IN SITU PHOTOPOLYMERIZED HYDROGELS FOR ENHANCING PROTEIN DELIVERY

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IN SITU PHOTOPOLYMERIZED HYDROGELS FOR ENHANCING PROTEIN DELIVERY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Chien-Chi Lin
August 2007

Accepted by:
Dr. Andrew T. Metters, Committee Chair
Dr. Naren R. Vyavahare
Dr. Karen J.L. Burg
Dr. Ken Webb
ABSTRACT

In recent years, there has been immense interest in the utilization of photopolymerized hydrogels as carriers for controlled protein delivery and cell scaffolds for tissue engineering applications. Although poly(ethylene glycol) (PEG)-based hydrogels formed from mild photopolymerization methods have been suggested as biocompatible matrices that allow for safely encapsulating biomolecules including proteins, peptides, DNA, and cells, the adverse effects of photopolymerization reactions on the encapsulated proteins have largely been overlooked. In addition, conventional hydrophilic hydrogels fail to effectively control protein delivery rates due to their high permeability. These two problems are critical since the delivery of protein therapeutics from hydrogel matrices in their active form and in optimal rates usually determine whether a device performs successfully in a given application.

The development of ideal hydrogel matrices requires a thorough understanding of protein-polymer interactions and the mechanisms governing protein-delivery rates from a crosslinked polymer network. The primary foci of this dissertation were to evaluate free radical-mediated protein-polymer conjugation and to develop synthetic affinity hydrogels for systematically controlling single and multiple-protein delivery. These research objectives combine the knowledge of protein chemistry, polymer science and engineering, molecular transport kinetics, and mathematical modeling.

The initial research efforts were to evaluate the factors causing protein inactivation during in situ photopolymerization, with the primary focus on photoinitiator chemistry and concentration (Chapter 3). Next, the undesirable formation of protein-polymer conjugates
during in situ photopolymerization and their effects on total protein release were investigated (Chapter 3, 4). Once the adverse effects of protein-polymer conjugates were identified, a pseudo-specific metal-ion chelating ligand was used to enhance protein bioavailability (Chapter 4).

Another challenge of using hydrophilic hydrogels for controlled protein delivery is the networks’ high permeability to encapsulated proteins. This limitation was circumvented by synthesizing affinity ligands that bind to target proteins and immobilizing them within otherwise inert hydrogel networks (Chapter 5). This modification provided a unique method for tuning the protein delivery rates. Two protein-binding mechanisms, namely electrostatic interaction and metal-ion chelation, were used separately to evaluate the efficacy of protein-ligand binding for controlling protein delivery (Chapter 5, 6). A mathematical model was also developed to predict the release of histidine-tagged protein from metal-chelating ligand imprinted affinity hydrogels (Chapter 5). Finally, these two binding mechanisms were used together in a one-step photopolymerized hydrogel matrix to independently control the delivery rates of two proteins encapsulated simultaneously (Chapter 7).
DEDICATION

To Tsai-Yu.
ACKNOWLEDGMENTS

I would first like to thank my dissertation committee members, Dr. Andrew Metters, Dr. Naren Vyavahare, Dr. Karen Burg, and Dr. Ken Webb, for their helpful discussions and precious advice during my graduate research. In particular, I would like to express my sincere gratitude to my dissertation advisor, Dr. Andrew Metters. As a mentor, Andrew has not only given me invaluable guidance but also surprised me constantly with numerous new ideas on this research project. As a scientist, Andrew has served as a role model for me and inspired me greatly on the path of research. I would also like to thank Dr. Martine LaBerge, Dr. Alexey Vertegel, and Dr. Jiro Nagatomi, for their valuable input during my qualifier and comprehensive examinations that made me realizing the importance of critical thinking.

I have been fortunate to study and conduct biomaterials research in a department full with knowledgeable faculties, staffs, and fellow students. Special thanks are given to the Metters research group – Santosh Rahane, Nihar Shah, Brad Harris, Ed Fritz, Betsy Metters, and two incredible undergraduates Suzanne Sawicki and Thomas Moore. Thank you all for giving me a memorable time during my graduate studies.

Great appreciations are given to the ChBE department at Clemson University and the Juvenile Diabetes Research Foundation for the financial support to this research project.

Finally, I would like to convey my deepest gratefulness to my family, especially my beloved mother and Tsai-Yu. Mom, thank you for raising me good and for supporting my decision on pursuing my dreams thousands of miles away from home. Tsai-Yu, thank you for everything you have given me; without you, I could not continue on this research, in this country, and in this life.
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CHAPTER ONE
INTRODUCTION

Synthetic hydrogels polymerized from poly(ethylene glycol) (PEG) derivatives have been extensively used in controlled release [1-8], tissue engineering [1, 4, 9-15], biosensors [16-18], and other biomedical applications. PEG-based hydrogels are widely used owing to their demonstrated biocompatibility, non-fouling properties, and well-understood structure-function relationships. To increase the applicability and cytocompatibility of inert PEG hydrogels, multiple functionalities including stimuli-responsiveness [8, 19-21] and ligands for biorecognition and protein-binding [22-24] can be readily tailored into hydrogel networks through the copolymerization of acrylated/methacrylated monomers, peptides, proteins, or other functional molecules with vinyl end groups.

A convenient yet efficient route of synthesizing covalently crosslinked PEG-based hydrogels is through photo-initiated solution polymerization or photopolymerization. Photopolymerization possesses numerous advantages including its aqueous and ambient polymerization conditions as well as rapid and facile spatial/temporal control over reaction kinetics. The incorporation of functional moieties (e.g. stimuli-responsiveness, affinity/biorecognition sites) as well as the encapsulation of biomolecules into PEG-based hydrogels can be achieved simultaneously via one-step photopolymerization. Due to the mild polymerization conditions and the unique sol-gel transition properties, hydrogels formed from in situ photopolymerization have been successfully used as injectable biomaterials for implantable tissue-engineered scaffolds and as vehicles for delivery of cells and/or therapeutic agents via a minimally invasive manner. For controlled delivery
applications, controlled amounts of therapeutic agents can be easily and precisely incorporated into the in situ-curable gels in a single processing step.

Although in situ photopolymerization offers many advantages, it may lead to undesired side reactions with encapsulated biomolecules such as DNA [25, 26] and proteins [27, 28]. These unwanted reactions include protein denaturation/inactivation and protein-polymer conjugation. The denaturation or inactivation of protein therapeutics not only causes loss in bioactivity and therapeutic efficacy, but the denatured proteins may also induce a host immune response. On the other hand, the primary consequence of undesired protein-polymer conjugation is reduced or incomplete protein release since the polymer in these materials is at least originally part of a crosslinked, insoluble network. Although the use of degradable matrices can ensure complete release of the encapsulated proteins, a portion of these released proteins are in the form of protein-polymer conjugates that may not be as active as native proteins and may also induce host immune response.

Prior studies on in situ photopolymerization have revealed the detrimental effects of photoinitiators on DNA [25, 26] and cells [29, 30]. However, damage to encapsulated proteins has not been extensively studied [27, 28]. The fact that more recombinant protein therapeutics are becoming indispensable for tissue engineering applications and clinical treatments makes it critical to develop methods that can safely encapsulate fragile protein therapeutics. Hydrogels are ideal candidates for protein encapsulation due to their hydrophilic and non-fouling properties that help to preserve protein bioactivity. However, there have been increasing concerns regarding protein stability and bioactivity during in situ photopolymerization. The first part of this dissertation aims to evaluate this critical phenomenon. The objectives are (1) to understand the mechanisms causing protein
inactivation and incomplete protein release, and (2) to enhance total protein delivery from in situ photopolymerized hydrogels using protein-binding ligands. It was hypothesized that incomplete protein release is due to the formation of protein-polymer conjugates within in situ curable hydrogels. Specifically, high-energy free radicals generated by photoinitiators during UV curing are responsible for these adverse conjugations and hence play a major role in protein inactivation and incomplete release. With this hypothesis in mind, we attempt to characterize the factors affecting protein activity during in situ photopolymerization. We then successfully employ a protein-binding ligand to reduce the extent to which protein-polymer conjugates are formed, ultimately leading to enhanced protein delivery efficiency.

PEG-based hydrogels are considered biocompatible not only due to their “stealth” character but also because of their high water contents that resemble natural tissue properties. The high water content of the hydrophilic hydrogels, however, leads to high permeability for the encapsulated proteins, which makes sustained protein delivery difficult. In view of this, several methodologies have been developed in an attempt to systematically decrease protein delivery rates from highly permeable hydrogels. Perhaps the most successful approach to date is the incorporation of protein-binding ligands into the otherwise inert hydrogel networks. These “affinity” ligands are either derived from natural polysaccharides such as heparin or cyclodextrin or from synthetic ligands including methacrylic acid. Despite a wide variety of synthesized affinity ligands, there still exists a demanding quest for more effective and biocompatible ligands that, upon facile incorporation into the polymer network, do not compromise the preferential hydrogel properties. The second part of this dissertation describes the design and application of novel PEG-based hydrogels copolymerized with synthetic protein binding ligands to address this
problem of obtaining sustained protein delivery from hydrogel carriers. The goals are (1) to systematically controll protein delivery using protein-binding ligands without sacrificing the preferential hydrophilic/biocompatible properties of hydrogels; (2) to predict protein delivery a priori through mathematical modeling; and (3) to design monolithic hydrogels for tunable dual-protein delivery.

The global objective of this phase of the research project is to enhance the performance of hydrogel-based controlled protein delivery devices with the specific aims of decreasing adverse protein-polymer conjugation and increasing the tunability of protein delivery from hydrophilic PEG-based hydrogels. The objectives of each chapter in this dissertation are outlined below:

Chapter 2 reviews the use of hydrogels in controlled release formulation, including the general design criteria/considerations as well as the mathematical modeling of hydrogel-based controlled delivery devices. Through this extensive review of hydrogel network design and mathematical modeling, we plan to identify some of the key issues that need to be addressed toward the development of an ideal hydrogel-based protein delivery device.

Chapter 3 discusses photoinitiator-mediated protein inactivation and its impact on controlled protein delivery from in situ photopolymerized hydrogels. Lysozyme was used as a model protein to investigate the adverse effects of photoinitiators during photopolymerization. Also discussed is the effect of nonacrylated poly(ethylene glycol) (PEG) and acrylated PEG macromers on the preservation of protein bioactivity. The specific aim of this chapter is to unravel the critical parameters for causing protein-inactivation and to optimize the photopolymerization conditions for preserving protein bioactivity.
Chapter 4 investigates the factors causing incomplete protein release from in situ photopolymerized hydrogels. Bovine serum albumin (BSA) was used as a model protein and a proof-of-principle protein-protection strategy based on pseudo-specific metal-ion chelating ligands was developed to enhance total protein release.

Chapter 5 describes the synthesis of affinity PEG hydrogels for sustained protein delivery. A methacrylated metal-ion chelating ligand was synthesized and copolymerized into the PEG hydrogels in an attempt to systematically decrease protein delivery rate by tuning protein-ligand binding affinity. Also developed is a mathematical model accounting for protein diffusion and reversible protein-ligand binding to enable prediction of total protein delivery from these affinity hydrogels.

Chapter 6 characterizes stimuli-responsive hydrogels for enhanced protein loading and sustained delivery. Two protein-loading techniques were used: post-loading and in situ-loading. The aims are to compare the controlled protein delivery performances using these two protein-loading techniques.

Chapter 7 details the use of monolithic affinity hydrogels for manipulating dual-protein delivery by selectively adjusting protein-ligand binding affinity. The objective is to use monolithic, highly swelling hydrogels for independently controlling delivery of one protein without affecting the release rate of a second, co-encapsulated protein. This novel strategy may potentially enhance hydrogel-based strategies for regenerating functional tissues by enabling localized, sustainable, and independently tunable delivery of multiple growth factors from an injectable tissue scaffold.

Chapter 8 outlines the major conclusions and recommendations for future studies on the use of in situ photopolymerized hydrogels for controlled protein delivery.
References


CHAPTER TWO
HYDROGELS IN CONTROLLED RELEASE FORMULATIONS: NETWORK DESIGN AND MATHEMATICAL MODELING


Abstract

Over the past few decades, advances in hydrogel technologies have spurred development in many biomedical applications including controlled drug delivery. Many novel hydrogel-based delivery matrices have been designed and fabricated to fulfill the ever-increasing needs of the pharmaceutical and medical fields. Mathematical modeling plays an important role in facilitating hydrogel network design by identifying key parameters and molecule release mechanisms. The objective of this article is to review the fundamentals and recent advances in hydrogel network design as well as mathematical modeling approaches related to controlled molecule release from hydrogels. In the first section, the niche roles of hydrogels in controlled release, molecule release mechanisms, and hydrogel design criteria for controlled release applications are discussed. Novel hydrogel systems for drug delivery including biodegradable, smart, and biomimetic hydrogels are reviewed in the second section. Several mechanisms have been elucidated to describe molecule release from polymer hydrogel systems including diffusion, swelling, and chemically-controlled release. The focus of the final part of this article is discussion of emerging hydrogel delivery systems and challenges associated with modeling the performance of these devices.

Keywords: Hydrogels, drug delivery, modeling, controlled release, diffusion, degradation
2.1 Introduction

2.1.1 Overview of manuscript / Scope of this review

Since the establishment of the first synthetic hydrogels by Wichterle and Lim in 1954 [1], the growth of hydrogel technologies has advanced many fields ranging from food additives [2] to pharmaceuticals [3] to biomedical implants [4]. In addition, the development of an ever-increasing spectrum of functional monomers and macromers continue to broaden the versatility of hydrogel applications. Hydrogels now play a critical role in many tissue engineering scaffolds, biosensor and BioMEMS devices, and drug carriers. Among these applications, hydrogel-based drug delivery devices have become a major area of research interest with several commercial products already developed [5]. A successful drug delivery device relies not only on intelligent network design but also on accurate a priori mathematical modeling of drug release profiles. An ordered polymer network composed of macromers with well understood chemistries yields hydrogels with well-defined physicochemical properties and reproducible drug-release profiles. In a complimentary fashion, a quantitative mathematical understanding of material properties, interaction parameters, kinetic events, and transport phenomena within complex hydrogel systems assists network design by identifying the key parameters and mechanisms that govern the rate and extent of drug release. In addition, mathematical modeling accelerates device design by limiting the number of experiments researchers must perform to understand the release mechanisms governing a particular delivery system.

Many excellent review articles have been published detailing the modeling of drug release from polymeric devices including hydrogels. This review builds on the established literature by not only tracking recent advances in the development of mathematical models
for quantitatively predicting drug delivery from hydrogel systems, but also highlights how these models are playing a critical role in the design of novel hydrogel networks for future applications. In addition to describing the mechanisms governing drug release from conventional hydrogels, the fabrication and modeling of several emerging and intelligently designed hydrogel systems for drug delivery applications are discussed. Specifically, these novel systems aim to incorporate advanced drug delivery strategies into tissue engineering scaffolds and other biomedical implants and require rigorous methods for quantifying multiple phenomena influencing molecule release.

2.1.2 Hydrogel – Definition, Classification, and Network structure

Hydrogels are polymeric networks that absorb large quantities of water while remaining insoluble in aqueous solutions due to chemical or physical crosslinking of individual polymer chains. Differing from hydrophobic polymeric networks such as poly(lactic acid) (PLA) or poly(lactide-co-glycolide) (PLGA) which have limited water-absorption capabilities (< 5-10 wt%), hydrophilic hydrogels exhibit many unique physicochemical properties that make them advantageous for biomedical applications including drug delivery. For example, hydrogels are excellent candidates for encapsulating biomacromolecules including proteins and DNA due to their lack of hydrophobic interactions which can denature these fragile species [6]. In addition, compared to commonly used hydrophobic polymers such as PLGA, the conditions for fabricating hydrogels are relatively mild. Gel formation usually proceeds at ambient temperature and organic solvents are rarely required. In situ gelation with cell and drug encapsulation capabilities further distinguishes hydrogels from the other hydrophobic polymers.
Hydrogels can be prepared from natural or synthetic polymers [7]. Although hydrogels made from natural polymers may not provide sufficient mechanical properties and may contain pathogens or evoke immune/inflammatory responses, they do offer several advantageous properties such as inherent biocompatibility, biodegradability, and biologically recognizable moieties that support cellular activities. Synthetic hydrogels, on the other hand, do not possess these inherent bio-active properties. Fortunately, synthetic polymers usually have well-defined structures that can be modified to yield tailorable degradability and functionality. **Table 2.1** lists natural polymers as well as synthetic monomers that are commonly used in hydrogel fabrication.

**Table 2.1** Natural polymers and synthetic monomers used in hydrogels fabrications [6, 7].

<table>
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<th>Natural polymer</th>
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<tr>
<td>Chitosan</td>
<td>Hydroxyethyl methacrylate (HEMA)</td>
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<td>Alginate</td>
<td>N-(2-hydroxypropyl) methacrylate (HPMA)</td>
</tr>
<tr>
<td>Fibrin</td>
<td>N-vinyl-2-pyrrolidone (NVP)</td>
</tr>
<tr>
<td>Collagen</td>
<td>N-isopropyl acrylamide (NIPAAm)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Vinyl acetate (VAe)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Acrylic acid (AA)</td>
</tr>
<tr>
<td>Dextran</td>
<td>Methacrylic acid (MAA)</td>
</tr>
<tr>
<td></td>
<td>Polyethylene glycol acrylate/methacrylate (PEGA/PEGMA)</td>
</tr>
<tr>
<td></td>
<td>Polyethylene glycol diacrylate/dimethacrylate (PEGDA/PEGDMA)</td>
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Since the favorable properties of hydrogels stem from their hydrophilicity, the characterization of their water-sorption capabilities is the first step towards understanding the nanoscopic structure of hydrogel networks. Generally, three parameters are critical in describing the nanostructure of crosslinked hydrogel networks: (1) polymer volume fraction in the swollen state, \( \nu_{2,s} \), (2) number average molecular weight between crosslinks, \( \overline{M}_c \), and (3) network mesh size, \( \xi \) [8]. For nonporous hydrogels, the amount of liquid being retained in the hydrogel, the distance between polymer chains, and the flexibility of those chains together determine the mobility of encapsulated molecules and their rates of diffusion within a swollen hydrogel matrix.

The polymer volume fraction in the swollen state \( \nu_{2,s} \) describes the amount of liquid that can be imbibed in hydrogels and is described as a ratio of the polymer volume \( (V_p) \) to the swollen gel volume \( (V_g) \). It is also a reciprocal of the volumetric swollen ratio \( (Q) \) which can be related to the densities of the solvent \( (\rho_1) \) and polymer \( (\rho_2) \) and the mass swollen ratio \( (Q_m) \) as described by Eq. (1):

\[
\nu_{2,s} = \frac{V_p}{V_g} = Q^{-1} = \frac{1/\rho_2}{Q_m / \rho_1 + 1/\rho_2}
\]  

The average molecular weight between two adjacent crosslinks \( (\overline{M}_c) \) represents the degree of crosslinking of the hydrogel networks. \( \overline{M}_c \) in a neutral, divinyl crosslinked network can be expressed as the following Flory-Rehner Equation [9].

\[
\frac{1}{\overline{M}_c} = 2 - \left( \frac{V}{V_L} \right) \left[ \ln \left( 1 - \nu_{2,s} \right) + \nu_{2,s} + \chi \nu_{2,s} \nu_{2,s} \right] \frac{\nu_{2,s}^{1/3} - \nu_{2,s}}{\nu_{2,s}^{1/3} - \nu_{2,s}}
\]  

(2)
Here, $\overline{M}_a$ is the average molecular weight of the linear polymer chains, $V$ is the specific volume of the polymer, $V_i$ is the molar volume of water, and $\chi_{12}$ is the polymer-water interaction parameter. More complex versions of the Flory-Rehner expression have been developed by Peppas and others to describe the swelling behavior of ionic gels or gels crosslinked during polymerization [8]. For neutral gels at highly swelling conditions ($Q>10$), Equation 2 can be simplified to illustrate how gel swelling scales with the average molecular weight between crosslinks ($\overline{M}_c$) [10]:

$$Q = \left[ \frac{\beta(1/2-2\chi_{12})\overline{M}_c}{V_i} \right]^{3/5} = \beta(\overline{M}_c)^{3/5}$$

(3)

Another important parameter used to describe hydrogel swelling is the network mesh size ($\xi$) which can be described as follows [11]:

$$\xi = v_{2,s}^{-1/3} \left( \overline{r_o^2} \right)^{1/2} = Q^{1/3} \left( \overline{r_o^2} \right)^{1/2}$$

(4)

Here, $\overline{r_o^2}$ is the root-mean-squared end-to-end distance of network chains between two adjacent crosslinks in the unperturbed state. It can be determined using the following relationship [11]:

$$\left( \overline{r_o^2} \right)^{1/2} = l(C_n N)^{1/2} = l \left( C_n \frac{2\overline{M}_c}{M_r} \right)^{1/2}$$

(5)

where $C_n$ is the Flory characteristic ratio, $l$ is the bond length along the polymer backbone, $N$ is the number of bonds between adjacent crosslinks, and $M_r$ is the molecular weight of the repeating units of the composed polymer.
Combining Eqs. (4) and (5), one can easily calculate the mesh size of a hydrogel network and further compare it with the hydrodynamic radii of the molecules to be delivered. Theoretically, no solute diffusion is possible within the hydrogel matrix when mesh size approaches the size of the solute as shown in Figure 2.1 [12]. Mesh size is affected by several factors including (1) degree of crosslinking of the gel; (2) chemical structure of the composing monomers; and (3) external stimuli such as temperature, pH and ionic strength. Mesh size is important in determining the physical properties of the hydrogels including mechanical strength, degradability, and diffusivity of the releasing molecule [10, 13]. Typical mesh sizes reported for biomedical hydrogels range from 5 – 100 nm in their swollen state [10, 14]. These size scales are much larger than most small-molecule drugs and therefore diffusion of these drugs are not significantly retarded in swollen hydrogel matrices. However, the release of macromolecules such as peptides, proteins, and oligonucleotides can be sustained from swollen hydrogels due to their significant hydrodynamic radii. When designed appropriately, the structure and mesh size of swollen hydrogels can be tailored to obtain desired rates of macromolecule diffusion [15]. Alternatively, the rate and degree of gel swelling or degradation can also be tailored to control the release of molecules much smaller than the gel mesh size.

2.1.3 Niche roles of hydrogels in drug delivery

The advance in recombinant protein technology has identified numerous protein and peptide therapeutics for disease treatment. However, the effective delivery of these biomolecules is challenging mainly because of their large molecular weights and unique three-dimensional structures. Intravenous or subcutaneous injection is by far the most commonly used route for drug administration. However, these biomolecules are prone to
proteolytic degradation and therefore experience extremely short plasma circulation times and rapid renal clearance, leading to multiple daily injections or increased dosage to maintain the drug concentration in the therapeutic window. Multiple daily injections decrease patient compliance while high doses may induce local toxicity and serious systemic immune responses. Polymeric controlled release formulations such as PLGA offer a sustained release mechanism in which the drug release rates can be controlled by changing polymer molecular weight and composition. However, it is well-recognized that these hydrophobic polymers induce detrimental effects to the encapsulated proteins or peptides during network preparation and delivery [16] as well as trigger the host immune response [17]. Hydrophilic hydrogels, on the other hand, provide relatively mild network fabrication and drug encapsulation conditions that make them suitable for protein delivery [6]. The common niche for hydrogels in controlled release is the encapsulation (and subsequent release) of bioactive materials. Therefore, the systems we will focus on in this review deal with delivery from matrix devices rather than membrane devices. Through proper design, hydrogels can be used in a variety of applications including sustained, targeted, or stealth biomolecule delivery.

![Figure 2.1](image-url)  
**Figure 2.1** Schematic of mesh size in hydrogels at swollen or shrunken states. Adapted from [12].
Several unique properties that hydrogels possess make them useful in delivering biomolecules. For example, stimuli responsiveness can be easily tailored into hydrogel networks during fabrication [18]. This enables sustained or bolus drug delivery corresponding to external stimuli such as pH or temperature. For example, pH-sensitive hydrogels are useful in oral drug delivery as they can protect peptide/protein drugs in the digestive track [19]. The pH responsiveness of hydrogels also facilitates lysosomal escape during gene delivery [20, 21]. Such responsiveness changes the mode of drug administration from merely passive release to active delivery. These exclusive properties of hydrogels can be attributed to the variety of available network precursors. Acrylic acid (AA) and methacrylic acid (MAA) [19, 22, 23] are the most commonly used monomers to fabricate anionic pH-sensitive hydrogels while 2-(dimethylamino)ethyl methacrylate (DMAEMA) [24, 25] is used for cationic hydrogel fabrication. N-isopropylacrylamide (NIPAAm) [26-28] and polypropylene oxide-polyethylene oxide-polypropylene oxide (PPO-PEO-PPO) block copolymers [28-30] are well-suited for the fabrication of temperature-sensitive hydrogels. The reversible swell-collapse transition modulates drug release rates and largely enhances the therapeutic efficacy of biomolecules.

Hydrogels can also be engineered to exhibit bioadhesiveness to facilitate drug targeting, especially through mucus membranes, for non-invasive drug administration [31-34]. Both natural polymers (e.g. chitosan) and synthetic monomers (e.g. AA) provide this advantageous property. Some bioadhesive polymers have been used to fabricate hydrogels for oral [6] and buccal drug delivery [35, 36].

Hydrogels offer an important “stealth” characteristic in vivo owing to their hydrophilicity which increases the in vivo circulation time of the delivery device by evading
the host immune response and decreasing phagocytic activities [37, 38]. For example, Hubbell and coworkers developed poly(ethylene glycol)-based hydrogel nanoparticles as colloidal drug carriers [39]. Several other stealth delivery systems, such as PEGylated gold nanoparticles [37, 40], have also been developed utilizing a PEG shell as a means of steric hindrance. This strategy exploits the hydrophilicity of PEG in excluding enzymatic degradation of the drug to be delivered. When conjugated with other protein therapeutics such as tumor necrosis factor (TNF), these PEGylated gold nanoparticles are good carriers for tumor-targeted delivery [41].

Another prospect of hydrogels is their role as scaffolding materials in tissue engineering applications [42-44]. Excellent examples are cartilage [45, 46] and nerve [47] tissue engineering. The mild gelling conditions and in situ polymerization capabilities of hydrogels enable the simultaneous encapsulation of cells and growth factors. Controlled release of encapsulated growth factors and other agents from these tissue constructs is critical to providing the necessary cues for cell migration, differentiation, angiogenesis, and upregulation of extracellular matrix production required for neotissue growth or regeneration [48, 49].

2.1.4 Drug release mechanisms from hydrogel devices

As discussed in the previous sections, hydrogels have a unique combination of characteristics that make them useful in drug delivery applications. Due to their hydrophilicity, hydrogels can imbibe large amounts of water (> 90 wt%). Therefore, the molecule release mechanisms from hydrogels are very different from hydrophobic polymers. Both simple and sophisticated models have been previously developed to predict the release
of an active agent from a hydrogel device as a function of time. These models are based on the rate-limiting step for controlled release and are therefore categorized as follows:

1. Diffusion-controlled
2. Swelling-controlled
3. Chemically-controlled

Diffusion-controlled is the most widely applicable mechanism for describing drug release from hydrogels. Fick’s law of diffusion with either constant or variable diffusion coefficients is commonly used in modeling diffusion-controlled release [13]. Drug diffusivities are generally determined empirically or estimated a priori using free volume, hydrodynamic, or obstruction-based theories [13].

Swelling-controlled release occurs when diffusion of drug is faster than hydrogel swelling. The modeling of this mechanism usually involves moving boundary conditions where molecules are released at the interface of rubbery and glassy phases of swollen hydrogels [50]. The release of many small molecule drugs from hydroxypropyl methylcellulose (HPMC) hydrogel tablets is commonly modeled using this mechanism. For example, Methocel® matrices, a combination of methylcellulose and HPMC, from Dow Chemical Company are commercially available for preparing swelling-controlled drug delivery formulations exhibiting a broad range of delivery timescales [50, 51].

Chemically-controlled release is used to describe molecule release determined by reactions occurring within a delivery matrix. The most common reactions that occur within hydrogel delivery systems are cleavage of polymer chains via hydrolytic or enzymatic degradation or reversible or irreversible reactions occurring between the polymer network and releasable drug. Under certain conditions the surface or bulk erosion of hydrogels will
control the rate of drug release. Alternatively, if drug-binding moieties are incorporated in the hydrogels, the binding equilibrium may determine the drug release rate. Chemically-controlled release can be further categorized according to the type of chemical reaction occurring during drug release. Generally, the liberation of encapsulated or tethered drugs can occur through the degradation of pendant chains or during surface-erosion or bulk-degradation of the polymer backbone. A more thorough discussion of these mechanisms can be seen in a later section of this review as well as in several other excellent reviews [6, 13, 52].

2.1.5 Design criteria for hydrogels in drug delivery formulations

Materials selection and network fabrication governs the rate and mode of drug release from hydrogel matrices. Several design criteria are crucial for drug delivery formulations and have to be evaluated prior to hydrogel fabrication and drug loading. These criteria are also important in mathematical modeling of drug release. Table 2.2 lists these important criteria and variables for designing hydrogel-based drug carriers. Within the realm of transport properties, the most notable variable is the drug diffusion coefficient, which is affected by the molecular size of the drug and characteristics of the polymer network. Hydrogel crosslinking density affects diffusivity to a large extent as shown in Figure 2.1 and as discussed previously. If special functionalities, such as ionic groups, are introduced into the hydrogel networks, interactions between these functionalities and encapsulated drugs certainly affect drug diffusivity. Physical properties of the hydrogel also affect drug release. For example, polymer molecular weights, composition, and polymer/initiator concentrations influence hydrogel swelling and also degradation. Finally, the stimuli-responsiveness of a
hydrogel network can also mediate the amount and rate of drug delivery. The understanding of transport and physical properties are especially crucial in modeling molecule release.

Even if a hydrogel delivery formulation is designed with the appropriate physical and transport properties, it may still fail to perform its therapeutic role when implanted in vivo due to a localized inflammatory response. The formation of a fibrous capsule surrounding the delivery device creates additional diffusion barriers that may limit drug release rates while increased proteolytic activity may increase rates of matrix and drug degradation. Proper material selection, fabrication process, and surface texture of the device are therefore always critical in designing biocompatible hydrogel formulations for controlled release.

Table 2.2 Design criteria for hydrogels in drug delivery formulations.

<table>
<thead>
<tr>
<th>Design criteria</th>
<th>Design variables</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transport properties</strong></td>
<td></td>
</tr>
<tr>
<td>Molecule diffusion</td>
<td>• Molecular weight and size of protein</td>
</tr>
<tr>
<td></td>
<td>• Molecular weight of polymer</td>
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<tr>
<td></td>
<td>• Crosslinking density</td>
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<tr>
<td></td>
<td>• Polymer-protein interactions</td>
</tr>
<tr>
<td></td>
<td>• Hydrogel degradation rate</td>
</tr>
<tr>
<td></td>
<td>• Additional functionalities</td>
</tr>
<tr>
<td><strong>Physical properties</strong></td>
<td></td>
</tr>
<tr>
<td>Gelling mechanisms /conditions</td>
<td>• Polymer/crosslinker/initiator concentrations</td>
</tr>
<tr>
<td>Structural properties</td>
<td>• Temperature, pH, ionic strength</td>
</tr>
<tr>
<td>Biodegradability</td>
<td>• Molecular weight of polymer</td>
</tr>
<tr>
<td>Stimuli-responsiveness</td>
<td>• Mechanical strength</td>
</tr>
<tr>
<td></td>
<td>• Concentration of degradable groups</td>
</tr>
<tr>
<td></td>
<td>• Concentration of responsive groups</td>
</tr>
<tr>
<td><strong>Biological properties</strong></td>
<td></td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>• Cytotoxicity of the hydrogel</td>
</tr>
<tr>
<td></td>
<td>• Capsule formation</td>
</tr>
</tbody>
</table>
2.2 Novel engineering of hydrogels for drug delivery

2.2.1 Biodegradable hydrogels

For most biomedical applications, biodegradable hydrogels are favored over non-degradable gels since they degrade in clinically relevant timescales under relatively mild conditions. Compared to non-degradable hydrogels, degradable carriers eliminate the need for additional surgeries to recover the implanted gels. However, proper techniques for predicting hydrogel degradation rates are critical for successful application of these degradable systems as they facilitate the design of implants with optimal degradation profiles that result in proper rates of drug release or tissue regeneration and hence maximize therapeutic effects.

The fabrication and modeling of hydrolytically degradable hydrogels are well-developed. For example, West and Hubbell fabricated PLA-\(b\)-PEG-\(b\)-PLA hydrogels composed of poly(lactic acid) (PLA) and poly(ethylene glycol) (PEG) block copolymers for protein release applications [53]. Metters et al. developed scaling laws to predict the degradation rates of PLA-\(b\)-PEG-\(b\)-PLA hydrogels based on macroscopic properties such as compressive modulus and volumetric swelling ratio [54-56]. Mason et al. further applied these scaling laws to predicting protein diffusion and release during bulk network degradation [10]. Using a more rigorous approach, Hennink and coworkers recently utilized a Monte Carlo simulation technique to microscopically predict the degradation and protein delivery behaviors of hydroxyethyl methacrylated dextran (dex-HEMA) microspheres [57].

In addition to hydrolytically degradable hydrogels, synthetic gels incorporating biological moieties that can be degraded enzymatically are also under intensive investigation. One way to fabricate this type of hydrogel is to incorporate peptide substrates for enzymatic
hydrogel formation [58] and degradation [59, 60]. Alternatively, polymers that can be naturally degraded by enzymes (e.g. polycaprolactone can be degraded by lipase) can be copolymerized with PEG to form enzymatically degradable gels [61]. Although hydrogels derived from natural sources (such as chitosan, gelatin, dextran, etc.) can also be degraded enzymatically, in many cases synthetic hydrogels containing defined biological moieties are more favorable because of their tunable physicochemical properties. For example, the degradation behavior can be more accurately tailored to obtain better control over gel degradation and drug release rates. Hubbell and colleagues have developed a hydrogel system containing integrin-binding sites for cell attachment and peptide substrates for matrix metalloproteinases (MMPs) or plasmin to mimic the natural bidirectional communication between extracellular matrix and migrating cells. Cells can only infiltrate the designer matrices once the gels are locally degraded in response to secretion of MMPs by invading cells [59, 60].

2.2.2 Smart hydrogels

“Smart” hydrogels, or stimuli-sensitive hydrogels, are very different from inert hydrogels in that they can “sense” changes in environmental properties such as pH and temperature and respond by increasing or decreasing their degree of swelling. These sensing capabilities are attractive in many biomedical applications and several review papers have been published in this field [18, 28]. The volume-changing behavior of ‘smart’ hydrogels is particularly useful in drug delivery applications as drug release can be triggered upon environmental changes [18, 29, 62]. For non-ionic hydrogels, the degree of swelling only depends on the chemical compositions of the polymers and does not respond to external pH
change. When ionic moieties are incorporated into hydrogels, the swelling depends not only on the chemical composition of the gel but also on the pH of the surrounding medium. Generally, anionic hydrogels deprotonate and swell more when external pH is higher than $pK_a$ of the ionizable groups tethered on polymer chains while cationic hydrogels protonate and swell more when external pH is lower than the $pK_b$ of the ionizable groups. Depending on the ionic monomers used to fabricate the gel, the pH-dependent swelling curves exhibit one or more inflection points near the $pK_a/pK_b$ of the ionizable groups as shown in Figure 2.2. Many modeling efforts have been devoted to predicting the dynamic and equilibrium swelling of ionic hydrogels [23, 63-66]. Because the swelling/deswelling behavior of the ionic hydrogels is closely related to ion movement, the swelling kinetics depends not only on the pH but also on the compositions of the external solutions. Hydrogels with pH-responsiveness have been used in a number of applications including oral peptide delivery [67-71], valves for microfluidic devices [72], and artificial muscles [73-75].

Figure 2.2. Schematic of relative ionic hydrogel swelling as a function of pH.
Another important stimulus for causing hydrogel responsiveness is temperature. The most commonly used synthetic polymer for fabricating temperature-sensitive hydrogels is poly(N-isopropylacrylamide) (poly(NIPAAm)), which possesses a lower critical solution temperature (LCST) at around 32°C. The value of the LCST can be increased or decreased by copolymerizing hydrophilic or hydrophobic polymers with poly(NIPAAm). When the bulk temperature is higher than the LCST of the polymer, the polymer chains lose their bound-water. Hydrophobic interactions between the polymer chains lead to a rapid collapse (deswelling) of the gel [76]. Readers are directed to other more thorough reviews discussing the mechanisms and applications of thermo-sensitive hydrogels [28, 30]. Temperature-responsiveness is particularly useful for in-situ formation of drug-delivery devices since it allows handling of the formulation in the sol-phase at room temperature and solidification of the carrier upon injection [28].

More recently, studies have been conducted to fabricate and characterize hydrogels with dual-sensitivities. This was accomplished by copolymerizing a temperature-sensitive monomer, usually N-isopropylacrylamide, and a pH-sensitive monomer such as acrylic acid or methacrylic acid [21, 77-82]. For example, Stayton’s group has investigated a series of co-polymers containing propylacrylic acid (PAA) and N-isopropylacrylamide pendant chains as pH- and thermo-sensitive moieties, respectively [20]. This new class of copolymers can sense environmental changes in the physiological range and has found usefulness in intracellular drug delivery in which subtle pH differences across the endosomal membrane triggers the delivery of protein or DNA.
2.2.3 Biomimetic hydrogels

One drawback of using synthetic and some natural hydrogels for in vivo applications is that they do not possess biological recognition sites for supporting cellular activities. For this reason, relatively inert polymer chains can be tailored with select biological moieties to yield bioactive hydrogels for tissue engineering applications. The Arginine-Glycine-Aspartic acid (RGD) tri-peptide derived from fibronectin is the most commonly used biological moiety in this regard as it mediates the adhesion of many cell types through integrin-binding without the need for protein adsorption on a hydrogel surface [83-86]. Through the selective presentation of bioactive ligands on otherwise bioinert hydrogel background, researchers are able to better control cell-hydrogel interactions to fulfill specific biomedical applications.

The controlled incorporation and presentation of biological cues within hydrogel matrices has also played a role in the development of novel controlled delivery devices. For example, in vivo observations of the sequestering and protection of proteins by the extracellular matrix (ECM) have inspired the design of novel biomimetic hydrogels with specific and reversible protein-binding capabilities [87-89]. This approach is especially useful in controlled release of growth factors for tissue regeneration as it mimics the mechanism and temporal profiles of endogenously produced growth factors. Through judicious selection of network-immobilized ligands with desired protein-binding affinities or by adjusting the molar ratio of protein to protein-binding ligand, researchers can readily manipulate protein release rates form these bioactive matrices.

Another biomimetic hydrogel system used in controlled release applications is the enzymatically-cleavable prodrug system. The main advantage of this approach is that the degradation rate of the prodrug linkage is directly proportional to the concentration of
specific enzymes secreted by local cells. Therefore the rate of drug release self-adjusts to the rate of cellular infiltration and cell-mediated matrix remodeling. Therapeutic proteins such as vascular endothelial growth factor (VEGF) have been covalently immobilized within hydrogel networks by enzyme-sensitive oligopeptides [90]. VEGF release is mediated by proteases (e.g. matrix metalloproteinases or MMPs) secreted by migrating fibroblast and endothelial cells and is therefore made available only when specific cellular processes occur. This cell-demanded VEGF release has been shown to not only preserve growth factor bioactivity but also promote localized angiogenesis.

2.3 Molecule release mechanisms for hydrogel formulations

The physicochemical properties of the hydrogel network as well as the selection of drug-loading method will determine the mechanism(s) by which the loaded drug is released from the crosslinked matrix. The incorporation of drugs into hydrogel delivery matrices can be performed via one of the following ways: (1) Post-loading: absorption of drugs is achieved after hydrogel networks are formed. If an inert hydrogel system is used, diffusion is the major driving force for drug uptake and release will be determined by diffusion and/or gel swelling. In the presence of hydrogels containing drug-binding ligands, terms accounting for drug-polymer interaction and drug diffusion must both be included in any model description of release; (2) In-situ loading: drugs or drug-polymer conjugates are mixed with polymer precursor solution and hydrogel network formation and drug encapsulation are accomplished simultaneously. In these systems, the release of drugs can be controlled by diffusion, hydrogel swelling, reversible drug-polymer interactions, or degradation of labile covalent bonds.
2.3.1 Diffusion-controlled delivery systems

Understanding the mechanisms and identifying the key parameters that govern drug release from hydrogels are the first step toward accurately predicting the entire release profile. For porous hydrogels, when pore sizes are much larger than the molecular dimensions of the drug, the diffusion coefficient can be related to the porosity and the tortuosity of the hydrogels [91]. However, for nonporous hydrogels and for porous gels with pore sizes comparable to the drug molecular size, drug diffusion coefficients are decreased due to steric hindrance provided by polymer chains within the crosslinked networks [13, 91, 92]. In these cases, the average free volume per molecule available to the drug is decreased and the hydrodynamic drag experienced by the drug is increased, leading to increased drug diffusion path length compared to porous hydrogels with pore sizes much larger than the encapsulated drug [93-95]. Due to the usually high permeabilities of hydrogel networks and the advantages of in situ fabrication, most research efforts are focused on understanding diffusion-controlled release of encapsulated drugs from three-dimensional hydrogel matrices.

Drug diffusion within highly swollen hydrogels is best described by Fick’s law of diffusion or Stefan-Maxwell equations [8]. Diffusion-controlled hydrogel delivery systems can be either reservoir or matrix systems [96]. For a reservoir system where the drug depot is surrounded by a polymeric hydrogel membrane, Fick’s first law of diffusion can be used to describe drug release through the membrane:

\[ J_A = -D \frac{dC_A}{dx} \]

Here, \( J_A \) is the flux of the drug, \( D \) is the drug diffusion coefficient, and \( C_A \) is drug concentration. In many cases, the drug diffusion coefficient is assumed constant to simplify
the modeling. However, in the general case it is a function of drug concentration and a special correlation incorporating the concentration-dependent drug diffusivity must be utilized to accurately predict drug flux. Another assumption of this expression is that \( J_A \) is the drug flux corresponding to the mass average velocity of the system.

For a matrix system where the drug is uniformly dispersed throughout the matrix, unsteady-state drug diffusion in a one-dimensional slap-shaped matrix can be described using Fick’s second law of diffusion:

\[
\frac{dC_A}{dt} = D \frac{d^2 C_A}{dx^2}
\]  

(7)

Here, the drug diffusion coefficient is again assumed as a constant. Other assumptions include sink condition and a thin planar geometry where the release through slab edges is neglected. When diffusivity is concentration-dependent the following equation is used:

\[
\frac{\partial C_A}{\partial t} = \frac{\partial}{\partial x} \left( D(C_A) \frac{\partial C_A}{\partial x} \right)
\]  

(8)

Many previous attempts to model diffusion-controlled drug delivery from hydrogels rely largely on empirically determined diffusion coefficients. Once the diffusion coefficient is determined, Eqs. (6) to (8) can be solved, together with proper initial and boundary conditions, to yield drug concentration profiles that dictate the release kinetics. For example, an exact analytical solution to Eq. (7) can be obtained using separation of variable technique. The ratio of the amount of molecule released up to any time \( t \) \( (M_t) \) to the final amount of molecule release \( (M_\infty) \) can be expressed as:

\[
\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \cdot \exp \left[ -\frac{8}{(2n+1)^2 \pi^2} \cdot \frac{D}{L^2} \cdot t \right]
\]  

(9)
This equation can be used to predict the diffusion of a broad range of molecules including small molecular weight drugs and biomacromolecules like proteins and DNA once an appropriate diffusion coefficient is obtained. Although this simple solution applies to many diffusion-controlled drug release systems, model complexity will increase as other mechanisms, polymer-drug interactions, and when non-spherical drugs are used [15].

Another empirical equation developed by Peppas et al. assumes a time-dependent power law function [6, 50]:

$$\frac{M_t}{M_\infty} = k \cdot t^n$$  \hspace{1cm} (10)

Here, $k$ is a structural/geometric constant for a particular system and $n$ is designated as release exponent representing the release mechanism. Table 2.3 lists the $n$ values for delivery matrices with different geometries and release mechanisms [50].

<table>
<thead>
<tr>
<th>Matrix Geometry</th>
<th>Diffusion-controlled delivery system (Case I)</th>
<th>Swelling-controlled delivery system (Case II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slab</td>
<td>$n = 0.5$</td>
<td>$n = 1$</td>
</tr>
<tr>
<td>Cylinder</td>
<td>$n = 0.45$</td>
<td>$n = 0.89$</td>
</tr>
<tr>
<td>Sphere</td>
<td>$n = 0.43$</td>
<td>$n = 85$</td>
</tr>
</tbody>
</table>

It is noteworthy that in a purely swelling-controlled slab-based delivery system, the drug fractional release ($M_t/M_\infty$) appears to be zero-order as the release exponent equals unity. The power law is easy to use and can be applied to most diffusion-controlled release systems. However, it is too simple to offer a robust prediction for complicated release phenomena. For example, in diffusion-controlled systems where $n = 0.5$, the power law is
only valid for the first 60% of the release profile. These empirical models can only predict the release profile after certain release experiments are conducted and have limited capability to predict how the release profiles will change as the chemical or network properties of the system are varied.

Analytical solutions to Fick’s law are not available when more complex geometries or non-constant drug diffusivities are incorporated into the model descriptions. Except in extremely dilute systems, drug diffusion coefficients will be a function of drug concentration. Additionally for hydrogel systems diffusivities of encapsulated molecules will depend on the degree of swelling and crosslinking density of the gels. Therefore the diffusion coefficient used to describe drug release will be sensitive to environmental changes or degradation of the polymer network and may vary over the timescale of release. Several theoretical models have been developed to relate molecule diffusion coefficients to fundamental hydrogel characteristics and have been reviewed elsewhere [6, 13]. Generally, theoretical models for predicting molecule diffusion coefficients have the following general form:

\[
\frac{D_g}{D_o} = f(r_s, v_{2G}, \xi)
\]  

(11)

Here, \(D_g\) and \(D_o\) are the drug diffusion coefficients in the swollen hydrogel network and in pure solvent, respectively. \(r_s\) is the size of the drug to be delivered. This general expression takes into account factors affecting drug release such as the structure of the gel, the polymer composition, the water content, and the size of the molecules. For a degradable hydrogel, \(D_g\) changes as the network degrades due to an increase in gel mesh size and a decrease in polymer volume fraction over time.
Several theories have been developed to correlate the relationship between drug diffusivity in the gels and in the solution [13]. For example, the following equation using a free-volume approach proposed by Lustig and Peppas can be used to describe the relationship between drug diffusivity and network structure [15]:

\[
\frac{D_g}{D_o} = \left(1 - \frac{r_s}{\xi}ight) \exp\left(-Y \left(\frac{v_{2,s}}{1 - v_{2,s}}\right)\right)
\]

(12)

Here, \(Y\) is defined as the ratio of the critical volume required for a translational movement of the encapsulated drug molecule and the average free volume per molecule of solvent. A good approximation for \(Y\) is unity. For highly swollen (\(Q > 10\)) hydrogels with degradable crosslinks the diffusivity correlation shown in Eq. (12) can be simplified during the initial stages of degradation to [10, 97]:

\[
1 - \frac{D_g}{D_o} = \frac{r_s}{\xi} \sim e^{-7/5 jk_e t}
\]

(13)

Here, the lumped parameter \(j k_E\) is the pseudo-first-order reaction rate constant for the hydrolysis of a labile crosslink. From this expression one can realize that mesh size is time-dependent due to network degradation. It is clear that \(D_g\) increases as degradation proceeds and approaches \(D_o\). The rate of increase in drug diffusivity depends on network structure and bond cleavage kinetics [10, 98].

2.3.2 Swelling-controlled delivery systems

Another mechanism for drug delivery is swelling-controlled delivery. As shown in Figure 2.3, hydrogels may undergo a swelling-driven phase transition from a glassy state where entrapped molecules remain immobile to a rubbery state where molecules rapidly diffuse. In these systems, the rate of molecule release depends on the rate of gel swelling.
One example of swelling-controlled drug delivery systems is hydroxypropyl methylcellulose (HPMC). Drug loaded HPMC tablets are three dimensional, hydrophilic matrices that are usually stored in a dry, glassy state. After oral administration, HPMC polymer absorbs liquid and a rapid glassy-to-rubbery phase-transition occurs once the glass transition temperature \(T_g\) is reached, causing the systematic release of loaded drugs. The drug release rates are modulated by the rate of water transport and the thickness of the gel layer.

![Schematic of HPMC hydrogel tablet in the glassy (left) and rubbery (right) state.](image)

**Figure 2.3.** Schematic of HPMC hydrogel tablet in the glassy (left) and rubbery (right) state.

Drug diffusion time and polymer chain relaxation time are two key parameters determining drug delivery from polymeric matrices. In diffusion-controlled delivery systems, the time-scale of drug diffusion, \(t\), (where \(t = \delta(t)^2 / D\) and \(\delta(t)\) is the time-dependent thickness of the swollen phase) is the rate-limiting step while in swelling-controlled delivery systems the time-scale for polymer relaxation (\(\lambda\)) is the rate-limiting step. The Deborah number \((De)\) is used to compare these two time-scales [99, 100]:

\[
De = \frac{\lambda}{t} = \frac{\lambda D}{\delta(t)^2}
\]

In diffusion-controlled delivery systems \((De \ll 1)\), Fickian diffusion dominates the molecule release process and diffusion equations described in the previous section can be
used to predict molecule release. In swelling-controlled delivery systems ($D_e >> 1$), the rate of molecule release depends on the swelling rate of polymer networks.

The empirical power law (Eq. (9)) used to describe diffusion-controlled drug release from hydrogel matrices can also be used comprehensively in swelling-controlled delivery systems. A modification of Eq. (9) takes into account both the drug diffusion and polymer relaxation [101]:

$$\frac{M_t}{M_\infty} = k_1 t^m + k_2 t^{2m} \quad (15)$$

where $k_1$, $k_2$, and $m$ are constants. The two terms on the right side represent the diffusion and polymer relaxation contribution to the release profile, respectively.

The above empirical relationship does not account for “moving-boundary” conditions in which the gel expands heterogeneously as water penetrates and swells the gels. For this more rigorous description, Korsmeyer and Peppas introduced a dimensionless swelling interface number, $Sw$, to correlate the moving boundary phenomena to hydrogel swelling [102-104]:

$$Sw = \frac{V \delta(t)}{D} \quad (16)$$

Here, $V$ is the velocity of the hydrogel swelling front and $D$ the drug diffusion coefficient in the swollen phase. For a slab system when $Sw << 1$, drug diffusion is much faster than the movement of glassy-rubbery interface and thus a zero-order release profile is expected.

Building on several modeling iterations [11, 15, 105-107], a more rigorous method for predicting molecule release from swelling-controlled systems is provided by a sequential layer model developed by Siepmann and Peppas [50, 108-112]. In this model, drug diffusion,
polymer relaxation and dissolution are all taken into account. Drug transport in both radial and axial directions is accounted for using Fick’s second law of diffusion in a cylindrical geometry with concentration-dependent diffusion coefficients as shown below [110, 111]:

\[
\frac{\partial C_k}{\partial t} = \frac{\partial}{\partial r} \left( D_k \frac{\partial C_k}{\partial r} \right) + \frac{D_k}{r} \frac{\partial C_k}{\partial r} + \frac{\partial}{\partial z} \left( D_k \frac{\partial C_k}{\partial z} \right)
\]  

(17)

Here, \( C_k \) and \( D_k \) are the concentration and diffusivity of the diffusible species (1: water; 2: drug), respectively. Concentration-dependent diffusivities derived by a “Fujita-like” free-volume model can be expressed as [113]:

\[
D_1 = D_{1eq} \exp \left( -\beta_1 \left( 1 - \frac{C_1}{C_{1eq}} \right) \right)
\]

(18)

\[
D_2 = D_{2eq} \exp \left( -\beta_2 \left( 1 - \frac{C_1}{C_{1eq}} \right) \right)
\]

(19)

where \( \beta_1 \) and \( \beta_2 \) are dimensionless constants and “eq” represents the equilibrium drug concentration at the water/matrix interface where polymer disentanglement occurs.

Due to the concentration-dependent diffusion coefficients, Eqs. (17) to (19) can only be solved numerically. Siepmann et al. demonstrated that these numerical solutions agreed well with experimental results [50, 108]. This model is therefore useful in predicting the shape and dimensions of HPMC tablets needed to acquire desired release profiles [109].

Stemming from the work of Siepmann and Peppas, Wu and coworkers [114] recently developed a mathematical model to describe swelling-controlled release. They introduced additional boundary conditions derived from a volume balance and accounted for two-dimensional movement of the swelling front in the radial or axial directions. This model assumes a homogeneous mixture of drug and polymer at \( t = 0 \), perfect sink conditions, and
geometrical symmetry of the tablet. Model predictions were verified using compressed poly(ethylene oxide) (PEO) hydrogel tablets with different molecular weights. The results of water uptake, swelling and dissolution of PEO matrices as well as drug release are shown to agree well with the mathematical model [114].

2.3.3 Chemically-controlled delivery systems

In addition to diffusion and swelling-controlled delivery systems discussed previously, a third type of molecule release mechanism is chemically-controlled delivery. The latter can be further classified as (1) purely kinetic-controlled release where polymer degradation (bond-cleavage) is the rate-determining step and diffusion term is assumed to be negligible; and (2) reaction-diffusion controlled release in which both reaction (e.g. polymer degradation, protein-drug interaction) and diffusion terms must be included in the model to accurately predict drug release. The reaction-diffusion controlled release is particularly intriguing as more synthetic hydrogel systems designed with drug-binding capacity are utilized in drug delivery [87, 88, 115] and tissue engineering [89].

2.3.3.1 Kinetic-controlled release – Pendant chain systems

There are two types of kinetic-controlled release systems: pendant chain (prodrugs) and surface-eroding systems. In pendant chain systems, drugs are covalently linked to the hydrogel network via cleavable spacers and drug release is controlled by the rate of spacer-bond cleavage. In surface-eroding systems, drug release is mediated by the rate of surface erosion. Drug diffusion does not determine the rate of drug release in either system.

Prodrugs or polymer-drug conjugates are designed to enhance the therapeutic efficacy of the drug. This strategy is especially useful when growth factors are to be delivered as most of them are susceptible to rapid proteolytic degradation. The design of prodrugs has
attracted much attention and extensive reviews on the design and therapeutic application of these systems can be found elsewhere [116, 117].

Generally, the release of covalently tethered prodrugs is determined by the degradation rate of the polymer-drug linkage [118-121]. Most of these linkages have been designed to be hydrolytically degradable allowing degradation and release rates to be characterized by fairly simple first-order kinetic relationships [59]. However, in particular applications, for example where a more targeted delivery profile is desired, it is advantageous to design enzymatically cleavable spacer bonds [122]. These chemistries lead to more complex release kinetics. Furthermore, in cases where the prodrugs are tethered to degradable hydrogel matrices, the kinetics of gel degradation may also play a significant role in determining overall drug release profiles. [118-123]

Ehrbar et al. recently developed fibrin matrices tethered with pendant vascular endothelial growth factor (VEGF) variants linked by plasmin-sensitive peptidyl substrates [122]. These covalently bound VEGF variants can only be liberated from the insoluble matrix through plasmin-mediated cleavage of the engineered peptide substrates. First-order cleavage kinetics were used to model the time-dependent VEGF release. Accurate prediction of VEGF release profiles also required a description of VEGF-release via matrix-mediated degradation. Two adjustable parameters were therefore used to accurately predict complete VEGF release profiles. The first parameter was the pseudo first-order degradation rate constant, \( k \). The degradation of bonds within the fibrin network and the plasmin-sensitive substrates used to link VEGF to the fibrin were assumed to follow the same first-order kinetics. The second adjustable parameter, \( N \), represented the number of fibrin repeat units between two crosslinks and was an indication of fibrin network structure. As shown in
Figure 2.4, the developed model accurately predicted release of cleavable and non-cleavable VEGF variants from both low and high-density fibrin matrices by accounting for both network structure and kinetics of individual bond cleavage.

Figure 2.4 Mathematical modeling predicts experimental observations of proteolysis-mediated release of fibrin-bound VEGF121 variants from low- or high-density fibrin gel networks. Reproduced from [122], Copyright (2005), with permission from Elsevier.

Unique release profiles unattainable with diffusion-controlled release mechanisms have also been demonstrated from hydrolytically degradable hydrogels with tethered agents. Dubose et al. covalently incorporated fluorescently labeled probe molecules within the three-dimensional network structure of PEG-based hydrogels formed via step-growth polymerizations [123]. As shown in Figure 2.5, they demonstrated that hydrolytic degradation of covalent bonds within the step-crosslinked PEG network as well as the cleavage of immobilized probe molecules resulted in a biphasic release profile in which a constant molecular release profile is obtained prior to gel dissolution and an almost instantaneous burst release following gel dissolution. The authors demonstrated that the
slope of the approximate zero-order delivery regime as well as the extent of the latent burst could be controlled by crosslinker functionality (tetra-functional versus octa-functional PEG, Figure 2.5a) and degradation kinetics (varying temperature, pH, or chemistry of the degradable bond, Figure 2.5b).

2.3.3.2 Kinetic-controlled release – Surface-eroding systems

Other kinetic-controlled systems occur when drug release is mediated by surface erosion of the polymer matrix. For hydrophobic polymer networks, surface erosion occurs when the rate of water transport into the polymer is much slower than the rate of bond hydrolysis. However, due to the inherently high water content of hydrogels, surface erosion only occurs in enzymatic-degrading systems where the transport of enzyme into the gel is slower than the rate of enzymatic degradation. While no hydrogels have been specifically designed to degrade in this fashion, surface erosion of enzymatically degradable poly(ethylene glycol)-polycaprolactone (PCL-b-PEG-b-PCL) block copolymer hydrogels has been observed in vitro by Rice et al. when exposed to relatively high concentrations of lipase [61].

![Fractional probe release from degradable PEG-acrylate/dithiol gels formed via step-growth polymerization](image)

Figure 2.5 Fractional probe release from degradable PEG-acrylate/dithiol gels formed via step-growth polymerization (a) Gels fabricated from 30 wt % eight-armed PEG-acrylate/DTT precursor solutions and degraded at varying temperatures: 37°C (▲), 46°C (♦), and 57°C (■). (b) Gels fabricated with either four-arm/10-kDa (■) or eight-arm/20-kDa (♦) PEG were measured and compared with model predictions (— — —). Reproduced with permission from [123]. Copyright 2005, John Wiley and Sons.
Most of the models focusing on surface-eroding polymers are based on hydrolytically-degrading polymers. These relationships, however, can also be applied to enzymatically degradable, surface-eroding hydrogel systems. Surface-eroding matrices are advantageous for drug delivery applications as the structural integrity of the carrier device is maintained during delivery and zero-order release of the encapsulated molecules can be readily obtained by appropriate choice of device geometry [7].

Hopfenberg initially developed a drug delivery model where the release only depends on matrix erosion rates. Eq. 22 describes the release from surface-eroding devices with an initial dimension \( a_0 \) (radius for a spherical or cylindrical geometry and half-thickness for slab geometry) and drug concentration \( C_0 \) [124]:

\[
\frac{M_t}{M_\infty} = 1 - \left( 1 - \frac{k_a t}{C_0 a_0} \right)^n
\]  

(20)

In this equation, \( n \) is a geometrical factor and a number of 1, 2, or 3 is used for a slab, cylinder, or sphere, respectively. It is clear that when a slab-shaped device is used (\( n = 1 \)), drug release appears to be a zero-order profile.

Following Hopfenberg’s work, Katzhendler, Hoffman, and coworkers further developed a general mathematical model for heterogeneous eroding networks accounting for different radial and vertical erosion rate constants for a flat tablet (\( k_a \) and \( k_b \) for radial and vertical degradation constant, respectively) [125]:

\[
\frac{M_t}{M_\infty} = 1 - \left( 1 - \frac{k_a t}{C_0 a_0} \right)^2 \left( 1 - \frac{2k_b t}{C_0 b_0} \right)
\]  

(21)

Here, \( a_0 \) is the initial radius of the tablet and \( b_0 \) is the thickness of the tablet. By changing the radius to thickness ratio of the device, one can easily obtain various drug release rates. It is
noteworthy that in these models, swelling of the matrices is either not considered or is assumed to occur prior to erosion and drug release. Stemming from these initial efforts, several additional models have been developed to predict molecule release via surface-erosion [108, 126, 127].

2.3.3.3 Reaction-diffusion controlled release – Bulk-degrading systems

Many of the approaches for modeling drug release from hydrogel networks assume only one mechanism, either diffusion, swelling, or degradation, dominates the release process. Although not realistic for many cases, this is one way to simplify the model and, in many cases, obtain a reasonable fit to experimental results. As more complicated drug delivery systems are designed to fulfill the ever-increasing needs for advanced drug delivery and tissue engineering, the assumption of a single dominant release mechanism will no longer be suitable. Overlooking the coupled effects of diffusion and matrix degradation within hydrogel matrices will result in significant deviations when comparing modeling and experimental results.

The coupling of reaction and diffusion phenomena is already notable in bulk degrading networks where drug release profiles are governed by both network degradation and molecule diffusion. Macroscopically, this degradation-diffusion coupling phenomena can be observed through the swelling characteristics and mechanical properties. The degradation behavior of chain-polymerized hydrogels with hydrolytically or enzymatically labile bonds can be tailored through a variety of parameters. Sawhney’s pioneering work incorporated degradable PLA moieties within hydrophilic PEG macromers [128]. The resulting PLA-PEG-PLA block copolymers can be polymerized to form hydrolytically degradable hydrogels. Metters et al. further described the release of encapsulated macromolecules from
bulk-degrading, covalently crosslinked PLA-PEG-PLA hydrogels considering network structure as well as degradation kinetics [55, 56]. Generally, molecule diffusivity decreases as crosslinking density increases ($\overline{M_c}$ decreases), as the molecular size ($r$) increases, and as the polymer volume fraction of the gel ($r_{w}$) increases [91, 129, 130]. In PLA-PEG-PLA hydrogel systems, molecule diffusivity can be correlated to gel degradation kinetics and can be used to predict drug release corresponding to gel degradation as shown in Figure 2.6 [10, 97]. The diffusion coefficient of a solute from the degrading network with time-dependent mesh size can then be obtained using Eq. (13) described in the previous section. As shown in Figure 2.6, the scaling model agreed well with the volumetric swelling ratio of the degrading gels while for solute release only a qualitative agreement was obtained.

The degradation behaviors described above are only valid for hydrogels made from di-vinyl macromers. For hydrogels formed via chain-polymerization of multifunctional macromers such as acrylated poly(vinyl alcohol) (PVA), Martens et al. developed a generalized statistical-co-kinetic model to predict their degradation behaviors [131-133]. In this model, a statistical approach was used to predict the different configurations of the crosslinking molecules and kinetic chains. It also accounts for the probability of an intact degradable linkage. The model was verified by experimental observation of gel swelling, mass loss and compressive modulus [133]. Combining the degradation kinetics provided by this model and the diffusivity estimated by Eq. (13), the release of a model protein, bovine serum albumin (BSA), was verified [97].

For hydrogels formed via step-growth polymerization, Metters and Hubbell have shown that the degradation rates of networks depend on molecular weight, hydrophilicity, and degree of functionality of the starting monomers [134].
Figure 2.6 (A) Volumetric swelling ratio and (B) fractional release of BSA as a function of degradation time from a series of PLA-\textit{b}-PEG-\textit{b}-PLA hydrogels polymerized from increasing concentrations of macromer: (●) 25 wt\%, (■) 35 wt\%, and (♦) 50 wt\%. Lines represent exponential fits to the swelling data (A) and solute release predictions based on scaling equations (B). $D_0 = 1.0 \times 10^{-5}$ mm$^2$/s for all curves. Reproduced with permission from [10]. Copyright 2001 American Chemical Society.
In addition to the statistical modeling approaches assuming homogeneous changes in gel properties, Monte Carlo simulations have also been used to predict protein release from degradable polymer networks at the microscopic level. Gopferich and Langer developed Monte Carlo simulations to predict polymer erosion and monomer release. Although this work was not for hydrogel systems, it allowed the calculation of porosity distributions within the polymer and was useful in predicting drug and degraded monomer release [135-138]. Monte Carlo simulation is good for describing network morphological changes, however it does not provide any information regarding molecule release. Diffusion equations (Fick’s law) must be incorporated in order to link the network degradation to molecule diffusion [138]. The following modified diffusion equation can be used to describe one-dimensional diffusion in porous polymers:

$$\frac{\partial}{\partial t} C(x,t)\varepsilon(x,t) = \frac{\partial}{\partial x} D_{\text{eff}}(C)\varepsilon(x,t) \frac{\partial C(x,t)}{\partial x}$$

(22)

Here, $C(x,t)$ is the concentration of diffusing monomer, $\varepsilon(x,t)$ is the porosity along the diffusion path, and $D_{\text{eff}}(C)$ is the effective concentration-dependent diffusion coefficient.

Recently, Vlugt-Wensink et al. developed kinetic Monte Carlo simulations to predict protein release from crosslinked dextran microspheres [57]. Although this approach, reasonably predicts protein release from degrading networks and incorporates spatial variations in the network microstructure that are not accounted for in the previously described macroscopic models of network degradation, some predictive limitations still exist. Most importantly, swelling of the hydrophilic microspheres and changes in swelling with matrix degradation were not accounted for in the described model.
The macroscopic models used to correlate solute release (diffusion) with gel degradation (reaction) provide a powerful tool for predicting protein release with changing network structure. However, macroscopic observations in gel swelling and mass erosion are not sufficient to obtain precise predictions due to the averaging of microscopic events. On the other hand, models describing network changes at a microscopic level may provide more accurate release predictions. However, gel swelling, a very important characteristic of hydrogel drug carriers, must be included during the simulation since solute diffusivity is tightly coupled to water content.

2.3.3.4 Reaction-diffusion controlled release – Affinity hydrogel systems

Inspired by the reversible sequestering of proteins to the extracellular matrix (ECM), researchers have developed biomimetic hydrogel carriers bearing reversible binding capacities to decrease release rates of target protein therapeutics. These so-called ‘affinity’ hydrogels can also be classified as reaction-diffusion controlled hydrogel delivery systems. The release kinetics of a molecule from affinity gels can be depicted by a model developed by Crank [139] where protein-ligand (P:L) binding equilibrium is described using simple binding kinetics:

\[ P + L \rightleftharpoons P \cdot L \]

\[
\frac{K_b}{k_r} = \frac{[L]}{[P]} = K_d
\]

Here, \( k_r \) and \( k_r \) are association and dissociation rate constants, respectively. In this model, binding of proteins to immobilized elements is considered reversible and a time-independent equilibrium constant \( K_b = C_{pl}/C_p \) is used to represent the concentration equilibrium between bound (PL) and free (P) proteins. \( K_s \) can be therefore also described as a ratio of free-receptor concentration to dissociation constant \( (K_s = [P]/K_d). \) Assuming that the reaction is
fast compared to protein diffusion, the following equation can be obtained for the transport of reversibly bound protein within an affinity hydrogel [140]:

\[
\frac{D}{K_b + 1} \nabla^2 C_p = \frac{\partial C_p}{\partial t}
\]  

(24)

Compared to the standard form of Fick’s law of diffusion (Eq. 7) the above equation illustrates that the presence of rapid and reversible protein-ligand binding retards the release of free protein by decreasing the apparent protein diffusivity by a factor of \((K_b+1)\).

From this simple reaction-diffusion model described above, one can easily obtain the concentration profile of free proteins in the affinity gels available for diffusion. However, due to the fact that this model assumes a time-independent equilibrium constant \((K_b)\) and a rapid binding equilibrium, the model is limited to describing systems with simple yet rapid binding mechanisms with high ratios of ligand to protein. These assumptions may not be valid in the hydrogel matrix where the mobility of therapeutic macromolecules and therefore the intrinsic reaction constants are retarded by their size and limited free volume.

Heparin, a highly sulfated glycosaminoglycan (GAG), is known to serve as a growth factor depot \textit{in vivo} owing to its electrostatic affinity to various basic growth factors including NGF, bFGF, VEGF, etc. Matrices containing heparin have been used as delivery depots to modulate the release rates of these growth factors through affinity binding [141, 142]. For example, Sakiyama-Elbert and Hubbell have developed affinity hydrogels composed of fibrin gels copolymerized with peptides that bind to heparin [87, 88, 115]. This system has been applied to deliver several growth factors including NGF [87], basic fibroblast growth factor (bFGF) [88], and neurotrophin-3 (NT-3) [89]. In order to incorporate heparin into the fibrin network and modulate growth factor release, a group of short peptide sequences with
different affinities for heparin have been identified and copolymerized into the fibrin gel networks. To model growth factor release from this tri-component delivery system, six partial differential equations based on diffusion-reaction kinetics were solved simultaneously [88]:

\[
\frac{\partial C_G}{\partial t} = D_G \frac{\partial^2 C_G}{\partial x^2} - k_F C_G C_H + k_R C_{GH} - k_F C_G C_{HP} + k_R C_{GHP} \tag{25}
\]

\[
\frac{\partial C_H}{\partial t} = D_H \frac{\partial^2 C_H}{\partial x^2} - k_F C_G C_H + k_R C_{GH} - \kappa_F C_H C_P + \kappa_R C_{HP} \tag{26}
\]

\[
\frac{\partial C_P}{\partial t} = -\kappa_F C_H C_P + \kappa_R C_{HP} - \kappa_F C_{GH} C_P + \kappa_R C_{GHP} \tag{27}
\]

\[
\frac{\partial C_{GH}}{\partial t} = D_{GH} \frac{\partial^2 C_{GH}}{\partial x^2} + k_F C_G C_H - k_R C_{GH} - \kappa_F C_{GH} C_P + \kappa_R C_{GHP} \tag{28}
\]

\[
\frac{\partial C_{HP}}{\partial t} = \kappa_F C_H C_P - \kappa_R C_{HP} - k_F C_G C_{HP} + k_R C_{GHP} \tag{29}
\]

\[
\frac{\partial C_{GHP}}{\partial t} = k_F C_G C_{HP} - k_R C_{GHP} + \kappa_F C_{GH} C_P - \kappa_R C_{GHP} \tag{30}
\]

In these equations, \( C_G, C_H \), and \( C_P \) represent the concentrations of growth factor (G), heparin (H), and heparin-binding peptide (P), respectively. Similarly, \( C_{GH}, C_{HP}, \) and \( C_{GHP} \) represent the concentrations of the possible biomolecule complexes.

Assuming the system is in equilibrium between the species initially, these equations, in conjunction with proper initial and boundary conditions, can be solved numerically and used to predict the fraction of growth factor present in its freely diffusible and bound state (Figure 2.7 [89]) and the ratio of heparin to growth factor needed to obtain sustained growth factor release (Figure 2.8. [88]). As can be seen from the above equations, there are four kinetic constants (\( k_F, k_R, \kappa_F, \kappa_R \)) and three diffusion coefficients (\( D_G, D_H, D_{GH} \)) required to solve the equations which largely complicate this modeling approach. Furthermore, while
experimental results and model predictions agree qualitatively, these results were never directly compared to the theoretical predictions obtained from this model [87-89].

Figure 2.7 Predicted initial equilibrium fractions of NT-3-containing species versus initial heparin to NT-3 ratio. Reproduced from [89], Copyright (2004), with permission from Elsevier.
Figure 2.8 Theoretical concentration of matrix-bound bFGF as a function of distance from the midline of a model tubular nerve growth guide, 6 mm long and open at both ends. Concentration is shown as percentage of the initial bound concentration, which was $5.7 \times 10^8$ M. The ratio of heparin to growth factor modeled was ~500. The decreasing concentration profile propagates inward over time, as one would expect. Reproduced from [88], Copyright (2000), with permission from Elsevier.

2.4 Emerging Systems and Remaining Challenges

Although mathematical simulations have been performed extensively to predict and design better hydrogel systems, there are still many challenges associated with the modeling of drug delivery phenomena and accurate prediction of release profiles from complex hydrogel systems. Creating a fundamental understanding of drug transport processes is the first step towards developing a suitable mathematical model. Mass transport governs the translocation of drug from the interior of hydrogels to the surrounding environments.
Multiple factors affect the mass transport of encapsulated molecules including the network crosslinking density, extent of swelling, gel degradation, the size and charge of the encapsulated molecules, and the physical interactions these molecules exhibit for themselves and for the polymer matrix. If specific drug-binding motifs are present within the hydrogels, then the kinetics and/or thermodynamics of drug-ligand binding must also be understood and quantified to predict the controlled release of the encapsulated molecules. In this final section, the network design and mathematical modeling of several emerging hydrogel-based delivery systems as well as the challenges associated with these systems are discussed.

2.4.1 Dynamic hydrogel delivery systems

2.4.1.1 Degradable hydrogels

Previous sections have detailed the fabrication, degradation, and molecule release from degradable hydrogels. Understanding degradation mechanisms is critical in designing hydrogels for drug delivery applications since the rates of matrix swelling and degradation govern the diffusion of encapsulated or tethered molecules. Via appropriate design of polymer chemistries and network structure, degradable hydrogel matrices can be engineered with proper degradation profiles for achieving previously unattainable molecule release regimes.

Mathematical modeling of molecule release has provided much information to facilitate the design of degradable hydrogels and identify key parameters dictating molecule release profiles. However, to accurately predict the unique molecule release profiles that occur with many degradable hydrogels, additional parameters not commonly found in previous release models must be included. For example, as discussed in the previous section, enzymatically degradable hydrogels are becoming more important in controlled release
applications. One challenge for this novel class of hydrogel is how to model the rate of enzyme (e.g. MMPs) production by invading cells. As discussed before, enzyme concentration determines whether gel degradation occurs via surface-erosion (rate of enzyme/substrate reaction greater than rate of enzyme transport) or bulk-degradation (rate of enzyme transport greater than rate of enzyme/substrate reaction). Therefore, the accuracy of predicting gel degradation and molecule release from enzymatically degradable hydrogels largely depends on correctly understanding cellular physiology and cell-material interactions and properly incorporating these phenomena in a quantitative model along with molecule transport and enzyme-substrate kinetics.

2.4.1.2 Stimuli-sensitive hydrogels

Stimuli-sensitive hydrogels represent another advanced hydrogel system that, under intelligent design, can sense changes in complex in vivo environments and utilize these triggers to modify drug release rates. Since the swelling or deswelling of these hydrogels is controlled by external stimuli, it is critical to model the dynamic swelling response in order to predict solute release. Several review articles have been published detailing the fabrication and application of stimuli-sensitive hydrogels [18, 62, 143]. Ionic or pH-sensitive hydrogels are probably the most studied stimuli-sensitive gels. At a fixed pH and salt concentration, the swelling of ionic hydrogels is balanced by the osmotic pressure and the relaxation of the polymer chains. Thermodynamically, the total free energy can be expressed as:

$$
\Delta G_T = \Delta G_e + \Delta G_m + \Delta G_o
$$

(31)

Here, $\Delta G_T$ is the total Gibbs free energy, $\Delta G_e$ is the free energy contributed by elastic force of the polymer chains, $\Delta G_m$ is the free energy of mixing, and $\Delta G_o$ is the free energy due to
osmotic pressure. When the swelling of an ionic hydrogel is in equilibrium ($\Delta G_T = 0$), the decreased elastic free energy is balanced by the free energy of mixing and osmotic pressure. Based on this concept, the simulations of ionic hydrogel swelling have been derived in many reports [66, 144-146]. Grimshaw et al. developed a continuum model to describe the macroscopic behaviors of pH-responsive poly(methacrylic acid) (PMAA) hydrogel membranes accounting for charge density, ionic strength, stress, strain, and electric field [144]. The simulation results were used to compare experimentally determined PMAA swelling and shrinking. It was found that the membrane swelling was slower than shrinking. Following Grimshaw’s work, De et al. derived an equilibrium model to predict the degree of hydrogel swelling at given pH and ionic strength and a kinetic model to predict the rate of swelling under changing pH. Their simulation results agreed well with experimental observations. The equilibrium swelling of anionic pH-responsive hydrogels appears to be proportional to the pH with a sharp increase around the $pK_a$ of the charge group.

For molecule release from pH-sensitive hydrogels, Peppas and coworkers developed a series of models focusing on ionic hydrogel swelling, water transport, and molecule release [106, 107, 147, 148]. For example, a concentration-dependent solute diffusion coefficient $D_i$ was used to predict cationic hydrogel swelling and solute release upon pH changes induced by the production of gluconic acids [148]:

$$D_i = D_{i,0} \exp(\alpha_d \nu_1)$$}

(32)

where $D_{i,0}$ is the solute diffusion coefficient in the dry state. From this expression, it is clear that the diffusion coefficient changes exponentially with the water volume fraction $\nu_1$ and experimentally determined water-polymer interaction parameter $\alpha_d$. The modeling of cationic
hydrogel swelling agreed well with experimental data. For insulin release, however, no experimental results were compared to model predictions [148] indicating that verification of this modeling approach is still required.

While the benefits of using thermo-sensitive hydrogels are widely acknowledged, mathematical simulation of molecular release from these “smart” hydrogels is still very limited. A strategy correlating gel swelling and diffusion-controlled molecule release can be readily constructed using equations for estimating molecule diffusivity. Amsden [13] reviewed a variety of hydrogel diffusivity models related to fundamental characteristics such as hydrogel water content and molecule free volume. Andersson et al. applied one such expression for assessing glucose and insulin diffusivities in n-isopropylacrylamide gels [26]:

\[
\frac{D_e}{D_0} = \frac{(I - \Phi)^3}{(I + \Phi)^2}
\]  

where \(D_e\) and \(D_0\) are the effective molecule diffusivities in the gel and in pure solvent, respectively. \(\Phi\) is the polymer volume fraction of the gel. Since the swelling of thermo-sensitive hydrogels depends on temperature changes, one can readily obtain the polymer volume fraction at the tested temperature. Using this equation, the effective diffusivities of molecules encapsulated within thermo-sensitive hydrogels can be estimated as a function of temperature. Once the molecule diffusivity is determined, a release profile can then be predicted using Fick’s law of diffusion [26]. Figure 2.9 shows one comparison of simulated and experimental results [26].

Finally, several groups have devoted significant efforts to the fabrication and characterization of dual-stimuli responsive hydrogels that respond to changes in pH and temperature [79-82]. Although the unique drug release profiles observed from these novel
carriers have revealed the usefulness of this exciting new strategy of hydrogel design, mathematical modeling of drug release from these dual-responsive networks has yet to be developed.

![Figure 2.9](image)

**Figure 2.9** Experimental and simulated concentration profiles for one of the glucose diffusion experiments at 10°C. Reproduced from [26], Copyright (1997), with permission from Elsevier.

### 2.4.2 Composite hydrogel delivery systems

Modeling drug release from composite hydrogel systems has proven to be challenging due to the fact that their material and molecule transport properties change dramatically with spatial location within the device. Two primary types of composite hydrogel delivery systems have been investigated, multilayer and multiphase systems. These composite systems have great potential in delivering multiple protein therapeutics for tissue engineering applications where temporal and spatial control over drug delivery is desirable. The simultaneous delivery of multiple proteins is known to occur in vivo during angiogenesis, bone remodeling, and
nerve regeneration. For example, several angiogenic proteins including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and matrix metalloproteinases (MMPs) are involved in the angiogenesis process. Marui et al. discovered that the dual delivery of bFGF and hepatocyte growth factor (HGF) from collagen microspheres greatly increased blood vessel formation in an animal model [149]. Peattie et al. utilized crosslinked hyaluronan (HA) hydrogels to simultaneous deliver VEGF and keratinocyte growth factor (KGF) to enhance angiogenesis [150]. Simmons et al. used alginate hydrogels to deliver bone morphogenetic protein-2 (BMP2) and transforming growth factor-β (TGF-β3) and showed enhanced bone formation compared to delivery of either single protein [49]. Although in vivo tissue growth was improved in animal models using these dual-protein delivery systems, it is not clear whether tissue growth would be further enhanced if the proteins were delivered at optimized rates since no independent control over the release profiles has been shown in these studies. Therefore, the development of models that can relate drug transport and release in these composite systems to their fundamental properties would prove valuable and possibly lead to the engineering of devices capable of independently tunable delivery of multiple proteins for modulating cell behavior and tissue growth.

2.4.2.1 Multi-layer hydrogel delivery systems

In multi-layer systems, a basal polymer layer is fabricated, followed by lamination of subsequent layers. Different proteins can be encapsulated into each layer during fabrication and tunable multiple-protein release or unique single-protein release profiles are made possible by independently adjusting the crosslinking density of each layer. Many models have been developed for predicting drug release from multi-layer hydrogel composites. For
example, Streubel et al. developed a multi-layer system to achieve bimodal drug release [151]. Fick’s second law of diffusion was used to predict drug release profiles. They derived diffusion equations accounting for constant or non-constant diffusivities, as well as stationary or moving boundary conditions. Grassi et al. fit their experimental data into a semi-empirical model accounting for the resistance the drug experienced when diffusing through the multi-layer system [152]. They started the modeling with an equation governing the dissolution of solid drug and accounted for the gel layer resistance (R) and drug dissolution resistance (1/K):

\[
\frac{dC}{dt} = \frac{\varphi_d A}{V} \left( C_s - C \right) C - \frac{C}{\left( 1/K \right) + R}
\]

(34)

where \( C \) is the drug concentration, \( t \) is the dissolution time, \( C_s \) is the solubility of the drug in the dissolution medium, \( \varphi_d \) is the drug volume fraction, \( A \) is the surface area at the solid/liquid interface, and \( V \) is the volume of the medium. The release of some small molecular weight drugs from partially coated matrices containing different drug to polymer fraction can be fit into the analytical solution of this model.

Sohier and colleagues developed a porous scaffold containing three hydrogel layers with different porosities to simultaneously deliver lysozyme and myoglobin [153]. The governing equations used to model this system were again based on Fick’s second law with a time-dependent diffusion coefficient related to the rate of polymer degradation. Although this model successfully predicted the release of lysozyme from a multi-layer polymer construct, it did not provide an accurate description of dual-protein delivery.

In addition to multiple-protein delivery, multilayer matrices can also be used to decrease the problematic burst release, a common challenge facing drug delivery. For
example, Lu and Anseth developed a multi-laminated hydrogel system prepared by photopolymerization. A desirable, zero-order release profile was obtained through non-uniform initial drug loading in multi-laminated hydrogels and the results were verified by a diffusion model [154-156]. Their model was based on the well-known diffusion model first developed by Crank [139]. Assuming a constant diffusion coefficient and one-dimensional release under sink conditions, the fractional passive release of drug \( \frac{M_t}{M_\infty} \) from these composite hydrogels can be analytically derived from Fick’s second law of diffusion and expressed as the following equation:

\[
\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{(-1)^n}{\lambda_n} e^{-\lambda_n^2 D t} \left( \int_0^L f(x) \sin(\lambda_n x) dx \right)
\]

\[(35)\]

where \( \lambda_n = \left( n + 0.5 \right) \pi / L \), and \( f(x) \) is the initial concentration profile, \( D \) is the molecule diffusion coefficient, and \( L \) is the thickness of the gel. As shown in Figure 2.10, experimental results verified the accuracy of this model and indicate that the initial burst was nearly eliminated.

### 2.4.2.2 Multi-phase hydrogel delivery systems

Another strategy for multiple-protein delivery is multi-phase systems. In this approach, prefabricated microspheres containing one or more proteins are uniformly embedded within a hydrogel containing a second protein [157-159]. The release of the microsphere-encapsulated protein is delayed due to the combined diffusional resistances of the microsphere polymer and surrounding gel. Richardson and colleagues prepared a composite polymeric scaffold containing PLGA microspheres embedded in porous PLGA
matrices with different intrinsic viscosities to simultaneous deliver VEGF and PDGF. The in vitro and in vivo results using this approach have shown promising results in an animal model to enhance the maturation of vasculatures [48]. Although this multi-phase formulation is not considered to be a hydrogel system, it was the first heterogeneous polymeric system for delivering two proteins with distinct release profiles. Holland et al. also fabricated degradable oligo(poly(ethylene glycol) fumarate) hydrogels containing gelatin microspheres to independently control the delivery of insulin-like growth factor-1 (IGF-1) and transforming growth factor-β1 (TGF-β1). Release profiles can be adjusted by varying the protein loading in each polymer phase [157]. These multi-phase dual delivery systems have achieved substantial success, however, to date no rigorous mathematical models for predicting molecule release from these composite networks have been developed.

![Figure 2.10](image.png)

**Figure 2.10** Comparison of theoretical and experimental solute release. The initial concentration profile used, from center outward, was: 1.2 wt%, 0.55 wt%, 0.2 wt%, and 0 wt%, respectively. Model results (——), experimental results (—●—). Reproduced from [154], Copyright (1999), with permission from Elsevier.
2.4.2.3 Challenges facing composite hydrogel delivery systems

The design and application of composite hydrogel delivery systems have attracted much attention due to their multifaceted roles in advanced drug delivery and tissue engineering. However, many challenges facing the design and modeling of these novel systems remain largely unattended and need to be addressed to optimize their application as drug carriers. First, these systems have complex network geometries and phase morphologies that must be properly parameterized to quantify diffusion length scales in each phase. The individually tailored physicochemical properties of each layer, which results in heterogeneous transport properties within a single matrix, must also be evaluated in the context of the overall device. For example, as shown by Sohier et al. the swelling, and therefore permeability, of a highly hydrophilic layer can be limited by its attachment to layers exhibiting a lower degree of swelling [153]. Once identified, the positional dependence of drug diffusion coefficients as well as drug-polymer interaction parameters must be taken into account during the development of any rigorous mathematical model describing these composite systems.

2.4.3 Micro/Nano-scaled hydrogel delivery systems

Over the past few decades, polymeric microspheres and, more recently, nanoparticles have been widely used for sustained or targeted drug delivery [160] as well as cell encapsulation [161-163]. Numerous studies have been conducted using PLGA as a matrix for encapsulating proteins, peptides, DNA, and small molecular weight drugs. However, the hydrophobicity, acidic degradation products, and harsh fabrication/encapsulation processes of PLGA micro/nanoparticles make them unfavorable as carriers for biomacromolecules such as protein and DNA [164]. Alternatively, micro/nanoparticles made from hydrophilic
hydrogels are more suitable for encapsulating these fragile biomacromolecules. These miniaturized drug-containing vehicles can be fabricated in vitro and then administered via oral [165, 166] or nasal route [167, 168] or injected into the patients in a minimally invasive manner to increase patient compliance. Protein-containing microparticles can also be fabricated and loaded into a bulk gel containing a second protein for dual-protein delivery as discussed in the previous section. It is beyond the scope of this review to thoroughly discuss the fabrication and application of micro/nanoparticles and readers are advised to look to the cited references for more information [169, 170].

Two types of mathematical approaches have been used to predict molecule release from hydrogel microspheres: macroscopic diffusion models and microscopic Monte Carlo simulations. For macroscopic modeling, the most applicable models are still based on Fick’s second law of diffusion. Particle size and geometry are the most important parameters in this type of modeling as well as surface area since this appears to correlate to observed burst effects. Additionally molecule diffusivities must be accurately determined. As with other diffusion-controlled delivery systems, simple empirical relationships have been used to estimate molecule diffusivity [171]. Other more rigorous expressions for molecule diffusivity such as those discussed in Section 3.1 for degradable gels, can also be applied to these systems. The accuracy of any diffusion model to predict molecule release from hydrogel-based microparticle systems will directly depend on the accuracy of the diffusivity estimation.

Another technique to model molecule release from hydrogel microspheres is Monte Carlo simulation. This method has proven itself valuable for describing the transport behavior of molecules within degradable microsphere systems and has been widely applied to hydrophobic polymer networks such as PLGA [172, 173]. As mentioned earlier, Vlugt-
Wensink et al. recently utilized Monte Carlo simulations to predict protein release from degradable dextran microspheres [57]. Unfortunately, the accuracy of the model is highly protein specific. For example, for larger proteins such as IgG, model predictions only agreed with experiments qualitatively in most cases. This may be due to the fact that swelling of the dextran gels was not accounted for in the Monte Carlo description of the degrading hydrogel network.

One of the unique challenges facing micro-scaled matrix delivery systems is burst release due to the high surface-to-volume ratio of these particulate systems [174, 175]. Burst release may cause a “dose-dumping” effect and is potentially harmful to patients in clinical applications. Several possible causes of burst release have been identified including material/drug interactions, fabrication conditions, and sample geometry and/or morphology [174]. Although not completely understood, burst release has been taken into consideration during the design of delivery matrices as well as in modeling approaches [154-156, 176, 177]. Several methodologies have been developed in an attempt to decrease the degree of burst release. These include increasing crosslinking density of the matrix surface [178, 179], coating additional drug-free layers [152, 177, 180], embedding the drug-containing particles within a bulk polymeric matrix [157-159, 181], and loading drug unevenly with higher concentrations toward the center of the matrix [182, 183].

The prediction of burst release is problematic as the exact mechanism has not been elucidated. Typically, diffusion-controlled release can be divided into two phases: a rapid burst phase and a prolonged diffusion-controlled phase. The later can be modeled by conventional diffusion theories while the prediction of initial burst release is not readily attainable. Models in this area are therefore very limited. Several attempts have been made to
predict burst release in polymeric delivery matrices. For example, the simplest model employed to describe the impact of burst release on drug delivery profiles is to add an extra parameter, namely $\alpha$, into the well-known fractional release equation [174]:

$$\frac{M_t}{M_{\infty}} = kt^n + \alpha$$  \hspace{1cm} (36)

In previous applications of this expression, the experimental release data were simply shifted a certain fraction to fit the model prediction. If no burst release exists, $\alpha$ equals zero and the equation is reduced to the original fractional release equation. However, this empirical model fails to relate the extent of burst release to quantifiable system parameters. Thus, this simple model is not practical for extrapolating results between different device designs or optimizing delivery profiles.

2.4.4 In-situ forming hydrogels

Recent advances in polymer chemistry and hydrogel engineering have promoted the development of in-situ forming hydrogels for drug delivery applications. Through intelligent design of monomers/macromers with desired functionalities, hydrogel precursor solutions can be injected and subsequently polymerized in situ. This in situ sol-gel transition enables the surgery or implantation procedure to be performed in a minimally invasive manner. Several physical or chemical crosslinking mechanisms have been used for in-situ network formation. Physically, in-situ forming gels are formed by one of the following mechanisms: hydrogen bonding, hydrophobic-hydrophobic interactions, or electrostatic interactions. Sodium alginate hydrogels, for example, can be physically crosslinked through the addition of calcium ions [160]. The common disadvantage of physical crosslinking, however, is that the gels thus formed are unstable and may disintegrate rapidly and unpredictably.
For long-term drug delivery applications, covalent crosslinking methods performed under physiological conditions, such as photopolymerization of multi-vinyl macromers, are more favorable compared to physical crosslinking methods as they produce relatively stable hydrogel networks with predictable degradation behaviors. The photocuring process, for example, is fast, usually taking only seconds to minutes to complete, and can be conducted at room temperature without organic solvents [53]. Photopolymerization of degradable hydrogels has been applied in protein [71, 184, 185] and gene delivery [186-188] and permits in situ encapsulation of these species during network fabrication. These advantages overcomes the complexities and limitations associated with post-loading techniques and provides a convenient and efficient way of loading high concentrations of proteins and other releasable solutes for subsequent long-term delivery.

When in-situ forming hydrogels are used to deliver macromolecules such as DNA and protein, reduced or incomplete release of these biomolecules is commonly observed [185-188]. Incomplete protein release decreases the bioavailability of the therapeutic agent and alters the overall delivery profile. In addition, the protein trapped within the gel is generally modified or denatured, which can lead to undesirable antigenic responses when applied in vivo. The factors influencing incomplete biomolecule release from these hydrogel carriers has commonly been attributed to the fabrication processes. For example, several researchers have studied the effect of drug-polymer interactions on molecule release using thermally responsive poly(N-isopropylacrylamide) hydrogels [189, 190] and alginate microparticles [191]. Although these studies observed and verified the incomplete release phenomena, no mathematical model was derived for predicting molecular release.
When in-situ forming gels are used to deliver proteins, irreversible interactions between the encapsulated proteins and polymerizing polymer chains decrease the efficacy of the therapeutic agent. van de Wetering et al. identified the modification of hGH by reactive thiol macromers in a PEG-based hydrogel system prepared via Michael-type addition reaction [192]. Additionally, Quick and Anseth specified free radicals as the major source of incomplete DNA release when photopolymerization was used to fabricate DNA-containing hydrogels [186-188]. According to the authors, free radicals produced from the photoinitiation process attacked DNA molecules during UV irradiation, leading to DNA damage. Based on similar observations during protein encapsulation, Lin and Metters utilized a metal-ion-chelating molecule, iminodiacetic acid (IDA), to block undesirable protein-polymer conjugation reactions mediated by free radicals. This protective agent increased the fractional release of target proteins such as bovine serum albumin (BSA) from 40% to 100% following in situ photocuring of PEG-diacrylate hydrogels [185]. A mathematical model accounting for reversible protein-IDA binding directly correlated the extent of BSA release to the degree of protein-IDA binding.

Modeling drug release from in-situ forming hydrogels is challenging due to several reasons. First, the effects of reduced/incomplete protein release discussed above can only be taken into account after identifying the sources of protein destabilization and quantifying the extent of interaction. These interactions will greatly depend on the selected polymer and drug chemistries as well as the method of gel fabrication. Secondly, in-situ forming gels assume irregular geometries at the implant site which are difficult to predict prior to injection. This irregular geometry will increase model complexity and may also contribute to non-uniform drug distribution within the gels, which further increases the difficulty to
accurately represent the real system in a mathematical construct. Finally, experimental measurement of release profiles is usually accomplished through in vitro release studies. These in vitro systems must be designed to include as many complexities of the in vivo environment as possible if these experiments are to accurately represent what will occur during clinical application.

2.5 Conclusion

Hydrogels have played a very important role in biomedical applications. With increasing efforts devoted to controlled molecule release, the applications of hydrogels will continue to grow in the future. Proper network design and accurate mathematical modeling are keys to tuning the drug release rates as well as to modulating tissue regeneration. Although many fundamental studies have revealed the basic molecule release mechanisms from hydrogel-based controlled release devices, many parameters in the current models are unknown and/or change with time or position and need to be identified in order to accurately predict drug release profiles. Reduced release efficiency, burst effects, complex geometries, and unknown correlations between in vitro and in vivo release further complicate our understanding of these materials as delivery devices and present difficult challenges to developing mathematical models that accurately describe the transport and release of molecules from these systems. Furthermore, as more advanced release devices are developed such as affinity hydrogels, microparticle systems, and in-situ forming gels, more rigorous mathematical modeling approaches are needed to describe the coupled mechanisms governing molecule release from these systems.

2.6 References


CHAPTER THREE
FREE RADICAL-MEDIATED PROTEIN INACTIVATION AND RECOVERY DURING PROTEIN PHOTOPOLYMERIZATION

Abstract

Photoencapsulation is very attractive for preparing biomolecule-loaded hydrogels for a variety of biomedical applications. However, detrimental effects of highly active radical species generated during photoencapsulation must be carefully evaluated to maintain efficient hydrogel crosslinking while preserving the stability of encapsulated biomolecules. Here, we examine free radical-mediated inactivation and incomplete release of proteins from photocurable hydrogels utilizing lysozyme as a conservative model system. Various photoencapsulation conditions were tested to determine the factors affecting lysozyme structural stability and bioactivity. It was found that a portion of the lysozyme becomes conjugated to polymer chains at high photoinitiator concentrations and long polymerization times. We also found that the more hydrophilic photoinitiator Irgacure-2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone I-2959) causes more damage to lysozyme, compared to the hydrophobic photoinitiator Irgacure-651 (2,2-dimethoxy-2-phenylacetophenone or I-651), even though I-2959 has been previously shown to be more cytocompatible. Furthermore, while non-acrylated PEG provides only limited protection from the denaturing free-radicals that are present during hydrogel curing, acrylated PEG macromers effectively preserve lysozyme stability in the presence of either photoinitiator. Overall, these findings indicate how hotopolymerization conditions must be optimized to obtain a functional hydrogel device that can preserve protein bioactivity and provide maximal protein release.
3.1 Introduction

Photopolymerization has emerged as a favorable method for preparing hydrogels [1-3], membranes/films [4-9], surface coatings [10-12] and other functional materials. Photopolymerization possesses many advantages including its facile spatial and temporal control over reaction kinetics and its rapid and mild reaction conditions that are feasible under ambient environments. Beginning with an aqueous prepolymer solution containing monomers, biomolecules, and photoinitiator, photopolymerization is completed in a single, rapid-step process and therefore has achieved substantial success in fabricating drug delivery carriers and tissue engineering scaffolds. Depending on the type of photoinitiator used, the prepolymer solution is irradiated with ultraviolet or visible light for photo-curing over the timescale of a few seconds to several minutes. These favorable properties of photopolymerization not only make it a simple method for preparing a variety of polymeric devices but also allow it to uniformly encapsulate biomolecules in a single processing step. Although some photoinitiator-free reaction schemes have been recently proposed [13-18], the use of biocompatible photoinitiator to rapidly initiate polymerization reactions is still the most popular method for simultaneous network polymerization and crosslinking. Biomaterials prepared via in situ photopolymerization with encapsulated biomolecules have been used extensively for controlled protein/gene delivery [19-22], cell encapsulation [3, 8, 23, 24], and tissue engineering applications [2, 3, 23].

Although most therapeutic proteins and other bioactive agents are not destabilized by short exposure to low-energy UV or visible light, encapsulation of these materials via photopolymerization still causes problems due to the use of light-sensitive photoinitiators. Photolysis of photoinitiators generates free radicals, which enable network formation by
initiating chain polymerization. High-energy free radicals react with not only polymerizable monomers but also attack the co-encapsulated biomolecules, leading to DNA fragmentation, protein denaturation, protein-polymer conjugation, or cell damage. Efforts have therefore been devoted to investigate the effects of photoencapsulation on the stability and bioactivity of these biomolecules. For example, Bryant et al. examined and compared the cytocompatibility of several photoinitiators during in situ cell encapsulation [25]. Williams et al. performed similar studies and revealed parallel results as Bryant et al. that photoinitiator Irgacure-2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone), when used under optimal conditions, is more cytocompatible than Irgacure-651 (2,2-dimethoxy-2-phenylacetophenone). As our results will demonstrate, the conclusions from these previous studies are not necessarily indicative of photoencapsulation systems where photoinitiators, acrylated macromers and protein or DNA/RNA therapeutics are all present during UV exposure.

With the objective of improving the delivery of various macromolecular therapeutics, Quick and Anseth studied the effects of photoinitiators on the structural integrity of plasmid DNA during in-situ photoencapsulation. Their results show that the detrimental effects of photoinitiators and free radicals can be decreased by adding free-radical scavengers [21, 22]. However, this method decreases the total radical concentration and rate of polymerization, necessitating longer reaction times or incomplete network formation.

To preserve high polymerization rates while minimizing detrimental interactions of encapsulated species with photo-initiated free-radicals, Lin and Metters recently proposed a protection mechanism using metal-ion chelators [20]. They demonstrated that for select proteins exhibiting a moderate affinity for various metal ions, such a mechanism could be
used to effectively prevent the significant amount of protein-polymer conjugation that routinely occurs during photoencapsulation. More recently, Gu et al. discussed the damaging effects of I-651 on the bioactivity of vascular endothelial growth factor (VEGF) and bovine serum albumin (BSA) [26]. They concluded that while free radicals generated from I-651 damage protein structure and bioactivity in the absence of reactive monomer, the presence of acrylated macromers effectively preserves protein stability during photopolymerization.

Proteins have long been considered major biological targets for free-radical induced oxidative damage. In biological systems, the major sources of free radicals include the primary hydroxyl radicals (•OH) and nitric oxide (•NO), as well as other secondary radicals. These radicals generate oxidative stress to proteins through modification of amino acid residues such as lysine, arginine, tryptophan, tyrosine, proline, cysteine, threonine, leucine, and histidine [27-29]. These modifications, in turn, result in peptide bond cleavage, site-specific oxidation, and protein-protein crosslinking. Several reaction routes have been suggested for free radical-mediated oxidative modifications in which the abstraction of hydrogen atoms from the α-carbon of amino acids or protein polypeptide backbone is the initial site of free-radical attack [28]. Although protein damage resulting from carbon radicals generated by ultraviolet and visible-light photoinitiators has not been extensively studied, it is reasonable to hypothesize a mechanism similar to the reaction generated by hydroxyl radicals and nitric oxide. Among the several possible reaction pathways, carbon-centered radicals transferred to proteins during photoencapsulation can react with propagating carbon radicals on growing polymer chains, resulting in covalent conjugation of the encapsulated proteins to the polymer matrix.

Although the importance and advantages of photopolymerization are wellrecognized
[1-3, 19, 23, 24], the potential for adverse effects on encapsulated species limit the advantageous use of photopolymerization for simultaneous network formation and biomolecule encapsulation. We aim to discover photoinitiator-induced protein-polymer interactions leading to protein inactivation and to provide generalized protection strategies for enhancing protein bioactivity and bioavailability [20]. Here, we design a series of protein photoencapsulation experiments to investigate protein stability and bioactivity under various reaction conditions, including the type and concentration of photoinitiator, UV-irradiation time, and the presence of acrylated versus non-acrylated polyethylene glycol (PEG). The often overlooked phenomenon of protein-polymer conjugation induced by free-radicals is also discussed.

3.2 Materials and Methods

3.2.1 Materials

Photoinitiators 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959 or I-2959) and 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651 or I-651) were obtained from Ciba Specialty Chemicals. Chicken Egg White Lysozyme, *Micrococcus lysodeicticus*, and hydroxyl-terminated poly(ethylene glycol) (MW. 8000) were obtained from Sigma-Aldrich. Poly(ethylene glycol) monoacrylate (PEGMA, MW. 3000) was supplied by Monomer-Polymer & Dajac Labs Inc. All other chemicals were obtained from Sigma-Aldrich unless otherwise specified.

3.2.2 Protein photoencapsulation

Protein photoencapsulation reactions were performed in 50mM, pH 7.4 phosphate-buffer solution (PBS) at room temperature. Photoinitiator stock solutions (I-2959 or I-651) were dissolved in ethanol at a concentration of 10 wt% and diluted in PBS to obtain required
concentrations. Lysozyme stock solution at 40 mg/mL was prepared in PBS and a final concentration of 2 mg/mL (with or without the presence of polymer) was used for UV irradiation. A low-energy ultraviolet light (BLACK-RAY®, intensity 8mW/cm² at 365nm) was used as source of UV irradiation.

### 3.2.3 Fluorescence spectroscopy

Fluorescence spectroscopy has been widely used as a facile method of detecting alterations in intrinsic protein fluorescence. The later characterizes changes in protein tertiary structure due to protein-ligand interaction as well as protein aggregation or denaturation. Lysozyme fluorescence measurements were carried out in a GeminiEM Spectramax plate reader (Molecular Devices, CA). The excitation wavelength was set at 295nm and the emission spectra recorded in the range of 300 to 400nm. Accurate measurements of lysozyme intrinsic fluorescence spectra are not possible in the presence of different concentrations of photoinitiators and PEGMA due to the high molar extinction coefficients of photoinitiators and auto-fluorescence of PEGMA.

### 3.2.4 Lysozyme bioactivity assay

Following photopolymerization, a portion of the reacted polymer-protein mixture was diluted 100-fold to obtain a solution with 0.02 mg/mL lysozyme for assaying bioactivity. *Micrococcus lysodeicticus* at a concentration of 0.4 mg/mL was used as lysozyme substrate. The lysozyme bioactivity assay was performed in a 96-well microplate where 20 µL lysozyme sample solution was added to 200 µL *Micrococcus lysodeicticus* solution. The turbidity of the combined solution was monitored kinetically using a UV-Vis spectrophotometer (µQuant, Biotek Instruments, Inc.) at a wavelength of 450 nm. The slope obtained from the linear
portion of the decreasing optical density (OD450) due to enzymatic degradation of the bacterial cell wall was used to represent lysozyme bioactivity. Native lysozyme solution was used as positive control for all samples and the ratio of the two slopes (sample lysozyme to native lysozyme) was calculated as relative bioactivity.

3.2.5 Circular dichroism (CD) spectroscopy

CD measurements were carried out in quartz cuvettes using a Jasco 810 CD spectrophotometer (Jasco Inc., Bethesda, MD). All measurements were carried out at room temperature and in double-distilled water. Lysozyme solutions at a concentration of 10 µg/mL were used for CD measurements. To decrease the influence of polymer chains (PEGMA) on CD measurements, samples were further diluted to 5 µg/mL lysozyme when PEGMA was present. CD spectra were recorded over a wavelength range of 190 to 240 nm.

3.2.6 Non-reduced SDS-PAGE

Non-reduced SDS-PAGE has been successfully used to determine protein molecular weight changes due to protein-polymer conjugation [20, 28]. Briefly, lysozyme (2 mg/mL), 10 wt% linear poly(ethylene glycol) monoacrylate (PEGMA), and required amount of photoinitiator I-2959 were mixed and subjected to a prescribed does of low-energy UV irradiation. Following exposure, protein-polymer solutions were analyzed according to standard SDS-PAGE procedure with the exception that lysozyme was not heat-denatured. Protein samples in the gel were visualized using Coomassie Blue staining.

3.2.7 Hydrogel fabrication, characterization, and protein release

Polyethylene glycol diacrylate monomers (PEGDA, M.W. 3400) were synthesized following an established protocol [7]. PEG hydrogels were prepared from solution photopolymerization of 10 wt% PEGDA monomers and required amount of photoinitiator.
The photopolymerization was carried out between glass slides separated by 0.55 mm Teflon spacers. The photopolymerized hydrogels were cut into rectangular shape (~0.8 × 0.8 cm). 0.3 wt% photoinitiator (I-2959 or I-651) was used to fabricate hydrogels for comparison of gelation efficiency through direct measurements of equilibrium swelling ratios (Q) and gel fractions ($f_{gel}$).

Equilibrium swelling ratio measurements: PEG hydrogels were washed, after gelation, in deionized water for 24 hours to remove any unreacted monomers, followed by drying in air (24-hr) and then in vacuum (24-hr). The dried gel weights were measured gravimetrically, after which the dried gels were placed in deionized water for 48 hours to obtain equilibrium swollen weight. Equilibrium swollen ratio (Q) was then determined by:

$$Q = \frac{\text{Equilibrium swollen weight}}{\text{Dried gel weight}}$$

Gel fraction measurements: the photo-cured gels were dried directly after gelation without the washing step. The obtained dried gel weight represents crosslinked gel and unreacted monomers. The gels were then swelled in deionized water for 48 hours and then dried again to obtain dried gel weight containing only crosslinked polymer. Gel fraction ($f_{gel}$) was determined using the following equation:

$$f_{gel} = \frac{\text{Dried gel wt. after swelling}}{\text{Dried gel wt. before swelling}} \times 100\%$$

### 3.2.8 Lysozyme release from in situ photopolymerized PEG hydrogels

Lysozyme at a final concentration of 1 wt% was mixed with 10 wt% PEGDA crosslinker solution. The lysozyme was then encapsulated in situ by exposing the precursor mixture with 0.1, 0.2, or 0.3 wt% I-2959 for 10-min to low-energy UV-irradiation to form
partially swollen hydrogels. This strategy permitted the effect of photoinitiator concentration on the released lysozyme structure and bioactivity to be investigated. Furthermore, hydrogels with 1 wt% lysozyme cured with 0.2 wt% I-2959 for 10 or 20-min gelation time were used to evaluate the effect of gelation time on lysozyme structure and bioactivity. Release studies were conducted in 3 mL, 50 mM PBS (pH 7.4) at 37°C. Total lysozyme release was characterized using a fluorescamine assay (3 mg/mL in acetone). Bioactivity assay and fluorescence spectroscopy were performed on released lysozyme samples with diluted concentrations (bioactivity assay: 0.02 mg/mL; fluorescence spectroscopy: 0.1 mg/mL).

3.3 Results and Discussion

3.3.1 Effect of photoinitiator and UV irradiation on lysozyme bioactivity

Based on prior experimental observations [20], we hypothesized that incomplete release of encapsulated proteins from photopolymerized hydrogels results, at least in part, from free-radical induced protein-polymer conjugation during protein photoencapsulation. Mechanistically, this is a consequence of radical species generated by photoinitiators and propagated by (meth)acrylated monomers that irreversibly conjugate proteins in the precursor solution to growing polymer chains. To examine the effects of photoinitiator chemistry and concentration on protein bioactivity during protein photoencapsulation, lysozyme was utilized as a representative protein due to its well-understood structure-function relationships. Prior to assessing protein inactivation during photoencapsulation, it was necessary to examine the effect of low-energy UV irradiation on protein structure and bioactivity. To achieve this, lysozyme solutions at 2 mg/mL were exposed to low-energy UV for up to 20 minutes. Figure 3.1 shows the intrinsic fluorescence (Excitation
wavelength: 295 nm) of lysozyme following increasing UV exposure time. Clearly, no substantial differences were found, suggesting the tertiary structure of lysozyme is unaffected by the low-energy UV irradiation commonly used to cure the hydrogel networks. The bioactivity of the UV-irradiated lysozyme samples is also comparable to that of native lysozyme. It is important to note that, although the low intensity UV used throughout this study does not cause any apparent protein damage, protein denaturation does occur at higher intensities, shorter wavelengths or with significantly prolonged exposure times [27, 29].

We next examined the effect of photoinitiator chemistry on lysozyme bioactivity at different UV exposure times. I-2959 and I-651 are two photoinitiators widely used to prepare hydrogel scaffolds for encapsulating cells, proteins, and other biologically active agents [19, 25, 27]. As shown in Figure 3.2, it is clear that, regardless of the type of photoinitiator used (0.05 wt% I-2959 or I-651), lysozyme activity decreases rapidly with increasing UV exposure time. For example, after 7 minutes of UV irradiation in the presence of 0.05 wt% I-2959 or I-651, lysozyme bioactivity decreases rapidly to only 5% or 12%, respectively. During UV irradiation, the presence of photoinitiator leads to protein inactivity and denaturation due to the generation of high-energy free radicals. Free-radical mediated protein denaturation is substantiated by observing increased turbidity of the protein solution with increasing UV exposure time (data not shown).

When comparing the photo-inactivation effects of I-651 and I-2959 on lysozyme, it is interesting to note that I-651 causes less damage to lysozyme (except at 1-min UV irradiation). This is an interesting result as prior studies by Bryant et al. [25] and Williams et al. [26] suggested that photoinitiator I-2959, when used at optimal conditions, is more cytocompatible than I-651 for mammalian cells. However, for in situ lysozyme
encapsulation, I-651 is at least as protein-friendly as I-2959. We hypothesize that this may be due to the increased phase separation of the hydrophobic, less water-soluble I-651 in aqueous solutions compared to I-2959. Therefore I-651 will not contact the exposed, hydrophilic residues of lysozyme (e.g. lysine or arginine) as extensively as I-2959 during UV exposure. Future studies, however, are needed to verify this hypothesis and to elucidate the underlying mechanisms. Nevertheless, the results presented in Figure 3.1 and 3.2 demonstrate that, although low-energy UV irradiation is harmless to protein structure and bioactivity, it can trigger serious protein inactivation during photoencapsulation by generating high-energy free radicals through photolysis of photoinitiators.

![Figure 3.1](image)

Figure 3.1 Fluorescence spectra (Excitation wavelength: 295 nm; emission wavelength: 310 - 400 nm) of lysozyme subjected to low-energy UV irradiation (BLACK-RAY®, intensity 8mW/cm² at 365 nm) without the presence of photoinitiator for up to 20 min (Lysozyme concentration: 2 mg/mL for UV exposure; 0.2 mg/mL for fluorescence spectra detection).
3.3.2 Effect of PEG on lysozyme bioactivity during protein photoencapsulation

It is well-known that PEG is a hydrophilic molecule that stabilizes proteins owing to its “stealth” effect [30-32]. Because of its volume-exclusion effect, PEG has also been used to precipitate proteins prior to encapsulation and hence can increase protein bioavailability [28]. Taken together, adding hydroxyl-terminated PEG (PEG-OH) into UV-irradiated protein-photoinitiator precursor solutions can presumably decrease free-radical mediated inactivation of proteins. To verify this hypothesis, we systematically increased the concentration of 8 kDa linear PEG-OH from 5 wt% to 20 wt% in lysozyme solutions containing 0.05 wt% I-2959 or I-651 and irradiated the mixture with UV light for 7-min. Table 3.1 reveals that a significant (PEG-OH)-induced protection effect (i.e., retention of
protein bioactivity) was found only in the most concentrated PEG-OH solutions (20 wt% PEG-OH). (PEG-OH)-induced inhibition of protein photo-inactivation can be attributed to: (1) increased solution viscosity that restricts the diffusion of free radicals, and (2) (PEG-OH)-induced precipitation of lysozyme that decreases the protein’s exposure to free radicals in the aqueous phase. In support of these proposed protection mechanisms, we also found that the addition of linear PEG-OH with molecular weight 3.4 kDa or 4.6 kDa did not provide significant retention of protein bioactivity at similar mass concentrations (5-20 wt%) and UV irradiation conditions (data not shown). Overall, these results indicate that addition of non-reactive PEG, even at relatively high concentrations, can only provide encapsulated proteins with limited protection from attack by free-radicals.

It has been previously shown that protein bioactivity can be retained during photocuring in the presence of acrylated macromers in organic solvent [27]. For example, Gu et al showed that the addition of acrylated star(ε-caprolactone-co-D,L-lactide) preserves the bioactivity of the photoencapsulated proteins (VEGF and BSA) initiated by high-energy UV (50-1000mW/cm\(^2\)). We confirmed this general result by investigating the effect of PEGMA on the inhibition of lysozyme photo-inactivation in aqueous solutions. As shown in Figure 3.3(a), the addition of 10wt% PEG monoacrylate (PEGMA, 3kDa) effectively eliminates the previously discussed photo-inactivation of lysozyme and increases its bioactivity to over 100% at all UV exposure times (up to 20-min) regardless of which photoinitiator is used.

To further elucidate the effect of PEGMA on inhibiting photoinitiator-induced protein inactivation and to examine whether I-651 indeed causes less damage to lysozyme, we varied the concentration of I-2959 or I-651 while keeping a constant UV exposure time
of 10-min. Surprisingly, the addition of 10 wt% PEGMA effectively inhibits photo-inactivation of lysozyme induced by I-651 at all concentrations (up to 0.3 wt%) while lysozyme bioactivity decreases when corresponding concentrations of I-2959 were used (Figure 3.3b). In fact, the addition of PEGMA increases lysozyme bioactivity to 130% when 0.3 wt% I-651 is used while at the same conditions with I-2959, PEGMA can only preserve lysozyme bioactivity to about 80%.

### Table 3.1 Effect of PEG macromer (8 kDa) on lysozyme bioactivity after 7-min UV irradiation.

<table>
<thead>
<tr>
<th>[PEG] (8 kDa)</th>
<th>Relative lysozyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 wt% I-2959</td>
</tr>
<tr>
<td>0%</td>
<td>6.15 ± 0.97</td>
</tr>
<tr>
<td>5%</td>
<td>14.7 ± 1.42</td>
</tr>
<tr>
<td>10%</td>
<td>22.9 ± 0.82</td>
</tr>
<tr>
<td>15%</td>
<td>29.1 ± 1.44</td>
</tr>
<tr>
<td>20%</td>
<td>48.0 ± 2.79</td>
</tr>
</tbody>
</table>
Figure 3.3 Addition of 10 wt% PEG-monoacrylate (PEGMA, 3 kDa) on lysozyme bioactivity. Effect of: (a) UV exposure time (photoinitiator: 0.05 wt% I-2959 (□) and 0.05 wt% I-651 (■)), and (b) photoinitiator concentration (I-2959 (□) and I-651 (■) for 10-min UV exposure). Bioactivity of native lysozyme was used as positive control (100%).
Comparing the two photoinitiators used in this study, I-651 has higher molar extinction coefficient (94.6 and 6.7 l mol⁻¹ cm⁻¹ for I-651 and I-2959, respectively [25]) and quantum yield (ranging from 0.1 to 0.7 for I-651 [33, 34] and 0.05 for I-2959 [35]) that make it more efficient in initiating photopolymerization. Thus at the same concentration and irradiation dose, I-651 generates more free radicals than I-2959. To indirectly verify this, we fabricated PEG hydrogels using PEGDA with either 0.3 wt% I-2959 or I-651 as photoinitiator (10-min low-energy UV exposure, 8 mW/cm² at 365 nm). As shown in Table 3.2, the equilibrium swelling ratio of gels cured with 0.3 wt% I-651 is significantly lower than those of gels cured with 0.3 wt% I-2959 (8.78 ± 0.61 and 9.86 ± 0.12, respectively, p<0.04) while their gel fractions are relatively identical (95.34 ± 5.63 and 93.88 ± 4.68 % for I-2959 and I-651, respectively). This suggests that I-651 results in gels with higher crosslinking densities and is at least as efficient as I-2959 in initiating polymerization under the given conditions. Although I-651 is considered more efficient than I-2959 in generating free radicals, it does not decrease protein bioactivity as significantly as I-2959 in the presence of PEGMA.

**Table 3.2** Equilibrium swelling ratio and gel fraction of PEGDA hydrogels prepared from different photoinitiators.

<table>
<thead>
<tr>
<th>Photoinitiator</th>
<th>Q [a]</th>
<th>Gel fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3wt% I-2959</td>
<td>9.86 ± 0.12 [b]</td>
<td>95.34 ± 5.63</td>
</tr>
<tr>
<td>0.3wt% I-651</td>
<td>8.78 ± 0.61 [b]</td>
<td>93.88 ± 4.68</td>
</tr>
</tbody>
</table>

[a] Q: equilibrium mass swelling ratio of hydrogels  
[b] Statistically significant. (Student’s T-test, n=3, p<0.04)
3.3.3 Lysozyme structural integrity

In order to gain more insights into the structural changes of lysozyme caused by photoinitiators and UV-irradiation, we examined lysozyme secondary structure by circular dichroism (CD) spectroscopy. Far-UV CD spectra shown in Figure 3.4a illustrate the secondary structure of lysozyme in its native form (curve a), incubated with 0.2 wt% I-2959 without (curve b), and with (curve c) 10-min UV-irradiation. Solely incubating lysozyme with 0.2 wt% I-2959 without UV-irradiation slightly decreases CD spectra intensity in the region between 190nm and 200nm, indicating moderate changes in lysozyme secondary structure (β-sheet). No differences were found in the two spectra above 200nm. However, the CD spectrum for lysozyme was altered dramatically after 10-min UV irradiation (curve c), suggesting significant modification of lysozyme secondary structure (both in α-helix and β-sheet). These results are in line with lysozyme bioactivity assays where moderate bioactivity decrease (to 69.4 ± 1.57%) was found for lysozyme incubated with 0.2 wt% I-2959 without UV-irradiation while no lysozyme activity was detected after 10-min UV.

Figure 3.4b shows that when 10 wt% PEGMA is added into 0.2 wt% I-2959-containing lysozyme solution, the secondary structure of lysozyme is not significantly altered (compared to native lysozyme, curve a) even after 20-min UV exposure (curve b), supporting the earlier argument that PEGMA provides some degree of protection from free-radical exposure during photopolymerization. Note that the differences in absolute CD signal occurring between Figures 3.4a and 3.4b were due to the use of different lysozyme concentrations (Figure 3.4a: 10 µg/ml; Figure 3.4b: 5 µg/ml).
Figure 3.4 Effect of photopolymerization conditions on lysozyme structure examined by circular dichroism spectroscopy. (a) In the absence of PEGMA. Curve a: native lysozyme; Curve b: lysozyme + 0.2 wt% I-2959 without UV exposure; Curve c: lysozyme + 0.2 wt% I-2959 with 10-min UV exposure (BLACK-RAY®, intensity 8mW/cm² at 365nm). (b) In the presence of 10 wt% PEGMA. Curve a: native lysozyme; Curve b: lysozyme + 0.2 wt% I-2959 + 20-min UV exposure.
While CD spectra reveal the alteration in protein secondary structure due to the presence of photoinitiators and UV irradiation, they do not provide information regarding whether protein is conjugated onto polymer chains [31, 36, 37]. Protein-polymer conjugation can be beneficial and lead to enhanced bioactivity via so-called PEGylation in cases where the site of conjugation is precisely controlled [38, 39]. However, the free-radicals present during photopolymerization can react with a number of sites on the protein backbone in an uncontrollable manner. Random protein-polymer conjugations have been shown to take place on the active sites of protein surfaces and results in reduced bioactivity [40]. In addition, protein-polymer conjugation within a crosslinked hydrogel network will result in incomplete protein release [20]. We have previously used non-reduced SDS-PAGE to display protein molecular weight changes due to protein-polymer conjugation [20]. This technique was used here to detect the molecular weight changes of lysozyme due to free radical-mediated protein-polymer conjugation. As shown in Figure 3.5a, solely irradiating lysozyme solution with low-energy UV for 10-min (Figure 3.5a, lane 2) or solely incubating lysozyme with 0.2 wt% I-2959 (Figure 3.5a, lane 3) does not induce any lysozyme molecular weight change. However, when combining these two conditions to generate free-radicals (Figure 3.5a, lane 4), the total amount of monomeric lysozyme decreases significantly, most likely due to the formation of large protein inactive aggregates at these conditions preventing its elution on the acrylamide gel. When PEGMA was added at 10 wt% without free-radicals present, some lysozyme can be seen at higher molecular weight positions (Figure 3.5a, lane 5 and 6), presumably due to non-specific adsorption of PEGMA monomers on lysozyme. These complexes are replaced by higher molecular weight products, however, upon the introduction of propagating free-radicals (i.e., UV, I-2959, and PEGMA). The amount of
Figure 3.5 Protein-polymer conjugation-induced lysozyme molecular weight changes examined by non-reduced SDS-PAGE. (a) Effect of I-2959 and PEGMA. (b) Effect of UV exposure time.
higher molecular weight species increases with increasing I-2959 concentration in conjunction with UV-irradiation. It can be seen that an almost uniform band of higher molecular weight protein products forms at higher photoinitiator concentration (Figure 3.5a, lane 9), suggesting the formation of polydispersed protein-polymer conjugates at this photopolymerization condition (0.3 wt% I-2959, 10 wt% PEGMA, and 10-min UV exposure).

In addition to photoinitiator concentration, another important parameter that determines the extent of protein-polymer conjugation is UV exposure time. As show in Figure 3.5b, non-reduced SDS-PAGE results reveal that higher molecular weight protein products increase dramatically with UV exposure time in the presence of 10 wt% PEGMA (0.2 wt% I-2959), indicating increased conjugation between the encapsulated proteins and the in-situ polymerized polymer chains. Although the SDS-PAGE results presented in Figure 3.5 substantiates the formation of protein-polymer conjugates in certain photopolymerization conditions, it is important to note that these conjugates may still remain protein bioactivity. However, these covalent irreversible conjugates will result in permanent immobilization in a crosslinked and non-degradable hydrogel network. The consequence of this is the incomplete protein release as discussed earlier.

The occurrence of protein-polymer conjugation during photocuring is also supported by fluorescence spectroscopy data which quantify the extent of protein tertiary structure changes compared to native protein. As shown in Figure 3.6, a decreasing lysozyme intrinsic fluorescence was observed at increasing UV exposure time in the presence of 0.2 wt% I-2959 and 10 wt% PEGMA, confirming changes in protein tertiary structure consistent with protein-polymer conjugation. Although conjugation of PEG onto protein
has been shown to enhance protein structural and functional stability [30, 41], nonspecific protein-PEG conjugations occurring during in-situ polymerization are not desirable in the cases where crosslinked hydrogels are to be used for controlled protein delivery. These results are important in that, although the conjugation of PEGMA chains to lysozyme does not necessarily decrease protein bioactivity (Figure 3.3), it does result in decreased protein bioavailability or incomplete protein release from in situ photo-cured hydrogels. The nonspecific conjugation of encapsulated proteins to the polymer chains will result in decreased bioavailability or incomplete release if the gels are non-degradable (See Section 3.3.4 and Figure 3.7). Even if degradable gels can be used, the release of these protein-polymer conjugates may not be as active as native proteins and may induce unpredictable physiological reactions including inflammation and immune response. Therefore, careful optimization of photopolymerization conditions is especially critical when photopolymerization is used to encapsulate protein therapeutics for controlled release applications.
Figure 3.6 Influence of photopolymerization time on lysozyme structural change in the presence of 0.2 wt% I-2959 and 10 wt% PEGMA examined by fluorescence spectroscopy. Increasing photopolymerization time (5-min incremental) results in decreased lysozyme intrinsic fluorescence.

3.3.4 In situ photo-cured hydrogels for protein delivery

Even though the changes in tertiary protein structure generated by protein-polymer conjugation during free-radical polymerization may, for proteins such as lysozyme, not elicit functional damage to encapsulated agents, covalent immobilization of the proteins onto a crosslinked polymer scaffold will inevitably cause incomplete protein release from the photopolymerized controlled delivery matrices. SDS-PAGE results from Figures 3.5, as well as previous reports in the literature, demonstrate that free-radical species generated by photoinitiators immobilize some fraction of encapsulated proteins to the growing polymer chains [20]. This phenomenon is likely to be universal for protein therapeutics and therefore precautions must be taken when encapsulating therapeutic agents within photopolymerized
delivery vehicles. As shown in Figure 3.7 and Table 3.3, we prepared 10 wt% PEGDA hydrogels by exposing the precursor solutions to UV light for 10-min with 0.1, 0.2 or 0.3 wt% I-2959, respectively. We found that the release kinetics and total release of lysozyme after 24 hours remain unaffected in all cases (Figure 3.7a) while the bioactivity of the released lysozyme decreases dramatically when higher concentrations of I-2959 were used to fabricate the hydrogels (Table 3.3). When examining the tertiary structure of the released lysozyme using fluorescence spectroscopy (Figure 3.7b, curve a, b, and c), it was found that the intrinsic fluorescence of released lysozyme decreases with increasing I-2959 concentration. These results reveal that although the release rates and total amount of released protein is not affected by the range of hydrogel fabrication conditions used in this study, the released protein may not be as active as native protein due to modifications caused by photoinitiator-generated radical species.

Table 3.3 Characteristics of lysozyme releasing from hydrogels with different fabrication conditions

<table>
<thead>
<tr>
<th>[I-2959]</th>
<th>Gelation time</th>
<th>Total release (%) [a]</th>
<th>Activity (%) [b]</th>
<th>(Q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 wt%</td>
<td>10-min</td>
<td>70.2 ± 1.07</td>
<td>92.0 ± 1.38</td>
<td>8.27 ± 0.04</td>
</tr>
<tr>
<td>0.2 wt%</td>
<td>10-min</td>
<td>72.0 ± 7.00</td>
<td>76.5 ± 4.80</td>
<td>8.29 ± 0.05</td>
</tr>
<tr>
<td>0.3 wt%</td>
<td>10-min</td>
<td>66.8 ± 4.29</td>
<td>73.2 ± 3.88</td>
<td>8.33 ± 0.09</td>
</tr>
<tr>
<td>0.2 wt%</td>
<td>20-min</td>
<td>54.9 ± 5.19</td>
<td>69.2 ± 3.00</td>
<td>8.76 ± 0.08</td>
</tr>
</tbody>
</table>

[a] Total release: obtained after releasing 24-hr, no significant increase was found afterward. [b] Activity of 24-hr release samples.
Figure 3.7 (a) Effects of I-2959 concentration and gelation time on lysozyme release from 10 wt% PEGDA hydrogels. (b) Representative intrinsic fluorescence spectra of lysozyme (24-hr released samples) released from PEGDA hydrogels cured from: 0.1 wt% I-2959, 10-min gelation (curve a); 0.2 wt% I-2959, 10-min gelation (curve b); 0.3 wt% I-2959, 10-min gelation (curve c); 0.2 wt% I-2959, 20-min gelation (curve d).
To strengthen the argument that protein-polymer conjugation decreases total protein release, we prepared non-degradable PEG hydrogels with in situ loaded lysozyme by photocuring for 10-min or 20-min. The results shown in Figure 3.7a demonstrate that the total amount of lysozyme released after 24 hours decreases from 72% (10-min exposure) to 55% (20-min exposure) but the bioactivity of the released lysozyme shows no significant changes (Table 3.3). Fluorescence spectroscopy of the released protein shown in Figure 3.7b reveals parallel results to the bioactivity assay and indicates no substantial differences in tertiary protein structure were found (curve b and d). These results suggest that while the gelation time does not sabotage the bioactivity of released lysozyme, a larger fraction of encapsulated lysozyme becomes permanently immobilized within hydrogel networks photocured for longer periods due to protein-polymer conjugation. Note that the hydrogels prepared for this study were non-degradable and exhibited identical crosslinking densities, irregardless of exposure time (Table 3.3).

3.3.5 Proposed protein inactivation mechanisms

Several studies, including the present one, have examined the detrimental effects of photoinitiators on protein denaturation, DNA fragmentation, and cell death during in situ photoencapsulation. One common and important feature of the photo-curable systems studied is the presence of highly reactive free-radical species, including primary radicals produced directly from photoinitiator fragments and propagating radicals located at the ends of actively growing polymer chains. These radical species either directly or indirectly damage the encapsulated biomolecules. Stemming from our previous work as well as studies established by others, the present study further reveals that the use of I-651 is not necessarily harmful to the in situ encapsulated proteins when applied in a suitable environment. This
interesting finding leads us to consider the supramolecular structure of an aqueous precursor solution consisting of PEGMA or PEGDA, a photoinitiator, and the therapeutic protein of interest. Figure 3.8 illustrates our hypothesis regarding the differential protein inactivation phenomena caused by I-2959 and I-651. Given that linear PEGMA is an amphiphilic molecule with a hydrophilic PEG tail and relatively hydrophobic acrylate head-group, at sufficiently high concentrations these functional macromers may form a micellar structure with a PEG shell and acrylate core. Since I-651 is more hydrophobic compared to I-2959, we hypothesize that I-651 will selectively partition into the acrylate core in PEGMA solutions to a greater extent than I-2959 (Figure 3.8a). This partitioning decreases I-651 exposure to predominantly hydrophilic proteins such as lysozyme while also facilitating polymerization due to the co-localization of initiator and reactive units. Overall this proposed architecture should lead to less protein inactivation as well as protein-polymer conjugation during UV irradiation. In contrast, the more hydrophilic I-2959 partitions to a greater extent into the protein-rich phase, increasing its exposure to encapsulated proteins leading to more significant protein inactivation (Figure 3.8b).

3.4 Conclusion

In summary, we have investigated the role of two photoinitiators, I-2959 and I-651, on the inactivation of lysozyme during photoencapsulation and hydrogel formation using fluorescence spectroscopy, circular dichroism, non-reduced SDS-PAGE, and a bioactivity assay. We found that I-651, compared to I-2959, causes less functional damage to the photoencapsulated lysozyme. Furthermore, while linear, non-acrylated PEG-OH provides only moderate protection against free-radical induced protein inactivation, the presence of acrylated PEG macromers (PEGMA) effectively retains lysozyme bioactivity following
Figure 3.8 Proposed mechanisms of (a) I-651 and (d) I-2959 induced protein inactivation in the presence of PEGMA and UV irradiation.

Exposure to photoinitiator-generated free radicals. We hypothesize that the addition of reactive acrylate monomers such as PEGMA do not lower the total radical concentration as radical scavengers have been shown to do, but rather convert the primary radical chemistry into a propagating species that is either less reactive or less damaging to encapsulated proteins. Furthermore, the degree to which PEGMA helps maintain protein bioactivity was shown to be dependent on the type of photoinitiator present with I-651 providing a greater level of lysozyme bioactivity compared to I-2959. These results are contrary to cytocompatibility trends published by previous researchers [25, 26]. It was also shown that extensive protein-polymer conjugates resulting in incomplete protein release will form at high photoinitiator concentrations (0.3 wt% I-2959) and/or long UV exposure times (20 minutes). Understanding the interactions that occur between polymers, free-radicals, and proteins during photoencapsulation of proteins and the effects of these interactions on protein bioactivity and bioavailability are critical to rationally optimizing protein
encapsulation conditions and successfully implementing photocurable hydrogels as protein delivery devices.

Acknowledgement

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3.5 References


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CHAPTER FOUR
ENHANCED PROTEIN DELIVERY FROM PHOTOPOLYMERIZED HYDROGELS
USING A PSEUDOSPECIFIC METAL CHELATING LIGAND

(As appearing in Pharmaceutical Research 2006(23):614-622)

Abstract

**Purpose.** This study was conducted to investigate the cause of incomplete protein release from photopolymerized poly(ethylene glycol) (PEG) hydrogels and verify the protein-protection mechanism provided by iminodiacetic acid (IDA). **Methods.** The in vitro release of bovine serum albumin (BSA) from PEG hydrogels prepared under different conditions was studied. Photoinitiator and initial protein concentrations were varied as well as the addition of IDA and metal ions. Protein immobilization within the nondegradable networks via free radical reaction was demonstrated by gel electrophoresis. **Results.** Protein release efficiency was shown to be dependent on photoinitiator and initial protein concentration. Gel electrophoresis results revealed immobilization of protein to the polymer network and further indicated the detrimental role of free radicals in lowering protein-release efficiency. Adding IDA to the prepolymer solution enhanced total protein release from the subsequently photopolymerized network in a dose-dependent manner. The addition of metal ions including Cu$^{2+}$, Zn$^{2+}$, and Ni$^{2+}$ further increased BSA release efficiency. Agreement between the protein release data and theoretical model predictions accounting for reversible protein-IDA binding further validated the protection effect provided by IDA and IDA-transition metal complexes. **Conclusions.** The protection effect described in this study offers a novel strategy for increasing the delivery efficiencies of many therapeutically valuable proteins.
4.1 Introduction

Controlled release of bioactive proteins is becoming attractive for disease treatments requiring extended therapeutic effects. Injectable depots of human growth hormone (hGH), for example, are administered in both adults and children to treat chronic growth hormone deficiency [1], whereas slow-acting formulations of insulin are used to prevent hyperglycemia in patients suffering from type I or type II diabetes. Differing from synthetic drugs with small molecular weights, proteins have complex three-dimensional structures and characteristics that provide added challenges for their effective delivery. The correct structural folding determines the bioactivity of a protein and its therapeutic efficiency [2] and therefore must be maintained. Furthermore, environmental factors such as pH, temperature, and solvent composition can have profound effects on the physical stability of a protein [3].

Poly(ethylene glycol) (PEG) hydrogels are excellent carriers for protein delivery because of their high hydrophilicity and aqueous gel-forming environments that help to retain protein stability and bioactivity [4]. Furthermore, by altering the composition and the degree of cross-linking, one can readily control the release rate of protein drugs from PEG hydrogels [5]. Another advantage of PEG hydrogels is that protein encapsulation and network formation can be achieved simultaneously via in situ photopolymerization [6]. This provides a convenient and efficient method for loading high concentrations of proteins for subsequent controlled release. The photocuring process is fast, mild, and can be performed under room temperature in aqueous solution. Moreover, spatial and temporal control of the photopolymerization enables hydrogels fulfilling specific physicochemical requirements to be fabricated. Photopolymerization of hydrogels has been applied in tissue engineering [7-11] as well as protein [12-14] and gene delivery [15-17].
The main disadvantage of photopolymerizations comes from the necessary production of highly reactive free radicals that facilitate network polymerization and cross-linking. These free radicals can also induce side reactions between encapsulated protein or DNA and polymer chains during network formation. Quick et al. [15-17] used photopolymerized hydrogels for DNA encapsulation and delivery. They showed that without the presence of monomer, free radicals attack and damage DNA molecules. Through the use of vitamin C, a radical scavenger, and protamine sulfate, a transfection agent, they were able to preserve the structural integrity of encapsulated DNA [15]. These studies suggest that free radicals are responsible for the reduced amount of DNA delivery.

Similar to the DNA damage caused by free radicals, proteins are susceptible to undesirable reactions during polymer network formation that limit protein bioavailability. Research has been conducted in the development of suitable polymeric matrices for protein delivery based on several chemistries including poly(lactide-co-glycolide) (PLGA) [18,19], poly(anhydride) [20], and poly(ethylene glycol) [4]. However, proteins may still be unstable in these synthetic polymeric matrices because of physical or chemical interactions with the encapsulating network or because of physical or chemical stresses experienced by the protein during matrix fabrication and protein encapsulation. Previous studies focused on PLGA delivery systems show that proper protein stabilization strategies are needed to increase the delivery efficiency and bioactivity of encapsulated proteins [21-28]. However, a protein stabilization strategy to overcome free-radical-induced protein-polymer reactions has not been discovered. Reactions with free radicals can reduce the bioactivity and bioavailability of the encapsulated protein. The decreased total protein delivery is attributable to protein denaturation, aggregation, or conjugation to the polymer network.
It is well known that recombinant proteins with polyhistidine tags on their N- or C-terminals can be easily purified by using immobilized metal ion chelating affinity chromatography (IMAC) [29-31]. It has been shown that histidine residues offer the strongest interaction with chelators such as nitrolothiuric acid (NTA) [32-35] or iminodiacetic acid (IDA) [36-38] in the presence of divalent transition metal ions including Cu$^{2+}$ and Ni$^{2+}$. In particular, the three-amino-acid sequence Asp-Ala-His on the N terminus of bovine serum albumin (BSA) is the strongest binding site for copper ions [39-42]. The strong binding between BSA and the chelator-metal ion complex is associated with the deprotonated Asp-Ala [43]. We hypothesize that the same mechanism is responsible for the reaction between protein and the monomeric or polymeric free radicals during photopolymerization. Therefore, when highly reactive free radicals are generated from photoinitiators, reactions with the deprotonated amide on BSA may occur and form protein-polymer conjugates, which result in permanent, irreversible changes to the protein chemistry, structure, and bioavailability.

To increase the delivery efficiency of BSA from photopolymerized networks, the factors affecting total protein release from nondegradable PEG hydrogels are evaluated as part of this study. A strategy for protecting BSA from freeradical reaction during polymer network formation and protein encapsulation is proposed by adding a known protein-binding ligand, IDA, to the prepolymer solution. BSA-IDA binding should minimize the exposure of highly reactive N-terminal residues of BSA to the free radicals generated during hydrogel formation. BSA was chosen because of its known affinity to both IDA and IDA-metal ion complexes. Nondegradable PEG hydrogels are used to eliminate any release due to
gel degradation. A theoretical model for estimating total protein release efficiency is derived based on the equilibrium dissociation constant ($K_d$) of the protein-IDA complex.

4.2 Materials and Methods

4.2.1 Formation of protein-loaded PEG hydrogel

PEG diacrylates (PEGDA) were synthesized by reacting linear 3.4 kDa PEG (Sigma-Aldrich) with acryloyl chloride (Sigma-Aldrich) as described elsewhere [44]. PEG hydrogels were formed via solution photopolymerization of PEGDA monomers. Briefly, PEGDA macromer was dissolved in 50mM, pH 7.4 phosphate buffered solution (PBS) to a concentration of 10 wt%. Required stoichiometric amounts of BSA (Sigma-Aldrich), IDA (Fisher Scientific), and metal ions including cupric sulfate pentahydrate, zinc sulfate heptahydrate, or nickel sulfate hexahydrate (Fisher Scientific) were mixed with PEGDA precursor solutions. Prior to photopolymerization, Irgacure 2959 (I-2959), a photoinitiator, was also added at the desired concentration. 50µl of the mixed precursor solution was injected between glass slides separated by 0.8mm Teflon spacers and was exposed to a 100-W UV lamp (BLACK-RAY®) with a maximum intensity of 8.4 mW/cm² at 365nm for a total of 20 minutes.

4.2.2 In vitro protein release

PEG hydrogels (weighted ~50mg) loaded with BSA, with or without chelating ligands, were placed into a 5mL of pH 7.4 PBS for *in vitro* protein release at 37°C. At specified time intervals, 200 µl supernatant solutions were sampled and replaced with an equal amount of fresh PBS. The cumulative percent of protein released was quantified by a fluorescamine assay. Briefly, fluorescamine (Sigma-Aldrich) was dissolved in acetone to a final concentration of 3 mg/ml. 150 µl of sample solution was mixed with 50 µl of fluorescamine
solution for fluorescence quantification at an excitation wavelength of 395 nm and an emission wavelength of 475 nm using a micro-plate reader (Spectramax GeminiEM, Molecular Devices). Protein concentrations in measured samples were quantified using the linear portion of a standard fluorescence curve constructed from solutions of known concentrations.

**4.2.3 Non-Reducing SDS-PAGE experiments**

The effect of free-radical induced BSA immobilization to PEG monomers was examined by SDS-PAGE. Linear 3.0 kDa PEG monoacrylate (PEGMA) was used to replace PEG3400 diacrylate to prevent photocrosslinking and gel formation during photopolymerization of samples. The amount of PEGMA used in these experiments was calculated based on the same concentration of acrylate bonds present during hydrogel formation and protein encapsulation. Varying amounts of IDA or IDA-Cu$^{2+}$ complex were added. The samples prepared for SDS-PAGE were loaded without any denaturation treatments so that any changes in the molecular weight or conformation of the BSA molecules can be detected.

**4.2.4 Swelling ratio measurements**

The swelling ratios of PEG hydrogels were measured to characterize the gel cross-linking density. After photopolymerization, gels were placed into an excess amount of pH 7.4 PBS for swelling. Gels were allowed to swell for 2 days to reach equilibrium and then dried completely under reduced pressure at room temperature. Gel weights before and after drying were taken and the mass swelling ratios (Q) were determined by the following equation:
Swelling Ratio \( (Q) = \frac{Swollen Weight}{Dried Weight} \)

4.2.5 Theoretical model of protein release efficiency

The affinity between BSA and metal-ion charged ligands such as iminodiacetic acid is well documented [36]. The binding of IDA to BSA is believed to reduce the protein-polymer conjugation caused by free radicals produced during network formation and thus increase the total amount of protein released from PEG hydrogels. In the model development, two assumptions were made: (1) BSA has only a single binding site for IDA-Cu\(^{2+}\), and (2) BSA is released in a non-aggregated state. The shielding effects provided by IDA and various IDA-metal ion complexes were modeled by assuming a reversible association between the two components given by:

\[
\text{Protein} + \text{Ligand} \rightleftharpoons K_d \rightarrow \text{Protein} \cdot \text{Ligand Complex} \quad (1)
\]

The dissociation constant \((K_d)\) of the protein-ligand interaction can be expressed as the following equation:

\[
K_d = \frac{[P][L]}{[P \cdot L]} = \frac{([P]_0 - x)([L]_0 - x)}{x} \quad (2)
\]

Where \([P]_0\) and \([L]_0\) are the initial concentrations of protein and ligand in the precursor solution and \(x\) is the concentration of associated protein, ligand or protein-ligand complex.

The dissociation constants for BSA-IDA and BSA-IDA-Cu\(^{2+}\) are 0.4 and 0.0082 mM [43], respectively. By knowing \(K_d\), Equation (2) can be used to determine \(x / [P]_0\), the fraction of protein bound to the ligand at equilibrium, for any combination of \([P]_0\) and \([L]_0\).

Increasing the ligand-protein ratio or decreasing \(K_d\) shifts the equilibrium such that a greater fraction of protein is present in the bound or “protected” form.
Furthermore, total release ($T_R$) is defined as the percentage of protein released after an infinite amount of time compared to the amount initially present in the gel and can be expressed as:

$$T_R = \left[ \frac{[P]}{[P]_0} \right] \times 100\% = \left[ \frac{[P]_0 - i([P]_0 - x)}{[P]_0} \right] \times 100\% = \left[ 1 - \frac{i x K_d}{([L]_0 - x)[P]_0} \right] \times 100\%$$

(3)

Where $i$ is the fraction of immobilized protein obtained when no ligand is present during gel formation. $i$ is obtained by experimentally measuring total protein release ($T_R$) when $[L]_0 = 0$ and is equivalent to $(100\% - T_R)$ under these conditions. Therefore $i$ accounts for any non-specific adsorption of protein to the polymer network as well as the presence of any aggregated protein that cannot be released. All other parameters needed to solve Equation (3) are determined $a$ priori based on ligand selection and composition of the gel precursor solution.

4.3 Results and Discussion

4.3.1 Photoinitiator effects on BSA total release

Several critical factors lead to incomplete release of proteins encapsulated during hydrogel photocuring. These factors can be either directly or indirectly related to the free-radical induced crosslinking in the hydrogels. Under UV exposure, free radicals generated from photoinitiators propagate through the vinyl bonds on di-acrylated PEG molecules and form cross-linked hydrogels. However, if other reactive sites on protein surfaces are present during photopolymerization, free radicals will also propagate through these sites. Although the exact mechanisms of interaction between these free radicals and encapsulated proteins remains unresolved and most likely varies dramatically with protein surface chemistry, we
hypothesize that these interactions lead to the irreversible immobilization of proteins within the hydrogel networks.

The most apparent variable affecting free radical concentration is the photoinitiator concentration. Excessive amounts of free radicals generated from photoinitiators play a critical role in limiting the bioactivity and bioavailability of encapsulated objects such as DNA, proteins and cells. As shown in Figure 4.1, incomplete release of BSA from photocured gels is observed at every photoinitiator (I-2959) concentration. As higher photoinitiator concentrations produce higher concentrations of free radicals during UV irradiation, a greater number of undesirable protein-radical interactions and a lower total release of BSA is expected as I-2959 concentration increases. This trend is confirmed by the experimental results in Figure 4.1, which show total release of BSA decreases from 42 % to 21 % after 24 hours as the concentration of I-2959 is increased from 0.2 wt% to 1.0 wt%.

The decrease in BSA release with an increase in photoinitiator concentration cannot entirely be attributed to changes in gel crosslinking density. The hydrogel mass swelling ratio decreases slightly and reaches a plateau with increasing initiator concentration (7.43 ± 0.16, 6.64 ± 0.28, and 6.75 ± 0.34 respectively for hydrogels cured with 0.2 %, 0.5 %, and 1 % photoinitiator). Furthermore, based on the release profiles, the diffusivities of releasable BSA within the gels of varying initiator concentration were found to be identical (~1.0×10⁻⁷ cm²/sec). These results suggest that the decrease in the total release of BSA with initiator concentration is due to increased protein-polymer coupling rather than changes in gel swelling.

To further investigate the cause of incomplete release, the total release of BSA from photopolymerized PEG hydrogels loaded with different concentrations of BSA was studied.
The results in Figure 4.2 indicate that total release of BSA is also a function of initial protein concentration. When BSA is initially loaded at 15 wt%, over 80% of the protein is released within 24 hours. However, less than 50% of the protein is released when BSA is loaded at 5 wt%. Therefore, the absolute amount of released protein increases with initial protein concentration. It is interesting to note, however, that the amount of unreleased protein within each of these networks remains relatively constant as can be seen in Table 4.1. This observation is attributed to the fact that all of these networks were photopolymerized under identical conditions (photoinitiator concentration, light intensity, etc.) and thus should present the same concentration of free radicals to encapsulated BSA during hydrogel formation.

![Figure 4.1](image)

**Figure 4.1** The effect of photoinitiator concentration on BSA release. BSA (5 wt %) released from 10 wt% PEGDA hydrogels polymerized with 0.2 wt % (●), 0.5 wt % (○) and 1.0 wt % (▲) of photoinitiator (I-2959). (n = 3, mean ± SD)
Figure 4.2 The effect of BSA initial loading concentration on its total release. Different amounts of BSA (wt %) were loaded into 10 wt% PEGDA gels. (n = 3, mean ± SD. Concentration of I-2959: 0.2 wt%)

Table 4.1 Total amount of released and unreleased BSA from photopolymerized PEG networks as a function of protein loading. (n = 3, mean ± SD)

<table>
<thead>
<tr>
<th>Concentration of Loading BSA (wt %)</th>
<th>Amount of loaded BSA (mg)</th>
<th>Total release of BSA (%)</th>
<th>Amount of unreleased BSA (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>51 ± 3.0</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>55 ± 0.7</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>73 ± 1.4</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>80 ± 1.0</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>85 ± 5.0</td>
<td>4.6 ± 1.5</td>
</tr>
</tbody>
</table>
4.3.2 BSA-polymer conjugation

The structural integrity of BSA was examined by SDS-PAGE to demonstrate the coupling of BSA with PEG macromers via free radicals during photopolymerization (Figure 4.3). The rationale for choosing SDS-PAGE instead of other molecular weight determination techniques such as MALDI-TOF is that the polymerization process yields protein-polymer conjugates with a wide distribution of molecular weights due to the polydispersity of the polymer chains. PEG monoacrylate (PEGMA, MW = 3000 Da) is used instead of PEGDA to prevent gel formation and allow sample elution on the PAGE gel.

4.3.2.1 Photoinitiator induced BSA-polymer conjugation

As shown in Figure 4.3a, when BSA and PEGMA are subjected to free-radical induced photopolymerization, broad distributions of higher molecular weight products were produced, suggesting that a large portion of the encapsulated BSA is covalently bound to polymerized oligomers of PEG. This results in higher molecular weight products (Lane 5 – 7) compared to native BSA (Lane 1). When higher concentrations of I-2959 were added to the protein-polymer mixture and subjected to UV exposure, the fraction of unmodified BSA monomer was decreased. Assuming any modified protein would not be released from a crosslinked polymer due to its immobilization onto the network this result agrees with the release trends of Figure 4.1 that demonstrate decreased BSA release with increasing photoinitiator concentration. This phenomenon clearly demonstrates the significant structural modification of BSA that occurs only in the combined presence of photoinitiator, monomer, and UV light.
The determination of BSA structural integrity using SDS-PAGE. A significant amount of higher molecular weight products appear when BSA is mixed with PEG3000MA and photoinitiator (I-2959) and exposed to 365nm UV light for 20 minutes. **(a)** Lanes: (1) Native BSA; (2) 5 wt% BSA, 0.2 wt% I-2959 and PEG3000MA without UV exposure; (3) 5 wt% BSA and 0.2 wt% I-2959 subjected to UV exposure; (4) 5 wt% BSA and PEG3000MA subjected to UV exposure; (5) 5 wt% BSA, PEG3000MA and 0.2 wt% I-2959 subjected to UV exposure; (6) BSA, PEG3000MA and 1.0 wt% I-2959 subjected to UV exposure; (7) BSA, PEG3000MA and 4.0 wt% I-2959 subjected to UV exposure. **(b)** Lanes: (1) Native BSA; (2) 5 wt% BSA, PEG3000MA and 0.2 wt% I-2959 subjected to UV exposure; (3) 5 wt% BSA and molar equivalent of IDA, PEG3000MA, and 0.2 wt% I-2959 subjected to UV exposure; (4) 5 wt% BSA and molar equivalent of IDA-Cu$^{2+}$, PEG3000MA, and 0.2 wt% I-2959 subjected to UV exposure.

### 4.3.2.2 Polydispersity of BSA-polymer conjugates

The broad distributions of BSA molecular weight products that occur during free-radical photopolymerization (Lanes 5-7 in **Figure 4.3a**) are due to the high polydispersity (PDI) of the conjugated PEG chains. BSA was conjugated onto uncrosslinked PEG molecules that polymerize with a high polydispersity, leading to a broad distribution of high molecular weight products. Therefore, no specific bands at molecular weights above...
monomeric BSA (MW ~60kD) are seen. Without UV exposure, BSA remains unmodified with the presence of I-2959 and PEGMA (Lane 2). Also, when BSA and I-2959 (Lane 3) or BSA and PEGMA (Lane 4) were subjected to UV exposure, no molecular weight modification was found in either case. This suggests that all three components are required for free-radical induced protein-polymer conjugation to occur.

4.3.2.3 BSA aggregates

As shown in Lane 1, an extremely small amount of BSA dimers, trimers, and aggregates appear above monomeric BSA (120kD, 180kD, etc). Furthermore, Figure 2 indicates that the total BSA release increases with initial BSA concentration. Combining these results with Figure 4.3a suggest that aggregates do not contribute to the fraction of unreleased BSA in the hydrogel systems studied. The results in Lanes 5-7 reveal that even relatively stable proteins such as BSA are modified under standard photopolymerization reaction conditions. Therefore, proper protein stabilization strategies are needed when encapsulating proteins into photopolymerized hydrogels.

4.3.2.4 Ligand-mediated protein protection

The ligand-mediated protection effect of BSA protein is directly demonstrated in Figure 4.3b. The addition of IDA (Lane 3) or IDA-Cu$^{2+}$ complex (Lane 4) prevents the polymerization of BSA molecules with PEG3000MA macromers as indicated by the absence of any significant molecular weight modification. This result qualitatively supports our hypothesis of IDA-mediated protein protection.

4.3.3 Effects of metal chelating-ligand on BSA total release

Enhanced protein delivery is achieved by adding IDA to the prepolymer solution prior to photopolymerization. As shown in Figure 4.4, the total release of BSA is increased
from about 40% to 60% when IDA is added in a molar ratio of 0.5 (to BSA). Total BSA release is further increased to almost 70% when the IDA to BSA molar ratio is increased to 1.0.

**Figure 4.4** The effect of IDA concentration on BSA total release. BSA (5 wt %) was mixed with PEGDA (10 wt %) and IDA in molar ratios (IDA:BSA) of 1 (▲), 0.5 (●), and 0 (●) in the prepolymer solution and released from PEGDA hydrogels. (n = 3, mean ± SD. Concentration of I-2959: 0.2 wt %)

Adding transition metal ions such as Cu2+ can further increase the affinity between BSA and IDA. The coupling of Cu2+ to metal-ion chelators like IDA has been well studied in the field of immobilized metal ion chelating affinity chromatography (IMAC). The dissociation constant of IDA-Cu2+ and BSA coupling is 0.0082mM [45] compared to a value of 0.4mM for the coupling of IDA and BSA. In another words, IDA-Cu2+ has a greater affinity for BSA than IDA alone. As shown in **Figure 4.5**, while the BSA to IDA molar ratio is held constant at 1, total BSA release increases as more Cu2+ ions are added. When Cu2+ is added in a molar ratio of one, the total release of BSA is increased to almost 100%.
No significant increase in total release is observed when Cu\(^{2+}\) is added to the prepolymer solution without IDA present (data not shown).

**Figure 4.5** The effect of Cu\(^{2+}\) concentration on BSA total release. BSA (5 wt %) was mixed with PEGDA (10 wt %), IDA and different amounts of Cu\(^{2+}\) ions. Molar ratio of IDA to BSA is held at 1. Molar ratios of Cu\(^{2+}\) ions to BSA are 1 ( ), 0.5 ( ), and 0 ( ). (n = 3, mean ± SD. Concentration of I-2959: 0.2 wt%)

### 4.3.4 Metal-ion effects on BSA total release

From IMAC studies it is well known that the affinity between protein and ligand can be varied by using different transition-metal ions [46-48]. For example, Cu\(^{2+}\) provides the strongest binding affinity to BSA, followed by Ni\(^{2+}\) and Zn\(^{2+}\) [48]. Ideally, at the same IDA concentration, BSA release efficiency should increase with protein-ligand binding strength. As shown in **Figure 4.6**, the total release of BSA increases when Zn\(^{2+}\) or Ni\(^{2+}\) are added along with IDA, compared to adding IDA alone. Furthermore, total release is even higher
when Cu$^{2+}$ ions are added and approaches 100%. Total release of BSA from all ligand-containing systems is significantly higher than when no ligand is present.

**Figure 4.6** also shows the mass swelling ratio of the ligand-containing gels. The swelling ratio of all gels remains relatively constant and suggests that the crosslinking density of the hydrogel is not affected by the addition of IDA or any metal ions. Thus the observed, enhanced release of BSA is solely due to the association of BSA with IDA and not changes in gel crosslinking density and protein diffusivity.

![Bar chart showing total release and swelling ratio](chart.png)

**Figure 4.6** The effect of different metal ions on total BSA release and hydrogel swelling. BSA (5 wt %) was mixed with equal moles of IDA and metal ions in 10 wt % PEGDA prepolymer solutions. The swelling ratios of each nondegradable gel were measured after protein release. ($n = 3$, mean ± SD. Concentration of I-2959: 0.2 wt%; * and ** indicate statistical significance with P<0.05)
The data in Figure 4.6 further demonstrate a direct correlation between the extent of protein-ligand binding and protein release efficiency. The results presented in Figure 4.3 demonstrate an inverse correlation between the extent of protein-ligand binding and the extent of protein-polymer coupling. Taken together, the results in Figure 4.3 and 4.6 support the conclusion that BSA is protected from adverse side reactions with free radicals during gel formation by complexing with the small-molecule IDA ligand. The higher the fraction of protein bound to the ligand, the greater the observed increase in release efficiency. Although the addition of metal ions can be beneficial to the therapeutic efficacy when released in vitro, a potential problem behind this approach is the toxicity of metal ions in vivo. Nonetheless, the increased amount of protein delivery proved by this research does open an avenue for enhancing protein stability during hydrogel formation and protein encapsulation.

4.3.5 Prediction of reversible IDA-BSA binding

A theoretical prediction of the extent of BSA-IDA-Cu$^{2+}$ association based on the binding equilibrium shown in Equations (1) and (2) is presented in Figure 4.7. At equilibrium, binding between BSA and the IDA-Cu$^{2+}$ complex is considered reversible and is determined, in part, by the $K_d$ value and total BSA concentration. The degree of association decreases with $K_d$ and increases with BSA concentration. BSA is far more concentrated in the prepolymer solution than in the swelled gel or the surrounding media. Therefore, compared to the protein-rich hydrogel (50 µl), the relatively protein and ligand-free supernatant solution (5 ml) can be treated as a “sink” where the BSA concentration is dramatically lower than in the gel. In Figure 4.7, the solid curve represents the theoretical extent of BSA-IDA-Cu$^{2+}$ association present in a 5 wt % BSA prepolymer solution at different BSA-IDA-Cu$^{2+}$ molar ratios ($K_d = 0.0082$ mM). The extent of BSA-IDA-Cu$^{2+}$
association is approximately 90% when a one to one molar ratio of IDA-Cu$^{2+}$ to BSA is used. However, when BSA-IDA-Cu$^{2+}$ complex is released from the hydrogel, its bulk concentration rapidly decreases. For example, under these same conditions, a drop in BSA concentration from 5% (solid curve) to 0.01% (dotted curve) also lowers the degree of association from 90% to only 13%. In other words, most of the released BSA will dissociate from the BSA-IDA-Cu$^{2+}$ complex due to its diluted concentration and make BSA available to its target in its uncomplexed, native form. The general trends in BSA-(IDA-Cu$^{2+}$) binding shown in Figure 4.7 can be readily extrapolated to other protein-ligands pairs.

Figure 4.7 Predicted extent of association between BSA and IDA-Cu$^{2+}$ complex ($K_a=0.0082\text{mM}$) as a function of total protein concentration. Solid curve: 5wt% BSA; Dashed curve: 0.5wt% BSA; Dotted curve: 0.01wt% BSA. The extent of association is defined as the molar ratio of IDA-Cu$^{2+}$-BSA complex to unbounded BSA.
4.3.6 Prediction of BSA total release

To further understand the mechanism of BSA protection provided by IDA-metal ion complexes during free-radical polymerization, a theoretical model was developed to predict total BSA release from gels where IDA-based ligands with different protein affinities are incorporated in the prepolymer solutions at various concentrations. The total release of BSA can be modeled by using known dissociation constant values and a single experimentally determined variable, the fraction of protein nonspecifically immobilized in the hydrogel ($i$). Figure 4.8 illustrates the ability of this model to predict total BSA release from systems exhibiting different protein affinities as well as ligand-protein ratios. The agreement between predictions of this theoretical model and experimental BSA release data shown in Figure 4.8 support the proposed mechanism of BSA immobilization and IDA-mediated protection within the photopolymerized networks.

Once validated, the developed protein-ligand binding model can be used to explain experimental observations. As shown in Figure 4.8, the total release of BSA depends upon the initial BSA loading concentration as well as the ligand-protein ratio. Recall from Figure 4.2, BSA total release increases with the protein loading concentration. Therefore, $i$ values were obtained from the experimentally determined total BSA release at 10 wt% and 5 wt% in Figure 4.2 and used to predict release at all ligand-protein ratios (dashed and solid lines) using Equation (3).
Figure 4.8  Comparison of experimentally determined total BSA release (symbols) with predictions of a theoretical model (curves). Release data obtained with various molar ratios of ligand to protein are used to verify the accuracy of the model. Ligand-protein ratios, protein loading concentration, and ligand-protein affinity all affect total BSA release. Release data and predictions from three systems are compared: (a) IDA-Cu$^{2+}$ with K$_d$ = 0.0082mM, [BSA]$_0$ = 10 wt% (♦ and dotted curve); (b) IDA- Cu$^{2+}$ with K$_d$ = 0.0082mM, [BSA]$_0$ = 5 wt% (▲ and dashed curve); and (c) IDA alone with K$_d$ = 0.4mM and [BSA]$_0$ = 5 wt% (△ and solid curve). (n = 3, mean ± SD. Concentration of I-2959: 0.2 wt%)

From Equation (3) it can be understood that the relatively weak binding affinity between BSA and IDA leading to low total BSA release can be countered by increasing IDA concentration. The increased ligand concentration forces the free protein into the associated protein-ligand state and therefore increases its degree of protection and total release. As shown by the solid line (model prediction) and open triangles (experimental data) in Figure 4.8, when the molar ratio of IDA to BSA is increased to a ratio of 4:1, the total release of BSA is increased to almost 90% compared to only 45% when no IDA is used. As predicted by the model, further increasing the IDA concentration to a molar ratio of 10 does not significantly increase the total release of BSA (data not shown).
Total release of BSA also increases with initial BSA loading concentration. As shown by the dotted (10 wt% loaded BSA) and dashed (5 wt% loaded BSA) lines in Figure 4.8, the more BSA loaded into the prepolymer solution, the greater its total fractional release. Additionally, the model predicts differences in total BSA release when a variety of protein-binding ligands are utilized. For example, incorporating the IDA-Cu$^{2+}$ ligand into a 5 wt% BSA prepolymer solution leads to higher total BSA release values than incorporation of IDA alone at similar ligand-protein ratios (solid triangles versus open triangles). This increase is due to the higher affinity of the IDA-Cu$^{2+}$ complex for BSA compared to IDA. Release data from systems incorporating high concentrations of IDA-Cu$^{2+}$ ligand are not obtained due to the fact that high Cu$^{2+}$ concentrations prevented gel formation.

Though the exact mechanism in which IDA-Cu$^{2+}$ complex provides a protection effect on BSA during polymer network formation remains undetermined, we speculate this protection effect is due to the fact that the copper-binding site on BSA also acts as a reactive center for protein-polymer conjugation as discussed previously. Therefore, when IDA-Cu$^{2+}$ complex is added, immobilization of BSA to the crosslinked polymer network is reduced which leads to an increased amount of BSA release. An alternative hypothesis is that binding of the IDA or IDA-Cu$^{2+}$ complex to BSA leads to a change in protein conformation that hinders accessibility of free radicals to any number of reactive sites on the BSA surface.

Although the model can accurately predict the total release of BSA in the presence of IDA or IDA-Cu$^{2+}$ complex, there are limitations to the model predictions. The model was developed under the assumption of a single IDA-Cu$^{2+}$ binding site per BSA molecule. The binding equilibrium shown in Equations (1) and (2) is valid only when the single binding site assumption holds. Some research suggests, however, that a second, weaker copper binding
site exists on BSA [43]. It is also assumed that the BSA is present in a non-aggregated state. If BSA forms aggregates under certain unfavorable conditions, which is not the case in this study as shown in **Figure 4.3**, the predicted binding equilibrium based on total protein concentration will not be valid. In the present model, purely empirical values for the model parameter \(i\) were used to generate the theoretical release curves only applicable to the system analyzed in this study. The amount of protein nonspecifically immobilized in the polymer networks will vary with external conditions such as the photopolymerization reaction parameters as well as inherent biochemical properties such as the extent of protein-polymer interaction. A method or model is needed to predict the amount of protein immobilized under specific photopolymerization conditions in the absence of protein-binding ligand. This is possible only when the exact mechanism of protein-free radical conjugation is resolved. Nevertheless, the results presented here demonstrate a possible mechanism of protein-polymer interaction and provide a solution that minimizes this undesired interaction.

### 4.4 Conclusions

The release efficiency of BSA from photopolymerized PEG hydrogels is increased by incorporating a soluble transition-metal chelator (iminodiacetic acid) into the prepolymer solutions. The significance of this strategy is that the protein protection effect is achieved by simply adding IDA and metal ions while maintaining the advantages provided by *in situ* photopolymerization and simultaneous protein encapsulation. The undesired protein-radical interactions commonly observed during PEG hydrogel formation and the reduction in total protein release were minimized via ligand incorporation. Specifically, BSA release efficiency was enhanced by adding IDA and different transition-metal ions to the prepolymer solution. Although BSA is used in this study, it is hypothesized that similar protection strategies can
be applied to other therapeutically valuable proteins that display an affinity for IDA. This includes proteins exhibiting transition-metal binding domains such as hepatocyte growth factor (HGF) or human growth hormone (hGH) and recombinant proteins engineered with histidine tags [49]. Furthermore, specialized ligands other than IDA can be engineered to display affinities for specific proteins of interest and thereby increase their release efficiency in a manner similar to that seen with BSA.

Acknowledgements

The authors wish to acknowledge Drs. Sarah Harcum, James Morris and Meredith Morris for discussion in SDS-PAGE. The project was supported in part by funding from NSF-EPSCoR.

4.5 References


CHAPTER FIVE
METAL-CHELATING AFFINITY HYDROGELS FOR SUSTAINED PROTEIN
RELEASE

(As appearing in Journal of Biomedical Materials Research A 2007, in press)

Abstract

Affinity hydrogels based on poly(ethylene glycol) diacrylate (PEGDA) and a metal-ion-chelating ligand, glycidyl methacrylate-iminodiacetic acid (GMIDA), have been developed to systematically decrease protein release rates from hydrophilic tissue engineering scaffolds formed in situ. In the current work, tunable and sustained release of a model protein, hexa-histidine tagged green fluorescence protein (hisGFP), is accomplished by judiciously increasing ligand:protein ratio or replacing low-affinity nickel ions with high affinity copper ions. Agreement between theoretical predictions of a reaction-diffusion model and experimental measurements confirm metal-ion-mediated sustained protein release from these affinity hydrogels is governed by equilibrium protein-ligand binding affinity (dissociation constant, $K_d$) as well as protein-ligand dissociation kinetics (protein debinding rate constant, $k_{off}$). The former dictates the release rate in the early period of protein release while the latter determines the long-term sustained release effect. While metal-ion affinity binding has been widely used for various purposes including protein purification and surface patterning, this is the first report describing its application in systematically controlling protein release from hydrophilic PEG networks suitable for cell encapsulation. By using ligands with proper binding kinetic constants ($K_d$ and $k_{off}$), localized protein delivery can be sustained over clinically relevant timescales while maintaining a favorable environment for cell encapsulation and viability.
5.1 Introduction

The development of affinity hydrogels is becoming increasingly important due to the emerging field of tissue engineering. Spatially and temporally controlled delivery of biomacromolecules such as growth factors and cytokines from artificial scaffolds is a critical technique for the successful implementation of tissue engineering strategies as these biomolecules are known to stimulate cell growth and tissue regeneration after injury [1-3]. Synthetic hydrogels are excellent candidates for delivering proteins and peptides due to highly controllable and relatively mild network fabrication conditions compared to hydrophobic polymers [4]. However, one of the limitations facing hydrogel-based protein delivery systems is their rapid release rate due to high water content and gel permeability. Protein release rates can be decreased by substantially increasing the hydrogel crosslinking density [5, 6]. However, this results in decreased amounts of loaded protein as well as decreased matrix hydrophilicity. Therefore, efforts have been directed towards the development of affinity hydrogels to systematically decrease release rates of therapeutically relevant proteins via mild and reversible protein-matrix binding. This unique strategy maintains hydrogel properties that are advantageous for tissue engineering and protein delivery applications.

Inspired by the reversible sequestering of proteins to the extracellular matrix (ECM), researchers have previously developed biomimetic hydrogel carriers bearing reversible binding capacities to decrease release rates of target protein therapeutics [7-9]. ECM is full of anionic polysaccharides including hyaluronic acid and proteoglycans. The abundance of negative charges on the native matrix modulates release of cationic growth factors and also protects them from proteolytic degradation. For example, heparin, a highly sulfated
glycosaminoglycan (GAG), serves as a growth factor depot in vivo owing to its electrostatic affinity to various cationic growth factors such as nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) [10, 11]. Matrices containing passively adsorbed heparin have been used as delivery depots to modulate release rates of these growth factors [12-16]. In addition to the physical adsorption of heparin, Sakiyama-Elbert and Hubbell developed fibrin-based hydrogels containing affinity peptides for reversibly immobilizing heparin [7, 8]. The transiently-immobilized heparin acts as an intermediate component that binds to growth factors and covalently-immobilized affinity peptides. These researchers showed that growth factor release rates could be adjusted by incorporating peptides with different heparin affinities. This system has been applied to the delivery of several growth factors including NGF [7], basic fibroblast growth factor (bFGF) [8], and neurotrophin-3 (NT-3) [9].

Heparin can also be covalently immobilized within the gel network. Yamaguchi and Kiick conjugated low molecular weight heparin (LMWH) onto multifunctional PEG hydrogel network to bind and release bFGF in a sustained manner [17, 18]. Benoit and Anseth copolymerized methacrylated heparin with dimethacrylated poly(ethylene glycol) (PEGDMA) to form heparin-containing PEG hydrogels [19] for releasing bFGF and stimulating hMSC adhesion, proliferation, and differentiation. Tae et al. also fabricated heparin-containing affinity hydrogels [20] to deliver VEGF for several weeks with low initial burst release.

While the development of heparin-based matrices for controlled protein release has been substantial, there is considerable room for improvement in regards to material selection and network fabrication. First, the electrostatic binding of growth factors to artificial heparin-containing hydrogels can be weak and unstable in complex biological environments.
In addition, to obtain significantly delayed growth factor release, an extremely excessive amount of heparin is required to maintain effective growth factor binding [8, 9]. Multiple releasable species further complicate growth factor release profiles and theoretical predictions. Finally, physiological complications associated with the use of heparin include serious side effects such as heparin-induced thrombocytopenia (HIT syndrome) [21].

In the current work we describe the development and characterization of an affinity hydrogel system based on the well-established mechanism of metal-ion-chelation and binding of histidine-tagged proteins [22, 23]. This binding mechanism is highly specific and stable under a broad range of physiologically relevant conditions. In addition to their widespread use in protein purification, matrix-immobilized metal-chelating moieties have been used to fabricate stimuli-sensitive hydrogels [24], and functionalize organic and inorganic surfaces [25, 26]. They have also been used to increase protein bioavailability during photoencapsulation [27]. Although several hydrogel systems with metal-ion-chelating capability have been developed and characterized previously [28-32], these systems were primarily used for enhancing metal-ion uptake [28-30, 32, 33], protein absorption [30, 31], or enzyme immobilization [34, 35]. Only a few studies have utilized these hydrogels to release small molecular weight drugs based on the non-specific electrostatic interactions and pH-responsiveness of the hydrogels [36-39].

In this study, methacrylated iminodiacetic acid (GMIDA) was synthesized and copolymerized with PEG-diacrylate (PEGDA) macromer to yield PEG-ω-GMIDA hydrogels. The affinity binding of GMIDA functionalities to a model recombinant protein, hexa-histidine tagged green fluorescent protein (hisGFP), was mediated by divalent metal ions such as nickel and copper. We demonstrate that hisGFP release rates can be readily
controlled by judiciously adjusting GMIDA concentration or type of chelated metal ion.

Finally, a mathematical model accounting for protein diffusion and protein-ligand binding kinetics is developed and shown to accurately predict protein release from these affinity matrices. This is the first such model to successfully predict quantitative protein release from affinity hydrogels.

5.2 Materials and Methods

5.2.1 Materials

Irgacure 2959 (2-Hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone; I-2959) obtained from Ciba Specialty Chemicals was used as a photoinitiator. All other chemicals were obtained from Sigma-Aldrich unless otherwise specified. pGFPuv6His plasmid was a generous gift from Professor Marcus Textor and Eva Kuennemann from ETH Zurich.

5.2.2 Synthesis of poly(ethylene glycol)-diacrylate (PEGDA)

PEG-diacrylate (PEGDA) was synthesized as previously described by Cruise et al. by reacting PEG macromer with an average molecular weight of 3400 Da with acryloyl chloride [40]. Briefly, measured amount of PEG was dried in toluene under N₂ atmosphere then reacted with 8 molar excess of acryloyl chloride for 4 hours at room temperature in dark. The acrylated PEG was filtered through alumina to remove triethylamine-HCl complex. Toluene was then removed from the product mixture under vacuum using a rotovap. To obtain pure PEGDA, the crude product was dissolved in dichloromethane and precipitated in chilled diethyl ether. The purified PEGDA was then filtered and dried under vacuum at room temperature. A degree of acrylation above 95% was determined using ¹H NMR.

5.2.3 Synthesis and characterization of methacyrlated iminodiacetic acid
Methacrylated iminodiacetic acid (GMIDA) was synthesized as shown in Scheme 5.1 following a previously established protocol [32]. Iminodiacetic acid (obtained from Fisher Scientific) was dissolved in double distilled water and neutralized with two molar excess of NaOH to keep acetic acid moieties from reacting with epoxy ring of glycidyl methacrylate. With powerful stirring, an equimolar amount of GMA was added drop-wise to the IDA disodium salt solution and allowed to react for one hour at 65°C. The product was then purified by precipitating in acetone and dissolving in double-distilled H₂O repeatedly. The final product was obtained by drying under vacuum at 65°C. A 90% degree of IDA methacrylation was determined by ¹H NMR (Bruker 300MHz). The ability of GMIDA to bind nickel ions was quantified by scanning the absorbance of GMIDA-nickel complex solution with different molar ratios of GMIDA to nickel using a UV-Vis spectrophotometer (μQuant, Biotek Instruments, Inc., USA).

![Scheme 5.1](image-url)  
**Scheme 5.1** Reaction scheme of glycidyl methacrylate-iminodiacetic acid (GMIDA).

### 5.2.4 Expression and purification of hexa-histidine tagged green fluorescent protein

The hisGFP expression and purification process was modified according to a previous report [26]. The plasmid (1 ng/µL) was transformed into MDS41E *E. coli* by electroporation. The transformed *E. coli* cells were expressed in DYT (double yeast tryptone: 1% Bacto yeast extract, 1.6% Bacto tryptone, 0.5% NaCl) broth containing
100µg/mL ampicillin to an optical density of 0.9 at 600nm at 37°C and then induced with 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside). After induction, the temperature was dropped to 25°C and the cells were allowed to grow for 18 hours. The cells were harvested by centrifugation and re-suspended in 20mM, pH 8.0 Tris-HCl buffer containing 1mg/mL lysozyme and 10µg/mL DNase at 4°C. The supernatant was collected by centrifuge at 4°C (30min, 17000g). The purification of hisGFP was performed with QIAexpressionist® (Qiagen) according to the manufacturer’s protocol. The hisGFP was obtained by dialysis against 10mM, pH8.0 HEPES buffer overnight and freeze-dried. The maximum excitation and emission wavelengths (395nm and 510nm, respectively) of hisGFP were determined by a full-scale scan using a microplate reader (Spectramax GeminiEM, Molecular Devices, CA, USA).

5.2.5 Fabrication of PEG-co-GMIDA hydrogels

PEG-co-GMIDA affinity hydrogels containing proteins were fabricated in situ via photopolymerization. Briefly, a 10wt% PEGDA macromer solution was produced by dissolving the dry macromer in 10mM, pH8.0 HEPES buffer. Required amounts of GMIDA monomer, metal ions, and hisGFP were added to the macromer solution. The mixed protein-monomer solution was then incubated at 4°C for at least 30 minutes to allow binding equilibrium to be obtained between hisGFP and metal ion-GMIDA complexes. After incubation, photoinitiator (I-2959) was added at a final concentration of 0.2wt%. The mixed prepolymer solution was then injected between two glass slides separated by 0.55mm Teflon spacers. The assembled apparatus was then exposed to UV-light (365nm, 8mW/cm²) for 8 minutes to form affinity hydrogels. The resulting gel was cut into 5mm×5mm
rectangular pieces. After swelling, the surface area of the gel is approximately 1 cm². The selected UV exposure was sufficient for gel formation without affecting hisGFP fluorescence.

5.2.6 Characterization of hydrogel swelling

The swelling ratios of the PEG-ε-GMIDA affinity hydrogels were measured gravimetrically. After photopolymerization, gels with different concentrations of GMIDA were placed into an excess amount of PBS buffer solution at pH 7.4 and 37°C. Gels were allowed to swell for 48 hours to reach equilibrium and then dried completely under reduced pressure at room temperature. Gel weights before and after drying were taken and the mass swelling ratios \(Q\) were determined by the following equation:

\[
Swelling Ratio (Q) = \frac{\text{Swollen Weight}}{\text{Dried Weight}}
\]

5.2.7 Mathematical modeling of hisGFP release

A mathematical model was developed to predict hisGFP release and to identify key parameters controlling protein release rates from affinity hydrogels. In its simplest case, the reversible protein-ligand binding can be described as the following equation:

\[
\text{Protein} + \text{Ligand} \leftrightarrow \text{Protein} \cdot \text{Ligand}
\]

where \(k_{on}\) and \(k_{off}\) are protein-ligand association and dissociation rate constants, respectively. The equilibrium dissociation constant \(K_d\) is the ratio of \(k_{off}\) to \(k_{on}\) and is inversely proportional to the affinity between protein and ligand. The initial protein-ligand binding equilibrium can be described by the following equation:

\[
K_d = \frac{k_{off}}{k_{on}} = \frac{[P]_{eq} [L]_{eq}}{[PL]_{eq}}
\]
Here, \([P]_{\text{eq}}\), \([L]_{\text{eq}}\), and \([PL]_{\text{eq}}\) are the equilibrium concentrations of free protein, unbound ligand, and protein-ligand complex (or bound ligand), respectively. Equation (2) dictates the initial equilibrium binding of proteins to the immobilized ligands. It was assumed that the protein-ligand binding reaction achieved equilibrium before protein release. The fraction of bound and free protein can be readily calculated based on the known value of \(K_d\) as well as the total amounts of protein and ligand initially loaded into the matrix. Mass balances for each species described in Equation 1 are given as:

\[
[P]_{\text{total}} = [P]_{\text{released}} + [P]_{\text{free}} + [P]_{\text{bound}} \tag{3}
\]

\[
[L]_{\text{total}} = [L]_{\text{unbound}} + [L]_{\text{bound}} \tag{4}
\]

\[
[PL]_{\text{total}} = [P]_{\text{bound}} = [L]_{\text{bound}} \tag{5}
\]

During the period of protein release, the concentrations of free protein, unbound ligand and bound ligand within the gel constantly change. It was assumed that sink conditions are maintained in the release medium and the release is one-dimensional owing to the use of high aspect-ratio gel slabs in which edge effects are neglected. It is also assumed that all ligand is immobilized in the network and the only diffusible species in the system is free protein due to the non-degradable PEG-based hydrogels used. With these assumptions, the following partial differential equations with proper initial and boundary conditions describe the concentration changes of free protein \([P]\), unbound ligand \([L]\), and bound protein-ligand \([PL]\) during the period of protein release from the gel matrix:

\[
\frac{\partial[P]}{\partial t} = D \frac{\partial^2[P]}{\partial x^2} - k_{\text{on}} [P][L] + k_{\text{off}} [PL] \tag{6}
\]

\[
\frac{\partial[L]}{\partial t} = -k_{\text{on}} [P][L] + k_{\text{off}} [PL] \tag{7}
\]
\[
\frac{\partial [PL]}{\partial t} = k_{on} [P][L] - k_{off} [PL] \tag{8}
\]

Initial and boundary conditions:
\[
[P]=[P]_{eq} \quad @ \quad t=0 \tag{9}
\]
\[
[L]=[L]_{eq} \quad @ \quad t=0 \tag{10}
\]
\[
[PL]=[PL]_{eq} \quad @ \quad t=0 \tag{11}
\]
\[
[P]=0 \quad @ \quad x=\delta \tag{12}
\]
\[
\frac{\partial [P]}{\partial x}=0 \quad @ \quad x=0 \tag{13}
\]
where \(D_0\) is the protein diffusivity within the crosslinked, nondegradable gel. \(\delta\) is the half-thickness of the swollen hydrogel slab, \(x\) is the spatial coordinate in the gel perpendicular to the gel surface (assuming one-dimensional release) and \(t\) is time. Equation 6 describes the change in free protein concentration within the hydrogel due to both diffusion and reversible ligand binding. When inert hydrogels are used (no ligand binding), Equation 6 can be simplified to the well-known Fick’s second law of diffusion.

Equations (6) to (8) can be solved numerically using a finite-difference method (Polymath 5.0 Software, Willimantic, CT) to obtain the time-dependent distributions of \([P]\), \([L]\), and \([PL]\) within the swollen hydrogel. After solving for these concentration distributions, the fractional release of protein at any time can be obtained by the following equation:
\[
{f}_{release} = 1 - 2\times\frac{\int_{x=0}^{x=\delta} [P]dx}{P_0} \tag{14}
\]
Here, $f_{\text{release}}$ is the cumulative fraction of protein released from the hydrogel slab after a given time, $A$ is the surface area of the gel, and $P_0$ is the initial amount of loaded protein. The factor of 2 is due to the symmetry of the gel slab.

5.2.8 In vitro release of hisGFP

For in vitro protein release, PEG-$\alpha$-GMIDA affinity hydrogels (~40 mg/gel) containing 0.25 wt% hisGFP were placed in 10 mM, pH 8.0 HEPES buffer (3 mL) at 37°C with constant stirring. 200 µL samples of the supernatant were taken at specified time points and an equal amount of fresh buffer was added after sampling to maintain constant solution volume. Total hisGFP release was quantified by fluorescence (Ex: 395 nm, Em: 510 nm) using a microplate reader and interpolated to a standard curve. Fractional protein release was obtained by dividing the total amount of hisGFP released up to a given time with the maximum amount released. For release studies with different ligand to protein ratios, different concentrations of GMIDA(Ni) ligand were added accordingly. GMIDA(Cu) complex was used as the higher affinity ligand.

5.3 Results and Discussion

5.3.1 Synthesis and Characterization of GMIDA monomer

To fabricate affinity hydrogels, methacrylated iminodiacetic acid (GMIDA) was first synthesized by reacting glycidyl methacrylate with iminodiacetic acid in basic aqueous solution. The methacrylate bond of GMIDA facilitates its copolymerization into the hydrogel network while the pendant iminodiacetic acid group acts as a metal-ion chelator that binds to hexa-histidine tagged proteins with high and stable affinity [22, 23]. The reaction between the epoxy ring on GMA and the secondary amine on IDA was confirmed
by visual inspection of the disappearance of the oil (GMA) – aqueous (IDA) interface during reaction. The structure of the purified monomer (Scheme 5.1) was then confirmed by $^1$H NMR with the presence of both methacrylate bond and iminodiacetic acid group.

After reaction and purification, GMIDA monomer retains its ability to chelate divalent metal ions such as nickel as shown in Figure 5.1. The wavelength at which maximum absorbance of Ni$^{2+}$ occurs shifts from 720nm (Ni$^{2+}$ only) to 639nm (GMIDA:Ni$^{2+}$ molar ratio of 0.5) to 626nm (GMIDA:Ni$^{2+}$ molar ratio of 1) as more GMIDA is added to the Ni$^{2+}$ solution. The ligand-metal bonding can also be substantiated by the color change of GMIDA-Ni$^{2+}$ complex solutions from light green (Ni$^{2+}$ only) to light blue (GMIDA molar ratio of 1). The chelation of transition metal ions to ionic ligands as well as the color spectra shift has been reported [32]. In this study, the wavelength shift of the Ni$^{2+}$ absorption maxima upon GMIDA addition demonstrates the metal-ion-chelating ability of GMIDA monomer.

5.3.2 Fabrication and characterization of photopolymerized affinity hydrogels

Photopolymerization is one of the most favorable methods for in situ hydrogel fabrication and protein loading [4]. Photopolymerized PEG-based hydrogels for controlled release applications can be formed under mild, physiological conditions which protects fragile biomolecules such as proteins. Furthermore, hydrogels formed via this route usually have readily tailorable physicochemical properties through adjustment of parameters such as macromer concentration or functionality. Several acrylate and methacrylate monomers including acrylic acid (AA) and methacrylic acid (MAA) have been used to expand the functionality of otherwise inert PEG hydrogels. For example, Peppas and coworkers have
extensively investigated PEG-g-MAA hydrogels for oral insulin delivery. The grafted MAA pendant chains enable pH-responsiveness in otherwise inert PEG hydrogels through hydrogen bonding between the PEG and anionic repeat groups [41].

![Figure 5.1](image)

**Figure 5.1** UV-vis spectra of (A) pure nickel solution ($\lambda_{\text{max}} = 721\text{nm}$), (B) GMIDA-Ni mixture solution with a molar ratio of 0.5 ($\lambda_{\text{max}} = 639\text{nm}$), and (C) GMIDA-Ni mixture solution with a molar ratio of 1 ($\lambda_{\text{max}} = 625\text{nm}$).

Here, we copolymerized PEGDA macromers with GMIDA monomers to form metal-ion-chelating hydrogels for controlled protein release. The resulting PEG-\textit{co}-GMIDA affinity hydrogels were first characterized by gel swelling. **Figure 5.2** shows the equilibrium mass swelling ratio of 10wt\% PEG-\textit{co}-GMIDA hydrogels as a function of copolymer composition. The swelling behaviors of similar anionic hydrogels have been well-characterized and reported in the literature [42-46]. GMIDA is a diprotic acid with two carboxylic acids and therefore the swelling of PEG-\textit{co}-GMIDA hydrogels increases as the concentration of GMIDA increases due to electrostatic repulsion between anionic pendent
groups (Figure 5.2). However, it is noteworthy that the effects of GMIDA on increasing equilibrium gel swelling were only observed at high GMIDA concentrations ([GMIDA] >10mM). At relatively low GMIDA concentrations there is no significant difference in gel swelling (Figure 5.2 inset). This can be explained by the following equation showing the total free energy balance of the hydrogel system [45].

\[ \Delta G = \Delta G_{\text{mix}} + \Delta G_{\text{gel}} + \Delta G_{\text{ion}} \]  

(15)

The three terms on the right hand side of the above equation represent the free energy change of polymer mixing, elastic-retractive force of the gel, and ionic force caused by GMIDA, respectively. For non-ionic PEG hydrogels or in the presence of relatively low concentrations of GMIDA, \( \Delta G_{\text{ion}} \) can be ignored and hence it does not contribute to increased gel swelling.

![Figure 5.2](image)

Figure 5.2  Mass swelling ratio of 10wt% PEG hydrogels copolymerized with different concentration of GMIDA monomers with (●) or without (▲) nickel ions.
In addition, while the swelling of PEG-ω-GMIDA hydrogels increases with GMIDA concentration, this trend is not as significant when transient metal ions such as nickel are added to the prepolymer solutions (Figure 5.2). This can be explained by the fact that the chelation of metal ions to GMIDA decreases the electrostatic repulsion between the charged groups.

5.3.3 Model prediction – Effects of binding kinetics on protein release

The design of affinity hydrogel networks and systematic control of protein release from these networks can be laborious without the help of mathematical modeling. To identify the values of various system parameters needed to achieve desired protein release profiles, a reaction-diffusion model is used to describe hisGFP release from PEG-ω-GMIDA affinity hydrogels. In our system, initial protein-ligand binding equilibrium was achieved by incubating the prepolymer-protein mixture solution for a sufficient time (30 min) prior to photopolymerization and subsequent placement into the release medium. At this stage, the percentage of bound protein can be calculated from the simple concentration equilibrium dictated by the dissociation constant as shown in Equation (2).

Figure 5.3 shows that the theoretical fraction of protein bound to matrix-immobilized ligand is determined not only by the affinity to the ligand \( K_j \), but also by the ligand to protein ratio (R). For example, at a fixed total protein concentration \( 1.82 \times 10^{-4} \) M or 0.25 wt\% about 50\% of the protein is bound to the immobilized ligand when R=1 and \( K_j = 10^{-4} \) M while over 95\% of the total protein is bound to ligand when R is increased to 10 at the same affinity. The equilibrium fraction of bound protein is important as it determines the initial protein release rate. It is critical to note that protein-ligand binding equilibrium was only assumed prior to release. During the period of protein release, Equations (6) to (8)
were used to describe the time and position-dependent free protein and unbound ligand concentrations considering both protein diffusion and protein-ligand binding.

![Graph showing the fraction of ligand-bound protein at equilibrium. The percentage of bound protein increases as (1) higher affinity (lower $K_d$) ligands and (2) higher ligand to protein ratios ($R$) are used. Total protein concentration: $1.82 \times 10^{-4}$ M. Solid line: $R=1$; Dashed line: $R=10$; Dotted line: $R=100$.]

**Figure 5.3** Fraction of ligand-bound protein at equilibrium. The percentage of bound protein increases as (1) higher affinity (lower $K_d$) ligands and (2) higher ligand to protein ratios ($R$) are used. Total protein concentration: $1.82 \times 10^{-4}$ M. Solid line: $R=1$; Dashed line: $R=10$; Dotted line: $R=100$.

To accurately predict protein release, it is crucial to understand the mechanisms governing protein transport within the affinity hydrogels. Three parameters are important in describing protein release from these gels, namely protein diffusivity ($D_0$), $K_d$, and $k_{dp}$. For inert hydrogels, protein diffusivity is determined by the protein size and the gel mesh size or gel swelling ratio [47]. As shown in **Figure 5.2**, the mass swelling ratios of our affinity hydrogels are not affected by the incorporation of up to 10mM GMIDA monomer. Since the maximum GMIDA concentration used in all release experiments was 9.4mM (equivalent
to 100 molar excess of hisGFP), it is therefore reasonable to assume that the diffusivity of free protein in the affinity hydrogels used in the current studies is independent of GMIDA concentration. Therefore, a protein diffusivity value of $10^{-7}$ cm$^2$/sec was empirically obtained by fitting an analytical solution of Fick’s second law of diffusion to the hisGFP release profile from comparable PEGDA gels without GMIDA. This value agrees with previous protein diffusivity measurements in the literature and was applied to all subsequent model predictions.

The second system parameter important in determining protein release rates from affinity networks is the dissociation constant for protein-ligand binding, $K_d$. As mentioned earlier, $K_d$ determines the equilibrium free protein concentration. As $K_d$ increases (lower affinity), a greater fraction of total protein will exist in its freely diffusible, unbound form which increases the rate of protein release during the initial release period. During release, protein is constantly removed from the affinity gel through diffusion. If the rates of protein-ligand binding and debinding are sufficiently faster than the rate at which protein is lost from the gel system due to diffusion, then binding equilibrium is maintained during protein delivery and the concentration of free protein within the gel can be readily predicted by the reaction equilibrium equation and the value of $K_d$. However, as free protein diffuses out, the rate at which bound protein dissociates from the immobilized ligand can affect the overall protein release rate. Therefore, the ligand-protein dissociation rate constant, ($k_{off}$), becomes important for determining protein release kinetics. It is important to note that for the binding of his-tagged proteins to metal-ion-chelating ligands, $k_{off}$ is usually much lower than $k_{on}$ [48]. Hence, the rate of ligand-protein dissociation is the rate-determining step.
Figure 5.4  (A) Kinetics of bound protein concentration as a function of dissociation rate constant ($k_{off}$) at a fixed $K_d$ ($10^{-4}$M). $k_{off} = 10^2$, $10^3$, $10^4$, and $10^5$ sec$^{-1}$ for curve 2, 3, 4, and 5, respectively. (B) Fractional release of hisGFP with different $K_d$ (Curve 1: no ligand; Curve 2-5, $K_d=10^{-4}$M; Curve 6, $K_d=10^{-5}$M) and $k_{off}$ ($10^2$, $10^3$, $10^4$, and $10^5$ sec$^{-1}$ for curves 2, 3, 4, and 5, respectively ($K_d=10^{-4}$M).)
To illustrate the impact of the dissociation rate constant \( (k_{\text{off}}) \) on protein release profiles, we simulated protein binding and release as a function of time at constant \( K_d (10^{-4} \text{ M}) \) but different dissociation rates (varying \( k_{\text{off}} \)). It is evident, as showed in Figure 5.4, that the concentration of bound protein ([PL]) decreases with time at all \( k_{\text{off}} \) values used. It is interesting to note that [PL] decreases much slower over time at lower \( k_{\text{off}} \) values. This can be explained by comparing the characteristic time for protein diffusion \( (t_d) \) and dissociation \( (t_{\text{off}}) \) described by the following equations:

\[
    t_d = \frac{\delta^2}{D} \quad (17)
\]

\[
    t_{\text{off}} = \frac{1}{k_{\text{off}}} \quad (18)
\]

where \( \delta \) is the protein diffusion length (the half-thickness of the gel). For example, for a protein diffusivity of \( 10^{-7} \text{ cm}^2/\text{sec} \) and a gel half-thickness of 0.28mm, the characteristic diffusion time \( (t_d) \) is \( 7.8 \times 10^3 \text{ sec} \). When \( t_{\text{off}} \) is at least one order of magnitude smaller than \( t_d \) \((k_{\text{off}} = 10^{-2} \text{ s}^{-1}; \text{ Curve 2 in Figure 5.4A})\), the concentrations of bound protein decrease rapidly at a rate controlled by protein diffusion. However, when \( t_{\text{off}} \) is comparable or larger than \( t_d \) \((k_{\text{off}} = 10^{-4} \text{ and } 10^{-5} \text{ s}^{-1}; \text{ Curves 4 and 5 in Figure 5.4A})\), the time needed for protein dissociation slows the decrease of [PL]. In summary, curves 2 and 3 in Figure 5.4A are almost identical because, although \( k_{\text{off}} \) decreases by an order of magnitude, protein release is diffusion-controlled in both cases. However, the [PL] profile changes significantly with \( k_{\text{off}} \) once protein release becomes kinetically controlled (Curves 4 and 5).
Figure 5.5 In vitro hisGFP release from PEG-$\omega$-GMIDA hydrogels as functions of: (A) Ligand (GMIDA(Ni)) concentration with $R=0$ (◆), 1(▲), 10(●), and 100 (■), and (B) Ligand affinity. No ligand (◆), $R=1$, GMIDA(Ni) (▲) and $R=1$, GMIDA(Cu) (●). Symbols are experimental data and curves are theoretical predictions as indicated in the figure. Protein loading concentration: $1.82\times10^{-3}$M; [PEGDA]: 10wt%.
Figure 5.6 Effect of metal ions on in vitro hisGFP release from PEG-co-GMIDA hydrogels. (A) No metal ions added: $R_{G}=0$ ( ), $R_{G}=1$ (▲), and $R_{G}=10$ ( ). (B) Excess amount of EDTA added in the precursor solution together with nickel ions. $R_{G}=0$ ( ), $R_{GNI}=10$ (▲), and $R_{GNI+EDTA}=10$ ( ).
Figure 5.4B shows the predicted protein release profiles from hydrogels without ligand (Curve 1) and with lower affinity ligand \((K_d = 10^4 \text{M})\); Curves 2-5) or higher affinity ligand \((K_d = 10^5 \text{M})\); Curve 6). Protein release rates are slower when ligand is incorporated. Generally, higher affinity ligand (smaller \(K_d\)) offers stronger protein binding and results in slower release rates. However, at the same \(K_d\) the rate of protein dissociation from ligand can significantly decrease protein release rates during later stages of release. This trend is clear when comparing Curves 2 and 5 in Figure 5.4B where they both have same \(K_d\) but different \(k_{off}\). Furthermore, protein release rates can be tuned with higher initial ‘burst’ but more sustained release afterward by using lower affinity ligands with small \(k_{off}\) values (Curve 5). Although the theoretical observations mentioned above have not yet been verified experimentally, they offer general gel design criteria regarding the selection of protein-binding ligands with proper affinity characteristics (\(K_d\) and \(k_{off}\)). In addition, once these parameters are determined for a given ligand system, release rates for a variety of proteins can be readily predicted.

5.3.4 In vitro release and verification of model predictions

The effectiveness of obtaining sustained protein release via GMIDA incorporation was demonstrated by quantifying his-tagged protein (hisGFP) delivery from PEG-co-GMIDA hydrogel networks. The impact of two parameters on hisGFP release profiles from highly permeable PEG networks was evaluated: (1) ligand-protein molar ratio \(R_G\) for GMIDA; \(R_{G,Ni}\) for GMIDA(Ni); \(R_{G,Cu}\) for GMIDA(Cu)) and (2) ligand-protein affinity \(K_d\). As shown in Figure 5.5A, when GMIDA(Ni) complex was copolymerized into the crosslinked PEG networks, the release rate of hisGFP systematically decreased with increasing ligand concentration. Specifically, without ligand incorporation, 80% of the
protein rapidly diffused out of the hydrogel within an hour. However, when 9.4 mM GMIDA(Ni) (corresponding to $R_{GNi} = 100$) was copolymerized into the network, the time required to release 80% of the protein was delayed to approximately 24 hours, indicating over an order of magnitude decrease in the overall rate of protein delivery. The burst-release behavior commonly observed with traditional hydrogel matrices was also greatly decreased in the presence of the copolymerized GMIDA(Ni) complex. As $R_{GNi}$ increases, the protein release rate and burst effect decrease because GMIDA-hisGFP binding equilibrium dictates that a greater fraction of encapsulated protein is reversibly immobilized to the insoluble PEG-$\omega$-GMIDA network. This conclusion of reaction-diffusion controlled release is supported by the fact that the swelling of the described hydrogel formulations does not decrease upon incorporation of GMIDA (Figure 5.2), indicating that the decreased release rates are not due to decreases in soluble protein diffusivity.

Predicted release profiles obtained using the reaction-diffusion transport model developed in this contribution were compared to the experimental hisGFP release data. Although the exact values of $K_d$, $k_{on}$, and $k_{off}$ for our affinity hydrogel system are yet to be determined, reasonable parameters were obtained from the literature. For example, $K_d$ values of $10^{-3}$ M and $10^{-4}$ M were reported for the binding of single histidine residues to IDA(Ni) and IDA(Cu), respectively [49, 50]. A range of $10^{-5}$ to $10^{-7}$ M has also been reported for the binding of a multi-histidine tag to IDA(Cu) [49]. Therefore, we chose $K_d = 10^{-4}$ M and $k_{off} = 10^{-2}$ sec$^{-1}$ to obtain model predictions of hisGFP release data from hydrogels incorporated with GMIDA(Ni) affinity ligands. A diffusivity value of $10^{-7}$ cm$^2$/sec was also used in the model as discussed in the previous section. As shown in Figure 5.5A, it is clear that the resulting theoretical predictions agree with experimental data at low ligand concentrations.
(R_{GNi}=0 and 1). However, the model overpredicts the amount of released protein at high ligand concentrations (R_{GNI} =10 and 100). A primary reason for this selective discrepancy is that the free-radical mediated hydrogel polymerization efficiency is significantly decreased in the presence of high concentrations of transition metals such as Ni and Cu as these metal ions are known to be efficient free radical scavengers [51].

To overcome the limitations presented by high ligand concentrations, model predictions indicate that low concentrations of high-affinity ligands can be used to achieve similar sustained release effects. For a given ligand to protein ratio, the fraction of hisGFP bound to an immobilized ligand should also increase as the hisGFP-GMIDA binding affinity increases. The binding affinity of PEG-ω-GMIDA hydrogels for encapsulated proteins can be readily tailored through exchange of chelated metal ions. As shown in Figure 5.5B, 80% of encapsulated hisGFP was released in 4 hours from gels prepared with 0.094mM GMIDA(Ni) (R_{GNi} = 1); however, this time-point was extended four-fold to approximately 24 hours when the same concentration of GMIDA(Cu) (R_{GCu} = 1) was used. This can be attributed to the fact that IDA-Cu, compared to IDA-Ni, provides a higher affinity for 6×his-tagged proteins [49, 52].

The developed theoretical model is used to fit the experimental release data when nickel and copper ions are used with the GMIDA. As shown in Figure 5.5B, a $K_d$ of $10^{-5}$ M and $k_{off}=10^{3}$ sec$^{-1}$ were used to generate the theoretical curve for GMIDA(Cu)-hisGFP release. The experimental data and theoretical predictions agree very well and indicate how higher affinity ligands extend the timescale of sustained protein release.

To further support the metal-ion-mediated protein release mechanism, additional hisGFP release experiments were conducted. Figure 5.6A shows hisGFP release profiles
obtained from PEG-ω-GMIDA hydrogels without metal ions. Fractional hisGFP release remains unaltered at different GMIDA to protein ratios ($R_G = 0, 1, 10$) indicating the indispensable role of metal ions in mediating binding of the negatively charged hisGFP. Additionally, when excess EDTA ($R_{{EDTA}} = 10$), a strong metal-ion-chelator, was added to the prepolymer solution together with hisGFP and GMIDA(Ni) ($R_{GNi} = 10$) during gelation, Ni$^{2+}$-mediated hisGFP binding to immobilized GMIDA sites is eliminated via competitive binding. The subsequent fractional hisGFP release profile is identical to the case where no ligand was used (Figure 5.6B). Taken together, these release results suggest that binding of hisGFP to metal-ion-chelating ligands plays a critical role in decreasing protein release rates from these affinity hydrogels.

### 5.3.5 Apparent diffusivity calculation

Protein release rates can be expressed in terms of an apparent diffusivity ($D_{app}$), a coefficient that can be measured experimentally. When the fractional release curves are plotted versus $t^{1/2}$, apparent diffusivities can be calculated from the slope of the linear portion of the curves (fractional release<0.6). Figure 5.7 shows the apparent diffusivities of hisGFP obtained from gels of various compositions. As can be seen, $D_{app}$ of hisGFP in PEGDA hydrogels (without ligand) is around $1 \times 10^{-7}$ cm$^2$/sec and decreases slightly when GMIDA alone is added ($R_G = 1$ and 10). As Ni$^{2+}$ is used synergistically with GMIDA at $R_{GNi} = 1$, $D_{app}$ drops to $0.58 \times 10^{-7}$ cm$^2$/sec. At $R_{GNi} = 10$ or $R_{GCa} = 1$, $D_{app}$ further decreases to $0.22 \times 10^{-7}$ cm$^2$/sec indicating the effects of using high ligand concentration or affinity as discussed before. However, further increasing ligand concentration to $R_{GNi} = 100$ does not substantially decrease $D_{app}$, contrary to theoretical predictions. As briefly discussed earlier, this is most likely an artifact due to limited ligand incorporation within the crosslinked...
networks. Furthermore, when EDTA was added to the prepolymer solution, the apparent diffusivity increases to the level comparable to when no Ni\(^{2+}\) was used. Although Cu\(^{2+}\) has been shown to greatly decrease \(D_{app}\) at low concentration (\(R_{Cu} = 1\)), further decreasing release rates using higher \(R_{Cu}\) was not possible due to the fact that during the preparation of PEG-\(\omega\)-GMIDA hydrogels, high concentrations of copper ions hinder gel formation (Cu\(^{2+}\) is known to be an efficient radical scavenger) [51].

![Calculated apparent diffusivity of hisGFP.](image)

**Figure 5.7** Calculated apparent diffusivity of hisGFP.

### 5.4 Conclusion

PEG-based affinity hydrogels containing immobilized metal-ion-chelating ligands have been successfully fabricated to deliver his-tagged GFP in a sustained manner. A mathematical model accounting for protein diffusion and dissociation from the ligand-bound state has also been developed to identify important parameters governing protein
release. The modeling of protein release reveals the importance of $K_d$ on the initial binding equilibrium and release in the early period while $k_{off}$ dominates the rates and amounts of protein release in the later period. Experimentally, decreased release rates can be obtained by utilizing ligands at higher concentrations or with higher affinities. Taken together, the modeling and experimental studies presented in this contribution broaden the understanding of the mechanisms governing protein release from affinity hydrogels. With this knowledge, these systems can be better utilized for wound healing and tissue regeneration applications.

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5.5 References


CHAPTER SIX
CHARACTERIZATION OF PROTEIN LOADING AND RELEASE FROM PHOTOPOYMERIZED IONIC POLY(ETHYLENE GLYCOL-CO-
GLYCIDYL METHACRYLATE IMINODIACETIC ACID) HYDROGELS

Abstract

Ionic hydrogels have been recognized as important biomaterials that are indispensable in controlled drug delivery applications. We describe herein the characterization and controlled protein delivery application of novel pH-responsive hydrogels prepared from solution photopolymerization. The hydrogels were copolymerized from poly(ethylene glycol) diacrylate (PEGDA) and anionic glycidyl methacrylate-iminodiacetic acid (GMIDA). PEGDA was used due to its demonstrated biocompatibility and non-fouling properties. The incorporation of anionic GMIDA monomers provides the pH-sensitivity of the resulting PEG-co-GMIDA hydrogels. The presence of anionic GMIDA in the hydrogel network enhances the post-loading efficiency of cationic protein lysozyme while decreasing its subsequent release rate due to electrostatic binding. However, the post-loading technique leads to relatively high rates of protein release. To overcome these disadvantages, we encapsulated lysozyme via in situ photopolymerization of PEG-co-GMIDA hydrogels. Results show that in situ-loaded gels deliver lysozyme at a significantly slower rate than post-loaded gels. We also utilized the pH-responsiveness of PEG-co-GMIDA hydrogels for controlled protein delivery - lysozyme is retained within the hydrogels at low pH (pH 5) and released at higher pH (pH 7.4).
6.1 Introduction

Hydrogels are crosslinked polymeric networks that absorb and retain a great amount of water [1, 2]. Because of this, hydrogels are important biomaterials for controlled drug delivery [1, 2], bioanalytical devices [3-5], and tissue engineering applications [6-9]. The “tissue-like” property of hydrogels not only contributes largely to device biocompatibility but also helps to maintain the structural and functional stability of encapsulated proteins. However, high water contents in hydrogels also result in high permeabilities that make it difficult to sustain protein delivery. One easy way to overcome this is to increase diffusional resistance encountered by the encapsulated proteins by increasing the network crosslinking density [10, 11] or utilizing more hydrophobic polymer precursors [12, 13]. Although these approaches are commonly used when designing polymeric carriers for controlled delivery, the decreased water content that results from these methods may destroy the preferential “tissue-like” properties of the original hydrogels. Moreover, the bioactivity and bioavailability of fragile encapsulated protein therapeutics can be significantly limited under these conditions due to increased chances for unfavorable protein-polymer interactions [14].

To maintain the preferential high water-content of hydrogels while permitting dynamic control over protein delivery rates and sustained release, research efforts have focused on the development of hydrogels bearing protein-binding affinity sites [15-22]. So-called “affinity” hydrogels can be designed to decrease protein release rates due to the formation of reversible protein-matrix complexes. By tailoring the concentration or affinity of the protein-binding ligands, protein binding and its release can be systematically controlled. Since many of the therapeutic proteins, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF), are
cationic in biological milieu, it is natural to incorporate anionic components into hydrogel networks to provide protein-matrix binding and subsequent sustained protein release. The most commonly used natural anionic ligand is highly sulfated heparin derived from extracellular matrix [17, 20-22]. Heparin binds to cationic growth factors and synchronizes their release in vivo. To mimic this controlling mechanism, heparin has been incorporated into many hydrogel systems for sustaining growth factor release in vitro and in vivo.

Through intelligent design of hydrogel compositions and architectures, stimuli-responsiveness can also be readily tailored into polymeric hydrogel networks [23-28]. Stimuli-responsive hydrogels have been widely used in controlled delivery owing to their ability to transform hydrogel structure between collapsed and swollen states corresponding to environmental changes including chemical (pH, ionic strength, glucose), physical (temperature, mechanical stress, ultrasound), or electrical cues. The encapsulated proteins are initially retained in the collapsed matrices and the release of loaded proteins is triggered when the matrices become swollen upon changes in the external stimuli.

Synthetic anionic monomers such as acrylic acid (AA) and methacrylic acid (MAA) are attractive in a wide variety of hydrogel-based applications due to their well-defined physical and chemical properties [11, 26, 29-31]. These anionic monomers are deprotonated or ionized when pH of the surrounding solution is higher than the pKa of the acidic groups. The deprotonated groups then contribute to the electrostatic protein-binding as well as the pH-responsive hydrogel swelling. When these anionic moieties are protonated, they are able to form hydrogen bonds with the hydrogel backbone through ether groups. Utilizing these protonation-deprotonation transitions, Peppas and colleagues have prepared a series of complexation PEG-g-MAA hydrogels that shrink in response to acidic environments due to
extensive hydrogen bonding and swell at neutral to basic environments due to electrostatic repulsion between deprotonated MAA groups [29-31]. These complexation hydrogels were used to entrap and protect insulin in acidic environments (stomach) and subsequently release it in the more neutral environments of the gastrointestinal tract (intestines). However, similar to less elegant methods for increasing network crosslinking density, this “on-off” control over protein delivery kinetics still relies on physically entrapping proteins within dehydrated matrices with mesh sizes smaller than the sizes of the encapsulated proteins and may cause protein denaturation due to extensive protein-polymer interactions.

Furthermore, the incorporation of ionic groups into neutral polymeric networks usually requires extended polymerization times or increased monomer concentrations that can be detrimental to the stability of in situ-encapsulated proteins. For example, the photopolymerized PEG-g-MAA hydrogels prepared by Peppas et al. usually take 30-min for gelation [30-33]. This long period of UV-exposure, together with high monomer content (40-60 wt%) [32, 33], will result in significant protein-polymer conjugation if protein is present during the curing process [34]. Therefore, a post-loading technique becomes the only choice for the incorporation of bioactive protein therapeutics into these anionic hydrogels.

Previously, we have described the synthesis, characterization, mathematical modeling, and controlled protein delivery applications of anionic poly(ethylene glycol)-co-(glycidyl methacrylated iminodiacetate) (PEG-ω-GMIDA) hydrogels copolymerized from diacrylated PEG (or PEGDA) and methacrylated IDA (or GMIDA) [15]. The synthetic ligand GMIDA is highly water-soluble and can be readily copolymerized into PEG-based hydrogel networks at high concentrations via photopolymerization. These features permit the rapid fabrication
of highly swollen (water content: > 90%) ionic hydrogels that allow for in situ encapsulation and retention of bioactive proteins.

We have detailed in earlier reports the applications of highly swollen PEG-co-GMIDA hydrogels for achieving sustained release of hexahistidine-tagged proteins over timescales suitable for tissue engineering applications [15]. In this work we demonstrate how these gels can also be used to enhance the loading and pH-responsive delivery of cationic proteins through straightforward electrostatic interactions. Differing from the PEG-g-MAA hydrogels developed by Peppas et al., the PEG-co-GMIDA hydrogels maintain high (> 90%) water contents at all pH values. Control over protein delivery kinetics in PEG-co-GMIDA gels relies predominantly on protein-matrix binding rather than network volume transitions. The objectives of this paper are to characterize the stimuli-responsive swelling of PEG-co-GMIDA hydrogels and to compare the controlled protein delivery performances from these anionic hydrogels using two protein-loading techniques: post-loading and in situ-loading.

6.2 Materials and Methods

6.2.1 Materials

Photoinitiator – Irgacure 2959 (or I-2959, 2-Hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone) was supplied from Ciba Specialty Chemicals. Iminodiacetic acid and fluorescamine were obtained from Fisher Scientific. All other chemicals were obtained from Sigma-Aldrich unless otherwise specified.

6.2.2 Monomer and hydrogel synthesis

PEGDA macromer (average molecular weight: 3.4kDa) was synthesized by reacting toluene-dried PEG macromer with 8 molar excess of acryloyl chloride for 4 hours in the
dark. The desired PEGDA was purified by dissolving crude products in dichloromethane followed by precipitation in cold diethyl ether [35]. GMIDA monomer was synthesized by reacting the amine group of iminodiacetic acid with epoxy ring of glycidyl methacrylate and purified by precipitation in acetone [15, 36]. The structure and purity of PEGDA (>95%) and GMIDA (>90%) were determined by $^1$H NMR (Bruker 300MHz).

To synthesize hydrogels, required amounts of PEGDA macromer (10 – 20 wt%) and GMIDA monomer (0 – 5 wt%) along with photoinitiator I-2959 at a final concentration of 0.2 wt% were mixed to obtain prepolymer solution. 40 mL hydrogel specimens were photopolymerized using low-energy UV-light (Black-Ray®, intensity: 8 mW/cm² at 365 nm) for 10 min from prepolymer solutions injected between two glass slides separated with 0.55 mm-thick Teflon spacers. For post-loading experiments, gels were synthesized without the presence of lysozyme. For in situ-loading experiments, 1 wt% of lysozyme was mixed with the prepolymer solution and subsequently photopolymerized to obtain the in gels with encapsulated protein.

6.2.3 Characterization of hydrogel swelling

PEG-co-GMIDA hydrogels with different GMIDA concentrations were retrieved from the assembled glass slides after photopolymerization and washed in de-ionized water at room temperature for 48 hours to remove un-reacted monomers. The gels were then dried in a vacuum desiccator for 48 hours. The dried gel mass ($W_D$) was measured gravimetrically and then placed in 50mM phosphate buffer solution (PBS) with different pH values or salt (NaCl) concentrations for 48 hours to obtain swollen gel mass ($W_S$). The equilibrium mass swelling ratio ($Q_w$) is defined as:
\[ Q_m = \frac{W_s}{W_D} \]

6.2.4 Protein loading and in vitro delivery

Hen egg white lysozyme (MW: 14.3kDa, pI: 11) was used as a model cationic protein for in vitro release studies. Two protein loading techniques were used in this study, namely in situ-loading and post-loading. For in situ-loading, a known amount of lysozyme was mixed with prepolymer solutions and exposed to low-energy UV light for in-situ photopolymerization. For post-loading, PEG-co-GMIDA hydrogels were synthesized from solution photopolymerization as described in the previous section. The polymerized hydrogels were washed in D.I. water for 48 hours to remove unreacted monomers and then dried in vacuum at room temperature for another 48 hours. The dried gels were weighed and immersed into concentrated lysozyme solution (500 µL, 10 mg/mL or 20 mg/mL) for one week or until fully saturated (longer incubation times did not increase protein loading). Protein loading efficiency was determined via mass balance calculations. After post-loading, the gel swelling ratio \( Q_m \) was determined with the weight of loaded lysozyme subtracted from the total swollen weight of the gel. Lysozyme-loaded PEG-co-GMIDA hydrogels (40 L) were each placed in 3mL of 50mM PBS at 37°C for in vitro release experiments. At predetermined time intervals, 200µL of released lysozyme solutions were sampled and stored at 4°C until quantification of protein concentration. After sampling, an equal amount of fresh PBS was added into the release buffer to maintain constant solution volume. Total release of lysozyme was determined using fluorescamine (3mg/mL acetone) assay as described in earlier publications [14, 15].

6.2.5 Apparent diffusivity calculation
In highly swollen hydrogels, protein release can be categorized as a diffusion-controlled mechanism and the time-dependent protein distribution in the gel can be described by Fick’s second law of diffusion:

\[
\frac{\partial C}{\partial t} = D_{\text{app}} \frac{\partial^2 C}{\partial x^2}
\]

(2)

where \( C \) is the protein concentration in the gel, \( D_{\text{app}} \) is the apparent protein diffusivity in the gel, \( t \) is time, and \( x \) is the diffusion coordinate. In this study, protein apparent diffusivity was assumed to be concentration-independent and the delivery was considered one-dimensional due to high aspect ratio (length/thickness ~ 20) of the hydrogel slabs. A sink condition is also assumed due to the excess volume of the incubating buffer used compared to the size of the gel (~75-fold excess). Equation (2) can be solved analytically and the apparent protein diffusivity can be estimated based on the linear portion of the fractional protein release profiles plotted against the square root of time according to the following equation [37]:

\[
\frac{M_t}{M_\infty} = \frac{4}{\pi^{1/2}} \left( \frac{D_{\text{app}} \cdot t}{\delta^2} \right)^{1/2}
\]

(3)

where \( M_t \) is the amount of protein released at any given time point, \( M_\infty \) is the amount of total releasable protein, \( \delta \) is the half-thickness of the hydrogel slab.

6.3 Results and Discussion

6.3.1 pH-responsive hydrogel swelling

We have previously described the synthesis and characterization of GMIDA monomers as well as the fabrication of PEG-co-GMIDA hydrogels [15]. Due to the copolymerization of anionic ligand – GMIDA, the equilibrium swelling of PEG-co-GMIDA hydrogels increases dramatically at higher GMIDA concentrations due to electrostatic
repulsion between anionic carboxylic acid groups. **Figure 6.1(A)** shows the pH-responsive equilibrium swelling of PEG-co-GMIDA hydrogels (10 wt% PEGDA, 10-min gelation time). Without the presence of GMIDA, the swelling of 10 wt% PEG hydrogels remains relatively constant at all pH values tested. With the copolymerization of GMIDA monomers, however, the degree of pH-responsive swelling depends largely on GMIDA concentration in the prepolymer solution. The swelling of PEG-co-GMIDA gels containing 1 wt% GMIDA (mole fraction of GMIDA: 51.4%) show no statistical difference compared to 10 wt% PEG hydrogels while the swelling of PEG-co-GMIDA hydrogels containing 3 wt% (mole fraction of GMIDA: 76.1%) or 5 wt% GMIDA (mole fraction of GMIDA: 84.1%) increases gradually with increasing pH in the buffer solutions. GMIDA is a diprotic acid containing two carboxylic acids with two pKa values, respectively 3.06 and 8.5. When GMIDA is polymerized into poly(GMIDA), the pKa of these acid groups shift to higher values of 3.81 and 9.54, respectively [36]. The presence of two different pKa values on a single GMIDA monomer can have profound influences not only on copolymer hydrogel swelling but also on the release of encapsulated protein therapeutics. For example, a cationic protein can be complexed with the anionic GMIDA immobilized within the hydrogel networks to minimize its release at neutral pH. The retained cationic protein can then be liberated at a lower pH due to the increased protonation of the anionic GMIDA, or at a higher pH due to decreased protein-ligand binding (cationic protein is less charged at high pH) as well as increased hydrogel swelling.

In order to compare the swelling behavior of PEG-co-GMIDA hydrogels to that of other ionic hydrogels, we also fabricated anionic hydrogels incorporating a commonly used anionic monomer – methacrylic acid (MAA). **Figure 6.1(B)** reveals the pH-dependent
swelling behavior of both PEG-co-MAA and PEG-co-GMIDA hydrogels. As expected, the increase of swelling of PEG-co-MAA hydrogels occurs at slightly higher pH values due to the higher established pKa values for pMAA compared to pGMIDA (5.5 [38] vs 3.81 [36]). The results also demonstrate significantly higher degrees of equilibrium swelling for PEG-co-GMIDA hydrogels at all pH values tested, compared to PEG-co-MAA hydrogels with the same anionic monomer concentration (93.5mM) and gelation time (15-min). It is also interesting to note that the swelling ratios of PEG-co-MAA hydrogels were lower than PEGDA hydrogels (monomer content: 10 wt%) at lower pH (2~6), suggesting significant hydrogen bond formation between protonated carboxylic acid groups on MAA and ether groups on PEG crosslinks, a phenomenon previously described by Peppas and colleagues [29, 30]. Furthermore, PEG-co-MAA hydrogels only swell slightly higher than PEG hydrogels at pH 7.4 and 8.8. However, the potential formation of hydrogen bonds at lower pH values does not appear to significantly restrict the swelling of PEG-co-GMIDA hydrogels. The higher degrees of swelling and hence larger mesh sizes of the PEG-co-GMIDA hydrogels in acidic and basic environments (Q_m>9, see Figure 6.1) allow us to readily evaluate protein delivery behaviors under the effects of protein-matrix binding without artifacts due to physical protein entrapment.
Figure 6.1 (A) pH-responsive swelling of anionic PEG-co-GMIDA hydrogels containing 10 wt% PEGDA and 0 wt% (*), 1 wt% (○), 3 wt% (●), and 5 wt% GMIDA (▲). (n=4, average ± standard deviation, gelation time: 10-min) (B) Comparison of pH-responsive swelling of PEG-co-GMIDA an PEG-co-MAA hydrogels at the same anionic monomer concentration (93.5mM, corresponding to 3 wt% of GMIDA). (n=3, average ± standard deviation, gelation time: 15-min)
6.3.2 Effect of salt concentration on hydrogel swelling

The swelling of anionic hydrogels can be affected by the salt concentration in the bathing medium. Figure 6.2 shows the equilibrium swelling of PEG-co-GMIDA (3wt% GMIDA) and non-ionic PEG hydrogels responding to various salt (NaCl) concentrations at pH 7.4. Clearly, the salt-induced swelling behavior of anionic PEG-co-GMIDA hydrogels is different from that of non-ionic PEG hydrogels. First, the addition of salt at lower concentrations (<200mM) decreases anionic hydrogel swelling but almost has no effect on non-ionic PEG hydrogel swelling. On the contrary, addition of higher salt concentrations (400 - 800mM) decreases the swelling of non-ionic PEG hydrogels but does not further decrease anionic hydrogel swelling. The fundamental difference in the swelling behavior of these ionic and non-ionic hydrogels can be explained by the balance of total free energy ($\Delta G$) in the hydrogel system described by:

$$\Delta G = \Delta G_{mix} + \Delta G_{gel} + \Delta G_{ion} + \Delta G_{osm}$$

where $\Delta G_{mix}$, $\Delta G_{gel}$, $\Delta G_{ion}$, and $\Delta G_{osm}$ are free energy of polymer mixing, elastic-retractive force of the hydrogel, electrostatic repulsion force caused by GMIDA, and osmotic pressure caused by salt ions, respectively. For non-ionic PEG hydrogels at fixed compositions (10 wt% PEGDA), no free energy is contributed by electrostatic repulsion force and hence $\Delta G_{osm}$ is the only variable term that affects the energy balance of the non-ionic hydrogel system. $\Delta G_{osm}$ is almost negligible at lower salt concentrations (>200 mM) and does not change the equilibrium gel swelling significantly. At elevated salt concentrations (400 – 800 mM), however, the increased osmotic pressure in the surrounding buffer cannot be omitted.
and this in turn “squeezes” water out of the non-ionic PEG hydrogels and decreases their equilibrium swelling.

![Figure 6.2](image)

**Figure 6.2** Salt-responsive swelling of non-ionic PEG hydrogels (▲) and anionic PEG-co-GMIDA hydrogels containing 10 wt% PEGDA and 3 wt% GMIDA (▲). (n=3, average ± standard deviation)

For anionic hydrogels containing fixed concentrations (3 wt%) of GMIDA, their equilibrium swelling is determined by the balance of $\Delta G_{\text{ion}}$ and $\Delta G_{\text{osm}}$. The increase in salt concentrations decreases anionic hydrogel swelling even at low concentration (<200 mM) due to the electrostatic shielding effect provided by counterions that partially offset the charge repulsion between anionic GMIDA. At elevated salt concentrations, one expects to see a further decrease in hydrogel swelling similar to that of the non-ionic hydrogels, due to increasing $\Delta G_{\text{osm}}$. However, the swelling of anionic PEG-co-GMIDA hydrogels does not show a monotonic decrease with increasing salt concentration. Instead, PEG-co-GMIDA
gel swelling ratio becomes insensitive to salt concentrations at high salinity. In an earlier study, Ostroha et al. concluded that the degree of anionic gel swelling is not affected by salinity when pH value of the bathing buffer is far from the transition regions or the pKa of the charged groups [39]. As can be seen in Figure 6.1, pH 7.4 is indeed away from the swelling transition regions of the PEG-co-GMIDA hydrogels (the two pKa for poly(GMIDA) are 3.81 and 9.54, respectively [36]). The fact that the increased ΔG_{osm} does not contribute to the decreasing gel swelling at high salinity suggests the important role of electrostatic interactions even when most of the ionic GMIDA groups are “screened” by soluble ions [39].

6.3.3 Post-loaded hydrogels: Protein loading characteristics

It is well-known that the incorporation of anionic groups into hydrogel networks will enhance the loading of cationic proteins via electrostatic binding[19]. The enhanced protein loading capability of our PEG-co-GMIDA hydrogels was examined by incubating dried gels in the concentrated lysozyme solution (500 µL, 10 mg/mL) for one week. As shown in Figure 6.3, very limited amount (26.3 ± 2.5 mg/g polymer) of lysozyme was imbibed into non-ionic PEG hydrogels, presumably due to the protein-excluding properties of PEG hydrogels. The incorporation of 1 wt% anionic GMIDA into the hydrogel network only slightly increases lysozyme loading to 40.8 ± 3.4 mg/g polymer. On the other hand, when GMIDA was copolymerized into the hydrogel network at 3 wt% and 5 wt%, dramatically increased lysozyme loadings were observed (178 ± 14.3 and 553 ± 33.9 mg/g polymer, respectively). Figure 6.3 also shows that the amounts of lysozyme loaded into PEG-co-GMIDA hydrogels increase significantly as the concentration of the bathing lysozyme solution is increased from 10 mg/mL to 20 mg/mL.
6.3.4 Post-loaded hydrogels: Controlled lysozyme delivery

Figure 6.4 shows the fractional release of lysozyme from post-loaded hydrogels using two different loading solutions. Due to the electrostatic binding of anionic GMIDA to cationic lysozyme, one expects decreased lysozyme release rates from hydrogels with increasing anionic contents. As expected, lysozyme release rates decreased as more GMIDA monomer was copolymerized into the PEG hydrogels, regardless of lysozyme loading. Lysozyme loading in PEG-co-GMIDA hydrogels increases with the lysozyme concentration of the bath solution (Figure 6.3). However, the lysozyme fractional release
profiles (Figure 6.4) and apparent diffusivities (Figure 6.5(A)) show no dependence on the loading conditions. Comparing Figures 6.4A and 6.4B, one sees that the sustained release effect is solely controlled by the hydrogel composition and is independent of the protein loading buffer. The sustained release effect, however, appears to saturate at higher GMIDA concentrations as almost identical fractional release profiles were obtained for 3 wt% and 5 wt% GMIDA-incorporated PEG-co-GMIDA hydrogels. One possible explanation for this is that the sustained release effect provided by increased electrostatic binding at higher GMIDA concentrations is offset by increases in hydrogel swelling and network mesh size. These results suggest that the use of hydrogel matrices that induce non-specific electrostatic binding to achieve sustained protein release may be inherently limited.

6.3.5 In situ-loaded hydrogels: Controlled lysozyme delivery

Although post-loading provides a means of achieving high protein loading into anionic PEG-co-GMIDA hydrogels (Figure 6.3), the exact dose of payload is difficult to control and is easily affected by several internal (hydrogel network) and external (bathing buffer) parameters. The internal parameters include hydrogel crosslinking density, specific protein-ligand (e.g. GMIDA) interactions, and non-specific protein-matrix interactions. The external parameters include pH, temperature, ionic strength, and composition of the bathing buffer. Thus, the appropriate combination of numerous conditions must be identified and multiple steps following matrix fabrication must be conducted to prepare matrices in this fashion with therapeutically relevant drug loadings.
Figure 6.4 Fractional lysozyme release from post-loaded PEG-co-GMIDA hydrogels containing 10 wt% PEGDA and 0wt% (*), 1wt% (○), 3wt% (□), and 5wt% (△) GMIDA. (A) Post-loading in 10mg/mL lysozyme solution, and (B) Post-loading in 20mg/mL lysozyme solution. (n=3, average ± standard deviation)
Figure 6.5 Apparent diffusivities of lysozyme as a function of GMIDA concentration. (A) Post-loading: PEG-co-GMIDA hydrogels containing 10 wt% PEGDA were post-loaded in 10mg/mL (■) or 20mg/mL (■) lysozyme solution. (B) In situ-loading: PEG-co-GMIDA hydrogels containing 10 wt% PEGDA (■), or 20 wt% PEGDA (■). Note that the apparent diffusivities for hydrogels containing 20wt% PEGDA and 3wt% or 5wt% GMIDA were calculated from the linear portion of fractional release shown in Figure 6.6 (between 1.5 and 3-hr). (n=3, average ± standard deviation)
On the other hand, in situ-loading - the encapsulation of protein during network formation, provides a convenient and efficient way of loading bioactive agents within crosslinked hydrogels. Not only can the payload doses be readily controlled, but the loaded protein distributions and swelling properties of the anionic PEG-co-GMIDA hydrogels can also be well-predicted, which subsequently determine the release rates of the loaded proteins.

The difference in delivery performance between post-loaded and in-situ loaded gels can be further appreciated by examining the lysozyme release characteristics from in situ-loaded hydrogels. As shown in Figure 6.6A, lysozyme release rates are again systematically decreased at higher GMIDA concentrations. Interestingly, PEG-co-GMIDA hydrogels with 5wt% GMIDA also cannot further decrease lysozyme release rate, similar to the release profiles in post-loaded gels. We have previously hypothesized that this phenomenon is likely due to either increased gel swelling that leads to increased protein diffusivity or decreased polymerization efficiency at higher GMIDA concentrations. The release results in Figure 6.6B reveal that, although the use of higher wt% PEG restricts the swelling of PEG-co-GMIDA hydrogels at higher GMIDA concentrations, the release rate of lysozyme still saturates at a lower limit as GMIDA concentration is increased. These results, in conjunction with the results presented in Figure 6.4, further solidify our conclusion that the use of an electrostatic protein-binding mechanism has its inherent limits. It is also interesting to note that at higher GMIDA concentrations (3 wt% and 5 wt%), the release of lysozyme is very limited in the first two hours with increased release rates observed afterward (Figure 6.6B). This sigmoidal profile is more typically observed during protein release from moderately crosslinked hydrogels.
Figure 6.6 Fractional lysozyme release from in situ-loaded PEG-co-GMIDA hydrogels containing 10 wt% PEGDA and 0 wt% (*), 1 wt% (◊), 3 wt% (○), and 5 wt% (△) GMIDA. Hydrogels composed of (A) 10 wt% PEG, and (B) 20 wt% PEG. (n=3, average ± standard deviation)
6.3.6 Comparison of lysozyme delivery from in situ and post-loaded hydrogels

Figure 6.5(B) reveals the apparent diffusivity of lysozyme released from in situ-loaded PEG-co-GMIDA hydrogels. When comparing Figures 6.5(A) and 6.5(B), it is clear that, at the same GMIDA concentration, the apparent protein diffusivities from in situ-loaded gels are all smaller than those obtained from post-loaded gels. In order to gain more insight into lysozyme release using the two different loading techniques and to determine whether the lower diffusivities from in situ-loaded gels are due to lower amount of lysozyme loading, we compare the release characteristics from two sets of data with similar lysozyme loadings. As shown in Table 6.1, similar lysozyme loading is obtained for both post-loaded and in situ-loaded gels (0.36 ± 0.03 and 0.39 ± 0.01 mg/gel, respectively). However, the apparent diffusivities obtained from the release data using these gels show a 52% decline using in situ-loaded gels.

Although the apparent diffusivities of lysozyme released from in situ-loaded gels were lower than those of post-loaded gels, it is interesting to note that the total percentage of released lysozyme was also lower from in situ-loaded gels compared to post-loaded gels (80.2% vs. 98.3%; Table 6.1). This unfavorable phenomenon can be attributed to the irreversible protein-polymer conjugation that decreases the amount of releasable lysozyme from photopolymerized, in situ-loaded gels [14, 34]. Decreased protein release rate from in situ-loaded gels has been previously observed in cationic hydrogel systems where the measured diffusivities of in situ-loaded insulin were lower than those of post-loaded insulin.[40] The authors attributed this to (1) the irreversible immobilization of insulin to the hydrogel network due to protein-polymer interaction and (2) the tighter gel crosslinking that restricts the diffusivity of in situ-loaded insulin. However, the decreased protein diffusivity
cannot be reasoned by the irreversible conjugation of protein to the hydrogel network as this only decreases the total amount of releasable protein but not the diffusivity of the releasable protein. In earlier reports, we have shown that irreversible protein-matrix conjugation binding determines the total amount of releasable protein[14] and reversible protein-ligand interaction determines the rate of protein delivery [15]. It is also unlikely that the decreased protein diffusivity is due to the entrapment of the in situ-loaded protein in the PEG-co-GMIDA hydrogel network, as from the typical swelling ratio values for the gels used in the current study at all GMIDA concentrations indicate network mesh sizes significantly larger than the size of lysozyme (26 x 45 Å).

**Table 6.1** Comparison of lysozyme release characteristics using two loading techniques.

<table>
<thead>
<tr>
<th>Loading technique[a]</th>
<th>Lysozyme loading (mg/gel)</th>
<th>Apparent diffusivity ($\times 10^7$, cm$^2$/sec)</th>
<th>Total release at 72-hr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Post-loading</td>
<td>0.36 ± 0.03</td>
<td>1.13 ± 0.18</td>
<td>98.3 ± 4.59</td>
</tr>
<tr>
<td>(2) In situ-loading</td>
<td>0.39 ± 0.01</td>
<td>0.59 ± 0.08</td>
<td>80.2 ± 1.56</td>
</tr>
</tbody>
</table>

[a] PEG-co-GMIDA hydrogels with 10 wt% PEG and 1 wt% GMIDA.  
(1) Post-loading in 20 mg/mL lysozyme bathing buffer.  
(2) In situ-loading with 1 wt% lysozyme.

In contrast to the unsupported reasons given by the previous authors, we suggest that the increase in protein diffusivities from post-loaded hydrogels observed in this study is due to the pre-swelling artifact obtained when using the post-loading technique. Post-loaded gels are fully swollen to their equilibrium values during the protein-loading process. On the other hand, in situ-loaded gels are only allowed to partially swell (below their equilibrium
values) after gelation and protein loading. Therefore it is not surprising to observe higher protein diffusivities for the post-loaded networks compared to identical in-situ loaded gels when both are placed in the releasing buffer.

6.3.7 pH-responsive controlled protein delivery

Reversible, electrostatic protein-ligand binding not only permits the control of protein delivery rates, but also allows one to temporally modulate protein delivery. As show in Figure 6.7, when in situ-loaded PEG-co-GMIDA hydrogels were incubated in buffered medium at pH 5, a very limited amount (<20%) of lysozyme was released, most likely from the release of surface-bound protein. The delivery of lysozyme can be triggered by placing the gels into pH 7.4 buffer. The mechanisms governing this pH-responsive release behavior can be explained by the swelling of the hydrogels as well as the state of GMIDA and lysozyme protonation or ionization. At pH 5, the PEG-co-GMIDA hydrogels swell less than at pH 7.4. In the mean time, considerable amounts of GMIDA are still deprotonated at pH 5 and can bind electrostatically to cationic lysozyme (which is charged to a greater extent at pH 5 than pH 7.4). Taken together, a transient retention of lysozyme within the hydrogel network is obtained. On the other hand, when hydrogels were placed into PBS buffer at pH 7.4, the hydrogel swelling increases and hence results in increased lysozyme release. A potential advantage for the PEG-co-GMIDA hydrogels compared to other anionic systems is their dual pH-responsiveness due to GMIDA’s multivalent character and its two pKa values (3.81 and 9.54, respectively [36]) which could potentially be utilized as two distinct, triggerable delivery windows. Future studies are needed to explore this added advantage.
Figure 6.7 pH-responsive lysozyme delivery from in situ-loaded PEG-co-GMIDA hydrogels containing 10 wt% PEGDA and 5 wt% GMIDA. Lysozyme-loaded hydrogels were gelled at pH 7.4 PBS and first placed into pH 5 buffer. After 120 hr, gels were transferred into pH 7.4 buffer. (n=3, average ± standard deviation)

6.4 Conclusions

In conclusion, we have prepared anionic PEG-co-GMIDA hydrogels via photopolymerization and utilized them as controlled protein delivery devices. The pH- and salt-dependent swelling of the anionic hydrogels are important characteristics that determine their protein loading and release properties under a variety of environmental conditions. From the perspective of anionic monomer selection, GMIDA can be copolymerized into hydrogel networks at sufficiently high concentrations. This permits fabrication of hydrogels with increased protein retention over clinically relevant time scales (i.e., less than 10-min). Enhanced post-loading capability for a cationic protein (lysozyme) is achieved by the addition of anionic GMIDA monomer compared to non-ionic PEG hydrogels. While post-
loaded hydrogels produce high protein release rates, significantly lower protein diffusivities can be obtained from in situ-loaded gels. These enhanced features can be attributed to not only the protein-loading technique but also the molecular architecture of GMIDA that allows for multi-valent protein interactions while minimizing dilution of the PEGDA crosslinks. Overall, the control over protein delivery rate from ionic hydrogels requires intelligent network design that delicately balances hydrogel swelling and protein-hydrogel interactions to achieve desired delivery performance.

6.5 References


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CHAPTER SEVEN
BI-FUNCTIONAL MONOLITHIC AFFINITY HYDROGELS FOR DUAL-PROTEIN DELIVERY

Abstract

Multiple-protein delivery has been proven to be a critical consideration for promoting tissue regeneration. Many polymeric composite biomaterials have been designed and used for modulating dual-protein delivery to enhance tissue regeneration in vitro or in vivo. However, the fabrication conditions and low water contents within the portions of these composite matrices that determine protein release rates are not optimal for maintaining the stability of encapsulated macromolecular therapeutics. In this proof-of-concept work, we aim to improve this deficiency by fabricating bi-functional, monolithic affinity hydrogels capable of independently delivering two or more proteins. Selective protein-binding sites were incorporated into poly(ethylene glycol) hydrogels via copolymerization with glycidyl methacrylate-iminodiacetic acid (GMIDA) ligands to modulate release of two model proteins, lysozyme and hexa-histidine tagged green fluorescent protein (hisGFP), via two distinct matrix-binding mechanisms – electrostatic interaction and metal-ion chelation. Results indicate that these unique, injectable affinity hydrogels are capable of uniformly encapsulating multiple therapeutic agents in a single step under mild physiological conditions and independently controlling their localized delivery. Differing from composite matrices for dual-protein delivery, the monolithic affinity hydrogels developed in this report can be fabricated in a single step while retaining high water permeabilities throughout the entire device – characteristics that are necessary for maintaining the stability and viability of encapsulated proteins and cells.
7.1 Introduction

The simultaneous appearance of multiple growth factors is known to occur in vivo during wound healing and other tissue regeneration processes [1-3]. Several polymeric delivery composites capable of releasing two or more proteins with distinct release profiles have been developed to mimic this physiological event [2, 4-6]. However, these heterogeneous, composite matrices require multiple fabrication steps and cumbersome processes to yield non-uniform distributions of loaded proteins. The use of multiple emulsion and polymerization processes in the presence of the loaded proteins as well as contact with the high-energy interfaces within these delivery devices often lead to protein denaturation and loss of bioactivity [7]. These characteristic deficiencies also hinder in situ device formation and injectability. Mechanistically, current composite delivery matrices rely on degradative and diffusional resistances presented in series to modulate molecular transport and hence fail to provide diverse protein release profiles.

We hypothesize that all the aforementioned limitations can be overcome by co-encapsulating and subsequently releasing proteins from a monolithic hydrogel network presenting distinct affinity sites for each of the co-encapsulated proteins. Here, we present the first report on bi-functional, monolithic affinity hydrogels designed for tailorable dual-protein delivery. Two affinity binding mechanisms, namely metal-ion-chelation and electrostatic interaction, are simultaneously employed within a homogeneous and otherwise inert poly(ethylene glycol)-diacrylate (PEGDA) hydrogel network to independently modulate delivery of two proteins without sacrificing the high water content of the hydrogel matrix or the need for composite polymer layers. Due to their structural simplicity, these affinity gels are readily fabricated in aqueous solution in a single processing step which, contrary to
existing composite delivery systems, greatly facilitates in situ encapsulation of bioactive proteins and living cells [8-12].

While spatially and temporally controlled delivery of multiple proteins is known to benefit tissue regeneration, the performance of current polymeric hydrogel delivery devices is far from ideal. Traditional hydrogel fail to produce versatile delivery profiles for coencapsulated multiple proteins due to the inherent constrains of size excluding effect. For example, one can only significantly delay the release rate of a protein with a dimension similar or larger to the mesh size of the inert hydrogel. In other words, these inert hydrogels do not have the capability to (1) distinguish the release rates of two proteins with similar sizes, or (2) delay the release rate of a smaller protein while keeping the release rate of a larger protein constant. Prior attempts at controlling dual-protein delivery have relied upon manipulating protein loading sites [2] (in microspheres or bulk polymer) or implementing selective matrix degradation to accelerate the release of one protein while keeping the release rate of the second protein unchanged [4]. One recently developed composite delivery system is based on degradable gelatin microspheres contained within oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels [4]. In this portioned three-phase system, insulin-like growth factor-1 (IGF-1) was loaded in densely crosslinked gelatin microspheres while transforming growth factor-β1 (TGF-β1) was loaded either in loosely crosslinked gelatin microspheres or in the bulk OPF gel. The release of TGF-β1 was accelerated by the addition of gelatin-degrading collagenase. Although this approach provides a means of tuning TGF-β1 release by changing its loading site, it requires the incorporation of two types of protein-loaded microspheres within a third crosslinked polymer phase. There are also concerns regarding the stability of proteins transported across microsphere-bulk polymer interfaces. From the
view point of molecular transport, unpredictable discontinuities in protein release profiles may also arise due to the presence of these interfaces [13-15].

Alternatively, polymers bearing a single type of affinity site for an encapsulated drug have been extensively studied. These affinity matrices have the capability of retarding the release of a particular target drug due to reversible drug-matrix binding that lowers the apparent diffusivity of the therapeutic molecule. Several binding mechanisms, including electrostatic [16-18], van der Waals, and hydrophobic interactions [19, 20] have been proposed and exploited to facilitate sustained drug release from affinity matrices. For example, synthetic monomers with acidic or basic functionalities have been tailored into a wide variety of polymeric drug carriers to sustain the release of oppositely charged molecules [16-18]. Natural macromolecules such as heparin and cyclodextrins (CD) have also been used extensively as drug-binders. Heparin is a highly sulfated glycosaminoglycan (GAG) that has the demonstrated capability to reduce cationic growth factor release rates when incorporated, either by physical adsorption or covalent conjugation, into polymer networks [8, 11, 21-24]. Cyclodextrins are another widely used drug-binder that can accommodate “guest” molecules by forming drug-CD complexes [25-28]. Although matrices incorporating these two types of affinity sites have achieved some success in sustaining protein release, cumbersome chemical modifications are generally required to utilize these macromolecules in hydrophilic polymer matrices. The mechanical and biological properties of the delivery matrices made from these affinity agents are also not ideal. For example, heparin is a polydisperse macromolecule with high molecular weight (ranging from 5,000 to 40,000 Daltons) that greatly limits its incorporation into polymer networks at sufficiently high concentrations. Administration and handling of heparin-based gels are difficult due to their
low mechanical strength. In addition, improper release of heparin may also cause serious physiological complications [29]. The difficulties presented by cyclodextrins, on the other hand, include their solubility, toxicity, and decreased drug-CD complex formation during network fabrication [25]. When incorporated into polymeric hydrogels, these properties make it difficult to systematically control protein release rates, to generate reproducible release profiles, and to provide distinct release profiles for multiple, co-encapsulated proteins.

Current research efforts are focused on developing synthetic affinity hydrogels with alternative ligand chemistries for enhancing protein delivery performance including enhanced bioavailability [30] and sustained delivery [31]. In an earlier report, we detailed the synthesis, characterization, as well as mathematical modeling and experimental verification of controlled protein release using a highly biocompatible and permeable PEG-co-GMIDA (glycidyl methacrylate-iminodiacetic acid) hydrogel network [31]. The metal-chelating GMIDA ligands were shown to reversibly bind to a genetically engineered hexahistidine-tagged green fluorescent protein (hisGFP). The sustained release of his-tagged proteins from highly swollen PEG-co-GMIDA hydrogels (water contents ~90%) reveals the usefulness of this unique and specific binding mechanism for controlling protein delivery from biocompatible hydrophilic networks. In this proof-of-concept work, we develop a platform alternative to the existing composite matrices for dual-protein delivery. The goals of this work are to obtain independently tunable, dual-protein delivery from structurally well-defined, highly permeable affinity hydrogels without sacrificing the preferential hydrophilicity and permeability of the monolithic hydrogel matrices.
7.2 Materials and Methods

7.2.1 Materials

All chemicals were obtained from Sigma-Aldrich unless otherwise specified.

7.2.2 Synthesis of poly(ethylene glycol) diacrylate (PEGDA) macromer

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized as previously described [32]. Briefly, toluene-dried PEG macromer with an average molecular weight of 3400 Da was reacted with 8 molar excess of acryloyl chloride for 4 hours at room temperature in dark. The triethylamine-HCl complex was removed from acrylated PEG by filtering the product through alumina. Toluene was then removed from the product mixture under vacuum. The crude product was dissolved in dichloromethane and precipitated in chilled diethyl ether to obtain pure PEGDA. The purified PEGDA was filtered and dried under vacuum at room temperature. $^1$H NMR (Bruker 300MHz) was used to determine the degree of acrylation (>95%).

7.2.3 Synthesis of methacrylated iminodiacetic acid (GMIDA) monomer

GMIDA monomer was synthesized by reacting glycidyl methacrylate (GMA) with iminodiacetic acid (IDA) as shown in Scheme 7.1. IDA was dissolved in double distilled water and neutralized with two molar excess of NaOH to keep carboxylic acids from reacting with epoxy ring of GMA. Under constant stirring, an equimolar amount of GMA was added drop-wise to the IDA solution and allowed to react for one hour at 65°C. The product was then purified by precipitating in acetone and dissolving in double-distilled H$_2$O repeatedly. The final product was obtained by drying in vacuum oven below 60°C. A degree of IDA methacrylation of 90% was determined by $^1$H NMR.
The synthesis of methacrylated iminodiacetic acid.

**Scheme 7.1** The synthesis of methacrylated iminodiacetic acid.

### 7.2.4 Expression and purification of hexahistidine-tagged green fluorescent protein (hisGFP)

6xhisGFPuv plasmid was a generous gift from Prof. M. Textor at ETH, Zurich. The expression and purification process was modified according to a previous report [33]. The 6xhisGFPuv plasmid (1ng/µL) was transformed into MDS41E *E. coli* by electroporation. The transformed *E. coli* cells were expressed in DYT (Double Yeast Tryptone: 1% Bacto yeast extract, 1.6% Bacto tryptone, 0.5% NaCl) broth containing 100µg/mL ampicillin to an optical density of 0.9 at 600nm at 37°C and then induced with IPTG (final concentration 1mM). After induction, the temperature was dropped to 25°C and the cells were allowed to grow for 18 hours. The cells were harvested by centrifugation and re-suspended in 20mM, pH8.0 Tris-HCl buffer containing 1mg/mL lysozyme and 10µg/mL DNase at 4°C. The supernatant was collected by ultracentrifuge at 4°C (30min, 180000g). The purification of hisGFP was performed with QIAexpressionist® (Qiagen) according to the manufacturer’s protocol. The hisGFP was obtained by dialysis against 10mM, pH8.0 HEPES buffer overnight and freeze-dried. The maximum excitation and emission wavelengths (395nm and 510nm, respectively) of hisGFP were determined by a full-scale scan using a microplate reader (Spectramax GeminiEM, Molecular Devices, CA, USA). The binding of his-tagged
protein to GMIDA-nickel complex was confirmed by decreased nickel absorbance (wavelength: 721nm) upon protein-ligand binding using a UV-vis spectrophotometer [31].

7.2.5 PEG-co-GMIDA hydrogel fabrications and characterizations

PEG-co-GMIDA affinity hydrogels containing proteins were fabricated in situ via photopolymerization. Briefly, a 10 wt% PEGDA macromer solution was produced by dissolving the dry macromer in 10 mM, pH 8.0 HEPES buffer. Required amounts of GMIDA monomer, metal ions, hisGFP (5 µg/mL), and lysozyme (10mg/mL) were added to the macromer solution. The mixed protein-monomer solution was then incubated at 4°C for at least 30 minutes to allow binding equilibrium to be obtained between hisGFP and metal ion-GMIDA complexes. After incubation, photoinitiator Irgacure-2959 (I-2959, Ciba Specialty Chemicals) was added to a final concentration of 0.2 wt%. The mixed prepolymer solution was then injected between two glass slides separated by 0.55mm Teflon spacers. The assembled apparatus was then exposed to UV-light (BLACK-RAY®, 8 mW/cm² at 365 nm) for 8 minutes to form affinity hydrogels. The time for photopolymerization was selected so that the fluorescence of his-GFP was not affected. For equilibrium swelling ratio measurements, the swollen weights of PEG-co-GMIDA hydrogels were measured gravimetrically to obtain swollen gel weights. After which the gels were dried in vacuum for 48 hours to obtain dried weights. Equilibrium swollen ratio ($Q$) was then determined by:

$$ Q = \frac{\text{Equilibrium swollen weight}}{\text{Dried gel weight}} $$
7.2.6 In vitro dual-protein delivery and quantification of released protein concentrations

In vitro dual-protein release was performed in 3 mL HEPES buffer (10mM, pH 8.0). At predetermined time intervals, release media were replenished with fresh buffer. hisGFP and lysozyme concentrations were quantified immediately after sampling. GFP intrinsic fluorescence was used to quantify its total amount while a fluorescamine (3 mg/mL acetone) assay was employed to determine lysozyme release. The fluorescamine signal contributed from hisGFP was subtracted from the total fluorescamine signal of the released protein mixture, based on a fluorescamine signal – hisGFP fluorescence calibration curve, to determine the amount of lysozyme release. However, due to the excessive amount of lysozyme loaded in the gel, released hisGFP contributed to less than 5% of the total fluorescamine signal. After completing the release studies, the swollen and dried gel weights were measured gravimetrically and used to determine equilibrium mass swelling ratio ($Q_m$) and water content. Equilibrium mass swelling ratio was used for the calculation of volumetric swelling ratio ($Q$) and gel mesh size ($\xi$) according to established equations [34, 35]. Total protein release was converted to fractional release ($f=M_t/M_\infty$) and plotted against the square root of time for apparent diffusivity calculations (see supporting materials). The slopes of the linear portion of these modified release curves were used to calculate apparent protein diffusivities [36]. After releasing 240 hours, dual-protein loaded affinity hydrogels were transferred from 10mM HEPES buffer into 100mM EDTA containing HEPES buffer to trigger the release of remaining hisGFP. Total protein release was quantified prior to and 24 hours after EDTA addition to determine the stimuli-responsiveness of affinity hydrogels.
7.3 Results and Discussion

The metal-chelation binding mechanism based on the use of IDA and NTA (nitrilotriacetic acid) ligand chemistries has been extensively used for protein purification [37] as well as for hydrogel fabrication [38] and functional surface-patterning [33, 39]. The common feature for these studies is the utilization of IDA or NTA functionalities to chelate metal ions for his-tagged-protein immobilization. In the current work we take advantage of not only the metal-ion chelating ability of IDA but also its anionic nature to fabricate bifunctional monolithic hydrogels capable of simultaneously yet independently tuning delivery of two representative proteins, hisGFP and lysozyme (Table 7.1). Our strategy for controlling dual-protein release is based on selective protein-ligand binding which significantly decouples protein release rates from matrix crosslinking density and degradation rate.

Table 7.1 Characteristics of proteins used for dual-delivery.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (kDa)</th>
<th>Isoelectric point (pI)</th>
<th>Stokes radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysozyme</td>
<td>14.1</td>
<td>11</td>
<td>16.0</td>
</tr>
<tr>
<td>hisGFP</td>
<td>27.4</td>
<td>5.5</td>
<td>28.2</td>
</tr>
</tbody>
</table>

Figure 7.1 illustrates the fabrication of dual ligand-protein loaded monolithic affinity hydrogels. PEG hydrogels are used due to their demonstrated biocompatibility [40], nonspecific protein-excluding property [41-43], and well-documented structure-property relationships [44, 45]. While degradable moieties can be readily incorporated into these devices, the use of non-degradable PEG hydrogels eliminates protein release resulting from
network degradation and facilitates the observation of sustained release effects due to affinity-binding [30, 31]. The use of diacrylated PEG macromers permits rapid network formation via photopolymerization as well as copolymerization with other (meth)acrylated monomers such as GMIDA. The GMIDA ligand monomer is a highly water-soluble monomer that complexes transition metal ions with high affinity \((K_d \sim 10^{-12}\text{M})\). GMIDA(Ni\(^{2+}\)) complexes copolymerized into the hydrogel network chelate his-tagged proteins such as hisGFP with moderate affinity \((K_d = 10^{-4} \sim 10^{-6}\text{M})\), providing sufficient ligand-protein binding while allowing diffusion-driven sustained protein release [31]. In addition, without metal ions present, anionic GMIDA monomers contribute to the delayed release of cationic proteins such as lysozyme via electrostatic binding of the diffusible protein to the insoluble matrix.

**Figure 7.1** Schematic representation of the proposed monolithic affinity hydrogel system for tailororable dual-protein delivery. The affinity hydrogels are prepared from one-step photopolymerization of PEGDA and methacrylated affinity ligands –GMIDA(Ni\(^{2+}\)) and GMIDA that reversibly complex with hisGFP and lysozyme, respectively.
Before demonstrating the ability of our affinity hydrogels on independently controlling dual-protein delivery, we first fabricated inert PEG hydrogels with different polymer contents (10 wt% and 20 wt%) in the prepolymer solution and used these inert hydrogels for simultaneously delivering the co-encapsulated proteins – lysozyme and hisGFP. Clearly shown in Figure 7.2, it is difficult to independently control dual-protein delivery by changing gel crosslinking density as the delivery rates of both proteins simultaneously decrease with increasing polymer content (Figure 7.2C). Furthermore, since hisGFP is a larger protein compared to lysozyme (Table 7.1), the apparent diffusivity of hisGFP is affected to a greater extent. An 11-fold decrease in hisGFP apparent diffusivity (from 0.63 to $0.06 \times 10^8$ cm$^2$/sec) is observed when polymer content of the PEG hydrogel is increased from 10 to 20 wt%. On the other hand, an approximately 3-fold decrease in lysozyme apparent diffusivity (from $8.43 \times 10^8$ cm$^2$/sec) is observed with the same increase polymer content.

Figure 7.2 (Continue on next page)
Figure 7.2 Dual-protein release from inert PEG hydrogels with different PEG content (10 vs. 20 wt%) in the prepolymer solution. (A) hisGFP release and (B) lysozyme release. (C) Apparent diffusivity of hisGFP and lysozyme is calculated from the initial portion of the fractional protein release curves according to established method [48]. (Average ± Standard deviation, n=3, * p<0.05).
Figure 7.2 reveals that the use of inert hydrogels provides very limited control over the relative delivery rates of multiple proteins. To promote tissue regeneration that requires sequential delivery of multiple growth factors, one may wish to deliver a larger protein (e.g. VEGF: M.W. ~45 kDa) faster than a co-encapsulated smaller protein (e.g. PDGF-BB: ~25 kDa) from a single delivery device. Apparently, it is very difficult to achieve this goal by using inert hydrogel matrices. By tailoring GMIDA and metal ion concentrations, however, dual delivery of cationic and his-tagged proteins with independently controllable release rates can be readily achieved from bifunctional, monolithic PEG-ω-GMIDA hydrogels. Figure 7.3 demonstrates that his-tagged protein (hisGFP) release from PEG-ω-GMIDA hydrogels can be independently and systematically controlled without altering the co-encapsulated cationic protein (lysozyme) release profile. By gradually increasing Ni^{2+} concentration while keeping the GMIDA concentration constant during in situ photopolymerization, GMIDA-metal ion complexes are formed which bind strongly and specifically to his-tagged proteins within the highly hydrophilic PEG-based hydrogel environment. This behavior is similar to the binding mechanism used in the purification of his-tagged proteins. Since the affinity binding between his-tagged proteins and GMIDA ligands is mediated by metal ions such as nickel, one expectedly sees a sharp decrease in hisGFP release (from 75 ± 5.0% for GMIDA10 gels to 20 ± 5.2% for GMIDA10Ni10 gels at 24-hr) when nickel ions are added in a stoichiometric ratio to GMIDA (Figure 7.3A). However, the addition of nickel ions does not significantly affect lysozyme release (Figure 7.3B). The non-responsiveness of lysozyme to the presence of nickel ions is also observed when lysozyme is solely released from PEG-ω-GMIDA hydrogels containing 10mM GMIDA (data not shown).
Figure 7.3 (Continue on next page)
Figure 7.3 Dual-protein release from monolithic PEG-\text{-co-}GMIDA hydrogels containing 10mM GMIDA only (GMIDA10), 10mM GMIDA and 5mM Ni (GMIDA10Ni5), or 10mM GMIDA(Ni) (GMIDA10Ni10) ligands demonstrating (A) tailorable hisGFP release and (B) constant lysozyme release. (C) Apparent protein diffusivity. (Average $\pm$ Standard deviation, n=3, * $p<0.02$).

When comparing apparent protein diffusivities obtained from PEG-\text{-co-}GMIDA gels with and without nickel ions present, one can see that the diffusivities of hisGFP decrease dramatically from $0.89 \pm 0.29 \times 10^{-8}$ to $0.15 \pm 0.02 \times 10^{-8}$ cm$^2$/sec ($p<0.02$) when nickel ions are added in a stoichiometric ratio to GMIDA concentration (Figure 7.3C). In contrast, the diffusivity of the co-encapsulated lysozyme shows no significant change when nickel ions are added. It is also important to note that the mass swelling ratios of the PEG-\text{-co-}GMIDA (10mM GMIDA) gels with and without nickel ions are 10.0$\pm$0.14 and 9.41$\pm$0.37, respectively (Table 7.2), implying consistently high gel water contents (90.0$\pm$0.14\% and 89.4$\pm$0.42\%). Taken together, these data suggest that the release of his-tagged proteins from a mixture of co-encapsulated proteins can be independently regulated by simply adjusting the
concentration or chemistry of copolymerized GMIDA-metal ion complexes without sacrificing the preferential high water content of these in situ deliverable hydrogels.

### Table 7.2 Characteristics of affinity PEG-co-GMIDA hydrogels used in dual-protein delivery.

<table>
<thead>
<tr>
<th>Hydrogel formulation</th>
<th>Ligand (mM)</th>
<th>Mass swelling ratio</th>
<th>Water content (%)</th>
<th>Mesh size (Å)[a]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GMIDA</td>
<td>Ni^{2+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMIDA0</td>
<td>0</td>
<td>0</td>
<td>9.84±0.13</td>
<td>89.8±0.13</td>
</tr>
<tr>
<td>GMIDA10</td>
<td>10</td>
<td>0</td>
<td>9.41±0.37</td>
<td>89.4±0.42</td>
</tr>
<tr>
<td>GMIDA10-Ni10</td>
<td>10</td>
<td>10</td>
<td>10.0±0.14</td>
<td>90.0±0.14</td>
</tr>
<tr>
<td>GMIDA15-Ni5</td>
<td>15</td>
<td>5</td>
<td>10.4±0.51</td>
<td>90.4±0.47</td>
</tr>
</tbody>
</table>

[a] Mesh size (ξ) is calculated from measured equilibrium gel swelling ratio. Equations for mesh size calculation can be found elsewhere [34].

We also performed additional release studies to demonstrate the ability of PEG-co-GMIDA affinity hydrogels to independently control the release of a cationic protein – lysozyme. As shown in **Figure 7.4A**, the release profile of hisGFP from PEG-co-GMIDA15Ni5 gels (15mM GMIDA and 5mM Ni^{2+}) is almost identical to that obtained with homopolymer PEGDA hydrogels. In hydrogels containing high GMIDA concentrations (15mM), one would expect to see faster hisGFP release due to electrostatic GMIDA-hisGFP repulsion (both components are anionic). This trend, however, is offset by adding a small amount of nickel ions (5mM). Comparing **Figures 7.4A** and 7.4B, it is clear that the lysozyme release is independently decreased due to increased electrostatic GMIDA-lysozyme binding without affecting the release of hisGFP. Further comparison of apparent protein diffusivities within the inert PEG hydrogels and PEG-co-GMIDA15Ni5 hydrogels (**Figure 7.4C** and supporting materials), a selective and dramatic decrease in lysozyme diffusivity
(from $8.43 \pm 1.80 \times 10^{-8}$ to $0.61 \pm 0.07 \times 10^{-8}$ cm$^2$/sec, $p<0.002$) demonstrates the ability of these bifunctional affinity hydrogels to independently control the release of co-encapsulated cationic proteins. When examining the release profiles shown in Figures 7.2 to 7.4, one can clearly see that a significant portion of lysozyme (at least 30%) was retained in the hydrogels. When the polymer content in the prepolymer solution was increased from 10 wt% to 20 wt% (Figure 7.2B), or when the anionic GMIDA content was increased to 15mM (Figure 3B), an even greater portion of lysozyme becomes unreleasable. This incomplete release phenomenon is most likely due to the following two reasons: (1) The formation of irreversible protein-polymer conjugates during photopolymerization that prevents their complete release [30]. This incomplete release phenomenon is routinely observed in polymeric protein delivery devices due to unfavorable protein-polymer interactions. (2) The irreversible electrostatic binding between cationic protein and anionic ligand. Although the electrostatic binding can be used to significantly decrease protein release rates (Figure 7.3C), it will gradually produce higher degrees of binding as the local protein concentration within the anionic gels decreases during the release process.
Figure 7.4 (Continue on next page)
Figure 7.4 Dual-protein release from monolithic PEG-co-GMIDA hydrogels containing no ligand (GMIDA0) or 15 mM GMIDA and 5 mM Ni\(^{2+}\) (GMIDA15Ni5) ligands demonstrating (A) constant hisGFP release and (B) tailorable lysozyme release. (C) Apparent protein diffusivity. (Average±Standard deviation, n=3, * \(p<0.002\))

The facts that the equilibrium water contents of all the described affinity hydrogels remain at a high level (Table 7.2) and no correlation was found between gel swelling and diffusivities of either protein suggest that the observed sustained protein delivery effects are due to the degree of selective protein-GMIDA binding. Furthermore, from the characteristic parameters of proteins and hydrogels listed in Table 7.1 and Table 7.2, one can clearly see that the network mesh sizes calculated for the hydrogels (~60Å [34]) are much larger than the sizes of either encapsulated protein (16.0Å for lysozyme, 28.2Å for hisGFP). Due to its smaller molecular size, lysozyme diffuses rapidly when it is co-encapsulated and delivered with hisGFP from inert PEGDA hydrogels (Figure 7.4, GMIDA0 gels). For practical applications, however, one may wish to deliver larger proteins at comparable or faster rates than smaller proteins. This type of dual-delivery profile is impossible to accomplish from traditional gel matrices where release rates are controlled by
size-exclusion effects. As described previously, the only existing method for achieving selective release of the larger protein is to use complex composite delivery vehicles where smaller proteins are encapsulated within microspheres or slow-degrading polymers incorporated within a second polymer matrix. As demonstrated by the data in Figures 7.3 and 7.4, the bifunctional affinity gels described in this work overcome the limitations presented by composite systems and are capable of successfully delivering proteins at controlled rates irrespective of their relative sizes.

An additional advantage of the PEG-ω-GMIDA affinity hydrogels as dual-delivery devices is their protein-selective stimuli-responsive behavior. This is achieved by selectively triggering the release of hisGFP (Figure 7.5A) but not lysozyme (Figure 7.5B) through the addition of EDTA (ethylenediamine tetraacetic acid), a strong metal-ion chelator, to the release medium. As can be seen in Figure 7.3A, hisGFP release reaches a plateau after 120 hours. Prolonged incubation of the hydrogels in the release medium does not yield significant increase in total hisGFP release, presumably due to exceptionally strong binding between the GMIDA(Ni^{2+}) ligands and a fraction of the encapsulated hisGFP molecules. As shown in Figure 7.5A, the complete release of this tightly-bound hisGFP fraction can be triggered via the addition of EDTA to the release medium. Complete release of hisGFP is observed within 24 hours of EDTA addition. In contrast, the release of lysozyme is not affected by the addition of EDTA (Figure 7.5B). Although not intent to work as an actual triggerable release mechanism in vivo, this unique feature demonstrates that the retained proteins are recoverable from the hydrogel matrices. The reversible GMIDA(Ni)-hisGFP binding can be destroyed by applying proper stimuli to fully recover the tightly bound protein. Most importantly, the retention of these tightly bound proteins is due to the
reversible protein-ligand binding, but not due to the additional diffusional resistance imposed by increasing polymer crosslinking density. Therefore, more sustained release profiles can be designed by incorporating degradable hydrogel chemistry into these affinity hydrogels without sacrificing the preferencial hydrophilicity of the delivery device.

The current work demonstrates that by preparing the protein-loaded hydrogels with well-defined hydrogel chemistry and ligand quantities, one can easily and independently control the delivery of either one protein from the mixture of multiple protein solutions. To date, there are very limited, if any, reports on the use of affinity hydrogels to deliver multiple-protein independently. The affinity-based dual-protein delivery strategy reported herein opens new avenue toward the design of multi-functional, biocompatible hydrogels capable of delivering multiple therapeutically relevant proteins to promote tissue regeneration and wound healing. Future studies will focus on the design and synthesis of hydrogels copolymerized with other biological ligands, such as oligo-peptides, that yield affinity to other therapeutically relevant growth factors.
Figure 7.5 Selective stimuli-responsive hisGFP release from monolithic affinity hydrogels containing 10mM GMIDA (GMIDA10) or GMIDA(Ni) (GMIDA10Ni10) ligands. After releasing 10 days, gels with residual proteins were transferred into release medium (10mM HEPES, pH8.0) containing 100mM EDTA for 24 hours. Protein release was quantified prior to and 24-hr after EDTA addition. (Average ± Standard deviation, n=3, * p<0.0004)
7.4 Conclusion

In conclusion, we have developed monolithic affinity hydrogels bearing well-defined protein-binding sites that can simultaneously and selectively bind to native cationic proteins and recombinant his-tagged proteins under physiological conditions. Since many therapeutic growth factors (e.g. basic fibroblast growth factor (bFGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), etc) are cationic at physiological conditions and numerous proteins can be recombinantly engineered with hexahistidine tags without altering the folding or bioactivity of the native protein, the versatility and biocompatibility of this delivery scheme is advantageous for many tissue engineering applications that require complex, simultaneous delivery of multiple proteins [46, 47]. To the best of our knowledge, this is the first published hydrogel delivery system with protein-binding affinity that can significantly sustain protein release, independently control delivery of multiple proteins, and exhibit selective stimuli-responsive behavior. The monolithic affinity hydrogels developed herein discard the need for complex composite matrix fabrication with non-uniform protein distributions, crosslinking densities or degradation rates. The one-step, biocompatible hydrogel fabrication method described in this work can also be easily modified, based on well-established chemistries, to accommodate matrix degradation or specific protein-binding mechanisms to achieve more sophisticated protein delivery profiles and therapeutic needs.

7.5 References


8.1 Conclusions

The rational design and facile fabrication of biocompatible yet versatile protein delivery matrices require the cooperative knowledge of biocompatibility, protein/peptide chemistry, polymer science and engineering, and molecular transport theories. To this end, the major objectives of this dissertation were to understand protein-polymer interactions during in situ photopolymerization and to design/fabricate affinity hydrogels that allow for effectively tuning delivery of bioactive proteins without sacrificing the preferential hydrophilicity of the hydrogel carriers. In this research project, the investigation and optimization of reaction conditions were performed for in situ photopolymerization in an attempt to design a better hydrogel-based protein delivery system that can prevent adverse protein-polymer conjugation and hence increase the bioavailability of the loaded protein therapeutics. Furthermore, systematically controlled single or multiple protein delivery from highly permeable hydrogel matrices was made possible by incorporating robust protein-binding ligands into the otherwise inert PEG hydrogel networks. Collectively, these efforts will lead to the successful development of biocompatible hydrogel matrices that can simultaneously deliver multiple bioactive protein therapeutics at optimal doses and delivery rates.

A better understanding of protein stability and their potential conjugation to the growing polymer chains during in situ photopolymerization permits one to evaluate the critical factors affecting the structural integrity and bioactivity of the encapsulated proteins. The detrimental effects of photoinitiators and free-radicals on protein bioactivity and
structural integrity during in situ photopolymerization were analyzed and demonstrated to be
directly related to the type and concentration of photoinitiator used during the UV-
irradiation process. The addition of hydroxyl-terminated high molecular weight
poly(ethylene glycol) at high concentrations only slightly increased lysozyme bioactivity,
presumably due to a molecular crowding effect that decreases the exposure of lysozyme to
free radicals generated from photoinitiators. On the other hand, the addition of acrylated
PEG macromers effectively decreases lysozyme damages caused by free-radicals and
enhances its bioactivity during in situ photopolymerization. Although the use of acrylated
polymers preserves the bioactivity of lysozyme, it does not prevent the formation of protein-
polymer conjugates that decrease total protein release. To further eliminate protein-polymer
conjugation, a pseudo-specific metal-ion chelating ligand was used for binding to
encapsulated proteins such as bovine serum albumin (BSA). This ligand binds to the
copper-binding site of BSA, which was suggested to be the site vulnerable to free-radical
attack, and prevents the growing of polymer chain from there. The prevention of protein-
polymer conjugation is critical for in situ photopolymerization as it not only increases
protein bioavailability but also decreases the risk of undesired immune response induced by
the conjugates.

An ideal carrier loaded with protein therapeutics must deliver its payloads with the
appropriate spatial and temporal control in order to maximize therapeutic efficacy while
preventing harmful side effects. Being a class of biomaterial that resembles natural tissue,
hydrogels have drawn tremendous interest and have been used extensively in controlled-
delivery and tissue-engineering applications. Locally delivering protein therapeutics is less of
an obstacle due to the advances in in situ polymerization techniques. However, a more
challenging task is how to systematically control the delivery of single or multiple proteins with distinct and highly tunable release profiles. The novel affinity hydrogel platform based on reversible and specific protein-ligand binding developed in this dissertation work demonstrated that protein delivery rate can indeed be systematically controlled without changing the matrix crosslinking density or degradation rate. In conjunction with experimental results, a mathematical model accounting for protein diffusion and reversible protein-ligand binding was developed to predict protein delivery profiles from this novel affinity hydrogel platform.

A thorough assessment of any protein delivery matrix should include an evaluation of the routes of network fabrication and the safety of the device (both to the host and to the encapsulated proteins) as well as the available methods of protein encapsulation and their subsequent release rate and therapeutic efficacy. In the context of protein-loading and its subsequent release, it was found that the delivery rates of protein from post-loaded gels were faster than the in situ-loaded gels. This phenomenon can be attributed to the excessive swelling of affinity hydrogels caused by the influx of not only proteins but also other electrolytes and water molecules. The increased mesh size has profound effects on the release rate of the encapsulated protein and therefore must be carefully evaluated in order to obtain optimal protein release profiles.

The sequential appearance of multiple proteins responding to tissue injury is an important characteristic of in vivo wound healing. The one-step in situ photopolymerized affinity hydrogel platform developed in this dissertation greatly simplifies matrix fabrication yet still permits independent control over dual-delivered proteins. This novel dual-protein
delivery strategy offers immense opportunities for tissue engineering applications requiring temporal control over multiple biological agents such as growth factors and other proteins.

8.2 Recommendations

Future work in the area of protein stabilization should be focused on the evaluation of other biocompatible protein-binding ligands for therapeutic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and so on. The delivery of these growth factors from injectable polymeric scaffolds for regenerative medicine has evolved as a standard strategy. However, their conjugation to the chemically crosslinked polymer networks has largely being ignored. Hence, challenges and opportunities exist for improving the bioavailability and safety of these therapeutic proteins. Since every protein possesses a distinct tertiary/quaternary structure and exposed active amino acid residues, it is not surprising to observe different extents of protein-polymer interaction/conjugation for each therapeutic growth factor during in situ photopolymerization. Therefore, it is expected that different protective ligands will be needed for each protein and techniques for identifying these ligands will be needed. A high-throughput screening technique that allows for the screening of peptide ligands expressed on the cell surface of bacteria, phages, or other microorganisms can be utilized for this purpose. Highly efficient screening methodologies will greatly facilitate the design of protein-binding ligands. Once appropriate peptide ligands are identified for therapeutically-relevant proteins, the hydrogel matrices can be designed and fabricated in a more biocompatible manner that allow for the delivery of optimal amounts of protein therapeutics to prevent waste of the valuable therapeutic agents and hence provide maximal therapeutic efficacy.
Future efforts in fabricating affinity hydrogels for protein delivery should focus on identifying unique peptide-ligand chemistries for use with native protein therapeutics. Although the binding of hexa-histidine tagged protein to the metal-ion chelating ligand is shown by the current research to be robust and reversible, it requires modification of proteins through genetic engineering and may not be acceptable when used clinically. Therefore, opportunities for the high-throughput screening and rational design of polymerizable peptide ligands that can reversibly bind to native protein therapeutics with adjustable affinity also exist in the arena of controlled drug delivery. Once identified, the potential of peptide ligands can be further enhanced by manipulating the chemistry by which the ligand is incorporated within the polymer network. For example, a peptide with a flexible PEG spacer arm can be synthesized, based on established chemistries, to improve protein binding and provide more predictable release profiles. Alternatively, multi-valent ligand-binding strategies can be integrated into the design of crosslinkable ligands or interpenetrating networks (IPNs). This would allow for strong protein-binding through the collaborative efforts of several neighboring, low-affinity ligands. Finally, biodegradable hydrogels should be used, in conjunction with the versatile protein-binding affinity mechanism, to maximize the clinical efficacy of the hydrogel matrices.

An important application for this type of affinity hydrogels is to promote or accelerate chronic/impaired wound healing such as diabetic foot ulcers. Impaired wound healing is characterized by an imbalance of growth factors and proteases at the wound site. One available treatment for impaired wounds is to locally apply growth factors, such as platelet-derived growth factor (PDGF). However, due to the extreme short half-lives of growth factors (only few minutes) in the biological environment, this strategy achieves very limited
clinical success even at high dosing. Furthermore, single growth factor therapy does not mimic the synergistic production of multiple growth factors that naturally occurs during normal wound healing. The novel concept of *injectable affinity hydrogels* capable of delivering multiple fragile protein therapeutics sequentially in distinct release profiles enables the rationale design of wound dressing and tissue filler loaded with multiple growth factors. For in vivo testing of this multiple protein delivery strategy, a diabetic animal model can be used to promote impaired wound healing by locally photo-curing affinity hydrogels that sequentially deliver two key growth factors – basic fibroblast growth factor (bFGF) and PDGF – at rates and doses that optimize clinical efficacy.
APPENDIX
A.1 Introduction

Hydrogels are water-swollen polymeric networks. They remain insoluble when placed in aqueous environments due to chemical or physical crosslinking of individual polymer chains. Chemical crosslinks may be ionic or covalent while physical crosslinks may be entanglements, crystallites, or weak associations such as hydrogen bonding or van der Waals interactions (Lowman and Peppas 1999).

Hydrogels have played an important role in biomedical applications since the late 1950s and the development of poly(2-hydroxyethyl methacrylate) (PHEMA) as a soft contact lens material (Wichterle and Lim 1960). Since that time they have found application in drug delivery. In the last decade hydrogels have played an ever-increasing role in the revolutionary field of tissue engineering where they are used as scaffolds to guide the growth of new tissues. Their widespread acceptance in these fields is primarily due to the structural similarity hydrogels exhibit compared to macromolecular-based components in the body and the ability of hydrogels to replicate the properties of natural tissue better than any synthetic material. Hydrogels such as those produced from PHEMA have high water contents at equilibrium, exhibit rubbery behavior, and show low surface coefficients of friction. Biocompatible hydrogels are currently used in numerous biomedical applications including ophthalmological devices, biosensors, biomembranes, and carriers for controlled delivery of drugs and proteins (Wichterle and Lim 1960; Andrade 1976; Lowman and Peppas 1999; Peppas, Huang et al. 2000).
More recently, the design and application of biodegradable hydrogels has dramatically increased the potential impact of hydrogel materials in the biomedical field and enabled the development of exciting advances in controlled drug delivery and tissue engineering. While all polymers will eventually degrade under extreme environmental conditions (i.e., high temperatures or low pH solutions), biodegradable hydrogels of interest to this discussion degrade over clinically relevant timescales under relatively mild conditions (i.e., aqueous solutions, physiological temperature and pH). This degradation capability eliminates the need for long-term in vivo biocompatibility or surgical removal of the gels. Biodegradable hydrogels, if correctly designed, will break down into lower molecular weight, water-soluble fragments in vivo that can then be resorbed or excreted by the body once the desired function of the gel is accomplished. In addition to minimizing surgical invasiveness, the use of these erodible gels facilitates a wide variety of new applications and delivery strategies such as degradation-controlled drug delivery, in situ scaffold formation and tissue regeneration, and controlled release via intravenous or pulmonary administration of degradable polymeric microspheres.

This chapter provides an overview of the chemistry, design, fabrication, and application of biodegradable hydrogels for drug delivery and tissue engineering. The first section briefly describes the various structural classes of degradable hydrogels that exist. Following an overview of the most commonly used gel types, the next section focuses on the fabrication and characterization of bulk-degrading, covalently crosslinked hydrogels. The chemical and structural parameters that quantify the physicochemical properties of these gels before and during gel degradation are identified and detailed from an experimental and theoretical perspective. The inherent factors that are known to significantly impact
degradation rates and macroscopic degradation behavior are also described in an effort to demonstrate how efforts to intelligently engineer the chemistry and structure of these gels on the molecular level directly correlate with improved device performance in tissue engineering and drug delivery applications.

The final section of this chapter delivers an overview of degradable hydrogel chemistries with proven or potential applications in tissue engineering and/or drug delivery. Gels are divided into two categories according to the natural or synthetic origin of their predominant polymer chemistry. Hydrogels from natural polymers have already gained widespread use in the biomedical field. However, gels obtained from natural polymers exhibit distinct limitations that have motivated approaches to modify these naturally occurring polymers as well as to develop synthetic derivatives and entirely novel synthetic chemistries. An emphasis of this concluding section is to highlight recent efforts to develop hybrid hydrogel systems that display the most advantageous properties derived from both natural and synthetic materials.

A.2 Hydrogel Classifications

A.2.1 Degradation versus Erosion

For the discussions in this chapter, degradation refers to bond cleavage or crosslink dissolution within a network, while erosion refers to the subsequent mass loss from the network that occurs as a result of gel degradation. Degradation can occur via dissolution of physical crosslinks. It can also occur in covalently crosslinked systems through the cleavage of hydrolytically labile bonds such as anhydride or ester groups, or enzymatically cleavable peptide or protein linkages. These labile bonds can be present in the crosslink segments
(predominant in synthetic polymer networks) or along the backbone chains (predominant in naturally derived polymer networks).

The *degradation behavior* of a hydrogel pertains to the time-dependent evolution of the chemical, physical, and structural properties of the crosslinked network that occur as labile bonds on the surface or within the bulk of the gel are cleaved. Important phenomena that occur to varying extents during gel degradation include changes in hydrogel swelling ratios or equilibrium water contents, network mechanics, and solute diffusivities within the swollen matrices. The rate and profile of mass loss from the hydrogel is also important. One or more of these listed properties will play a role in determining the successful function of a degradable gel for a particular drug delivery or tissue engineering application. In addition, the molecular weight, chemistry, and local concentration of the degradation products produced by an eroding gel must always be considered to ensure complete biocompatibility.

In addition to the macroscopic gel properties themselves, the rate of hydrogel degradation must be carefully controlled both in drug delivery and tissue engineering applications. In drug delivery systems, hydrogel degradation and erosion rates help determine drug availability and pharmacokinetic effects on surrounding cells and tissues. For tissue engineering applications, it is usually desirable to have biodegradable scaffolding to promote cell infiltration and tissue growth. It is a commonly believed that the degradation rates of tissue scaffolds must be matched to the rates of various cellular processes in order to optimize tissue regeneration (Hubbell 1999; Lee, Alsberg et al. 2001). Therefore, the degradation behavior of all biodegradable hydrogels should be well defined, reproducible and tunable via hydrogel chemistry or structure.
For physically crosslinked gels, the degradation kinetics of dissolving crosslinks are hard to define and control. The degradation kinetics of chemically crosslinked gels are, however, more easily defined and much work has recently been done to link the kinetics of labile bond degradation to the overall gel degradation behavior in hopes of engineering biodegradable hydrogels with precise properties.

A.2.2 Bulk versus Surface Degradation

In general, degradation of crosslinked networks occurs in one of two forms: non-uniform, surface degradation or uniform, bulk degradation (Kohn and Langer 1996). Surface-degrading networks maintain their crosslinking density and structural integrity throughout the degradation process, because degradation is limited to the surface of the material. As an example, surface degradation often results when the rate of bond hydrolysis is much faster than the rate of water transport into a polymeric device. Surface degrading networks are advantageous for drug delivery applications because zero-order release of entrapped species at a desired rate can be obtained by choosing the appropriate device geometry or altering the kinetics of degradation (Davis and Anseth 2002).

In bulk-degrading networks, infiltration and transport of species critical to the particular degradation mechanism employed by the hydrogel chemistry are faster than the inherent degradation kinetics. By definition, all hydrolytically degradable hydrogels will exhibit bulk-degrading characteristics due to the presence of a relatively high concentration of water molecules throughout the gel architecture. The same water that swells these gels will also homogeneously degrade the labile bonds present throughout the network. However, hydrogels that are degraded by species other than water may degrade by a bulk, surface, or combined mechanism depending on the permeability of the degrading species
within the gel. For example, peptide-crosslinked hydrogels that degrade through the actions of a particular enzyme may exhibit extremely high water contents in excess of 90% by volume. However, because the gel chemistry or limited mesh size prevents the uptake of the degradative, macromolecular enzyme within the bulk of the gel, only the labile peptide bonds at the surface will be exposed to the enzyme. The limited enzyme permeation will produce the observation of surface-mediated gel degradation and erosion.

While the crosslinking density and physical properties of surface-degrading gels remain constant during the biodegradation process, the properties of bulk-degrading gels are altered in a systematic fashion. The evolution of microscopic and macroscopic properties in bulk-degrading hydrogels is inherently tied to their polymer chemistry, network structure, and degradation kinetics. For example, highly swollen hydrogels formed from dimethacrylated poly(lactic acid)-b-poly(ethylene glycol)-b-poly(lactic acid) (PLA-PEG-PLA) undergo bulk degradation. The modulus, solute permeability, water content, and many other gel properties depend on the crosslinking density of the PEG-based hydrogel (Sawhney, Pathak et al. 1993; Metters, Anseth et al. 2000). As the lactic acid bonds are cleaved uniformly throughout the gel via hydrolysis, the crosslinking density of the still insoluble network systematically decreases, increasing swelling and network mesh size while lowering gel modulus. These property changes then, in turn, lead to macroscopically observable changes in the water content, permeability, and elasticity of the hydrogel as it degrades. The quantitative relationships between these dynamic properties during hydrogel degradation are detailed later in the chapter.

A.2.3 Homogeneous versus Heterogeneous Networks
Because the microscopic structure of a degradable hydrogel network plays such an important role in its degradation behavior, degradable hydrogels can be classified according to their network structure. Hydrogel network morphologies can be described as being homogeneous or heterogeneous. Homogeneous gels exhibit a random distribution of relatively mobile chains and pores within the crosslinked network. Examples of homogeneous gels include networks derived from synthetic polymers such as poly(ethylene glycol), poly(vinyl alcohol), or poly(acrylamide). Homogeneous gels may be amorphous or semi-crystalline. They may be crosslinked using ionic, covalent, or non-covalent methods. They can also be either neutral or ionic depending on the ionization of their pendant groups (Peppas 1986).

Heterogeneous hydrogels, on the other hand, exhibit an anisotropic network structure characterized by a high degree of inter-polymer interaction. Examples of heterogeneous hydrogels include many insoluble networks derived from naturally occurring polymers such as calcium alginate, agarose, and \( \kappa \)-carrageenan (Muhr and Blanshard 1982). Additionally, supermolecular fibrils and fiber bundles of size-scales much greater than individual polymer chains can be formed in these networks via complex, thermodynamically driven self-assembly processes (Stupp 2005). While such structures exhibit high porosities between immobile, large-scale fiber bundles, their overall degradation more closely resembles the characteristics of surface-eroding systems due to the inability of water or macromolecular enzymes to penetrate within the small-scale, self-assembled architectures (Ehrbar, Metters et al. 2005). Fibrous, anisotropic networks are found in gels made of natural or synthetic macromolecules such as collagen, fibrin, and synthetic polypeptides (Voet and Voet 1995; Lutolf and Hubbell 2005).
Both homogeneous and heterogeneous networks may be cast in the form of macroporous, microporous, or nonporous gels (Peppas 1986). Both types of morphologies may also display anisotropies in macroscopic properties such as swelling, elasticity, and porosity. Furthermore the degradability of both gel types is based on a limited number of biodegradable bonds. Therefore their degradation during biomedical application occurs due to identical mechanisms – hydrolytic or enzymatic cleavage of covalent crosslinks or dissolution of physical crosslinks (Shalaby, Blevins et al. 1991; Sawhney, Pathak et al. 1993; West and Hubbell 1999; Jeong, Kim et al. 2002).

However, the effect of labile bond cleavage on the evolution of macroscopic gel properties differs significantly between homogeneous and heterogeneous gels due to differences in crosslinking, supermolecular polymer-chain organization, and overall network structure. Thermodynamic relationships developed for non-degradable, homogeneous gels have been applied to help understand the dynamic degradation behavior of homogeneous gels. These relationships are detailed in the next section of this chapter. However, the anisotropic network structure present in heterogeneous gels greatly limits our current ability to correlate the extent of gel degradation with predicted or experimentally measurable changes in gel properties. While advances in the design and application of heterogeneous hydrogels for biomedical applications have been made and will be discussed in this review, the overview of structural characteristics presented in the next section focuses on identifying the key parameters that determine the microscopic and macroscopic behavior of bulk-degrading, homogeneous hydrogels.
A.3 Bulk-Degrading, Covalently Crosslinked Hydrogels

As detailed in the final section of this chapter, a large array of techniques exists for creating biodegradable hydrogels from both synthetic and naturally derived polymer chemistries. In recent years, covalent crosslinking has emerged as a preferred method due to its wide compatibility with a number of polymer chemistries and its ability to fine tune hydrogel properties (Hennink and van Nostrum 2002). Therefore in this section the fabrication and characterization of covalently crosslinked, degradable hydrogels are detailed. The quantitative analyses provided for correlating degradation behavior to labile bond cleavage kinetics apply to a specific class of covalently crosslinked, bulk-degrading hydrogels with hydrolytically degradable crosslinks. However, the observed degradation behavior is not necessarily unique to this class of hydrogel and many of the structure-function relationships developed to describe this system can readily be extended to describe the degradation behavior of other types of degradable hydrogels.

A.3.1 Fabrication and Network Structure

The degradation behavior of a biodegradable hydrogel depends significantly on its method of fabrication. Various mechanisms for forming these materials have been investigated, including ionic crosslinking, thermally induced physical crosslinking, and enzymatic or pH-induced gelation. Unfortunately, most of these methodologies yield limited control over the gelation kinetics, material properties, and degradation behavior. In contrast, covalent crosslinking methods remove the need for interpolymer interactions and lead to the formation of homogeneous networks with uniform and precise crosslinking densities. This high degree of engineerability permits fine-tuning of polymer diffusivity and permeability, degradation rate, equilibrium water content, elasticity, and modulus.
Three main polymerization mechanisms are used to form covalently crosslinked, degradable hydrogels including step-growth, chain-growth, and mixed-mode chain and step growth mechanisms. **Figure A.1** illustrates the methods of gel fabrication and potential degradation for each of these polymerization techniques (Rydholm, Bowman et al. 2005). It should be noted that the distinct site of bond cleavage within each of these biodegradable networks depends on the method of polymerization as well as on the chemistry and functionality of the chosen macromers and/or monomers. Modifications to the monomer chemistry or reaction conditions directly impacts the density and/or degradability of the network crosslinks and allows the degradation behavior as well as the moduli, elasticity, permeability, and gel water content to be tailored in each system.

Network formation via the step-growth mechanism is based on the reaction of gel precursors exhibiting a stoichiometric ratio of at least two mutually reactive chemical groups (**Figure A.1a**). These traditional A-B type polymerizations lead to the formation of an insoluble network if the average precursor functionality is greater than or equal to two as first dictated by Flory (Flory 1953). The simplest form of the step-growth mechanism related to biodegradable hydrogel fabrication is the straightforward crosslinking of highly multifunctional natural polymers using small, bifunctional crosslinking agents. For example, hyaluronic acid or other polysaccharides can be crosslinked with glutaraldehyde. For these networks, degradation will occur via cleavage of chemical bonds along the backbone of the naturally derived polymer chain. Alternatively, biodegradable hydrogels can also be formed via the step-wise crosslinking of nondegradable, synthetic polymers using degradable crosslinkers such as peptides, proteins, or even cells.
As the size and number of reactive functionalities per monomer molecule decrease, the step-growth mechanism assumes the character of a true polymerization rather than a simple crosslinking reaction. Degradable networks that result from step-growth polymerizations of small, multifunctional monomers have been developed. For example, as illustrated in Figure A.1a, Hubbell and coworkers developed degradable networks using Michael-type addition reactions between thiol and acrylate-functionalized monomers (West and Hubbell 1999; Elbert, Pratt et al. 2001; Lutolf and Hubbell 2003; Lutolf, Lauer-Fields et al. 2003; Pratt, Weber et al. 2004; Seliktar, Zisch et al. 2004). Since gel degradation occurs by hydrolytic cleavage of a thio-ether ester bond formed during thiol-acrylate coupling, low molecular weight, nondegradable monomers can be used to form degradable hydrogels. Elbert et al. fabricated hydrolytically degradable hydrogels from multi-armed PEG acrylates and linear PEG di-thiols. Additionally, enzymatic degradability was imparted to these gels through the use of protease-sensitive di-thiols made from short oligopeptide sequences (Lutolf and Hubbell 2003; Lutolf, Lauer-Fields et al. 2003).

As shown by Metters and Hubbell, the degradation rates of networks formed via the step-growth mechanism depend on the molecular weight, hydrophilicity, and degree of functionality of the starting monomers (Metters and Hubbell 2005). Step-growth polymerizations are known to produce few structural defects during network formation which permits precise control of the crosslinking density and degradation behavior (Dusek and Duskov-Smrckova 2000). Additionally, using relatively low molecular weight monomers to form the degradable, Michael-type gels eliminates the subsequent production of high molecular weight degradation products that commonly result from using polymeric gel precursors (Metters, Bowman et al. 2000; Lovestead, Burdick et al. 2002).
Contrary to the high concentration of reactive intermediates present during step-growth network formation, a low concentration of active centers is generated during formation of a typical chain-growth network (Figure A.1b). These active centers are typically radicals and are generated by a variety of methods including thermal energy, redox reactions, and cleavage of a photoinitiator molecule when irradiated with UV or visible light (Odian 1991). They rapidly propagate through monomers containing multiple carbon-carbon double bonds to form high molecular weight, kinetic chains that are covalently crosslinked. The highly stable carbon-carbon bonds that result from chain-growth polymerization are generally non-degradable under biological conditions. Rather, degradation is incorporated into the networks through specially designed multi-vinyl macromers with hydrolytically or enzymatically cleavable segments (Sawhney, Pathak et al. 1993; Davis, Burdick et al. 2003; Lutolf and Hubbell 2005). Upon network formation, these linkages are present in the network crosslinks. The degradation products from such networks are comprised of the degraded segments from the crosslinking molecules as well as the higher molecular weight kinetic chains generated during polymerization (Anseth, Metters et al. 2002).

An illustration of degradable, chain-polymerized networks comes from the pioneering work of Sawhney et al. where linear PEGs of various molecular weights were used as initiators for the ring-opening polymerization of a-hydroxy acids (lactic, glycolic), followed by reaction with acid halides to produce vinyl terminated macromonomers containing degradable ester linkages (Figure A.2) (Sawhney, Pathak et al. 1993). Chain-growth polymerization of the poly(lactic acid)-b-poly(ethylene glycol)-b-poly(lactic acid) (PLA-PEG-
PLA) tri-block copolymer macromers was accomplished using mild photopolymerization conditions that permitted in situ network formation under physiological conditions.

Figure A.1 Pictorial representation of the initial monomer molecules, cross-linked polymer networks, and degradation products for materials formed from (a) step-growth polymerization mechanism, (b) chain-growth polymerization mechanism, and (c) mixed-mode chain and step-growth mechanism. (From Rydholm, A.E., Bowman, C.N., et al. 2005. Biomaterials 26: 4495–4506. With permission.)
Figure A.2 Illustration of three different stages during the bulk degradation of a PLA-b-PEG-b-PLA hydrogel network: (a) initial, nondegraded PLA-b-PEG-b-PLA network, (b) primary erosion products that are released during degradation, and (c) final degradation products after complete hydrolysis. (From Metters, A.T., Bowman, C.N., et al. 2000. *J. Phys. Chem. B* 104: 7043–7049. With permission.)

The degradation behavior of chain-polymerized networks with hydrolytically or enzymatically labile crosslinks can be tailored through a variety of parameters. Although physically crosslinked networks can also be formed using degradable ABA block copolymers such as non-acrylated PLA-PEG-PLA, Sawhney’s work was the first to incorporate polymerizable moieties into the macromer design. Covalent crosslinking of the degradable macromers provides dramatically improved control over the resulting network structure and subsequent degradation rate. For example, the degradation rate of covalently crosslinked (PLA-PEG-PLA) hydrogels can be tailored by varying the molecular weights of the PEG and/or PLA copolymer blocks within the crosslinker, the chemistry and degree of vinyl group functionalization, or the type and amount of comonomers added to the system (Metters, Anseth et al. 2000; Metters, Bowman et al. 2000; Metters, Anseth et al. 2001).
Finally, as their name suggests, networks formed from mixed-mode polymerizations exhibit characteristics between chain and step-growth polymerizations (Figure A.1c). One relatively new type of degradable hydrogel based on the mixed-mode polymerization of acrylated PLA-PEG-PLA monomers and multifunctional thiols has been developed by Bowman and coworkers (Cramer, Reddy et al. 2004; Reddy, Cramer et al. 2004; Lu, Carioscia et al. 2005; Okay and Bowman 2005; Okay, Reddy et al. 2005; Reddy, Anseth et al. 2005). The network structure that results from this mixed-mode polymerization mechanism is unique from networks formed by chain and step-growth polymerizations and is directly impacted by reactive group ratios. As the ratio of thiol to acrylate groups increases in the system, the networks transition from being chain-like to more step-like. In addition, the erosion profile and swelling changes that occur during degradation are controlled by variations in thiol-acrylate ratios that impact network structure. Additionally, changing the thiol mole fraction in the network provides control of the degradation products’ molecular weight distributions (Reddy, Anseth et al. 2005).

A.3.2 Function and Degradation

In addition to the obvious need for biocompatibility during the lifetime of the degradable hydrogel, three material properties critical to the successful biomedical application of any hydrogel are water content, mechanical stiffness or elasticity, and permeability. By definition hydrogels must exhibit high water contents. While no exact water content value is required to describe a hydrophilic, crosslinked material as a hydrogel, most hydrogels currently used to encapsulate living cells, for example, swell to greater than 90% water by weight when placed in suitable physiological fluids. The highly solvated gel environment is critical to maintaining cell viability and also minimizes nonspecific adsorption
of proteins and other macromolecules present in a biological environment that would otherwise lead to harmful inflammatory responses (Bryant and Anseth 2002).

The mechanical properties of hydrogels are also particularly important in tissue engineering applications where the gel must create and maintain a space for cell infiltration and tissue development. In addition, results from a number of investigations have demonstrated that the adhesion, structure, metabolism, and gene expression of encapsulated cells are strongly influenced by the mechanical properties of the polymer scaffold (Huang and Ingber 1999).

Finally, gel permeability is important to successful gel function. Controlled gel permeability permits sustained drug release over long periods as well as prevents the infiltration of harmful species such as enzymes or inflammatory/immune cells that would affect the stability of encapsulated proteins or transplanted cells respectively (Langer 1990; Langer 1991). In addition, correct gel permeability is also important for the transportation of nutrients and metabolic wastes to support growth of gel-encapsulated cells and tissues.

The swelling characteristics, mechanical properties, and permeability of hydrogels depend on several factors including the supermolecular structure of the original polymer chains; the type of crosslinking molecules and the crosslinking density; and the hydrophilic/hydrophobic balance of the crosslinks and backbone polymer chains within the crosslinked network (Lee and Mooney 2001). As previously mentioned, the high degree of interpolymer interactions and structural anisotropies that occur in heterogeneous gels can be extremely difficult to characterize. However, to describe the structure, chemistry, and resultant material properties of homogeneous gels, the multitude of system design variables can generally be condensed to a few critical parameters: (1) the polymer volume fraction in
the swollen state, \( v_{2,s} \), (2) the number average molecular weight between crosslinks, \( \overline{M}_c \), (3) the degree of polymer-solvent interaction, \( \chi_{12} \), and (4) the network mesh size, \( \zeta \) (Peppas 1986).

### A.3.3 Swelling

By definition, the equilibrium polymer volume fraction in a degradable or nondegradable hydrogel, \( v_{2,s} \), is the ratio of the volume of polymer, \( V_p \), to the volume of the swollen gel, \( V_{gel} \), and the reciprocal of the volume swelling ratio \( Q \):

\[
v_{2,s} = \frac{V_p}{V_{gel}} = Q^{-1}
\]

The polymer volume fraction can be determined by equilibrium swelling measurements before or during degradation (Peppas 1986; Metters, Anseth et al. 2000). The degree of swelling is also commonly reported as a mass swelling ratio, \( Q_m \) or \( q \), which can also be related to \( v_{2,s} \) as follows (Kong, Lee et al. 2002):

\[
v_{2,s} = Q^{-1} = \frac{1/\rho_2}{\left[ Q_m / \rho_1 + 1/\rho_2 \right]}
\]

where

\[
Q_m = \frac{M_{gel}}{M_p}
\]

Here, \( M_{gel} \) and \( M_p \) are the masses of the swollen gel and dried polymer respectively. \( \rho_1 \) and \( \rho_2 \) are the densities of the solvent and polymer respectively.

For further hydrogel characterization, the number-average molecular weight between crosslinks, \( \overline{M}_c \), is the most common parameter used to represent the level of crosslinking within the network. Assuming an ideal network structure is formed, \( \overline{M}_c \) can be theoretically calculated based on the size, chemistry, and functionality of the gel precursors. However,
defects in network structure that increase its value occur during the fabrication of almost every hydrogel (Dusek and Duskov-Smrekova 2000; Elliott, Nie et al. 2003; Elliott, Macdonald et al. 2004; DuBose, Cutshall et al. 2005). Therefore, $M_c$ is best-determined using theories that correlate its true value to experimental measurements of gel swelling and mechanical strength.

The swelling behavior of hydrogels in biological fluids can be reasonably described by a variety of non-ideal thermodynamic models. Due to the highly complex behavior of polymer networks in electrolyte solutions, no theory can predict exact swelling behavior. However, the Flory-Rehner analysis and its various modifications, continues to be used with reasonable success (Flory and Rehner 1943). This theoretical framework describes gels as neutral, crosslinked networks with a Gaussian distribution of polymer chains. When placed in aqueous solution this model assumes that swelling equilibrium will occur at the point where the swelling force due to the thermodynamic compatibility of the polymer and water balances the retractive force induced by the stretching of the network crosslinks. This analysis leads to the Flory-Rehner expression for the true $M_c$ of a nonionized hydrogel:

$$\frac{1}{M_c} = \frac{2}{M_n} \left( \frac{v}{V_i} \left[ \ln\left(1 - \frac{v_{2,a}}{v_{1,a}}\right) + \frac{v_{2,a}}{v_{1,a}} + \chi_{12} v_{2,a}^2 \right] \right)$$

Here, $\chi_{12}$ is the polymer-water interaction parameter, $V_i$ is the molar volume of water, $v$ is the specific volume of the polymer, $M_n$ is the average molecular weight of linear polymer chains prepared at the same conditions without crosslinking, and $\phi$ is the functionality of the crosslinker (e.g., $\phi = 4$ for a chain-polymerized, divinyl crosslinker).
Under the common conditions of high network swelling where $Q > 10$ ($\nu_{2i} < 10\%$) the original Flory-Rehner Equation can be simplified to show a more direct relationship between $Q$ and $\overline{M}_c$:

$$Q = \left[ \frac{V}{\nu} \left( \frac{1}{2} - 2 \frac{Z_{12}}{V} \overline{M}_c \right) \right]^{1/5} = \beta \left( \frac{\overline{M}_c}{M_n} \right)^{1/5}$$

Here, $\beta$ is a constant. This simplification assumes that chain-end effects can be neglected ($\overline{M}_c << M_n$) and that all physical parameters remain constant (Flory 1953).

Although $\overline{M}_c$ cannot be directly measured, the power-law relationship between $Q$ and $\overline{M}_c$ outlined above is indirectly evident in the experimentally observed swelling behavior of degrading hydrogels. In a bulk-degrading hydrogel, degradable linkages present along network crosslinks or backbone polymer chains will be cleaved homogeneously throughout the entire gel at a rate controlled by the reaction kinetics of labile bond cleavage (e.g., hydrolysis of PLA ester bonds within PLA-PEG-PLA crosslinks). This ongoing bond cleavage systematically decreases the crosslinking density of the overall network and increases $\overline{M}_c$. As predicted by the simplified equation given above, the hydrogel swelling ratio will increase as degradation proceeds and $\overline{M}_c$ increases. This behavior is observed during the bulk degradation of a wide variety of hydrogels where $Q$ and $Q_n$ are seen to increase with degradation time (Figure A.3) (Lee, Bouhadir et al. 2000; Metters, Anseth et al. 2000; Elbert, Pratt et al. 2001; Lutolf, Lauer-Fields et al. 2003; Metters and Hubbell 2005). The exact function describing the rate of increase in gel swelling with degradation time will depend on the kinetics of individual bond cleavage as well as the gel structure (Metters,
Figure A.3 Examples of systematically increasing network swelling ratio with degradation time for different bulk-degrading hydrogels. (a) As the molecular weight of an octa-acrylate PEG precursor is increased, both the initially observed swelling ratio (at $t_0$) and the apparent rate of degradation (slope of swelling curve) increase (Metters, A. and Hubbell, J. 2005. Biomacromolecules 6: 290–301.). (b) Swelling measurements of enzymatically degradable hydrogels with three different peptide cross-linkers exhibiting varying susceptibility to exogenously added proteases (Lutolf, M.P., Lauer-fields, J.L., et al. 2003. Proc. Natl Acad. Sci. USA 100: 5413–5418.). (c) Gel swelling and (d) calculated polymer volume fraction during degradation of gels made from PEG-dithiol and PEG-multiacylates (Elbert, D.L., Pratt, A.B., et al. 2001. J. Control. Release 76:11–25.)

In some covalently crosslinked, degradable hydrogels the swelling behavior as a function of degradation and the dependence of $\overline{M}_c$ on the labile-bond cleavage kinetics can be described more quantitatively. For example, dimethacrylated PLA-b-PEG-b-PLA
macromers, once polymerized, form hydrolytically degradable crosslinks within swollen hydrogel structures as shown in Figures A.1b and A.2 (Metters, Anseth et al. 2000; Rydholm, Bowman et al. 2005). Assuming pseudo first-order hydrolysis kinetics of the individual PLA ester bonds leads to a first order decrease in the gel crosslinking density since the cleavage of any ester bond in the macromer will lead to crosslink cleavage (Metters, Bowman et al. 2000). Combining the ester-bond hydrolysis kinetics with knowledge of the triblock crosslink structure yields the following exponential relationship for $M_c$ as a function of degradation time ($t$):

$$M_c(t) = M_c \left|_{t=0} \right. e^{2j\bar{k}E t}$$

Where $t=0$ represents the initial time prior to any network degradation, $j$ is the degree of polymerization of the two PLA blocks in the PLA-PEG-PLA macromer (equivalent to the number of ester bonds per block), and $\bar{k}_E$ is the pseudo first-order kinetic rate constant for hydrolysis of those ester bonds. Combining this time-dependent expression for $M_c$ with the simplified form of the Flory-Rehner Equation provided above yields an equation predicting a similar exponential increase in gel swelling with degradation time:

$$Q(t) = Q \left|_{t=0} \right. e^{6/5j\bar{k}E t}$$

Thus, for a system where mass-transfer limitations are not significant and the system is reaction controlled, the swelling ratio of the hydrogel at any point during degradation can be predicted based on knowledge of the hydrolysis kinetics of the individual bonds as well as the composition of the crosslinks and overall network structure of the degradable gel. As shown in Figure A.4, the typical swelling behavior of a degrading PLA-PEG-PLA hydrogel
exhibits this predicted exponential increase in gel swelling ratio with degradation time (Metters, Anseth et al. 2000; Anseth, Metters et al. 2002).

The time or degradation-dependent swelling behavior of highly permeable, degradable hydrogels formed via step-growth polymerizations can also be predicted with adequate knowledge of bond cleavage kinetics and network structure. DuBose et al. showed how the bond hydrolysis kinetics and network structure could be varied independently to affect the dynamic swelling profiles of PEG-based hydrogels formed using Michael-type addition reactions with small-molecule di-thiols (DuBose, Cutshall et al. 2005). **Figure A.5** demonstrates how the rate of swelling can be increased in a predictable fashion by increasing temperature during degradation (i.e., increasing the rate constant for bond hydrolysis) or by decreasing the number of acrylate groups per PEG crosslink in the gel network.

![Figure A.4](image)

**Figure A.4** Typical *in vitro* degradation behavior of a PLA-b-PEG-b-PLA hydrogel: compressive modulus (●) and volumetric swelling ratio (■). The solid and dashed lines are exponential curves fit to each property with time constants of $\tau_Q = 4200$ min and $\tau_K = 2000$ min. (From Anseth, K.S., Metters, A.T., et al. 2002. *J. Control. Release* 78: 199–209. With permission.)
Figure A.5 Swelling of degradable PEG-acrylate/dithiol gels formed via step-growth polymerization. (a) Gels fabricated from 30 wt % eight-armed PEG-acrylate/DTT precursor solutions and degraded at varying temperatures: 37°C (▲), 46°C (♦), and 57°C (■). (b) Gels fabricated with either four-arm/10 kDa (■) or eight-arm/20 kDa (♦) PEG were measured and compared with model predictions (— —). (From DuBose, J.W., Cutshall, C., et al. 2005. J. Biomed. Mater. Res. A 74A: 104–116. With permission.)

A.3.4 Mechanics

The mechanical behavior of hydrogels is best described using the theories of rubber elasticity and viscoelasticity (Flory 1953; Treloar 1975; Aklonis and MacKnight 1983). These theories are based on the time-independent and time-dependent recovery of the chain orientation and structure, respectively. Rubbers are materials that respond to stresses with nearly instantaneous and fully reversible deformation (Bueche 1962). Normal rubbers are lightly crosslinked networks with a rather large free volume that allows them to respond to external stresses with a rapid rearrangement of the polymer segments. In their swollen state, most hydrogels satisfy these criteria. When a hydrogel is in the region of rubber-like behavior, the mechanical behavior of the gel is dependent mainly on the architecture of the polymer network. Only at very low temperatures will these gels lose their rubber elastic properties and exhibit viscoelastic behavior. General characteristics of rubber elastic behavior include high extensibility generated by low mechanical stress, complete recovery
after removal of the deformation, and high extensibility and recovery that are driven by entropic rather than enthalpic changes.

By using rubber elasticity theory to describe the mechanical behaviors of biomedical hydrogels, it is possible to analyze the polymer structure and determine the effective molecular weight between crosslinks ($\bar{M}_c$) as well as elucidate information about the number of elastically active chains and deviations from ideal network structure (defects) that occur under a variety of reaction conditions. Using this theory, the shear modulus of an unswollen, dry gel ($G_d$) depends on the average molecular weight between crosslinks as follows (Anseth, Bowman et al. 1996):

$$G_d = \frac{\rho R T}{M_c} \left(1 - \frac{2\bar{M}_c}{M_s}\right)$$

Here $\rho$ is the polymer density, $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$), and $T$ is temperature (K). The effects of chain ends have been included. This equation is easily modified to describe the mechanical behavior of swollen hydrogels. The shear modulus of a swollen gel ($G_s$) is dependent on its network structure and degree of swelling as given by:

$$G_s = \frac{\rho R T}{M_c} \left(1 - \frac{2\bar{M}_c}{M_s}\right)(Q)^{1/3} = \frac{\rho R T}{M_c} \left(1 - \frac{2\bar{M}_c}{M_s}\right)\frac{1}{Q^{1/3}}$$

Therefore, for a given hydrogel at a fixed temperature, a higher degree of swelling results in a reduction of the shear modulus. Alternatively, if the degree of crosslinking is increased (i.e., $\bar{M}_c$ is decreased), the modulus is increased. This interpretation is adequate for low strains.

Assuming analysis of a bulk-degrading hydrogel exhibiting a high degree of swelling ($Q > 10$), as well as neglecting the influence of chain ends, the relationship between $Q$ and
$\overline{M}_c$ obtained from using the simplified Flory-Rehner Equation can be inserted to yield $G_s$ as a function of $\overline{M}_c$:

$$G_s = \frac{\rho RT}{\overline{M}_c} \left( \frac{1}{\beta(\overline{M}_c)} \right)^{1/3} = \frac{\gamma}{\overline{M}_c^{5/6}}$$

Here $\beta$ and $\gamma$ are constants. This equation explains why the modulus of a bulk-degrading PLA-PEG-PLA hydrogel decreases exponentially with time as shown in Figure A.4. As $\overline{M}_c$ increases exponentially with time for the PLA-PEG-PLA hydrogel, $G_s$ decreases in the following manner:

$$G_s(t) = \frac{G_{s0}}{e^{12/5 \beta t}} = G_{s0} e^{-12/5 \beta t}$$

Here, $G_{s0}$ is the initial shear modulus of the swollen hydrogel network prior to degradation.

As shown by the equation above, the elasticity of a bulk-degrading network is dependent upon bond cleavage kinetics and gel microstructure. These dependencies are similar to what were previously calculated for gel swelling. However, comparison of the exponential rate constants for the modulus and swelling degradation curves reported in Figure A.4 indicate that the rate of modulus decay is approximately twice as fast as the rate at which swelling increases for degrading PLA-PEG-PLA gels. These experimental results are supported through first-principle predictions based on the simplified thermodynamic relationships given above which relate both gel swelling and modulus to $\overline{M}_c$ and predict (Metters, Bowman et al. 2001).

$$G_s = \frac{\gamma}{\overline{M}_c^{5/6}} = \frac{\gamma}{(\frac{Q}{\beta})^{5/3} \alpha^{1/3}} = \frac{1}{Q^{2/3}}$$
This scaling argument shows that for degradable and non-degradable hydrogels, the shear modulus of the gel ($G_s$) is approximately twice as sensitive to changes in $\overline{M}$ as the volumetric swelling ($Q$).

It is important to note that while the results presented in Figure A.4 illustrate good agreement between experimental observations of hydrogel degradation and the predictive thermodynamic equations, many assumptions were made to reach the scaling argument provided above. These assumptions include a high degree of swelling, constant physical and thermodynamic parameters, and an ideal network structure founded on degradable crosslinks. The presence of non-idealities in the network structure, changes in hydrogel chemistry during degradation (e.g., production of numerous carboxylic acid end groups during PLA ester bond cleavage), mass transfer limitations, or auto-catalytic effects will produce characteristics in the gel degradation behavior that cannot be appropriately described by the simplified analysis presented in this section (Metters, Anseth et al. 2001). For example, Amsden et al. have developed an alternative strategy for modeling the degradation of hydroxyethylmethacrylate-grafted dextran hydrogels where bond cleavage occurs along the backbone chains of the network and changes in polymer chemistry during degradation are significant (Amsden, Stubbe et al. 2004).

A.3.5 Diffusivity

The ability of a hydrogel to restrict the diffusive movement of a solute plays a key role in applications as diverse as cell encapsulation, chromatography, biosensors, and drug delivery (Langer and Peppas 1981). Diffusion-controlled drug-delivery systems based on hydrogel materials, for example, can be matrix or reservoir systems (Lowman and Peppas 1999; Mallapragada and Narasimhan 1999). In the reservoir system, the active agent is
located in a core and a polymer membrane surrounds it. In matrix systems, the drug or protein is homogeneously distributed throughout the membrane and is slowly released from it. In either system the hydrogel membrane can be biodegradable (Heller 1980).

It is important to have an understanding of the mechanisms and underlying parameters governing solute diffusion within hydrogels. For this reason, a number of mathematical relationships have been developed in an effort to model solute diffusion in hydrogels. Drug or protein diffusion within swollen hydrogel networks is best described by Fick’s equation or by the Stefan-Maxwell equations that correlate the flux of a particular solute with its chemical-potential gradient in the system (Peppas, Huang et al. 2000). For porous gels with pore sizes much larger than the molecular dimensions of the solute, the diffusion coefficient is related to the porosity and the tortuosity of the porous structure (Peppas 1986). For porous hydrogels with pore sizes comparable to the solute molecular size and for nonporous hydrogels, various expressions have been proposed for the diffusion coefficients (Peppas 1986; Amsden 1998; Masaro and Zhu 1999). The polymer chains within these crosslinked networks have been proposed to retard solute movement by reducing the average free volume per molecule available to the solute, by increasing the hydrodynamic drag experienced by the solute, or by acting as physical obstructions that increase the solute path length (Mackie and Meares 1955; Cohen and Turnbull 1959; Bird, Stewart et al. 1960). Model complexity increases as these mechanisms are combined as well as when non-spherical solutes (e.g., linear polymer chains of high molecular weight) and polymer-solute interactions are considered (Muhr and Blanshard 1982). An excellent review of the most prevalent models along with a quantitative assessment of their predictive abilities has been presented by Amsden (Amsden 1998).
Solute transport within hydrogels is assumed to occur primarily within the water-filled regions delineated by the polymer chains. The average size of these spaces can be quantified through the correlation length, $\xi$, also known as the network mesh size (Peppas 1986; Lustig and Peppas 1988). The mesh size of a hydrogel network can be determined as described by Canal and Peppas (Canal and Peppas 1989).

$$\xi = v_{2,s}^{-1/3} \left( \frac{r_s}{\bar{r}_o} \right)^{1/2} = Q^{1/3} \left( \frac{r_s}{\bar{r}_o} \right)^{1/2}$$

where $\left( \frac{r_s}{\bar{r}_o} \right)^{1/2}$ is the root-mean-squared end-to-end distance of network chains in the unperturbed state and is directly proportional to $\bar{M}_c$. Any factor that reduces the relative size of these solvent-filled spaces compared to the size of the solute will further retard solute diffusion. In general, the diffusivity of a solute through a covalently crosslinked hydrogel decreases as crosslinking density increases ($\bar{M}_c$ decreases), as the size of the solute ($r_s$) increases, and as the volume fraction of polymer within the gel ($v_{2,s}$) increases (Yasuda, Lamaze et al. 1968; Peppas 1986; Johansson, Skantze et al. 1991).

An exponential increase in both $Q$ and $\bar{M}_c$ with degradation time has already been predicted for bulk degrading gels with crosslinks that degrade according to pseudo-first order kinetics. Therefore the network mesh size for these systems will also increase in an exponential manner with degradation time as shown below (Mason, Metters et al. 2001):

$$\xi = Q^{1/3} \left( \frac{r_s}{\bar{r}_o} \right) \sim \bar{M}_c^{7/10} = \left[ \bar{M}_c \right]_{t=0} e^{2j\kappa t} = e^{7/5 j\kappa t}$$

where $\eta$ is a constant.

Theoretical models for predicting solute diffusion coefficients take the form:
\[
\frac{D_s}{D_o} = f\left(r, v_{2,s}, \xi\right)
\]

where, $D_s$ is the solute diffusion coefficient in the swollen hydrogel network, $D_o$ is the diffusion coefficient of solute in pure solvent, and $r$ is the size of the solute molecules. Thus, the structure and pore size of the gel, the polymer composition, the water content, and the nature and size of the solutes are all taken into account by the diffusion coefficient of the solute (Peppas, Huang et al. 2000). This general framework can be used to predict the rate of diffusion of all species within the gel network including the influx of degrading moieties (e.g., water or enzymes) as well as the release rate of encapsulated species such as drugs and other therapeutic agents. For a degradable hydrogel the previously discussed increase in mesh size and decrease in polymer volume fraction with network degradation will influence $D_s$ and cause it to change with the extent of degradation as well.

Numerous variations of the general framework given above have been developed by several research groups to more clearly describe the relationship between solute diffusivity and network structure for non-degrading gels. As an example, one such model is given by:

\[
\frac{D_s}{D_o} = \left(1 - \frac{\xi}{\xi_c}\right)\exp\left(-Y\frac{v_{2,s}}{1-v_{2,s}}\right)
\]

Here $Y$ is physically defined as the ratio of the critical volume required for a successful translational movement of the solute molecule and the average free volume per molecule of solvent. For most purposes a good approximation for $Y$ is unity. This model was developed by Lustig and Peppas using a free-volume approach and has proven useful for predicting solute diffusivities within poly(ethylene glycol) (PEG) networks (Lustig and Peppas 1988).
For highly swollen, degradable PLA-PEG-PLA gels the diffusivity correlation provided by Lustig and Peppas can be simplified to:

\[
\frac{D_t}{D_o} = \left(1 - \frac{r_s}{\xi}\right) = \left(1 - \frac{r_s}{\eta e^{-\frac{7}{5} \mu_x t}}\right)
\]

using the time-dependent expression for the mesh size given above. From this modified expression it can be clearly observed that as degradation proceeds, solute diffusivity within the gel \((D_t)\) will increase in a systematic, predictable fashion and approach \(D_o\). Like the shear modulus of the degrading gel \((G)\), the rate of decrease in solute diffusivity depends on network structure and bond cleavage kinetics. This type of analysis has been used to explain the diffusivities and release profiles of various drugs from bulk degrading networks compared to non-degrading matrices (Lu and Anseth 2000; Mason, Metters et al. 2001). It can also be used with alternative free-volume approaches, hydrodynamic scaling models, and obstruction theories to predict the time-dependent solute diffusivities within other degradable gels.

**A.3.6 Characteristic Erosion**

Mass loss or erosion from bulk-degrading, covalently crosslinked hydrogels is a complex process that depends upon the network structure and degradation kinetics (Heller 1980; Metters, Anseth et al. 2000; Metters, Bowman et al. 2000). In this respect, mass loss from degrading hydrogels is similar to the swelling or mechanical properties. However, unlike most other macroscopic gel properties that are only related to the time-dependent crosslinking density or average molecular weight between crosslinks \((M_c)\), mass loss also relies upon additional structural parameters. For example, models developed to predict mass loss from photocrosslinked PLA-PEG-PLA hydrogels show that the erosion profiles for
these systems are linked to network parameters such as the number of crosslinks per backbone chain and the mass fraction of the network contained in the backbone chains relative to the crosslinks (Metters, Bowman et al. 2000; Martens, Metters et al. 2001; Metters, Anseth et al. 2001; Martens, Bowman et al. 2004). Predictions of mass loss versus degradation time also depend upon kinetic parameters such as the order and rate constant of the degradation reaction.

In Figure A.6, experimental and predicted mass loss profiles are plotted versus degradation time for two PLA-PEG-PLA hydrogels polymerized in solution at different macromer concentrations (Metters, Bowman et al. 2000). These degrading hydrogels have the same chemical composition, yet different initial crosslinking densities and microstructures that result from the behavior of the chain-growth polymerization used in their fabrication. Increasing the initial macromer concentration during polymerization increases the average length and functionality of the backbone polymer chains and decreases the number of structural defects within the network. These architectural changes are reflected in an increased time until complete network dissolution as well as a lower apparent degradation rate (slope of linear portion of curve) due to the additional crosslinking and lower swelling ratios of these networks.

A characteristic of bulk-degrading gels that is evident in Figure A.6 is the occurrence of gel dissolution prior to 100% bond degradation. Gel dissolution, or reverse gelation as it has been termed, is an abrupt solid to liquid transition that leads to almost instantaneous erosion of a substantial fraction of the crosslinked gel mass (Metters, Bowman et al. 2000). It is analogous to the liquid to solid transition that occurs at the gel point during hydrogel formation (Flory 1953). The exact fraction of the total network mass lost during reverse
gelation increases as the number of crosslinks per backbone chain decreases as shown in Figure A.6.

**Figure A.6** Experimental mass loss data as a function of degradation time for two identical PLA-PEG-PLA macromers polymerized at different concentrations to form gels with unique degradation behavior: (■) 25 wt% and (●) 50 wt%. The solid and dashed lines represent the percent mass loss predicted by a statistical model: (dashed) 25 wt% and (solid) 50 wt%. (From Metters, A.T., Bowman, C.N., et al. 2000. *J. Phys. Chem. B* 104: 7043–7049. With permission.)

The burst of mass loss that occurs at the onset of reverse gelation can have broad implications on the successful application of the degradable hydrogel. A large fraction of network mass lost during reverse gelation will result in a large, localized concentration of potentially cytotoxic degradation products. For example, there is evidence that the macrophage response to implanted PLA-PEG-PLA gels appears strongest immediately following gel dissolution. This is most likely a response to the high concentration of soluble, acidic degradation products that are commonly seen with PLA-based materials and can lead to adverse cellular responses *in vivo*. These acidic degradation products can also decrease the stability of encapsulated proteins when these hydrogels are used for the controlled release of pharmaceutics. Therefore to ensure biocompatibility and proper application of any
degradable hydrogel it is important to understand the factors that determine when gel dissolution will occur.

Finally, the erosion profiles of bulk degrading hydrogels can also be engineered for specific drug delivery applications. The same kinetic and structural factors that impact mass loss from a crosslinked gel can also be used to control the delivery of therapeutic biomolecules covalently attached to the gel network (Heller 1980; Zhao and Harris 1997; Zhao and Harris 1998; Seliktar, Zisch et al. 2004; DuBose, Cutshall et al. 2005). For example, fluorescently labeled probe molecules resembling clinically relevant peptide drugs were covalently incorporated within the three-dimensional network structure of PEG-based hydrogels formed via step-growth polymerizations. As shown in Figure A.7, hydrolytic cleavage of the covalent bonds within the crosslinked PEG network, including those used to immobilize the probe molecule, resulted in a biphasic release profile consisting of a relatively slow, constant release rate of probe prior to gel dissolution and an almost instantaneous release following the onset of gel dissolution (DuBose, Cutshall et al. 2005). The various release profiles shown in Figure A.7 demonstrate how the release rate as well as the fraction of probe released prior to network dissolution can be controlled through intelligent choice of crosslinker functionality (tetra-functional PEG versus an octa-functional PEG in Figure A.7a) or degradation kinetics (varying temperature, pH, or chemistry of the degradable thioether ester bond in the network in Figure A.7b). Such characteristic erosion profiles are seen in a variety of bulk-degrading hydrogel systems.
Figure A.7 Fractional probe release from degradable PEG-acrylate/dithiol gels formed via step-growth polymerization (a) Gels fabricated from 30 wt % eight-armed PEG-acrylate/DIT precursor solutions and degraded at varying temperatures: 37°C (▲), 46°C (◇), and 57°C (■). (b) Gels fabricated with either four-arm/10 kDa (■) or eight-arm/20 kDa (◇) PEG were measured and compared with model predictions (— — —). (From DuBose, J.W., Cutshall, C., et al. 2005. J. Biomed. Mater. Res. A 74A: 104–116. With permission.)

A.4 Degradable Hydrogel Chemistries

Degradable hydrogels can be prepared from naturally derived biopolymers, synthetic polymers or the combination of the two. The major advantages of using naturally derived biopolymers such as chitosan, alginate, fibrin, collagen, gelatin and hyaluronic acid derivatives include their inherent biodegradability and biocompatibility. However, natural biopolymers may not provide adequate mechanical strength as well as precise functionalities compared to synthetic polymers. Moreover, precautions against pathogenic contamination must be taken when many natural biopolymers are used in clinical applications.

On the other hand, many synthetic polymers, although not inherently degradable themselves, can be modified or copolymerized with labile groups to create degradable hydrogels. The good mechanical properties and well-defined chemistries enable hydrogels made from synthetic polymers such as poly(ethylene glycol) (PEG), poly(vinyl alcohol)
(PVA), poly(N-isopropylacrylamide) (PNIPAAm), and poly(methyl methacrylate) (PMMA) to be used for many biomedical applications.

Recently, many fundamental studies as well as clinical applications using degradable hydrogels derived from natural or synthetic polymers have been conducted. In this final section of the chapter, the chemistries as well as applications that comprise the most common degradable hydrogels are described.

A.5 Degradable Hydrogels Derived from Natural Biomaterials

A.5.1 Chitosan-based Hydrogels

Chitosan, with a subunit of β-(1,4)-2-amido-2-deoxy-D-glucopyranose, is a form of deacetylated chitin that has been shown to be biocompatible. Chitosan can be dissolved in weak acidic solution and is positively charged in natural or basic environment due to its amino groups. It has been demonstrated that chitosan can accelerate wound healing processes and therefore is a well accepted material for various biomedical applications including tissue engineering, controlled delivery of proteins, and gene delivery. Several review papers have been published addressing the chemistry and applications of chitosan hydrogels. For example, Berger et al. have reviewed the chemistry of chitosan hydrogels formed via electrostatic assembly (Berger, Reist et al. 2004). Kumar et al. extensively reviewed chitosan chemistry as well as its pharmaceutical applications (Kumar, Muzzarelli et al. 2004).

Chitosan hydrogels can be fabricated via either physical or chemical crosslinking. In previously used methods to induce physical crosslinking, positively charged chitosan was mixed with negatively charged polymers including alginate (Murata, Kontani et al. 2002; Lee, Yoon et al. 2004; Lin, Liang et al. 2005) and poly(vinyl alcohol) (Koyano, Minoura et al.
to form a hydrogel network via electrostatic interaction. By adjusting the ratio of the positive and negative charged components during the fabrication process, a tunable gel swelling behavior can be readily achieved. Although physical crosslinking may be a convenient way to prepare chitosan hydrogels, disadvantages also exist such as poor mechanical strength and uncontrollable dissolution.

Chemical or covalent crosslinking is favorable for tissue engineering applications when adequate mechanical strength is required. In order to form covalent crosslinks, additional functionalities must be introduced onto the chitosan backbone. Modifications have been made to the hydroxyl or amino groups on chitosan to facilitate covalent crosslinking. For example, several chemistries have been proposed to graft synthetic polymers or reactive groups onto chitosan including PEG (Gupta and Kumar 2001; Park, Kim et al. 2001; Hu, Jiang et al. 2005), PNIPAAM (Cho, Kim et al. 2004; Lee, Ha et al. 2004), polyurethane (Gong, Zhang et al. 1998; Silva, Menezes et al. 2003), and glycidyl methacrylate (GMA) (Navarro and Tatsumi 2001; Flores-Ramirez, Elizalde-Pena et al. 2005). Figure A.8 illustrates the chemical structure of chitosan and summarizes chitosan-based hydrogels fabricated via both electrostatic and covalent crosslinking.

Chitosan hydrogels can be degraded via its β-1,4-glycosidic linkage by various enzymes including chitosanase and lysozyme. The degradability of chitosan-based hydrogels can be controlled by the degree of substitution of grafted side chains such as PEG. For example, the degradation rate of chitosan-g-PEG hydrogels by lysozyme has been shown to decrease with increasing degree of substitution of PEG on chitosan backbone (Hu, Jiang et al. 2005). This behavior is attributed to the fact that the grafted PEG increases the hydrostability of
chitosan which disrupts the accessibility of lysozyme to chitosan and thus decreases its enzymatic degradability.

![Figure A.8](image)

**Figure A.8** Structure of chitosan and its derivatives for hydrogel synthesis.

### A.5.2 Alginate-based Hydrogels

Alginate is a linear polysaccharide composed of 1,4-linked poly(α-L-guluronic acid) and poly(β-D-mannuronic acid). Alginate can be physically crosslinked through its poly(guluronic acid) residues by adding calcium ions. Ionically crosslinked alginate does not form a stable hydrogel because the gel can be disintegrated or dissolved once calcium ions diffusive away or are stripped off by chelating agents. This disadvantage can be overcome by introducing cationic chitosan or polylysine to form a polyelectrolyte reinforced composite, or by grafting covalent crosslinking functionalities to alginate chains. The use of alginate hydrogels has been primarily in the field of controlled release of growth factors such as VEGF (Lee, Yoon et al. 2004), BMP2 and TGF-β3 (Simmons, Alsberg et al. 2004) as well as the encapsulation and transplantation of pancreatic islet cells for diabetes treatments (Sun 1988).

Differing from chitosan, alginate is negatively charged at physiological pH because of the carboxylic acid groups on the backbone. Because of this negative charge, alginate can be fabricated electrostatically via self-assembly. Multilayers of hydrogel microspheres have been
successfully produced by non-covalent conjugation of cationic polymers such as chitosan and polylysine to the anionic alginate.

In addition to ionic crosslinking, alginate hydrogels can also be formed via covalent crosslinking. As shown in Figure A.9, Mooney and coworkers developed a chemical crosslinking method for alginate-based hydrogels where they oxidized poly(guluronate), the crosslinking portion of alginate, and used adipic dihydrazide as the crosslinking agent to form poly(aldehyde guluronate) hydrogels (Bouhadir, Hausman et al. 1999; Lee, Bouhadir et al. 2000). The resulting alginate-derived hydrogels possess a wide range of mechanical properties and are suitable for tissue engineering application when cell-adhesive peptides were incorporated onto the otherwise non-adhesive alginate backbone.

![Figure A.9 Scheme for the synthesis and cross-linking of poly(aldehyde guluronate).](image)

While physical and chemical crosslinking of alginate hydrogels have been explored extensively by several research groups, few reports have been made regarding the biological crosslinking of alginate gels. In this particular area, Mooney’s group developed a novel
crosslinking method based on cell-ligand interaction (Lee, Kong et al. 2003). They first immobilized RGD peptide sequences on an alginate backbone and then utilized the integrin receptors on cell surfaces to crosslink the alginate into a three-dimensional hydrogel structure. There are several advantages of using this novel method for gel crosslinking. First of all, the immobilization of the target cells can be achieved simultaneously during gel formation. Secondly, this biological crosslinking system can be used to study cell-ligand interactions. Figure A.10 shows the structure of sodium alginate and the methods for hydrogel fabrication including electrostatic, chemical, and cellular crosslinking.

![Diagram of Sodium Alginate and Crosslinking Methods](image)

**Figure A.10** Structure of sodium alginate and methods for hydrogel fabrication.

Unlike chitosan hydrogels, which degrade by bond cleavage along the chitosan backbone, the degradation of ionically crosslinked alginate hydrogels occurs when calcium ions, the crosslinker, are removed from the hydrogel network. If alginate is oxidized to form a covalent crosslinked network, the hydrolytic degradation takes place on the acyl hydrazone bonds grafted onto alginate (Bouhadir, Hausman et al. 1999). Mooney and colleagues have extensively studied the degradation behavior (Kong, Alsberg et al. 2004; Kong, Kaigler et al. 2004) as well as biomedical applications of alginate hydrogels (Simmons, Alsberg et al. 2004). They showed that the mechanical rigidity and the degradation rate of alginate hydrogels can
be controlled by adjusting the molecular weight distribution of alginate hydrogels assembled from ionic or covalent crosslinking (Kong, Alsberg et al. 2004; Kong, Kaigler et al. 2004). By controlling the molecular weights of alginate hydrogels in conjunction with delivery of multiple growth factors (TGF-β3 and BMP2), they were able to enhance bone regeneration in vivo (Simmons, Alsberg et al. 2004).

**A.5.3 Fibrin-based Hydrogels**

Fibrin, another source of polymer for fabricating degradable hydrogels, has undergone extensive investigation. Fibrin, derived from fibrinogen, is found in the blood and polymerized by factor XIIIa to form a clot in response to injuries. Fibrin clot degradation is associated with a series of cellular enzymatic activities during wound healing. The most important fibrin-degrading enzyme is plasmin. Clinically, fibrin is applied to wound sites as a glue to stop bleeding after surgeries or dental procedures. Fibrin glue is obtained from mixing fibrinogen and thrombin solutions to form a fibrin clot (Thompson, Letassy et al. 1988). Although not approved in the US because of the potential for blood-borne transmission of diseases, fibrin glue is commonly used in Europe for controlling blood loss.

Due to the delicate design of fibrin activation and degradation by nature, researchers are able to fabricate degradable hydrogels based on fibrin chemistry. Hubbell and coworkers utilized modified fibrin hydrogels as matrices for various biomedical applications such as the controlled delivery of vascular growth factor (VEGF) (Zisch, Schenk et al. 2001; Hubbell, Zisch et al. 2003; Zisch, Lutolf et al. 2003; Ehrbar, Djonov et al. 2004; Ehrbar, Metters et al. 2005; Urech, Bittermann et al. 2005). There are several novelties of their fibrin-based materials. First, VEGF was covalently incorporated into fibrin hydrogels utilizing the transglutaminase activity of factor XIIIa. Second, the delivery of VEGF was mediated by
cellular activity. As shown in Figure A.11, the liberation of growth factors was achieved either non-specifically by the degradation of fibrin network by plasmin (Zisch, Schenk et al. 2001; Hubbell, Zisch et al. 2003; Zisch, Lutolf et al. 2003; Ehrbar, Djonov et al. 2004) or specifically by the incorporation of substrate peptides for enzymes such as matrix metalloproteinases (MMPs) secreted by cells (Ehrbar, Metters et al. 2005; Urech, Bittermann et al. 2005). Not only can this release strategy largely preserve the bioactivity of the growth factors, the cell-demanded release profile can also match to the rate of cell infiltration or tissue regeneration.

Figure A.11 Model schemes: Differential control of VEGF release from fibrin gel matrices by differential susceptibility to local cell-associated proteolytic activities. Top box. Native VEGF_{121} is freely diffusible in the aqueous milieu of the fibrin matrix. Middle box, VEGF variant, a_P1_{1-8}-VEGF_{121}, is protected from diffusion, and its liberation is dependent on the cleavage of the fiber network by cell-associated fibrinolytic enzymes (slow, cell-demanded release). Bottom box, a new VEGF variant, a_P1_{1-8}Pla-VEGF_{121}, was designed to couple to fibrin networks via a plasmin-sensitive anchor. Cleavage of this plasmin-sensitive site by low and local plasmin could occur independent of fiber network degradation and enhance VEGF release rate (accelerated, cell-demanded release). (From Ehrbar, M., Metters, A., et al. 2005. J. Control. Release 101: 93–109. With permission)
Sakiyama-Elbert and Hubbell have developed fibrin hydrogels containing linker peptides and heparins for affinity-based drug delivery specifically used in promoting nerve regeneration (Sakiyama-Elbert and Hubbell 2000; Sakiyama-Elbert and Hubbell 2000). Theoretical model predictions indicate that the release rate of growth factors from these gels can be modulated through the affinity between the peptides and heparin and the concentration of heparin presented in the fibrin gels (Taylor, McDonald et al. 2004). Figure A.12 shows the schematic diagram of the heparin-containing fibrin hydrogels for growth factor delivery (Taylor, McDonald et al. 2004).

**Figure A.12** Diagram showing the components of the heparin-binding delivery system. a2PI1–5–ATIII121–134 peptide is cross-linked into the fibrin gel via the transglutaminase activity of Factor XIII; heparin can bind to the peptide by electrostatic interactions. NT-3 can bind to the bound heparin, creating a gel-bound ternary complex that is not diffusible. NT-3 can also exist in the diffusible state, alone, or in a complex with free heparin. (From Taylor, S.J., McDonald, J.W., et al. 2004. *J. Control. Release* 98: 281–294. With permission.)
In addition to controlled growth-factor delivery, fibrin hydrogels have also been used as tissue engineering scaffolds. For example, fibrin hydrogels have been used as 3-dimensional cell-culture matrices for cardiovascular tissue engineering. It was shown that fibrin gels can support homogenous cell growth, confluent collagen production, and tissue development (Ye, Zund et al. 2000; Jockenhoevel, Zund et al. 2001).

Fibrin hydrogels have also been shown to be biocompatible and can be formed in situ during cartilage repair (Homminga, Buma et al. 1993). However, a potential shortcoming of fibrin hydrogels is that they do not possess significant mechanical strength when applied on load-bearing tissue such as cartilage. Composite scaffolds made form fibrin-polyglycolic acid (Ameer, Mahmood et al. 2002) and fibrin-polyurethane (Lee, Grad et al. 2005) have been developed to increase the mechanical properties of fibrin-based gels for articular cartilage tissue engineering.

A.5.4 Collagen and Gelatin-based Hydrogels

Collagen, a triple helix protein, is the major component of connective tissues. Due to its physiological abundance, collagen is considered biocompatible. Collagen can be biodegraded by enzymes such as collagenase. The application of collagen hydrogels, however, is limited due to its laborious, batch-production procedures as well as the inconsistency of its biological and mechanical properties between batches.

Modified collagen gels are still favored for many tissue-engineering applications. Composite scaffolds such as collagen-alginate (Bohl, Shon et al. 1998) or collagen-hyaluronan (Segura, Anderson et al. 2005; Segura, Chung et al. 2005) have been fabricated and used for several tissue engineering and DNA delivery applications. Matrigel, a type IV collagen-based and a commercially available hydrogel, mimics the ECM environment and is
commonly used in *in vivo* or *in vitro* studies including cell growth and differentiation (Taub, Wang et al. 1990), angiogenesis, and tumor augmentation (Benelli and Albini 1999; Auerbach, Lewis et al. 2003). Collagen hydrogels have also been used to immobilize human neuroblastoma cells for cell-based biosensing (Mao and Kisaalita 2004).

An important consideration of using collagen hydrogels in tissue engineering is that the gels significantly shrink after cell-seeding (Bell, Ivarsson et al. 1979; Nakagawa, Pawelek et al. 1989). Several methods have been developed to suppress the contraction of the collagen hydrogels such as increased crosslinking with glutaraldehyde (Torres, Freyman et al. 2000) or incorporation of short collagen fibers (Gentleman, Nauman et al. 2004). Increasing the crosslinking density of collagen in the scaffold has also been shown to decrease the degradation rate of the gels (Meinel, Hofmann et al. 2004; Meinel, Karageorgiou et al. 2004; Meinel, Karageorgiou et al. 2004; Hu, Jiang et al. 2005).

Gelatin, a natural glycine-rich polymer derived from hydrolyzed collagen, is widely used in food industry as well as in pharmaceutical devices for the controlled release of growth factors. Besides its advantageous biodegradability and biocompatibility, the most attractive characteristic of gelatin is its ability to form polyion complexes. Depending on the manufacturing process, the isoelectric point (PI) of gelatin can be adjusted to yield a positively or negatively charged polymer. This flexibility makes gelatin a suitable matrix for controlled delivery of charged growth factors such as anionic basic fibroblast growth factor (bFGF) (Tabata, Hijikata et al. 1999) and bone morphogenetic protein-2 (BMP-2) (Yamamoto, Takahashi et al. 2003).

Different gelatin formulations have been studied to evaluate the drug loading capacity and release rate. Like the other hydrogels, drug release profiles obtained from gelatin
hydrogels can be readily adjusted by changing the network crosslinking density. Several methods have been developed to crosslink gelatin hydrogels including glutaraldehyde, dehydrothermal treatment, UV or electron beam irradiation (Liang, Chang et al. 2004).

The preparation of gelatin hydrogels is rather easy compared to other natural polymers. For example, gelatin hydrogels can be formed by simply mixing gelatin solution with small amount of the crosslinker (e.g., glutaraldehyde) and left for several hours at 4°C. In addition to the conventional gelation methods such as freeze-drying, gelatin hydrogels can also be prepared via photopolymerization. For example, Matsuda and colleagues have synthesized photocurable, tissue-adhesive gelatin hydrogels for drug release (Okino, Nakayama et al. 2002; Okino, Manabe et al. 2003; Manabe, Okino et al. 2004; Masuda, Furue et al. 2004), arterial repair (Li, Sajiki et al. 2003) as well as for nerve-guidance prosthetic scaffolds (Gamez, Ikezaki et al. 2003). The major advantage of these gelatin hydrogels is that they are photocurable and can be formed via in situ polymerization.

Burmania et al. prepared interpenetrating networks containing gelatin and PEG-diacrylate and further characterized the protein release, fibroblast adhesion, and in vivo host response to these gels (Burmania, Martinez-Diaz et al. 2003; Burmania, Stevens et al. 2003). The chemical and mechanical properties of these gelatin hydrogels can be controlled by changing the weight percentage of gelatin in the IPN network or through chemical modifications of the gel precursors.

**A.5.5 Dextran-based Hydrogels**

Dextran, with subunits consisting of α-1,6-linked D-glucopyranose, is another common, naturally occurring polymer used in the fabrication of degradable hydrogels. Dextran can be readily produced by bacteria or yeast via fermentation and is therefore an
ideal polysaccharide for industrial as well as clinical usage. Dextran is also water-soluble and has been widely used in surgery owing to its antithrombotic effect.

Similar to other hydrogels, dextran hydrogels can be formed via physical or chemical crosslinking. Physically crosslinked dextran hydrogels can be fabricated via electrostatic interaction. Hennink and coworkers have fabricated microspheres with positive and negative charges by modifying dextran with dimethylaminoethyl methacrylate (DMAEMA) and methacrylic acid (MAA), respectively (Figure A.13) (Van Tomme, van Steenbergen et al. 2005). Dextran hydrogels are formed when microspheres with opposite charges were mixed together. One interesting characteristic of this physical hydrogel is that, when sufficient shear is applied, the viscosity of the gel decreases, rendering the gel injectable. After injection the shear is removed and the hydrogel spontaneously reforms.

![Figure A.13 Chemical structures of dex-HEMA (A), methacrylic acid (B) and dimethylaminoethyl methacrylate (C). (From Van Tomme, S.R., van Steenbergen, M.J., et al. 2005. Biomaterials 26: 2129–2135. With permission.)](image)
Another method for fabricating physically crosslinked dextran hydrogels was developed by Hennink and coworkers. It is based on stereocomplexation between D-lactate and L-lactate oligomers (de Jong, De Smedt et al. 2000; Hennink, De Jong et al. 2004). Enantiomeric lactic acid oligomers were grafted to dextran and the dex-lactate hydrogels were formed by stereocomplex formation between D-lactate and L-lactate. The sustained release of pharmaceutical proteins (Cadee, de Groot et al. 2002; Hennink, De Jong et al. 2004) as well as the biocompatibility (Cadee, van Luyn et al. 2000; Cadee, Brouwer et al. 2001) have been demonstrated for these dextran-based hydrogels.

Several modification schemes have been proposed to fabricate degradable dextran hydrogels via chemical crosslinking. For instance, Hennink and coworkers have modified dextran with hydroxyethyl methacrylate (HEMA). The resulting dex-HEMA hydrogels can be formed via free-radical polymerization and crosslinking of the methacrylate side groups (van Dijk Wolthuis, Tsang et al. 1997). Degradable hydrogels were formed by incorporating lactate into the modified dextran chains to form dex-lactate-HEMA hydrogels (Figure A.14A). The degradation rate of these hydrogels can be tailored by varying the length of a spacer unit within the crosslink (Cadee, De Kerf et al. 1999; Cadee, van Luyn et al. 2000; Cadee, Brouwer et al. 2001; Cadee, de Groot et al. 2002). Increasing the size of the crosslink lowers the overall crosslinking density, increases the degree of swelling and water content within the gel, and results in a faster rate of degradation.

PEGylation of dextran is another means of fabricating dextran hydrogels. Moriyama et al. have prepared multi-layered PEG-g-dextran hydrogels for pulsatile drug delivery. In this two-phase system, grafted-PEG chains act as an insulin depot while dextran domains form the main matrix. Upon surface-limited degradation by dextranase, a pulsatile release
profile appears due to the multi-layered structure of the polymer formulation (Moriyama and Yui 1996; Moriyama, Ooya et al. 1999).

![Figure A.14](image_url) Structures of (A) Dex-lactate-HEMA, (B) Dex-maleic acid, and (C) Dex-methacrylate.

Dextran hydrogels can also be prepared via photopolymerization. For example, Kim et al. synthesized dex-maleic acid macromers by reacting dextran with maleic anhydride (Figure A.14B) (Kim, Won et al. 1999). Glycidyl methacrylate has also been reacted with dextran to form dex-GMA photocrosslinkable hydrogels (Figure A.14C) (Pitarresi, Palumbo et al. 2003; Li, Williams et al. 2004).

Interpenetrating networks (IPN) (Kurisawa, Terano et al. 1995; Yamamoto, Kurisawa et al. 1996; Kurisawa, Terano et al. 1997) or semi-IPNs (Kumashiro, Lee et al. 2002) consisting of dextran and other components provide another route for dextran hydrogel preparation. Kurisawa et al. have prepared a series of dextran-based IPNs including Dex-
PEG (Kurisawa, Terano et al. 1995; Kurisawa, Terano et al. 1997) and Dex-Gelatin (Yamamoto, Kurisawa et al. 1996; Kurisawa and Yui 1998; Kurisawa and Yui 1998). These dextran-based hydrogels have been shown to exhibit a double-stimuli-response function and are degradable only when two enzymes, which independently degrade distinctly different substrates, are both present.

**A.5.6 Hyaluronic Acid**

Hyaluronic acid (HA) is another natural polymer derived from glycosaminoglycan composed of D-glucuronic acid and N-acetylglucosamine. Physiologically, HA is the backbone of connective tissues such as cartilage. In addition to its structural importance, HA also possesses many characteristics that make it suitable for biomedical application. For example, HA mediates angiogenesis, wound healing, metastasis, inflammation, and granulation (Chen and Abatangelo 1999). Because of these favorable properties, HA has been used in many clinical applications. However, disadvantages of using HA as tissue engineering scaffolds include its non-adhesive property which prohibits cell adhesion. Furthermore, when HA is used as a scaffolding material, it does not provide enough mechanical strength, which largely limits its application. Fortunately, the first disadvantage can be overcome by grafting cell-adhesive peptides, such as arginine-glycine-aspartic acid (RGD), to HA molecules. By incorporating reactive side functionalities such as acrylate or methacrylate groups (Figure A.15), linear HA chains can be crosslinked to form water-swellable hydrogels with adequate mechanical strengths.

Collagen, glycidyl methacrylate, methacrylic anhydride, and gelatin have been used to crosslink HA chains and form cell-adhesive, mechanically stable hydrogels. For example, Park et al. have prepared methacrylated, RGD-containing HA-based hydrogels via
photopolymerization (Park, Tirelli et al. 2003). The cellular response as well as the gel swelling ratio and mechanical properties were extensively studied. Leach et al. have synthesized a photocrosslinkable HA by reacting it with the epoxy group of glycidyl methacrylate (GMA) (Leach, Bivens et al. 2003). The pendant methacrylate bonds present on the resulting GMA-modified HA molecules (GMHA) facilitate photopolymerization and crosslinking of the polymer chains. Furthermore, an acrylated PEG-peptide can be copolymerized into the GMHA hydrogels to render them cell adhesive (Leach, Bivens et al. 2004).

![Figure A.15](image_url)  
**Figure A.15** Reaction schemes for methacrylation of hyaluronic acid through (A) N-(3-aminopropyl)-methacrylamide, (B) glycidyl methacrylate, and (C) methacrylic anhydride.

When presented in vivo, hyaluronic acid hydrogels can be degraded by hyaluronidase (Menzel and Farr 1998) or hydroxyl radicals (Yui, Nihira et al. 1993; Hawkins and Davies 1998) produced at the inflammation sites. Hyaluronidase-mediated degradation is relatively slow compared to degradation by hydroxyl radicals. This is mainly because of the low physiological concentration of hyaluronidase. On the other hand, hydroxyl radicals generated in the inflammation sites can effectively degrade HA via glycosidic cleavage (Yui,
Nihira et al. 1993; Hawkins and Davies 1998). As shown in Figure A.16, the degradation rate of GMHA hydrogels is slower than native HA hydrogels (Leach, Bivens et al. 2003). Leach et al. have also tested the in vitro degradability of the GMHA hydrogels using hyaluronidase (Leach, Bivens et al. 2003; Leach, Bivens et al. 2004). Finally, Langer and coworkers were able to better control the resulting GMHA-hydrogel swelling and degradation behavior by partially oxidizing or grafting an oligomer onto the multifunctional HA chains prior to conjugation with GMA (Jia, Burdick et al. 2004).

![Figure A.16](image)

**Figure A.16** Enzymatic degradation of HA hydrogels. (a) Native HA (shown schematically) is quickly degraded in vivo by the enzyme hyaluronidase. (b) Cross-linking the HA chains forms an insoluble hydrogel matrix that is more resistant to enzymatic degradation. (From Leach, J.B., Bivens, K.A., et al. 2003. Biotechnol. Bioeng. 82: 578–589. With permission.)

Methacrylic anhydride has also been reacted with HA to form methacrylated HA (HAMA) for subsequent gel formation (Masters, Shah et al. 2004; Burdick, Chung et al. 2005; Masters, Shah et al. 2005). Applications of HA hydrogels based on this chemistry include the incorporation of specialized peptides to enhance the adhesion and proliferation of valvular interstitial cells (Masters, Shah et al. 2004; Masters, Shah et al. 2005) as well as the
photoencapsulation of chondrocytes for cartilage regeneration (Burdick, Chung et al. 2005). Smeds and Grinstaff have prepared photocrosslinkable HA hydrogels by reacting HA with excess methacrylic anhydride (Smeds and Grinstaff 2001). The gel formation was rapid and its \textit{in situ} photopolymerization enables minimally invasive implantation when used on an articular cartilage repair application (Nettles, Vail et al. 2004).

A.6 Degradable Hydrogels Derived from Synthetic Biomaterials

Degradable hydrogels made from synthetic polymers have recently gained considerable interest. The most common synthetic polymers used to construct hydrogel networks include poly(ethylene glycol) or poly(ethylene oxide), poly(vinyl alcohol), poly(N-isopropylacrylamide), and others. The most apparent advantage of using synthetic polymers is the high degree of control over the polymer chemistry, architecture, and physical hydrogel properties including degradation behavior. Hydrogels made from synthetic polymers can be engineered with tailorable hydrophilicity, crosslinking density, and degradability. In addition, when degraded \textit{in vivo}, there is no immune recognition associated with the low molecular weight degradation products if the polymer and hydrogel fabrication technique are carefully chosen.

A.6.1 Poly(ethylene glycol)-based Hydrogels

Poly(ethylene glycol) is a non-ionic, hydrophilic polymer. Because of this, PEG is widely used to create nonfouling surfaces which repel non-specific protein adsorption and cell adhesion. Many researchers have utilized the terminal hydroxyl groups on PEG molecules to modify PEG into a crosslinkable material. The most commonly used chemistry is the acrylation or methacrylation to endcap linear, branched, or star-shaped PEG molecules with reactive vinyl species (Figure A.17). Free-radical polymerization of these methacrylate
or acrylate groups results in crosslinked hydrogels that are stable over clinically relevant timescales.

Hydrolytically or enzymatically labile groups can be introduced into crosslinked PEG networks by modifying the soluble PEG precursors with ester-containing \( \square \)-hydroxy acid oligomers or protease-sensitive peptide blocks prior to vinyl-group endcapping. Upon polymerization and crosslinking, these bonds will render the resulting hydrogels degradable. In particular, the incorporation of hydrolytically degradable poly(lactic acid) (PLA) groups greatly increase the potential applications of PEG hydrogels (Figure A.2). Sawhney et al. pioneered the acrylation of PLA-PEG-PLA diblock macromers for \textit{in situ} formation of degradable hydrogels (Sawhney, Pathak et al. 1993). Since that time, Bowman and Anseth and their colleagues have synthesized a series of degradable PLA-PEG-PLA hydrogels and extensively characterized their degradability via experimental observation as well as theoretical verification (Metters, Anseth et al. 2000; Metters, Anseth et al. 2000; Metters, Bowman et al. 2000; Mason, Metters et al. 2001; Metters, Anseth et al. 2001; Metters, Bowman et al. 2001).

\[ \text{Poly(ethylene glycol)} \quad \xrightarrow{\text{PEG diacrylate}} \quad \text{H}_2\text{C} \cdots \text{C} \cdots \text{O} \cdots \text{O} \left( \text{CH}_2\text{CH}_2\text{O} \right)_n \text{C} \cdots \text{C} \cdots \text{CH}_2 \]

\[ \text{PEG Methacrylate} \]

\[ \text{Poly(propylene fumarate)-co-ethylene glycol} \]

\[ \text{Figure A.17} \] Poly(ethylene glycol) and its derivatives for PEG-based hydrogel synthesis.
Mikos and colleagues have synthesized a series of PEG-containing, water-soluble block copolymers, including poly(propylene fumarate)-co-ethylene glycol) (Suggs, Kao et al. 1998; Behravesh, Jo et al. 2002; Tanahashi, Jo et al. 2002) and oligo(poly(ethylene glycol) fumarate) (Figure A.17) (Jo, Shin et al. 2001; Shin, Jo et al. 2002; Temenoff, Athanasiou et al. 2002). These hydrogels are biodegradable, injectable, in situ photocrosslinkable, and biocompatible. Because of these properties, they hold great potential both in drug delivery (Holland, Tabata et al. 2003; Kasper, Kushibiki et al. 2005; Park, Temenoff et al. 2005) and tissue engineering applications (Shin, Jo et al. 2002; Behravesh and Mikos 2003; Shin, Ruhe et al. 2003; Temenoff, Shin et al. 2003; Fisher, Jo et al. 2004; Shin, Zygourakis et al. 2004). The degradation of these hydrogels occurs at the ester linkage within the poly(propylene fumarate) block (Suggs, Krishnan et al. 1998) and can be accelerated by decreasing pH and crosslinking density (Timmer, Ambrose et al. 2003).

Recently, Hubbell and coworkers have used Michael-type addition reactions to form PEG-based hydrogels by reacting vinyl-sulfone functionalized PEG chains with thiol groups present on other macromers. This conjugation reaction can be carried out under physiological conditions without the use of initiators (Elbert, Pratt et al. 2001; Heggli, Tirelli et al. 2003; Lutolf and Hubbell 2003; van de Wetering, Metters et al. 2005) which produce free radicals and often induce damage to the encapsulated protein (Lin and Metters 2005), DNA (Quick and Anseth 2003; Quick and Anseth 2004; Quick, Macdonald et al. 2004) or cells (Quick and Anseth 2003).

West and colleagues have incorporated enzyme-sensitive peptides into PEG-based hydrogels. The resulting peptide-incorporated PEG-based hydrogels can be degraded by collagenase or plasmin (West and Hubbell 1999; Mann, Gobin et al. 2001; Gobin and West
The incorporation of enzyme-sensitive peptides not only facilitates gel degradation but also enhances cell migration (Gobin and West 2002). Hubbell and coworkers have also modified PEG hydrogels with recombinantly produced peptides that are proteolytically labile (Park, Lutolf et al. 2004). The main advantage of these approaches is that the cellularly controlled gel-degradation rate self-adjusts to the rate of cell infiltration, making these gels extremely attractive for tissue regeneration applications.

**A.6.2 Poly(vinyl alcohol)-based Hydrogels**

Poly(vinyl alcohol) hydrogels were one of the earliest hydrogels to play an important role in biomedical applications (Brinkman, Vanderdoes et al. 1991). Unlike PEG, which has at most two derivatizable hydroxyl groups at the chain termini, PVA possesses numerous pendant hydroxyl groups which can be modified for crosslinking or ligand attachment. Both physical and chemical crosslinking methods can be used to fabricate PVA hydrogels. Peppas pioneered the fabrication of PVA hydrogels by the freeze-thaw process (Peppas and Scott 1992; Stauffer and Peppas 1992; Mongia, Anseth et al. 1996). The resulting PVA hydrogels were used for controlled release (Peppas and Scott 1992) and wound healing applications (Mongia, Anseth et al. 1996).

In addition to the freeze-thaw process, PVA hydrogels can be physically crosslinked using blend copolymers. Some examples of polymers that can be blended with PVA to create stable hydrogels are polysaccharides including chitosan and dextran. Cascone et al. reviewed the fabrication and applications of several PVA/polysaccharide blend hydrogels (Cascone, Barbani et al. 2001). These hydrogels could be used in drug delivery systems and are capable of delivering human growth hormone in physiological amounts.
To create chemically crosslinked PVA hydrogels that can be formed in situ, Anseth and coworkers synthesized a series of photocurable PVA-based hydrogels and characterized their mechanical properties as well as degradability (Martens and Anseth 2000; Nuttelman, Henry et al. 2002; Bryant, Davis-Arheart et al. 2004). Specifically, fibronectin-modified PVA hydrogels were found to enhance NIH3T3 cell adhesion, proliferation, and migration (Nuttelman, Mortisen et al. 2001). PLA-g-PVA hydrogels were found to improve valve interstitial cell adhesion by increasing gel hydrophobicity (Nuttelman, Henry et al. 2002). PEG-PVA hydrogels with tailorable characteristics were used in cartilage tissue engineering (Figure A.18) (Martens, Bryant et al. 2003). West and coworkers also prepared photocrosslinkable PVA hydrogels modified with cell-adhesive peptides for encouraging cell attachment (Schmedlen, Masters et al. 2002).

Figure A.18 An idealized schematic of the structure of hydrogels formulated by copolymerizing poly(ethylene glycol)-lactic acid-dimethacrylate (PEG-LA-DM) with acrylate-ester-poly(vinyl alcohol) (Acr-Ester-PVA). The network consists of kinetic chains (light lines) connected via PEG cross-links (dotted lines) and the multifunctional PVA chains (bold lines). (From Martens, P.J., Bryant, S.J., et al. 2003. Biomacromolecules 4: 283–292. With permission.)
A.6.3 Polyacrylamide-based Hydrogels

Polyacrylamide gels are widely used for protein separation (e.g., SDS-PAGE) and are prepared from free-radical polymerization of acrylamide monomer and N,N’-methylene bisacrylamide (BIS) crosslinker. The free radicals are typically generated via a redox reaction involving two components, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). To fulfill the desired protein separation requirements, the crosslinking density of these gels can be increased by increasing the monomer concentration or crosslinker to monomer ratio. While acrylamide bonds are considered to be stable in aqueous environments, the unique architecture of the bisacrylamide crosslinker enables it to be cleaved via hydrolytic attack. Hydrolytic cleavage of the crosslinker leads to eventual degradation of the polyacrylamide gel.

Hydrogels made from N-(2-hydroxypropyl)-methacrylamide (HPMA) have been prepared via radical polymerization (Figure A.19) (Ulbrich, Subr et al. 1993; Ulbrich, Subr et al. 1995). The resulting poly(HPMA) hydrogels have been shown to be stable in acidic environments but hydrolytically degradable at pH 7.4 (Ulbrich, Subr et al. 1995). Applications of these degradable methacrylamide-based hydrogels include controlled drug delivery (Ulbrich, Subr et al. 1995; Stastny, Plocova et al. 2002) and gene therapy (Howard, Dash et al. 2000; Oupicky, Ogris et al. 2002). Poly(HPMA) hydrogels have also been used to conjugate doxorubicin, an anti-cancer drug, via enzymatically or hydrolytically labile linkages (St'astny, Plocova et al. 2002; Stastny, Plocova et al. 2002). Prolonged, degradation-controlled release of this drug from poly(HPMA) gels has been shown to effectively reduce tumor size as well as increase survival time in an animal model (St'astny, Plocova et al. 2002).
Another well-known application of polyacrylamide-based polymers is the fabrication of thermally responsive poly(N-isopropylacrylamide) (PNIPAAM) hydrogels. PNIPAAM, with a lower critical solution temperature (LCST) around 32°C, exhibits a reversible thermo-sensitive swelling behavior. Above the LCST, the PNIPAAM polymer collapses and precipitates from the surrounding solution due to strong intra-molecular hydrophobic interactions. Below the LCST, the polymer chain swells and is soluble. This attractive feature allows PNIPAAM at a temperature below the LCST to be readily injected into the body. Immediately following injection, the system temperature increases to 37°C and the PNIPAAM gel forms in situ.

![Figure A.19](image_url)  

Although PNIPAAM itself is a non-degradable polymer, many efforts have been made to incorporate degradable functionalities to render degradable PNIPAAM hydrogels.
These copolymers include dextran (Huh, Hashi et al. 2000; Kumashiro, Huh et al. 2001; Kumashiro, Lee et al. 2002; Huang and Lowe 2005), acrylic acid (Kim and Healy 2003), dimethylacrylamide (Kurisawa, Matsuo et al. 1998; Kurisawa and Yui 1998), poly(amine acids) (Yoshida, Aoyagi et al. 2003), and poly(lactic acid) (Xiao, Nayak et al. 2004). The temperature-responsiveness and biodegradability of these gels make them unique and in many cases allows better control over physical gel properties and function. For example, the degree of swelling, degradation rate, and release rate of drugs from hydrogels containing PNIPAAm can be modulated by small temperature changes (Kurisawa, Matsuo et al. 1998).

A.6.4 Polyphosphazene-based Hydrogels

As a degradable synthetic hydrogel for biomedical applications, polyphosphazene has gained much attention for controlled drug release. Readers are referred to recent review articles for a more in-depth discussion of polyphosphazene polymers (Andrianov and Payne 1998). Unlike most of the other synthetic polymers with carbon-carbon backbones, polyphosphazene, on the other hand, possesses a backbone with alternative phosphorus and nitrogen atoms and two substitutive groups on phosphorus atoms. Polyphosphazenes can be synthesized from precursor macromers poly(dichlorophosphazene) into water soluble polyphosphazene polyacid. The resulting polyacid can then be crosslinked into ionic hydrogels (Figure A.20) (Andrianov, Chen et al. 1998; Andrianov, Svirkin et al. 2004). The degradation rate of polyphosphazene has been shown to depend on the degree of substitution of the pendant groups. Specifically, the degradation rate decreases with a decreasing degree of substitution (Andrianov, Svirkin et al. 2004).
Many modifications have been proposed in the fabrication of polyphosphazene hydrogels. For example, Allcock et al. have synthesized a series of phosphazene hydrogels for biomedical applications including, but not limited to, poly[(amino acid-ester) phosphazenes] (Allcock, Pucher et al. 1994), poly(alkyl oxybenzoate) phosphazene (Greish, Bender et al. 2005), tyrosine-bearing polyphosphazenes (Allcock, Singh et al. 2003), and polyphosphazene blend copolymers (Ibim, Ambrosio et al. 1997; Ambrosio, Allcock et al. 2002). Polyphosphazene can also be fabricated as thermo- or pH-responsive hydrogels.

A.6.5 Protein-Crosslinked Hydrogels

Hydrogels made from synthetic polymers exhibit many favorable characteristics as discussed in the previous sections. However, one of the drawbacks of using pure synthetic hydrogels is that they do not possess biological recognition sites for cell-material interactions that may be advantageous for tissue engineering applications. For this reason, efforts have been made to develop hydrogels with synthetic polymer backbones and biologically derived crosslinks. The use of proteins as crosslinkers for synthetic hydrogels can be traced back to the early 90’s where albumin was used to crosslink poly(1-vinyl-2-pyrrolidinone) (PVP) hydrogels (Shalaby, Blevins et al. 1990; Shalaby, Blevins et al. 1991) The crosslinking density
of the PVP hydrogels can be controlled by the degree of albumin functionality and the concentration of albumin. Furthermore, albumin-crosslinked hydrogels can be enzymatically degraded by pepsin via surface erosion or bulk degradation depending on the functionality of the albumin and the overall crosslinking density of the gel.

More recently, PEGylated fibrinogen has also been used to crosslink PEG hydrogels. Several advantages exist for using fibrinogen as a hydrogel crosslinking agent. For example, fibrinogen possesses inherent cell-recognition sites for cellular ingrowth as well as enzymatically degradable peptide sequences for gel degradation (Almany and Seliktar 2005).

While protein-crosslinked hydrogels provide sites for cell-material interactions, genetically engineered or artificial proteins have emerged as alternative tools for crosslinking synthetic, polymer-based hydrogels. Recombinant DNA technology was used to create artificial proteins or peptides as building blocks within otherwise synthetic hydrogels. Hydrogels composed or crosslinked by genetically engineered proteins preserve all the favored characteristics of natural-protein crosslinkers while eliminating excessive recognition sites that may prove detrimental to successful application. Differing from conventional synthetic polymers and natural proteins, genetically engineered proteins have monodisperse molecular weights and precisely engineered functionalities.

Owing to this fine tuning over polymer size and chemistry, researchers are able to better predict and determine the physiological or biological fate of the artificial biopolymers (Haider, Megeed et al. 2004). Examples of genetically engineered proteins used for degradable hydrogels are elastin-like proteins (ELPs) (Megeed, Haider et al. 2004), silk-elastinlike proteins (SELPs) (Megeed, Cappello et al. 2002) and coiled-coil proteins (Petka, Harden et al. 1998; Wang, Stewart et al. 1999; Wang, Kopecek et al. 2001; Kopecek 2003).
Hydrogels made from these materials have been used in many biomedical applications focusing on drug release (Kopecek 2003; Haider, Megeed et al. 2004), gene delivery (Megeed, Cappello et al. 2002; Megeed, Haider et al. 2004), and tissue engineering (Panitch, Yamaoka et al. 1999).

Tirrell and coworkers pioneered the study of artificial-protein hydrogels in which the synthetically engineered proteins retain at least two domains, one for water retention and another for hydrogel network formation (Petka, Harden et al. 1998). The resulting hydrogels can be delicately designed so that the gel is stimuli-sensitive. The mechanical properties of artificial-protein matrices were also determined and used to control cell and tissue behavior (Di Zio and Tirrell 2003). In addition to the stimuli-controlled swelling, another advantage of incorporating artificial proteins into synthetic hydrogels is that many key features of the extracellular matrix, including fibrinogen and elastin domains, can be engineered in vitro and used to promote tissue regeneration in vivo (Di Zio and Tirrell 2003).

Kopecek and coworkers have developed genetically engineered stimuli-sensitive hydrogels based on coiled-coil proteins for controlled release and tissue engineering (Wang, Stewart et al. 1999). As shown in Figure A.21, recombinant coiled-coil protein was used to crosslink N-(2-hydroxypropyl)-methacrylamide (HPMA) hydrogels containing metal-chelating monomer N-(N',N'-dicarboxymethylaminopropyl) methacrylamide (DAMA) (Wang, Stewart et al. 1999). The resulting poly(HPMA-co-DAMA) hydrogels can swell or shrink in response to environmental changes and are very useful in stimuli-sensitive drug release (Xu, Breedveld et al. 2005). On the other hand, the designed protein or peptide sequence can be used as an epitope when incorporated into hydrogels for cellular recognition (Tang, Wang et al. 2000; Tang, Wang et al. 2001).
Hubbell and coworkers have also devoted significant efforts to the design and fabrication of PEG-based hydrogels containing artificial-proteins. They created several model synthetic polymeric systems containing molecularly engineered peptides or proteins for mimicking the natural ECM (Halstenberg, Panitch et al. 2002; Lutolf, Lauer-Fields et al. 2003; Lutolf, Weber et al. 2003; Seliktar, Zisch et al. 2004; Raeber, Lutolf et al. 2005). In these systems, the proteins are incorporated as highly mobile pendant chains rather than as crosslinks. The pendant protein systems are not stimuli-sensitive, but do facilitate the development of synthetic scaffolds for use in tissue engineering. They also provide a
platform for investigating some of the fundamental yet critical cellular behavior such as migration, secretion, and proliferation. For example, a PEG-based hydrogel bearing a matrix metalloproteinase (MMP) inhibitor and tumor necrosis factor-alpha (TNF-α) was used to investigate cellular protease activity as well as cell migration (Raeber, Lutolf et al. 2005).

A.7 Conclusions

Degradable hydrogels have already been successfully employed in numerous biomedical applications. Because of their unique combination of properties, they have the potential to dramatically impact the future of biomaterials and biomedicine, especially in the fields of controlled drug delivery and tissue engineering. Irregardless of their chosen application, degradable hydrogels must meet a number of design criteria to function appropriately in complex biological environments. They must be biocompatible, mechanically resilient, selectively permeable, and degradable over appropriate timescales. Furthermore, the degradation behavior of all biodegradable hydrogels should be well defined, reproducible and tunable via precursor chemistry and structure.

The chemistries of all hydrogel components are critical to maintaining sufficient biocompatibility, degradability and hydrophilicity. Many polymers derived from biological sources are readily crosslinked into biocompatible, resorbable hydrogels. However, these materials suffer from concerns of reproducibility and engineerability. Synthetic polymers offer better engineerability in the chemistry, structure, and physicochemical properties of biodegradable hydrogels, but are not necessarily biodegradable nor biocompatible. Degradability is typically engineered into these systems via unique macromonomer designs (e.g., dimethacrylated PLA-PEG-PLA triblock copolymers) or specialized polymerization reactions that create labile bonds (e.g., Michael-type additions between an acrylate and thiol).
Future designs of biodegradable hydrogels will rely on synthesizing gels exhibiting the advantages of both natural and synthetic polymers. Novel methods of genetic engineering and controlled polymerizations such as atom-transfer radical polymerizations (ATRP) are currently producing polymers with precisely defined molecular weights and functionalities that can be used to fabricate biodegradable hydrogels with exact physicochemical properties and degradation behaviors.

Finally, network structure also plays a key role in determining the elasticity, water content, permeability, and degradation behavior of biodegradable hydrogels. The significant majority of biodegradable gels currently used in biomedical applications degrade via cleavage of a small number of known hydrolytically or enzymatically labile bonds. However, the progression in observable hydrogel properties such as degree of swelling, modulus, and mass loss during degradation varies greatly due to differences in the location, arrangement, distribution, and total concentration of these labile bonds within the network structure. Network structure is a function of precursor chemistry, precursor architecture, and method of gel fabrication (e.g., chain versus step-growth polymerization). In most cases it cannot be experimentally characterized and is made more complex by interpolymer interactions as well as the occurrence of structural defects during fabrication. While physical crosslinking is readily accomplished in a number of systems, covalent crosslinking methods remove the need for interpolymer interactions and lead to the formation of homogeneous networks with relatively uniform chain distributions and crosslinking densities. In addition, fundamental thermodynamic principles can be used to relate experimentally observable gel properties during degradation of these gels to their time-dependent crosslinking density or average molecular weight between crosslinks (Mc). These relationships have already been verified.
for covalently crosslinked PLA-PEG-PLA gels. Development of the next generation of biodegradable hydrogels with precise combinations of properties (e.g., in situ formation, cell-mediated degradation, high mechanical strength and solute permeability, etc.) will depend on furthering our understanding of hydrogel structure while also increasing our library of available biodegradable and biocompatible polymer chemistries.

A.8 Reference


