

Development and Implementation of Clickable Amino Acids

by

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Abstract

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The Development and Implementation of Clickable Amino Acids

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Peptides present an enormous potential for therapeutic and material science applications. However, in their native format, due to a limited number of chemical handles useful for structural manipulation to address stability issues and facilitate inclusion into other materials, the ability to completely access and implement the potential wealth peptides have to offer is restricted. The overall objective of this thesis endeavored to expand the utility of peptides through the development of synthetic amino acids compatible with click chemistry. Specifically, this work investigated the development of maleimide and furan derivatized amino acids for implementation in the Michael addition and Diels-Alder click reactions to manipulate peptide structure, properties and function.

Click chemistry handles are typically added or incorporated into a peptide sequence post-synthetically or through the use of side chain protecting groups. By developing amino acids expressing click moieties, the furan and maleimide functionalities could be directly incorporated into a peptide chain during solid phase synthesis reducing both time and expense. The synthetic amino acids were created using amino ethyl glycine as a template to attach furan and maleimide functional groups. Through the addition of orthogonal protecting groups to amino ethyl glycine, it was possible to attach furan and maleimide carboxylic acids to an amine on the backbone molecule by means of well developed coupling chemistry. Additionally, selection of the proper

orthogonal protecting groups for the amino ethyl glycine molecule simultaneously made the synthetic maleimide and furan amino acids compatible with standard Fmoc mediated solid phase peptide synthesis.

Once developed, the ability to make use of the clickable amino acids to exercise control over peptide structure, properties and function was assessed. To this end, the ability to use the maleimide amino acid as a handle to conduct Michael-type addition reactions for the modification of peptides was first considered. Through the use of a Diels-Alder/retro-Diels-Alder protecting scheme, the possibility to directly incorporate the maleimide amino acid into a growing peptide sequence while maintaining maleimide activity was demonstrated. Due to synthetic difficulties, maleimide functionalities have traditionally been restricted to post-synthetic incorporation into a peptide sequence to afford a reactive moiety. With an active maleimide group present on the peptide, the potential for conformation manipulation was shown by inciting an intramolecular Michael addition between the thiol of a cysteine residue and maleimide to form cyclic structure from a linear sequence. In addition, the maleimide amino acid handle was also exploited to covalently attach peptides to surfaces and into other materials. Finally, the bioactivity of a peptide that had made use of the maleimide functionality as a handle to incorporate the sequence into another material was assessed.

The ability to implement the furan amino acid as a handle to influence peptide function was also investigated. The introduction of a furan functionality to a peptide granted the ability of the given sequence to participate in the Diels-Alder reaction. This feature was harnessed to controllable release peptidyl materials from a poly(ethylene glycol) (PEG) hydrogel platform by means of Diels-Alder/retro-Diels-Alder mediated reaction diffusion process. A polymer constructed by off stoichiometrically reacting a tetrafunctional maleimide PEG macromer with

various multifunctional thiol crosslinkers such that the thiol species was the limiting reagent allowed for the creation of Diels-Alder tethering sites within the network. The validity of a Diels-Alder release mechanism as well as the ability to predict the release were verified through the use of a reaction-diffusion mathematical model. Furthermore, as the impact of unreacted maleimide and furan species on cell viability is largely unknown, the bioactivity of both the hydrogel release platform containing excess maleimide groups and furan functionalized peptide sequences were evaluated.

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Chapter 1

Introduction and Background

1.1. Motivational Aspects

Peptide sequences possess enormous potential for use in a number of different biological endeavors, from applications in materials science to the development of new therapeutics.^{1,2} In addition, short peptide sequences have demonstrated the ability to elicit activity comparable to a larger protein from which they were derived. In this vein, peptides have been incorporated into polymeric and other materials to achieve bioactive properties. For instance, antimicrobial peptide sequences have been applied to materials to prevent the proliferation of bacteria^{3,4}. Conversely, other peptides, such as RGD, which is a fraction of the protein sequence fibronectin, have been included in polymer networks to foster and promote cellular growth.⁵

Unfortunately, in their native format many peptide sequences suffer limitations that preclude implementation in material science and therapeutic applications. Some of the problems encountered with naturally occurring peptide sequences include stability, low cellular permeability and rapid degradation by enzymatic proteases.^{6,7} To address these issues, feasible solutions have typically involved changing the sequence through the addition/deletion of residues or altering structural conformation of the peptide. The latter approach involves making use of chemical moieties present on the peptide sequence to alter the peptide conformation (linear to cyclic, for instance⁸) through the use of intramolecular reactions.

The use of functionalities on the peptide to accomplish conformational changes presents another problem commonly associated with naturally occurring sequences: the limited number of moieties useful as chemical reaction handles. Reactive groups present on the peptide not only

enable conformational manipulations but also facilitate the incorporation of the sequence into other materials. Naturally occurring peptide sequences are limited to several synthetically useful chemical handles, including amines, carboxylic acids, thiols and alcohols, which have been used both in the process of modifying and including a peptide sequence into other materials.

Through the inclusion of non-native amino acids, the use of protecting groups, and the attachment of non-native functionalities to compatible side chains, a number of other functional groups can and have been introduced into peptides. The functionalities expressed in this manner diversify the number and type of reactive species present on a sequence and allow for other reactions to be employed in the manipulation and control of peptides and peptide-modified materials. Of particular interest is the ability to incorporate functionalities conducive to click chemistry (See Section 1.3). As defined, these reactions allow for specific and efficient alteration of a peptide's structure or inclusion of that biofunctionality into other materials.

However, incorporating reactive handles through the modification of side chains or addition of special protecting groups does have disadvantages. The use of side chain decoration to introduce other functionalities often requires post synthetic modification of a given peptide and also adds time and expense to the synthesis. Additionally, these non-natural chemical functionalities are not always adaptable to or compatible with every situation. For instance, the use of a copper catalyzed azide/alkyne reaction (See Section 1.3) has demonstrated cytotoxicity and the use of this reaction to incorporate peptides into other materials in the presence of cells may have a detrimental impact.

Increasing the number of methods available to incorporate clickable moieties and implement these functionalities in manipulative reactions augments the ability to employ peptide sequences in material and therapeutic applications. One way to expand the use of click

chemistry and engage some of the issues of peptide manipulation is through the direct incorporation of clickable species in a growing sequence during peptide synthesis. By directly placing the click functionality in the sequence during synthesis, the need to post-synthetically modify side chains is addressed. Further, a greater degree of control is granted in terms of placement of the clickable group; the functionality could be placed anywhere in the peptide sequence and would no longer be restricted to one of the terminal ends or a compatible side chain tethering site. The simplest means by which to accomplish this aim would be to place the clickable functionality on an amino acid whereby the carboxylic acid and amine groups would allow for incorporation into and continued growth of a peptide sequence. The main premise of this thesis therefore focuses on the development of non-natural, synthetic amino acids capable of participating in click chemistry, specifically the Michael addition and Diels-Alder reactions, to allow for a greater degree of control to be exercised over peptide structure, properties and function.

1.2. Peptide Synthesis

Amino acids possess the potential to react with one another by means of a condensation reaction where the carboxylic acid of one amino acid reacts with the amine on another to form a peptide bond⁷. By making use of the condensation reaction, the possibility exists to react multiple amino acid monomer units together and form a larger molecule. This reaction can occur multiple times, resulting in the formation of a polymeric species. Depending upon the size (molecular weight) of the amino acid conglomerate, the polymeric sequence can be categorized as either a peptide or protein. Though the molecular weight cutoffs are arbitrary, typically amino acid polymer sequences containing fewer than 50 amino acid residues are categorized as peptides and those composed of a greater number as proteins⁷. Unlike most proteins, which are derived or

require isolation from biological sources, many peptide sequences can be generated by synthetic means. Over the past century, the techniques pertaining to the synthesis of peptides have evolved from a solution-based methodology to the utilization of a solid support or resin.

Initial attempts to synthesize peptides made use of solution-based methodologies. In utilizing this technique, amino acids were placed in solution where the amine and carboxylic acid functionalities were allowed to react. One problem encountered with the solution-phase approach was solubility. As peptide sequences were synthesized they would lose solubility and precipitate out of solution⁹. With the growing peptide not in solution the efficiency by which additional amino acid residues could attach was significantly decreased. Another major limitation with this approach, at least initially, related to the inability to control the specific sequence of the peptide synthesized. In early attempts at peptide synthesis, the amino acids, possessing both amine and carboxylic acid functionalities were present in solution. With both the amine and carboxylic acid groups in solution, peptides were able to form; however, there was little to no control over the length or sequence obtained. The introduction of protecting groups on the amino acids aided in the mitigation of the problem of sequence control by enabling only the unprotected functionalities to react^{10,11}. In addition to solubility and limited sequence control, the solution-based method also presented problems for purification and isolation; lengthy and often difficult chromatographic means were required to separate the product sequence after each residue coupling⁹⁻¹¹.

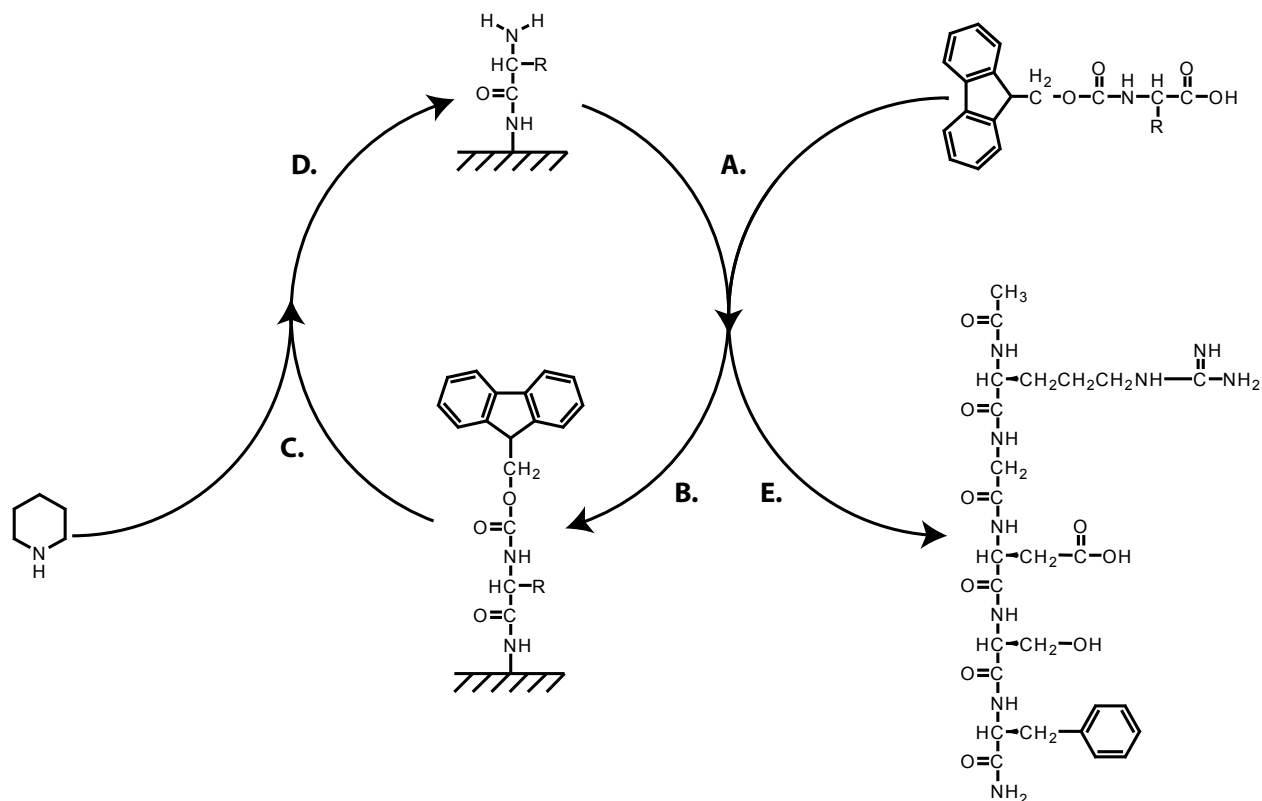


Figure 1.1. Standard Fmoc mediated solid phase peptide synthesis making use of a generic α -amino acid. A. An exposed secondary amine bound to an insoluble solid substrate is exposed to an Fmoc protected amino acid. B. The protected amino acid is anchored to the solid support. Typically the attachment is facilitated through the use of a coupling agent such as a carbodiimide. C. The Fmoc group is removed from the now surface-bound amino acid by using a base, such as piperidine. D. A new primary amine is exposed and ready for subsequent reaction with another amino acid residue. This cycle can be repeated until the desired peptide sequence is attained. E. Once the target sequence is grown, the peptide can be removed and isolated from the solid support. Several means exist to sever the linkage between the solid support and peptide, the most common being the use of trifluoroacetic acid.

The advent of solid phase peptide synthesis (See Figure 1.1), introduced by Merrifield in the 1960's, revolutionized peptide synthesis.^{9,12,13} Rather than construct a peptide in solution, Merrifield's approach covalently attached the carboxylic acid of the first amino acid to an insoluble solid support. The amino acids Merrifield employed possessed protecting groups on the amine functionality, preventing additional, undesired coupling that could potentially lead to the formation of branched and erroneous peptide sequences. Once attached to the support, the

protecting group of the anchored molecule was removed and another amino acid was introduced for coupling to the terminal amine. This sequential addition-deprotection process was repeated multiple times until the target peptide sequence was achieved. After attachment of the final amino acid residue, the peptide was removed from the solid support by the introduction of a cleavage mixture that severed the link between the peptide and the resin.

By constructing the peptide in this manner, Merrifield's approach was able to address and overcome many of the limitations plaguing the solution-based method. Covalently attaching the growing peptide sequence to a solid support facilitated and simplified the removal of unreacted species and impurities. These impurities, which remain in solution and do not attach to the resin, could now be readily removed and separated from the solid support containing the peptide, negating the need for difficult isolation and purification steps between each coupling step. The ease of removing unwanted/unreacted materials also allowed for the use of an excess of a given amino acid residue. The use of an excess drives the coupling of the residue to the growing peptide chain to completion.

Since being introduced, many changes and improvements have been made to the solid phase peptide synthesis methodology. One major deviation from the solid phase technique outlined by Merrifield is the adoption of a fluorenylmethyloxycarbonyl (Fmoc) over the original tert-butyloxycarbonyl (Boc) mediated synthesis as the dominant protection scheme¹⁴. Fmoc mediated synthesis presents a safer and more orthogonal approach to the solid phase peptide synthesis methodology¹⁵. In terms of safety, Boc mediated synthesis requires the use of hydrofluoric acid for the liberation of the peptide from the solid support. In contrast, the Fmoc approach employs trifluoroacetic acid to cleave the peptide sequence, a milder acid than hydrofluoric acid from a health and safety standpoint. In addition to using an acid to cleave the

peptide from the resin, the Boc methodology makes use of acid to cleave the protecting group- a Boc group from which the method's name is derived- on the terminal amine. The use of acid to remove both the protecting group and the finished peptide from the solid support leads to potential cleavage of the peptide during each deprotecting step, resulting in lower yield of the target sequence. Conversely, Fmoc mediated solid phase peptide synthesis relies upon an orthogonal protection scheme; a base is used to remove the Fmoc protecting group on the N-terminal amine and an acid cleaves the peptide from the resin.

In addition to the Fmoc solid phase synthesis strategy, other advances in peptide synthesis have been developed including new solid supports and other orthogonal side chain protecting groups. New solid supports possessing different reaction conditions for peptide cleavage and varying swelling characteristics and degrees of functionalization have been devised. These advances in solid support technology have helped to minimize undesired reactions, which have previously lowered the overall yield of the target sequence. For example, changing the characteristics of the solid support, such as using a lower surface functionalization and/or increasing ability of the resin to swell increases the distance between adjacent, growing peptide chains¹⁵. The increased spacing resulting from a lower surface functionalization density helps to minimize undesired reactions between neighboring peptide sequences that lead to dimers and higher molecular weight species formation. New linkers, which attach the peptide to the solid substrate, have also been developed that allow for different reaction conditions to cleave the sequence from the resin. For instance, a photodegradable linker has been created that allows for the liberation of peptides from the solid support without the use of strong acidic conditions, such as those present when using trifluoroacetic acid¹⁶.

Other protecting groups, which offer another layer of orthogonality to Fmoc solid phase peptide synthesis, have also been created. These protecting groups are not cleaved under the traditional conditions used to remove the Fmoc protecting group (basic) or the peptide from the solid support (strong acid). Instead, these orthogonal protecting groups are typically removed using mildly acidic conditions (much less acidic than required to cleave the peptide from the solid substrate), hydrazine or catalytically with palladium.^{17,18} The use of these protecting groups have allowed for more complex peptide structures, such as cyclic architectures, to be synthesized while the molecule is anchored to the solid support.⁸

1.3. Click Chemistry

Initially conceived with the aim of producing new pharmaceutical compounds at a rapid pace, Sharpless introduced the paradigm of click chemistry consisting of reactions with the ability to produce complex molecular structures in a simple yet robust manner. In particular, reactions associated with click chemistry are reputed to achieve quantitative conversion, undergo relatively few (if any) side reactions, allow for simple, non-chromatographic isolation of the product species and occur under mild reaction conditions such as ambient temperature, atmospheric pressure and in aqueous media^{19,20}. These attributes, coupled with their need for high specificity, tight control and inherent high expense have led to the implementation of click chemistry in materials science and biological applications.

The Huisgen reaction or copper catalyzed 1,3-dipolar cycloaddition reaction between azides and alkynes has come to represent the quintessential click reaction, demonstrating many of the tenants of the click paradigm^{21,22} (See Figure 1.2).

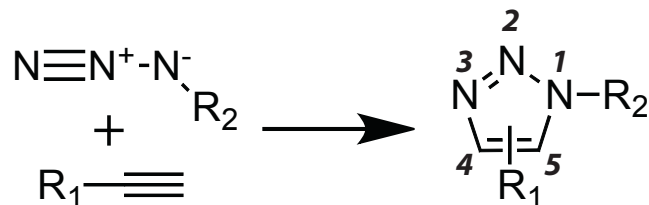


Figure 1.2. The “premier” click reaction or Huisgen reaction. A 1,3-dipolar cycloaddition takes place between an azide functionality and an alkyne to produce the cyclic product. When catalyzed by copper(I), the 1,4-cyclic product is the preferentially formed product whereas the non-catalyzed version forms a mixture of the 4 and 5 substituted species.

Pertaining to accomplishing the requirements associated with click chemistry, when the Huisgen reaction is performed, the cycloaddition occurs orthogonally; few to zero extraneous reactions involving the azide and alkyne take place. Additionally, minimal further purification is required, near complete conversion, formation of regioselective product (the 1,4-disubstituted heterocycle) and high yield are attained. The Huisgen reaction can also take place in mild solvents like water, which has been found to facilitate the reaction²².

The 1,3-dipolar cycloaddition reaction has been implemented for many applications, both non-biological and biological. From a materials science standpoint, this reaction has been used in a number of facets including polymer synthesis²³, dendrimer synthesis²⁴, and nanomaterial fabrication and functionalization^{25,26}. In terms of biological applications, this premier click reaction has come to use as a means to ligate peptides and other molecules²⁷, anchor biomolecules to surfaces²⁸ and label biological species with fluorophore tags or other motifs^{29,30}. With regard to biological pursuits it is important to note that the 1,3-dipolar cycloaddition does have limitations. In order for the reaction to progress at an appreciable rate, copper(I) is generally required to catalyze the reaction for most alkynes. As copper is cytotoxic, the use of copper(I) as a catalyst limits the use of the azide alkyne reaction in biological applications where cells are present. Methods to circumvent or minimize the use of copper in the Huisgen reaction,

such as the use of ring strained alkynes or metal chelating agents³¹⁻³³, have been developed and applied with promising results.

The Huisgen reaction, as evidenced by the characteristics it displays, meets the demanding requirements associated with the click philosophy and has come to be synonymous with click chemistry. However, the click paradigm, as conceived of by Sharpless and co-workers, was not intended to be restricted to a single reaction. Rather, click chemistry was designed and envisioned to consist of and encompass a large set of reactions fulfilling certain stipulations (high yield, facile purification, mild reaction conditions, etc.) that could be used to efficiently synthesize complex materials. Other reactions, in addition to the Huisgen cycloaddition, have been identified that meet the stringent criteria associated with click chemistry. The Michael addition and Diels-Alder reactions^{19,34} are two prime examples of other reactions that are capable of meeting the rigorous standards of click chemistry. These two reactions will be discussed and were extensively used in the work associated with this thesis.

1.4. The Michael Addition Reaction

The Michael addition, which was first noted in the 1940's³⁵, is one reaction that has come to be associated with click chemistry. One specific form of this reaction makes use of a thiol species as one of the reactants and is commonly referred to as thiol-Michael addition. As depicted in Figure 1.3, the Michael addition occurs between a nucleophilic species, such as a thiol, and an electron deficient carbon-carbon double bond typically referenced as an α,β -unsaturated carbonyl compound.

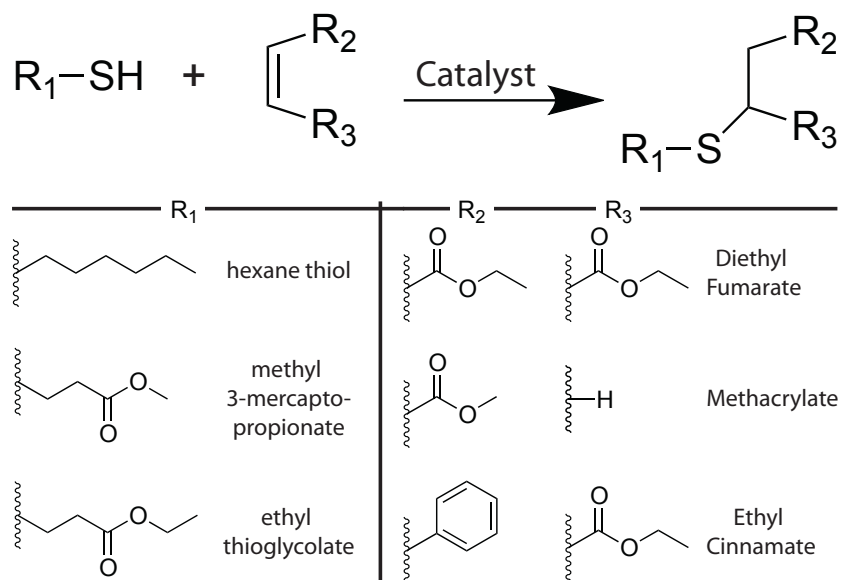


Figure 1.3. The thiol-Michael addition reaction. The reaction scheme is presented in the top portion of the figure. Beneath the reaction scheme, some potential substituents, which have an influence on the reaction, for the thiol and –ene species are shown.

In many cases a catalyst, in the form of a base or phosphine, is added to the reaction system to promote the Michael addition. Several factors have been demonstrated to impart a strong influence on the efficiency of the thiol-Michael addition. These include the structure and substitution of the thiol and –ene species participating in the reaction as well as the specific base or phosphine catalyst selected^{36–38}.

Before examining the Michael addition's dependence on the selection of catalyst, the details of the mechanisms associated with the base and phosphine mediated reaction pathways will be discussed. Mechanistically, the use of a base or phosphine catalyst takes different routes to accomplish the formation of the enolate ion responsible for the Michael addition reaction. For the case of a base catalyst, the alkaline conditions initiate the reaction by deprotonation of the thiol species, leading to the generation of a thiolate anion. The anion then adds to the electron deficient –ene at the β -position resulting in the formation of an enolate intermediate and a

thioether bond between the two reacting species. The enolate intermediate exhibits basic character and reacts to abstract a proton from the base, thereby regenerating the catalyst and allowing for propagation and continuation of the reaction.

When a phosphine is employed as the catalyst, the reaction mechanism is slightly different. In contrast to the base catalyzed mechanism, the initiation of the reaction relies upon the nucleophilicity of the phosphine to attack the carbon-carbon double bond at the β -position. This reaction gives rise to a basic zwitterionic phosphonium enolate intermediate, which deprotonates the thiol species. This allows the thiolate anion to attack other α -ene molecules at the β -position and generate another enolate intermediate. Formation of the enolate allows for the reaction to propagate and, ideally, react until the limiting reagent(s) is exhausted.

Depending on which mechanism is employed, the catalyst selected has an impact on the reaction kinetics. With regard to the phosphine-catalyzed approach, the ability to act as a nucleophile directly coincides with a given phosphine molecule's effectiveness as a catalyst in the Michael addition. When the ability of the phosphine to attack the β -carbon of the double bond is hindered, the speed of the reaction is reduced. Therefore, by placing substituents on the phosphine that influence the molecule's nucleophilic character, the rate of the Michael addition can be altered. A prime example as shown by Chan and co-workers³⁸ can be seen through the systematic addition of electron withdrawing phenyl rings, which diminish the nucleophilicity of the phosphine. When only one phenyl ring was attached to the phosphorous atom, the Michael addition proceeded rapidly. Upon the addition of two and three phenyl groups, however, the rate of the Michael addition was found to drop by approximately a factor of two.

Pertaining to the base-catalyzed reaction mechanism, with the use of stronger base a more effective deprotonation of the thiol is achieved. Deprotonation allows the thiol to react with the

β -carbon of the α,β -unsaturated carbonyl compound and incite the formation of the enolate intermediate. The formation of the enolate leads to the regeneration of the base catalyst and the continued progression of the reaction. If the pKa value of the base selected for the catalyst in the Michael addition is less than the thiol, deprotonation of the thiol will not readily occur and the reaction will not take place by means of the base catalyzed mechanism. Provided the base does not have a sufficient enough pKa, it is still possible for the Michael addition to take place. Depending upon the nucleophilic character of the base, the possibility exists for the reaction to proceed down the pathway employed by phosphine compounds. The uses of primary and secondary amines as well as other amine species with pKa values lower than the thiol, such as 1-methyl imidazole, have demonstrated this ability³⁷.

As has been demonstrated, not all thiol species exhibit the same reactivity when implemented in Michael addition reactions. In particular, the structure and substituents present on the thiol species have been found to greatly influence the Michael addition. When participating in the Michael addition, the thiol species must first undergo deprotonation for the reaction with an activated (electron deficient) α,β -unsaturated carbonyl compound to occur. The ease with which deprotonation takes place correlates to how well a thiol will participate in the Michael addition. In general, the higher the pKa value of the thiol, the easier the separation of the sulfur atom from the hydrogen atom and the more effective the thiol becomes in the Michael addition reaction. Changing the substituents on a given thiol species allows for a simple means to alter the pKa value of the molecule. The inclusion of electron withdrawing species (See Figure 1.3), such as phenyls or carbonyls, increases the pKa value associated with a molecule and facilitates deprotonation. For example, ethyl thioglycolate (See Structure in Figure 1.3), which contains an electron

withdrawing carbonyl, has been shown to react orders of magnitude faster in a Michael addition than hexane thiol³⁸.

In addition to the role of the catalyst and thiol species, the configuration of the electron deficient α -ene also influences the rate of the Michael addition reaction. The electron deficiency of an α -ene is increased when conjugation of an electron withdrawing species with the carbon-carbon double bond occurs. In general, an increase in the electron deficiency of an α -ene results in a higher reactivity towards Michael addition type reactions. For example, the placement of carbonyl groups about the carbon-carbon double bond bolster the reactivity of diethyl fumarate in the Michael addition reaction relative to materials lacking the symmetric electron withdrawing groups. Though the possibility exists to increase the reactivity of an α -ene by adding electron withdrawing substituents, the influence of these groups can be negated through the use and introduction of stabilizing or bulky steric groups (See Figure 1.3).

There exist a number of methods that allow for the control and tuning of the thiol-Michael addition, including: changing the structure of the α -ene and thiol, placing different functional group substituents on the reactive species and making use of different catalysts. These handles can be exploited to enable reactions that occur rapidly, in high yield with minimal side products and in mild solvent conditions, keeping the Michael addition in line with the principles of click chemistry. A number of applications have made use of the efficiency the thiol-Michael click reaction, which is exemplified in terms of polymer and material science. In particular, the ability to select reactants that are conducive to the biological pursuits have lead to the implementation of the thiol click reaction in biological applications. The thiol-Michael addition has found use in a number of different applications in the biological realm including surface functionalization^{25,39}, disulfide bridge replacement^{40,41} and the labeling and crosslinking

of peptides and proteins^{42,43}.

1.5. The Diels-Alder Reaction

Discovered in the late 1920's⁴⁴, the Diels-Alder (DA) reaction is another reaction that often fulfills the requirements associated with click chemistry. Presented in Figure 1.4, the classic form of the DA reaction results in the concerted 2+4 cycloaddition between a conjugated diene species(A) and an electron deficient dienophile(B) to form a six member ring as the product or adduct.

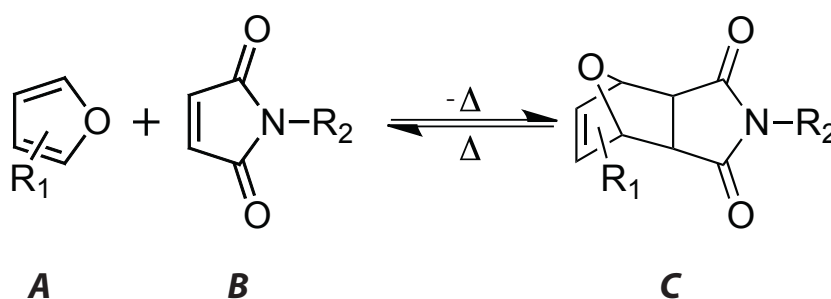


Figure 1.4. The Diels-Alder and retro-Diels-Alder reactions between a generic furan (A), the diene, and a maleimide (B), the dienophile, to form the adduct (C). The forward Diels-Alder reaction and subsequent formation of adduct is favored at lower temperatures. An increase in temperature promotes the retro-Diels-Alder reaction and leads to a shift of the equilibrium to favor the diene and dienophile material.

An interesting feature, as noted in Figure 1.4, is the dynamic nature of the DA reaction. Low temperatures favor the formation of adduct, whereas elevated temperatures promote reversion to the initial diene and dienophile species, a process referred to as the retro-Diels-Alder (rDA) reaction. In addition to and in conjunction with temperature, several other aspects can influence the DA reaction including the diene conformation, substituents on the reactant species and the solvent in which the reaction is carried out. These features can be implemented to tune the equilibrium of the DA reaction.

There exist a large number of conjugated diene species, however, not all are compatible with the DA and rDA reactions⁴⁵. In order for a conjugated diene to participate in the DA

reaction it must have or be able to attain the proper conformation: the carbon-carbon double bonds of the diene must be in the cis configuration. Linear diene species are capable of undergoing the DA reaction, provided the molecule can rotate about the carbon-carbon double bond to attain the cis conformation. In general, linear dienes display a reduced reactivity in the DA reaction as compared to structures, such as cyclic species, that lock the diene into the cis conformation.

In addition to conformation, the placement of substituents on the diene also influences the DA reaction.⁴⁵⁻⁴⁷ The diene, as exhibited by the two carbon-carbon double bonds (See Figure 1.4), is an electron rich species. In the DA reaction, the diene serves as the electron donor, forming covalent bonds with the electron deficient dienophile. Incorporating an electron withdrawing species, when conjugated to the diene, reduces the electron density of the diene and decreases the ability of the molecule to donate electrons. With a diminished ability to donate electrons, the diene typically exhibits lowered reactivity towards participation in the forward DA reaction. Placement of substituents on the dienophile also has an impact on the DA reaction. In contrast to the diene, the addition of electron-withdrawing species to the dienophile promotes the adduct formation. The addition of an electron-withdrawing group increases the electron deficiency of the dienophile, which facilitates the acceptance of electrons from the diene.

Another parameter, aside from the diene structure and substituents placed on the reactants pertains to the solvent system in which the DA reaction is conducted^{45,48,49}. Conventionally, the DA reaction has been carried out in organic media; however, as with most reactions, the solvent system has been found to have a significant impact on the reaction. When the reaction between some diene and dienophile species is executed in an aqueous environment, the DA reaction has been shown to progress at a faster rate than if run in a nonpolar organic solvent. The higher DA

reaction rate observed in aqueous conditions has been attributed to a combination of hydrogen bonding and “enforced” hydrophobic interactions. These influences are envisioned to help to stabilize the reaction transition state leading to the formation of adduct as well as enhance the electron withdrawing and donating capacity of the dieneophile and diene.

The DA reaction presents the ability to be controlled not only by means of temperature, but also through changes in diene conformation, the solvent system in which the reaction is run and the decoration of the reactants with substituents expressing varying degrees of electron withdrawing character. Use of these attributes enables the attainment of a fast and selective reaction that, in addition to the ability to carry the reaction out in a mild solvent such as water, enables the DA reaction to meet the other standards associated with click chemistry. A variety of applications have made use of the orthogonality and efficiency of the DA click reaction in the field of material science as well as in the biological realm. In terms of material science, the DA/rDA reactions have been taken advantage of to create functional group protecting schemes, removable coatings and mendable materials⁵⁰⁻⁵³. Many of the materials science applications make use of and rely upon the thermoreversibility of the DA reaction. In a number of biological situations, such as the ligation and surface binding of peptides and proteins^{54,55}, the dynamic nature of the DA is not the primary objective and has not been harnessed. Other biological applications, in particular the formation of protecting groups⁵⁶, have made central use of the reversible nature of the DA reaction.

1.6. Project Overview

The main and overarching goal of this thesis was to investigate and expand the use of click chemistry with regard to the manipulation and control of peptide structure, function and properties. To achieve this overall goal, the focus was placed on two particular click reactions:

the Diels-Alder and Michael addition reactions. In the second chapter of this thesis, the framework for accomplishing the main objectives is provided through the establishment of several key, measurable objectives.

The third chapter investigates the thermodynamic and kinetic parameters of the Diels-Alder reaction between furan and maleimide molecules. To aid in the selection of a diene and dienophile pair for use in a given application, this study measured and presents the impact on these properties of the placement of carboxylic acid functionalities at various substitutional positions on Diels-Alder reactants. The carboxylic acid moiety, as demonstrated in Chapter 3, facilitates the incorporation of the maleimide and furan functionality into other molecules and materials.

Chapter 4 presents the development of a new, synthetic maleimide amino acid and explores its applications in peptide chemistry. The direct incorporation of the new maleimide amino acid in a growing peptide chain is demonstrated. Also shown is the potential to make use of the maleimide functionality as a handle to alter a peptide's conformation and incorporate the peptide into other materials. Further, the bioactivity of a peptide that has made use of the maleimide amino acid to attach the sequence to another material is assessed.

In the fifth chapter, the ability to utilize the Diels-Alder reaction to controllably release material from a polymer network is demonstrated. Releasing materials through a Diels-Alder/retro-Diels-Alder reaction scheme in this manner has not been examined previously. In addition, Chapter 5 also presents the ability to tune the release through changes in temperature and the number of Diels-Alder reactive sites. The predictability of the release by means of a Diels-Alder reaction with a reaction-diffusion mathematical model is also discussed.

Following up on the proof of concept presented in Chapter 5, the sixth chapter explores the bioactivity of the release system; specifically the impact of the Diels-Alder moieties on cell viability. The impact of excess, unreacted maleimide pendant groups in the polymer network on cell longevity is investigated. Additionally, influence of the incorporation furan on the bio-
viability and activity of a pharmaceutical compound is considered.

The final chapter, Chapter 7, presents conclusions and future directions for the use of the clickable amino acid species developed as part of this thesis.

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Chapter 2

Project Objectives

Peptides have come to represent a large potential source for the development and control of innovative biomaterials and therapeutics. Unfortunately, in their native format peptides exhibit limited applicability due to low stability, rapid enzymatic breakdown and a lack of an efficient means by which to incorporate them into other materials. These limitations hinder the use of peptides in biological and biomaterials applications. Reactions belonging to the paradigm of click chemistry, first introduced by Sharpless, have been used to address these shortcomings.

In order to be associated with click chemistry a reaction must have certain characteristics including: high yield of the target species, few (if any) side reactions, and simple, non-chromatographic purification. In addition, the tenants of click chemistry also require a given reaction to occur under mild conditions such as ambient temperature, atmospheric pressure and in aqueous media. With these features, click style reactions are well suited to address the modification and manipulation of biological materials, which are often expensive and highly susceptible to degradation. When a functional group associated with click chemistry is introduced to a peptide, the moiety can be implemented to induce conformational changes that increase stability or be used to incorporate the sequence into other molecules or materials.

Two click reactions commonly exploited to modify peptides or facilitate their incorporation into other materials are the maleimide-thiol Michael addition and the Diels-Alder (DA) reactions. The Michael addition reaction takes advantage of the high and fast reactivity of the maleimide functionality with thiol species to efficiently form a thioether linkage. Not only can the maleimide functionality participate in the Michael addition, but it also has the capacity to

partake in another click reaction, the Diels-Alder reaction. In this reaction, the maleimide functional group serves as a dienophile and reacts with diene species, such as furans, to form the Diels-Alder adduct. Unlike the Michael addition, the DA reaction is dynamic: low temperatures promote the formation of the adduct whereas elevated temperatures incite the regeneration of the reactant materials through the retro-Diels-Alder (rDA) reaction. Depending on the conditions and target application, the diene (i.e. furan) and dienophile (i.e. maleimide) pair can be selected to give the DA reaction the ability to serve as either a protecting group or a stable ligation between multiple species.

Despite all the advantages click chemistry offers, the maleimide functionality, whether implemented in the Michael addition or DA reaction, still has significant limitations with regard to the modification and control of peptides. One significant encumbrance associated with this reaction is the ability to incorporate an active maleimide group into a growing peptide chain. If any thiol groups are present, the maleimide will irreversibly react with them by means of a Michael addition. In addition to thiol groups, maleimides also have the potential to react with primary or secondary amines. Amines, such as a primary amine present on the N-terminus of the growing peptide chain or the secondary amine piperidine, are constantly present during solid phase peptide synthesis. Reaction through either of these pathways precludes the use of maleimide for further manipulation and control of a peptide's structure and function.

With the possibility of these undesired reactions taking place, the placement of the maleimide functionality in the peptide has also been constrained to certain locations in the sequence. To avoid the premature reaction with thiols, amines or other species present during solid phase synthesis, the maleimide functionality has been attached to the sequence post synthetically. Not only does this limit the placement of the maleimide to the terminus of the

sequence or the end of a compatible side chain, but also requires the use of an additional deprotection step which can add time and expense to the synthesis. The side chains of the amino acids are often protected to minimize undesired branching of the peptide during its synthesis. To deprotect one amino acid residue and not the others requires the introduction of an orthogonal, and often more costly, protecting group.

Similar to the problems surrounding the incorporation of maleimide moieties, the inclusion of a diene species into a peptide sequence for use in the DA reaction also has limitations. The primary difficulty that arises when attempting to place a diene in a peptide sequence is the incompatibility of many forms of this moiety with traditional solid phase peptide synthesis. For instance, some dienes, such as 3-(2-furyl) propionic acid, degrade upon the exposure to trifluoroacetic acid, which is employed to liberate the peptide from the solid support. When the diene degrades, it becomes incompatible with DA chemistry and can no longer undergo the DA/rDA reactions. The potential degradation can be averted by either the post synthetic addition of the diene or through the introduction of protecting groups. Both these potential solutions further complicate the peptide synthesis.

In light of these observations and needs, the global objective of this thesis, the investigation and expansion of click chemistry for peptide manipulation and control, addresses these apparent limitations of the maleimide and furan functionalities and can be disintegrated into two key components. One part of the overall goal pertains to extending the use of the maleimide functionality's ability to participate in the Michael addition reaction, which can be used to exercise control over peptide structure and provide the ability to be incorporated into other materials. The second aspect of the global objective looks into the development and characterization of a previously unexplored use of the DA/rDA reactions as a potential means to

controllably release peptidyl material from a polymer network. By making use of the DA reaction liberation of peptides or, potentially, other materials from the polymer network will be governed by a combination of reaction and diffusion granting increased control over their release profiles.

To investigate these two aspects of the global objective, the following specific aims were established:

1. Design and synthesize novel amino acid structures possessing maleimide and furan moieties capable of participating in the Michael addition and Diels-Alder reactions.
 - a. As not all maleimide and furan species react at the same rate or to the same extent in the DA and rDA reactions, evaluate the thermodynamic and kinetic behavior associated with a range of different furan and maleimide species.
 - b. After screening the kinetic and thermodynamic properties of the different maleimide and furan molecules in the DA/rDA reactions, incorporate the furan and maleimide species with desirable reaction characteristics onto amino acid backbones.
2. Implement the new maleimide functionalized amino acid as a click chemistry handle in the Michael addition reaction to:
 - a. Manipulate and control peptide conformation, and
 - b. Covalently incorporate peptide sequences into other materials such as polymer networks or onto surfaces.
3. Promote the controlled release of furan-functionalized peptides from a hydrogel containing DA compatible tethering sites.

Chapter 3

Kinetic and Thermodynamic Measurements for the Facile Property Prediction of Diels-Alder-Conjugated Material Behavior*

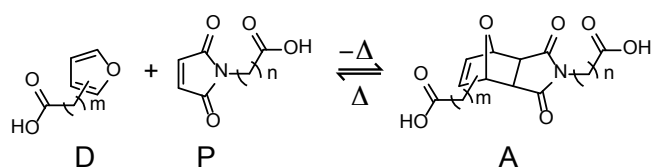
The Diels-Alder reaction between maleimide and furan moieties possessing various substitutions was performed as a means for developing predictive capabilities for temperature and conversion-dependent material properties in networks comprised of Diels-Alder moieties. Using ^1H NMR spectroscopy, the reactions of maleimide and furan-containing molecules further functionalized with carboxylic acids were monitored to ascertain the impact that substitutional changes had on the thermodynamic and kinetic behavior of the Diels-Alder reaction. The reaction rate and equilibrium conversion of the furan and maleimide increased when the carboxylic acid functional group directly connected to the furan ring was moved from the two to the three position. When an aliphatic two-carbon spacer was employed, such that the π -electrons of the carboxylic acid and furan were no longer conjugated, the reaction rate increased further. We also report the reactivity effect on the distance between the carboxylic acid functional group and the maleimide, which yielded little impact on the reaction rate, but exhibited increased equilibrium conversion with increasing distance. Additionally, the impact on the kinetic and thermodynamic properties of coupling the carboxylic acid to another molecule, t-butyl glycine, was also determined. When the carboxylic acid was coupled to an amine, the Diels-Alder reaction between the furan and maleimide was generally found to have similar kinetic and thermodynamic behavior as compared to their uncoupled, carboxylic acid equivalents. Thus, the

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characterized and tabulated kinetic and thermodynamic data presented herein, enables the prediction of a broad set of temperature dependent chemical and material properties. Finally, we discuss practical limitations and nuances of the Diels-Alder reaction, such as the potential for the maleimide to ring-open in aqueous media via hydrolysis.

3.1. Introduction

The Diels-Alder (DA) reaction is one of the premier ‘click’ reactions¹ owing to its exceptional ability to efficiently and orthogonally form carbon-carbon bonds.² For example, the direct organic synthesis of several natural products, such as Oseltamivir Phosphate³ utilizes the DA reaction to create carbon-carbon linkages. While in much of conventional organic synthesis it is desirable for the diene and dienophile pair (D/P in Scheme 3.1) to be essentially irreversible and favor the forward reaction to the exclusion of any significant reversibility, frequently, the DA adduct possesses the capability to revert back to the starting diene and dienophile pair at elevated temperatures via the retro Diels-Alder (rDA) reaction.



Scheme 3.1. The Diels-Alder carboxylic acid species studies here, where furan (Diene) and maleimide (dienoPhile) species react to form the Diels-Alder Adduct. Low temperatures promote the forward reaction to form the DA Adduct while elevated temperatures cause reversion to the initial diene and dienophile pair. The nature of the forward and reverse reactions and their thermoreversibility are controlled by the diene and dienophile chemical structure.

Recently, several research groups have utilized the DA and the subsequent rDA reaction for several applications, ranging from protection/deprotection of functional groups to the

formation of thermoreversible crosslinks in polymer networks⁴⁻⁶. For example, the DA and rDA reactions have been used to protect maleimides, which are used in a variety of applications, including fluorescent staining, antibody coupling, and surface functionalization.⁷⁻⁹ As the maleimide readily reacts in the presence of free radical species and organic bases,^{5,10,11} the DA adduct formed by the reaction with a diene moiety is used to prevent undesired side-reactions during synthetic transformations.^{10,12} After synthesis, the maleimide is reformed and isolated by simply heating the product and liberating the diene moiety.

Thermoreversible polymers have also been produced from multifunctional diene and dienophile monomers or oligomers.^{13,14} Unlike conventional polymeric network materials, covalent networks composed of DA adduct crosslinks are capable of complete structural reversion, including having the capacity to depolymerize completely. Such materials have found prominent applications in covalent adaptable networks (CANs), allowing for the creation of various technologies, including removable coatings, recyclable polymers, and self-healing materials.^{6,15}

Current applications that utilize the forward and reverse DA reaction typically employ a trial and error process to select the diene and dienophile pairs that exhibit the desired thermodynamic and kinetic properties. The examination and understanding of thermodynamic and kinetic properties for furan and maleimide pairings would facilitate informed selection of the correct DA functionalities for a given application. Since these species exemplify the archetypal DA thermoreversible characteristics over moderate temperatures (from room temperature to 100°C), the DA reaction between maleimide and furan are commonly exploited throughout the literature in a range of applications, such as those mentioned above^{6,15}. In particular, our studies will focus on carboxylic acid functionalize maleimide and furan molecules. The carboxylic acid

form of the maleimide and furan are readily available either commercially or by means of simple synthetic measures. In addition, the carboxylic acid provides a convenient synthetic handle through which the furan or maleimide is incorporated into other structures or materials having amines and hydroxyl groups, such as polymer networks or peptides, via well-developed coupling chemistries.

Herein, a comprehensive set of kinetic and thermodynamic data are presented and discussed, enabling the selection of furan and maleimide functional groups for the *a priori* design of functional materials and other applications, including self-healing networks and protecting groups. To accomplish this outcome, the impact of systematic substitutional changes on the thermodynamic and kinetic parameters of the DA reaction between the furan, a diene, and the maleimide, a dienophile, functional carboxylic acids was evaluated. Additionally, to investigate the role that solvent plays in the DA reaction, thermodynamic and kinetic data were collected from experiments conducted in two solvents, namely dimethylformamide (DMF) and water.

3.2. Experimental

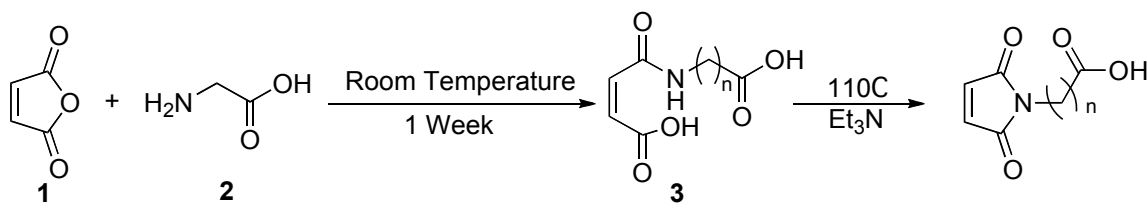
3.2.1. Materials

The following compounds were purchased from the specified vendor and, unless otherwise indicated, directly used without further purification. Dienes employed in this study, 2-furoic acid, 3-furoic acid and 3-(2-furyl) propionic acid, were all purchased from Sigma-Aldrich. To remove a brown discoloration and any impurities, 3-(2-furyl) propionic acid was recrystallized from hexanes (30 to 50°C)¹⁶. Maleic anhydride and the amino acid precursors: β -alanine, glycine, and 6-aminohexanoic acid involved in the synthesis of the maleimides considered in this investigation were purchased from Fisher Scientific and Sigma-Aldrich,

respectively. To investigate the amine coupling of furan and maleimide, thionyl chloride was purchased from Sigma Aldrich and glycine tert-butyl ester HCl was acquired from AK Scientific. Deuterated solvents, dimethyl formamide d7 (DMF) and deuterium oxide, d2, for use in NMR spectroscopy were acquired from Cambridge Isotope Laboratories.

3.2.2. Synthetic Methodology

3.2.2.1. Maleimide Carboxylic Acids. Scheme 3.2 depicts the general reaction scheme to synthesize maleimide carboxylic acids with n carbons separating the maleimide ring and the carboxylic acid functionality.



Scheme 3.2. Maleimide Carboxylic Acid Synthesis Overview

Synthesis of the diacid (3) was performed by mixing the desired amino acid (2) with maleic anhydride (1) in a 1:1 stoichiometric ratio in tetrahydrofuran (THF) and allowing the mixture to react for one week. Vacuum filtration, followed by rinsing of the white solid precipitate with THF yielded the diacid product. Contrary to the synthetic procedure reported in the literature,¹ the THF was not dried prior to use, nor was the reaction flask purged with argon, though a high yield was achieved (~98% for each of the precursors synthesized).

Glycine Diacid ($n=1$): ^1H NMR (500 MHz, DMSO) δ 9.24 (s, -N-H-, 1H), 6.40 (d, -HC=CH-, $J = 12.4$, 1H), 6.29 (d, -HC=CH-, $J = 12.4$, 1H), 3.89 (d, -H₂C-CH₂- $J = 5.8$, 2H).

β-Alanine Diacid (n=2): $^1\text{H NMR}$ (500 MHz, DMSO) δ 9.12 (t, -N-H-, $J = 5.3$, 1H), 6.40 (m, -HC=CH-, 1H), 6.24 (d, -HC=CH-, $J = 12.5$, 1H), 3.37 (dd, -H₂C-CH₂-, $J = 6.7, 12.4$, 2H), 2.47 (d, -H₂C-CH₂-, $J = 6.8$, 2H).

Caproic Diacid (n=5): $^1\text{H NMR}$ (500 MHz, DMSO) δ 9.11 (s, -N-H-, 1H), 6.39 (d, $J = 12.6$, -HC=CH-, 1H), 6.23 (d, -HC=CH-, $J = 12.5$, 1H), 3.16 (dd, -H₂C-CH₂-, $J = 6.9, 12.8$, 2H), 2.19 (t, -H₂C-CH₂-, $J = 7.4$, 2H), 1.47 (dt, -H₂C-CH₂-, $J = 7.5, 24.8$, 4H), 1.28 (dd, -H₂C-CH₂-, $J = 8.3, 15.6$, 2H).

For each of the diacid spectra reported, the -COOH peak is broad and downfield at approximately 12ppm.

The second step of the maleimide acid synthesis is ring closure.² (Step 2 Scheme 3.2) The diacid precursor (**3**) was suspended in toluene. To the diacid suspension, a two molar excess of triethylamine was added, deprotonating the diacid's amine and facilitating ring closure. The reaction mixture was stirred vigorously and heated to reflux, while water byproduct was collected overhead with a Dean-Stark apparatus. After refluxing for ninety minutes, the hot toluene product mixture was decanted into another flask, separating it from a red-orange oil residue. The decanted toluene was removed via rotary evaporation to leave a white crystalline powder for $n=1$, and a pale yellow oily residue for the cases when $n>1$. Prior to acidification with 1M HCl, the pale yellow residue was taken into approximately 100mL of water. From the acidified mixture, product maleimide was extracted using 3x100mL ethyl acetate fractions. The combined ethyl acetate washes were dried with sodium sulfate and rotary evaporated to yield a pale yellow to white solid as the product. As with the synthesis of the diacid precursor, the ring

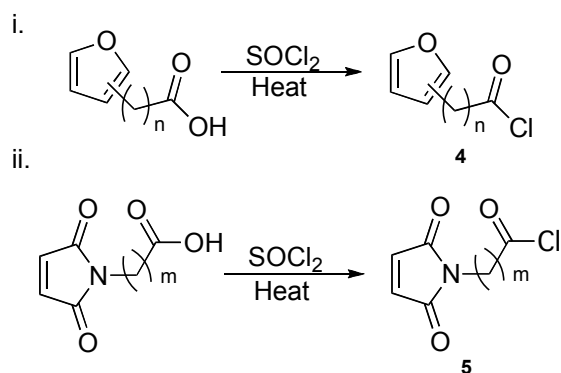
closure step did not use dry toluene and attained a similar yield (~30%) compared to values reported in the literature.

Glycine (n=1) Maleimide: ¹H NMR (500 MHz, DMSO) δ 7.13 (s, -HC=CH-, 2H), 4.13 (s, -H₂C-C₂H-, 2H).

β-Alanine (n=2) Maleimide: ¹H NMR (500 MHz, CDCl₃) δ 6.73 (d, -HC=CH-, J = 3.3, 1H), 3.84 (t, -H₂C-C₂H-, J = 7.2, 2H), 2.71 (t, -H₂C-C₂H-, J = 7.2, 2H).

Caproic (n=5) Maleimide: ¹H NMR (500 MHz, CD₃OD) δ 6.80 (s, -HC=CH-, 2H), 3.50 (t, -H₂C-C₂H-, J = 7.1, 2H), 2.28 (t, -H₂C-C₂H-, J = 7.4, 2H), 1.61 (tt, -H₂C-C₂H-, J = 7.4, 14.7, 4H), 1.32 (m, -H₂C-C₂H-, 2H).

3.2.2.2 Acid Chloride Synthesis. To facilitate incorporation onto an amine, the carboxylic acid functionality of the diene and dienophile were first transformed into acid chlorides. Synthetic route appended and adapted from Camper et al³. (See Scheme 3.3.)



Scheme 3.3. Furan (i) and Maleimide (ii) Acid Chloride Synthesis

3.2.2.2.a. Furoic Acid Chloride Synthesis. 3-Furoic or 3-(2-furyl) propionic carboxylic acids were dissolved in approximately 150mL of dichloromethane while purging with argon gas. To this solution, a tenfold excess of thionyl chloride was added. After stopping the argon purge, the reaction mixture was brought to reflux for three hours. Removal of dichloromethane and excess thionyl chloride via evaporation yielded the desired furan acid chloride, which was reacted with glycine without further purification.

3-Furoic Acid Chloride: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 8.22 (dd, $J = 0.8, 1.4, 1\text{H}$), 7.54 – 7.50 (m, 1H), 6.82 – 6.79 (m, 1H).

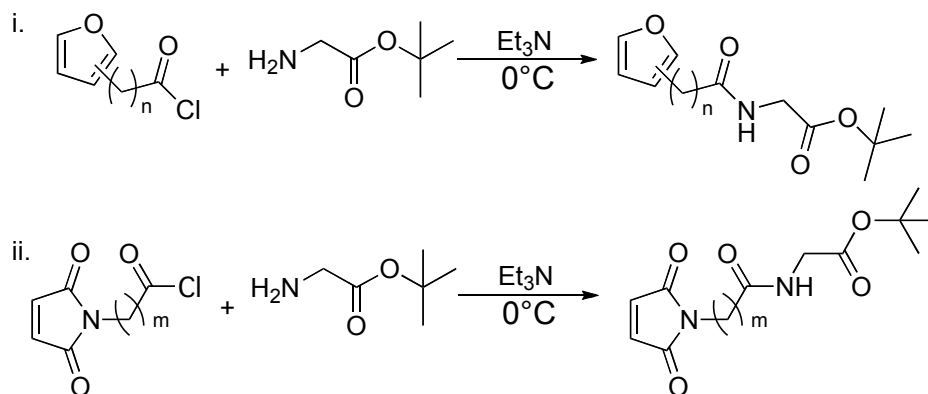
3-(2-Furyl) Propionic Acid Chloride: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 7.33 (dd, $J = 0.8, 1.9, 1\text{H}$), 6.30 (dd, $J = 1.9, 3.2, 1\text{H}$), 6.08 (ddd, $J = 0.8, 1.7, 3.1, 1\text{H}$), 3.27 – 3.22 (m, 2H), 3.07 – 3.00 (m, 2H).

3.2.2.2.b. Maleimide Acid Chloride Synthesis. Glycine maleimide ($m=1$) or β -alanine maleimide ($m=2$) carboxylic acids were dissolved in approximately 200mL of dichloromethane while purging with argon gas. To this solution, a tenfold excess of thionyl chloride was added. After stopping the argon purge, the reaction mixture was brought to reflux for twenty-four hours. Removal of dichloromethane and excess thionyl chloride via evaporation yielded the desired maleimide acid chlorides, which were reacted with glycine without further purification.

Glycine Maleimide Acid Chloride ($m=1$): $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.84 (d, $J = 3.5, 2\text{H}$), 4.66 (s, 2H).

β -Alanine Maleimide Acid Chloride ($m=2$): $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.74 (s, 2H), 3.86 (t, $J = 6.9, 2\text{H}$), 3.25 (t, $J = 6.9, 2\text{H}$).

3.2.2.3. Diels-Alder Moiety Coupling to Glycine. After conversion of the carboxylic acid to acid chloride, the diene and dienophile were coupled to t-butyl glycine (see Scheme 3.4).



Scheme 3.4. Furan (i) and Maleimide (ii) Coupling to Glycine

3.2.2.3.a. Furan Coupling to Glycine. The 3-furoic or 3-(2-furyl) propionic acid chloride synthesized previously (**4**) was taken into approximately 200mL of dichloromethane. To the acid chloride solution at $\sim 0^\circ\text{C}$, tert-butyl glycine ester in an amount equivalent to the initial quantity of carboxylic acid used to synthesize the acid chloride was added. Triethylamine in a 1:1 stoichiometric ratio to the tert-butyl glycine ester was also added to the reaction vessel. After reacting overnight, the dichloromethane mixture was washed with 3x300mL 1M HCl. Residual water was removed with a 1x500mL brine wash followed by the addition of sodium sulfate. Removal of dichloromethane by means of rotary vacuum evaporation yielded the target coupled furan glycine.

3-Furoic t-Butyl Glycine: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 7.98 – 7.93 (m, 1H), 7.43 (t, $J = 1.7$, 1H), 6.65 (dd, $J = 0.8, 1.9$, 1H), 6.39 (s, 1H), 4.08 (d, $J = 5.0$, 2H), 1.49 (s, 9H).

3-(2-Furyl) Propionic t-Butyl Glycine: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 7.30 (d, $J = 1.1$, 1H), 6.27 (dd, $J = 2.0, 3.0$, 1H), 6.03 (dd, $J = 0.8, 3.2$, 1H), 6.01 (s, 1H), 3.93 (t, $J = 4.9$, 2H), 3.03 – 2.96 (m, 2H), 2.61 – 2.55 (m, 2H), 1.47 (d, $J = 2.1$, 9H).

3.2.2.3.b. Maleimide Coupling to Glycine. The glycine ($m=1$) or β -alanine maleimide acid chloride synthesized previously (**5**) was taken into approximately 200mL of dichloromethane. To the acid chloride solution at $\sim 0^\circ\text{C}$, a stoichiometric equivalent of tert-butyl glycine ester (relative to the carboxylic acid used in the previous step) was added. Triethylamine, in a 1:1 stoichiometric ratio to the tert-butyl glycine ester, was also added to the reaction vessel. After reacting overnight, the light red dichloromethane mixture was washed with 3x300mL 1M HCl. Residual water was removed from the now yellow organic layer with a 1x500mL brine wash followed by the addition of sodium sulfate. Removal of dichloromethane by means of rotary vacuum evaporation granted the coupled maleimide glycine product.

Glycine Maleimide t-Butyl Glycine: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.80 (s, 2H), 6.25 (s, 1H), 4.24 (s, 2H), 3.95 (d, $J = 4.8$, 2H), 1.47 (s, 9H).

β -Alanine Maleimide t-Butyl Glycine: $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.67 (s, 2H), 6.27 (s, 1H), 3.87 (d, $J = 5.1$, 2H), 3.81 (t, $J = 7.2$, 2H), 2.55 (t, $J = 4.9, 12.1$, 2H), 1.42 (s, 9H).

3.2.3. NMR Spectroscopy

NMR spectroscopic measurements were acquired using a Varian 500MHz NMR spectrometer equipped with a Nalorac probe. Samples studied via NMR spectroscopy were prepared by measuring equimolar quantities of the diene and dienophile under consideration

directly into a 5mm NMR tube. Immediately following the addition of deuterated solvent, an NMR spectrum was acquired to serve as a reference point in equilibrium measurements and the first time point in kinetic studies. To control the temperature while spectra were acquired, samples were kept in the heating block between NMR experiments. As the NMR experiments took approximately 1 minute to complete and as the DA and rDA reactions between the dienes and dienophiles are relatively slow at ambient temperature with half-lives of hours to days, the subsequent reaction during this interim was deemed negligible. The progress of the DA reaction was monitored by measuring the consumption of the furan and maleimide functionalities while subsequently monitoring the formation of the adduct peaks in the NMR spectrum. The location of each peak in the spectrum (diene, dienophile or adduct) depended on the particular furan and maleimide reacted and the deuterated solvent used. A singlet associated with the maleimide carbon-carbon double bond was observed around 7 ppm, multiplets corresponding to the furan could be found from 6 to 7.5 ppm, and characteristic peaks of the endo and exo isomers of the adduct were typically located between 4.5 and 5.5 ppm. Detailed spectra cataloguing the endo and exo forms of adduct may be found in the supplemental material.

3.2.3.1. Furan and Maleimide Diels-Alder H-NMR Spectra. The characteristic maleimide – ene proton singlet occurred at approximately the same spectral position (around 7 ppm) for each of the maleimides considered. Each spectrum below portrays the Diels-Alder reaction where β -Alanine has been used as the dienophile. The following figures (Figure 3.1, 3.2, and 3.3) illustrate the location of the furan ring protons for the different furan moieties considered as well as the location of key adduct peaks in deuterated dimethylformamide. (Residual DMF solvent peaks at 8.03, 2.92 and 2.75ppm.)

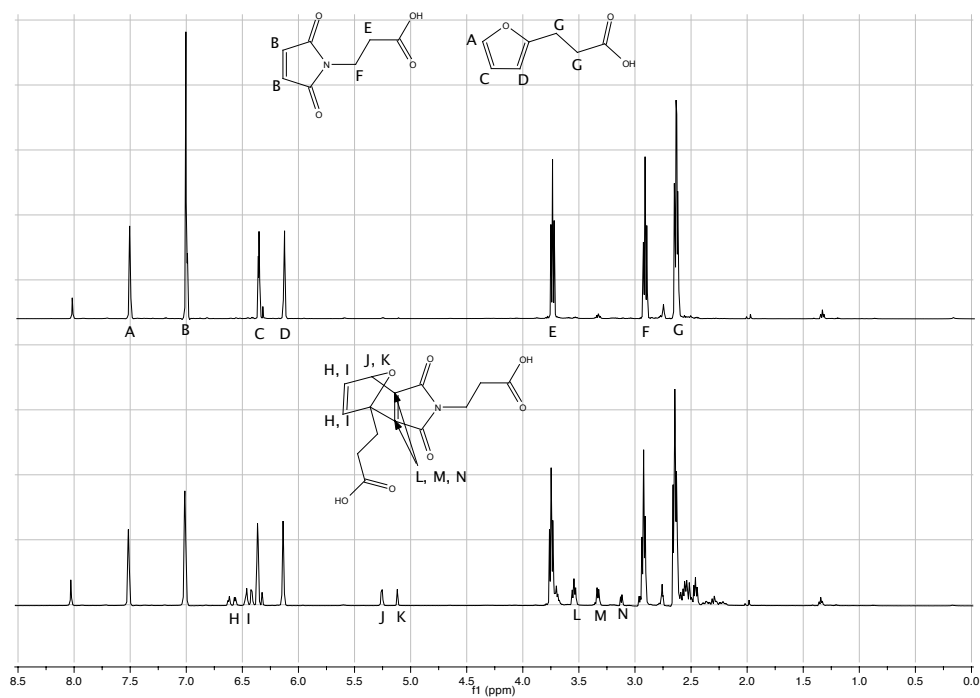


Figure 3.1. 3-(2-furyl propionic) acid Diels-Alder reaction with β -alanine maleimide at 40°C. The top spectrum depicts the start of the reaction whereas the bottom shows the progress after approximately 1 hour. The multiple peaks present in the product are due to the presence of the endo and exo isomers of the Diels-Alder adduct.

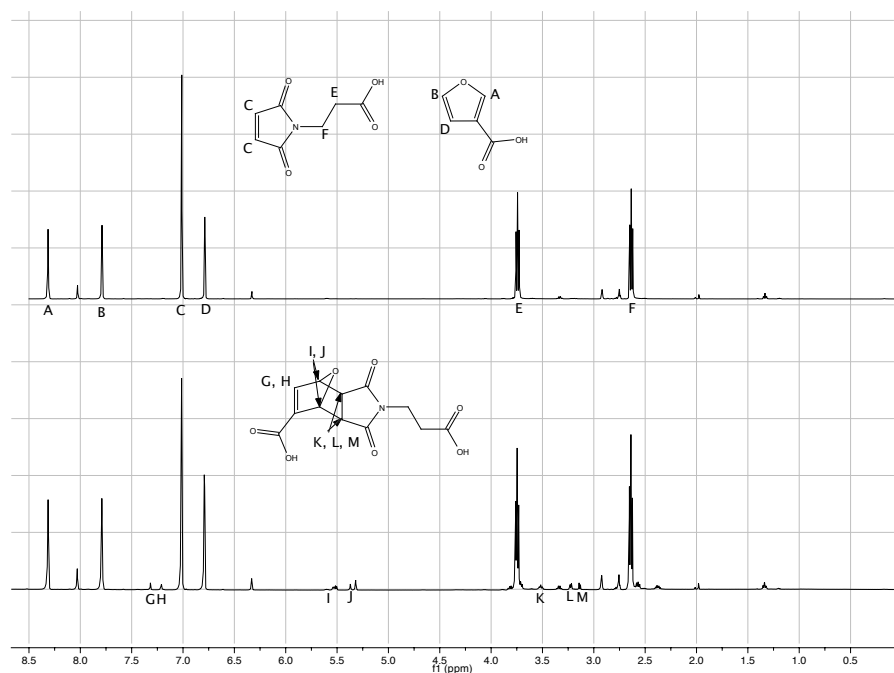


Figure 3.2. 3-furoic acid Diels-Alder reaction with β -alanine maleimide at 40°C. The top spectrum depicts the start of the reaction whereas the bottom shows the progress after approximately 8 hours. The multiple peaks present in the product are due to the presence of the endo and exo isomers of the Diels-Alder adduct.

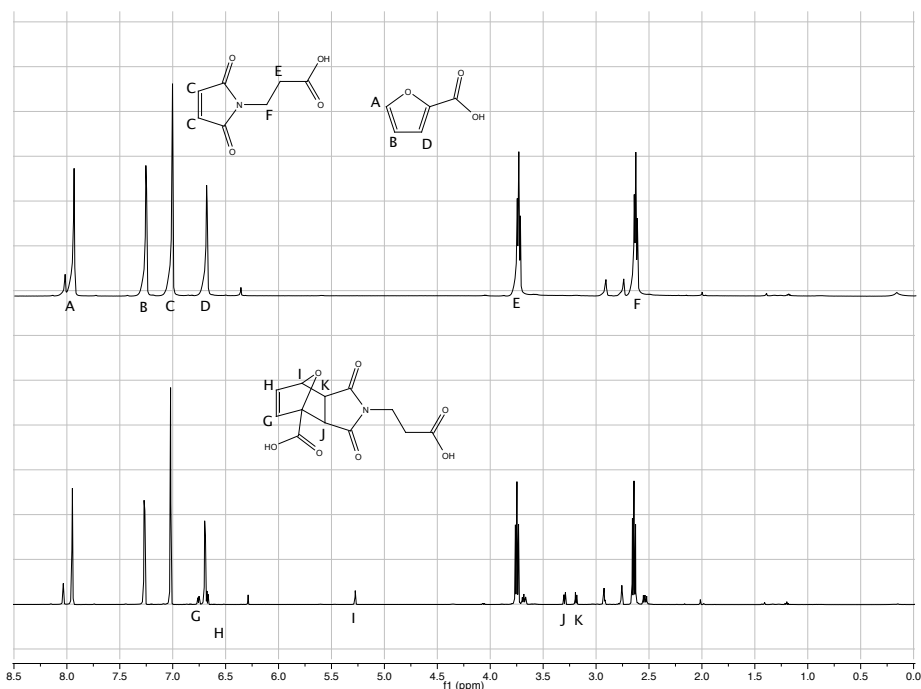


Figure 3.3. 2-furoic acid Diels-Alder reaction with β -alanine maleimide at 40°C. The top spectrum depicts the start of the reaction whereas the bottom shows the progress after approximately 128 hours. Unlike the Diels-Alder reactions presented in Figures 3.1 and 3.2, only one form of the adduct is present. This is most likely due to a combination the diminished reactivity of 2-furoic acid in the Diels-Alder reaction and the length of time the reaction proceeded to form notable adduct quantities.

3.2.4. Analysis

To determine the thermodynamic and kinetic properties, the DA reaction between the various maleimide and furan functionalized molecules (see Scheme 3.1) was monitored as a function of temperature using NMR spectroscopy. NMR spectroscopy enabled the monitoring of maleimide and furan moiety consumption while simultaneously permitting the observation of adduct formation. Each DA reaction studied was monitored via NMR until equilibrium was achieved, allowing for thermodynamic and kinetic parameter determination. A given reaction mixture was considered to have reached equilibrium when the conversion of maleimide and furan remained constant over multiple time points (See Figure 3.4).

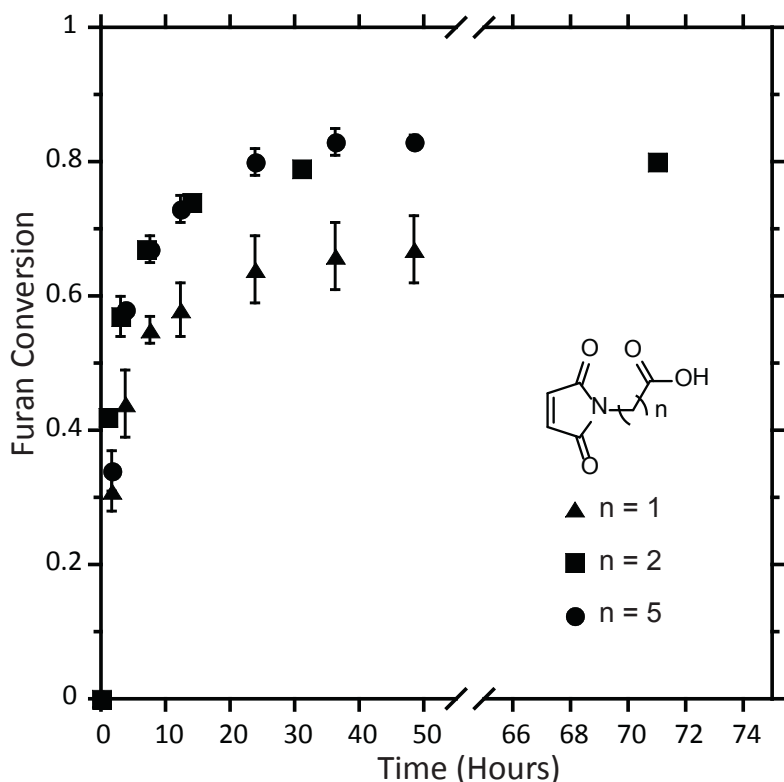


Figure 3.4. Progression of the Diels-Alder reaction between 3-(2-furyl) propionic acid and various maleimides at 60°C.

Once known, the equilibrium conversion in conjunction with the initial reactant concentration is used to calculate the equilibrium constant, K_c , which may be expressed in terms of diene conversion as

$$K_c = \frac{X_{\text{Diene}}}{C_{0,\text{Diene}} (1 - X_{\text{Diene}}) \left(\frac{1}{r} - X_{\text{Diene}} \right)}, \quad (1)$$

where r is the stoichiometric ratio of the initial diene to dienophile concentrations (i.e., $r = C_{0,\text{Diene}}/C_{0,\text{Dienophile}}$, where $r = 1$ since all studies performed herein used a stoichiometric equivalent of furan to maleimide), and X_{Diene} is the equilibrium diene conversion. Calculation of the equilibrium constant at several temperatures provides sufficient data to determine ΔH_{rxn} and ΔS_{rxn} from the Van't Hoff equation,

$$\ln K_c = \frac{\Delta S_{\text{rxn}}}{R} - \frac{\Delta H_{\text{rxn}}}{RT}. \quad (2)$$

Once the equilibrium constant, and thus the ratio of the forward and reverse rate constants, is known, the time dependent diene concentration is used to determine the forward and reverse rate constants. Where the mass balance on dienes is

$$\frac{dD}{dt} = -k_f [D][P] + k_r [A], \quad (3)$$

where D, P, and A denote the diene, dienophile, and adduct concentrations, respectively. As with determination of the thermodynamic properties, NMR spectroscopy allows the simultaneous monitoring of diene and dienophile functional group conversion as well as the adduct formation over time. To determine the individual rate constants, the integral form of equation (3), which is presented by equation (4), was used to obtain a best fit of the data over the experimental time range.

$$t_2 - t_1 = \frac{1}{k_r \sqrt{4F_0K + 1}} \ln \left(\frac{\sqrt{4F_0K + 1} + 2F_0(x_2 - 1) - 1}{\sqrt{4F_0K + 1} - 2F_0(x_2 - 1) - 1} \cdot \frac{\sqrt{4F_0K + 1} + 2F_0(x_1 - 1) - 1}{\sqrt{4F_0K + 1} - 2F_0(x_1 - 1) - 1} \right) \quad (4)$$

3.3. Results and Discussion

3.3.1. Thermodynamic Experiments

A linear regression of the natural logarithm of K versus 1/T, as depicted in an Arrhenius plot (for example, see Figure 3.5), yielded both the enthalpy and entropy of reaction from the slope and intercept, respectively, via the Van't Hoff equation (2), for each of the maleimide-furan combinations studied here.

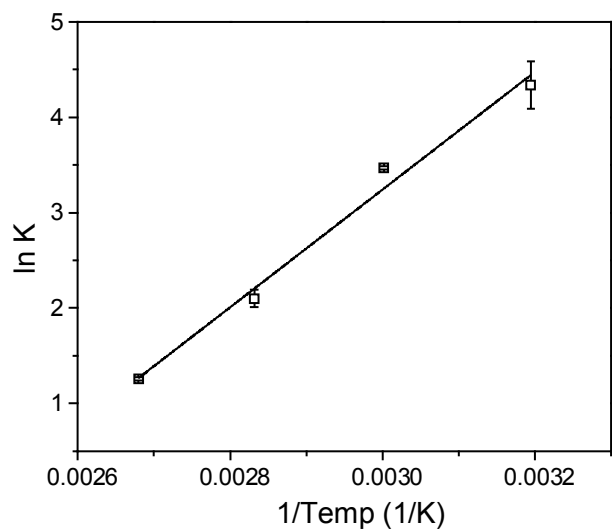


Figure 3.5. Arrhenius plot for the reaction of 3-(2-furyl) propionic acid with β -alanine maleimide acid allowing for determination of ΔH_{rxn} and ΔS_{rxn} from the slope and intercept, respectively.

The reaction entropies and enthalpies for the reaction between the carboxylic acid functionalized maleimide and furan are presented in Table 3.1 and 3.2. Using the Van't Hoff equation, this data is used to calculate the equilibrium conversion for a given furan-maleimide pair throughout the temperature range studied (40 to 100°C). The ability to predict the temperature dependent equilibrium conversion is critical in the design of DA-based materials. For example, these thermodynamic parameters enable the prediction of temperature-dependent phenomena such as the gel point conversion and crosslink density in thermoreversible covalent adaptable networks.¹⁵

Table 3.1. Summarized Furan and Maleimide Carboxylic Acid Enthalpies in DMF (kJ/mol)

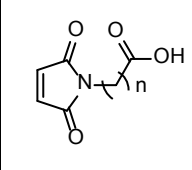
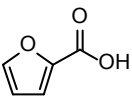
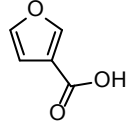
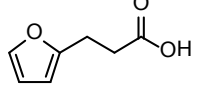
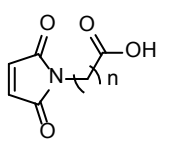
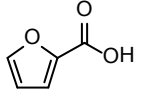
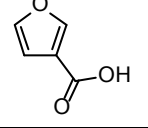
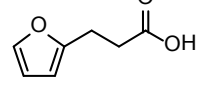
Dienophile		<i>n</i>		
		1	2	5
Diene		-48±3	-42±6	-43±2
		-34±6	-53±2	-56±2
		-59±2	-51±3	-40±3

Table 3.2. Summarized Furan and Maleimide Carboxylic Acid Entropies in DMF (J/mol-K)

Dienophile		<i>n</i>		
		1	2	5
Diene		-167±6	-150±20	-139±12
		-80±20	-128±7	-138±10
		-154±7	-138±8	-95±8

To emphasize trends in the thermodynamic properties resulting from substitutional changes in the maleimide and furan carboxylic acids, Figure 3.6 presents the equilibrium constants at 80°C for the DA reaction between each of the possible diene and dienophile combinations considered in this study. The 2-furoic acid has the smallest equilibrium constant, regardless of the dienophile and measured temperature, which is attributed to the strong electron

withdrawing capacity of the carbonyl group in the two-position of the furan ring. Delocalization of the electron density of the furan ring to the carbonyl of the carboxylic acid reduces the electron donating potential¹⁷ and retards the DA reaction. Shifting the carboxyl electron-withdrawing group to the three-position (i.e., 3-furoic acid) or adding a hydrocarbon spacer (i.e., 3-(2-furyl propionic acid)), favors the DA adduct given by the higher equilibrium constants.

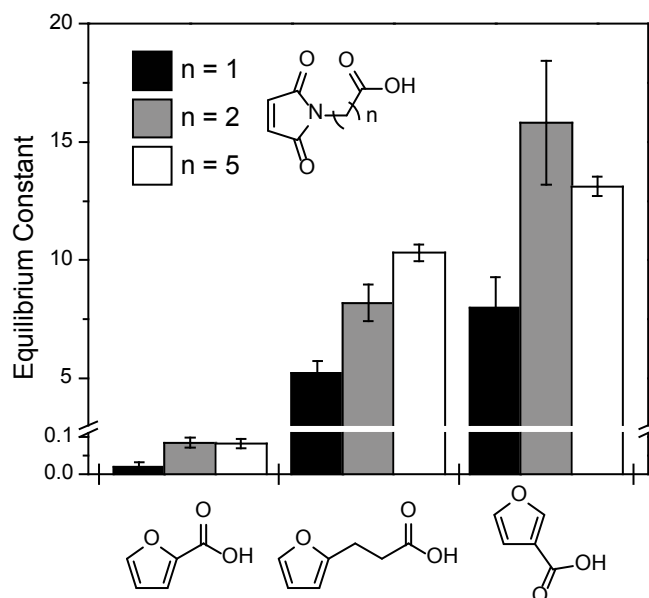


Figure 3.6. Furan and maleimide carboxylic acid equilibrium constants at 80°C in DMF. Each clustered group presents one of the furoic acids considered in the study. Each of the furans was reacted with one of the three maleimides with different hydrocarbon spacers.

In addition to electron withdrawing effects, the side chain can have steric effects that may impact the progress of the reaction. In order for the DA reaction to occur, the furan molecule must be able to form a bridgehead; thus, the rigidity of 2-furoic acid may also contribute to the low equilibrium constant. In contrast, 3-furoic and 3-(2-furyl) propionic acids with increased distance of the carboxyl group from the reactive center would have less steric hindrance in the DA adduct formation.

Changing the hydrocarbon chain length between the carboxylic acid and maleimide ring also had an impact on the equilibrium constant. For each of the furans studied, the equilibrium constant increased with increasing hydrocarbon chain length. A larger increase in equilibrium conversion was observed when the spacer was increased from 1 to 2 atoms than from 2 to 5. This trend either owes to unfavorable steric *intermolecular* interactions with the furan or *intramolecular* interactions that stabilize the maleimide. With a carbon spacer of $n = 1$, the maleimide species has the capacity to form hydrogen bonds between the carboxylic acid hydrogen and a carbonyl oxygen on the maleimide heterocycle, resulting in a seven-membered ring configuration. When the carbon spacer length increases to 2 or 5, a less stable eight or eleven-membered ring would be necessary to achieve this intramolecular arrangement.

An interesting observation is that the solvent plays a considerable role in the equilibrium conversion of the DA reaction.^{14,18,19} While we observed a very low equilibrium constant for the reaction between 2-furoic acid and β -alanine maleimide in DMF (Figure 3.6), the equilibrium constant in water was at least two orders of magnitude greater. As shown in Table 3.3 and 3.4, the water has a significant effect on the entropy of reaction.

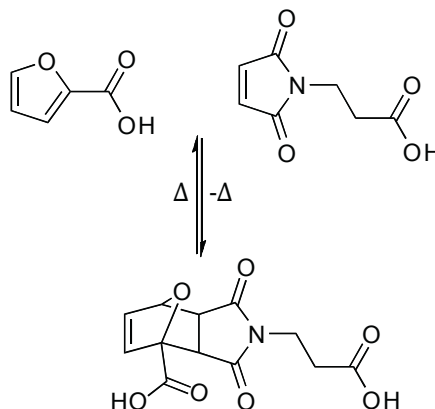
Tables 3.3 and 3.4: Solvent Comparison of Thermodynamic Parameters- Reaction of 2-Furoic Acid with β -Alanine Maleimide. The top panel (Table 3.3) presents the experimentally determined thermodynamic parameters of the DA reaction while the bottom (Table 3.4) illustrates the predicted equilibrium constants at 25 and 80°C using the empirical parameters. The reaction scheme is presented to the right of the two tables.

Table 3.3: Thermodynamic Data Comparison

	ΔH (kJ/mol)	ΔS (J/mol*K)
DMF	-42 \pm 8	-160 \pm 20
Water	-41 \pm 3	-110 \pm 10

Table 3.4: Equilibrium Constant Comparison

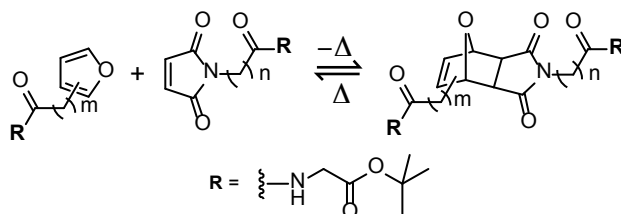
Temperature (°C)	Predicted Equilibrium Constant	
	DMF	Water
25	0.10	27
80	0.01	2.1



This increase in reaction entropy most likely stems from hydrophobic interaction between the solvent and reactants- the diene and dienophile. To minimize free energy, the solvent/reactant system arranges itself such that hydrophobic components are in close proximity to other hydrophobic entities and similarly with hydrophilic groups. While the diene and dienophile considered in this study possess hydrophobic character, the carboxylic acid moiety promotes hydrogen bonding. Hydrogen bonding most likely enhances the electron donating capacity of the diene, which may also explain the increase in conversion. Water hydrogen bonds well with the carbonyl and hydroxyl groups of a carboxylic acid such as those present in the furans studied. Hydrogen bonding to the carbonyl oxygen of the carboxylic acid likely delocalizes the extended conjugation of the furan ring and the carbonyl, which for a compound like 2-furoic acid enables the electrons of the furan ring to participate more readily in the DA reaction. The conversion for the reaction 3-(2-furyl) propionic acid with β -alanine maleimide

also increased in water; however it achieves nearly complete conversion for each temperature, making the determination of thermodynamic parameters impractical using this method. An irreversible side reaction was found to dominate the study concerning 3-Furoic Acid and β -Alanine maleimide (See Section 3.3).

In a large number of materials applications, the DA species are incorporated by covalently coupling these reactive functional groups into the material. As stated above, the carboxylic acid functional group is a convenient handle as it is readily coupled to amine functional groups to form an amide bond, which are found in a large set of commercially available materials, including proteins and DNA. As a result, we also explored the thermodynamic and kinetic differences between coupled and uncoupled furan and maleimide functional groups. With the exception of 2-furoic acid, which as noted earlier does not participate extensively in the DA reaction, the maleimides and furans were coupled to the amine of t-butyl glycine. Upon their incorporation onto t-butyl glycine, the diene and dienophile (Scheme 3.5) were permitted to react at various temperatures in DMF allowing for the analysis of the thermodynamic properties presented in Figure 3.7.



Scheme 3.5. Summarized t-Butyl Glycine Coupled Diels-Alder Reaction

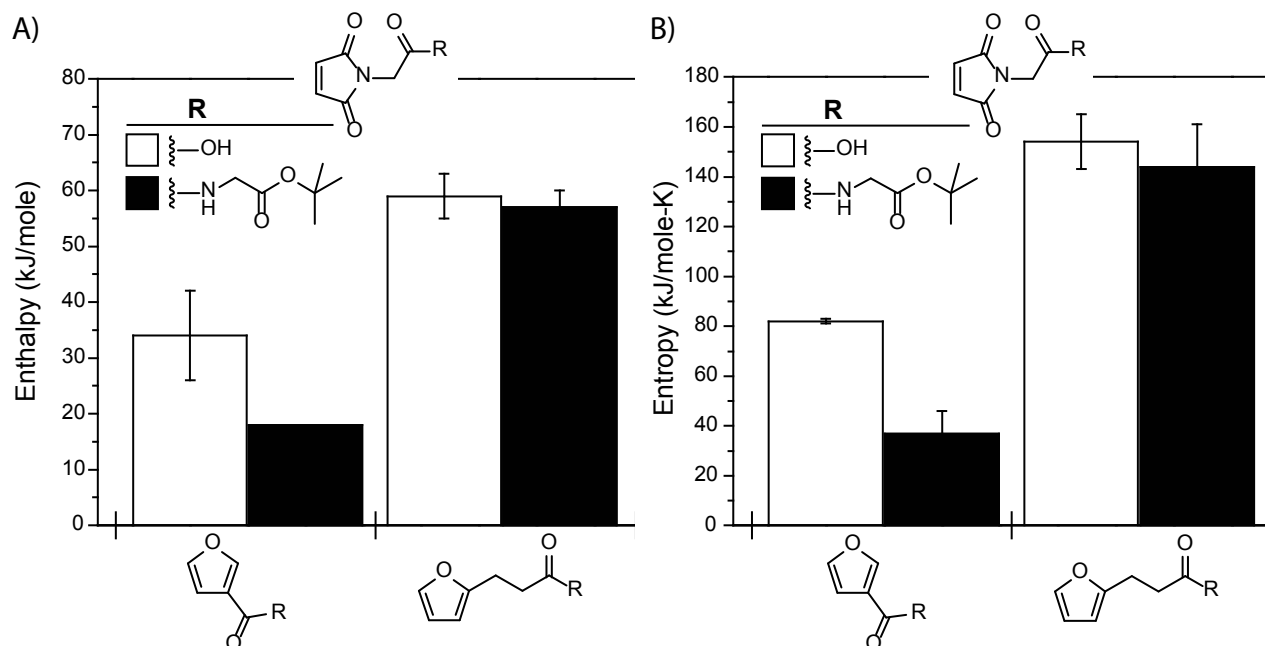


Figure 3.7. Summarized t-butyl glycine coupled furan and maleimide enthalpies (A) and entropies (B) in DMF (kJ/mole) compared to their carboxylic acid equivalents for the case where the maleimide spacer, n , equals 1. The side-by-side bars show the uncoupled (carboxylic acid, open bars) and coupled (amide, dark bars) species (left to right, respectively).

Maleimides with a hydrocarbon spacer two carbon units in length exhibited similar entropy and enthalpy values for both coupled and non-coupled carboxylic acids (See Figure 3.8).

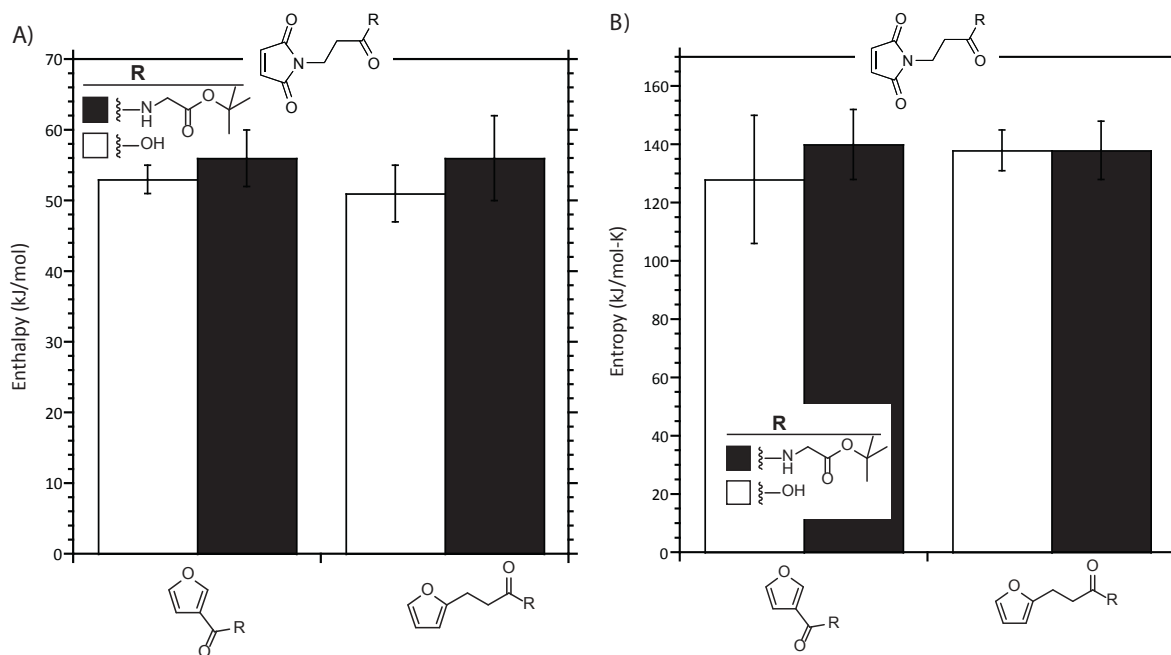


Figure 3.8. Summarized t-butyl glycine coupled furan and maleimide enthalpies (A) and entropies (B) in DMF (kJ/mole) compared to their carboxylic acid equivalents for the case where the maleimide spacer, n , equals 2. The side-by-side bars show the uncoupled (carboxylic acid, open bars) and coupled (amide, dark bars) species (left to right, respectively).

Likewise, when the furan hydrocarbon spacer was increased beyond one, amide coupling had a negligible effect on the entropy and enthalpy (right bars in Figure 3.8a and b). In contrast, the entropies and enthalpies of maleimides with a single carbon spacer were significantly different when an amide was coupled to the 3-furoic acid (left bars in Figure 3.8a and b). As the carbonyl in a three-substituted furan is in resonance within the furan ring, it is perhaps not surprising that a change in electronegativity (amide versus carboxylic acid) has a significant effect on the equilibrium conversion. For example, the enthalpy and entropy found for the 3-furoic acid were -53 ± 2 kJ/mole and -128 ± 7 J/mol-K, respectively, compared to -18 ± 0 kJ/mole and -37 ± 1 J/mol-K for their glycine coupled equivalents. The more positive enthalpy and entropy illustrate that the electron withdrawing group and its proximity to the diene or dienophile significantly influence

the DA reaction and the properties of materials that incorporate these moieties. With the electron-withdrawing group far enough removed from the diene and/or dienophile functionalities, the furan and maleimide exhibit similar thermodynamic properties.

3.3.2. Kinetic Experiments.

While the thermodynamic parameters provide information about the maximum extent of reaction, the reaction rate is often critical in employing DA-based synthetic strategies or designing DA-based materials. For example, the healing kinetics of a covalent adaptable network must be on a time-scale dictated by the application and the rheological behavior of polymer networks that incorporate DA structures is directly controlled by the thermoreversion kinetics.⁶ Here, rate constants were determined as a function of temperature using equation (3), as discussed above, which enabled the determination of the activation energy and pre-exponential terms via an Arrhenius plot (see Table 3.5 and Table 3.6). Expressing the reaction kinetics in terms of activation energy and a pre-exponential term enables the rate constants to be estimated over the entire experimental range of temperatures. The ability to estimate the rate at which adducts form or revert back to the diene and dienophile will facilitate the *a priori* design for new materials¹⁵. In particular, knowing these rates will be of great benefit when designing a mendable material or a protecting group as this determines the rate of material healing and reaction time, respectively.

Table 3.5: Kinetic Parameters Associated with Adduct Formation (Forward Reaction)

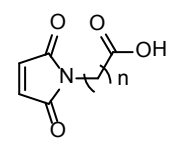
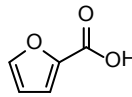
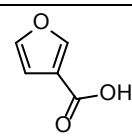
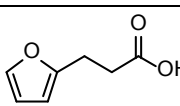
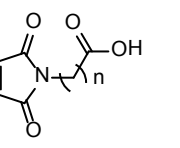
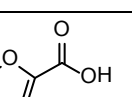
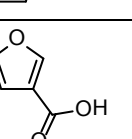
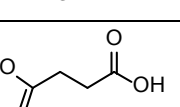
Acid Dienophile		Activation Energies (kJ/mol)			Frequency Factor (Hour ⁻¹)		
		n			n		
		1	2	5	1	2	5
Acid Diene		69±7	24±11	57±6	(4±7)x10 ⁸	30±70	(1±1)x10 ⁷
		55±13	69±3	55±5	(2±4)x10 ⁷	(6±3)x10 ⁹	(4±4)x10 ⁷
		44±6	23±7	60±4	(3±4)x10 ⁶	(3±6)x10 ⁵	(1±1)x10 ⁹

Table 3.6: Kinetic Parameters Associated with Adduct Degradation (Reverse Reaction)

Acid Dienophile		Activation Energies (kJ/mol)			Frequency Factor (Hour ⁻¹)		
		n			n		
		1	2	5	1	2	5
Acid Diene		115±11	62±28	99±7	(1±4)x10 ¹⁷	(3±20)x10 ⁸	(2±3)x10 ¹⁴
		89±6	123±2	110±6	(3±4)x10 ¹¹	(1.8±0.6)x10 ¹⁷	(4±4)x10 ¹⁴
		107±7	74±4	95±4	(8±10)x10 ¹⁴	(1±1)x10 ¹⁰	(1±1)x10 ¹³

To visualize better the trends in the data, the reverse reaction rate constant at 80°C is shown in Figure 3.9. The hydrocarbon spacer in the maleimide has a similar effect on the DA equilibrium constant as the reaction rate. Specifically, once the hydrocarbon spacer is greater

than one carbon, the reaction rate is dictated by the furan structure. In a trend opposite to that observed with the equilibrium constant, as the carboxylic acid is shifted from the two- to three-position on the furan ring, there is a significant decrease in the reaction rate (i.e., the equilibrium constant was largest for the 3-furoic acid whereas 3-furoic acid had the slowest reverse rate constant). While this behavior is at least partially explained by the differences in the position of the carbonyl electron-withdrawing group, steric hindrance may also play a significant role. Thus, the location of the electron-withdrawing group is more significant to the reverse reaction rate than any benefits resulting from the distance between the ring to the carboxylic acid.

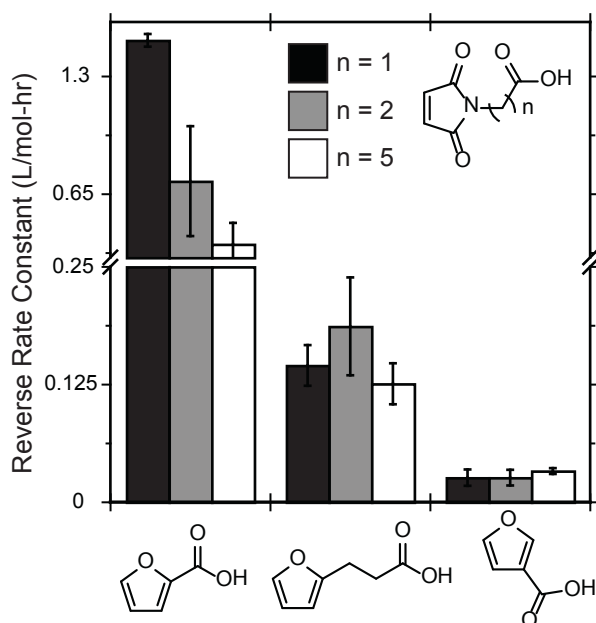


Figure 3.9. The reverse reaction rate constant at 80°C for the Diels-Alder reaction of maleimide and furan carboxylic acids possessing various substitutional positions.

As observed with the equilibrium constant in the previous section, the solvent has a significant effect on the reaction rate. When water was utilized as the solvent, the forward reaction rate was seen to increase markedly. For instance, the forward reaction rate constant for

the 2-furoic acid with β -alanine maleimide in water was found to be $0.36 \pm 0.02 \text{ L}/(\text{mole}\cdot\text{hour})$ compared to a forward rate constant of $0.002 \pm 0.001 \text{ L}/(\text{mole}\cdot\text{hour})$ for the same reaction in DMF. As stated earlier, water has the capacity to hydrogen bond with the carbonyl oxygen, which could potentially make 2-Furoic Acid a more reactive diene in the DA reaction. Additionally, hydrophobic effects also have the potential to bring the non-polar diene and dienophiles into close proximity to one another in a hydrophilic environment, which could allow for a greater number of collisions and therefore chances to form the adduct.

The reverse kinetic parameters associated with furan and maleimide coupled to another species (glycine) compared to their carboxylic acid equivalents were also considered. It was found that the reverse rate constants for the carboxylic acids were very similar to their glycine coupled equivalents (See Figure 3.10). When the carbon chain length on the maleimide was increased, the trend of similar reverse rate constants in the carboxylic acids compared to the glycine-coupled counterparts held true (See Figure 3.10b).

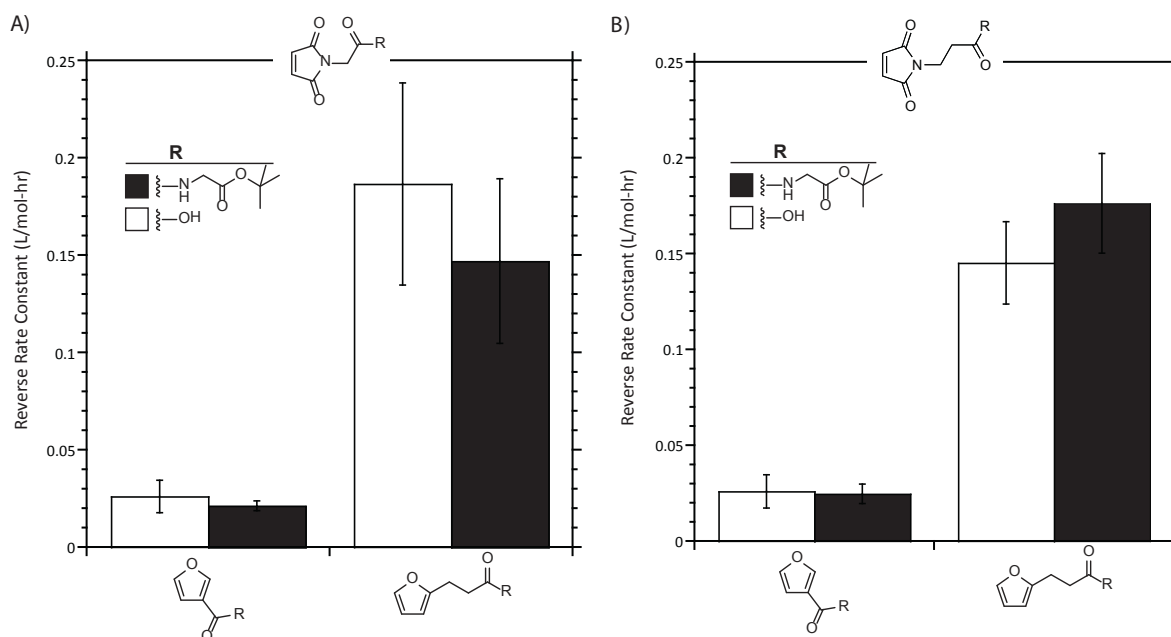


Figure 3.10. Comparison of the Reverse Rate Constant at 80°C for Carboxylic Acids and their Coupled Glycine Equivalents. The plot on the left (A) depicts the reverse rate constant for the different furan moieties in carboxylic acid and glycine coupled form reacted with maleimide having an aliphatic spacer (See Scheme 6) of 1. The right hand side (B) illustrates the same reaction of the same furan moieties with a maleimide possessing a side chain with a spacer of two.

3.3.3. Side Reactions

Monitoring the reactions considered in this study via NMR in lieu of other techniques, such as Fourier Transform Infrared Spectroscopy (FTIR), allowed the detection of the DA adduct as well as the formation of other products. The reactions involving 3-furoic acid and 3-furan functionalized t-butyl glycine were observed to undergo an irreversible side reaction. Though the DA adduct was still the dominant product, the peaks of the new material were present in the NMR spectrum. This side reaction is facilitated by the presence of the maleimide; since it does not occur in its absence, and may occur in adduct form. Figure 3.11 depicts the structure of a 3-furoic acid and 2-furoic acid with β -alanine maleimide in the adduct form. One possible explanation is that the 3-furoic acid forms a conjugated electron poor vinyl bond, which may be susceptible to nucleophilic attack.

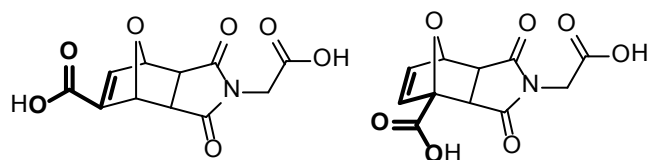


Figure 3.11. Adducts of 3-furoic acid and 2-furoic acid with β -alanine maleimide. The structure on the left, an adduct with 3-furoic acid, possesses a conjugated electron poor vinyl functional group (in bold) while the structure on the right does not.

In addition, a separate side reaction was found to occur when the reaction was examined in water. This side reaction was observed to occur with all diene and dienophile pairs at higher temperature. A control study was conducted where the dienes and dienophiles were prepared separately in D₂O and placed in separate NMR tubes. The study revealed that the furan alone did not undergo a side reaction. NMR of the maleimide dienophiles, however, illustrated the formation of a new compound. HNMR spectra are presented in the supplemental information for 3-furoic acid and β -alanine maleimide carboxylic acids illustrating their stability, or lack thereof, upon exposure to water at elevated temperatures.

This side reaction has also been reported in the literature where maleimide has been shown to hydrolyze (ring open) when subjected to basic conditions²⁰. An analogous mechanism whereby the maleimide hydrolyzes in acidic environments is also a potential pathway.

3.4. Conclusions

By systematically varying the substitutional position of carboxylic acid moieties on furan and maleimide molecules, this study has presented thermodynamic and kinetic data that facilitate the selection and implementation of furan and maleimide pairs for various polymeric materials applications. Ultimately, selection of a substituent and a hydrocarbon spacer on the furan and maleimide depends on the final target application and the specific requirements of that application. An abridged list of criteria that may come into play when selecting the appropriate furan and maleimide includes: reversibility of the DA reaction, the desired time scale for the reaction, and the final conversion achieved for the forward and reverse reactions. Given that the thermodynamic and kinetic properties of the carboxylic acid functionalized furan and maleimide molecules are similar once incorporated into other molecules, the data presented in this study grant some guidance for selection of the proper DA pair. For instance, construction of a

mendable polymer material would require thermodynamic properties that maintain a high conversion (high adduct population) at the desired normal operating temperature, providing an acceptable tolerance above and below this point. At the same time, if the material sustains damage, the reaction behavior must also allow reversion back to near complete diene and dienophile species upon heating above some threshold temperature to allow for healing to take place. In addition to these thermodynamic properties, the selected system for a mendable material must also have desirable kinetics; the system must be able to reach its final forward and reverse conversion on a reasonable time scale. In terms of the carboxylic acids dienes and dienophiles considered here, a large ΔH and small ΔS value (both in magnitude, not sign) would be desirable to achieve the desired reaction behavior and enable formation of a mendable material. Additionally, the solvent system selected can also act as a handle to tune the reaction behavior. For instance, aqueous conditions drive the DA reaction to higher conversion faster than if the reaction were carried out in an aprotic solvent like DMF, providing a protection/deprotection strategy based solely on changing solvents.

3.5. Acknowledgments

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3.6. References

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Chapter 4

Development of a Maleimide Amino Acid for use as a Tool for Peptide Conjugation and Modification *

An amino acid possessing a maleimide side chain was developed and synthesized in good yield. With a propensity to undergo the Michael addition reaction, the creation of a maleimide amino acid derivative was targeted for use as a highly functional tool for enabling peptide conjugation and structural modifications. After addressing the inherent potential side reactions of maleimides during solid phase peptide synthesis, the ability to incorporate the maleimide amino acid in an RGDS peptide sequence was demonstrated. ¹H NMR and mass spectroscopic techniques enabled thorough characterization of the peptide sequence, confirming the presence of the maleimide functionality. Once characterized, the ability to use the maleimide moiety as a peptide modification tool was investigated. Specifically, it was shown that the maleimide functional group could be exploited, given the proper reaction conditions, to anchor a peptide to a surface and create a cyclic conformation from a linear sequence. Furthermore, bioactivity of the peptide containing maleimide amino acid was evaluated by studying cellular interactions with surfaces functionalized with an integrin binding sequence.

*Koehler, K.C., Alge, D.L., Anseth, K.S. and Bowman, C.N. *Bioconjugate Chemistry* Submitted

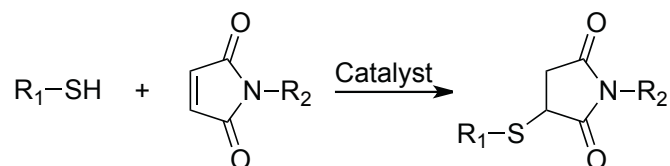
4.1. Introduction

Peptides represent a large potential source for the development of therapeutic compounds and biologically responsive materials. In their native conformation many peptide sequences suffer setbacks, such as low bioavailability, decreased stability and rapid enzymatic breakdown¹, which hinder their use in therapeutic applications. These setbacks can often be mitigated or removed completely by synthetically altering the peptide's sequence or conformation. However, when composed of naturally occurring amino acids, a limited number of functional chemical handles exist, such as amines, thiols and carboxylic acids, to enable the ability to conjugate the sequence to another biomolecule or material and/or change a peptide's conformation. Exploitation of protecting groups commonly used in peptide synthesis and the introduction of synthetic, non-natural residues have increased the number of reactions that are available to facilitate the modification of a given peptide structure.

One approach to increase the viability of incorporating peptides into therapeutic compounds and materials involves the cyclization of a linear sequence to form macrocycles or staples. As compared to their linear counterparts, peptides presented in cyclic or stapled format have been shown to exhibit increased stability, higher binding efficiency and a decreased susceptibility to degradation by enzymatic proteases.¹⁻³ Many strategies, including lactam formation, photochemical techniques⁴⁻⁶, olefin metathesis¹⁻³ and extended native chemical ligation⁷, have been developed relevant to the formation of cyclic and stapled peptides. In addition to conformational modifications, the inclusion of environmentally responsive sequences, such as those that degrade in the presence of matrix metalloproteinases^{8,9}, also facilitates the use of peptides in materials. Responsive sequences have been applied in therapeutic and material's applications ranging from the masking/unmasking of chemical functionalities¹⁰ to the release from a polymeric substrate.¹¹

Some commonly employed chemical moieties for peptide modification include vinyl, amine, thiol, carboxylic acid, azide and alkyne groups. Of these groups, the functionalities that are capable of undergoing what Sharpless termed ‘click’ chemistry, such as the Huisgen 1,3-dipolar cycloaddition reaction between an azide and alkyne, are of particularly great interest. By definition, reactions belonging to the click paradigm attain high yield, form few to no side products, occur at ambient conditions and are easily purified.¹² These reaction criteria make click chemistry an ideal strategy for peptide functionalization, synthesis, and modification.

A prime example of a click reaction that has been used to modify peptides is the thiol-Michael addition reaction. The thiol-Michael addition reaction makes use of a catalyst, typically an organic base or nucleophile¹³, to create a thio-ether bond (See Scheme 4.1).



Scheme 4.1. A typical thiol-Michael addition reaction between a generic thiol and maleimide (alkene) resulting in the formation of a thiol-ether bond. The catalyst used in this reaction is typically either a nucleophile like an alkylphosphine or organic base, such as triethylamine.

In particular, the efficient, fast and highly selective Michael addition reaction between maleimide and thiol moieties has been used extensively in biological applications. Over the years, this specific type of Michael addition reaction has come to be known by a number of different names ranging from bioconjugation to the thiol-based click reaction.^{14, 15} Some examples of the use of the maleimide/thiol Michael addition include protein crosslinking¹⁶, disulfide bridge replacement¹⁷, antibody staining, surface functionalization^{18, 19} and fluorescent labeling of peptides and proteins.²⁰

With regard to peptides, the maleimide functionality is often introduced post-synthetically where it undergoes a Michael addition with a thiol present in the side chain of cysteine residues.^{17, 21} Having undergone the Michael addition reaction, post-synthetic incorporation in this fashion prevents the use of maleimide as an effective handle for incorporation into another material or attachment to a surface. Through the use of a maleimide possessing a carboxylic acid moiety, it has been demonstrated that maleimides are capable of undergoing additional reactions when incorporated on the N-terminus of a peptide¹⁷; however, use of the carboxylic acid to amine coupling technique to incorporate an active maleimide functionality has limitations and drawbacks. One constraint involves the placement of the maleimide functionality. By using a carboxylic acid, incorporation of the maleimide is limited to the N-terminus or an amine containing side chain, such as the ϵ amine of a lysine residue. Placing the maleimide carboxylic acid on a growing peptide sequence acts as a capping agent; further growth from the terminus containing the maleimide moiety is effectively extinguished. Additionally, this technique is susceptible to side reactions between maleimide and an exposed secondary amine or thiol on the peptide competing with the desired carboxylic acid coupling.^{18, 22}

A maleimide-bearing amino acid would address these shortcomings and improve accessibility of the maleimide-thiol click reaction in peptide chemistry. By proper selection of protecting groups on the maleimide moiety, a maleimide amino acid could be used in solid phase peptide synthesis, removing the need for post-synthetic modification. Possessing an amino acid handle not only enables the facile incorporation of maleimide moieties anywhere in the peptide sequence, but is also available for use as a site for further, future reactions such as the formation of macrocycles and tethering to other materials/surfaces

Herein, the development of a novel maleoyl amino acid for use in solid phase peptide synthesis is discussed. Prior to the construction of peptides with the maleoyl amino acid, the prevention of inherent undesired reactions of the maleimide moiety with organic bases during solid phase peptide synthesis is addressed. After resolving side reactions, the ability to place the maleimide moiety anywhere in the peptide sequence *via* this chemistry is demonstrated. In addition, it is shown that the Michael addition between the maleimide substituent on the amino acid allows subsequent peptide modification in two distinct manners: anchoring a peptide to a surface and the formation of an intramolecular cycle from a linear sequence.

4.2. Materials and Methods

4.2.1. Materials

For the synthesis of the target maleoyl amino acid, the compounds that follow were acquired from their respective vendors and, unless otherwise noted, used without further purification. Diisopropylethylamine (DIEA), tert-butyl bromoacetate, N-(9H-fluoren-2-ylmethoxycarbonyloxy)succinimide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) were purchased from Ak Scientific, Inc. (Union City, CA). The solvents used in syntheses or assays, including dichloromethane, methanol, tetrahydrofuran, diethyl ether, toluene and pentane, were bought from Fisher Scientific (Waltham, MA). Additional reagents acquired from Fisher included ethylene diamine, maleic anhydride, β -Alanine, triethylamine, furan, sodium bicarbonate, sodium chloride, sodium sulfate, trifluoroacetic acid (TFA), and 5,5'-dithiobis(2-nitrobenzoic acid). Triisopropylsilane (TIPS) was purchased from Sigma Aldrich (Saint Louis, MO) and used as a radical scavenger. Deuterated dimethylsulfoxide, DMSO-d₆, containing 0.05% v/v TMS internal standard, was acquired from Cambridge Isotope Laboratories (Andover, MA).

4.2.2. Peptide Synthesis

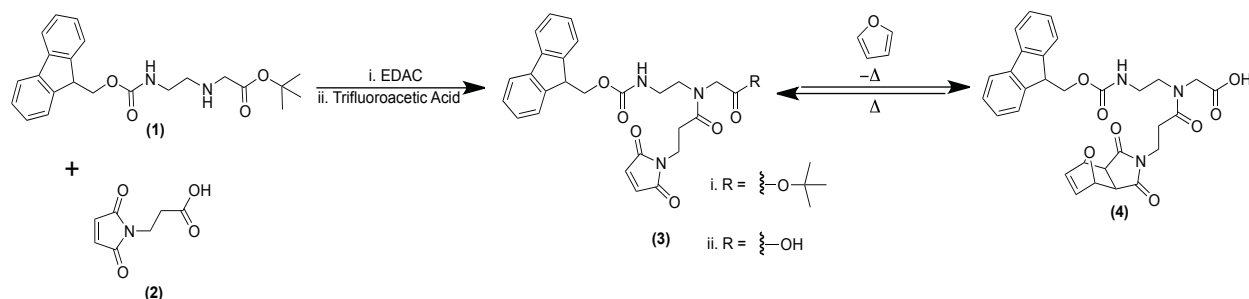
The peptide sequences considered in this study were created using standard Fmoc mediated solid phase synthesis on a Protein Technologies (Tucson, AZ) Tribute Benchtop automated synthesizer. During this study, several different amine functionalized resins were used to synthesis peptides by means of Fmoc-mediated solid phase synthesis. Polystyrene resin (Rink Amide MBHA) with an amine loading ranging from 0.5 to 0.85mmol NH₂/g was purchased from ChemPep, Inc. (Wellington, FL). Resins possessing this range of amine functional loading were primarily used for the synthesis of linear maleimide RGD peptide sequences such as the control RGDS and maleimide RGDS sequences. In an effort to avoid intermolecular reactions, the on-resin formation of macrocycles was performed on ChemMatrix resin, a PEG based substrate. ChemMatrix resin has a lower loading (0.21mmol NH₂/g) and better swelling characteristics as compared with polystyrene resin and was obtained from PCAS Biomatrix, Inc. (Saint-Jean-sur-Richelieu, Quebec).

Amino acids used to construct the peptide sequences considered in this study including N- α -Fmoc-L-phenylalanine, N- α -Fmoc-D-phenylalanine, N- α -Fmoc-O-t-butyl-L-serine, N- α -Fmoc-L-aspartic acid t-butyl ester, N- α -Fmoc-glycine, N- α -Fmoc-N-g-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl-L-arginine and N- α -Fmoc-S-p-methoxytrityl-L-cysteine were acquired from ChemPep Inc. (Wellington, FL). To facilitate the formation peptide bonds, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) purchased from AK Scientific (Palo Alto, CA) was used as an activating agent. Solvents and reagents compatible with solid phase peptide synthesis and the Tribute Automated peptide synthesizer were bought from AB Applied Biosystems (Carlsbad, CA). These included dimethylformamide (DMF), piperidine, n-methylpyrrolidone (NMP), n-methylmorpholine

(NMM), 1,8-Diazabicycloundec-7-ene (DBU) and 2,6-lutidine. Acetic anhydride was acquired from Sigma Aldrich (Saint Louis, MO) and used as a capping reagent to terminate uncoupled peptide chains. The peptides were synthesized on either a 0.25 or 0.5mmol scale using ChemMatrix PEG or Rink Amide MBHA resins respectively. The lower scale (0.25mmol) associated with the ChemMatrix resin was due to the high swelling characteristics of the substrate. A solution consisting of 20% piperidine (v/v) and 2% DBU (v/v) in NMP was added to the resin (2x5minute additions) to remove the Fmoc protecting group. To the deprotected resin, activated Fmoc protected amino acids were added and allowed to react for 35minutes. Activation was accomplished by mixing HATU and NMM with the amino acid in NMP (1:1:2 amino acid:HATU:NMM). Four equivalents of the commercially available amino acid relative to the resin substitution were used in the solid phase peptide synthesis. For the case of the synthetic amino acid (i.e. maleimide AEG), 2 equivalents relative to the resin functionality were used. After the coupling of each residue, unreacted amine sites were capped with a solution of 5% (v/v) acetic anhydride 6% (v/v) 2,6-lutidine in NMP for 10minutes. The final peptide sequence was cleaved from the solid support by the introducing of a solution of TFA, TIPS and H₂O (95:2.5:2.5 v/v) to the resin and allowing the mixture to react for 90minutes.

The peptide sequence was isolated from the cleavage cocktail by three precipitations and centrifugations in ice-cold diethyl ether. The resulting solid peptide was dried in a desiccator over night. The dried peptide was then purified by means of reversed phase high performance liquid chromatography (RP-HPLC) using a Waters Delta Prep 4000 HPLC system and Waters X-Select C-18 column. Purification of the target peptide sequences was accomplished using a linear gradient elution from 5 to 95% v/v acetonitrile in water over 70minutes.

4.2.3. N-maleoyl-beta-alanine tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl) aminoethyl] glycinate synthesis



Scheme 4.2. N-maleoyl-beta-alanine (1) coupling to AEG (2) to form a maleimide functionalized amino acid. i. The coupling of the maleimide functionality to the amino acid backbone, AEG, was facilitated using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) to yield the maleimide t-butyl AEG. ii. Removal of t-butyl protecting group from maleimide AEG to expose a carboxylic acid functionality, making the molecule compatible with standard Fmoc mediated solid phase peptide synthesis. Compound 4 represents the protection/deprotection of the maleimide functionality through a Diels-Alder/retro-Diels-Alder reaction. Lower temperatures, including ambient temperature, favor the forward reaction and the formation of Diels-Alder Adduct. When heated, the retro-Diels-Alder reaction dominates, resulting in the formation of starting material, thereby exposing the maleimide moiety. Furan makes an excellent protecting group in this reaction scheme due to its low boiling point; once the retro-Diels-Alder reaction occurs, furan typically evaporates and is removed from the system.

The formation of an amino acid with a maleimide functionality was accomplished by coupling n-maleoyl-beta-alanine to tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate hydrochloride (AEG), an orthogonally protected amino acid backbone commonly used in the synthesis of peptide nucleic acids.²³ Detailed information pertaining to the synthesis of AEG (1) and n-maleoyl-beta-alanine (2) is provided elsewhere.^{21, 23, 24} To a solution of N-maleoyl-beta-alanine (7.23g, 43mmol) and AEG (8.87g, 21mmol) in 200 mL dichloromethane was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (8.60g, 44mmol) in two equal portions 30 minutes apart (See Scheme 4.2). The reaction mixture was allowed to stir overnight at ambient temperature (approximately 14 hours). The solvent was then removed by

reduced pressure, and the crude product was re-dissolved in diethyl ether (~500mL) and washed with water (3x300mL), saturated sodium bicarbonate (3x500mL) and brine (1x500mL). The product solution was then dried over sodium sulfate, filtered and concentrated under vacuum to afford **(3)** n-maleoyl-beta-alanine tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl) aminoethyl] glycinate (8.00g, 14.61mmol, 71% yield) as a white foam.

1H NMR (300 MHz, DMSO-*d*6): δ 7.89 (*d*, *J* = 7.4, 2H), 7.68 (*dd*, *J* = 3.7, 7.2, 2H), 7.41 (*dd*, *J* = 6.7, 7.4, 2H), 7.33 (*tt*, *J* = 1.4, 7.4, 2H), 7.50 – 7.25 (*broad/overlap*, 1H), 7.01 (*d*, *J* = 1.3, 2H), 4.31 (*t*, *J* = 5.5, 2H), 4.21 (*t*, *J* = 6.7, 1H), 3.95 (*d*, *J* = 47.2, 2H), 3.68 – 3.52 (*m*, 2H), 3.11 (*dd*, *J* = 6.4, 13.3, 2H), 2.72 – 2.59 (*m*, 2H), 2.45 (*d*, *J* = 7.5, 2H), 1.40 (*d*, *J* = 8.5, 9H).

4.2.4. N-maleoyl-beta-alanine n-[2-(n-9-fluorenylmethoxycarbonyl) aminoethyl] glycine Synthesis

To make the maleimide t-butyl AEG compatible with standard Fmoc solid phase peptide synthesis, removal of the t-butyl protecting group masking the carboxylic acid was required (See Scheme 4.2). To a solution of **3**, maleimide t-butyl AEG (0.55g, 1.0 mmol) in approximately 20 mL of dichloromethane, was added to trifluoroacetic acid (30g, 260mmol) and allowed to react at ambient temperature for approximately 4 hours. The resulting yellow to brown solution was concentrated in vacuo, producing a dark oil. The oil was dissolved into a minimal amount of dichloromethane. Addition of the dichloromethane solution to a mixture of diethyl ether and n-pentane (1:4 v/v) resulted in a white precipitate. Centrifugation, decanting of the supernate and removal of excess solvent by vacuum yielded the target product **4** as a white solid (0.37g, 0.75 mmol, 75% yield).

¹H NMR (300 MHz, DMSO-d₆): δ 7.89 (d, J = 7.3, 2H), 7.68 (dd, J = 3.6, 7.2, 2H), 7.41 (dd, J = 6.7, 7.4, 2H), 7.33 (ddd, J = 1.4, 4.4, 7.4, 2H), 7.01 (d, J = 6.3, 2H), 4.30 (t, J = 5.8, 2H), 4.22 (d, J = 6.6, 1H), 3.99 (d, J = 41.4, 2H), 3.66 – 3.52 (m, 2H), 3.35 (dd, J = 8.6, 15.6, 2H), 3.22 – 3.05 (m, 2H), 2.71 – 2.42 (m, 2H).

4.2.5. Diels-Alder Protection of N-maleoyl-beta-alanine n-[2-(n-9-fluorenylmethoxycarbonyl) aminoethyl] glycine

To a stirred vial of furan (5g, 73mmol), **4** (0.25g, 0.51mmol), was taken into solution. The mixture was allowed to react for approximately 12 hours, during which time an off-white to brown precipitate formed. Following the reaction period, excess furan was removed by means of rotary evaporation yielding an endo and exo mixture of **5** as an off-white to brown solid which was directly used in peptide synthesis.

4.2.6 Thiolated Surface Fabrication.

Thiol functionalized surfaces used in this investigation for surface modification or anchoring of hydrogels were created by modifying standard pre-cleaned Corning glass microscope slides (Corning, NY). Prior to modification, the glass slides were first passed several times per side through a flame to further clean the surface. While the slides were cooling, a solution of the silating agent, 3-mercaptoptrimethoxysilane (Gelest, Inc. - Morrisville, PA) was prepared. An approximately 30mM solution of 3-mercaptoptrimethoxysilane was made by adding the silating agent to 190 proof ethyl alcohol (Decon Laboratories, Inc. – King of Prussia, PA) adjusted to a pH between 4.5 and 5.5 with glacial acetic acid (Mallinckrodt Chemicals – Phillipsburg, NJ). After allowing the silating solution to react for five minutes it was applied directly to the glass slides, completely covering the surface. The solution was left on the slides

for approximately 2 to 3 minutes, after which time the slides were rinsed in fresh 190 proof ethyl alcohol and dried in an 80°C oven for at least 15 minutes.

4.2.7. Maleimide Thiol Michael Addition Hydrogel Formation

To consider the bioactivity of the maleimide RGD sequence, polyethylene glycol (PEG) hydrogels were constructed that allowed for the covalent incorporation of the maleimide RGD peptide. To accomplish this, maleimide and thiol functionalized PEG macromers, acquired from Laysan Bio Inc. (Arab, AL), were used to create a polymer network by means of a Michael addition reaction. Figure S8 presents the 10k molecular weight maleimide and thiol macromer structures.

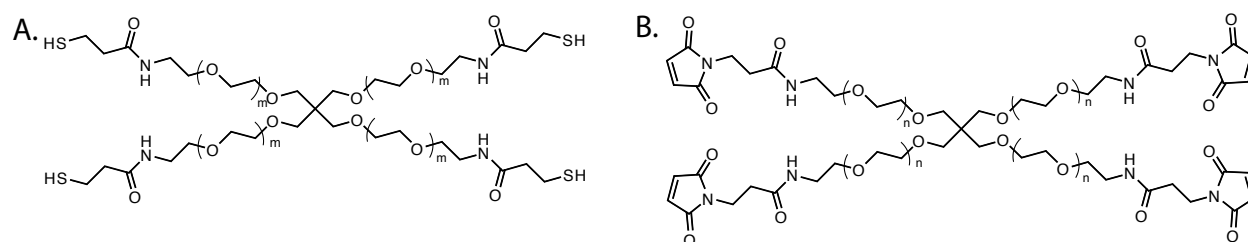


Figure 4.1. Polyethylene glycol thiol (A) and maleimide (B) macromer structures. These monomers were used to create hydrogels by means of a Michael addition reaction. Conducting the polymerization in an off stoichiometric manner allowed for the incorporation of either a cysteine or maleimide RGDS sequence.

By reacting the macromers in an off stoichiometric ratio, it was possible to covalently incorporate the maleimide RGD sequence into the polymer network. In this study, three gel formulations were considered: a negative control without RGD incorporated into the network, a positive control with a cysteine RGD sequence attached to the gel and the test case where the maleimide RGD is incorporated in the polymer.

Stock solutions (20 weight percent) of maleimide and thiol macromers in phosphate buffered saline (PBS) solution adjusted to a pH of approximately 2 were made. The lower pH

decreased the rate of the Michael addition polymerization and allowed time to mix the two macromer solutions. Aliquots of the two stock solutions were mixed with additional PBS buffer in a ratio to yield a 30 μ L gel. When RGDS was incorporated into the network, the additional PBS buffer contained approximately 2mM of either the cysteine or maleimide RGDS. The PEG macromer mixture was transferred via pipette to a thiol functionalized glass cover slide. During the polymerization, the thiolated surface enabled the hydrogel to covalently attach, which anchored the gel to the cover slide and helped to restrict movement of the material (See Section 4.2.6 for additional information on the fabrication of the thiol surface.). Approximately 3 μ L of 300mM triethanol amine was added to the macromixture to raise the pH and thereby incite the Michael addition polymerization. Immediately after the addition of the base catalyst, a Sigmacote coated cover slide was placed on top of the polymerizing material to create a flat, uniform surface for the seeding of cells.

After approximately 15minutes, the Sigmacote coated glass cover slides were removed, resulting in a PEG hydrogel tethered to a glass surface. Once fabricated, the gels were allowed to swell in PBS buffer overnight. To sterilize the gels and ready them for cell seeding, isopropanol was added to the PBS buffer for one hour. Following through washing with sterile PBS buffer, NIH 3T3 fibroblast cells were seeded on top of the hydrogels.

4.2.8. Linear Peptide Sequence Characterization

Prior to incorporating the synthetic maleimide amino acid residue in a peptide, work was done to thoroughly characterize the RGDS sequence into which it would be incorporated. To this end, an RGD sequence was constructed by means of solid phase peptide synthesis and analyzed with matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry and proton nuclear magnetic resonance (¹HNMR) spectroscopy. Figures 4.2 and 4.3 below depict

the MALDI spectrum and fully characterized NMR spectrum respectively. A 2D-COSY spectra (not shown) aided in the peak assignment of the one-dimensional proton spectra.

The complete analysis of a RGD sequence greatly facilitated the validation of the inclusion of the synthetic maleimide residue into an RGD peptide. The MALDI spectrum is presented in Figure 4.2, confirming the synthesis of a peptide bearing the correct molecular weight. Figure 4.3 presents the NMR spectrum of the maleimide RGD sequence with complete peak assignment. The complete assignment of the protons confirms the successful synthesis of the maleimide RGD peptide.

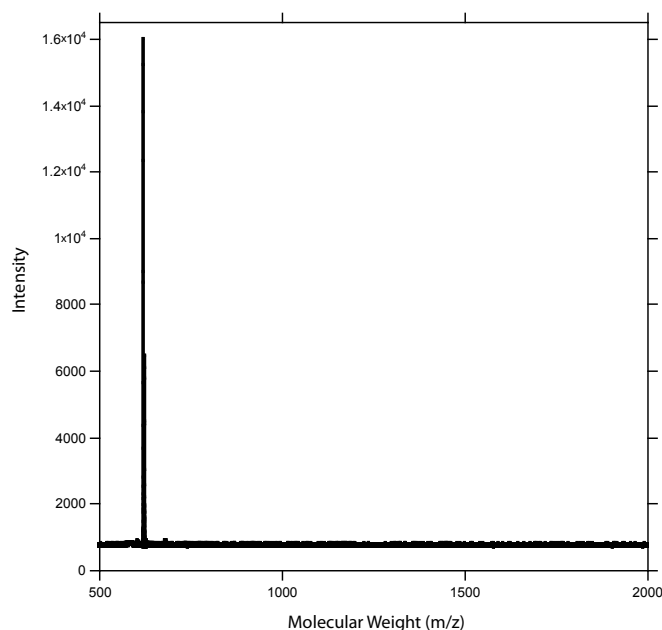


Figure 4.2. MALDI spectrum confirming the correct molecular weight of the RGDS sequence synthesized. Target molecular weight: $[M-H^+] = 621$.

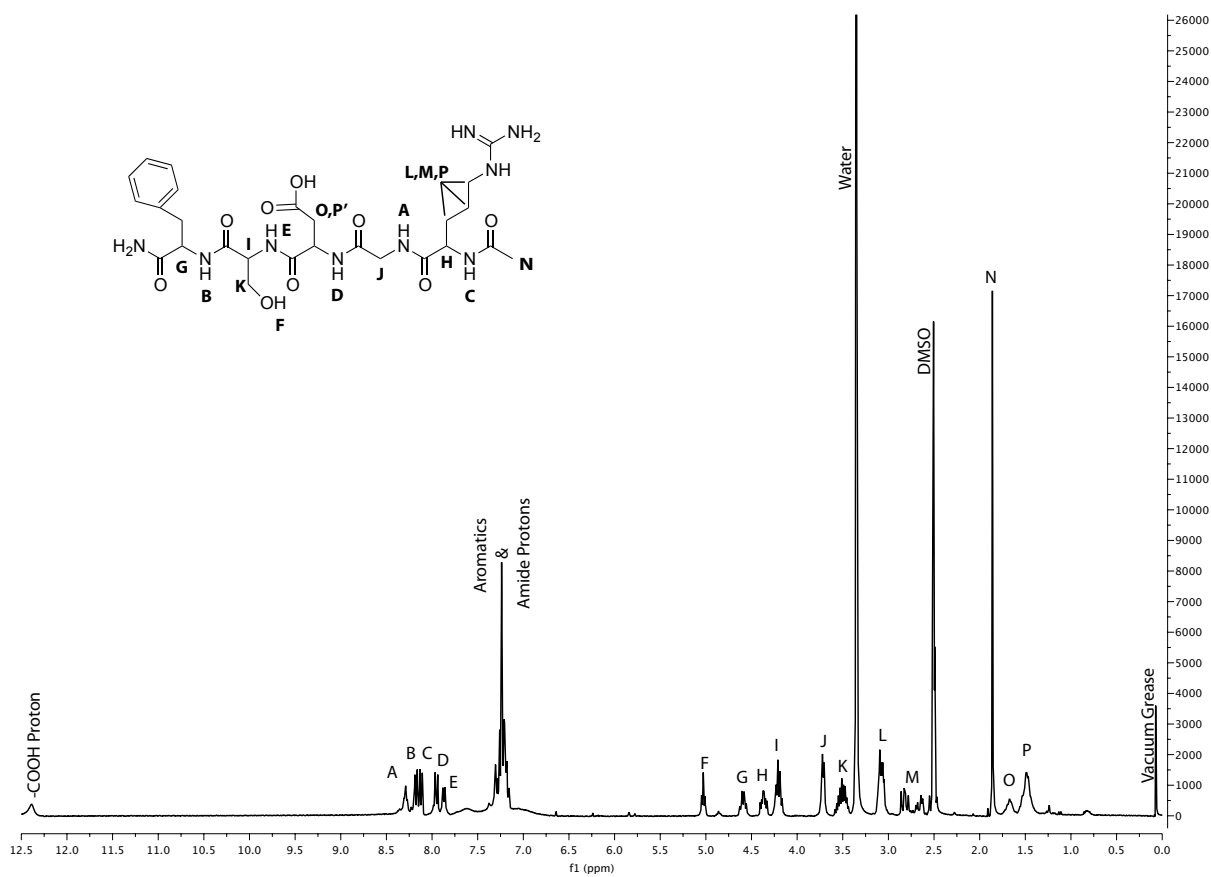


Figure 4.3. Labeled ¹H NMR spectrum of RGDS peptide sequence in DMSO-d₆.

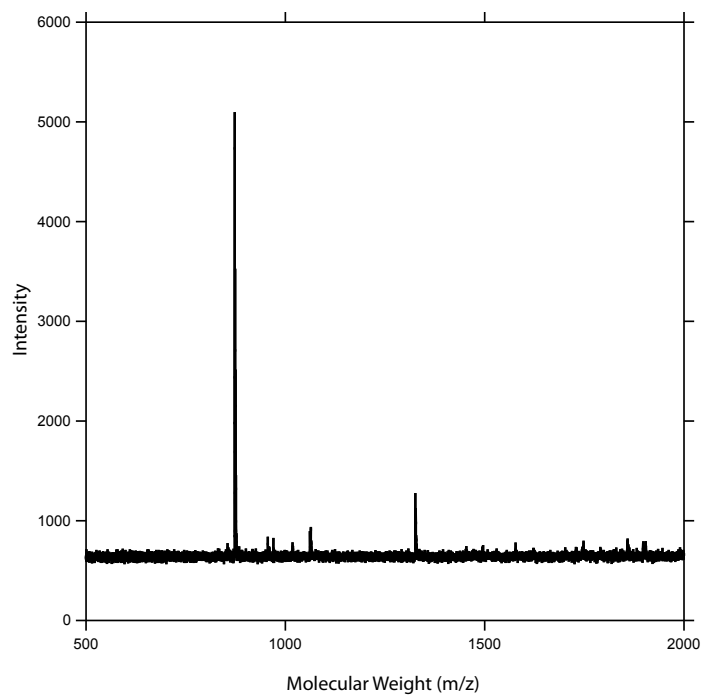


Figure 4.4. MALDI mass spectrum confirming the molecular weight of the target maleimide RGDS sequence at $[M-H]^+ = 873$.

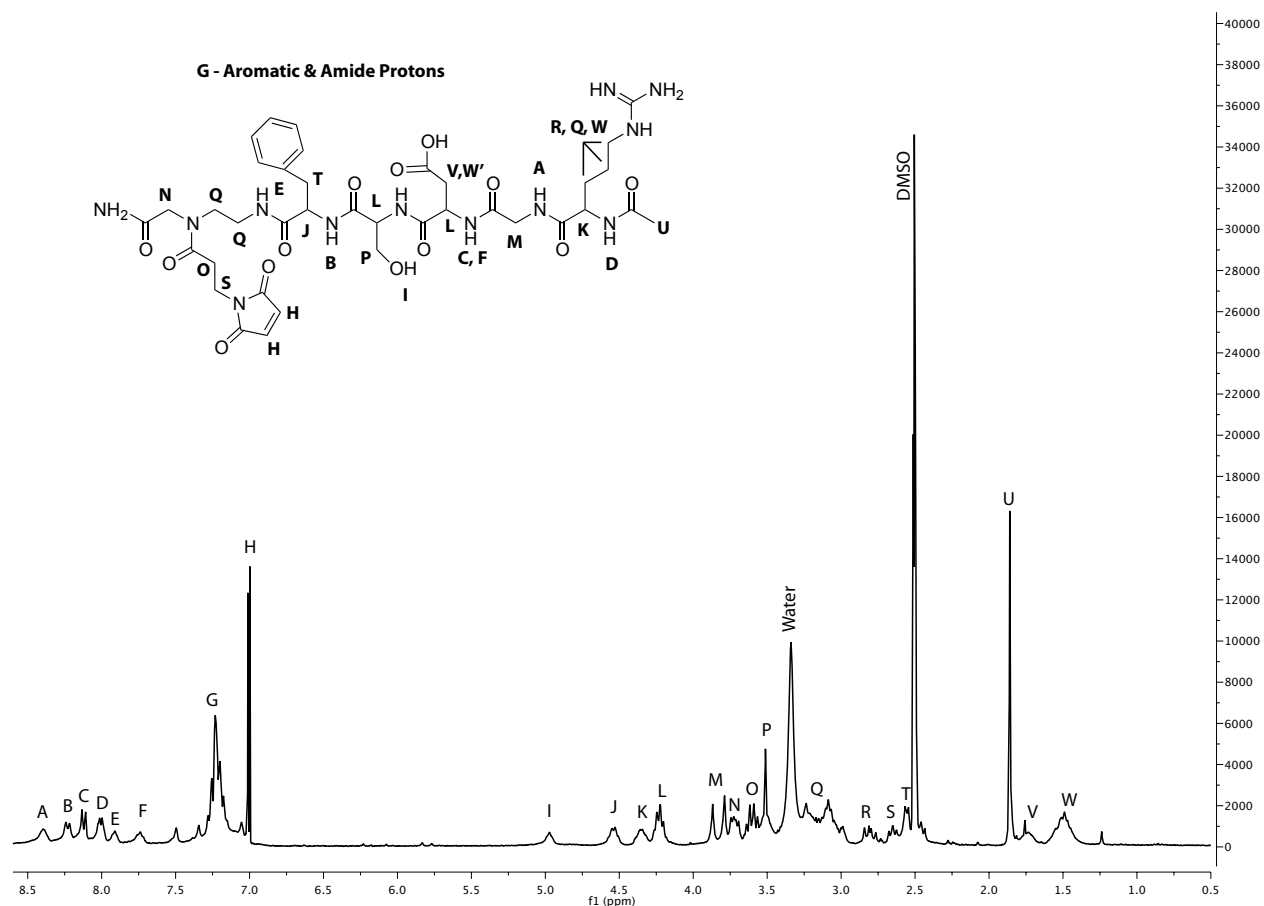


Figure 4.5. Fully characterized ^1H NMR of maleimide RGDS in DMSO- d_6 . The characteristic maleimide peak is clearly seen around 7ppm.

4.3. Results and Discussion

Maleimide motifs are known to readily undergo various reactions, which can be undesired depending on the synthetic objective, when exposed to a number of different conditions including high temperature, radicals and amines.^{18, 22, 25} Organic bases, namely secondary amines, were of primary concern for the incorporation of a maleimide amino acid in a peptide by means of standard Fmoc solid phase peptide synthesis, as the route relies upon piperidine to remove the Fmoc protecting group and exposes a free amine. To prevent undesired

reactions with piperidine or amines on the N-terminus during solid phase peptide synthesis, a Diels-Alder/retro Diels-Alder (DA/rDA) reaction scheme was employed to protect the maleimide moiety (See Scheme 4.2). Past work has demonstrated the feasibility of using sequential DA/rDA reactions to preserve the maleimide functionality in polymer systems where radicals, heat and other maleimide-reactive species are present.^{26, 27}

To demonstrate the ability to incorporate the maleimide amino acid in a peptide chain, a DA protected maleimide AEG residue was placed directly adjacent to the solid support, upon which the remainder of the sequence was built. In this demonstration, a peptide containing an RGDS sequence (See Figure 4.6) was constructed. The RGDS peptide sequence, which was chosen because of its widespread use in mimicking tissue extracellular matrix proteins that facilitate integrin-mediated cell attachment to synthetic biomaterials²⁸, was grown from the N-terminus of the maleimide-functionalized AEG. Upon refluxing the resin-bound peptide in toluene for approximately 4 hours and cleaving from the resin, the desired peptide sequence was obtained with the maleimide functionality intact.

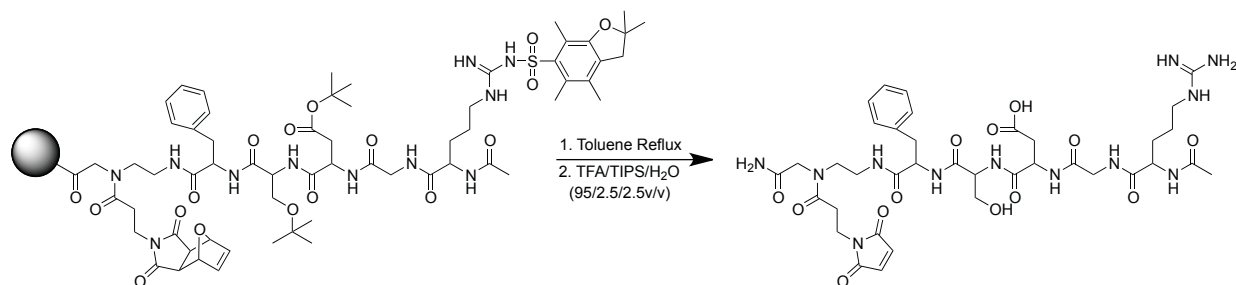


Figure 4.6. Portrayal of the construction of a RGDS peptide sequence containing the novel maleimide amino acid. The maleimide amino acid in Diels-Alder protected format was placed directly adjacent to the solid support (denoted by shaded circle) and from here the remaining RGDS sequence was built. Once the final residue was attached, the Diels-Alder reaction was reversed and the peptide cleaved from the solid support to yield the target maleimide-RGDS sequence.

Verification of the incorporation of the maleimide moiety in a peptide sequence was obtained through ^1H NMR and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry. Previous attempts were made to incorporate the maleimide amino acid in a non-DA protected format into a peptide; however, these efforts resulted in lower to no yield of the target sequence. With placement directly adjacent to the solid support representing the most difficult test of stability, it is not difficult to conceive of placing the maleimide moiety anywhere along the growing peptide chain by making use of the DA/rDA protection strategy.

After positive confirmation of incorporation of maleimide AEG in the peptide sequence, the reactivity of the maleimide functionality was assessed. First, the amino acid-bound maleimide was used to tether the peptide sequence to a surface by exposing a solution of the peptide to a thiol functionalized glass slide in the presence of triethylamine. This surface study was accomplished by printing a microarray on the thiolated surface using a $\sim 5\text{mM}$ solution of the maleimide RGDS sequence (See Figure 4.7) labeled with a carboxyfluorescein fluorophore in PBS buffer.

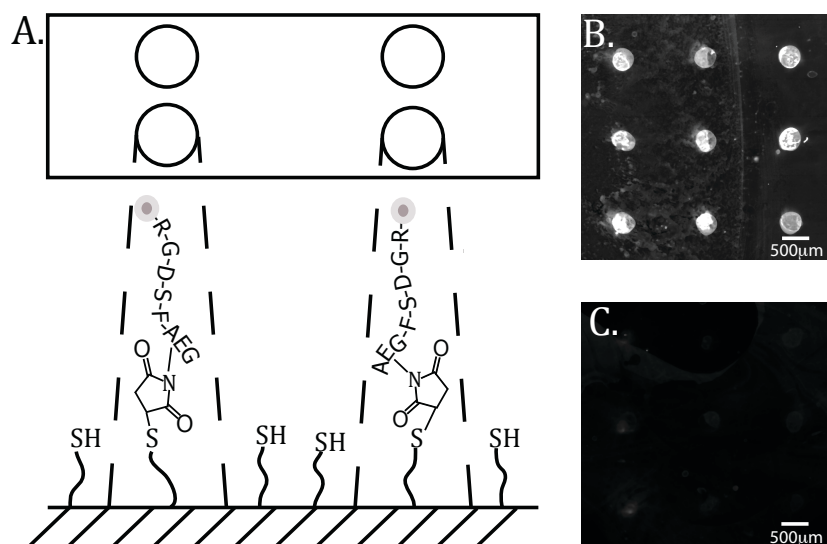


Figure 4.7. The left figure (A) shows a schematic depicting microarray printing. Panel B on the top right shows the ability to print a microarray pattern on a thiol surface making use of the maleimide handle in a Michael addition reaction. Below, panel C illustrates a thiol surface that was blocked with acrylate prior to printing the microarray. The lack of fluorescent spots indicates little to no nonspecific surface binding.

The attachment of the carboxyfluorescein label rendered the peptide sparingly soluble in PBS; however, upon addition of the triethylamine required to catalyze the Michael addition, the fluorescently labeled peptide sequence was readily solubilized. After allowing the Michael addition reaction to proceed for 15 to 20 minutes, the slide was washed with Tween80 (~5%v/v), isopropanol and PBS buffer. The slide was imaged using an Agilent microarray scanner, demonstrating a successful Michael addition in the thiol-patterned area of the glass slide (See Figure 4.6). As a control, a second thiol functionalized slide was reacted with 2-ethylhexyl acrylate prior to microarray patterning with the maleimide RGDS sequence. Reaction of the thiol surface was done in an effort to assess whether or not the binding was attributable to the Michael addition between maleimide and thiol or if there was another, nonspecific reaction occurring. No fluorescence was observed with this negative control. This test demonstrated that the maleimide functionality is not only intact, but also capable of serving as a specific, selective reactive handle via the Michael addition reaction.

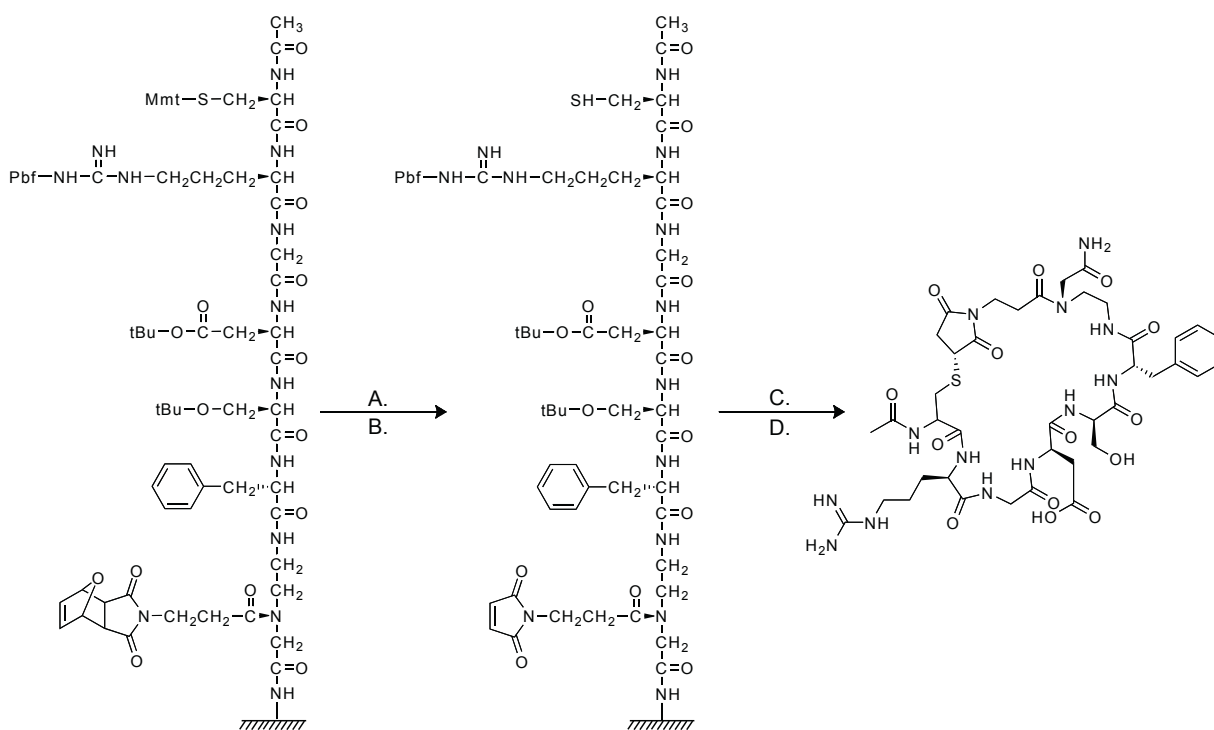
In addition to investigating the possibility of incorporating a maleimide amino acid anywhere along a given peptide sequence and further demonstrating its use as a modification handle, the ability to exploit the maleimide functionality to form cyclic conformations was also considered. To accomplish the formation of a macrocycle, the maleimide was reacted in a Michael addition reaction with the thiol of a cysteine residue. By taking advantage of orthogonal protecting chemistries, it was possible to introduce both a cysteine residue and maleimide AEG into a peptide sequence. For this part of the investigation, RGDS was again considered for the

peptide sequence (See Figure 4.3). Past studies have demonstrated enhanced bioactivity of cyclic RGDS peptides.²⁸ For the purposes of cyclization, it should be noted that, unlike the remainder of the peptide sequence, the phenyl alanine residue was replaced with the D-enantiomer which has been demonstrated to aid in the formation of cyclic conformations.⁶

Once synthesized, the peptide sequence containing both Diels-Alder protected maleimide and thiol masked with the monomethoxytrityl (mmt) protecting group was left on the solid support. The peptidyl resin was washed with isopropanol (3x50mL) and dichloromethane (3x50mL) to remove any residual reagents remaining from solid phase peptide synthesis. Given that toluene does not optimally swell the solid support, which allows adjacent peptide chains to be in close proximity and to prevent the potential Michael addition between those adjacent peptide chains (i.e., formation of dimer), the DA adduct was removed prior to the mmt protecting group. Following removal of the DA adduct by means of toluene reflux, the mmt protecting the thiol was removed using a (95/5)5 v/v (DCM/TIPS)TFA solution while leaving the remaining protecting groups intact.²⁹ The removal of the mmt group was confirmed by observing the presence of a yellow-orange liquid when washing the resin and with a qualitative modified on-resin Ellman's Assay.³⁰ Sequentially removing the protecting groups in this order was done to promote cyclization and minimize the potential occurrence of undesired oligomerization.

Cyclization of the peptide sequence was accomplished by means of a Michael addition between the exposed maleimide and cysteine functionalities. Prior to the addition of DIEA to catalyze the Michael addition, the resin was washed thoroughly with water to remove residual TFA. The presence of acid in the reaction mixture not only negates the effect of the base catalyst, but also inhibits the Michael addition. Upon acid removal, the peptidyl resin was re-swollen in DCM and 9 to 18 equivalents of DIEA were added to the stirred reaction vessel. The progress of

the cyclization reaction was monitored qualitatively using a modified Ellman's Assay to assess whether or not free, unreacted thiols remained on the solid support. After approximately 24 to 48 hours, an Ellman's Assay negative for thiol was attained, signifying completion of the Michael addition cyclization. Scheme 4.3 summarizes the cyclization methodology starting with the systematic orthogonal deprotection steps through the Michael addition reaction to the final cleavage from the solid phase.



Scheme 4.3. On-resin methodology leading to the formation of a macrocycle through the use of a maleimide-thiol Michael addition. Steps: A. Exposure of the maleimide functionality by removal of the Diels-Alder adduct protecting group (toluene reflux, 4 hours). B. Addition of DCM/TIPS/TFA to remove the mmt protecting group. C. Swelling of the resin in DCM followed by the addition of DIEA (9-18 equivalents) to incite cyclization (24-48 hours). D. Cleavage of the macrocycle from the resin using TFA/H₂O/TIPS.

After on-resin cyclization, the macrocycle sequence was cleaved from the solid support and purified by means of HPLC. Figure 4.7 displays the chromatograph for the HPLC purification.

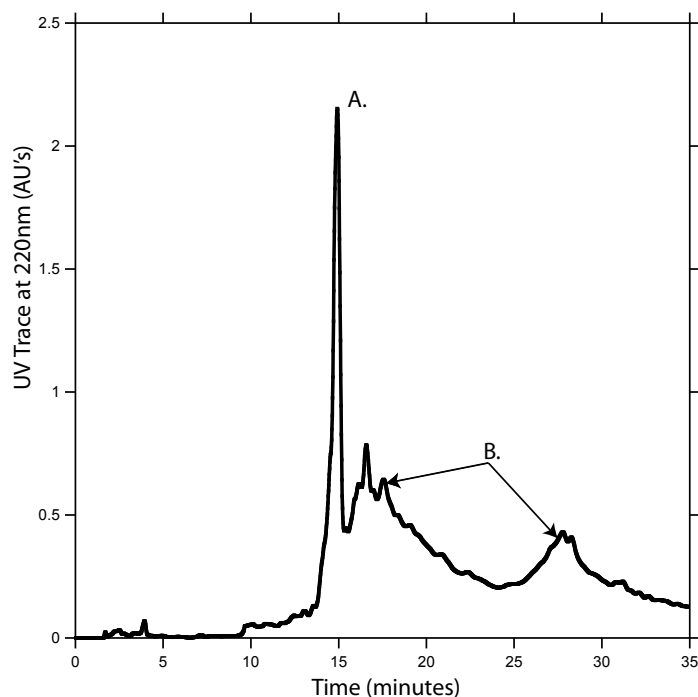


Figure 4.7. Cyclized maleimide-cysteine RGDS RP-HPLC UV trace. Peak A represents the target cyclized product whereas the peaks denoted by B correspond to higher molecular weight materials such as dimerized peptide sequences.

A denotes the molecular weight of the target species whereas species of higher molecular weight are presented by B. Characterization of the elution volumes corresponding to the peaks in the HPLC UV trace by means of MALDI confirmed the presence of the molecular weight associated with the macrocycle. Additionally, MALDI confirmed that the higher molecular species corresponded to oligomers, such as dimers, likely formed by the Michael addition of adjacent or near neighbor side chains. Figure 4.8 presents the MALDI spectrum of the target macrocycle following HPLC purification.

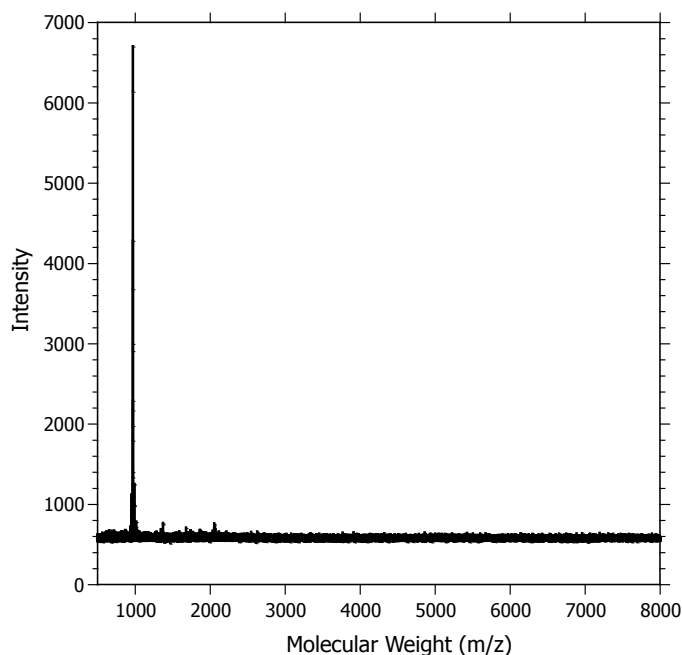


Figure 4.8. MALDI spectrum of cyclized RGDS after HPLC purification. The target product is the dominate species in the spectrum at $[M-H^+] = 976$.

Once cleaved from the resin and purified via reverse phase HPLC, the cyclic RGDS peptide sequence was obtained in approximately 30% yield, which is comparable to other cyclization techniques.^{4, 7, 17}

After cleavage from the solid phase, characterization to determine whether or not cyclization of the linear sequence occurred was required. MALDI-TOF mass spectroscopy verified the molecular weight of the target species. However, since the Michael addition reaction used to form the macrocycle does not result in a molecular weight difference when progressing from reactants to products, on its own MALDI-TOF does not positively confirm the presence of a cyclic structure. The MALDI spectrum verifying the correct molecular weight, in conjunction with a negative Ellman's Test for thiol, supported the formation of a macrocycle. Further proof supporting the formation of a macrocycle was obtained through NMR spectroscopy. A 1D ^1H NMR spectrum of the purified material clearly illustrated the disappearance of the

characteristic maleimide alkene peak (7ppm) and the formation of new peaks corresponding to a Michael addition across the heterocycle's double bond. Compared to the bottom spectrum in Figure 4.9, the top HNMR spectrum clearly shows the disappearance of the characteristic maleimide peak at around 7ppm.

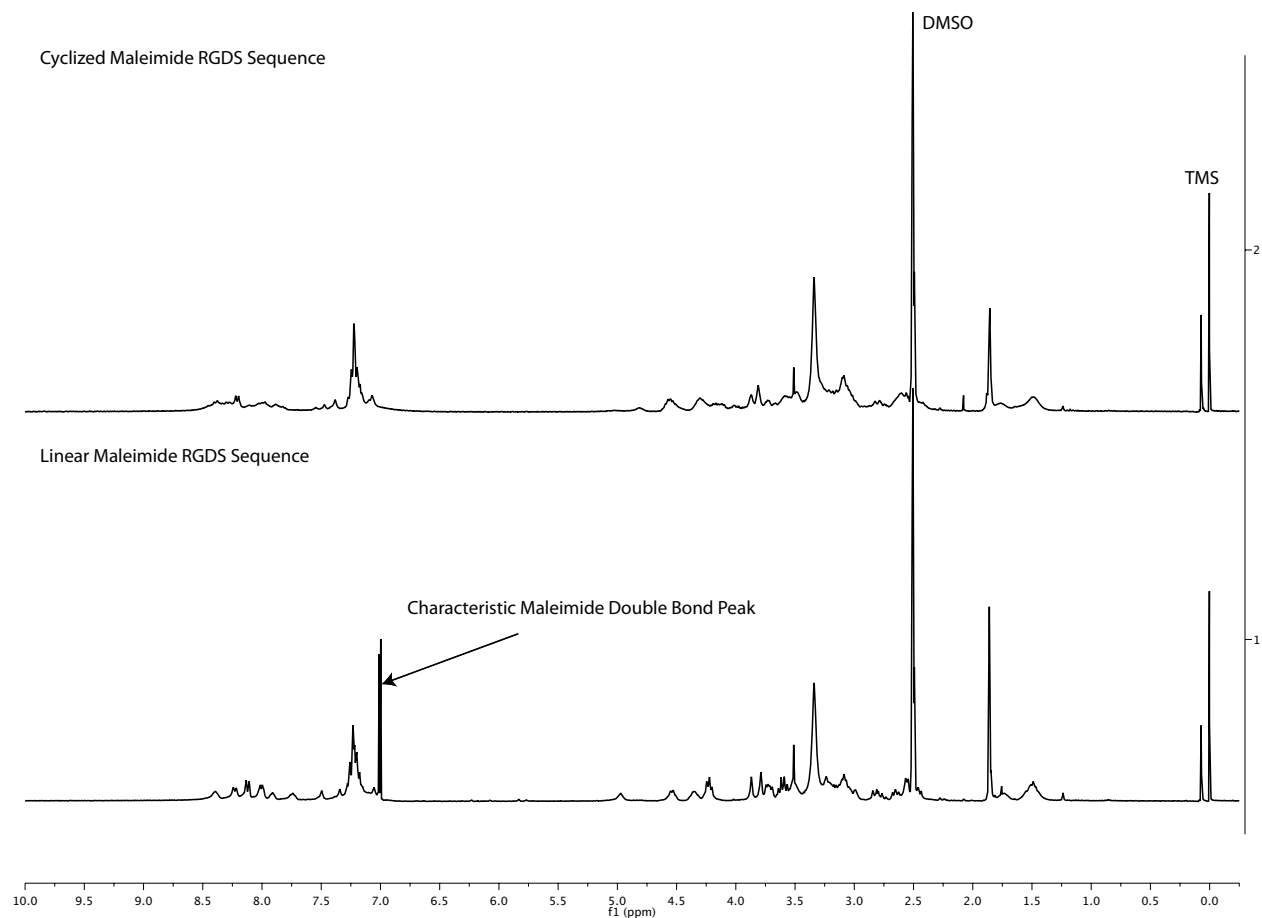


Figure 4.9. ¹H NMR spectra of a linear maleimide RGDS sequence lacking cysteine (top) and cyclized RGDS peptide (bottom). The characteristic maleimide double bond peak is not present in the cyclized RGDS, further supporting the formation of a macrocycle.

The absence of this peak in conjunction with the MALDI spectra support further supports the formation of a macrocycle. Additionally, a two-dimensional ROSSY spectrum was acquired to demonstrate further the formation of a thio-ether bond comprising the cyclic peptide. Figure 4.10

presents the ROSEY spectrum along with numbered chemical structures and the chemical shifts corresponding to the protons of interest.

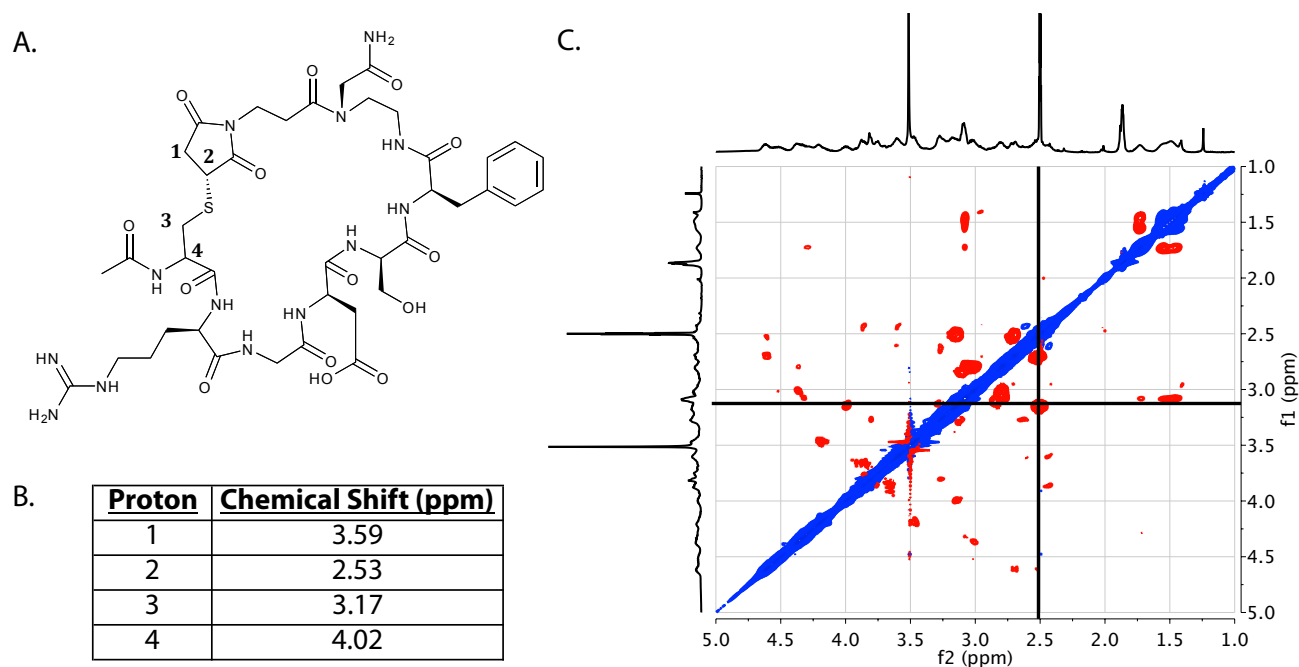


Figure 4.10. The chemical structure in the top left (A) portrays the Michael addition formed RGDS macrocycle with protons labeled around the cyclization point. B presents the chemical shifts for the labeled protons. The intersecting lines on the ROSEY spectrum on the right (C) indicate a through space correlation between protons 2 and 3 that form the thio-ether bond. Correlations between the other protons numbered on the cycle and presented in the table on the lower left may also be found in the ROSEY spectrum.

In addition to providing another means by which to construct cyclic peptides, the developed maleoyl amino acid allows for greater freedom in the placement of the maleimide functional group in a peptide sequence. With this increased versatility, the possibility exists to make use of the maleimide amino acid to manipulate and control peptides in a broader range of applications. One potential use afforded by the placement of maleimide anywhere in a given peptide sequence would be the formation of staples. Additionally, multiple maleimide

functionalities could readily be incorporated in a peptide sequence, effectively creating a peptide cross linker for the development of biomaterials.

With the ability to implement successfully the maleimide amino acid as a functional group for the subsequent modification of peptides, the question remained as to the biological activity of the sequences into which it is incorporated. The high reactivity of the maleimide functionality coupled with the elevated temperature required to remove the DA protecting group lends concern over the potential loss of bioactivity in a given peptide sequence. To verify peptide bioactivity, the model RGDS sequence was again considered. Synthetic biomaterials, which lack biological epitopes recognized by cells, are routinely functionalized with RGDS to mimic adhesive proteins and promote cell-material interactions, specifically cellular adhesion and spreading.^{9, 31, 32}

To demonstrate the activity of a peptide containing a maleimide residue, a linear maleimide RGDS sequence was synthesized and incorporated into a poly(ethylene glycol) (PEG) hydrogel. The hydrogels in this study were synthesized by means of Michael addition from maleimide and thiol functionalized PEG macromers (See Supporting Information), which facilitated the incorporation of maleimide and cysteine (positive control) RGDS at a concentration of approximately 2mM into the network. After synthesis and equilibrating in phosphate buffer solution, fibroblast cells (NIH3T3) were seeded onto the surface of the hydrogels. Importantly, because of the hydrophilicity and non-protein binding attributes of PEG gels and surfaces, cells interact minimally with pure PEG surfaces and remain weakly interacting in the absence of the RGDS sequence. However, provided the maleimide RGDS retains its biological activity, cells seeded on to hydrogels possessing that peptide sequence should adhere strongly and spread on the hydrogel surface.

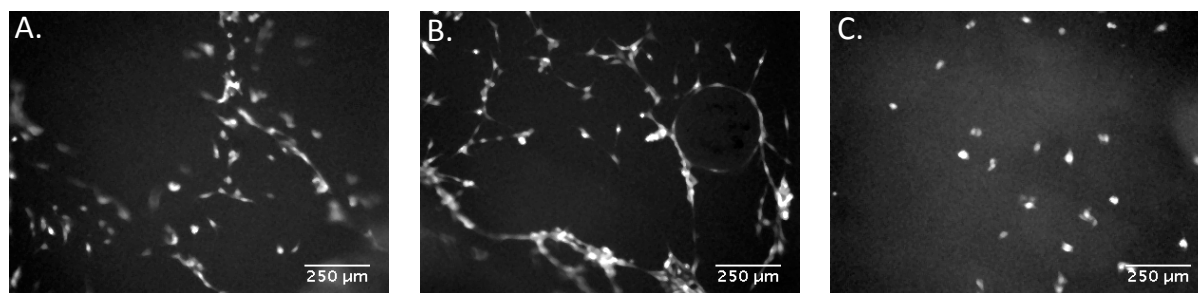


Figure 4.11. Fluorescently stained NIH3T3 fibroblast cells seeded onto poly(ethylene glycol) hydrogels formed via Michael addition of maleimide and thiol PEG macromers after incubation for 24 hours at 37°C. When incorporated, RGDS was present at approximately 2mM concentration in the hydrogel formulation. (A) depicts cells on the surface of a hydrogel with the maleimide RGDS incorporated into the polymer network, (B) shows cells on a hydrogel containing a positive control cysteine RGDS covalently incorporated in the network and (C) presents cells on a hydrogel that contains no RGDS. The presence of the RGDS sequence enables cells to interact with and bind to the hydrogel resulting in the morphological differences from A and B to C.

Figure 4.11 presents the two-dimensional morphology of fluorescently stained NIH3T3s on a maleimide-RGDS equipped hydrogel (4.11A) and clearly demonstrates cell attachment and spreading. To illustrate morphological changes that typically occur when RGD is present, a positive control hydrogel sample was prepared. The positive control consisted of CRGDS, which was incorporated into a PEG hydrogel using thiol-ene chemistry, as previously described⁹ (4.11B). Similar spreading was observed for the hydrogels containing maleimide and cysteine RGD. When no RGDS (Figure 4.11C) was present in the network, some non-specific adhesion to the hydrogel surface was observed. However, cell spreading was minimal, as the cells presented a distinct rounded morphology. Details pertaining to the formulation and preparation of the hydrogels may be found in the Supporting Information.

In addition, cell spreading was quantified using MetaMorph image analysis software to calculate an elliptical form factor of the attached cells. The elliptical form factor measures the

relative circularity of an object, such as the 3T3 fibroblast cells studied here. Average elliptical form factors of 2.0 ± 0.2 and 1.9 ± 0.2 were calculated for 3T3s seeded on hydrogels containing cysteine and maleimide RGDS, respectively (panels A and B of Figure 4.11), whereas a value of around 1.4 ± 0.1 was observed when RGDS was absent (panel C of Figure 4.11). The visual observation of cell interaction in conjunction with image analysis supports the availability and bioactivity of RGDS peptide capable of binding to cell integrins.

4.4. Conclusion

The efficiency of the reaction between maleimide and thiol in a Michael addition is of great use in biological applications. In terms of its use with respect to peptides, the maleimide motif has been limited to placement at the N-terminus of a peptidyl sequence or post-synthetic incorporation, whereby the maleimide is typically reacted with another functional group on the chain. In this study, a maleimide amino acid was developed with the ability to overcome these limitations. By employing a DA/tDA protection scheme, it was demonstrated that the maleimide moiety could be placed adjacent to the solid support and endure the rigors of peptide synthesis. By placing the moiety directly adjacent to the solid support, it is demonstrated that incorporation of the maleimide anywhere along a peptide chain is feasible without loss of activity. The presentation of the maleimide moiety in its reactive state provides for a unique handle, which allows for subsequent modification of a peptide chain. Results further illustrated that the maleimide amino acid, when incorporated in a peptide chain, allowed for the tethering of a sequence to surfaces and the formation of macrocycles. The ease with which the maleimide amino acid can be synthesized and incorporated in a peptide chain, while maintaining key functional properties, makes it a viable and useful tool for synthetic peptide modifications.

4.5 Acknowledgments

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Chapter 5

Diels-Alder Mediated Controlled Release from a Poly(ethylene glycol) Based Hydrogel*

A synthetic amino acid bearing a furan functionality was developed and incorporated into peptide sequences. Peptides expressing the furan moiety were attached to poly(ethylene glycol) (PEG) hydrogels through a thermally reversible covalent bond formed by a Diels-Alder reaction. Reactions of thiol and maleimide PEG macromers in an off-stoichiometric Michael addition were performed such that the maleimide moiety was in excess to create hydrogel networks with pendant Diels-Alder compatible, i.e., the maleimide, tethering sites. By making use of the Diels-Alder reaction, it was possible to control the release rate of material from the hydrogel by changing the temperature; higher temperatures favor a faster retro-Diels-Alder reaction and therefore a faster release from the polymer network. Incorporation of a fluorescently labeled furan-RGDS sequence into a hydrogel possessing excess maleimides and monitoring its subsequent liberation at various temperatures supported a Diels-Alder mediated release mechanism. The release was studied at temperatures ranging from physiological (37°C) to 80°C. By changing the temperature, varying extents of release were attained, ranging from 40% release for lower temperatures to complete release for the highest temperature considered. Further confirmation of a reaction-controlled release mechanism was obtained through comparison of the experimental release data to a reaction-diffusion model of the release. In addition to thermal modulation, tuning of the release rate was accomplished by altering the number of possible Diels-Alder tethering sites present in the hydrogel. Increasing the amount of free maleimide and therefore the number of potential Diels-Alder reaction sites effectively slowed the release of

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material from the polymer. For instance, doubling the amount of maleimide sites present in the release platform decreased the amount of peptide released from approximately 60% to about 40% in the same span of time.

5.1. Introduction.

Poly(ethylene) glycol (PEG) hydrogels possess a number of key attributes that lend to their implementation in biological applications. In particular, PEG based materials have a demonstrated history of exhibiting low toxicity and eliciting a limited immune response¹ which has made them well suited for cellular scaffolds^{2,3} and *in vivo* applications like implantable medical devices⁴. The monomers comprising PEG hydrogels are also highly versatile allowing for different chemical functionalities to be readily incorporated. The facile alteration of PEG monomers has allowed for the formation of hydrogels by a number of different biologically viable chemistries including the azide/alkyne^{5,6}, thiol-ene⁷ and Michael addition reactions^{8,9}. Not only has the incorporation of a wide range of functional groups been used to synthesize hydrogels by a number of different reactions, but it has also enabled the finished polymer to be adapted to the demands of a specific task by allowing for the decoration of the polymer with different chemical and biological moieties.

This inclusion of different epitopes as either pendent groups or crosslinks has further enhanced the use of PEG in the biological realm. For instance, enzymatically cleavable crosslinks such as matrix metalloproteinase¹⁰ and the inclusion of the cellular adhesion peptide sequence RGD have been implemented to foster differentiation and improve cell viability within polymer networks^{2,11}. In addition to these properties, PEG hydrogels also exhibit a high rate of material exchange between the polymer network and the surrounding environment. By making use of their high permeability, the potential application of PEG-based materials as a delivery

release platform has been demonstrated through the release of such compounds as dexamethasone^{12,13}, fluvastatin¹⁴, and nerve growth factor¹⁵.

In the simplest of cases and in many forms, diffusion has been used to govern the release of materials, such as peptides and proteins from hydrogel networks³. By changing the crosslink density of the polymer, it is possible to exercise some degree of control over the release rate from the hydrogel³. A higher crosslink density generally reduces the polymer mesh size and thereby hinders the diffusion of materials out of the network. In other applications, however, a release dictated by size, and therefore diffusion does not afford sufficient control. Consequently, a need for tighter regulation over the rate and mechanism by which a compound releases from the hydrogel exists. Taking advantage of the ease with which PEG-based materials can be modified, moieties contained in crosslinks or expressed as pendant groups have been incorporated in the polymer network and enabled a greater degree of control over the release of biomolecules and other compounds. In this vein, hydrogels have been developed that allow for the release of compounds covalently attached to the network in response to spatial and temporal environmental queues such as the addition of an enzyme^{16,17}, application of an electric field¹⁸ or exposure to light¹⁹. In the case of these adaptations, covalent bonds linking the compound to the network are severed, ultimate release from the hydrogel is then controlled by its ability to diffuse out of the polymer. Typically, after liberation from the network, molecules released by these means are not covalently re-incorporated into the polymer structure. Granting more control over release, hydrogels have been developed that allow for species to detach and re-bind to the polymer network through non-covalent interactions. For example, metal-ion chelating ligands^{20,21} and other compounds such as herapin¹⁵ have been incorporated into hydrogel networks, exploiting non-covalent, electrostatic interactions to tailor the release profile of compounds from the

polymer. In these hydrogel systems, increasing or decreasing the ligand density was demonstrated to provide control over the release rate. Unfortunately these affinity-based systems were mainly developed for the release of large molecules, such as proteins. The development and implementation of a dynamic reaction that allows for the covalent capture or re-incorporation of compounds of a wide range of molecular weights would extend additional control over the release profile for small molecule pharmaceuticals and peptides to longer timescales.

One potential set of reactions that fulfill the requirement for a reversible release whereby covalent bonds are broken and regenerated is the Diels-Alder (DA) reaction and its counterpart, the retro-Diels-Alder (rDA) reaction (See Figure 5.2). The DA/rDA reactions belong to the ‘click’ paradigm of chemistry outlined by Sharpless²³. Reactions associated with click chemistry boast the ability to occur orthogonally, without the generation of appreciable side products and in aqueous media at physiologically compatible conditions^{22,23} making reactions belonging to this ideology highly suited for biological uses. The thermally reversible DA reaction possesses the desired dynamic character to enable release and covalent re-incorporation of materials from a suitably fabricated polymer network. As with other delivery and release methods, the rDA reaction liberates material from the polymer; however, once decoupled from the network the material’s release is not solely dictated by the ease with which it traverses through the hydrogel. If released by the rDA reaction, the compound has the potential to re-attach covalently to the network via the forward DA reaction prior to metabolism or diffusion out of the hydrogel, provided that available DA binding sites exist. Although the DA/rDA mechanism is thermally modulated, the ability to control the reaction from an equilibrium perspective is of greater importance and necessity to biological applications. In an equilibrium reaction, removal of the

starting material, as would be done by diffusion in the case of a hydrogel, causes the product to revert back to reactant form. Exploiting Le Châtelier's principle in this manner negates the need for elevated temperatures to incite the rDA reaction, favoring the viability of biomaterials and biologically active compounds.

Past efforts have been made to employ the DA/rDa reactions in the fabrication of degradable hydrogel materials. These attempts successfully focused on the creation of a hydrogel where the crosslinks of the polymer were comprised of Diels-Alder adducts between maleimide and furan^{16,24}. Unfortunately these materials did not readily degrade by a rDA process as the kinetics of the reaction were too slow under physiologically compatible conditions. In order to achieve significant reversion of the crosslinks to incite degradation of these materials, multiple rDA reactions needed to occur simultaneously at a rate surpassing the forward DA reaction. Therefore, to release materials from or degrade the network of these DA crosslinked materials, other measures not favorable to biological applications, such as the use of high temperatures and concentrations of organic solvent were implemented.

Here, this work investigates the development of a PEG hydrogel DA/rDA controlled release system making use of furan containing peptide sequences, namely an RGDS derivative. Rather than construct a DA crosslinked degradable polymer, the potential use of a hydrogel containing pendent DA reaction sites to control the release of molecules from an irreversibly crosslinked network was studied. To accomplish this, a hydrogel was constructed by means of a thiol-Michael addition reaction between maleimide and thiol PEG macromers to create DA compatible tethering sites. The PEG hydrogels were constructed in an off-stoichiometric fashion such that excess, unreacted maleimide groups remain in the polymer network which represent sites for potential DA reactions. Constructing the hydrogel in this manner had the advantage of

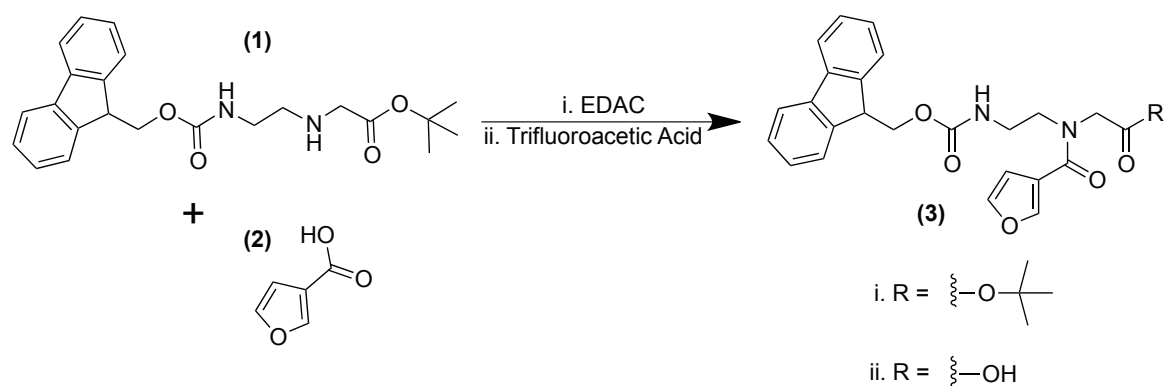
requiring only one, not multiple, DA adducts to undergo the rDA reaction. The ability to control the release rate by varying the amount of available maleimide DA tethering sites while maintaining a constant crosslink density was explored by the introduction of cysteine as a mono-thiol blocking agent. Both investigating the thermal dependence of the release profiles at several temperatures and comparing the empirical release data to a reaction diffusion model assessed the validity of a DA/rDA reaction mechanism.

5.2. Materials and Methods.

5.2.1. Materials. The following reagents were purchased from their respective vendors and, unless otherwise specified, used without further purification. Material for use in the creation of a 3-furan amino acid compatible with solid phase peptide synthesis including 3-furoic acid, tert-butyl bromoacetate, diisopropylethylamine (DIEA) N-(9H-fluoren-2-ylmethoxycarbonyloxy)succinimide (Fmoc-o-su), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) were purchased from AK Scientific, Inc. (Union City, CA). Ethylene diamine used as the backbone in the 3-furan amino acid was purchased from Fisher Scientific (Waltham, MA). Other materials acquired from Fisher Scientific included 10x concentrated phosphate buffered saline (PBS), dichloromethane, and triethanol amine (TEA). The fluorophore, 5(6)-carboxyfluorescein, used to label peptide sequences in this study, was obtained from Nova Biochem (Darmstadt, Germany). Trifluoroacetic acid (TFA) and cysteine were acquired from Sigma Aldrich (St. Louis, MO). Multi-arm maleimide and thiol functionalized PEG macromers were bought from JenKem (Allen, TX) for the formation of hydrogel polymer networks.

5.2.2. Peptide Synthesis. Standard Fmoc mediated solid phase synthesis making use of a Protein Technologies Tribute Benchtop automated peptide synthesizer (Tucson, AZ) was performed to construct the peptide sequences considered in this study. The amino acids used in this study, arginine, glycine, aspartic acid and serine, were acquired from ChemPep Inc. (Wellington, FL). Manual, post-synthetic modification of the peptide sequences while they were attached to the solid phase was performed to insert a fluorescent label, 5(6)-carboxyfluorescein on the N-terminus.

5.2.3. 3-furoic n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate Synthesis. Tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate hydrochloride (AEG), an orthogonally protected amino acid commonly associated with peptide nucleic acid synthesis²⁵, provided a backbone that allowed for both the facile incorporation of a furan moiety and use in solid phase peptide synthesis (See Scheme 5.1).



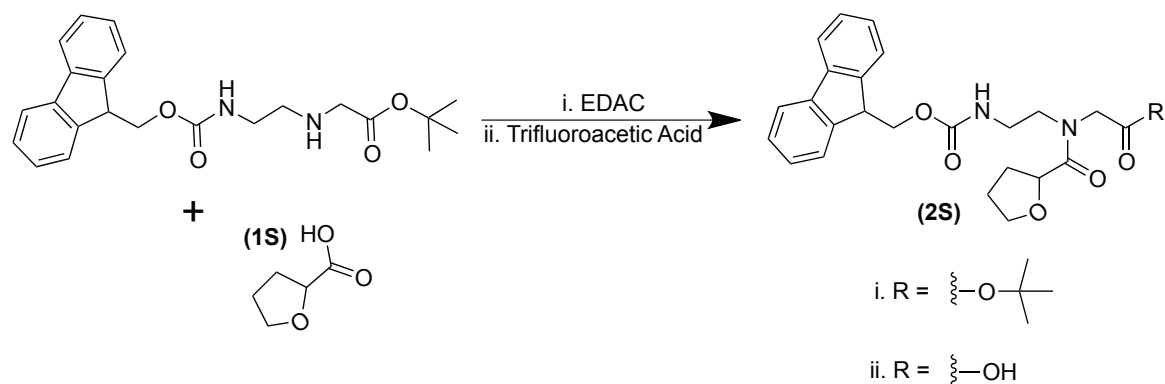
Scheme 5.1. The synthetic route to the formation of a 3-furan amino acid compatible with Fmoc mediated solid phase peptide synthesis. EDAC coupling is used to introduce the 3-furoic moiety onto an orthogonally protected amino acid backbone. Once coupled, to render the furan amino acid compatible with Fmoc mediated solid phase peptide synthesis, the t-butyl group protecting the carboxylic acid is removed by exposing the molecule to a solution of trifluoroacetic acid.

The synthetic route to the formation of AEG is detailed elsewhere²⁶. To a solution of **(1)** AEG (3.60g, 8.33mmol) in dichloromethane (60mL) was added **(2)** 3-furoic acid (1.12g, 10.0mmol). After dissolution of the AEG and 3-furoic acid, EDAC was introduced to the mixture in two equal additions (0.95g, 0.01mmol) spaced approximately thirty minutes apart. The reaction was allowed to proceed overnight (approximately 14 hours) at ambient conditions. Dichloromethane was then removed under reduced pressure to afford an oily residue, which was subsequently taken into anhydrous diethyl ether. The crude product was washed with water (3 by 100mL) and saturated sodium bicarbonate (3 by 100mL). Following the washes, the organic layer was polished with brine, dried over sodium sulfate and filtered. Concentration in vacuo yielded **(3i)** 3-furoic tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate which was used directly in the next step without further purification

In order to use the 3-furoic AEG **(3i)** in solid phase peptide synthesis, the t-butyl protecting the carboxylic acid moiety was removed. Trifluoroacetic acid (40mL, 0.52mmol) was added to a solution of **(3i)** 3-furoic AEG in dichloromethane (40mL). To ensure complete removal of the t-butyl group, the deprotection reaction was carried out for approximately 3 hours. Removal of dichloromethane and trifluoroacetic acid under reduced pressure resulted in a dark oil. The oil was immediately taken back into a minimal amount of dichloromethane and added to a solution of n-pentane and diethyl ether (4:1 v/v) resulting in the formation of an off-white precipitate. The off white solid was isolated by means of centrifugation and dried to afford **(3ii)**, the title product (2.64g, 6.10mmol, 73% yield).

¹H NMR (300 MHz, DMSO-d₆) δ 8.10-7.84 (d, 1H), 7.96-7.84 (m, 2H), 7.77-7.70 (m, 1H), 7.67 (d, J = 7.4 Hz, 2H), 7.47-7.25 (m, 5H), 6.62 (d, J = 38.8 Hz, 1H), 4.31 (d, J = 6.8 Hz, 2H), 4.20 (dd, J = 12.3, 5.1 Hz, 1H), 4.07 (s, 2H), 3.50 (dq, J = 12.8, 6.4 Hz, 2H), 3.23 (q, J = 6.6 Hz, 2H).

5.2.4. Tert-butyl tetrahydro 2-furoic n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl]

glycinate Synthesis.

Scheme 5.2. Synthetic route to the formation of a tetrahydrofuran amino acid compatible with standard Fmoc mediated solid phase peptide synthesis. The first step involves the coupling of a tetrahydrofuran carboxylic acid (**1S**) to an orthogonally protected aminoethylglycine backbone using EDC chemistry (**2Si**). Following the coupling, the t-butyl group on the C-terminus is removed by exposing the molecule to a solution of trifluoroacetic acid (**2Sii**).

Tetrahydro-2-furoic acid was acquired from Sigma Aldrich (St. Louis, MO) and used without further purification. The tetrahydro-2-furoic acid (**1S**) was attached to a tert-butyl n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate hydrochloride (AEG) backbone by means of EDAC coupling. (See Scheme S1.) To a stirred suspension of AEG (5.0g, 11.6mmol) in dichloromethane (DCM) (200mL) was added **1S** (1.6g, 13.8mmol). Upon dissolution, two equal fractions of EDAC (2.7g, 13.9mmol) were added approximately half an hour apart. The reaction mixture was allowed to stir at room temperature overnight (approximately 14hours) after which time the mixture was concentrated under reduced pressure. The resulting oil was taken into diethyl ether and washed 3x150mL water and 3x150mL saturated sodium bicarbonate. After washing, the organic layer was polished with brine and drying over sodium sulfate. Concentration of the organic layer by rotary evaporation afforded the target product (**2Si**), which was used directly in the next step.

$^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 7.95 – 7.84 (m, 2H), 7.73 – 7.60 (m, 2H), 7.46 – 7.29 (m, 4H), 7.29 – 7.23 (m, 1H), 4.59 (ddd, $J = 61.4, 7.4, 5.5$ Hz, 1H), 4.30 (dd, $J = 10.7, 6.3$ Hz, 2H), 4.24

(s, 1H), 4.12 (s, 1H), 3.81 – 3.61 (m, 2H), 3.50 – 3.34 (m, 3H), 3.26 – 3.01 (m, 2H), 2.12 – 1.68 (m, 4H), 1.41 (d, J = 10.5 Hz, 9H).

5.2.5. Tetrahydro 2-furoic n-[2-(n-9-fluorenylmethoxycarbonyl)aminoethyl] glycinate Synthesis.

To a solution of **2Si** in dichloromethane (30mL) was added trifluoroacetic acid (TFA) (30mL). The mixture of DCM, TFA and **2Si** were allowed to react at ambient temperature for five hours to remove the t-butyl group and expose a carboxylic acid functionality. After the five-hour period, excess TFA and DCM were removed under reduced pressure. The resulting oil was taken back into a minimum amount of DCM and added to mixture of diethyl ether and pentane (ratio v/v). The target compound formed a white precipitate upon addition to the ether/pentane mixture. Centrifugation and decantation of the supernatant yielded the title product, **2Sii** (0.9g, 2.0mmol and 17% yield).

¹H NMR (300 MHz, DMSO-d₆) δ 7.89 (dt, J = 7.5, 0.9 Hz, 2H), 7.72 – 7.58 (m, 2H), 7.48 – 7.20 (m, 5H), 4.69 - 4.47 (m, 1H), 4.39 – 4.10 (m, 4H), 3.91 (d, J = 8.5 Hz, 1H), 3.82 – 3.62 (m, 2H), 3.59 – 2.98 (m, 4H), 2.11 – 1.65 (m, 4H), –OH peak broad at δ13.00 – 12.00.

5.2.6. Hydrogel Formulations.

For this study 10 weight percent PEG, 30μL hydrogels were fabricated from 10kDa tetrafunctional maleimide and thiol PEG macromers. To facilitate the creation of hydrogels, 20 wt% stock solutions of the PEG macromers were prepared in PBS buffer. In an effort to react approximately ten percent of the maleimide functionality with the releasable compound, 3-furan labeled peptide sequences were first introduced to the maleimide stock solution at least 24hours prior to polymerization to allow attainment of equilibrium. Structures for the PEG macromers and a peptide sequence, RGDS, incorporating 3-Furoic AEG are presented in Figure 5.1.

macromer solution, a method to decrease the polymerization rate was required, and thus, pH was used to control the rate of the Michael addition. Adjusting the pH to approximately 2 of the make-up PBS buffer added to decrease the weight percent of the hydrogels slowed the Michael addition and afforded sufficient time to mix the materials uniformly.

After addition of PBS buffer to bring the PEG content to 10 wt%, the solution of PEG macromer was transferred to 1mL syringes, which had been modified by removal of their tips. The addition of 3 μ L of 0.3M triethanol amine solution increased the pH of the solution, promoting the Michael addition reaction to polymerize the thiol/maleimide macromer mixture. The syringe barrels served as a mold for the polymerization, facilitating the creation of hydrogels with a uniform cylindrical geometry. In their swollen state, the finished hydrogels were found to have a height of 1.3 \pm 0.1mm and diameter of 6.0 \pm 0.1mm on average.

After initiating the polymerization with TEA, the reaction was found to be complete after fifteen minutes by performing an Ellman's assay of the finished hydrogel for free thiol. For experiments where the amount of maleimide in the finished hydrogel was decreased by the introduction of a monofunctional thiol, cysteine, the Michael addition was allowed to proceed overnight to insure complete reaction (approximately 12 hours).

5.2.7. Release Studies. Hydrogels loaded with carboxyfluorescein labeled 3-furan RGDS were immersed in 1mL aliquots of PBS buffer. The hydrogels were maintained at a constant temperature by placing the samples in an Oakton High Performance StableTemp Utility bath (Model #12501-00). The 1mL PBS buffer media in which the hydrogel samples were kept was exchanged with fresh solution at regular intervals. A BioTek Synergy H1 Hybrid microplate reader (Winooski, VT) detecting fluorescent emission at 518nm from a 494nm excitation was

used to determine the amount of carboxyfluorescein present in the 1mL PBS buffer aliquots and thereby monitor the release of peptide from the hydrogels.

5.3. Results and Discussion.

5.3.1. Hydrogel Formulation and Initial Release Studies. Many chemical reactions are available for hydrogel formation, however, not all are compatible with the DA and rDA reactions. To create a hydrogel containing DA compatible tethering sites, this investigation took advantage of another click-style reaction, the thiol-Michael addition^{27,28}. Typically initiated with the use of a base or phosphine catalyst, the thiol-Michael addition reaction occurs between a nucleophile, such as a thiol, and an activated carbon-carbon double or triple bond²⁹ (See Figure 5.2a). The maleimide moiety, in addition to serving as a dienophile in the DA reaction (See Figure 5.2b), can also participate in a Michael addition reaction, an ability which has been exploited in a number of different biological applications including surface functionalization^{30,31}, disulfide bridge replacement^{32,33}, and the crosslinking and labeling of peptides and proteins^{34,35}.

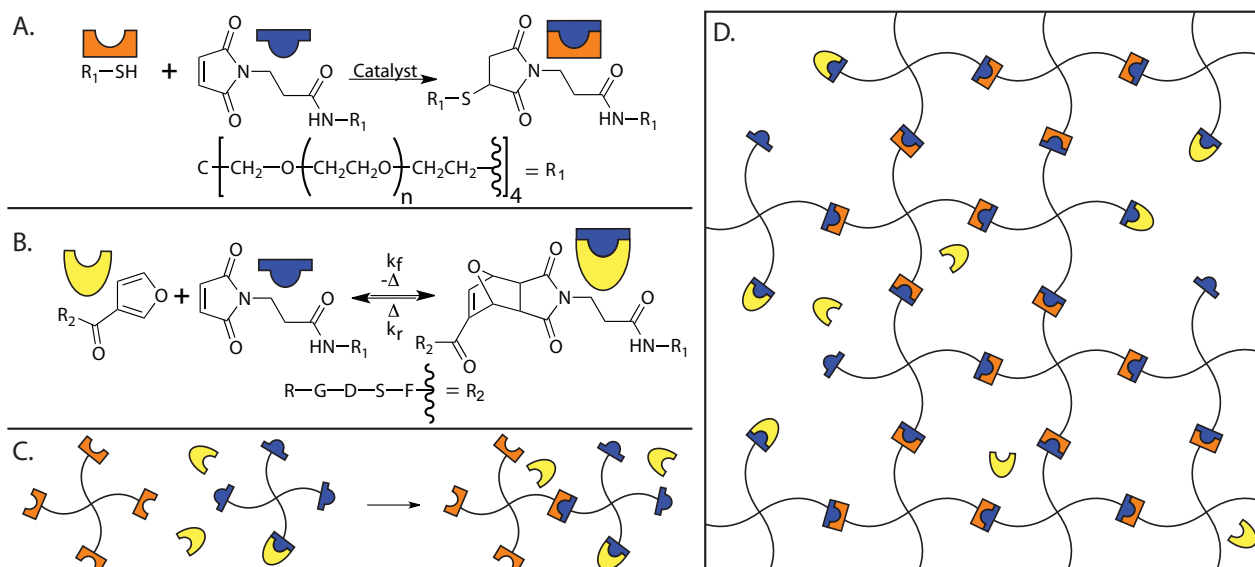


Figure 5.2. The creation of a PEG hydrogel with Diels-Alder compatible tethering sites. A. To create the hydrogel, a thiol-Michael addition reaction between multifunctional maleimide and thiol PEG macromers was performed. The reaction was conducted in an off-stoichiometric ratio to allow for excess maleimide groups to be present as pendent groups throughout the polymer network. B. By making use of the excess maleimide groups, the Diels-Alder reaction could take place allowing for a releasable species to be covalently tethered to the network by a reversible bond. C. Schematic representation of the Michael addition and Diels-Alder reactions employed to create the hydrogel release platform. D. Portrayal of the overall polymer network crosslinked via Michael addition. Furan peptide sequences are present in both their bound and unbound states.

The high reactivity of the maleimide attributed to ring strain and the electron withdrawing carbonyls adjacent to the carbon-carbon double bond has further contributed to the use of the Michael addition reaction. The increased reactivity of the maleimide reduces the necessary amount of catalyst, which has the potential to be detrimental to biological applications, compared to the quantity required for other Michael addition reactions, such as those involving acrylate or vinyl sulfone groups⁸. Reacting maleimide and thiol functionalized PEG macromers in an off-stoichiometric Michael addition reaction such that thiol was the limiting reagent allowed for the formation of a hydrogel containing pendent dienophile DA tethering sites throughout the network (See Figure 5.2c and d).

To enable a rDA mediated release, a functionality compatible with the Diels-Alder reaction needed to be incorporated in the releasable species. As the dieneophile, the maleimide was present in the polymer network, necessitating that the diene component of the DA reaction be present in the releasing material. To accomplish this, a 3-furan moiety, a diene capable of participating in the DA and rDA reactions with the network-bound maleimide, was attached to a peptide commonly used in biological pursuits, RGDS. The RGDS sequence has been extensively studied and, as a derivative of the extracellular matrix (ECM) protein fibronectin, its inclusion in polymeric materials has been established as a means to promote cellular adhesion and spreading in synthetic environments¹¹.

3-Furoic AEG was directly placed at the C-terminus during solid phase synthesis of the RGDS sequence, effectively imparting the diene moiety terminally on the peptide sequence (See Figure 5.3). By making use of 3-furoic AEG, it was possible to bypass the need to post-synthetically add a diene by sequential deprotection/addition to chemically compatible side chains. Additionally, placement of the 3-furan moiety at the C-terminus allowed for further modification of the N-terminus. This feature was exploited to attach a carboxyfluorescein label to the RGDS sequence, enabling facile detection of the peptide sequence as it released from the hydrogel.

Prior to the hydrogel formation, the fluorescently labeled 3-furan RGDS sequence was introduced to the maleimide PEG macromer stock solution. Addition of the furan functionalized peptide to the PEG macromer before polymerization enabled the amount of peptide in the finished hydrogel to be controlled and known. Additionally, with the dynamic nature of the DA/rDA reaction, pre-mixing of the PEG macromer with the 3-furan RGDS sequence also allowed time for the reaction between the maleimide and furan to reach equilibrium.

After allowing the furan labeled peptide to react with the maleimide PEG macromer and the subsequent Michael addition to form a hydrogel, the feasibility of DA reaction-mediated release was investigated. As a reservoir for the release, hydrogel samples were immersed in phosphate buffered saline (PBS) solution. The PBS media was removed and replaced at regular intervals to maintain infinite sink conditions for the release. Monitoring the fluorescence of the collected aliquots obtained while changing out the PBS buffer revealed carboxyfluorescein labeled material releasing from the hydrogel. In comparing the release of the 3-furan RGDS to that of an analogous tetrahydrofuran (THF) RGDS peptide sequence (See Supporting Information) incapable of participating in the DA/rDA reactions but otherwise identical, two distinct profiles were observed (See Figure 5.3).

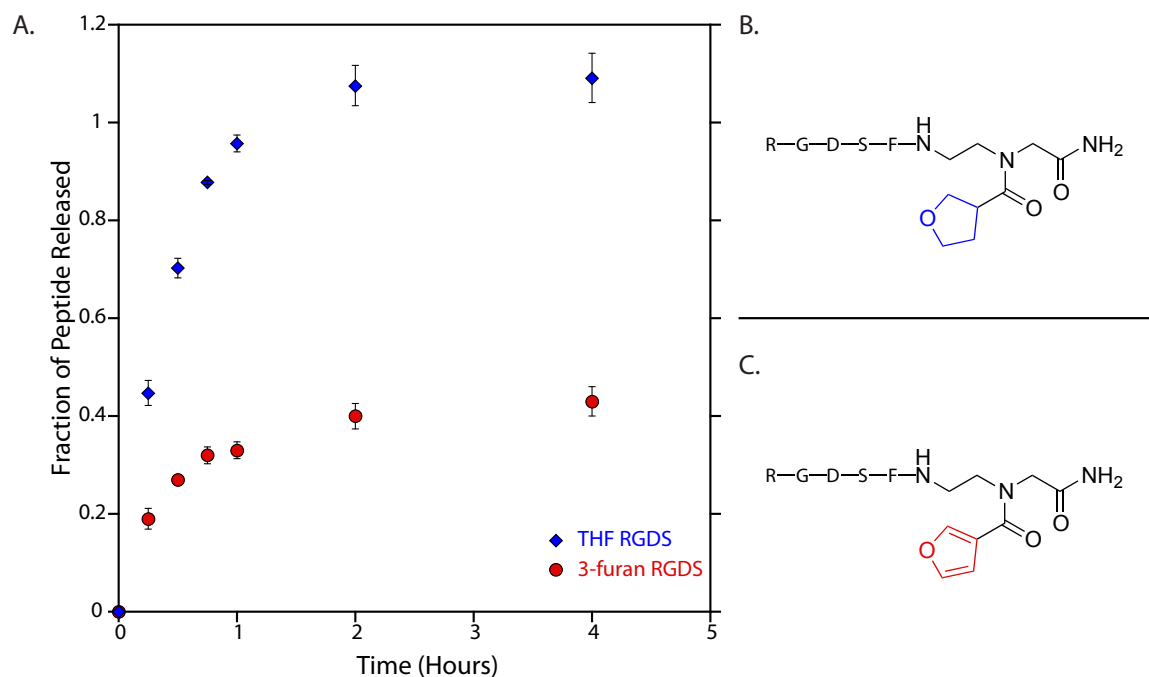


Figure 5.3. A. Comparison of the Diels-Alder controlled release of a 3-furan RGDS peptide to the structurally similar tetrahydrofuran RGDS sequence. The THF species, which does not participate in the DA reaction or couple to the network in any manner, releases from the material by a purely diffusive mechanism. As a result, the THF sequence releases from the material at a much higher rate than the 3-furan peptide whose release from the material is governed by the DA/rDA reactions. B and C illustrate the structure of the THF and 3-furan species, respectively,

and their placement in the RGDS peptide sequence. Note the conjugation of the furan in panel C which allows the molecule to participate in the Diels-Alder reaction.

At a physiologically relevant temperature, 37°C, the 3-furan sequence has an initial burst followed by a slow, sustained release dictated by the DA/rDA reactions. In contrast, at the same temperature, the THF peptide, which lacks the ability to interact with the hydrogel by means of the DA reaction, releases from the network by a diffusion controlled process. As diffusion is the release mechanism, the THF-RGDS peptide releases at a much higher rate and subsequently achieves complete release before the 3-furan sequence which has the ability to interact with the network through the DA/rDA reactions.

5.3.2. Diels-Alder Controlled Release. The equilibrium of the Diels-Alder reaction is thermally controlled; elevated temperatures induce the reverse reaction and the reformation of maleimide and furan moieties whereas lower temperatures favor the adduct formation. If peptide release from the hydrogel is governed by a DA/rDA mechanism, exposing the polymer networks to different temperatures should dramatically influence the rate of the DA and rDA reactions and therefore the rate and quantity of material released from the network. Increasing the temperature at which the release studies were conducted resulted in a change in the amount and rate of liberation of the 3-furoic RGDS from the hydrogel networks (See Figure 5.4). As illustrated in Figure 5.4, significantly different profiles were obtained for the three temperatures considered with the peptide releasing more than twice as rapidly at 80°C as compared to the release at 37°C. The higher release rate observed at elevated temperatures corresponds to an increased reverse rate in the rDA reaction relative to the forward reaction. Figure 5.4a highlights the data in the initial period of the release, depicting different release rates at each temperature for the initial

portion of the study. Figure 5.4b depicts the long time release behavior. The reduced rate observed for longer times at all temperatures is attributable to interaction of the 3-furoic peptide sequence with the hydrogel through the DA/rDA reactions which hinders the release, preventing it from achieving complete delivery as seen with a non-reactive species (See Figure 3). The higher than unity fractional release observed in the 80°C experiment is most likely attributed to instabilities of both the peptide material and the maleimide functionality in the network at elevated temperatures in an aqueous environment.

To provide further evidence of the Diels-Alder mechanism by which material is released from the hydrogel, a fit of the empirical data to a reaction-diffusion model was conducted. In terms of reaction-diffusion controlled release, the use of the DA/rDA reaction considered in this study is analogous to the model developed by Lin and coworkers for protein delivery from an affinity hydrogel system²⁰. Unlike the affinity-based systems, which rely upon electrostatic interactions, release from hydrogels by means of a DA/rDA scheme occurs through a series of covalent bonds forming and breaking.

The DA reaction is responsible for covalent interactions between the releasing material and the polymer, thereby comprising the reactive component of the model and may be expressed as



where F, M, and A denote the furan, maleimide and adduct species, respectively. The reverse and forward rate constants associated with the DA and rDA reactions are denoted by k_f and k_r . Chemical structures for these components are presented in Figure 5.2. For the case of the hydrogels studied here, maleimide functionalities were irreversibly bound to the network during the Michael addition polymerization. Additionally, adduct species can only be present when a

furan labeled peptide is covalently attached to the network by means of the DA reaction. As a result, maleimide and adduct cannot be found in the bulk solution outside of the hydrogel; only the furan containing peptide sequence can release from the material. Coupling the expression for the DA reaction (equation 5.1) with Fick's law leads to:

$$\frac{\partial[F]}{\partial t} = D_0 \nabla^2 [F] - k_f [F][M] + k_r [A], \quad (\text{Eq. 5.2})$$

which describes the furan concentration at any time and location inside the polymer. With a diameter to height aspect ratio of approximately 4.5 ± 0.3 , a one-dimensional model could not accurately portray the release from the hydrogels³⁶. Therefore, the radial and axial directions were also included in modeling the release behavior though the symmetry of the hydrogel allows for the removal of the θ component from the equation.

As the peptide traverses through the hydrogel by means of a reaction-diffusion mediated mechanism, the amount of adduct and the concentration of possible maleimide sites for the DA reaction to occur are also in a state of flux. As a result, the spatial and temporal distributions for the maleimide and adduct must be solved simultaneously with the equation describing the furan-labeled peptide's profile in the hydrogel. The remaining partial differential equations describing the maleimide and adduct distributions over time are given by

$$\frac{\partial[M]}{\partial t} = k_r [A] - k_f [F][M], \quad (\text{Eq. 5.3})$$

$$\frac{\partial[A]}{\partial t} = k_f [F][M] - k_r [A]. \quad (\text{Eq. 5.4})$$

These partial differential equations describe the concentration profiles of the furan, maleimide and adduct species at any given time and position within the confines of the hydrogel. To obtain the amount of material released from the polymer, an overall mass balance on furan-labeled peptide was performed. The total amount of furan present in the system is equivalent to the

quantity of furan in the hydrogel and that released to the surroundings. Integration of the furan distribution over space, which was obtained by simultaneously solving equations 5.2, 3 and 4 yields the amount of furan within the boundaries of the hydrogel at any particular time. Subtraction of the amount of 3-furan RGDS present in the gel from the initial amount of the peptide loaded gives the amount of material released from the hydrogel.

To solve these equations, an algorithm was developed in Matlab (The Mathworks, Natick, MA) making use of Euler's method to obtain numerical solutions for the concentration profiles of furan, maleimide and adduct in the hydrogel. These solutions were used to determine the amount of 3-furan RGDS released. By appending the algorithm, it was possible to make use of a nonlinear fitting function built into the Matlab environment to approximate the diffusion coefficient, forward rate constant and reverse rate constant from the empirical data associated with the release of the 3-furan RGDS sequence. Figure 5.4 presents the reaction-diffusion model fit superimposed upon the experimental data.

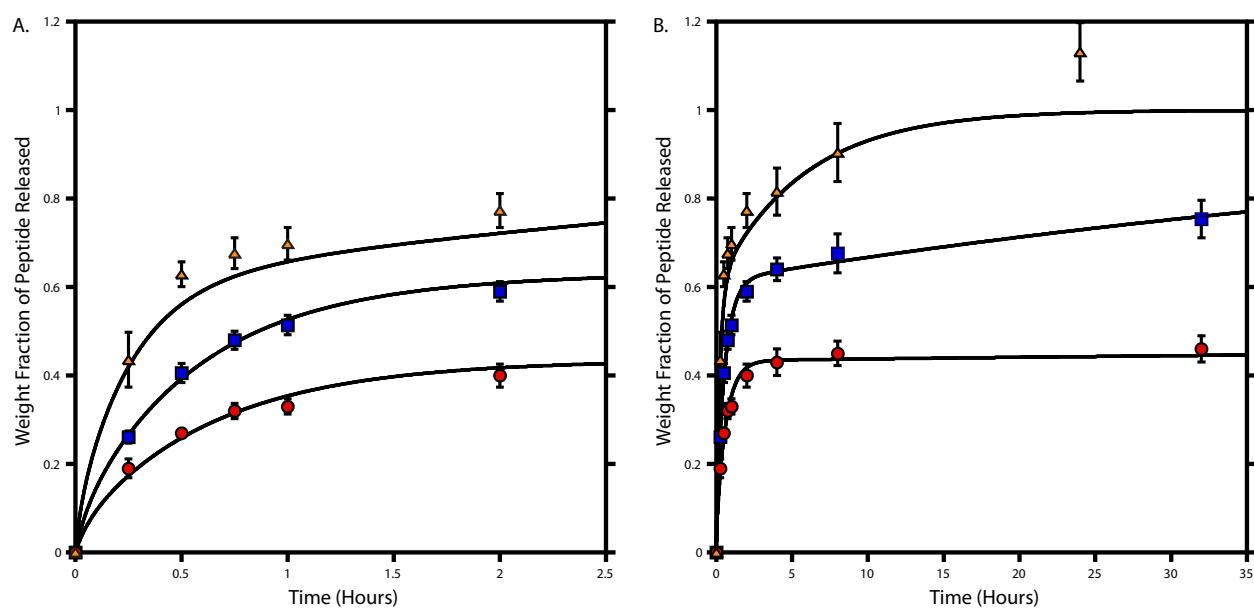


Figure 5.4. Release of 3-furan RGDS from thiol Michael addition hydrogels at several temperatures. ● 37°C, ■ 60°C and ▲ 80°C. By changing the temperature, the release profile of the

peptides labeled with a 3-furan functionality can be tailored, demonstrating the ability to release material from the hydrogels through a DA/rDA reaction. Higher temperatures incite the rDA reaction resulting in a higher quantity of free furan and maleimide species that can release from the hydrogel. This trend is supported in the figure as at 80°C the highest release rate is observed whereas at physiological temperature, the release rate is much slower. A. presents the initial release, highlighting the different release behavior at short times. B. portrays the release of 3-Furan RGDS over a longer period of time.

The quality of the model fit, possessing the same general contour and temperature dependence as the experimental data, supports a DA reaction-diffusion mechanism for the mode of release from the hydrogel.

From the nonlinear fit of the experimental data, the diffusion coefficient at 37°C for the 3-furan RGDS peptide was found to be on the order of 10^{-5} cm²/sec. This value is within the range of other small molecule species, which have diffusion coefficients ranging from 10^{-5} to 10^{-6} cm²/sec³⁷. In addition, the values of the forward (k_f) and reverse (k_r) reaction rates at various temperatures were used to approximate the activation energy for the DA and rDA reactions taking place within the polymer network. For the release from the hydrogel, the activation energy associated with the forward DA reaction was found to be 84±10kJ/mol and a value of 120±16kJ/mol was obtained for the reverse rate. These values are comparable to the activation energy reported for the DA and rDA reactions between analogous small molecules, 3-furoic and β-alanine maleimide carboxylic acids. For direct comparison, when dimethylformamide (DMF) was used as the solvent, the activation energies associated with the reaction of the small molecules were found to be 69±3kJ/mol and 123±2kJ/mol for the DA and rDA reactions, respectively³⁸. The release of the 3-furan peptide may take place in an aqueous solution; however, the carbon-based backbone of a PEG hydrogel presents an environment similar to the organic solvent DMF. Possessing similar activation energies to those exhibited by analogous

small molecules further demonstrates that the release of 3-furan RGDS from the hydrogel was mediated by the rDA reaction.

5.3.3. Control of the Diels-Alder Release Profile. In addition to making use of temperature as a means to tune the DA mediated release, it was also possible to control the release rate by varying the ratio of maleimide tethering sites in the gel to the 3-furan RGDS loading which changes the fraction of vacant maleimide sites available for recombining with furan-labeled peptide. Increasing the concentration of free maleimide groups within the hydrogel shifts the equilibrium of the reaction within the gel, resulting in an increased formation of adduct. An increase in the amount of adduct translates to a greater amount of furan-labeled peptide attached to the polymer at equilibrium, and therefore, a slower release from the hydrogel. In this case, not only does the bound material need to undergo the rDA to decouple from the hydrogel, but it has a greater probability of encountering another maleimide as it traverses through the network, resulting in reattachment to the polymer via the DA reaction. Conversely, if the amount of maleimide present in the gel is decreased a more rapid release from the hydrogel is achieved. When the free maleimide concentration is decreased, the potential for a released or free furan peptide to encounter maleimide and undergo the DA reaction to re-attach to the network is lowered, allowing for diffusion and the kinetics of the rDA reaction to dominate over the forward DA reaction and dictate the release.

To demonstrate the ability to influence the release rate by changing the number of available DA/rDA reaction sites, the amount of free maleimide in the gel was reduced by the addition of a monofunctional thiol. For this purpose cysteine, a naturally occurring amino acid that has been demonstrated to react efficiently with maleimides by means of a Michael addition

reaction^{30-32,34,35,39}, was selected as the monofunctional thiol species. As has been shown in other studies, increasing or decreasing the crosslink density of the network can have a significant impact on material release from the polymer³. By making use of a monofunctional thiol to block maleimide sites in the gel, the crosslink density of the polymer was maintained constant and only the concentration of free maleimide was varied. Figure 5.5 presents the ability to control the amount and rate of material released from a DA compatible hydrogel simply by altering the number of maleimide tethering sites present in the polymer. When the number of potential maleimide DA tethering sites within the hydrogel was decreased (lower θ value in Figure 5.5) the furan RGDS peptide was able to release from gel at a higher rate. The faster rate in this instance is attributable to a smaller number of DA/rDA reactions taking place, which impede the release of the furan labeled peptide. In contrast, when more maleimide was present (higher theta value), the release from the gel was hindered by the need for the releasing species to undergo a greater number of DA/rDA reactions prior to extricating itself from the gel.

Furthermore, the reaction-diffusion model in conjunction with the estimated parameters determined from a fit to the experimental data at 37°C, including the diffusion coefficient and forward and reverse rate constants, were used to approximate the release for the cases where the maleimide concentration was approximately 1.5 times greater and equal to the concentration of the furan peptide loaded into the hydrogel ($\theta = 1.5$ and 1 in Figure 5.5).

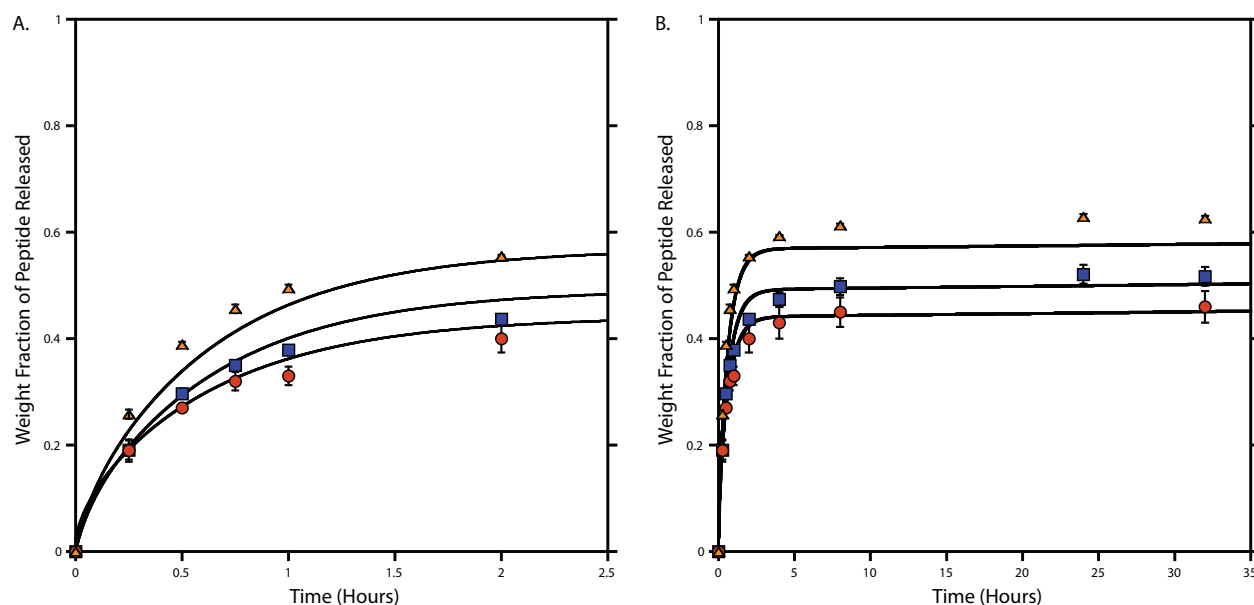


Figure 5.5. The tailoring of the Diels-Alder mediated release by reducing the amount of potential maleimide reaction sites by introduction of a monofunctional thiol, cysteine. Theta (θ) represents the ratio of potential maleimide binding sites in the gel to the initial amount of furan labeled peptide loaded. With each gel containing a 3-furan RGDS concentration of approximately $4\mu\text{M}$, θ values of 2 (●), 1.5 (■) and 1 (▲) were considered. By reducing the amount of maleimide sites at which the DA reaction could occur, the number of DA/rDA reactions that had to occur was decreased, resulting in a faster release rate.

As can be seen from Figure 5.5, the model approximation attains the same general profile as the experimental data. The model reaction diffusion equation follows the same general trajectory as the empirical data for the initial (Figure 5.5a) and long-term release (Figure 5.5b). The model fit deviates slightly from the empirical data, which is most likely due to the slow degradation of maleimide in an aqueous environment through processes such as ring-opening hydrolysis⁴⁰. When in a ring open format, the maleimide is incapable of participating in the Diels-Alder reaction, further decreasing the number of tethering sites in the network and effectively increasing the release rate.

5.4. Conclusions.

The high permeability of PEG based hydrogels allows for a high exchange rate between material in the surrounding environment and the interior of the polymer making them ideal candidates for the design and development of drug delivery platforms. To extend and apply a dynamic release mechanism from a PEG hydrogel, this study employed the thermally controlled Diels-Alder and retro-Diels-Alder reactions in the development of a controlled release platform. Rather than simply diffusing out of the network, molecules making use of the Diels-Alder reaction as a release mechanism have the ability to attach to the network by means of a reversible covalent bond. The dynamic Diels-Alder bond attaching the releasable species to the network breaks to allow release, but also has the potential to reform and reincorporate material into the polymer. By making use of a synthetic furan amino acid, a diene was readily incorporated into an RGDS peptide with the ability to partake in the DA and rDA reactions. The furan-RGDS sequence was introduced to a PEG hydrogel containing unreacted maleimide sites for the DA reaction which enabled tethering of the peptide to occur. Exposure of the hydrogel to several distinct temperatures demonstrated strongly temperature dependent release of the furan-RGDS from the hydrogel, supporting a DA/rDA mechanism. In addition to thermal control, the ability to tune the release profile of the furan-RGDS by changing the number of potential maleimide binding sites was investigated. Lowering the concentration of maleimide in the finished hydrogel was found to result in a faster release, as there were fewer sites for the DA/rDA reactions to occur. Further support of a DA/rDA reaction mechanism controlling the release was obtained by comparing the data to a reaction-diffusion model. Through the use of DA and rDA reactions between maleimide and furan, this investigation has presented a unique and facile method of extending control over the release of material from a PEG hydrogel.

5.5. Acknowledgements

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Chapter 6

A Diels-Alder Modulated Approach to Control and Sustain the Release of Dexamethasone and Induce Osteogenic Differentiation of Human Mesenchymal Stem Cells*

In this study, we report a new approach to controlled drug release based upon exploiting the dynamic equilibrium that exists between Diels-Alder reactants and products, which we have used to control the release of a furan containing dexamethasone peptide (dex-KGPQG-furan) from a maleimide containing hydrogel. Using a reaction-diffusion model, we were able to tune the release kinetics to achieve sustained concentrations conducive to osteogenic differentiation of human mesenchymal stem cells (hMSCs). Efficacy was first demonstrated in a 2D culture model, in which dexamethasone release induced significant increases in alkaline phosphatase (ALP) activity and mineral deposition compared to a dexamethasone free treatment. The results were similar to what was observed with soluble dexamethasone treatment. More drastic results were seen in 3D culture, where co-encapsulation of a dexamethasone releasing hydrogel within an hMSC-laden extracellular matrix mimetic poly(ethylene glycol) hydrogel resulted in robust osteogenic differentiation. ALP activity reached levels that were up to six times higher than the dexamethasone free treatment. Interestingly, at 5 and 10 day time points the ALP activity exceeded the dexamethasone positive control, suggesting a potential benefit of sustained release in 3D culture. At 21 days, substantial mineralization comparable to the positive control was also observed in the hydrogels. Collectively, these results demonstrate Diels-Alder modulated release as an effective and powerful new platform for controlled drug delivery.

* Collaboration between Koehler, K.C. and Alge, D.L.

6.1. Introduction

Dexamethasone is a potent synthetic corticosteroid that has found widespread use in a variety of medical and biological applications. Clinically, dexamethasone has been used as an anti-inflammatory agent to treat conditions such as rheumatoid arthritis^{1,2}, cerebral edema³ and altitude sickness⁴. Dexamethasone has also found utility in oncology, as it has been shown to alleviate treatment side effects^{5,6} and bolster the efficacy of the cancer therapy⁷. In addition to these clinical uses, dexamethasone is also known to be a powerful morphogen and is routinely used to induce the differentiation of multipotent mesenchymal stem cells (MSCs). For example, dexamethasone is a key ingredient in osteogenic differentiation medium used to differentiate MSCs into osteoblasts. Typical dexamethasone concentrations for inducing osteogenic differentiation of MSCs are on the order of 100nM⁸⁻¹⁰. Dexamethasone is also used to induce adipogenic differentiation of MSCs. However, the concentration used to induce adipogenesis is an order of magnitude higher than for osteogenic differentiation⁸, highlighting the dose dependent effects of dexamethasone.

In order to maximize therapeutic efficacy and mitigate undesired side effects⁹⁻¹¹, there is a critical need for the development of biomaterial strategies to control the time-dependent release of pharmaceuticals. To address this need, a myriad of polymeric controlled release platforms have been developed and applied to dexamethasone. Perhaps the simplest technique for modulating release entails loading dexamethasone into a polymer substrate from which the pharmaceutical is able to diffuse and reach cells in the surrounding vicinity¹¹⁻¹³. In this approach, the diffusional release can be controlled to a certain extent by varying the composition of the polymer, by changing the crosslink density, or through the inclusion of other materials such as

organosilicates^{14,15}. However, only limited control over the release kinetics is possible with this approach, where an initial burst release and an inability to sustain delivery of a given material for a long period of time can be problematic.

Another approach has been to covalently attach dexamethasone to a polymer network through degradable linkages. For example, Nuttelmann *et al.* made use of hydrolytically labile lactide ester bonds to control the release of dexamethasone from hydrogels and showed that the length of the linker and number of ester bonds could be used to tune the release kinetics¹⁶. More recently, Webber, Stupp, and co-workers reported dexamethasone conjugation to self-assembling peptide amphiphile gels via hydrolysable hydrazone linkages as a means for sustained dexamethasone release¹⁷. A similar strategy for dexamethasone release was reported by Liu *et al.*¹⁸. However, the dependence on pH and susceptibility to degradation by enzymes are important limitations of these approaches that can potentially result in deviations from predicted release kinetics. More specific release of covalently tethered dexamethasone has been accomplished through the use of enzymatically degradable linkages. Yang *et al.* used this approach to release dexamethasone in response to matrix metalloproteinase (MMP) secretion by cells¹⁹. While this cell-mediated release mechanism overcomes some of the limitations associated with hydrolytically cleavable bonds, MMP expression is ubiquitous in tissue remodeling and can vary widely^{20,21}, making it difficult to predictably tune the drug release kinetics with this approach.

Recognizing the limitations of diffusion, hydrolytic, and enzymatic based release mechanisms, we sought to develop a biomaterial platform that would allow for tunable, predictable, and sustainable dexamethasone release. To achieve this goal we turned to the use of a Diels-Alder reaction. In a traditional sense, the Diels-Alder (See Figure 6.1b) reaction is

thermally reversible: low temperatures promote the generation of the Diels-Alder adduct or product whereas elevated temperatures incite reversion to the reactant species. However, because a dynamic equilibrium exists between the product and reactants, we hypothesized that the reversibility of the Diels-Alder reaction could be exploited to tune and sustain dexamethasone release from a hydrogel without the need to apply potentially detrimental elevated temperatures. Importantly, removal of reactants as accomplished by either diffusion out of the network or through cellular metabolism will result in reversion of the adduct to the reactants, thereby promoting continual release. However, unlike other controlled delivery platforms in which release is diffusion controlled once hydrolytically or enzymatically labile bonds are severed, the Diels-Alder mechanism allows for bonds to reform and the releasing species can be re-incorporated into the network, presenting a unique and powerful means for tuning drug release kinetics. To demonstrate proof of concept for Diels-Alder mediated dexamethasone release, we have synthesized a furan-modified dexamethasone that was readily incorporated into maleimide containing poly(ethylene glycol) (PEG) hydrogels. As this release platform has not been previously implemented in biological applications, the bioactivity of the furan modified dexamethasone was first verified. The ability to sustain release from a hydrogel and induce osteogenic differentiation of human MSCs (hMSCs) *in vitro* was then explored in 2D and 3D culture.

6.2. Materials and Methods.

6.2.1. Materials.

The following materials were acquired from their respective vendors and, unless otherwise indicated, used without further purification or modification. The main components of

the hydrogel networks created as part of this investigation, a tetrafunctional maleimide and thiol PEG macromers were acquired from Laysan Bio (Arab, AL). Octa-functional PEG norbornene was synthesized from a hydroxylated PEG precursor purchased from JenKem Technology USA (Allen, TX) following a published synthetic route²². The di-cysteine peptide crosslinker KCGPQGIWGQCK and mono-cysteine cell adhesive peptide CRGDS were purchased from American Peptide Company (Sunnyvale, CA). The active pharmaceutical considered in the release studies, dexamethasone, was bought from Enzo Life Sciences (Farmingdale, NY). Fmoc protected amino acid residues O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate activator (HBTU), and Rink Amide MBHA resin used for solid phase peptide synthesis were purchased from ChemPep, Inc. (Wellington, FL). All other chemicals required for peptide synthesis were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture reagents were purchased from Life Technologies (Grand Island, NY) except for recombinant human fibroblast growth factor-2 (FGF-2), which was purchased from Peprotech (Oak Park, CA). The alkaline phosphatase staining kit (i.e., Leukocyte Alkaline Phosphatase Kit) and alkaline phosphatase substrate solution (i.e., p-nitrophenyl phosphate) were purchased from Sigma-Aldrich. dsDNA in cell lysates was quantified using the Quant-iT™ Picogreen kit from Life Technologies. The calcium detection kit for measuring mineral deposition was purchased from Point Scientific (Canton, MI).

6.2.2. Synthesis of Dexamethasone Functionalized, Diels-Alder Reactive Peptide

To achieve Diels-Alder mediated dexamethasone release, a peptide with the sequence KGPPQ-furan was synthesized using standard Fmoc-mediated solid phase peptide synthesis methods on a Protein Technologies (Tucson, AZ) automated Tribute Bench Peptide Synthesizer.

The 3-furoic functionality was directly incorporated during the solid phase synthesis using a synthetic Fmoc protected amino acid analogue. Details pertaining to the synthetic route to obtain the Fmoc protected 3-furoic amino acid and its inclusion into the peptide are provided elsewhere²³. Dexamethasone NHS ester was then introduced to the resin-bound peptide sequence and allowed to react with the N-terminal primary amine overnight. Carbamate bond formation and dexamethasone conjugation was confirmed via a qualitative Kaiser test. Importantly, similar conjugation schemes have previously been reported and demonstrated no adverse affect on dexamethasone bioactivity. The reactive dexamethasone-NHS ester was prepared according to procedures previously presented^{19,24}.

After synthesis and final modification, the dexamethasone-GQPGK-3-furan peptide was cleaved from the solid support using a mixture of trifluoroacetic acid, triisopropyl silane and water (95/2.5/2.5 v/v) and precipitated in cold diethyl ether. The crude peptide was purified by means of high performance liquid chromatography (HPLC) using a reverse-phase C-18 column on a Waters Delta Prep 4000 system and a linear gradient beginning at a ratio of 95/5 water/acetonitrile ramping over 70 minutes to pure acetonitrile. Confirmation of synthesis and isolation of the target peptide species was achieved through matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry. Prior to performing release studies, bioactivity of the dexamethasone peptide was confirmed via an *in vitro* osteogenic differentiation assay with hMSCs (see Section 2.5).

6.2.3 Diels-Alder Compatible Hydrogels for Dexamethasone Release

Maleimides can participate in Diels-Alder reactions with furans. Thus, to be compatible with our Diels-Alder modulated release mechanism, the base hydrogels for dexamethasone

release were crosslinked through the Michael addition reaction of 10k molecular weight tetrafunctional maleimide and thiol PEG macromers (see Figure 6.1, Section 6.3.1). Prior to hydrogel polymerization, the furan modified dexamethasone peptide sequence (see Section 2.4.) was introduced to a 20 wt. % stock solution of PEG tetra-maleimide macromer, creating an initial mixture containing furan at a concentration of approximately 5mM (see Section 3.1). The resulting maleimide PEG macromer and furan dexamethasone peptide were allowed to react for at least 24 hours at ambient temperature. This process allowed for the formation of covalent Diels-Alder linkages between the furan-modified dexamethasone and maleimide macromer. Briefly, the PEG tetra-maleimide was used at a working concentration of 7.5wt. % (23 mM maleimide) and PEG tetra-thiol working concentration was 2.5wt. % (17mM thiol) in phosphate buffered saline (PBS). In order to prevent premature polymerization, the pH of the macromer solution was adjusted to approximately 2 to inhibit the thiol-maleimide Michael addition reaction. The reaction was catalyzed by the addition of 3 μ L of 300mM triethanolamine to 30 μ L of hydrogel precursor solution. Polymerization was complete within approximately 15 minutes, as ascertained by a negative qualitative Ellman's assay. Prior to their use in cell studies, the hydrogels were stored in PBS for 24-48hrs and then washed twice with PBS to remove triethanolamine.

6.2.4 Cell Culture

hMSCs were isolated from the mononuclear fraction of human bone marrow specimens (purchased from Lonza) based on their adherence in culture, as previously described. Cells were subcultured under standard conditions of 37°C and 5% CO₂ in medium consisting of low glucose Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum, 1%

penn/strep, and 1ng/ml recombinant human FGF-2. The cells were passaged via treatment with trypsin/EDTA upon reaching approximately 70-80% confluence. Passage 3-4 cells were used for all experiments.

6.2.5 2D Osteogenic Differentiation

hMSCs were seeded in 24 well plates at 18,500 cells/cm² and cultured overnight in growth media (GM) consisting of low glucose DMEM supplemented with 10% FBS and 50U/mL each penicillin/streptomycin. The media was then replaced with a base osteogenic differentiation media (OST) consisting of high glucose DMEM supplemented with 10% FBS, 50U/mL each penicillin/streptomycin, 20mM β -glycerophosphate, and 50 μ M ascorbic acid. Hydrogels releasing dexamethasone, which were polymerized in 1 ml syringe molds (i.e., syringes with tips removed) in order to have a uniform cylindrical geometry, were then suspended in the media above the plated cells using BD Falcon cell culture transwell inserts (transparent, PET membranes with 1.0 μ m pores). OST media supplemented with 0 and 100nM dexamethasone were used as negative and positive controls, respectively. GM was also used as a negative control.

6.2.6 3D Osteogenic Differentiation

Dexamethasone releasing hydrogels were formed by placing a rubber gasket mold (5 mm diameter, 500 μ m thickness) on a thiolated glass cover slip and filling them with the PEG macromer solution. Glass thiolation was achieved using 3-mercaptoptrimethoxysilane and a liquid phase silanization protocol, as previously described²⁵. This procedure covalently attached the cylindrical hydrogel to the glass surface through a thioether linkage. hMSC laden

extracellular matrix mimetic hydrogels were subsequently polymerized around the dexamethasone releasing hydrogel. These hydrogels were polymerized using photoinitiated thiol-ene chemistry, as previously described^{22,26}. Briefly, 40 kDa octa-functional PEG-norbornene at 7.5 wt. % (15 mM norbornene) was combined with 7 mM KCGPQGIWGQCK crosslinker peptide, 1mM CRGDS cell adhesive peptide, and 2 mM lithium acylphosphinate photoinitiator²⁷. hMSCs suspended in PBS were added to the macromer solution so that the final cell density was 2.5×10^6 cells/mL. 100 μ L of thiol-ene hydrogel solution was transferred to rubber gasket molds (10 mm diameter, 4 mm thickness) that were placed on top of the dexamethasone releasing hydrogels. The cell-laden hydrogels were then polymerized under UV light (365 nm, ~ 4 mW/cm²) for 3 min and transferred to OST media (0 mM dexamethasone) for culturing. As controls, hMSC-laden hydrogels were polymerized using an identical formulation and cultured in GM and OST media with 0 and 100 nM dexamethasone.

6.2.7 Alkaline Phosphatase (ALP) Activity

For the 2D osteogenic differentiation experiment, hMSC differentiation was qualitatively assessed after 14 days by staining for alkaline phosphatase (ALP). Prior to staining, the cells were fixed in neutral buffered formalin for 30 min at 4°C. The cells were then washed with PBS and stained for approximately 45 min using a commercially available histochemical staining kit for alkaline phosphatase according to the manufacturer's protocol.

For both the 2D and 3D osteogenic differentiation experiments, ALP activity was quantitatively evaluated using a colorimetric assay that is based on the conversion of p-nitrophenyl phosphate to p-nitrophenol by alkaline phosphatase. For the 2D experiment, the cells were washed with PBS and then lysed by treatment with 500 μ L of 1X RIPA buffer. For the 3D

experiment, the gels were washed twice for 20 min with PBS, transferred to a microtube with 300 μ L of RIPA buffer, and then homogenized with a pestle. The ALP activity of all samples was determined by combining 50 μ L of lysate, 50 μ L of 1X RIPA buffer, and 100 μ L of alkaline phosphatase substrate (i.e., p-nitrophenyl phosphate) in a clear bottom 96 well plate and monitoring the change in absorbance at 405nm over 10 min with 1 min intervals on a BioTek Synergy H plate reader. Special care was taken to ensure that the absorbance versus time curves were linear for all samples. The mean slopes of the curves were taken to be the relative ALP activities. To account for variation in cell number between gel samples, the 3D data were normalized to the dsDNA concentration in the cell lysate. Data normalization was not performed for the 2D study. All ALP activity values are reported as fold increases relative to day 0.

6.2.8 Mineral Deposition

Osteogenic differentiation was also quantitatively assessed on the basis of mineral deposition. For the 2D experiment, wells were treated with 250 μ L of 1 M HCl at 4°C for 72 hrs to dissolve any calcium phosphate mineral present. For the 3D experiment, the remaining volume of homogenized hydrogel samples after performing the ALP activity assay was acidified with 50 μ L of 12 M HCl and stored at 4°C for 72 hrs. To quantify mineralization, the acidified solutions were diluted with phosphate buffered saline (PBS; dilutions were 1:4 and 1:10 for the 2D and 3D experiments, respectively). 50 μ L of diluted solution was then combined with 100 μ L of calcium reagent solution in a clear bottom 96 well plate. The absorbance values at 570nm for each sample were then measured on a plate reader and compared to a calcium phosphate standard curve in order to determine the Ca²⁺ concentration. Special care was taken to ensure

that the most concentrated samples fell within the linear response range of the assay. Final values are reported as the total mass of calcium deposited for each sample.

6.2.9 Statistical Analysis

Quantitative data for ALP activity and mineral deposition are presented as the average \pm standard error. Data analysis was performed using the open source statistical software package R (available for free at www.r-project.org). At each time point, the data were analyzed by means of a one-way ANOVA to determine the effects of media treatment. Significant differences between groups were determined by post hoc Tukey comparisons ($\alpha = 0.05$).

6.3. Results and Discussion

6.3.1. Diels-Alder Chemistry Enables Sustained Release

Numerous controlled drug release strategies have been developed and are described in the literature. For dexamethasone specifically, hydrolytically mediated release and cell-mediated release from biomaterials have been achieved via covalent tethering of the drug molecule through labile ester and hydrazine bonds and enzymatically degradable peptide sequences, respectively^{16,17,19}. Although effective, these approaches are limited in their ability to predictably tune the release kinetics. Ester and hydrazone hydrolysis are pH dependent. Therefore, hydrolytically mediated release is sensitive to changes in the local cellular microenvironment, which can lead to deviations from predicted release profiles. While controlled release through enzymatically degradable linkers mitigates this problem to a certain extent, this approach is still dependent on cellular activity, which can vary widely and is difficult to accurately model mathematically. In an effort to circumvent these problems and develop a platform capable of

sustained release, we have turned to Diels-Alder chemistry. Importantly, a dynamic equilibrium exists between Diels-Alder reactants and products. This equilibrium can be exploited to control the release of covalently tethered molecules from a hydrogel biomaterial in a predictable manner²³. Furthermore, because Diels-Alder reactive moieties are not naturally present in biological systems, this chemistry is pseudo-bioorthogonal and is insensitive to changes in the cellular microenvironment.

To achieve a Diels-Alder modulated release, a suitably functionalized hydrogel platform was developed using PEG maleimide and thiol functionalized macromers, which were reacted via standard Michael addition chemistry (Figure 6.1A). The Michael addition between these two species is a fast and efficient reaction that has been implemented in a number of reactions including surface functionalization²⁸, protein crosslinking²⁹ and fluorescent labeling³⁰. In our application, however, the maleimide and thiol groups were reacted in an off-stoichiometric manner such that thiol was the limiting reagent. Consequently, hydrogels with residual, unreacted maleimide functional groups were formed. The presence of unreacted maleimide was a key aspect of the network design, as this functionality not only possesses the ability to react with thiols to form the crosslinked network, but can also serve as the dienophile in a Diels-Alder reaction (Figure 6.1B). Thus, when a molecule bearing a diene functionality (e.g., furan) comes into contact with the active maleimide sites in the hydrogel, there exists the potential for the formation of a Diels-Alder adduct, thereby reversibly anchoring the species to the network through a covalent bond (Figure 6.1F).

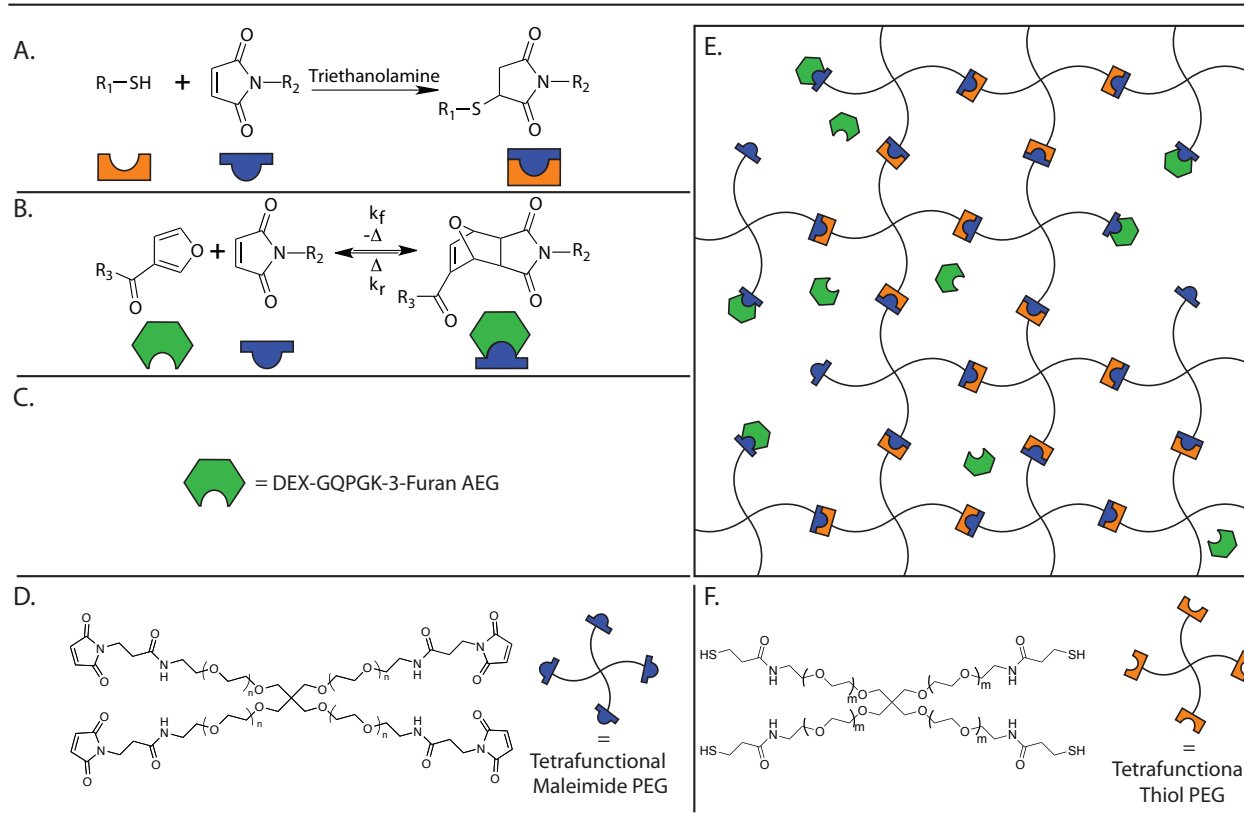


Figure 6.1. Design and development of a hydrogel compatible with Diels-Alder modulated release. A. The Michael addition reaction between thiol and maleimide functionalities. B. The Diels-Alder reaction between a generic furan (diene) and maleimide (dienophile) species. C. A dexamethasone labeled peptide sequence equipped with a Diels-Alder reactive furan functionality. D and F. Tetrafunctional maleimide and thiol PEG macromers. Reaction of these species in an off stoichiometric ratio where thiol was the limiting reactant allowed for the creation of sites at which the Diels-Alder reaction could occur. E. Schematic representation of the network created as a result of a Michael addition between (D) and (E) and including (C) as a releasable species.

To predict dexamethasone release using Diels-Alder chemistry, we employed a Diels-Alder reaction diffusion mathematical model that was previously developed based on experimental data²³. Although the experimental data was not collected for our dexamethasone peptide, the model peptide (i.e., fluorescein-RGDSF) was nearly identical in molecular weight. The hydrogel network was also identical to what we have used here, and the model peptide bore the same 3-furoic amino ethyl glycine functionality, as well as a large hydrophobic moiety similar to dexamethasone. For these reasons, the model is suitable as a predictive tool for furan

dexamethasone peptide release. An initial loading concentration of 0.5mM dexamethasone peptide was chosen and used to predict the release of 5 days (Figure 6.2). Analyzing the release trajectory, it is clear that an initial burst of furan dexamethasone peptide occurs over the first 24 hours, during which approximately 50% of the loaded peptide is released. This burst is most likely due to the equilibrium position of the Diels-Alder reaction, whereby some of the peptide is not bound to the hydrogel and rapidly leaves the system by a diffusion driven process. Importantly, the initial burst release is followed by a linear, long-term release profile that allows for a predictable concentration of the furan dexamethasone peptide to be maintained. Based on the model, over the course of twenty-four hours the concentration of peptide will rise to a value on the order of 100nM, which is the concentration typically used to induce hMSC to undergo osteogenic differentiation⁸. Furthermore, according to the model, release at this rate can be maintained almost indefinitely, illustrating that Diels-Alder modulated release is suitable for sustaining dexamethasone release.

6.3.2. Diels-Alder Reactive Dexamethasone is Bioactive

The Diels-Alder reactive dexamethasone peptide designed in this study was synthesized via coupling of the primary hydroxyl functionality of dexamethasone to the N-terminus of a synthetic peptide (Figure 6.2A). Multiple studies have reported that dexamethasone modification at this location is not detrimental to bioactivity. Nevertheless, prior to testing the efficacy of the Diels-Alder modulated release platform, it was first necessary to verify the bioactivity of our dexamethasone peptide. In addition to the peptide modification, which is similar to that used by Yang *et al.*¹⁹, the inclusion of the 3-furoic functionality was of particular concern, as this functionality has not previously been utilized in cellular applications. Importantly, after 7 days

in 2D culture, hMSCs treated with osteogenic media containing 100nM dexamethasone-KGPGK-furan were healthy and appeared morphologically identical to control cells (data not shown).

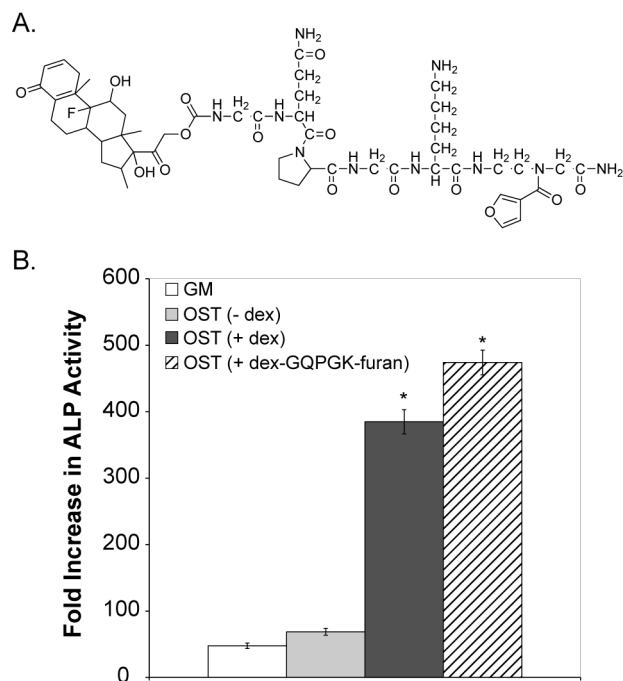


Figure 6.2. Diels-Alder reactive dexamethasone peptide induces hMSC osteogenic differentiation. A. Structure of the furan functionalized dexamethasone peptide. B. Fold increase in ALP activity after hMSCs were treated for 7 days. The concentration of dex-GQPGK-furan was 100 nM. * indicates statistical significance compared to the other experimental groups ($n = 5$; $p < 0.05$).

Furthermore, the dexamethasone peptide treatment significantly increased the ALP activity compared to the negative controls and the effect was similar but slightly increased compared to that observed for cells treated with 100nM dexamethasone (Figure 6.2B). As ALP activity is a key indicator of hMSC osteogenic differentiation³¹, these results indicate that the peptide modification and furan functionalization did not impair the drug's bioactivity.

6.3.3 Diels-Alder Modulated Dexamethasone Release Induces Osteogenic Differentiation of hMSCs in 2D and 3D Culture

To test the efficacy of the Diels-Alder modulated release platform, transwell inserts were used to suspend dexamethasone peptide releasing hydrogels above hMSCs in 2D culture. To avoid the elevated concentrations associated with the initial burst release and operate the Diels-Alder release solely in the linear region, furan dexamethasone peptide loaded hydrogels were placed in PBS buffer solution for approximately 36 hours prior to introduction to cell culture. As can be noted by the vertical line in Figure 6.2, the release is well into the linear phase after 36 hours.

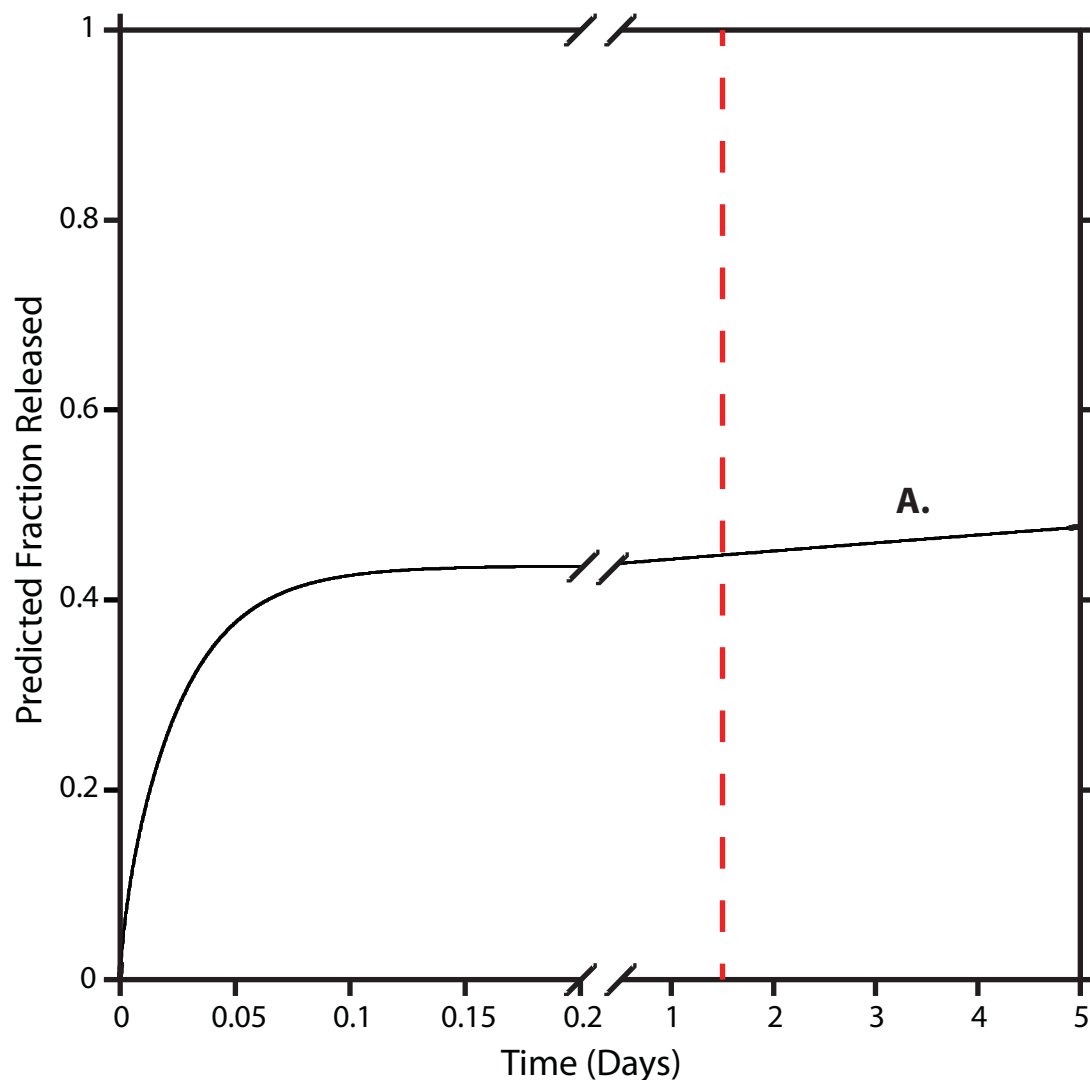


Figure 6.3. Simulated results predicting the release profile of the furan dexamethasone peptide sequence from the maleimide-thiol hydrogel platform. The red dashed line indicates the time at which the furan dexamethasone loaded hydrogels were introduced to two and three dimensionally cultured hMSC's after being introduced to PBS buffer to avoid the large initial burst of dexamethasone and operate in the linear release regime. A. The dexamethasone release rate in the linear regime was calculated to be approximately $0.5\mu\text{g/day}$.

Thus, introduction of the hydrogels at this time allowed for the release to gradually increase the amount of dexamethasone present in the media over time. Given the results forecast by the model, the sustained release achieved using the Diels-Alder platform results in hMSCs being continually supplied with dexamethasone at levels conducive to osteogenic differentiation.

Indeed, robust hMSC osteogenic differentiation in standard 2D culture conditions was achieved using Diels-Alder modulated dexamethasone release from hydrogels. Osteogenic differentiation was first observed qualitatively by staining for ALP after 14 days in culture (Figure 6.4A). As expected, minimal staining was observed for cells cultured in GM, as well as OST media lacking dexamethasone. However, intense blue/purple staining was seen for cells cultured in OST media and exposed to dexamethasone releasing hydrogels. Similar staining was observed for hMSC cultured in OST media supplemented with 100nM dexamethasone. Quantitative analysis of ALP activity further supported the efficacy of Diels-Alder modulated dexamethasone release platform.

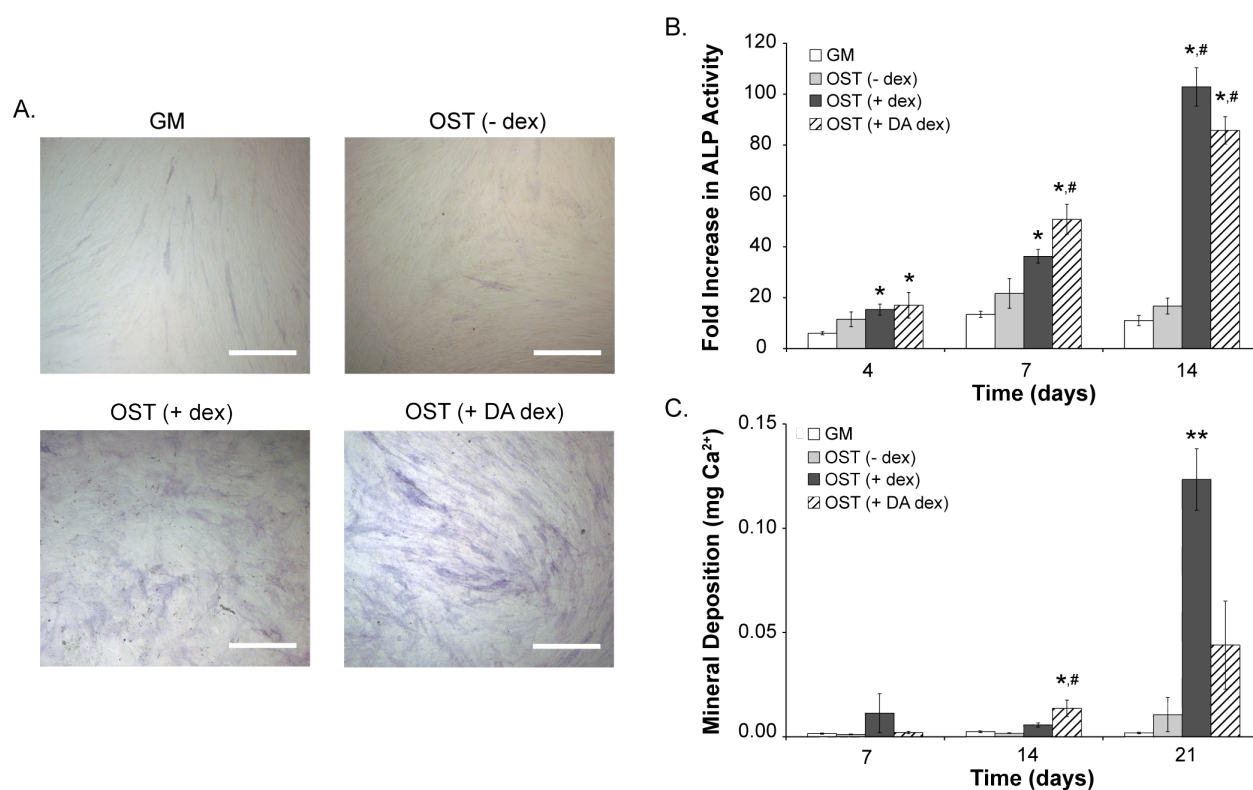


Figure 6.4. Diels-Alder modulated dexamethasone release induces hMSC osteogenic differentiation in 2D culture. A. Representative images from ALP stain after 14 days (scalebars = 500 μm). B. Fold increase in ALP activity after hMSCs were treated for 4, 7, and 14 days. C. Mineral deposition after hMSCs were treated for 7, 14, and 21 days. Note: DA = Diels-Alder; * indicates significance compared to the GM treatment; # indicates significance compared to the

OST(- dex) treatment; ** indicates significance compared to all other treatments (n = 6 samples pooled from three independent experiments; p < 0.05).

While hMSCs cultured in GM and OST minus dexamethasone only showed marginal increases in ALP activity from baseline levels over the 14 day time course, exposure to Diels-Alder modulated dexamethasone peptide releasing hydrogels resulted in a significant increases in ALP activity at 7 and 14 days, similar to what was observed for the positive control (Figure 6.4B). Significantly increased mineral deposition by the cells cultured with dexamethasone releasing hydrogels was seen at 14 days. However, at 21 days the extent of mineralization was lower than that seen for cells cultured OST with 100nM dexamethasone (Figure 6.4C).

While the results of the 2D study support the ability of the Diels-Alder reaction to controllably release and sustain the levels of a bioactive material from a suitably functionalized network, we also sought to demonstrate efficacy in 3D cell culture. Unfortunately, despite published work on maleimide-thiol Michael addition hydrogels³², this crosslinking chemistry was not suitable for 3D culture in our work due to poor cytocompatibility, presumably due to the high concentration of unreacted maleimides in our hydrogel formulation. Thus, to investigate osteogenic differentiation in 3D culture, we turned to a gel within a gel system in which a dexamethasone peptide releasing hydrogel was encapsulated within an hMSC-laden hydrogel (Figure 6.5A). The hMSC-laden hydrogels, which consisted of PEG as well as ECM mimetic synthetic peptides to allow for cell adhesion and cell-mediated degradation, were photocrosslinked with cytocompatible thiol-ene chemistry²². This approach minimized cellular exposure to unreacted maleimides and enabled us to test the effects of Diels-Alder modulated dexamethasone release in 3D. The dexamethasone releasing hydrogels were fabricated using the

same formulation as for the 2D experiments, and were pre-treated similarly in PBS to maintain a steady dexamethasone release rate at osteoinductive concentrations.

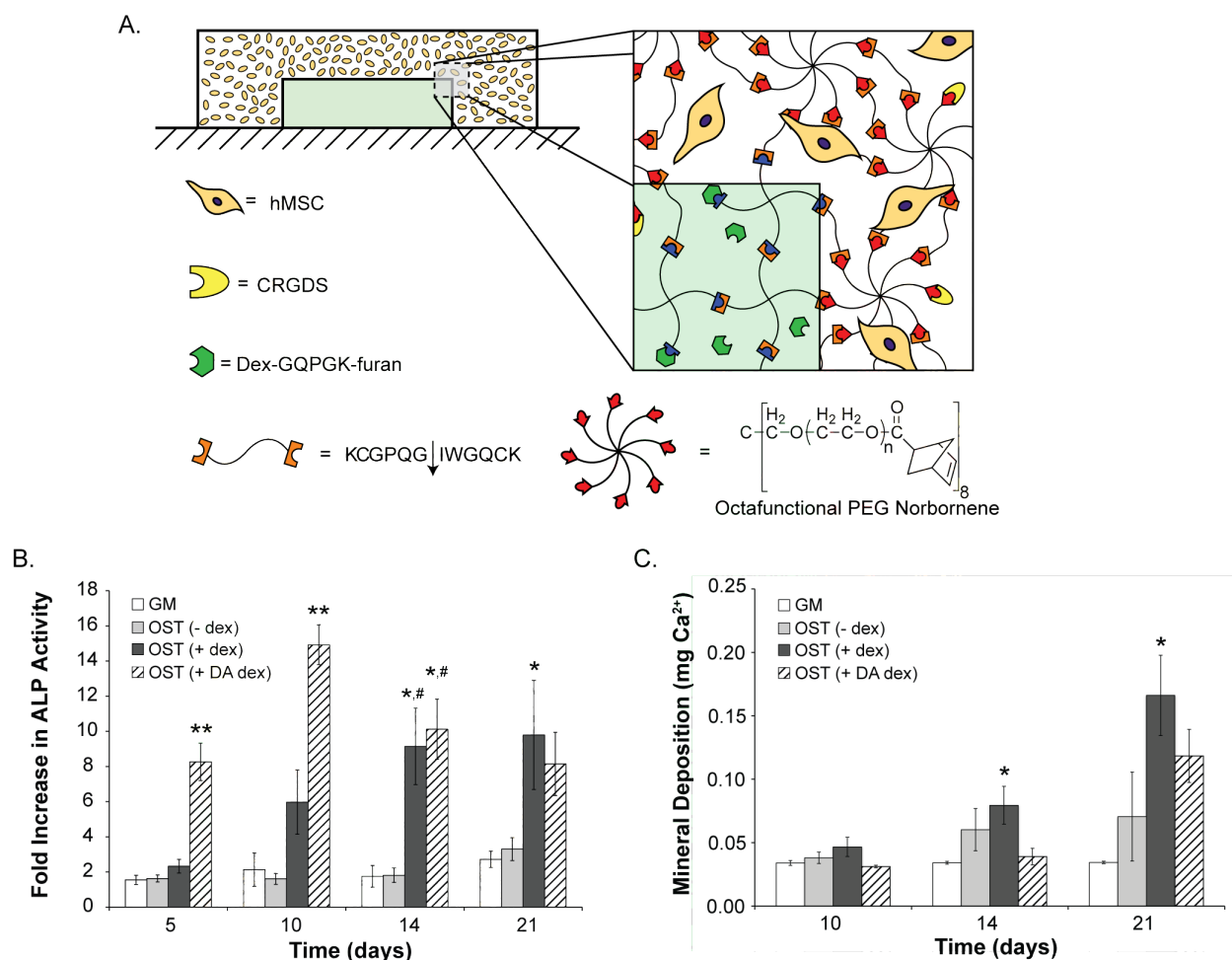


Figure 6.5. Diels-Alder modulated dexamethasone release induces hMSC osteogenic differentiation in 3D culture. **A.** Schematic of the experimental setup. Dexamethasone releasing hydrogels were encapsulated within an hMSC laden, peptide functionalized PEG hydrogel. **B.** Fold increase in ALP activity after hMSCs were treated for 5, 10, 14, and 21 days. ALP activity was normalized to dsDNA content to account for differences in cell seeding number. **C.** Mineral deposition in the hydrogels after hMSCs were treated for 10, 14, and 21 days. Note: DA = Diels-Alder; * indicates significance compared to the GM treatment; # indicates significance compared to the OST(- dex) treatment; ** indicates significance compared to all other treatments (n = 6 samples pooled from three independent experiments; p < 0.05).

As in the 2D study, Diels-Alder modulated sustained release of dexamethasone efficiently induced osteogenic differentiation of hMSCs in 3D culture. Measurement of ALP activity, which was normalized to dsDNA content in order to account for any differences in cell

number within the hydrogel samples, showed significant increases in differentiation compared to the negative controls (i.e., GM and OST media without dexamethasone) at 5, 10, and 14 days (Figure 6.5B). Interestingly, the ALP activity values for the Diels-Alder modulated dexamethasone release exceeded those of the OST plus 100 nM dexamethasone treatment at the 5 and 10 day time points, suggesting enhanced efficacy in 3D culture. This difference could possibly be due to enhanced cellular uptake resulting from the peptide modification. When added in solution, the dexamethasone peptide slightly increased ALP activity at 7 days compared to an equal concentration of unmodified dexamethasone. Alternatively, the increased ALP activity in the 3D study could also be the result of proximity and increased bioavailability in the 3D system, since dexamethasone was continually diffusing out to the encapsulated hMSC from the inner hydrogel. In addition to the increased ALP activity, substantial mineralization was also observed at 21 days. Mineralization in the OST minus dex treatment was also observed, most likely due to the presence of β -glycerophosphate in the media. However, the extent of mineral deposition in response to the Diels-Alder mediated dexamethasone release was comparable to the positive control, and these two groups were not significantly different. Collectively, the results of both the 2D and 3D studies clearly demonstrate that the Diels-Alder modulated release platform was effective for controlling dexamethasone release and inducing hMSC osteogenic differentiation.

6.4. Conclusions

The dynamic nature of the Diels-Alder reaction can be exploited to controllably release covalently tethered drug molecules in a manner uncharacteristic of traditional platforms. Here, we have demonstrated proof of concept for Diels-Alder modulated controlled release using

dexamethasone. Specifically, we conjugated dexamethasone to a furan containing synthetic peptide to achieve sustained release from a maleimide containing hydrogel. Using a predictive model, we were able to tune the dexamethasone release rate and achieve concentrations suitable for inducing osteogenic differentiation of hMSCs. In 2D culture, Diels-Alder mediated dexamethasone release stimulated hMSCs to undergo osteogenic differentiation, as indicated by increased ALP activity and mineral deposition. Robust osteogenic differentiation was also achieved in 3D culture when a dexamethasone releasing hydrogel was encapsulated within an hMSC-laden hydrogel. ALP activity was significantly increased, even compared to the positive control at 5 and 10 days. Substantial mineralization was also observed after 21 days. Based on these results, the dexamethasone release platform we have developed here could be useful for bone tissue engineering applications. However, it should be noted that the release could just as easily be tuned for other applications in which sustained dexamethasone release is required, such as suppressing chronic inflammation. In addition, it may be possible to further tune drug release using other diene/dienophile species that participate in Diels-Alder and retro-Diels-Alder reactions. Importantly, opportunities beyond dexamethasone also exist, as a similar Diels-Alder strategy could feasibly be applied to control the release of other drug molecules.

6.5. Acknowledgements

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Chapter 6: A Diels-Alder Modulated Approach to Control the Release of Dexamethasone to Induce Osteogenic Differentiation in Human Mesenchymal Stem Cells

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Chapter 7

Conclusions and Recommendations for Future Work

7.1. Introduction

Peptides present the capability to elicit biological responses comparable to a larger protein sequence from which they were derived; a feat smaller synthetic molecules can only attempt to imitate^{1,2}. In addition, peptide sequences can easily be synthesized and produced due to the advent and advances in solid phase synthetic techniques^{3,4}. The use of peptides in a number of biological applications, including the development of therapeutics and materials is often limited due to stability issues and insufficient or incompatible functional groups for the manipulation of the sequence. Much work has been done to address the shortcomings associated with peptide stability and functionality. The main approach has been to incorporate new functionalities onto the peptide as either side chain protecting groups or through the post-synthetic addition of a new reactive group to other, compatible moieties on the amino acid residues comprising the sequence⁵⁻⁷. In particular, interest has arisen in the decoration of a peptide sequence with click chemistry compatible functional groups. Click chemistry, with reactions that rapidly reach high conversion, produces few or easily removed side products, occurs under mild reaction conditions such as in an aqueous environment⁸, and is well suited for biological applications. This thesis sought to expand the use and accessibility of click chemistry to promote the structural manipulation and incorporation of peptides into other materials. To this end, new synthetic amino acids compatible with the Michael addition and Diels-Alder click reactions were developed to foster manipulation and control over peptide properties, structure and function.

7.2. Clickable Amino Acid Development

The first goal associated with achieving the overall thesis objective entailed the design and development of amino acid species capable of participating in the Michael addition and Diels-Alder reactions. By developing clickable amino acids, rather than post synthetically modifying a peptide, the potential to incorporate a given click functionality directly into a growing peptide sequence was realized. In this investigation, maleimide and furan functional groups were selected and implemented for a majority of the click reactions considered. These functionalities have demonstrated both the ability to participate in the Diels-Alder reactions in a number of different pursuits⁹⁻¹². In addition, the maleimide functional group has the capacity to react not only as a dienophile in the Diels-Alder reaction, but also as an electron deficient vinyl species in a Michael addition. Examples of the use of the reaction between maleimide and thiol can be seen in a number of applications including polymer science^{13,14}, surface modification, and various biological endeavors^{5,13,15,16}.

As a number of different furan and maleimide derivatized species exist, the first step involved determining a pair that could participate in the Diels-Alder reaction. Portrayed in Chapter Three, the Diels-Alder reaction between various maleimide and furan species was assessed in terms of their thermodynamic and kinetic properties in an effort to identify potential candidates for the creation of synthetic clickable amino acids. To facilitate their incorporation onto an amino acid backbone, each of the furan and maleimide groups contained a carboxylic acid moiety. From the data obtained during these studies, the impact of an electron-withdrawing substituent, the carboxylic acid, and the solvent were found to influence the rate and extent to which a given maleimide and furan pair participated in the Diels-Alder reaction. Maleimide species were found to react more favorably in the Diels-Alder reaction if the carboxylic acid was

separated from the maleimide heterocycle by at least two carbon spacers. Increasing the distance by the addition of more carbon atoms, such as from two to five, did not result in a significant improvement in the speed and final conversion of the Diels-Alder reaction.

Similarly, conjugating the carbonyl of a carboxylic acid group into the diene (furan) also influenced the Diels-Alder reaction. A furan species with a carbon atom spacer separating the diene from the carbonyl was found to react faster and to a greater conversion than when the carboxylic acid functionality was placed at the two or three position of the ring. The placement of the carbon spacer effectively disrupted the conjugation between the furan ring and the carbonyl of the carboxylic acid, maintaining the electron density of the diene. Furthermore, solvent choice also has the potential to influence the Diels-Alder reaction: the forward reaction was noted to proceed at a higher speed when conducted in water compared to an aprotic solvent such as dimethylformamide (DMF). These features, placement of an electron-withdrawing species and solvent selection, provide the ability to tune the Diels-Alder reaction. The required rate and final conversion of the Diels-Alder reaction, however, ultimately depend upon the target application. Investigating thermodynamic and kinetic properties allowed for screening of the Diels-Alder reaction between various maleimide and furan species, which enabled the selection of a given pair suitable for use in a controlled release application or as a protecting group.

As described in Chapters 4 and 5, the synthetic amino acids were constructed by attaching maleimide and furan carboxylic acids to an aminoethylglycine backbone. Well-developed carbodiimide coupling chemistry allowed for the joining of a carboxylic acid to the aminoethylglycine backbone, facilitating the attachment of click functionalities. Aminoethylglycine has commonly been used in the development of peptide nucleic acids (PNA) whereby the base pairs of DNA have been conjugated to the molecule¹⁷. To create PNAs, an

orthogonal protecting group scheme is employed whereby the N-terminus of the aminoethylglycine molecule is protected with an Fmoc group and a t-butyl group conceals and prevents reaction of the C-terminus carboxylic acid. Once functionalized with the desired base, the t-butyl group can be selectively removed while maintaining the integrity of the Fmoc protecting group by exposure of the molecule to acid. In this manner the use of aminoethylglycine as the backbone not only enabled the creation of synthetic furan and maleimide amino acids, but also simultaneously fashioned clickable amino acids directly compatible with Fmoc-mediated solid phase peptide synthesis.

With the ease by which maleimide and furan carboxylic acids were attached to aminoethylglycine, the potential exists to incorporate and create other novel synthetic amino acids. Future work could investigate the creation of other synthetic amino acids compatible with not only the Diels-Alder and Michael addition reactions, but also other click chemistry such as the Huisgen azide-alkyne, thiol-ene and thiol-yne reactions. Placement of synthetic amino acids possessing functional groups compatible with these chemistries directly into the sequence during solid phase peptide synthesis facilitates a reduction in the time and expense incurred with typical post-synthetic modification or use of rare protecting groups. Additionally, if these functional residues are developed, the use of amino acids greatly facilitates and simplifies the process of incorporating click reagents into a peptide. At the same time this technique also potentially permits the placement of multiple orthogonal click functionalities in the same sequence simultaneously.

7.3. The Maleimide Amino Acid as a Handle for Peptide Modification

After developing amino acids eliciting click chemistry functionalities, the ability to implement them to exercise control over peptide structure, properties and function was assessed. Depicted in Chapter 4, the potential use of the maleimide amino acids in a Michael addition to manipulate a peptide's structure and facilitate the incorporation into other materials was first investigated. Due to synthetic difficulties, namely the tendency for maleimide to react with primary or secondary amines or with thiols commonly expressed on cysteine residues, maleimide species have traditionally been restricted to post-synthetic incorporation into a peptide sequence to afford and maintain a reactive functionality^{5,18}. To maintain the integrity of an active maleimide functionality, a Diels-Alder/retro-Diels-Alder protecting scheme was employed to directly incorporate the maleimide amino acid into a peptide sequence during solid phase synthesis. When formed, the adduct formed from the maleimide and furan does not undergo the undesired reaction with amines or thiol groups. After the solid phase peptide synthesis process and the application of heat to induce the retro-Diels-Alder reaction, the intact maleimide functionality was exposed and ready for use.

By promoting an intramolecular Michael addition, the activity of the maleimide functionality and ability to manipulate the conformation of a peptide sequence was demonstrated. This outcome was achieved by reacting a maleimide present at the C-terminus with the thiol of a cysteine residue at the N-terminus to create a cyclic peptide from a linear sequence. The C-terminal maleimide was the first residue placed on the solid support during peptide synthesis, a possibility attainable only via post-synthetic modification in previous endeavors.

The use of the maleimide functional amino acid as a means to incorporate peptide sequences into other materials was also illustrated. Specifically, the ability to attach and pattern peptides onto surfaces as well as decorate hydrogels with biological epitopes through the Michael addition of the maleimide species was considered. A solution of peptide containing the maleimide amino acid residue was exposed to a thiolated surface in the presence of base catalyst in nanoliter drops with the aim of fabricating a microarray pattern. Upon washing excess solution away, the microarray pattern was observed, indicating the Michael addition between the maleimide equipped peptide and surface was successful. Furthermore, the ability to attach an adhesive peptide containing a maleimide amino acid residue to a hydrogel was also possible. The peptide sequence exhibiting maleimide was allowed to react with a multifunctional thiolated poly(ethylene glycol) (PEG) macromer. After using the thiol PEG macromer that had been modified to contain an adhesive peptide sequence by a Michael addition reaction with the maleimide, the interaction between cells and the polymer were observed. The cells seeded onto the hydrogel surface demonstrated the ability to interact with the polymer network in a manner comparable to a control where the same sequence was incorporated by means of a different functional group. Additionally, the interaction of cells with the polymer that made use of the maleimide group to attach an adhesive sequence showed a greater degree of spreading/cellular interaction compared to a hydrogel that did not contain any peptide. This behavior not only indicated the presence and ability to incorporate the peptide into the polymer, but also demonstrated the bioactivity of the sequence that had made use of the maleimide amino acid as a handle.

These uses, the ability to alter confirmation and incorporate a peptide into other materials, exhibit the power and potential of the maleimide amino acid with regard to the

Michael addition reaction. There still exist other potential avenues to consider that could make use of the maleimide amino acid and Michael addition reaction. Since the maleimide amino acid has the capability of being directly introduced during solid phase peptide synthesis, the potential for the creation of multifunctional maleimide peptide sequences is a possibility (See Figure 7.1).

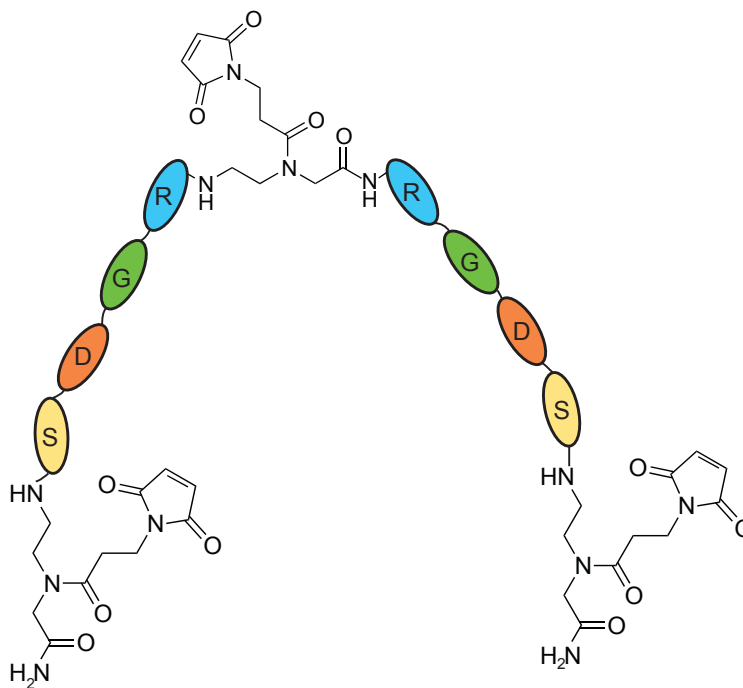


Figure 7.1. A potential multifunctional maleimide peptide crosslinker for application in network design. Here, the maleimide amino acid has been incorporated into an RGD peptide sequence. There exists the possibility to use the maleimide functionality to create other peptide crosslinking agents, such as protease cleavable crosslinkers, which typically make use of thiol as the reactive agent to incorporate the peptide into a network.

A peptide possessing multiple maleimide functional groups could serve as a crosslinking agent in the formation of polymer networks. For instance a crosslinker such as the dithiol matrix metalloproteinase degradable peptide could replace the cysteine amino acids expressing thiol groups with maleimide residues to achieve polymerization. The maleimide amino acid could

also be employed as a ligation handle to attach peptides to other materials not explored in this thesis or create more complex peptide architectures such as branched or stapled sequences.

7.4. A Furan Amino Acid for Diels-Alder Modulated Controlled Release

In addition to the implementation of the maleimide-equipped amino acid, the application of the furan bearing amino acid was also considered to manipulate peptide function. With regard to this synthetic amino acid, the ability to release furan functionalized peptide sequences controllably from a suitably fabricated hydrogel platform, as presented in Chapters 5 and 6, was successfully established. The presence of a furan moiety enabled peptide sequences to attach to a hydrogel exhibiting maleimide tethering sites by means of a thermally reversible covalent bond formed through a Diels-Alder reaction. Exploitation of the equilibrium based nature of the Diels-Alder reaction allowed for a release at physiologically compatible temperatures without the need for the application of elevated, and potentially biologically adverse temperatures. Other than thermal conditions, control over the release rate was dictated and tuned by the number of maleimide or Diels-Alder reaction sites available in the polymer. As a greater number of Diels-Alder/retro-Diels-Alder reactions were required for release to occur, an increase in the number of maleimide groups present in the network was found to slow the liberation of furan-labeled peptides.

The design of most delivery platforms attempts to control the release of a given material, which necessitates the need for the ability to forecast, as least to some degree, the amount and rate of the releasing species. To determine the predictability of the release of furan functionalized peptides from a hydrogel platform and also assess the validity of a Diels-Alder release mechanism, a reaction diffusion model was developed. The projected model release was

found to correlate well with the empirical data, supporting a Diels-Alder mechanism and illustrating the potential to control the release trajectory in a targeted manner.

Finally, the impacts of maleimide and furan functionalities on biological systems, such as cells, are largely unknown. Therefore, the bioactivity of both the release platform exhibiting unreacted maleimide functionalities as well as the furan bearing peptide sequences were explored and found to be conducive to biological applications.

The use of a Diels-Alder based release platform offers potential for future research directions beyond the scope of this work. One potential area that could be explored would be the use of different diene/dienophile pairs. Initial studies with a different furan, substitution of the carbonyl at the two position instead of the three position, were performed. As can be seen in Figure 7.2, there was a slight difference between the 2 and 3 furan governed release profiles.

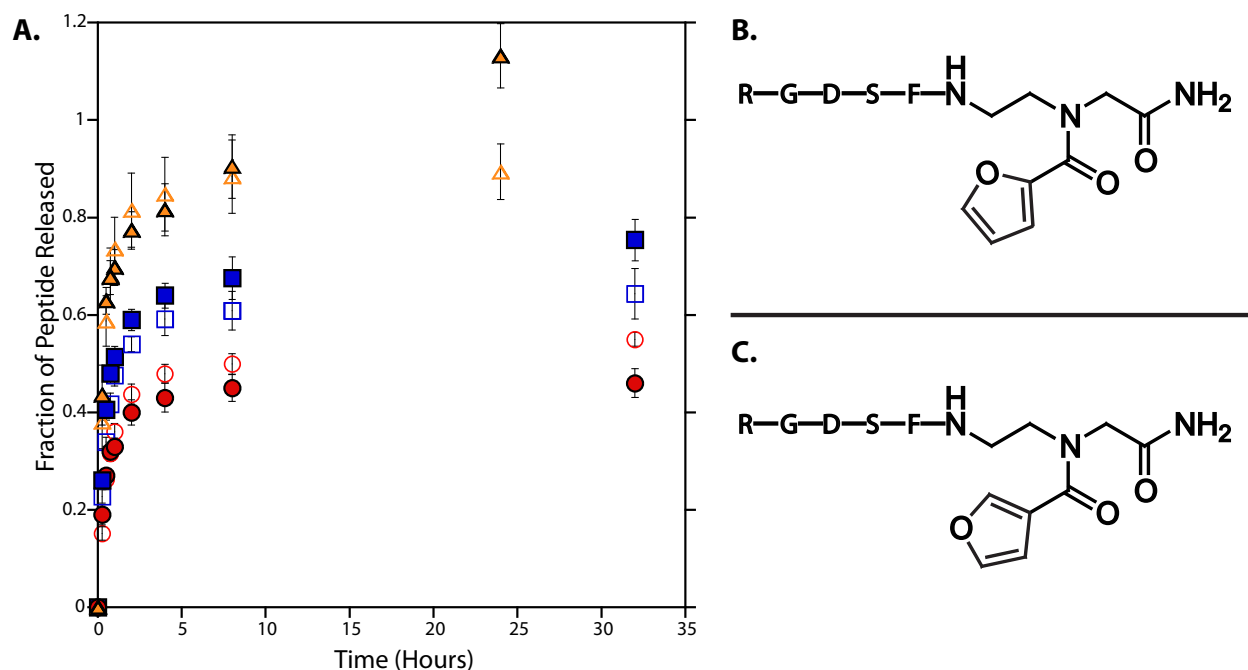


Figure 7.2. The release of RGD peptide sequences bearing two similar furan functionalities. A. The release profiles for the two peptide species at various temperatures (● 37°C, ■ 60°C and ▲ 80°C) from a hydrogel platform. Closed shapes (● ■ ▲) represent the release of the 3-furan

containing species whereas open markers (○□△) correspond to the 2-furan labeled peptide. B. An RGD peptide sequence labeled with a 2-furoic diene functionality. C. The structure of a 3-furoic diene attached to the RGD peptide considered.

The similarity of the two species is most likely due to rate acceleration caused by conducting the Diels-Alder reaction in aqueous media (See Chapter 3), as was done for the case of the release studies. Though there were not major differences between the release profiles of the two different furan species, other diene and dienophile pairs offering more distinct release rates exist. The use of other diene and dienophile species could potentially be coupled to the amino acid backbone and subsequently incorporated into peptide sequences. Figure 7.3 presents other diene and dienophile carboxylic acids that could potentially be added to couple to an amino acid backbone, providing the ability for modification of their structure to allow for incorporation, and subsequently employed in controlled release applications.

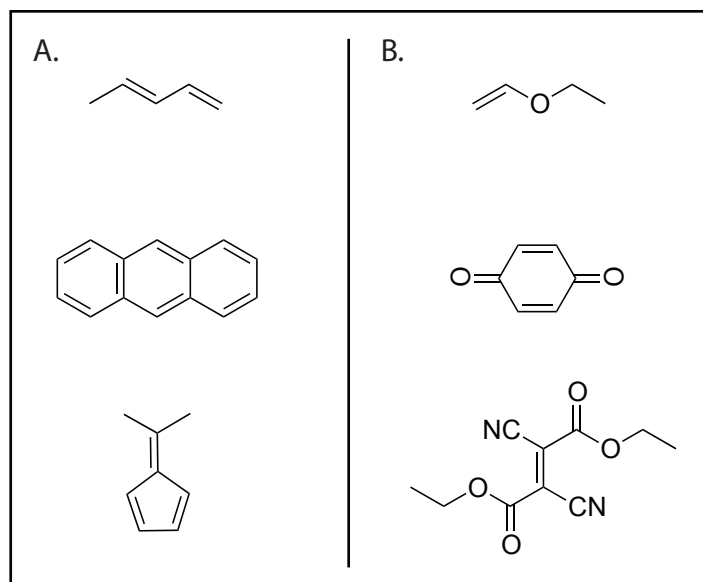


Figure 7.3. Various diene and dienophile species that could potentially be attached to an amino acid backbone. By implementing different diene and dienophile pairs, different thermodynamic and kinetic properties for the Diels-Alder reaction can be obtained. These factors influence the Diels-Alder reaction and allow for a means to control the release from a hydrogel platform. Panel A denotes potential dienes whereas B portrays dienophile species.

Through the use of other Diels-Alder pairs, different release profiles should be possible, allowing for further means to exercise control of release. Provided significantly different release kinetics, there also exists the potential to simultaneously release multiple compounds at various rates through the use of other diene and dienophile species.

Another area to investigate relates to the encapsulation of cells in a Diels-Alder releasing platform. The bioactivity of the hydrogel release platform and furan containing peptide sequences has been demonstrated in previous studies (See Chapter 6). Encapsulating cells in a hydrogel could allow for interesting studies making use of a Diels-Alder release mechanism. One example involves the use of the use of an adhesion sequence, such as the peptide RGD. Equipping this sequence with a furan functionality (See Chapter 5) and incorporation into a hydrogel containing cells may exhibit a unique release profile and cellular response. As the concentration of RGD decreases in the hydrogel due to release via a retro-Diels-Alder mechanism, a change in cell morphology and decrease in viability should be observed. Additionally, as cells integrin bind and attach to RGD through focal adhesions, they will exert a force on the Diels-Alder bond linking the adhesive peptide to the network. This force will likely influence the Diels-Alder reaction and could possibly accelerate the reverse reaction and subsequent release of RGD from the network. If this takes place, cells would likely be observed to attach to the network through focal adhesions to RGD sequences. Once the Diels-Alder bond reverses, the extension may likely be seen to retract, as has been witnessed in other cases where the site containing the focal adhesion was degraded¹⁹.

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Appendix

An Attempt to Implement the Diels-Alder and retro-Diels-Alder Reactions Between Maleimide and Furan Species as a Means for Peptide Purification

Solid phase synthesis has enabled a simple and robust process by which to synthesize peptide sequences. Compared to the preceding solution based technique, solid phase synthesis allows for a facile method to obtain high yields of the target peptide. During the solid phase synthesis process there are, however, still undesired reactions, such as incomplete residue coupling which leads to the formation of erroneous deletion sequences or the formation of cleavage byproducts that result from the liberation of the peptide from the resin. The presence of these impurities necessitates further purification and isolation of the target product. The conventional method employed to purify and isolate the target sequence from the crude peptide is through the use of high performance liquid chromatography (HPLC).

Typically, HPLC entails the use of a reversed (nonpolar) stationary phase which interacts with the peptide species in accord with polarity; the more polar the peptide sequence, the weaker the interaction with the solid support and the faster the elution. Additional modifications to the solid support, such as the inclusion of charged groups, have also been made to allow for a greater control over and degree of separation. The mobile phase also plays a role in the ability to separate and purify material. When more polar solvents introduced to the mobile phase composition, there is a little interaction between the nonpolar solid support and the solvent system. In this case, the mobile phase does not compete with the peptide and other solute molecules/impurities for binding sites. In contrast, if the polarity of the mobile phase is adjusted such that it becomes more nonpolar, the interactions between the peptide and solid phase

diminish resulting in faster elution times. A combination of solvents eliciting different polarities, such as a mixture of acetonitrile and water, are therefore implanted as the mobile phase to control the separation of the target peptide sequence from impurities.

Though HPLC does allow for the purification of peptides and has been extensively applied to achieve this objective, there are several limitations associated with this method. One shortcoming surrounding HPLC pertains to time. For small amounts of material, HPLC can be performed in a relatively short timeframe to purify the batch. However, when a large quantity of material requires purification, multiple injections and hence multiple elutions are required to obtain the finished product. If an attempt were made to load all of a large batch of crude material onto the column, a diminished or no separation would result. The column would be overloaded where the number of adsorption sites would be completely bound, unable to bind any additional material. Any material not bound to or able to interact with column simply elutes with no separation or purification benefits. Depending on the length of elution coupled with the need to regenerate and re-equilibrate the stationary phase before the next injection, the use of HPLC to purify a large batch of crude peptide can be time consuming.

In addition to time, the expense of materials required to operate and maintain an HPLC purification system can also be limiting. Aside from the obvious initial capital investments associated with an HPLC system that exist, such as the solvent pumps, column and UV-detector, there are also consumable materials. The expense related to these consumables is proportional to the extent to which the HPLC system is used. Solvent presents the main consumable cost associated with HPLC operation. Each loading and subsequent elution requires a certain volume of solvent as the mobile phase. The greater the number of runs to purify a batch of material, the larger the volume of mobile phase consumed.

To address the time and expense limitations of HPLC purification of peptides, other techniques have been proposed. Another technique that has been explored to purify peptides, very similar to HPLC, is affinity chromatography¹⁻³. This method involves labeling a peptide sequence with a functionality that strongly interacts with a solid support. Species, such as impurities, not possessing the affinity tag lack the ability to interact with the solid support and are subsequently washed away, resulting in purification of the desired peptide. Changing the pH of the mobile phase, which reduces the interaction between the solid support and desired peptide, can be employed to elute the target molecule. In addition to affinity-based approaches, the formation of covalent bonds between the desired sequence and a solid support has also been implemented to purify peptides⁴⁻⁶. The approach of covalent capture typically relies upon adding a cysteine residue or threonine group to the terminus of the desired peptide. Sequences expressing one of these moieties react with surface bound aldehydes and covalently attach to the solid phase. The covalent attachment of the desired sequence enables washing of the solid support to remove undesired impurities. Through the addition of a basic solution, such as *o*-methylhydroxylamine, cleavage of the bond linking the peptide to the solid support is achieved. With the ability to form thermally reversible bonds, the Diels-Alder reaction could also potentially be implemented as a method to covalently capture and purify peptides where the use of an additional reagent would not be necessary to liberate the target molecule from the solid support. This study therefore attempted to make use of the Diels-Alder reaction to develop a new covalent capture strategy for the purification of peptides.

The Diels-Alder reaction takes place between an electron rich diene and an electron accepting dienophile to form an adduct. Unlike most reaction whose equilibrium resides predominantly toward the product species, the Diels-Alder reaction is dynamic; the covalent

bond comprising the adduct can reverse to form the starting diene and dienophile. The dynamic nature of the this reaction is governed largely by temperature. Elevated temperatures promote the retro-Diels-Alder reaction and lower temperatures favor the formation of adduct. Through the use of appropriately functionalized peptides and solid supports, it was theorized that a Diels-Alder/retro-Diels-Alder reaction scheme could be used to purify peptide sequences.

For the Diels-Alder reaction used in this study, maleimide and furan functionalities were selected for the dienophile and diene, respectively. Both of these functional groups can be synthesized such that they possess a carboxylic acid moiety and are available in this format either through commercial sources or through simple synthetic means (See Chapter 3). The carboxylic acid functionality, with the ability to efficiently react with amines in the presence of activating agents like carbodiimides, enabled the incorporation of the furan and maleimide groups onto peptide sequences during solid phase synthesis.

Figure A1 presents the concept behind employing the Diels-Alder reaction as a purification handle for peptides.

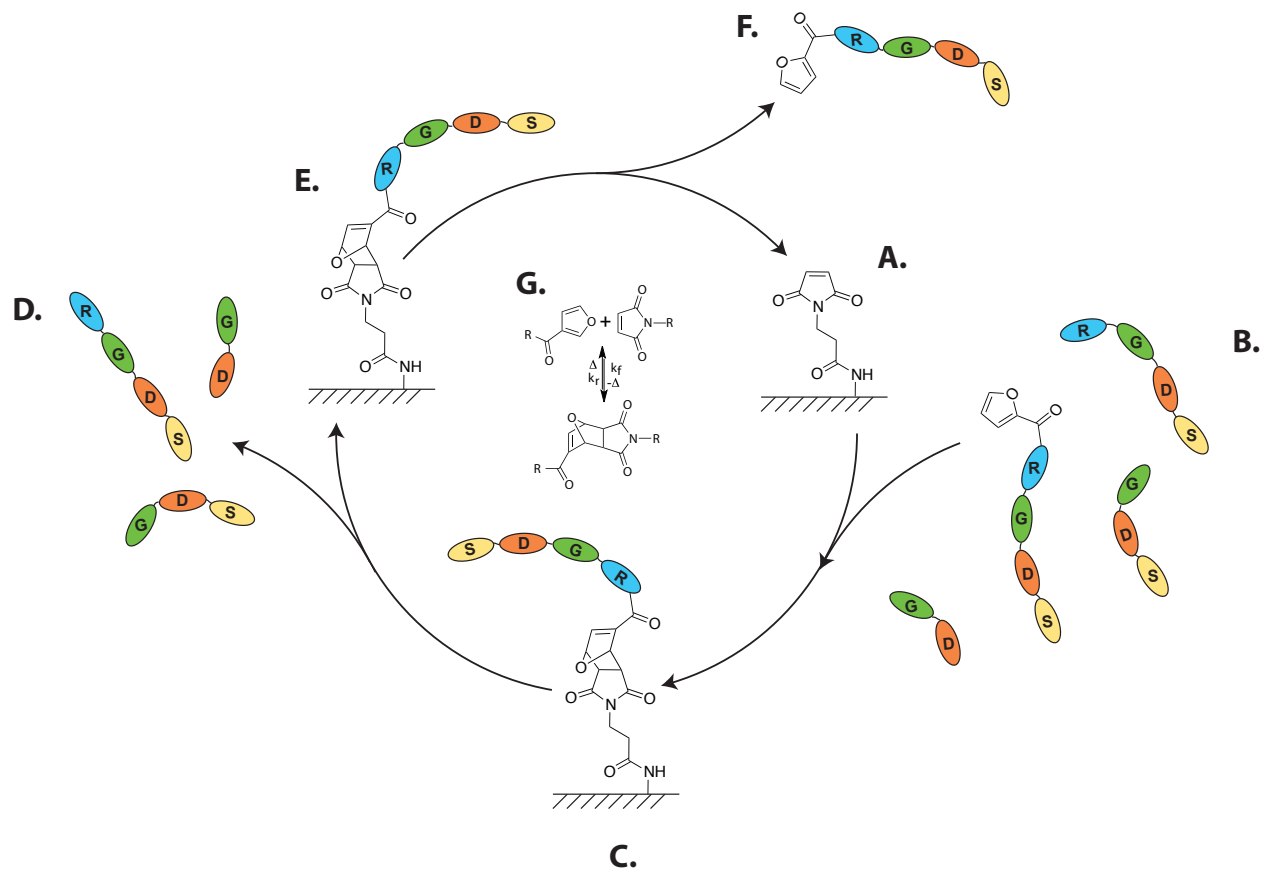


Figure A1. Proposed Diels-Alder mediated approach to the purification of peptide sequences. A. A solid phase support or resin that has been functionalized with a dienophile (maleimide) capable of participating in the Diels-Alder reaction. B. Crude peptide material. The target sequence RGDS labeled with a furan functionality in the presence of other deletion sequences—species that are missing one or more of amino acid residue. In this example, RGDS without the furan functionality is considered a deletion sequence. C. Introduction of the crude peptide mixture to the maleimide functionalized resin. Only sequences containing the furan functional group are capable of reacting in the Diels-Alder reaction to tether to the solid support. D. Washing of the resin. Any sequences not bound to the resin are washed away, removing impurities from the target species. E. After washing, only the Diels-Alder adduct resin-bound peptide sequence remains. F. Heating the resin incites the retro-Diels-Alder reaction and subsequently liberates the now purified peptide sequence. G. The Diels-Alder reaction between generic maleimide and furan species.

A solid support exhibiting a Diels-Alder compatible functionality was fabricated by attaching a maleimide moiety to the surface of a resin. With the maleimide bound to the surface, the furan functionality can be incorporated into the peptide sequence to enable the formation of a thermally reversible covalent bond through the Diels-Alder reaction and subsequent purification.

For simplicity, a peptide can be modified through the attachment of a furan bearing a carboxylic acid moiety to the N-terminus of the peptide. While the peptide sequences possessing a furan species are attached to the solid support, the impurities remain unbound and in solution. Washing the solid support containing the Diels-Alder ligated peptide sequences can selectively remove the impurities from the target material. After washing, heating the resin promotes the retro-Diels-Alder reaction and thereby releases the isolated, target peptide sequence.

Unfortunately in practice, the use of the Diels-Alder reaction between furan and maleimide to accomplish purification of peptide sequences was not practical from a thermodynamic and kinetic standpoint. As an initial proof of concept, a short RGDS sequence was synthesized and equipped with a furan functional group. Additionally, the RGDS sequence has demonstrated thermal stability (See Chapter 4), a feature that facilitates in the application of heat to incite the retro-Diels-Alder reaction and removal of the peptide from the surface. In the preliminary investigation, a solution of the furan RGDS peptide in water, as water has been demonstrated to accelerate the Diels-Alder reaction, was exposed to a maleimide-functionalized solid support and allowed to react for approximately 48 hours. A polystyrene based support was first explored, however the hydrophobicity of the resin prevented adequate dispersion and suspension of the solid phase in an aqueous medium. The inability to form a good suspension likely prevented or hindered the Diels-Alder reaction between the peptidyl furan and resin-bound maleimide. To address this issue, the experiment was repeated where a poly(ethylene glycol) based hydrophilic resin was functionalized with maleimide and then exposed to an aqueous solution containing the furan-RGDS peptide.

After rinsing the equilibrated solid support with cold solvents (dichloromethane, isopropanol and water), the resin was re-suspended in toluene and heated to reflux. A sample for analysis was obtained by separating the toluene from the solid support via filtration and followed by removal of solvent under reduced pressure. Upon examination of the resulting material with matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy, the target peptide sequence was found, however it exhibited a weak signal convoluted with other species that may have leached out of the resin during the toluene reflux (See Figure A2).

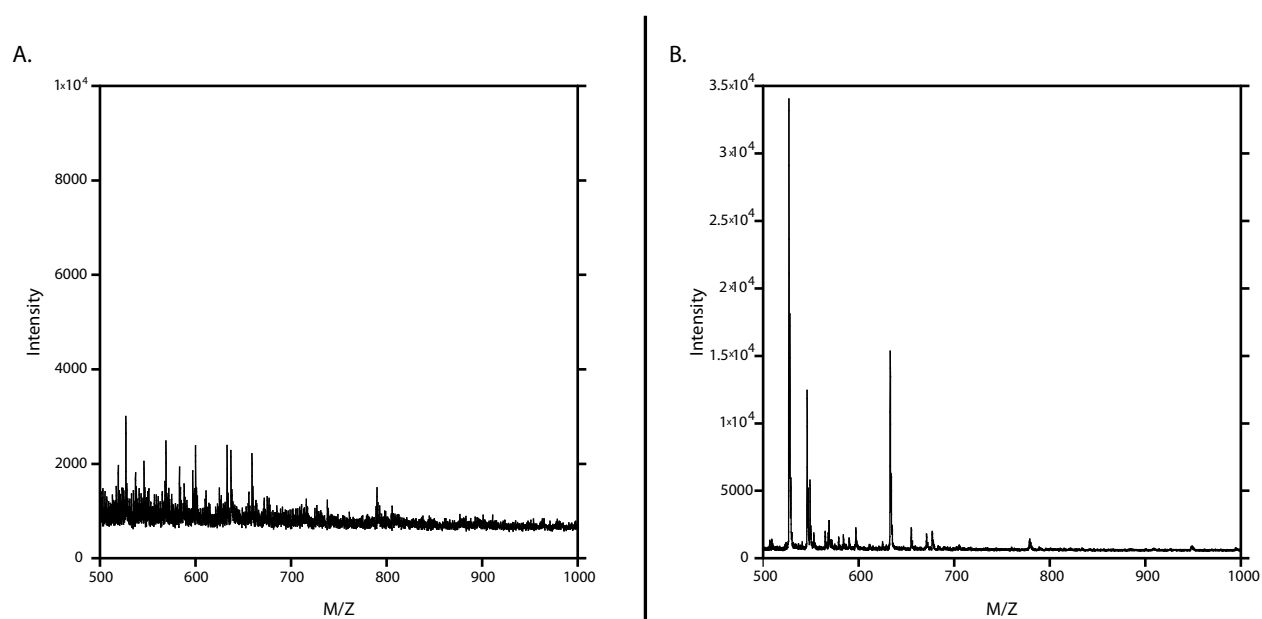


Figure A2. Representative mass spectrum results for the use of the Diels-Alder reaction between a furan functionalized RGDS sequence and a surface bound maleimide as means to purify peptides. The molecular weight of the protonated species is 527. Panel A depicts the material that resulted from heating the solid support after allowing the Diels-Alder reaction to proceed for approximately 48hours. The spectrum portrayed in B shows the material present in the solution that was exposed to the resin for 48hours, showing that the target sequence was still present in the solution and did not bind to the solid support.

The solution of furan exposed to the resin was also subjected to mass spectroscopy. The resulting mass spectrum presented a strong signal indicative of the target molecule. As the target species was still present in the solution, the Diels-Alder reaction did not completely tether the peptide to the surface and was not removed during the washing steps. Longer times may have

increased the amount of surface bound Diels-Alder tethered peptide, however, this could potentially preclude the use of this technique as an effective purification strategy. In order to serve as an efficient, implementable purification platform the binding to the surface needs to be rapid and preferably require less than the 48hours initially employed in this experiment.

In an effort to circumvent these difficulties, other modifications to the Diels-Alder reaction purification methodology were executed. One alteration involved the use of different furan functionalities: dienes such as 2-furan, 3-furan and 3-(2-furfuryl) propionic acids were selected and implemented in the Diels-Alder purification strategy. The 3-(2-furfuryl) propionic species was found to undergo a ring-opening reaction when exposed to trifluoroacetic acid (TFA), rendering it inert to the Diels-Alder reaction. This susceptibility of the 3-(2-furfuryl) moiety precluded the inclusion of this furan on a peptide, as TFA is commonly used to release a peptide from the solid support. This led to another variation in the efforts to successfully implement the Diels-Alder/retro-Diels-Alder purification strategy. Rather than place the maleimide on the solid support, it was attached to the peptide chain and the furan functionality was fixed to the resin. Similar results were obtained for each of these modifications, where little to no Diels-Alder functionalized peptide was found to bind to the solid support.

Despite these unsuccessful attempts, the use of the Diels-Alder reaction presents an interesting potential technique for the purification of peptides that could save time and reduce solvent based expenses. Future efforts to develop a successful Diels-Alder purification strategy and platform could look into several areas. One item that could be investigated, which lies outside the realm of the work presented in this thesis, would be the use of other Diels-Alder compatible species. Furan and maleimide are not the only two species capable of participating in the Diels-Alder and retro-Diels-Alder reactions; other potential diene and dienophile pairs exist

that may be amenable to incorporation into peptide sequences and onto solid supports. The selection of different diene and dienophile pairs will result in different kinetic and thermodynamic properties that could potentially make the Diels-Alder and retro-Diels-Alder purification platform feasible. An additional modification may be seen in terms of the solid support. The alteration of the functionalization and distance of the functional groups from the surface of the solid support could be altered to promote a more feasible reaction. For instance, increasing the distance of the surface bound diene or dienophile would decrease the steric hindrance and potentially better enable the peptide to attach via the Diels-Alder reaction.

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