Drug Delivery Coatings for Cardiovascular Stents: Silicone Elastomer and Thrombin Responsive Hydrogel Coatings

Katherine S. Walline

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This is to certify that I have examined this copy of a master's thesis by

Katherine S. Walline

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

Committee Members:

_____
Buddy D. Ratner

_____
François Baneyx

Date:_____

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1 INTRODUCTION

1.1 Stents and Restenosis: The Problem

Percutaneous transluminal coronary revascularization (PTCR) is a widely performed procedure used to open coronary arteries that have been blocked due to atherosclerotic plaque. PTCR is performed more than 800,000 times annually in the United States [1] and more than 2,000,000 times annually worldwide [2]. PTCR is most commonly performed by a method called balloon angioplasty, where a small balloon is threaded into the blocked artery and inflated.

Stents are metal mesh tubes that were developed to prevent the artery from collapsing upon itself immediately after PTCR. Stents are implanted during PTCR and remain in the artery permanently to hold the artery open. Implantation is performed by placing a collapsed stent onto a balloon angioplasty device, then expanding the stent at the site of blockage by inflating the balloon. The stent then remains in its expanded state, holding the artery open. Stents reduce acute and long-term risk of complications such as restenosis, or reclosure of the artery, but have not eliminated the problem [3]. Stents were first implanted into humans in 1986 [2] and were approved by the FDA in 1994 [4]. Today, stents are implanted in more than 70% of PTCR procedures [3]. Figure 1.1.1 shows a diagram of a healthy artery, one that is partially blocked, and what an artery might look like after PTCR with and without a stent.

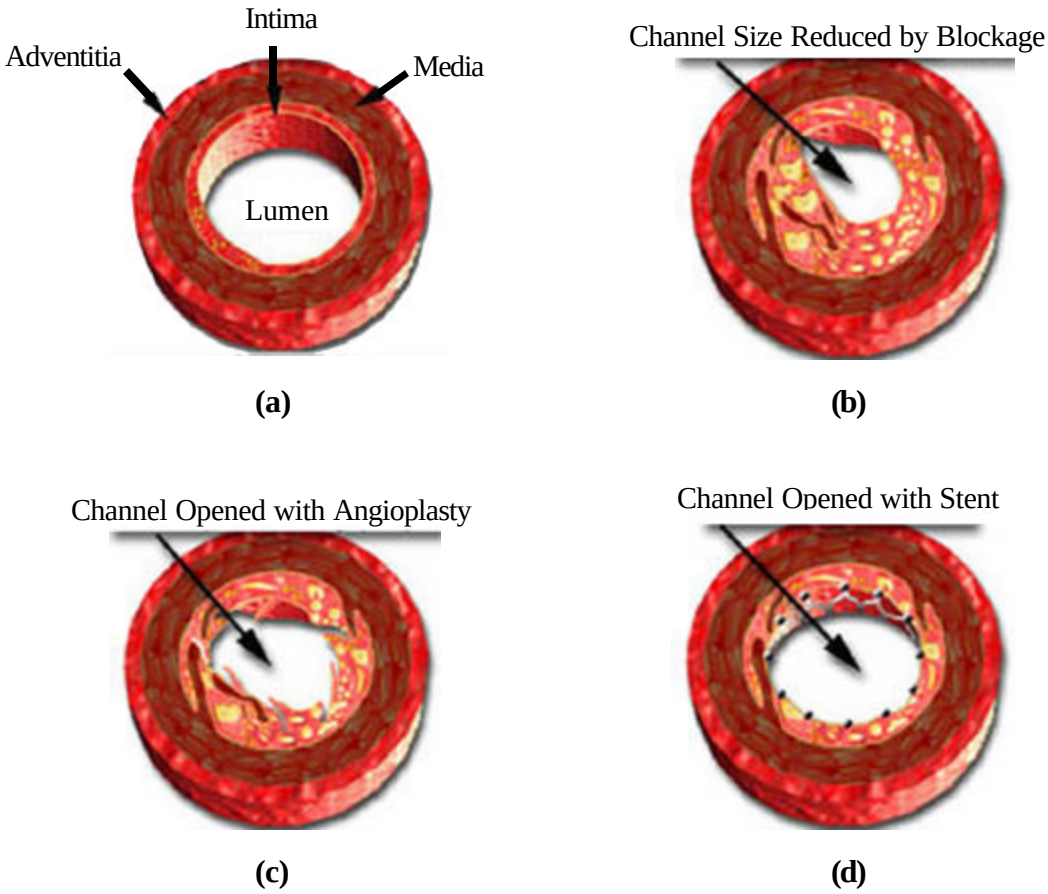

Figure 1.1.1: Cross-sectional views of a coronary artery. **(a)** Healthy coronary artery. **(b)** Partially blocked coronary artery. **(c)** Coronary artery after balloon angioplasty. **(d)** Coronary artery after angioplasty with stent implantation. Pictures adapted from [5], with permission.

In the weeks or months after a PTCR procedure, restenosis, or reclosure of the artery, can occur. Although stents help alleviate the problem of restenosis, in-stent restenosis still occurs. For statistical purposes, restenosis can be defined more specifically in several ways. Two of the most common ways are 1) in terms of an angiographic result or 2) a clinical result. Angiographic restenosis is defined as a certain percent loss in lumen diameter, as determined by an angiogram at some time point after the PTCR procedure. Clinical restenosis can be defined as the need for reopening of the artery, by a repeat PTCR or by bypass surgery [2, 3]. Depending on the definition used,

restenosis occurs in 30-60% of patients who have PTCR without the implantation of a stent and in 15-30% of patients receiving stents [1, 6].

In the absence of a stent, the primary causes of restenosis after angioplasty are elastic recoil, or vasoconstriction due to endothelial disruption, and long term remodeling, or shrinkage, of the vessel [2, 7]. When a stent is implanted, restenosis (in-stent restenosis) is primarily caused by neointimal proliferation [8, 9], whereby the intimal layer becomes thicker due to proliferating smooth muscle cells (SMCs) and extracellular matrix formation [10-12]. Restenosis is the primary cause of long-term complications once short term success has been achieved [3]. The course of events of restenosis is as follows: [13]

- Within the first 24 hours, elastic recoil, or constriction of the vessel due to endothelial disruption occurs.
- Within the first few weeks, thrombus formation can occur with local platelet activation and thrombin secretion; along with an increase in SMC activation, proliferation, and migration; and leukocyte recruitment. This induces neointimal hyperplasia, which occurs for weeks or months.
- Smooth muscle cell proliferation continues and a large amount of extracellular matrix is produced, causing neointimal growth.

In-stent restenotic lesions have a larger content of smooth muscle cells than restenotic lesions from balloon PTCR without stents. Smooth muscle cells occupy $96 \pm 2\%$ of total plaque area in lesions from humans with in-stent restenosis and $46 \pm 6\%$ of plaque area in post-PTCR lesions without stents. This proliferation of SMCs indicates that administration of antiproliferative drugs may be particularly beneficial in the treatment of in-stent restenosis [14].

1.2 Local Drug Delivery as a Potential Solution

General Principles of Local Delivery

A potential solution to the problem of in-stent restenosis is local delivery of antiproliferative agents to the artery post-PTCR. Studies have shown local delivery of antiproliferatives to be effective at decreasing SMC proliferation [15-33]. Local delivery of a drug has several advantages over systemic delivery. For example, high concentrations of the drug can be delivered directly to the tissue of interest, minimizing the negative side effects associated with systemic delivery.

When looking at the artery as a potential site for local drug delivery, there are several options for the route of delivery. The drug could be delivered during the angioplasty procedure, via administration to the arterial wall from the angioplasty device. A second option is to deliver the drug from a coating on the stent itself. The advantage of the second option is that the delivery can be sustained much longer than from administration during the angioplasty procedure. Additionally, the drug is delivered right where restenosis would take place, that is, directly around the stent.

Drug eluting stents have been the subject of much current research. Several drug eluting stents have been approved for clinical use in Europe and one has been approved for use in the United States. The first FDA approved drug eluting stent was the Cypher sirolimus (Rapamycin®) eluting stent, made by Cordis, Corp., a Johnson and Johnson company. It received FDA approval in April 2003 [1]. The clinical studies of this stent showed that it reduced restenosis by two thirds [1].

Two other drug eluting stents have been approved in Europe and are awaiting US approval: the Cook V-Flex Plus and the Boston Scientific Taxus stent [34]. These two stents release the drug paclitaxel (Taxol®). The Cook V-Flex stent releases paclitaxel from a polymer free coating, and the Boston Scientific Taxus stent releases paclitaxel from a proprietary polymeric coating over the course of several weeks. In the clinical trial that studied the V-Flex stent, a 3.1% restenosis rate was reported [35]. In the clinical trial studying the Taxus stent, a 4% restenosis rate was reported [36].

There are several important design characteristics for a drug eluting stent, which include a biocompatible, thin coating, so as not to interfere with blood flow in the artery. Additionally, the coating should have maximal drug loading capacity, to ensure that efficacious levels are reached, and to minimize the coating thickness. The coating also should not interfere with the performance of the stent. Finally, the coating should be tailored to release the drug with optimal kinetics, such that drug concentrations in the affected tissue are at efficacious, but not toxic, levels for a beneficial amount of time.

Paclitaxel

Paclitaxel (Taxol®) is a lipophilic drug that has been shown to prevent restenosis both with oral administration [37] and local delivery [16-19, 21, 37, 38]. Paclitaxel prevents the proliferation of human arterial smooth muscle cells by shifting the balance of intramolecular microtubule assembly and disassembly towards assembly, thus producing extremely stable, unorganized microtubules inside the cytoplasm. Microtubules have many functions in the cell, the primary one being miotic spindle formation during cell division. Additionally, microtubules are involved in functions such as migration, signal transduction, shape changes, and intracellular transport. The formation of extremely stable and unorganized microtubules results in cell death and the cessation of cell replication [15, 39, 40].

Paclitaxel is an ideal candidate for local delivery because it can easily pass through the hydrophobic barrier of cell membranes, due to its lipophilic nature, leading to rapid cellular uptake. This mechanism can lead to long lasting effects, even with small doses. Furthermore, a significant amount of paclitaxel may be retained by membrane lipids, resulting in continuous release even after the drug has been completely released from the device [26]. Creel et al. [41] investigated the arterial distribution and deposition of paclitaxel ex vivo and observed tissue concentrations of the drug to be 100-fold higher than perfusate concentration. This observation indicates that a significant amount of drug binds to the tissue near the site of delivery.

Axel and Sollott showed that paclitaxel inhibits the proliferation and migration of human, rat, and rabbit arterial smooth muscle cells in a dose-dependent manner [15, 37].

Axel et al. showed inhibition of proliferation of human arterial smooth muscle cells at concentrations between 0.01 and 10 μM [15]. Clinical trials have shown restenosis rates ranging from 0-4% for paclitaxel coated stents compared to 11-27% for stents without paclitaxel in patients six months post implantation [42].

Tranilast

The drug tranilast (Rizaben®) has gained attention as a treatment for restenosis. Tranilast is a hydrophilic drug that has been shown to inhibit migration and proliferation of vascular smooth muscle cells induced by platelet-derived growth factor and transforming growth factor-beta (TGF-β) [43, 44]. Tranilast also inhibits collagen synthesis by these cells [45-47]. Tranilast inhibits cytokine and chemical mediator production and release by inflammatory cells and macrophages [43, 44]. Several clinical trials have shown that oral administration of 300 and 600 mg/day of tranilast reduced restenosis rates in patients after PTCR [45, 48]. However, in one clinical trial, tranilast in doses of 300 and 450 mg/day was not effective in treating restenosis [49]. Because of these conflicting results, research on effective dosing has continued. Ward et al. [44] showed that tranilast, given at a dose of 10 mg/kg orally, twice daily, resulted in a 48% reduction in maximal neointimal cross sectional area 28 days after implantation of a stent in Boston minipigs. It has been found that maximal effectiveness of the drug to inhibit SMC migration in vitro occurs between 100 and 300 μM [50, 51]. Local delivery of this drug would allow greater concentration of the drug within the artery without increasing systemic levels.

1.3 Principles of Controlled Drug Release

The controlled release of drugs for medical applications has been investigated since the mid-1960's [52]. The concept of controlled release is to release a bioactive agent over a designated period of time at a particular rate. The time course of release could be days or years. Controlled release devices most commonly utilize polymeric systems to control the release kinetics. Table 1.1 shows a classification system,

developed by Langer and Peppas [52] and later modified by Narasimhan [53], of controlled release devices.

Table 1.1: Classification of Controlled Release Systems.

- **Diffusion Controlled Systems** – Diffusion of the drug through a polymer controls the release rate.
  - Reservoir (Membrane) Devices
  - Monolithic (Matrix) Devices
- **Swelling Controlled Systems** – Swelling of the polymer, and subsequent diffusion of the drug through the polymer controls the release rate.
- **Chemically Controlled Systems** – Hydrolytic or enzymatic cleavage results in polymer swelling, erosion, or release of covalently bonded drug by cleavage of a pendent chain.
- **Osmotically Controlled Systems** – Osmotic forces control the release of drug.
- **Dissolution Controlled Systems** – Dissolution of the polymeric carrier results in release of drug.
- **Externally Controlled Systems** – An external stimuli results in release of drug.
  - Magnetically Controlled Systems
  - Ultrasound Controlled Systems

The research presented in this thesis involves silicone diffusion controlled systems (Chapter 2), poly(hydroxyethyl acrylate) hydrogel swelling controlled systems (Chapter 3), and chemically controlled hydrogel systems (Chapter 4).

Diffusion Controlled Systems

Fick's law of diffusion can often be used to describe the transport of a bioactive agent through a diffusion controlled polymer system. Fick's law in one dimension can be represented by the following two equations [54]:

$$J_1 = -D_{12}\frac{dc_1}{dz} \tag{1.1}$$

$$\frac{\partial c_1}{\partial t} = D_{12}\frac{\partial^2 c_1}{\partial z^2} \tag{1.2}$$

Where J_1 = molar flux of the drug (mol/cm^2 s)

D_{12} = diffusion coefficient of the drug in the polymer (cm^2/s)

c_1 = drug concentration (mol/cm^3)

z = length (cm)

t = time (s)

In the above equations, it is assumed that the diffusion coefficient is independent of concentration. For nonporous, homogeneous, polymeric films, Equation 1.1 can be rewritten as:

$$J_1 = D_{12}K\frac{\Delta c_1}{\delta} \tag{1.3}$$

Where K = drug partition coefficient (ratio of drug concentration at the interface and drug concentration in the bulk)

δ = device thickness (cm)

Δc_1 = drug concentration difference of the solutions on either side of the membrane (mol/cm^3)

There are many factors that affect the diffusion coefficient of the drug in the polymer, D_{12}, including the properties of the polymer and the drug. For semicrystalline polymers, the degree of crystallinity and crystallite size affect the diffusion coefficient. For swollen polymers, the degree of swelling, and the translational and relaxational behavior of the polymer affect the coefficient. For crosslinked polymer networks, the degree of crosslinking and mesh size (the distance between adjacent crosslinks) have an

effect. For porous polymers, the porous structure and tortuosity affect drug diffusion [53]. Properties of the drug also affect D_{12}, such as the drug's size and affinity for the polymer. Typical values of D_{12} are $10^{-6} – 10^{-8}$ cm^2/s for rubbery polymers and $10^{-10} – 10^{-12}$ cm^2/s for glassy polymers [53].

Reservoir systems are those in which drug diffuses through a membrane or polymer film from a core of drug. Figure 1.3.1 is a schematic of drug release from a reservoir device.

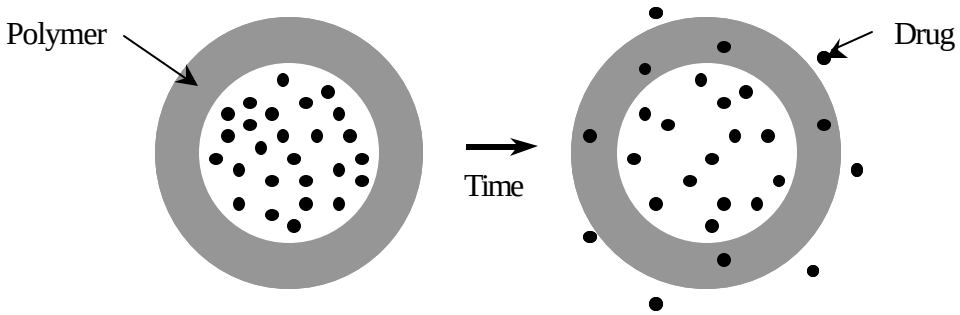

Figure 1.3.1: Schematic of drug release from reservoir devices.

If the transmembrane concentration difference, Δc_1, is kept constant, zero-order release can be achieved. For Δc_1 to be approximated as constant, a high drug concentration must be maintained on the inner wall of the membrane. To achieve this, a powdered drug can be loaded to give high concentrations above the solubility limit of the drug.

Monolithic devices are ones in which a drug is either dispersed or dissolved in a polymer carrier. Such devices have the advantage of being easy and inexpensive to fabricate. If the drug is loaded below the solubility limit in the polymer, release is controlled by simple diffusion through the polymer. If the drug is loaded above its solubility limit, however, dissolution of the drug becomes the controlling factor. Figure 1.3.2 shows a schematic of drug release from a monolithic device.

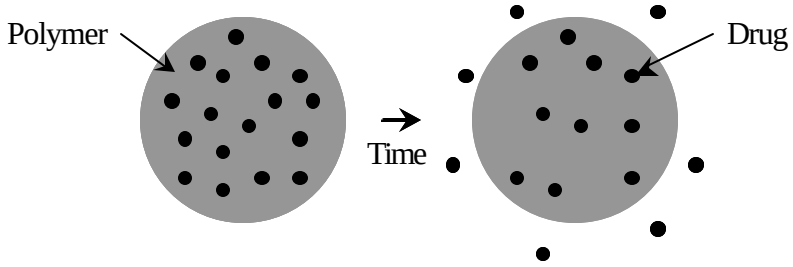

Figure 1.3.2: Schematic of drug release from monolithic devices.

Different modeling techniques have been used for different types of monolithic systems. The type of model used depends on whether the drug is dissolved or dispersed in the device and whether the device is porous or nonporous. The modeling of drug diffusion from porous systems can be very difficult due to the random nature of the pore network and the dynamic nature of the pore structure that constantly changes over the course of the drug release. Here, I will briefly highlight on several models for drug delivery from monolithic devices. For further detail, see [54-59].

For many monolithic systems, the drug release rate is proportional to $t^{-1/2}$, where t = time. The release rate of drug decreases with time because there is no rate controlling membrane. For the simple geometry of a slab of thickness δ, and based on the following assumptions: (1) no edge effects and (2) the boundary condition of zero drug concentration at the interface (no boundary layer effects), we obtain the following initial release rates (up to 40% release) [52, 53]:

Case 1: Release of dispersed drug from a nonporous polymer, where the drug is dispersed at a concentration, c_d, greater than the solubility in the polymer, c_s:

$$\frac{dM_t}{Adt} = \frac{1}{2}\left[D_{12}c_{sp}\left(2c_d - c_{sp}\right)\right]^{1/2} t^{-1/2} \tag{1.4}$$

Where $\frac{dM_t}{Adt}$ = release rate of drug per unit area of exposure (mol/cm^2·s)

D_{12} = diffusion coefficient of the drug in the polymer (cm^2/s)

c_{sp} = drug solubility in polymer (mol/cm^3)

c_d = initial drug loading in polymer (mol/cm^3)

t = time (s)

Case 2: Release of dissolved drug from a nonporous polymer, where the drug is dispersed below the solubility of the polymer and the drug diffuses via a solution-diffusion mechanism:

$$\frac{dM_t}{Adt} = 2c_d\left(\frac{D_{12}}{\pi}\right)^{1/2} t^{-1/2} \tag{1.5}$$

Case 3: Release of dispersed drug from a porous polymer where diffusion occurs through water-filled pores in the matrix:

$$\frac{dM_t}{Adt} = \frac{1}{2}\left[\frac{D_{12}\varepsilon}{\tau}c_{sw}\left(2c_d - \varepsilon c_{sw}\right)\right]^{1/2} t^{-1/2} \tag{1.6}$$

Where ε = porosity

τ = tortuosity

c_{sw} = drug solubility in water (mol/cm^3)

Case 4: Release of dissolved drug from a porous polymer where diffusion occurs through water-filled pores in the matrix:

$$\frac{dM_t}{Adt} = 2c_d\left(\frac{D_{12}\varepsilon}{\tau\pi}\right)^{1/2} t^{-1/2} \qquad (1.7)$$

Swelling Controlled Systems

Swelling controlled systems, such as hydrogel release systems, are those where the drug is incorporated into the swellable polymer, and then diffuses out upon swelling of the polymer. Figure 1.3.3 is a schematic of drug release from a crosslinked hydrogel.

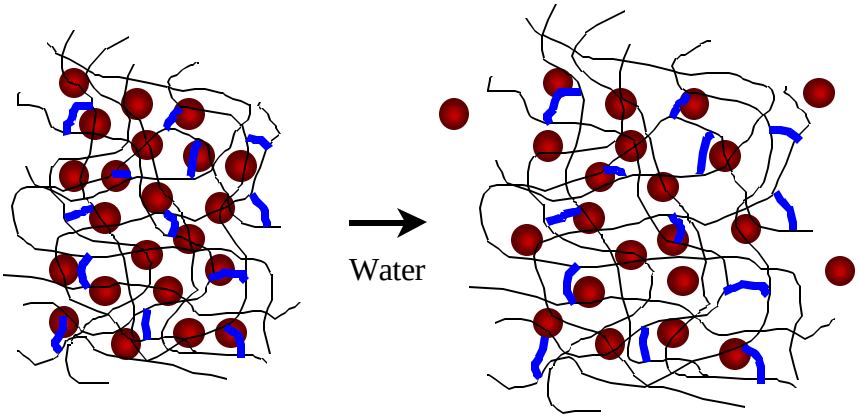

Figure 1.3.3: Schematic of drug release from hydrogels. Drug shown in red, hydrogel backbone shown in black, crosslinks shown in blue.

There are five main parameters that affect the release of a drug from a hydrogel: 1) the polymer composition, 2) the releasing agent, 3) the water content, either as bound or free form, 4) the crosslink density, and 5) the solvent-polymer interaction parameter (χ) [60-62]. These parameters are not independent of one another. Specifically, the polymer composition and crosslink density affect the swelling of the polymer, and thus, its water content.

When soaked in aqueous solution, the hydrogel eventually reaches its equilibrium swollen state. For hydrogels that don't contain ionic moieties, the Flory-Rehner theory

applies: at equilibrium, the forces of chemical potential, which drive water into the polymer, and the elastic retractive forces of the hydrogel, which restrict water absorption, balance out [62-64]. As the level of crosslinking increases, the amount of water absorbed into the hydrogel decreases. The equilibrium swelling ratio can be measured either as a mass swelling ratio (q) or a volume swelling ratio (Q) [63].

$$q = \frac{M_s}{M_d} \tag{1.8}$$

Where q = equilibrium mass swelling ratio

M_s = mass of swollen polymer (g)

M_d = mass of dry polymer (g)

$$Q = \frac{V_s}{V_d} = 1 + \frac{\rho_p}{\rho_s}(q - 1) \tag{1.9}$$

Where Q = equilibrium volume swelling ratio

V_s = volume of swollen polymer (cm^3)

V_d = volume of dry polymer (cm^3)

ρ_p = density of the polymer (g/mL)

ρ_s = density of solvent (g/mL)

Crosslink density can be calculated using the following equation, derived by Flory and Rehner [63, 64]:

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\left(\overline{\upsilon}/V_1\right)\left[\ln\left(1-\upsilon_{2,s}\right)+\upsilon_{2,s}+\chi\upsilon_{2,s}^2\right]}{\left[\upsilon_{2,s}^{1/3}-\frac{\upsilon_{2,s}}{2}\right]} \tag{1.10}$$

Where $\overline{M}_c$ = Average molecular weight between crosslinks (g/mol)

$\overline{M}_n$ = Number average molecular weight in the absence of crosslinking (g/mol)

$\overline{\upsilon}$ = specific volume of the polymer, $1/\rho_p$ (mL/g)

V_1 = molar volume of the solvent (mL/mol)

$\upsilon_{2,s}$ = equilibrium polymer volume fraction, 1/Q

χ = solvent polymer interaction parameter

The diffusivity of a solute within a hydrogel (D_g) can be estimated by [63, 65]:

$$\frac{D_g}{D_o} = \left(1-\frac{r_s}{\xi}\right)\exp\left(-Y\frac{\upsilon_{2,s}}{1-\upsilon_{2,s}}\right) \tag{1.11}$$

Where D_g = solute diffusivity in hydrogel (m^2/s)

D_o = solute diffusivity in pure solvent, e.g. water (m^2/s, see Eqn. 1.12)

r_s = Stokes-Einstein hydrodynamic radius of solute (m)

ξ = mesh size, distance between two adjacent crosslinks (m, see Eqn. 1.13)

Y = ratio of the critical volume required for a successful translational movement of the solute molecule to the average free volume per molecule of the liquid (usually = 1)

The solute diffusivity (D_o) in pure solvent is calculated by:

$$D_o = \frac{k_B T}{6\pi\eta r_s} \tag{1.12}$$

Where k_B = Boltzmann's constant (1.3807×10^{-23} J/K)

T = temperature (K)

η = viscosity of water at T (Pa·s)

The mesh size of the polymer (ξ) can be calculated by [63, 66]:

$$\xi = \upsilon_{2,s}^{-1/3} C_n^{1/2} \ln^{1/2} \tag{1.13}$$

Where C_n = characteristic ratio of the polymer

l = bond length (m)

n = number of bonds between crosslinks

From Equations 1.10 – 1.13, we can see that diffusion coefficient of a solute through a polymer is dependent on the solute's diffusivity in the solvent, the size of the solute molecule, the crosslink density, the strength of interactions between the solute and the polymer, and the equilibrium swelling ratio of the polymer.

In some cases, hydrogel drug delivery systems are dried to remove all of the solvent before use. When considering the swelling of glassy (solvent-free) polymers, drug release is controlled by the velocity of the front of water penetrating the polymer. This is due to the fact that the drug diffuses through the water and rubbery polymer that results when the polymer swells. Diffusion of drug through the glassy polymer is negligible. As the solvent penetrates the polymer, the glass transition temperature is lowered and the swollen polymer is in a rubbery state. Modeling of such a phenomena requires the use of moving boundary conditions. Fick's diffusion equation is solved with concentration dependent or independent diffusion coefficients and moving boundary conditions. Sometimes, it is necessary to consider the macromolecular relaxation that

accompanies the polymer swelling in the region near the interface between the glassy and rubbery state of the polymer. This results in non-Fickian diffusion and the analysis can be quite complicated. A more detailed description of this behavior has been reported by Peppas *et al*. [67, 68].

Chemically Controlled Systems

Chemically controlled drug delivery systems are ones where an outside chemical stimulus creates a change in the swelling or degradation behavior of the polymer, and this change triggers the release of the drug. Figure 1.3.4 is a schematic of one type of stimulus responsive hydrogel drug delivery system.

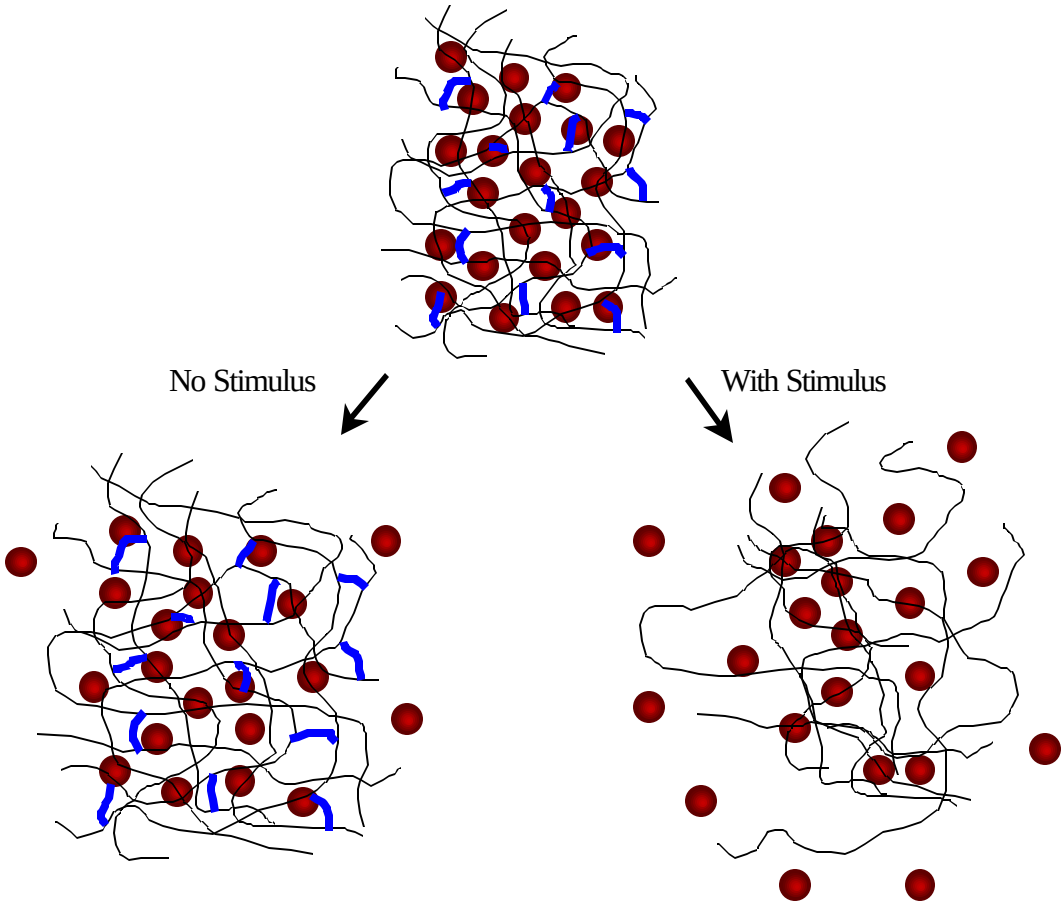

Figure 1.3.4: Schematic of drug release from stimulus responsive hydrogel. Drug shown in red, hydrogel backbone shown in black, crosslinks shown in blue. The stimulus cleaves the crosslinks, resulting in swelling and degradation of the hydrogel.

In the example shown in Figure 1.3.4, the hydrogel swells to some degree in the absence of a stimulant, but when the appropriate stimulent is present, the crosslinks are broken, which creates an increase in swelling, and potentially the degradation of the polymer, resulting in a faster drug release rate.

In chemically controlled systems, degradation of the polymer can occur at the surface of the crosslinked polymer or throughout the bulk of the polymer network. Surface erosion has been investigated more than bulk erosion for drug delivery systems because surface erosion can result in near zero-order release kinetics for constant delivery of the drug [62].

1.4 Silicone Drug Delivery Coatings

Silicones, or polydimethylsiloxanes, have a number of advantages for use in medical devices and drug delivery systems. They are biocompatible, easy to fabricate, relatively inexpensive, resistant to sterilization procedures, and are mechanically flexible and strong [69-72]. Silicone has been investigated for drug delivery applications for the last 40 years [52, 69, 70, 73-79]. Several silicone drug delivery systems have been used clinically. Norplant® [80] has been used to deliver levonorgestrel over the course of 5 years from a capsule formulation inserted under the skin, and Compudose® [81] has been used to deliver estradiol to cattle from a matrix formulation over a 100 day period.

Release of proteins from silicone systems has also been investigated. Hsieh *et al*. and Fujiokia *et al*. reported that proteins could be continuously released from silicone matrices when incorporated in the polymer at concentrations of 30-50% (w/w) [70, 73, 76, 82]. Release of more potent protein drugs has been achieved by mixing the potent drug with albumin before incorporating it into the polymer to dilute the drug [73, 76]. Controlled release of antigens from silicone has been investigated as a way to develop new vaccine formulations [83].

Drug release profiles from silicone are affected by the physiochemical properties of the drug and any additives used in the formulation, as well as the polymer geometry and the drug and additive loading [69, 75, 77, 78]. Ghannam *et al*. [79] showed a

correlation between a drug's hydrophilicity or lipophilicity and its permeation through silicone membranes. Silicone's hydrophobicity makes it particularly suitable for the delivery of lipophilic drugs [78]. Silicones generally are impermeable to polar compounds, which results in low permeability of water-soluble drugs [70].

One way to affect the release of both lipophilic and hydrophilic drugs has been to disperse various compounds within the silicone [14, 69, 70, 75, 77, 84, 85]. Kajihara *et al*. [75] investigated the effect of adding human serum albumin (HSA) to silicone matrices. They showed that the addition of HSA enhanced the release rate, while reducing the initial burst, which led to a more constant drug release rate. They proposed that the decreased initial burst was due to the HSA blocking the diffusion of the drug, because it does not have affinity for either the drug or the silicone. Later in the process, the HSA itself diffused out of the polymer, causing the drug to be released more quickly. Glycerin and poly(ethylene glycol) additives have been shown to promote the release of lipophilic drugs by causing swelling of the polymer formulation [77, 78].

When lipophilic compounds are incorporated into silicone, they diffuse through the silicone matrix and out into the surrounding media, as shown in Figure 1.4.1 (a). When hydrophilic compounds are incorporated into silicone, the drug compound exists as solid particles that must first dissolve before being released. This occurs first near the surface of the polymer. Upon releasing the drug near the surface, water fills the resulting pore space, causing dissolution and release of drug from nearby areas. Finally, interconnected water-filled channels result [69, 73, 75, 76]. This is shown schematically in Figure 1.4.1 (b).

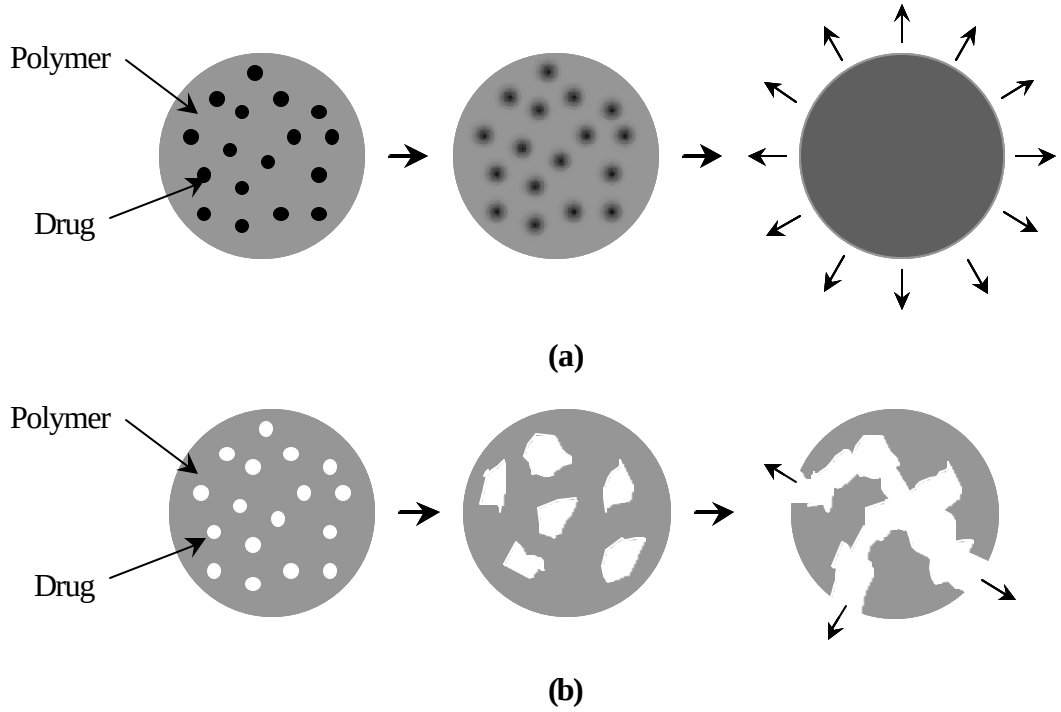

Figure 1.4.1: **(a)** Diffusion mechanism of lipophilic drugs from silicone. **(b)** Diffusion mechanism of hydrophilic drugs from silicone [69, 73, 75, 76].

1.5 Hydrogel Drug Delivery Coatings

Hydrogels as Drug Delivery Systems

Hydrogels are crosslinked networks of hydrophilic polymer that readily absorb water. Hydrogels can absorb many times their weight in water. Crosslinks prevent the dissolution of the hydrophilic polymer chains [86]. Crosslinking can either occur by covalent bonds, ionic bonds, or physical interactions between polymer chains. These physical interactions can be molecular entanglements or secondary forces, like hydrogen bonding or hydrophobic forces [61]. It is common for a monomer or macromer with a single vinyl group to be covalently polymerized with a crosslinking agent containing two or more vinyl groups to create a hydrogel.

One early medical application of hydrogels was contact lenses. Poly(hydroxyethyl methacrylate) hydrogels were first developed into contact lenses in the

1960s by Wichterle and Lim [87]. Hydrogels have been developed into many biomedical and drug delivery systems since then. These materials have proven to be biocompatible, due to their similarity to living tissue. Hydrogels, like living tissue, have a high water content and are generally soft and rubbery.

Hydroxyethyl Acrylate Hydrogels

Poly(hydroxyethyl acrylate) (pHEA) hydrogels have had a history of biocompatibility, which make them appropriate for many medical applications, including drug delivery. Additionally, pHEA swelling is dependent on crosslink density, which allows for the tuning of drug delivery rates with crosslink density. Finally, the linear chain of pHEA is soluble in water, making it a good candidate for signal responsive drug delivery. Figure 1.5.1 (a) shows the chemical structure of HEA monomer, and Figure 1.5.1 (b) shows the chemical structure polymerized HEA, pHEA.

(a) **(b)**

Figure 1.5.1: Chemical structures of **(a)** Hydroxyethyl acrylate monomer and **(b)** Poly(hydroxyethyl acrylate).

Hydroxyethyl acrylate can be polymerized using free radical initiator to link the monomer together into linear chains. In the presence of difunctional crosslinkers, however, covalent links between the straight chains form, resulting in a crosslinked hydrogel. Figure 1.5.2 is a schematic of crosslinked pHEA.

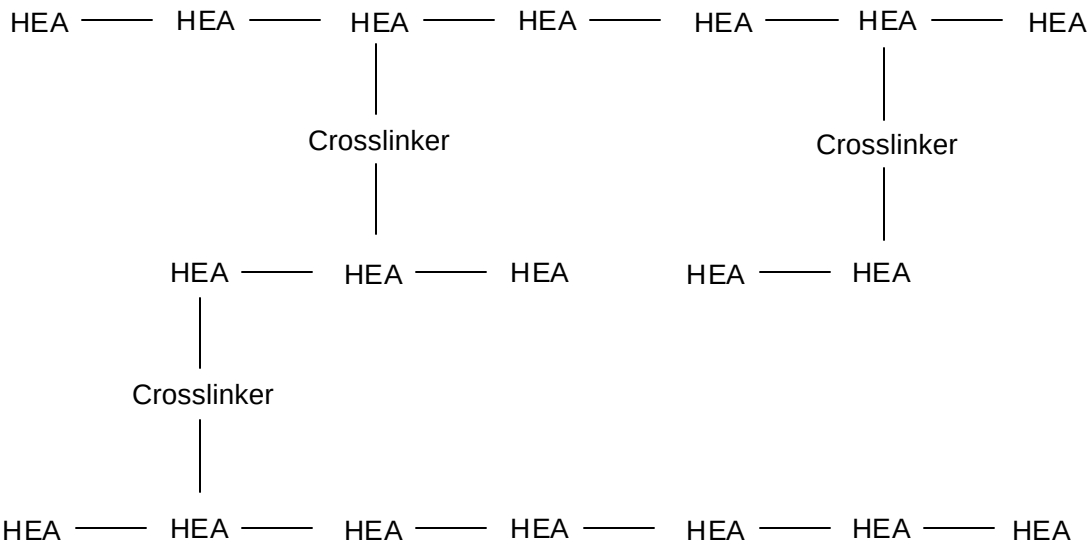

Figure 1.5.2: Schematic of crosslinked poly(hydroxyethyl acrylate).

1.6 Signal Responsive Drug Delivery Coatings

Signal Responsive Hydrogels

Signal responsive drug delivery systems have the potential advantage of delivering drug only when needed. This reduces the cost of the device by minimizing the drug required, and it can improve the effectiveness of the drug by delivering it only when needed. Signal responsive hydrogel drug delivery systems can be created by synthesizing a crosslinker that degrades in the presence of a specific stimulus. If a drug is incorporated into a hydrogel made with such a crosslinker, then as the crosslinks are broken, the hydrogel will swell or degrade, resulting in drug release.

The design of a signal responsive hydrogel system for the treatment of restenosis involves finding a signal that exists while restenosis is happening and synthesizing a crosslinker that degrades in the presence of that signal. Presented here is a method for the synthesis of a thrombin degradable crosslinker.

Thrombin as a Signal

Thrombin is present in elevated levels during smooth muscle cell (SMC) proliferation [88]. It is formed by the prothrombinase complex on activated platelets and is involved in restenosis and thrombosis in several ways. Thrombin cleaves fibrinogen into fibrin and it's the platelet-fibrin complex that results in thrombus formation. Thrombus formation results in thrombosis and may also provide a matrix for SMC migration [89]. Thrombin is also an important signal in that it expresses platelet derived growth factor [90], secretes collagen [91], and stimulates SMC proliferation [92].

Thrombin has been used as a signal for signal-responsive drug delivery in the past by Tanihara *et al*. [93-96]. A thrombin sensitive peptide linker was used to connect a PVA (polyvinyl alcohol) hydrogel and a drug, for use as a drug delivery treatment for wound infection. In that system, thrombin cleaved a drug from the hydrogel, leaving a peptide linker attached to the drug. The peptide linker was then cleaved by leucine aminopeptidase. In that regard, the drug delivery system required the presence of both thrombin and leucine aminopeptidase, not thrombin alone.

By creating a crosslinker with a thrombin cleavable peptide, it is possible to create a thrombin responsive drug delivery hydrogel that can be used as a coating or as a drug delivery device on its own. In this work, a thrombin degradable crosslinker is synthesized by adding acrylate end-groups to a thrombin cleavable peptide.

1.7 Project Overview

The overall research goal of this thesis was to develop a drug delivery coating for stents, as a treatment for in-stent restenosis. To accomplish this goal, we examined three different types of systems: silicone drug delivery coatings, poly(hydroxyethyl acrylate) hydrogel drug delivery coatings and a signal responsive hydrogel drug delivery coating.

Various silicone elastomer coatings with and without poly(ethylene glycol), as a drug compatilizer and pore former, were evaluated for their ability to release paclitaxel and tranilast for an extended period of time. Both monolithic and reservoir type coatings were investigated. The goal of these experiments was to create a tunable drug delivery coating, where the release kinetics of both hydrophobic and hydrophilic drugs can be

optimized by changing the type and composition of the coating. The optimal drug release kinetics for the treatment of restenosis is not known, but these coatings could be used to test the effects of various release patterns in vivo.

Poly(hydroxyethyl acrylate) hydrogels were investigated as a drug delivery system. In order to investigate the affect of pHEA hydrogel properties on drug release, experiments were done to compare release of several model drugs from hydrogels made with two different crosslinkers, tetraethylene glycol diacrylate (TEGDA) and 1,4-butanediol diacrylate, at various crosslinker concentrations. The goal of these experiments was to determine how the above mentioned factors affect drug release from pHEA hydrogels in preparation for the creation of a signal responsive hydrogel drug delivery system.

The goal of the last part of this research was to create a thrombin responsive hydrogel drug delivery coating. A thrombin cleavable crosslinker was synthesized that could, when incorporated into a hydrogel, be used as a thrombin responsive drug delivery coating. Thrombin has been shown to stimulate smooth muscle cell proliferation following vessel wall injury and is present at increased levels after vessel wall injury [88]. The crosslinker is a thrombin degradable peptide sequence with acrylate end-groups. Because of the acrylate end-groups, the crosslinker can be incorporated into hydrogels like poly(hydroxyethyl acrylate). The crosslinker was synthesized and its degradation performance was evaluated.

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2 SILICONE/PEG DRUG DELIVERY COATINGS

2.1 Introduction

Various silicone elastomer coatings with and without poly(ethylene glycol) (PEG) were evaluated for their ability to release paclitaxel and tranilast for an extended period of time. Poly(ethylene glycol) was added to the silicone matrix to act as a pore-former and compatabilizer between the drug and the polymer [1-3]. Silicones, or polydimethylsiloxanes, have a number of advantages for use in medical devices and drug delivery systems. They are biocompatible, easy to fabricate, relatively inexpensive, resistant to sterilization procedures, and are mechanically flexible and strong [4-7].

Both paclitaxel and tranilast have been shown to inhibit restenosis following PTCR procedures [8-20]. The chemical structures of paclitaxel and tranilast are given in Figure 2.1.1 and Figure 2.1.2, respectively.

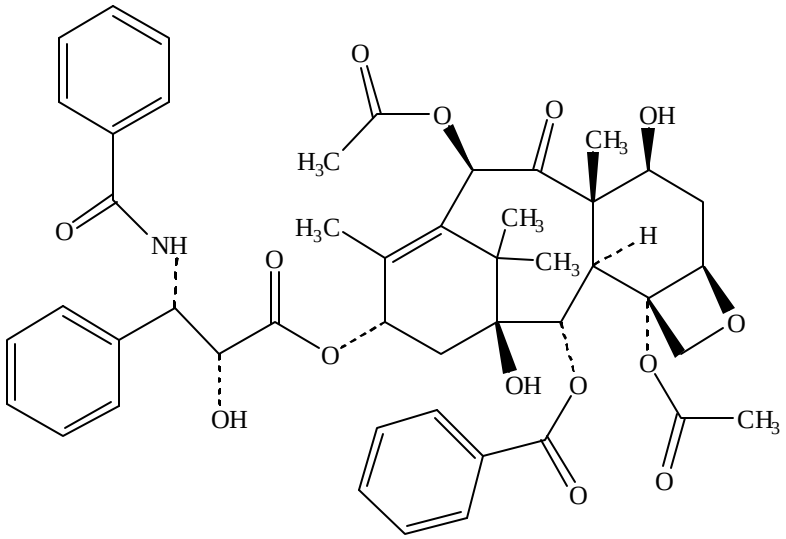

Figure 2.1.1: Chemical structure of paclitaxel.

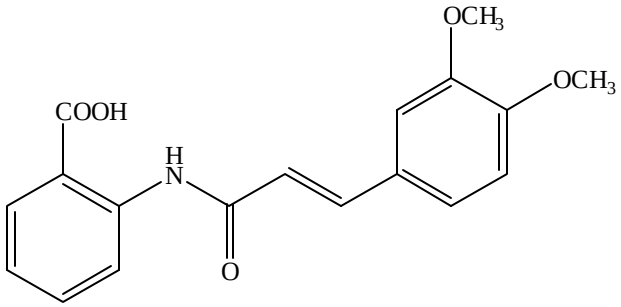

Figure 2.1.2: Chemical structure of tranilast.

The release of these two drugs from coatings made of silicone and PEG is interesting to compare because paclitaxel is hydrophobic while tranilast is hydrophilic. Additionally, different coating types, monolithic and reservoir, were compared.

Drug release profiles from silicone are affected by the physiochemical properties of the drug and any additives used in the formulation, as well as the polymer geometry and the drug and additive loading [4, 21-23]. Silicone's hydrophobicity makes it particularly suitable for the delivery of lipophilic drugs [22]. Silicones generally are impermeable to polar compounds, which results in low permeability of water-soluble drugs [5]. One way to affect the release of both lipophilic and hydrophilic drugs has been to disperse various compounds within the silicone [4, 5, 21, 23-26].

When lipophilic compounds are incorporated into silicone, they diffuse through the silicone matrix and out into the surrounding media, as shown in Figure 2.1.3 (a). When hydrophilic compounds are incorporated into silicone, the drug compound exists as solid particles that must first dissolve before being released. This occurs first near the surface of the polymer. Upon releasing the drug near the surface, water fills the resulting pore space, causing dissolution and release of drug from nearby areas. Finally, interconnected water-filled channels result [4, 21, 27, 28]. This is shown schematically in Figure 2.1.3 (b).

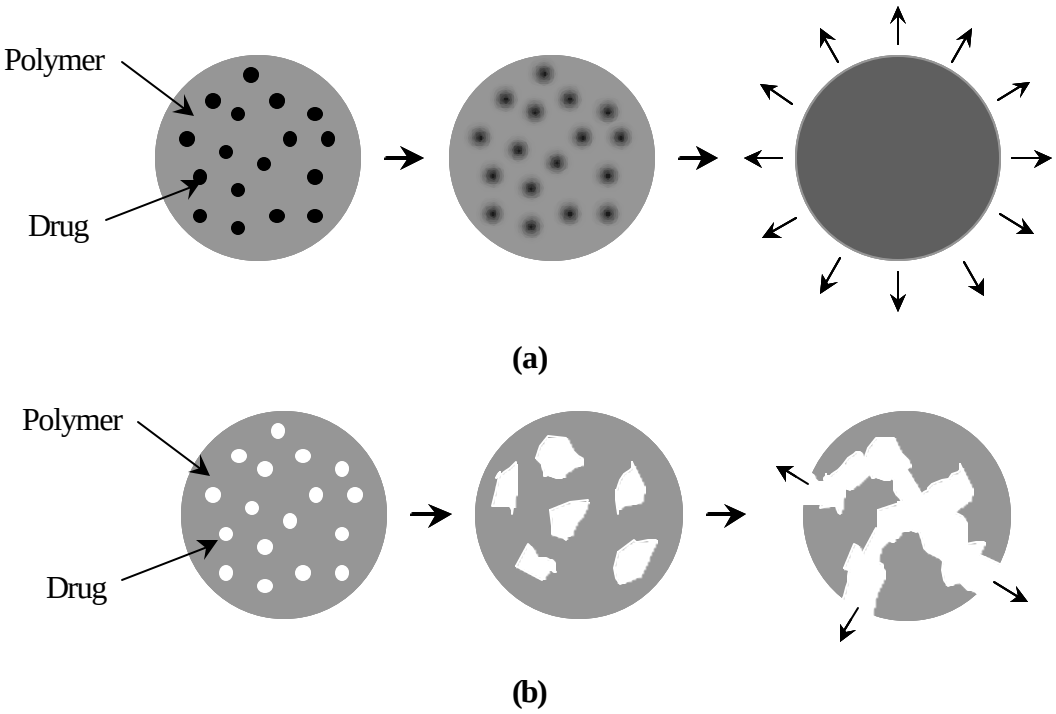

Figure 2.1.3: **(a)** Diffusion mechanism of lipophilic drugs from silicone. **(b)** Diffusion mechanism of hydrophilic drugs from silicone.

Monolithic devices are ones in which a drug is either dispersed or dissolved in a polymer carrier. Monolithic devices have the advantage of being easy and inexpensive to fabricate. If the drug is loaded below the solubility limit in the polymer, release is controlled by simple diffusion through the polymer. If the drug is loaded above its solubility limit, however, dissolution of the drug becomes the controlling factor. Figure 2.1.4 shows a schematic of drug release from a monolithic device.

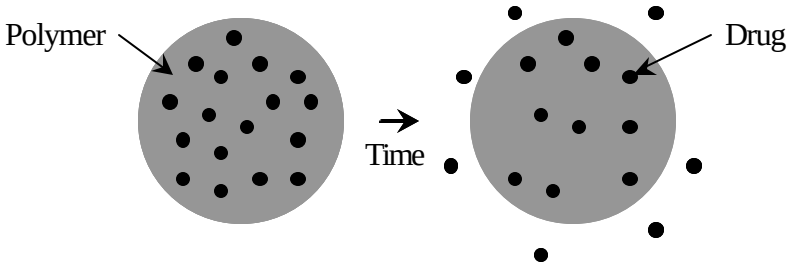

Figure 2.1.4: Schematic of drug release from monolithic devices.

Reservoir systems are those in which drug diffuses through a membrane or polymer film from a core of drug. Figure 2.1.5 is a schematic of drug release from a reservoir device.

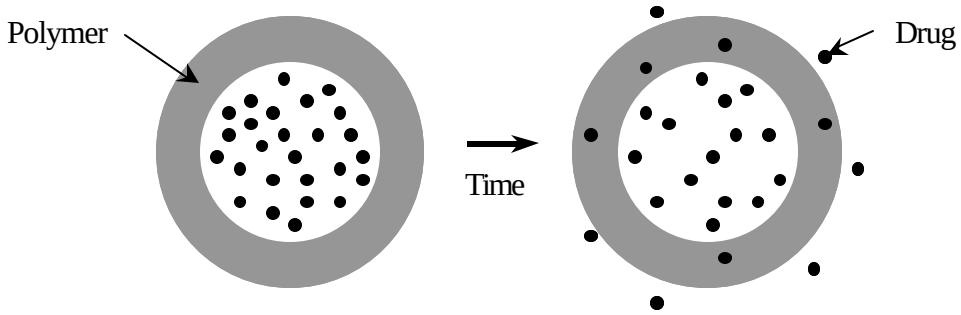

Figure 2.1.5: Schematic of drug release from reservoir devices.

2.2 Materials and Methods

Materials

Two drugs, paclitaxel and tranilast, were used in the controlled release experiments. Both drugs were donated by Genzyme, Inc. Polymer formulations were made with DAP 100% silicone sealant (Dow Corning), poly(ethylene glycol), molecular weight 3400 (Polysciences), and either methylene chloride or toluene (HPLC grade, Aldrich). Coatings were applied to 316L stainless steel pieces (Alaskan Copper and Brass, Co.).

Release Coating Formulation

Samples for drug elution studies were made by coating pieces of 316L stainless steel (1 cm x 1 cm x 0.4 mm), with various formulations of silicone, poly(ethylene glycol) (PEG), and drug. Two different types of coatings were studied: monolithic and reservoir. Monolithic coatings are those in which the drug is dispersed evenly throughout the polymer matrix. Reservoir coatings are those where a drug layer is coated by a polymer layer [29]. Additionally, studies were performed with monolithic coatings that

had an additional topcoat of polymer. Figure 2.2.1 is a schematic of the three different coatings used in these studies.

Monolithic: Drug dispersed in polymer

Monolithic with topcoat: Drug dispersed in polymer with polymeric topcoat

Reservoir: Drug layer with polymer topcoat

Figure 2.2.1: Schematic of different types of drug release coatings.

Polymer formulations were made by codissolving silicone, PEG, and drug in either methylene chloride or toluene. Table 2.1 shows the formulations used for the drug layer in these studies. Table 2.2 shows the formulations of topcoats used over the drug layer. The thickness of the polymer coatings is also shown in the tables. Silicone was measured as a weight to volume percentage with respect to solvent. Poly(ethylene glycol) and drug were measured in terms of weight percent with respect to uncured silicone except in the case where no silicone was present in the formulation; in that case the drug is shown as a percentage with respect to solvent.

Table 2.1: Drug layer formulations used for silicone release studies. The solvent used for all of these coatings was methylene chloride.

Formulation:	**Silicone %:[1]**	**Drug:**	**Drug %:[2]**	**PEG %:[3]**	**Coating thickness:**
A	10	paclitaxel	2	20	37.2 ± 11.9 ìm
B	10	paclitaxel	2	0	15.3 ± 3.8 ìm
C	10	tranilast	5	20	48.4 ± 1.6 ìm
D	10	tranilast	5	0	26.6 ± 1.6 ìm
E	0	paclitaxel	0.33*	0	n/a
F	0	tranilast	0.16*	0	n/a

[1]Silicone % is w/v with respect to solvent. [2]Drug % is w/w with respect to silicone.
[3]PEG % is w/w with respect to silicone. [*]Drug in solvent only.

Table 2.2: Topcoat formulations used for silicone release studies.

Formulation:	Silicone %:[1]	PEG %:[2]	Solvent:	Coating thickness:
a	10	20	methylene chloride	16.0 ± 3.7 ìm
b	10	0	methylene chloride	8.9 ± 0.9 ìm
c	1	20	methylene chloride	1.1 ± 1.6 ìm
d	1	0	methylene chloride	0.66 ± 1.2 ìm
e	10	20	toluene	43.9 ± 4.1 ìm
f	10	0	toluene	40.1 ± 4.4 ìm

[1]Silicone % is w/v with respect to solvent. [2]PEG % is w/w with respect to silicone.

Stainless steel pieces were cleaned by sonicating in 3% Isopanasol® (C.R. Callen Corp.), water, and acetone for six minutes each. For studies of monolithic coatings, stainless steel pieces were weighed and then dip-coated in the polymer/drug solution and allowed to cure at room temperature. The entire piece of stainless steel was coated on both sides, except for a small section of one corner, where an alligator clip was used to hold the sample during dipping and curing. For studies of reservoir coatings, samples were made by pipetting drug solution onto one side of the stainless steel piece, letting it dry, and then pipetting polymer solution on top of the drug coating. To prevent the drug from dissolving into the polymer solution, the polymer solution was prepared using toluene as a solvent. Neither drug is soluble in toluene. The drug and topcoat formulations used for each release study are shown in Table 2.3.

Table 2.3: Monolithic and reservoir release studies. Drug layer and topcoat formulations from Table 2.1 and Table 2.2, respectively. Drug loading is per stainless steel sample.

Release Study:	Drug:	Drug Layer:	Drug Loading:	Topcoat:
M-1	paclitaxel	A, B	120 ± 35 µg	None
M-2	tranilast	C, D	325 ± 22 µg	None
M-3	tranilast	D	167 ± 17 µg	a, b, c, d
R-1	paclitaxel	E	100 ± 15 µg	e, f
R-2	tranilast	F	100 ± 17 µg	e, f

Elution Studies

Elution studies were conducted by soaking drug-containing samples in elution media in a shaking water bath at 37 ^{o}C and monitoring drug release at certain time intervals. At each time point the old elution media was removed for evaluation of drug release and replaced with fresh media. Elution media consisted of 0.01 M phosphate buffered saline solution (PBS, pH = 7.4) for tranilast studies and bovine serum (Sigma) for paclitaxel studies. Phosphate buffered saline was made by dissolving PBS tablets (Sigma) in deionized water. Elution studies were carried out until the drug release rate reached zero. Drug concentration was determined using a competitive inhibition enzyme immunoassay (CIEIA) for paclitaxel and using UV spectrophotometry for tranilast. The total amount of drug released, the % release, and the release rate were calculated using Equations 2.1 and 2.2, and 2.5, respectively:

$$M_t = \sum_t V_e C_t \qquad (2.1)$$

where M_t = cumulative amount of drug released at time t (μg)

V_e = volume of elution medium (mL)

C_t = concentration of drug in elution medium at time t (μg/mL)

$$\%\,\text{Release} = M_t / M_i \times 100 \qquad (2.2)$$

where M_t = cumulative amount of drug released at time t (îg)

M_i = initial mass of drug in sample (îg), calculated using Equation 2.3 for monolithic coatings and Equation 2.4 for reservoir coatings:

$$M_{i,m} = M_c C_{d,w} \qquad (2.3)$$

where $M_{i,m}$ = initial mass of drug in monolithic coatings (îg)

M_c = weight of coating (îg)

$C_{d,w}$ = w/w % of drug in coating

$$M_{i,r} = V_d C_{d,v} \times 10^{-3} \quad (2.4)$$

where $M_{i,r}$ = initial mass of drug in reservoir coatings (ìg)

V_d = volume of drug solution (ìl)

$C_{d,v}$ = w/v % of drug in coating (mg/mL)

$$\text{Release Rate} = \frac{\Delta M_t}{\Delta t} \quad (2.5)$$

where ΔM_t = difference in drug cumulative mass between two consecutive time points

Δt = time elapsed between two consecutive time points

Paclitaxel Assay

Commonly, paclitaxel concentration is determined by high performance liquid chromatography (HPLC) [30, 31]. Due to limited availability of an HPLC system, an alternative method was used to measure paclitaxel concentrations. Paclitaxel concentrations were determined using a competitive inhibition enzyme immunoassay (CIEIA) from Hawaii Biotechnology Group, Inc. The assay kit contains paclitaxel-protein coating antigen, anti-paclitaxel antibody, and a paclitaxel standard. Goat anti-mouse IgG-alkaline phosphatase conjugate was obtained from Caltag Laboratories. All other reagents were purchased from Sigma. Figure 2.2.2 illustrates the assay procedure.

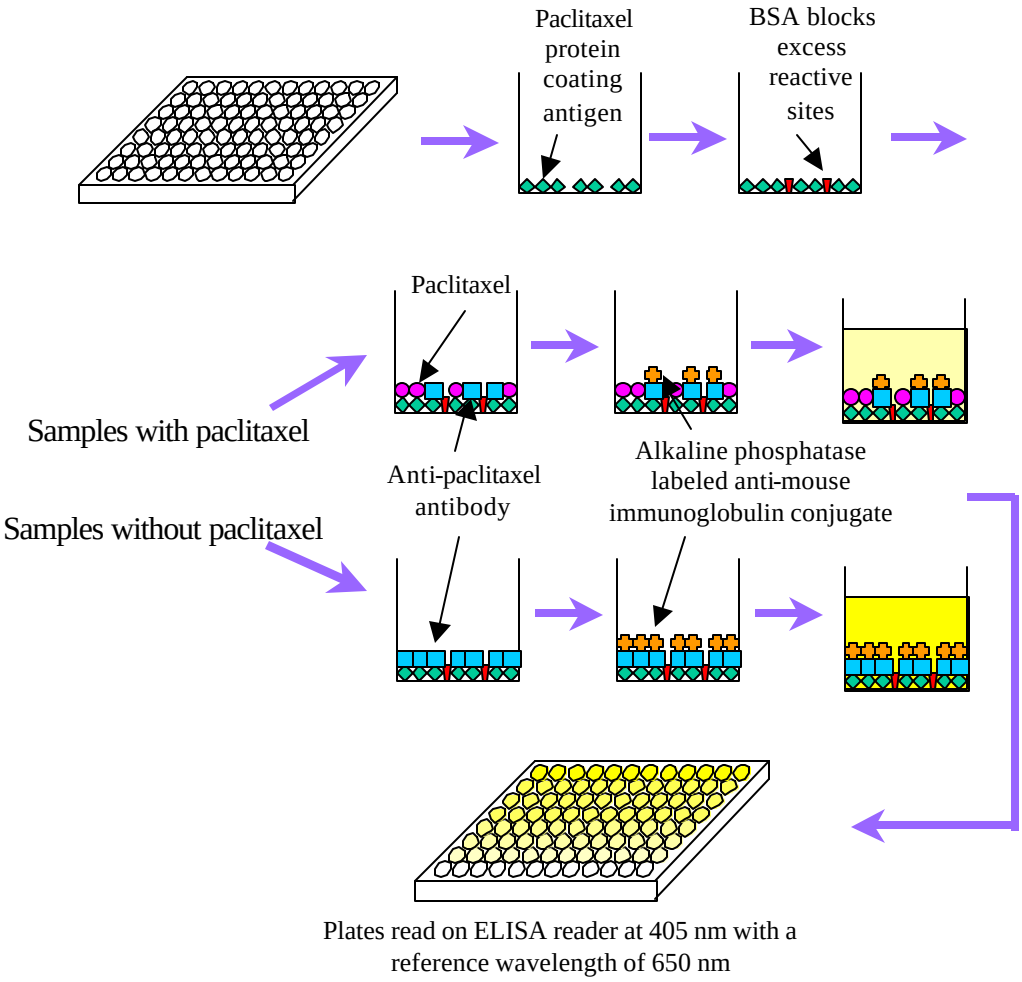

Figure 2.2.2: Competitive inhibition enzyme immunoassay for paclitaxel.

The assay is conducted in Immulon™ 2 flat bottom 96 well plates (VWR). First, a paclitaxel protein coating antigen is adsorbed to each well, followed by a bovine serum albumin (BSA) solution that is used to block any excess reactive sites. In the third step, the paclitaxel containing serum and an anti-paclitaxel antibody are added to the wells. The paclitaxel competitively adsorbs along with the anti-paclitaxel antibody. The concentration of paclitaxel in the sample solution is directly proportional to the amount of inhibition of the reaction between solid-phase paclitaxel-protein conjugate and anti-paclitaxel antibody. An alkaline phosphatase labeled anti-mouse immumoglobulin conjugate is then added, which binds to the anti-paclitaxel antibody. Finally, an enzyme

substrate is added to the well, which causes the solution to turn yellow in response to the concentration of anti-paclitaxel antibody. In this way, if there is no paclitaxel present, the solution will turn yellow, and if there is paclitaxel in the solution the solution will be a less intense shade of yellow. The concentration of paclitaxel is proportional to the inhibition of the free paclitaxel binding. During each step, the plates are left for one hour to allow for the necessary adsorption and between each step, the wells are washed thoroughly with a wash buffer. Wells are washed by shaking out the contents of each, refilling to overflowing with wash buffer, then emptying by shaking out. This procedure is repeated six times per wash step.

The absorbance of each well at 405 nm was measured using a VersaMax ELISA reader. A standard curve is produced along with each assay run. A sample standard curve is shown in Figure 2.2.3. Each test sample is diluted to four different concentrations with buffer to ensure that one will fall within the linear portion of the standard curve. The sensitivity of this assay is 3.5 ng/mL for paclitaxel and can be used to analyze paclitaxel in crude bark extracts, plant tissue extracts, and human plasma samples with no sample pretreatment. Details of the assay procedure can be found in [32].

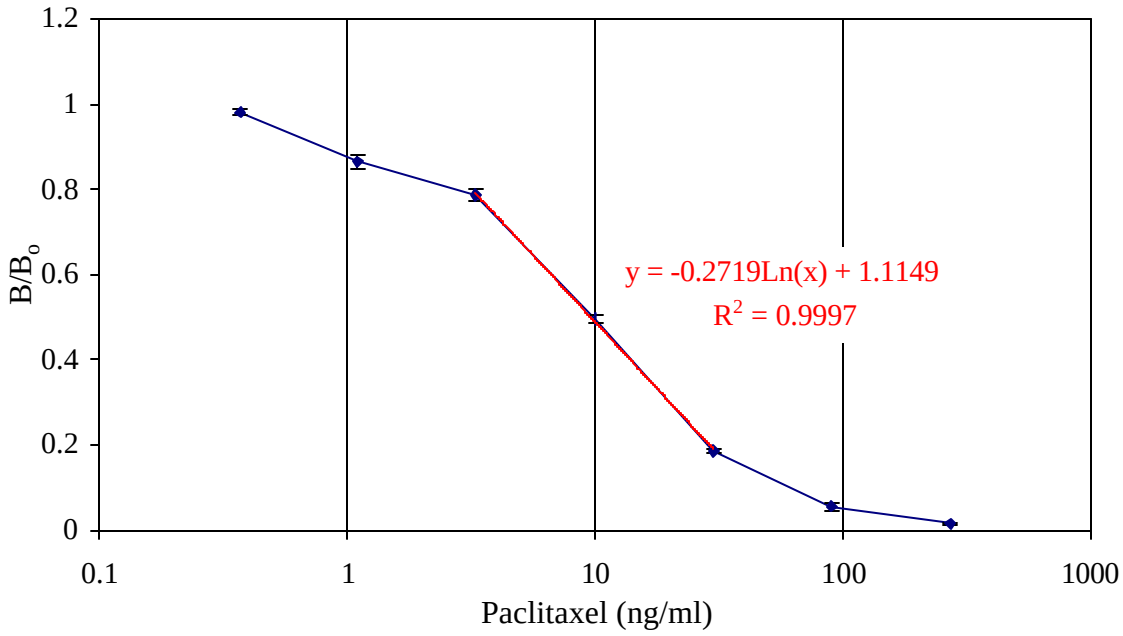

Figure 2.2.3: Sample standard curve for paclitaxel assay. B is the mean absorbance of the appropriate dilution of inhibitor paclitaxel standard. B_o is the mean absorbance of the uninhibited reference wells.

UV Spectrophotometry of Tranilast

Tranilast concentrations were determined using UV-vis spectrophotometry. The peak absorbance of tranilast was determined using a HP 8452A diode array spectrophotometer and found to be 340 nm. The UV-vis spectrum for tranilast is shown in Figure 2.2.4.

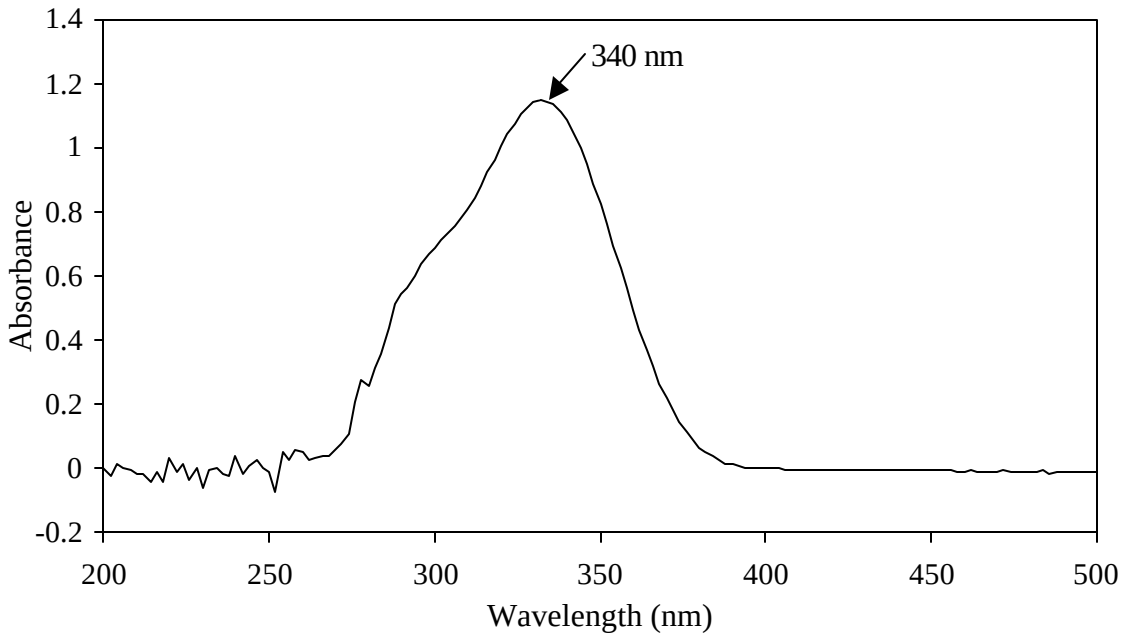

Figure 2.2.4: UV-vis spectrum of tranilast.

To determine tranilast concentrations of samples in the drug release experiments, absorbance at 340 nm was determined for 200μL of solution using 96 well plates and a VersaMax tunable microplate reader. A standard curve for tranilast is shown in Figure 2.2.5.

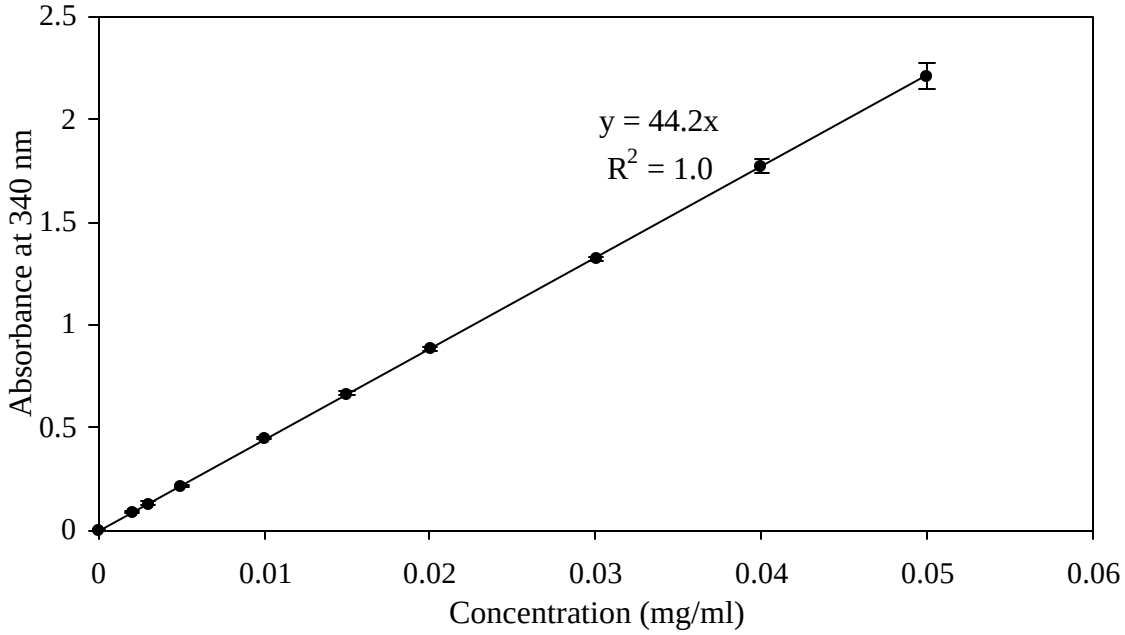

Figure 2.2.5: Standard curve of tranilast absorbance at 340 nm vs. concentration.

2.3 Results: Monolithic Coatings

Figure 2.3.1 compares percent paclitaxel release vs. time from monolithic coatings made of silicone with and without 20% PEG. The incorporation of PEG decreased the initial burst of drug release and increased the release rate after the initial burst. After the initial burst of drug, the release rate for coatings with PEG was 0.38 ± 0.03 μg/day and the release rate for coatings made without PEG was 0.21 ± 0.03 μg/day. After the initial burst, near zero-order release was achieved for 60 days.

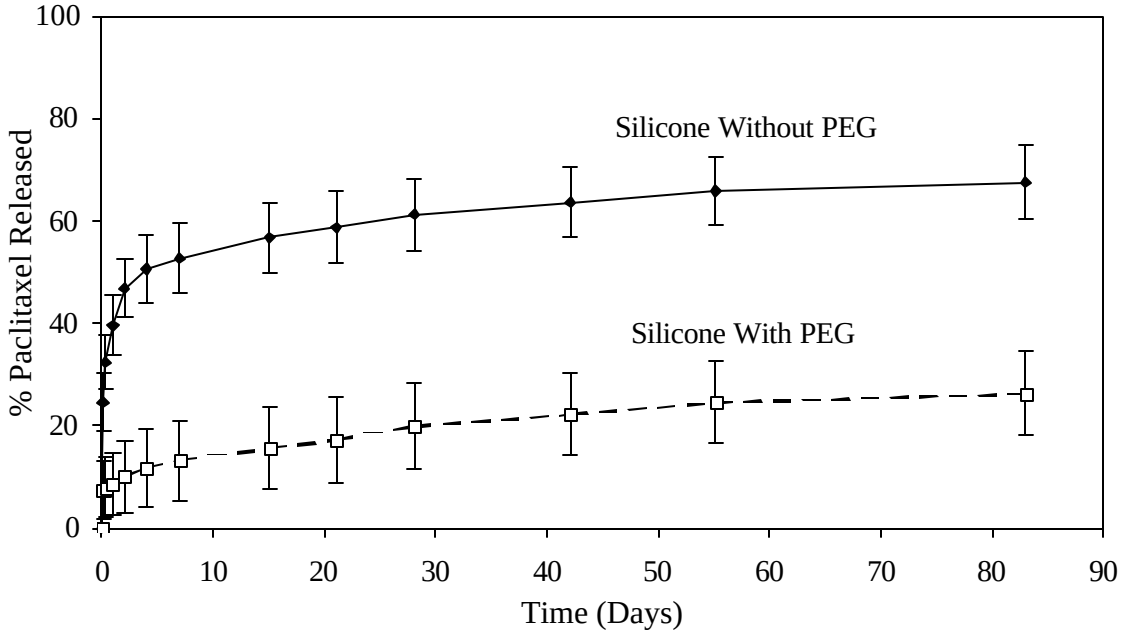

Figure 2.3.1: Release study M-1. Paclitaxel release from monolithic silicone coatings fabricated with or without PEG.

Tranilast was also released from monolithic silicone coatings made with and without PEG, as shown in Figure 2.3.2. In the absence of PEG, the total initial burst was similar to paclitaxel. The incorporation of PEG reduced the initial burst of tranilast, although this reduction was not as significant as with paclitaxel due to the increased hydrophilicity of tranilast. The release rate after the initial burst was higher and more extended from coatings made without PEG.

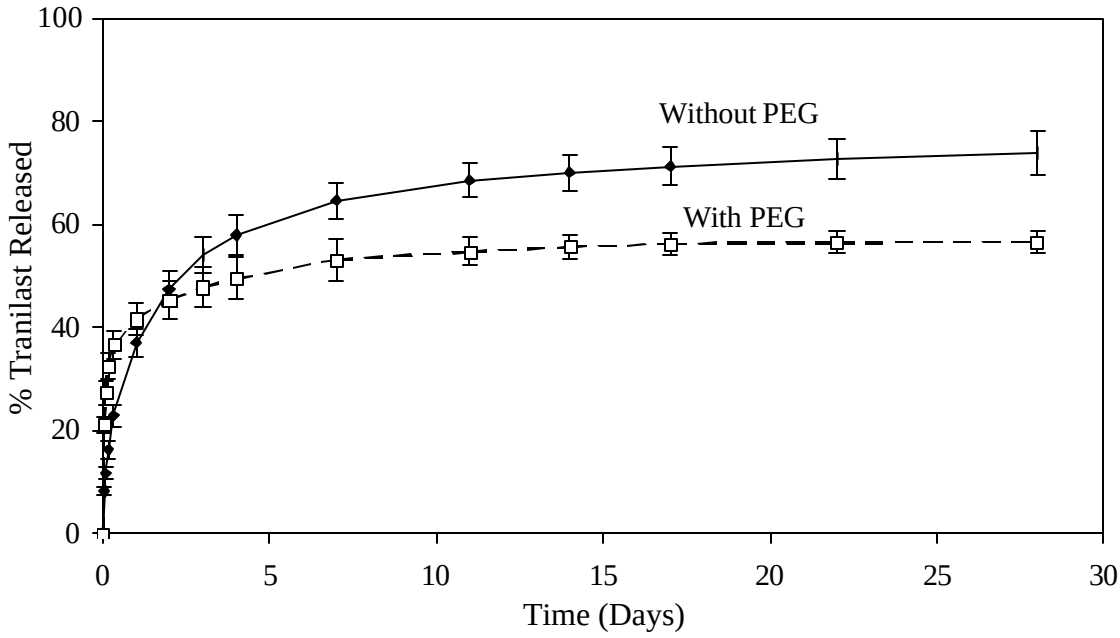

Figure 2.3.2: Release study M-2. Comparison of tranilast release from monolithic silicone coatings made with and without PEG.

The effects of various topcoats were investigated on the release of tranilast from monolithic coatings fabricated without PEG. The topcoats were made with either 1 or 10% silicone, resulting in two different thicknesses of coatings. Additionally, topcoats were made with or without PEG. As shown in Figure 2.3.3, there was no statistical difference between the release from coatings with various topcoats, but all of the topcoats did decrease the initial burst of drug. However, the top coats did not cause the release to be extended past 21 days.

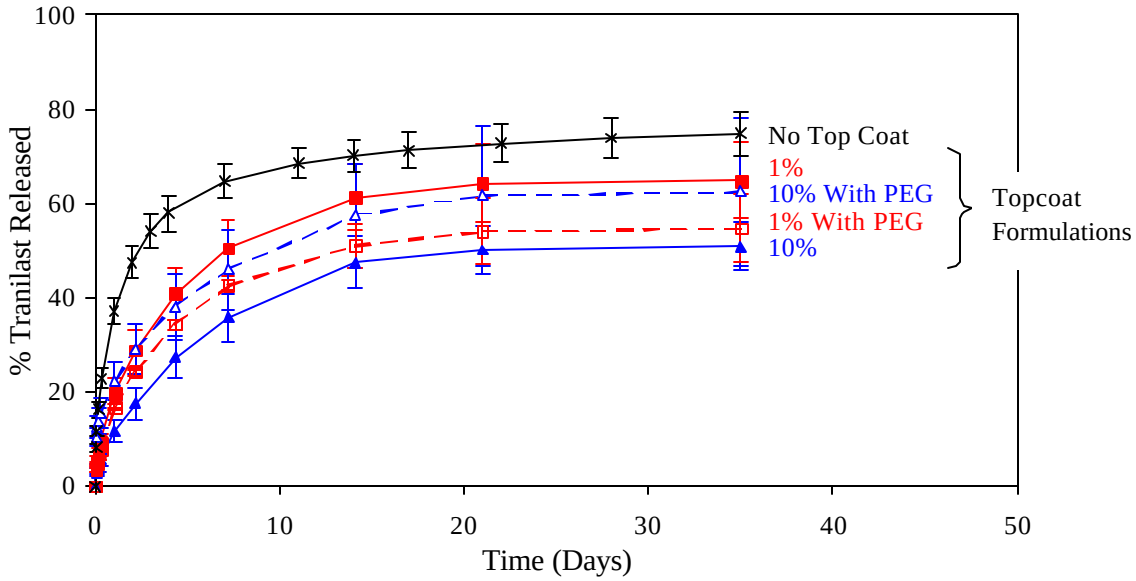

Figure 2.3.3: Release study M-3. Tranilast release from monolithic silicone coatings with various topcoats.

2.4 Results: Reservoir Coatings

The release rates of paclitaxel from reservoir devices with various topcoats were investigated. The topcoats were made of silicone with and without PEG and were fabricated in two different thicknesses. Additionally, a device with no topcoat was examined. Figure 2.4.1 shows that with no topcoat, the paclitaxel was completely released within the first four hours. However, with the addition of a topcoat, the release of paclitaxel was significantly decreased. Interestingly, the silicone topcoats without PEG resulted in the lowest initial burst.

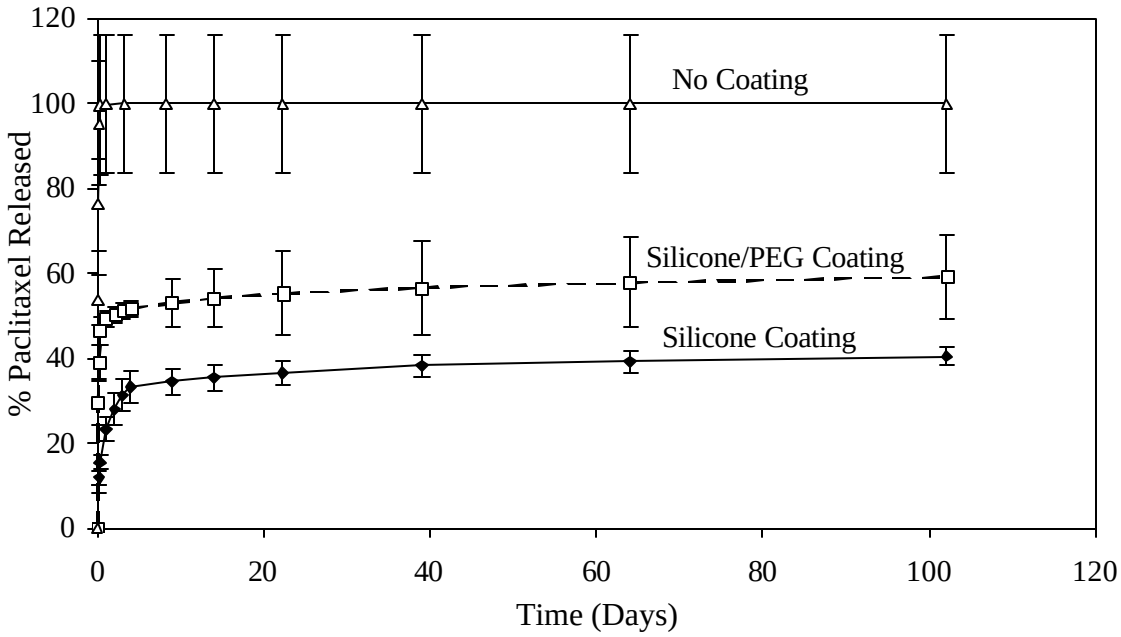

Figure 2.4.1: Release study R-1. Paclitaxel release from a reservoir with silicone coatings fabricated with and without PEG.

The release of tranilast from a reservoir with no coating, a PEG-containing silicone coating, and a silicone coating without PEG was investigated. As shown in Figure 2.4.2, the incorporation of PEG increased the initial burst significantly. The drug was completely released from a reservoir with a silicone coating in about 15 days and was released from a reservoir with a silicone/PEG coating in about 3 days. In the absence of any coating, all of the drug was released within the first 2 hours.

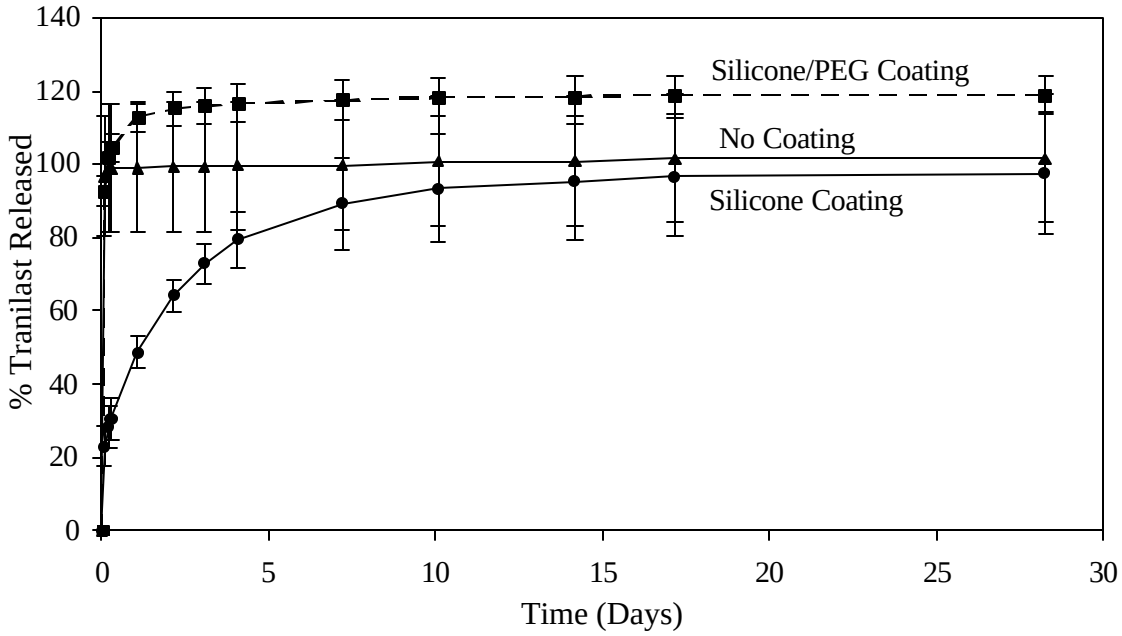

Figure 2.4.2: Release study R-2. Tranilast release from a reservoir with silicone coatings fabricated with or without PEG.

2.5 Discussion

Various silicone elastomer coatings with and without poly(ethylene glycol), as a drug compatilizer and pore former, were evaluated for their ability to release paclitaxel and tranilast for an extended period of time. Both monolithic and reservoir type coatings were investigated. Table 2.4 shows the release rates of drug from silicone coatings for various time intervals.

Table 2.4: Release rates in μg/day of drug for silicone drug release coatings for various time intervals.

Release Study	Drug Layer	Topcoat	0-1 day Release Rate	1-7 day Release Rate	7-14 day Release Rate	14-28 day Release Rate
M-1	A	None	13.4 ± 8.7	1.2 ± 0.4	0.46 ± 0.03	0.52 ± 0.08
	B	None	32.2 ± 4.2	1.8 ± 0.1	0.41 ± 0.07	0.28 ± 0.01
M-2	C	None	146.0 ± 7.8	6.8 ± 0.3	1.3 ± 1.0	0.27 ± 0.15
	D	None	111.3 ± 5.8	13.8 ± 1.7	2.4 ± 0.7	0.81 ± 0.38
M-3	D	a	31.9 ± 2.4	5.8 ± 0.9	2.5 ± 0.3	0.44 ± 0.42
	D	b	18.8 ± 1.8	6.5 ± 0.9	2.6 ± 0.5	0.27 ± 0.18
	D	c	32.2 ± 1.9	8.5 ± 0.5	2.6 ± 1.5	0.50 ± 0.47
	D	d	31.6 ± 2.3	8.6 ± 0.4	2.7 ± 0.2	0.41 ± 0.30
R-1	E	e	136.0 ± 5.3	2.5 ± 2.0	1.5 ± 1.6	0.60 ± 0.61
	E	f	65.3 ± 8.1	5.0 ± 0.3	0.63 ± 0.12	0.31 ± 0.05
R-2	F	e	87.6 ± 3.3	0.61 ± 0.31	0.13 ± 0.03	0.02 ± 0.02
	F	f	37.7 ± 3.5	5.6 ± 1.3	0.75 ± 0.43	0.13 ± 0.07

It was shown that for the hydrophobic drug paclitaxel, the incorporation of PEG into monolithic silicone elastomer coatings decreased the initial burst rate and raised the steady state release rate of the drug. Near zero-order release rates were achieved for paclitaxel after the initial burst and sustained for 60 days, with continued but decreased release continuing for as long as 140 days.

For the hydrophilic drug tranilast, it was shown that the incorporation of PEG into monolithic coatings increased the initial burst rate while decreasing the subsequent steady state release rate. Release of the drug did not follow zero-order release kinetics and leveled off to zero after 21 days. Adding a topcoat to the tranilast/silicone coating reduced the initial burst, but did not extend the release past 21 days.

Lipophilic drugs, such as tranilast, are released from silicone by dissolving into and then diffusing out of the silicone. When a hydrophilic additive (PEG) is added to the silicone, it presents a barrier to diffusion of the lipophilic drug out of the silicone. Eventually, the PEG diffuses out of the silicone, leaving behind water-filled channels, which increase the subsequent release rate of the lipophilic drug. As expected, topcoats

on top of the tranilast monolithic coatings resulted in lower total release and decreased initial burst. This is due to the fact that the drug has an additional barrier to diffusion.

Hydrophilic drugs, such as paclitaxel, are released from silicone by forming water-filled channels in the silicone and then diffusing through these channels. The drug on the surface is released quickly and then the drug further inside the polymer diffuses through the channels formed by the already diffused drug. In the case of a hydrophilic drug with silicone, PEG can act as a compatilizer between the silicone and the drug, resulting in some diffusion through the polymer as well as through the water-filled channels.

Reservoir coatings involving silicone/PEG topcoats also gave interesting results when comparing the release of a hydrophilic drug versus a lipophilic drug. The silicone coating presented a more difficult diffusion barrier for both drugs than the silicone/PEG coating. It should be noted that more of the paclitaxel was trapped completely by the topcoats than tranilast.

Drug release kinetics is an important consideration when designing a drug delivery system. It has been hypothesized that an initial burst of drug, followed by a long slow release may be the best clinical answer to restenosis when treating with paclitaxel. In other drug delivery situations, it is useful to minimize initial burst, but to maximize the release rate over a long period of time. Also, it is beneficial for all of the drug to be released over the course of the delivery process, so as to reduce the cost associated with drug loading of the device. By tuning the above-mentioned parameters and by using PEG as a compatilizer and pore-former in silicone coatings, a variety of different release kinetics can be achieved.

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3 HYDROGEL DRUG DELIVERY COATINGS

3.1 Introduction

In an effort to investigate the effect of poly(hydroxyethyl acrylate) (pHEA) hydrogel properties on drug release, experiments were conducted to compare release of several model drugs: methylene blue, 4',5'-dibromofluorescein, and various molecular weight FITC-labeled dextrans. Release from hydrogels made with two different cross-linkers, tetraethylene glycol diacrylate (TEGDA), and 1,4-butanediol diacrylate was also compared. Additionally, crosslinker concentration was varied.

Poly(hydroxyethyl acrylate) hydrogels have a history of biocompatibility, which makes them appropriate for many medical applications, including drug delivery. In addition, pHEA swelling is dependent on crosslink density, allowing drug delivery rates to be tuned with crosslink density. Finally, the linear chain of pHEA is soluble in water, making it a good candidate for signal responsive drug delivery. Figure 3.1.1 (a) shows the chemical structure of HEA monomer, and Figure 3.1.1 (b) shows the chemical structure polymerized HEA (pHEA).

Figure 3.1.1: Chemical structures of **(a)** Hydroxyethyl acrylate monomer and **(b)** Poly(hydroxyethyl acrylate)

Hydroxyethyl acrylate can be polymerized using free radical initiator to link the monomer together into linear chains. In the presence of difunctional crosslinkers, however, covalent links between the straight chains form, resulting in a crosslinked hydrogel. Figure 3.1.2 is a schematic of crosslinked pHEA.

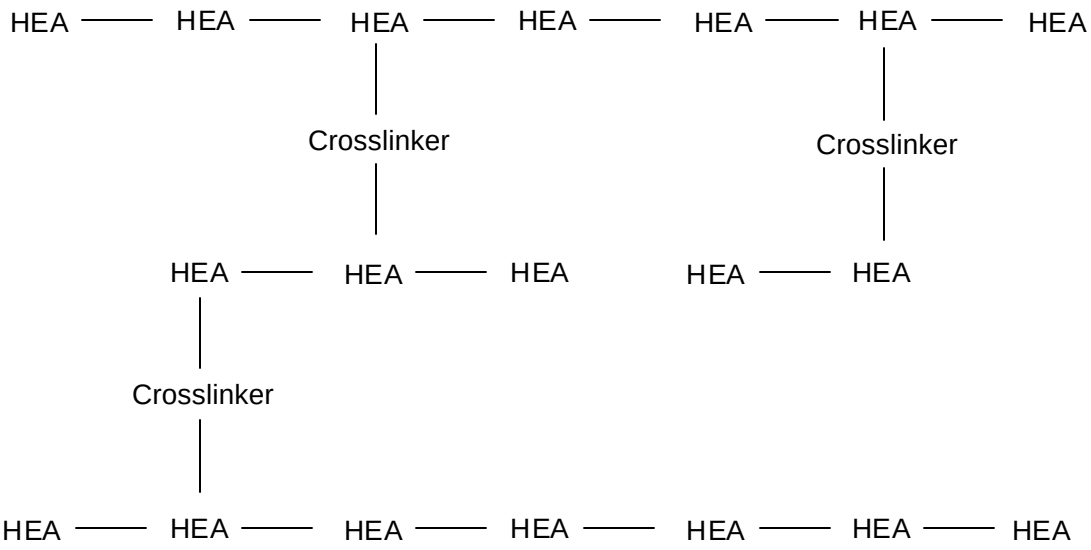


Figure 3.1.2: Schematic of crosslinked poly(hydroxyethyl acrylate).

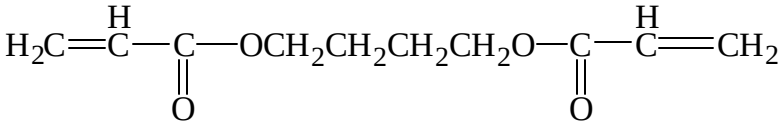
The diffusion coefficient of a drug through a hydrogel is dependent on the drug's diffusivity in the solvent, the size of the drug molecule, the crosslink density, the strength of interactions between the drug and the polymer, and the equilibrium swelling ratio of the polymer.

3.2 Materials and Methods

Materials

All hydrogels used in these studies were made with hydroxyethyl acrylate (HEA) monomer from Polysciences. Two different crosslinkers were used, tetraethylene glycol diacrylate (TEGDA) and 1,4-butanedioldiacrylate (BDDA), both from Polysciences. The chemical structures of the two crosslinkers are shown Figure 3.2.1. The crosslinkers have different levels of hydrophilicity, allowing us to affect the properties of the hydrogel.

Tetraethylene glycol diacrylate (TEGDA)



1,4-Butanediol diacrylate (BDDA)

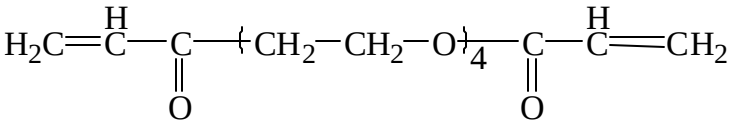


Figure 3.2.1: Bifunctional crosslinkers used in hydrogel release studies.

The gels were polymerized by free radical initiation using a dual component redox initiating system composed of ammonium persulfate and sodium metabisulfite (Aldrich) [1]. Water and ethylene glycol (Fisher) were used as solvents. The model drugs used in the release experiments were methylene blue, 4',5'-dibromofluorescein, and fluorescein labeled dextrans (FITC-dextrans). Three molecular weights of dextran were used, 4300, 10500, and 19500 (Sigma). These model drugs are shown in Figure 3.2.2.

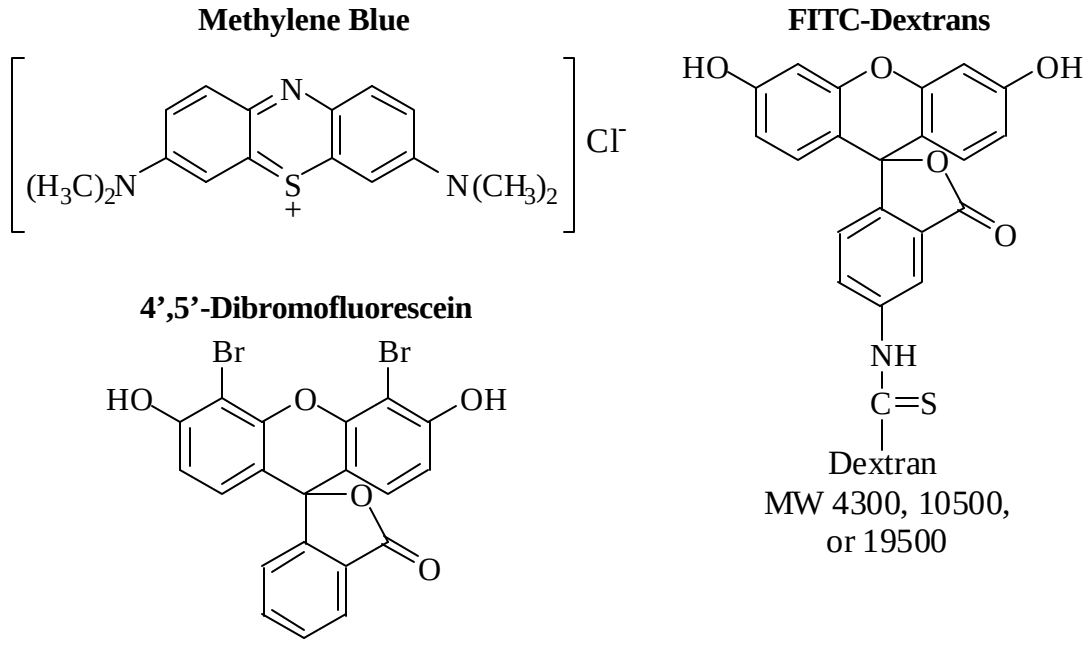


Figure 3.2.2: Model drugs used in hydrogel release studies.

Hydrogel Formulation

Model drug-containing hydrogels were prepared by mixing HEA, crosslinker, model drug, and initiator in a solvent mixture of water and ethylene glycol. The mixtures were then cast between glass plates separated by 0.03 inch Teflon® spacers and allowed to polymerize for 24 hours. Table 3.1 lists the hydrogel formulations used for these release studies. Table 3.2 contains the drug/crosslinker combinations that were investigated in each of the studies.

Table 3.1: Solvent, drug, and crosslinker formulations for hydrogel release studies. Each formulation also included 1 mL HEA monomer and 0.03 mL of each 0.4 g/mL $(NH_4)_2S_2O_8$ and 0.15 g/mL $Na_2S_2O_5$.

Formulation	Ethylene Glycol (mL)	Water (mL)	Drug Solution* (mL)	Crosslinker (mL)
I	0.3	0.042	0.158	0.02
II	0.3	0.040	0.160	0.04
III	0.3	0.036	0.164	0.08
IV	0.142	0.2	0.158	0.02
V	0.140	0.2	0.160	0.04
VI	0.136	0.2	0.164	0.08
VII	0.124	0.2	0.176	0.20
VIII	0.3	1.0	0.2	0.04
IX	0.3	1.0	0.2	0.08

*1% w/v in water for formulations I-VII and 0.0016M for formulations VIII and IX.

Table 3.2: Drugs, crosslinker, and formulations used for hydrogel release studies. MB is methylene blue; DBF is 4',5'-dibromofluorescein; Dex is FITC-labeled dextran; TEGDA is tetraethylene glycol diacrylate; BDDA is 1,4-butanediol diacrylate.

Release Study:	Drug(s) Used	Crosslinker Used	Formulations
H-1	MB	TEGDA	I, II, III
H-2	DBF	TEGDA	IV, V, VI
H-3	DBF	BDDA	IV, V, VI, VII
H-4	Dex 4300, 10500, 19500	TEGDA	VIII, IX
H-5	Dex 4300, 10500, 19500	BDDA	VIII, IX

Hydrogels used in swelling studies were made in the same manner as those used in the release studies, but without model drug. Table 3.3 shows the formulations used in the swelling studies.

Table 3.3: Formulations used for swelling study. Each formulation involved 1 mL HEA monomer, and 0.03 mL of each 0.4 g/mL $(NH_4)_2S_2O_8$ and 0.15 g/mL $Na_2S_2O_5$.

Formulation	Ethylene Glycol (mL)	Water (mL)	Crosslinker (mL)
a	0.3	0.2	0.02
b	0.3	0.2	0.04
c	0.3	0.2	0.08
d	0.3	0.2	0.2
e	0.3	1.2	0.02
f	0.3	1.2	0.04
g	0.3	1.2	0.08
h	0.3	1.2	0.2

Elution Studies

After polymerization, the hydrogel slabs were removed from the glass plates and sample disks, 17/32 inch or 10 mm diameter, were cut from the hydrogels. These disks were then placed in 20 mL vials with 2.0 mL of phosphate buffered saline solution (PBS), pH = 7.4. At appropriate time intervals the PBS was removed, replaced with fresh PBS, and the model drug concentration was determined using UV-spectrophotometry. The amount of drug released and the % release of drug were calculated using Equations 3.1 and 3.2, respectively. The initial mass of drug in each hydrogel sample was calculated using Equation 3.3.

$$M_t = \sum_t V_e C_t \qquad (3.1)$$

Where M_t = cumulative amount of drug released at time t (ìg)

V_e = volume of elution medium (mL)

C_t = Concentration of drug in elution medium at time t (ìg/mL)

$$\%\,\text{Release} = M_t / M_i \times 100 \qquad (3.2)$$

Where M_t = cumulative amount of drug released at time t (ìg)

M_i = initial mass of drug in sample (ìg)

$$M_i = \frac{M_c C_{d,v} C_{d,s}}{\rho_{h,s}} \quad (3.3)$$

Where M_i = initial mass of drug in hydrogel samples (g)

M_c = weight of sample (g)

$C_{d,v}$ = v/v % of drug solution in hydrogel solution

$C_{d,s}$ = w/v % of drug in drug solution (g/ml)

$\rho_{h,s}$ = density of hydrogel solution (g/ml)

UV Spectrophotometry of Model Drugs

The concentration of all model drugs was determined using UV-vis spectrophotometry at wavelengths of 609 nm, 502 nm, and 495 nm for methylene blue, 4',5'-dibromoflourescein, and FITC-dextrans respectively. The peak wavelength of each model drug was determined by using a Hewlett Packard HP 8452A diode array spectrophotometer. A standard curve was created for each drug by measuring the absorbance of 200 μL solution in a 96 well plate at the above-mentioned wavelength for a range of known concentrations. The UV-vis spectra and standard curve for each drug are shown in Appendix B. Concentration of the released drug in the eluent was then determined by measuring the absorbance of 200 μL of the eluent in 96 well plates, subtracting the absorbance of a blank solution (PBS, pH = 7.4) and referencing to the standard curve.

Hydrogel Swelling Studies

After polymerization, the hydrogels were removed from the glass plates and cut into 5 mm diameter sample disks. The disks were weighed and placed in 2.5 mL sample cups with 1 mL PBS, pH = 7.4. At each time point, three sample disks of each type were removed from the sample cups, blotted dry, and placed into pre-weighed 1.5 mL microfuge tubes. The tubes were weighed again, to determine the wet weight of the hydrogel samples. The samples were then frozen in a –80 ^{o}C freezer overnight and lyophilized for 3 days. The lyophilized hydrogel samples were weighed to determine

their dry weights. The equilibrium mass swelling ratio was calculated using the following equation:

$$q = \frac{M_s}{M_d} \tag{3.4}$$

Where q = equilibrium mass swelling ratio

M_s = mass of swollen polymer (g)

M_d = mass of dry polymer (g)

3.3 Results: Effect of Crosslinker Concentration and Crosslinker Type on Release of Low Molecular Weight Model Drugs

The effect of crosslinker concentration on release of methylene blue and 4',5'-dibromofluorescein was investigated. Two different crosslinkers, tetraethylene glycol diacrylate (TEGDA) and 1,4-butanediol diacrylate (BDDA), were used in these studies.

Crosslinker concentration did not affect the release of methylene blue from gels made with a TEGDA crosslinker concentration of 0.78-3.10 mol %, as shown in Figure 3.3.1. Note that about 25% of the drug remained in the gels at the end of the experiment.

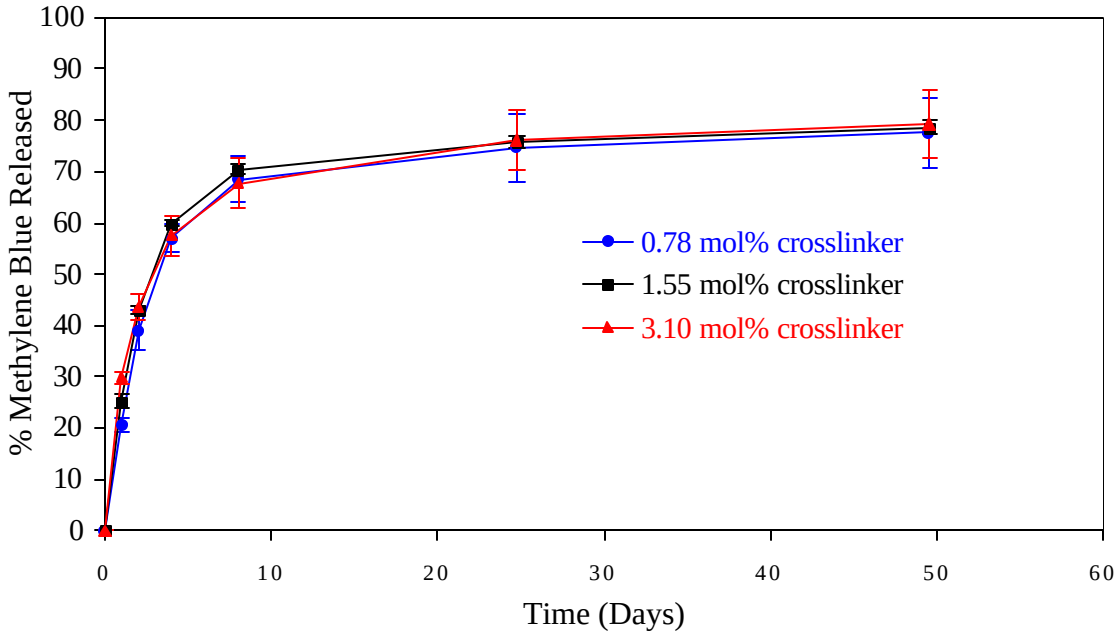


Figure 3.3.1: Release Study H-1. Effect of crosslinker concentration on release of methylene blue from gels made with TEGDA.

Figure 3.3.2 shows how crosslinker concentration affected the release of the more hydrophobic 4',5'-dibromofluorescein from HEA hydrogels made with the more hydrophilic TEGDA crosslinker concentration of 0.78-3.10 mol %. In this case, almost 100% of the drug was released from the gel, but there was no statistically significant difference in the release rates of the drug from gels made with different crosslinker concentrations.

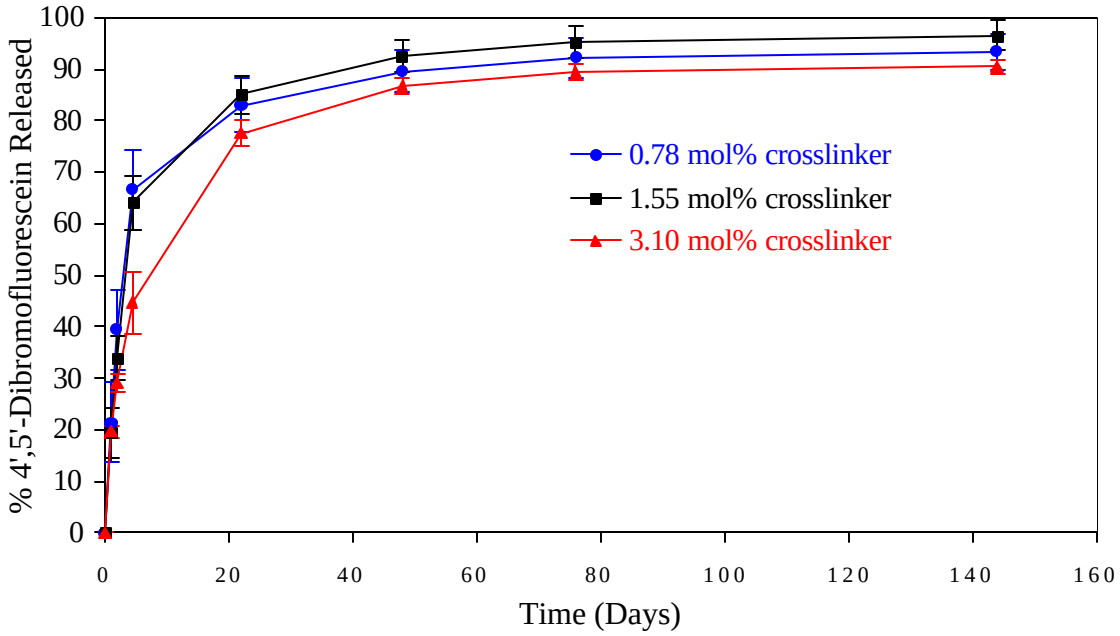


Figure 3.3.2: Release Study H-2. Effect of crosslinker concentration on release of 4',5'-dibromofluorescein from gels made with TEGDA.

Figure 3.3.3 compares the release of 4',5'-dibromofluorescein from gels made with various concentrations of 1,4-butanediol diacrylate crosslinker. In this case, there was a difference in the release rates in relation to crosslinker concentration. Gels made with a higher crosslinker concentration released the drug more slowly than gels made with less crosslinker.

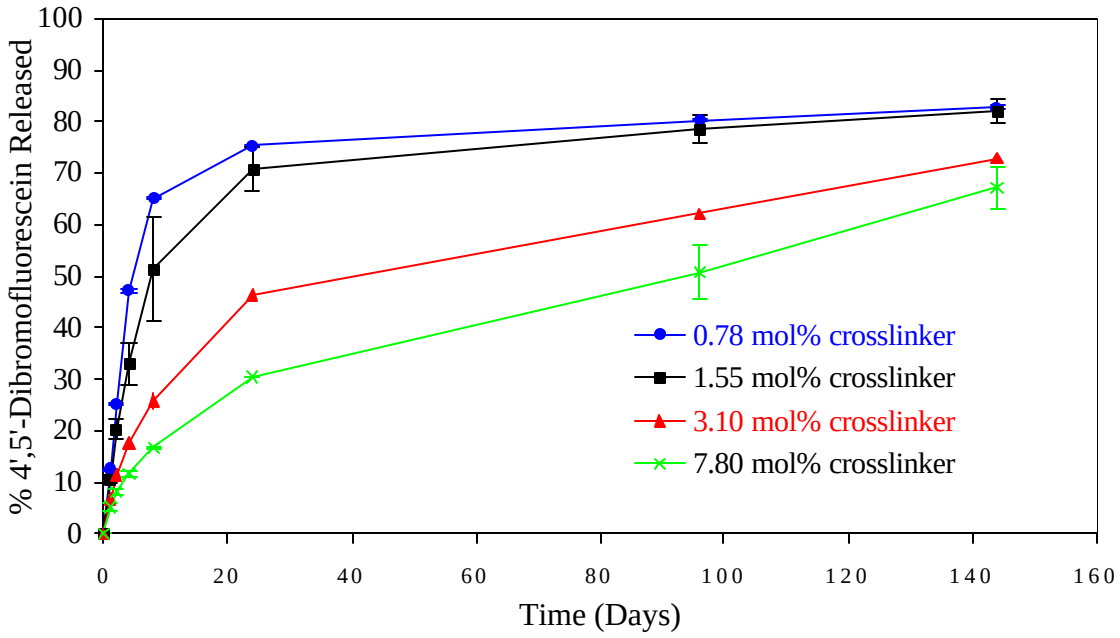


Figure 3.3.3: Release Study H-3. Effect of crosslinker concentration on release of 4',5'-dibromofluorescein from gels made with 1,4-butanedioldiacrylate.

3.4 Results: Effect of Drug Molecular Weight on Release of FITC-Dextrans

Three different molecular weight FITC-labeled dextrans were used to evaluate the effect of drug molecular weight on its release from HEA hydrogels. Dextrans with molecular weights of 4300, 10500, and 19500 were used. Figure 3.4.1 and Figure 3.4.2 compare the percent release of these three model drugs from HEA hydrogels made with two different amounts of TEGDA crosslinker and 1,4-butanediol diacrylate crosslinker, respectively.

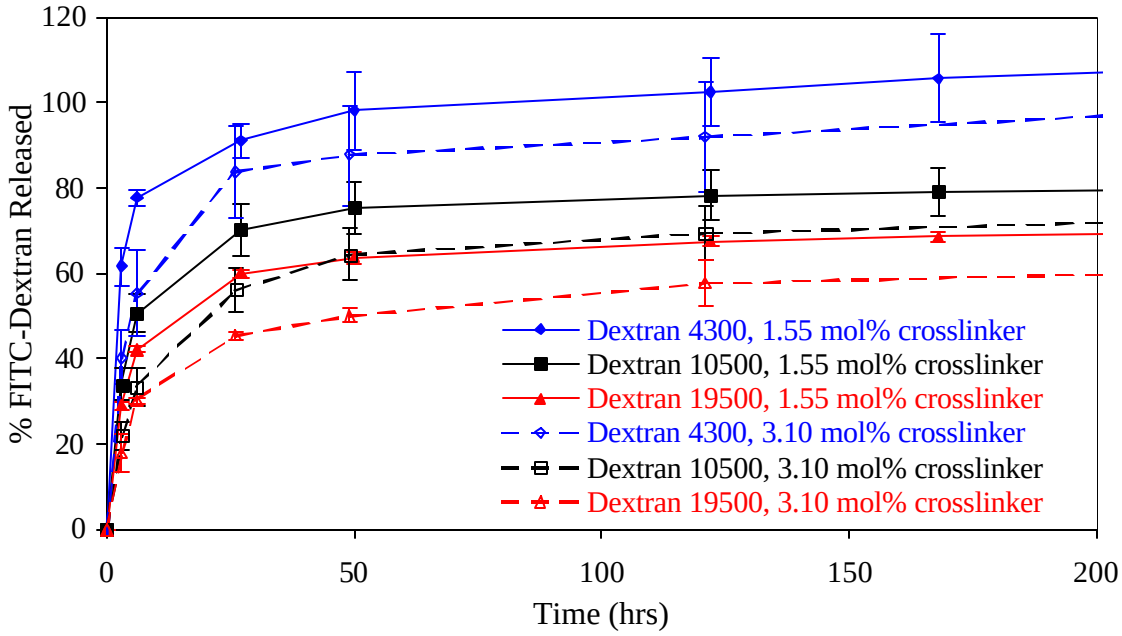


Figure 3.4.1: Release Study H-4. Effect of drug molecular weight and crosslinker concentration on the release of various FITC-labeled dextrans from gels made with TEGDA.

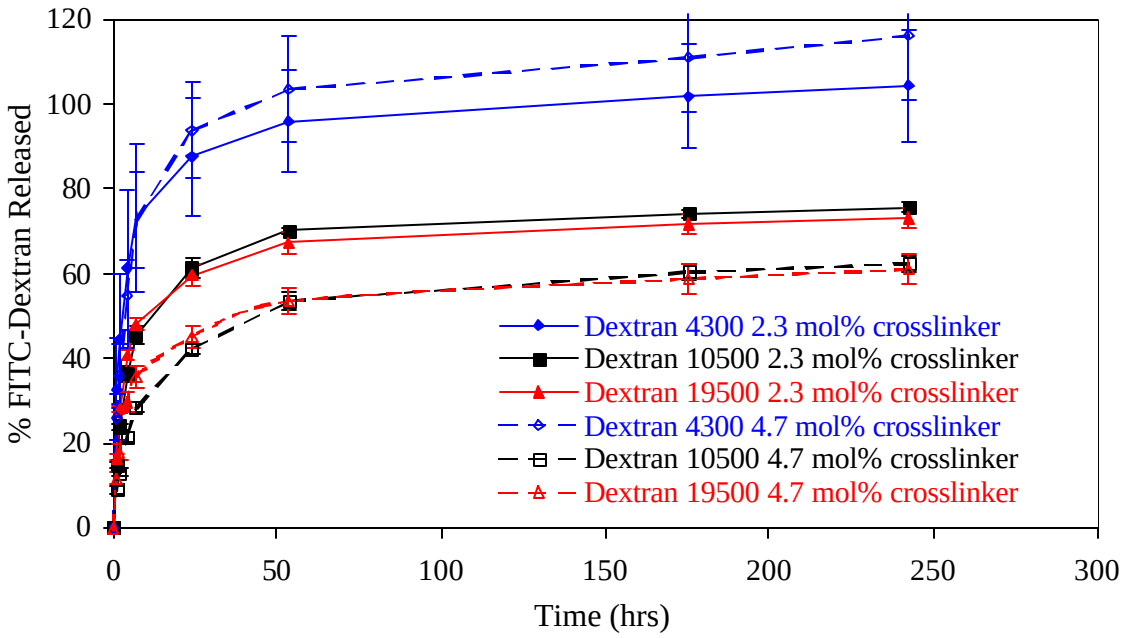


Figure 3.4.2: Release Study H-5. Effect of drug molecular weight and crosslinker concentration on the release of various FITC-labeled dextrans from gels made with 1,4-butanediol diacrylate.

These results demonstrate that molecular weight does affect the rate of dextran release from the hydrogels. An increase in drug molecular weight resulted in decreased release from the gels. Also, higher crosslinker concentration resulted in lower drug release levels.

3.5 Results: Effect of Crosslinker Concentration and Crosslinker Type on Swelling of HEA Hydrogels

In order to see how swelling of the hydrogels related to drug release from the hydrogels, a swelling study conducted and the equilibrium mass swelling ratios of hydrogels made with TEGDA and 1,4-butanediol diacrylate were compared. Four different crosslinker concentrations were investigated. Figure 3.5.1 shows the results of this study.

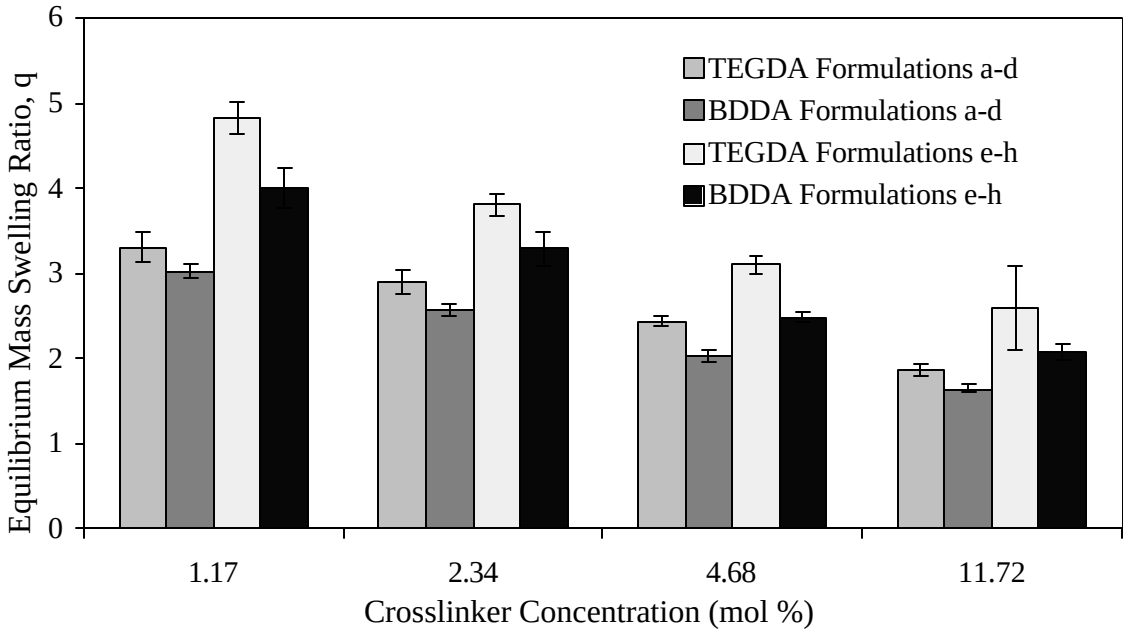


Figure 3.5.1: Effect of crosslinker concentration and crosslinker type on equilibrium mass swelling ratio of pHEA hydrogels.

An increase of crosslinker concentration resulted in a decrease in equilibrium mass swelling ratios of the gels, as expected. Additionally, the use of a less hydrophilic crosslinker, 1,4-butanediol diacrylate, results in lower swelling ratios than for gels made with TEGDA. These results correlate with the drug release results presented above in that drug was released fastest from gels that swell the most.

3.6 Discussion

In these studies, we looked at the effects of crosslinker concentration, crosslinker hydrophilicity, drug hydrophilicity, and drug molecular weight on drug release rates from poly(hydroxyethyl acrylate) (pHEA) hydrogels. We showed that crosslinker concentration has an inverse relationship with delivery rates of drugs when the drug is sufficiently large such that its diffusion through the hydrogel can be inhibited by the additional crosslinks present or when the drug is hydrophobic. This finding is consistent with hydrogel controlled release theory. We also showed that gels made with higher crosslinker concentrations have lower equilibrium swelling ratios than gels made with lower crosslinker concentrations. These swelling studies help to explain the observation that the drug release rate from the more highly crosslinked gels is lower. Additionally, we showed that drugs of a higher molecular weight diffuse out of the gel more slowly than those of lower molecular weight. This also is consistent with hydrogel controlled release theory.

One of the goals of this part of the project was to determine if an optimal crosslinker concentration could be achieved to hold the drug in the gel for a period of time that would allow signal responsive drug delivery to be effective. We have shown that crosslinker concentration affects the release of 4',5'-dibromofluorescein and higher molecular weight drugs. 4'5'-dibromofluorescein has similar structure and size to paclitaxel, so it is likely that crosslinker concentration would affect the release of paclitaxel from pHEA gels. The next chapter describes how this could be used in a signal responsive drug delivery system.

Notes to Chapter 3

1. Ratner, B.D., I.F. Miller, *Journal of Polymer Science: Part A-1* **10**, 2425-2445 (1972).

4 SIGNAL RESPONSIVE DRUG DELIVERY COATINGS

4.1 Introduction

Thrombin Responsive Hydrogels

Presented here is a method for synthesis of a thrombin degradable crosslinker that can be used to create a signal responsive drug delivery coating for cardiovascular stents. Thrombin has been shown to stimulate smooth muscle cell (SMC) proliferation following vessel wall injury [1], and is present in elevated levels during SMC proliferation [1].

Thrombin is formed by the prothrombinase complex on activated platelets and is involved in restenosis and thrombosis in several ways. Thrombin cleaves fibrinogen into fibrin and forms a platelet-fibrin complex that causes thrombus formation. Thrombus formation in turn causes thrombosis and may also provide a matrix for SMC migration [2]. In addition, thrombin is an important signal in that triggers the expression of platelet-derived growth factor [3], induces the secretion of collagen [4], and stimulates SMC proliferation [5].

By creating a crosslinker with a thrombin cleavable peptide, it is possible to create a thrombin responsive drug delivery hydrogel that can be used as a coating or as a stand alone drug delivery device. There are many peptide sequences that are cleaved by thrombin [6-8]. Lottenberg et al. showed that the best substrates for thrombin were ones with arginine in the P_1 amino acid position, proline or a proline homolog in the P_2 position, and an apolar amino acid in the P_3 position. Val-Pro-Arg-Gly was chosen here because of its ease of synthesis. Thrombin is known to cleave this sequence between the arginine and glycine residues.

The thrombin cleavable peptide was synthesized using solid phase peptide synthesis (SPPS), and the acrylate end-groups were coupled to the peptide using carbodiimide chemistry. The peptide crosslinker's performance was tested by determining if it was cleaved by thrombin at the appropriate location. Next, hydrogels

were created with the crosslinker, and finally the swelling or degradation of these hydrogels in the presence of thrombin was determined.

Peptide Synthesis

Since the invention of solid phase peptide synthesis (SPPS) by Merrifield [9], the synthesis of small peptides has been considerably easier than by solution phase methods. SPPS involves the stepwise coupling of amino acids to a growing peptide chain covalently attached to a polymeric support resin. Each amino acid has its reactive side chains protected until the peptide is cleaved from the resin. The N-terminus of the amino acid is typically protected with the acid-labile *tert*-butyloxycarbonyl (Boc) protecting group or the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group. After the coupling has taken place, the N-terminus is deprotected so that the reactive amine group is available for the next coupling. Because this takes place on a solid support, all of the reagents and by-products can be removed by filtration. Additionally, a large excess of reagents can be used, resulting in high yields [10]. Figure 4.1.1 shows a schematic of the general solid phase peptide synthesis process.

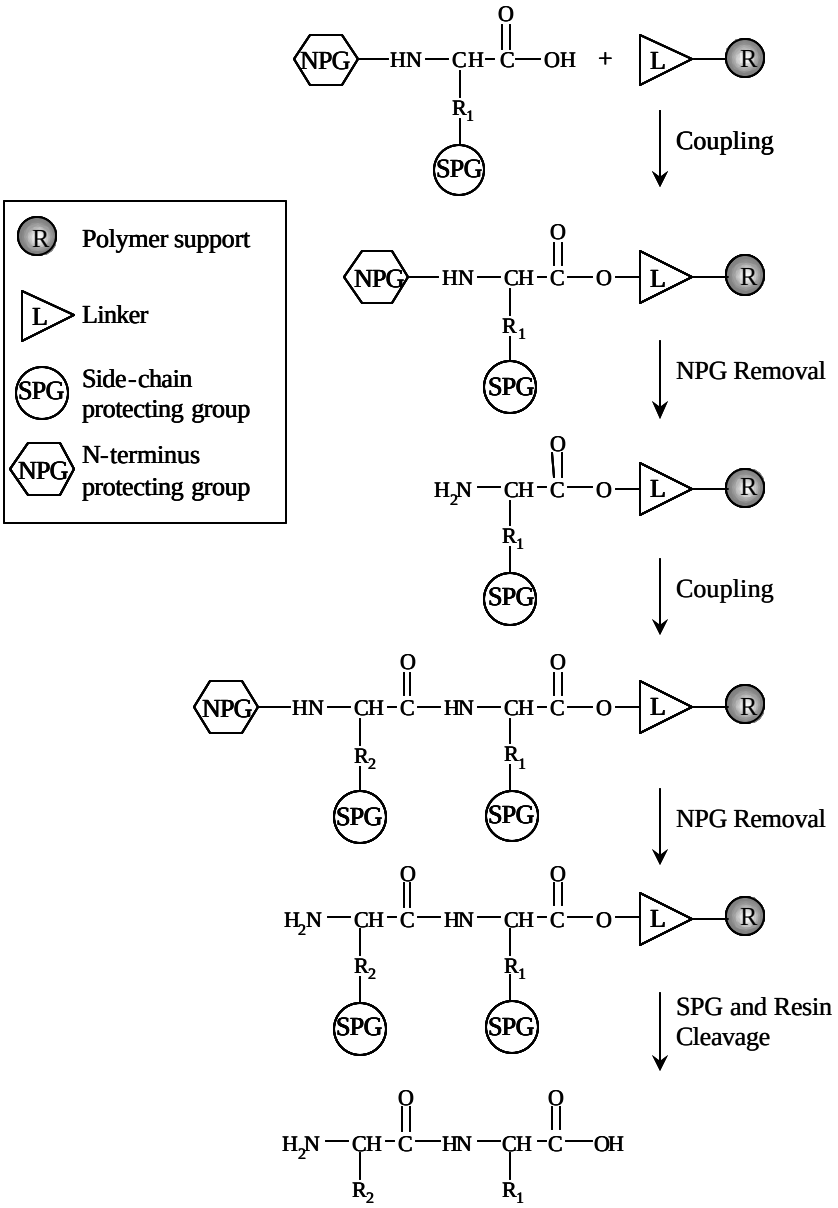


Figure 4.1.1: Schematic of solid phase peptide synthesis.

4.2 Crosslinker Synthesis

Overall Scheme

The crosslinker described here is a tetrapeptide with acrylate end-groups. The molecular structure of the crosslinker is shown in Figure 4.2.1.

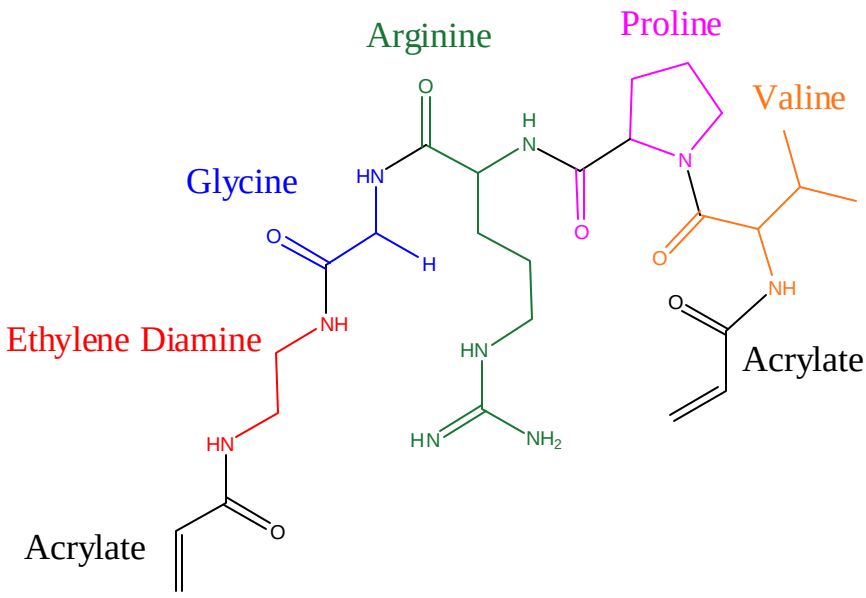


Figure 4.2.1: Chemical structure of peptide crosslinker.

The synthesis of the crosslinker involves Fmoc (9-fluorenylmethyloxycarbonyl) solid phase peptide synthesis (SPPS) of the four residue peptide sequence on 1,2-diaminoethane trityl resin resulting in amine groups on both the C- and N- terminus of the peptide upon cleavage from the resin. After cleavage (by dilute trifluoroacetic acid (TFA)), the reactive arginine side chain remains protected by a 2,2,5,7,8-pentomethylchroman-6-sulfonyl (Pmc) protecting group. Next, acrylate end-groups are added to the peptide using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Finally, the arginine protecting group is cleaved with 95% TFA, 2.5% triethylsilane (TES), 2.5% water. Figure 4.2.2 shows a schematic of the reaction scheme.

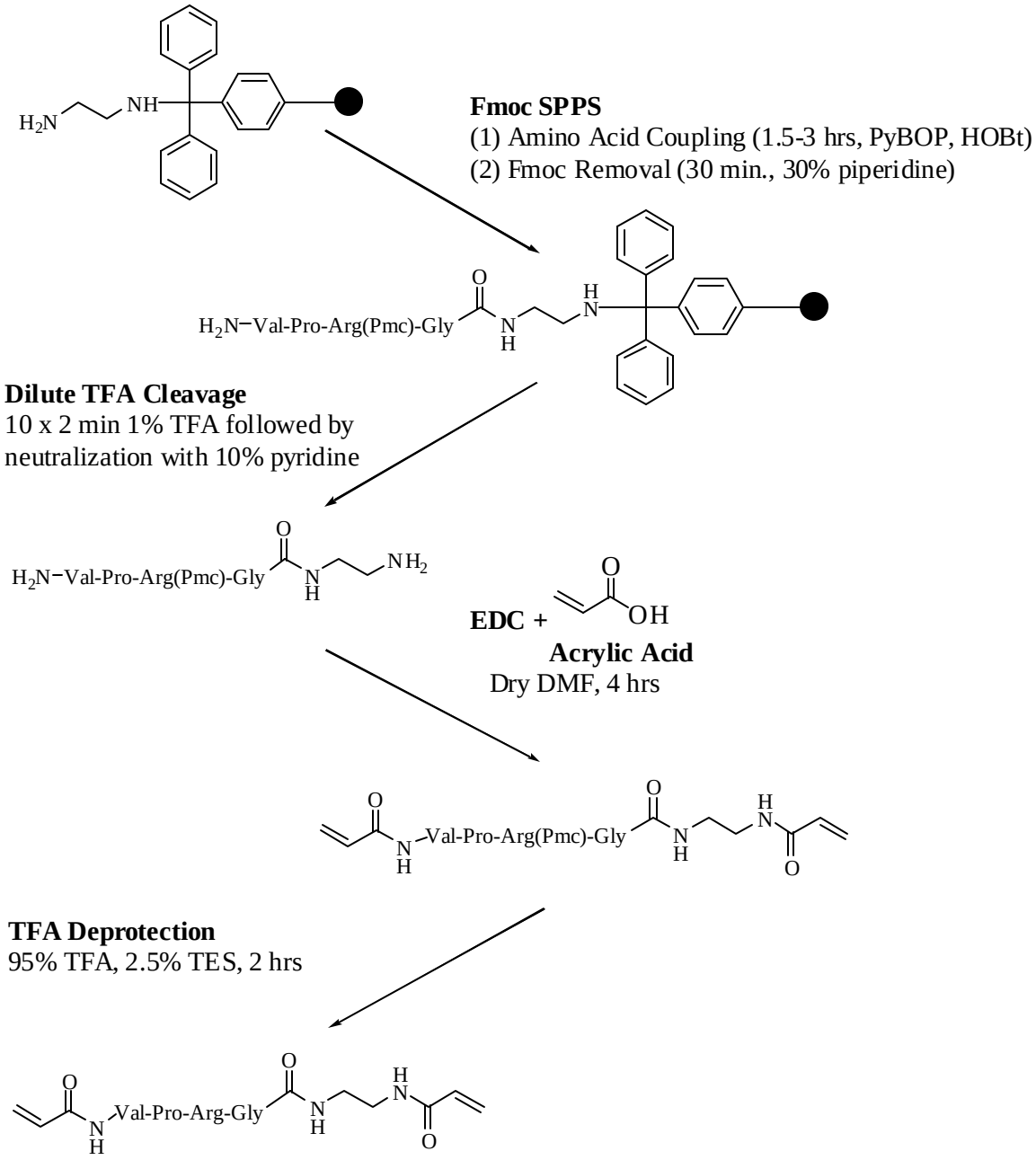


Figure 4.2.2: Crosslinker Synthesis Scheme.

Fmoc chemistry was chosen for the SPPS process. The cleavage of Boc (*tert*-butyloxycarbonyl) protecting groups requires the use of hydrofluoric acid and special lab equipment, whereas Fmoc deprotection is facilitated by use of a mild base procedure

using 30% piperidine. The synthesis described here was slightly modified from that shown in Figure 4.1.1. In the synthesis of this peptide crosslinker, there were two major goals in the basic peptide synthesis: to synthesize a peptide with two reactive amine groups, one on each end, and to protect any other reactive groups on the molecule through the resin cleavage. With these two goals met, it would be possible to couple acrylate groups onto both ends of the peptide in one step without modifying the amine group of the arginine. For clarity, Figure 4.2.3 shows a general schematic of this procedure that could be applied to other peptide sequences.

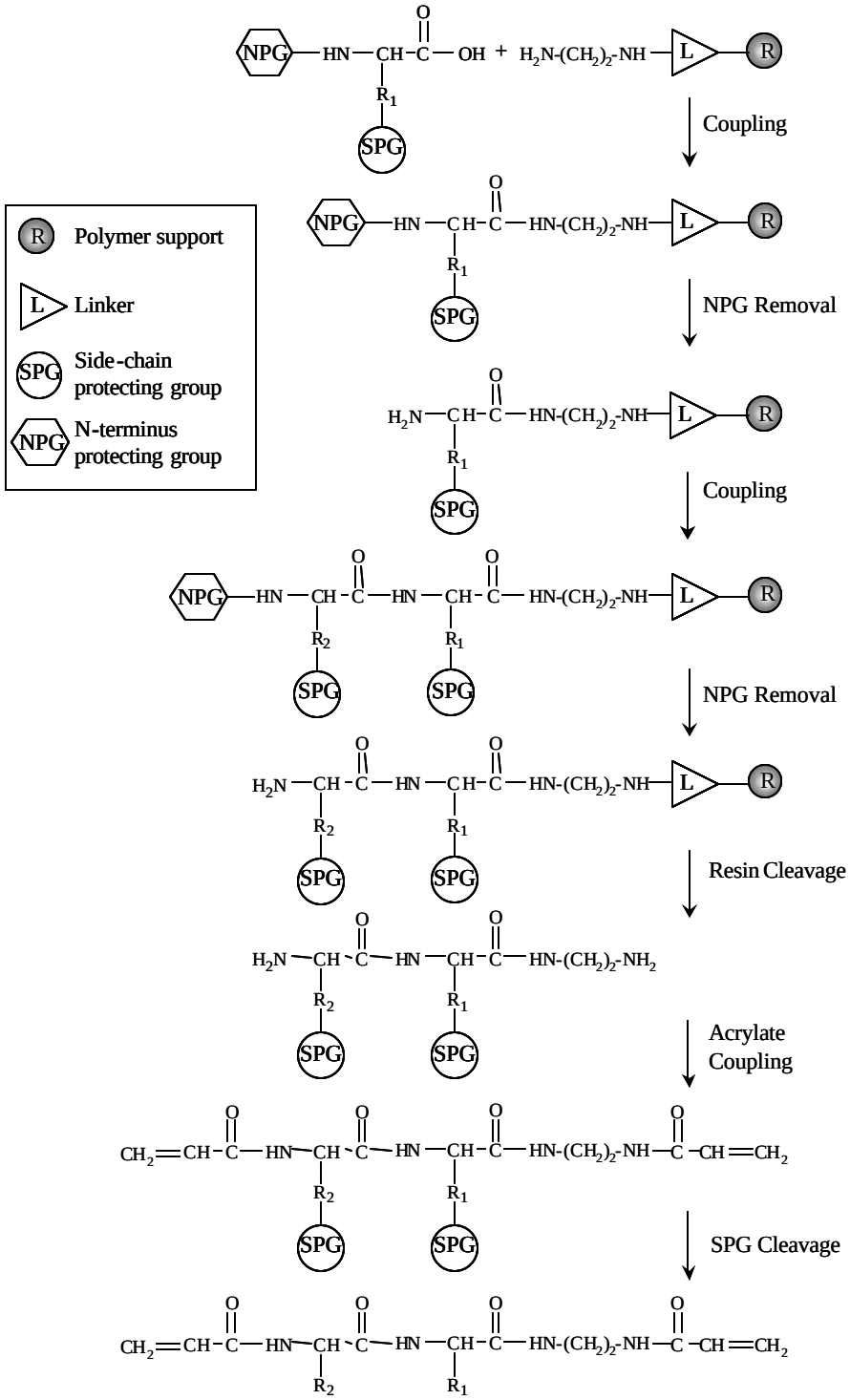


Figure 4.2.3: Schematic of SPPS scheme for the synthesis of a peptide diacrylate.

The goals of having reactive amine groups on both ends of the peptide, and of keeping the side chain protecting groups in tact through the resin cleavage were accomplished by using 1,2-diaminoethane trityl resin, shown in Figure 4.2.4. When cleaved, this resin leaves behind an ethylene diamine group on the C-terminus of the peptide, thus creating a peptide with amine groups on both ends of the peptide. Also, the resin can be cleaved with dilute TFA [11, 12], which some side-chain protecting groups can withstand.

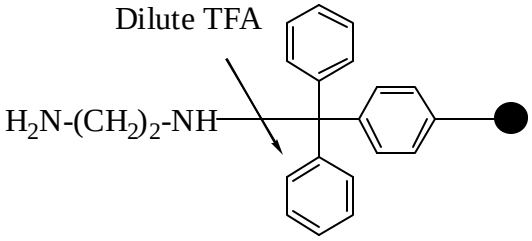


Figure 4.2.4: 1,2-diaminoethane trityl resin. The resin can be cleaved using dilute TFA, leaving an ethylene diamine group attached to the peptide.

The thrombin cleavable peptide sequence Val-Pro-Arg-Gly has a reactive guanidine group on the side chain of arginine that required protection during the EDC coupling of the peptide end groups with acrylic acid after the completed peptide synthesis. The Pmc side chain protecting group would remain in tact during the dilute TFA resin cleavage [13, 14]. Figure 4.2.5 shows the molecular structure of Fmoc-Arg(Pmc)-OH. The Fmoc group is cleaved during the SPPS process with piperidine and the Pmc protecting group is cleaved in the last step of the crosslinker synthesis with 95% TFA [15].

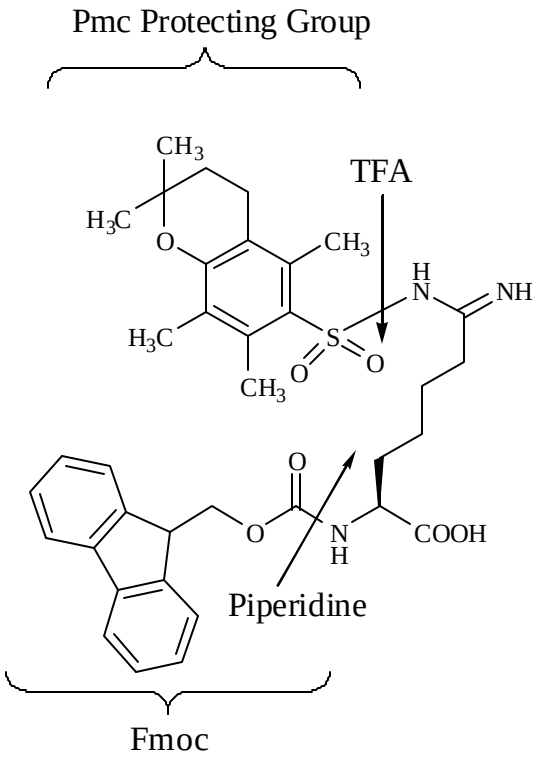


Figure 4.2.5: Fmoc-Arg(Pmc)-OH. Fmoc protects the N-terminus and is removed by 30% piperidine and Pmc protects the side chain and is removed with 95% TFA.

PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) was chosen as a coupling reagent because it is widely available, easy to use, provides fast reaction times, and is safer to use than the common BOP (benzotriazole-7-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate). HOBt (1-hydroxybenzotriazole) is used to accelerate the reaction. Figure 4.2.6 shows a schematic of PyBOP and HOBt.

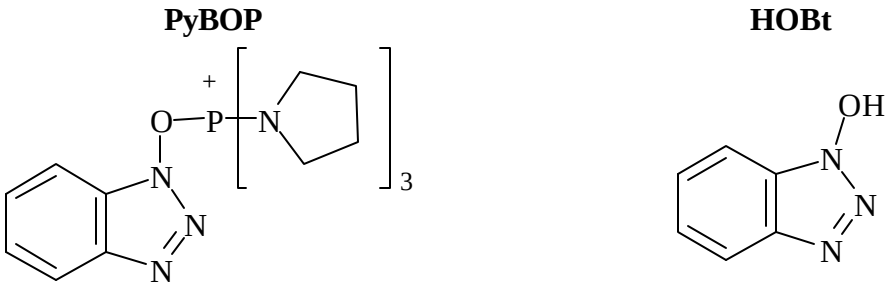


Figure 4.2.6: Coupling reagents used in SPPS.

EDC Coupling

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) can be used to conjugate a molecule containing a primary amine or hydroxyl to another molecule containing a carboxylic acid. Carbodiimides react with carboxylic acids to form an O-acylisourea intermediate. This intermediate then reacts with a primary amine to produce an amide bond. Figure 4.2.7 shows the EDC reaction between acrylic acid and the peptide synthesized here.

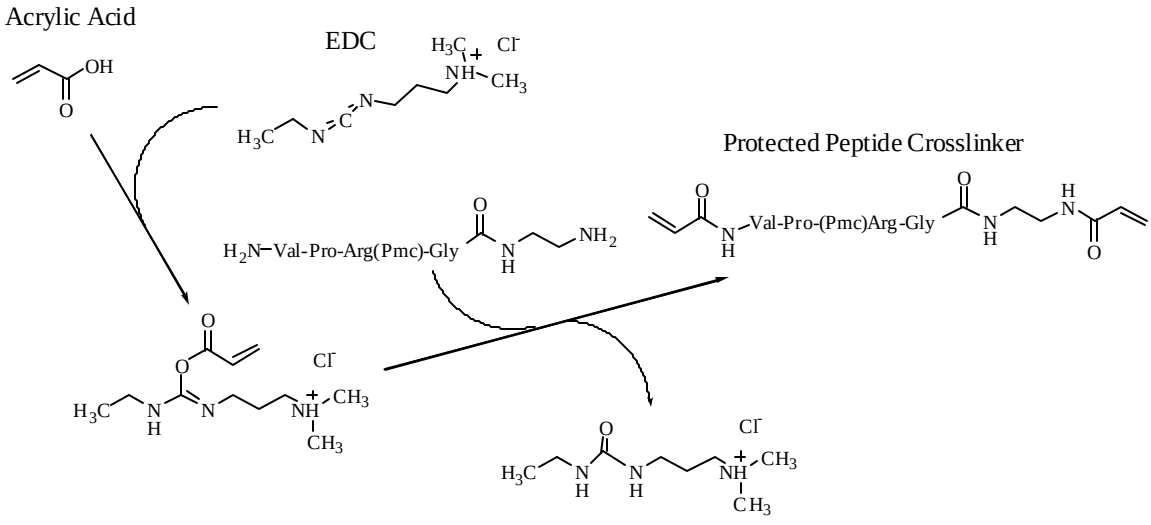


Figure 4.2.7: Schematic of the EDC coupling between acrylic acid and protected peptide.

Materials

1,2-diaminoethane trityl resin, Fmoc protected amino acids, PyBOP, and HOBt were purchased from Novabiochem. All other chemicals were purchased from Aldrich or Fisher. N,N-diisopropylethylamine (DIEA) was 99.5% biotech grade and was checked for purity by performing the ninhydrin test (described below) before each use.

Reaction Specifics: Fmoc SPPS

The Fmoc SPPS procedure was as follows. 0.5 g of 1,2-diaminoethane trityl resin (0.6-1.5 mmol/g loading) was swollen by washing three times with 10 mL methylene chloride in a peptide reaction vessel. The resin was then washed three times with 10 mL dimethylformamide (DMF). For each coupling, a three molar excess of each Fmoc protected amino acid, PyBOP, and HOBt, and a five molar excess of DIEA were sonicated in 10 mL DMF for 5 minutes, before adding the solution to the resin and placing the reaction vessel on a shaker for 1.5-2.5 hours. The resin was then washed three times with 10 mL DMF and a small number of resin beads were tested with either the ninhydrin or *p*-chloranil test, described below, to ensure that the coupling was complete. If the test provided a negative result for amines, the reaction was complete, and Fmoc removal was performed. If the test provided a positive test for amines, the coupling was repeated. In general, the arginine coupling needed to be performed twice and all other couplings only once.

Fmoc removal was accomplished by adding 10 mL of 30% piperidine in DMF to the peptide vessel and shaking for 30 minutes. The resin was then washed three times with 10 mL DMF and the ninhydrin or *p*-chloranil test was performed to determine if the Fmoc group had been removed. If a positive result was obtained from the test, the next coupling was done, if a negative result was obtained, the Fmoc removal procedure was repeated. All steps were performed at room temperature.

Reaction Specifics: Ninhydrin (Kaiser) Test

The ninhydrin, or Kaiser test, is a qualitative test for the presence of free amino groups. The ninhydrin test works well for most amino acids, but not all. Serine,

asparagines, and aspartic acid don't return positive results, and proline, being a secondary amino acid, also doesn't give a positive result. Three test solutions were prepared: (a) 5 g ninhydrin in 100 mL butanol, (b) 80 g phenol in 20 mL butanol, and (c) 2 mL 0.01 M aqueous solution of potassium cyanide in 98 mL pyridine [16]. The test was performed by removing 10-20 resin beads from the peptide vessel and washing them once with 50:50 acetic acid/ethanol and then three times with 100% ethanol. Two drops of each test solution were then added to the tube with the beads and heated to 100 ^{o}C for 5 minutes. A positive result was indicated by blue/purple resin beads.

Reaction Specifics: *p*-Chloranil Test

P-chloranil is used to test for secondary amino groups, in this case, to detect deprotected proline. The resin beads were washed once with 50:50 acetic acid/ethanol and three times with ethanol, and then two drops of each 2% acetaldehyde in DMF and 2% *p*-chloranil in DMF were added. After 5 minutes, blue colored beads indicated a positive result.

Reaction Specifics: TFA Cleavage

After all four of the couplings were complete, the peptide was cleaved from the resin, using a dilute TFA cleavage method described here. The cleavage was repeated ten times: 5 mL 1% TFA in methylene chloride was added to the peptide reaction vessel, the vessel was shaken by hand for 2 minutes, and the TFA solution was removed to a flask containing 1 mL 10% pyridine in methanol under nitrogen pressure. After cleavage, the resin was washed three times with 15 mL methylene chloride, three times with 15 mL methanol, two times with 15 mL methylene chloride, and three times with 15 mL methanol. All of the wash solution was added to the flask with the TFA and pyridine solution. The solution was then reduced to approximately 2-3 mL on a rotary evaporator. The reduced solution was added drop-wise to at least a ten-fold amount of cold ether, and the precipitate was collected and dried under vacuum.

Reaction Specifics: EDC Reaction

The EDC reaction was performed with a twelve molar excess of EDC and a ten molar excess of acrylic acid, in anhydrous DMF. The EDC and acrylic acid were dissolved in DMF and allowed to react for 10 minutes. The peptide was then added to the solution, and allowed to react for four hours. The volume of the solution was doubled with methanol and reduced to approximately 2-3 mL on a rotary evaporator. The reduced solution was added drop-wise to cold ether and the precipitate was collected and dried under vacuum.

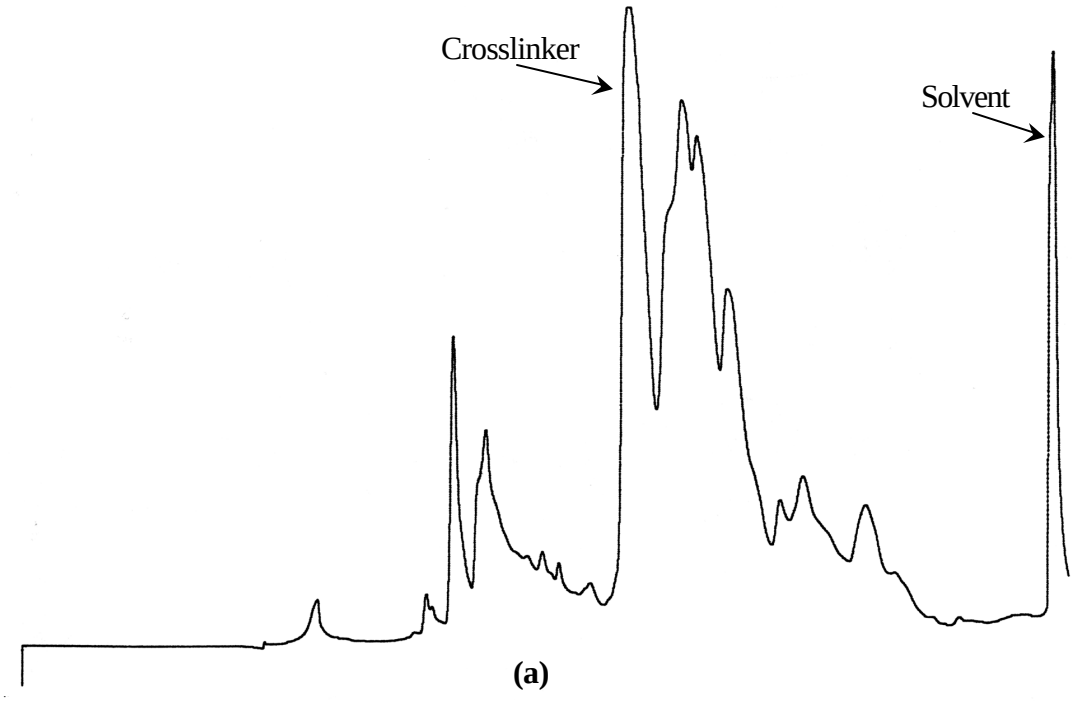
Reaction Specifics: Side Chain Protecting Group Removal

The Pmc arginine protecting group was removed using TFA. A solution of 10 mL of 95% TFA, 2.5% triethylsilane (TES), 2.5% water was added to the protected peptide crosslinker and was allowed to react for 2.5 hours. The solution was reduced to 2-3 mL by evaporation and the reduced solution was added drop-wise to at least a ten fold amount of cold ether. The precipitate was collected and dried under vacuum.

4.3 Crosslinker Purification and Characterization

High Performance Liquid Chromatography Purification

The peptide crosslinker was purified using reversed phase high-performance liquid chromatography (RP-HPLC) [16, 17]. RP-HPLC was carried out using a Rainin HPXL system equipped with a UV/vis detector operated at 220 nm. A 100 mm x 22 mm I.D. platinum EPS C_{18}, 5 μm particle size semi-preparative column from Alltech was used for the purification. A solvent system consisting of solvent A: 95% water, 5% acetonitrile, 0.02% TFA and solvent B: 95% acetonitrile, 5% water, 0.02% TFA was used. A gradient of these solvents running first from 100% A to 95% B in 15 minutes, holding at 95% B for 2 minutes, and then running back to 100% A over 3 minutes was used. Figure 4.3.1 (a) shows the HPLC chromatogram at before purification and Figure 4.3.1 (b) shows the HPLC chromatogram of the purified crosslinker.



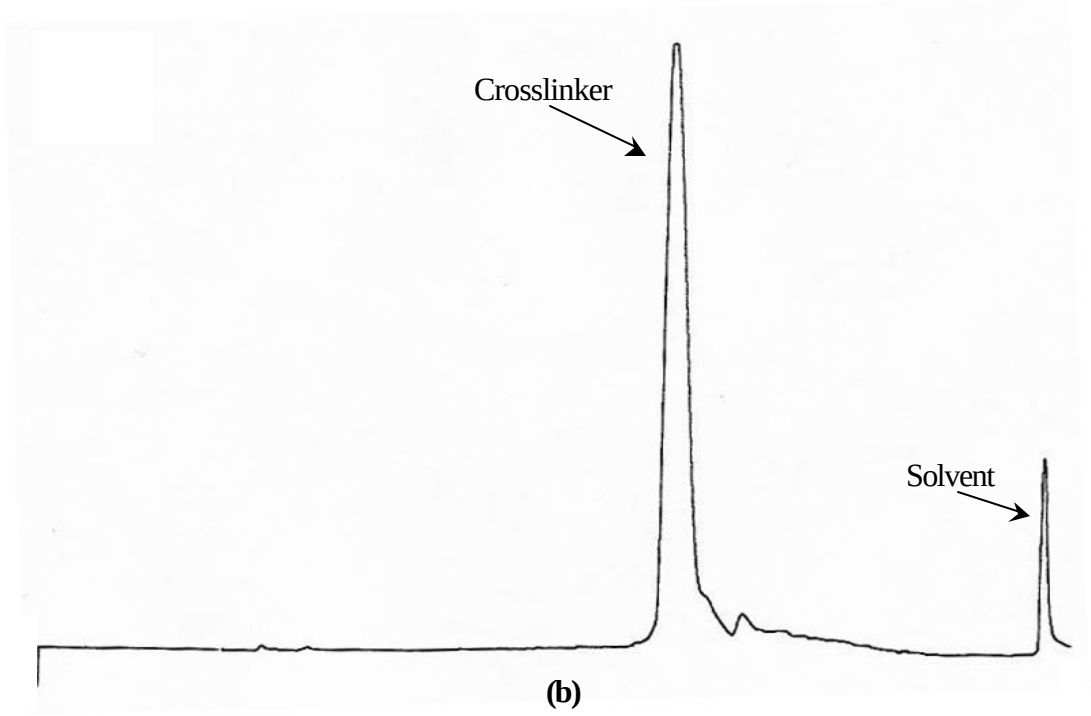


Figure 4.3.1: HPLC spectra of **(a)** unpurified and **(b)** purified peptide crosslinker.

Mass Spectroscopy

Mass spectroscopy was used to characterize the peptide crosslinker at each step of the synthesis, and to determine the degradation products of the peptide crosslinker when cleaved by thrombin. Mass spectroscopy is a powerful technique by which we can determine the molecular composition of a compound [18]. The mass spectrum consists of the masses of the positively charged molecular ion and fragments versus their concentrations. The molecular ion peak is important, because it gives us the mass of the molecule, while the fragment peaks give insight into the structure of the molecule. Additionally, a technique called ms/ms can be used to isolate the molecular ion peak, and then fragment the molecule further, to give additional fragmentation data.

For these experiments, a Bruker Esquire ion trap mass spectrometer with electrospray, APCI, and nanospray ionization sources was used, in positive ion mode. The mass spectrum of the purified peptide crosslinker is shown in Figure 4.3.2. The molecular ion peak of the crosslinker has a m/z of 578.

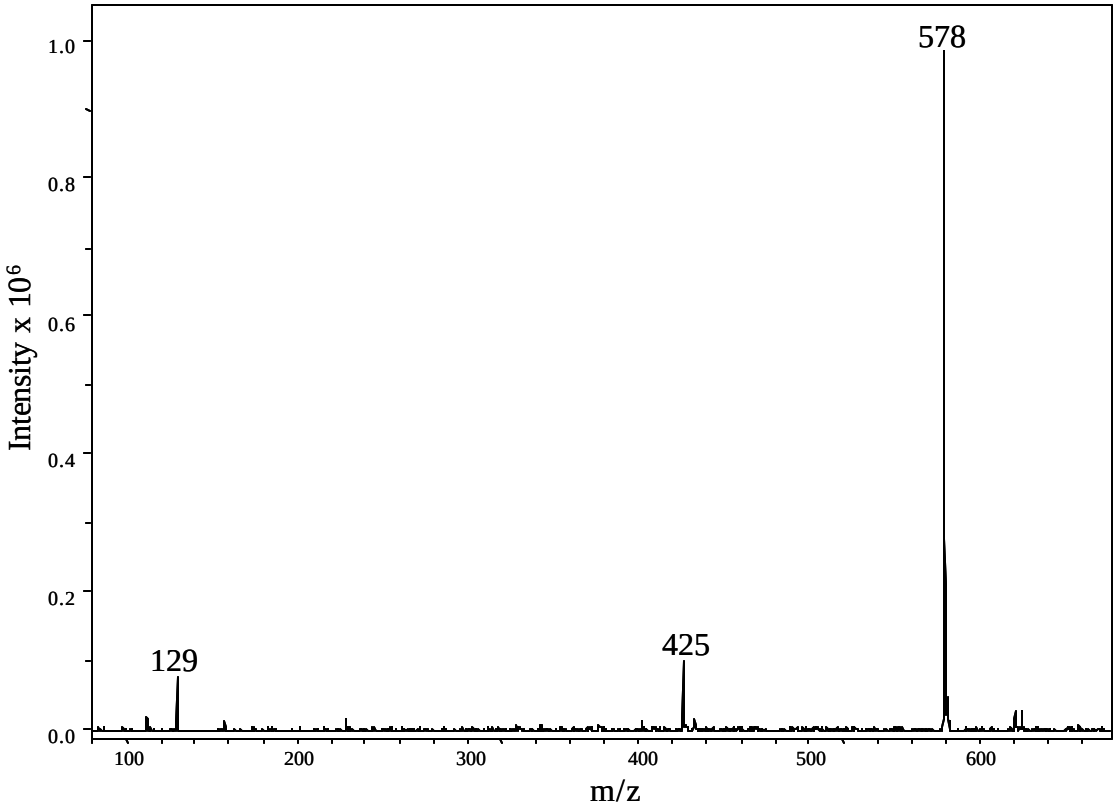


Figure 4.3.2: Mass spectrum of peptide crosslinker.

In order to confirm that the peak with m/z of 578 represented the molecular ion peak of the peptide crosslinker, it was isolated and ms/ms was performed. Figure 4.3.3 shows the ms/ms spectrum of the peptide crosslinker.

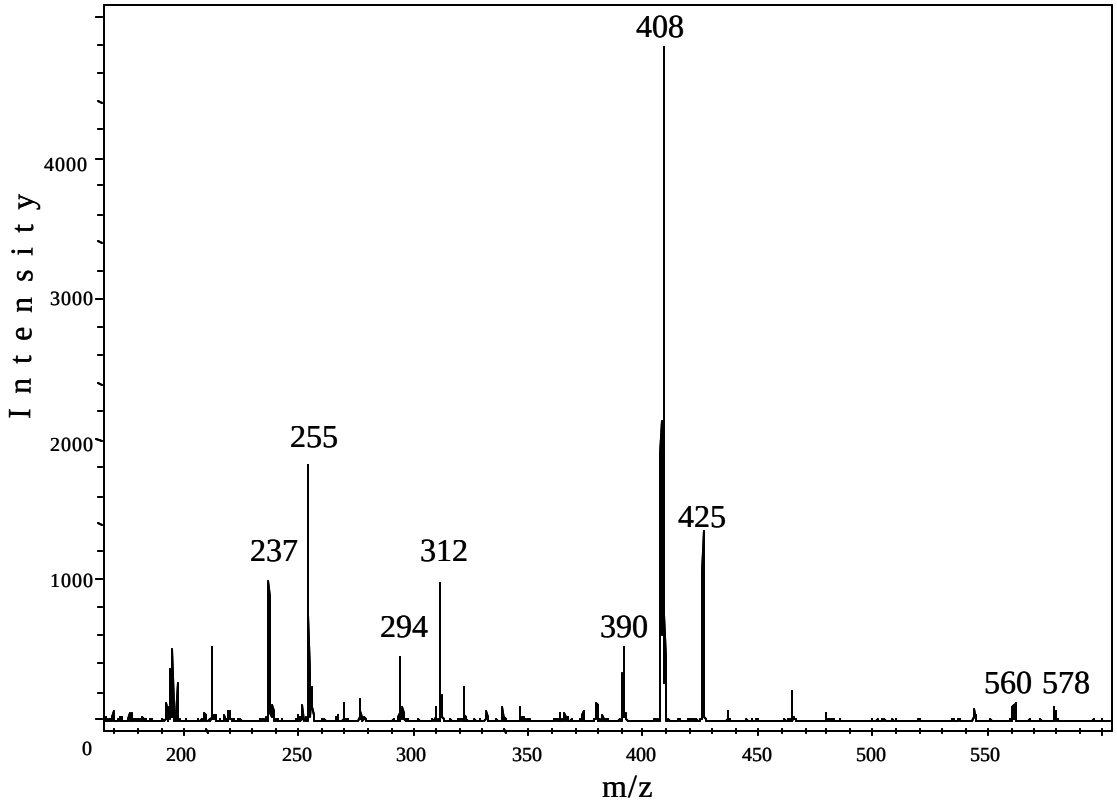


Figure 4.3.3: Ms/ms fragmentation spectrum of peptide crosslinker, with initial isolation of 578.

Table 4.1 contains a list of the m/z values of fragments of the peptide crosslinker and the fragments that they represent.

Table 4.1: Exact masses of fragments of the peptide crosslinker.

m/z:	**Fragment:**
578	$[M + H]^+$
560	$[M + H - H_2O]^+$
425	$[M + H$ - Acrylate - Ethylene Glycol - Gly $+ H_2O]^+$
408	$[M + H$ - Acrylate - Ethylene Glycol - Gly$]^+$
390	$[M + H$ - Acrylate - Ethylene Glycol - Gly $- H_2O]^+$
312	$[M + H$ - Acrylate - Val - Pro$]^+$
294	$[M + H$ - Acrylate - Val - Pro $- H_2O]^+$
255	$[M + H$ - 2 Acrylate - Ethylene Glycol - Gly - Val$]^+$
237	$[M + H$ - 2 Acrylate - Ethylene Glycol - Gly - Val $- H_2O]^+$

4.4 Crosslinker Performance

Crosslinker Degradation

To determine if thrombin cleaves the peptide at the amide bond between the arginine and glycine residues, and to examine the time scale of the cleavage, the crosslinker was dissolved in two solutions, a control solution of PBS and PBS with 10 U/mL human thrombin. All solutions were immersed in a 37 ^{o}C shaking water bath. At 6 and 24 hours, samples were analyzed with mass spectroscopy to determine the fate of the crosslinker.

Thrombin was expected to cleave the peptide sequence Val-Pro-Arg-Gly between the glycine and arginine residues. Figure 4.4.1 shows a schematic of the crosslinker's expected degradation site. The m/z of each fragment is also listed.

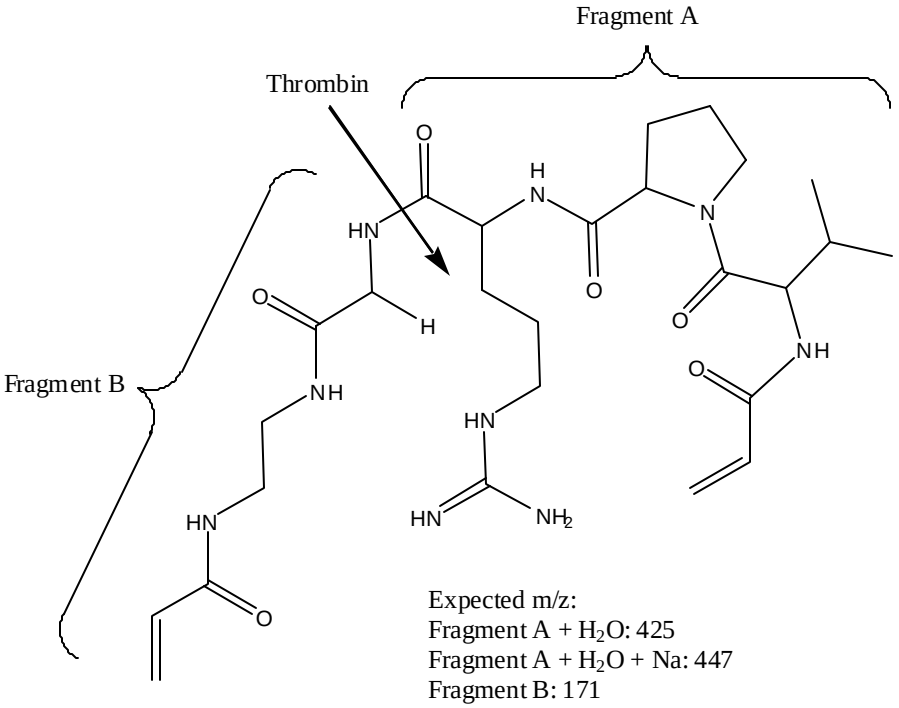


Figure 4.4.1: Schematic of crosslinker degradation site and products.

The mass spectrum of the crosslinker solution before the addition of thrombin is shown in Figure 4.4.2.

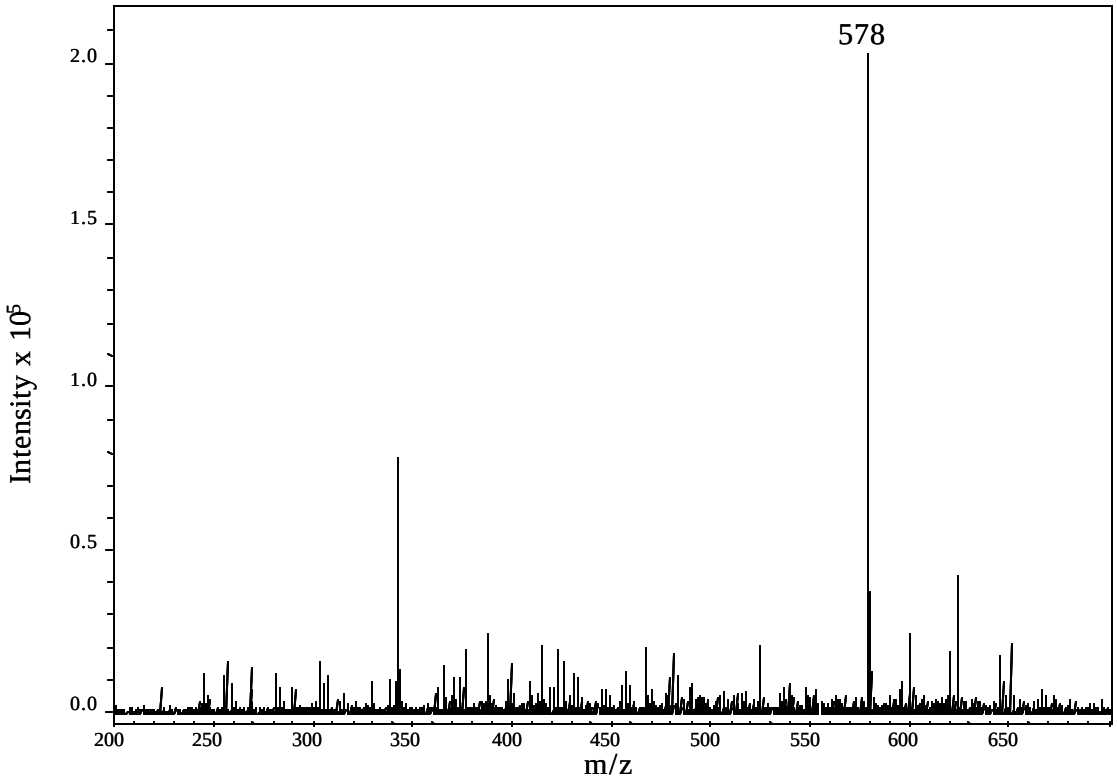


Figure 4.4.2: Mass spectrum of crosslinker before cleavage experiment.

Figure 4.4.3 shows the mass spectrum of the solution that contained thrombin after 6 hours. Note that the peak with m/z = 578 is shrinking, while the m/z 447 and 425 peaks, are growing. These masses are representative of the crosslinker degradation products, [Fragment A + H_2O + Na]$^+$ and [Fragment A + H_2O]$^+$, respectively (see Figure 4.4.1).

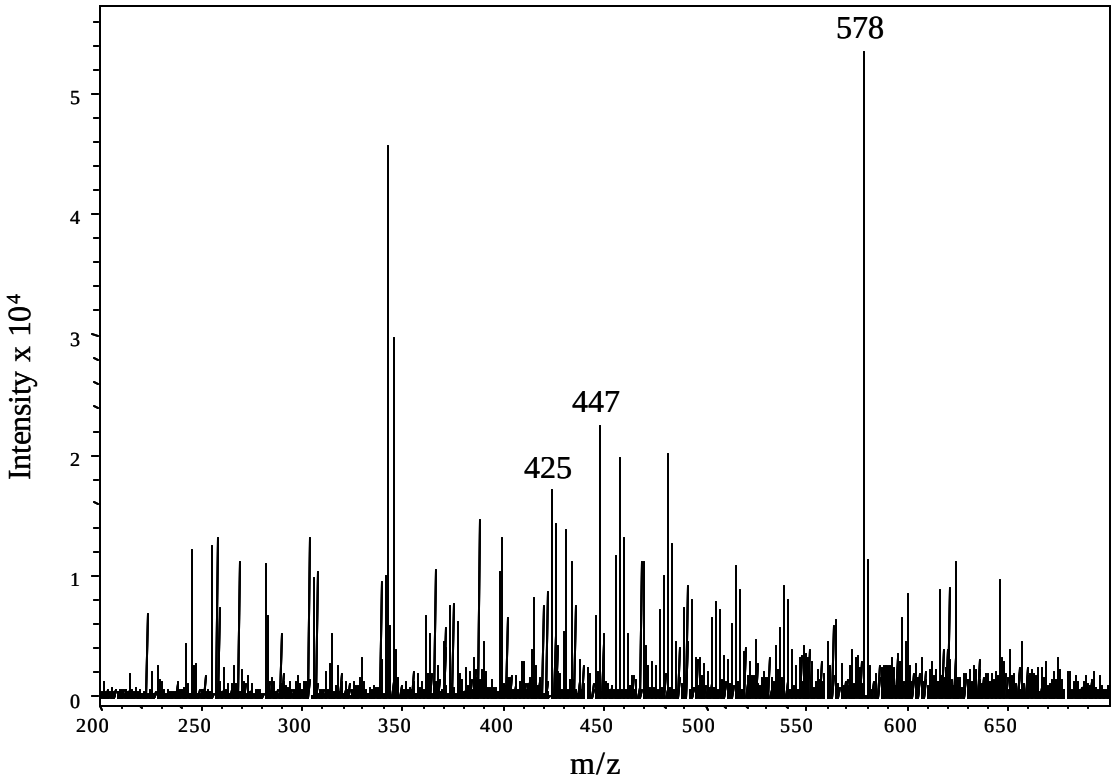


Figure 4.4.3: Mass spectrum of peptide crosslinker in solution with thrombin after 6 hours at 37 ^oC.

Figure 4.4.4 shows the mass spectrum for the solution containing thrombin after 24 hours. The m/z 578 peak has decreased further, while the peaks with m/z = 425 and 447 have continued to increase. Figure 4.4.2 through Figure 4.4.4 show that thrombin does degrade the crosslinker and they provide some clues about the time dependence of this degradation.

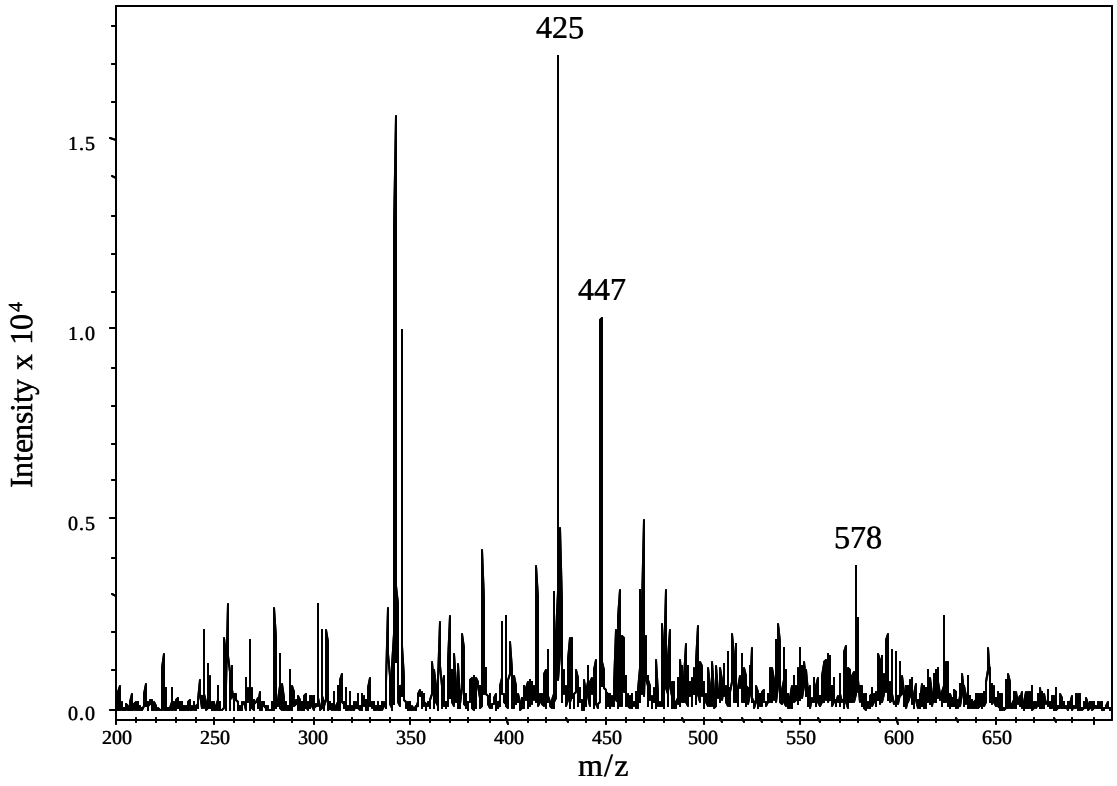


Figure 4.4.4: Mass spectrum of peptide crosslinker in solution with thrombin after 24 hours at 37 ^{o}C.

Figure 4.4.5 shows the mass spectrum for the control solution, crosslinker dissolved in PBS without thrombin, after 24 hours. Note that there is no degradation of the crosslinker, as illustrated by the large peak with m/z = 578 and the absence of the m/z 447 or 425 that correspond to the degradation products.

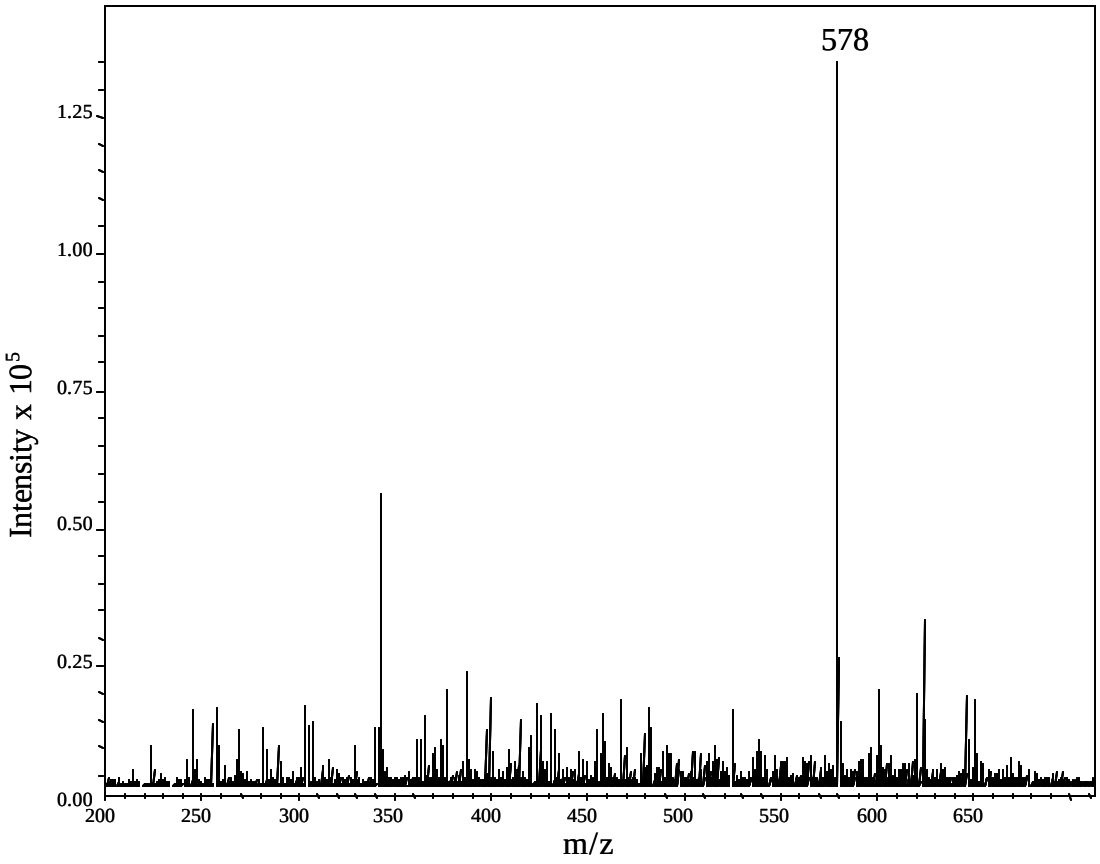


Figure 4.4.5: Mass spectrum of peptide crosslinker in PBS solution after 24 hours at 37 ^{o}C.

Peptide Crosslinker Incorporation Into HEA Hydrogels

After determining that the crosslinker can be cleaved by thrombin, hydrogels were made with the purified peptide crosslinker. For hydrogel synthesis, a formulation similar to that used in previous hydrogel experiments was used. A hydrogel solution was prepared containing 1 mL HEA, 0.2 mL water, 0.3 mL ethylene glycol, 85.6 mg peptide crosslinker, and 0.03 mL of each 0.4 g/mL ammonium persulfate and 0.15 g/mL sodium metabisulfite. This formulation has a crosslinker concentration of 1.57 mol %. Once the hydrogel had formed, qualitative observations were made. When fabricating hydrogels in the past, it was noted that increased crosslinker concentration gave more brittle, less flexible gels. This is indicative of higher crosslink densities. The hydrogel formed in this experiment had a mid-range level of flexibility, indicating that crosslinking had occurred. The next step was to determine if the gels made with the peptide crosslinker degrade in the presence of thrombin.

HEA/Peptide Crosslinker Degradation Experiment

An experiment was performed to determine if the hydrogels formed in the previous section with the peptide crosslinker would swell or degrade in the presence of thrombin. This experiment was carried out by placing hydrogel samples in one of three solutions, PBS, pH = 7.4, PBS with 10 U/mL thrombin, and PBS with 50 U/mL thrombin. All samples were placed in a 37 ^{o}C shaking water bath for the duration of the experiment. At one week intervals, the solutions were replaced with fresh solutions and the wet weight of the hydrogels was determined. After three weeks, the hydrogels were lyophilized and weighed to determine their final dry weights.

There was no statistically significant difference in swelling between gels soaked in PBS control or thrombin test solutions. The mass swelling ratio, q, was determined to be 7.0 ± 0.4 for test solutions. Additionally, no degradation of the gels was observed.

4.5 Discussion

A thrombin degradable peptide crosslinker was successfully synthesized. The synthesis described here can be used to synthesize a variety of peptide crosslinkers. This

flexibility provides options for optimizing the thrombin degradable crosslinker as well as for creating different peptide based crosslinkers.

The performance of the crosslinker was examined by testing three different factors. The crosslinker was degraded by thrombin when in solution, and the crosslinker was successfully incorporated into a pHEA hydrogel, however the crosslinked hydrogel did not degrade or swell to a greater degree in the presence than in the absence of thrombin. There are several reasons why this could be the case. First, it's possible that thrombin isn't able to penetrate the hydrogel far enough to cleave the crosslinks. Second, when the crosslinker is incorporated into the hydrogel, the active site of the peptide may no longer be available to the thrombin due to conformational changes.

Another possibility is that the peptide crosslinks are being broken, but that there are competing crosslinks formed during the polymerization process that are not degradable. It is possible that the HEA monomer forms a dimer with two vinyl groups and that this dimer forms non-degradable crosslinks. This could be due to impurities in the HEA monomer, or the mechanism of polymerization being used. Other researchers have shown that poly(hydroxyethyl methacrylate), pHEMA, gels with a polycaprolactone crosslinker show better hydrolytic degradation when polymerized with a photoinitiator than when polymerized using redox initiation [19]. The reason for this could be the increased speed of polymerization with photoinitiation. With the slower redox polymerization, there may be increased opportunity for dimer formation and chain transfer reactions. The next chapter offers some recommendations for determining what is preventing the crosslinked hydrogels from degrading and some options for correcting the problem.

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5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Silicone Drug Delivery Coatings

Conclusions

Various silicone elastomer coatings with and without poly(ethylene glycol), as a drug compatilizer and pore former, were evaluated for their ability to release paclitaxel and tranilast for an extended period of time. Both monolithic and reservoir type coatings were investigated.

It was shown that for the hydrophobic drug paclitaxel, the incorporation of PEG into silicone elastomer coatings decreased the initial burst rate and raised the steady state release rate of the drug. Near zero-order release rates were achieved for paclitaxel after the initial burst for 60 days, with continued but decreased release continuing for as long as 140 days.

For the hydrophilic drug tranilast, it was shown that the incorporation of PEG increased the initial burst rate while decreasing the subsequent steady state release rate. Release of the drug did not follow zero-order release kinetics and leveled off to zero after 21 days. Adding a topcoat to the tranilast/silicone coating reduced the initial burst, but did not extend the release past 21 days.

As expected, topcoats on top of the tranilast monolithic coatings resulted in lower total release and decreased initial burst. This is due to the fact that the drug has an additional barrier to diffusion.

Reservoir coatings involving silicone/PEG topcoats also gave interesting results when comparing the release of a hydrophilic drug versus a hydrophobic drug. The silicone coating presented a more difficult diffusion barrier for both drugs than the silicone/PEG coating.

Recommendations

An important next step in developing drug delivery coatings for stents would be to determine optimal release kinetics for restenosis inhibiting drugs. Both human and

animal studies thus far have focused on dosing, but not on release kinetics. Silicone coatings with and without PEG could be used to test the efficacy of a wide range of release kinetics for the treatment of restenosis in vivo.

Another interesting aspect to look into for silicone drug delivery coatings is whether or not phase separation among the drug, PEG, and silicone is occurring. Time of flight secondary ion mass spectroscopy (TOF-SIMS) would be a useful technique to investigate this. TOF-SIMS can give detailed molecular information about the surface of a material. The coatings could be cross-sectioned to investigate phase separation on the inside of the coating as well. This analysis would provide more information about the mechanism of drug release from these coatings.

5.2 Hydrogel Drug Release Coatings

Conclusions

In these studies, we looked at the effects of crosslinker concentration, crosslinker hydrophilicity, drug hydrophilicity, and drug molecular weight on drug release rates from poly(hydroxyethyl acrylate) hydrogels. We showed that crosslinker concentration has an inverse relationship with delivery rates of drugs when the drug is sufficiently large such that its diffusion through the hydrogel can be inhibited by the additional crosslinks present or when the drug is hydrophobic. This finding is consistent with hydrogel controlled release theory. We also showed that gels made with higher crosslinker concentrations have lower equilibrium swelling ratios than gels made with lower crosslinker concentrations. These swelling studies help to explain the observation that the release rate from the more highly crosslinked gels was lower. Additionally, we showed that drugs of a higher molecular weight diffuse out of the gel more slowly than those of lower molecular weight. This also is consistent with hydrogel controlled release theory.

One of the goals of this part of the project was to determine if an optimal crosslinker concentration could be achieved to hold the drug in the gel for a period of time that would allow signal responsive drug delivery to be effective. We have shown

that crosslinker concentration affects the release of 4',5'-dibromofluorescein and higher molecular weight drugs. 4'5'-dibromofluorescein has similar structure and size to paclitaxel, so it is likely that crosslinker concentration would affect the release of paclitaxel from pHEA gels.

Recommendations

In order to fully determine if pHEA hydrogels can hold drug in the gel for a period of time that would allow for signal responsive delivery, two additional factors must be investigated. First, baseline drug release from hydrogels made with the thrombin responsive crosslinker needs to be investigated. This can be done in the same manner that elution studies were done with the non-degradable crosslinkers. Elution studies with drug-impregnated gels made with the peptide crosslinker can be done with PBS as the elution media can be done to determine how much drug is released without the crosslinks being broken.

Additionally, drug release of actual restenosis inhibiting drugs should be investigated. Paclitaxel and tranilast release should be investigated, as well as the release of other drugs that may be useful in treating restenosis.

5.3 Thrombin Responsive Drug Release Coatings

Conclusions

A thrombin degradable peptide crosslinker was successfully synthesized. The performance of the crosslinker was examined by testing three different factors. The crosslinker was degraded by thrombin when in solution, and the crosslinker was successfully incorporated into a pHEA hydrogel, however the crosslinked hydrogel did not degrade or swell to a greater degree in the presence than in the absence of thrombin. There are several reasons why this could be the case. First, it's possible that thrombin isn't able to penetrate the hydrogel far enough to cleave the crosslinks. Second, when the crosslinker is incorporated into the hydrogel, the active site of the peptide may no longer be available to the thrombin due to conformational changes.

Another possibility is that the peptide crosslinks are being broken, but that there are crosslinks formed during the polymerization process that are not degradable. It is possible that the HEA monomer forms a dimer with two vinyl groups and that this dimer forms non-degradable crosslinks. This could be due to impurities in the HEA monomer, or the mechanism of polymerization being used. Stephanie Bryant and Andrew Marshall, in unpublished results, have shown that poly(hydroxyethyl methacrylate), pHEMA, gels made with degradable crosslinks show better degradation when polymerized with a photoinitiator than when polymerized using redox initiation. This could be due to the increased speed of polymerization with photoinitiation. With the slower redox polymerization, there may be increased opportunity for dimer formation.

Recommendations

An experiment that could answer the question of what is preventing the degradation of the thrombin responsive hydrogel would be to hydrolyze the peptide bonds of the crosslinked gel. This could be done with a procedure used for amino acid analysis where the peptide bonds are hydrolyzed with 6N HCl at an elevated temperature (110 ^{o}C) for about 24 hours [1]. This procedure would break apart the peptide bonds, but leave the HEA chain in tact. If the hydrogel does not degrade, it would indicate that competing non-degradable crosslinks are the problem.

If the issue is competing non-degradable crosslinks that are being formed, there are two options for solving this problem. First, it may be necessary to purify the HEA monomer. It's possible that there is a small amount of HEA dimer, with two vinyl groups contaminating the HEA monomer. This would result in non-degradable crosslinks in the final crosslinked gel. It's also possible that there is dimer formation or chain transfer reaction during the polymerization process. It may be advantageous to speed up the polymerization to prevent dimer formation. Photopolymerization using the common 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651®) is completed in under an hour, whereas redox polymerization takes several hours.

To address the issue of thrombin not being able to penetrate the polymer, it may be useful to lengthen the crosslinker. Hern et al. [2] illustrate a method of adding PEG

lengtheners to peptide acrylates. This involves the use of acryloyl-PEG-N-hydroxysuccinimide ester from Shearwater Corp. The acryloyl-PEG-N-hydroxysuccinimide reacts with the amine groups on the peptide and forms a peptide-acrylate with a PEG spacer arm. This may allow the thrombin to more easily penetrate the hydrogel and cleave the peptide.

If the hydrogel degradation is hindered by conformational changes in the peptide sequence due to crosslinking, it may be useful to use a different, and perhaps longer, peptide sequence. Tanihara et al. [3] have investigated a variety of peptide sequences, and have shown that longer peptide sequences are more easily and quickly degraded by thrombin. The only downside to this option would be the cost associated with synthesizing longer peptide sequences.

The crosslinker synthesis described here can be used to synthesize a variety of peptide crosslinkers. This flexibility provides options for optimizing the thrombin degradable crosslinker as well as for creating different peptide based crosslinkers. There are also other potential applications for peptide based degradable hydrogel systems. Thrombin degradable drug delivery hydrogels could be used in a variety of wound healing applications. Additionally, it would be easy to synthesize peptide diacrylate crosslinkers with other peptide sequences using the synthesis described here.

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APPENDIX A – ABBREVIATIONS AND ACRONYMS

A	Area
Arg	Arginine
BDDA	1,4-Butanediol diacrylate
Boc	*tert*-butyloxycarbonyl
BOP	Benzotriazole-7-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate
BSA	Bovine serum albumin
c_1	Drug concentration
c_d	Initial drug loading in polymer
$C_{d,s}$	w/v % of drug in drug solution
$C_{d,v}$	w/v % of drug in coating
$C_{d,w}$	w/w % of drug in coating
c_{sp}	Drug solubility in polymer
c_{sw}	Drug solubility in water
CIEIA	Competitive inhibition enzyme immunoassay
δ	Device thickness
D_{12}	Diffusion coefficient of drug in polymer
D_g	Solute diffusivity in hydrogel
D_o	Solute diffusivity in pure solvent
DBF	Dibromofluorescein
DIEA	N,N-diisopropylethylamine
DMF	Dimethylformamide
ε	Porosity
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FITC	Fluorescein isothiocyanate
FDA	Food and Drug Administration
Fmoc	9-fluorenylmethyloxycarbonyl
Gly	Glycine
HEA	2-Hydroxyethyl acrylate
HOBt	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
HSA	Human serum albumin
J_1	Molar flux of drug
K	Drug partition coefficient
k_B	Boltzmann's constant
l	Bond length
M_c	Initial weight of polymer coating or sample
$\overline{M}_c$	Average molecular weight between crosslinks
M_d	Mass of dry polymer
M_i	Initial mass of drug in sample
$M_{i,m}$	Initial mass of drug in monolithic coatings

$M_{i,r}$	Initial mass of drug in reservoir coatings
$\overline{M}_n$	Number average molecular weight in the absence of crosslinking
M_s	Mass of swollen polymer
M_t	Cumulative mass of drug released at time t
MB	Methylene blue
η	Viscosity of water
n	Number of bonds between crosslinks
$\rho_{h,s}$	Density of hydrogel solution
ρ_p	Density of the polymer
ρ_s	Density of the solvent
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
pHEA	Poly(2-hydroxyethyl acrylate)
Pmc	2,2,5,7,8-Pentomethylchroman-6-sulfonyl
Pro	Proline
PTCR	Percutaneous transluminal coronary revascularization
PVA	Polyvinyl alcohol
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
q	Equilibrium mass swelling ratio
Q	Equilibrium volume swelling ratio
r_s	Stokes-Einstein hydrodynamic radius of the solute
RP-HPLC	Reverse phase high performance liquid chromatography
SMC	Smooth muscle cell
SPPS	Solid phase peptide synthesis
τ	Tortuosity
t	Time
T	Temperature
TEGDA	Tetraethylene glycol diacrylate
TES	Triethylsilane
TFA	Trifluoroacetic acid
$\overline{\upsilon}$	Specific volume of the polymer
$\upsilon_{2,s}$	Equilibrium polymer volume fraction
V_1	Molar volume of the solvent
V_d	Volume of dry polymer
V_d	Volume of drug solution
V_s	Volume of swollen polymer
Val	Valine
ξ	Mesh size
Y	Ratio of the critical volume required for a successful translational movement of the solute molecule to the average free volume per molecule of the liquid
z	Length

APPENDIX B – UV SPECTROPHOTOMETRY OF DRUGS AND MODEL DRUGS

For each model drug, the peak absorbance was determined by measuring the UV spectrum using a HP 8452A diode array spectrophotometer. A standard curve was then created by measuring the absorbance of 200 μL of various concentrations of model drug in 96 well plates. The absorbance of each concentration was measured in triplicate. A standard curve of absorbance vs. concentration was then created. This appendix contains the UV spectra and standard curves for each model drug.

Methylene Blue

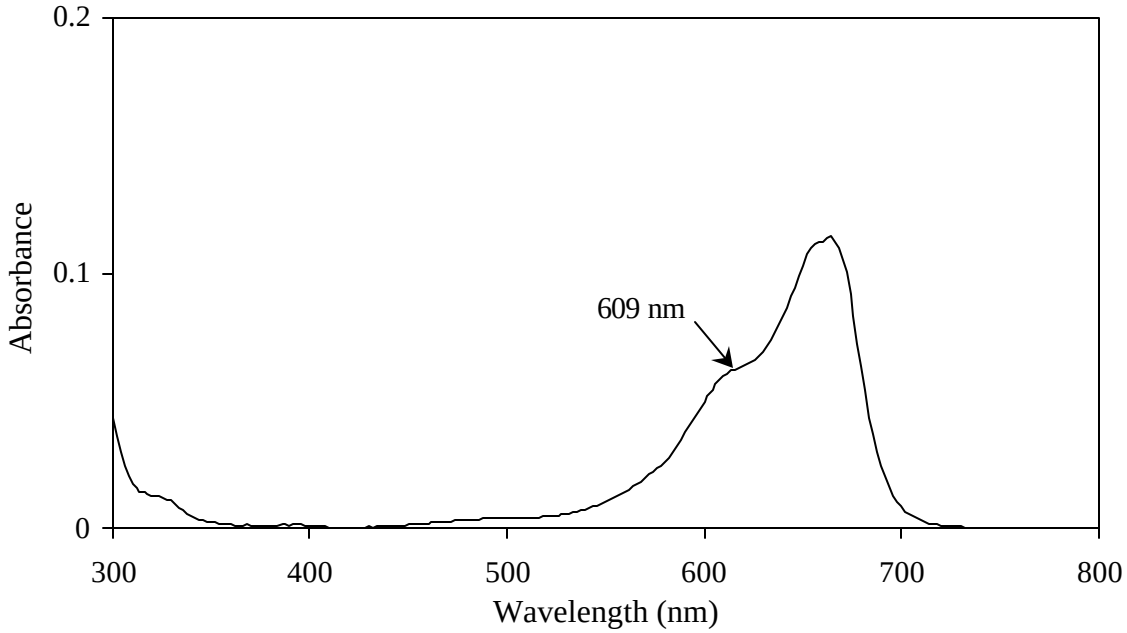

Figure B.1: UV Spectrum of methylene blue.

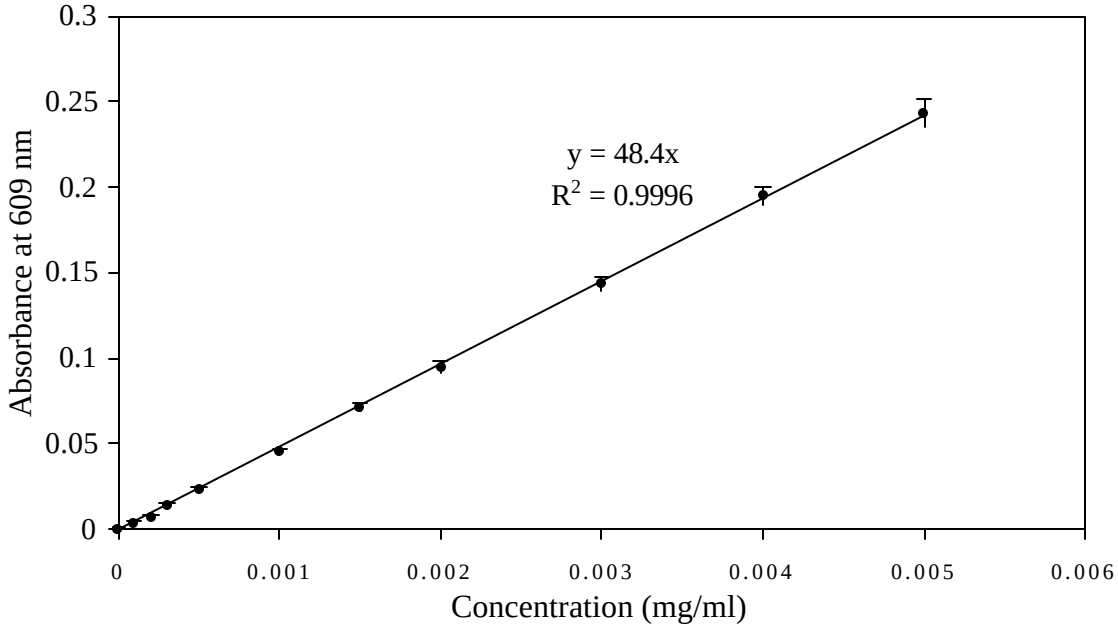

Figure B.2: Standard curve for methylene blue. Error bars: 95% confidence limit, n=3.

4',5'-Dibromofluorescein

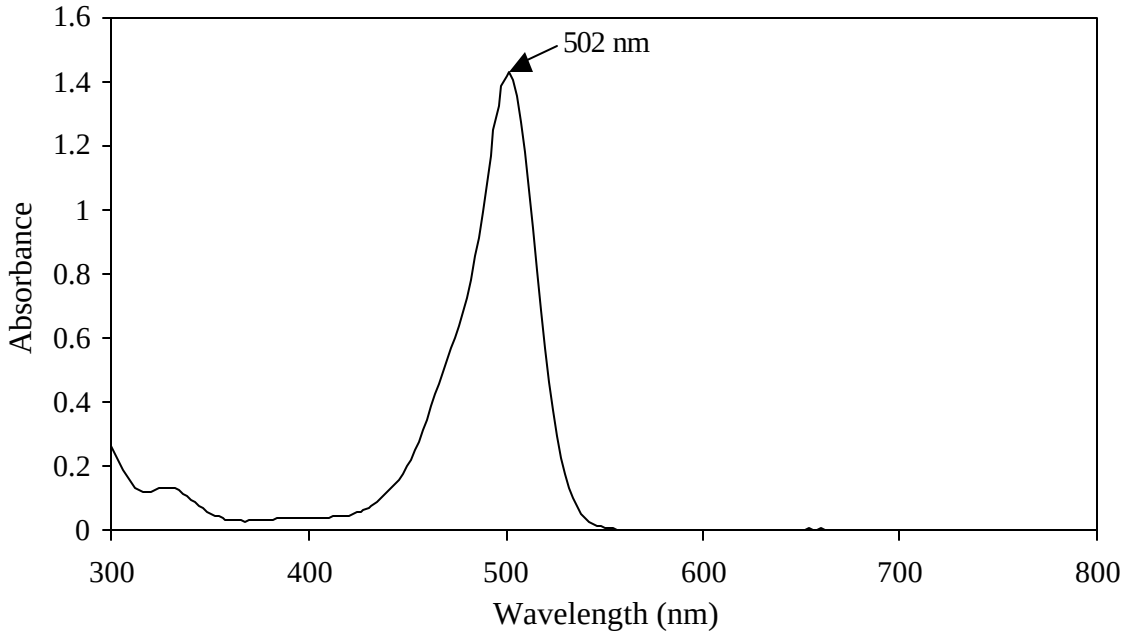

Figure B.3: UV spectrum of 4',5'-dibromofluorescein.

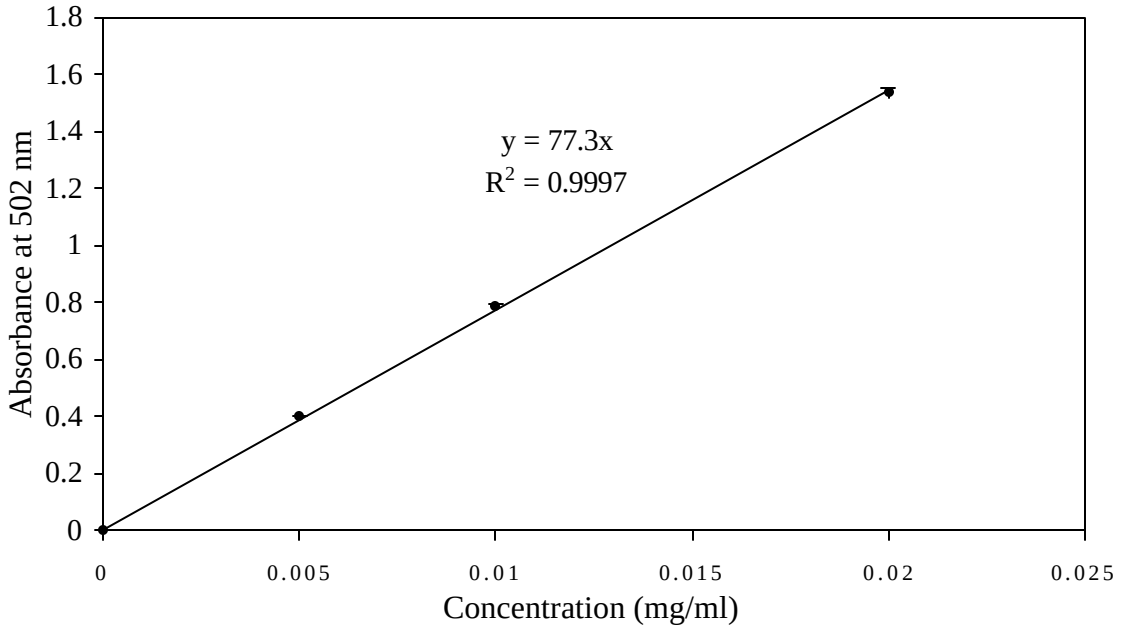

Figure B.4: Standard curve for 4',5'-dibromofluorescein. Error bars: 95% confidence limit, n=3.

FITC-Dextran, MW 4300

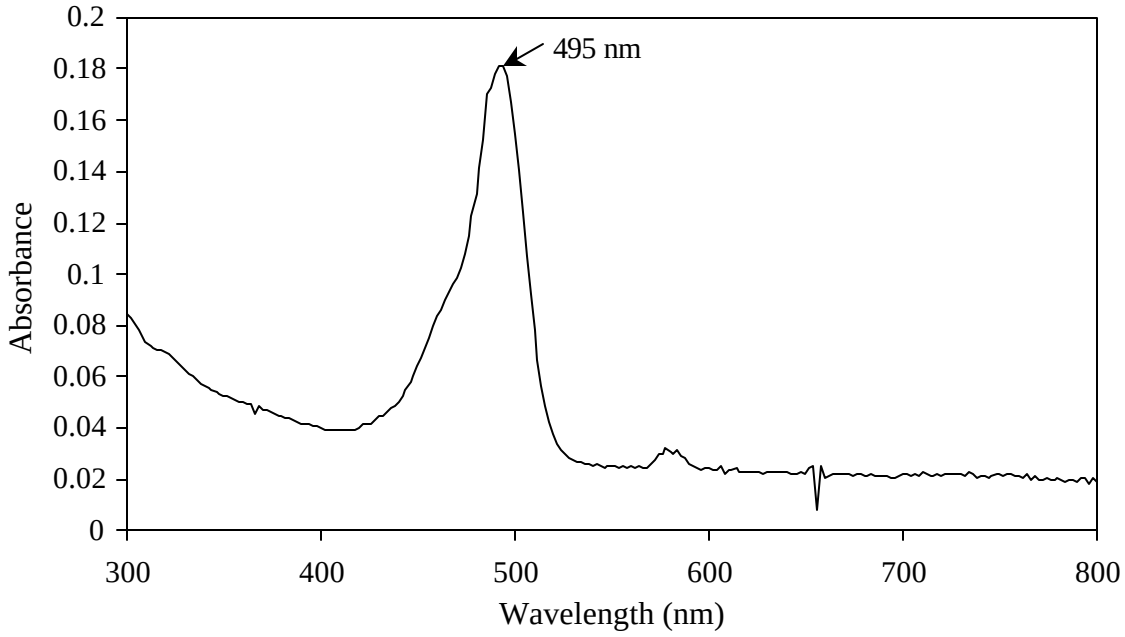

Figure B.5: UV spectrum of FITC-dextran, MW 4300.

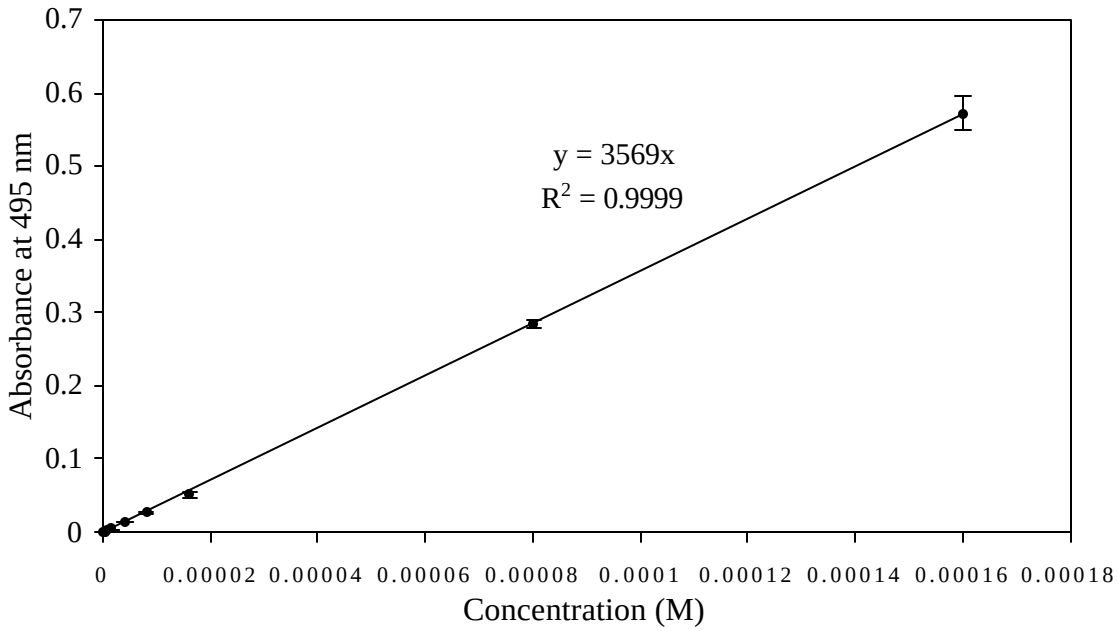

Figure B.6: Standard curve for FITC-dextran, MW 4300. Error bars: 95% confidence limit, n=3.

FITC-Dextran, MW 10500

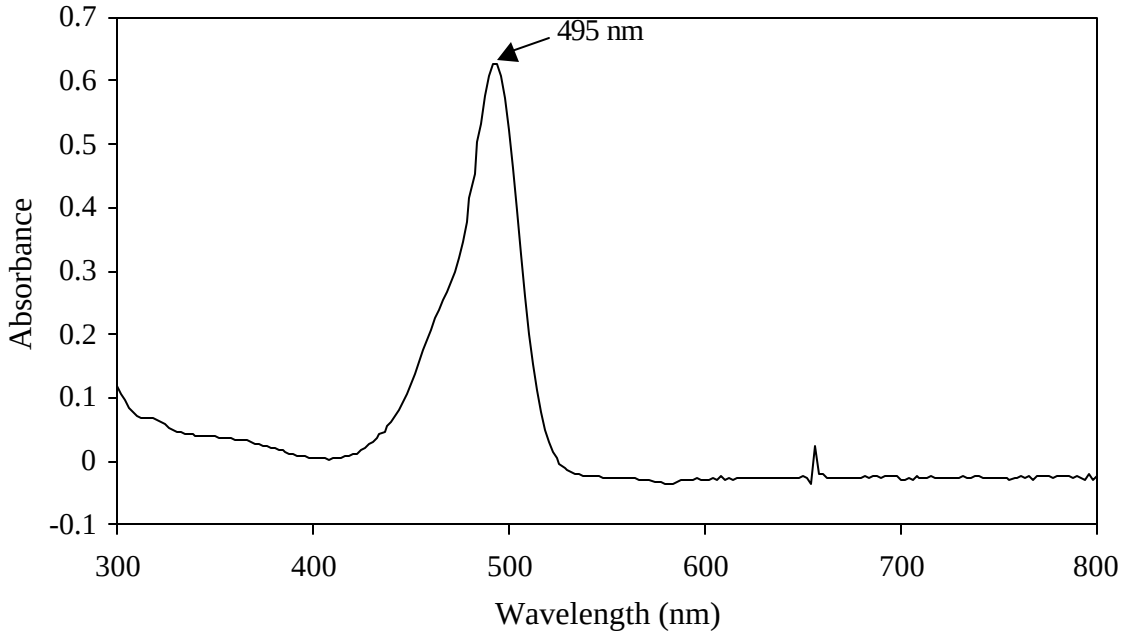

Figure B.7: UV spectrum of FITC-Dextran, MW 10500.

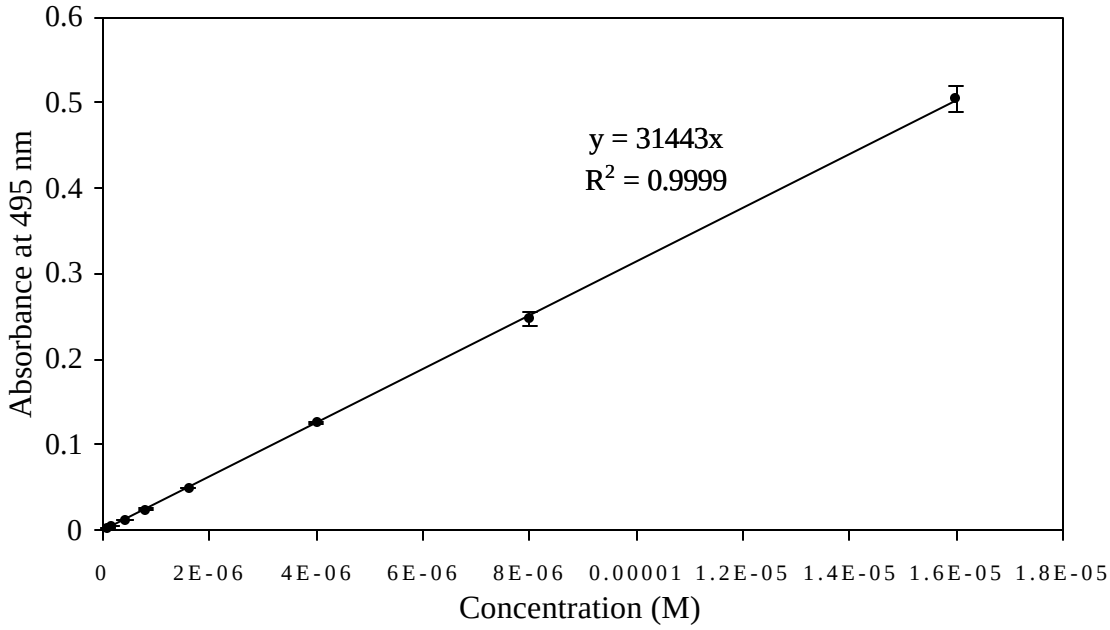

Figure B.8: Standard curve for FITC-Dextran, MW 10500. Error bars: 95% confidence limit, n=3.

FITC-Dextran, MW 19500

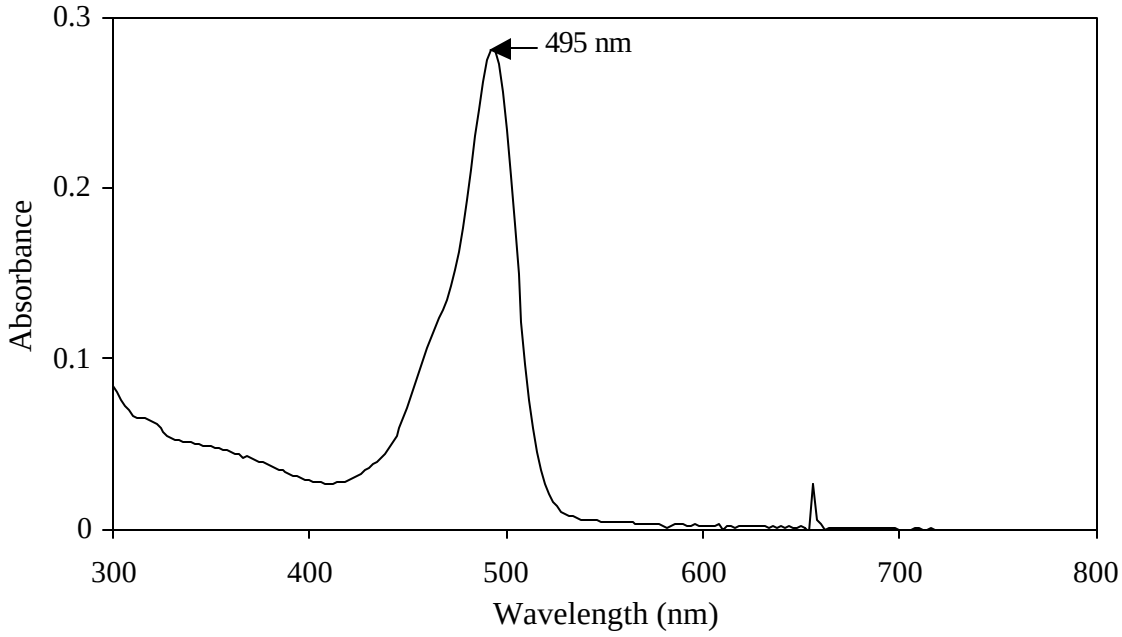

Figure B.9: UV spectrum of FITC-dextran, MW 19500.

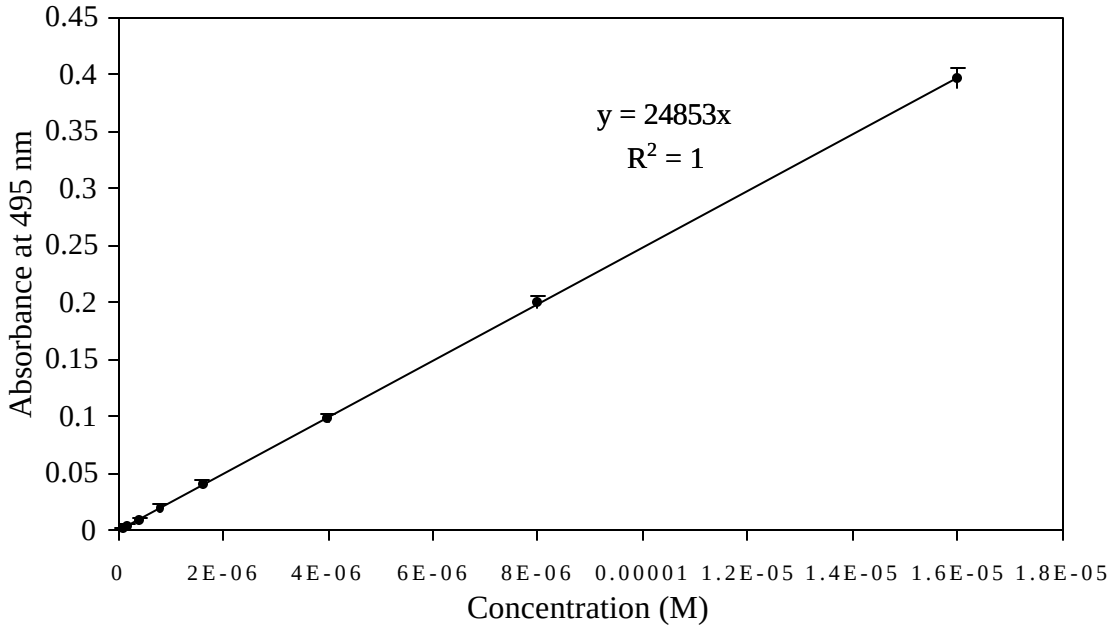

Figure B.10: Standard curve for FITC-dextran, MW 19500. Error bars: 95% confidence limit, n=3.