF). Ultrasonication with a Branson Sonifier 450 ultrasonic probe (10 pulses, 10% duty, output=1) was utilized to uniformly disperse the microparticles at the onset of the release study. The microparticles were then incubated at 37 °C in microcentrifuge tubes mounted on a rotating shaker. After various time-points, the release media was centrifuged to pellet the microparticles and the media was removed and stored at –20 °C until further analysis. Fresh PBS was added at each time-point to resuspend the microparticles and continue the release study. At the end of the study the microparticles were degraded by the addition of 1 N NaOH to verify the total amount of loaded protein. The release rate of VEGF was quantified using a Quantikine® human VEGF immunoassay per the manufacturers instructions. Triplicate samples were measured and expressed as an average plus or minus one standard deviation. Results were presented as an accumulative release of VEGF at each time point compared to blank microparticles containing no protein. The release rate of the carrier protein, BSA, was quantified using the BioRad DC colorimetric assay as described in chapter 5. Triplicate samples were measured and expressed as an average plus or minus one standard deviation. Results were presented as a percentage of BSA released at each time point compared to blank microparticles.

6.3 Results

6.3.1 Fabrication and Initial Testing of Cross-Linked PEG/PLA Microparticles

Microparticulate IPNs comprised of 40/60, 50/50, and 60/40 ratios of 550 Da PEGDMA and 38 kD PLA were successfully obtained utilizing a modified Prolease® technique. Microparticulate formulations containing less than 40% PEG or greater than 60% PEG were unsuccessful in producing microparticles. In these cases, a cloudy immiscible liquid was obtained during the 4 °C extraction. For formulations containing less than 40% PEG, the PLA concentration in the frozen microparticulates may be high enough to impede the extraction of the BB/BA solvent, whereas for formulations containing more than 60% PEG the concentration of PLA may have been insufficient to maintain the structural integrity of the microparticles. Successful formulations resulted in a free-flowing powder that did not fully dissolve in aqueous or organic solvent suggesting successful cross-linking of the microparticulates. The swollen microparticles ranged in size from 50 to 100 microns and were irregular in shape (Figure 6-2A). The irregularity may be due to difficulty in atomizing the high viscosity polymer solutions or to extraction of unreacted PEG domains from the microparticles. When the PLA component was extracted in BB/BA, a PEG network remained, demonstrating successful cross-linking of the microparticulate IPNs (Figure 6-2B)

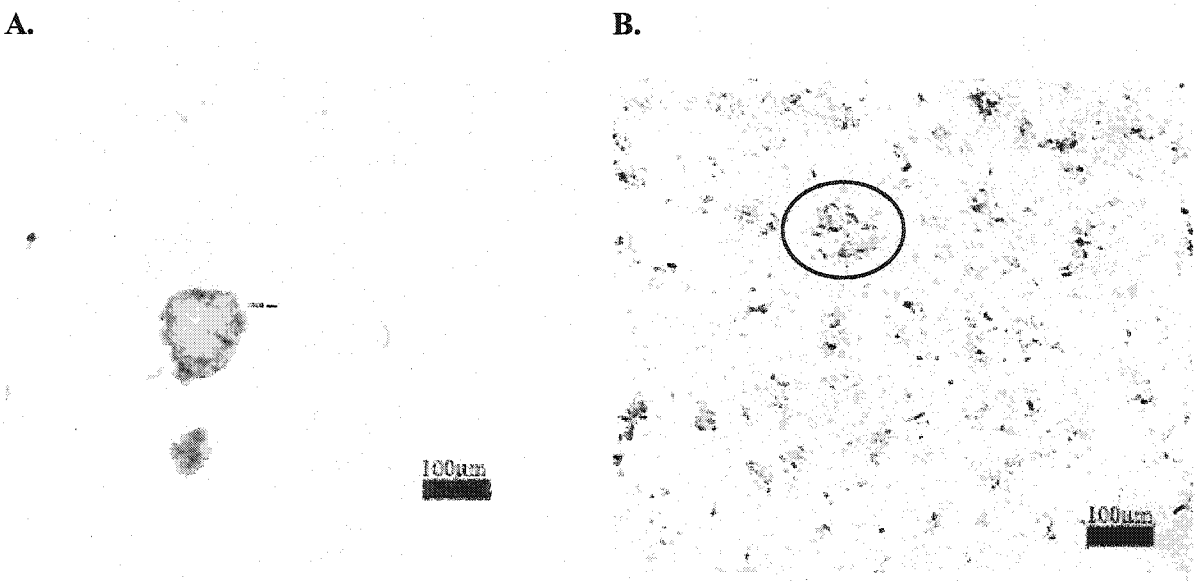


Figure 6-2: Microparticulate IPNs comprised of a 50/50 ratio of PEGDMA 550 Da and 38 kD PLA with 5% BSA loading resuspended in (A) PBS, pH=7.4 and (B) after extraction of PLA component in a 95/5 mixture of benzyl benzoate and benzyl alcohol. Image was taken using light transmittance microscopy at 10X magnification. Circle shows what appears to be remaining PEG network.

Microparticles comprised of a 50/50 ratio of 550 Da PEG and 38 kD PLA and loaded with 5% (w/w) BSA were placed into physiological PBS, pH=7.4 and analyzed for their ability to control the release of BSA (Figure 6-3). This microparticle formulation provided very good control over the rate of release of BSA with a dramatic decrease in the extent of the initial burst of protein (~15%). This improved initial burst is presumably due to the removal of the BB/BA component from the release system during microparticle fabrication. This moderate burst was followed by a slow linear release of BSA for the 5 day period of the study. Higuchi model analysis of the release profile demonstrated linear release over the entire 5 day period, suggesting that the protein release is diffusional in nature (Figure 6-4).

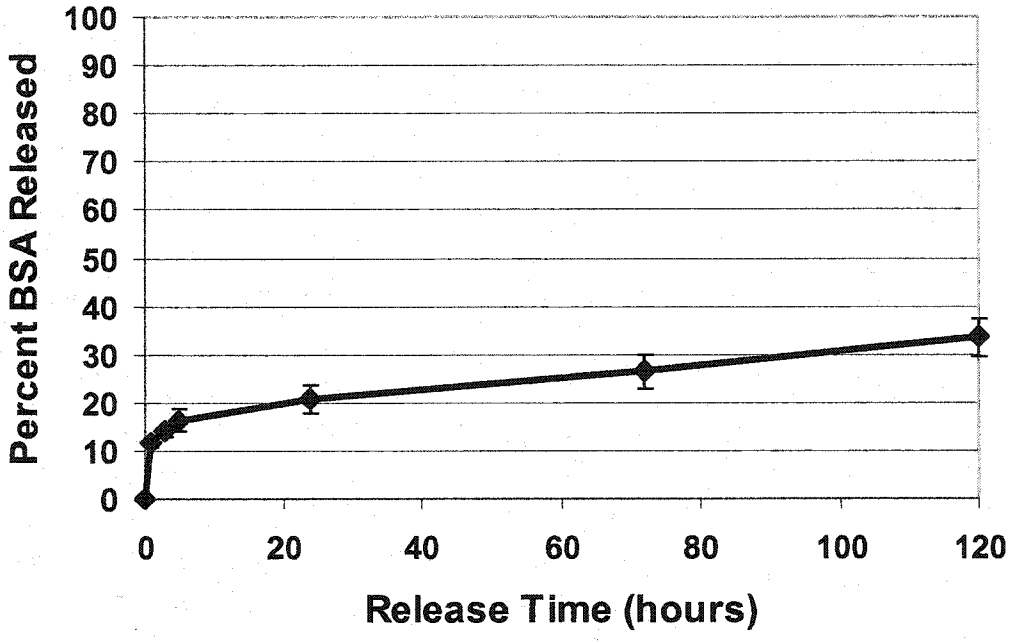


Figure 6-3: Release profile of BSA from IPN microparticles comprised of a 50/50 ratio of 550 Da PEG and 38 kD PLA containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C.

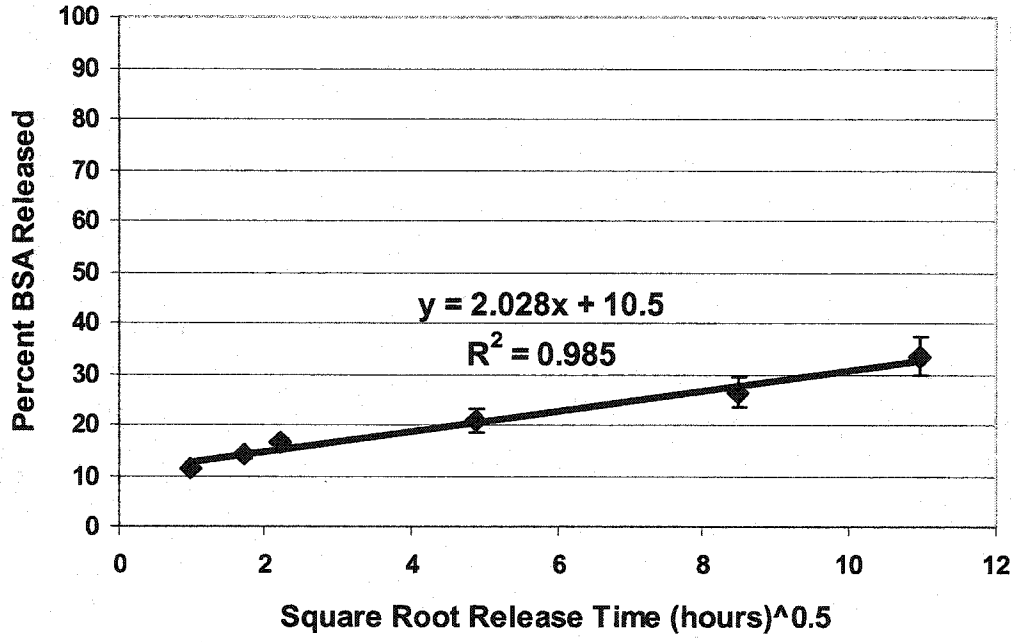


Figure 6-4: Higuchi model fits of release of BSA from 50/50 ratio of 550 Da PEG and 38 kD PLA microparticle IPNs containing 5% (w/w) BSA placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at 37°C.

6.3.2 Release of VEGF from Cross-Linked PEG/PLA Microparticles

IPN microparticles comprised of a 50:50 ratio of 550 Da PEG and 38 kD PLA have been shown to successfully control the release of a model protein BSA. Thus, this particle formulation was examined for its ability to control the release of VEGF. VEGF was added to the microparticle IPNs at a loading of 13ng of VEGF/mg of polymer. These microparticles were placed into physiological PBS, pH=7.4 containing 0.05% NaN_3 as an antibacterial and 0.25% BSA as a VEGF stabilizer. The release of VEGF was analyzed over a 5 day period using an ELISA assay. VEGF was released very slowly from the microparticles with only 50 pg of VEGF released during the first day (Figure 6-

5). After the 5 day study, only 60 pg of VEGF was released from the microparticulate system.

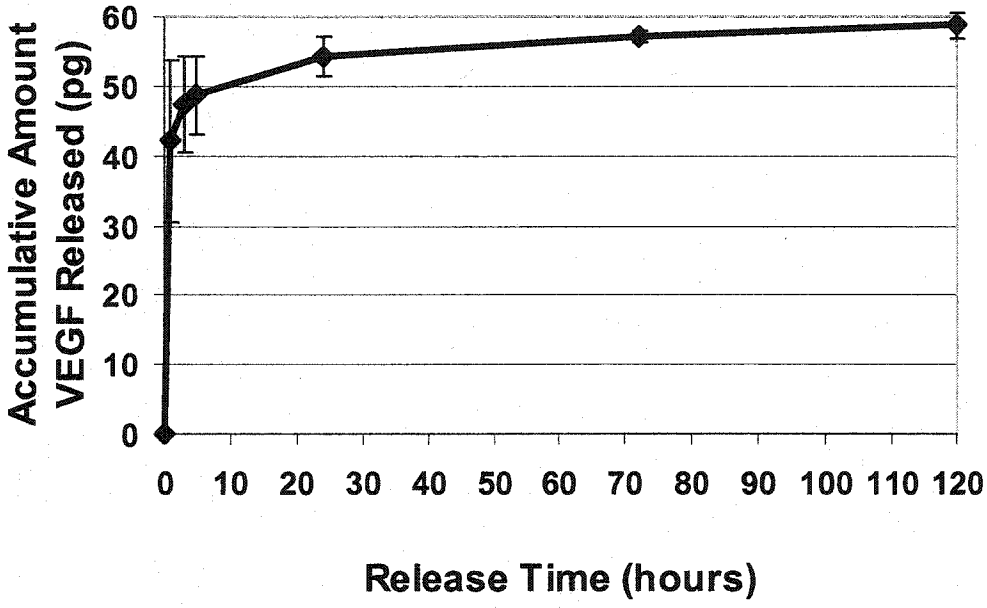


Figure 6-5: Release profile of VEGF from IPN microparticles comprised of a 50/50 ratio of 550 Da PEG and 38 kD PLA containing VEGF (13 ng/mg polymer) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% NaN_3, and 0.25% BSA) at 37°C.

This release is much lower than expected and may be due to multiple factors. First, the VEGF may not be loaded efficiently into the microparticles due to difficulty of dissolution into BB/BA or due to extraction during solvent removal. To further investigate this possibility, a sample of the VEGF loaded microparticle IPNs was sent to AAA Laboratories (Mercer Island, WA) for amino acid analysis. It was found that VEGF was successfully entrapped in the microparticles at a loading of 0.13% (w/w). Another possibility is that the protein loading may not be sufficient to obtain reasonable

release rates since protein release rates decrease with a decrease in protein loading. Thus, BSA at a 5% (w/w) loading was added to the VEGF containing microparticle IPNs as a carrier for VEGF. These microparticles were placed into physiological PBS, pH=7.4 containing 0.05% NaN_3 as an antibacterial and the release of VEGF analyzed. Again, approximately 50 pg of VEGF was released during the first day of release followed by very little additional release (Figure 6-6). This suggests that a low protein loading is not responsible for the slow release of protein from the microparticle IPNs.

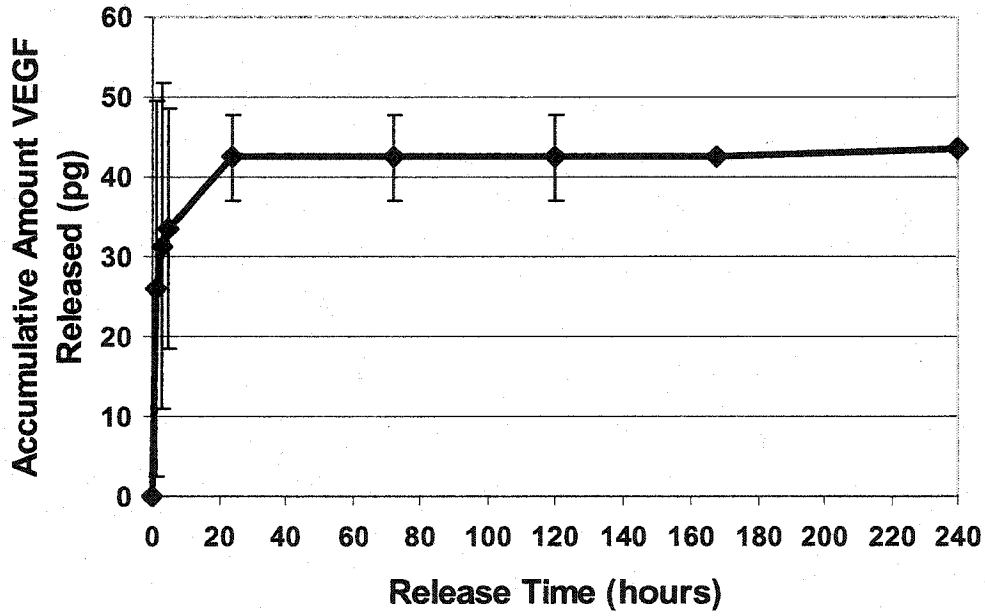


Figure 6-6: Release profile of VEGF from IPN microparticles comprised of a 50/50 ratio of 550 Da PEG and 38 kD PLA containing VEGF (13 ng/mg polymer) and BSA (5% w/w) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, and 0.05% NaN_3) at 37°C.

Since low protein loading was eliminated as a potential cause of low VEGF release, we hypothesized that the VEGF may be denatured during processing steps resulting in

lack of protein recognition by the immunoassay. Specifically, we believe that the benzyl alcohol in the formulation may be responsible for the denaturation of the VEGF. To this end, we prepared microparticle IPNs comprised of a 50:50 ratio of 550 Da PEG and 38 kD PLA in 100% benzyl benzoate without the addition of benzyl alcohol. The microparticle IPNs were loaded with VEGF (13 ng/mg polymer) and BSA (5% w/w). The microparticles were placed into physiological PBS, pH=7.4 containing 0.05% NaN_3 as an antibacterial and the release of VEGF analyzed. Again, the VEGF release was characterized by approximately 50-60 pg of VEGF being released during the first day (Figure 6-7). However, in contrast to previous studies, the VEGF release continued gradually throughout the 10 day release study with approximately 100 pg released after 10 days. This result suggests that benzyl alcohol may play a small role in VEGF stability, but the release of VEGF was still much lower than expected.

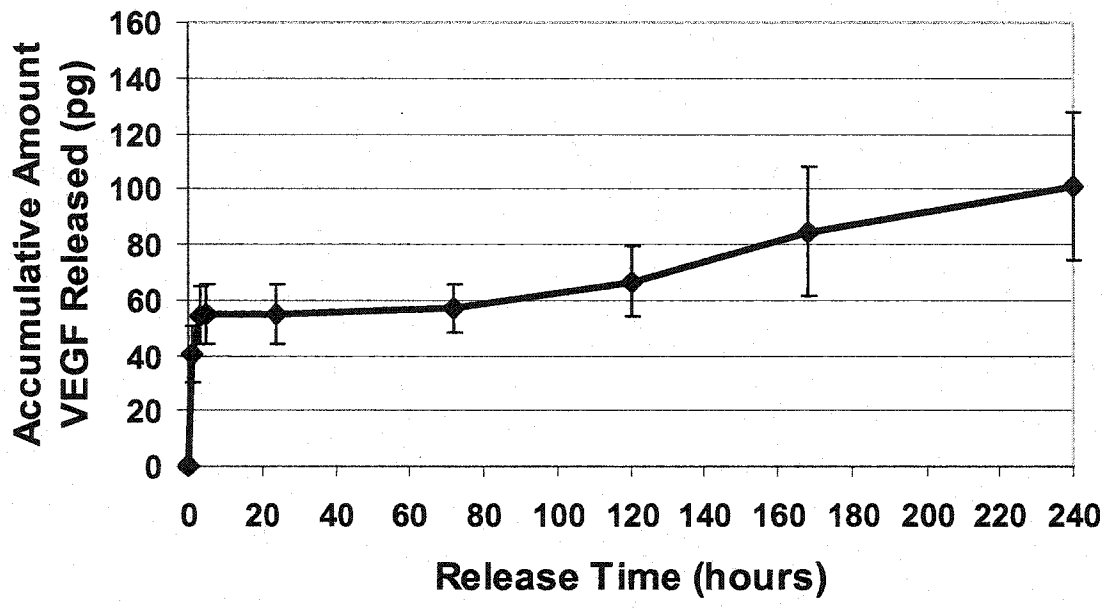


Figure 6-7: Release profile of VEGF from IPN microparticles comprised of a 50/50 ratio of 550 Da PEG and 38 kD PLA fabricated without benzyl alcohol. Particles loaded with VEGF (13 ng/mg polymer) and BSA (5% w/w) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, and 0.05% NaN_3) at 37°C.

As a final attempt to investigate the low release of VEGF from the PEG/PLA microparticle IPNs, microparticles comprised of PEG/PLA ratios of 40:60, 50:50, and 60:40 in benzyl benzoate without benzyl alcohol were examined for the release of both VEGF (13 ng/mg of polymer) and BSA (5% w/w). This study was to test whether the low release of VEGF from the microparticles was due to denaturation or naturally slow release of VEGF. If VEGF release is naturally slow, an increase in PEG content of the microparticle IPNs should result in an increase in VEGF release. Further, the percent release of VEGF can be checked against the release of BSA to verify relative percent release of the two proteins, which should be similar. The microparticles were placed

into physiological PBS, pH=7.4 containing 0.05% NaN_3 as an antibacterial and the release of VEGF and BSA analyzed.

The release of VEGF from the 50:50 PEG/PLA IPN microparticles was once again demonstrated to be low with approximately 50-60 pg released in the first day followed by a slow gradual release (Figure 6-8). An increase in VEGF release was observed when the PEG/PLA ratio is increased to 60:40 with approximately 120 pg of VEGF released. This is as expected due to the more hydrophilic nature of this formulation. However, when the PEG/PLA ratio was decreased to 40:60, the amount of VEGF released dramatically increased with approximately 1.2 ng of VEGF released within the first day (20-fold increase). Further, during the remaining 10 days of the study, another 150 pg of VEGF was released which is greater than the total VEGF release of any other formulation studied. This unexpected result may be due to aggregation of VEGF on the more hydrophobic surface of this formulation, which is then quickly released during the release study. However, this effect would also be seen in the BSA release profile. Instead, this result may suggest that the cross-linking chemistry utilized may be detrimental to the bioactivity of the encapsulated proteins. The vinyl density of this formulation is lower than that seen in the other formulations and may correlate with the increase in VEGF release.

Even though the release of VEGF is improved in this formulation (~1% released) it is still low in light of the amount of BSA release observed (Figure 6-9). PEG/PLA IPN microparticles of all three ratios released 30 to 40 percent of the entrapped BSA. Further,

the amount of BSA released increases with an increase in hydrophilicity of the IPN microparticle as afforded by an increase in the PEG/PLA ratio as expected.

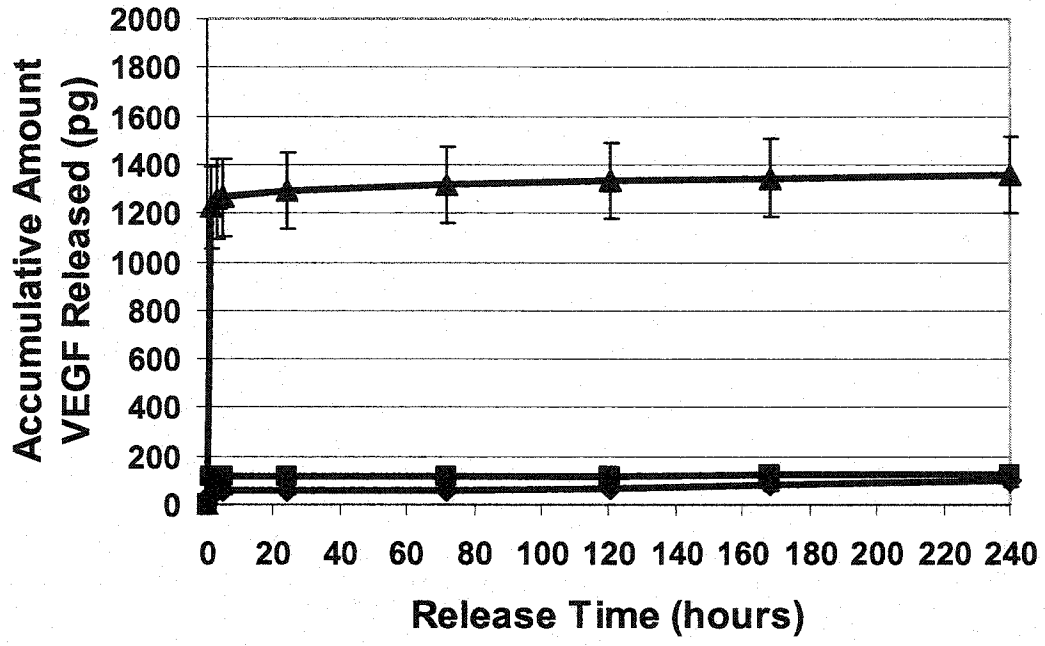


Figure 6-8: Release profile of VEGF from IPN microparticles comprised 550 Da PEG and 38 kD PLA fabricated without benzyl alcohol. Particles loaded with VEGF (13 ng/mg polymer) and BSA (5% w/w) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, and 0.05% NaN_3) at 37°C. PEG/PLA ratios of 40:60 (▲), 60:40 (■), and 50:50 (♦).

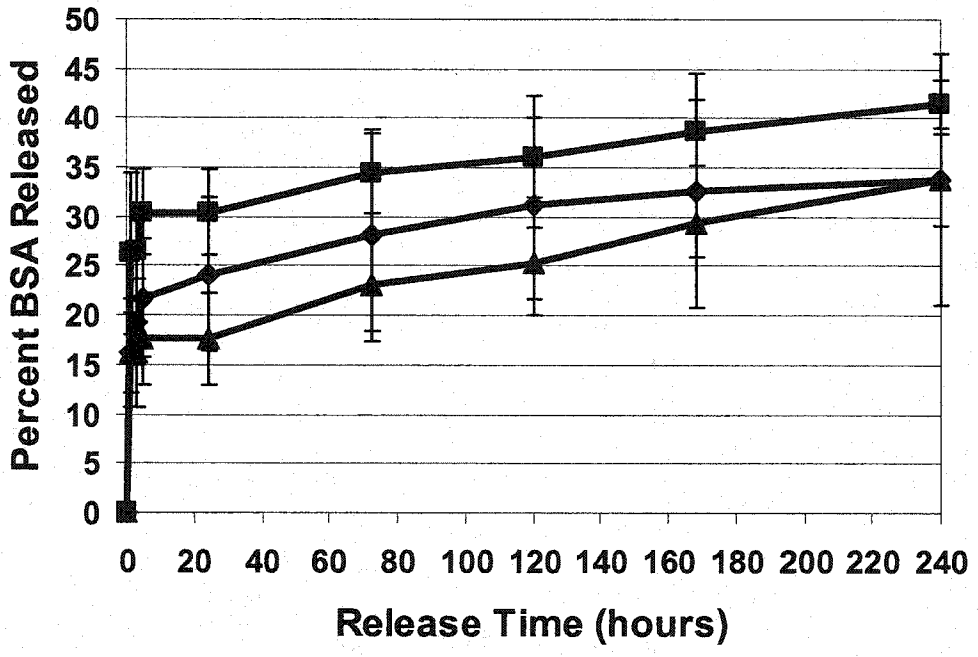


Figure 6-9: Release profile of BSA from IPN microparticles comprised 550 Da PEG and 38 kD PLA fabricated without benzyl alcohol. Particles loaded with VEGF (13 ng/mg polymer) and BSA (5% w/w) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, and 0.05% NaN_3) at 37°C. PEG/PLA ratios of 60:40 (■), 50:50 (♦), and 40:60 (▲).

Higuchi analysis of the BSA release from the PEG/PLA IPN microparticles with various ratios of PEG/PLA is shown in Figure 6-10. Interestingly, the slope is nearly identical with all three PEG/PLA ratios. This suggests that the differences in BSA release observed with these microparticles may be due to an increase in initial burst and not due to a change in release rate. Thus, unreacted PEGDMA may be acting as a pore former that aids in the initial release of protein followed by a constant release rate regardless of the initial composition. It may also be true that the difference in the

PEG/PLA ratio of the formulations presented here are not large enough to see a change in release rate.

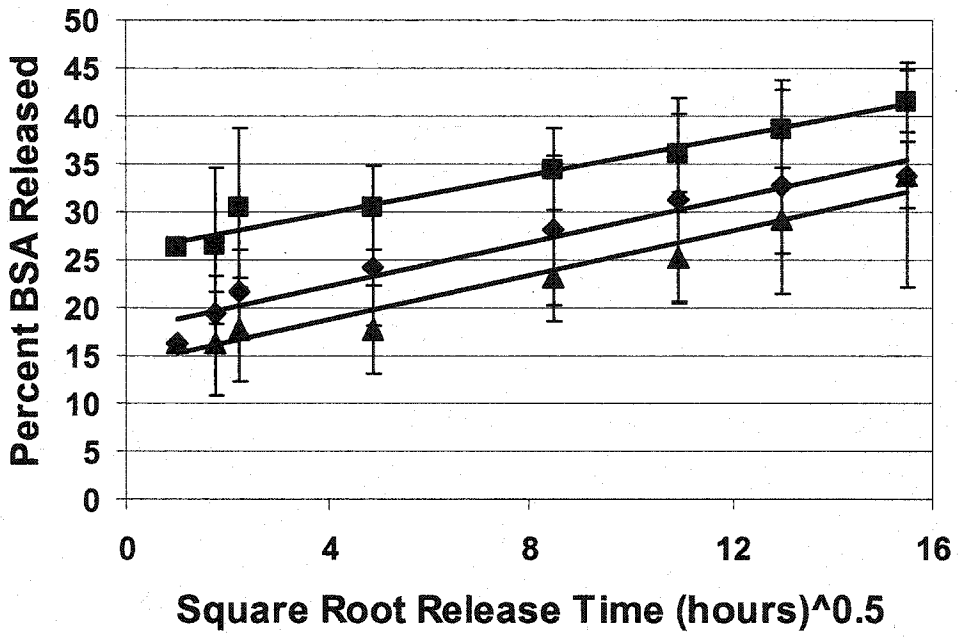


Figure 6-10: Higuchi model fits of release of BSA from IPN microparticles comprised 550 Da PEG and 38 kD PLA fabricated without benzyl alcohol. Particles loaded with VEGF (13 ng/mg polymer) and BSA (5% w/w) placed in release buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4, and 0.05% NaN_3) at 37°C. PEG/PLA ratios of 60:40 (■), 50:50 (♦), and 40:60 (▲).

6.3.3 Microscopic Analysis of Cross-Linked PEG/PLA Microparticles

To further characterize the 550 Da PEG and 38 kD PLA microparticle IPNs, light microscopy was utilized to image the microparticles at the various PEG/PLA ratios (Figure 6-11). In general, as the PEG/PLA ratio decreased from 60:40 to 40:60 the size and aggregation of the particles increased. Further, as the percent of PLA increased in the formulation, the microparticles appeared to be more irregular in shape. This suggests

that the increase in viscosity of the polymer solution may affect the uniformity of the microparticles during the atomization step. More uniform particles may be obtained by using lower molecular weight polymers or decreasing the polymer concentration to afford a less viscous solution for atomization.

Next, the PLA component was extracted into benzyl benzoate, in order to visualize the cross-linked PEG network of the PEG/PLA IPN microparticles. Qualitatively, more extensively cross-linked PEG networks were observed as the PEG/PLA ratio decreased from 60:40 to 40:60. This is contrary to expectations and suggests that the second cross-linking step after the 4°C extraction may be more important for the formation of the PEG network. In particles with higher PEG/PLA ratios, the unreacted PEG is easily extracted into the hexane bath, whereas in particles with lower PEG/PLA ratios the unreacted PEG can be entrapped in the PLA network and successfully cross-linked. Thus, unreacted PEG may act as a pore former in particles with higher PEG/PLA ratios leading to an increase in initial protein release.

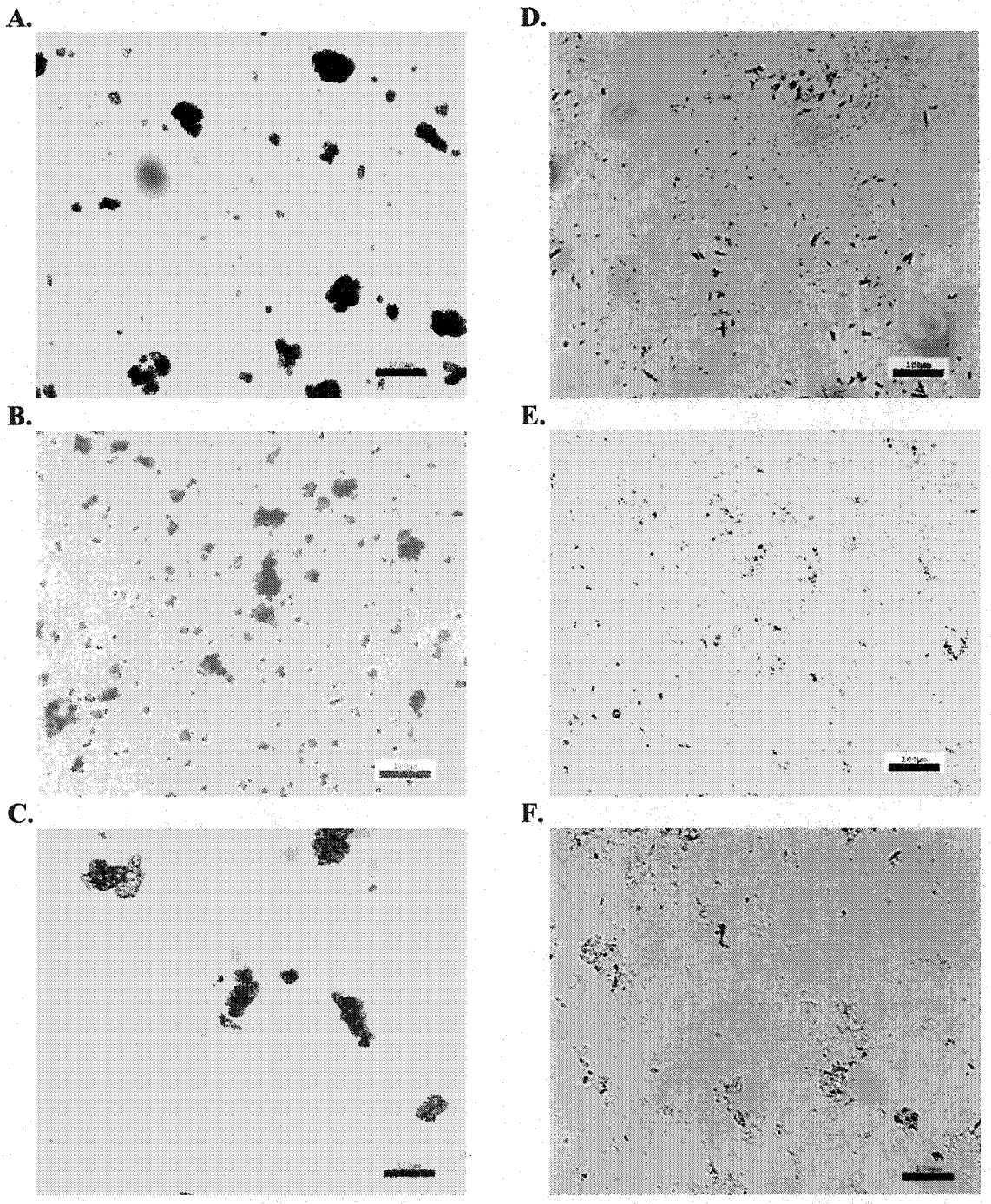


Figure 6-11: Microparticulate IPNs comprised of a various ratio of PEGDMA 550 Da and 38 kD PLA loaded with VEGF (13ng/mg polymer) and BSA (5% w/w) resuspended in (A-C) PBS, pH=7.4 and (D-F) after extraction of PLA component into benzyl benzoate. Image was taken using light transmittance microscopy at 10X magnification. PEG/PLA ratios of 60:40 (A,D), 50:50 (B,E), and 40:60 (C,F). Scale bar = 100μm

6.4 Discussion

Cross-Linked microparticulate IPNs of 550Da PEG and 38 kD PLA were successfully fabricated at PEG/PLA ratios between 40/60 and 60/40 using a modified Prolease® strategy. Microparticles with more than 60% PEG or less than 40% PEG were not obtained due to the formation of a cloudy immiscible phase. For formulations containing lower amounts of PEG, the concentration of PLA may be high enough to impede the efficient removal of the benzyl benzoate solvent causing aggregation of the thawed microparticles. At higher PEG percentages, unreacted PEGDMA in the system may cause aggregation of the thawed microparticles. PEG may also play a role in limiting the extraction of benzyl benzoate from the particles, in that formulations containing a higher PEG molecular weight (3400 Da) were unsuccessful due to the formation of the same cloudy immiscible phase. It may be possible to prepare PEG/PLA IPN microparticles at other PEG/PLA ratios by using lower molecular weight PLA. This will decrease the viscosity of the polymer solution and should aid in the extraction of benzyl benzoate from the microparticles.

The PEG/PLA IPN microparticles were examined for their ability to control the rate of release of VEGF with desired release rates of approximately 10 ng/day. VEGF is a potent biomolecule capable of initiating angiogenic events at concentrations as low as 1 ng/ml [114]. However, the release of VEGF from the 50/50 PEG/PLA IPNs was disappointingly low with only 60 pg of total VEGF release. Amino acid analysis verified that the low amount of release was not due to an insufficient loading of VEGF in the microparticles (0.13%). However, this level of loading would require approximately 50

mg of PEG/PLA IPN microparticles in order to provide sufficient levels of VEGF to release 10 ng of VEGF per day for a period of 7 days. This is equivalent to approximately 0.25 cm^3 of microparticles. Thus, the volume of a tissue engineering scaffold with dimensions of 1 cm X 1cm X 0.25 cm would consist almost entirely of microparticles. Obviously, techniques to increase the protein loading of the microspheres while maintaining reasonable fabrication costs must be investigated.

Next, a carrier protein, BSA, was added to increase the protein loading of the microparticles in an attempt to increase the release rate of VEGF; however, no increase in the amount of VEGF released was observed. This further suggests that the low release rate is not due to a low loading of protein in the IPN microparticles. It follows, that the formulation strategy utilized may be detrimental to the bioactivity of the entrapped VEGF. Thus, we hypothesized that the benzyl alcohol co-solvent may be leading to denaturation of VEGF. Thus, microparticles were prepared utilizing only benzyl benzoate and tested for their ability to release VEGF. A slight increase in VEGF release was observed, suggesting that benzyl alcohol may play a small role in the denaturation of VEGF. However, the release is still much lower than expected and other factors may be leading to the denaturation of the entrapped VEGF. Another possibility is that the hydrophobicity of the formulation may contribute to the loss of bioactivity. To this end, microparticles with various PEG/PLA ratios were examined. The release of VEGF from the 60:40 PEG/PLA microparticles was approximately twice that of the 50:50 formulation. This is as expected as the swelling and diffusion in these particles should be increased due to the higher hydrophilicity of this formulation. However, this release is

still much lower than expected. Unexpectedly, the 40:60 PEG/PLA formulation demonstrated a 20-fold increase in VEGF release with 1.2 ng of protein release within the first day. This was followed by an additional 150 pg of VEGF release, which was higher than that seen in any other formulation. This release may be due to surface aggregation of VEGF on the more hydrophobic 40/60 PEG/PLA microparticles; however, this extreme increase in VEGF release would be mirrored by an increase in the release of BSA from this formulation. It is more probable that the cross-linking chemistry is denaturing the entrapped VEGF and that the lower vinyl density of this formulation, due to the decrease in PEGDMA added, is allowing for an increase in VEGF bioactivity. VEGF contains numerous disulfides that are necessary for maintaining the tertiary and quaternary structures of the protein. Thiols are known scavengers for free radicals, thus thiol groups on VEGF may react with free radicals in solution, leading to a decrease in VEGF bioactivity. Even though the amount of VEGF released is much greater in this formulation, it is still less than anticipated. Further, sustained release of nanogram levels of VEGF is desired. In order to improve the release and stability of VEGF in the PEG/PLA IPN microparticles, known VEGF stabilizers such as trehalose or heparin could be added to the formulation [160]. Further, increases in release rates could be achieved by utilizing lower molecular weight PLA, which are known to degrade faster and release entrapped molecules at a greater rate.

Analysis of the release of BSA from the PEG/PLA IPN microparticles with various ratios of PEG/PLA demonstrated an increase in the amount of BSA release with an increase in PEG/PLA ratio. This is as expected due to an increase in swelling and

diffusion anticipated with the more hydrophilic formulation. Approximately 30 to 40 percent of total BSA release was seen in these studies. This is in sharp contrast to the approximately 1% release of VEGF seen above. This further suggests that the low release of VEGF is due to denaturation of VEGF and not due to slow release kinetics. Interestingly, Higuchi analysis of the BSA release profiles provides linear plots with nearly identical slopes. Thus, the release appears to be diffusional in nature, but the release rate is independent of the particle composition. This suggests that the change in PEG/PLA ratio may only have an effect on the initial burst of protein release from the scaffold. This could be the case if unreacted PEG is acting as a pore former in the formulation. Thus, formulations with a higher content of PEG would have more surface area for initial protein release. PEG/PLA IPN microparticulates with various release rates can still be achieved using PLA of various molecular weights. The release of growth factor should increase with a decrease in PLA molecular weight. Thus, the initial burst of growth factor release could be modulated using the PEG/PLA ratio and the underlying release rate finely tuned using the PLA molecular weight. This strategy should provide a versatile protein delivery strategy for many applications including the delivery of growth factors in tissue engineering, vaccine development, and other protein therapeutics.

Chapter 7: Conclusions and Future Directions

General Conclusions:

- Hydrogels with improved release characteristics can be fabricated using physical and chemical cross-linking techniques.
- Unique PEG/PLA IPNs allow for modulation of protein release rates by manipulating the hydrophobic/hydrophilic balance.
- PEG/PLA microparticulates may provide a versatile growth factor delivery system in tissue engineering.

Future Directions:

- Stabilization of VEGF in microparticulates using sugars, such as trehalose or mannitol, or VEGF affinity molecules, such as heparin.
- Further adjustment of protein release rate from microparticulates by varying the PLA molecular weight.
- Coencapsulation of growth factor loaded microparticulates with cells in a tissue engineering construct.

The ability to deliver proteins in a stable and controlled manner is an important technology that may have a great impact on the field of tissue engineering as well as in the development of protein therapeutics and vaccines. With the completion of the sequencing of the genome an abundance of therapeutic proteins and their targets of action

are likely to be discovered. The ability to deliver these potent biomolecules to local targets in a stable manner will be vital to the successful treatment of many diseases. Local delivery will be necessary in order to diminish the toxic side effects of systemic delivery of these important proteins. Further, controlled delivery formulations will be necessary to provide chemotactic gradients necessary for some cellular processes, while sustained delivery devices will be ideal for increased patient comfort and compliance.

In this dissertation we have focused on the controlled delivery of growth factors for the advancement of tissue engineering strategies. A variety of growth factors are available for the manipulation of various cell functions. However, the success of these important biomolecules is dependent on the amount delivered to the cell and the timing of their delivery. Important processes such as inflammation, wound healing and associated angiogenesis, immune regulation, and cellular differentiation and proliferation may be mediated when these factors are delivered in an appropriate manner. Many traditional tissue-engineering scaffolds, such as poly(lactide-co-glycolide), are relatively hydrophobic in nature and require processing conditions that can denature proteins. Further, protein delivery systems fabricated from these polymers result in non-uniform protein distribution and lead to non-ideal release characteristics. Hydrogels provide a more stable environment for proteins; however, they swell rapidly and release entrapped biomolecules quickly. It was our hypothesis that proteins could be delivered in a controlled and stable manner by combining appropriate ratios of hydrophobic and hydrophilic polymers in cross-linked networks, thus promoting beneficial biological functions. In this work, synthetic and natural polymers were utilized to develop

physically or chemically cross-linked protein delivery systems for the controlled delivery of growth factors.

7.1 Chitosan/Glycerol Matrices: Improved Protein Release from Hydrogels

First, a physically cross-linked system based on chitosan was developed for the controlled delivery of PEG-(GM-CSF). The swelling and protein release rates of this system were found to increase with an increase in the amount of the physical cross-linking agent, glycerol. Further, the release of PEG-(GM-CSF) could be further modulated by adjusting the molecular weight of the conjugated PEG molecule and thus altering the diffusional properties of the protein. Glycerol was found to act as a hydrogen bonding agent with the chitosan amines, leading to their deprotonation and allowing for the removal of residual acetic acid. This had the overall effect of increasing the hydrophobicity of the chitosan matrix resulting in the decreased swelling and protein release rates observed. Due to the structural similarities between glycosaminoglycans and chitosan and the accelerated wound healing affects of chitosan and GM-CSF, this formulation may be useful as a wound dressing. Further, due to the mucoadhesive properties of chitosan, chitosan/glycerol formulations may be interesting for the oral delivery of anionic proteins. Finally, due to the slower release of highly PEGylated proteins from the chitosan/glycerol matrix, the rate of release of protein therapeutics could possibly be modulated using degradable PEG linkages. Thus, the PEGylated protein would be released from the scaffold at release rates dictated by the rate of cleavage of the PEG linkages. Although, chitosan may be an interesting matrix for the

delivery of acidic proteins, the delivery of cationic proteins will require different formulation strategies. To this end, chemically cross-linked scaffolds of hyaluronic acid were prepared.

7.2 Hyaluronic Acid Hydrogels: Photo-Crosslinkable Cell Scaffolds

Methacrylate moieties were introduced on the backbone of hyaluronic acid after modification with glycidyl methacrylate. Glycidyl methacrylate modified hyaluronic acid (HA-GMA) of various degrees of methacrylation was developed. These scaffolds were easily cross-linked using photochemical techniques. Although the mechanical strength of these scaffolds was greatly increased with an increase in methacrylation density, the matrices proved inadequate for controlling the rate of protein release. Protein release from these scaffolds was characterized by a large initial burst of protein release with complete release within five days. However, the diffusional and mechanical properties of these matrices, along with the biological properties of HA, may make them ideal for the photo-encapsulation of cells for tissue engineering and regeneration strategies. The results of these studies suggest that some hydrophobic character may be desirable in hydrogel protein delivery devices in order to counteract the rapid rate of swelling of the hydrogel.

7.3 PEG/PLA IPNs: A Versatile and Tunable Protein Delivery System

In order to prepare a versatile protein delivery formulation with tunable release rates, chemically cross-linked interpenetrating networks of PEG dimethacrylate and

poly(lactide) were produced using photochemical techniques. Unique to this strategy was the utilization of a biocompatible benzyl benzoate/benzyl alcohol solvent mixture as a co-solvent for both of the polymers and the protein. This allowed for uniform dispersion of the protein throughout the crosslinked PEG/PLA IPN. The swelling of the PEG/PLA IPN and the rate of release of BSA were shown to decrease as the hydrophobic nature of the IPN increased. Thus, by increasing the hydrophobic character of the IPN through increases in PLA molecular weight, PLA concentration, or a decrease in the PEG/PLA ratio, the rate of protein release could be decreased. However, due to the inability to remove the nonvolatile benzyl benzoate and benzyl alcohol from the IPNs, a large initial burst of BSA release was exhibited. Thus, a strategy to remove this solvent from the formulation is necessary in order to gain control over the release of protein. We utilized a modified Prolease® strategy to form microparticulates of our PEG/PLA IPNs. This strategy allowed for the extraction of the benzyl benzoate and benzyl alcohol from the formulation upon the slow thawing of the microparticles in a non-solvent hexane bath. The microparticles produced were shown to exhibit a marked decrease in the initial burst of BSA release. This was followed by a gradual, diffusional based release of BSA over the 10 day period of the study. The amount of BSA released was increased when the PEG/PLA ratio of the formulation was increased; however, this was independent of the rate of protein release. Essentially, the PEG/PLA ratio acted to modulate the initial burst of protein release, suggesting that unreacted PEGDMA may be acting as a pore former in the formulation. The underlying rate of protein release may still be modulated using this strategy by adjusting the molecular weight of the PLA in the IPN. Thus, the

initial burst of protein release could be modulated using the PEG/PLA ratio and the release rate adjusted using the PLA molecular weight. This may be interesting in the development of protein vaccines where an initial burst of protein release is required, followed by a lower secondary release of protein.

The PEG/PLA IPN microparticles were examined for their ability to control the release of VEGF. The release of this protein was much lower than expected possibly due to denaturation of the VEGF. Surprisingly, a large increase in VEGF release was seen when utilizing the 40:60 PEG/PLA IPN microparticles in spite of the increased hydrophobicity of this formulation. This suggests that the cross-linking strategy employed here may be detrimental to the entrapped VEGF, due to an increase in vinyl group density as the PEG/PLA ratio is increased. Several strategies may be pursued to prevent this denaturation. First, compounds with the ability to stabilize VEGF, such as trehalose or heparin, could be added to the formulation. Further, PLA of lower molecular weights could be utilized in order to prepare microparticles at lower PEG/PLA ratios. This would allow for further investigation into the effect of vinyl group density on VEGF stability and would provide a mechanism to increase the release rate of protein. In the end, a systematic study of each component and procedure during the development of the PEG/PLA IPN microparticles should be conducted in order to correct the cause of VEGF denaturation.

In summary, the PEG/PLA IPN microparticles provide a versatile protein delivery system with many variables (PLA molecular weight, PEG molecular weight, polymer concentration, and PEG/PLA ratio) to tune the rate of protein release. This system should

be applicable to a variety of different proteins with various delivery requirements. In addition, the microparticulate nature of this delivery system should allow for its easy incorporation into many tissue engineering strategies. For example, microparticles can be co-encapsulated in a tissue engineering scaffold with the cell type of interest in photo-crosslinkable matrices, such as the glycidyl methacrylate modified hyaluronic acid described above. The microparticles can be designed to release VEGF and PDGF at rates optimal for the development of vasculature surrounding the tissue engineering implant. Other strategies may involve the injection of various PEG/PLA microparticle formulations near the site of an implant to aid in tissue regeneration or to control inflammation. Thus, once issues of growth factor stability are dealt with through the addition of protein stabilizers, a versatile protein delivery system can be established.

Endnotes

1. Cleland, J.L., A. Daugherty, and R. Mrsny, *Emerging protein delivery methods.* Current Opinion in Biotechnology, 2001. **12**: p. 212-219.

2. Tabata, Y., *The importance of drug delivery systems in tissue engineering.* Pharmaceutical Science & Technology Today, 2000. **3**(3): p. 80-89.

3. Whitaker, M.J., *et al.*, *Growth factor release from tissue engineering scaffolds.* Journal of Pharmacy and Pharmacology, 2001. **53**: p. 1427-1437.

4. Babensee, J.E., L.B. McIntire, and A.G. Mikos, *Growth factor delivery for tissue engineering.* Pharmaceutical Research, 2000. **17**(5): p. 497-504.

5. Baldwin, S.P. and W.M. Saltzman, *Materials for protein delivery in tissue engineering.* Advance Drug Delivery Reviews, 1998. **33**: p. 71-86.

6. Murphy, W.L. and D.J. Mooney, *Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices.* Journal of Periodontal Research, 1999. **34**: p. 413-419.

7. Richardson, T.P., W.L. Murphy, and D.J. Mooney, *Polymeric delivery of proteins and plasmid DNA for tissue engineering and gene therapy.* Critical Reviews in Eukaryotic Gene Expression, 2001. **11**: p. 47-58.

8. Krewson, C.E. and M.W. Saltzman, *Transport and elimination of recombinant human NGF during long-term delivery to the brain.* Brain Research, 1996. **727**: p. 169-181.

9. Maquet, V. and R. Jerome, *Design of macroporous biodegradable polymer scaffolds for cell transplantation.* Material Science, 1997. **250**: p. 15-42.

10. Kaufman, P.M., *et al.*, *Highly porous polymer matrices as a three-dimensional culture system for hepatocytes.* Cell Transplantation, 1997. **6**(5): p. 463-468.

11. Mooney, D.J., *et al.*, *Localized delivery of epidermal growth factor improves the survival of transplanted hepatocytes.* Biotechnology and Bioengineering, 1996. **50**: p. 422-429.

12. Mooney, D.J., *et al.*, *Long-term engraftment of hepatocytes transplanted on biodegradable polymer sponges.* Journal of Biomedical Materials Research, 1997. **37**: p. 413-420.

13. Lee, H., *et al.*, *local delivery of basic fibroblast growth factor increases both angiogenesis and engraftment of hepatocytes in tissue-engineered polymer devices.* Transplantation, 2002. **73**(10): p. 1589-1593.

14. Richardson, T.P., *et al.*, *Polymeric system for dual growth factor delivery.* Nature Biotechnology, 2001. **19**: p. 1029-1034.

15. Sheridan, M.H., *et al.*, *Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery.* Journal of Controlled Release, 2000. **64**: p. 91-102.

16. Park, Y.J., *et al.*, *Controlled release of platelet-derived growth factor from porous poly(L-lactide) membranes for guided tissue regeneration.* Journal of Controlled Release, 1998. **51**: p. 201-211.

17. Murphy, W.L., D.H. Kohn, and D.J. Mooney, *Growth of continuous bonelike mineral within porous poly(lactide-co-glycolide) scaffolds in vitro.* Journal of Biomaterials Research, 2000. **50**: p. 50-58.

18. Whang, K., *et al.*, *Ectopic bone formation via rhBMP-2 delivery from porous bioabsorbable polymer scaffolds.* Journal Biomedical Materials Research, 1998. **42**: p. 491-499.

19. Rotter, N., *et al.*, *Cartilage reconstruction in head and neck surgery: comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage.* Journal of Biomedical Materials Research, 1998. **42**: p. 347-356.

20. Mann, B.K., R.H. Schmedlen, and J.L. West, *Tethered TGF-β increases extracellular matrix production of vascular smooth muscle cells.* Biomaterials, 2001. **22**: p. 439-444.

21. Mann, B.K., *et al.*, *Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering.* Biomaterials, 2001. **22**: p. 3045-3051.

22. Elisseeff, J., *et al.*, *Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks.* Journal of Biomedical Materials Research, 2000. **51**: p. 164-171.

23. Bryant, S. and K. Anseth, *The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels.* Biomaterials, 2001. **22**: p. 619-626.

24. Bryant, S. and K.S. Anseth, *Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels.* Journal of Biomedical Materials Research, 2002. **59**: p. 63-72.

25. Anseth, K., *et al.*, *In situ forming degradable networks and their application in tissue engineering and drug delivery.* Journal of Controlled Release, 2002. **78**: p. 199-209.

26. Lee, K.Y., *et al.*, *Controlled growth factor release from synthetic extracellular matrices.* Nature, 2000. **408**: p. 998-1000.

27. Kuo, C.K. and P.X. Ma, *Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate, and mechanical properties.* Biomaterials, 2001. **22**: p. 511-521.

28. Elcin, Y.M., V. Dixet, and G. Gitnick, *Controlled release of endothelial cell growth factor from chitosan-albumin microspheres for localized angiogenesis: in vitro and in vivo studies.* Artificial Cells, Blood Substitutes, and Immobilization Biotechnology, 1996. **24**(3): p. 257-271.

29. Park, Y.J., *et al.*, *Platelet derived growth factor releasing chitosan sponge for periodontal bone regeneration.* Biomaterials, 2000. **21**: p. 153-159.

30. Lee, J.-Y., *et al.*, *Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials.* Journal of Controlled Release, 2002. **78**: p. 187-197.

31. Kim, H.D. and R.F. Valentini, *Retention and activity of BMP-2 in hyaluronic acid-based scaffolds in vitro.* Journal of Biomaterials Research, 2002. **59**: p. 573-584.

32. Lisignoli, G., *et al.*, *Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic based polymer scaffold.* Biomaterials, 2001. **22**: p. 2095-2105.

33. Gao, J., *et al.*, *Tissue-engineered fabrication of an osteochondral composite graft using rate bone marrow-derived mesenchymal stem cells.* Tissue Engineering, 2001. **7**(4): p. 363-371.

34. Sakiyama-Elbert, S.E. and J.A. Hubbell, *Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix.* Journal of Controlled Release, 2000. **69**: p. 149-158.

35. Sakiyama-Elbert, S.E. and J.A. Hubbell, *Development of fibrin derivatives for controlled release of heparin -binding growth factors.* Journal of Controlled Release, 2000. **65**: p. 389-402.

36. Allemann, F., *et al.*, *Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds.* Journal of Biomedical Materials Research, 2001. **55**: p. 13-19.

37. Yamamoto, M., Y. Tabata, and Y. Ikada, *Growth factor release from gelatin hydrogel for tissue engineering.* Journal of Bioactive and Compatible Polymers, 1999. **14**: p. 474-489.

38. Gilding, D.G., *Biocompatibility of clinical implant materials*, in *Biodegradable Polymers*, D.F. Williams, Editor. 1981, CRC Press: Boca Raton. p. 209-232.

39. Hora, M.S., *Release of human serum albumin from poly(lactide-co-glycolide) microspheres.* Pharmaceutical Research, 1990. **7**: p. 1190-1194.

40. Baker, R., *Biodegradable Systems*, in *Controlled Release of Biologically Active Agents*. 1987, John Wiley & Sons: New York. p. 84-136.

41. Zhu, G., S.R. Mallery, and S.P. Schwendeman, *Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide).* Nature Biotechnology, 2000. **18**: p. 52-57.

42. Fu, K., A.M. Klibanov, and R. Langer, *Protein stability in controlled-release systems.* Nature Biotechnology, 2000. **18**: p. 24-25.

43. Molina, I., *et al.*, *Protein release from physically crosslinked hydrogels of the PLA/PEO/PLA triblock copolymer-type.* Biomaterials, 2001. **22**: p. 363-369.

44. Han, D.K., *et al.*, *Surface characteristics and biocompatibility of lactide-based poly(ethylene glycol) scaffolds for tissue engineering.* Journal of Biomaterials Science Polymer Edition, 1998. **9**(7): p. 667-680.

45. Tomihata, K. and Y. Ikada, *In vitro and in vivo degradation of films of chitin and its deacetylated derivatives.* Biomaterials, 1997. **18**(7): p. 567-575.

46. Brown, C.D. and A.S. Hoffman, *Modification of natural polymers: chitosan*, in *Methods of Tissue Engineering*, A. Atala and R. Lanza, Editors. 2002, Academic Press: San Diego. p. 565-573.

47. Horowitz, S.T., S. Roseman, and H.J. Blumenthal, *The preparation of glucosamine oligosaccharides. I. Separation.* Journal of American Chemical Society, 1957. **79**: p. 5046-5049.

48. Horton, D. and D.R. Lineback, *N-Deacetylation: Chitosan from chitin*, in *Methods in Carbohydrate Chemistry*, R.L. Whistler, J.N. BeMiller, and M.L. Wolfrom, Editors. 1965, Academic Press: New York and London. p. 403-406.

49. Sandford, P.A. and A. Steinnes, *Biomedical applications of high-purity chitosan*, in *Water-Soluble Polymers: synthesis, solution properties, and applications*, S.W. Shalaby, C.L. McCormick, and G.B. Butler, Editors. 1991, American Chemical Society: Washington, DC. p. 430-445.

50. Onishi, H. and Y. Machida, *Biodegradation and distribution of water-soluble chitosan in mice.* Biomaterials, 1999. **20**: p. 175-182.

51. Pangburn, S.H., P.V. Trescony, and J. Heller, *Lysozyme degradation of partially deacetylated chitin, its films and hydrogels.* Biomaterials, 1982. **3**(2): p. 105-8.

52. Hirano, S., H. Tsuchida, and N. Nagao, *N-acetylation in chitosan and the rate of its enzymic hydrolysis.* Biomaterials, 1989. **10**(8): p. 574-576.

53. Demarger-Andre, S. and A. Domard, *Chitosan carboxylic acid salts in solution and in the solid state.* Carbohydrate Polymers, 1994. **23**: p. 211-219.

54. Illum, L., N.F. Farraj, and S.S. Davis, *Chitosan as a novel nasal delivery system for peptide drugs.* Pharmaceutical Research, 1994. **11**(8): p. 1186-1189.

55. Jameela, S.R., A. Misra, and A. Jayakrishnan, *Cross-linked microspheres as carriers for prolonged delivery of macromolecular drugs.* Journal of Biomaterial Science Polymer Edition, 1994. **6**(7): p. 621-632.

56. Calvo, P., *et al.*, *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers.* Journal of Applied Polymer Science, 1997. **63**: p. 125-132.

57. Lueben, H.L., *et al.*, *Mucoadhesive polymers in pleural peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosa in vitro.* Journal of Controlled Release, 1997. **45**: p. 15-23.

58. Kratz, G., *et al.*, *Heparin-chitosan complexes stimulate wound healing in human skin.* Scandavian Journal of Plastic Reconstructive Hand Surgery, 1997. **31**: p. 119-123.

59. Kratz, G., *et al.*, *Immobilized Heparin accelerates the healing of human wounds in vivo.* Scandavian Journal of Plastic Reconstructive Hand Surgery, 1998. **32**: p. 381-385.

60. Sechriest, V.F., *et al.*, *GAG-augmented polysaccharide hydrogel: A novel biocompatible and biodegradable material to support chondrogenesis.* Journal of Biomedical Materials Research, 2000. **49**: p. 534-541.

61. MacLaughlin, F.C., *et al.*, *Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery.* Journal of Controlled Release, 1998. **56**(1-3): p. 259-272.

62. Richardson, S.C.W., H.J.V. Kolbe, and R. Duncan, *Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA.* International Journal of Pharmaceutics, 1999. **178**(2): p. 231-243.

63. Roy, K., *et al.*, *Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy.* Nature Medicine, 1999. **5**(4): p. 387-391.

64. Ueno, H., *et al.*, *Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs.* Biomaterials, 1999. **20**: p. 1407-1414.

65. Howling, G.I., *et al.*, *The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro.* Biomaterials, 2001. **22**: p. 2959-2966.

66. Muzzarelli, R., *et al.*, *Biological activity of chitosan: ultrastructural study.* Biomaterials, 1988. **9**(3): p. 247-252.

67. Chandy, T. and C.P. Sharma, *Chitosan - as a biomaterial.* Biomaterials, Artificial cells, and Artificial Organs, 1990. **18**(1): p. 1-24.

68. Elcin, A.E., Y.M. Elcin, and G.D. Pappas, *Neural tissue engineering: Adrenal chromaffin cell attachment and viability on chitosan scaffolds.* Neurological Research, 1998. **20**: p. 648-654.

69. Tamura, H., *et al.*, *Carboxymethyl-chitin and hydroxyapatite composite for bone repairing.* Polymer Preprints, 2000. **41**(1): p. 1032-1033.

70. Yoshihara, Y., *et al.*, *Study of carboxymethyl-chitin and hydroxyapatite composite for bone repairing.* Advances in Chitin Science, 1997. **2**: p. 682-687.

71. Muzzarelli, R.A.A., *et al.*, *Osteoinduction by chitosan-complexed BMP: Morpho-structural responses in an osteoporotic model.* Journal of Bioactive and Compatible Polymers, 1997. **12**: p. 321-329.

72. Kawase, M., *et al.*, *Application of glutaraldehyde-crosslinked chitosan as a scaffold for hepatocyte attachment.* Biology and Pharmacology Bulletin, 1997. **20**(6): p. 708-710.

73. Yagi, K., *et al.*, *Effectiveness of fructose-modified chitosan as a scaffold for hepatocyte attachment.* Biology and Pharmacology Bulletin, 1997. **20**(12): p. 1290-1294.

74. Dillon, G.P., *et al.*, *The influence of physical structure and charge on neuritis extension in a 3D hydrogel scaffold.* Journal of Biomaterial Science Polymer Edition, 1998. **9**(10): p. 1049-1069.

75. Elcin, Y.M., V. Dixit, and G. Gitnick, *Hepatocyte attachment on biodegradable modified chitosan membranes: In vitro evaluation for the development of liver organoids.* Artificial Organs, 1998. **22**(10): p. 837-846.

76. Black, A.F., *et al.*, *In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent.* FASEB J., 1998. **12**: p. 1331-1340.

77. Duplan-Perrat, F., *et al.*, *Keratinocytes influence the maturation and organization of the elastin network in a skin equivalent.* Journal of Investigational Dermatology, 2000. **114**: p. 365-370.

78. Yamamura, K., *et al.*, *Sustained release of basic fibroblast growth factor from the synthetic vascular prosthesis using hydroxypropylchitosan acetate.* Journal of Biomedical Materials Research, 1995. **29**: p. 203-206.

79. Yamamura, K., T. Nabeshima, and T. Sakurai, *Use of hydroxypropylchitosan acetate as a carrier for growth factor release*, in *Tissue Engineering of Prosthetic Vascular Grafts*, P. Zilla and H.P. Greisler, Editors. 1999, R. G. Landes Company: Austin. p. 599-604.

80. Luo, Y., K.R. Kirker, and G.D. Prestwich, *Modification of Natural Polymers: Hyaluronic Acid*, in *Methods of Tissue Engineering*, A. Atala and R.P. Lanza, Editors. 2002, Academic Press: San Diego. p. 539-554.

81. Sutherland, I.W., *Novel and established applications of microbial polysaccharides.* Trends in Biotechnology, 1998. **16**(1): p. 41-46.

82. Bullard, K.M., M.T. Longaker, and H.P. Lorenz, *Fetal wound healing: current biology.* World Journal of Surgery, 2003. **27**(1): p. 54-61.

83. Collis, L., *et al.*, *Rapid hyaluronan uptake is associated with rapid motility: implications for a intracellular mode of action.* FEBS Letters, 1998. **440**: p. 444-449.

84. Entwistle, J., C.L. Hall, and E.A. Turley, *HA receptors: regulators of signaling to the cytoskeleton.* Journal of Cellular Biochemistry, 1996. **61**(4): p. 569-577.

85. Chen, W.Y.J. and G. Abatangelo, *Functions of hyaluronan in would repair.* Wound Repair and Regeneration, 1999. **7**(79-89).

86. Noble, P.W., *Hyaluronan and its catabolic products in tissue injury and repair.* Matrix Biology, 2002. **21**: p. 25-29.

87. Sattar, A., S. Kumar, and D.C. West, *Does hyaluronan have a role in endothelial cell proliferation of the synovium?* Seminars in Arthritis and Rheumatism, 1992. **22**(1): p. 37-43.

88. West, D.C., *et al.*, *Angiogenesis induced by degradation products of hyaluronic acid.* Science, 1985. **228**: p. 1324-1326.

89. Deed, R., *et al.*, *Early-response gene signaling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high molecular-weight hyaluronan.* International Journal of Cancer, 1997. **71**: p. 251-256.

90. Slevin, M., *et al.*, *Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation.* Laboratory Investigation, 1998. **78**(8): p. 987-1003.

91. Montesano, R., *et al.*, *Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis in vitro.* Laboratory Investigation, 1996. **75**(2): p. 249-262.

92. Vercruysse, K.P. and G.D. Prestwich, *Hyaluronate derivatives in drug delivery.* Critical Reviews in Therapeutic Drug Carrier Systems, 1998. **15**(5): p. 513-555.

93. Campoccia, D., *et al.*, *Semisynthetic resorbable materials from hyaluronan esterification.* Biomaterials, 1998. **19**: p. 2101-2127.

94. Valle, F.D. and A. Romeo. Esters of hyaluronic acid. United States Patent: 4,851,521 (1989).

95. Grigolo, B., *et al.*, *Transplantation of chondrocytes seeded on a hyaluronan derivative (Hyaff-11) into cartilage defects in rabbits.* Biomaterials, 2001. **22**: p. 2417-2424.

96. Aigner, J., *et al.*, *Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester.* Journal of Biomedical Materials Research, 1998. **42**: p. 172-181.

97. Ghezzo, E., *et al.*, *Hyaluronan derivative microsphere as NGF delivery devices: preparation methods and in vitro release characterization.* International Journal of Pharmaceutics, 1992. **87**: p. 21-29.

98. Rochira, M., *et al.*, *Novel vaginal delivery systems for calcitonin II. Preparation and characterization of Hyaff microspheres containing calcitonin.* International Journal of Pharmaceutics, 1996. **144**: p. 19-26.

99. Illum, L., *et al.*, *Hyaluronic acid ester microspheres as a nasal delivery system for insulin.* Journal of Controlled Release, 1994. **29**: p. 133-141.

100. Larsen, N.E. and E.A. Balazs, *Drug delivery systems using hyaluronan and its derivatives.* Advanced Drug Delivery Reviews, 1991. **7**: p. 279-293.

101. Prestwich, G.D., *et al.*, *Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivatives.* Journal of Controlled Release, 1998. **53**: p. 93-103.

102. Smeds, K.A. and M.W. Grinstaff, *Photocrosslinkable polysaccharides for in situ hydrogel formation.* Journal of Biomedical Materials Research, 2001. **54**: p. 115-121.

103. Smeds, K.A., *et al.*, *Synthesis of a novel polysaccharide hydrogel.* Journal of Macromolecular Science. Pure and Applied Chemistry, 1999. **A36**(7-8): p. 981-989.

104. Singer, A.J. and R.A.F. Clark, *Cutaneous wound healing.* The New England Journal of Medicine, 1999. **341**(10): p. 738-746.

105. Hill, A.D.K., *et al.*, *The effect of granulocyte-macrophage colony-stimulating factor on myeloid cells and its clinical applications.* Journal of Leukocyte Biology, 1995. **58**: p. 634-642.

106. Mann, A., *et al.*, *Keratinocyte-derived granulocyte-macrophage colony-stimulating factor accelerates wound healing: stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization.* Journal of Investigational Dermatology, 2001. **117**(6): p. 1382-1390.

107. Dorr, R.T., *Sargramotism*, in *Cancer Chemotherapy Handbook*, R.T. Dorr and D.D. VonHoff, Editors. 1994, Appleton and Lange: Norwalk. p. 829-837.

108. Niven, R.W., *et al.*, *Pulmonary absorption of polyethylene glycolated recombinant human granulocyte-colony stimulating factor (PEG-rhG-CSF).* Journal of Controlled Release, 1994. **32**: p. 177-189.

109. Colton, C.K., *Implantable biohybrid artificial organs.* Cell Transplantation, 1995. **4**: p. 415-436.

110. Ahrendt, G., D.E. Chickering, and J.P. Ranieri, *Angiogenic growth factors: a review for tissue engineering.* tissue engineering, 1998. **4**(2): p. 117-130.

111. Yancopoulos, G.D., *et al.*, *Vascular-specific growth factors and blood vessel formation.* Nature, 2000. **407**: p. 242-248.

112. Veikkola, T. and K. Alitalo, *VEGFs, receptors and angiogenesis.* Cancer Biology, 1999. **9**: p. 211-220.

113. Josko, J., *et al.*, *Vascular endothelial growth factor (VEGF) and its effect on angiogenesis.* Medical Science Monitor, 2000. **6**(5): p. 1047-1052.

114. Klagsbrun, M. and P.A. D'Amore, *Regulators of Angiogenesis.* Annual Reviews in Physiology, 1991. **53**: p. 217-239.

115. Lazarous, D.F., *et al.*, *Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury.* Circulation, 1996. **94**(5): p. 1074-1082.

116. Putney, S.D. and P.A. Burke, *Improving protein therapeutics with sustained-release formulations.* Nature Biotechnology, 1998. **16**: p. 153-157.

117. Harris, J.M. and S. Zalipsky, eds. *Poly(ethylene glycol): chemistry and biological applications*. ACS Symposium Series. 1997, American Chemical Society: Washington DC. 489.

118. Harris, J.M., ed. *Poly(ethylene glycol) chemistry: biotechnical and biomedical applications.* Topics in Applied Chemistry, ed. A.R. Katritsky and G.J. Sabongi. 1992, Plenum Press: New York. 385.

119. Delgado, C., G.E. Francis, and D. Fisher, *The uses and properties of PEG-linked proteins.* Critical Reviews in Therapeutic Drug Carrier Systems, 1992. **9**(3,4): p. 249-304.

120. Abuchowski, A., *et al.*, *Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol.* Journal of Biological Chemistry, 1977. **252**(11): p. 3578-3581.

121. Cunningham-Rundles, C., *et al.*, *Biological activities of polyethylene-glycol immunoglobulin conjugates.* Journal of Immunological Methods, 1992. **152**(2): p. 177-190.

122. Abuchowski, A., *et al.*, *Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase.* Journal of Biological Chemistry, 1977. **252**(11): p. 3582-3586.

123. Pettit, D.K., *et al.*, *Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling.* Journal of Biological Chemistry, 1997. **272**(4): p. 2312-2318.

124. Peppas, N.A., *Molecular calculations of poly(ethylene glycol) transport across a swollen poly(acrylic acid)/mucin interface.* Journal of Biomaterial Science Polymer Edition, 1998. **9**(6): p. 535-542.

125. Jensen-Pippo, K.E., *et al.*, *Enteral bioavailability of human granulocyte stimulating factor conjugated with poly(ethylene glycol).* Pharmaceutical Research, 1996. **13**(1): p. 102-107.

126. Suzuki, T., *et al.*, *Physicochemical and biological properties of poly(ethylene glycol)-coupled immunoglobulin G.* Biochimica et Biophysica Acta, 1984. **788**(2): p. 248-255.

127. Gombotz, W.R. and D.K. Pettit, *PEGylation: A tool to enhance protein delivery*, in *Controlled Drug Delivery: Designing Technologies for the Future*, K. Park and R.J. Mrsny, Editors. 2000, American Chemical Society: Washington, DC. p. 110-123.

128. Kinstler, O.B., *et al.*, *Characterization and Stability of N-terminally PEGylated rhG-CSF.* Pharmaceutical Research, 1996. **13**(7): p. 996-1002.

129. Wade, A. and P.J. Weller, eds. *Handbook of Pharmaceutical Excipients*. 2nd ed. . 1994, Alden Press: Oxford.

130. Working, P.K., *et al.*, *Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives*, in *Poly(ethylene glycol) Chemistry and Biological Applications*, J.M. Harris and S. Zalipsky, Editors. 1997, American Chemical Society: Washington DC. p. 45-57.

131. Park, Y.K., *et al.*, *Pharmacology of Escherichia coli-L-asparaginase polyethylene glycol adduct.* Anticancer Research, 1981. **1**: p. 373.

132. Hershfield, M.S., *et al.*, *Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase.* New England Journal of Medicine, 1987. **316**(10): p. 589-596.

133. Hadziyannis, S.J. and G.V. Papatheodoridis, *Peginterferon-alpha2a (40 kDa) for chronic hepatitis C.* Expert Opinion on Pharmacotherapy, 2003. **4**(4): p. 541-551.

134. Willis, F. and R. Pettengell, *Pegfilgrastim.* Expert Opinion On Biological Therapy, 2002. **2**(8): p. 985-992.

135. Jiang, W. and S.P. Schwendeman, *Stabilization and controlled release of bovine serum albumin encapsulated in poly(d,l-lactide) and poly(ethylene glycol) microsphere blends.* Pharmaceutical Research, 2001. **18**(6): p. 878-885.

136. Kissel, T., Y. Li, and F. Unger, *ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins.* Advanced Drug Delivery Reviews, 2002. **54**: p. 99-134.

137. Kissel, T., *et al.*, *Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks.* Journal of Controlled Release, 1996. **39**: p. 315-326.

138. Jeong, B., Y.H. Bae, and S.W. Kim, *In situ gelation of PEG-PLA-PEG triblock copolymers aqueous solutions and degradation thereof.* Journal of Biomedical Materials Research, 2000. **50**: p. 171-177.

139. Okumu, F.W., *et al. Evaluation of SABER as a local delivery system for rhVEGF-formulation design and in vitro assessment.* in *27th International Meeting of the Controlled Release Society*. 2000. Paris, France.

140. Darling, S., *et al. Extended release of human growth hormone suspended in SABER formulation design and in vitro assessment.* in *Millennial World Congress of Pharmaceutical Sciences.* 2000. San Francisco, CA.

141. Okumu, F.W., *et al. SABER provides burst free release of growth hormone from PLGA microspheres.* in *Millennial World Congress of Pharmaceutical Sciences.* 2000. San Francisco, CA.

142. Sullivan, S.A., *et al. Incorporation of polymer microparticles in sucrose acetate isobutyrate reduces burst and extends release.* in *27th International Meeting of the Controlled Release Society.* 2000. Paris, France.

143. Street, J., *et al., Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover.* Proceedings of the National Academy of Science, 2002. **99**(15): p. 9656-9661.

144. Nnoruka, E.N. and C.E. Agu, *Successful treatment of scabies with oral ivermectin in Nigeria.* Tropical Doctor, 2001. **31**: p. 15-18.

145. Ghosh, D. and J. Sengupta, *Anti-nidatory effect of a single, early post-ovulatory administration of mifepristone (RU-486) in the rhesus monkey.* Human Reproduction, 1993. **8**(4): p. 552-558.

146. Guterres, S.S., *et al., Poly(rac-lactide) nanocapsules containing diclofenac: protection against muscular damage in rats.* Journal of Biomaterials Science Polymer Edition, 2000. **11**(12): p. 1347-1355.

147. Guterres, S.S., *et al., Poly(DL-lactide) nanocapsules containing diclofenac: I. Formulation and stability study.* International Journal of Pharmaceutics, 1995. **113**: p. 57-63.

148. Fessi, H., *et al., Nanocapsule formation by interfacial polymer deposition following solvent displacement.* International Journal of Pharmaceutics, 1989. **55**: p. R1-R4.

149. Gombotz, W.R., M.S. Healy, and L.R. Brown. Very low temperature casting of controlled release microspheres. United States Patent: 5,019,400 (1991).

150. Berger, H.L., *Ultrasonic Liquid Atomization, Theory and Application.* 2002: Partridge Hill Publishers. 169.

151. Costantino, H.R., *et al., Protein spray-freeze drying. Effect of atomization conditions on particle size and stability.* Pharmaceutical Research, 2000. **17**(11): p. 1374-1383.

152. Costantino, H.R., *et al.* Method of producing sub-micron particles of biologically active agents and uses thereof. United States Patent: US 6,284,283 B1 (2001).

153. Costantino, H.R., *et al.*, *Protein spray freeze drying. 2. Effect of formulation variables on particle size and stability.* Journal of Pharmaceutical Sciences, 2002. **91**(2): p. 388-395.

154. Johnson, O.L., *et al.* Composition for sustained release of human growth factor. United States Patent: 6,051,259 (2000).

155. Sun, Y.-N., *et al.*, *A Pharmacokinetic/Pharmacodynamic model for recombinant human growth hormone effects on induction of insulin-like growth factor I in monkeys.* The Journal of Pharmacology and Experimental Therapeutics, 1999. **289**(3): p. 1523-1532.

156. Tracy, M.A., *Development and scale-up of a microsphere protein delivery system.* Biotechnology Progress, 1998. **14**: p. 108-115.

157. Lee, H.J., *et al.*, *In vivo characterization of sustained-release formulations of human growth hormone.* Journal of Pharmacology and Experimental Therapeutics, 1997. **281**: p. 1431-1439.

158. Johnson, O.L., *et al.*, *A month-long effect from a single injection of microencapsulated human growth hormone.* Nature Medicine, 1996. **2**(7): p. 795-799.

159. Lam, X.M., *et al.*, *Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes.* Journal of Controlled Release, 2000. **67**: p. 281-292.

160. Cleland, J.L., *et al.*, *Development of poly(D,L-lactide-co-glycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis.* Journal of Controlled Release, 2001. **72**: p. 13-24.

161. Cleland, J.L., *et al.*, *Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development.* Advanced Drug Delivery Reviews, 1997. **28**: p. 71-84.

162. Cleland, J.L. and A.J.S. Jones, *Stable Formulations of Recombinant Human Growth Hormone and Interferon-γ for microencapsulation in biodegradable microspheres.* Pharmaceutical Research, 1996. **13**(10): p. 1464-1475.

163. Johnson, O.L., *et al.*, *The stabilization and encapsulation of human growth hormone into biodegradable microspheres.* Pharmaceutical Research, 1997. **14**(6): p. 730-735.

164. Lam, X.M., E.T. Duenas, and J.L. Cleland, *Encapsulation and stabilization of nerve growth factor into Poly(lactic-co-glycolic) acid microspheres.* Journal of Pharmaceutical Sciences, 2001. **90**(9): p. 1356-1365.

165. Aspden, T.J., *et al.*, *Chitosan as a nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary rates in human turbinates and volunteers.* Journal of Pharmaceutical Sciences, 1997. **86**(4): p. 509-513.

166. Kitamura, T., *et al.*, *Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin.* Journal of Cellular Physiology, 1989. **140**(2): p. 323-334.

167. Wrzyszczynski, A., *et al.*, *Blends of poly(ethylene oxide) with chitosan acetate salt and with dibuturylchitin: structure and morphology.* Polymer Bulletin, 1995. **34**: p. 493-500.

168. Lewis, R.N.A.H. and R.N. McElhaney, *Fourier transform infrared spectroscopy in the study of hydrated lipids and lipid bilayer membranes*, in *Infrared spectroscopy of biomolecules*, H.H. Mantsch and D. Chapman, Editors. 1996, Wiley-Liss: New York. p. 159-202.

169. Balazs, E.A., *Hyaluronan Biomaterials: Medical Applications*, in *Handbook of Biomaterials and Applications*, D.L. Wise, Editor. 1995, Marcel Dekker: New York. p. 2719-2741.

170. Willoughby, D.A., ed. *First International Workshop on Hyaluronan in Drug Delivery*. . 1994, Royal Society of Medicine Press: Windsor, UK.

171. Bryant, S.J., C.R. Nuttelman, and K.S. Anseth, *Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro.* Journal of Biomaterials Science Polymer Edition, 2000. **11**(5): p. 439-457.

172. Mellot, M.B., K. Searcy, and M.V. Pishko, *Release of protein from highly cross-linked hydrogels of poly(ethylene glycol) diacrylate fabricated by UV polymerization.* Biomaterials, 2001. **22**: p. 929-941.

173. Sanders, J.E., *et al.*, *Testing of elastomeric liners used in limb prosthetics: classification of fifteen products by mechanical performance.* Journal of Rehabilitation Research and Development, 2003. **In Press**.

174. Smith, P.K., *et al.*, *Measurement of protein using bicinchoninic acid.* Analytical Biochemistry, 1985. **150**: p. 76-85.

175. Yonese, M., *et al.*, *Light Scattering studies of soluble complexes between hyaluronate and bovine serum albumin.* Progress in Colloid Polymer Science, 1997. **106**: p. 252-256.

176. Leach, J.B., *et al.*, *Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds.* Biotechnology and Bioengineering, 2003. **82**: p. 578-589.

177. Lowry, O.H., *et al.*, *Protein measurement with the folin phenol reagent.* Journal of Biological Chemistry, 1951. **193**: p. 265-275.

178. Mecerreyes, D., *et al.*, *Unimolecular combination of an atom transfer radical polymerization initiator and a lactone monomer as a route to new graft copolymers.* Macromolecules, 1999. **32**(16): p. 5175-5182.

179. Detrembleur, C., *et al.*, *New functional aliphatic polyesters by chemical modification of copolymers of ε-caprolactone, γ-bromo-ε-caprolactone, and a mixture of β- and γ–Ene-ε-caprolactone.* Macromolecules, 2000. **33**: p. 7751-7760.

180. Mecerreyes, D., *et al.*, *First example of an unsymmetrical difunctional monomer polymerizable by two living/controlled methods.* Macromolecular Rapid Communications, 2000. **21**: p. 779-784.

181. Elbert, D.L. and J.A. Hubbell, *Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering.* Biomacromolecules, 2001. **2**(2): p. 430-441.

References

Abuchowski, A., T. V. Es, et al. (1977). "Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol." Journal of Biological Chemistry **252**(11): 3578-3581.

Abuchowski, A., J. R. McCoy, et al. (1977). "Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase." Journal of Biological Chemistry **252**(11): 3582-3586.

Ahrendt, G., D. E. Chickering, et al. (1998). "Angiogenic growth factors: a review for tissue engineering." Tissue Engineering **4**(2): 117-130.

Aigner, J., J. Tegeler, et al. (1998). "Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester." Journal of Biomedical Materials Research **42**: 172-181.

Allemann, F., S. Mizuno, et al. (2001). "Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds." Journal of Biomedical Materials Research **55**: 13-19.

Anseth, K., A. T. Metters, et al. (2002). "In situ forming degradable networks and their application in tissue engineering and drug delivery." Journal of Controlled Release **78**: 199-209.

Aspden, T. J., J. D. T. Mason, et al. (1997). "Chitosan as a nasal delivery system: the effect of chitosan solutions on *in vitro* and *in vivo* mucociliary rates in human turbinates and volunteers." Journal of Pharmaceutical Sciences **86**(4): 509-513.

Babensee, J. E., L. B. McIntire, et al. (2000). "Growth factor delivery for tissue engineering." Pharmaceutical Research **17**(5): 497-504.

Baker, R. (1987). Biodegradable Systems. Controlled Release of Biologically Active Agents. New York, John Wiley & Sons: 84-136.

Balazs, E. A. (1995). Hyaluronan Biomaterials: Medical Applications. Handbook of Biomaterials and Applications. D. L. Wise. New York, Marcel Dekker: 2719-2741.

Baldwin, S. P. and W. M. Saltzman (1998). "Materials for protein delivery in tissue engineering." Advance Drug Delivery Reviews **33**: 71-86.

Berger, H. L. (2002). Ultrasonic Liquid Atomization, Theory and Application, Partridge Hill Publishers.

Black, A. F., F. Berthod, et al. (1998). "*In vitro* reconstruction of a human capillary-like network in a tissue-engineered skin equivalent." FASEB Journal **12**: 1331-1340.

Brown, C. D. and A. S. Hoffman (2002). Modification of natural polymers: chitosan. Methods of Tissue Engineering. A. Atala and R. Lanza. San Diego, Academic Press: 565-573.

Bryant, S. and K. Anseth (2001). "The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels." Biomaterials **22**: 619-626.

Bryant, S. and K. S. Anseth (2002). "Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels." Journal of Biomedical Materials Research **59**: 63-72.

Bryant, S. J., C. R. Nuttelman, et al. (2000). "Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts *in vitro*." Journal of Biomaterials Science Polymer Edition **11**(5): 439-457.

Bullard, K. M., M. T. Longaker, et al. (2003). "Fetal wound healing: current biology." World Journal of Surgery **27**(1): 54-61.

Calvo, P., C. Remunan-Lopez, et al. (1997). "Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers." Journal of Applied Polymer Science **63**: 125-132.

Campoccia, D., P. Doherty, et al. (1998). "Semisynthetic resorbable materials from hyaluronan esterification." Biomaterials **19**: 2101-2127.

Chandy, T. and C. P. Sharma (1990). "Chitosan - as a biomaterial." Biomaterials, Artificial cells, and Artificial Organs **18**(1): 1-24.

Chen, W. Y. J. and G. Abatangelo (1999). "Functions of hyaluronan in would repair." Wound Repair and Regeneration **7**(79-89).

Cleland, J. L., A. Daugherty, et al. (2001). "Emerging protein delivery methods." Current Opinion in Biotechnology **12**: 212-219.

Cleland, J. L., E. T. Duenas, et al. (2001). "Development of poly(D,L-lactide-co-glycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis." Journal of Controlled Release **72**: 13-24.

Cleland, J. L., O. L. Johnson, et al. (1997). "Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development." Advanced Drug Delivery Reviews **28**: 71-84.

Cleland, J. L. and A. J. S. Jones (1996). "Stable Formulations of Recombinant Human Growth Hormone and Interferon-γ for microencapsulation in biodegradable microspheres." Pharmaceutical Research **13**(10): 1464-1475.

Collis, L., C. Hall, et al. (1998). "Rapid hyaluronan uptake is associated with rapid motility: implications for a intracellular mode of action." FEBS Letters **440**: 444-449.

Colton, C. K. (1995). "Implantable biohybrid artificial organs." Cell Transplantation **4**: 415-436.

Costantino, H. R., L. Firouzabadian, et al. (2000). "Protein spray-freeze drying. Effect of atomization conditions on particle size and stability." Pharmaceutical Research **17**(11): 1374-1383.

Costantino, H. R., L. Firouzabadian, et al. (2002). "Protein spray freeze drying. 2. Effect of formulation variables on particle size and stability." Journal of Pharmaceutical Sciences **91**(2): 388-395.

Costantino, H. R., W. E. Jaworowicz, et al. (2001). Method of producing sub-micron particles of biologically active agents and uses thereof. United States Patent. United States, Alkermes Controlled Therapeutics.

Cunningham-Rundles, C., Z. Zhuo, et al. (1992). "Biological activities of polyethylene-glycol immunoglobulin conjugates." Journal of Immunological Methods **152**(2): 177-190.

Darling, S., F. W. Okumu, et al. (2000). Extended release of human growth hormone suspended in SABER formulation design and *in vitro* assessment. Millennial World Congress of Pharmaceutical Sciences, San Francisco, CA.

Deed, R., P. Rooney, et al. (1997). "Early-response gene signaling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high molecular-weight hyaluronan." International Journal of Cancer **71**: 251-256.

Delgado, C., G. E. Francis, et al. (1992). "The uses and properties of PEG-linked proteins." Critical Reviews in Therapeutic Drug Carrier Systems **9**(3,4): 249-304.

Demarger-Andre, S. and A. Domard (1994). "Chitosan carboxylic acid salts in solution and in the solid state." Carbohydrate Polymers **23**: 211-219.

Detrembleur, C., M. Mazza, et al. (2000). "New functional aliphatic polyesters by chemical modification of copolymers of ε-caprolactone, γ-bromo-ε-caprolactone, and a mixture of β- and γ–Ene-ε-caprolactone." Macromolecules **33**: 7751-7760.

Dillon, G. P., X. Yu, et al. (1998). "The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold." Journal of Biomaterial Science Polymer Edition **9**(10): 1049-1069.

Dorr, R. T. (1994). Sargramotism. Cancer Chemotherapy Handbook. R. T. Dorr and D. D. VonHoff. Norwalk, Appleton and Lange**:** 829-837.

Duplan-Perrat, F., O. Damour, et al. (2000). "Keratinocytes influence the maturation and organization of the elastin network in a skin equivalent." Journal of Investigational Dermatology **114**: 365-370.

Elbert, D. L. and J. A. Hubbell (2001). "Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering." Biomacromolecules **2**(2): 430-441.

Elcin, A. E., Y. M. Elcin, et al. (1998). "Neural tissue engineering: Adrenal chromaffin cell attachment and viability on chitosan scaffolds." Neurological Research **20**: 648-654.

Elcin, Y. M., V. Dixet, et al. (1996). "Controlled release of endothelial cell growth factor from chitosan-albumin microspheres for localized angiogenesis: *in vitro* and *in vivo* studies." Artificial Cells, Blood Substitutes, and Immobilization Biotechnology **24**(3): 257-271.

Elcin, Y. M., V. Dixit, et al. (1998). "Hepatocyte attachment on biodegradable modified chitosan membranes: *In vitro* evaluation for the development of liver organoids." Artificial Organs **22**(10): 837-846.

Elisseeff, J., W. McIntosh, et al. (2000). "Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks." Journal of Biomedical Materials Research **51**: 164-171.

Entwistle, J., C. L. Hall, et al. (1996). "HA receptors: regulators of signaling to the cytoskeleton." Journal of Cellular Biochemistry **61**(4): 569-577.

Fessi, H., F. Puisieux, et al. (1989). "Nanocapsule formation by interfacial polymer deposition following solvent displacement." International Journal of Pharmaceutics **55**: R1-R4.

Fu, K., A. M. Klibanov, et al. (2000). "Protein stability in controlled-release systems." Nature Biotechnology **18**: 24-25.

Gao, J., J. E. Dennis, et al. (2001). "Tissue-engineered fabrication of an osteochondral composite graft using rate bone marrow-derived mesenchymal stem cells." Tissue Engineering **7**(4): 363-371.

Ghezzo, E., L. Benedetti, et al. (1992). "Hyaluronan derivative microsphere as NGF delivery devices: preparation methods and in vitro release characterization." International Journal of Pharmaceutics **87**: 21-29.

Ghosh, D. and J. Sengupta (1993). "Anti-nidatory effect of a single, early post-ovulatory administration of mifepristone (RU-486) in the rhesus monkey." Human Reproduction **8**(4): 552-558.

Gilding, D. G. (1981). Biocompatibility of clinical implant materials. Biodegradable Polymers. D. F. Williams. Boca Raton, CRC Press: 209-232.

Gombotz, W. R., M. S. Healy, et al. (1991). Very low temperature casting of controlled release microspheres. United States Patent. United States, Enzytech, Inc.

Gombotz, W. R. and D. K. Pettit (2000). PEGylation: A tool to enhance protein delivery. Controlled Drug Delivery: Designing Technologies for the Future. K. Park and R. J. Mrsny. Washington, DC, American Chemical Society: 110-123.

Grigolo, B., L. Roseti, et al. (2001). "Transplantation of chondrocytes seeded on a hyaluronan derivative (Hyaff-11) into cartilage defects in rabbits." Biomaterials **22**: 2417-2424.

Guterres, S. S., H. Fessi, et al. (1995). "Poly(DL-lactide) nanocapsules containing diclofenac: I. Formulation and stability study." International Journal of Pharmaceutics **113**: 57-63.

Guterres, S. S., H. Fessi, et al. (2000). "Poly(rac-lactide) nanocapsules containing diclofenac: protection against muscular damage in rats." Journal of Biomaterials Science Polymer Edition **11**(12): 1347-1355.

Hadziyannis, S. J. and G. V. Papatheodoridis (2003). "Peginterferon-alpha2a (40 kDa) for chronic hepatitis C." Expert Opinion on Pharmacotherapy **4**(4): 541-551.

Han, D. K., K. D. Park, et al. (1998). "Surface characteristics and biocompatibility of lactide-based poly(ethylene glycol) scaffolds for tissue engineering." Journal of Biomaterials Science Polymer Edition **9**(7): 667-680.

Harris, J. M., Ed. (1992). Poly(ethylene glycol) chemistry: biotechnical and biomedical applications. Topics in Applied Chemistry. New York, Plenum Press.

Harris, J. M. and S. Zalipsky, Eds. (1997). Poly(ethylene glycol): chemistry and biological applications. ACS Symposium Series. Washington DC, American Chemical Society.

Hershfield, M. S., R. H. Buckley, et al. (1987). "Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase." New England Journal of Medicine **316**(10): 589-596.

Hill, A. D. K., H. A. Naama, et al. (1995). "The effect of granulocyte-macrophage colony-stimulating factor on myeloid cells and its clinical applications." Journal of Leukocyte Biology **58**: 634-642.

Hirano, S., H. Tsuchida, et al. (1989). "N-acetylation in chitosan and the rate of its enzymic hydrolysis." Biomaterials **10**(8): 574-576.

Hora, M. S. (1990). "Release of human serum albumin from poly(lactide-co-glycolide) microspheres." Pharmaceutical Research **7**: 1190-1194.

Horowitz, S. T., S. Roseman, et al. (1957). "The preparation of glucosamine oligosaccharides. I. Separation." Journal of American Chemical Society **79**: 5046-5049.

Horton, D. and D. R. Lineback (1965). N-Deacetylation: Chitosan from chitin. Methods in Carbohydrate Chemistry. R. L. Whistler, J. N. BeMiller and M. L. Wolfrom. New York and London, Academic Press. **V:** 403-406.

Howling, G. I., P. W. Dettmar, et al. (2001). "The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes *in vitro*." Biomaterials **22**: 2959-2966.

Illum, L., N. F. Farraj, et al. (1994). "Chitosan as a novel nasal delivery system for peptide drugs." Pharmaceutical Research **11**(8): 1186-1189.

Illum, L., N. F. Farraj, et al. (1994). "Hyaluronic acid ester microspheres as a nasal delivery system for insulin." Journal of Controlled Release **29**: 133-141.

Jameela, S. R., A. Misra, et al. (1994). "Cross-linked microspheres as carriers for prolonged delivery of macromolecular drugs." Journal of Biomaterial Science Polymer Edition **6**(7): 621-632.

Jensen-Pippo, K. E., K. L. Whitcomb, et al. (1996). "Enteral bioavailability of human granulocyte stimulating factor conjugated with poly(ethylene glycol)." Pharmaceutical Research **13**(1): 102-107.

Jeong, B., Y. H. Bae, et al. (2000). "*In situ* gelation of PEG-PLA-PEG triblock copolymers aqueous solutions and degradation thereof." Journal of Biomedical Materials Research **50**: 171-177.

Jiang, W. and S. P. Schwendeman (2001). "Stabilization and controlled release of bovine serum albumin encapsulated in poly(d,l-lactide) and poly(ethylene glycol) microsphere blends." Pharmaceutical Research **18**(6): 878-885.

Johnson, O. L., J. L. Cleland, et al. (1996). "A month-long effect from a single injection of microencapsulated human growth hormone." Nature Medicine **2**(7): 795-799.

Johnson, O. L., M. M. Ganmukhi, et al. (2000). Composition for sustained release of human growth factor. United States Patent. United States, Alkermes Controlled Therapeutics.

Johnson, O. L., W. Jaworowicz, et al. (1997). "The stabilization and encapsulation of human growth hormone into biodegradable microspheres." Pharmaceutical Research **14**(6): 730-735.

Josko, J., B. Gwozdz, et al. (2000). "Vascular endothelial growth factor (VEGF) and its effect on angiogenesis." Medical Science Monitor **6**(5): 1047-1052.

Kaufman, P. M., S. Heimrath, et al. (1997). "Highly porous polymer matrices as a three-dimensional culture system for hepatocytes." Cell Transplantation **6**(5): 463-468.

Kawase, M., N. Michibayashi, et al. (1997). "Application of glutaraldehyde-crosslinked chitosan as a scaffold for hepatocyte attachment." Biology and Pharmacology Bulletin **20**(6): 708-710.

Kim, H. D. and R. F. Valentini (2002). "Retention and activity of BMP-2 in hyaluronic acid-based scaffolds *in vitro*." Journal of Biomaterials Research **59**: 573-584.

Kinstler, O. B., D. N. Brems, et al. (1996). "Characterization and Stability of N-terminally PEGylated rhG-CSF." Pharmaceutical Research **13**(7): 996-1002.

Kissel, T., Y. Li, et al. (2002). "ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins." Advanced Drug Delivery Reviews **54**: 99-134.

Kissel, T., Y. X. Li, et al. (1996). "Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks." Journal of Controlled Release **39**: 315-326.

Kitamura, T., T. Tange, et al. (1989). "Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin." Journal of Cellular Physiology **140**(2): 323-334.

Klagsbrun, M. and P. A. D'Amore (1991). "Regulators of Angiogenesis." Annual Reviews in Physiology **53**: 217-239.

Kratz, G., C. Arnander, et al. (1997). "Heparin-chitosan complexes stimulate wound healing in human skin." Scandavian Journal of Plastic Reconstructive Hand Surgery **31**: 119-123.

Kratz, G., M. Back, et al. (1998). "Immobilized Heparin accelerates the healing of human wounds *in vivo*." Scandavian Journal of Plastic Reconstructive Hand Surgery **32**: 381-385.

Krewson, C. E. and M. W. Saltzman (1996). "Transport and elimination of recombinant human NGF during long-term delivery to the brain." Brain Research **727**: 169-181.

Kuo, C. K. and P. X. Ma (2001). "Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate, and mechanical properties." Biomaterials **22**: 511-521.

Lam, X. M., E. T. Duenas, et al. (2001). "Encapsulation and stabilization of nerve growth factor into Poly(lactic-co-glycolic) acid microspheres." Journal of Pharmaceutical Sciences **90**(9): 1356-1365.

Lam, X. M., E. T. Duenas, et al. (2000). "Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes." Journal of Controlled Release **67**: 281-292.

Larsen, N. E. and E. A. Balazs (1991). "Drug delivery systems using hyaluronan and its derivatives." Advanced Drug Delivery Reviews **7**: 279-293.

Lazarous, D. F., M. Shou, et al. (1996). "Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury." Circulation **94**(5): 1074-1082.

Leach, J. B., K. A. Bivens, et al. (2003). "Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds." Biotechnology and Bioengineering **82**: 578-589.

Lee, H., R. A. Cusick, et al. (2002). "local delivery of basic fibroblast growth factor increases both angiogenesis and engraftment of hepatocytes in tissue-engineered polymer devices." Transplantation **73**(10): 1589-1593.

Lee, H. J., G. Riley, et al. (1997). "*In vivo* characterization of sustained-release formulations of human growth hormone." Journal of Pharmacology and Experimental Therapeutics **281**: 1431-1439.

Lee, J.-Y., S.-H. Nam, et al. (2002). "Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials." Journal of Controlled Release **78**: 187-197.

Lee, K. Y., M. C. Peters, et al. (2000). "Controlled growth factor release from synthetic extracellular matrices." Nature **408**: 998-1000.

Lewis, R. N. A. H. and R. N. McElhaney (1996). Fourier transform infrared spectroscopy in the study of hydrated lipids and lipid bilayer membranes. Infrared spectroscopy of biomolecules. H. H. Mantsch and D. Chapman. New York, Wiley-Liss: 159-202.

Lisignoli, G., N. Zini, et al. (2001). "Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic based polymer scaffold." Biomaterials **22**: 2095-2105.

Lowry, O. H., N. J. Rosebrough, et al. (1951). "Protein measurement with the folin phenol reagent." Journal of Biological Chemistry **193**: 265-275.

Lueben, H. L., C. O. Rentel, et al. (1997). "Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae *in vitro*." Journal of Controlled Release **45**: 15-23.

Luo, Y., K. R. Kirker, et al. (2002). Modification of Natural Polymers: Hyaluronic Acid. Methods of Tissue Engineering. A. Atala and R. P. Lanza. San Diego, Academic Press: 539-554.

MacLaughlin, F. C., R. J. Mumper, et al. (1998). "Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery." Journal of Controlled Release **56**(1-3): 259-272.

Mann, A., K. Breuhahn, et al. (2001). "Keratinocyte-derived granulocyte-macrophage colony-stimulating factor accelerates wound healing: stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization." Journal of Investigational Dermatology **117**(6): 1382-1390.

Mann, B. K., A. S. Gobin, et al. (2001). "Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering." Biomaterials **22**: 3045-3051.

Mann, B. K., R. H. Schmedlen, et al. (2001). "Tethered TGF-β increases extracellular matrix production of vascular smooth muscle cells." Biomaterials **22**: 439-444.

Maquet, V. and R. Jerome (1997). "Design of macroporous biodegradable polymer scaffolds for cell transplantation." Material Science **250**: 15-42.

Mecerreyes, D., B. Atthoff, et al. (1999). "Unimolecular combination of an atom transfer radical polymerization initiator and a lactone monomer as a route to new graft copolymers." Macromolecules **32**(16): 5175-5182.

Mecerreyes, D., J. Humes, et al. (2000). "First example of an unsymmetrical difunctional monomer polymerizable by two living/controlled methods." Macromolecular Rapid Communications **21**: 779-784.

Mellot, M. B., K. Searcy, et al. (2001). "Release of protein from highly cross-linked hydrogels of poly(ethylene glycol) diacrylate fabricated by UV polymerization." Biomaterials **22**: 929-941.

Molina, I., S. Li, et al. (2001). "Protein release from physically crosslinked hydrogels of the PLA/PEO/PLA triblock copolymer-type." Biomaterials **22**: 363-369.

Montesano, R., S. Kumar, et al. (1996). "Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis in vitro." Laboratory Investigation **75**(2): 249-262.

Mooney, D. J., P. M. Kaufman, et al. (1996). "Localized delivery of epidermal growth factor improves the survival of transplanted hepatocytes." Biotechnology and Bioengineering **50**: 422-429.

Mooney, D. J., K. Sano, et al. (1997). "Long-term engraftment of hepatocytes transplanted on biodegradable polymer sponges." Journal of Biomedical Materials Research **37**: 413-420.

Murphy, W. L., D. H. Kohn, et al. (2000). "Growth of continuous bonelike mineral within porous poly(lactide-co-glycolide) scaffolds *in vitro*." Journal of Biomaterials Research **50**: 50-58.

Murphy, W. L. and D. J. Mooney (1999). "Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices." Journal of Periodontal Research **34**: 413-419.

Muzzarelli, R., V. Baldassare, et al. (1988). "Biological activity of chitosan: ultrastructural study." Biomaterials **9**(3): 247-252.

Muzzarelli, R. A. A., G. Biagini, et al. (1997). "Osteoinduction by chitosan-complexed BMP: Morpho-structural responses in an osteoporotic model." Journal of Bioactive and Compatible Polymers **12**: 321-329.

Niven, R. W., K. L. Whitcomb, et al. (1994). "Pulmonary absorption of polyethylene glycolated recombinant human granulocyte-colony stimulating factor (PEG-rhG-CSF)." Journal of Controlled Release **32**: 177-189.

Nnoruka, E. N. and C. E. Agu (2001). "Successful treatment of scabies with oral ivermectin in Nigeria." Tropical Doctor **31**: 15-18.

Noble, P. W. (2002). "Hyaluronan and its catabolic products in tissue injury and repair." Matrix Biology **21**: 25-29.

Okumu, F. W., S. Darling, et al. (2000). SABER provides burst free release of growth hormone from PLGA microspheres. Millennial World Congress of Pharmaceutical Sciences, San Francisco, CA.

Okumu, F. W., A. Daughtery, et al. (2000). Evaluation of SABER as a local delivery system for rhVEGF-formulation design and in vitro assessment. 27th International Meeting of the Controlled Release Society, Paris, France.

Onishi, H. and Y. Machida (1999). "Biodegradation and distribution of water-soluble chitosan in mice." Biomaterials **20**: 175-182.

Pangburn, S. H., P. V. Trescony, et al. (1982). "Lysozyme degradation of partially deacetylated chitin, its films and hydrogels." Biomaterials **3**(2): 105-8.

Park, Y. J., Y. Ku, et al. (1998). "Controlled release of platelet-derived growth factor from porous poly(L-lactide) membranes for guided tissue regeneration." Journal of Controlled Release **51**: 201-211.

Park, Y. J., Y. M. Lee, et al. (2000). "Platelet derived growth factor releasing chitosan sponge for periodontal bone regeneration." Biomaterials **21**: 153-159.

Park, Y. K., A. Abuchowski, et al. (1981). "Pharmacology of *Escherichia coli*-L-asparaginase polyethylene glycol adduct." Anticancer Research **1**: 373.

Peppas, N. A. (1998). "Molecular calculations of poly(ethylene glycol) transport across a swollen poly(acrylic acid)/mucin interface." Journal of Biomaterial Science Polymer Edition **9**(6): 535-542.

Pettit, D. K., T. P. Bonnert, et al. (1997). "Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling." Journal of Biological Chemistry **272**(4): 2312-2318.

Prestwich, G. D., D. M. Marecak, et al. (1998). "Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivatives." Journal of Controlled Release **53**: 93-103.

Putney, S. D. and P. A. Burke (1998). "Improving protein therapeutics with sustained-release formulations." Nature Biotechnology **16**: 153-157.

Richardson, S. C. W., H. J. V. Kolbe, et al. (1999). "Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA." International Journal of Pharmaceutics **178**(2): 231-243.

Richardson, T. P., W. L. Murphy, et al. (2001). "Polymeric delivery of proteins and plasmid DNA for tissue engineering and gene therapy." Critical Reviews in Eukaryotic Gene Expression **11**: 47-58.

Richardson, T. P., M. C. Peters, et al. (2001). "Polymeric system for dual growth factor delivery." Nature Biotechnology **19**: 1029-1034.

Rochira, M., M. R. Miglietta, et al. (1996). "Novel vaginal delivery systems for calcitonin II. Preparation and characterization of Hyaff microspheres containing calcitonin." International Journal of Pharmaceutics **144**: 19-26.

Rotter, N., J. Aigner, et al. (1998). "Cartilage reconstruction in head and neck surgery: comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage." Journal of Biomedical Materials Research **42**: 347-356.

Roy, K., H. Q. Mao, et al. (1999). "Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy." Nature Medicine **5**(4): 387-391.

Sakiyama-Elbert, S. E. and J. A. Hubbell (2000). "Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix." Journal of Controlled Release **69**: 149-158.

Sakiyama-Elbert, S. E. and J. A. Hubbell (2000). "Development of fibrin derivatives for controlled release of heparin -binding growth factors." Journal of Controlled Release **65**: 389-402.

Sanders, J. E., B. S. Nicholson, et al. (2003). "Testing of elastomeric liners used in limb prosthetics: classification of fifteen products by mechanical performance." Journal of Rehabilitation Research and Development **In Press**.

Sandford, P. A. and A. Steinnes (1991). Biomedical applications of high-purity chitosan. Water-Soluble Polymers: synthesis, solution properties, and applications. S. W. Shalaby, C. L. McCormick and G. B. Butler. Washington, DC, American Chemical Society: 430-445.

Sattar, A., S. Kumar, et al. (1992). "Does hyaluronan have a role in endothelial cell proliferation of the synovium?" Seminars in Arthritis and Rheumatism **22**(1): 37-43.

Sechriest, V. F., Y. J. Miao, et al. (2000). "GAG-augmented polysaccharide hydrogel: A novel biocompatible and biodegradable material to support chondrogenesis." Journal of Biomedical Materials Research **49**: 534-541.

Sheridan, M. H., L. C. Shea, et al. (2000). "Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery." Journal of Controlled Release **64**: 91-102.

Singer, A. J. and R. A. F. Clark (1999). "Cutaneous wound healing." The New England Journal of Medicine **341**(10): 738-746.

Slevin, M., J. Krupinski, et al. (1998). "Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation." Laboratory Investigation **78**(8): 987-1003.

Smeds, K. A. and M. W. Grinstaff (2001). "Photocrosslinkable polysaccharides for in situ hydrogel formation." Journal of Biomedical Materials Research **54**: 115-121.

Smeds, K. A., A. Pfister-Serres, et al. (1999). "Synthesis of a novel polysaccharide hydrogel." Journal of Macromolecular Science. Pure and Applied Chemistry **A36**(7-8): 981-989.

Smith, P. K., R. I. Krohn, et al. (1985). "Measurement of protein using bicinchoninic acid." Analytical Biochemistry **150**: 76-85.

Street, J., M. Bao, et al. (2002). "Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover." Proceedings of the National Academy of Science **99**(15): 9656-9661.

Sullivan, S. A., S. K. Meador, et al. (2000). Incorporation of polymer microparticles in sucrose acetate isobutyrate reduces burst and extends release. 27th International Meeting of the Controlled Release Society, Paris, France.

Sun, Y.-N., H. J. Lee, et al. (1999). "A Pharmacokinetic/Pharmacodynamic model for recombinant human growth hormone effects on induction of insulin-like growth factor I in monkeys." The Journal of Pharmacology and Experimental Therapeutics **289**(3): 1523-1532.

Sutherland, I. W. (1998). "Novel and established applications of microbial polysaccharides." Trends in Biotechnology **16**(1): 41-46.

Suzuki, T., N. Kanbara, et al. (1984). "Physicochemical and biological properties of poly(ethylene glycol)-coupled immunoglobulin G." Biochimica et Biophysica Acta **788**(2): 248-255.

Tabata, Y. (2000). "The importance of drug delivery systems in tissue engineering." Pharmaceutical Science & Technology Today **3**(3): 80-89.

Tamura, H., S. Tokura, et al. (2000). "Carboxymethyl-chitin and hydroxyapatite composite for bone repairing." Polymer Preprints **41**(1): 1032-1033.

Tomihata, K. and Y. Ikada (1997). "*In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives." Biomaterials **18**(7): 567-575.

Tracy, M. A. (1998). "Development and scale-up of a microsphere protein delivery system." Biotechnology Progress **14**: 108-115.

Ueno, H., H. Yamada, et al. (1999). "Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs." Biomaterials **20**: 1407-1414.

Valle, F. d. and A. Romeo (1989). Esters of hyaluronic acid. United States Patent. United States, Fidia.

Veikkola, T. and K. Alitalo (1999). "VEGFs, receptors and angiogenesis." Cancer Biology **9**: 211-220.

Vercruysse, K. P. and G. D. Prestwich (1998). "Hyaluronate derivatives in drug delivery." Critical Reviews in Therapeutic Drug Carrier Systems **15**(5): 513-555.

Wade, A. and P. J. Weller, Eds. (1994). Handbook of Pharmaceutical Excipients. Oxford, Alden Press.

West, D. C., I. N. Hampson, et al. (1985). "Angiogenesis induced by degradation products of hyaluronic acid." Science **228**: 1324-1326.

Whang, K., D. C. Tsai, et al. (1998). "Ectopic bone formation via rhBMP-2 delivery from porous bioabsorbable polymer scaffolds." Journal Biomedical Materials Research **42**: 491-499.

Whitaker, M. J., R. A. Quirk, et al. (2001). "Growth factor release from tissue engineering scaffolds." Journal of Pharmacy and Pharmacology **53**: 1427-1437.

Willis, F. and R. Pettengell (2002). "Pegfilgrastim." Expert Opinion On Biological Therapy **2**(8): 985-992.

Willoughby, D. A., Ed. (1994). First International Workshop on Hyaluronan in Drug Delivery. Windsor, UK, Royal Society of Medicine Press.

Working, P. K., M. S. Newman, et al. (1997). Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives. Poly(ethylene glycol) Chemistry and Biological Applications. J. M. Harris and S. Zalipsky. Washington DC, American Chemical Society. **680:** 45-57.

Wrzyszczynski, A., X. Qu, et al. (1995). "Blends of poly(ethylene oxide) with chitosan acetate salt and with dibuturylchitin: structure and morphology." Polymer Bulletin **34**: 493-500.

Yagi, K., N. Michibayashi, et al. (1997). "Effectiveness of fructose-modified chitosan as a scaffold for hepatocyte attachment." Biology and Pharmacology Bulletin **20**(12): 1290-1294.

Yamamoto, M., Y. Tabata, et al. (1999). "Growth factor release from gelatin hydrogel for tissue engineering." Journal of Bioactive and Compatible Polymers **14**: 474-489.

Yamamura, K., T. Nabeshima, et al. (1999). Use of hydroxypropylchitosan acetate as a carrier for growth factor release. Tissue Engineering of Prosthetic Vascular Grafts. P. Zilla and H. P. Greisler. Austin, R. G. Landes Company: 599-604.

Yamamura, K., T. Sakurai, et al. (1995). "Sustained release of basic fibroblast growth factor from the synthetic vascular prosthesis using hydroxypropylchitosan acetate." Journal of Biomedical Materials Research **29**: 203-206.

Yancopoulos, G. D., S. Davis, et al. (2000). "Vascular-specific growth factors and blood vessel formation." Nature **407**: 242-248.

Yonese, M., S. H. Xu, et al. (1997). "Light Scattering studies of soluble complexes between hyaluronate and bovine serum albumin." Progress in Colloid Polymer Science **106**: 252-256.

Yoshihara, Y., T. Ishii, et al. (1997). "Study of carboxymethyl-chitin and hydroxyapatite composite for bone repairing." Advances in Chitin Science **2**: 682-687.

Zhu, G., S. R. Mallery, et al. (2000). "Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide)." Nature Biotechnology **18**: 52-57.

Vita

Chad David Brown

chadbr@u.washington.edu

EDUCATION

UNIVERSITY OF WASHINGTON — Seattle, WA

Ph.D. in Bioengineering, August 2003.

Dissertation: Cross-Linked Hydrogels for the Delivery of Growth Factors in Tissue Engineering.

Advisors: Allan S. Hoffman, Sc.D. and Patrick S. Stayton, Ph.D.

Goal: To develop protein delivery systems comprised of synthetic and natural polymers that are capable of stable and controlled delivery of growth factors with appropriate release kinetics in order to promote proper wound healing events in tissue engineering.

- Developed a chitosan-based hydrogel delivery system that utilized glycerol to physically cross-link the matrix through hydrogen bonding and modulate the release of PEGylated granulocyte-macrophage colony-stimulating factor.
  - Release characterized through swelling and HPLC studies
  - Release mechanism elucidated using pH and FTIR measurements

- Developed a novel photo-crosslinkable protein delivery system based on poly(ethylene glycol) and poly(lactic acid) interpenetrating networks using benzyl benzoate/benzyl alcohol as a co-solvent for the polymers and protein.
  - Release rate adjusted by varying the PEG/PLA ratio and molecular weight
  - Developed a unique method for forming microparticles of the delivery system for the release of VEGF.

COLORADO STATE UNIVERSITY — Fort Collins, CO

Bachelors of Science in Chemical Engineering, Minor in Chemistry

Magna Cum Laude, GPA 3.97, June 1997.

AWARDS, HONORS and FELLOWSHIPS

UNIVERSITY OF WASHINGTON

Fellowship: Whitaker Foundation Graduate Fellowship
The Whitaker Foundation, 1997-2002.

Professional Societies: Biomedical Engineering Society (BMES)
Society for Biomaterials (SFB)

COLORADO STATE UNIVERSITY

Honors: Walter Scott Jr. Memorial Scholarship, 1993-1997
President's Scholarship, 1994-1997
Omega Chi Epsilon National Chemical Engineering Honor Society
Tau Beta Pi National Engineering Honor Society
Golden Key National Honor Society
Phi Kappa Phi National Honor Society

Awards: CRC Press Freshman Chemistry Achievement Award, 1993
AIChE Sophomore Chemical Engineer Excellence Award, 1994
Outstanding Senior Chemical Engineering Student, 1997

LABORATORY SKILLS

Chemistry	Monomer synthesis, purification (flash chromatography), and characterization (NMR, TLC), free-radical polymerization, ring-opening polymerization, polymer purification and characterization (GPC, NMR, UV, FTIR, viscometry), small molecule derivatization of polymers (peptide conjugation, polymerizable groups), electron spectroscopy for chemical analysis (ESCA), surface modification by plasma discharge, contact angle
Biochemical	Plasmid amplification and isolation, bacterial transformation, polyacrylamide gel electrophoresis, Western blots, gel extraction, plasmid subcloning, PCR, protein assays, ELISA
Cell Culture	Cell line maintenance (fibroblasts-NIH3T3 and insect-sf9), adhesion assays, cytoxicity assays, protein bioactivity assays

PUBLICATIONS

Brown, C.D., Stayton, P.S., and Hoffman, A.S. *Delivery of VEGF from photo-crosslinked microparticles of interpenetrating networks of PEG and PLA,* Biomaterials (2003, in preparation).

Cuy, J.L., Beckstead, B.L., Brown, C.D., Hoffman, A.S., and Giachelli, C.M. *Adhesive protein interactions with chitosan: consequences for valve endothelial cell growth on tissue-engineering materials,* Journal of Biomedical Materials Research (2003, submitted).

Brown, C.D. and Hoffman, A.S. *Modification of natural polymers: chitosan,* In: Methods of Tissue Engineering, A. Atala and R. Lanza, Editors. 2002, Academic Press: San Diego, p.565-573.

Brown, C.D., Kreilgaard, L., Nakakura, M., Caram-Lelham, N., Petit, D.K., Gombotz, W.R., and Hoffman A.S. *Release of PEGylated granulocyte-macrophage colony-stimulating factor from chitosan/glycerol films,* Journal of Controlled Release 72 (2001) p.35-46.

ABSTRACTS

Brown, C.D., Stayton, P.S., and Hoffman, A.S. (2003) Cross-linked PLA/PEG hydrogels for the controlled delivery of growth factors. Society for Biomaterials Annual Meeting. Reno, NV. Podium presentation. April 30 – May 3.

Brown, C.D., Stayton, P.S., and Hoffman, A.S. (2002) Cross-linked PLA/PEG hydrogels for the controlled delivery of growth factors. Gels, Genes, Grafts, and Giants: Transitioning into the 21st Century, University of Washington Engineered Biomaterials Symposium. Maui, HI. Poster presentation. December 17-20.

Brown, C.D., Hahn, S.K., Stayton, P.S., and Hoffman, A.S. (2002) Hyaluronic acid hydrogels as drug delivery carriers for tissue engineering. Society for Biomaterials Annual Meeting. Tampa Bay, FL. Podium presentation. April 24-27.

Brown, C.D., Kreilgaard, L., Nakakura, M., Caram-Lelham, N., Petit, D.K., Gombotz, W.R., and Hoffman A.S. (2000) Effect of glycerol on the release of PEGylated GM-CSF from chitosan/glycerol films. Biomedical Engineering Society Annual Meeting. Seattle, WA. Poster presentation. October 12-14.

Brown, C.D., and Hoffman, A.S. (2000) Controlled release strategies for protein therapeutics. The Whitaker Foundation Annual Biomedical Engineering Research Conference. La Jolla, CA. Poster presentation. August 10-13.

Brown, C.D., Kreilgaard, L., Nakakura, M., Caram-Lelham, N., Petit, D.K., Gombotz, W.R., and Hoffman A.S. (2000) Unusual effect of glycerol on release of PEGylated GM-CSF from chitosan/glycerol films. Sixth World Biomaterial Congress. Kamuela, HI. Poster presentation. May 15-20.