SYNTHESIS AND CHARACTERIZATION OF COLLAGEN-MIMICKING HYDROGELS TO EXAMINE LUNG FIBROSIS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) presents a challenge even in this age of advanced medical technology. The causes and progression of the disease in humans remain largely unknown as it is often diagnosed well after it has begun and is difficult to observe *in vivo*. This thesis presents work towards the development of a new model for the collagenous extra-cellular matrix (ECM) found in the interstitial space of the human lung to aid in the research of the cellular responses that contribute progression of IPF. First, collagen mimetic peptides that assemble into triple helices were synthesized for the incorporation into hydrogels. Challenges arose in the synthesis of initial peptides compositions of K(aloc)G$(POG)_7$GK(aloc), which included additional amino acid residues that contain orthogonal reactive groups. Consequently, subsequent testing focused on determining the best method to synthesize this new class of peptides, including synthesizing the sequences $(POG)_4$, $(POG)_7$, and K(alloc)$(POG)_7$ on different resins. Second, hydrogels were synthesized and characterized on a rheometer to determine the compositions with similar physical properties to those of healthy and diseased human lung tissue, from 10 to 20 kPa. Poly(ethylene glycol) compositions of 10% and 12.5% weight percent were identified to mimic these moduli, respectively, and these syntheses demonstrated the high level of control that can be exercised on the final properties of the hydrogel based on the concentration of reactants. Future directions for this work will include attempting new peptide sequences that do not include K(alloc) and incorporating these new peptides

into hydrogels that will be used to obtain the cellular responses to their physical environment.

Chapter 1

INTRODUCTION

1.1 Motivation: Idiopathic Pulmonary Fibrosis

1.1.1 Disease symptoms, diagnosis, prognosis, and treatment

Idiopathic pulmonary fibrosis (IPF) is characterized by localized patches of uncontrolled "wound healing" in the interstitial connective tissue surrounding the alveoli. Patients with IPF experience labored breathing and a persistent dry cough. These symptoms are the result of the excessive deposition of new extracellular matrix (ECM) material by pulmonary fibroblast cells[1]. The ECM is the complex, gel-like material that provides the structural support for cell attachments while at the same time allowing for the transport of vital materials like oxygen and cellular nutrients. The majority of the ECM is comprised of polysaccharides and fibrous proteins[2]. These fibrous proteins, especially collagen I are the primary contributors to the stiffening of the lung tissue. Figure 1.1.1 depicts the difference in the tissue a person with healthy lungs and a patient with IPF.

Currently, little is known about IPF. There is no known cause of the disease and a regular, detailed disease progression has not been established. Around 40% of those affected with IPF will die, usually within three years of diagnosis, from respiratory failure that results from the decreased oxygen transfer across the stiffened lung tissue. Despite the high mortality rates, there is still no known medicine or

treatment that is capable of arresting the progression of this disease, with the exception of lung transplant.[1]

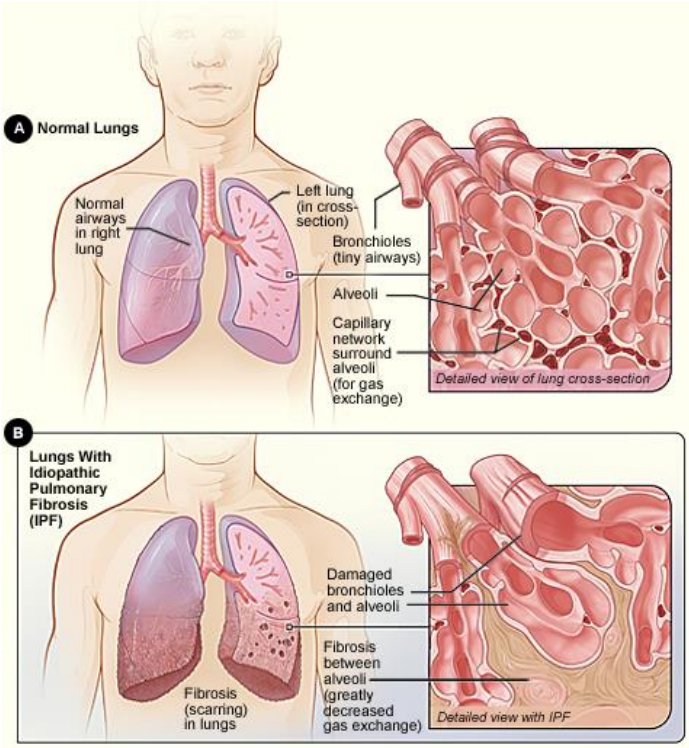

Figure 1.1.1 Development of scar tissue in lungs from IPF[16]

1.1.2 Affected populace

Around 30,000 new cases of IPF are diagnosed every year. Generally, the patients are 50 years and older and are more often men than women. Many of the people that are diagnosed with IPF have a history of smoking or exposure to environmental irritants like metal dusts[3]. Lung tissue will scar in response to damage caused by these sources; however, these types of injuries usually result in a normal immune response and healing with limited scar formation, whereas it is not clear what specific physical damage event(s) lead to IPF and uncontrolled healing and scarring.

1.1.3 Challenges facing disease research efforts

Because the symptoms of IPF are similar to those of more common ailments such as bronchitis or asthma, a proper diagnosis is frequently delayed by significant lengths of time[1]. As such, IPF is rarely observed in its earliest stages, which is part of the reason why the cause of the disease still has not been identified. Once the proper diagnosis is reached, the complexity of the natural ECM makes it exceedingly difficult to isolate individual factors for determining potential causes and for monitoring the disease's progression in humans. Because of this innate complexity, researchers have turned to models systems to study IPF.

1.1.4 Current model systems

There are currently three broad classes of models that are used to observe biological systems: animal models and in vitro culture models. Animal models provide the highest level of complexity, and therefore are the most similar to the human ECM. However, the disease progression of pulmonary fibrosis in other mammals does not perfectly describe what occurs in humans; for example, pulmonary fibrosis is reversible within typical mouse models of the disease, potentially limiting the applicability of the information gleaned[4]. Also, the inherent complexity of the animal models does not make observation and examination of the disease initiation or progression much easier than dealing with human ECM.

In vitro model systems often utilize mimics of the native ECM for the culture of cells in an environment that captures aspects of the in vivo environment (appropriate stiffness and biochemical cues) for the two- and three-dimensional culture of cells. Two classes of water-swollen materials (hydrogels) typically are used for this purpose: natural hydrogels and synthetic hydrogels. Natural hydrogels can

provide a more reasonable approximation of key biochemical and biophysical properties of the human ECM and have been utilized within in vitro models for the culture of cells[18]. Natural hydrogels have been obtained by harvesting the ECM from animals or that is produced by fibroblast cells in cultures[18]. Approximately mimicking cues from the native ECM is important when attempting to observe the progression of a disease like IPF because the behavior of the pulmonary fibroblast cells can be changed based on the environmental cues they are receiving. To get the most accurate idea of what is occurring, the hydrogel should be as similar as possible to the human ECM, making the data obtained from using it more useful. The problem with using natural hydrogels is that they have limited control of their biochemical and biophysical properties and batch-to-batch variability, making it difficult to conduct hypothesis testing[5].

Synthetic hydrogels introduce the controllability and simplicity that natural hydrogels lack. Consequently, synthetic hydrogels are useful for conducting hypothesis testing because their simplicity makes it easy to observe the cellular response to a controlled set of variables. This level of simplicity, while useful for conducting experiments, does not capture the complexity of the natural ECM, which means that the results obtained from experiments with synthetic hydrogels may only describe a part of the larger picture[5].

1.1.5 Need for better *in vitro* model systems

Synthetic hydrogels promise a highly controllable material that can be used to make important observations about the onset and progression of IPF, but current models have critical limitations that impact their adequacy; for example, many synthetic hydrogels that have a ‘stiffness’ similar to IPF-affected tissue lack the

appropriate nanostructure to mimic this heavily collagenous tissue. The more physical and biological cues that can be incorporated into the hydrogel, the more similar the hydrogel would be to natural ECM, but also the more difficult they become to synthesize and characterize. A balance between complexity and feasibility needs to be struck in order to make breakthroughs in the study of IPF. The purpose of this thesis is to provide a guide to obtaining these desired materials: hydrogels that have a nanostructure that mimics collagen and enable the controlled presentation of key biophysical and biochemical cues present in IPF.

1.2 Previous Work

1.2.1 Hydrogels

1.2.1.1 Mimic of the extracellular matrix

The properties of hydrogels make them the ideal material to mimic the extracellular matrix. The most important property of hydrogels that makes them useful for modeling biological systems is that their high water content is very similar to human tissue[5]. This high water content makes hydrogels very good at transporting materials like gases and small molecules through the material. Another property that makes hydrogels a good human ECM mimic is that they are highly conducive to mechanotransduction[5]. This is a result of the softness of the material. This is especially important for this thesis because the modulus (stiffness) of hydrogels spans the typical values of healthy to heavily scarred lung tissue. Finally, the ability of hydrogels to be made into different configurations for cellular culture makes them highly valuable as models of the ECM. Cells can be cultured on top of a hydrogel (2D), on top of a hydrogel that gets folded on top of itself (2.5D, a quasi-three

dimensional configuration), or within the hydrogel (3D). These different configurations can be used to test the impacts of different types of cues, such as stiffness or the presence of a chemical gradient.

1.2.1.2 Advantages of PEG-based hydrogels

Poly (ethylene glycol) (PEG) is a highly advantageous material to use in hydrogels for studying cellular interactions with the ECM. The first major benefit of using PEG is its bio-compatibility. The repeat unit of PEG ($-CH_2CH_2O-$) is naturally derived. This means there are no cyto-toxicity issues involved when using PEG in a hydrogel. PEG hydrogels allow for the easy transport of gases, nutrients, and biological waste through the material, which is necessary for the survival of cells cultured on or within the material. Because PEG hydrogels are so soft, they are readily broken down by cells to allow for mechanotaxis (cellular movement) within the gel. The combination of these three benefits allows for normal cellular function[5].

Another advantage of PEG-based hydrogels is their lack of interaction with cells. Because PEG was derived from naturally occurring materials, no unwanted interactions with cells occur. This allows PEG to provide a “blank slate” for control over cellular cues[5]. The “blank slate” means that the only interactions the cell can have with the hydrogel must be purposefully included when the hydrogel is synthesized. This provides the ability to controllably add more complexity to the hydrogel in order to better mimic the ECM.

The final major benefit of using PEG-based hydrogels is the ability to easily control the mechanical and chemical properties of the hydrogel. These properties are set when the hydrogel is synthesized. The mechanical properties are totally dependent on the relative composition of the monomers used to synthesize the hydrogel.

Increasing amounts of monomer in the pre-reaction mixture lead to increased crosslinking density, which translates directly to a stiffer material. The chemical properties are dependent on the chemical structure of the monomers themselves. The PEG monomer itself can be modified with different functionalities or other molecules that do not create a crosslink can be attached. These functional groups can be biochemical cues for the cells that will be cultured, which will allow for new cellular interaction with its environment. For this thesis, an assembling peptide was chosen to be the crosslinker so that cells could recognize the 3D structure of the molecule rather than a reactive group.

1.2.1.3 Thiol-ene chemistry

Thiol-ene chemistry is one class of the set of reactions called the "click reactions." Click reactions are fast reactions that occur under mild temperature and pH conditions and are orthogonal to many other reactions. This means that they are highly specific without being difficult to run. Thiol-ene chemistry specifically deals with the radical reaction between carbon-carbon double bonds and thiol groups. The reaction only occurs in the presence of ultraviolet light and requires a radical initiator to generate the radical species that propagate the reaction. This gives high levels of temporal control over reaction progress. Because the reaction is so specific, side reactions with other functional groups rarely occur. This is extremely important for hydrogel synthesis because it leads to a highly regular crosslinked network while allowing for the incorporation of biochemical cues. The thiol-ene reaction can be run at room temperature, has no pH requirements, and is cyto-compatible[6]. This is important especially if cells were to be cultured in the hydrogel in a 3D configuration.

The cells would simply be included in the reactant mixture prior to synthesis to accomplish this.

1.2.2 Collagen Mimetic Peptides

1.2.2.1 Significance in lung tissue environment

As mentioned in the earlier definition, one of the major components of the extracellular matrix is fibrous proteins. There several major types of these proteins, but the most important of them in the lung tissue environment is collagen. Collagen makes up about 30% of the total amount of the protein in the human body[7], but it plays a crucially important role in the lungs. Collagen plays a large part in wound healing, which is no longer under control in patients with IPF[8]. Therefore, incorporating collagen into a hydrogel for the study of IPF would be highly beneficial.

1.2.2.2 Design and current state of the art

Collagen has a unique structure that greatly influences its properties. A single collagen molecule has a secondary structure of an α helix (right-handed helix). Due to its amino acid sequence (primary structure), three collagen molecules will come together and wrap around each other in a left-handed helix to form a triple helix. The triple helical structure is the source of collagen's strength, which is why it is such an important component of the ECM[2]. Collagen is a very large molecule, so it would be difficult to use in a hydrogel. As such, prior experiments have used collagen-mimetic peptides (CMPs) to capture the triple helical structure while keeping the molecular size low. CMP sequences follow an $(X\text{-}Y\text{-}G)_n$ design, where X is almost always the amino acid proline, Y is almost always hydroxyproline (the hydroxyl-functionalized version of proline), G is the amino acid glycine, and n is usually between 4 and 10[8].

These CMP's are traditionally synthesized via solid phase peptide synthesis, which involves adding the amino acids of the sequence to a linearly growing chain that is attached to an anchor bead.

1.2.2.3 Molecular Assembly

Collagen and collagen mimetic peptides assemble into a triple helical structure as a result of two major factors. First, the structure of the amino acids proline and hydroxyproline cause the single strand of naturally coil into an α helix that consists of just three amino acids per turn. The three member rings of proline and hydroxyproline naturally face the outside of the helix because they would disrupt the folding of the helix if they faced the inside. Second, the hydrophilicity of the peptide bonds causes multiple strands to stick together to form the triple helix. The hydroxyl groups on the hydroxyprolines are also hydrophilic, so they face out into the surroundings, which causes the three strands to align themselves properly. Because the triple helix is the result of intermolecular forces, they can be disrupted by increased temperatures. This behavior is described by the "melting temperature," or temperature where the thermal excitation is enough to overwhelm the hydrophilic interactions. For collagen mimetic peptides, the melting temperatures range between 17 – 70 °C[8-13]. This value is dependent on the amino acids in the sequence and the length of the sequence. For a CMP to be useful for experimentation, the melting temperature should be significantly greater than the average human body temperature.

1.2.2.4 Potential for cell interaction

The incorporation of these CMPs into the hydrogel is for the purpose of creating a response from the cells. It is known that cell binding integrins (cellular

surface proteins) α1β1 and α2β1 can interact with several different types of collagen (primarily collagen I, II, and IV)[13]. These proteins interact with the collagen strands not at the terminus, but along the length of the strand, meaning that it recognizes the triple helical structure rather than functional groups. This is important for this work because the incorporation of triple helical CMPs might create a physical environment similar to the human ECM.

1.2.2.5 Engineering for incorporation within hydrogels or decoration with other moieties

In order to incorporate the CMPs into a hydrogel, an additional step must be taken to give the CMP the functionality needed to engage in thiol-ene chemistry. There are multiple ways of doing this, but the two most accepted ways of doing it are by using thiol-functionalized PEG with alkene-functionalized CMPs or by using thiol-functionalized CMPs with alkene-functionalized PEG[6]. To use thiol-functionalized PEG, alkenes can be introduced into the CMP by adding an alloc-functionalized lysine amino acid. At least two of these lysines need to be added in order for the peptide to be used as a crosslinker rather than a pendant. These functional groups should be placed at the termini of the sequence in order to not interfere with triple helix formation. The triple helix has to be fully formed before the hydrogel is synthesized, or the helix will not form at all because the CMP strands are locked into place.

1.3 Objectives

The first objective of this thesis was to design, synthesize, and characterize CMPs functionalized with orthogonal reactive groups. Figure 1.3-1 summarizes the process used to synthesize both the CMPs and non-assembling peptides.

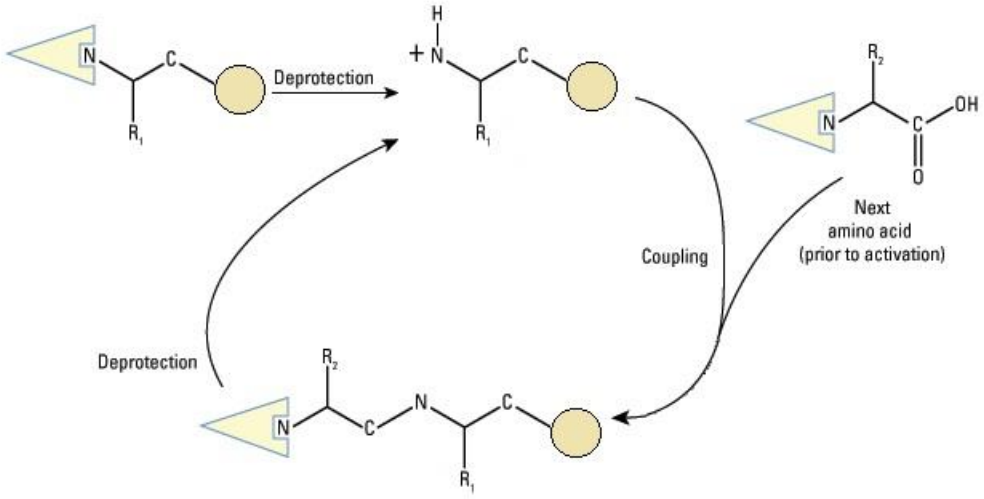

Figure 1.3-1 Method of solid phase peptide synthesis off of resin bead (tan)[17]

By adding lysines with the alloc-functionalized R group, an alkene is added to the peptide, which allowed for the incorporation of the peptide into a hydrogel. The second objective was to design, synthesize, and characterize photopolymerizable hydrogels. Hydrogels can be tuned by varying the weight percent of the PEG monomer in the reactant mixture. Before incorporating in CMPs, non-assembling peptides were used as the crosslinker to determine the tenability of the hydrogel. Figure 1.3-2 summarizes the approach used. In b), the crosslinker presented is a CMP, but the same type of reaction would occur with a non-assembling peptide.

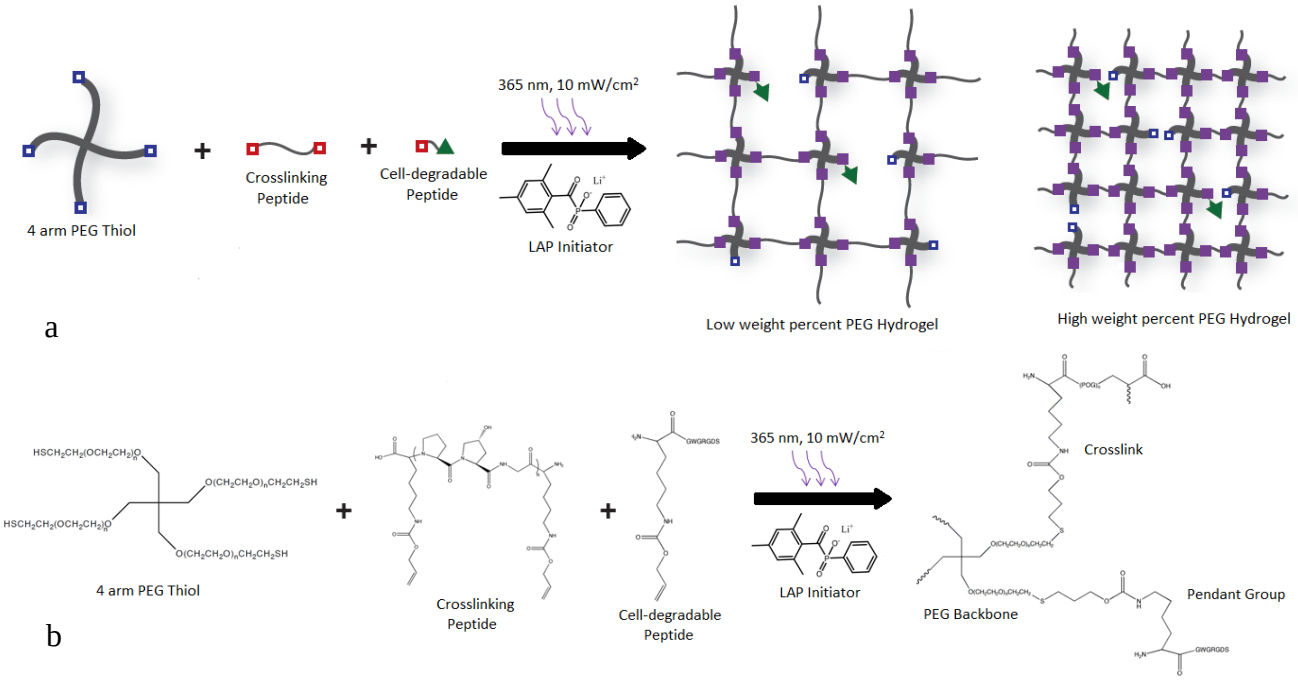

Figure 1.3-2 a) Structural representation of hydrogel formation b.) Chemical representation of hydrogel formation

Chapter 2

MATERIALS AND METHODS

2.1 Materials

All Fmoc-protected amino acids, Rink Amide MBHA resin, and HBTU ((O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) were purchased from Chem-Impex International (Wood Dale, IL). Table 2.1 details all of the amino acids utilized in this thesis.

Table 2.1 List of amino acids used

Amino Acid Name	Amino Acid Abbreviation	Used In
Arginine	R	Non-assembling, cell-degradable
Aspartic Acid	D	Cell-degradable
Cysteine	C	Cell-degradable
Glycine	G	Non-assembling, CMP, cell-degradable
Hydroxyproline	O	CMP
Lysine	K	Non-assembling
Lysine with alloc group	K(alloc)	Non-assembling, CMP, cell-degradable
Proline	P	CMP
Tryptophan	W	Cell-degradable

H-Rink Amide- ChemMatrix LL Resin was purchased from PCAS Biomatrix (Saint-Jean-sur-Richelieu, Quebec). Dimethyl formamide (DMF), diethyl ether, trifluoracetic acid Optima LC/MS, and HPLC grade acetonitrile was purchased from

Fischer Scientific (Fair Lawn, NJ). Piperidine, phenol, HPLC grade methanol, dimethyl phenylphosphonite, 2,4,6-trimethylbenzoyl chloride, lithium bromide, and 2-butanone were purchased from Sigma Aldrich (St. Louis, MO). Trifluoracetic acid (TFA) and triisopropylsilane (TIPS) were purchased from Acros Organics (Geel, Belgium). Dithiothreitol (DTT) was purchased from Research Productions International (Mount Prospect, IL) and 4 arm PEG thiol, MW 20000, was purchased from Jenkem USA (Allen, TX).

2.2 Peptide Synthesis

2.2.1 Collagen Mimetic Peptides

Fmoc-protected proline, hydroxyproline (O-tBu), alloc-protected lysine, and glycine were prepared for a 0.25 mmol scale synthesis by weighing out 1 mmol of amino acid and 1 mmol of HBTU into each amino acid vessel. All amino acids were double coupled during the synthesis, so two vessels of each amino acid to be attached had to be prepared. The synthesis vessel was loaded with approximately 1 gram of ChemMatrix resin to provide 0.25 mmol of active sites. Peptide syntheses were run on a Protein Technologies, Inc. (Tucson, AZ) Tribute automated synthesizer. Synthetic methods followed standard solid phase peptide synthesis methods[15] and the coupling time for each amino acid was one hour.

2.2.2 Non-assembling Peptides

Fmoc-protected arginine (pbf), lysine (boc), lysine (alloc), and glycine were prepared for a 0.25 mmol scale synthesis by weighing out 1 mmol of amino acid and 1 mmol of HBTU into each amino acid vessel. All amino acids were double coupled during the synthesis, so two vessels of each amino acid to be attached had to be

prepared. The synthesis vessel was loaded with approximately 1 gram of ChemMatrix resin to provide 0.25 mmol of active sites. Peptide syntheses were run on a Protein Technologies, Inc. (Tucson, AZ) Tribute automated synthesizer. Synthetic methods followed standard solid phase peptide synthesis methods[15] and the coupling time for each amino acid was one hour.

2.2.3 Cleavage

All peptides were cleaved from the resin using a 95:2.5:2.5 TFA/H_2O/TIPS cocktail. 5% (w/v) DTT and 2.5 (w/v) of phenol were dissolved into this cocktail. The cleavage cocktail was added to the resin and mixed for 2.5 hours. The cocktail was collected and separated into two 50 mL conicals filled with diethyl ether to precipitate the peptide. The conicals were centrifuged and the ethyl ether was decanted. The peptide pellet was dissolved in ethyl ether and centrifuged two more times.

2.2.4 Purification

The dried peptide pellets were dissolved in a 95:5 H_2O/acetonitrile mixture to a concentration of 20 mg/mL and were purified using a Waters (New Castle, DE) reverse-phase HPLC system. The gradient of solvent ran from 10% acetonitrile to 30% acetonitrile. The purified, dissolved peptides were frozen in a -85 °C and lyophilized to remove all solvent. Electrospray ionization mass spectrometry was performed on the dried peptide to verify the product's identity.

2.3 Hydrogel Synthesis

Hydrogel synthesis reactions were performed on a TA Instruments (New Castle, DE) ARES-G2 rheometer. All reactants were dissolved in 1x phosphate

buffered saline (PBS). The reactants for the hydrogel were 4-arm PEG thiol (20,000 g/mol), bi-functional peptide, mono-functional peptide, lithium acylphospinate (LAP) initiator, and PBS. 9.8 µL of 10 wt % and 12.5 wt % PEG mixture were dropped onto UV light base plate and the 8 mm steel Peltier plate was lowered until the gap between the plate and the base was completely filled with the mixture. The 365 nm light (10 mW/cm^2) was turned on after two minutes of 0.25 % strain on the mixture.

Chapter 3

RESULTS

3.1 Peptide Synthesis

3.1.1 Non-assembling Peptide Synthesis

The short non-assembling peptide sequence K(alloc)RGKGRKGK(alloc)G was synthesized for homogenous hydrogels formations to determine the weight percent of peptides and PEG necessary to obtain mechanical properties that match human lung tissue stiffness. Figure 3.1.1 shows the resulting HPLC and mass spectrometry traces of the peptide.

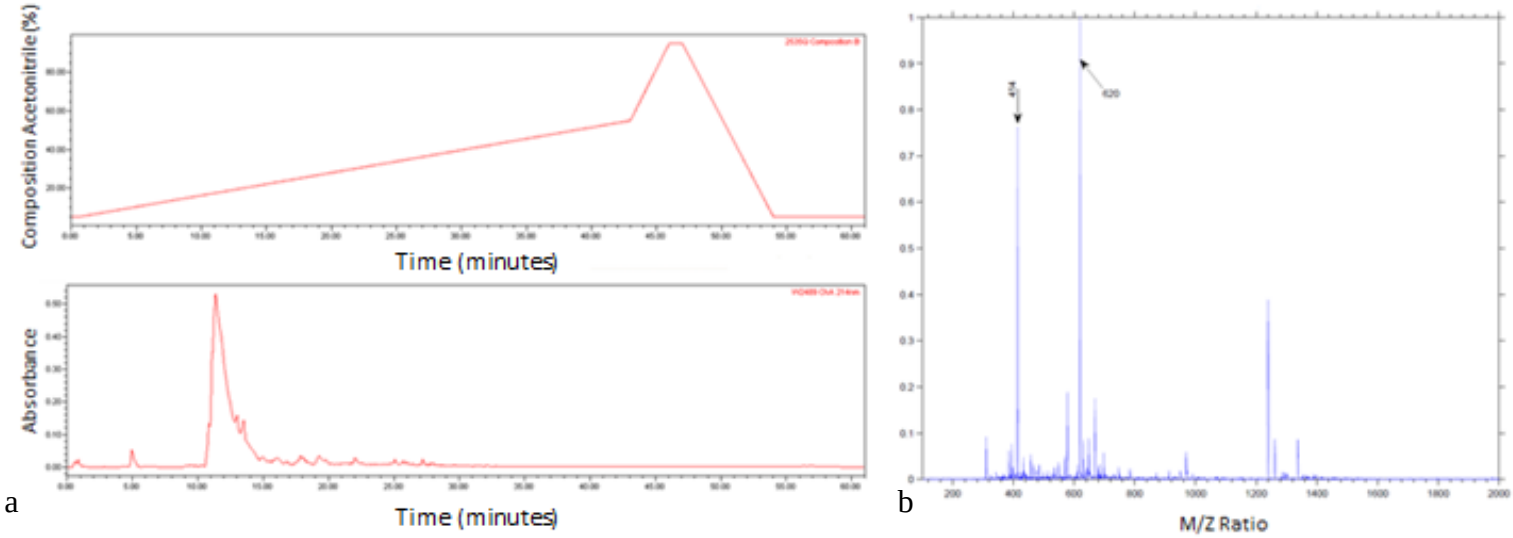

Figure 3.1.1 a) HPLC trace of K(alloc)RGKGRKGK(alloc)G b) Mass spectrometry trace of K(alloc)RGKGRKGK(alloc)G

K(alloc)RGKGRKGK(alloc)G had an expected molar mass of 1241.58 g/mol. The HPLC trace shows the elution of a single product in the peak that begins around 11 minutes into the run, and the mass spectrometry shows mass peaks occurring at 1241, 620, and 414 g/mol. These are the expected M/Z (mass/charge) ratios for the single-, double-, and triple-charged species of the desired peptide, respectively. These analysis methods verified the successful synthesis of the peptide.

3.1.2 Collagen Mimetic Peptide Synthesis

3.1.2.1 K(alloc)G(POG)$_7$GK(alloc)

The amino acid sequence K(alloc)G(POG)$_7$GK(alloc) was selected as the desired CMP for these experiments because it was determined that seven (POG) repeats would be necessary for the formation of the triple helix. The glycine spacers were added to prevent the long side chains of the alloc-protected lysines from interfering with the triple helix assembly. This peptide was also synthesized for the future incorporation in hydrogel synthesis. Figure 3.1.2.1 shows the HPLC and mass spectrometry traces for this synthesis. The HPLC trace suggested that there was a distribution of products rather than a single, dominant product. This is reinforced by the mass spectrometry trace, which shows a large number of significant (greater than 10% of the value of the largest signature) products that are close to the expected mass of the desired peptide, 2429.97 g/mol (shows up on the mass spectrometry trace as 1215, the double-charged species because 2000 is the maximum detectable M/Z ratio). This was interpreted to mean that there was a problem in the synthesis of the peptide. Two possible explanations for the failure in synthesis were:

1. The ChemMatrix resin was not allowing for the removal of the deprotection agent, causing amino acids to be able added at random.
2. The side chains of the alloc-functionalized lysines were disrupting the synthesis of the peptide on its own strand and possibly other strands.
3. The peptides begin assembling while still on the resin, leading to steric hindrance when attempting to attach the next amino acid on all strands.

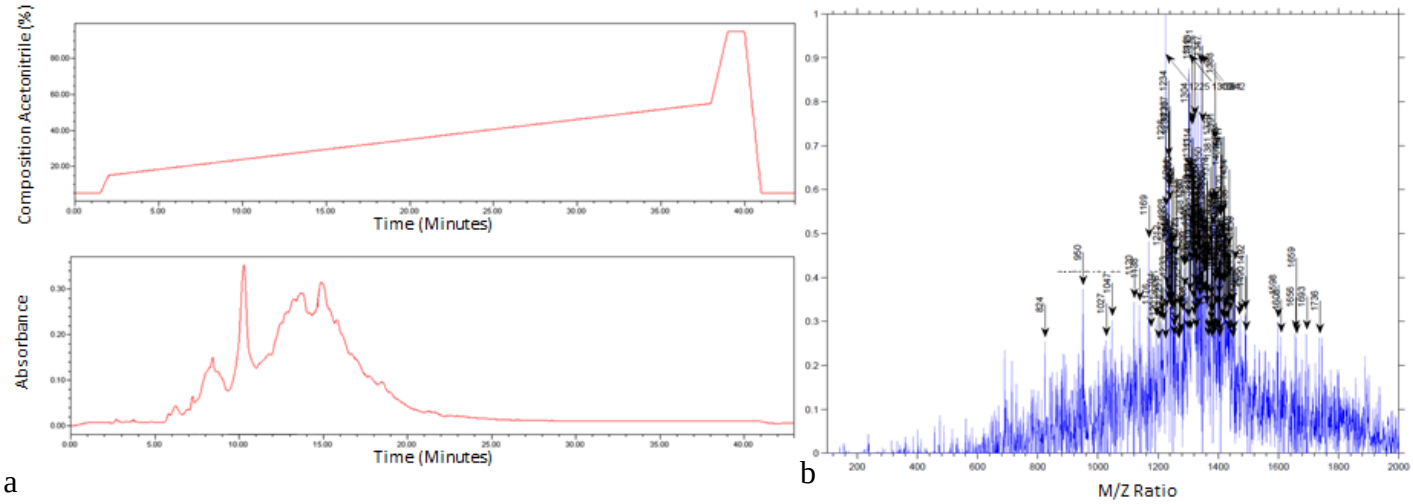

Figure 3.1.2.1 a) HPLC trace for K(alloc)G(POG)$_7$GK(alloc) b) Mass spectrometry trace for K(alloc)G(POG)$_7$GK(alloc)

3.1.2.2 Resin Comparisons

A new experiment was designed to compare the effectiveness of the Rink Amide MBHA Resin with H-Rink Amide-ChemMatrix resin for synthesizing CMPs. Two 0.1 mmol scale batches of the short collagen mimetic peptide (POG)$_4$ (1087 g/mol) were synthesized: one batch on MBHA resin and one batch on ChemMatrix resin. Figure 3.1.2.2 displays the results of these experiments. As can be seen from

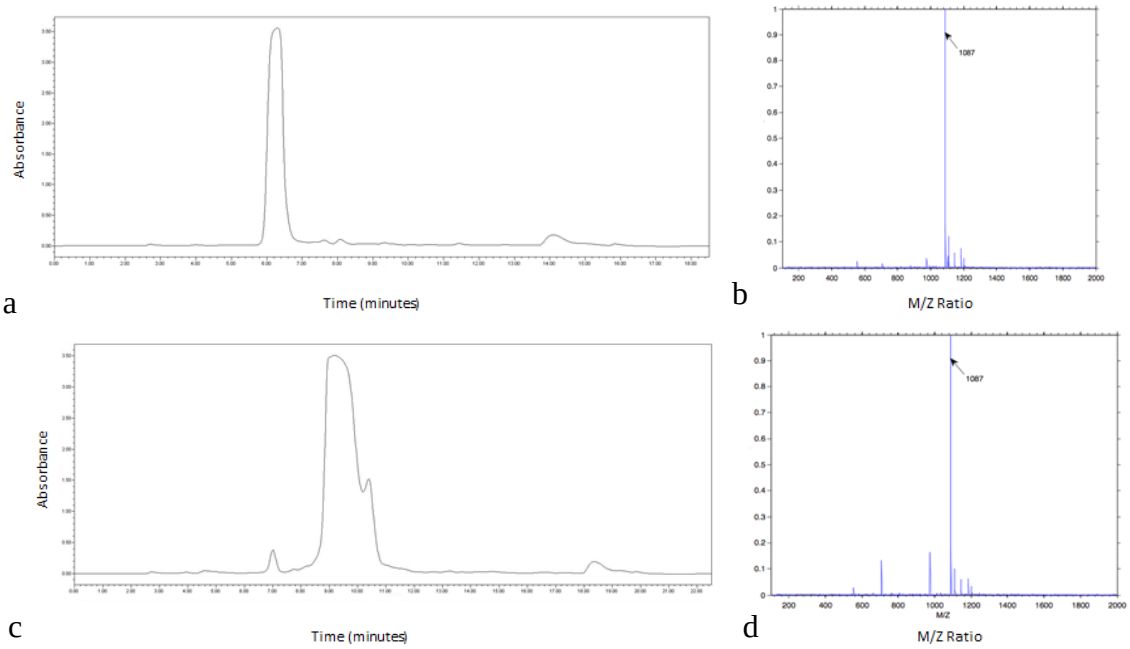

Figure 3.1.2.2 a) HPLC trace for ChemMatrix resin-based $(POG)_4$ b) Mass spectrometry trace for ChemMatrix resin-based $(POG)_4$ c) HPLC trace for MBHA resin-based $(POG)_4$ d) Mass spectrometry trace for MBHA resin-based $(POG)_4$

These results were unexpected in that there was very little difference between the two; both resins are equally effective at synthesizing short CMP sequences. Whether or not there was a performance difference for longer sequences, which were the most relevant for this thesis, remained unanswered.

3.1.2.3 K(alloc)$(POG)_7$

The next experiment had two functions:

1. Test the effectiveness of each resin for longer sequences $(POG)_7$
2. Determine the effect of the alloc-functionalized lysine on HPLC purification

Like the resin experiment, two 0.1 mmol batches of peptide were synthesized in this experiment. The peptide sequence that was synthesized on each resin was $(POG)_7$. An additional step called capping was added to the process. Capping is performed by adding a synthesis vessel with several milliliters of acetic anhydride (Sigma Aldrich) after each amino acid to be attached; growing chains that did not successfully attach the previous amino acid are terminated as a result. The benefit of using capping is observed when purifying the peptide.

Once the Tribute completed the synthesis of this sequence, the resin from each batch was removed from the Tribute and separated into two equal sub-batches. By using a manual peptide synthesis protocol, an alloc-functionalized lysine was added one sub-batch from each resin, resulting in the new sequence $K(alloc)(POG)_7$. This resulted in four batches of peptide. Table 3.1.2.3 summarizes the characteristics of each batch.

Table 3.1.2.3 Summary of Batches

Batch Name	**Resin**	**Sequence Contains K(alloc)?**
ChemMatrix+	ChemMatrix	Yes
ChemMatrix–	ChemMatrix	No
MBHA+	MBHA	Yes
MBHA–	MBHA	No

All four of the batches were then cleaved and purified by the standard methods detailed in Chapter 2. Figure 3.1.2.3-1 compares the HPLC traces of the ChemMatrix– batch and the MBHA– batch. The two traces are virtually indistinguishable, and the mass spectrometry data for these two batches, detailed in Figure 3.1.2.3-2, verified the identity of $(POG)_7$ as the main product by the presence of

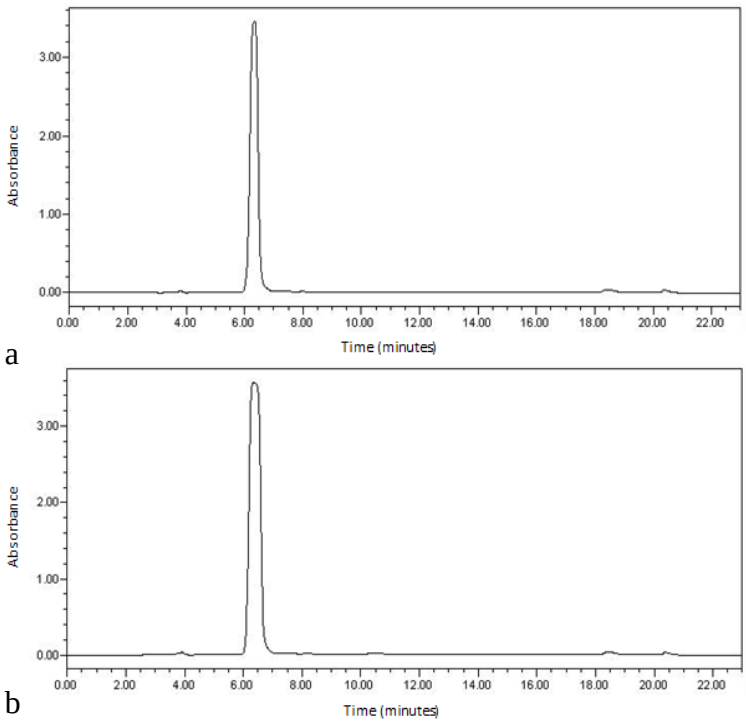

Figure 3.1.2.3-1 a) HPLC trace of ChemMatrix resin-based $(POG)_7$ b) HPLC trace of MBHA resin-based $(POG)_7$

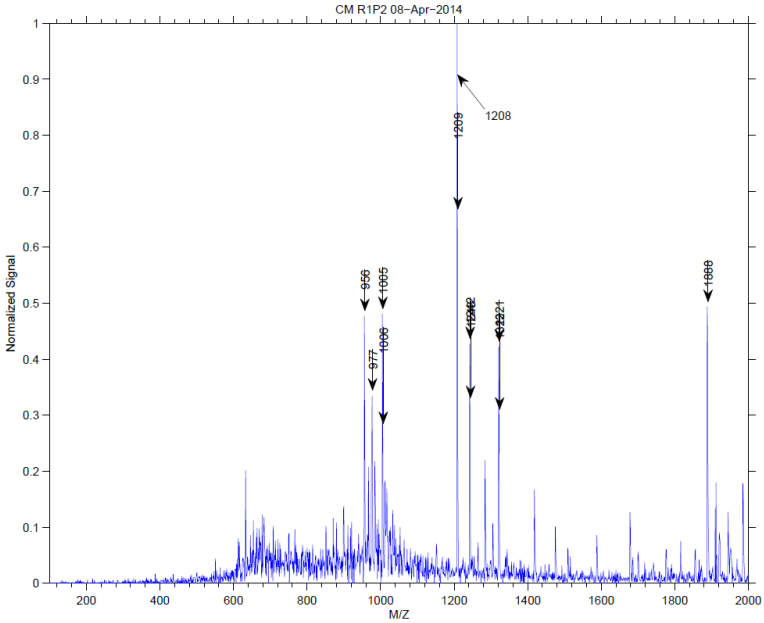

Figure 3.1.2.3-2 Mass spectrometry trace obtained for $(POG)_7$ from both resins

the peak at 1888 g/mol (singly charged species) and 956 g/mol (doubly charged species). This confirms the result of the previous experiment. Figure 3.1.2.3-3 compares the HPLC of the ChemMatrix+ and the ChemMatrix– batches. The clear difference between the two traces is the appearance of a second peak in the ChemMatrix+ trace.

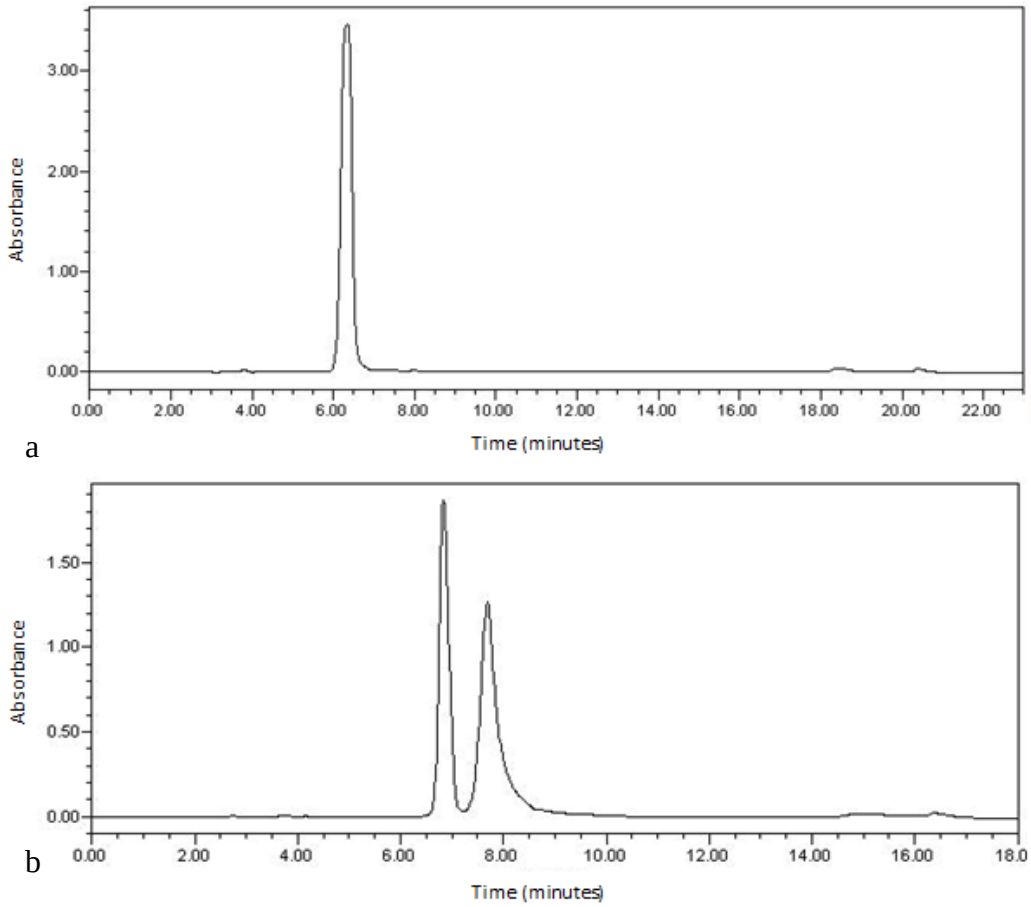

Figure 3.1.2.3-3 a) HPLC trace of ChemMatrix resin-based $(POG)_7$ b) HPLC trace of ChemMatrix resin-based K(alloc)$(POG)_7$

Because the double peak in the ChemMatrix+ is shifted to the right of the ChemMatrix- peak, this means that the ChemMatrix+ product is more hydrophobic than the first peak (due to the gradient of acetonitrile increasing with time), which is indicative of a successful attachment of the hydrophobic K(alloc) residue. Using the mass spectrometry data for the ChemMatrix+ species as shown in Figure 3.1.2.3-4, it was determined that the desired species had been obtained.

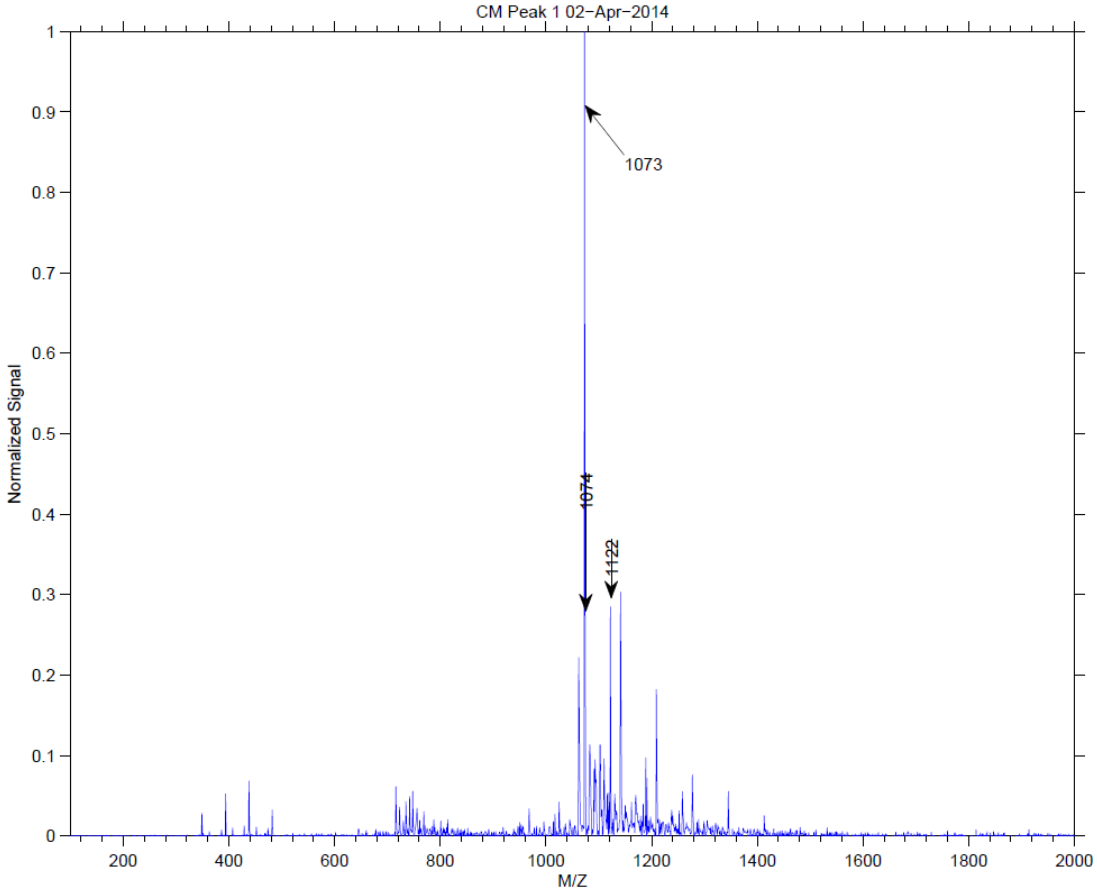

Figure 3.1.2.3-4 Mass spectrometry trace for the ChemMatrix+ batch, species appearing at 1073.

Both the first and second beak on the ChemMatrix+ batch contained the desired species, with the second peak containing more impurities than the first. Another possible explanation of this second peak is that triple helical assembly was occurring in the sample, which led to a longer elution time for the same species. Figure 3.1.2.3-5 shows the results of the HPLC traces for the $(POG)_7$ and $K(alloc)(POG)_7$ that were synthesized on the MBHA resin.

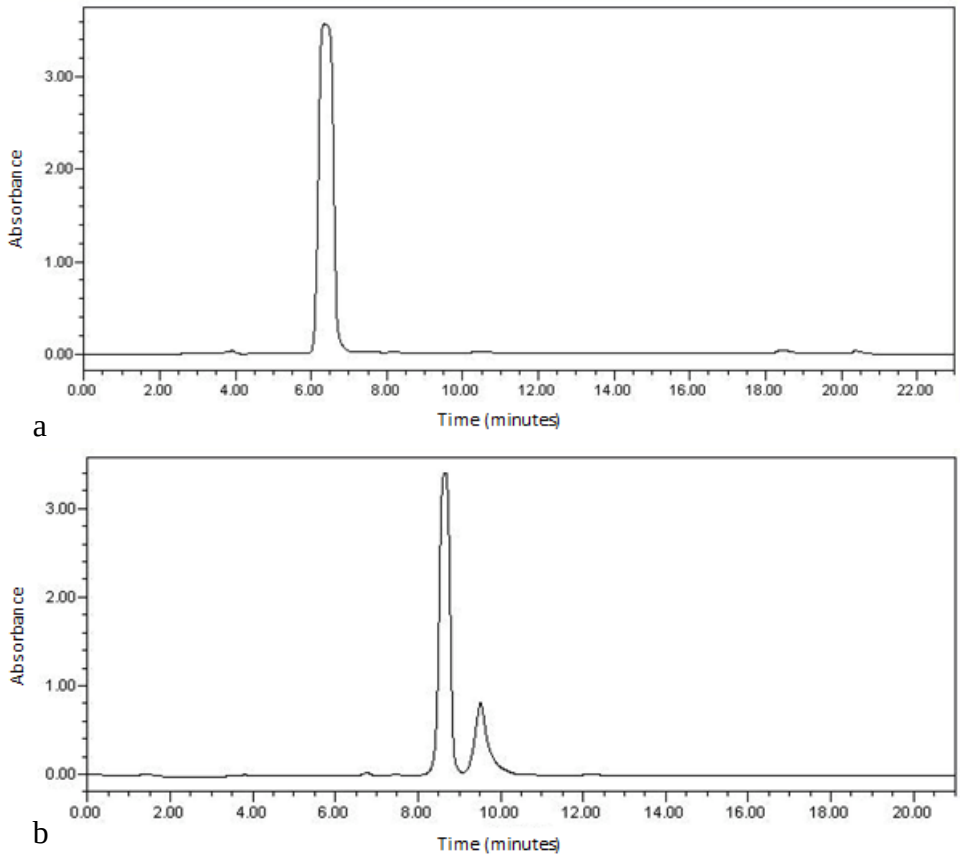

Figure 3.1.2.3-5 a) HPLC trace of MBHA resin-based $(POG)_7$ b) HPLC trace of MBHA resin-based $K(alloc)(POG)_7$

The HPLC traces of the two peptides made on the MBHA resin follows the form of those synthesized on the ChemMatrix resin, but the magnitudes of the peaks in the MBHA+ trace are significantly different, meaning that there was potentially more than one product. Similarly to the ChemMatrix+ batch though, the mass spectrometry trace, Figure 3.1.2.3-6, verified the presence of K(alloc)$(POG)_7$ (1074 g/mol for the doubly charged species) in both of the peaks. Again, this peak splitting may have been the result of triple helix assembly with impurities or with other K(alloc)$(POG)_7$ molecules.

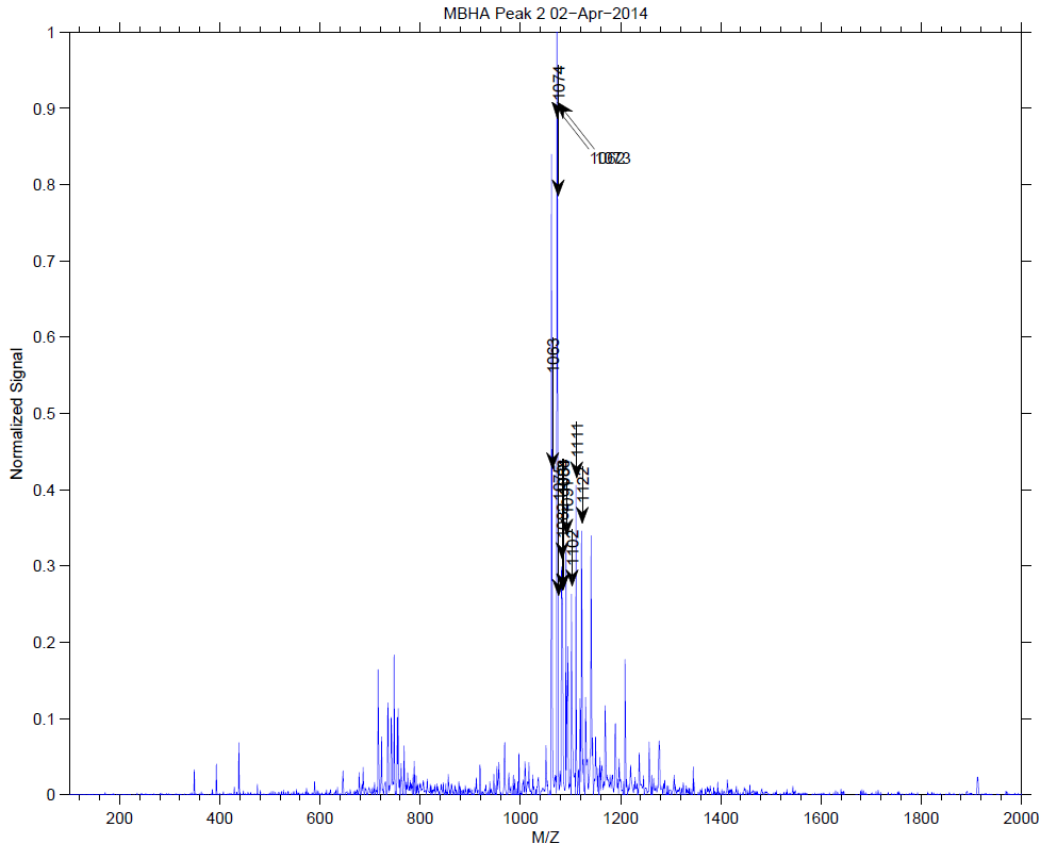

Figure 3.1.2.3-6 Mass spectrometry trace for the MBHA+ batch, species appearing at 1074.

This experiment determined that both resins are equally good at synthesizing the undecorated CMPs and K(alloc) functionalized CMPs. For the undecorated CMPs, the HPLC and mass spectrometry traces for both resins were virtually identical and the dominant product was determined to be the $(POG)_7$ sequence as desired. For the K(alloc) CMPs, the desired species was determined from the mass spectrometry to be the single major product despite the different shape of the HPLC traces. This leads to the conclusion that there is no measurable benefit to using ChemMatrix resin for the synthesis of any CMP variation for the current protocol and peptide synthesizer. Because ChemMatrix resin is much more expensive than MBHA resin and does not confer any additional benefits or chance of synthetic success for these peptides, it was determined that MBHA should be used for future CMP experimentation.

3.2 Hydrogel Synthesis

The goal of these hydrogel syntheses was to determine the proper concentrations of the PEG, crosslinker peptide, and cell-degradable peptide that would result in hydrogels with moduli of 10 and 20 kilopascals (kPa). These values are significant because they represent the modulus of healthy and scarred lung tissue, respectively. Using the experience of prior Kloxin group experiments, it was determined that a concentration of 10 % (w/w) PEG would result in a hydrogel with a modulus of 10 kPa and a concentration of 12.5 % (w/w) PEG would result in a hydrogel with a modulus of 20 kPa. The rheometer measures the shear modulus of the gels as the reaction proceeds, so the results obtained from the rheometer must be transformed to Young's Modulus by using Poisson's Ratio for this system, Equation 1.

$$Equation\ 1:\ E = 3G$$

where E is Young's Modulus and G is the shear modulus

The concentrations of PEG, the non-assembling peptide crosslinker K(alloc)RGKGRKGK(alloc)G, a cell-degradable peptide K(alloc)GWGRGDS, and the radical initiator (LAP) for both sets of trials are displayed in Table 3.2.

Table 3.2 Summary of Hydrogel Reactant Mixtures

	Concentration (wt %)			
Desired Modulus (kPa)	PEG	Crosslinker Peptide	Cell-degradable Peptide	Initiator
10	10	1.1178	0.1892	0.067
20	12.5	1.4283	0.1892	0.067

The initiator concentration was selected from the literature for polymerization of similar PEG-based hydrogels and the cell-degradable peptide was determined to be at a constant 2 mM, a typical concentration used for promoting cell adhesion to PEG-based hydrogels. With these concentrations determined, the hydrogel syntheses were conducted following the protocol detailed in Chapter 2, Section 2.3. Figure 3.2-1 shows the typical appearance of the curve that was produced.

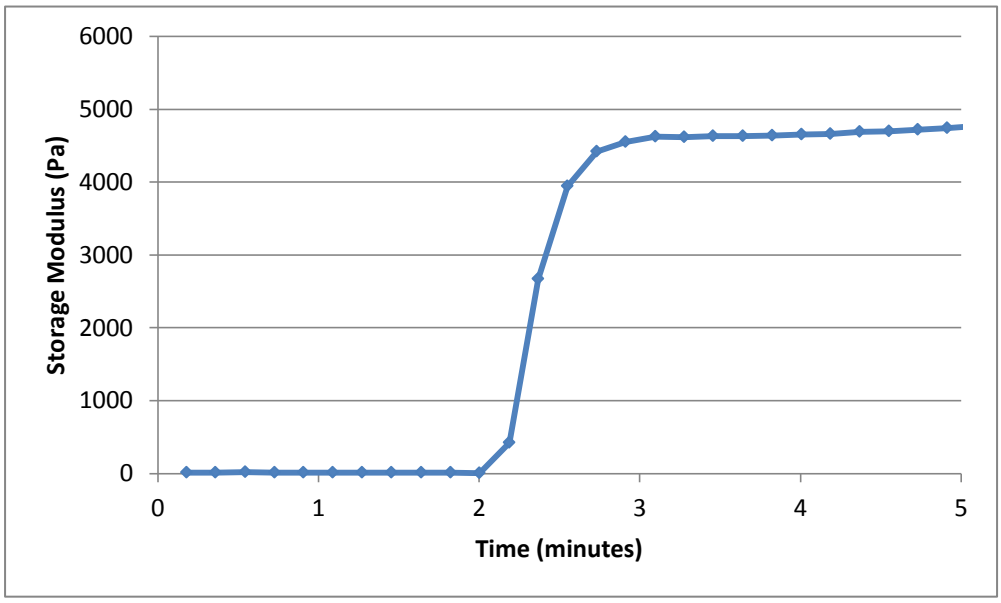

Figure 3.2-1 A typical curve of the evolution of hydrogel modulus during hydrogel synthesis, where increasing storage modulus correlates with hydrogel polymerization and increased crosslink density

The curve pictured above was performed with a 10 weight percent PEG mixture. The UV light was turned on at the two minute mark of each run in order to allow conditions to stabilize. As can be seen in the figure, the polymerization is largely complete within the first minute of ultraviolet exposure. The begins to increase again about three minutes after the light is turned on, but this is due to the hydrogel beginning to dry out. As the hydrogel dries out, the material becomes more solid and, therefore, stiffer.

Figure 3.2-2 summarizes the results of both the 10 and 12.5 weight percent PEG hydrogel syntheses. Both sets of hydrogels overshot their target values by about 4 kPa. The two values were determined to be statistically different at the 95% confidence level by using an unpaired Student-t test. Though the targets were not met, these experiments demonstrate the ability to finely control the physical properties of hydrogel by varying the relative composition of the reactant mixture.

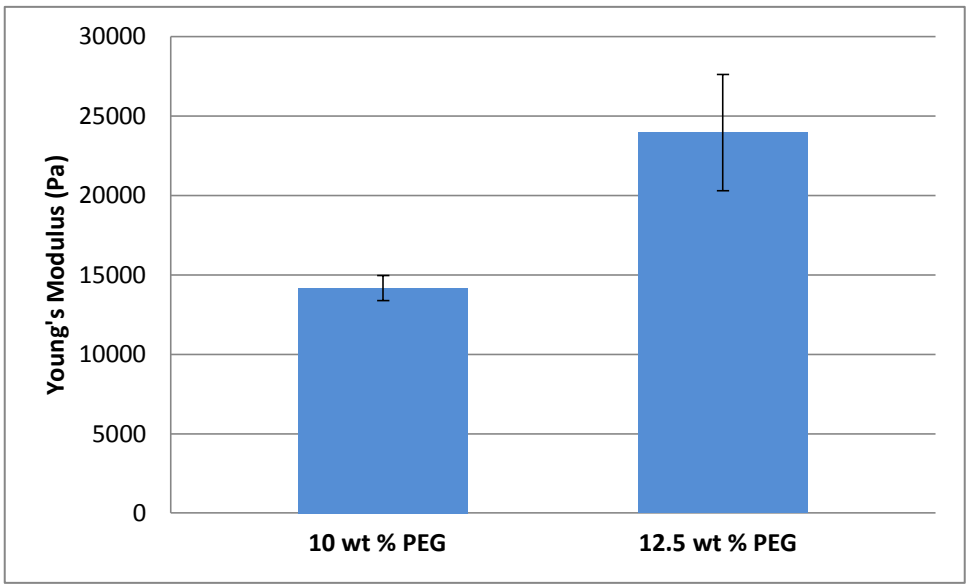

Figure 3.2-2 Average Young's Moduli for both the 10 and 12.5 wt% PEG hydrogels

Chapter 4

CONCLUSIONS AND FUTURE WORK

4.1 Peptide Synthesis

Non-assembling peptides with alloc-functionalized lysine were successfully synthesized on the Tribute automated synthesizer, but a distribution of partially formed species was produced when collagen mimetic peptides with the alloc-functionalized lysines were synthesized. Through subsequent experiments, we established that both resins (MBHA and ChemMatrix) were viable options for synthesizing CMPs, but due to cost considerations, MBHA resin would be the better choice for CMP synthesis moving forward.

The synthesis of a collagen mimetic peptide with two functional groups can be accomplished through further experimentation with the alloc-functionalized lysines and MBHA resin, or a new method can be introduced. One possible alternative would be to replace the K(alloc) residues with cysteines and replacing the 4 arm PEG thiol with 4 arm PEG norbornene or another 4 arm PEG with carbon-carbon double bonds. The alloc-functionalized lysines have proven difficult to work with when synthesizing CMPs because of their long side chains interfering with the addition of subsequent residues. By using cysteines, the problem of this steric inhibition would be completely avoided by the short, thiol-bearing side chain of the cysteine amino acid. Making this switch requires the PEG thiol to be switched as well in order to continue utilizing the highly effective thiol-ene chemistry in hydrogel synthesis.

4.2 Hydrogel Synthesis

The targeted moduli for both healthy and scarred lung tissue were overshot by their respective hydrogels. Though the targets were not met, the capability of to synthesize hydrogels with the desired range of moduli without additional, special conditions was thoroughly verified. The weight percent of PEG should be dropped by 0.5-2 weight percent in order to obtain the proper moduli. Once a bi-functional CMP has been successfully synthesized, they should be incorporated into hydrogel syntheses on the rheometer to verify that the weight percents of PEG in the hydrogel result in moduli that match both healthy and scarred lung tissue with the new crosslinker. Once this has been verified, the hydrogels with non-assembling peptide as the crosslinker and hydrogels with CMP as the crosslinker should be synthesized in a non-destructive manner so that cells can be cultured on them to determine a difference in cellular behavior.

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