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A novel iterative reducible ligation strategy for the synthesis of homogeneous gene delivery polypeptides

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A NOVEL ITERATIVE REDUCIBLE LIGATION STRATEGY FOR THE SYNTHESIS OF HOMOGENEOUS GENE DELIVERY POLYPEPTIDES

by

Mark David Ericson

An Abstract

Of a thesis submitted in partial fulfillment
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ABSTRACT

The ability to safely delivery efficacious amounts of nucleic acids to cells and tissues remains an important goal for the gene therapy field. Viruses are very efficient at delivering DNA, but safety concerns limit their clinical use. Nonviral vectors are not as efficient at DNA delivery, but have a better safety profile. Limiting the efficaciousness of nonviral vectors are the numerous extra and intracellular barriers that must be overcome for successful DNA delivery *in vivo*. While single polymers can successfully transfect immortalized cell lines *in vitro*, multicomponent gene delivery systems are required for delivery *in vivo*. Key in the development of multicomponent systems is their syntheses. Optimization of a nonviral gene delivery system requires the development of methodologies that incorporate the different components in a controlled fashion, generating homogeneous gene delivery vectors. Such syntheses ensure every polymer has the different components required for successful delivery. The amount of each component and location within the gene delivery system can also be varied systemically, allowing optimization of the vector.

The overall scope of this thesis is to develop a chemical method to iteratively couple gene delivery peptides through reducible disulfide bonds. The synthesis of such polypeptides allows the triggered disassembly of a polypeptide polyplexed with DNA upon cellular uptake. To synthesize homogeneous gene delivery polypeptides, a novel iterative reducible ligation strategy was developed, based upon the use of a thiazolidine masked cysteine. Initial studies demonstrated that a thiazolidine could be unmasked to a cysteine in the presence of a disulfide bond without side reaction, though the reported thiazolidine hydrolysis conditions of aqueous methoxyamine were insufficiently robust for high yielding ligations. Discovery of a novel silver trifluoromethanesulfonate hydrolysis led to an efficient process for generating reducible polypeptides, as evidenced in the synthesis of a 4 component polypeptide.

Due to the success of the thiazolidine mediated iterative ligation strategy, cysteines were replaced by penicillamines to produce more stable disulfide bonds. The mild thiazolidine hydrolysis and subsequent peptide conjugation reactions led to an attempt at an iterative ligation strategy on a solid support, eliminating purification steps that lowered the yields in the solution phase methodology. Initial progress at generating gene delivery peptides that could be incorporated into the synthetic strategy included the generation of a tri-orthogonal cysteine protecting scheme that allowed a third cysteine to be derivatized with a targeting ligand or stealthing polymer. Due to the use of terminal cysteines in the iterative ligation strategy, a PEG stealthing polymer could be placed in the center of a polyacridine gene delivery peptide with only a small decrease in the ability to condense and protect DNA during systemic circulation. A convergent synthesis was also developed that was able to synthesize large polypeptides in fewer linear steps. The synthetic methodology of thiazolidine mediated iterative reducible ligation developed in this thesis is important in the gene therapy field as it allows the construction of polypeptides that can be systemically optimized, potentially resulting in highly efficacious nonviral gene delivery.

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PH.D. THESIS

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has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
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To my wife, family, and friends who have supported me in my educational pursuits for far too many years

Scientific research was much like prospecting: you went out and you hunted, armed with your maps and instruments, but in the end your preparations did not matter, or even your intuition. You needed your luck, and whatever benefits accrued to the diligent, through sheer, grinding hard work.

Michael Crichton
The Andromeda Strain

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ABSTRACT

The ability to safely delivery efficacious amounts of nucleic acids to cells and tissues remains an important goal for the gene therapy field. Viruses are very efficient at delivering DNA, but safety concerns limit their clinical use. Nonviral vectors are not as efficient at DNA delivery, but have a better safety profile. Limiting the efficaciousness of nonviral vectors are the numerous extra and intracellular barriers that must be overcome for successful DNA delivery *in vivo*. While single polymers can successfully transfect immortalized cell lines *in vitro*, multicomponent gene delivery systems are required for delivery *in vivo*. Key in the development of multicomponent systems is their syntheses. Optimization of a nonviral gene delivery system requires the development of methodologies that incorporate the different components in a controlled fashion, generating homogeneous gene delivery vectors. Such syntheses ensure every polymer has the different components required for successful delivery. The amount of each component and location within the gene delivery system can also be varied systemically, allowing optimization of the vector.

The overall scope of this thesis is to develop a chemical method to iteratively couple gene delivery peptides through reducible disulfide bonds. The synthesis of such polypeptides allows the triggered disassembly of a polypeptide polyplexed with DNA upon cellular uptake. To synthesize homogeneous gene delivery polypeptides, a novel iterative reducible ligation strategy was developed, based upon the use of a thiazolidine masked cysteine. Initial studies demonstrated that a thiazolidine could be unmasked to a cysteine in the presence of a disulfide bond without side reaction, though the reported thiazolidine hydrolysis conditions of aqueous methoxyamine were insufficiently robust for high yielding ligations. Discovery of a novel silver trifluoromethanesulfonate hydrolysis led to an efficient process for generating reducible polypeptides, as evidenced in the synthesis of a 4 component polypeptide.

Due to the success of the thiazolidine mediated iterative ligation strategy, cysteines were replaced by penicillamines to produce more stable disulfide bonds. The mild thiazolidine hydrolysis and subsequent peptide conjugation reactions led to an attempt at an iterative ligation strategy on a solid support, eliminating purification steps that lowered the yields in the solution phase methodology. Initial progress at generating gene delivery peptides that could be incorporated into the synthetic strategy included the generation of a tri-orthogonal cysteine protecting scheme that allowed a third cysteine to be derivatized with a targeting ligand or stealthing polymer. Due to the use of terminal cysteines in the iterative ligation strategy, a PEG stealthing polymer could be placed in the center of a polyacridine gene delivery peptide with only a small decrease in the ability to condense and protect DNA during systemic circulation. A convergent synthesis was also developed that was able to synthesize large polypeptides in fewer linear steps. The synthetic methodology of thiazolidine mediated iterative reducible ligation developed in this thesis is important in the gene therapy field as it allows the construction of polypeptides that can be systemically optimized, potentially resulting in highly efficacious nonviral gene delivery.

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LIST OF ABBREVIATIONS

Acm .. .. acetamidomethyl

Acr ..... Nε-acridinyllysine

AgOTf.. ..... silver trifluoromethanesulfonate

Ala... ..... alanine

BLI..... bioluminescence imaging

Boc... ..... t-butyloxycarbonyl

Cys ..... cysteine

DCM..... dichloromethane

DIC..... diisopropylcarbodiimide

DIPEA.. ..... diisopropylethylamine

DMAP.. ..... 4-dimethylaminopyridine

DMF.. ..... *N,N*-dimethylformamide

DMSO.. ..... dimethylsulfoxide

DMT.. ..... 2,2-dimethylthiazolidine

DNA..... deoxyribonucleic acid

DTDP.. ..... 2-2'dithiodipyridine

DTNP.. ..... 2,2-dithiobis(5-nitropyridine)

EDTA..... ethylenediaminetetraacetic acid

EDT.. ..... 1,2-ethanedithiol

ESI... ..... electrospray ionization

Fmoc.. ..... 9-fluorenylmethoxycarbonyl

Gly... ..... glycine

HATU..O-(7-Azabenzo-triazol-1-yl)-N,N,N',N'-tetramethylurionium hexafluorophosphate

HBM ..... HEPES buffered manitol

HBTU.. ..... O-(Benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate

HD ..... hydrodynamic dose

HEPES ..... 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HMPA ..... hexamethylphosphoramide

HMPB ..... 4-(4-Hydroxymethyl-3-methoxyphenoxy) butyric acid

HMW ..... high molecular weight

HOBt.. ..... 1-hydroxybenzotriazole

HPLC.. ..... high performance liquid chromatography

i.p.… ..... intraperitoneal

i.v.… ..... intravenous

LC.... ..... liquid chromatography

LMW ..... low molecular weight

Lys... ..... lysine

MALDI-TOF.. ..... matrix assisted laser desorption ionization-time of flight

MS… ..... mass spectrometry

mV… ..... millivolt

nPys..... 3-nitro-2-sulfanylpyridine

NMR.. ..... nuclear magnetic resonance

PEG..... poly(ethylene)glycol

PEI... ..... polyethyleneimine

Pen... ..... penicillamine

Pys 2-S-sulfanylpyridine

QELS... ..... quasi-elastic light scattering

RNA... ..... ribonucleic acid

RP…. ..... reverse phase

RT..... room temperature

TCEP..... tris(2-carboxyethyl)phosphine

TFA... ..... trifluoroacetic acid

Thz.....thiazolidine

TIS... .....triisopropylsilane

TRI... ..... triantennary N-glycan

Trityl.....triphenylmethyl

Trp..... tryptophan

CHAPTER 1

LITERATURE REVIEW

Abstract

Chapter 1 reviews the literature for peptides used in non-viral gene delivery. Areas of discussion include the barriers of gene delivery and assembly of multicomponent peptide gene delivery systems to overcome these barriers. The discussion will also describe disulfide bond forming reactions utilized in peptide chemistry as well as a brief summary of cysteine protecting groups and their applicability to iterative reducible ligation chemistry.

Introduction

An elusive goal for the gene therapy community remains the ability to safely and efficaciously deliver nucleic acids into cells and tissues. Delivering exogenous DNA to correct genetic defects causing diseases such as phenylketonuria and cystic fibrosis was proposed 40 years ago.[1] The discovery of RNA-mediated knockdown of gene expression by Fire and Mello 14 years ago introduced a new mechanism to treat certain diseases by knocking down aberrant levels of gene expression by delivery of exogenous RNA.[2] While many advances have lead to a greater understanding of the barriers for nucleic acid delivery, the continued lack of a safe, efficacious delivery system has resulted in only one gene therapy treatment being recommended for regulatory approval in Europe or the US.[3]

There are two basic categories of vectors used to deliver DNA: viral and nonviral. Viruses are very efficient at delivering DNA into cells, having evolved for the explicit purpose of transfecting cells; however concerns over their immunogenicity, mutagenicity and cytotoxicity limit their clinical success.[4-8] Nonviral vectors are typically composed of polycationic polymers capable of binding and condensing DNA through ionic interactions with the anionic phosphate backbone of DNA. These include polymers such as PEI,[9] chitosan,[10] lipids,[11] basic peptides,[12] and other polyamine containing polymers.

Also included are intercalator based systems that interact with DNA via insertion between base pairs.[13] These carriers offer many advantages over viruses for delivering nucleic acids: they can bind any size plasmid, are readily chemically synthesized as stable polymers, and offer better safety profiles compared to viruses.[4-6] However, they are not as efficient at delivering DNA as viral vectors.

There are several barriers that must be overcome for efficient nonviral gene delivery. This chapter will review the *in vitro* and *in vivo* barriers for gene delivery and provide examples of different peptides overcoming these individual barriers. For successful *in vivo* delivery, multiple barriers must be overcome requiring many different components to be incorporated into a gene delivery system. The assembly of peptide nonviral gene delivery system containing multiple components will be discussed, emphasizing the advantages of linking components through reversible disulfide bonds. Attention will also be given to disulfide bond forming chemistries and cysteine (Cys) protecting groups.

Barriers to Nonviral Gene Delivery

To successfully deliver their nucleic acid cargo, nonviral carriers must overcome several barriers (Fig 1-1). For *in vitro* transfections of immortalized cell lines, these barriers are well understood. A carrier must be able to condense the DNA, cross the cellular membrane, escape from the endosome before degradation, and translocate into the nucleus. Cationic peptides bind and condense DNA into positively charged polyplexes, first described by Wu *et al.* using a heterogeneous poly-lysine polymer.[12, 14, 15] By varying the number of Lys residues in homogenously prepared poly-lysine chains, Wadhwa *et al.* established at least 13 Lys are required for polyplex formation and robust *in vitro* gene expression.[16] Following condensation, electropositive polyplexes can readily associate with the electronegative cell surface, due to the presence of sulfated

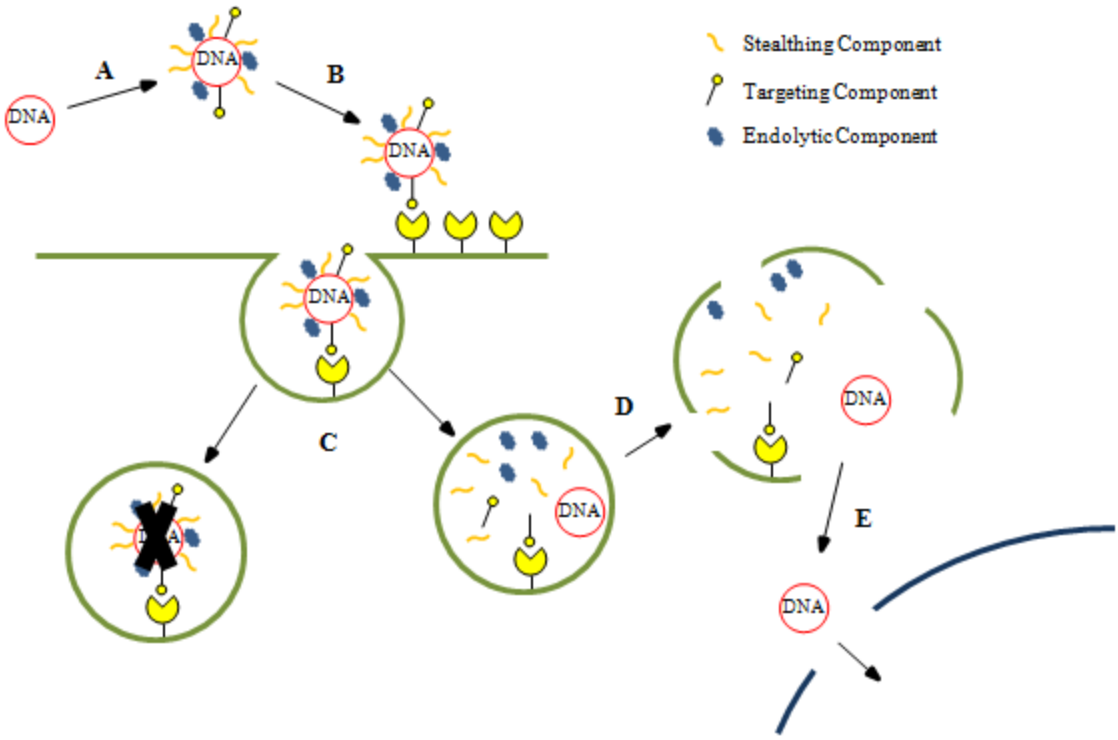


Figure 1-1: *Summary of the Barriers to Efficient Nonviral Gene Delivery*. (**A**) Plasmid DNA must be condensed into a polyplex to prevent nuclease degradation. (**B**) During systemic circulation, the charge of the particle must be masked to prevent aggregation and binding with serum proteins. Upon reaching the target site, the polyplex must bind and be internalized by a specific receptor. (**C**) Following internalization, the polyplex must dissociate to unmask the endolytic function before lysosomal degradation, allowing for endosomal escape (**D**). The plasmid DNA must then translocate across the cytoplasm and cross the nuclear membrane for gene expression (**E**).

proteoglycans on the cell membrane.[17] The resulting nonspecific uptake is enhanced during *in vitro* transfections as the polyplexes can sediment onto the surface of cells.

Following internalization, polyplexes must escape from the endosomal pathway before degradation by lysosomes. There are two different mechanisms by which peptides can achieve endosomal escape. The first takes advantage of the so called proton sponge effect, where a buffering reagent in the endosome causes an increased influx of protons and counter ions resulting in osmotic swelling and eventual rupturing of the endosome.[18, 19] The imidazole ring of histidine has a pKa of 6 and can buffer the endosome as the pH

drops from 7.4 to 4 in lysosomes.[20] As first reported by Midoux and Monsigny, the incorporation of histidine residues into a poly-lysine polymer resulted in a several order increase in gene expression.[21] The other mechanism involves fusogenic peptides that directly disrupt the endosomal membrane. Fusogenic peptides including influenza derived hemagglutinin[22] and bee venom melittin[23] have been successfully incorporated into gene delivery systems, enabling endosomal escape and increased gene expression. The endolytic effects of both peptide classes are improved *in vitro*, where a large excess of buffer or fusogenic peptide can be administered and co-internalized with the polyplexes. The resulting high concentration of either peptide promotes endolysis.

Upon release into the cytoplasm, a polyplex must still translocate into the nucleus for transcription. The nuclear membrane presents a formidable hurdle, as the relatively large size of a polyplex prevents it passive diffusion through the nuclear pore complex.[24] This can readily be overcome in immortalized cell line transfections, since the constant cell division results in a fluid restructuring of the nucleus including the nuclear membrane.[25] The polyplex does not have to traverse the membrane; during mitosis the polyplex can gain access to the nucleus when the nuclear membrane disassembles. Nuclear entry for primary cell transfections, including stem cells, remains more challenging since these cell types do not undergo the constant replication of immortalized cell lines. To address this challenge, a variety of peptide nuclear localization signals have been incorporated into gene delivery systems to increase nuclear uptake with varied degrees of modest success.[26]

The *in vitro* gene delivery barriers are well understood and different peptides can overcome these barriers and permit robust gene expression. Despite the advances made from transfecting cells, *in vivo* gene delivery remains much more difficult. While the same barriers exist, they are more complicated when transfecting an organism; hence optimization for *in vitro* transfection efficiency does not necessarily result in increased expression *in vivo*. A carrier must still bind and condense DNA into polyplexes. To

achieve successful gene expression *in vivo*, polyplexes need to be systemically circulated and must be stable in the presence of serum nucleases and the high 150 mM salt concentration found in blood. Both highly polymerized and discrete length poly-lysine peptides protect DNA from nuclease degradation.[27, 28] However poly-lysine delivery systems are not stable in physiological salt concentrations. High molecular weight (HMW) poly-lysine polymer polyplexes precipitate in 150 mM NaCl solutions,[29] resulting in diminished *in vivo* expression since the precipitates are trapped in the first capillary bed encountered. Low molecular weight (LMW) poly-lysine polyplexes do not have sufficient affinity for DNA in high salt concentrations and rapidly dissociate upon administration in mice, resulting in DNA degradation.[30] To overcome the premature dissociation of LMW poly-lysine vectors, a poly-intercalator system has recently been introduced containing multiple Nε-acridinyllysine (Acr) and Lys residues that form polyplexes by a combination of intercalation and ionic interaction and do not dissociate in physiological salt concentrations.[31, 32]

The charge of the polyplex must also be masked during circulation to prevent polyplex aggregation, binding of serum proteins such as albumin, and prevent opsonization.[33, 34] This is often done by attachment of an uncharged polymer such as PEG that creates a stealthing layer masking the charge of the polyplex. Incorporation of PEG onto either HMW or LMW poly-lysine systems prevents particle aggregation and can successfully mask the charge of the polyplexes, though enhancement of circulatory stability has not been demonstrated.[29, 33, 35] By contrast, PEG-polyacridine delivery systems can protect DNA during systemic circulation for several hours.[31, 32] Transfection component DNA can be observed 7 hrs after an i.v. dose of PEG-polyacridine polyplex in mice, while no expressible DNA was found after 1 hr when polyplexes were formed with a control polyacridine peptide lacking a PEG.[32] While PEG promotes longer circulation due to masking the charge of polyplexes, cellular uptake is also diminished because interactions with the sulfated proteoglycans are also inhibited.

Systemically circulated polyplexes must target specific cells in a tissue to promote polyplex accumulation and eventual gene expression. Numerous targeting ligands have been utilized for enhancing peptide-based nonviral gene delivery. Transferrin, targeting the transferrin receptor, has been attached to HMW poly-lysine and demonstrated receptor mediated expression in a chicken erythroblast cell line, though the ubiquitous distribution of this receptor in different tissues hinders its use *in vivo*.[36] Glycans such as asialoorosomucolid and triantennary that target the asialoglycoprotein receptor of hepatocytes have been incorporated into a HMW poly-lysine and a LMW CWK_{18} peptide with targeted uptake evidenced *in vivo*.[14, 30] Similarly, a high mannose glycan (Man9) conjugated to the CWK_{18} peptide showed selective uptake of radiolabeled polyplexes in Kuffer cells upon administration in mice.[37] A peptide ligand for the epidermal growth factor receptor demonstrated selective uptake into transplanted tumor cells in mice.[38] An important consideration for incorporating a targeting motif is that it must be large enough to penetrate the PEG stealthing layer, to allow recognition by the target receptor and internalization.

Upon internalization, polyplexes must be able to escape the endosome before lysosomal degradation. Endosomal escape is much more challenging *in vivo* because excess endolytic agent cannot reach the target tissue.[25] The significantly lower amount of buffering or fusogenic peptide present in targeted *in vivo* delivery is not enough to promote endolysis at therapeutic relevant dosages of polyplexes. As delivery of excess peptide is an unlikely solution, a catalytic method for disrupting the endosome is needed. A few groups have introduced the enzyme phospholipase A2 to achieve endolysis.[39, 40] This enzyme can puncture membranes by removing one of the two fatty acid tails of a phospholipid, destabilizing the membrane.[41] While promising, only early *in vitro* results have been reported and not with a peptide based system.[39, 40]

Following endosomal escape, the DNA must still be intracellularly trafficked to the nucleus and unpackaged to allow efficient expression. Like primary cell *in vitro*

transfections, most targeted cells are not rapidly dividing and mitosis cannot be utilized to achieve nuclear localization. Few reports have specifically examined nuclear targeting *in vivo*. Work by Dean *et al*. showed incorporation of DNA nuclear targeting sequences (sequences within a plasmid believed to target the plasmid to the nucleus) within a plasmid did show up to a 40-fold increase in luciferase expression in rat vascular tissue compared to DNA lacking the targeting sequence, though the DNA was delivered by a physical method of electroporation.[42] A multicomponent gene delivery system has not yet been demonstrated to achieve nuclear localization, because this requires a fully integrated and functional system to overcome the previous barriers. Nuclear targeting can only occur with a polyplex that is stable in blood, achieves specific uptake, and can escape from the endosome. Conversely if nuclear targeting is not robust, a polyplex that can selectively enter cells and promote endolysis will not express.

A non-viral gene delivery system has currently not been developed that can deliver efficacious amounts of DNA in an animal. Cell transfections have been more successful, because a single polymer such as PEI can readily overcome the different barriers to *in vitro* gene expression. The more varied and difficult barriers in an organism prevent a single polymer from achieving success. Gene delivery systems must incorporate many different components for efficacious *in vivo* expression, each designed to overcome one of the barriers. Key to these more complicated gene delivery systems is their assembly, to allow rationale modifications that can guide further improvements.

Assembly of Multicomponent Gene Delivery Systems

For optimization of a gene delivery system, structurally defined components need to be synthesized. Randomly assembled components may be able to overcome barriers to gene delivery, but replication of results and optimization are nearly impossible since the chemical structure can change with each synthesis. Due to the precise chemistry of solid phase peptide synthesis, pioneered by Merrifield,[43] peptides are ideal for generating

chemically defined structures for gene delivery. The need for homogeneous peptides was illustrated by McKenzie *et al.* comparing a chemically homogeneous CWK_{18} chain to a commercially available heterogeneous poly-lysine 20 chain, with the defined CWK_{18} peptide demonstrating a 4 log increase in gene expression.[44] This was a direct result of the heterogeneity of the commercially available poly-lysine, with the most prevalent form containing only 6 Lys (below the 13 required for polyplex formation),[16] illustrating the importance of accurately controlling the peptide composition. The CWK_{18} peptide has been further modified with a PEG stealthing conjugate,[33] as well as two different glycans to create chemically defined glycopeptides.[45] More recently, Wagner *et al.* introduced unnatural amino acids into a chemically defined polymer using solid phase synthesis.[46] The resulting polymers were shown to increase polyplex delivery, organ targeting, membrane lysis, and siRNA knockdown depending on the composition and structure of the polymer.

Successful *in vivo* delivery of a polyplex requires a carrier to contain multiple components and the assembly of the different components into a single gene delivery system is critical. The defined CWK_{18} glycan and PEG peptides are ideal for their precise structure, but readily dissociate from DNA when administered to mice from lack of affinity.[30] To create more stable poly-lysine delivery systems, various cross-linking strategies have been employed. One of the first introduced was by Adami and Rice, utilizing glutaraldehyde to form reversible Schiff bases between inter-peptide Lys residues after polyplex formation.[47] This was applied *in vivo* using PEG-CWK_{18} and two N-glycan CWK_{18} glycopeptides, where following DNA condensation polyplexes were stabilized with glutaraldehyde.[30, 37] In every case, the polyplexes showed increased stability towards metabolism by prolonging the circulatory half-life of radiolabeled DNA from minutes to several hours.[30, 37] However only a low magnitude of expression was observed, most likely due to polyplexes being too stable from the lack of Schiff base hydrolysis. Therefore not only must gene delivery systems contain components with

defined structure and be incorporated to form stable polyplexes, there still must be a triggered release of the DNA intracellularly to enable gene expression.

A commonly exploited biological stimulus for non-viral gene delivery is the intracellular reductive enviroment.[48, 49] The 1000-fold difference in intracellular versus extracellular glutathione concentration (10 mM compared 10 μM) creates a reducing environment within the cell that readily cleaves disulfide bonds.[50, 51] Cationic gene delivery systems that contain disulfide bonds advantageously are covalently linked together and have high affinity for DNA due to being large, charged polymers. Upon cellular uptake, the disulfide bonds are reduced, resulting in shorter cationic units that more readily dissociate from DNA and result in high expression. Also beneficial is the reduced toxicity that is observed compared to high molecular weight polycationic systems such as PEI and poly-lysine.[49]

The benefits to using Cys-flanked reducible peptides was first reported by McKenzie *et al*., with a $CWK_{17}C$ peptide that condensed DNA and was subsequently oxidatively cross-linked to form stable polyplexes.[52] A 60-fold increase for gene expression *in vitro* was observed compared to alkylated controls. A subsequent study demonstrated that even a short tetra-lysine peptide with terminal Cys could form stable DNA polyplexes and significantly improve *in vitro* gene transfer relative to longer poly-lysine peptides that lacked terminal Cys residues,[53] and were much shorter than the 13 Lys that were normally required to fully condense DNA.[16]

To develop more complex, multicomponent gene delivery systems, PEG and N-glycan functionalized disulfide cross-linking peptides were randomly co-polymerized using a plasmid DNA template.[54] The cross-linked polyplexes were administered in mice to target hepatocytes, one of the first examples of a disulfide bond cross-linked polyplex tested *in vivo*.[55] Modest levels of gene expression were observed, along with an increased circulatory half-life of radiolabeled DNA.

An alternative to the DNA template polymerization was developed by Seymour *et al.* through a solution phase random polymerization of disulfide cross-linking peptides.[56] A $CK_{10}C$ peptide was polymerized before condensation with DNA. This approach allowed characterization of the polymer and resulted in a stable product, since monomeric Cys flanked peptides will slowly polymerize over time even when stored in acidic conditions at low temperatures. Polyplexes formed with polymerized $CK_{10}C$ peptide and stabilized by surface coating with HMPA copolymers also showed increased circulatory half-life while increasing *in vitro* gene expression.[57] Expanding the solution phase polymerization to include fusogenic peptides, Chen *et al.* polymerized Cys-terminated melittin into a disulfide cross-linked fusogenic polypeptide that underwent glutathione mediated triggered release from DNA polyplexes.[58] Potent *in vitro* gene expression was observed compared to melittin without flanking Cys.

To generate a carrier that contained a stealthing domain, targeting ligand, and fusogenic peptide, a solution phase random co-polymerization of a Cys-terminated PEG-peptide, glycopeptide, and melittin produced disulfide cross-linked PEGylated glycopolypeptides.[59] The solution phase polymerization allowed incorporation of different components into the delivery system that have different affinities for DNA. Both the PEG and glycopeptides contained multiple Lys residues and readily interacted with DNA. The melittin employed did not contain as many charges and would not readily incorporate if a DNA template polymerization approach was used. Following condensation with DNA, the administered polyplexes were only able to mediate measurable gene expression *in vivo* following stimulation with a blank hydrodynamic stimulatory dose.[59] Transfection component DNA could only be demonstrated 5 min after the initial dose of polyplex.

An additional benefit to using reducible bonds is the ability to activate different gene delivery components upon cellular uptake. Baumhover *et al.* demonstrated a 100-fold increase of *in vitro* gene expression when melittin was linked to a polyacridine

peptide through a disulfide bond compared to a non-reversible maleimide bond.[60] Upon cellular uptake and entry to the endosomal pathway, the reversible disulfide bond was presumably cleaved, allowing melittin to dissociate and disrupt the endosomal membrane, resulting in high levels of expression. When the non-reducible maleimide form was used, melittin remained bound to the polyplex, unable to disrupt the membrane. Not only can the intracellular reducing environment trigger polyplex disassembly, but can also activate components of a gene delivery system.

With the exception of the polyacridine-melittin delivery system, each of the carriers described resulted from polymerization of one or more Cys-terminated peptides into heterogeneous disulfide cross-linked polypeptides. While individual components are chemically defined, the resulting polymerized polypeptides are a mixture that is not structurally defined. This prevents optimization of the architecture of the carrier, by not allowing control over the placement of a peptide within the carrier and by not allowing the amount of a peptide to be precisely controlled. For efficacious delivery, each component must be included within every polyplex, which cannot be guaranteed with a randomly polymerized carrier. Components and carriers must be arranged with precise chemistry to allow the optimization of the gene delivery systems. The polyacridine-melittin system represents a precise structure, but only contains one disulfide bond linking two components. The synthesis of a single disulfide bond is readily controlled, but it remains much more challenging to conjugate multiple peptides through reducible bonds to create chemically defined polypeptides.

Peptide Disulfide Bond Formation

There are many different methods for forming disulfide bonds that can be divided into two general types of reactions: random and directed (Fig 1-2). Random disulfide bond formation utilizes two or more Cys sulfhydryls that are allowed to randomly form bonds (Fig 1-2A). In the presence of atmospheric oxygen at slightly basic pH[61, 62] or a

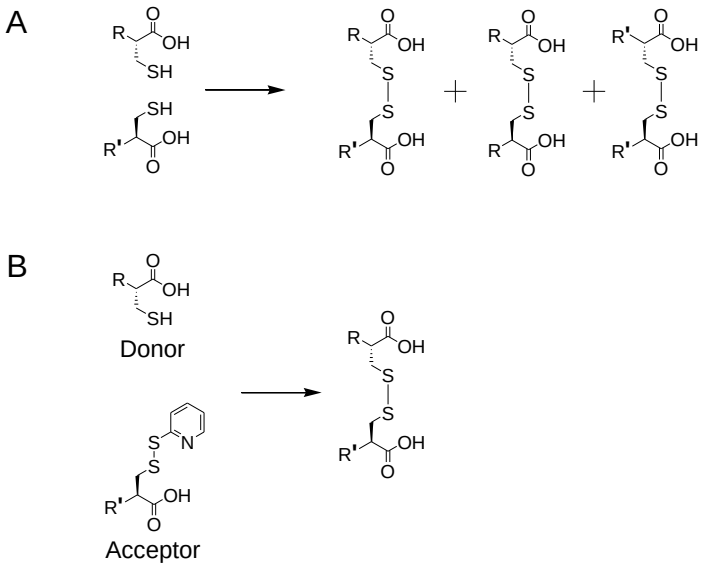


Figure 1-2: *Methods to form Disulfide Bonds*. (**A**) Random disulfide bond formation. Two free Cys will spontaneously form disulfide bonds. A mixture of products is observed. (**B**) Directed disulfide bond formation. An acceptor Cys activated as sulfanylpyridine will react with a free donor Cys to generate a disulfide bond. One product is observed if the reaction is performed in acidic conditions.

variety of oxidizing reagents such as potassium ferricyanide[63, 64] or DMSO,[65] free Cys will readily form disulfide bonds. Certain protected Cys can also be directly converted into disulfide bonds using reagents such as I_2[66, 67] or thallium (III) trifluoroacetate.[68, 69] Random reactions work well for intramolecular disulfide bonds, where peptides can be kept dilute to promote intramolecular bond formation when multiple Cys are present on one peptide. However this type of reactions is not efficient at generating a homogeneous intermolecular disulfide bond as the ratio of product formation is based upon the statistical distribution of one Cys encountering another.[62] Random polymerization of Cys flanked gene delivery peptides has generated a variety of polypeptides that condense DNA and demonstrate some selective uptake or increased stability.[30, 37, 52, 53, 55-59] The heterogeneous nature of the product polypeptides prevents further optimization.

By contrast, directed disulfide bond formation utilizes a free donor Cys sulfhydryl performing a nucleophilic attack on an activated Cys acceptor sulfhydryl to form a disulfide bond (Fig 1-2B). This type of reaction is favorable for intermolecular bond formation, as the acceptor Cys does not participate in side reactions and dimerization of the donor Cys can be minimized by performing the reaction under acidic conditions. The pioneering work of Harpp and Back first utilized a Cys-sulfanylphthalimide derivative that was stable, isolable, and would readily react with another Cys to generate an unsymmetric disulfide.[70] Numerous other reagents have been used to activate Cys, including dithiopyridines (2,2'-dithiodipyridine (DTDP)),[71, 72] sulfanyl chlorides (2-pyridinyl sulfanyl chloride (Pys-Cl)),[73] and bis(*tert*-butyl)azodicarboxylate.[74] The resulting sulfanylpyridine and sulfanyl hydrazide intermediates are stable and isolable, but are readily displaced by a free sulfhydryl. Directed disulfide bond formation has been utilized to generate a homogeneous two component gene delivery system that displayed potent gene transfer *in vitro*.[60] A reducible, homogeneous gene delivery system containing three or more components has not been reported.

This is partly due to the synthetic challenge of forming multiple disulfide bonds between different peptides in a directed fashion, a process which will be called iterative reducible ligation. For iterative reducible ligation, two Cys must be included within one peptide (Fig 1-3, Peptide 2). One Cys must incorporate an activating group (C1) to facilitate the formation of a disulfide bond with another peptide. The other Cys must be protected with a group (C2) that is stable during the initial disulfide bond formation. After generation of the first disulfide bond, the C2 group must be removed. The selection of the protecting group is critical for success; most Cys protecting groups are unsuitable to be used as the C2 group as will be discussed later. Despite the challenges, two groups have developed successful iterative reducible ligation strategies and applied these to generate model polypeptides.

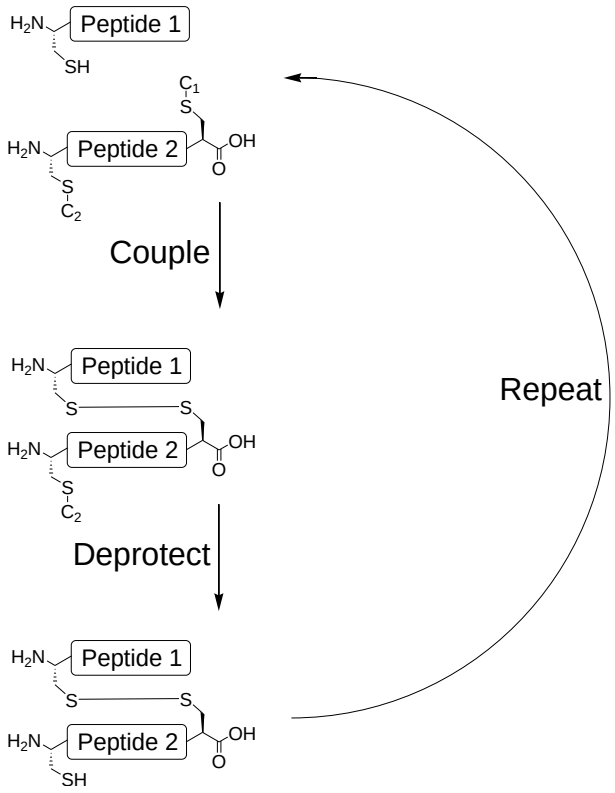


Figure 1-3: *Generalized Synthetic Approach for Iterative Reducible Ligation*. To conjugate multiple peptides through disulfide bonds, a peptide must contain two Cys with two different protecting/activating groups, C1 and C2. The C1 group must activate the Cys for directed disulfide bond formation with an incoming peptide. The C2 group must protect the second Cys during activation of the first Cys and disulfide bond formation. The subsequent removal of C2 must generate a free Cys and not disrupt the disulfide bond, allowing for repeated ligation steps.

Futaki and Kitagawa assembled four α-helical peptide units through selective disulfide bond formation, creating artificial 84-residue polypeptides to mimic four-helix-bundle proteins (Fig 1-4).[75-77] A convergent synthesis was undertaken, with initial disulfide bond formation utilizing Cys(trityl) residues deprotected and activated as 2-sulfanylpyridines to form the C1 activating group (**2** and **4**). The critical C2 protecting group was the acetamidomethyl (Acm) group. The Cys(Acm) were selectively deprotected with silver trifluoromethanesulfonate using neat TFA in the presence of a previously formed disulfide bond without any significant disulfide scrambling. Key to

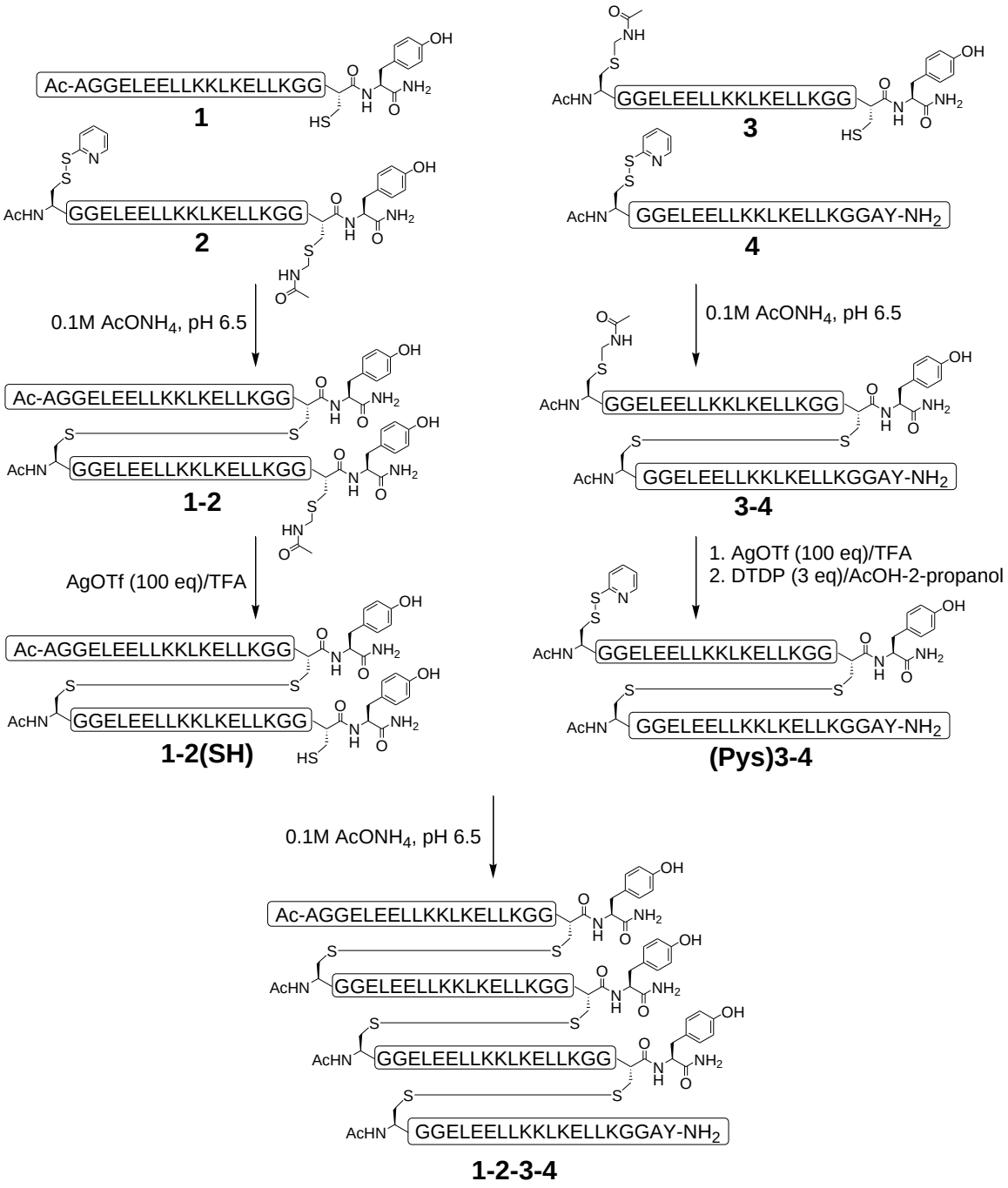


Figure 1-4: *Iterative Reducible Ligation Strategy Developed by Futaki and Kitagawa*.[75-77] Four peptides were successfully ligated to generate an artificial helix bundle protein.

their method was the omission of the traditional dithiothreitol reduction step used to remove excess silver ion resulting from the Acm deprotection, avoiding reduction of the disulfide bonds.[78] While the gel filtration purification step following this reaction was unlikely to remove the silver bound to Cys sulfhydryls ($-S^-Ag^+$), the presence of silver did not impact the nucleophilicity of the sulfhydryl towards reaction with DTDP or an activated peptide. The use of 100 mM ammonium acetate, pH 6.5 as the coupling buffer was sufficiently acidic to prevent disulfide scrambling in the formation of **1-2-3-4**. Coupling yields were approximately 75% for the first disulfide bonds from purified peptide intermediates and 41% for the ligation of the two polypeptides, including the Acm deprotection and DTDP activation reactions.

Another iteratively reducible ligation strategy was developed to synthesize artificial collagen, conjugating three peptide subunits together through a simplified cystine-knot as reported by Ottl and Moroder (Fig 1-5).[79, 80] This linear methodology was hypothesized to be adaptable to conjugating four or more subunits together for larger polypeptides.[81] Similar to the first strategy, an initial disulfide bond was formed between a donor peptide and an acceptor peptide with a Cys C1 activated with 2,2'dithio-di-(5-nitro)pyridine (DTNP), a more reactive form of DTDP.[82, 83] The C2 group was again selected to be an Acm (**5-6(Acm)**); however, instead of a two step removal of the protecting group followed by activation of the Cys, the Acm was directly displaced by 3-nitropyridyl-2-sulfanyl chloride (nPys-Cl) to form **5-6(nPys)**. The simplified scheme allowed deprotection and activation of the Cys in one step without generating a free Cys that could result in scrambling of the initial disulfide bond. Ligation with a third donor peptide in 50 mM ammonium acetate pH 5.5 resulted in the formation of the reducible polypeptide **5-6-7**, with the ligation buffer sufficiently acidic to prevent disulfide scrambling. Yields of 70 - 80% were reported for each ligation step.

Figure 1-5: *Iterative Reducible Ligation Strategy Developed by Ottl and Moroder*.[79-81] Three peptides were conjugated together to form artificial collagen strands through a simplified cystine knot. The chemistry allows further addition of more peptides by continual incorporation of two Cys within each new peptide.

While successfully applied to generate reducible polypeptides, these strategies are not robust enough for the generation of gene delivery polypeptides. Critical to the Futaki and Kitagawa process was the removal of an Acm in neat TFA. The attachment of a PEG or glycan results in peptides that are poorly soluble in TFA, resulting in less than stoichiometric Acm deprotection that would form truncated polypeptide species over multiple ligation cycles. The use of TFA as a solvent also complicates developing this strategy on a solid support. An iterative reducible ligation approach on a solid support offers many advantages to a solution phase approach, including eliminating intermediate purification steps, washing away of excess reagents, and generation of a single product that can be cleaved from the solid support.[84] Since TFA cleaves many of the commonly available resin linkers, polypeptides could be prematurely cleaved from the solid support.

The methodology developed by Ottl and Moroder directly transforms a Cys(Acm) into a reactive Cys(nPys). The activating reagent nPys-Cl is known to react with the indole ring of tryptophan.[85, 86] Consequently, gene delivery components containing tryptophan such as the fusogenic peptide melittin would also be chemically modified. Additionally nPys-Cl can react with peptides containing an Nε-acridinyllysine residue, which has been shown to be critical for increasing the circulatory stability of polyplexes.[31, 32] While both methodologies have produced structurally defined reducible polypeptides, neither is ideal for gene delivery polypeptides. A new iterative reducible ligation strategy is required, utilizing mild deprotection conditions of the C2 protecting group that are compatible with glycans, PEG, and Acr-containing peptides.

Cysteine Protecting Groups

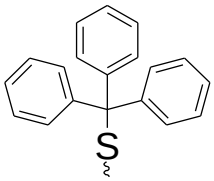
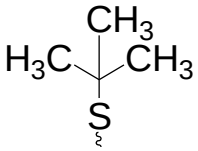
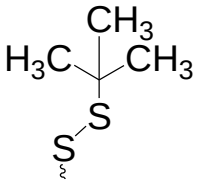
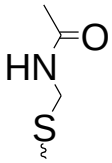
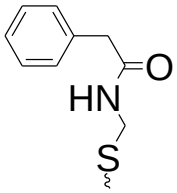
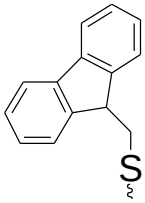
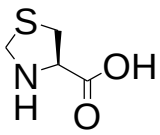
The ability to form multiple disulfide bonds in a directed, iterative fashion is dependent on the selection of appropriate C1 and C2 groups. The C1 group must activate the Cys for reaction with a donor Cys to generate a disulfide bond. This can be one of many different Cys activating groups, including a sulfanylpyridine (Pys). However these

groups cannot be incorporated as protected Cys residues during Fmoc solid phase peptide synthesis since these groups are labile to repeated base treatments.[87] These groups must be generated from Cys that are protected with other groups, which can be removed prior to activation or directly activated. Perhaps the most facile way is to use an acid labile group such as a triphenylmethyl (trityl)[88] group that is removed during the cleavage of the peptide from resin, generating a free Cys that can be reacted with DTDP to generate a C1 Pys group. Alternatively most Cys protecting groups can be removed post cleavage and then activated with DTDP. Direct conversions to activated Cys with sulfanyl chloride derivatives should be avoided since Trp residues are also modified.[85, 86] One interesting approach is the direct conversion of the Cys protecting group with DTNP in the presence of thioanisole, which reportedly can substitute a variety of protecting groups.[89, 90]

The choice of the C2 group requires more careful consideration, as this group must be selectively removed after the formation of a disulfide bond without side reaction. A variety of deleterious reactions are possible including dimerization, reduction of the disulfide bond, or disulfide scrambling following the removal of the C2 protecting group due to the presence of a free Cys and a disulfide bond. Additionally this group must be stable to resin cleavage conditions to avoid premature removal, not susceptible to activation when the C1 group is activated, and be unaffected during the formation of the initial disulfide bond. While many Cys protecting groups have been reported (Table 1-1), few are acceptable as a C2 protecting group.

There are many acid labile groups utilized in peptide synthesis, including the 9H-xanthen-9-yl (Xan),[91] 2,4,6-trimethoxybenzyl (Tmb),[92] 4-methoxytrityl (Mmt),[93] and triphenylmethyl (trityl)[88] groups. These groups are removed with mild acid treatment such as dilute TFA in the presence of scavengers such as ethanedithiol (EDT). Such groups are inappropriate for the C2 protecting group as they are removed during the cleavage of the peptide from resin and subsequently cannot protect the Cys through the first ligation reaction.

Table 1-1: *Structures and Deprotection Conditions for Common Cys Protecting Groups*.

Structure	Name	Removal
	S-Triphenylmethyl (trityl)	TFA, I_2, metals, RSCl
	S-*tert*-butyl (tBut)	HF, metals, RSCl
	S-*tert*-butylmercapto (StBu)	TFA, HF, base, RSCl
	S-Acetamidomethyl (Acm)	Metals, I_2, RSCl
	S-Phenylacetamidomethyl (Phacm)	Penicillin G Acylase (*E. coli*)
	S-Flourenylmethyl (Fm)	Base
	Thiazolidine (Thz)	Methoxyamine, Metals

Some acid labile groups are stable in high concentrations of TFA and require more harsh deprotection conditions and include the benzyl (Bzl),[94] 4-methoxybenzyl (Mob),[95] 4-methylbenzyl (Meb),[96] and *tert*-butyl (*t*Bu)[97] groups. These groups require the use of HF for removal, requiring specialized equipment for safe handling. The highly toxic and corrosive nature of HF limits the applicability for these groups to be utilized as the C2 protecting group.

While many disulfide reagents are not stable to multiple base treatments, hindered disulfides such as the *tert*-butylmercapto (S*t*Bu)[98] group can be employed with Fmoc peptide synthesis. This group can be removed by addition of reducing agents to generate a free Cys. For an iterative reducible ligation strategy, the introduction of reducing agents would also result in the reduction of all disulfide bonds and result in the degradation of the polypeptide. Reducible protecting groups can therefore not be utilized.

Metal labile protecting groups have been developed to offer base and acid stable Cys protecting groups, and include the acetamidomethyl (Acm),[99] trimethylacetamidomethyl (Tacm)[100], and phenylacetamidomethyl (Phacm)[101] groups. To date the only successful iterative reducible ligation strategies have employed the Acm protecting group as the critical C2 group, by omitting reduction steps or directly converting an Acm to nPys with a sulfanyl chloride. However the current approaches cannot be utilized for gene delivery polypeptides for the reasons described previously.

An interesting protecting group is the Phacm group,[101] which is chemically similar to the Acm and is labile in the presence of metals. This group is also cleavable by the *E. coli* enzyme penicillin amidohydrolase, which can be immobilized on a solid support.[102] Enzymatic removal of the group in the absence of a reducing agent results in the formation of the oxidized product and a free Cys can only be generated in the presence of reducing agents.[102]

In the design of an iterative reducible ligation strategy, the C2 protected Cys is located on the N-terminal. This allows the use of Boc-Cys residues, including base labile

groups such as the 9-fluorenylmethyl (Fm)[103] or 2-(2,4-ditnitrophenyl)ethyl (Dnpe)[104] groups. However, the increased reactivity of Cys in basic conditions results in dimerization during removal of these groups, requiring the use of a reducing agent to generate a free Cys.

While not exhaustive for every Cys protecting group, this list summarizes the most commonly utilized groups in Fmoc peptide chemistry with none suitable for an iterative reducible ligation strategy for gene delivery polypeptides. The ideal protecting group must be stable to acid and be removed by addition of a reagent in mild aqueous acidic conditions to ensure solubility of PEG and glycan containing peptides and prevent dimerization of the free Cys. The deprotection reaction must also result in a free Cys without a reduction step. This is not a common requirement for protected Cys.

Pioneered by Kent *et al.*,[105] another technique to ligate multiple peptides together is native chemical ligation in which an amide bond is formed by reaction of an N-terminal Cys with an activated carboxylic acid. A thioester is formed first by the sulfur of Cys attacking the carboxylic acid, followed by an S-to-N acyl shift resulting in the amide formation (Fig. 1-6). Similar to iterative reducible ligation is the use of an N-terminal Cys to mediate the ligation of two peptides, with a reduced Cys required for the ligation.

Numerous protecting groups have been employed successfully on the N-terminal Cys for native chemical ligation, including a trityl for a single ligation and Acm for multiple ligations.[106-108] One protected Cys used for native chemical ligation but not commonly found in other peptide chemistries is a thiazolidine,[109, 110] resulting from Cys condensed with formaldehyde to form a five member ring similar to proline. The reported deprotection involves treatment of a thiazolidine with high concentrations of methoxyamine, forming formaldehyde O-methyl-oxime and generating a free Cys.[110, 111] Methoxyamine is commercially available as an HCl salt, resulting in a typical reaction pH of approximately 4. In theory the low pH should inhibit dimerization of the liberated

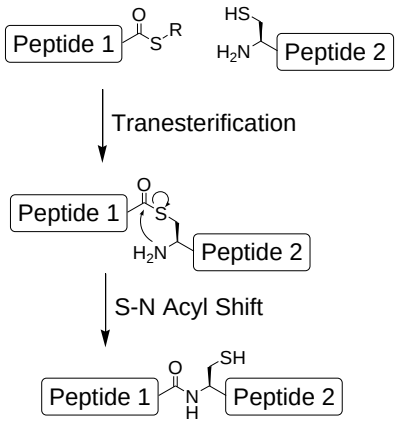


Figure 1-6: *Mechanism for Native Chemical Ligation*. An N-terminal Cys on one peptide reacts with an activated thioester to generate a new thioester between the two peptides. The terminal amine then promotes an S-N acyl shift to generate the more stable amide, resulting in the ligation of the two peptides through an amide bond.

Cys, though often a reducing agent is used after the thiazolidine hydrolysis to ensure a free Cys.[110] This protecting group has been used successfully in the synthesis of the small 46 amino acid protein crambin,[109] as well as in the complete chemical synthesis of a 304-amino acid tetra-ubiquitin representing the longest chemical synthesis of a polypeptide.[112] Thiazolidine based native chemical ligation has also been used in the successive ligation of glycopeptides, demonstrating the stability of glycans towards the thiazolidines and the methoxyamine deprotection conditions.[113] Additionally this methodology has been applied on a solid support successfully in the generation of a short enzyme, taking advantage of the benefits of using a solid support based chemistry.[114] Despite the mild deprotection conditions, a thiazolidine has never been utilized in an iterative reducible ligation strategy.

Research Objectives

While many multicomponent gene delivery polypeptides can generate high levels of gene expression *in vitro*, there is currently no system that can mediate therapeutic levels *in vivo*. This is partly due to the numerous barriers to gene delivery in an animal,

requiring a carrier to incorporate many different components. While multicomponent carriers have been designed and tested, none demonstrate efficacious delivery. The assembly of the carrier is critical for success and requires incorporation of a triggered release mechanism to disassemble the polyplex upon cellular uptake. The reducing environment within the cell affords such a mechanism, as disulfide bonds are readily cleaved. Generation of single disulfide bonds through directed chemistry is easily achieved, though the directed formation of multiple disulfide bonds is not as readily accomplished. While a few groups have successfully synthesized polypeptides composed of multiple iterative directed disulfide bonds, their methodologies are not suitable for gene delivery polypeptides. Generation of gene delivery systems incorporating reducible bonds must be done in a controlled, directed synthesis for optimization of the carrier; however, this has never been reported for gene delivery polypeptides.

The overall scope of this thesis is to develop a chemical methodology that iteratively couples peptides through reversible disulfide bonds in high yields. This methodology could then be used to generate homogeneous gene delivery polypeptides that can be optimized for *in vivo* gene delivery. The lack of a chemical means of selectively conjugating multiple peptides through reducible bonds is an unexplored area in the gene therapy field and one of critical importance in the generation of macromolecular gene delivery systems. Only by generating well characterized, defined systems can the different components within the system be optimized. Furthermore, optimization requires the use of precise chemistries that can install the desired components to specified locations within the carrier.

The central hypothesis of this thesis is that a novel iterative reducible ligation strategy based upon the thiazolidine protecting group used in native chemical ligation could be developed and optimized to generate a strategy of linking multiple peptides through reducible bonds with high yielding reactions. Important in the development of this chemistry is the use of mild conditions that are compatible with both PEGylated and

glycan modified peptides. Furthermore, to generate peptides linked together by disulfide bonds of varying strength, penicillamine analogues will replace Cys in the iterative coupling process. Due to the mild conditions of this methodology, the incorporation of a solid support will also be explored.

CHAPTER 2

THIAZOLIDINE MEDIATED ITERATIVE REDUCIBLE LIGATION

Abstract

Chapter 2 describes the application of thiazolidines in an iterative reducible ligation strategy. Initial studies highlighted the sensitivity of sulfanylpyridine activated peptides to treatment with methoxyamine and the lability of thiazolidines to treatment with dithiodipyridine. Optimization of thiazolidine hydrolysis using methoxyamine indicated high concentrations of methoxyamine coupled with low peptide concentrations were required for hydrolysis without concurrent dimerization. A strategy was developed that allowed the formation of a reducible polypeptide of three peptide subunits, though in less than desirable yields. Reduction of the polypeptide resulted in the generation of the constituent peptides with distinct RP-HPLC retention times.

Introduction

The condensation reaction of Cys with carbonyl groups to form a proline-like cyclic amino acid has been well documented, with the first evidence of thiazolidine formation done by Schubert in 1936 with Cys condensed with formaldehyde.[115, 116] The substitution of the carbonyl has been demonstrated to determine the stability of the thiazolidine. To hydrolyze a thiazolidine (Thz) derived from Cys and formaldehyde, Ratner and Clarke subjected the Thz to boiling HCl, treated with ferric chloride at pH 10, or reacted with sodium sulfite at pH 6.[117] Conversely, a 2,2-dimethylthiazolidine (DMT) derived from Cys condensed with acetone was hydrolyzed by addition of water.[118]

The use of a thiazolidine as a protected Cys for peptide chemistry was first purposed by Sheehan and Armstrong using DMT.[119] King *et al*. subsequently used DMT in the synthesis of glutathione, using mercury to mediate thiazolidine hydrolysis.[120] The mercury was subsequently removed with hydrogen sulfide. Further exploration demonstrated that following coupling of DMT to Gly, the substituted thiazolidine could

be hydrolyzed with HCl in MeOH without requiring a reduction step to generate the free Cys.[121]

The direct conversion of a thiazolidine to an activated Cys was first shown by Kemp and Carey, where DMT was attached to a Gly-Gly-Ala tripeptide and converted to the activated S-methoxycarbonylsulphenyl (Scm) derivative.[122] This was done by either DMT hydrolysis in 1:1 solutions of water:alcohol or by direct reaction with Scm-Cl. Thiazolidines have also been incorporated into peptides as pseudo-proline derivatives, improving the solvation and coupling kinetics of the peptides.[123, 124] One interesting study compared the stability of DMT and Thz in TFA, finding DMT labile after several hours while Thz was stable.[123] While many syntheses have benefited from the relative ease of DMT hydrolysis, this protected thiazolidine cannot be incorporated into an iterative reducible ligation strategy because it is too labile.

The first significant use of Thz as a temporary Cys protecting group was reported by Villain *et al.* in 2001.[110] The authors examined the hydrolysis of Thz incorporated in a ThzYAKYAKL octapeptide, using methoxyamine ($MeONH_2$) to mediate Thz hydrolysis. Methoxyamine had previously been used to release peptides with an N-terminal Cys bound to an aldehyde functionalized resin through the 5 member thiazolidine heterocyclic ring.[111] A pH and $MeONH_2$ concentration dependent hydrolysis was observed and the authors report using the chemistry to ligate four peptide fragments together through native chemical ligation, though details of the synthesis are not reported.[110] The first detailed synthesis utilizing this chemistry was by Bang and Kent in the total synthesis of the small protein Crambin.[109] A one-pot total synthesis was reported for the ligation of three peptides in higher yields compared to when a Cys(Acm) was employed.[108] Subsequent thiazolidine mediated native chemical ligations have resulted in the total syntheses of HIV type 1 protease,[125] human lysozyme,[126] and tetraubiquitin.[112]

Due to the many successes of native chemical ligation at utilizing an N-terminal thiazolidine to selectively mask a required Cys functionality, the applicability of a Thz to

an iterative reducible ligation strategy was explored. The reported unmasking conditions of methoxyamine in aqueous acidic conditions were expected to be compatible with previously formed disulfide bonds, as methoxyamine is not a reducing agent and acidic conditions should minimize the nucleophilicity of the resulting free Cys. The preliminary findings presented here demonstrate the suitability of a thiazolidine as a C2 protecting group in the formation of a reducible polypeptide containing three subunits. The low yielding synthesis highlighted the problems with this initial approach and led to the improvements in the strategy discussed in chapter 3.

A Note on Nomenclature Used in Chapter 2

A combination of 1 and 3 letter abbreviations is used to denote peptides used in model reactions. This is replaced by a numbering scheme when a reducible polypeptide is generated. An (a) will signify that a thiazolidine has been hydrolyzed. For reduced polypeptides, the number of the parent peptide is used though sulfanylpyridines and thiazolidines are no longer present.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, triethylamine, diisopropylethylamine, 2,2’-dithiodipyridine (DTDP), acetic anhydride, 9-chloroacridine, thiazolidine-4-carboxcylic acid (Thz), Di-*tert*-butyl dicarbonate, tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), methoxyamine hydrochloride, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, dichloromethane (DCM), dimethylformamide (DMF) and trifluoroacetic acid (TFA) were obtained from Fisher Scientific.

Synthesis of N-Boc-(R)-thiazolidine-4-carboxcylic acid (Boc-Thz-OH)

N-Boc-(R)-thiazolidine-4-carboxcylic acid was prepared according to prior published procedures.[127] Briefly, thiazolidine-4-carboxycylic acid (2.496 g, 18.74 mmol) was suspended in 25 ml DCM. Triethylamine (4.00 ml, 27.4 mmol) was added and the reaction was cooled to 0˚ C. Di-*tert*-butyl dicarbonate (4.500 g, 20.62 mmol) was dissolved in 50 ml DCM and added dropwise to the chilled suspension. After 12 hrs, the reaction was warmed to RT and the solvent evaporated under reduced pressure. Following addition of 50 ml EtOAc, the organic layer was washed with 20 ml 1 N HCl, dried over $MgSO_4$, and evaporated under reduced pressure. A white powder was precipitated from petroleum ether and filtered to yield 3.43 g (14.7 mmol, 79 %). (300 MHz ^{1}H NMR, $CDCl_3$): δ=4.4-4.9 (m, 3H), 3.3 (m, 2H), 1.5 (s, 9H).

Synthesis and Characterization of Peptide Subunits

Unsubstituted Wang resin was loaded with Fmoc-Lys(Boc)-OH or Fmoc-Cys(Trt)-OH using standard DIC coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. S-2-sulfanylpyridine protected peptides were generated by reacting crude thiol-deprotected peptides with 10 mol eq of DTDP in 2 M acetic acid/2-propanol (10:3 v/v) overnight. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak was collected and

pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Trp ε_{280nm} = 5600 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Acr ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Ligation Reactions to form Polypeptides

Ligation reactions were performed on a scale ranging from 0.050-2.5 μmol in 0.1 M ammonium acetate pH 5. A donor peptide containing free Cys (0.50 μmol) was reacted with an S-2-sulfanylpyridine acceptor peptide (0.75 μmol, 1.5 mol eq) for 12 hrs at RT in 0.5 ml total volume. The product was preparatively purified by injecting 0.25 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak from multiple runs was collected, pooled, and lyophilized. The ligated polypeptide product was reconstituted in 0.1 v/v % TFA and the yield determined by Abs. Purified polypeptides were characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Thiazolidine Hydrolysis

The thiazolidine was hydrolyzed on a 0.1 μmol scale by reaction with 1 M methoxyamine in 0.1 v/v % TFA for 12 hrs at RT in 1 ml total volume. For iterative ligation, the resulting donor polypeptide was purified by injecting 0.1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$. The major peak was collected and lyophilized. The donor polypeptide was reconstituted in 0.1 M ammonium acetate pH 5 and reacted with an S-2-sulfanylpyridine acceptor peptide as described above.

Results

The present study was undertaken to examine whether a thiazolidine was an appropriate C2 protecting group for an iterative reducible ligation strategy. The pioneering work of Villain *et al*. demonstrated that high concentrations of methoxyamine and long reaction times were required for thiazolidine hydrolysis.[110] An initial investigation was undertaken to see if high concentrations of $MeONH_2$ would disrupt a disulfide bond. A model $AcrK_6C$-C peptide was shown to be stable in 0.2 M $MeONH_2$ over several hours, indicating that $MeONH_2$ does not disrupt Cys-Cys disulfide bonds. However when an activated $AcrK_6C$(Pys) peptide was treated with 0.2 M $MeONH_2$, a new product was observed (Fig 2-1). This product was 32 amu heavier than the parent peptide without the sulfanylpyridine group (1195.5 amu versus the observed 1227.0), and most likely represented an oxidation of the Cys with the addition of two oxygen atoms. These results indicated that methoxyamine could potentially hydrolyze a thiazolidine in the presence of a disulfide bond, though a purification step would be required before the free Cys could undergo subsequent coupling with an activated peptide.

A peptide was designed to incorporate both an activating C1 sulfanylpyridine group and a C2 masking thiazolidine (Fig 2-2). Such a peptide would allow iterative coupling, with the assumption that the masked C2 thiazolidine would be stable during C1 activation and initial ligation. The Boc-Thz-OH amino acid was synthesized according to published procedures in 79 % yield and incorporated into a model $ThzWK_4C$ peptide. The model $ThzWK_4C$ peptide (935.2 amu) could be cleaved, stored, and analyzed with an intact thiazolidine (Fig 2-3A). Unfortunately the thiazolidine was not stable upon addition of DTDP. After addition of DTDP to activate the C-terminal Cys, an earlier eluting peak with a 14 amu loss was observed instead of the expected later eluting peak that was expected to be 110 amu heavier (Fig 2-3B). The lower mass indicated the loss of the thiazolidine with disulfide bond formation, resulting in the cyclic or polymerized product.

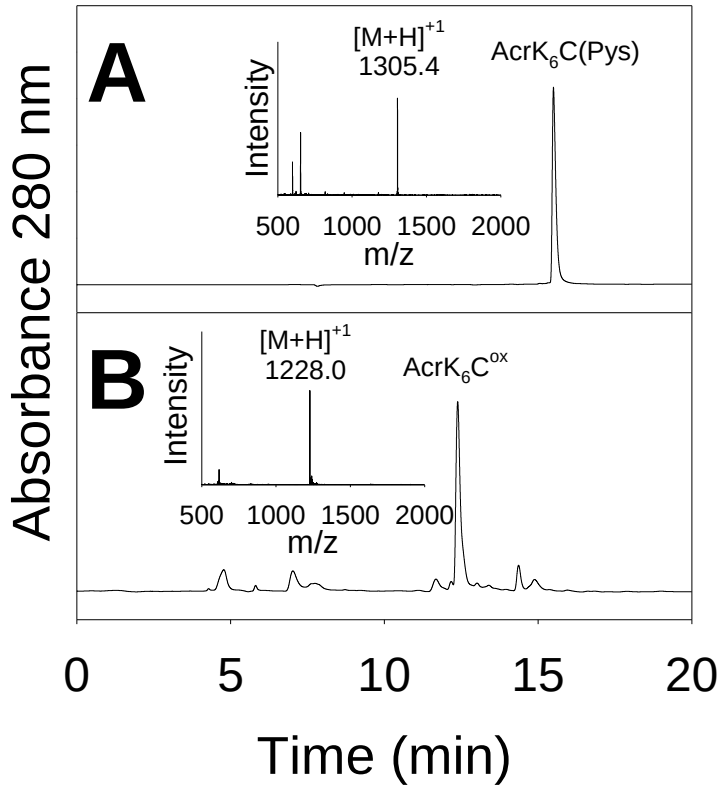


Figure 2-1: *LC-ESI-MS Analysis of the AcrK$_6$C(Pys) Treated with Methoxyamine*. Panels A & B illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates purified AcrK$_6$C(Pys). Panel B illustrates the treatment of AcrK$_6$C(Pys) in 0.2 M methoxyamine for 2 hrs, resulting in an oxidized product.

Due to the sharpness of the peak, the cyclic peptide was more likely. Using lower stoichiometric excesses of DTDP also resulted in the cyclized peptide indicating the Thz could not prevent the intramolecular oxidation reaction.

An interesting observation was made when the number of spacing amino acids was increased between the N-terminal C2 thiazolidine and the C-terminal C1 Cys(Pys). The synthesis of a ThzWK$_9$C peptide increased the number of spacing amino acids between the Thz and Cys from 5 to 10, which eluted as a relatively pure peak (Fig 2-4A). Addition of DTDP to ThzWK$_9$C resulted in the formation of two later eluting products

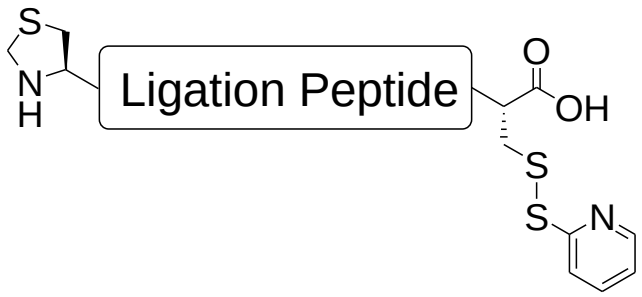


Figure 2-2: *Structure of General Ligation Peptide with C1 Cys(Pys) and C2 Thz Groups*. Ligation peptide with terminal Cys to undergo reducible iterative ligation. The C-terminal Cys has an activated C1 sulfanylpyridine. The N-terminal Cys is masked as a C2 thiazolidine.

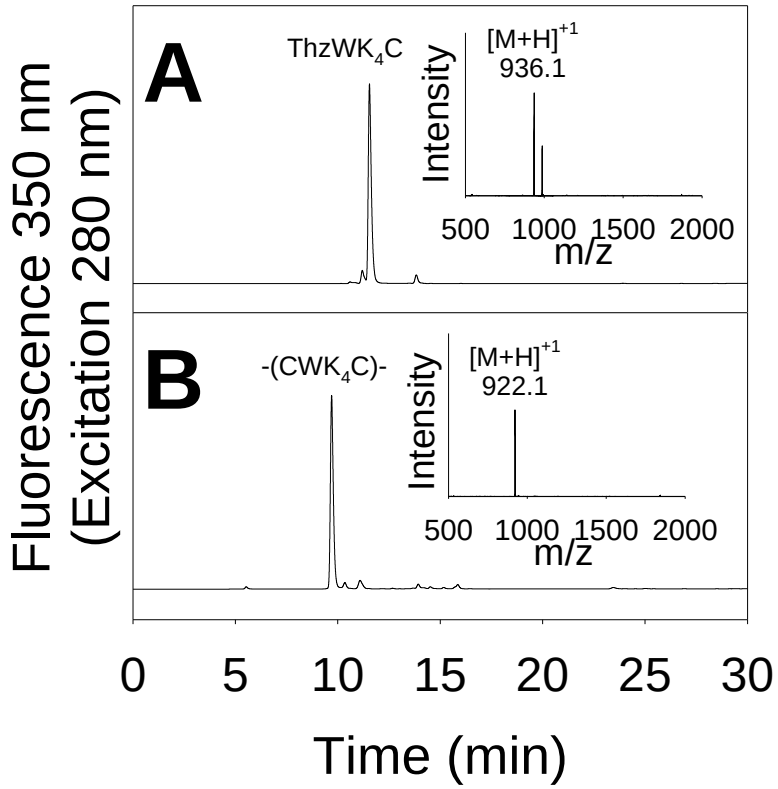


Figure 2-3: *Attempted Formation of a Thiazolidine C2 and Sulfanylpyridine C1 Model Peptide*. Panels A & B illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Fl_{Ex=280\ nm,\ Em=350\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates purified $ThzWK_4C$. Panel B illustrates the treatment of $ThzWK_4C$ with 10 mol eq DTDP, forming the cyclized peptide.

(Fig 2-4B). The first product was identified to be the desired ThzWK$_9$C(Pys) and the second as the doubly activated C(Pys)WK$_9$C(Pys), with no cyclization or polymerization observed.

The C2 Thz proved unstable to activation of C1 in a ThzWK$_x$C peptide, where x = 4 or 9. These results indicated that a thiazolidine could not be incorporated onto an acceptor peptide, as activation of the C1 concurrently resulted in reaction of the thiazolidine. To overcome this, the thiazolidine containing peptide would have to be the donor peptide in formation of a disulfide bond (Fig 2-5, **2**). This would allow formation

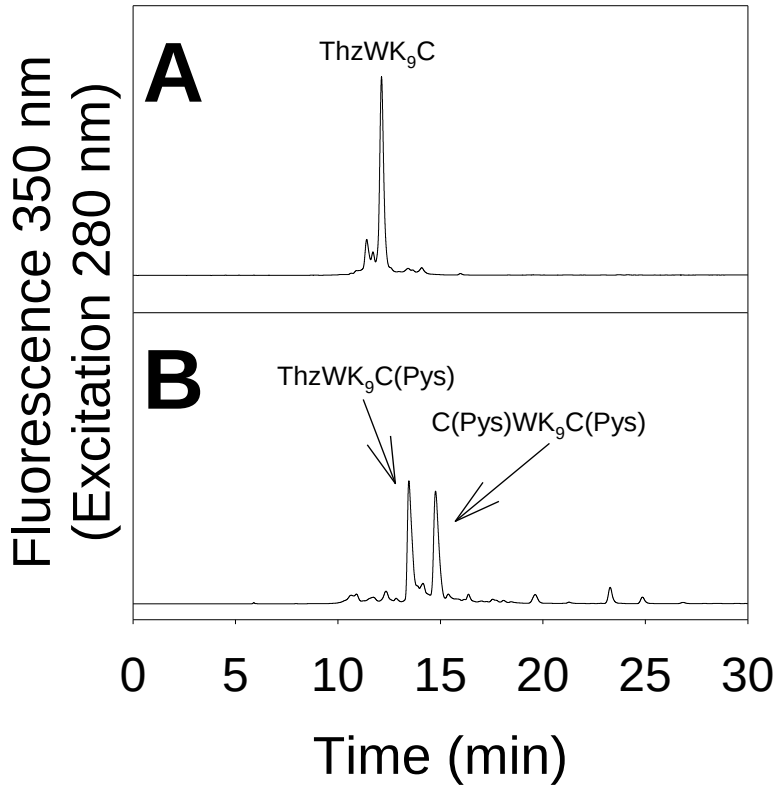


Figure 2-4: *Increasing Spacing Amino Acids Between Thiazolidine C2 and Sulfanylpyridine C2 Peptide*. Panels A & B illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Panel A illustrates purified ThzWK$_9$C. Panel B illustrates the treatment of ThzWK$_9$C with 10 mol eq DTDP, forming the singly and doubly activated ThzWK$_9$C(Pys) and C(Pys)WK$_9$C(Pys).

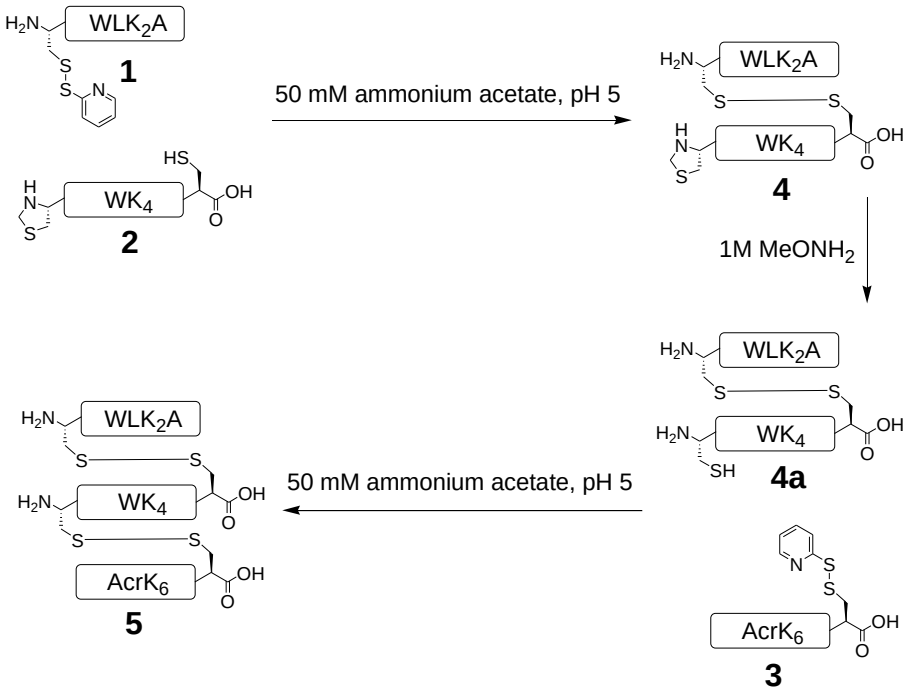


Figure 2-5: *Synthetic Scheme to Generate Reducible Polypeptide 5*. Acceptor peptide **1** and donor peptide **2** were conjugated to form protected polypeptide **4**. Removal of the Thz from **4** yielded donor polypeptide **4a**, which was subsequently ligated to acceptor peptide **3** to form reducible polypeptide **5**.

of the first disulfide bond with acceptor peptide **1**, forming polypeptide **4** with an intact thiazolidine. Subsequent thiazolidine removal would yield a donor polypeptide **4a** that could react with acceptor peptide **3** to form polypeptide **5** containing three peptides ligated through two disulfide bonds. If longer polypeptides were desired, the donor polypeptide **4a** could be converted into an acceptor polypeptide by addition of DTDP, followed by reaction with donor peptide **2**.

The synthesis of reducible polypeptide **5** was therefore undertaken (Fig 2-5). Purified peptides **1** (Fig 2-6A) and **2** (Fig 2-6B, 1.5 mol eq) were coupled in 100 mM ammonium acetate pH 5 to yield the polypeptide **4** (Table 2-1). Following preparative purification, **4** was recovered in 63 % purified yield and eluted as a single peak with the correct mass (Fig 2-6C).

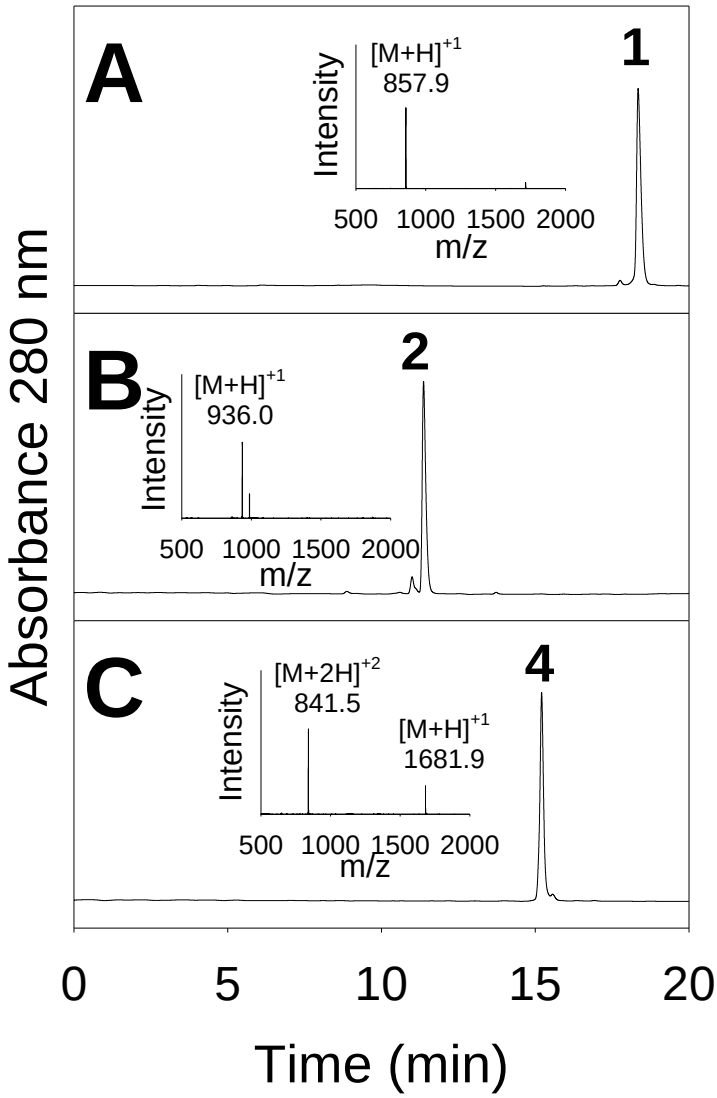


Figure 2-6: *Synthesis of Polypeptide **4***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified acceptor peptide **1** and donor peptide **2** to establish their retention time. Reaction of **1** and **2** (1.5 mol eq) in 50 mM ammonium acetate pH 6 yielded the polypeptide **4**, which was recovered in a 63 % purified yield as a single product (Panel C).

The hydrolysis of **4** to **4a** highlighted the difficulties of using methoxyamine to mediate thiazolidine hydrolysis. Initial studies using 0.2 M to 1.0 M $MeONH_2$ indicated only partial hydrolysis after 2 hrs and significant dimerization after 18 hrs. Performing

Table 2-1: *Synthesis and Characterization of Initial Ligation Peptides*.

Peptide[a]	Mass (calc / obs)	% Yield
1 (C(Pys)WLK$_2$A)	857.1 / 856.9	36[b]
2 (ThzWK$_4$C)	935.2 / 935.0	24[b]
3 (AcrK$_6$C(Pys))	1304.7 / 1304.2	32[b]
4 (ThzWK$_4$C-CWLK$_2$A)	1681.1 / 1680.9	63[c]
5 (AcrK$_6$C-CWK$_4$C-CWLK$_2$A)	2862.7 / 2862.4	8[c]

[a] Thz refers to thiazolidine, Pys to 2-sulfanylpyridine, and Acr to Nε-acridinyllysine.

[b] Purified yield based on initial resin substitution.

[c] Purified yield determined for the last ligation step.

the reaction in 0.1 v/v % TFA minimized dimerization after 18 hrs, but the presence of **4** was observed indicating the reaction was not complete. Longer reaction times in 0.1 v/v % TFA resulted in the disappearance of **4** concurrent with the formation of dimer **4a**. Dimerization could be minimized by keeping the peptide dilute, with no dimerization observed when the reaction was performed using a peptide concentration of 20 μM. A slightly higher concentration of 100 μM **4** was selected during the synthesis of **5**, balancing the dimerization with generating sufficient **4a** for purification and further conjugation.

Under optimized conditions, 100 nmol of **4** were treated with 1 M methoxyamine in 0.1 v/v % TFA for 18 hrs to generate donor polypeptide **4a** (Fig 2-7A & B). A slightly earlier retention time was observed as well as a loss of 12 amu from **4** (Fig2-7B). Starting peptide **4** and cyclized **2** were observed in the reaction, resulting in approximately 75 % conversion to **4a** (Fig 2-7B). Following preparative purification of **4a**, acceptor peptide **3** (Fig 2-7C, 1.5 mol eq) and donor polypeptide **4a** were reacted in 100 mM ammonium acetate, pH 5. Generation of reducible polypeptide **5** was observed, which following preparative purification was recovered in 7.5 % yield from intermediate **4** (Fig 2-7D).

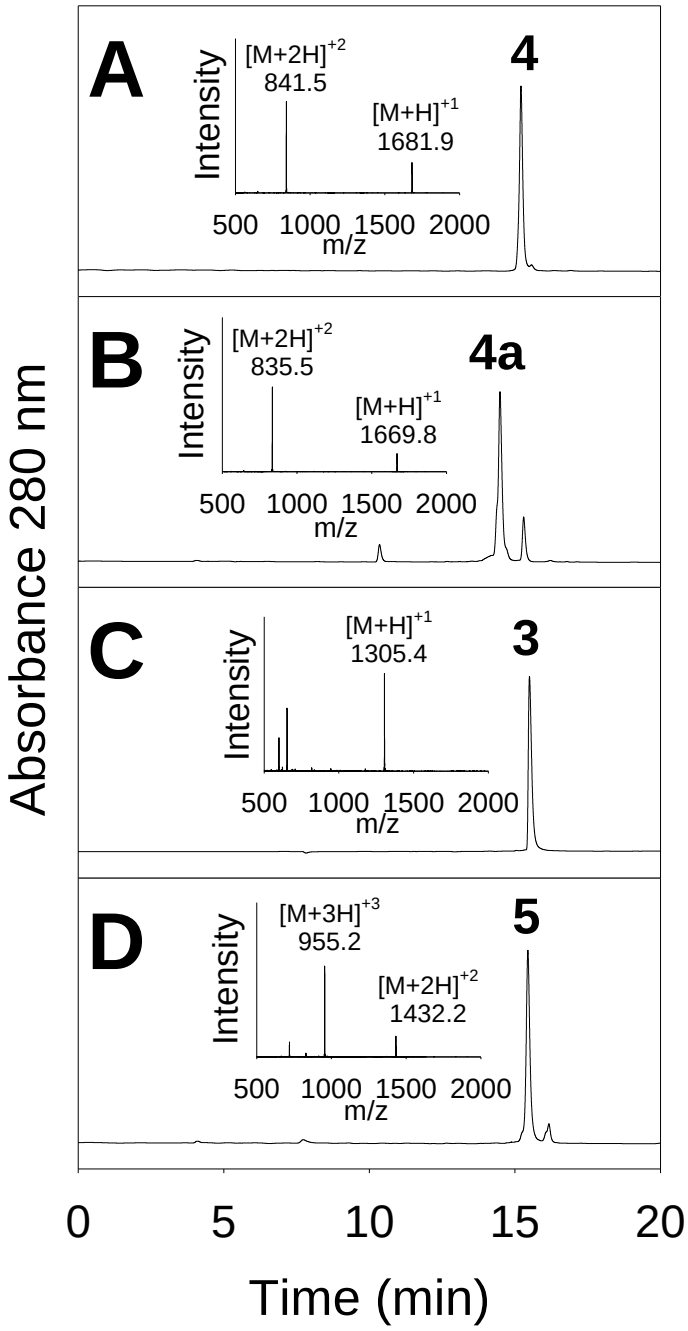


Figure 2-7: *Synthesis of Polypeptide **5***. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates the RP-HPLC analysis of Thz protected polypeptide **4**. 1 M methoxyamine was used to hydrolyze the Thz to generate donor polypeptide **4a** (Panel B). Following purification, **4a** was immediately reacted with acceptor peptide **3** (Panel C) to generate reducible polypeptide **5**, which was recovered in a 7.5 % yield following preparative purification (Panel D).

One interesting observation of this polypeptide was the unique fluorescence and absorption spectra (Fig 2-8). Before the final peptide coupling, **4** only contained tryptophans for chromophores, which have intrinsic 350 nm fluorescence and 280 nm absorbance. An acridinyllysine residue was incorporated into peptide **3**, which has an

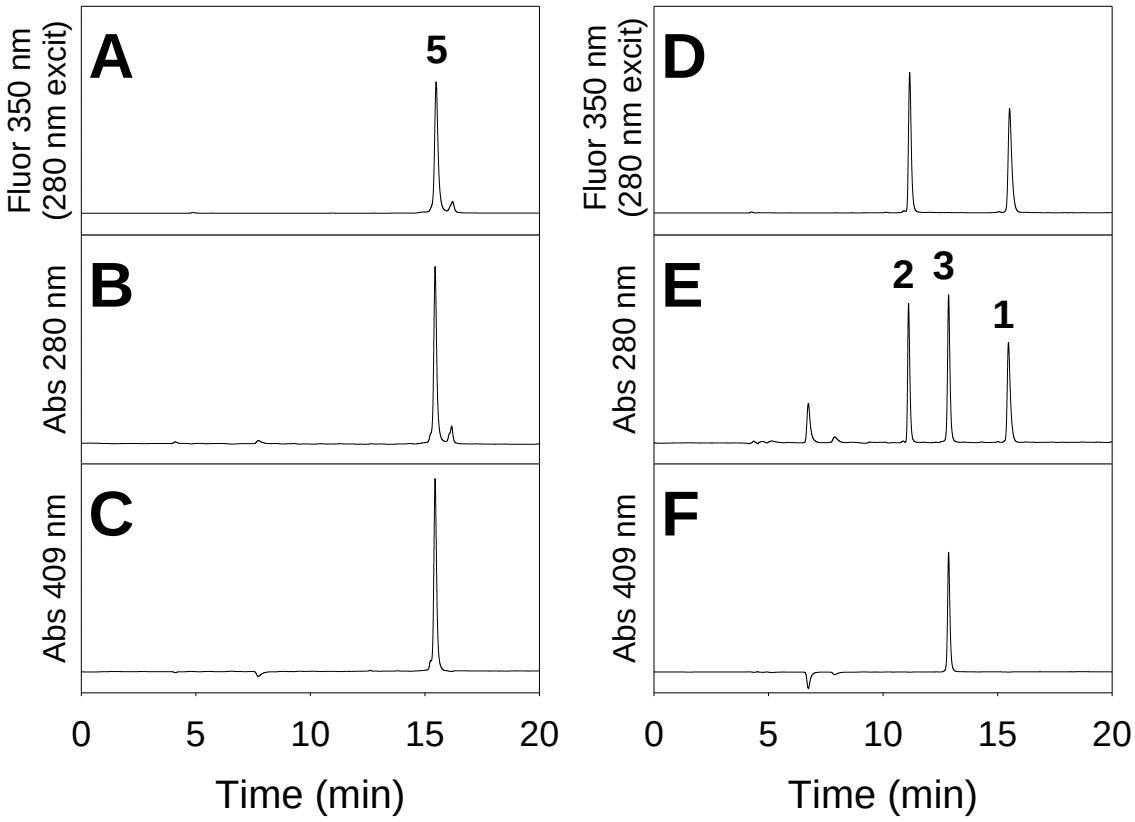


Figure 2-8: *Analytical RP-HPLC of Polypeptide **5** and Reduced **5***. Panels A-F illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Fl_{Ex=280\ nm,\ Em=350\ nm}$ (Panels A & D) and $Abs_{280\ nm}$ (Panels B & E) and $Abs_{409\ nm}$ (Panels C & F). **5** displays both 350 nm fluorescence and 409 nm absorbance from the tryptophan and acridinyllysine chromophores (Panels A-C). When reduced, component peptides **1** and **2** elute as the first peak and third peak and display 350 nm fluorescence and 280 nm absorbance due to the tryptophan chromophore. Peptide **3** elutes as the second peak with 280 nm and 409 nm absorbance due to the Acr chromophore.

intrinsic 280 and 409 nm absorbance but does not fluorescence at 350 nm. Unique to the final polypeptide was the 350 nm fluorescence and 409 nm absorbance, which could only result from the incorporation of both Trp and Acr into the final polypeptide (Fig 2-8A-C). This was clearly demonstrated with reduction of polypeptide **5** to the constituent peptides **1**, **2**, and **3** with 100 mol eq TCEP (Fig 2-8D-F, Fig 2-9). Tryptophan containing peptides **1** and **2** had intrinsic 350 nm fluorescence and 280 nm absorbance, but did not absorb at 409 nm. Peptide **3** containing an Acr did not fluorescence at 350 nm, but had both 280 nm and 409 nm absorbance.

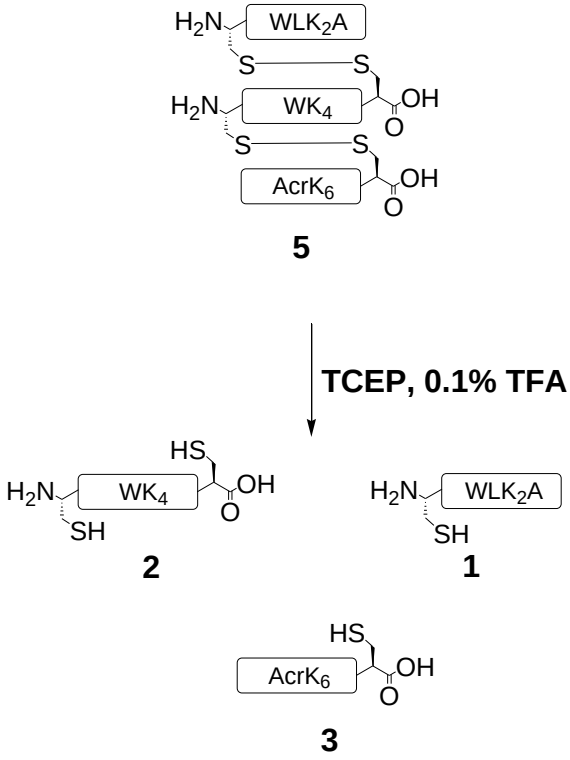


Figure 2-9: *Reduction of Polypeptide **5***. Polypeptide **5** was reduced with 100 mol eq TCEP to generate the constituent peptides. The peptides are numbered as their parent peptides, though **1** and **3** no longer are sulfanylpyridine derivatives and the Thz has been hydrolyzed from **2**.

Discussion

An N-terminal thiazolidine has been successfully used for native chemical ligation, but has never been utilized for the iterative formation of disulfide bonds. In evaluating the suitability of a thiazolidine for iterative reducible ligation, two conditions must be met for successful ligation: methoxyamine could not cleave a disulfide bond, and the hydrolysis of a thiazolidine must result in a stable Cys that does not dimerize or scramble the disulfide bonds.

Disulfide bonds were found to be stable by incubation of a model $AcrK_6C$-C peptide with 0.2 M $MeONH_2$. No reaction was observed, indicating the first requirement was achieved though with a caveat. An activated $AcrK_6C$(Pys) peptide was found to form an apparent oxidized product in 0.2 M $MeONH_2$, indicating activated acceptor peptides were unstable in the presence of methoxyamine. This did not present a problem for successful reducible ligation, since the hydrolysis product of a thiazolidine containing peptide can be purified before the next coupling step. However it eliminates the ability to perform a one-pot solution phase approach, since an activated peptide cannot be added following generation of a donor polypeptide from a thiazolidine. The use of a solid phase could potentially eliminate this problem, as excess $MeONH_2$ could be washed away, avoiding purifications.

The second condition of having a stable Cys following thiazolidine hydrolysis proved more difficult. Commercially available methoxyamine is sold as a hydrochloride salt, and a 0.2 M solution has a pH of approximately 4.[109] The relative acidity of the reaction should presumably hinder disulfide bond formation, though this was not observed even when reacting 1 M $MeONH_2$ in 0.1 v/v % TFA. The oxidization of the Cys to disulfides is commonly seen in native chemical ligation reactions, and is usually overcome by addition of reducing agents to break the disulfide bonds.[110, 114, 125] This poses no problem for native chemical ligation, as the first bond formed is an amide and stable to treatment with reducing agents. For an iterative reducible ligation strategy, the

first bond formed is a disulfide bond and treatment with a reducing agent would destroy the growing polypeptide chain.

As the dimer was the major side product formed, other ways to mitigate dimer formation were examined. Running the thiazolidine hydrolysis in acidic conditions prevented dimerization, but also impeded the hydrolysis reaction. More promising was running the reaction with dilute peptide concentrations. While this could prevent dimerization, the peptide had to be kept in very low concentrations of 20 μM. A reaction performed on a 1 ml scale translates to 20 nmol. Following thiazolidine hydrolysis, the methoxyamine from the reaction mixture had to be removed before the next coupling could occur due to instability of acceptor peptides in methoxyamine. To synthesize an appreciable amount of product, the reaction volume would have to be large; many purification techniques for peptides are volume dependent and are difficult for large quantities of dilute peptides. While not impossible to overcome, purifying dilute samples results in increased time and cost for the ligation strategy and would be best to avoid, therefore requiring a new approach to thiazolidine hydrolysis. A solid phase approach could take advantage of the pseudo-dilution phenomenon to prevent dimerization.[128] Simply described, if two substituents are sufficiently isolated on a solid support and cannot encounter one another, no dimerization can occur.

An iterative reducible ligation strategy would benefit from reaction on a solid support, but would require a donor Cys on resin to react with an incoming acceptor peptide with a C2 protecting and C1 activating groups. While an acceptor Cys could be generated on resin to mirror the chemical approach derived in this chapter (Fig 2-5, peptide **1** bound to resin), previous work has demonstrated that an incoming donor peptide dimerizes.[129] The present protecting scheme was insufficient to generate a C2 protected C1 activated ligation peptide, due to the instability of the thiazolidine. Therefore it is of critical importance to develop a way of further stabilizing the thiazolidine to develop a solid phase iterative reducible ligation strategy.

One interesting observation resulted from increasing the number of spacing amino acids between the Thz and Cys when reacting peptides with DTDP (Figs 2-3 & 2-4). When 5 spacing amino acids were used in $ThzWK_4C$, a cross-linked and most likely cyclic product was observed. Increasing the number of amino acids from 5 to 10 in $ThzWK_9C$ resulted in the desired $ThzWK_9C(Pys)$ and doubly activated $C(Pys)WK_9C(Pys)$ forming. In both peptides, the thiazolidine was not sufficiently stable to prevent unwanted products. However, the nature of the products suggested that there was a minimum number of spacing amino acids required between terminal Cys to prevent cyclization. During the synthesis of **5**, detectable amounts of the self-cleaved cyclic **2** were observed (Fig 2-7B), indicating the instability of **4a**. Increasing the number of amino acids may result in more stable polypeptides and will be incorporated into subsequent chapters.

Another observation was the utility of incorporating multiple chromophores within a polypeptide that can easily identify and characterize the final product (Fig 2-8). The addition of a peptide containing an Acr residue in the second ligation simplified the quantification of the final product. Only Acr had intrinsic 409 nm absorbance which could be used to determine the final yield without having to add absorbance ε values of multiple chromophores. By designing model peptides to contain Trp or Acr, there were two unique signals that only the final polypeptide could contain – the Acr 409 nm absorbance and the Trp 350 nm fluorescence. In the case of polypeptide **5**, MS clearly indicated the correct product and the chromatographic evidence were not needed. Full length gene delivery polypeptides often contain a polydisperse PEG_{5KDa} that cannot be characterized by ESI-MS. MALDI-TOF is usually used to characterize PEG containing gene delivery peptides, but the ionization energy is high and can cleave disulfide bonds, preventing the characterization of reducible gene delivery polypeptides. In developing gene delivery polypeptides, it is important to be able to monitor reactions and identify

products without relying on MS analysis. Incorporation of unique chromophores may be one useful method in the synthesis of more complicated gene delivery polypeptides.

CHAPTER 3

FMOC-THIAZOLIDINE ITERATIVE REDUCIBLE LIGATION MEDIATED BY SILVER TRIFLUOROMETHANESULFONATE

Abstract

Chapter 3 presents further optimization of thiazolidine mediated iterative reducible ligation. Replacing the Boc protecting group of the thiazolidine amine with an Fmoc led to a stabilized thiazolidine, which could be incorporated onto an acceptor peptide. A novel silver trifluoromethanesulfonate hydrolysis of the thiazolidine ring is also described, generating a donor polypeptide using higher peptide concentrations in good yield. To show the benefits of the new chemistry, a series of dodecapeptides were iteratively coupled through disulfide bonds, resulting in polypeptides of 2, 3, and 4 peptide subunits ligated through reducible bonds. Reduction of the polypeptides resulted in the generation of the constituent peptide subunits.

Introduction

The previous chapter introduced thiazolidine mediated coupling of multiple peptides through reducible bonds. While a model polypeptide consisting of three subunits was synthesized, the chemistry was not robust for an iterative reducible ligation synthesis of gene delivery polypeptides. The synthesis required dilute reaction conditions to hydrolyze the thiazolidine and resulted in yields of 7.5% during the second disulfide bond formation. Both limit the ability to synthesize polypeptides on an appreciable scale. Chapter 3 further develops the thiazolidine chemistry with two key improvements: the use of an Fmoc protecting group on the amine of the thiazolidine and the use of silver trifluoromethanesulfonate (AgOTf) to mediate the thiazolidine hydrolysis.

The selection of a thiazolidine was based upon its successful use in native chemical ligation. A Boc thiazolidine is often used for native chemical ligation because the required thioesters are labile in the nucleophilic bases required in Fmoc synthesis.[130,

[131] Additionally an N-terminal thiazolidine is stable in the presence of C-terminal thioesters, having been utilized in native chemical ligation in many syntheses without side reactions.[109, 112, 114, 126, 132, 133] While sufficiently stable for native chemical ligation, the susceptibility of a thiazolidine to side reactions with DTDP prohibited the use of an unprotected thiazolidine on an acceptor peptide. The thiazolidine must stay protected during addition of the C1 activating group and therefore an Fmoc-Thz was investigated. Unlike a Boc protecting group, an Fmoc will remain attached to a peptide during cleavage from resin. The use of an Fmoc-Thz is less common for native chemical ligation, since it requires a second deprotection step to generate a free Cys. A few instances of using an Fmoc-Thz have been reported for ligation reactions, through it is often removed before cleavage of the peptide from resin and therefore offers no advantage as compared to a Boc-Thz.[134, 135] In one instance where an Fmoc-Thz was incorporated and the Fmoc was retained during the first ligation, a two step deprotection generated the free Cys.[113] The yield for the two steps was low at 59 %, and would need to be increased for iterative ligation.

A second limiting factor in the thiazolidine mediated iterative reducible ligation was the use of methoxyamine to hydrolyze the thiazolidine. In reported thiazolidine mediated native chemical ligation, methoxyamine has been used exclusively to generate a free Cys.[109, 112-114, 126, 132-135] Methoxyamine is not efficient for thiazolidine hydrolysis since high concentrations of reagent and long reaction times are required. Methoxyamine hydrolysis also does not prevent dimerization, which can be solved in native chemical ligation by the use of reducing agents after hydrolysis. The dimerization is detrimental for iterative reducible ligation, since there is no way to recover the dimerized peptide without destroying the polypeptide. The reaction has to be performed in dilute conditions, limiting the practicability of performing the ligation strategy on an appreciable scale. Therefore other reagents were explored for their use in iterative reducible ligation. There is a literature precedent for utilizing metals to induce thiazolidine hydrolysis, including

Hg[120, 121] and Ag[136] for various substituted thiazolidines. However these reactions were performed in basic conditions, formed dimers and required the use of hydrogen sulfide to generate free sulfurs, and used the more labile DMT. Improved thiazolidine hydrolysis conditions and thiazolidine stability are developed in this chapter resulting in a successful, scalable iterative reducible ligation strategy, as previously published.[137]

A Note on Nomenclature Used in Chapter 3

A combination of 1 and 3 letter abbreviations to denote the peptides is used to describe initial experiments. The abbreviations are replaced by a numbering scheme during the generation of the polypeptide series. This allows the composition of the larger polypeptides to be readily described. To simplify the conversion of an Fmoc-Thz protected peptide to free Cys, letters following the number denotes the protecting group. A (b) will signify that the Fmoc was removed with the thiazolidine ring intact, while a (c) will signify the free Cys (hydrolyzed thiazolidine). For reduced polypeptides, the constituent peptides are indicated as the starting peptides though the C1 and C2 groups were removed during the ligation reactions.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, 9-chloroacridine, tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), methoxyamine, carboxymethoxyamine and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, trifluoroacetic acid (TFA), and glacial acetic acid were obtained from Fisher Scientific.

Synthesis and Characterization of Peptide Subunits

Unsubstituted Wang resin was loaded with Fmoc-Lys(Boc)-OH or Fmoc-Cys(Trt)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. S-2-sulfanylpyridine protected peptides were generated by reacting crude thiol-deprotected peptides with 10 mol eq of DTDP in 2 M acetic acid/2-propanol (10:3 v/v) overnight. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Trp ε_{280nm} = 5600 M^{-1} cm^{-1}, Fmoc-L-thiazolidine-4-carboxylic acid (Fmoc-Thz-OH) ε_{280nm} = 6550 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Lys(Acr) ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Donor and Acceptor Ligation Reactions

Ligation reactions were performed on a scale ranging from 0.15-5.0 μmol in 0.1 M ammonium acetate pH 5. A donor peptide containing free Cys (0.50 μmol) was reacted

with an S-2-sulfanylpyridine acceptor peptide (0.75 μmol, 1.5 mol eq) for 12 hrs at RT in 0.5 ml total volume. The product was preparatively purified by injecting 0.25 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring Abs_{280} or $Abs_{409\ nm}$. The major peak from multiple runs was collected, pooled, and lyophilized. The ligated polypeptide product was reconstituted in 0.1 v/v % TFA and the yield determined by Abs. Purified polypeptides were characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Model Thiazolidine Hydrolysis Reactions

Thiazolidine hydrolyses were performed by drying 5 nmol aliquots of polypeptide and reconstituting in 10 μL 0.1 % v/v TFA containing the indicated reagent. At the indicated time point, reaction mixtures were characterized by RP-HPLC by injecting 2 nmol onto an analytical C18 column eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min.

Optimized Conversion of Fmoc-Thz to Cys

Generation of a donor polypeptide required a two step deprotection of the C2 Fmoc-Thz. Lyophilized polypeptide (0.25 μmol) was reconstituted in 0.25 ml of 5 v/v % piperidine/DMF and reacted 5 min to remove the Fmoc. The reaction was quenched by the addition of 0.25 ml glacial acetic acid, then applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak eluting at 21 ml was collected and lyophilized.

The thiazolidine was then hydrolyzed by reaction with 50 mol eq of AgOTf in 0.25 ml 0.1 v/v % TFA for 1 hr. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing the newly formed donor polypeptide eluting at 21 ml was collected and lyophilized. The donor polypeptide was then reconstituted in 0.1 M ammonium acetate pH 5 and reacted with an S-2-sulfanylpyridine acceptor peptide as described above.

Reduction of Polypeptides

Reducible ligation polypeptide products were further characterized by reduction to component peptides by reacting 2 nmol in 0.1 ml of 0.1M $AcONH_4$, pH 5 containing 200 nmol TCEP (100 mol eq) for 1 hr. The product peptides were then analyzed by LC-ESI-MS by injecting the sample in a Vydac C-18 analytical column eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while monitoring $Fl_{Ex=280\ nm,\ Em=350\ nm}$, Abs_{280} and $Abs_{409\ nm}$.

Results

The last chapter demonstrated the feasibility of using a thiazolidine as a C2 protecting group to protect an N-terminal Cys and be selectively unmasked in the presence of an internal disulfide bond for iterative reducible ligation. However, the instability of the thiazolidine to DTDP and relatively inefficient hydrolysis of the thiazolidine with methoxyamine severely curtailed the yield. To overcome the thiazolidine instability, the amine protecting group of the thiazolidine ring was switched from the Boc to Fmoc (Fig 3-1). This substitution allowed the amine protecting group to be preserved during the acidic cleavage from the resin, further stabilizing the thiazolidine. An Fmoc-Thz was found to be stable in the presence DTDP, allowing the construction of acceptor peptides with this C2 protecting group. Optimization of the deprotection showed that the Fmoc could be completely removed when treated with a 5 v/v % piperidine/DMF solution for 5 min with a peptide concentration of 1 mM, followed by quenching with glacial acetic acid. The limited base treatment did not adversely affect previously formed disulfide bond.

To overcome the inefficient methoxyamine mediated thiazolidine hydrolysis, numerous reagents were tested for their ability to hydrolyze a thiazolidine. A model polypeptide $ThzWK_{14}C$-$CWLK_2A$ was synthesized to contain both a C2 Thz and internal disulfide bond, to determine conditions for efficient hydrolysis that did not concurrently

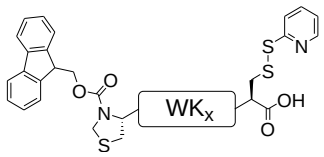


Figure 3-1: *Structure of General Ligation Peptide with C1 Cys(Pys) and C2 FmocThz Groups*. Ligation peptide with terminal Cys to undergo iterative reducible ligation. The C-terminal Cys has an activated C1 2-sulfanylpyridine. The N-terminal Cys is masked as a C2 Fmoc-thiazolidine.

disrupt a previously formed disulfide bond (Fig 3-2). Incubating the polypeptide in 0.1% TFA for 24 hrs resulted in no reaction (Fig 3-2A & D). A variety of oxyamines were examined, with many insoluble in aqueous conditions. The use of 1 M methoxyamine generated the expected partially deprotected polypeptide at 2 hrs with concurrent dimerization, and complete hydrolysis and significant dimerization observed after 24 hrs (Fig. 3-2B & E). The use of 1 M carboxymethoxyamine limited dimerization at 24 hrs, presumably due to the acidic nature of the reagent, but still required long reaction times and high concentrations of reagent (not shown). Other reagents tested were metals known to deprotect other Cys protecting groups. It was found that silver could efficiently hydrolyze a thiazolidine and prevent dimerization after 24 hrs (Fig 3-2C & F). As little as 10 mol eq AgOTf in 30 min could mediate complete hydrolysis without side reaction. With an improved protecting scheme and efficient deprotection and hydrolysis reactions, a series of model polypeptides were synthesized to demonstrate the improvements of the iterative reducible ligation strategy.

Model dodecapeptides **1**, **2**, and **3** were prepared using standard Fmoc solid phase peptide synthesis and characterized by LC-ESI-MS (Fig. 3-3, Table 3-1). Ten spacing amino acids between Cys were used to prevent the reaction of the donor Cys with an internal disulfide bond. The dodecapeptides were primarily composed of Lys residues designed to bind the anionic backbone of DNA. An Acr residue was incorporated into model peptide **3** to simplify final quantification of polypeptides and add a second

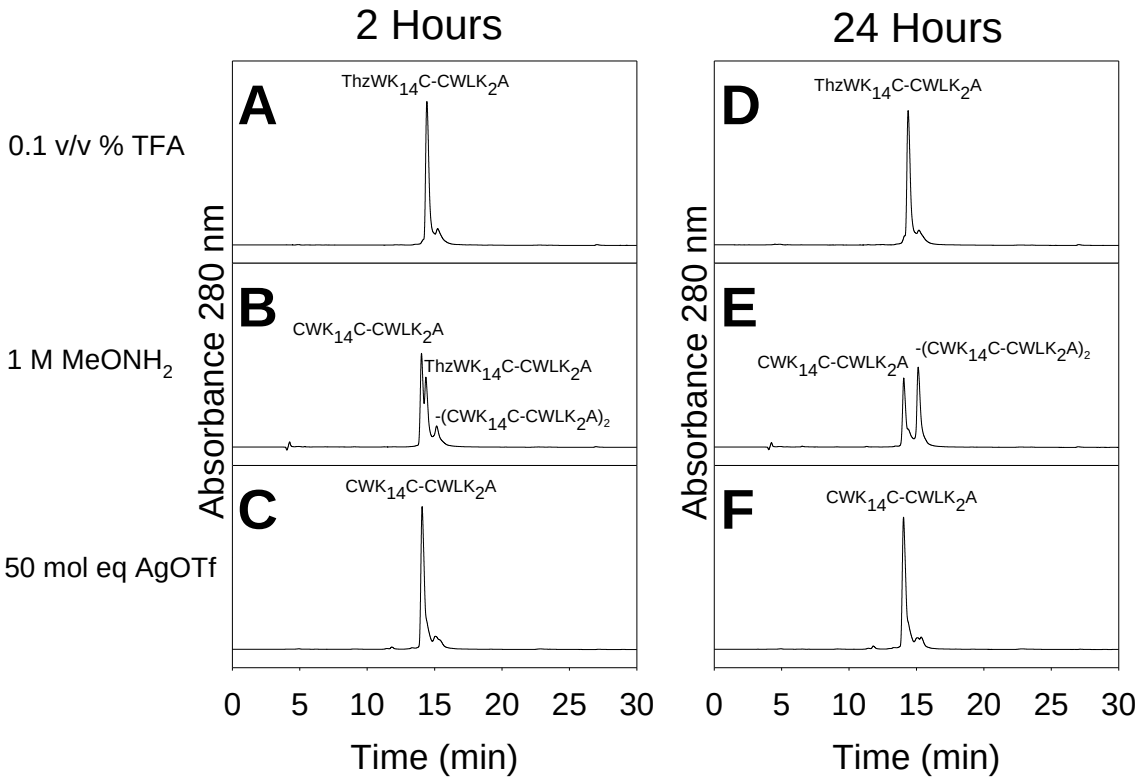


Figure 3-2: *Analytical HPLC Analysis of Thiazolidine Hydrolysis Reactions*. Panels A-F illustrate RP-HPLC chromatograms following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Panels A, B, and C illustrate the treatment of $ThzWK_{14}C\text{-}CWLK_2A$ with 0.1% v/v TFA, 1 M methoxyamine in 0.1% v/v TFA, and 50 eq AgOTf in 0.1% v/v TFA for 2 hrs respectively. Panels D, E, F illustrate the same treatments at 24 hrs. No reaction is observed when the peptide is treated with 0.1% TFA (Panels A and D). Treatment with methoxyamine results in hydrolysis and subsequent dimerization over time (Panels B and E). Only hydrolyzed product is observed when the peptide is treated with AgOTf (Panels C and F).

chromophore. A C-terminal Cys was incorporated into **2** and **3** and converted into the activated S-2-sulfanylpyridine C1 derivatives with DTDP to form acceptor peptides whereas an N-terminal Cys in peptide **1** allowed its use as a donor peptide. A key N-terminal Fmoc-thiazolidine as a masked Cys residue was incorporated in **2** to function as the C2 protecting group, to allow the selective unmasking of Cys in the presence of internal disulfide bonds.

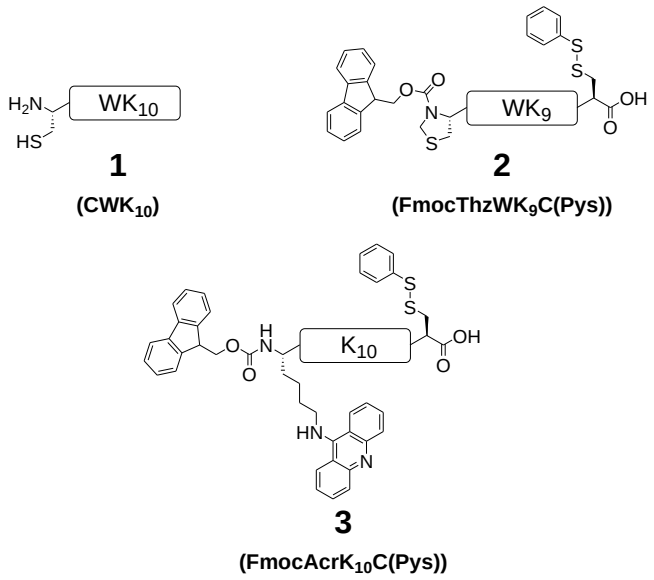


Figure 3-3: *Model Cross-linking Donor and Acceptor Peptides **1-3***. Peptide **1** is a donor peptide with an unprotected N-terminal Cys. Peptides **2** and **3** are acceptor peptides containing Cys activated with C1 Pys. Peptide **2** also possesses a Cys masked as an Fmoc-thiazolidine as the C2 protecting group.

Table 3-1: *Synthesis and Characterization of Thiazolidine Mediated Iterative Reducible Ligation Peptides*.

Peptide[a]	**Mass (calc / obs)**	**% Yield**
1 (CWK_{10})	1589.1 / 1588.8	28[b]
2 ($FmocThzWK_9C(Pys)$)	1907.5 / 1907.0	58[b]
3 ($FmocAcrK_{10}C(Pys)$)	2039.6 / 2039.2	42[b]
4 ($FmocAcrK_{10}C\text{-}CWK_{10}$)	3517.6 / 3517.2	53[c]
5 ($FmocThzWK_9C\text{-}CWK_{10}$)	3385.4 / 3385.2	49[c]
6 ($FmocAcrK_{10}C\text{-}CWK_9C\text{-}CWK_{10}$)	5079.6 / 5078.8	54[c]
7 ($FmocThzWK_9C\text{-}CWK_9C\text{-}CWK_{10}$)	4947.4 / 4946.4	55[c]
8 ($FmocAcrK_{10}C\text{-}CWK_9C\text{-}CWK_9C\text{-}CWK_{10}$)	6641.7 / 6640.5	43[c]

[a] Thz refers to thiazolidine, Pys to 2-sulfanylpyridine, and Acr to Nε-acridinyllysine.

[b] Purified yield based on initial resin substitution.

[c] Purified yield determined for the last ligation step.

Peptides **1**, **2**, and **3** were used to generate disulfide cross-linked polypeptides **4-8** (Fig. 3-4). The conjugation of donor peptide **1** with excess acceptor peptide **3** resulted in the formation of polypeptide **4** as determined by analytical LC-ESI-MS. The coupling reaction was studied using a buffer of 0.1 M ammonium acetate at a pH ranging from 3–6.5 to establish an optimal pH of 5. A 1.5 mol eq excess of acceptor peptide minimized the formation of the dimeric donor peptide side product. Under optimal conditions, donor peptide **1** reacted with 1.5 mol eq of acceptor **3** in 0.1 M ammonium acetate pH 5 to yield **4** (Fig. 3-5A-C). RP-HPLC analysis of the reaction product established the complete consumption of **1** and the presence of excess **3** (Fig. 3-5C). Polypeptide **4** was purified by RP-HPLC in 53 % yield and re-chromatographed using analytical LC-ESI-MS as a single polypeptide with mass closely matching that of the calculated value (Fig. 3-5D).

In an analogous fashion to **4**, polypeptide **5** was obtained by reacting donor **1** with 1.5 mol eq of acceptor **2** (Fig 3-6A-C). Analysis indicated the complete consumption of **1**, excess **2**, as well as the displacement of pyridine-1-(1H)-thione (Fig. 3-6C). Preparative purification of **5** resulted in a 49 % purified yield, which upon re-analysis produced a single RP-HPLC peak with a corresponding mass to the desired product (Fig 3-6D).

To apply iterative coupling, the C2 Fmoc-thiazolidine on polypeptide **5** must be removed without reduction or scrambling of the internal disulfide bond (Figure 3-7). Removal of the Fmoc and hydrolysis of the thiazolidine exposes an N-terminal Cys that can react with an acceptor peptide. Reaction of **5** (Fig 3-8A) with 5 v/v % piperidine/DMF for 5 min efficiently removed the Fmoc, resulting in the product **5b** which shifted to an earlier RP-HPLC retention time and loss of Fmoc absorbance. Following gel filtration purification, analytical LC-ESI-MS established the quantitative (97%) recovery of the desired **5b** product (Fig. 3-8B).

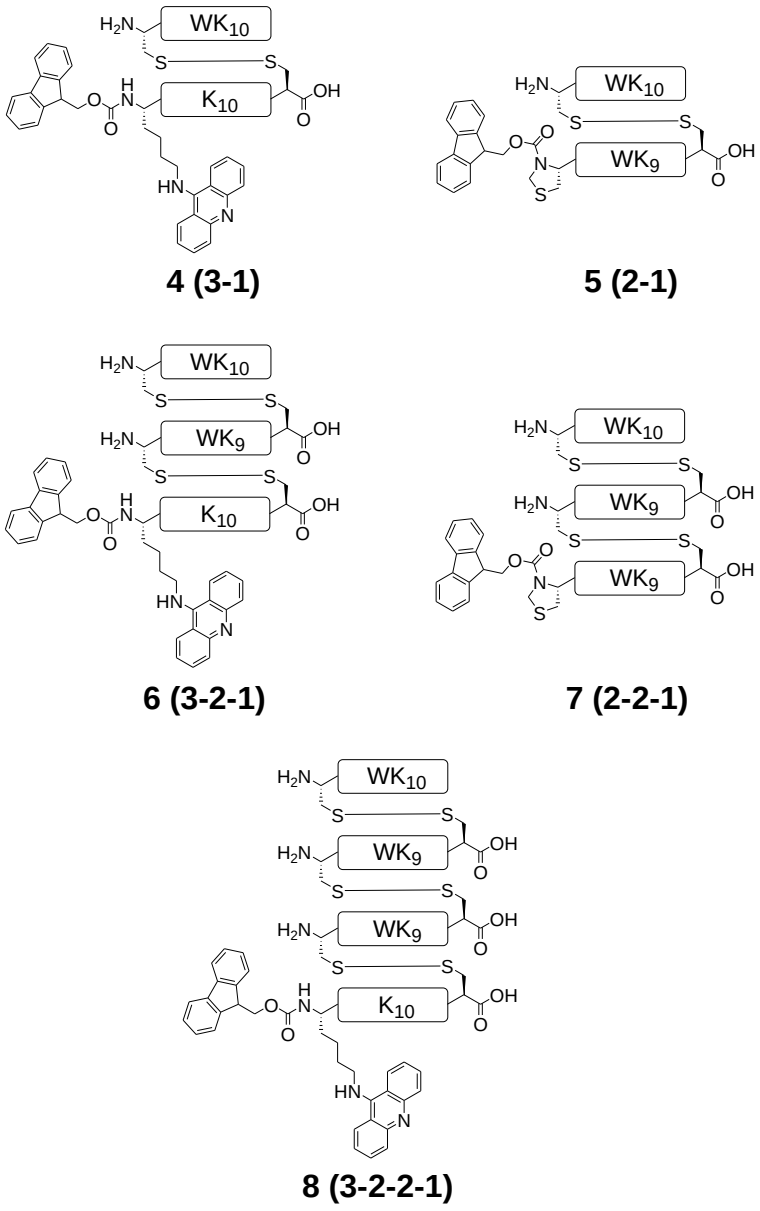


Figure 3-4: *Polypeptides Synthesized by Iterative Reducible Ligation*. Polypeptides **4-8** are illustrated along with their composition of peptides **1-3.** Polypeptide **8** contains 48 amino acids and is composed of peptides **3-2-2-1** ligated by three internal disulfide bonds.

Reaction of **5b** with AgOTf (50 mol eq) in 0.1 v/v % TFA for 1 hr resulted in the efficient conversion of the thiazolidine to form donor polypeptide **5c** with quantitative recovery following gel filtration. Analytical LC-ESI-MS established the recovery of the

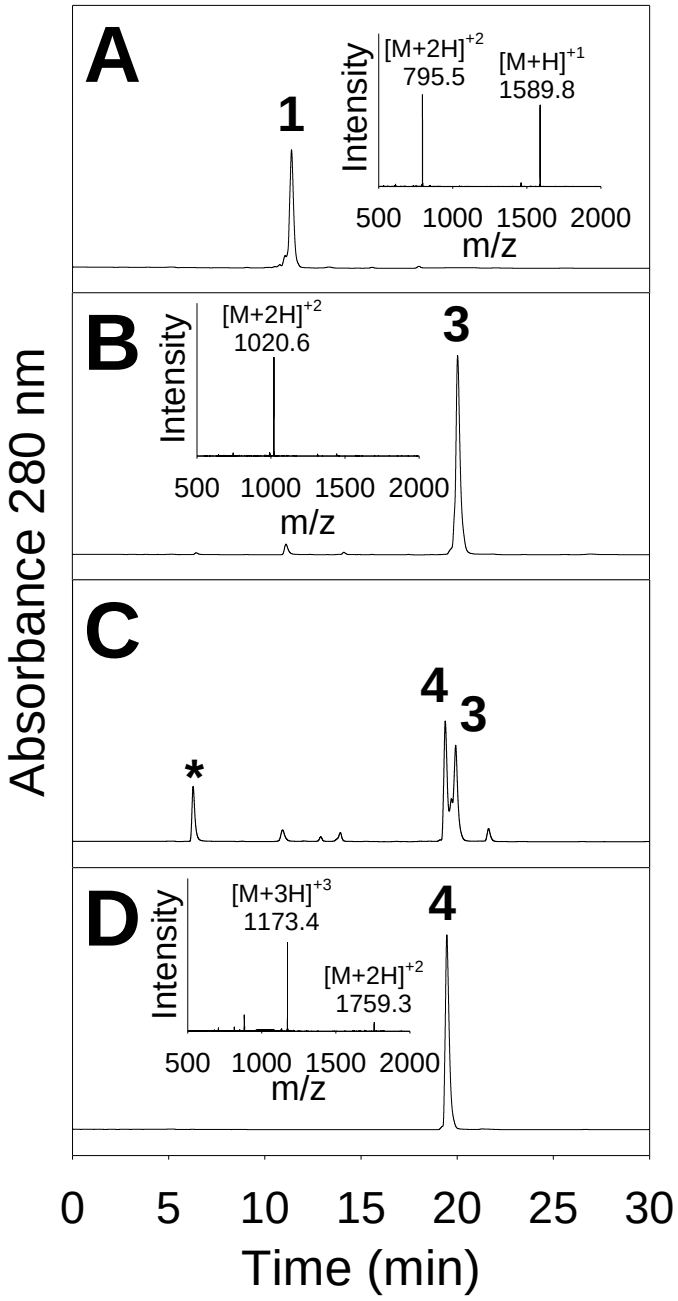


Figure 3-5: *Synthesis of Polypeptide **4***. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified donor peptide **1** and acceptor peptide **3** to establish their retention time. Following reaction of **1** and **3** (1.5 mol eq), the product mixture demonstrates the complete loss of **1**, the recovery of residual **3**, the generation of (*) pyridine-2-(1H)-thione, and the formation of polypeptide **4** at a new retention time (Panel C). Following preparative purification with 53% recovery, re-analysis of polypeptide **4** establishes its purity (Panel D).

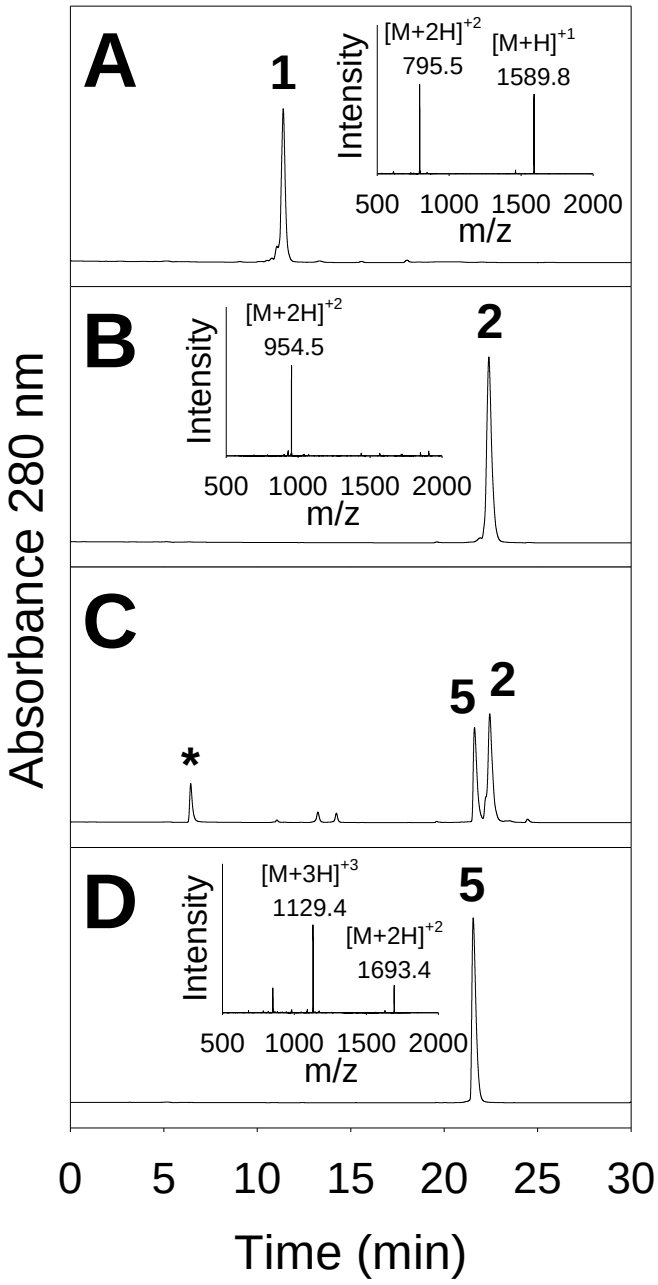


Figure 3-6: *Synthesis of Polypeptide **5***. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified donor peptide **1** and acceptor peptide **2** to establish their retention time. Following reaction of **1** and **2** (1.5 mol eq), the product mixture demonstrates the complete loss of **1**, the recovery of residual **2**, the generation of (*) pyridine-2-(1H)-thione, and the formation of polypeptide **5** at a new retention time (Panel C). Following preparative purification with 49% recovery, re-analysis of polypeptide **5** establishes its purity (Panel D).

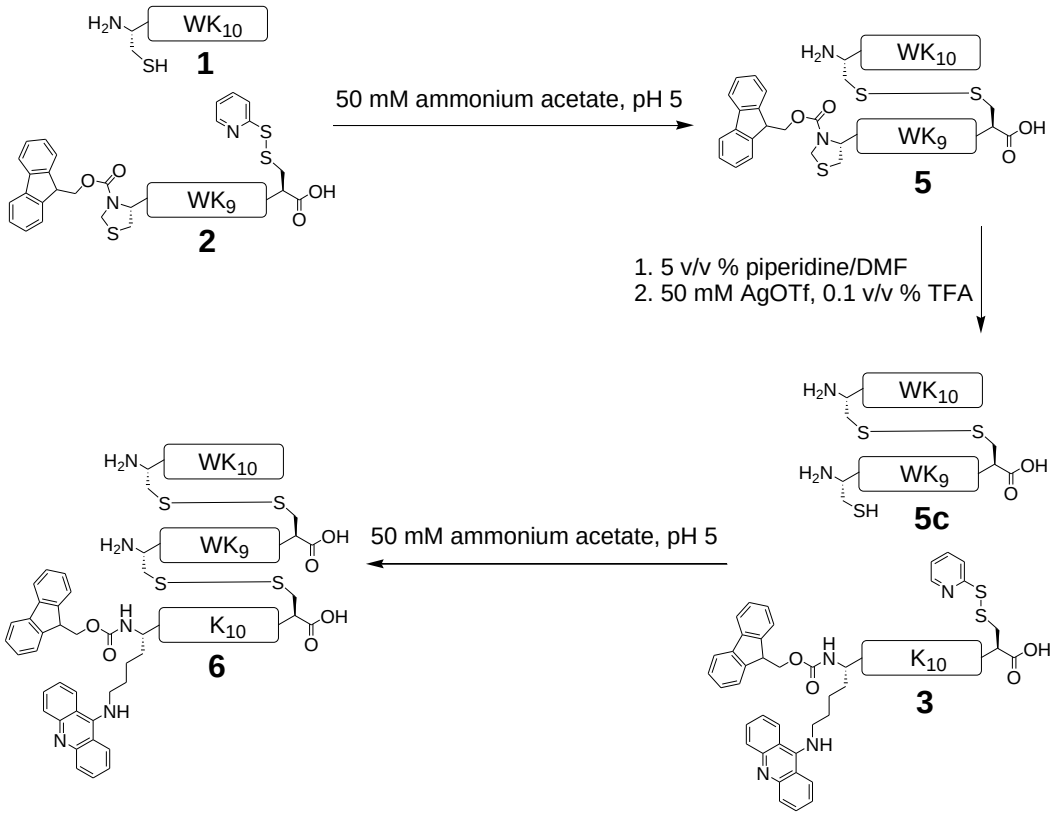


Figure 3-7: *Synthetic Scheme to Generate Polypeptide **6***. Donor peptide **1** and acceptor peptide **2** were ligated to form protected polypeptide **5**. Polypeptide **5** was reacted with 5 v/v % piperidine/DMF to remove the Fmoc resulting in the formation of **5b**. The thiazolidine was hydrolyzed using 50 mol eq of AgOTf in 0.1 v/v % TFA to form the donor polypeptide **5c**. **5c** was ligated with acceptor peptide **3** to generate polypeptide **6**.

desired polypeptide **5c** as a peak eluting at an earlier retention time with a loss of 12 amu due to hydrolysis of the thiazolidine (Fig. 3-8C).

The donor polypeptide **5c** was immediately reacted with acceptor peptide **3** to form polypeptide **6**. A 1.5 mol eq excess of acceptor peptide (**3**) was used to minimize donor dimerization and donor polypeptide scrambling (Fig. 3-8D). Upon purification, **6** was obtained in 54 % yield from **5** and re-chromatographed as a single polypeptide on analytical LC-ESI-MS (Fig. 3-8E).

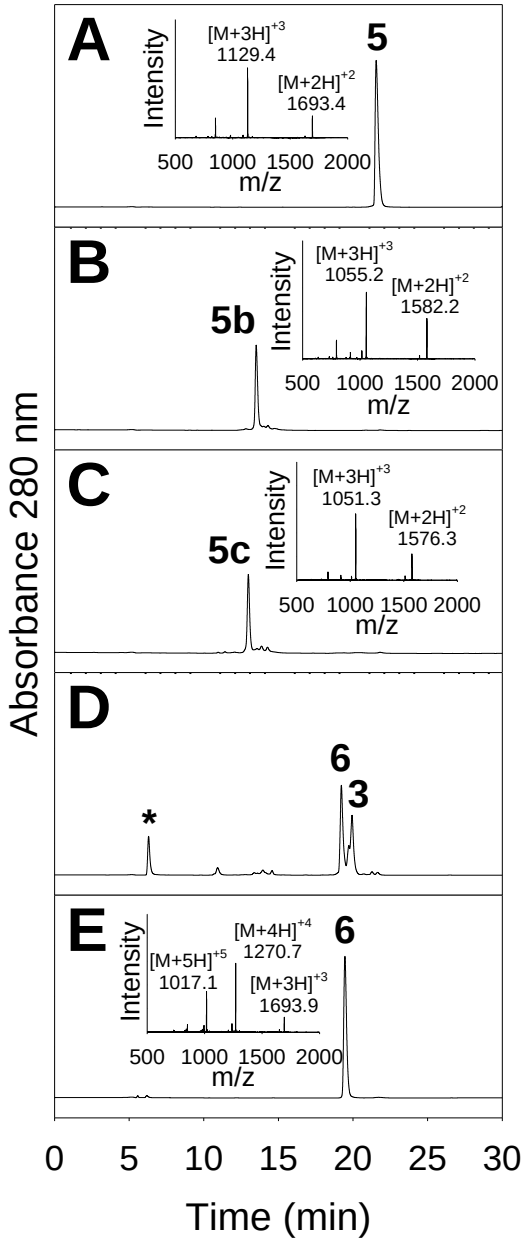


Figure 3-8: *Synthesis of Polypeptide **6***. Panels A-E illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates polypeptide **5**. Removal of Fmoc using 5 % piperidine in DMF results in the formation of **5b** possessing a shorter retention time (Panel B). Subsequent thiazolidine hydrolysis using 50 mol eq of AgOTf in 0.1 v/v % TFA led to the formation of **5c** (Panel C). Reaction of **5c** and acceptor peptide **3** (1.5 mol eq) results in the complete consumption of the donor polypeptide **5c**, generation of (*) pyridine-2-(1H)-thione, recovery of residual acceptor peptide **3**, and the formation of polypeptide **6** with a new retention time (Panel D). Purification of polypeptide **6** resulted in 54% yield (Panel E).

To demonstrate the robustness of the ligation, donor polypeptide **5c** was also reacted with acceptor peptide **2** to yield polypeptide **7**. Following purification, analytical LC-ESI-MS demonstrated the formation of **7** with an observed mass of 4946.4 amu corresponding to the desired polypeptide in 55 % purified yield (Fig. 3-9B). **7** was then deprotected to generate donor polypeptide **7c**, which was reacted with acceptor **3** to generate tetra-dodecapeptide **8** (Fig 3-9C). Polypeptide **8** was purified in 43% yield and analytical LC-ESI-MS demonstrated a single product peak with an observed mass of 6640.5 closely corresponding to that of the calculated mass (Fig. 3-9D). A complete list of synthesized polypeptides, yields, and calculated and observed masses can be found in Table 3-1.

Due to the presence of both the tryptophan and acridine chromophores in **6**, this compound displayed the unique tryptophan 350 nm fluorescence and acridine 409 nm absorbance (Fig 3-10A-C). When **6** was reduced using TCEP, the constituent peptides demonstrated the correct chromophore absorbance and fluorescence signals (Fig 3-10D-F, Fig 3-11). Peptides **1** and **2** co-elute under HPLC conditions, but can be identified by their masses. The co-eluted **1** and **2** containing tryptophan retained 350 nm fluorescence, while **3** containing acridine displayed 409 nm absorbance. The labels of **2** and **3** indicated the starting peptides that have been chemically altered during the ligation process, losing Fmoc, thiazolidine, or 2-sulfanylpyridine functionalities.

Comparing $Abs_{280\ nm}$ of reduced **4**, **6**, and **8** demonstrates the incorporation of more **2** in the polypeptides (Fig. 3-12). This is seen in the increase in the first peak absorbance (containing **1** and **2**) relative to the later eluting peak (**3**). Additionally, the intensity of peptide **2** mass can be seen increasing in the mass spectra insets relative to **1**, indicating an increased amount of **2**.

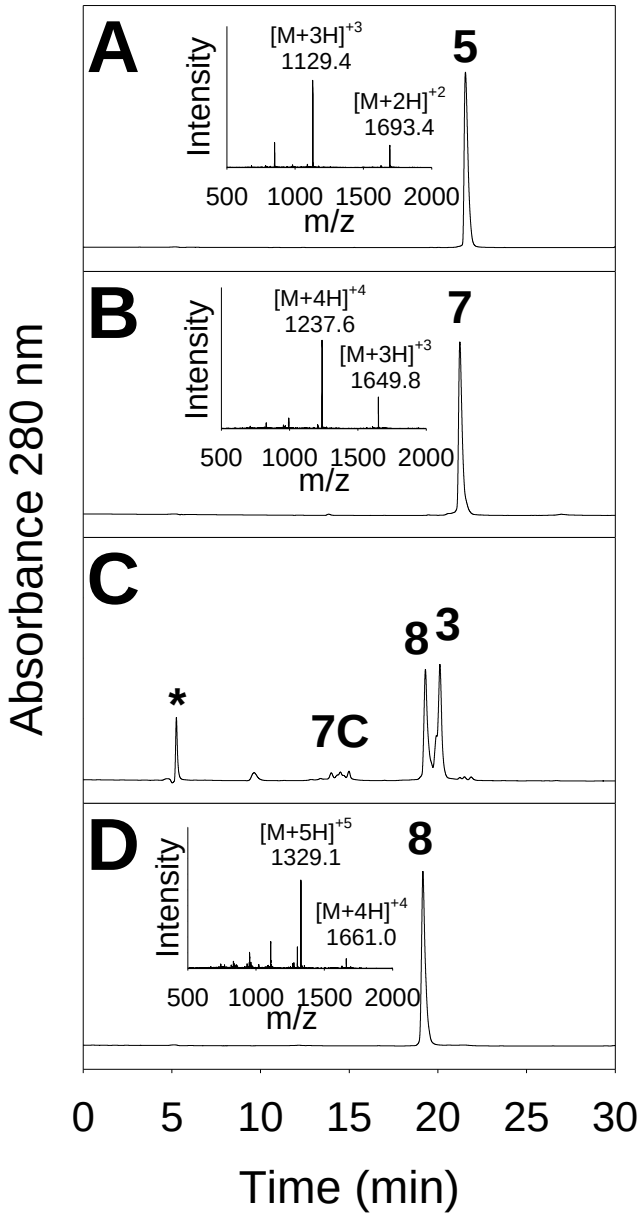


Figure 3-9: *Syntheses of Polypeptides **7** & **8***. Panels A-D illustrate RP-HPLC chromatograms following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting Abs_{280nm}. The insets represent the ESI-MS for the product peak. Peptides **5** (Panel A) and **2** were ligated in an analogous fashion to **6**, generating polypeptide **7** (Panel B) in a 55% purified yield. Following the removal of Fmoc and thiazolidine hydrolysis from **7** and reaction with **3**, analysis of the reaction product illustrates the consumption of the Fmoc deprotected, Thz hydrolyzed, donor polypeptide **7c**, the formation of (*) pyridine-2-(1H)-thione, recovery of residual **3**, and the formation of polypeptide **8** (Panel C). Preparative purification of polypeptide **8** in 43% isolated yield and re-analysis established its purity (Panel D).

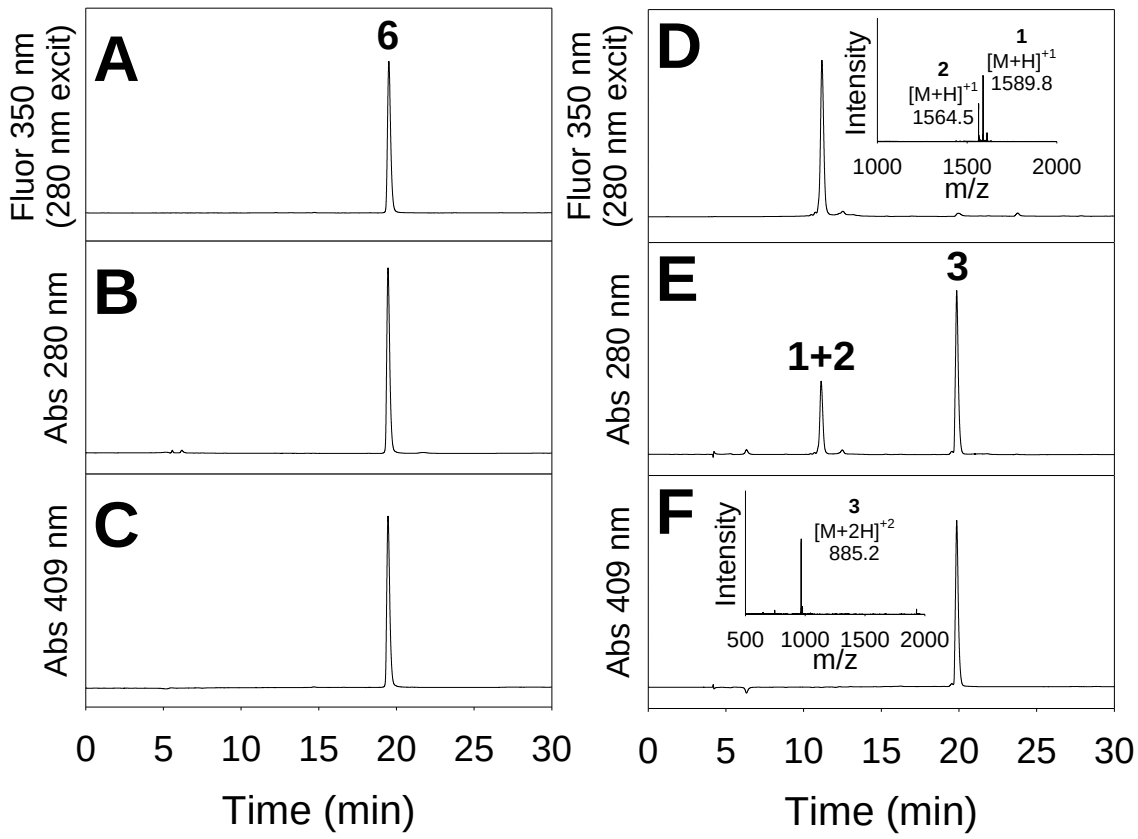


Figure 3-10: *Analytical RP-HPLC of Polypeptide **6** and Reduced **6***. Panels A-F illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Fl_{Ex=280\ nm,\ Em=350\ nm}$ (Panels A & D) and $Abs_{280\ nm}$ (Panels B & E) and $Abs_{409\ nm}$ (Panels C & F). The insets show the ESI-MS for the product peaks. **6** displays both 350 nm fluorescence and 409 nm absorbance from the tryptophan and acridine chromophores (Panels A-C). When reduced, component peptides **1** and **2** co-elute as the first peak and display 350 nm fluorescence and 280 nm absorbance due to the tryptophan chromophore. Both peptides can be identified in the MS inset. Peptide **3** elutes as the second peak with 280 nm and 409 nm absorbance due to the acridine chromophore.

Discussion

Initial attempts at thiazolidine mediated reducible ligation in chapter 2 resulted in the successful ligation of a polypeptide containing three peptide subunits linked together through two disulfide bonds. However the chemistry was not robust enough for a large scale synthesis of reducible polypeptides. The coupling yield of the third peptide was

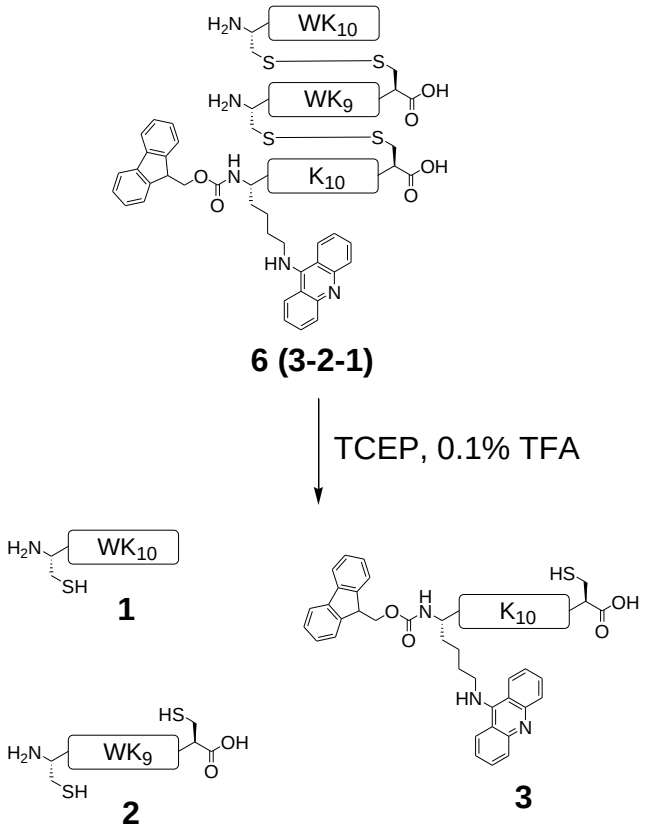


Figure 3-11: *Reduction of Polypeptide **6** to Component Peptides*. Polypeptide **6** was reacted with 100 mol eq TCEP to reduce the polypeptide into its component peptides. Due to the ligation reactions to form **6**, peptides **2** and **3** no longer contain the C1 and C2 groups (2-sulfanylpyridine and Fmoc-Thz).

7.5 %; if a fourth peptide was added with a similar yield, the combined reaction yields for both steps would be under 1 %, unacceptable for creating gene delivery polypeptides that contain valuable N-glycans and complex peptides such as melittin

The first improvement was to develop a protecting scheme that allowed the thiazolidine to be incorporated onto an acceptor peptide activated with DTDP. A Boc group was initially incorporated and was removed when the peptide was cleaved from the resin. The thiazolidine was found to be susceptible to side reactions during subsequent activation of the peptide with DTDP. Replacing the Boc protecting group on the thiazolidine amine with Fmoc preserved the amine protecting group during resin cleavage

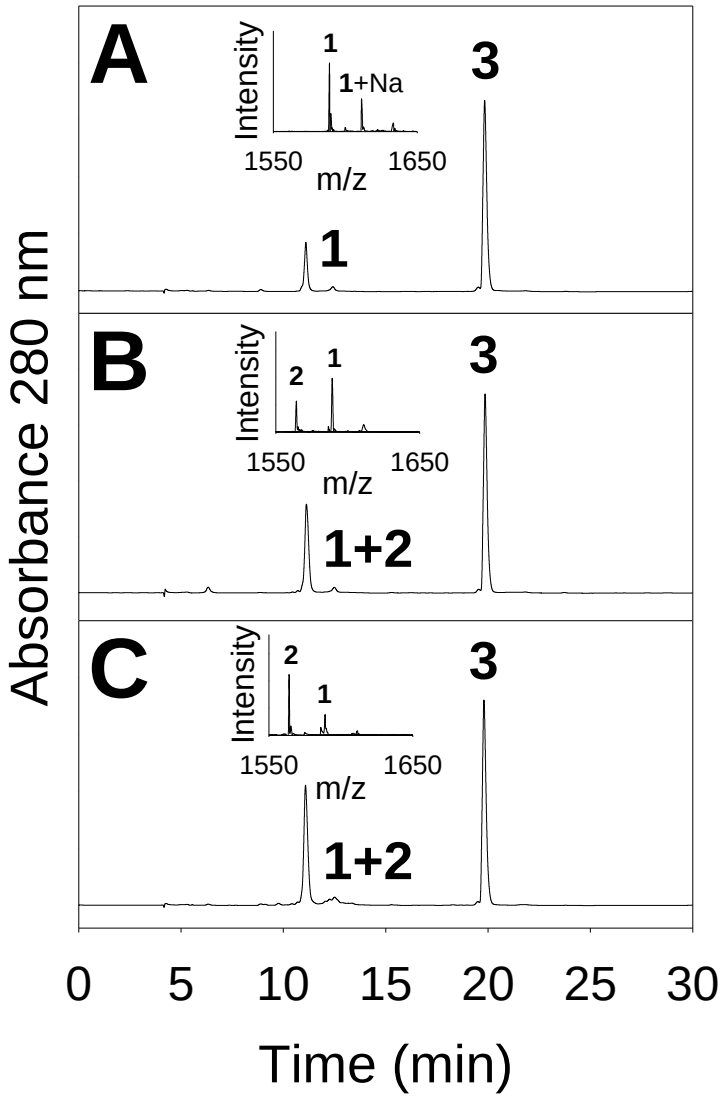


Figure 3-12: *LC-ESI-MS Analysis of Reduced **4**, **6**, and **8***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of polypeptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the first peak. Reduced polypeptides **4**, **6**, and **8** are represented in Panels A-C respectively. The component peptides are indicated, with increasing amounts of **2** observed by a corresponding increase in the first peak relative to the second from Panels A-C and by the increasing ion intensity of **2** in the MS insets.

of the peptide. Subsequent activation of the C1 Cys with DTDP could proceed without side reactions. Additional protecting groups are to be avoided when possible, as the extra deprotection and purification steps lower yields. However a short treatment with low

amounts of piperidine was sufficient to remove the group and the liberated thiazolidine polypeptide could be purified and recovered in almost quantitative yield following gel filtration chromatography. The short basic treatment also did not result in scrambling of disulfide bonds. The use of the hydrophobic Fmoc group had the unexpected advantage of creating dramatic RP-HPLC retention time shifts that facilitated purification, as well as providing an additional chromophore to boost the absorbance signal.

The second major challenge was to find other thiazolidine hydrolysis conditions that efficiently unmasked the Thz without reaction at internal disulfide bonds or result in dimerization. Initially a panel of oxyamines derivatives was tested. The only compound to hydrolyze better than methoxyamine was the acid-containing carboxymethoxyamine. This reagent was able to generate the free Cys using higher peptide concentrations without dimerization seen with methoxyamine. The relatively high 1 M concentration resulted in the presence of a 1 M carboxylic acid, resulting in a low pH, highly buffered reaction. This combination seemingly prevented dimerization, which may be possible to achieve with methoxyamine if run in 1M acetic acid. The reaction still required several hours to go to completion and a high reagent concentration.

Due to the inefficient hydrolysis reactions with oxyamines, a series of metals was examined. Metals have been utilized in the past for the removal of various Cys protecting groups and the key to one previous iterative reducible ligation strategy with a C2 Cys(Acm) was the use of AgOTf.[75-77] The key discovery was that silver could efficiently hydrolyze the thiazolidine in acidic aqueous conditions without dimerization or cleavage of disulfide bonds. There is a precedence for using metals to mediate thiazolidine hydrolysis, as both Hg(II)[120, 121] and Ag[136] have been used to hydrolyze substituted thiazolidines. The use of Hg required the use of hydrogen sulfide to generate the free thiol, a reduction step that would destroy any internal disulfide bonds. The prior use of AgCl was done in basic conditions; if applied under basic conditions, this approach would fail because the nucleophilic donor Cys would be too reactive. Both metals also

utilized substituted thiazolidines including the relatively labile dimethylthiazolidine. The importance of this novel deprotection step was the use of a metal in slightly acidic aqueous conditions. The acidity ensured the preservation of the generated Cys and the use of water as the solvent ensured fully deprotected peptides, including PEGylated and N-glycan modified peptides, would be soluble. While silver could efficiently hydrolyze a thiazolidine, a doubly protected Fmoc-Thz containing polypeptide was found to be stable.

With the revised C2 protecting group of Fmoc-Thz and optimized conditions to generate the free Cys, a series of model polypeptides was synthesized to mimic the generation of reducible polypeptides in a gene delivery system. Polypeptides up to four peptide units were synthesized and theoretically could be extended to any desired number of peptide subunits. The optimized deprotection and coupling conditions led to consistent yields of over 40 % per coupling cycle. The use of gel-filtration chromatography minimized losses from multiple intermediate purifications, with most of the material lost from the preparative purification step. An excess of the acceptor peptide was required to prevent dimerization of the donor peptide but could be recovered during purification (around 33 % of the initial acceptor peptide was consistently recovered). The high yielding unmasking of the Fmoc-Thz to Cys was in contrast to a previous report, where a two step deprotection using a 10 % morpholine base treatment followed by 0.4 M methoxyamine hydrolysis resulted in a 59 % recovery of a free Cys, subsequently used in a native chemical ligation reaction.[113] The developed deprotection strategy greatly improved the yield for the two step deprotection, from 59 % to around 96 %, and may be applied advantageously to the field of native chemical ligation.

Work in this chapter demonstrated the successful synthesis of model polypeptides by a thiazolidine mediated iterative reducible ligation strategy. Subsequent chapters will further expand the scope of this synthetic methodology. The stability of disulfide bonds can be increased by replacing Cys with penicillamines and the chemical compatibility of penicillamines to reducible ligation is explored. Despite mild conditions, the solution

strategy requires a number of purification steps that lower yields. While the purifications steps are unavoidable for solution chemistry, the compatibility of the ligation technique with a solid support and the advantages of a solid phase ligation reaction are explored. Application to gene therapy peptides requires the attachment of N-glycans and PEG moieties, often attached through Cys residues. Therefore an additional level of Cys protecting group orthogonality is required and a C3 protecting group is developed, as well as some initial SAR of placement of the Cys. Due to the orthogonality of an Acm removal to an Fmoc-Thz, a convergent synthesis utilizing both is also considered.

CHAPTER 4

INCORPORATION OF PENICILLAMINES INTO THIAZOLIDINE MEDIATED ITERATIVE REDUCIBLE LIGATION

Abstract

Chapter 4 advances the thiazolidine mediated iterative chemical ligation chemistry by including penicillamines to create more stable disulfide bonds. Reducible polypeptides with N or C-terminal Pen were generated, with inverted donor and acceptor peptides for C-terminal Pen polypeptides. To generate a Pen-Pen polypeptide, random disulfide bond formation had to be utilized due to the stability of an activated Pen.

Introduction

One of the first reports of penicillamine, a β,β-dimethyl Cys derivative, involved its discovery in the urine of patients treated with penicillin.[138] It has since been used to treat a variety of disease, including Wilson's disease,[139] heavy metal poisoning,[140] and cystinuria.[141] For the first two cases, oral dosing of penicillamine is able to chelate copper and lead within the body, causing a marked increase in the urine for both metals. It can also undergo a disulfide bond exchange reaction with cystine, resulting in a more soluble disulfide in patients with cystinuria.

Disulfide bonds formed with penicillamines are more difficult to reduce than Cys bonds.[142] This is a result of the β,β-dimethyl groups adding steric bulk adjacent to the sulfhydryls. Reducing agents are quite poor at reducing a Pen-Pen disulfide. Treatment with 25 mol eq of Cys, glutathione, or dithioerythritol resulted in less than 20% reduction after 2 hrs at pH 8.[142] Increased stability was also reported for a Pen-Cys mixed disulfide. The increased stability of a mixed Pen disulfide bond also results in it being more difficult to form by a directed synthesis, most likely because the dimethyls also stabilize a Pen bound activating group such as sulfanylpyridine. There are no reports of a directed Pen-Pen disulfide bond reaction, though Pen will auto-dimerize if exposed to

air.[143] Disulfide exchange between Pen and mixed Pen-Cys disulfides to generate a Pen-Pen bond has also been reported to be unfavorable.[143, 144]

The increased stability of disulfide bonds incorporating Pen has rarely been exploited in gene delivery systems. One report utilized Pen containing cross-linking peptides to condense with Cys containing PEG and glycopeptides to mediate targeted *in vivo* delivery to the liver.[55] These peptides were allowed to undergo template polymerization on DNA. When compared to Cys containing cross-linking peptides, the Pen containing peptide polyplexes were demonstrated to be more stable to TCEP reduction. Despite the increased reductive stability of the formulation, it did not show an increased circulatory half-life of radiolabeled DNA. A more recent investigation by Khargharia *et al.* demonstrated the effect of chemical linkage of PEG to a polyacridine delivery system.[145] When PEG was attached via a Cys-sulfhydryl bond, transfection component DNA was maximally found for 2 hrs before apparent release of the PEG. If a Pen-sulfhydryl bond was used, the DNA was found to be maximally protected for 5 hrs, indicating a more stable disulfide bond could extend the circulatory half-life of DNA.

The last chapter demonstrated the successful development of thiazolidine mediated iterative reducible ligation using silver trifluoromethanesulfonate to link polypeptides through Cys residues. This chapter explores the incorporation of Pen into the iterative ligation strategy to form more stable disulfide bonds. Incorporation of Pen into gene delivery polypeptides would result in more reductively stable polypeptides, potentially offering an additional layer of triggered release during delivery.

A Note on Nomenclature in Chapter 4

In Chapter 4, all peptides are referred to by a numbering scheme. Additionally, a (b) will signify a peptide with Fmoc removed, (c) will reference peptides with hydrolyzed thiazolidines, and (d) will label peptides activated as sulfanylpyridine derivatives following Fmoc removal and thiazolidine hydrolysis.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, cysteine hydrochloride (H_2N-Cys-OH), penicillamine hydrochloride (N_2N-Pen-OH), diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), 2,2'-dithiobis(5-nitropyridine) (DTNP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, 9-chloroacridine, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, trifluoroacetic acid (TFA), and glacial acetic acid were obtained from Fisher Scientific. Fmoc-Pen(trityl)-Wang resin was obtained from Bachem. Fmoc-(R)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic-acid (Fmoc-Pen(Thz)-OH) was purchased from Chem-Impex International.

Synthesis and Characterization of Peptide Subunits

Unsubstituted Wang resin was loaded with Fmoc-Lys(Boc)-OH or Fmoc-Cys(Trt)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. S-2-sulfanylpyridine protected peptides were generated by reacting crude thiol-deprotected peptides with 10 mol eq of DTDP in 2 M acetic acid/2-propanol (10:3 v/v) overnight. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a

Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Trp ε_{280nm} = 5600 M^{-1} cm^{-1}, Fmoc-Pen(Thz)-OH ε_{280nm} = 6550 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Acr ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Synthesis of Polypeptide **5**

Ligation reactions were performed in 0.1 M ammonium acetate pH 5. Donor peptide **1** containing free Pen (1.0 μmol) was reacted with an S-2-sulfanylpyridine acceptor peptide **2** (1.5 μmol, 1.5 mol eq) for 12 hrs at RT in 2 ml total volume. Product polypeptide **4** was preparatively purified by injecting 0.5 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$. The major peak from multiple runs was collected, pooled, and lyophilized. Polypeptide **4** was reconstituted in 0.1 v/v % TFA and the yield determined by Abs. Purified **4** were characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Lyophilized **4** (0.25 μmol) was reconstituted in 0.25 ml of 5 v/v % piperidine/DMF and reacted 5 min to remove the Fmoc. The reaction was quenched by the addition of 0.25 ml glacial acetic acid, then applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak **4b** eluting at 21 ml was collected and lyophilized.

The thiazolidine was then hydrolyzed by reaction with 50 mol eq of AgOTf in 0.25 ml 0.1 v/v % TFA for 1 hr. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing donor polypeptide **4c** eluting at 21 ml was collected and lyophilized. The donor polypeptide **4c** was then reconstituted in 0.1 M ammonium acetate pH 5 and reacted with an S-2-sulfanylpyridine acceptor peptide **3** as described above to generate reducible polypeptide **5**.

Synthesis of $FmocAcrK_{10}Pen(nPys)$

Crude $FmocAcrK_{10}Pen$ was reacted with 5 mol eq DTNP in 3:1 acetic acid:H_2O for 72 hrs. $FmocAcrK_{10}Pen(nPys)$ were then purified to homogeneity on RP-HPLC by injecting 0.5 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C.

Synthesis of Polypeptide **10**

Ligation reactions were performed in 0.1 M ammonium acetate pH 5. Donor peptide **7** containing free Pen (1.0 μmol) was reacted with an S-2-sulfanylpyridine acceptor peptide **6** (1.5 μmol, 1.5 mol eq) for 12 hrs at RT in 2 ml total volume. Product polypeptide **9** was preparatively purified by injecting 0.5 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$. The major peak from multiple runs was collected, pooled, and lyophilized. Polypeptide **9** was reconstituted in 0.1 v/v % TFA and the yield determined by Abs. Purified **9** were characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Lyophilized **9** (0.25 μmol) was reconstituted in 0.25 ml of 5 v/v % piperidine/DMF and reacted 5 min to remove the Fmoc. The reaction was quenched by

the addition of 0.25 ml glacial acetic acid, then applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak **9b** eluting at 21 ml was collected and lyophilized.

The thiazolidine was then hydrolyzed by reaction with 50 mol eq of AgOTf in 0.25 ml 0.1 v/v % TFA for 1 hr. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing donor polypeptide **9c** eluting at 21 ml was collected and lyophilized.

Polypeptide **9c** was then activated to an acceptor peptide with 10 mol eq DTDP in 0.5 ml 2 M acetic acid/2-propanol (10:3 v/v) overnight. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing acceptor polypeptide **9d** eluting at 21 ml was collected and lyophilized.

Donor peptide **8** and acceptor polypeptide **9d** were ligated as described above to generate polypeptide **10**.

Synthesis of Polypeptide **13**

Ligation reactions were performed in 0.1 M ammonium acetate pH 5. Peptide **1** (0.15 μmol) was reacted **11** (1.5 μmol, 10 mol eq) for 12 hrs at RT in 0.05 ml total volume. Product polypeptide **12** was preparatively purified by injecting 0.15 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$. The polypeptide peak was collected and lyophilized. Polypeptide **12** was reconstituted in 0.1 v/v % TFA and the yield determined by $Abs_{280\ nm}$. Purified **12** was characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Lyophilized **12** (0.100 μmol) was reconstituted in 0.1 ml of 5 v/v % piperidine/DMF and reacted 5 min to remove the Fmoc. The reaction was quenched by the addition of 0.1 ml glacial acetic acid, then applied to a Sephadex G-10 column (1.5 x

50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak **12b** eluting at 21 ml was collected and lyophilized.

The thiazolidine was then hydrolyzed by reaction of **12b** (0.025 μmol) with 10 mol eq of AgOTf in 0.25 ml 0.1 v/v % TFA for 1 hr. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing donor polypeptide **12c** eluting at 21 ml was collected and lyophilized.

Peptide **8** (0.25 μmol) and polypeptide **12c** (0.025 μmol) were ligated as described above to generate polypeptide **13**.

Results

The last chapter demonstrated that a thiazolidine could be incorporated in an iterative reducible ligation to generate polypeptides linked together by disulfide bonds. To expand this methodology, a series of polypeptides replacing Cys with Pen were designed and synthesized, to generate polypeptides with more stable disulfide bonds. The peptides were designed using polypeptide **6** from chapter 3 as a template. The series consisted of three polypeptides: a polypeptide with N-terminal Pen (**5**), a polypeptide with C-terminal Pen (**10**), and a polypeptide with both N & C-terminal Pen (**13**). With the polypeptides in this chapter, every combination of Cys-Cys, Cys-Pen, Pen-Cys, and Pen-Pen would have been synthesized and the ease of Pen incorporation at different positions and combinations could be evaluated.

The first polypeptide in the series consisted of C-terminal Cys and N-terminal Pen, protected as C1 Cys(Pys) and C2 Fmoc-Pen(Thz) (Fig 4-1). Donor peptide **1** and acceptor peptide **2** (Fig 4-2A & B) were conjugated to form protected polypeptide **4**. Following preparative purification, **4** was recovered in 57% purified yield and eluted as a single peak (Fig 4-2C). The Fmoc was removed using 5% v/v piperidine/DMF solution for 5 min followed by purification by a Sephadex G-10 column, resulting in almost

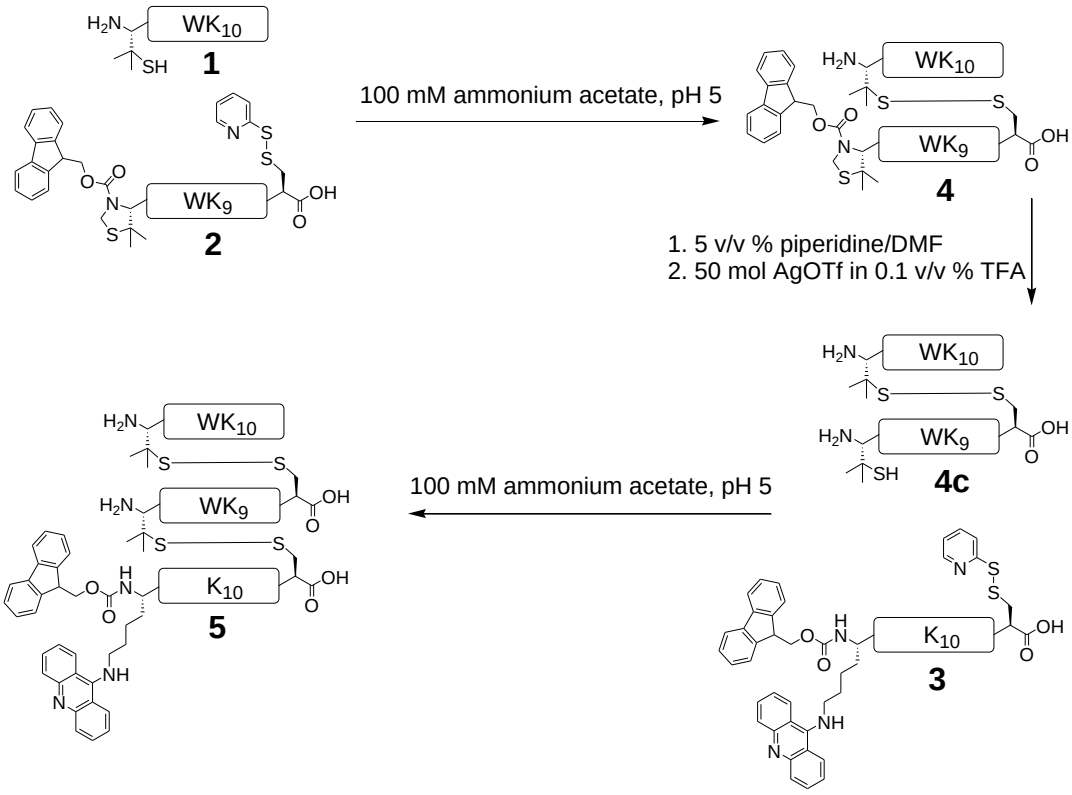


Figure 4-1: *Synthetic Scheme to Generate Polypeptide 5*. Donor peptide **1** and acceptor peptide **2** were ligated to form protected polypeptide **4**. Subsequent Fmoc removal with 5 v/v % piperidine/DMF and Thz hydrolysis with 50 mol eq AgOTf in 0.1% TFA yielded donor polypeptide **4c**. **4c** was ligated with acceptor peptide **3** to generate polypeptide **5** containing 2 N-terminal Pen.

quantitative conversion to **4b**. The thiazolidine was hydrolyzed using 50 mM AgOTf for 1 hr and purified by gel filtration. The hydrolysis of the Pen(Thz) to Pen resulted in quantitative conversion without side reaction. Following purification, donor polypeptide **4c** was reacted with acceptor peptide **3** (Fig 4-2D, 1.5 mol eq) to form polypeptide **5**. Preparative purification resulted in **5** eluting as a single peak, with a recovered yield of 50 % (Fig 4-2E). Polypeptide **5** contained two N-terminal Pen (Fig 4-1) and the incorporation of Pen is clearly seen by MS (5134.4 amu for **5** versus 5078.8 for the Cys containing analogue from chapter 3).

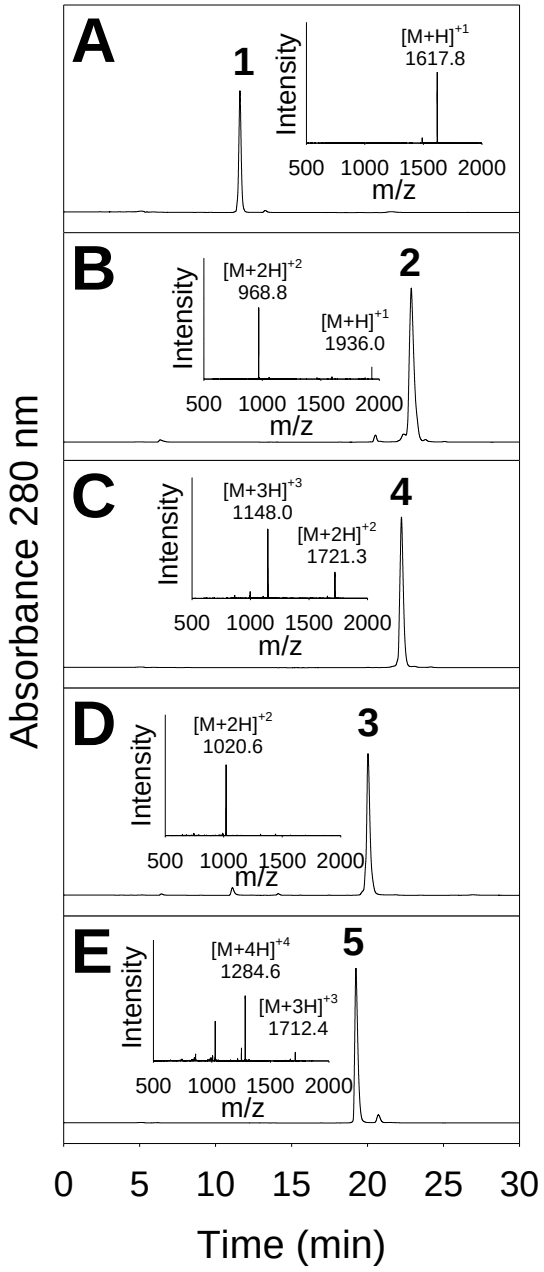


Figure 4-2: *Synthesis of Polypeptide **5***. Panels A-E illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified donor peptide **1** and acceptor peptide **2** to establish their retention times. **1** and **2** (1.5 mol eq) were ligated to form protected polypeptide **4** in a 57 % yield following preparative purification (Panel C). Following Fmoc removal and thiazolidine hydrolysis, donor polypeptide **4c** was coupled with 1.5 mol eq of acceptor **3** (Panel D) to form polypeptide **5**. Re-analysis following purification showed a single peak in 50 % yield (Panel E).

The second polypeptide consisted of C-terminal Pen and N-terminal Cys. Before following the same donor and acceptor peptide roles used in the synthesis of **5**, the reactivity of a Pen substituted with sulfanylpyridine analogues was explored. The reaction of DTDP with a model FmocAcrK$_{10}$Pen proceeded in good yield, and the derivatized FmocAcrK$_{10}$Pen(Pys) could be recovered as a single product (Fig 4-3A). Experiments in chapter 3 demonstrated that a donor Cys could be fully conjugated with a slight excess of acceptor Cys(Pys) (1.5 mol eq) in 100 mm ammonium acetate, pH 5. Similar experiments showed complete reaction of an acceptor Cys(Pys) with 1.5 mol eq of a donor Cys. Unlike Cys, the Pen stabilized the sulfanylpyridine group to such a degree that large excess of reagent had to be used to fully react the Pen(Pys). Reaction of acceptor FmocAcrK$_{10}$Pen(Pys) with 10 mol eq H$_2$N-Cys-OH in 100 mM ammonium acetate pH 5 only resulted in 50% of the peptide reacting to form the Pen-Cys mix disulfide (Fig 4-3B). Complete reaction could be achieved using 50 mol eq H$_2$N-Cys-OH in 100 mM ammonium acetate pH 5 or by increasing the pH of the reaction to 7 with a 10-fold excess of H$_2$N-Cys-OH (Fig 4-3C & D).

Due to the lack of reactivity of the Pen(Pys), a more reactive nitro-sulfanylpyridine (nPys) was incorporated into the FmocAcrK$_{10}$Pen model peptide (Fig 4-4A). Despite the reported increased reactivity of the nPys group, the resulting FmocAcrK$_{10}$Pen(nPys) proved to be too stable. A 5-fold excess of H$_2$N-Cys-OH in 100 mM HEPES pH 7 only resulted in approximately 70 % reaction (Fig 4-4B). Like the Pys activated peptide, 10 mol eq of H$_2$N-Cys-OH was required for complete formation of the mixed disulfide (Fig 4-4C).

The large excess of donor peptide required to react with an acceptor Pen prevented the use of Pen as an accepting sulfhydryl for directed disulfide bond formation. To incorporate a C-terminal Pen and an N-terminal Cys, the Pen peptides served as donor peptides without a C1 group and the C2 position were activated as Cys(Pys), inverting the usual donor and acceptor peptides (Fig 4-5). Acceptor **6** and Pen containing donor **7**

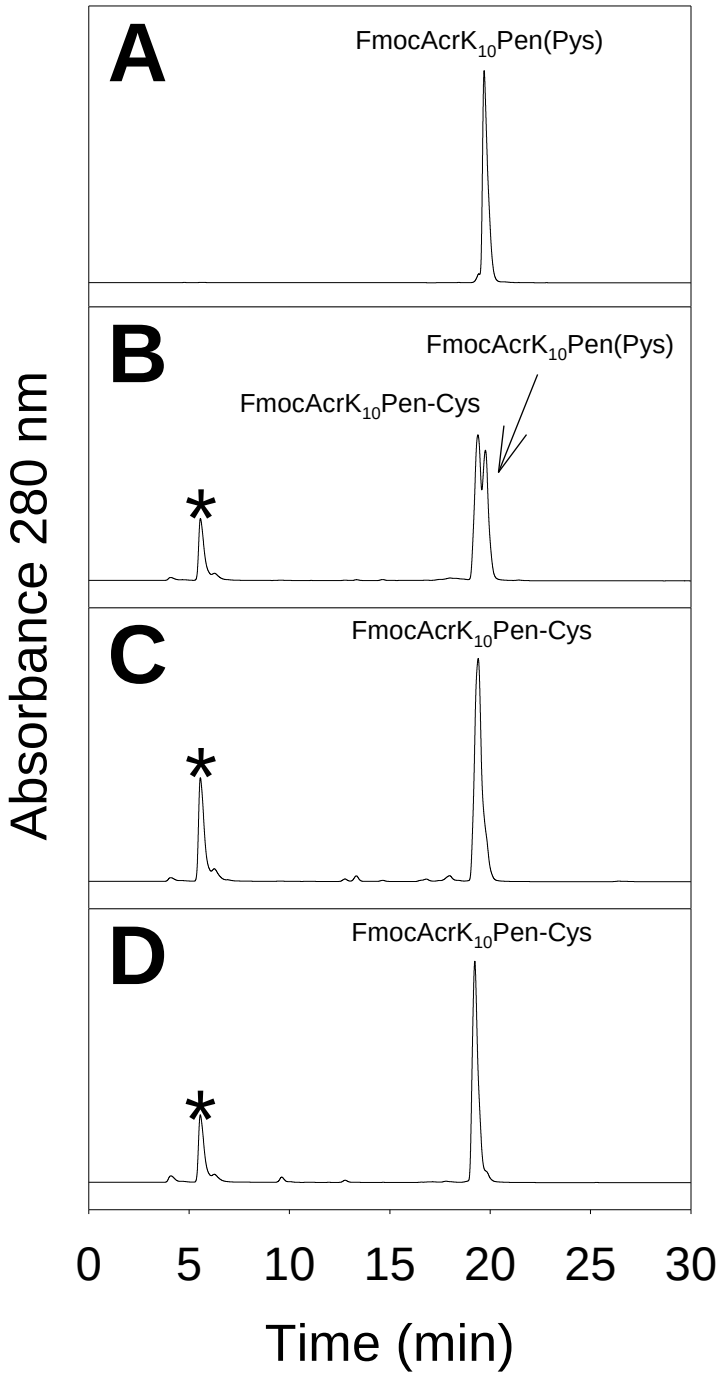


Figure 4-3: *RP-HPLC Analysis of FmocAcrK$_{10}$Pen(Pys) Reacting with Cys*. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting Abs$_{280\ nm}$. Panel A illustrates purified FmocAcrK$_{10}$Pen(Pys). Panels B, C, and D represent reaction of FmocAcrK$_{10}$Pen(Pys) with 10 mol eq Cys in 100 mM ammonium acetate pH 5, 50 mol eq Cys in 100 mM ammonium acetate pH 5, and 10 mol eq Cys in 100 mM HEPES pH 7. The asterisk (*) represents displaced pyridine-2-(1H)-thione.

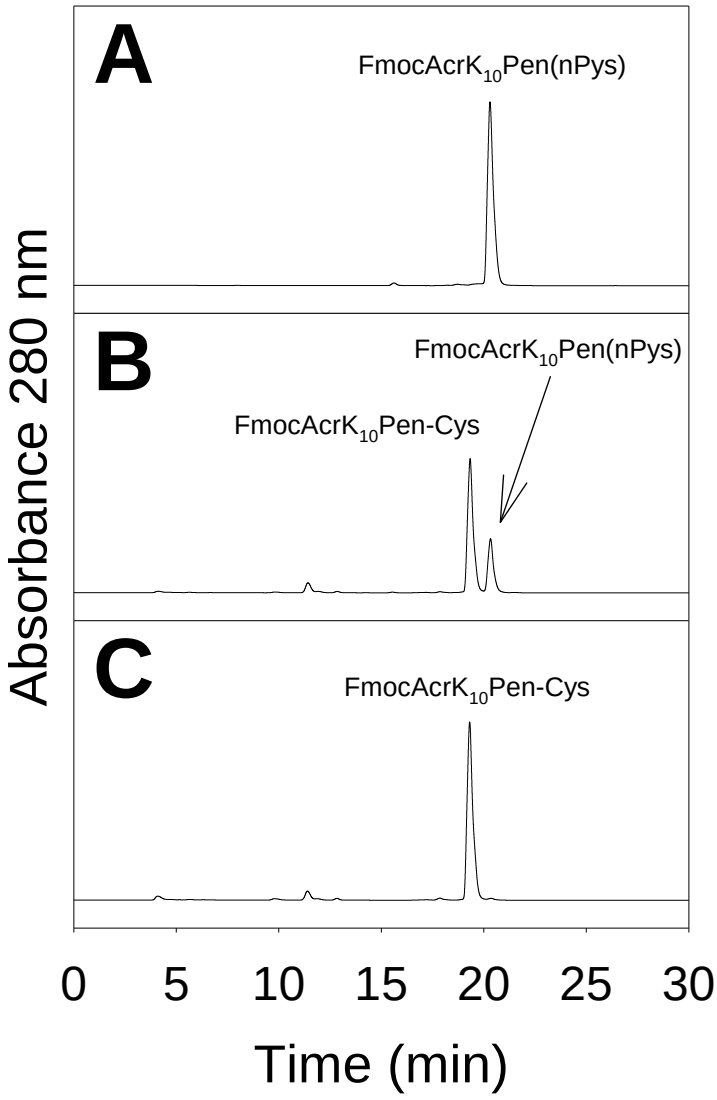


Figure 4-4: *RP-HPLC Analysis of FmocAcrK$_{10}$Pen(nPys) Reacting with Cys*. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting Abs$_{280\ nm}$. Panel A illustrates purified FmocAcrK$_{10}$Pen(nPys). Panels B and C represent reaction of FmocAcrK$_{10}$Pen(Pys) with 5 mol eq and 10 mol eq Cys in 100 mM HEPES pH 7.

were ligated to yield protected polypeptide **9** (Fig 4-6A & B). Following purification, **9** was recovered as a single peak in 72 % purified yield (Fig 4-6C). Subsequent Fmoc removal by 5% v/v piperidine/DMF followed by thiazolidine hydrolysis with 50 mM AgOTf in 0.1% TFA generated the free Cys polypeptide **9c**. The N-terminal Cys was

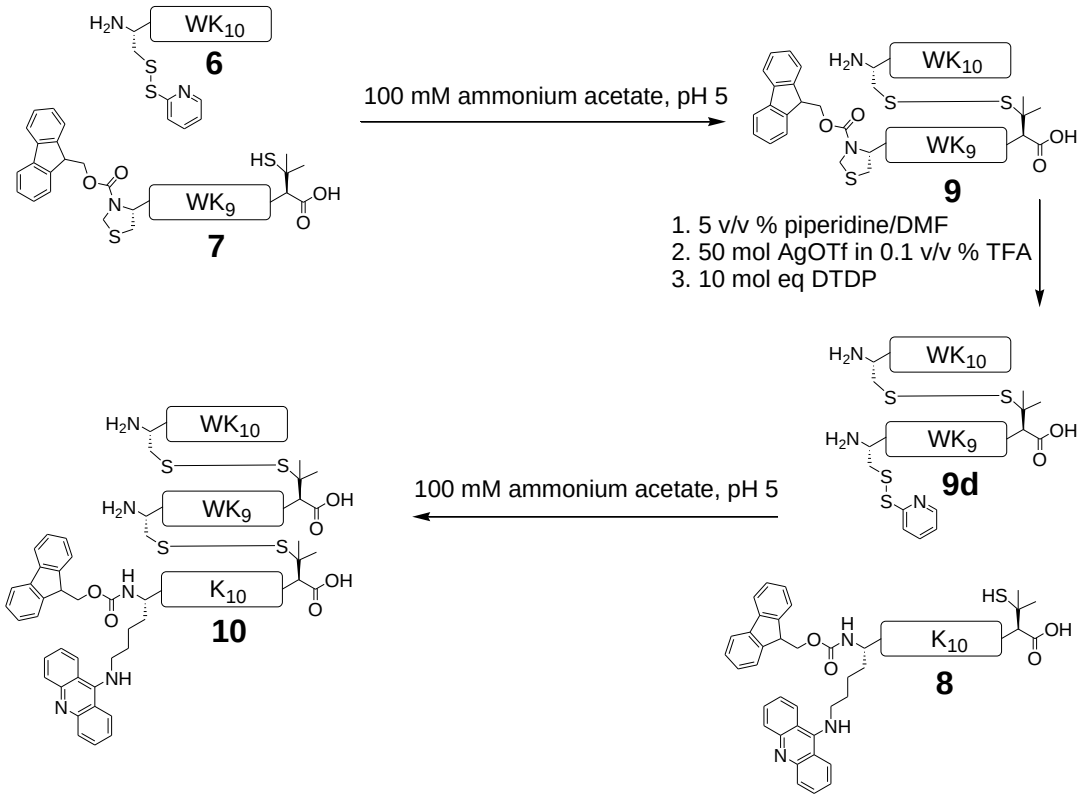


Figure 4-5: *Synthetic Scheme to Generate Polypeptide **10**.* Acceptor peptide **6** and donor peptide **7** were ligated to form protected polypeptide **9**. Subsequent Fmoc removal with 5 v/v % piperidine/DMF, Thz hydrolysis with 50 mol eq AgOTf in 0.1 % TFA, and 2-sulfanylpyridine activation with DTDP yielded acceptor polypeptide **9d**. **9d** was ligated with donor peptide **8** to generate polypeptide **10** containing 2 C-terminal Pen.

then activated with DTDP to yield acceptor polypeptide **9d**. Reaction of donor peptide **8** (Fig 4-6D, 1.5 mol eq) with acceptor peptide **9d** formed polypeptide **10** containing 2 C-terminal Pen. Following preparative purification, polypeptide **10** was recovered in 45% yield with an identical mass to regioisomer **5** (Fig 4-6E).

The final desired model polypeptide consisted of every disulfide bond being between two Pen. The directed formation of a Pen-Pen bond proved to even more difficult than the corresponding Pen-Cys disulfide. The steric bulk of a donor Pen further impeded disulfide bond formation (Fig 4-7). The use of 10 mol eq of H_2N-Cys-OH could completely react with Pen(Pys) or Pen(nPys) in 100 mM HEPES pH 7 (Fig 4-3D & Fig

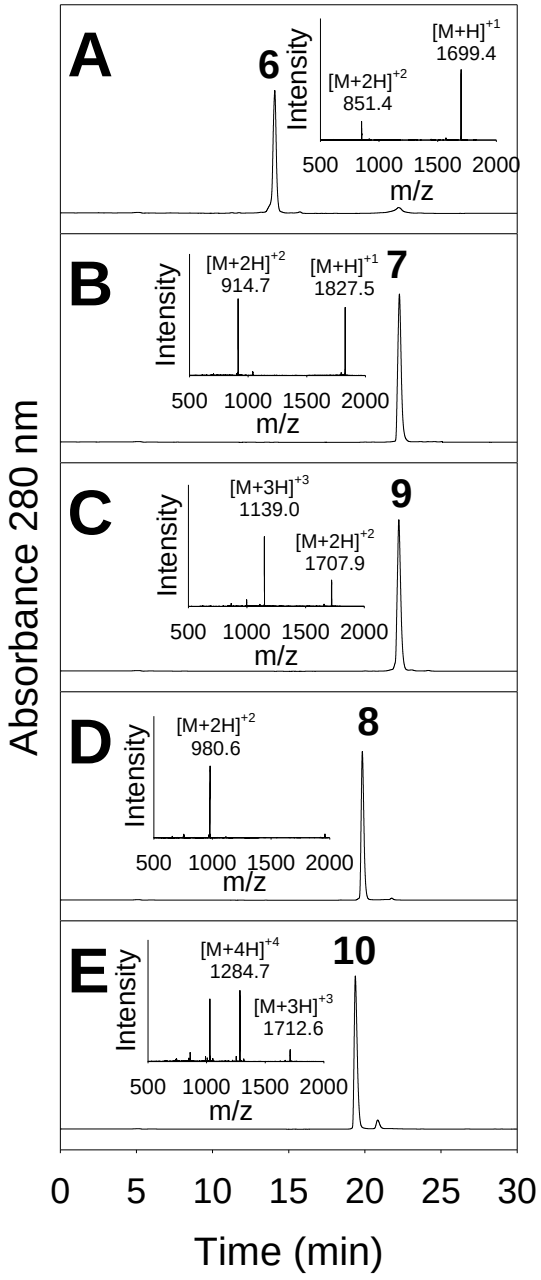


Figure 4-6: *Synthesis of Polypeptide **10***. Panels A-E illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified acceptor peptide **6** and donor peptide **7**. **7** and **6** (1.5 mol eq) were ligated to form protected polypeptide **9** in a 72 % yield following preparative purification (Panel C). Following Fmoc removal, thiazolidine hydrolysis, and 2-sulfanylpyridine activation, acceptor polypeptide **9d** was coupled with 1.5 mol eq of donor **8** (Panel D) to form polypeptide **10**. Re-analysis following purification showed a single peak eluting at 21 min and recovered in 45 % yield (Panel E).

4-4C). However, both the Pen(nPys) and Pen(Pys) proved stable to the presence of large excesses of free H_2N-Pen-OH. When 20 mol eq of H_2N-Pen-OH was added to either $FmocAcrK_{10}Pen(nPys)$ or $FmocAcrK_{10}Pen(Pys)$, at best 70 % of the desired Pen-Pen bond was formed (Fig 4-7B & D), despite reaction in 100 mM HEPES pH 7 and incubation for 18 hrs. Further complicating directed Pen-Pen bond formation was the dimerization of the donor Pen. After 30 min incubation in 100 mM ammonium acetate pH 5, peptide **1** had undergone significant dimerization (~33 %). Dimerization could be avoided by diluting Pen containing peptides or by acidifying the solution, though these

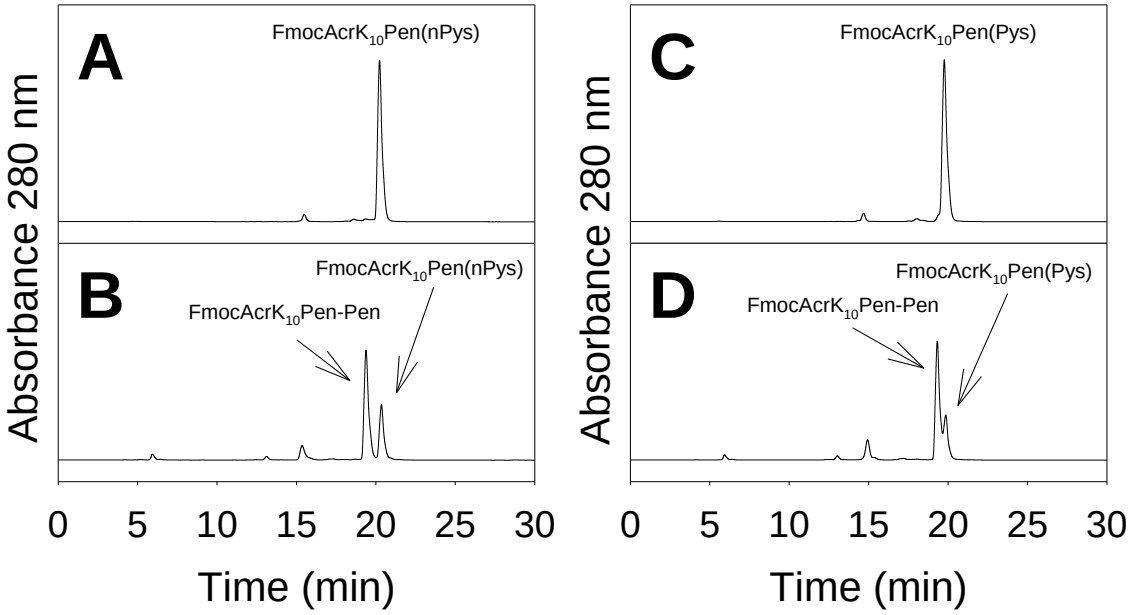


Figure 4-7: *RP-HPLC Analysis of $FmocAcrK_{10}Pen((n)Pys)$ Reacting with Pen*. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Panel A illustrates purified $FmocAcrK_{10}Pen(nPys)$. Panel B represents reaction of $FmocAcrK_{10}Pen(nPys)$ with 20 mol eq Pen in 100 mM HEPES pH 7. Panel C illustrates purified $FmocAcrK_{10}Pen(Pys)$. Panel D represents reaction of $FmocAcrK_{10}Pen(Pys)$ with 20 mol eq Pen in 100 mM HEPES pH 7.

also impeded directed disulfide bond formation. Physical methods of removing oxygen from the solvent, such as bubbling Ar and freeze-pump-thaw methods, did not prevent dimerization. Due to the poor reactivity of an acceptor Pen peptide and propensity of a donor Pen to dimerize, the directed disulfide bond formation was abandoned for random disulfide bond formation (Fig 4-8).

Purified peptides **1** and **11** (10 mol eq) were conjugated in 100 mM ammonium acetate pH 5 overnight to form a variety of peptide dimers (Fig 4-9 A-C). A large excess of **11** was used due to the observation that the peptides formed homodimers

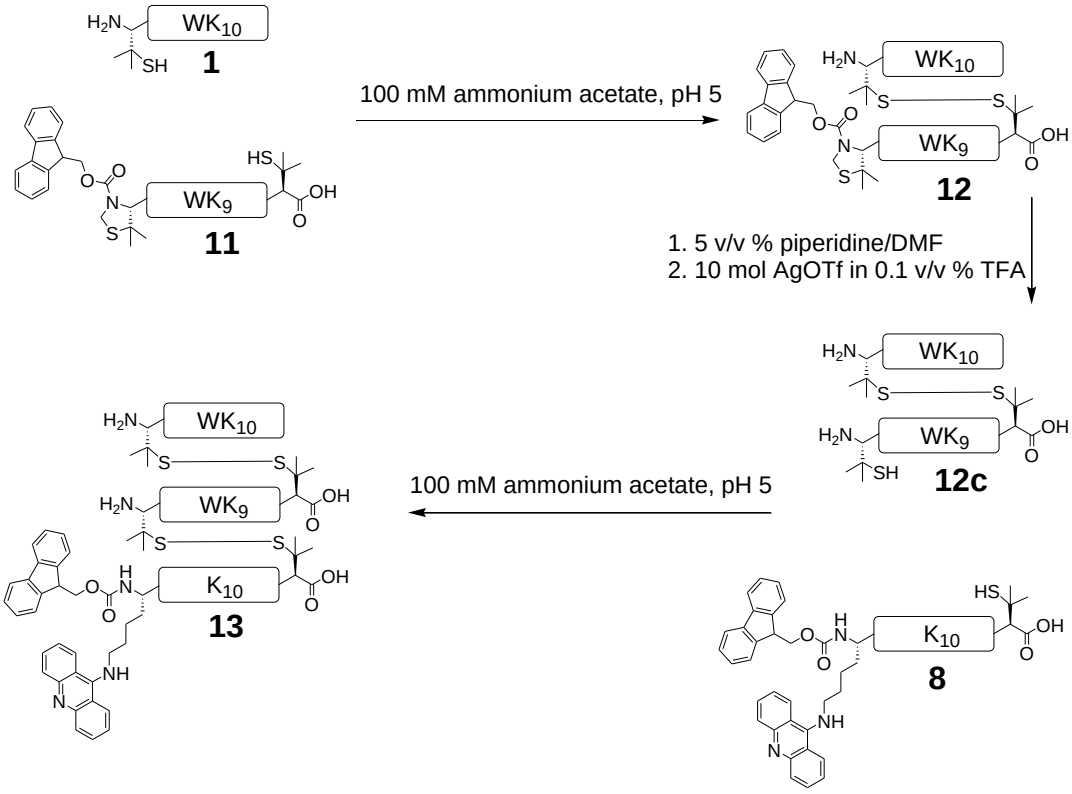


Figure 4-8: *Synthetic Scheme to Generate Polypeptide **13***. Peptides **1** and **11** were ligated to form protected polypeptide **12**. Subsequent Fmoc removal with 5 v/v % piperidine/DMF and Thz hydrolysis with 10 mol eq AgOTf in 0.1 v/v % TFA yielded polypeptide **12c**, which was ligated with **8** to generate polypeptide **13** containing 4 Pen forming 2 disulfide bonds.

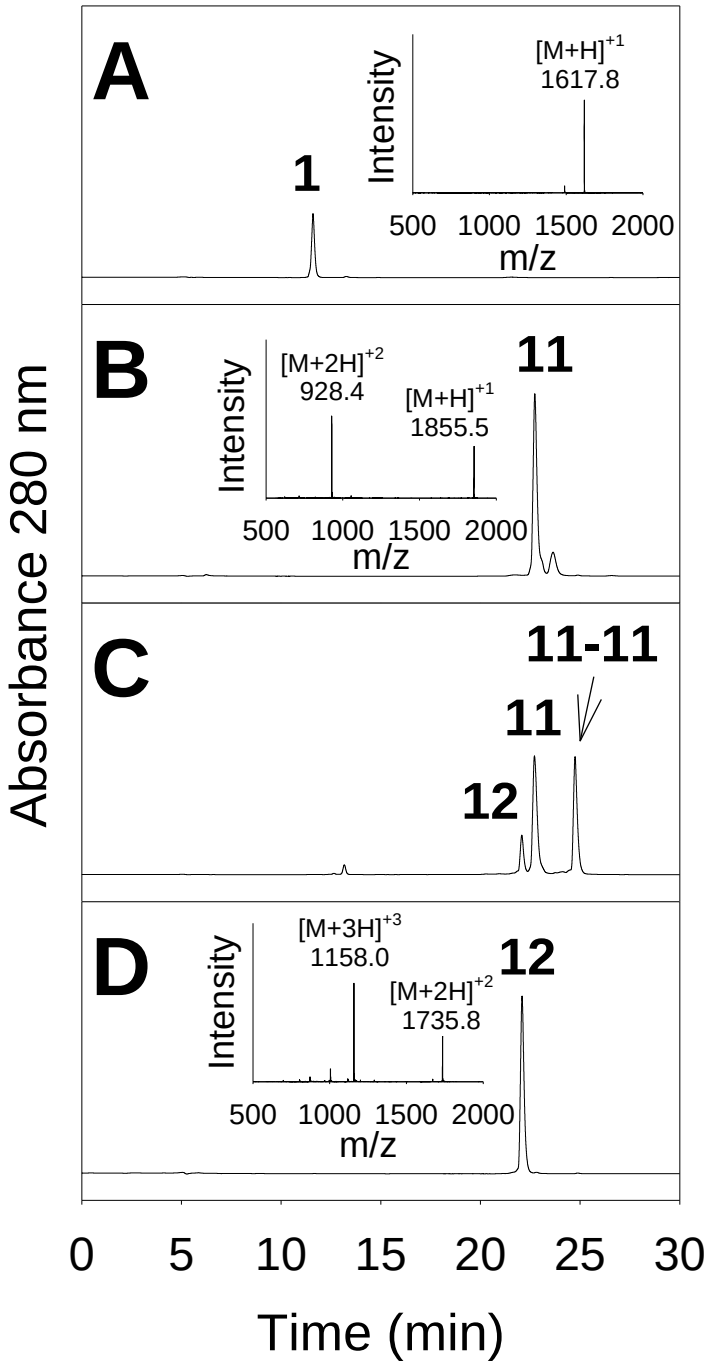


Figure 4-9: *Synthesis of Polypeptide **12***. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the re-analysis of purified peptides **1** and **11**. **1** and **11** (10 mol eq) were allowed to randomly ligate to form protected polypeptide **12** along with significant homodimerization of **11**. (Panel C). **12** was preparatively purified, resulting in a single peak in 72 % yield from the limiting peptide **1** (Panel D).

preferentially. The large excess of **11** also consumed most of **1**, resulting in the formation of two major polypeptides (Fig 4-9C, **12** & **11-11**). Following purification, the desired polypeptide **12** was recovered in 72% yield from limiting peptide **1** (Fig 4-9D).

Generating the free Pen polypeptide **12c** proved to be difficult. Removing the Fmoc with 5% v/v piperidine/DMF for 5 min followed by Sephadex G-10 purification yielded the desired polypeptide **12b** in good yield and purity (Fig 4-10A & B). Treatment of the polypeptide with 50 mM AgOTf was expected to generate the **12c** polypeptide; unexpectedly this caused the cleavage of the Pen-Pen bond to form the constituent peptides (Fig 4-10C). This is evident in the 3 min change in elution time between Fig 4-10B & C, much more than the typical 0.5 min change for a thiazolidine hydrolysis. The MS insets also provided evidence, with the $[M+3H]^{+3}$ ion disappearing after thiazolidine hydrolysis. A mass was observed close to the expected value following thiazolidine hydrolysis (m/z = 1624.8 in Fig 4-10B and m/z = 1618.7 in Fig 4-10C). If read as the $[M+2H]^{+2}$ ion, it would indicate the loss of the thiazolidine with a 12 amu difference. The similar mass is a result of a Pen and a Lys residue having almost the same molecular weight, and peptides **1** (calculated mass = 1617.1 amu) and cyclic **11** (calculated mass = 1618.1) would show a similar mass to the $[M+2H]^{+2}$ ion of **12c**. The lack of the $[M+3H]^{+3}$ indicated that the Pen-Pen disulfide was broken, likely from the liberated Pen reacting with the internal disulfide bond to form cyclic **11** and displaced **1**.

In attempts to hydrolyze the Pen(Thz), varying concentrations of AgOTf were used. The instability of **12c** to high concentrations of AgOTf was evident (Fig 4-11). Treatment of the polypeptide with low a concentration of AgOTf (2 mM) caused only partial hydrolysis of the thiazolidine (Fig 4-11B). Increasing the concentration to 10 mM resulted in the formation of the desired polypeptide **12c** as a stable intermediate (Fig 4-11C). Increasing the concentration to 50 mM and 250 mM resulted in the breakdown of the Pen-Pen disulfide bond (Fig 4-11D & E). A narrow concentration range could therefore generate **12c**.

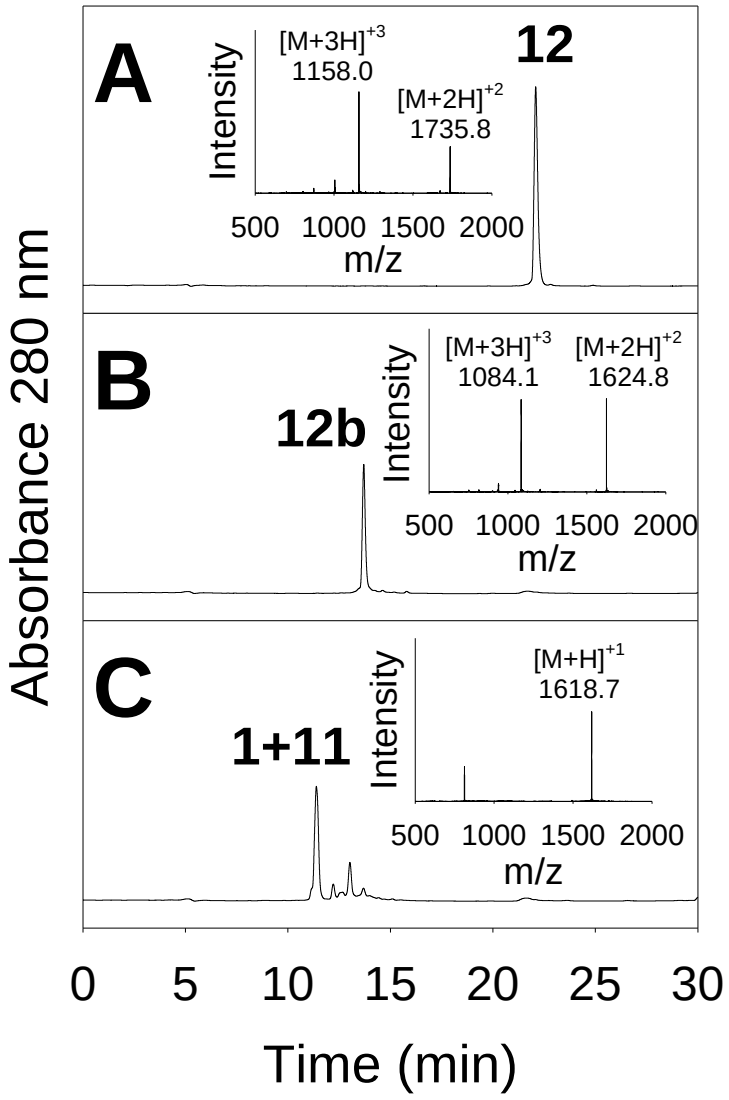


Figure 4-10: *LC-ESI-MS Analysis of Fmoc Removal and Thiazolidine Hydrolysis from **12***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates purified **12**. The Fmoc was removed by reaction with 5% v/v piperidine/DMF, and following G-10 purification eluted as a single peak **12b** (Panel B). **12b** was subsequently treated with 50 mM AgOTf in 0.1% TFA, which unexpectedly generated component peptides **1** and cyclic **11** (Panel C).

Using the lower 10 mM AgOTf in 0.1 v/v % TFA, **12b** could be converted into polypeptide **12c**, which was subsequently purified on a Sephadex G-10 column. Reaction

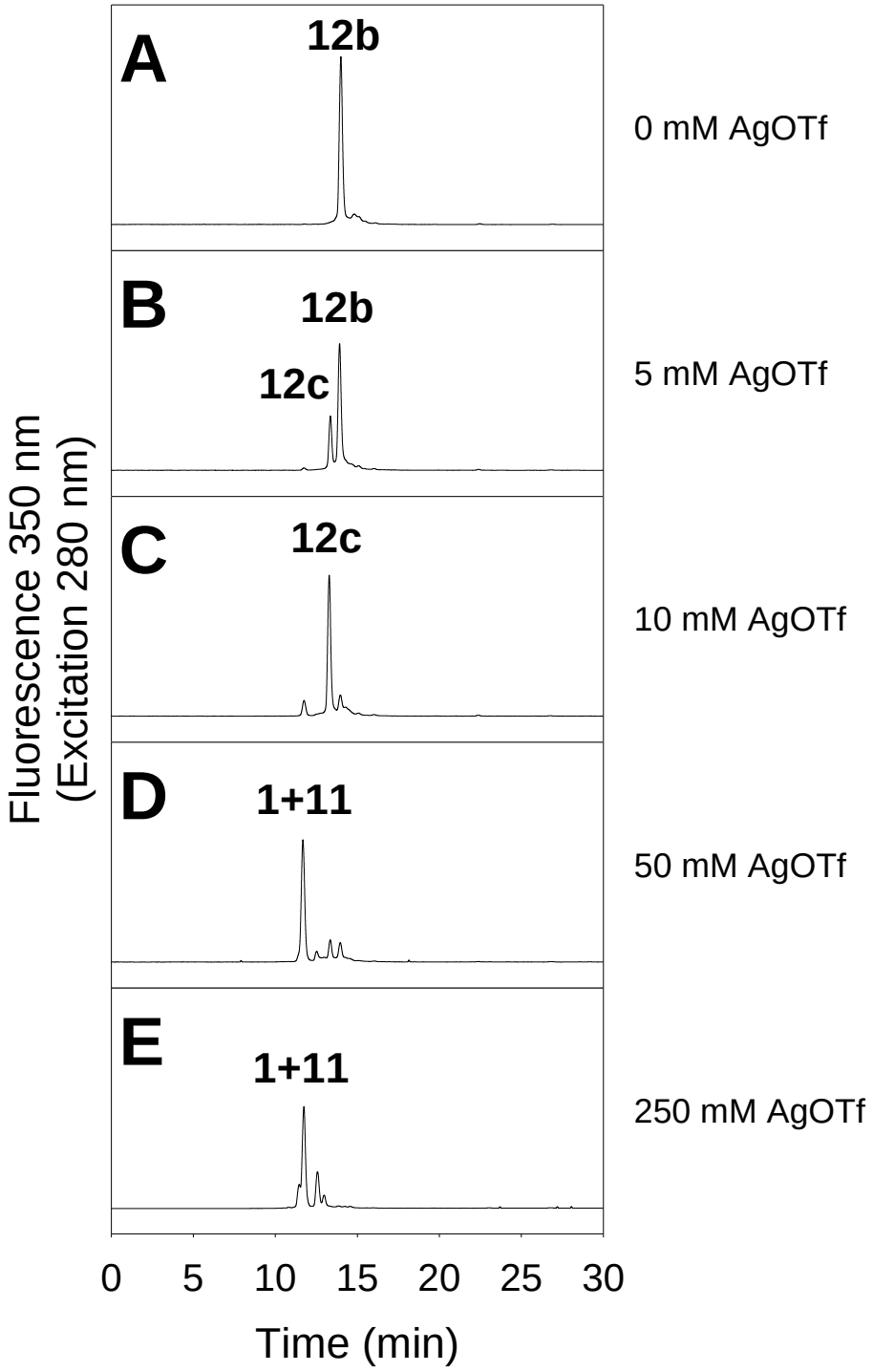


Figure 4-11: *RP-HPLC Analysis of Thiazolidine Hydrolysis from **12b***. Panels A-E illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Panel A illustrates purified **12b**. Panels B-E illustrate reaction of **12b** with 2, 10, 50, and 250 mM AgOTf in 0.1% TFA.

of **12c** with 10 mol eq **8** in 100 mM ammonium acetate pH 5 resulted in the formation of the desired polypeptide **13** containing 4 Pen linking 3 peptides through 2 disulfide bonds. Following preparative purification, **13** was recovered in 12 % purified yield from **12** (Fig 4-12). A complete list of peptides, masses, and yields can be found in Table 4-1.

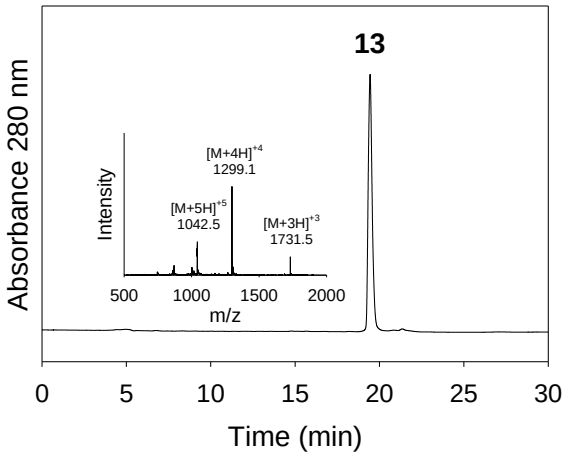


Figure 4-12: *LC-ESI-MS Analysis of Purified **13***. The chromatogram illustrates RP-HPLC analysis following injection of 2 nmol of polypeptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peak. Polypeptide **13** is observed as a single peak following preparative purification in 12 % yield.

Discussion

Disulfide bonds are a commonly used method for triggering polyplex dissociation upon cellular uptake for nonviral gene delivery.[48, 49] Incorporation of different strength disulfide bonds would allow more control over the disassembly of the polyplex. Cys-Cys disulfide bonds would quickly dissociate, followed by Cys-Pen or Pen-Cys bonds, with Pen-Pen bonds remaining intact the longest because they are the most stable. Therefore components could be released immediately or with some delay time based upon the disulfide bond. Such intricate control over polyplex disassembly has never been reported

Table 4-1: *Synthesis and Characterization of Penicillamine Peptides.*

Peptide[a]	Mass (calc / obs)	% Yield
1 ($PenWK_{10}$)	1617.1 / 1616.8	26[b]
2 (FmocPen(Thz)WK_9C(Pys))	1935.5 / 1935.4	52[b]
3 (Fmoc$AcrK_{10}C$(Pys))	2039.6 / 2039.2	42[b]
4 (FmocPen(Thz)K_9C-$PenWK_{10}$)	3441.5 / 3441.0	57[c]
5 (Fmoc$AcrK_{10}C$-$PenWK_9C$-$PenWK_{10}$)	5135.7 / 5134.4	50[c]
6 (C(Pys)WK_{10})	1698.2 / 1698.0	29[b]
7 (FmocThzWK_9Pen)	1826.4 / 1826.1	35[b]
8 (Fmoc$AcrK_{10}$Pen)	1985.6 / 1958.6	37[b]
9 (FmocThzK_9Pen-CWK_{10})	3413.4 / 3412.6	72[c]
10 (Fmoc$AcrK_{10}$Pen-CWK_9Pen-CWK_{10})	5135.7 / 5135.0	45[c]
11 (FmocPen(Thz)WK_9Pen)	1854.4 / 1854.5	43[b]
12 (FmocPen(Thz)K_9Pen-$PenWK_{10}$)	3469.6 / 3469.6	72[c]
13 (Fmoc$AcrK_{10}$Pen-$PenWK_9$Pen-$PenWK_{10}$)	5191.8 / 5191.5	12[c]

[a] Thz refers to thiazolidine, Pen(Thz) to 5,5-dimethylthiazolidine, Pys to 2-sulfanylpyridine, and Acr to Nε-acridinyllysine.

[b] Purified yield based on initial resin substitution.

[c] Purified yield determined for the last ligation step.

and the ability to ligate specific peptides with specific disulfide bonds would represent a new application of iterative reducible ligation. The replacement of Cys with Pen was attempted, to determine the chemical feasibility of incorporating Pen at various positions within a model gene delivery polypeptide.

Replacement of N-terminal Cys with Pen resulted in the formation of polypeptide **5**. There was a commercial source for the Fmoc-Pen(Thz)-OH and generation of a free Pen mirrored the chemistry to generate a free Cys from a Thz. Ligation yields for this polypeptide were 57 and 50 %, indicating the β,β-dimethyls steric bulk of Pen did not negatively affect the nucleophilicity of the sulfhydryl when reacting with an activated Cys(Pys).

Generation of C-terminal Pen polypeptides was possible, but required the inversion of donor and acceptor peptides. The β,β-dimethyls of the Pen provided too much steric hindrance that lowered the reactivity of a Pen(Pys). This was not unexpected, given the low reactivity of Pen-Cys mixed disulfides.[142] Generation of the Pen(nPys) peptide also did not result in more robust disulfide bond formation, despite the presence of the nitro group known to provide a better leaving group.[81, 82, 146] Reaction with a 10-fold excess of H_2N-Cys-OH was sufficient for complete reaction at pH 7. Using a 10-fold excess of donor peptide was not feasible, especially for the subsequent ligation where the polypeptide would serve as the donor. Use of a 10-fold excess of the polypeptide would be wasteful; therefore a strategy of activating the N-terminal Cys with DTDP while keeping a free Pen at the C-terminal was chosen. The synthesis of polypeptide **10** was achieved with 72 and 45 % yield, with the additional DTDP activation of the polypeptide not resulting in greatly diminished yields.

Generating the N & C-terminal Pen polypeptide was possible for a directed reaction, but very inefficient. A 20-fold excess of H_2N-Pen-OH only resulted in about 70 % of the Pen-Pen bond formation, most likely due to stabilization of Pen((n)Pys) disulfide and decreased nucleophilicity of the free Pen. This was further complicated by a free Pen showing the tendency to dimerize in solution. Dimerization could be inhibited by lowering the pH or diluting the sample, but both approaches also led to decreased directed disulfide bond formation. Despite a previous report of the physical method of freeze-pump-thaw to deoxygenate the solvent successfully preventing Pen dimerization,[143] this technique was not able to prevent peptide **1** from dimerizing. This might be due to the presence of Lys residues on peptide **1**, which have previously been observed in the Rice lab to increase the reactivity of Cys.

Rather than prevent dimerization and promote an unfavorable reaction, a random Pen-Pen disulfide bond approach was selected. An excess of one peptide was used to drive the consumption of the other peptide. When a 1:1 stoichiometry of Pen-containing

peptides was used, only about 10 % of the heterodimer was formed with homodimerization of both peptides preferentially occurring. The use of 10 mol eq was still less than the 20 mol eq required for 70% coupling using the directed Pen approach. While inefficient, the desired product polypeptide could be obtained in high purity.

The second coupling proved to be more difficult, mostly due to the unexpected cleavage of the Pen-Pen disulfide bond during treatment with AgOTf. The breakdown of a Cys-Cys polypeptide can be observed when using AgOTf, but only at high concentrations (1 M or approximately 1000 mol eq relative to peptide). The more stable Pen-Pen was not expected to breakdown under these conditions and it would be interesting to see if this is a general way of cleaving a Pen-Pen disulfide bond or if it is the result of having a third Pen in close proximity to the Pen-Pen bond. Only a small variance in AgOTf concentration was tolerated, as a 5-fold increase or decrease in concentration led to product breakdown or incomplete hydrolysis. The poor second coupling yield of 12 % can be partially attributed to the sensitive hydrolysis reaction, as slight differences in the AgOTf concentration when scaling up the reaction resulted in less than stoichiometric free Pen polypeptide formation.

Thiazolidine mediated iterative reducible ligation was shown to be tolerant to incorporation of either an N or C-terminal Pen, indicating more stable disulfide bonds could be incorporated into gene delivery polypeptides. While a polypeptide can be synthesized containing multiple Pen-Pen bonds, more efficient ligation chemistry is needed before this could be used for more expensive gene delivery polypeptides, as 10-fold excess and less than 15 % yield would not be tolerated.

CHAPTER 5

PROGRESS TOWARDS THIAZOLIDINE MEDIATED ITERATIVE REDUCIBLE LIGATION ON A SOLID SUPPORT

Abstract

Chapter 5 incorporates a solid support for thiazolidine mediated iterative reducible ligation. A resin consisting of cross-linked ethylene glycol units resulted in the highest peptide ligation. Optimization of the coupling conditions resulted in efficient coupling of a single peptide to a peptide bound to the solid support. Conjugation of a third peptide resulted in a mixture of polypeptide products. A C1 Cys(Pys) and C2 Thz protecting scheme was shown to be stable, removing a polypeptide Fmoc deprotection and purification step.

Introduction

Previous chapters have detailed the development of a thiazolidine iterative reducible ligation strategy. While the methodology was able to generate polypeptides containing multiple subunits, a major limitation was the preparative purification following a ligation reaction. The removal of the Fmoc and thiazolidine hydrolysis were both high yielding reactions, aided by near quantitative recovery of the products following gel filtration purifications. The conjugation reaction product was preparatively purified, due to the required use of an excess of an acceptor peptide to prevent donor peptide dimerization. This purification resulted in the majority of the coupling reaction yield loss. One way to avoid the yield-lowering purifications is to perform the reaction on a solid support. Polypeptides would be covalently attached to the solid support and excess reagents and acceptor peptide could be removed by extensive washing, bypassing intermediate purification steps. The final product could be cleaved and used without further purifications.

The use of a polymeric support in peptide synthesis was originally developed by Merrifield in 1963.[84] In this landmark paper, a tetra-peptide was synthesized on a polystyrene resin to overcome the problems in solution phase peptide synthesis of low solubility of long protected chains and large number of purifications. It was also correctly noted that this approach simplified the chemical manipulations, shortened the time required to synthesize a peptide, and that the entire process could be automated.

The original proposal for solid phase peptide synthesis (SPPS) involved the sequential coupling of protected amino acids into peptide chains. A more advanced form of SPPS involved the coupling of protected peptide sequences to a solid support, in a process coined convergent solid phase peptide synthesis.[147-149] The advantages of this technique include fewer conjugation reactions and simpler purification because truncated forms are missing several sequential amino acids and are more easily separated from the final product.[148] The major limitation of convergent SPPS is that it requires the use of protected peptide segments, which depending on the length are very difficult to purify to homogeneity due to their low solubility in organic solvents.[147]

To overcome poorly soluble protected fragments, peptide conjugation techniques such as native chemical ligation utilize fully deprotected peptide segments that are readily purified. The application of native chemical ligation on a solid support has been reported in the synthesis of a small protein EETI-II, a 28-amino acid trypsin inhibitor.[114] Two ligation steps resulted in the successful formation of the protein, which showed the ability to inhibit trypsin both on and off resin. The authors' utilized thiazolidine mediated native chemical ligation with methoxyamine deprotection, with TCEP added following thiazolidine hydrolysis to reduce any disulfide bonds. Syntheses of larger proteins have not been reported.

Ligation reactions of unprotected peptide segments in aqueous solvents on a solid support require the use of specialized resins, as normal polystyrene resins used in SPPS do not swell in aqueous conditions.[150] Reagents cannot diffuse through a resin that does

not swell, as first reported by Merrifield,[84] resulting in low yielding reactions. Many resins that can swell in water have been developed. An early type of water miscible resin involved grafting PEG onto a polystyrene core, creating the commercially available PEG-PS and TentaGel resins.[151-153] To further improve swelling capacities, resins composed of ether bonds and ethylene glycol repeats were developed and are commercially available as NovaPEG and ChemMatrix resins.[154-156]

Previous groups have successfully formed disulfide bonds on resin, though these often involved the formation of intramolecular bonds.[62, 157] Intermolecular disulfide bond formation on resin has been reported, but involved the pre-formation of the disulfide bond before peptide elongation.[158] A donor peptide has also been used to form a disulfide to a resin attached sulfhydryl acceptor, with dimerization of the donor peptide also observed.[129] To date there are no reports of iteratively conjugating peptides through reducible disulfide bonds on a solid support. Due to the mild conditions of thiazolidine mediated iterative reducible ligation and similarity to native chemical ligation that has successfully been applied to a solid support, this chapter examined if a solid support is compatible with iterative reducible ligation.

A Note on Nomenclature in Chapter 5

Due to the variety of peptides that were used in examining iterative reducible ligation on a solid support, a new nomenclature is used in chapter 5. Peptides are numbered by addition to the solid support and are denoted with a P. The P1 peptide is the peptide synthesized on the solid support and has an N-terminal Fmoc-Thz. P2 represents the second peptide conjugated, and P3 the third. A (b) will denote the removal of Fmoc and (c) will indicate thiazolidine hydrolysis.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-

hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, 9-chloroacridine, methoxyamine hydrochloride, triisopropylsilane (TIS), 4-dimethylaminopyridine (DMAP), H-Gly-TentaGel, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, trifluoroacetic acid (TFA), dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Fisher Scientific. Unsubstituted Wang-NovaPEG resin was purchased from NovaBioChem **(**Billerica, MA). H-Gly-ChemMatrix was generously provided by PCAS BioMatrix Inc (Saint-Jean-sur-Richelieu, Quebec).

Synthesis and Characterization of Peptide Subunits

Unsubstituted Wang resin was loaded with Fmoc-Cys(Trt)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. S-2-sulfanylpyridine protected peptides were generated by reacting crude thiol-deprotected peptides with 10 mol eq of DTDP in 2 M acetic acid/2-propanol (10:3 v/v) overnight. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides

were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Trp ε_{280nm} = 5600 M^{-1} cm^{-1}, Fmoc-L-thiazolidine-4-carboxylic acid (Fmoc-Thz-OH) ε_{280nm} = 6550 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Acr ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Initial Resin Substitution

Unloaded Wang-NovaPEG resin (1 gr, substitution = 0.60 mmol/g) was swelled in 9:1 DCM:DMF solution. Fmoc-Ala-OH (560 mg, 1.8 mmol) and HOBt (490 mg, 3.6 mmol) were combined in 3 ml DMF and added to the resin. DIC (0.56 ml, 3.6 mmol) and DMAP (7.3 mg, 0.060 mmol) were dissolved in 2 ml DMF and added to resin suspension. The reaction was agitated for 2 hrs and then additional equivalents of Fmoc-Ala-OH, HOBt, and DIC were added. The reaction was allowed to continue another 12 hrs. To cap resin, acetic anhydride (4 ml) and pyridine (4 ml) were added and agitated for 45 min. The resin was washed with 300 ml DMF, DCM, MeOH and then dried with 100 ml ethyl ether. Initial resin substitution was checked by weighing small aliquots of resin in triplicate, cleaving the sample with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution for 2 hrs and measuring UV-Vis absorbance assuming Fmoc-Ala-OH ε_{265nm} = 15560 M^{-1} cm^{-1}. Initial substitution was 0.11 mmol/g.

Substitution of Fmoc-Thz-OH onto Resin

Procedure A (H-Ala-NovaPEG and H-Gly-TentaGel):

Loaded resin (100 mg) was suspended in 0.5 ml DMF. A loading of 0.11 mmol/g H-Ala-NovaPEG and 0.22 mmol/g H-Gly-TentaGel (from company analysis) was

assumed. Fmoc-Thz-OH (5 mol eq), HOBt (10 mol eq), HBTU (4.5 mol eq) and DIPEA (10 eq) were combined in 2 ml DMF and added to resin suspension. Reaction was shaken for 10 min, filtered, and resin double coupled with fresh equivalents of all reagents. Following washing with 100 ml DMF, DCM, MeOH, and 50 ml ethyl ether, a Kaiser test was negative for the presence of amines.[159] Cleavage of resin aliquots with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution for 2 hrs and measuring UV-Vis absorbance indicated loadings of 0.035 mmol/g FmocThzA-NovaPEG and 0.05 mmol/g FmocThzG-TentaGel.

Procedure B (H-Gly-ChemMatrix):

Loaded resin (200 mg) was suspended in 3 ml DMF. A loading of 0.44 mmol/g H-Gly-ChemMatrix was assumed from company analysis. Fmoc-Thz-OH (5 mol eq), HOBt (10 mol eq), and HATU (4.5 mol eq) were combined in 1.5 ml DMF and added to resin. Following addition of DIPEA (10 eq), the reaction was agitated for 2 hrs. The resin was then washed with 150 ml DMF, DCM, MeOH, and 50 ml ethyl ether. Cleavage of resin aliquots with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution for 2 hrs and measuring UV-Vis absorbance indicated loadings of 0.078 mmol/g FmocThzG-ChemMatrix. Subsequent double coupling resulted in the formation of equal parts FmocThzG- and FmocThz-ChemMatrix.

Resin Coupling Reactions

1.0 mg resin was weighed into a 1.5 ml microcentrifuge tube and swelled 30 min in 100 µl DMF. The resin was centrifuged for 5 min and DMF removed by pipette. The Fmoc was removed following addition of 100 µl 5 v/v % piperidine/DMF for 5 min, followed by centrifugation of the resin and removal of the piperidine/DMF solution by pipette. The resin was then washed 2 x with 100 µl DMF and 3 x with 100 µl 0.1 v/v % TFA. The thiazolidine was then hydrolyzed using 0.5 ml 1 M $MeONH_2$ for 12 hrs or 100 µl 50 mM AgOTf for 1.5 hrs. The resin was centrifuged, solvent removed by pipette, and washed 3 x with 100 µl H_2O. The resin was then dried on a Lab Conco Centrivap

Concentrator (Kansas City, MO). Acceptor peptide **P2** (100 nmol) was added to dry resin with 10 µl 100 mM ammonium acetate pH 5 and allowed to react 1.5 hrs. Excess peptide was removed from resin by washing 3 x with 100 µl H_2O. For the conjugation of a third peptide, the above procedure was repeated. Polypeptides were cleaved from dry resin using 150 µl 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS for 2 hrs, followed by drying on a Centrivap Concentrator. Polypeptides were reconstituted in 0.5 ml 0.1 v/v % TFA and were characterized on an Agilent 1100 series LC by injecting 100 µl onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min.

Fmoc Removal from **P2**

Lyophilized **P2** (0.5 µmol) was reconstituted in 0.5 ml 5 v/v % piperidine/DMF and reacted 5 min to remove Fmoc. The reaction was quenched by addition of 0.5 ml glacial acetic acid and then applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak eluting at 21 ml was collected, lyophilized and reconstituted in 0.1 v/v % TFA for LC-ESI-MS analysis as described above. A variable 70 - 95 % recovery of **P2b** was observed.

Results

Previous chapters have described a novel iterative reducible ligation strategy that was used to synthesize polypeptides conjugated through reducible disulfide bonds. While polypeptides containing up to 4 subunits were synthesized, yields of each coupling step were lower due to a preparative purification step. In attempts to improve the yields, a solid phase approach utilizing the thiazolidine chemistry was undertaken. The development of this approach was concurrent with the chemistry in chapters 2 and 3.

Most resins commonly used for solid phase peptide synthesis are composed of polystyrene and do not swell in water. Therefore, two types of commercially available resins that swell in water were tested for the conjugation of an acceptor peptide to a donor

peptide synthesized on the solid support. The first resin type was a PEG-grafted polystyrene resin, commercially known as a TentaGel resin (Fig 5-1A). This was purchased preloaded as an H-Gly-TentaGel resin. The second resin type consists of ethylene glycol units and can be purchased as NovaPEG or ChemMatrix (Fig 5-1B). An unloaded Wang-NovaPEG resin was purchased and loaded with Fmoc-Ala-OH. The loading of the first amino acid onto this resin proved difficult with a low 0.11 mmol/g substitution observed compared to a typically loadings of over 0.50 mmol/g on a polystyrene resin. Attempts at loading using other techniques including HBTU, HATU, and the PFP ester did not result in higher substitution.

The addition of Fmoc-Thz-OH to either resin was also difficult. Despite double coupling using 5 mol eq, approximately 25 – 30 % of the dipeptide was observed on either resin based upon initial loading calculations. While the resulting dipeptide resin loadings were low, this presumably also increased the spacing of thiazolidines and decreased the opportunities for unproductive dimerization on resin to occur. A lower substitution also required fewer mol eq of an acceptor peptide for each conjugation step. The FmocThzA-NovaPEG and FmocThzG-TentaGel resins were therefore used with

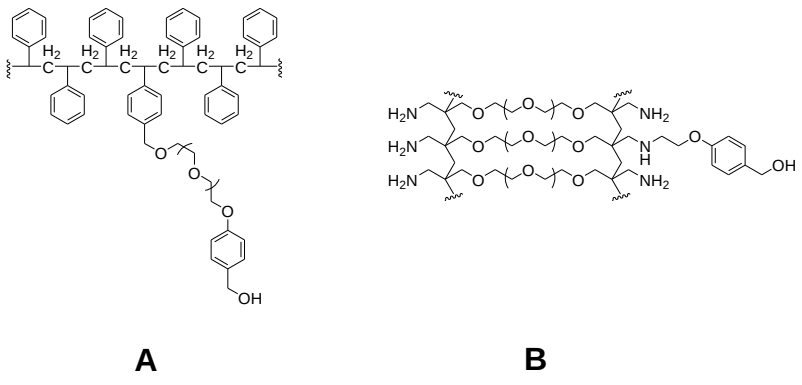


Figure 5-1: *Structures of Water Soluble Resins*. (**A**) The structure of a TentaGel resin, a PEG-grafted polystyrene resin. (**B**) The structure of NovaPEG and ChemMatrix resins, composed of ethylene glycol units.

substitutions around 0.035 and 0.05 mmol/g. Recovery of a free-flowing resin following amino acid addition also proved difficult due to the inability of the washing steps to completely dry the resins. All PEG resins examined showed a tendency to clump after extensive drying, complicating precise weighing and substitution estimations of the resins. This resulted in a qualitative approach to estimating polypeptide formation on resin.

Ligation of an acceptor peptide through a disulfide bond first required the removal of Fmoc and Thz hydrolysis from the dipeptide **P1** on resin (Fig 5-2). Subsequent addition of an acceptor peptide **P2** would result in the formation of a polypeptide **P2-P1**. Initial coupling experiments utilizing the Fmoc-Thz loaded TentaGel and NovaPEG resins indicated that substantially more product was observed with the NovaPEG resin (Fig 5-3). Due to the higher substitution, the NovaPEG resin was selected for further optimization.

Numerous parameters were examined for affecting the reducible ligation on resin. The pH of the reaction buffer was examined, with no improvement found between 5 and 7. However changing the reaction volume had a pronounced effect on the amount of polypeptide formed (Fig 5-4). Coupling of a FmocThzWK_9C(Pys) **P2** peptide (5 mol eq) to deprotected **P1** peptide on resin (0.5 mg) in 20 µl total volume led to less polypeptide compared to 10 µl, which was less than when the reaction was performed in 5 µl. The decreased reaction volume increased the relative acceptor peptide concentration and the more concentrated peptide solution resulted in more polypeptide formation. This resulted in the minimal amount of solvent required to completely suspend the resin be employed in future conjugation reactions.

The equivalents of **P2** peptide also had a noticeable effect on polypeptide formation (Fig 5-5). While keeping the reaction volume constant, 1, 2, 3, 4 and 5 mol eq of **P2** peptide were used to form polypeptide on resin (0.5 mg). An increased amount of polypeptide was observed when increasing mol eq were used up to 3 (Fig 5-5A – C). No

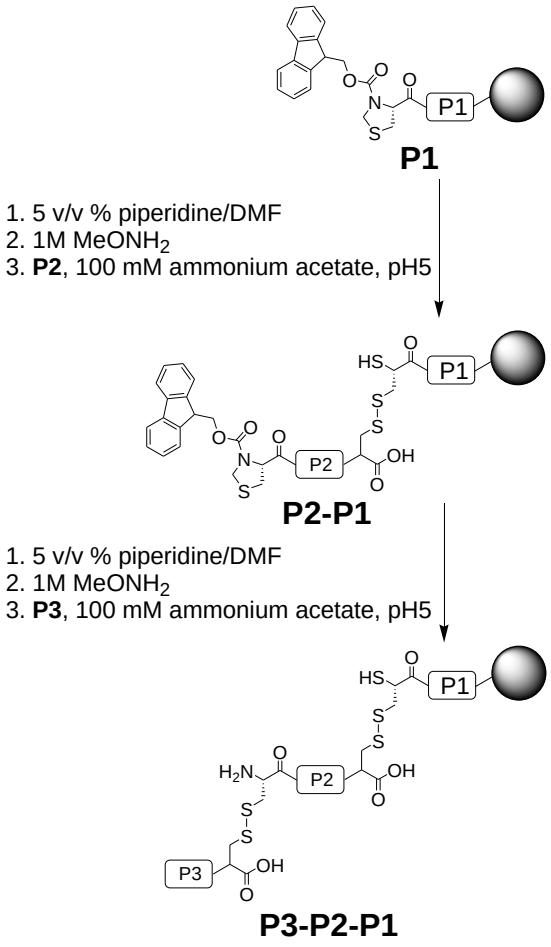


Figure 5-2: *Synthetic Schematic of Solid Phase Reducible Iterative Ligation*. An initial **P1** peptide is synthesized on resin to contain a C2 Fmoc-Thz. Removal of the Fmoc and Thz hydrolysis of **P1** precede coupling of an activated **P2**, forming a **P2-P1** polypeptide on resin. Excess peptide can be washed after the conjugation reaction and the process repeated to form the polypeptide **P3-P2-P1**.

improvement was observed increasing to 4 and 5 mol eq (Fig 5-5C - E). This resulted in 3 mol eq of acceptor peptide to be used in future experiments.

With successful deprotection and conjugation conditions to form a **P2-P1** polypeptide, further ligation to form a **P3-P2-P1** was examined. This required the Fmoc removal and Thz hydrolysis from the **P2** peptide and subsequent conjugation with a **P3** peptide. Attempts to form the **P3-P2-P1** polypeptide resulted in minimal desired product

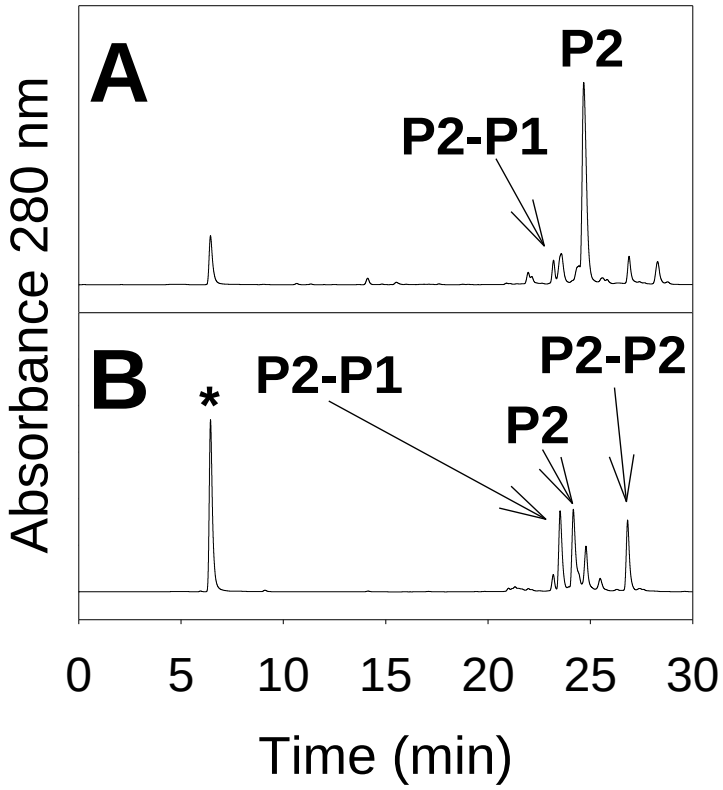


Figure 5-3: *Comparison of TentaGel and NovaPEG Resin Couplings.* Panels A & B illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Following Fmoc removal and Thz hydrolysis of the resin bound **P1** peptides, 100 nmol $FmocThzWK_4C(Pys)$ **P2** were coupled to 0.6 mg TentaGel (Panel A) or 1.2 mg NovaPEG (Panel B). The asterisk (*) denotes pyridine-2-(1H)-thione. Following coupling, the resin was cleaved with 95:2.5:2.5 (v/v/v) $TFA:H_2O$:TIS solution. Significantly more polypeptide was formed on the NovaPEG resin.

with the major product a **P3-P1** polypeptide. Examining the reaction steps demonstrated that the polypeptide **P2-P1** could be successfully formed on resin (Fig 5-6A). Subsequent Fmoc removal with 5 v/v % piperidine/DMF resulted in the formation of **P2b-P1** with an earlier retention time (Fig 5-6B). The expected loss of Fmoc 280 nm absorbance was observed. Treatment with 1 M methoxyamine resulted in the almost complete loss of peptide signal, though some **P2c-P1** was detected (Fig 5-6C). Subsequent conjugation

with an FmocThzWK$_4$C(Pys) **P3** peptide resulted in the formation of both **P3-P2-P1** and **P3-P1** polypeptides, though the **P3-P1** peptide was the major product (Fig 5-6D).

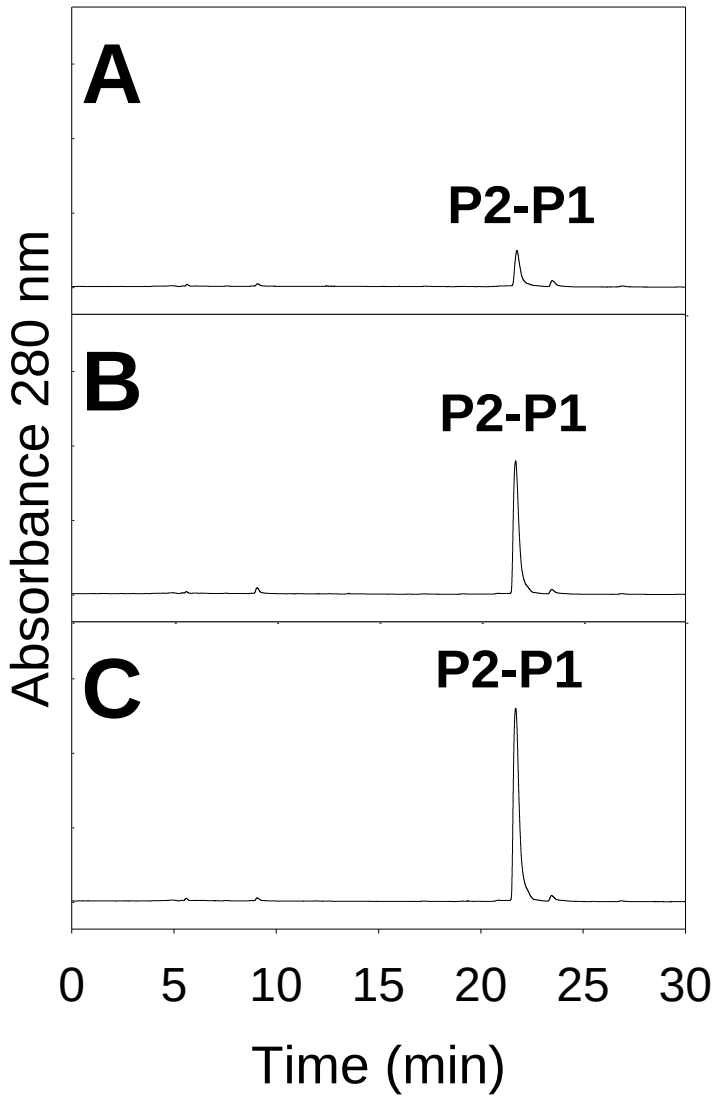


Figure 5-4: *Optimization of NovaPEG Resin Coupling by Varying Reaction Volume.* Panels A-C illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. NovaPEG resin substituted with FmocThzA **P1** was deprotected to form the free Cys, followed by coupling with a FmocThzWK$_9$C(Pys) **P2** peptide (5 mol eq) in 20, 10, or 5 µL 100 mM ammonium acetate pH 5 (Panels A-C, respectively). Following coupling, the resin was cleaved with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution. Decreasing the volume increased the amount of polypeptide formed.

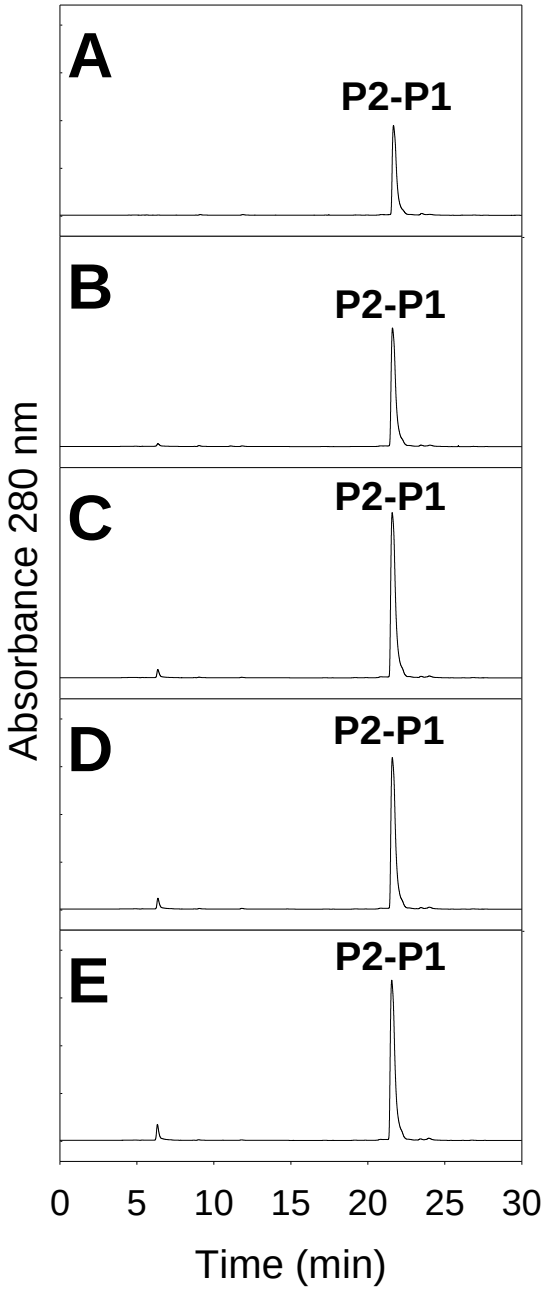


Figure 5-5: *Optimization of NovaPEG Resin Coupling Varying Peptide Equivalents.* Panels A-E illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. NovaPEG resin (0.5 mg) substituted with FmocThzA **P1** was deprotected to form the free Cys, followed by coupling with 1, 2, 3, 4, or 5 mol eq FmocThzWK_9C(Pys) **P2** peptide in 5 µL 100 mM ammonium acetate pH 5 (Panels A-E, respectively). Following coupling, the resin was cleaved with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution. At least three equivalents of peptides were required to form the maximal amount of polypeptide.

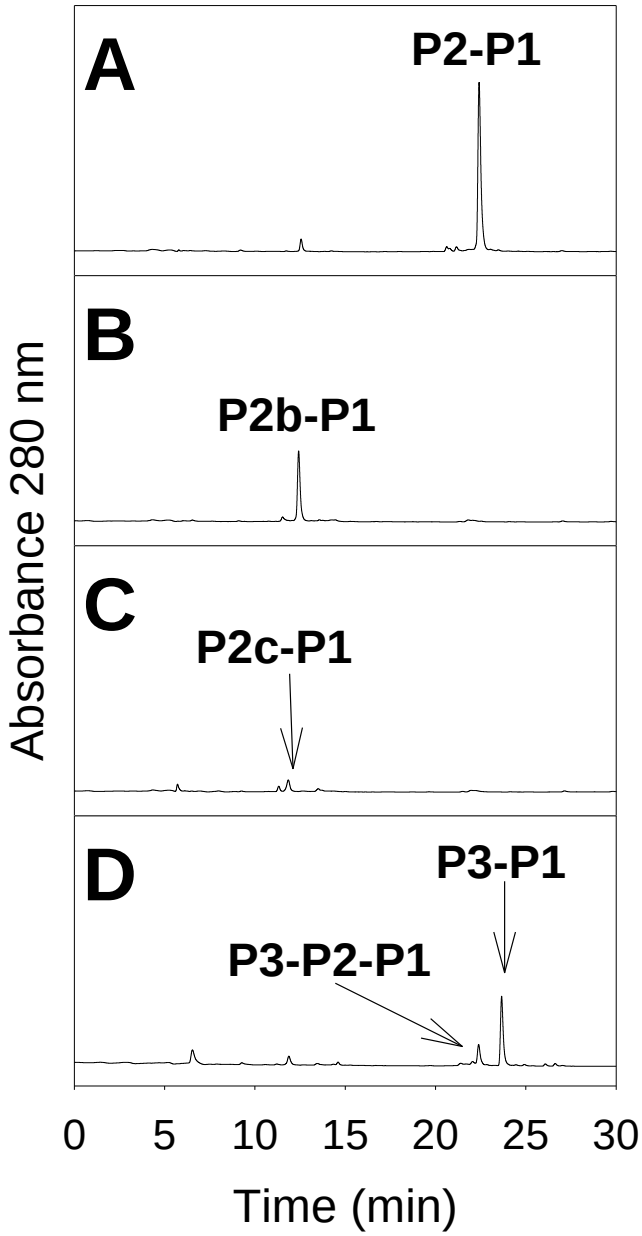


Figure 5-6: *Synthesis of a **P3-P2-P1** Polypeptide on a Solid Support*. Panels A-D illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Optimized conditions were used to conjugate FmocThzA **P1** and FmocThzWK$_9$C(Pys) **P2** to form the polypeptide **P2-P1** on NovaPEG resin (Panel A). The Fmoc was removed by treatment of 5 v/v % piperidine/DMF for 5 min to generate **P2b-P1** (Panel B). The thiazolidine was hydrolyzed using 1 M methoxyamine overnight to yield the free Cys containing **P2c-P1** (Panel C). An FmocThzWK$_4$C(Pys) **P3** was then conjugated, forming both **P3-P2-P1** and **P3-P1** polypeptides (Paned D). After formation of each intermediate, the resin was cleaved with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution.

These results indicated that the methoxyamine treatment resulted in the premature cleavage of the peptide from resin, due to the loss of polypeptide signal.

The silver trifluoromethanesulfonate chemistry was concurrently being developed and was used to mediate the **P2** thiazolidine hydrolysis. The resulting conjugation of the FmocThzWK$_4$C(Pys) **P3** peptide resulted in significant formation of the **P3-P2-P1** polypeptide, though **P3-P1** were still observed (Fig 5-7). While promising, the further optimization was hindered by the lack of FmocThzA-NovaPEG resin. After an initial batch of resin was substituted, further attempts at resin substitution failed at loading an initial amino acid on the resin. Coupling procedures using DIC, HBTU, HATU, activated PFP esters, and 2,6-dichlorobenzyol chloride were unsuccessful at loading an Fmoc amino acid.

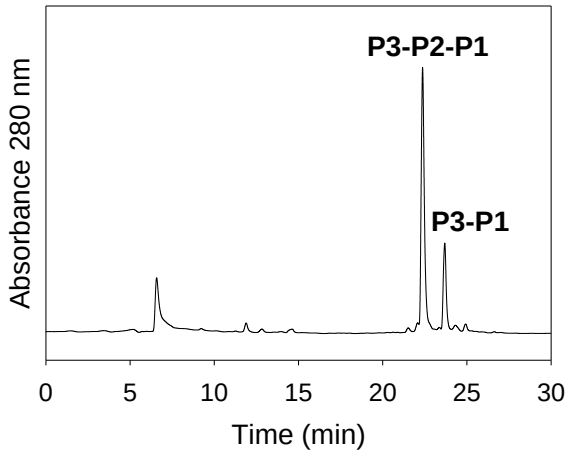


Figure 5-7: *Synthesis of a **P3-P2-P1** Polypeptide on a Solid Support Using AgOTf.* The panel illustrates the RP-HPLC chromatogram of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. Optimized conditions were used to conjugate FmocThzA **P1** and FmocThzWK$_9$C(Pys) **P2** to form the polypeptide **P2-P1** on NovaPEG resin. The Fmoc was removed with 5 v/v % piperidine/DMF, followed by 50 mM AgOTf mediated hydrolysis replacing 1 M $MeONH_2$. An FmocThzWK$_4$C(Pys) **P3** was then conjugated to resin, forming a mixture of **P3-P2-P1** and **P3-P1**. More three component polypeptide was observed. The resin was cleaved with 95:2.5:2.5 (v/v/v) TFA:H_2O:TIS solution.

The Wang-NovaPEG resin could not be purchased preloaded with an amino acid, though a NovaPEG with a more acid labile 4-hydroxymethyl-3-methoxyphenoxybutyric (4-HMPB) linker preloaded with an amino acid could be obtained. This linker was not stable in the presence of 0.1 v/v % TFA and therefore was ineffective due to premature cleavage during the thiazolidine hydrolysis.

Another vendor of the PEG resin was therefore selected which could preload the Wang linker resin with an amino acid. A H-Gly-ChemMatrix was obtained and substituted with Fmoc-Thz-OH using company protocols, resulting in FmocThzG-ChemMatrix resin with a substitution of 0.08 mmol/g. Fmoc removal was performed using 5 v/v % piperidine/DMF, followed by treatment with 50 mM AgOTf to hydrolyze the thiazolidine. Subsequent conjugation with a FmocThzWK$_9$C(Pys) **P2** peptide resulted in efficient ligation to form polypeptide **P2-P1** (Fig 5-8A). Attempts at forming a **P3-P2-P1** polypeptide were unsuccessful at synthesizing significant product. Further examination indicated that removal of the Fmoc from **P2-P1** to form the thiazolidine **P2b-P1** also resulted in the free Cys **P2c-P1** (Fig 5-8B). The free Cys in basic conditions could scramble the disulfide bonds and self cleave from resin, explaining the low yield. This side reaction was not observed with methoxyamine and most likely was due to silver retention in the resin despite repeated washes. The use of a lower concentration of AgOTf (2 mM) decreased the amount of free Cys formed, but could not eliminate it. Attempts at washing with EDTA to chelate the silver also did not produce less free Cys.

To avoid exposing the resin to basic conditions, removal of the Fmoc on the FmocThzWK$_9$C(Pys) **P2** peptide was done off resin to generate the **P2b** peptide (Fig 5-9). Conjugation of the **P2** peptide was unaffected by the presence of the retained silver and pre-removal of the Fmoc would avoid the basic conditions causing premature thiazolidine hydrolysis and cleavage. Removal of the Fmoc was undertaken using 5 v/v % piperidine/DMF for 5 min followed by quenching with glacial acetic acid. The reaction mixture was applied to a G-10 Sephadex column, where the **P2b** peptide was recovered

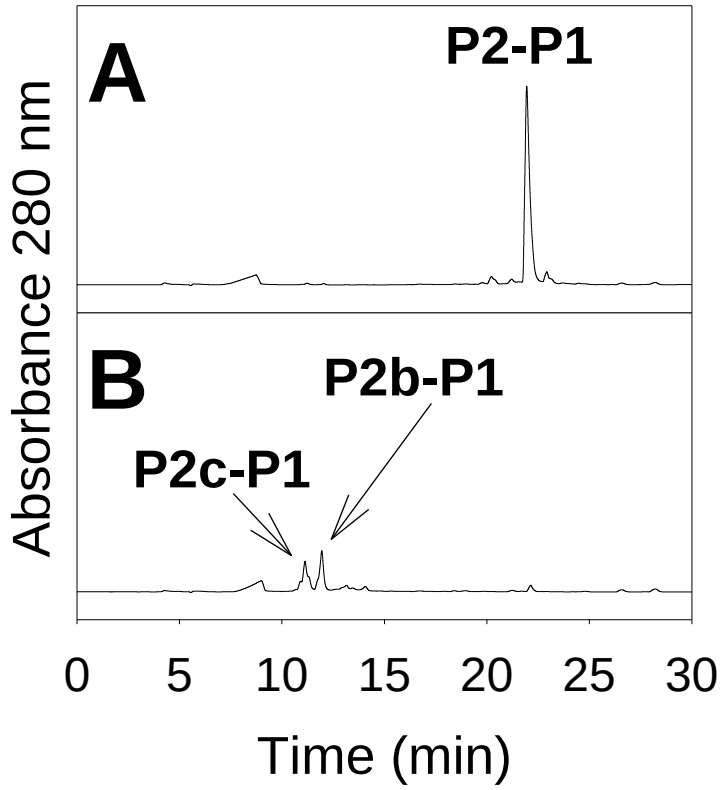


Figure 5-8: *Synthesis of a **P2-P1** Polypeptide on ChemMatrix Resin with AgOTf.* Panels A & B illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. An FmocThzG **P1** was synthesized on ChemMatrix resin. Following Fmoc removal with 5 v/v % piperidine/DMF, the thiazolidine was hydrolyzed with 50 mM AgOTf in 0.1 v/v % TFA. Subsequent conjugation with an FmocThzWK_9C(Pys) **P2** resulted in the formation of **P2-P1** polypeptide (Panel A). Treatment of the **P2-P1** polypeptide with 5 v/v % piperidine/DMF resulted in the formation of **P2b-P1** and **P2c-P1** (Panel B), indicating retained AgOTf in the solid support.

in excellent purity (Fig 5-10). The loss of the Fmoc absorbance signal, weight by MS, and early retention time were evident (Fig 5-10B).

Comparison of coupling **P2** and **P2b** peptides to 2 mM AgOTf thiazolidine hydrolyzed ChemMatrix-bound **P1** peptide indicated that while the coupling of **P2** was relatively unaffected by retained Ag on the resin, significantly less **P2b** was conjugated (Fig 5-11A & B). However if methoxyamine was used to hydrolyze the thiazolidine on the **P1** peptide, more **P2b** polypeptide was formed and resulted in a cleaner product

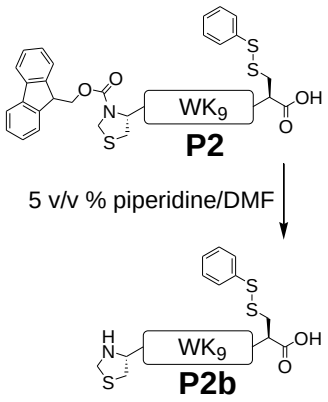


Figure 5-9: *Synthetic Scheme for Pre-resin Removal of Fmoc from **P2***. Coupling peptide **P2** was treated with 5 v/v % piperidine/DMF followed by gel filtration purification to yield the **P2b** peptide that can be conjugated to a solid support. This approach omits a deprotection step and avoids basic reactions on resin.

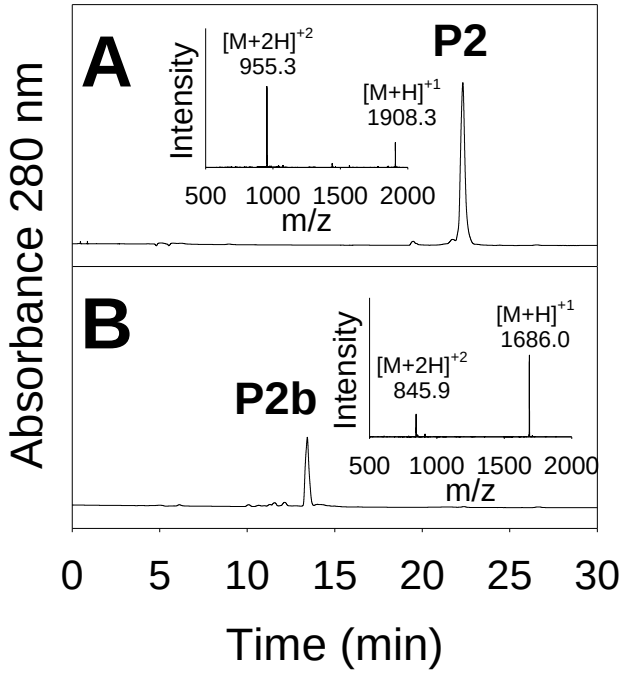


Figure 5-10: *Generation of **P2b** Peptide*. Panels A & B illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates the re-analysis of purified peptide FmocThzWK$_9$C(Pys) **P2**. Removal of Fmoc using 5 v/v % piperidine in DMF results in the formation of **P2b** possessing a shorter retention time and loss of Fmoc mass (Panel B).

mixture (Fig 5-11B & D). Methoxyamine treatment also led to comparable **P2-P1** polypeptide formation when **P2** was coupled (Fig 5-11A & C), indicating methoxyamine could hydrolyze the thiazolidine to the same extent as AgOTf. The **P2-P1** polypeptide was also observed when coupling **P2b** due to incomplete removal of the Fmoc when generating **P2b**.

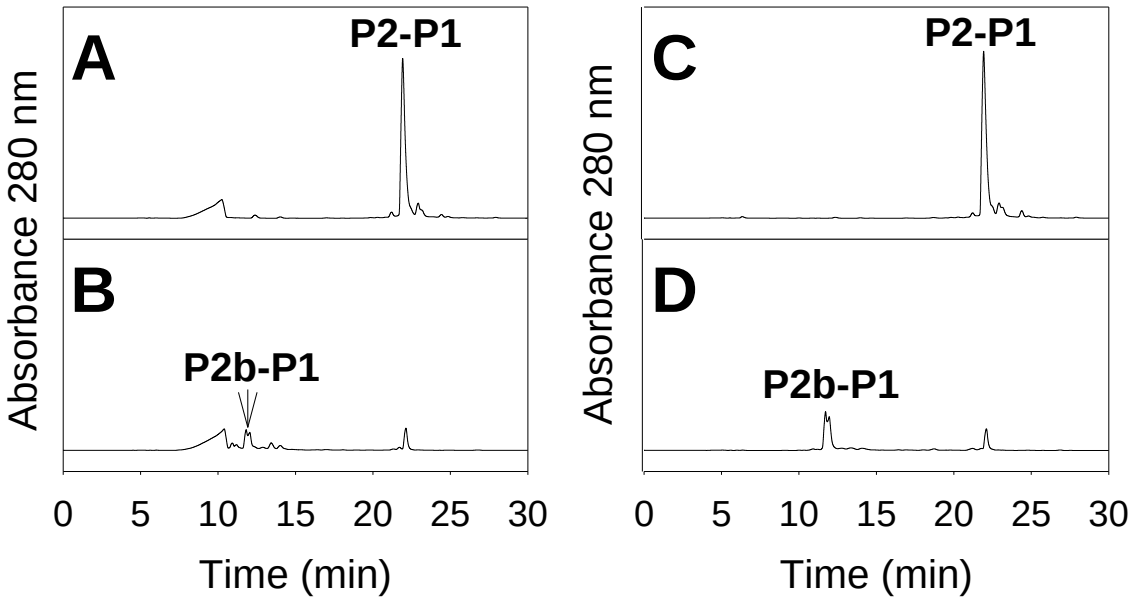


Figure 5-11: *Synthesis of **P2-P1** and **P2b-P1** Polypeptide.* Panels A-D illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. A mixture of FmocThz and FmocThzG **P1** peptide was synthesized on resin. Following removal of Fmoc using 5 v/v % piperidine/DMF, either 2 mM AgOTf in 0.1 v/v % TFA (Panels A & B) or 1 M $MeONH_2$ (Panels C & D) was used to hydrolyze the thiazolidine. An FmocThzWK_9C(Pys) **P2** (Panels A & C) or ThzWK_9C(Pys) **P2b** (Panels B & D) was then conjugated to the resin bound **P1**. Conjugation of a **P2** peptide resulted in similar amounts of polypeptide formation regardless of thiazolidine hydrolysis conditions (Panels A & C). More polypeptide was formed when 1 M $MeONH_2$ was used to hydrolyze the thiazolidine than AgOTf when coupling a **P2b** peptide with prior cleavage of the Fmoc (Panels B & D).

A final experiment examined the formation of the polypeptide **P3-P2-P1** using 1 M methoxyamine to hydrolyze the thiazolidine of **P1** and either 2 mM AgOTf or 1 M methoxyamine to hydrolyze the thiazolidine of **P2**. Deprotection of the **P1** peptide and subsequent coupling of the **P2b** peptide resulted in formation of the polypeptide **P2b-P1** in good purity (Fig 5-12A). Thiazolidine hydrolysis by AgOTf and subsequent ligation with $AcrK_{10}C(Pys)$ **P3** peptide resulted in an assortment of products, including unreacted **P2c-P1**, trace amounts of **P3-P1**, and the desired **P3-P2-P1** polypeptide (Fig 5-12B). Removal of the **P2b** thiazolidine by 1 M $MeONH_2$ and subsequent ligation with $AcrK_{10}C(Pys)$ resulted in the formation of unwanted **P3-P1** and the desired **P3-P2-P1** polypeptide in greater yield than when AgOTf was used (Fig 5-12C).

Discussion

While thiazolidine mediated iterative reducible ligation has been successful at synthesizing polypeptides, incorporating a solid support could potentially offer better yields and purity of the final products. The use of fully deprotected peptides required the use of resins that could swell in water, most of which contain the water soluble polymer PEG. The loading and drying of these resins are more complicated than normal polystyrene resins that have well established loading procedures and can be efficiently dried with MeOH. While Fmoc-Thz-OH could be directly loaded onto a resin, it was desirable to incorporate another amino acid first to simulate the synthesis of a peptide on the solid support. The small amino acids Ala and Gly were selected because they do not contain protecting groups that may lead to solubility concerns of the **P1** peptide when attempting to conjugate with a **P2** peptide in water. The loading of small amino acids onto PEGylated resins has been reported to be difficult and the subsequent loaded resins shown to have a fusion temperature between 19-25˚C resulting in a sticky resin (PCAS BioMatrix, personal communication). The resulting low resin substitutions may be advantageous for iterative reducible ligation by spacing the peptides on the resin and

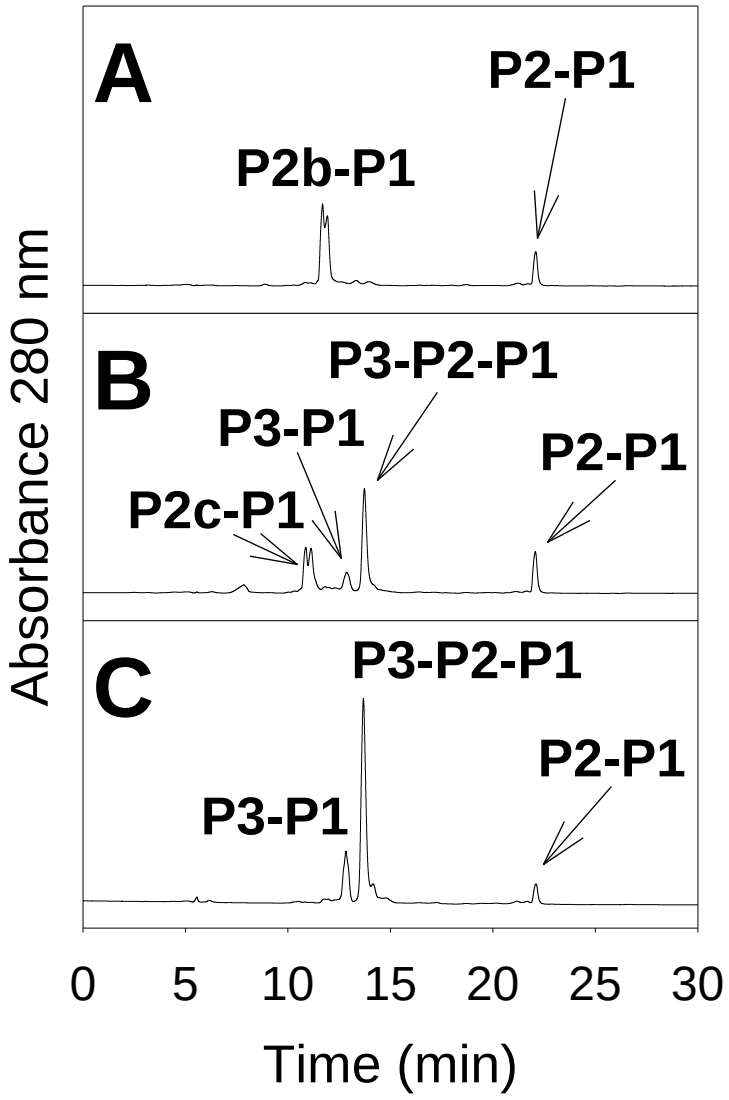


Figure 5-12: *Synthesis of a **P3-P2-P1** Polypeptide on ChemMatrix Resin.* Panels A-C illustrate RP-HPLC chromatograms of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. A mixture of FmocThz and FmocThzG **P1** peptide was synthesized on resin. Following removal of Fmoc using 5 v/v % piperidine/DMF, 1 M $MeONH_2$ was used to hydrolyze the thiazolidine. Subsequent conjugation with $ThzWK_9C$ **P2b** peptide resulted in the formation of **P2b-P1** (Panel A). Subsequent thiazolidine hydrolysis using 2 mM AgOTf in 0.1 v/v % TFA and conjugation with $AcrK_{10}C(Pys)$ **P3** resulted in the formation of the desired **P3-P2-P1** polypeptide, **P3-P1** polypeptide, and unreacted donor **P2c-P1** polypeptide (Panel B). Thiazolidine hydrolysis using 1 M $MeONH_2$ and subsequent conjugation of **P3** resulted in the formation of **P3-P2-P1** polypeptide with **P3-P1** formation also evident (Panel C).

preventing dimerization, though repeatable and consistent loadings were difficult. The low substitution was advantageous in requiring less **P2** and **P3** peptides equivalents for conjugations. The low coupling yields also prevented the incorporation of a chromophore such as Acr onto the **P1** peptide, since a robust amino acid coupling reaction was not found.

An initial experiment examined two different classes of PEG-containing resins, and found that a PEG-grafted polystyrene resin did not form significant amounts of reducible polypeptide (Fig 5-3). This could be a direct result of the PEG linker offering too much conformational flexibility to the Cys on resin and allow the deprotected Cys on resin to form a disulfide bond. Another experiment utilizing AgOTf to hydrolyze the **P1** on the TentaGel resin also resulted in a similar low yield.

Optimization of the **P2** coupling indicated the importance of volume and peptide equivalents to efficient ligation. Excess reagent is usually required to drive a solid phase reaction to completion and large excesses would be prohibitive using gene delivery polypeptides. A three-fold excess was shown to result in maximal coupling and excess of peptide could be recovered following filtering from the resin.

Conjugation beyond two peptides was difficult and could not be achieved with good purity. In the **P3-P2-P1** conjugations resulting in the most polypeptide formation, significant amounts of **P3-P1** peptide were observed (Fig 5-7, 5-12C). The formation of **P3-P1** could be the result of incomplete thiazolidine hydrolysis of **P1**, incomplete reaction of **P2** with deprotected **P1**, or premature cleavage of **P2** from **P2-P1** resulting in the regeneration of **P1** on resin capable of conjugating with **P3**. An efficient way to cap a free Cys could help distinguish which mode is responsible for generating **P3-P1** and may help overcome the side reaction.

The failure of methoxyamine mediated thiazolidine hydrolysis to generate a **P3-P2-P1** when utilizing the NovaPEG resin and success with ChemMatrix resin was unexpected, as the resins are chemically similar. The omission of the base treatment

when using ChemMatrix was the most likely explanation, as a **P2b** peptide was conjugated that did not need to undergo basic Fmoc removal. While extensive washing was performed after the on resin Fmoc removal to remove the piperidine and equilibrate the resin with 0.1 v/v % TFA, residual base may have been present that promoted disulfide scrambling after methoxyamine mediated thiazolidine hydrolysis. The removal of Fmoc from an acceptor peptide to generate C1 Cys(Pys) and C2 Thz groups may have advantages in solution phase iterative reducible ligation by removing a deprotection and purification step. In the synthesis of poly-lysine polypeptides, the Fmoc was still required due to RP-HPLC retention time shifts that allowed purification of the polypeptides, though would not be required for more chemically diverse polypeptides.

Though AgOTf was critical in the development of solution phase iterative reducible ligation, it hindered the solid phase approach. This was observed in premature thiazolidine hydrolysis (Fig 5-8B), low coupling of a **P2b** peptide (Fig 5-11B), and limited **P3** conjugation (Fig 5-12B), all likely due to the retention of the silver to the solid support. Lowering the concentrations of AgOTf from 50 mM to 2 mM resulted in similar yields of **P2-P1** polypeptides, though premature thiazolidine hydrolysis was still observed upon Fmoc removal from **P2**. Utilizing EDTA in attempts to chelate excess silver did not prevent thiazolidine hydrolysis, though EDTA is known to form relatively weak complexes with silver.[160, 161] Other means of completely removing excess silver from the resin could be considered to enable the use of AgOTf developed iterative reducible ligation chemistry. While reducible polypeptides can be synthesized on a solid support, improvements in resin loading, thiazolidine hydrolysis, and conjugation yields are required before application with gene delivery polypeptides.

CHAPTER 6

OTHER ITERATIVE REDUCIBLE LIGATION EXPERIMENTS

Abstract

Chapter 6 describes three additional experiments involving iterative reducible ligation and gene delivery peptides. The first investigated a tri-orthogonal Cys protecting scheme that was used to generate an N-glycan modified peptide through a third Cys not involved with reducible ligation. The second set of experiments investigated the effect of inserting a PEG_{5KDa} in the middle of an $AcrK_4Acr$ repeat that previously has been shown to mediate high levels of gene expression. The final experiments developed a convergent iterative reducible ligation approach to generate large polypeptides.

Triorthogonal Protection Scheme

Introduction

Chapters 2 and 3 described the development of a novel iterative reducible ligation strategy that could conjugate multiple peptides through disulfide bonds. In these chapters, model poly-lysine peptides were used to simulate gene delivery polypeptides that are necessarily cationic to bind and condense DNA. A Trp or Acr residue was incorporated as a chromophore, easing purification and quantification of the resulting peptides and polypeptides. Two terminal Cys were also incorporated to ligate the different peptides through reducible disulfide bonds. Absent in the model polypeptides was a chemical handle that could be used to generate targeting or PEGylated peptides, necessary components of a gene delivery polypeptide.

The importance of generating chemically defined peptides for gene delivery was discussed in chapter 1. A variety of methods can be used to incorporate a targeting ligand is a site specific fashion. The incorporation of an alkyne allows subsequent specific reaction with an azide,[162, 163] and has been used to incorporate the epidermal growth

factor (EGF) targeting ligand onto a dendron for targeted gene delivery.[164] The high affinity of streptavidin for biotin has also been utilized for nonviral gene therapy, following EGF-PEG-biotin complexed with PEI derivatized with streptavidin.[165-167]

One approach amendable to peptide synthesis is the incorporation of a Cys that can be derivatized with targeting ligands or PEG. The sulfhydryl of Cys can react with maleimides,[168] vinyl-sulfones,[169] iodoacetyl groups,[170] and pyridyl disulfides[83] to form semi-stable linkages for the first three and a reducible linkage for the fourth. Cys has been used extensively as a chemical handle for gene delivery peptides. Targeting N-glycans functionalized with an iodoacetyl group have been conjugated to a CWK_{18} gene delivery peptide.[45] PEG has been added to the same CWK_{18} peptide through a disulfide bond and vinyl sulfones,[33] and more efficiently through a maleimide.[171] Polyacridine gene delivery systems have also incorporated PEG through a terminal Cys, resulting in prolonged circulatory stability of polyplexes.[31, 32]

While Cys can advantageously be used as a chemical handle to integrate targeting ligands and PEG, the incorporation of a labeling Cys into an iterative reducible ligation peptide is complicated by the other two Cys required for iteratively coupling the peptide. Therefore a tri-orthogonal Cys protecting scheme is needed, requiring a Cys for selective coupling of the targeting ligand or PEG, a Cys for activation to form an initial directed disulfide bond, and a masked Cys for further ligation steps. The experiments described in this section involved developing a protecting scheme that could successfully generate PEG and N-glycan derivatized peptides which could be introduced into an iterative reducible ligation for the synthesis of gene delivery polypeptides.

A Note on Nomenclature

A numbering scheme was adopted in this section to avoid lengthy descriptions of the peptides. The C1 and C2 nomenclature remain the same from other chapters, with the

third Cys protecting group labeled as C3. An (a) will signify peptides that have been modified at the C3 Cys. A (b) will signify peptides with a C1 Cys(Pys).

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, iodoacetic acid, diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, 9-chloroacridine, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Fisher Scientific.

Synthesis and Characterization of Peptide Subunits

Unsubstituted Wang resin was loaded with Fmoc-Cys(Acm)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$ or $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Trp ε_{280nm} = 5600

M^{-1} cm^{-1}, Fmoc-Thz ε_{280nm} = 6550 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Acr ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Synthesis of Peptide **1b**

Iodoacetic acid (50 mol eq) was dissolved in 3 ml 500 mM HEPES, adjusted to pH 8. Crude peptide **1** was reconstituted in 1 ml 500 mM HEPES pH 8, transferred into the iodoacetic acid solution, and reacted at RT for 12 hrs. Peptide **1a** was then preparatively purified as described above.

Lyophilized **1a** was dissolved in 9:1 TFA:anisole solution containing 50 mM AgOTf in 75 µL and reacted 2 hrs at 0˚C. 750 ml cold ethyl ether was used to precipitate the peptide for 30 min, followed by centrifugation for 5 min to pellet the peptide. Ether/TFA was decanted and the peptide allowed to air dry for 30 min. The peptide was then reconstituted in 0.4 ml 0.1% TFA containing 10 mol eq DTDP. The resulting peptide **1b** was then purified to homogeneity as described above.

Synthesis of Peptide **2b**

The synthesis of iodoacetamide triantennary (I-Tri) has previously been described.[45, 172] Reaction of I-Tri and purified **2** (1.5 mol eq) occurred in 100 mM Tris, pH 8 for 12 hrs. The mixture was then preparatively purified as described above to yield glycopeptide **2a**.

Subsequent removal of the Acm in **2a** occurred in 95:5 TFA:anisole solution containing 0.4 M AgOTf (200 mol eq) for 2 hrs at 0˚C. The glycopeptide was then precipitated in chilled ethyl ether for 30 min, centrifuged, followed by TFA/ether being removed by decantation. The deprotected glycopeptide was allowed to air dry for 30 min

and was then brought up in 10:3 2 M acetic acid:isopropanol containing 10 mol eq DTDP. The final peptide concentration was 0.2 mM and allowed to react 12 hrs. The final product peptide **2b** was then preparatively purified as described above.

Results

The development of a tri-orthogonal Cys protecting scheme was important to the synthesis of PEG and N-glycan peptides to be used for iterative reducible ligation of gene delivery polypeptides. A model peptide was first synthesized to incorporate three orthogonal protected Cys to test different Cys protecting groups (Fig 6-1). Two Cys were required for iterative reducible ligation. The development of the C2 Fmoc-Thz has been the subject of other chapters in this thesis and was chosen for this protecting group in the model peptide. The C1 has been a trityl that is removed during cleavage of the peptide from resin and subsequently activated with DTDP to Cys(Pys). However in the synthesis of gene delivery peptides, the PEG or glycan would be conjugated first to the peptide. Therefore a C3 trityl was incorporated to allow derivatization first. A C1 was then selected to be an acetamidomethyl (Acm), which could be selectively removed following conjugation of the C3 Cys. The model peptide also contained four Lys residues to mimic the use of basic amino acids on gene delivery peptides, as well as a Trp to simplify purification and characterization.

Following synthesis, crude peptide **1** was reacted with 50 mol eq iodoacetic acid to yield the carboxymethylated peptide, which upon preparative purification was obtained in 37 % yield and eluted as a single peak (Fig 6-2A & B). The addition of the carboxymethyl group corresponded to the increased mass in the MS, with no evidence for reaction with either the C1 or C2 groups (Fig 6-2B). The C1 Acm was then removed using 100 mol eq AgOTf in a 9:1 TFA:anisole solution forming a free Cys at the C1 position. Subsequent activation with DTDP generated the C1 Pys peptide **1b**, which was preparatively purified in 23 % yield (Fig 6-2C). This product was observed as a single

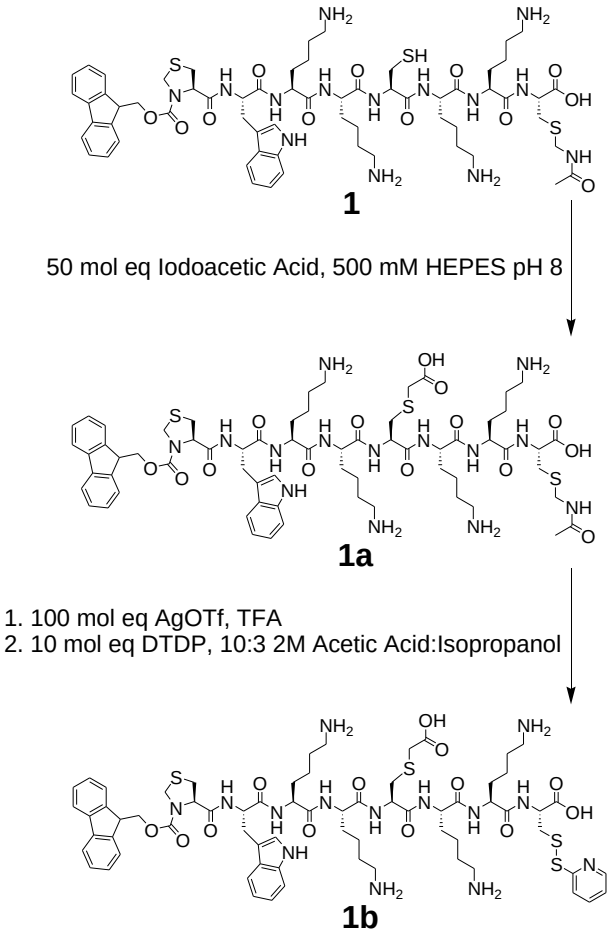


Figure 6-1: *Synthetic Strategy for Model Peptide **1b***. Peptide **1** was a model peptide containing three Cys with orthogonal protecting groups. The C1 group was an Acm, the C2 an Fmoc-Thz, and the C3 a trityl that was removed during workup. Following cleavage from resin, **1** was reacted with 50 mol eq iodoacetic acid to model reaction of the C3 Cys with a PEG or N-glycan, forming the carboxymethylated **1a** peptide. The C1 Acm was subsequently removed with 100 mol eq AgOTf in TFA to generate the free Cys, which was subsequently activated with DTDP to generate the Cys(Pys) containing **1b**. The product peptide **1b**, containing a C1 Cys(Pys) and C2 Fmoc-Thz, had the proper Cys activating/protecting groups for iterative reducible ligation.

peak, with corresponding MS indicating loss of Acm and activation with DTDP. The late eluting peak indicated the continued presence of the Fmoc with MS indicating no loss of the thiazolidine.

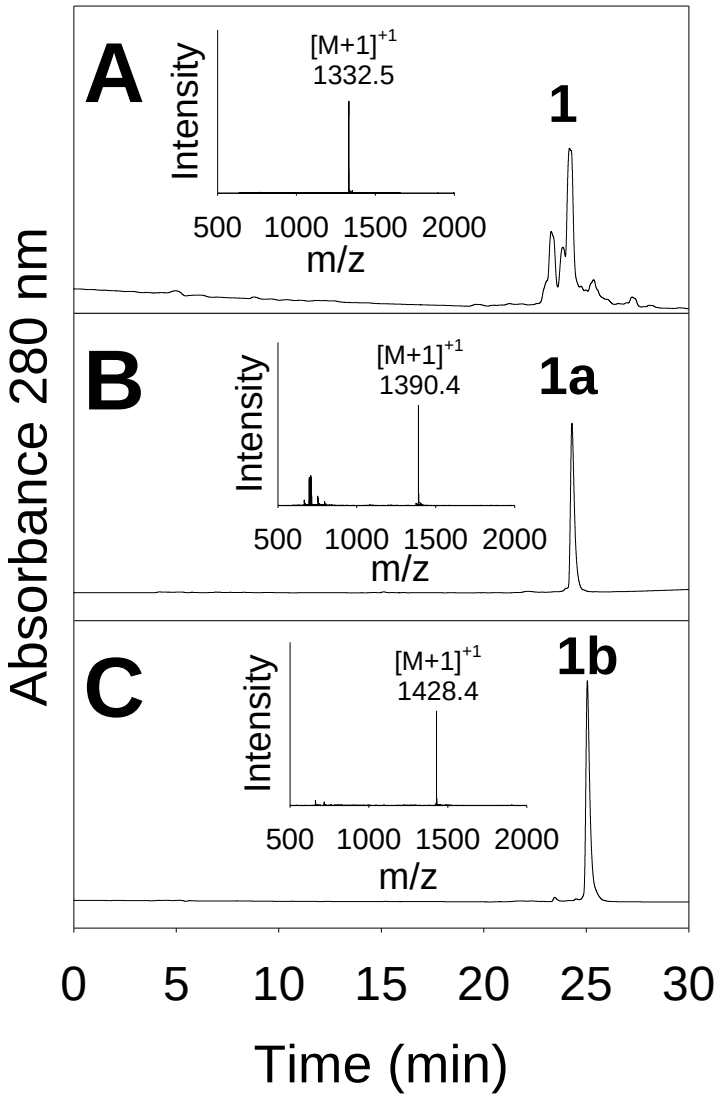


Figure 6-2: *Synthesis of Model Peptide **1b***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates crude peptide **1** which was used without purification. Reaction of **1** with iodoacetic acid formed the carboxymethylated product **1a** in 38 % yield following preparative purification (Panel B). Subsequent Acm removal and activation with DTDP resulted in the formation of ligation model peptide **1b** in 23 % yield (Panel C).

The synthesis of peptide **1b** demonstrated an appropriate selection of the C1 Acm, C2 Fmoc-Thz, C3 trityl protecting scheme that could sequentially be modified to

generate gene delivery peptides. This was demonstrated in the synthesis of peptide **2** (Fig 6-3), a full length gene delivery peptide containing $AcrK_4Acr$ repeats, that has previously been shown to mediate high levels of stimulated gene expression.[32] The C3 Cys(trityl) was incorporated into the middle of the peptide, disrupting one of the three $AcrK_4Acr$ repeats; the next section will explore the impact of such a disruption on the ability of a peptide to form stable polyplexes. A C1 Acm and a C2 Fmoc-Thz were incorporated as before.

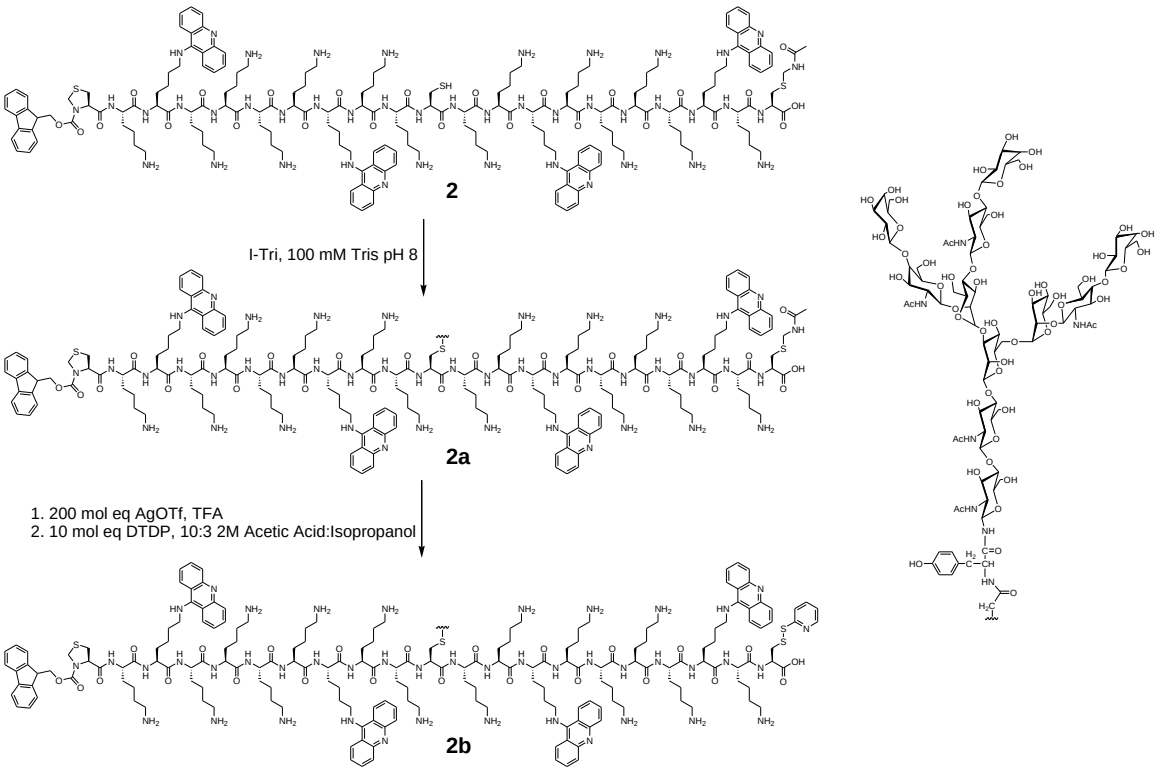


Figure 6-3: *Synthetic Strategy for Tri-Polyacridine **2b** Ligation Peptide*: Peptide **2** was a polyacridine peptide designed for iterative reducible ligation containing a C1 Acm group, a C2 Fmoc-Thz, and a C3 trityl that was removed during resin cleavage to generate a free Cys. Iodoacetamide triantennary N-glycan (I-Tri) is reacted with **2** (1.5 mol eq) to generate glycopeptide **2a**, with Tri attached through a thiol ether linkage at the C3 Cys. The structure of triantennary is depicted to the right. The C1 Acm is subsequently removed and the free Cys activated as a sulfanylpyridine to generate **2b**. This glycopeptide contains a C1 Pys and C2 Fmoc-Thz, allowing incorporation into reducible iterative ligation for generating gene delivery polypeptides.

Peptide **2** was first purified to homogeneity (Fig 6-4A). Reaction of I-Tri with **2** (1.5 mol eq) yielded glycopeptide **2a** which was recovered in 27 % yield following preparative purification (Fig 6-4B). The large increase in mass corresponded to the addition of Tri to **2** with retention of the Acm and Fmoc-Thz protecting groups. Removal of the C1 Acm was then achieved using 100 mol eq AgOTf in a 95:5 TFA:anisole solution, followed by subsequent activation of the C1 Cys with DTDP. The resulting glycopeptide **2b** was preparatively purified in 25 % yield and contained a C1 Cys(Pys) and C2 Fmoc-Thz, the Cys protecting groups used in chapter 3 for iterative reducible ligation.

Discussion

The goal of the current section was to incorporate a third Cys into a reducible ligation peptide that could be used to add an N-glycan or PEG moiety for a gene delivery peptide. A model peptide was synthesized containing a C1 Acm, C2 Fmoc-Thz, and C3 trityl. Since a trityl group is removed during work-up, the C3 Cys could be derivatized first. The C1 was subsequently converted from an Acm to Pys in a two step reaction, generating a model peptide appropriate for iterative ligation containing a modified Cys. These protected groups were then incorporated into a gene delivery peptide, first modified with Tri and subsequently to an activated C1 Cys(Pys) to generate a glycopeptide that could be incorporated into a gene delivery iteratively ligated polypeptide.

The conjugation of I-Tri to **2** resulted in slightly lower yields than a previous report to attach I-Tri to CWK_{18} (27 % versus 40 %).[45] This might be the result of the location of the derivatizing Cys. In the original report the Cys is on the N-terminus while in peptide **2** it is located in the middle of the peptide, surrounded by Lys and Acr residues that may impede conjugation. Incorporation of Gly or Ala around the C3 Cys may ameliorate this by forming a more exposed Cys for conjugation.

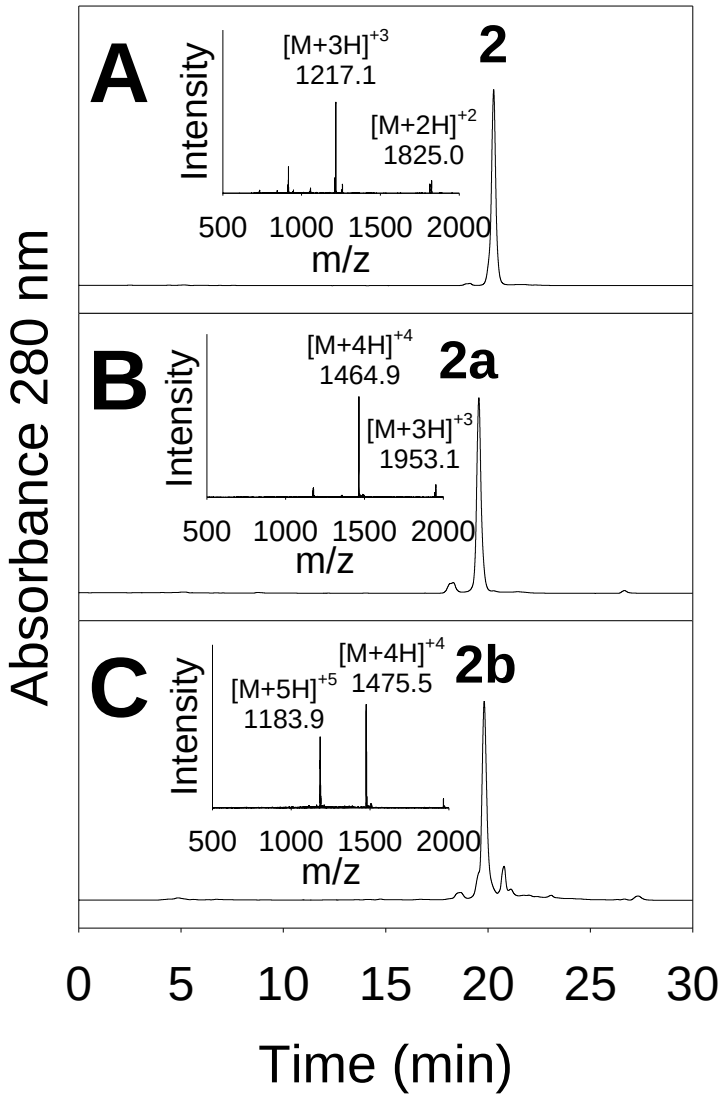


Figure 6-4: *Synthesis of Tri-Polyacridine Ligation Peptide **2b***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panel A illustrates purified **2**. Reaction of I-Tri with **2** formed glycopeptide **2a** in 27 % purified yield (Panel B). Subsequent Acm removal and activation with DTDP resulted in the formation of Tri-polyacridine ligation peptide **2b** in 25 % yield (Panel C).

The incorporation of C1 Acm and conversion to the activated Pys was tolerated by the glycopeptide, though the reaction yield was lower than optimal (25 %). While terminal Cys(Acm) have previously been incorporated into gene delivery peptides

containing Tri and PEG,[59] the complete removal of an Acm from PEG and glycan modified peptides is difficult, due to solubility of PEG and glycan peptides in TFA. Other C1 protecting groups may therefore be preferentially incorporated that are more compatible with PEG and glycopeptides. The *tert*-butylmercapto group that is removed by reduction in aqueous conditions may prove easier to transform into an activated Pys.

A Mal-PEG_{5KDa} was also incorporated into the model peptide **2**, but characterization of the subsequent Acm removal and Pys activation proved difficult. The polydispersity of the PEG prevented the use of MS characterization of the intermediate. The Mal-PEG_{5KDa} has a mass range of about 3000 amu and an Acm weighs 72 amu; the loss of such as small group is hard to discern in such a mixture, especially when quantitative conversion is unlikely from solubility concerns. The best evidence for removal is during the subsequent activation of the Cys with DTDP, when pyridine-2-(1H)-thione is also produced and identifiable by RP-HPLC. Ellman's reagent could also be used to assess free Cys,[173] though the absorbance of the resulting 2-nitro-5-thiobenzoic acid overlaps with the signal of acridine.

Effect of Cys Location on PEGylated Polyacridine Polyplexes

Introduction

PEGylation is required for gene delivery polypeptides to mask the charge of polyplexes to avoid aggregation and interaction with serum proteins.[33, 35, 171] Low molecular weight poly-lysine carriers traditionally have incorporated the PEG onto the N-terminus of the peptide, such as CWK_{18}.[33, 59, 171] Cross-linking of this peptide to other peptides containing targeting ligands was originally done with glutaraldehyde.[30, 37] Linking a LMW PEGylated peptide to targeting glycopeptides through disulfide bonds was achieved by incorporation of Cys within the poly-lysine chain, such as PEG-$CysW(CysK_3)_4CysK$[55] or PEG-$CysWCysK_{15}CysK$.[54] In both cases the PEG was attached

to an N-terminal Cys with cross-linking Cys interspersed within the peptide. One example of an internal Cys can be found in disulfide cross-linked polypeptides containing PEG, N-glycan, and melittin.[59] The PEGylated $CWK_8C(PEG)K_8C$ attached the PEG in the middle of the peptide, without significant loss of affinity of the peptide to DNA. This is likely due to the peptide condensing DNA through ionic interactions, with the number of charges determining the relative binding affinity of the peptide and not dependent on the sequence of the charges.

Despite condensing DNA into small polyplexes, sulfhydryl cross-linked poly-lysine delivery systems do not result in high levels of expression *in vivo*.[30, 37, 55, 59] These peptides do not have sufficient affinity for DNA following administration because of the high salt concentrations found in blood. A new class of LMW peptides has recently been introduced to overcome the lack of *in vivo* affinity demonstrated by poly-lysine peptides.[31] These PEGylated peptides were designed based upon the principle of poly-intercalation and poly-ionic interactions, containing both Nε-acridinyllysine (Acr) residues and Lys residues. Their resulting high affinity towards DNA and polyplex formation is not entirely on ionic interactions and have been demonstrated to protect the DNA for several hrs during circulation in mice.[31, 32]

Limited SAR has been performed on the polyacridine peptides. The initial study varied the number of $(Acr\text{-}Lys)_2$ repeats from 1-4, finding that increasing the number of repeats to 3 lead to an increase in stability, with 4 repeats not contributing further blood stability of polyplexes.[31] A follow-up study investigated the number of spacing Lys between 4 Acr subunits, finding an optimal of 4-5 Lys resulted in prolonged polyplex stability.[32] In every case a PEG was attached to a C-terminal Cys with the generalized structure $(AcrK_x)_yAcrKC\text{-}PEG$, where x represents the number of spacing Lys and y the number of Acr repeats.

For a reducible iterative ligation strategy, C and N-terminal Cys are used for conjugating multiple peptides together. It is beneficial to link through the terminal amino

acids as these are the most exposed residues. Incorporation of a third Cys for conjugation to PEG or a targeting ligand presents a choice of where in the peptide this Cys should be incorporated. Incorporation in the middle of the peptide allows the relative large glycan or PEG to be removed from Cys required for conjugation and limiting steric bulk around ligating Cys. However this could result in disruption of the peptide binding to DNA, as the effect of PEGylation in the middle of a polyacridine peptide is unknown. The experiments in this section were designed to investigate the effect of PEG location in a polyacridine peptide, comparing the relative stability of polyplexes *in vivo* based upon a C-terminal or mid-peptide PEG location.

A Note on Nomenclature

The peptides in this subchapter are assigned a number to simplify identification. A (a) will indicate the addition of PEG to the starting peptide.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, 9-chloroacridine, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were obtained from Fisher Scientific. Mal-PEG_{5KDa} was obtained from Laysen Bio (Avab, AL). D-Luciferin was purchased from Roche Applied Science (Indianapolis, IN). pGL3 control vector, a 5.3 kb luciferase plasmid containing a SV40 promoter and enhancer, was obtained from Promega (Madison, WI). pGL3 was amplified in a DH5α strain of *Escherichia coli* and purified according to manufacturer's instructions.

Synthesis and Characterization of Peptides

Unsubstituted Wang resin was loaded with Fmoc-Lys(Boc)-OH or Fmoc-Cys(Trt)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. The synthesis of Fmoc-protected Nε-acridinyllysine (Acr) has been previously reported.[60] Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{409\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by Abs, assuming Acr ε_{409nm} = 9266 M^{-1} cm^{-1}, to determine isolated yield. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Synthesis of Peptides **3a** and **4a** (In collaboration with Koby Kizzire)

Purified **3** or **4** and Mal-PEG_{5KDa} (1.1 mol eq) were reacted in 4 ml of 100 mM HEPES pH 7 for 12 hrs at room temperature to generate the PEGylated **3a** and **4a**. These products were purified by semi-preparative RP-HPLC as described above. PEG-peptides were characterized by matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF) by combining 1 nmol with 10 μL of 2 mg ml^{-1} α-cyano-4-

hydroxycinnamic acid (CHCA) in 50 v/v % acetonitrile and 0.1 v/v % TFA. Samples were spotted onto the target and ionized on a Bruker Biflex III Mass Spectrometer.

Characterization of PEGylated Polyacridine Peptide Polyplexes (In collaboration with Koby Kizzire)

The relative binding affinity of PEGylated polyacridine peptides for pGL3 was determined by a fluorophore exclusion assay.[16] pGL3 (200 µl of 5 µg ml^{-1} in 5 mM HEPES pH 7.5 containing 0.1 µM thiazole orange) was combined with 0, 0.05, 0.1, 0.13, 0.15, 0.18, 0.2, 0.25, 0.3, 0.4, or 0.5 nmol of PEGylated **3a** or **4a** in 300 µl of HEPES and allowed to bind at room temperature for 30 min. Thiazole orange fluorescence was measured using an LS50B fluorometer (Perkin-Elmer, Cambridge, UK) by exciting at 498 nm while monitoring emission at 546 nm with the slit widths set at 10 nm. A fluorescence blank of thiazole orange in the absence of pGL3 was subtracted from all values before data analysis.

The particle size and zeta potential of polyplexes was determined by preparing 2 ml of polyplex in 5 mM HEPES pH 7.5 at a pGL3 concentration of 30 µg ml^{-1} and a PEGylated polyacridine peptide stoichiometry of 0.8 nmol μg^{-1} of pGL3. The particle size was measured by quasi-elastic light scattering (QELS) at a scatter angle of 90˚ on a Brookhaven ZetaPlus particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The zeta potential was determined as the mean of ten measurements immediately following acquisition of the particle size.

Pharmacokinetic Analysis of PEGylated Polyacridine Polyplexes (In collaboration with Sanjib Khargharia)

Radioiodinated pGL3 was prepared as previously described.[174] Triplicate mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg per kg) and xylazine hydrochloride (10 mg per kg), then underwent a dual cannulation of the right and left jugular veins. An i.v. dose of I^{125}pGL3 or polyplex (3 µg:2.4 nmol peptide

in 50 µl of HBM, 1.2µC) was administered via the left catheter, and blood samples (10 µl) were drawn from the right catheter at 1, 3, 6, 10, 20, 30, 60, 90, and 120 min and immediately frozen, then replaced with 10 µl of normal saline. The amount of radioactivity in each blood time point was quantified by direct γ-counting.

Hydrodynamic Stimulation and Bioluminescence Imaging

(In Collaboration with Sanjib Khargharia)

Polyplexed pGL3 (1 µg:0.8 nmol peptide) was tail vein dosed in triplicate mice in 50 µl HBM. At times ranging from 1 hr to 9 hrs, a HD-stimulatory dose of normal saline (9 wt/vol % of the body weight) was administered over 5 sec, according to published procedure.[31, 32] At 24 hrs post HD stimulation, mice were anesthetized by 3 % isofluorane, then administer an i.p. dose of 80 µL of D-luciferin (30 µg per µl in phosphate-buffered saline). At 5 min following the D-luciferin, mice were imaged for bioluminescence (BLI) on an IVIS Imaging 200 Series (Xenogen). BLI was performed in a light-tight chamber on a temperature-controlled, adjustable stage while isofluorane was administered by a gas manifold at a flow rate of 3%. Images were acquired at a 'medium' binning level and a 24.6 cm field of view with 10 sec acquisition time. The Xenogen system reported BLI as photons/sec/cm^2/seradian in a 2.86 cm diameter region of interest covering the liver.

Results

Preliminary structure activity relationships on a LMW polyacridine gene delivery peptide led to the development of **4a**, an 18 amino acid peptide that can successfully bind and protect DNA for several hrs during circulation in the blood of mice (Fig 6-5).[32] A PEG is necessarily attached to the peptide to shield the polyplex charge and permit circulation, with the PEG attached at the C-terminus in the published results. For incorporation into homogeneous gene delivery polypeptides, the PEG must be moved to another part of the peptide to allow ligation of the terminal Cys. There are no reports

examining the affect of disrupting an acridine repeat sequence. The incorporation of PEG into the middle of the sequence could potentially lower the binding affinity of the peptide to DNA by disrupting the binding domain.

To test this hypothesis, peptide **3a** was synthesized to be as structurally similar to **4a** as permitted (Fig 6-5). Both peptides contained the same number of Lys and Acr residues (13 and 4, respectively) along with 1 Cys. Acr residues were spaced by 4 Lys residues. The difference was the location of the Cys residue use to attach PEG: the Cys was attached at the C-terminal in **4a** and in the middle of the peptide in **3a**, introducing one more spacing amino acid in the middle $AcrK_4Acr$ repeat along with the PEG_{5KDa}. Both peptides were synthesized and purified in 16 % (**3**) and 20 % (**4**) yield and characterized by LC-ESI-MS (Fig 6-6A for **3a**; the synthesis of **4a** has previously been published).[32] Peptides were reacted with Mal-PEG_{5KDa} in 100 mM HEPES pH 7 to yield the PEGylated peptides along with the formation of dimer peptide (Fig 6-6B). Following preparative purification, the PEGylated peptides **3a** and **4a** were recovered in 72 % and 77 % yield and RP-HPLC analysis indicated a single peak (Fig 6-6C, Table 6-1). The difference between the observed and calculated mass of the PEGylated peptides was the result of PEG polydispersity between different lots of PEG.

The ability of PEGylated **3a** and **4a** to bind plasmid DNA was evaluated using a thiazole orange dye exclusion assay. The results established that both peptides could fully displace the fluorophore at a stoichiometry of 0.15 nmol μg^{-1} of DNA, though **3a** more weakly displaced at lower stoichiometries (Fig 6-7). Further experiments were done at a 0.8 nmol μg^{-1} of DNA. Particle size and zeta potential of the resulting polyplexes were similar, with slightly larger particles formed with **3a** (Fig 6-8).

Analysis of the pharmacokinetic profiles for **3a** and **4a** polyplexes established that both circulate with a long half-life and are cleared at a slow and coincident rate from the blood (Fig 6-9, Table 6-2). The location of the PEG had seemingly little impact on the polyplex stability for the 2 hr profile compared to naked DNA that was rapidly degraded.

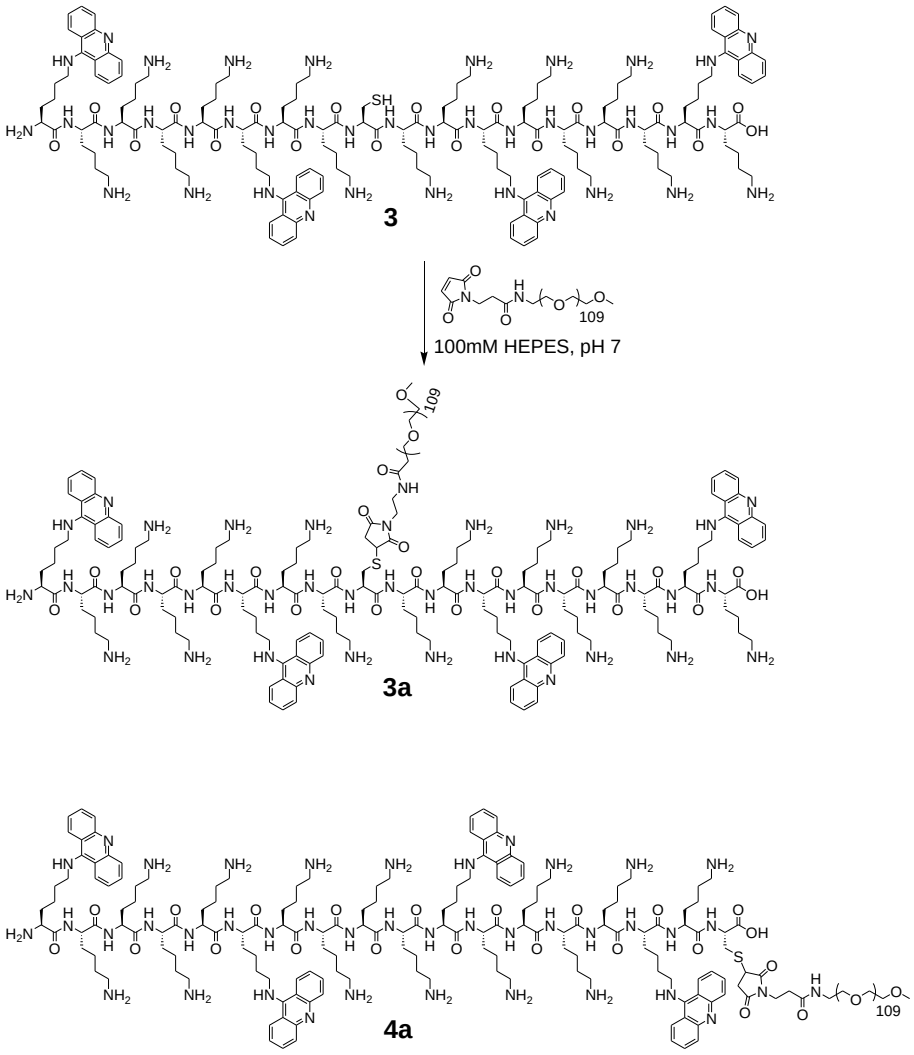


Figure 6-5: *Synthetic Strategy for PEGylated Polyacridine Peptide **3a***. Peptide **3** was synthesized to determine if a Cys placed in the middle of a polyacridine peptide disrupted its ability to bind, condense, and protect DNA *in vivo*. Following purification, the Cys of **3** was derivatized with a Mal-PEG$_{5KDa}$ to form PEGylated **3a**. This PEGylated peptide was compared to **4a**, a peptide previously demonstrated to produce polyplexes with favorable *in vivo* characteristics.

The stimulation profile of the polyplexes indicated a slightly different result. Mice were administered a tail-vein dose of 1 μg of pGL3 polyplexed with 0.8 nmol peptide. At select time points, a blank stimulatory hydrodynamic dose of saline was administered. This allowed circulating transfection competent DNA to be uptaken into the liver and be

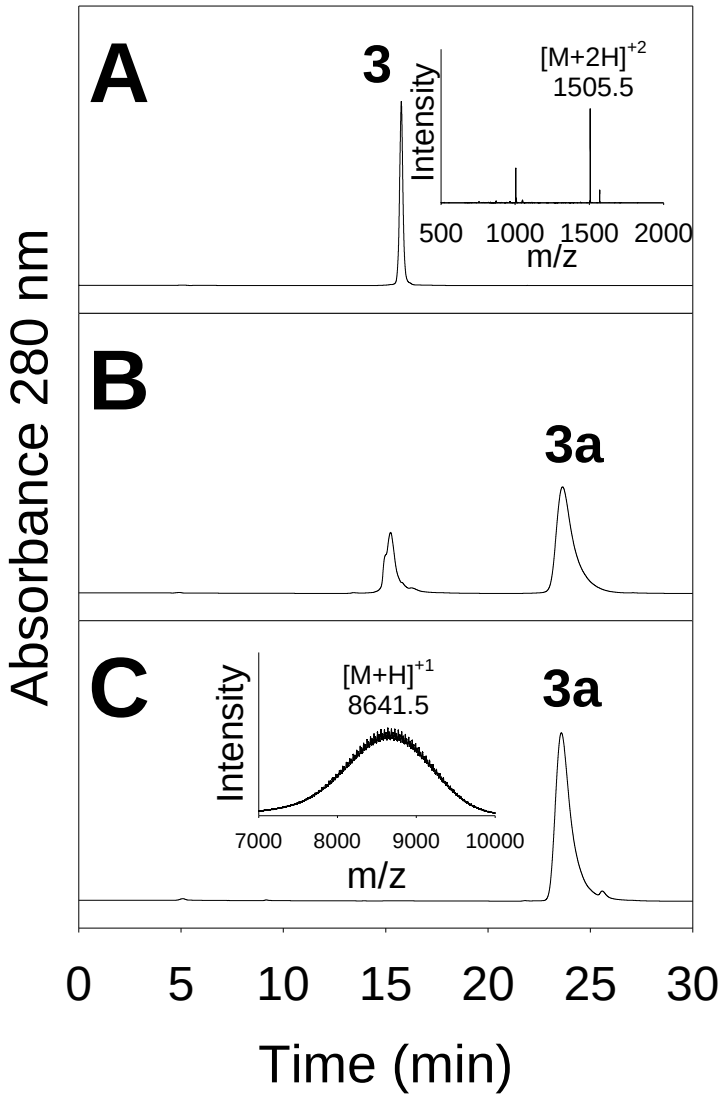


Figure 6-6: *Synthesis of PEGylated Peptide **3a***. Panels A-C illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS (A) or MALDI-TOF-MS (C) for the product peaks. Panel A illustrates purified peptide **3**. Reaction of Mal-PEG_{5KDa} with **3** results in the formation of PEGylated peptide **3a** and **3-3** dimer (Panel B). Following preparative purification, **3a** is recovered in 72 % yield as a polydisperse product, with an average molecule weight of 8641.5 amu (Panel C).

expressed, allowing a comparison on the *in vivo* stability of the polyplexes for several hrs. Starting at 2 hrs, polyplexes formed with **3a** resulted in a detectable decrease in gene expression compared to **4a** (Fig 6-10). Examination of the stimulated gene expression

Table 6-1: *Synthesis and Characterization of PEGylated Polyacridine Peptides*

Peptide	% Yield	Mass (calc / obs)
3	16[a]	3008.9 / 3009.0[c]
4	20[a]	3008.9 / 3008.8[c]
3a	72[b]	8909 / 8645[d]
4a	77[b]	8909 / 8697[d]

[a] Purified yield based on initial resin substitution.

[b] Purified yield from PEGylation

[c] Measured by ESI-MS

[d] Measured by MALDI-TOF

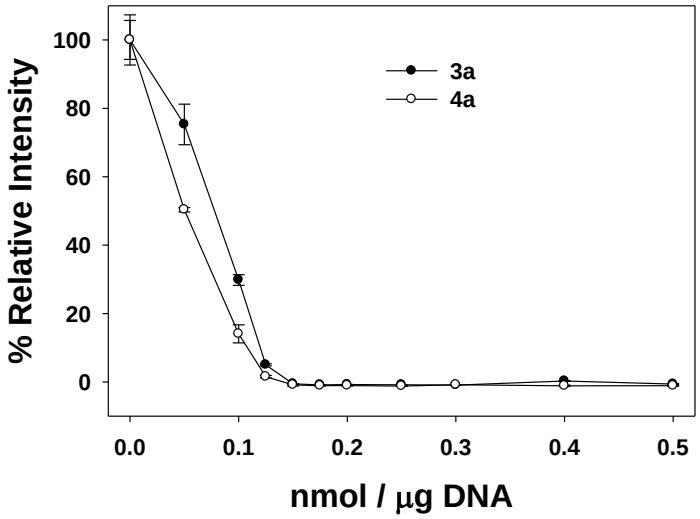


Figure 6-7: *Binding of PEGylated Polyacridine Peptides to DNA*. The displacement of thiazole orange from pGL3 was monitored by loss of fluorescence intensity as a function of peptide to DNA stoichiometry. Peptide **3a** displaced slightly worse at low peptide stoichiometries, though both fully displace thiazole orange at 0.15 nmol. Each result represents the mean and standard deviation of three determinations.

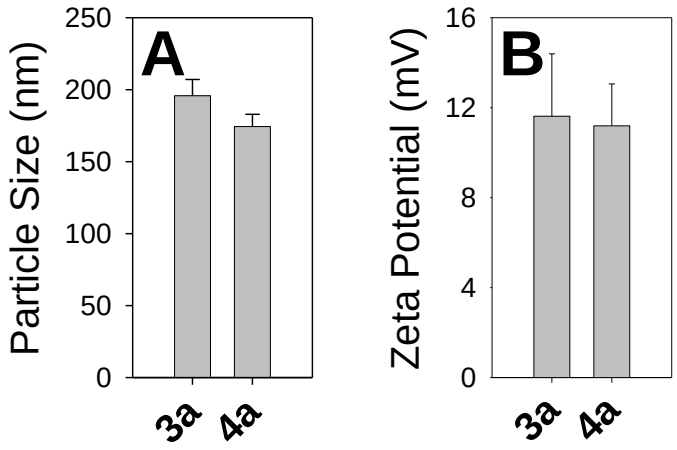


Figure 6-8: *Size and Charge of PEGylated Polyacridine Peptide Polyplex*. The mean particle size and charge for 30 μg ml^{-1} of pGL3 polyplexes prepared at 0.8 nmol of PEG-peptide per μg of pGL3 are illustrated. Panel A establishes the mean particle size of 195 nm (**3a**) and 175 nm (**4a**). Panel B establishes mean zeta potential of +11 mV for each polyplex. Each result represents the mean and standard deviation of three determinations.

profile demonstrated a 5-fold loss of activity for **3a** compared to **4a** at 4, 5, and 7 hrs. A measurable effect was observed from the relocation of PEG, though polyplexes from either peptide remained stable for several hrs.

Discussion

The only LMW gene delivery peptide that has shown ability to protect DNA in systemic circulation contains a combination of Acr and Lys residues, as well as a PEG_{5KDa}.[31, 32] Adoption of this class of peptides to an iterative reducible ligation strategy is complicated by the location of the PEG moiety, located on the C-terminal end of the peptide. As the terminal ends of the peptide present the most advantageous location for sequential ligation, the PEG moiety must be moved. The new location of the PEG is preferentially away from either terminus in order to prevent PEG interfering with the

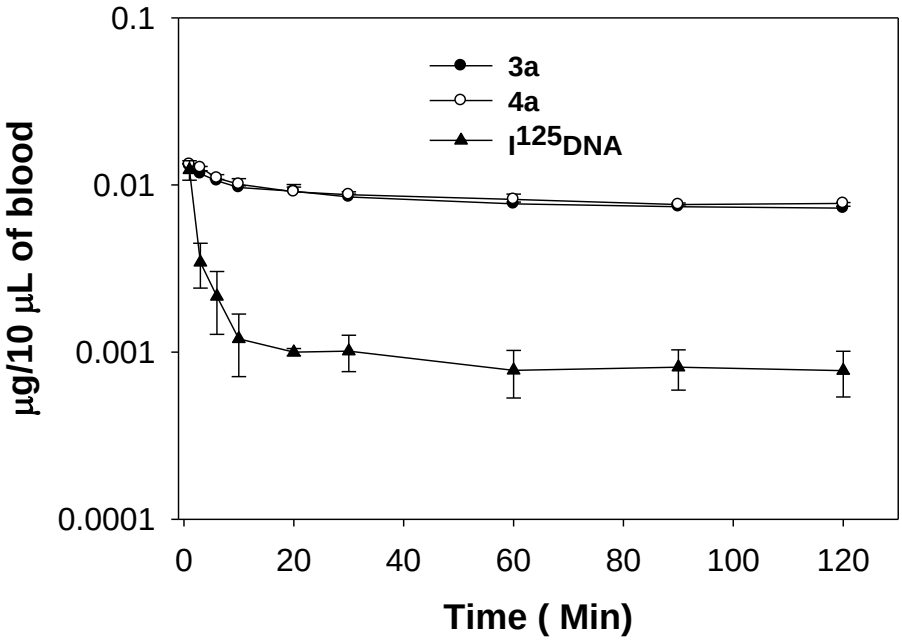


Figure 6-9: *Pharmacokinetic Profiles of PEGylated Polyacridine Polyplexes*. The concentration of ^{125}I-pGL3 and polyplexes in blood versus time is compared as a function of PEG-peptide. The corresponding calculated pharmacokinetic parameters are included in Table 6-2. Each result represents the mean and standard deviation of three determinations. Polyplexes are formed using a 1 µg pGL3:0.8 nmol peptide stoichiometry. Polyplexes condensed with **3a** (closed circle) and **4a** (open circle) showed essentially identical profiles, indicating both could stabilize polyplexes up to 2 hrs in the blood, wherein naked DNA was rapidly degraded (closed triangle).

conjugation reactions. However movement of PEG could also disrupt the polyacridine binding core.

This study examined the location of the PEG in terms of the ability to bind DNA and form polyplexes, and test the stability of the polyplexes *in vivo*. The most efficacious polyacridine peptide, **4a**, was tested against a geometric isomer with the PEG located in the middle of the peptide (**3a**). Both peptides were synthesized in similar yields and purity, and both condensed DNA to a similar extent as evidenced by the thiazole orange assay, although **3a** demonstrated weaker displacement at lower stoichiometries. The particles did not show significant differences in size or surface charge.

Table 6-2: *PEGylated Polyacridine Peptide Polyplex Pharmacokinetics*.

Peptide	$t_{1/2}\alpha$[a] (min)	$t_{1/2}\beta$[b] (min)	V_d[c] (ml/kg)	Cl[d] (ml/min/kg)	MRT[e] (min)	AUC[f] (μg*min/ml)
3a	3.6±0.5	330±40	160±10	0.4±0.1	470±70	440±40
4a	4±2	370±40	160±10	0.3±0.0	540±60	510±40

[a] Calculated α-half life.

[b] Calculated β-half life.

[c] Volume of distribution.

[d] Total body clearance rate.

[e] Mean residence time.

[f] Area under curve.

The stability of the resulting polyplexes was tested *in vivo*, first by using radiolabeled pGL3 polyplexes. Both peptides produced polyplexes that had similar pharmacokinetic profiles for the 2 hrs examined, producing similar half-life and clearance rates. More interesting was the stimulated expression profile, which examined polyplex stability up to 9 hrs. Polyplexes formed from **3a** demonstrated a small, marked decrease in gene expression, although this is still significantly higher than either naked DNA or poly-lysine delivery systems (which cannot be stimulated after 0 or 5 min).[59] Although the decrease suggested that **3a** had a slightly lower ability to protect plasmid DNA *in vivo* than **4a**, the peptide still produced stable polyplexes that could be stimulated after 7 hrs of circulation. With the location of PEG in the middle of the molecule, a new structural analogue is possible that contains terminal Cys that could be used for forming disulfide bonds between multiple PEGylated peptides. The resulting polypeptides may recover and

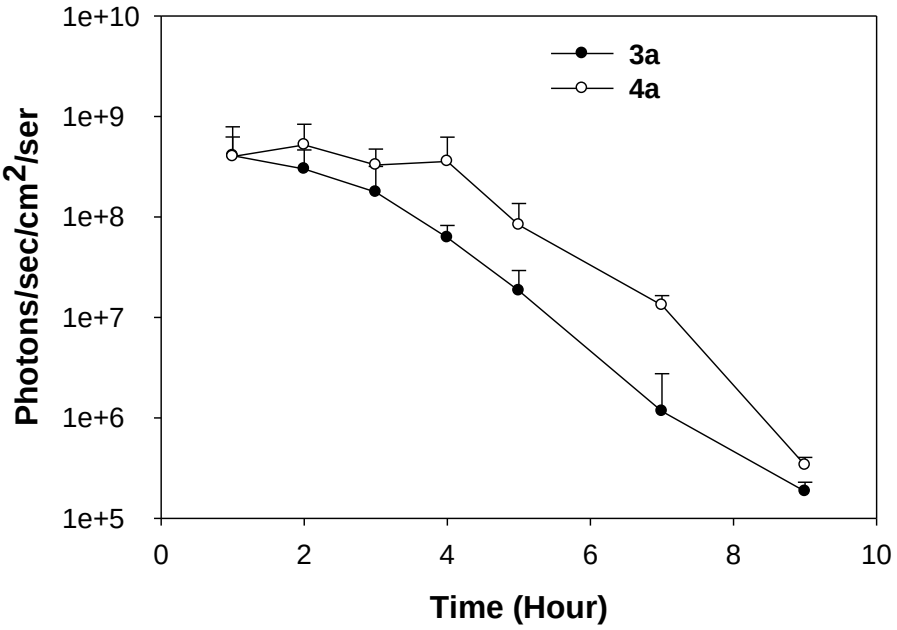


Figure 6-10: *Stimulated Gene Expression for **3a** and **4a** pGL3 Polyplexes*. The relative gene expression in the liver of mice was determined after tail vein injection of a 1 μg dose of pGL3 peptide polyplex and delayed hydrodynamic stimulation (tail vein administration of 1.5-2 ml of saline in 5 sec) at times ranging from 1-9 hrs. The magnitude of luciferase expression was determined by BLI at 24 hrs following hydrodynamic stimulation. Each result represents the mean and standard deviation of three determinations. Polyplexes were formed using 1 μg pGL3:0.8 nmol peptide stoichiometry. At two hours, less expression was observed with **3a** (closed circle) compared to **4a** (open circle), with a 5-fold decrease observed at 4, 5 and 7 hrs. **3a** polyplexed DNA could be stimulated at 7 hrs.

further extend gene expression window, by affording a stealthed polypeptide that has increased reversible binding affinity from multiple PEG containing polyacridine peptides.

A Convergent Iterative Reducible Ligation Synthesis

Introduction

One of the limitations of the solution phase iterative reducible ligation strategy introduced in chapter 3 was the required preparative purification of a polypeptide after formation of a disulfide bond. This purification technique is inefficient, resulting in losses of 30 – 40 %. In the linear synthesis developed, each conjugation step can therefore at

best only yield about 60 %. In the synthesis of a polypeptide containing 4 peptide subunits, the overall yield can only be 22 %; if the polypeptide sequence is extended to 8 subunits, the overall yield decreases to 3 %. While individual conjugation steps permit acceptable yields, the overall synthetic yield becomes costly, especially if gene delivery peptides are used containing N-glycans and long fusogenic peptides such as melittin.

One approach to overcome low yields due to purification losses is to perform the chemistry on a solid support. The progress for applying iterative reducible ligation to a solid support was explored in chapter 5 and requires further optimization. Another approach would be to synthesize polypeptides in a convergent synthesis. If two polypeptides containing two peptide subunits can be conjugated, the formation of a four unit polypeptide can be synthesized in two linear steps. The synthesis of an 8 subunit polypeptide could then theoretically be synthesized in three steps from purified starting peptides. The geometric growth possible by such a synthetic approach would decrease the number of purification steps. Assuming a 60 % coupling yield, a convergent approach would produce a 4 subunit polypeptide in about 36 % overall yield, and an 8 subunit in 22 %. While purifications still result in lowered yields, the overall yield would be higher compared to a linear approach.

The first section in chapter 6 resulted in the development of a tri-orthogonal Cys protecting scheme used to generate gene delivery peptides that could undergo iterative reducible ligation. Correct placement of these Cys protecting groups would also permit a convergent synthesis of reducible polypeptides. This section investigated a convergent synthetic strategy for reducible polypeptides using a combination of Cys(trityl), Cys(Acm) and an Fmoc-Thz masked Cys.

A Note on Nomenclature

The peptides in this subchapter are assigned a number to simplify identification. Similar to prior chapters, a (c) will denote a polypeptide transformed into a donor

polypeptide. A (d) will denote a polypeptide activated with DTDP into an acceptor polypeptide.

Materials and Methods

Unsubstituted Wang resin for peptide synthesis and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Fmoc-Lys(Boc)-OH, 9-hydroxybenzotriazole (HOBt), and O-(benzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Aapptec (Louisville, KY). 1,3-Diisopropylcarbodiimide (DIC), piperidine, diisopropylethylamine, 2,2'-dithiodipyridine (DTDP), silver trifluoromethanesulfonate (AgOTf), Sephadex G-10, acetic anhydride, and 1,2-ethanedithiol (EDT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were obtained from Fisher Scientific.

Synthesis and Characterization of Peptides

Unsubstituted Wang resin was loaded with Fmoc-Lys(Boc)-OH, Fmoc-Cys(Acm)-OH or Fmoc-Cys(Trt)-OH using standard coupling procedures. Peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with HOBt and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. Peptides were cleaved from resin and side chain deprotected using a cleavage cocktail of TFA/H_2O/EDT (93:4:3 v/v/v), followed by precipitation in cold ether. Precipitates were pelleted by centrifugation for 30 min at 5000 x g at 4˚C and the supernatant decanted. Peptides were then purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) eluted at 10 ml/min with 0.1 v/v % TFA and acetonitrile, detecting $Abs_{280\ nm}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20˚C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by $Abs_{280\ nm}$, assuming Trp ε_{280nm} = 5600 M^{-1} cm^{-1}, Cys(Pys) ε_{280nm} = 5315 M^{-1} cm^{-1}, and Fmoc-Thz ε_{280nm} = 6550 M^{-1} cm^{-1}, to determine isolated yield. The ε values for peptides

containing multiple chromophores were assumed to be additive. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 ml/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while acquiring ESI-MS in the positive mode.

Donor and Acceptor Ligation Reactions

Ligation reactions were performed on a scale ranging from 0.10-6 μmol in 0.1 M ammonium acetate pH 5. A donor peptide containing free Cys (0.50 μmol) was reacted with an S-2-sulfanylpyridine acceptor peptide (0.75 μmol, 1.5 mol eq) for 12 hrs at RT in 0.5 ml total volume. The product was preparatively purified by injecting 0.25 μmol onto a Vydac C18 semi-preparative column (2 x 25 cm) while monitoring $Abs_{280\ nm}$. The major peak from multiple runs was collected, pooled, and lyophilized. The ligated polypeptide product was reconstituted in 0.1 v/v % TFA and the yield determined by $Abs_{280\ nm}$. Purified polypeptides were characterized by LC-ESI-MS by injecting 2 nmol onto an analytical C18 column eluted and detected as described above.

Synthesis of **9d**

Lyophilized polypeptide **9** (0.25 μmol) was reconstituted in 0.25 ml of 5 v/v % piperidine/DMF and reacted 5 min to remove the Fmoc. The reaction was quenched by the addition of 0.25 ml glacial acetic acid, then applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak eluting at 21 ml was collected and lyophilized.

The thiazolidine was then hydrolyzed by reaction with 50 mol eq of AgOTf in 0.25 ml 0.1 v/v % TFA for 1 hr. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak eluting at 21 ml was collected and lyophilized.

Lyophilized **9c** was then activated to an acceptor peptide with 10 mol eq DTDP in 0.5 ml 2 M acetic acid/2-propanol (10:3 v/v) overnight. The reaction mixture was applied to a Sephadex G-10 column (1.5 x 50 cm) eluted with 0.1 v/v % TFA while monitoring $Abs_{280\ nm}$. The major peak representing acceptor polypeptide **9d** eluting at 21 ml was collected and lyophilized.

Synthesis of **10c**

Lyophilized **10** (0.25 μmol) was reconstituted in 0.125 ml 95:5 v/v % TFA/anisole solution containing 200 mol eq (50 μmol) AgOTf and allowed to react 1.5 hrs at 0˚C. 1 ml cold ethyl ether was used to precipitate the peptide for 30 min, followed by centrifugation for 5 min to pellet the peptide. Ether/TFA was decanted and the peptide allowed to air dry for 30 min. The resulting donor polypeptide **10c** was used without further purification.

Results

A series of four model peptides (**5** - **8**) were synthesized to demonstrate the feasibility of a convergent synthetic route for synthesizing polypeptides (Fig 6-11). An increased number of spacing amino acids (18) between Cys was selected to more closely approximate the size of gene delivery polypeptides. Peptides were synthesized to contain Cys protecting groups that allowed selective activation and transient protection that could be removed in the presence of a disulfide bond.

Peptide **5** contained one Cys at the N-terminal, protected as a trityl group that was removed during resin cleavage to generate the free Cys (Fig 6-11, Table 6-3). This peptide served as a donor peptide in reaction with **6**, an acceptor peptide containing a C1 Cys(Pys) and a C2 Fmoc-Thz. Conjugation of these two peptides resulted in the formation of polypeptide **9**. After preparative purification, the polypeptide **9** was

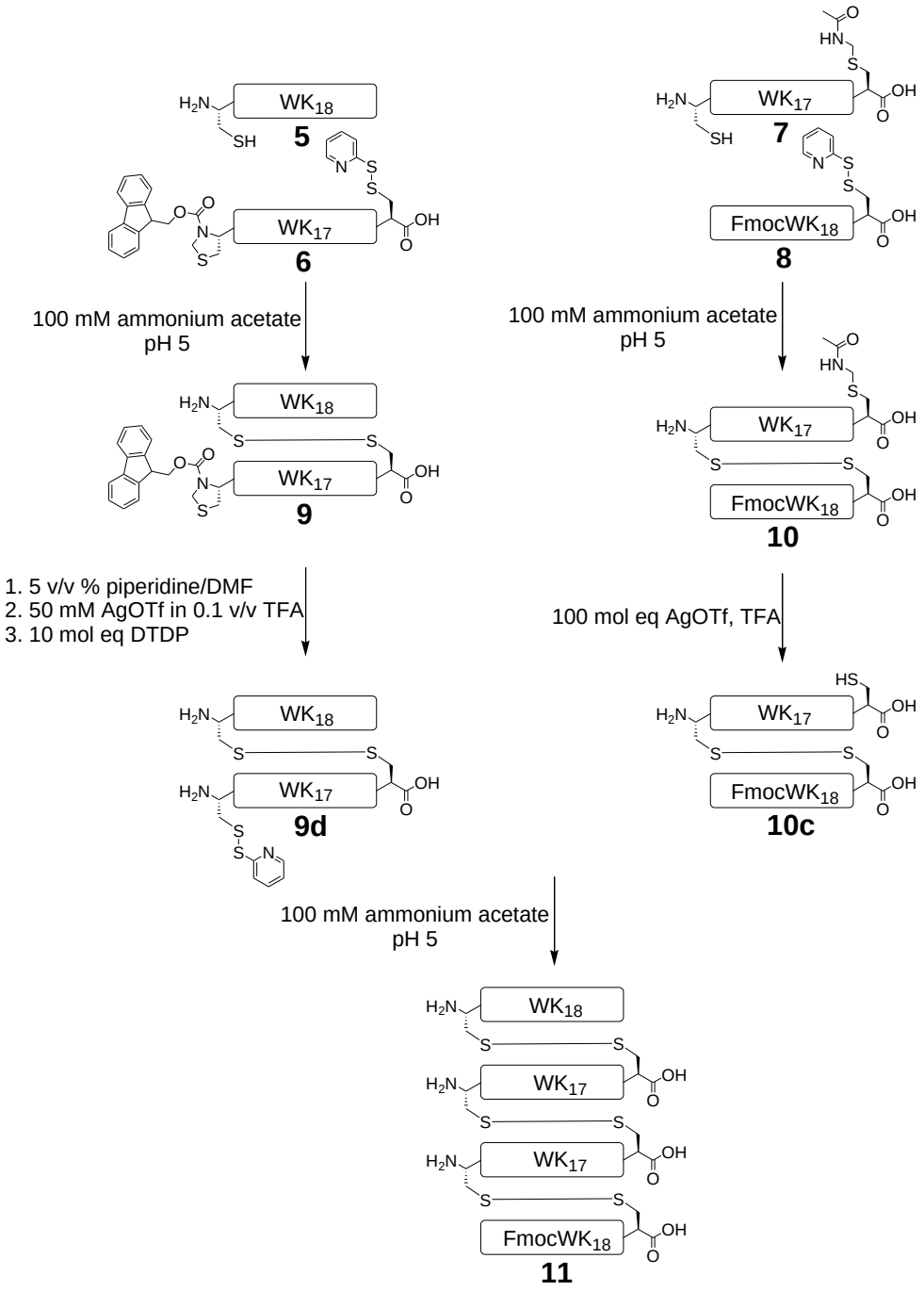


Figure 6-11: *Convergent Synthetic Strategy for Polypeptide **11***. Purified peptides **5** with **6** and **7** with **8** were conjugated to form polypeptides **9** and **10** respectively. Polypeptide **9** was converted to acceptor **9d** by removal of the Fmoc with 5 v/v % piperidine in DMF, followed by thiazolidine hydrolysis with 50 mM AgOTf in 0.1 v/v % TFA and subsequent activation with 10 mol eq DTDP. Polypeptide **10** was converted to donor polypeptide **10c** by removing an Acm with 100 mol eq AgOTf in TFA. Donor **10c** and acceptor **9d** were then conjugated in 100 mM ammonium acetate pH 5 to generate polypeptide **11**.

Table 6-3: *Synthesis and Characterization of Peptides in the Convergent Synthesis of **11***.

Peptide[a]	**Mass (calc / obs)**	**% Yield**
5 (CWK_{18})	2614.5 / 2614.0	24[b]
6 (FmocThz$WK_{17}C$(Pys))	2932.8 / 2932.2	30[b]
7 ($CWK_{17}C$(Acm))	2660.5 / 2660.2	33[b]
8 (Fmoc$WK_{18}C$(Pys))	2945.9 / 2944.8	30[b]
9 (FmocThz$WK_{17}C$-CWK_{18})	5436.2 / 5436.8	59[c]
10 (Fmoc$WK_{18}C$-$CWK_{17}C$(Acm))	5495.2 / 5496.3	62[c]
11 (Fmoc$WK_{18}C$-$(CWK_{17}C)_2$-CWK_{18})	10624.0 / 10623.6	27[c]

[a] Thz to thiazolidine, Pys to 2-sulfanylpyridine, and Acm to acetamidomethyl

[b] Purified yield based on initial resin substitution.

[c] Purified yield determined for the last ligation step.

recovered in 59% yield and re-chromatographed as a single peak by LC-ESI-MS (Fig 6-12A). This polypeptide contained an Fmoc-Thz that previous chapters demonstrated could be removed in the presence of a disulfide bond.

Donor peptide **7**, containing a C1 Acm and C2 trityl that was removed during resin cleavage, was ligated to acceptor peptide **8**, containing a C1 Cys(Pys). Following conjugation and purification, the polypeptide **10** was obtained in 63% yield and analysis indicated a single peak with the correct mass (Fig 6-12B). This polypeptide contained a Cys(Acm) that previous groups have proven can be removed without side reaction in the presence of a disulfide bond.[75-77]

Polypeptides **9** and **10** then had to be converted from protected to donor and acceptor polypeptides. Both polypeptides could be converted into a donor or acceptor polypeptide based upon reported chemistries. When attempting to convert **10** into an acceptor polypeptide (with the structure Fmoc$WK_{18}C$-$CWK_{17}C$(Pys)), low yields were observed (<50%). Higher yields were observed upon conversion of **9** to an acceptor

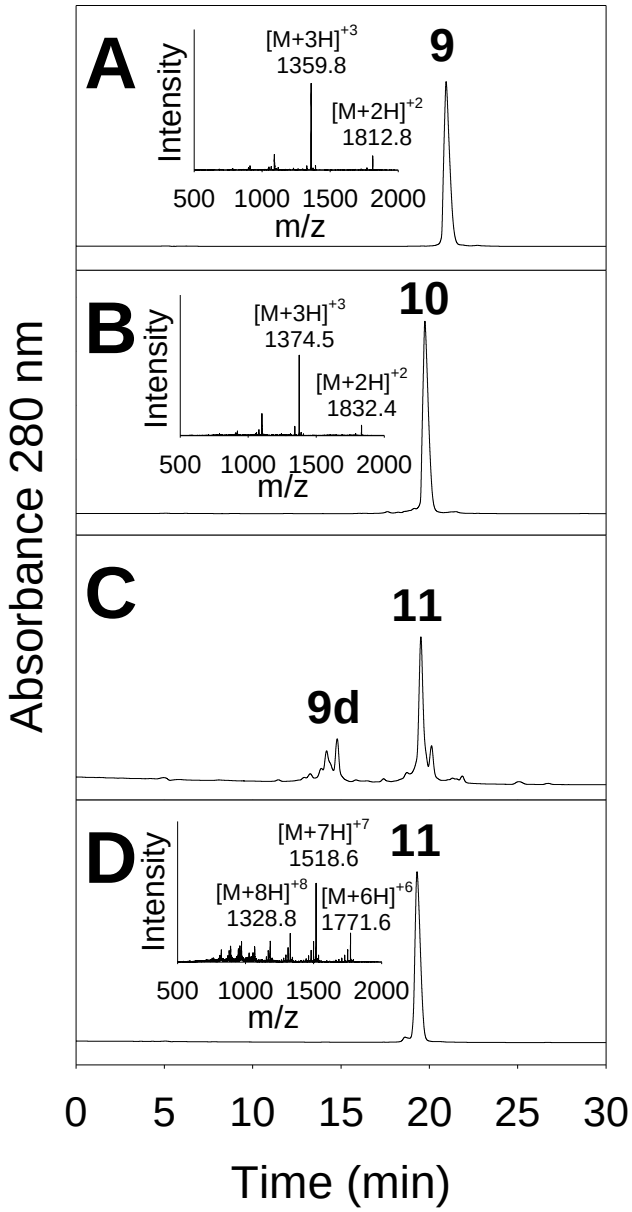


Figure 6-12: *Synthesis of Polypeptide **11***. Panels A-D illustrate RP-HPLC analyses following injection of 2 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while detecting $Abs_{280\ nm}$. The insets show the ESI-MS for the product peaks. Panels A and B illustrate the purity of polypeptides **9** and **10** following ligation of **5** with **6** and **7** with **8** in 59 % and 62 % yields, respectively. Polypeptide **9** was converted to acceptor polypeptide **9d** by Fmoc removal with 5 v/v % piperidine/DMF, thiazolidine hydrolysis by 50 mM AgOTf in 0.1 v/v % TFA, and reaction with 10 mol eq DTDP in 10:3 2 M acetic acid:isopropanol. Polypeptide **10** was converted to donor polypeptide **10c** by reaction with 200 mol eq AgOTf in TFA. Donor **10c** and acceptor **9d** (1.5 mol eq) were then conjugated to form polypeptide **11** with a new retention time (Panel C). Following preparative purification with 27 % yield, re-analysis of polypeptide **11** establishes its purity (Panel D).

polypeptide, resulting in the strategy of **9** being transformed to acceptor **9d** and **10** being converted into a donor **10c** (Fig 6-11).

Polypeptide **9** was converted into acceptor **9d** through a series of reactions. The Fmoc group was removed in a 5 v/v % piperidine/DMF treated for 5 min, followed by quenching with glacial acetic acid. Next, the thiazolidine was hydrolyzed to the free Cys with treatment of 50 mol eq AgOTf in 0.1 v/v % TFA for 1 hr, followed by G-10 purification. The free Cys intermediate was then activated to form acceptor polypeptide **9d** by treatment with 10 mol eq DTDP in 10:3 2 M acetic acid:isopropanol, followed by gel filtration purification. The overall yield for the three reaction steps was approximately 75 %.

Polypeptide **10** was converted into the donor polypeptide **10c** by reaction with 200 mol eq of AgOTf in a 95:5 v/v % TFA/anisole solution. This reaction was carried out at 0˚ C for 1.5 hrs, followed by precipitation in chilled ethyl ether. Centrifugation pelleted the precipitated polypeptide, the TFA/ether layer decanted, and the resulting pellet allowed to air dry for 30 min. The polypeptide was used without further purification.

Donor **10c** and acceptor **9d** were then coupled to form the polypeptide **11** (Fig 6-12C). An excess of the acceptor polypeptide was used (1.5 mol eq) and can be seen in the crude reaction mixture (Fig 6-12C). Following preparative purification, the four member polypeptide was observed as a single peak with a mass of over 10 kDa, recovered in a 26 % yield (Fig 6-12D).

While the desired ion envelope of the polypeptide is observed (Fig 6-12D), other ions within each charged state of the MS were observed (Fig 6-13). Magnifying the $[M+6H]^{+6}$ ion demonstrated each of these peaks is approximately 21.2 mass units lower, deconvoluting to mass differences of 127.2 amu. A Lys residue within a peptide has a mass of approximately 128 amu, indicating the presence of truncated forms of the polypeptide lacking 1-5 Lys residues.

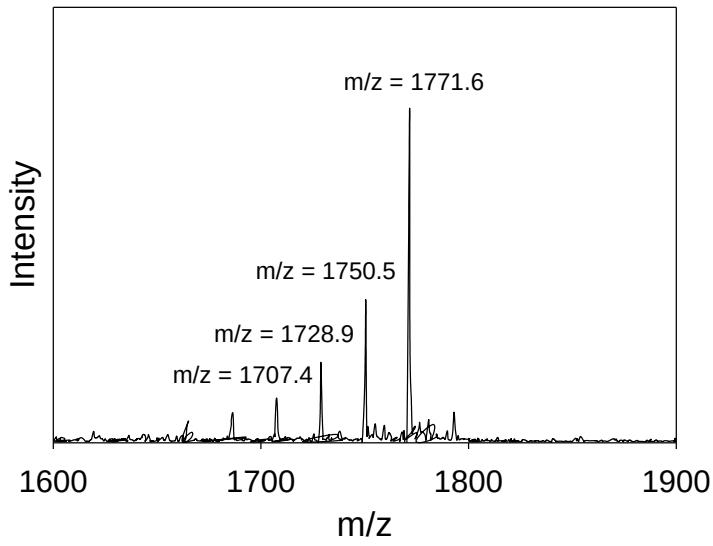


Figure 6-13: *Magnified ESI-MS Trace for the $[M+6H]^{+6}$ Ion of **11***. The enlarged window of the $[M+6H]^{+6}$ ion also demonstrates a series of evenly spaced ions. The difference between peaks deconvolutes to a mass difference of a Lys residue, signifying the presence of truncated forms of the polypeptide.

Discussion

The synthesis of homogeneous, reducible gene delivery polypeptides requires efficient ligation chemistry that can iterative couple multiple peptides in good yields. The size and required number of components for an optimal gene delivery polypeptide are unknown; multiple fusogenic peptides or targeting ligands may be required for efficacious gene delivery *in vivo*. The linear iterative reducible ligation approach developed in chapters 2 and 3 resulted in the formation of polypeptides containing three or four peptide subunits. While the linear approach can accommodate larger polypeptides, the purification yields limit the adaptation to gene delivery polypeptides beyond three or four components. Incorporation of other iterative ligation strategies that were developed using different Cys protecting groups allowed the development of a convergent synthesis, limiting the number of linear steps to large polypeptides.

Previous reducible ligation strategies involved the use of an Acm as the key protecting group for Cys.[75-77, 79-81] This thesis developed the use of an Fmoc-Thz that can

also be selectively deprotected in the presence of a disulfide bond and subsequent form new disulfide bonds in a directed fashion. These two protecting groups are stable to the removal conditions of the other. An Acm requires the use of TFA for deprotection and is stable in aqueous solutions containing AgOTf. Conversely, an Fmoc-Thz is stable to the presence of AgOTf in TFA as long as the Fmoc is attached, though a thiazolidine is hydrolyzed in TFA with AgOTf. Since the two groups can be selectively deprotected, a convergent strategy could be developed with both groups.

A model polypeptide containing 4 subunits was synthesized using both protecting groups. Two polypeptides were synthesized, each protected selectively with an Acm or an Fmoc-Thz. Selective removal and activation generated the model polypeptide in two conjugation steps. The yields of the first step were consistently high at around 60 %. While the yield for the second conjugation was lower than desired at 27 %, it still represents the conjugation of two peptide fragments weighing over 5KDa. Optimization of deprotection and conjugation conditions for such large polypeptides may result in further improvements in yields and improve the applicability of such a technique to gene delivery polypeptides.

One limitation of the ligation protocol was observed in the formation of the 10 KDa polypeptide, where truncated forms of the polypeptide were observed by MS. The truncated forms were present in the starting peptides before conjugation, though their presence was not detected after preparative purification or after the first conjugation step. These truncated forms represent incomplete coupling reactions of the peptides during their solid phase synthesis, and improvements in synthesis or purification would be required to omit such forms in future syntheses.

The presently synthesized polypeptide does not offer any advantages to the synthesis developed by Futaki, where two polypeptide fragments were ligated after deprotection of two Acm groups protecting the polypeptides.[75-77] The advantage to the present approach is that a polypeptide can be synthesized to contain both an Fmoc-Thz

and a Cys(Acm) and be selectively removed. For example, peptides **6** and **7** could be conjugated. Subsequent activation and conjugation of **6**-**7** to both **9** and **10**, with one additional ligation would result in the formation of an eight subunit polypeptide derived in only three linear steps. Such a synthesis was attempted, though the truncated forms and lowered ionizability due to the large size prevented polypeptide identification by MS. The advantage to this convergent iterative reducible ligation strategy over previously reported ones is large polypeptides containing more than 4 subunits could be synthesized.

CHAPTER 7

RESEARCH SUMMARY

Nonviral gene delivery polypeptides have made several advances that routinely overcome many of the barriers for *in vitro* gene delivery and can mediate high levels of expression in immortalized cell lines. Despite these advances, there is currently no efficacious gene delivery system that can work *in vivo,* due to the many technical challenges of delivering DNA within an organism. The many barriers require the careful construction of gene delivery polypeptides to incorporate different components that can overcome the different barriers. Such multicomponent polypeptides need to be constructed to disassemble upon polyplex uptake, which can be done by ligating peptides through reducible bonds. Most importantly, the assembly of the polypeptides must use controlled synthetic techniques to allow optimization of both the amount and location of the different components within the structure of the polypeptide. No multicomponent gene delivery polypeptide has been synthesized using robust chemistry to synthesize homogeneous characterized polypeptides, the overarching topic of this thesis.

Chapter 2 described an initial attempt at iterative reducible ligation, or a strategy of linking multiple peptides through disulfide bonds in a controlled synthesis. Key to any iterative strategy is the protecting group on a Cys that must be present when an initial disulfide bond is formed, but removed without affecting the previously formed disulfide bond. A thiazolidine has been used successfully with native chemical ligation and was selected as this key protecting group. Using the reported methoxyamine-mediated hydrolysis of a thiazolidine, a polypeptide consisting of three peptide subunits was constructed, though with disappointing yields following the second disulfide bond formation.

Two key improvements were made in chapter 3 allowing for expansion of the iterative reducible ligation strategy. An Fmoc protected thiazolidine was incorporated

resulting in a more stably protected thiazolidine and a novel silver trifluoromethanesulfonate thiazolidine hydrolysis was discovered. During the synthesis of a model set of gene delivery polypeptide, relatively high ligation reaction yields were reported, and polypeptides up to 4 peptide subunits were synthesized. Synthesis of even larger polypeptides was possible using this chemistry as well as the synthesis of gene delivery polypeptides.

To take advantage of the greater stability of disulfide bonds containing β,β-dimethyl penicillamines, chapter 4 explored the incorporation of penicillamines into the thiazolidine mediated iterative reducible ligation strategy. Incorporation of Pen could occur at either the N or C terminal position and iterative formation of Pen-Cys and Cys-Pen bonds was possible. The limited reactivity of an activated Pen meant that a Pen-Pen bond had to be formed using random oxidation reactions; though iterative formation of Pen-Pen bonds could be shown to occur, the reactions were costly for the amount of excess peptide required to drive the reactions. The more stable Pen-Cys and Cys-Pen bonds could easily be incorporated into gene delivery polypeptides, affording an additional amount of triggered release within a cell.

The relative mild conditions required for Fmoc removal, Thz hydrolysis, and peptide conjugation permitted the use of a solid support for thiazolidine mediated iterative reducible ligation, representing the work contained in chapter 5. A single disulfide bond could readily be formed on a solid support, but extending the synthesis to two disulfide bonds could not be achieved with good polypeptide product purity. The key silver thiazolidine hydrolysis from chapter 3 appeared to interfere with the reaction on a PEG containing resin because silver could not be washed from the resin. Further optimization is required for the use of solid support iterative reducible ligation, though the potential benefits of carrying out the protocol on a solid support makes this an attractive avenue for future research.

A variety of other experiments were also performed to expand the ligation strategy, both to include the synthesis of gene delivery peptides and by making polypeptides in a more efficient manner. The first two sections in chapter 6 described progress towards gene delivery polypeptides that incorporate triantennary and PEG_{5KDa} and can be used in an iterative reducible ligation strategy. A tri-orthogonal Cys protecting scheme was described, allowing selective incorporation of the triantennary or PEG into a gene delivery polypeptide that could be included into an iterative ligation strategy. The placement of the Tri or PEG Cys was also shown to be tolerated in the center of a polyacridine peptide, with slightly diminished stimulated gene expression observed after 4 hrs. While a single peptide showed diminished expression, the ligation of multiple peptides could potentially overcome this lowered response and result in prolonged expression. These results indicated the structures of a targeting peptide and a stealthing PEG peptide that could be incorporated into a homogeneous gene delivery polypeptide, with the chemistry to control the location and number of peptides of each component within the polypeptide.

Also included in chapter 6 was a convergent synthetic approach, which could be designed to generate polypeptides in solution in a more efficient strategy. This benefited from previous iterative ligation strategies that utilize an Acm protected Cys, and polypeptides could be synthesized to contain both FmocThz and Acm protected terminal Cys that allow selective elongation through either terminal. While a solution approach still suffers from low purification yields, the total number of conjugation steps decreases for increasingly long polypeptides.

The purpose of this thesis was to develop an iterative ligation strategy that allowed the conjugation of multiple gene delivery peptides to form homogeneous polypeptides that could be used for optimization of *in vivo* gene delivery. This was achieved in the synthesis of model polypeptides in thiazolidine mediated iterative reducible ligation syntheses. The next logical progression for this synthetic methodology

involves the incorporation of full length polyacridine gene delivery polypeptides derivatized with targeting ligands such as triantennary and stealthing domains such as PEG, as well as fusogenic peptides. Such gene delivery polypeptides would be the first multicomponent chemically defined polypeptides that contain a triggered release mechanism. Due to the precise assembly of the polypeptides, the peptide components could be optimized, including their location within the polypeptide, the individual amount of each component peptide, as well as the relative strength of the linking disulfide bonds. Such structure-activity relationships would result in the ability to further enhance gene expression by systemically changing the various compositions of gene delivery polypeptides and would lead to a greater understanding of overcoming the numerous delivery barriers *in vivo*. For example, multiple melittin peptides may be required within the delivery polypeptide to ensure a sufficient concentration of melittin upon polyplex disassembly to promote endolysis. The improvements made through the development of chemically defined reducible polypeptides may eventually result in efficacious gene delivery *in vivo*.

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