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**GRAFT THROUGH POLYMERIZATION OF PEPTIDES: APPLICATIONS IN
PEPTIDE THERAPEUTICS AND IN VIVO TISSUE TARGETING**

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

CHEMISTRY

by

JACQUELIN KAY KAMMEYER

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2016

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Chair

University of California, San Diego

2016

DEDICATION

I dedicate this thesis to my Father

Jay Randal Kammeyer, Mother Andrea Kay Kammyer, and siblings Jason William

Kammeyer and Krystal Kay Wyer

For their unconditional support

EPIGRAPH

*“Most great people have attained
their greatest success just
one step beyond their greatest failure”*

- Napoleon Hill

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LIST OF ABBREVIATIONS

ACM	=	Acetamidomethyl
AMPs	=	Antimicrobial peptides
ATRP	=	Atom transfer radical polymerization
BBB	=	Blood brain barrier
Boc	=	tert-Butyloxycarbonyl
CLI	=	Critical limb ischemia
CNS	=	Central nervous system
CPP	=	Cell penetrating peptide
CTA	=	Chain transfer agent
DCM	=	Dichloromethane
DIPEA	=	Diisopropylethyl amine
DLS	=	Dynamic light scattering
DMF	=	Dimethyl formamide
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribose nucleic acid
DP	=	Degree of polymerization
DPBS	=	Dulbecco's phosphate-buffered saline
ECM	=	Extracellular matrix
ELPs	=	Elastin-like polypeptides
EPR	=	Enhanced permeability and retention effect
ESI-MS	=	Electrospray ionization-mass spectrometry
EVE	=	Ethyl vinyl ether

FaSSGF = Fasted state simulated gastric fluid
 Fmoc = Fluorenylmethyloxycarbonyl chloride
 GLP-1 = Glucagon like peptide 1
 GnRH = Gonadotropin hormone releasing hormone
 GPCRs = G protein-coupled receptors
 HBTU = O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
 HDBPs = High density brush polymers
 HS = Heparan sulfate
 hTS = Human thymidylate synthase
 IP = Intraaperitoneal injection
 LCMS = Liquid chromatography-mass spectrometry
 M:I = Monomer to initiator ratio
 MAP = Model amphipathic peptide
 MbCD = Methyl beta cyclodextran
 MMP = Matrix metalloproteinase
 mRNA = Messenger ribonucleic acid
 MTT = 4-Methyltrityl
 MW = Molecular weight
 NCAs = α -amino acid-N-carboxyanhydrides
 NET = Neuroendocrine tumors
 NMP = Nitroxide-mediated radical polymerization
 NMR = Nuclear magnetic resonance
 OEG = Oligoethylene glycol
 PAD = Peripheral artery disease

PEG = Polyethylene glycol

PI = Propidium Iodide

PLGA = Polylactic-co-glycolic acid

PPA = Peptide polymer amphiphile

RAFT = Reverse addition-fragmentation chain transfer

ROMP = Ring opening metathesis polymerization

ROP = Ring opening polymerization

RP-HPLC = Reverse phase high performance liquid chromatography

SEC-MALS = Size exclusion chromatography- multiangle light scattering

siRNA = Small interfering ribonucleic acid

SLS = Static light scattering

SPPS = Solid phase peptide synthesis

SUV = Standard uptake value

Tat = Trans-activator of transcription

TFA = Trifluoroacetic acid

TIPS = Triisopropyl silane

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

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PEPTIDE THERAPEUTICS AND IN VIVO TISSUE TARGETING**

by

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A strategy for the synthesis of peptides displayed as high density brush polymers is detailed. With this strategy, numerous high density brush polymers displaying peptides were prepared and studied for their ability to protect the peptides from proteolysis. Furthermore, strategies to enable cellular internalization of peptide high density brush polymers were

investigated. Finally, peptides were incorporated into polymeric nanomaterials and used for tumor and ischemic tissue targeting.

Peptide high density brush polymers were prepared using *graft through* ring opening metathesis polymerization (ROMP) to investigate which amino acids could be incorporated into the peptide and polymerized successfully using a ruthenium-based initiator. It was found that only cysteine was unable to be polymerized, whereas lysine and methionine showed difficulty. Strategies to incorporate these problematic amino acids include maintaining protecting groups on the side chains, and incorporating a longer linker between the peptide and the polymerizable moiety.

With this strategy in hand, a variety of high density brush polymers bearing peptides that are enzyme substrates were prepared. These peptides were investigated for their ability to be proteolytically degraded by thrombin, trypsin and pepsin. It was found that in general, peptides displayed as high density brush polymers were protected from proteolysis compared to their monomeric counterparts. When the recognition sequence was spaced closer to the norbornene backbone, the peptide was more protected from proteolysis. Furthermore, peptides were more protected from proteolysis when polymerized to higher molecular weights. With these data, it was believed that polymerizing peptides into high density brushes could protect peptides from proteolysis, a major problem in peptide therapeutics.

A second problem in peptide therapeutics is the inability to internalize into cells. Using high density brushed polymers displaying cationic peptides, the polymeric material is able to internalize into cells in a concentration and degree of polymerization dependent process. Furthermore, a therapeutic peptide was displayed as a high density brush polymer and internalized into cells, and maintained its bioactivity of inducing mitochondrial dependent apoptosis.

Matrix metalloproteinases (MMPs) are enzymes that are upregulated in many diseased states such as hind limb ischemia and cancer. A peptide polymer amphiphile that displayed an MMP substrate as the hydrophilic block, and was labeled with a near infrared dye was prepared. Peptide polymer amphiphiles are able to self-assemble into micellar nanoparticles. It was previously shown that when the nanomaterial is incubated with MMPs, the enzyme cleaves off the peptide, resulting in a change in polymer amphiphilicity inducing an aggregation event. Near infrared labeled polymeric materials were intravenously administered to animal models of cancer and peripheral artery disease, two diseases where MMPs are upregulated, and the particles were able to accumulate into the diseased tissue, albeit not significantly over the non-responsive control. It was found, however, that when incorporating a zwitterionic dye to the polymeric material, there was a significant decrease in off target accumulation.

Chapter 1

Peptides in Therapeutics and Nanomedicine

1.1 Introduction

The Gianneschi laboratory has ongoing interest in programming polymeric nanomaterials with biological molecules. These nanomaterials are used to interface with, manipulate and respond to biology in manners that include responding to enzymes and DNA,^{1,2} manipulating the expression of mRNA³, and more recently serving as responsive therapeutic delivery vehicles.⁴ Polymers are attractive scaffolds for interfacing with biology and nanomedicine due to their tailorable size, shape, charge, loading capacities and diverse chemical functionality. Polymers can be used to improve the delivery of small molecule drugs by overcoming their poor tissue specificity, low water solubility, and low bioavailability due to rapid elimination.

Pre-clinically, biological therapeutics such as peptides excel in the treatment of diseases due to their high specificity, potency and biocompatibility, unlike small molecule drugs, yet they often fail clinical trials due to their instability *in vivo*. For example, peptides are rapidly degraded by proteolysis, generally have an inability to cross biological membranes, and have extremely short circulation half-lives. However, because of their promise *in vitro*, decades of research have been performed at improving the utility of peptide therapeutics *in vivo*. Herein, the attributes and functions of peptides will be described in regard to their roles as clinical therapeutics, strategies for stabilizing peptide therapeutics, and the incorporation of peptides into nanomaterials for improved tissue targeting.

1.2 The Invention of Solid Phase Peptide Synthesis

Many of the achievements of peptide therapeutics would not have been possible without the ease of synthesis and high yield enabled through the use of solid phase peptide synthesis (SPPS). Prior to SPPS, all peptide bonds were formed in solution phase and necessitated purification. In 1901 the first peptide, Gly-Gly, was synthesized using solution phase by Emil Fischer and Ernest Fourneau.⁵ In 1932, Bergmann, a former student of Fischer, utilized a carbobenzoxy group for protection of the amine allowing for the synthesis of more complex peptides.⁶ Using this protection method, du Vigneaud synthesized the first peptide hormone in 1953, a synthetic version of oxytocin.⁷ Many original reports indicated that synthetic peptides maintained the bioactivity of their naturally occurring counterparts; however, the solubility and purity of these materials decrease dramatically as peptide length increased, obviating the need for new method to be developed.⁸

In 1963, Robert Merrifield reported a new synthetic technique to prepare peptides in which he conjugated the first amino acid to a solid polymer, followed by sequential addition of the amino acids, then finally removal of the peptide from the solid support.⁹ This new method, which he termed solid phase synthesis, allowed for the synthesis of longer peptides with higher purity. In recent years, SPPS technology has advanced in many ways, but especially as result of the creation of new polymer supports, the use of a variety of side chain protecting groups, the development of highly active coupling agents and the use of non-natural amino acids. These advances allow for almost any complex peptide to be synthesized on solid support in high yield and large quantities.

Limitations still exist when using SPPS. Typically, SPPS is not used to synthesize peptides longer than 70 amino acids due to the limitations of yield at each successive amino acid coupling step. Typically, a 99% yield is achieved at each step, but the total peptide yield

deteriorates exponentially with each step. Therefore, shorter peptides can be synthesized in high yield, but peptides longer than 70 amino acids would result in a less than 50% yield, assuming each step is 99% yield. Peptides longer than 70 amino acids typically synthesized in bacteria cultures.

1.3 Therapeutic Peptides

Peptides have numerous innate functions in biology, which include acting as hormones^{10–14} and antibiotics.^{15–17} In recent years, researchers have also utilized peptides as targeting agents to specific tissues^{18–22} and as anticancer agents.^{23,24} One important use of peptides is as therapeutic agents due to the high specificity and selectivity of peptides to their targets, as well as their biocompatibility and low immunogenicity.^{25–29} These features of peptides often result in low off target effects, making peptides viable and enticing therapeutic options. As of 2010 there were 60+ peptide therapeutics in the American, European or Japanese clinical markets,²⁹ and though many peptides continue to reach the market, the development and clinical utility of peptide therapeutics is often thwarted due to poor bioavailability. Below, the problems facing peptide therapeutics will be highlighted, and some key peptide therapeutics currently on the market will be described.

1.3.1 Problems with Peptide Therapeutics

Once passing phase 1 clinical trials, peptides have a higher probability of reaching the market compared to small molecule drugs because of their favorable *in vivo* properties. However, passing phase 1 clinical trials is extremely difficult due to the low bioavailability of peptide therapeutics. This results from rapid degradation by endogenous proteases, fast size-dependent renal clearance, and an inability to penetrate biological barriers.

To prevent proteolysis of a peptide therapeutic, the peptide must be modified so that it is unrecognizable by endogenous proteases. Researchers can modify different aspects of the peptide sequence by techniques such as *N*-terminal acetylation,³⁰ incorporation of non-natural amino acids,^{31–36} modification of the backbone,^{37–39} cyclization,^{40–43} or the synthesis of peptide mimics.^{32,44–46} Each of these modifications occurs at the site of proteolytic susceptibility, and prevents the enzyme from recognizing its substrate, thereby, improving the *in vivo* half-life of the therapeutic. Utilizing these strategies, however, necessitates thorough structure-function assays to ensure the modification to the peptide does not reduce its biological function.

The majority of peptide therapeutics lack the ability to cross biological barriers such as cellular membranes, especially in the case of the lumen of the small intestine and the blood brain barrier (BBB). The BBB is a layer of endothelial cells that surround all of the capillaries in the brain. This membrane allows the passive diffusion of gasses (CO₂, O₂), as well as small hydrophobic molecules (hormones). The BBB is thought to be impermeable to large molecules (>500Da), especially hydrophilic molecules.^{47,48} For this reason, the BBB is decorated in proteins that assist in the active transport of hydrophilic molecules such as glucose and amino acids, which are necessary for brain function.

The holy grail of peptide therapeutic delivery is through the oral route. However, the stomach and gastrointestinal track have numerous protective mechanisms to prevent agents such as antigens and pathogens from crossing the intestinal epithelial cell wall. Thus, these protective mechanisms often thwart viable oral drug and peptide delivery.⁴⁹ Physical barriers in the intestine include the epithelial cells and tight junctions between them, and the mucus and stagnant water layer. Furthermore, there exists an enzymatic barrier where numerous promiscuous luminal enzymes break down peptides and proteins.⁵⁰ A peptide must evade the

enzymes, and transverse through the mucosal and epithelial cell layers to reach the blood for proper circulation.⁵⁰

A common strategy employed to help agents cross all these barriers is to append a cationic cell-penetrating peptide (CPP) to the therapeutic.^{51–54} CPPs, discussed in more detail later in Chapter 4, typically have a multivalent display of cationic amino acids, most importantly arginine, that can undergo favorable hydrogen bonding with cellular membranes, allowing for passage into the interior of the cell.^{55–59} CPPs have been used successfully for delivery of insulin and other peptide drugs across the intestinal wall.^{51,60,61} Indeed, the intestinal absorption of insulin increases 6–8 fold when conjugated to a canonical CPP, Tat.⁶⁰ CPPs have also been used for delivery of memapsin, a therapeutic peptide, across the BBB.⁶² Furthermore, by incorporating multiple copies of a cationic moiety such as guanidinium, the functional group present in Arg, groups have had success delivering antibiotics, albumin, nerve growth factors and other peptides through biological barriers.^{28,63–66}

Typically, for a material to passively diffuse across these barriers, it must be lipophilic and low in molecular weight. There is a strong positive correlation between lipophilicity and the ability to cross the BBB.⁶⁷ Peptides inherently have low lipophilicity, but conjugating a peptide to lipid moieties such as lipophilic triglycerides increases the lipophilicity of peptides and improves their ability to cross the BBB.^{68,69} However, crossing the intestinal epithelial wall does not seem to improve with increasing lipophilicity of a peptide, but rather by decreasing the hydrogen bonding capabilities of the sequence.^{70,71}

Peptides can also be transported through biological barriers via an active transport mechanism. Biological barriers are decorated with active protein transporters for a variety of small molecules that cannot otherwise cross such as D-Glucose and vitamin B12. Researchers have taken advantage of these transporters by appending receptor binders to the peptide of interest, allowing the transport of the peptide across the barrier through the transporter.⁷² For

example, peptides that are glycosylated with D-glucose can be transported across the BBB using the glucose carrier Glut-1.^{73,74} Peptides can also be transported across the brush border membranes of epithelial cells of the small intestine if appended to vitamin B12, which allows for receptor-mediated endocytosis.⁷⁵⁻⁷⁷ In particular, this has been shown effective for delivering luteinizing hormone releasing hormone across the epithelial layer.⁷⁶ The small intestine also has oligopeptide transporters, however, delivery using these transporters is limited to di- and tripeptides and other small peptide based antibiotics.^{78,79}

1.3.2 Peptide Therapeutics in the Market

A common theme used in the development of therapeutic peptides is modifying the peptide to improve its biological function. For example, to improve the stability, many therapeutic peptides incorporate substitutions to the amino acid sequence that include non-natural or D-amino acids,⁸⁰⁻⁸⁴ and some are cyclized.^{40,43} Moreover, to improve tissue targeting, therapeutic peptides can be conjugated to targeting peptides for better biodistribution.^{19,85,86} Therapeutic peptides that are either used in the clinic or in preclinical and clinical trials are described below.

An important class of peptide therapeutics targets G protein-coupled receptors (GPCRs). The majority of GPCRs expressed on the cell surface bind to peptide hormones or other small proteins. These ligands can range anywhere from 3-60 amino acids. Within this class of peptide therapeutics are those that target and treat HIV infection. Fuzeon®, a 36 amino acid modified peptide, is used to prevent HIV infection into host cells by inhibiting HIV cellular entry. For HIV to enter cells, the fusogenic protein gp41 on HIV binds with CD4+ receptors on immune cells. Fuzeon® works by binding to the fusogenic protein gp41 on HIV, blocking HIV from binding to immune cells, and therefore preventing HIV infection.^{84,87-89} Fuzeon® was discovered in 1996 and approved by the FDA in 2003, and

was the first in its class of virus entry inhibitors. Unlike the remainder of the peptides discussed in this section, Fuzeon® has only simple modifications: acetylation of the *N*-terminal nitrogen and amidation of the *C*-terminus. These modifications do improve the bioavailability of the peptide, but the peptide is still susceptible to proteolysis, and it is usually only used as a salvage treatment when nothing else has worked. Typically, using Fuzeon® requires the patient to self-administer *via* multiple subcutaneous injections daily.

Many GPCR binding peptides on the market help treat diabetes. Byetta® and Symlin® are used to treat both type 1 and type 2 diabetes. Byetta® acts as a glucagon like peptide 1 (GLP-1) mimic.²⁹ GLP-1 is a peptide endogenously expressed to control insulin levels.⁹⁰ However, this peptide cannot be used as a therapeutic due to its half-life of 2 minutes and poor therapeutic index due to the cleavage of the agonist by DPP-4 protease.^{91,92} Byetta®, also known as exendin-4, is a 39-amino acid peptide that mimics GLP-1, and has been shown in to improve insulin biosynthesis in animal and cell models,^{83,93} to lower glucose in several animal models,⁹⁴ and to improve diabetic outcomes in patients where other drugs were ineffective.⁹⁵ These effects are seen due to the fact that Byetta® is not recognized by DPP-4 leading to an extension of the peptide's half-life *in vivo*. Similar to Fuzeon®, Byetta® is a linear, naturally occurring peptide with no synthetic modifications to improve its stability, but because this peptide acts similar to GLP-1, yet is not recognized by DPP-4, the half-life is improved from 2 minutes to 2.5 hrs. Even with this extended half-life, Byetta® still has to be administered twice daily via subcutaneous injections.

Symlin®, also known as pramlintide acetate, differs from the other drugs in that it is a synthetic mimic of the hormone amylin. This hormone is coexcreted with insulin and complements insulin's effect of maintaining stable blood glucose levels. Many patients with type 1 and type 2 diabetes are amylin deficient, therefore, amylin replacement therapy is necessary to improve insulin's effects in these patients.⁹⁶ Amylin is rapidly degraded *in vivo*

and suffers from aggregation, rendering it ineffective. Symlin® differs from amylin in that 3 amino acids are replaced with proline, which leads to improved solubility and decreased aggregation.

Angiomax® is a peptide therapeutic for the treatment of cardiovascular disease. Angiomax®, also known as bivalirudin trifluoroacetate hydrate, is used as an anticoagulant that inhibits the action of thrombin, the enzyme responsible for blood coagulation.⁸¹ Prior to Angiomax®, heparin was used as an anticoagulant factor, however the pharmacokinetics and pharmacodynamics of heparin were unpredictable due to the inherent heterogeneity of heparin. Angiomax® contains a single D-amino acid that improves its bioavailability, yet the peptide still has a short half-life. Regardless, Angiomax® has a more predictable anticoagulation effect than that of heparin and is now used in the clinic for patients undergoing percutaneous coronary intervention.⁸¹

Peptide hormone mimics are a very commonly used in the treatment of cancer.⁹⁷ One such peptide mimetic that is approved by the FDA is a class of gonadotropin hormone releasing hormone (GnRH) agonist such as Zoladex®, Decapeptyl®, and Ovuplant®.⁸⁰ In each of these, the peptide sequence is modified to contain non-natural amino acids to prevent degradation. Native GnRH has the sequence pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂. Simple changes in the peptide sequence, such as replacing Gly⁶ with D-Ala increased the potency of GnRH by 300-400%.⁹⁸ For the above examples, Zoladex® incorporates D-Ser(But)⁶, and Aza-Gly¹⁰, Decapeptyl® incorporates D-Trp⁶, and Ovuplant® incorporates D-Trp⁶ and Pro⁹-NEt, where the amide nitrogen is ethylated.⁸⁰ Each of these modifications improves the bioavailability of the peptide without diminishing its therapeutic effect.

Lanreotide and Octreotide are two somatostatin analogs that can bind to the somatostatin receptors overexpressed on neuroendocrine tumors (NET). When somatostatin

and these analogs bind to the receptors, there is a decrease in production of other hormones that can slow the progression of the tumor. Native somatostatin has a half-life of a few minutes, and therefore is not an effective therapeutic. Both Lanreotide and Octreotide are cyclized variations of somatostatin with improved half-lives of 1-2 hours.^{40,99,100} Landreotide also contains non-natural and D-amino acids, which reduces degradation by proteolysis.

RGD and NGR peptides were the first in class of tumor-targeting peptides discovered through phage display.¹⁰¹ These peptides bind to cell surface integrins, which are overexpressed in many cells undergoing angiogenesis.¹⁰¹ They show very little selectivity in tumor type in that they target breast cancer, malignant melanoma, and in some cases arthritis due to the increased expression of integrins in these tissues. Cilengitide is a synthetic RGD peptide in clinical trials that actually exhibits a therapeutic function other than targeting tissue.⁴³ Due to the cyclization, Cilengitide is more selective than its “promiscuous” linear counterpart and targets integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ specifically. These integrins are known to be upregulated in many cancers.^{102,103} Cilengitide can bind to these integrins at sub nanomolar levels, inhibiting the interactions of these integrins with the extracellular matrix (ECM), which is necessary for angiogenesis.^{104,105} This inhibition can cause apoptosis of endothelial cells, which decreases vessel density and prevents tumor growth.¹⁰⁶ As of 2010, Cilengitide is in phase III clinical trials for the treatment of glioblastoma, and Phase II for many other types of cancers.⁴³

Another class of therapeutic peptides is known generically as anticancer peptides. These peptides can have numerous functions such as restricting tumor growth through preventing angiogenesis or inducing apoptosis. These peptides can also inhibit various protein-protein interactions, enzymes, and signal transduction pathways.^{24,86,107–111} For example, eight peptides have been discovered that inhibits the activity of human Thymidylate Synthase (hTS), an enzyme crucial for the synthesis of thymidine. Peptides can be appended

to cell specific targeting peptides and have shown to inhibit cell growth on numerous cancerous cell lines that are either drug sensitive or resistant.¹¹² Antimicrobial peptides have also been exploited as anticancer peptides.²³ Antimicrobial peptides (AMPs) typically bind to and lyse bacterial membranes, but when conjugated to cancer cell specific targeting peptides, AMPs can internalize and cause mitochondrial dependent apoptosis due to the resemblance of mitochondrial membranes to bacterial membranes.^{21,24,85,86} In each of these cases, the peptides are indiscriminant and must be appended to a targeting peptide in order to reach their therapeutic targets.

1.4 Peptides in Nanomaterials for Biological Applications

Nature has utilized peptide based nanomaterials as the machinery of cells, such as structural proteins, receptors in cell signaling, and those that catalyze reactions. Many researchers are using nature as inspiration in the synthesis and development of nanomaterials. Due to the inherent chemical diversity of amino acids, peptides are great candidates in nanomedicine because they offer the potential to endow nanomaterials with a variety of properties, such as self-assembly and tissue targeting.

Peptides are often used to generate self-assembled nanomaterials due to their ability to form alpha helicies and beta sheets. Beta sheet forming peptides are often used to form peptide based hydrogels that are used in regenerative medicine. These materials can encapsulate a variety of cells allowing them to form a synthetic ECM with regenerative properties. One example of a beta sheet forming peptide that forms a hydrogel is the peptide known as KLD12 (sequence: KLDLKLDLKLDLKLDL). When chondrocytes were encapsulated in the hydrogel and cultured for 4 weeks, they developed ECM like properties such as the development of proteoglycans and collogens and the hydrogel has been used for cartilage tissue repair.¹¹³ The peptide RADA16-I (sequence: RADARADARADARADA)

self assembles into a soft hydrogel that can be used for tissue regeneration as well. When coupled with an angiogenic peptide, the hydrogel allowed for enhanced endothelial cell survival, along with proliferation and migration, which likely resulted from the proangiogenic properties of the appended peptide.¹¹⁴ Another type of peptide hydrogel is that formed from Fmoc-protected di and tri peptides.^{115–118} Ulijn and co-workers formed a mixed hydrogel with Fmoc-FF and Fmoc-RGD, where the gel was able to encapsulate dermal fibroblasts. The hydrogel allowed for adhesion, proliferation and cell spreading.¹¹⁹

Self-assembled peptide amphiphiles have also been used in numerous biomedical applications.¹²⁰ Certain peptide amphiphiles developed by Stupp and co-workers have 4 key features that allow for their self-assembly: the first is a hydrophobic domain consisting of a long carbon tail, followed by a beta sheet forming domain comprised of Val₃Ala₃, then a hydrophilic charged peptide that allows for pH and salt responsive self-assembly, and finally the bioactive peptide.¹²⁰ For example, Stupp and co-workers were able to improve angiogenesis in an animal model of critical limb ischemia by incorporating a small VEGF mimicking peptide to a peptide amphiphile. The amphiphiles formed a hydrogel, and were injected in ischemic muscle. Over the course of 28 days, the VEGF mimicking peptide displayed on the amphiphiles induced angiogenesis, increased perfusion of blood to the limb, and improved outcomes such as limb survival.¹²¹ Stupp and co-workers have also used self-assembled peptide amphiphiles for bone,^{122–124} tissue,^{121,125,126} and neural¹²⁷ regeneration.

Elastin-like polypeptides (ELPs) have also been used as a peptide based material for biological applications.¹²⁸ ELPs have very interesting properties such as temperature responsiveness, as well as high sequence control and biocompatibility, which makes them very useful in biomedicine.¹²⁹ ELPs exhibit a unique temperature dependent phase transition where at low temperatures they are soluble, however, at higher temperatures they undergo hydrophobic collapse and aggregate.^{130,131} Chilkoti and co-workers exploited this property to

accumulate ELP nanoparticles in tumor tissues.¹³² ELPs were administered to tumor bearing mice, and ELP nanoparticles showed a 2-fold increase in tumor accumulation when the tumors were undergoing hyperthermia.¹³² Since this development, ELP nanoparticles have been used in cancer therapy through imaging, drug delivery, and other therapies.^{133–136}

Finally, groups have utilized polymerization of α -amino acid-*N*-carboxyanhydrides (NCAs) to form large polypeptide polymers either as homo-, blend, and more recently block copolymers.^{137–139} Using NCAs, peptide based materials can be synthesized into vesicles and hydrogels with biomedical applications. For example, polypeptide block copolymers comprised of arginine and leucine (R₆₀L₂₀) can self-assemble into vesicles. Due to the polycationic nature, the polymers can condense DNA and transfect mammalian cells with lower cytotoxicity and immunogenicity compared to other transfection agents.¹⁴⁰ Furthermore, peptide based materials derived from polymerization of NCAs can also form biocompatible hydrogels.¹³⁹ Hydrogels formed from lysine-leucine or aspartic acid-leucine (K₁₈₀L₂₀ or E₁₈₀L₂₀) were shown to be biocompatible in the central nervous system, and could be used to deliver protein therapeutics such as a nerve growth factor through slow release to the central nervous system (CNS).^{141,142}

Hybrid materials can also be formed by conjugating peptides to nanomaterials. In most cases, the peptide functions as a targeting ligand for active targeting of specific tissues. Traditional nanomaterials used the enhanced permeability and retention (EPR) effect, which is defined by an enhanced permeability of diseased tissues due to leaky vessels and enhanced retention due to poor lymphatic drainage. The EPR effect is seen in many cancerous animal models, however, the utility of this effect in human carcinoma is controversial, and more active targeting strategies are therefore desired. Many groups have turned to using peptides as the targeting unit due to their small size, ease of chemical synthesis and good tissue specificity. Numerous peptides that can target specific cells and receptors have been

identified such as integrin receptors,¹⁰¹ thrombin receptors,¹⁴³ and cells such as cardiomyocytes,¹⁴⁴ and tumor cells and vasculature.^{19,21,145} Researchers can use these peptides that target specific cells to append them to a nanocarrier that serves as a therapeutic or imaging agent to enable higher accumulation of the material at the site of preference.

One of the most common targeting peptides is the RGD peptide that targets integrins (discussed above).¹⁰¹ RGD has been incorporated into numerous nanomaterial drug delivery vehicles. For example, a cyclic RGD analog was attached to doxorubicin loaded liposomes and the resulting conjugates showed improved efficacy *in vivo* in a colon cancer mouse model when compared to free doxorubicin and doxorubicin loaded liposomes without the RGD peptide.¹⁴⁶

Using phage display against MDA-MB-435 human carcinoma xenograft tumors, the tumor-homing peptide LyP-1 was identified.²¹ This peptide is a 9 amino acid cyclic peptide that targets tumor lymphatic vessels. Groups have utilized nanoparticle LyP-1 conjugates to improve tumor targeting.¹⁴⁷ For example, nanoparticles formed from polyethylene glycol (PEG) and polylactic-co-glycolic acid (PLGA) block copolymers and coated with LyP-1 showed an 8-fold increase in targeting metastatic lymph nodes compared to uncoated PEG-PLGA nanoparticles.¹⁴⁷ Furthermore LyP-1 can also target macrophages in atherosclerosis plaques. When a heat shock protein was genetically encoded to express LyP-1 on its outermost surface and labeled with a near infrared fluorophore, the LyP-1-HSP conjugate was able to selectively accumulate in macrophage rich carotid lesions.¹⁴⁸ Thus, the use of targeting peptides can improve the targeting of nanomaterials *in vivo*.

1.5 Conclusion and Outlook

The development of peptide therapeutics and the incorporation of peptides into nanomaterials has opened doors to new avenues in the targeting and treatment of disease due

to the chemical diversity present in the side chains of amino. This thesis describes the development of a strategy for incorporating peptides into a polymer scaffold. This strategy proved to be beneficial in the field of peptide therapeutics, in that packaging peptides in a polymeric scaffold can protect peptides from proteolytic degradation, as well as activate peptides for cellular uptake. Furthermore, this strategy is also used in the preparation of nanomaterials that can potentially target MMP activity *in vivo*.

1.6 References

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Chapter 2

Preparation of High Density Peptide Brush

Polymers *via* Graft-through Ring Opening

Metathesis Polymerization

2.1 Introduction

Polymers and polymeric nanomaterials have gained much attention recently for applications in biology and medicine.¹ This stems from the ability of polymers to contain multiple functionalities, as well as the ease of tailoring their size, shape, and chemical functionality.² With this increasing interest in synthetic polymers, new strategies must be developed for the synthesis and preparation of highly reproducible polymers. Historically, the most common polymerization techniques, such as condensation polymerization, have given rise to essential polymeric materials needed in everyday life such as plastics. However, most early techniques were done in an uncontrolled non-living fashion resulting in materials that were ill-defined and polydispersed. For applications in interfacing with biology, it is therefore necessary to create a polymerization technique that is controlled and reproducible that results in a highly characterized and defined polymer material.

To achieve this, groups have more recently relied on controlled living polymerization methods, which allow for the synthesis of ordered, well defined polymers. The most common of these techniques are atom transfer radical polymerization (ATRP),³ reverse addition-

fragmentation chain transfer (RAFT) polymerization,⁴ nitroxide-mediated radical polymerization (NMP),⁵ ring-opening polymerization (ROP),⁶ and ring opening metathesis polymerization (ROMP).⁷ Each of these techniques involves a living polymerization, meaning the growing polymer chain does not undergo premature termination. This allows for block copolymer synthesis because the polymer can be synthesized in stages where each individual monomer is added, and consumed without any termination. Living polymerizations also occur via a fast initiation and slow propagation resulting in low dispersity of polymer molecular weights.

To display functional groups such as peptides along a polymer backbone (brush polymer), 3 different strategies can be used: *grafting to*, *grafting through*, and *grafting from* (Figure 2.1). In the *grafting to* approach, peptides are appended to a preformed polymer post-polymerization. This strategy, though useful, often leads to unpredictable or low yielding conjugation reactions, requiring excess peptides and, in many cases, multiple purification steps.^{8,9} As such, in the hopes of generating monodisperse, highly reproducible polymers, a different strategy should be used.

As an alternative, many researchers have utilized a *grafting from* strategy where the peptides or proteins themselves are used as the initiators of polymerization. In this context, a functional initiator is added to the peptide or protein, then a nonfunctional polymer is polymerized off at the initiator point.^{8,10-12} *Grafting from* typically produces heterogeneous mixtures of polymers due to differences in the initiation rates of the various initiators. *Grafting from* can be used to generate a brush polymer where a preformed polymer contains multiple initiation points (Figure 2.1), however it has not been applied to the generation of brush polymers displaying peptides as the brushes.¹³

For the synthesis of brush polymers that contain peptides as the brush, we envision the best strategy is using a *grafting through* approach. In this approach, peptide monomers

are directly polymerized into a polymer. Unlike *grafting to* and *grafting from*, this approach typically yields homogeneous polymers (depending on the initiator selection), and does not need any excess of peptide, or any post polymerization purification.^{14–20}

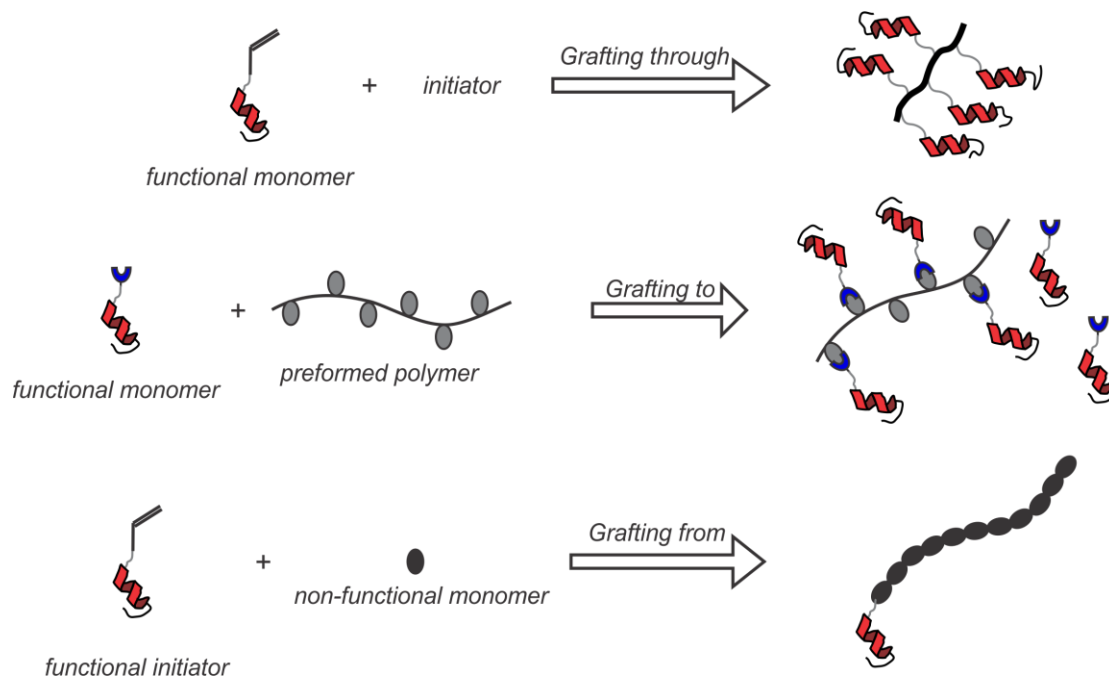


Figure 2.1 General polymerization strategies for incorporating functionalities into polymeric materials.

ROMP is an ideal polymerization technique for the *graft through* polymerization of peptides into brush polymers. Indeed, it has been widely used to synthesize polymers that incorporate peptides, DNA, sugars, and other biologically relevant molecules and drugs.^{7,21–29} The utility of ROMP in these contexts is largely thanks to the availability of excellent catalyst initiators.^{30,31} In particular Ru-based catalysts out of the Grubbs lab are typically highly functional group tolerant and have been demonstrated to be useful for the incorporation of peptides in a *graft through* polymerization. In order to undergo *graft through* polymerization of peptides, a strained cyclic olefin, such as norbornene or transcyclooctene, should be incorporated into the peptide (Figure 2.2).³² However, previous reports concerned peptides

containing only aliphatic and aromatic amino acids, or those with side chain protecting groups.^{27,33,34} In fact, when more complex peptides were needed, a *grafting to* approach was used.³⁵ We sought to explore the range of peptides that can be prepared via graft through ROMP by investigating the tolerance of a common ROMP initiator to amino acid identity.

2.2 *Graft through Polymerization of Protecting-Group Free*

Pentapeptides

The incorporation of complex peptides into high density brushes via *graft through* polymerization is thought to be possible due to the highly functional group tolerant ruthenium based initiators.^{30,36} Though these initiators are known to be functional group tolerant, they still have difficulty polymerizing strongly nucleophilic functional groups such as amines and imidazoles. However, as part of a peptide sequence functional groups present on the side chains of amino acids might exhibit less of an effect on the initiator due to spacing. In order to synthesize a more diverse set of peptide based polymers via *graft through* ROMP, the tolerance to amino acid identity must first be determined.

The functional group tolerance of a common ROMP initiator (IMesH₂)(C₅H₅N)₂(Cl)₂Ru=CHPh (I, Figure 2.3) to all 20 canonical amino acids was determined through the synthesis and polymerization of a library of penta-peptide sequences (Figure 2.3). The library was based on the pentapeptide sequence Norbornyl-Gly-Phe-Pro-Leu-Ile, a short aliphatic and aromatic peptide known to be polymerized by ROMP rapidly and reproducibly.²⁵ The peptide was modified at the X₂ or X₅ position to contain each canonical amino acid not already present in the sequence (for example G, P, and L). These positions were chosen because X₂ is positioned nearest to the olefin and therefore can potentially interact with the catalyst and inhibit polymerization. X₅ is positioned at the

[illegible]

Figure 2.2 Scheme for the incorporation of a norbornene unit into a peptide. *N*-(glycine)-*cis*-5-norbornene-*exo*-dicarboximide is coupled to the *N*-terminus of a peptide using standard solid phase peptide synthesis (SPPS) conditions.

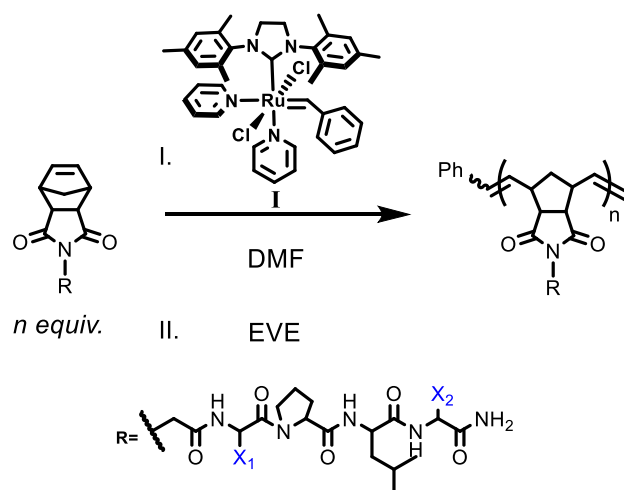


Figure 2.3 Scheme of the polymerization of peptide brush polymers via *graft through* ROMP using initiator **I**. Note that n equivalents of peptide monomer added to the catalyst afforded a polymer with n repeating units.

To determine the tolerance of the initiator **I** to amino acid identity, each peptide monomer was polymerized to a relatively low degree of polymerization (DP; 20) or a high DP (200) using the monomer to initiator ratio (M:I) of either 20:1 or 200:1 respectively. Each polymerization reaction was monitored by ^1H NMR at various time points with the terminal time point being 24 hours. With NMR, we can monitor the disappearance of the norbornyl monomer olefin that appears as a singlet around $\delta = 6.5$ ppm in DMF_{d7} concurrently with the appearance of the polynornornyl-olefin protons around $\delta = 5.6$ and 5.8 ppm. Upon complete polymerization, or reaching the 24-hour time point, polymers were quenched with the termination agent ethyl vinyl ether (EVE) to release the catalyst from the polymer. Molecular weight and polymer dispersity were determined using size-exclusion chromatography multi-angle light scattering (SEC-MALS). For each polymerization, percent conversion was determined through integration of the monomer and polymer olefin resonances by NMR.

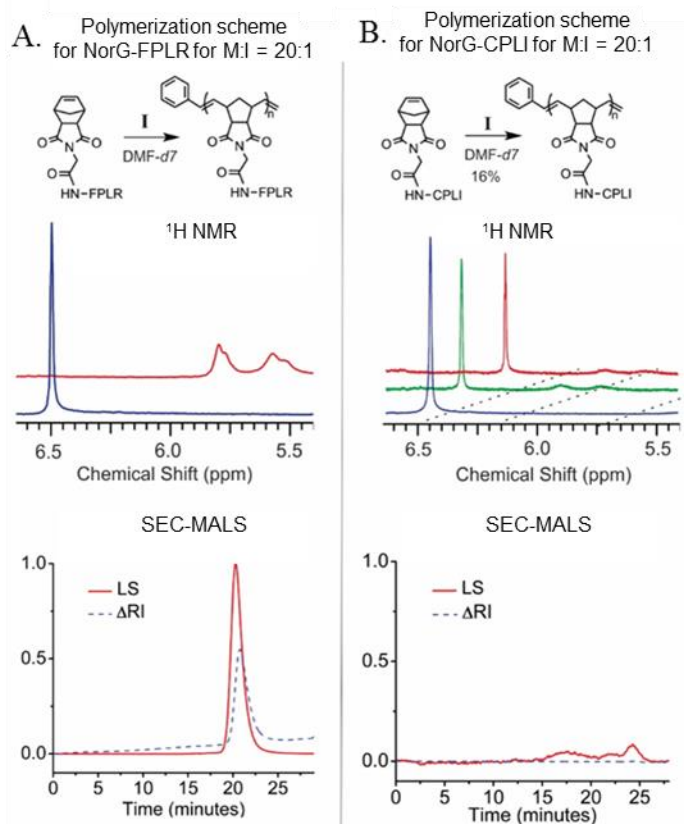


Figure 2.4 Example polymerizations of peptide monomers from the library. A. An example showing a complete polymerization of the peptide monomer NorG-FPLR. The ^1H NMR shows the spectrum of the monomer before addition of the initiator in blue and the spectrum of the polymer after addition of the initiator in red. Notice that the monomer olefin proton at δ 6.5 is no longer present in the final NMR, and there is an appearance of polymer olefin proton between δ 5.5 and 6.0. After quenching the polymerization, the polymer was characterized by SEC-MALS to determine molecular weight and dispersity. B. An example of an incomplete polymerization of the peptide monomer NorG-CPLI. Note that in the spectrum, there is no significant disappearance of the monomer olefin at 6.5 ppm suggesting that the polymer did not reach complete polymerization. Likewise, no light scattering events were detected when the resulting material was characterized by SEC-MALS further confirming no polymer was formed.

After subjecting each monomer to standard polymerization conditions, almost all amino acids were tolerated by the ROMP initiator and could be incorporated into a polymer (Table 2.1). In figure 2.3, examples of a complete polymerization and an incomplete polymerization are shown. For a complete polymerization of the peptide sequence GFPLR, there is a clear disappearance of the monomer olefin proton at 6.5ppm and an appearance of a

broad olefin resonance between 5.5 and 6.0 ppm confirming the monomer was completely consumed (Figure 2.3A). For a typical incomplete polymerization such as for the peptide Nor-GCPLI, the monomer olefin was still present after 24 hours, signifying that the polymerization never reached completion. This was confirmed by the SEC-MALS trace in which there was no polymer detected. Table 2.1 summarizes the findings from the polymerization study.

Table 2.1 Summary of NMR and SEC-MALS data obtained from the polymers.

Monomer	Peptide Sequence	M : I of 20 : 1			M : I of 200 : 1		
		% conversion ^a	DP ^b	M _w /M _n ^b	% conversion ^a	DP ^b	M _w /M _n ^b
1	NorGFPLI	100	15	1.02	100	191	1.31
2	NorGAPLI	100	22	1.03	100	194	1.02
3	NorGVPLI	100	23	1.06	100	187	1.03
4	NorGRPLI	100	15	1.1	96	155	1.09
5	NorGSPLI	100	23	1.02	100	125	1
6	NorGKPLI	100	15	1.19	83	62	1.02
7	NorGNPLI	100	19	1.05	100	179	1.02
8	NorGTPLI	100	20	1.03	100	159	1.04
9	NorGMPLI	100	15	1.04	76	102	1.01
10	NorGQPLI	100	22	1.05	100	165	1.03
11	NorGHPLI	100	21	1.1	100	182	1.2
12	NorGWPLI	100	16	1.13	100	189	1.1
13	NorGCPLI	16	-	-	-	-	-
14	NorGYPLI	100	20	1.1	100	188	1.02
15	NorGDPLI	100	ND ^c		100	ND ^c	
16	NorGEPLI	100	ND ^c		100	ND ^c	
17	NorGFPLA	100	19	1.13	100	175	1.03
18	NorGFPLV	100	18	1.11	100	193	1.04
19	NorGFPLR	100	30	1.03	98	134	1.36
20	NorGFPLS	100	17	1.24	100	182	1
21	NorGFPLK	100	12	1.05	87	132	1.2
22	NorGFPLN	100	14	1.04	98	193	1.03
23	NorGFPLM	100	15	1.04	100	157	1.03
24	NorGFPLQ	100	15	1.04	100	163	1.31
25	NorGFPLE	100	ND ^c		100	ND ^c	
26	NorGFPLW	100	25	1.02	99	216	1.02
27	NorGFPLT	100	14	1.22	100	187	1.02
28	NorGFPLH	100	17	1.1	100	182	1.03
29	NorGFPLY	100	20	1.2	100	192	1.02
30	NorGFPLD	100	ND ^c		100	ND ^c	
30*	NorGFPLD(tBu)	100	12	1.02	100	189	1.162
31	NorGFPLC	22	-	-	-	-	-
31*	NorGFPLC(acm)	100	21	1.1			
32	Nor(GFPLI) ₃	100	18	1.04			
33	Nor(GFPLI) ₆	100	18	1.04			

a : determined by ¹H NMR. *b*: determined by SEC-MALS. *c*: could not be determined by SEC-MALS

When targeting a M:I ratio of 20:1, every peptide monomer except those that contain cysteine (**13** and **31**) was completely polymerized. Cysteine contains a sulfhydryl group, which are known to be problematic with ruthenium based initiators due to catalyst coordination. If cysteine needs to be incorporated into peptide sequence, we found that

peptides can be synthesized using Seiber Amide resin, which allows for the removal of the peptide from resin using mildly acidic conditions, keeping the side chain protecting groups intact. Indeed, when NorG-FPLC(Acm) was subjected to standard polymerization conditions, quantitative conversion of the monomer to polymer was achieved (**31***).

Cysteine was the only amino acid that could not be incorporated into peptide polymers without modification; however, there did exist other problematic amino acids. Peptide monomers containing a guanidinium moiety (Arg- **4**, **19**, Table 2.1), or primary amine (Lys- **6**, **21**, Table 2.1), went to complete polymerization when targeting lower degrees of polymerization (M : I of 20 : 1), however for the higher degree of polymerization (M : I of 200 :), they underwent high yielding but incomplete polymerization. Furthermore, when the amino acid methionine was at position X₂ and polymerized to M : I of 200 : 1, the peptide monomer never reached completion, which was not the case when methionine was located at position X₅. We have since determined that either using protecting groups, or incorporating a 5 methylene spacer between the norbornene unit and the peptide monomer enables peptides that contained problematic amino acids to be polymerized to completion.³⁷

Other issues were encountered in the characterization of the polymers where the peptide contained a carboxylic acid side chain (polymers **15**, **16**, **25**, and **30**, Table 2.1). These polymers exhibited gel-like properties in DMF post termination with EVE. These gel-like polymers did not perform well on the SEC-MALS column due to aggregation, however, when the carboxylic acid containing peptides were synthesized with the protecting groups still intact, the polymers were able to be characterized by SEC-MALS (**30***).

2.3 *Graft Through Polymerization of More Complex Peptides*

Peptides used in biomedical applications are often longer than 5 amino acids. It was therefore necessary to determine if longer peptides could be polymerized using initiator **I**. A 15 (**32**) and a 30 amino acid long monomer (**33**) containing the sequence GFPLI_n, where $n = 3$ for **32** and $n = 6$ for **33**, were subjected to standard polymerization conditions and both went to complete polymerization within 2 hours when targeting M : I of 20 : 1 (Table 2.1).

Many bioactive peptides often have complex structures and functions that need to be taken into consideration when preparing as a high density brush polymer. For example, the gonadotropin releasing hormone (GnRH) is a peptide where the *N*- and *C*- termini are necessary for hormone receptor binding.³⁸ Native GnRH has the sequence pyroGlu-His-Trp-Ser-Tyr-Lys-Leu-Arg-Pro-Gly-NH₂. Previous researchers found that modifications to Lys₆ were tolerable and did not decrease the function of the peptide.³⁸ If GnRH is to be incorporated into a peptide brush polymer, the norbornene moiety cannot be conjugated to the *N*- terminus, however it can be conjugated to the Lys₆. A GnRH analog that contained a norbornene modified Lys was synthesized, where norbornene was conjugated to the ϵ amine on resin (Figure 2.4). The peptide monomer was subjected to standard polymerization conditions where M : I ratios of 10 : 1 and 15 : 1 were targeted. Unfortunately, the peptide did not reach complete polymerization, likely due to the presence of multiple complex functional groups on the amino acid side chains. The peptide could not be synthesized with side chain protecting groups due to the synthetic difficulty to maintain protecting groups on the side chains and incorporate a norbornene unit on the Lys. The peptide did however reach high yielding polymerization conversions of 90 and 93% respectively. GnRH is a very complex peptide to polymerize due to its hairpin like nature and multiple complex functional groups. It is therefore promising that such a complex peptide can be incorporated into a dense brush polymer via *graft through* ROMP.

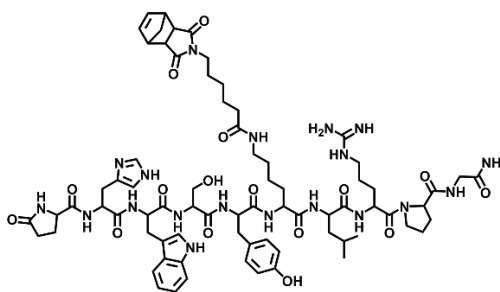


Figure 2.5 Structure of the GnRH peptide monomer.

Table 2.2 GnRH polymer characterizations.

Monomer	Peptide Sequence	M : I of 10 : 1			M : I of 15 : 1		
		% conversion ^a	DP ^b	M _w /M _n ^b	% conversion ^a	DP ^b	M _w /M _n ^b
34	NorGnRH	90	8	1.098	93	15	1.159

2.4 Conclusion

In conclusion, we believe that the initiator (IMesH₂)(C₅H₅N)₂(Cl)₂Ru=CHPh (I, figure 2.2) is tolerant to most peptide based norbornene monomers. When peptides contain one of the problematic amino acids (Lys, Arg, Met, and Cys), and therefore cannot be polymerized to completion, either the use of side chain protecting groups or longer spacers between the monomer unit and the peptide can be employed to increase the rate of polymerization and likelihood of completion. Furthermore, using initiator I we can polymerize very long peptides (30 amino acids long), as well as non-linear peptides (GnRH). We believe that using the common ROMP initiator I and the strategies developed herein, we can polymerize any peptide substrate that we want, so long as it is soluble in a solvent compatible with the catalyst initiator. Note that these results are indeed surprising in that many of the amino acids, such as His and Lys, have side chains that contain functional groups that were once considered not polymerizable by these Ruthenium based initiators. These

results will now allow the facile *graft through* synthesis of high density brush polymers where the brushes contain peptides.

2.5 Methods

2.5.1 General Methods

All reagents were purchased from commercial sources and used without further purification. Peptides were synthesized using an AAPTEC Focus XC automated synthesizer. Amino acids were purchased from AAPTEC and NovaBiochem. *N*-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide,²⁶ (IMesH₂)(C₅H₅N)₂(Cl)₂Ru=CHPh³⁹ and *N*-(hexanoic acid)-*cis*-5-norbornene-*exo*-dicarboximide²⁷ were prepared according to published protocols. All polymerizations were set up in J. Young NMR tubes (5 mm diameter) in a glove box under a dinitrogen atmosphere using DMF-*d*₇ drawn from sealed ampules (Cambridge Isotopes). HPLC analysis was performed on a Jupiter Proteo90A phenomenex column (150 x 4.60 mm) using a Hitachi-Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi-Elite LaChrome L-2420). Purification was done using a Jupiter Proteo90A Phenomenex column (2050 x 25.0 mm) on a Waters DeltaPrep 300 system. Buffer A was 0.1% TFA in water and Buffer B was 99.9% ACN and 0.1% TFA. The sequence identities of purified peptide-monomers were confirmed using ESI-MS in the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Polymer dispersities and molecular weights were determined by size-exclusion chromatography (phenomenex Phenogel 5u 10, 1k-75k, 300 x 7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF)) using a Shimadzu pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 MW polystyrene standard. In the situations where a multimodal distribution is observed by light scattering but not in the RI chromatogram, we

analyzed only the peak width that has an associated RI component (*i.e.* S4 d). ^1H (400 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts were reported in ppm relative to the DMF residual proton peaks.

2.5.2 Peptide Synthesis

Peptide monomers were synthesized via standard Fmoc-based solid phase synthesis using Rink Amide MBHA resin (AAPTEC) via automated synthesis. In brief, Fmoc deprotection was performed using 20% 4-methylpiperidine in DMF. Amino acid couplings were carried out using HBTU and DIPEA (resin/amino acid/HBTU/DIPEA 1:3.5:3.4:4). After each peptide was synthesized, a final amino acid coupling with *N*-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide was performed. The final peptide monomers were cleaved from the resin using a mixture of TFA/H₂O/TIPS (95:2.5:2.5) for 45 minutes. The peptides were precipitated and washed with cold ether. The peptides were dissolved in buffer A with minimal amounts of buffer B. Peptides were analyzed using RP-HPLC and purified using preparative HPLC. Peptide identity and purities were confirmed using ESI-MS and RP-HPLC monitoring at $\lambda_{\text{abs}}=214$ nm.

Peptide monomers that required a protected amino acid side chain were synthesized via standard Fmoc-based solid phase synthesis using Sieber Amide Resin (AAPTEC). Standard solid phase peptide synthesis protocols were followed for the preparation of the side-chain protected monomers with the following exceptions. The resin was swelled for 1 hr in DMF and then the initial N-terminal Fmoc protecting group was removed by shaking in a solution of 20% 4-methylpiperidine in DMF for 5 min and then again for 1 hr. The initial amino acid was loaded onto the resin for 1 hr, the solution was drained and then a fresh set of amino acid/coupling agent was added and mixed for 1 hr to ensure complete loading. Peptides were cleaved from the resin using 2% TFA in DCM for 45 minutes. The peptides

were precipitated and washed with cold ether. The peptides were dissolved in buffer A with minimal amounts of buffer B. Peptides were analyzed using RP-HPLC and purified using preparative HPLC. Peptide identity and purities were confirmed using ESI-MS and RP-HPLC monitoring at $\lambda_{\text{abs}} = 214 \text{ nm}$.

2.5.3 GnRH Peptide Synthesis

The GnRH peptide was synthesized using standard SPPS protocol above using Rink Amide MBHA resin. The peptide was synthesized using a Lys (MTT) derivative. Post complete peptide synthesis, the MTT group on the Lysine side chain was removed by first swelling the resin in DCM, followed by 15 x 5 minute washes with 2%TFA in DCM. After the final wash, the resin was washed thoroughly with DCM, then DMF, then DCM. The final peptide monomers were cleaved from the resin using a mixture of TFA/H₂O/TIPS (95:2.5:2.5) for 45 minutes. The peptides were precipitated and washed with cold ether. The peptides were dissolved in buffer A with minimal amounts of buffer B. Peptides were analyzed using RP-HPLC and purified using preparative HPLC. Peptide identity and purities were confirmed using ESI-MS and RP-HPLC monitoring at $\lambda_{\text{abs}} = 214 \text{ nm}$.

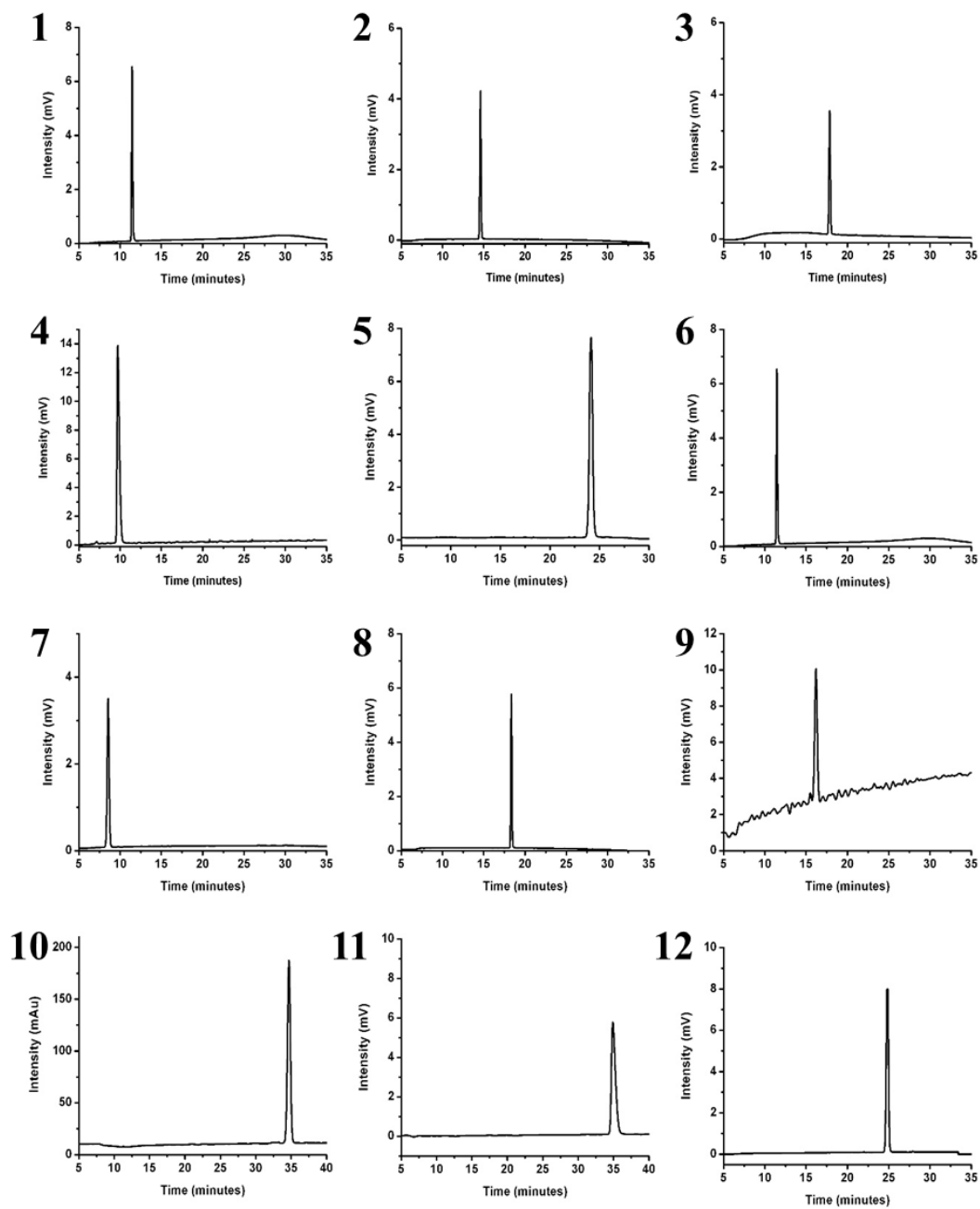


Figure 2.6 Purified HPLC chromatograms for monomers 1-12.

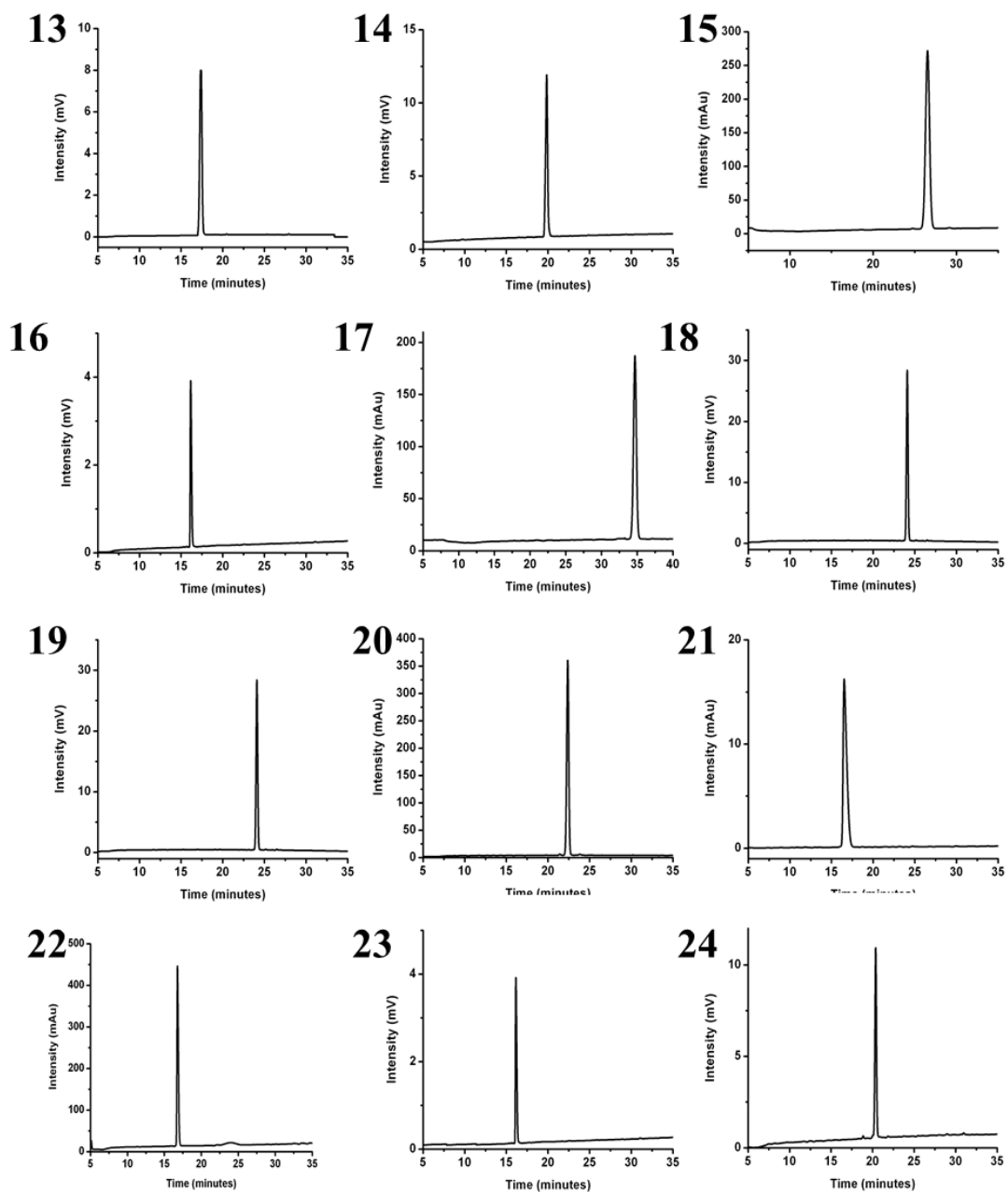


Figure 2.7 Purified HPLC chromatograms for monomers 13-24.

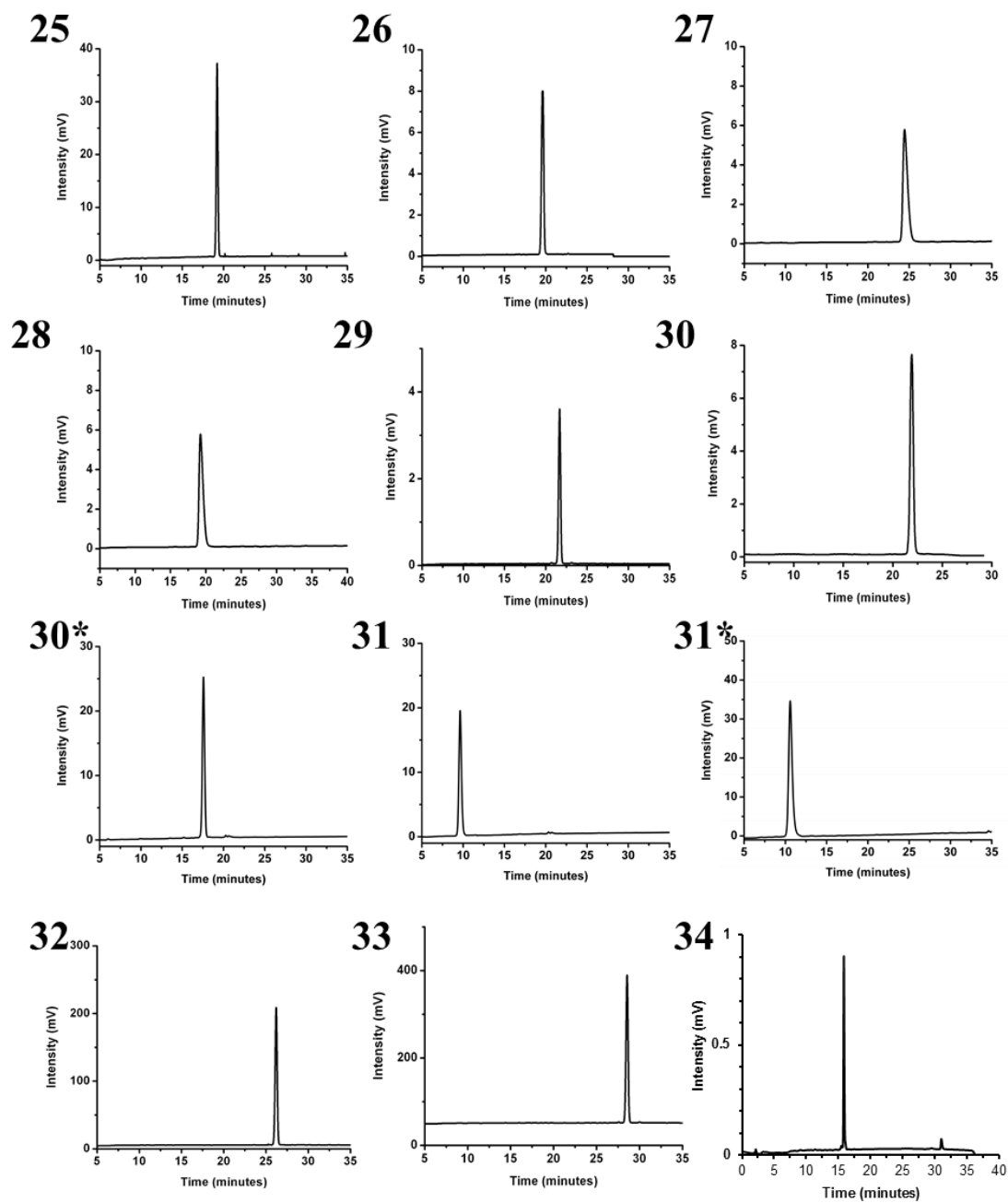


Figure 2.8 Purified HPLC chromatograms of peptide monomers 25-34.

Table 2.3 Summary of peptide monomer characterization.

Monomer	Peptide Sequence	ESI Calc	ESI Obs
1	NorGFPLI	690.31	691.4
2	NorGAPLI	614.34	615.5
3	NorGVPLI	642.37	643.5
4	NorGRPLI	699.41	700.64
5	NorGSPLI	630.73	631.64
6	NorGKPLI	671.4	672.86
7	NorGNPLI	657.34	658.75
8	NorGTPLI	644.35	645.94
9	NorGMPLI	674.35	675.44
10	NorGQPLI	671.33	672.56
11	NorGHPLI	682.81	683.38
12	NorGWPLI	729.86	730.38
13	NorGCPLI	646.31	647.79
14	NorGYPLI	706.54	707.77
15	NorGDPLI	658.33	657.89
16	NorGEPLI	672.35	671.67
17	NorGFPLA	648.33	649.27
18	NorGFPLV	677.35	678.4
19	NorGFPLR	733.39	734.52
20	NorGFPLS	664.32	665.3
21	NorGFPLK	705.38	706.2
22	NorGFPLN	691.71	692.1
23	NorGFPLM	708.33	709.1
24	NorGFPLQ	705.8	706.3
25	NorGFPLE	706.79	707.2
26	NorGFPLW	763.88	764.2
27	NorGFPLT	678.34	678.8
28	NorGFPLH	714.35	714.8
29	NorGFPLY	740.35	741.3
30	NorGFPLD	682.32	682.9
30*	NorGFPLD(tBu)	748.38	748.7
31	NorGFPLC	680.3	680.9
31*	NorGFPLC(acm)	751.34	751.34
32	Nor(GFPLI) ₃	1077.5	1077.9
33	Nor(GFPLI) ₆	2707.5	2707.9
34	NorGnRH	1511.7	1510.9

2.5.4 Polymer Synthesis

The peptide monomers were polymerized via ROMP using Grubbs' modified second generation catalyst $[(\text{H}_2\text{IMES})(\text{pyr})_2(\text{Cl})_2\text{Ru}=\text{CHPh}]$. The catalyst (1 equiv) was dissolved in $\text{DMF-}d_7$ and added to peptide-norbornene monomer (20 or 200 equiv) in $\text{DMF-}d_7$ to a final

volume of 500 μ L in a J. Young NMR tube under inert environment. The resulting mixture was monitored by NMR and checked for completion at 1 hour, 2 hours, 3 hours and 24 hours. Once complete, or at 24 hours, the polymerization was quenched with ethyl vinyl ether (2 equiv). The polymers were characterized via SEC-MALS.

2.5.5 Standard SEC-MALS Conditions

Phenomenex Phenogel 5u 10, 1k-75k, 300 x 7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF)) using a Shimatzu pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 MW polystyrene standard at a flow rate of 0.75 mL/min.

2.6 Acknowledgments

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Chapter 3

Peptides Prepared as a High Density Brush

Polymer are Protected from Proteolysis

3.1 Introduction

Though there is an ever increasing number of peptide therapeutics reaching the market, there are numerous fundamental problems that limit the utility of peptides, and other biologics, *in vivo*. The main limitations that researchers have to overcome are low bioavailability resulting from the short half-life of peptides.¹⁻⁶ These are typically the result of instability of the peptides *in vivo* due to proteolytic degradation, as well as their poor ability to cross cell membranes, and rapid size dependent renal clearance. Of these, one of the largest obstacles to overcome is the proteolytic instability of peptides. There exist numerous endogenous enzymes *in vivo* whose purpose is to degrade foreign peptide material. Indeed, blood is 55% plasma, and plasma is composed of 120 different proteins, many of which are proteolytic enzymes.⁵ Peptides administered *in vivo* will circulate through the blood are therefore susceptible to cleavage by a wide variety of proteases, limiting their bioavailability. Orally ingested peptide therapeutics face the biggest barrier in the stomach where there exist gram quantities of pepsin.⁵ If peptides happen to survive the stomach conditions, they will then encounter 15 more peptidases as they pass through the small intestine.⁵ Furthermore, some peptide therapeutic targets reside in the cytosol of cells. Therefore, these materials need to access the interior of cells and often do so through endocytic pathways, along which there

also exists numerous lysosomal peptidases that can degrade peptides. Finally, many tissues and cells express a variety of peptidases extracellularly that can also degrade small peptides before they reach their therapeutic target. All in all, there is an extreme number of peptidases *in vivo* that a peptide must evade as it transverses through the biological milieu to reach its therapeutic target, and often times evading these proteases is not possible. Therefore, to advance the field of peptide therapeutics, strategies need to be developed to increase the stability of peptides by decreasing their proteolytic susceptibility.

There are numerous structural modifications to peptides that improve stability. Some of these modifications either change selected attributes of the parent peptide like the amino acid identity, whereas others completely change the chemistry of the peptide such as changing the chemical composition of the backbone. Figure 3.1 shows examples of modifications that will be outlined below, with the area that was modified highlighted in red.

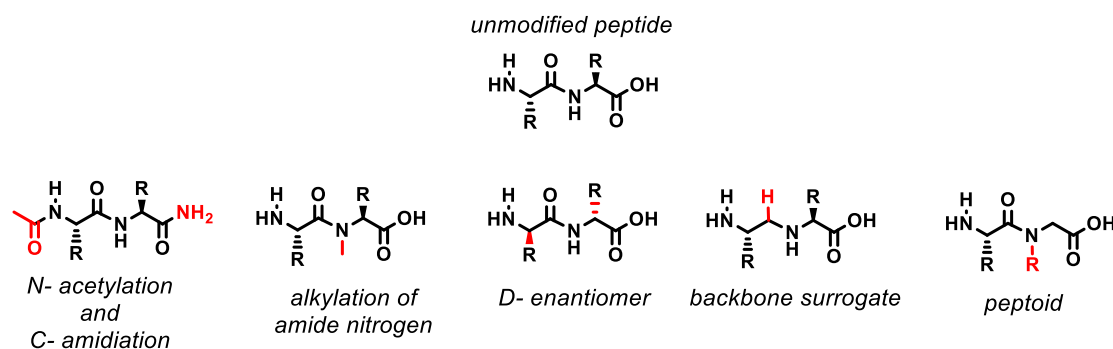


Figure 3.1 Common modifications to peptides that increase the stability.

Synthetically, one of the easiest approaches to reducing proteolysis is to simply modify select amino acids in the peptide sequence. These modifications can limit the proteolysis of endo- and exopeptidases. Exopeptidases cleave peptides starting at either the *N*- or *C*- terminus, whereas endopeptidases cleave specific sequences inside a peptide. To prevent exopeptidase cleavage, the *N*- or the *C*- terminus of the peptide can be modified so

that it is no longer recognized by enzymes. Typically, peptides with unmodified *N*- and *C*-termini are extremely susceptible, and many naturally occurring peptides have *N*- and *C*-termini modifications present to increase stability. For example, many neurological and hormonal peptides contain an *N*-terminal pyroglutamic acid that protects the free amine.^{7,8} Some peptides contain other protecting groups such as *N*-acetylation and *C*-amidation.⁹ For example, the melanocyte-releasing hormone peptide has these naturally occurring protective measures and therefore is only susceptible to endopeptidase degradation.¹⁰ Using nature as an inspiration, many groups have synthetically modified the termini of peptides, and using these simple protecting groups, the half-lives of peptides can be increased dramatically. For example, the *in vivo* half-life of a somatostatin analogue, a peptide hormone, was improved from 3 minutes to 400 minutes by simply acetylating the *N*-terminus,¹¹ and when peptide analogs of thymopoietin were doubly protected with *N*-acetylation and *C*-amidation, the peptide had no detectable degradation, a major improvement from a 1 minute half-life.¹² Though these simple modifications offer some protection, they typically exhibit no effect on the activity of endopeptidases.

There are significantly more strategies to protect peptides from endopeptidases. Before a peptide can be modified to prevent proteolytic instability, the peptide must be analyzed to find its most susceptible points. To do this, researchers typically incubate the peptide with various enzymes or tissue homogenates and analyze the product mixture by liquid chromatography-mass spectrometry (LCMS) to identify cleavage fragments.⁴ Once these points of susceptibility are determined, modifications can be made at those locations. One such modification is alkylation of the backbone amide nitrogen.^{13–15} This type of modification changes the chemistry along the amide backbone so that endopeptidases can no longer recognize and hydrolyze the backbone. For example, when the amide nitrogen groups are alkylated in Enkephalin, an endorphin-like peptide, the peptide had improved stability.¹⁶

In this case, there was also no loss of bioactivity, however, in some cases the decrease in the flexibility of peptides post alkylation can reduce activity of the peptide. N-methylation also often favors a *cis amide* conformation, which can result in a significant change in the structure of the peptide.¹⁷

Another simple approach to reducing peptide degradation involves replacing amino acids at susceptible points with their D enantiomers, or a different natural or non-natural amino acid. When modifying the side chain, researchers try to closely mimic the original amino acid to preserve the activity of the peptide. These approaches have been successful in preventing degradation of prosaposine, a neuronal peptide. Researchers changed two leucine residues to isoleucine, and an arginine residue to alanine. With these modifications, the peptide they called TX15-2 showed higher activity and stability with no loss of biological function.¹⁸ Replacing an amino acid with its D-enantiomer¹⁹⁻²¹ has also proven successful in reducing proteolysis. For example, the GNRH peptide was found to be cleaved at a lysine at the 6th position. When this lysine was replaced with D-Lys, the peptide showed higher activity due to decreased proteolysis.²²

Other modifications to peptides can be introduced to change the physical conformation of the peptide such as cyclizing or stapling the peptide. Cyclizing the peptide either through disulfide formation (heterodetic), or *N*- to *C*- termini amide bond formation (homodetic)²³⁻²⁶ can actually render the peptide more stable by reducing the conformational flexibility. For example, RGD, an integrin binding peptide, is more stable and active in its cyclized form.²⁷ Furthermore, many cyclized versions of vasopressin are also in clinical trials due to their increased in stability.²⁸ Stapling peptides, where a peptide is held together via a hydrocarbon brace, has been used to improve stability, decrease proteolysis, and improve binding stability of peptides to their targets.²⁹⁻³³ This has been successfully applied to numerous peptides, one of which is a stapled variant of the BCL-2 domain, which showed an

increase in proteolytic stability and binding target affinity. When this peptide was administered *in vivo* it showed improved half-life and better efficacy.³³

Many groups have also found success in reducing proteolysis by synthesizing peptide mimics. A peptide mimic can be synthesized where the peptide contains a backbone surrogate in which the amide bond is replaced with one that cannot be hydrolyzed by enzymes (i.e, by replacing the CONH with CH₂SH or CH₂NH).⁴ For example, replacement of the amide bond with CH₂NH in the octapeptide of equine angiotensinogen conferred the peptide with an increased binding affinity to its receptor and rendered it resistant to cleavage.³⁴ This modification can cause a peptide to lose activity because the partial double bond character of the amide bond confers the peptide with rigidity that might be necessary for its function.³⁵ Furthermore, these types of modifications can also alter the hydrophobicity of the peptide.³⁵

Another peptide mimic modification is a class of peptides called peptoids developed by Simon and coworkers. Peptoids are peptide mimics where the side chain of the amino acid is moved to the nitrogen of the amide.³⁶ Peptoids have been shown to increase the stability of the peptide because naturally occurring enzymes do not recognize these substitution patterns.³⁷ However, due to the removal of the hydrogen present on the nitrogen, the peptoid backbones can no longer serve as hydrogen bond donors, thus limiting their ability to form secondary structures and creating a more flexible peptide.⁴ This could potentially limit the function of the peptide *in vivo*. Nonetheless, peptoids have been used in peptide therapeutics, specifically in creating antibacterial peptides that display activity against some bacteria that are antibiotic resistant.³⁸

The underlying theme with all of the strategies outlined above is changing the peptide chemistry so that it is no longer recognized by enzymes. These strategies often modify the connectivity, parent peptide sequence, and backbone of the peptide, which can result in a

peptide with reduced activity. Multiple rounds of structure-function assays are often needed in order to optimize and restore the peptide's activity.³⁹

Often, a peptide cannot function with such extreme modifications, therefore many researchers have improved the stability of the peptide by conjugating the peptide to polyethylene glycol (PEG)^{40–44}, other polymer scaffolds,⁴⁵ or small molecule scaffolds.^{46–48} Using this conjugation strategy, the peptide sequence itself is not modified, however, synthesizing these materials often requires specific conjugation chemistries and multiple purification steps, which often result in low yields of the conjugate.

Herein, a new strategy for protecting peptides from proteolysis will be discussed. This strategy involves packaging peptides as high-density brush polymers (HDBPs) using *graft through* ROMP as described in Chapter 2. This technique offers many advantages in that only a very simple modification to the peptide is made, addition of an norbornene polymerization handle. Moreover, these peptides are directly incorporated into the material during polymerization, obviating the need for conjugation reactions and multiple purification steps.

Peptides packaged as HDBPs are investigated for their resistance to proteolysis by pepsin, trypsin and thrombin. Pepsin is an aspartic protease that is excreted in gram quantities in the stomach and is responsible for breaking down proteins into smaller peptides, allowing for further processing in the small intestine.^{5,49,50} Pepsin is active at very low pH values (less than 2) and preferentially cleaves at the *N*- terminal side of aromatic amino acids (tyrosine, phenylalanine and tryptophan). Pepsin can cleave after hydrophobic amino acids, albeit with lower fidelity. Trypsin is responsible for breaking down peptides even further into smaller oligoamino acids in the small intestine that can then be broken down into amino acids by other proteases to allow for absorption. Trypsin is a canonical serine protease that has a well-studied active site.⁵¹ Trypsin cleaves at the *C*- terminal side of arginine and lysine, except

when followed by a proline. Thrombin is another serine protease that cleaves preferentially between Arg-Gly bonds.⁵¹ Though thrombin is similar to the other serine proteases, it has a stronger substrate preference than more promiscuous serine proteases such as trypsin and chymotrypsin and therefore is the most extensively studied serine protease.⁵¹⁻⁵³ Thrombin is responsible for cleaving fibrinogen, resulting in blood coagulation. In table 3.1, some of the key features of these enzymes are listed in Table 3.1.

Table 3.1 Summary of Enzymes tested.

Enzyme	Type	MW (kDA)	Optimal pH	pI
Pepsin	Aspartic	34.6	1.5	2.2-3.0
Trypsin	Serine	23.3	7-9	10.1-10.5
Thrombin	Serine	36	8.3	6.35-7.6

3.2 Proteolytic Stability of Peptides Displayed as a HDBP to Trypsin

It was hypothesized that peptides displayed as HDBPs might be resistant to proteolysis due to steric exclusion of the enzyme from reaching its recognition sequence. To test this, two different trypsin substrates were tested where the location of the cleavage sequence was varied. Trypsin recognizes and cleaves after lysine (K) or arginine (R). The sequences tested were Nor-G**K**QLISGSGS as trypsin monomer 1 (TP1) and Nor-GSGSG**K**QLIS as trypsin monomer 2 (TP2) where K (bolded) is the recognition sequence for trypsin. When polymerized, TP1 will contain the recognition sequence closest to the backbone of the polymer, which is buried and less accessible to the solvent, and might therefore prevent trypsin access. On the other hand, TP2 has a recognition sequence that is

spaced further away from the backbone of the polymer and might be more accessible to the enzyme. These sequences also contained an asparagine, and a series of serine residues that were known to be easily polymerized by ROMP (see Chapter 2), and provided necessary hydrophilicity to ensure solubility in aqueous buffers. Each monomer was subjected to standard polymerization conditions with the ROMP initiator $(\text{IMesH}_2)(\text{C}_5\text{H}_5\text{N})_2(\text{Cl})_2\text{Ru}=\text{CHPh}$ with an M:I of 20:1 (Figure 3.2). Monomers were successfully purified and polymerized to completion (Figure 3.3, for polymer characterization data see Table 3.2).

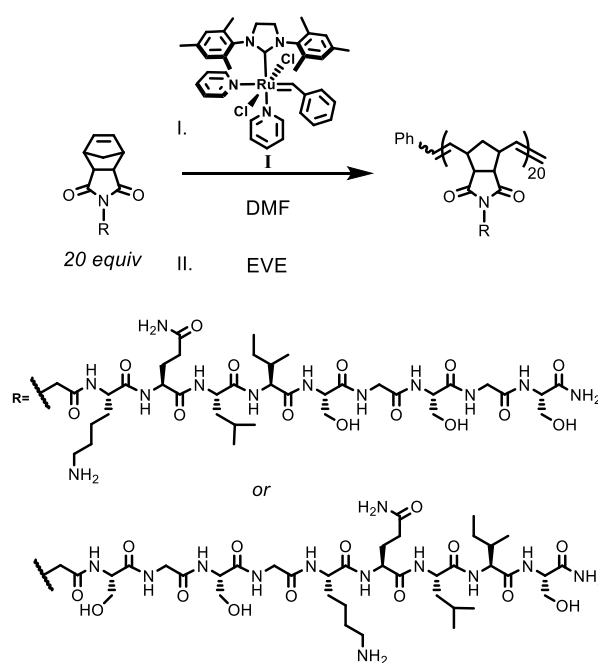


Figure 3.2 Polymerization scheme for peptide HDBP containing trypsin recognition sequences. The top sequence represents GKQLISGSGS and the bottom sequence represents GSGSGKQLIS.

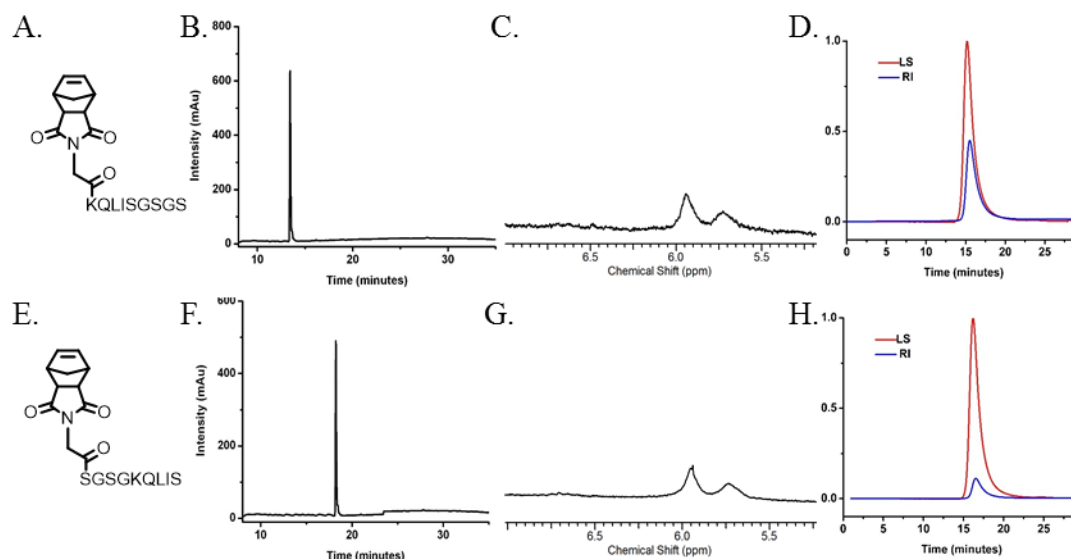


Figure 3.3 Characterization of trypsin monomers and polymers. A. Structure of trypsin monomer 1 (TP1). B. Purified HPLC chromatogram. C. ^1H NMR of final polymer showing complete polymerization of the monomer. D. SEC-MALS trace of the polymer. E. Structure of trypsin monomer 2 (TP2). F. Purified HPLC chromatogram. G. ^1H NMR of final polymer showing complete polymerization of the monomer. H. SEC-MALS trace of the polymer.

Table 3.2 Polymer characterization for trypsin peptide monomers.

Polymer	Peptide Sequence	M:I Targeted	M:I Actual	M_w/M_n
TP1	NorG-KQLISGSGS	20	21	1.04
TP2	NorG-SGSGKQLIS	20	19	1.02

Monomeric peptides and peptides packaged as HDBPs were subjected to trypsin cleavage at a ratio of 400:1 peptide/polymer:enzyme. Cleavage was analyzed every 45 minutes by HPLC. Monomeric peptides of TP1 and TP2 were readily cleaved by trypsin reaching 100% cleavage in less than 90 minutes (Figure 3.4). As expected, when polymeric TP1 was subjected to proteolysis, the polymer only reached ~6% cleavage after 550 minutes (Figure 3.4). When the trypsin recognition sequence was spaced further away from the backbone as in polymeric TP2, the peptide on the polymer was cleaved to ~95% after 550 minutes (Figure 3.4). In the case of TP1, the recognition sequence for trypsin was buried in the polymer, and likely sterically excluded the enzyme from reaching the K residue. Peptides might be most rigid nearest to the polymer scaffold, but more flexible towards the end of the

peptide, therefore when K is spaced further down the peptide sequence like in TP2, there exhibits more flexibility and less steric hindrance. Note that even in the case where cleavage of the peptide is seen in TP2, the efficiency is still not of that of the monomeric peptide. These results do suggest that peptides displayed as HDBP are protected from proteolysis. Furthermore, these data show that the location of the recognition sequence plays a key role in the amount of protection in that burying the recognition sequence nearer to the backbone can allow for a greater protection from proteolysis.

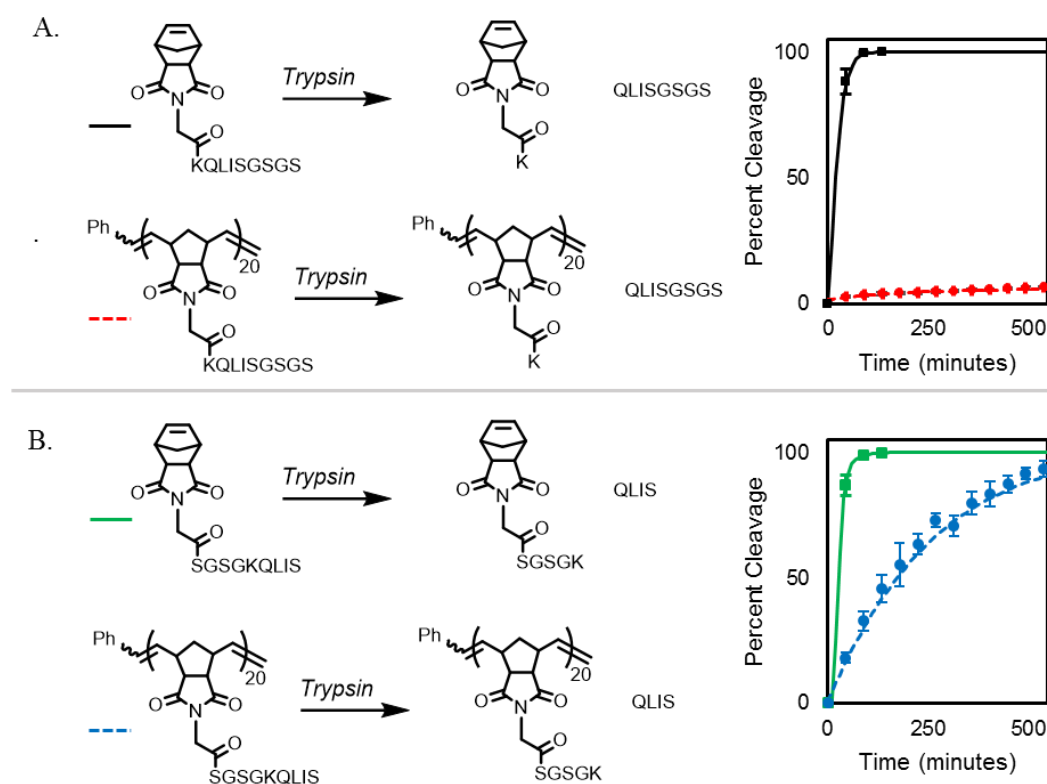


Figure 3.4 Proteolysis of A. TP1 peptide monomer and polymer, and B. TP2 peptide monomer and polymer.

3.3 Proteolytic Stability of Peptides Displayed as HDBPs to Pepsin

Pepsin, similar to trypsin, is a fairly promiscuous enzyme that cleaves after aromatic amino acids such as phenylalanine, tryptophan, and tyrosine. Here, a pepsin recognition sequence was synthesized that incorporates a tyrosine residue (Y), as tyrosine is also amenable to ROMP. The sequence chosen was Nor-Aha-GSGYGRR, which contains hydrophilic amino acids that are well tolerated by ROMP with our initiator (Figure 3.5).

The peptide was synthesized with the protecting groups present on the side chains of the residues S, Y, and R in order to target high degrees of polymerization in good yield and low dispersity. After purification, the peptide was subjected to standard polymerization procedures targeting an M:I of 15:1 and 60:1. Upon complete monomer consumption as indicated in ^1H NMR, polymers were terminated with ethyl vinyl ether (EVE) and analyzed by SEC-MALS to determine molecular weight and dispersity (Figure 3.6 C-F and Table 3.3).

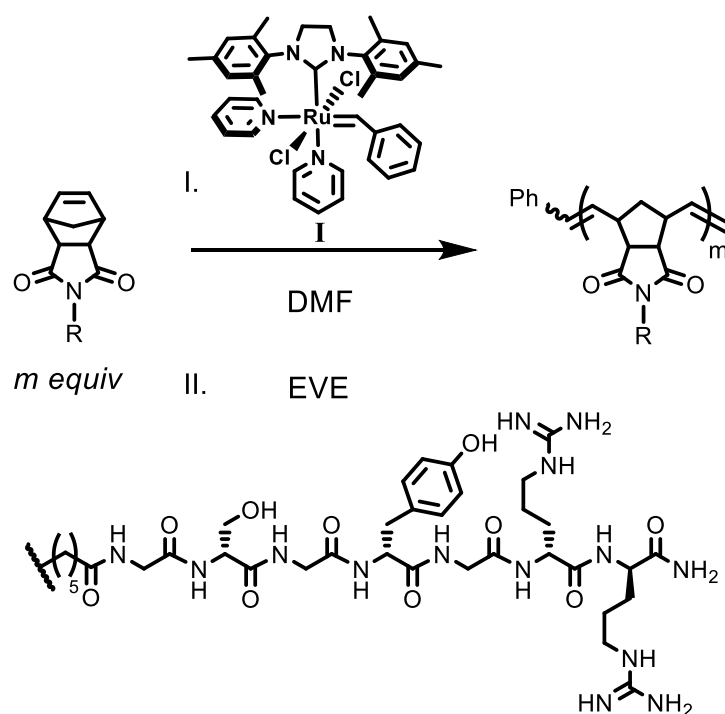


Figure 3.5 Polymerization scheme for the pepsin peptide where $m=15$ (PE1) or $m=60$ (PE2).

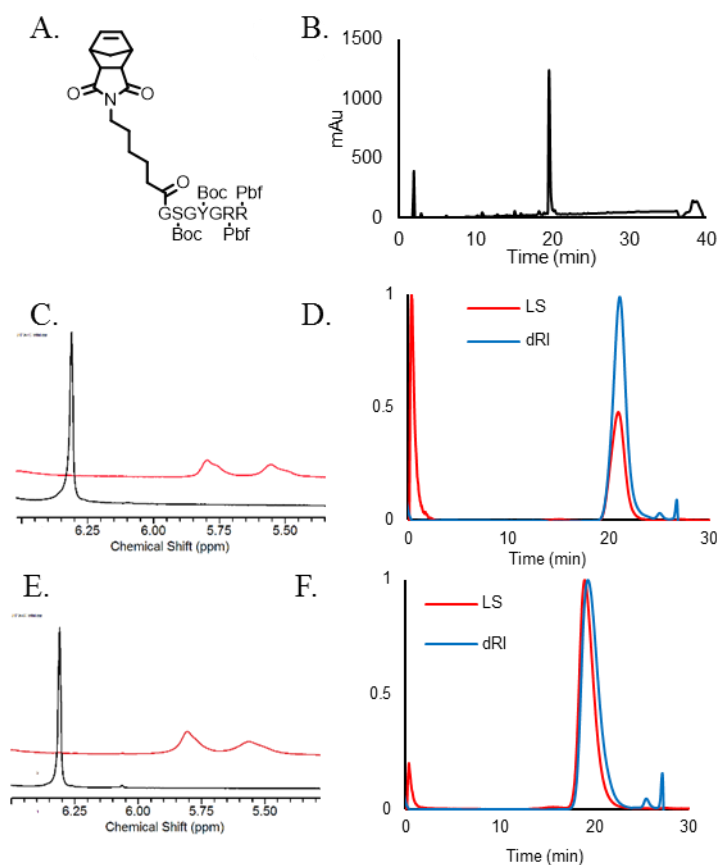


Figure 3.6 Characterization of NorAha-GSGYGRR peptide and polymers.

Table 3.3 Polymer characterization for pepsin enzyme substrate.

Polymer	Peptide	M:I Targeted	M:I Actual	M_w/M_n
PE1	NorAha-GSGYGRR	15	15	1.07
PE2	NorAha-GSGYGRR	60	63	1.15

In order to test the activity of pepsin in an authentic setting, the enzyme assay was conducted in a simulated stomach environment. To do so, a buffer using fasted state simulated gastric fluid (FaSSGF) powder was prepared. This powder contained all necessary salts that are present in the stomach during the fasted state and has shown to better predict the stomach environment than other simulated gastric fluids.⁵⁴ Peptide monomer and polymers were incubated with pepsin in FaSSGF buffer at pH 1.2 at a ratio of 20:1 peptide:enzyme. At

various timepoints (up to 180 minutes) aliquots were removed and quenched by adding enough base to reach pH of 8 that denatures pepsin, then analyzed by HPLC.

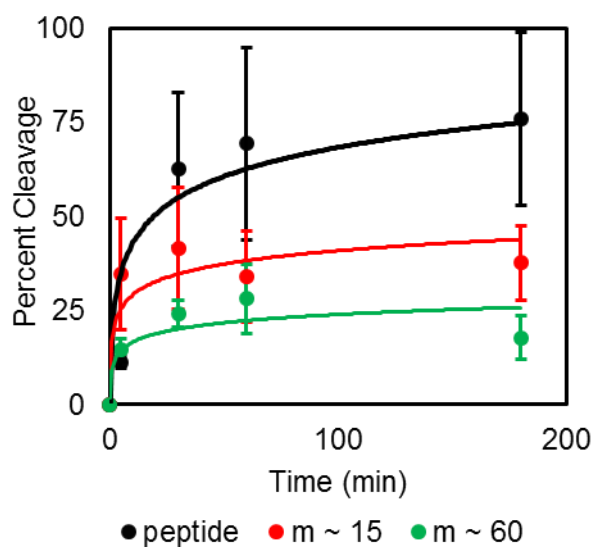


Figure 3.7 Pepsin cleavage of NorAha-GSGYGRR peptide and polymers.

When subjected to pepsin cleavage, the peptide reached ~75% cleavage after 180 minutes, however PE1 and PE2 only reached ~38% and ~18% respectively (Figure 3.7). The polymer that had a lower DP was cleaved to a greater extent than the higher DP polymer, suggesting that increasing the DP could increase the protection from proteolysis. For each polymer, the peptides were more resistant to pepsin when displayed on an HDBP than when free in solution.

Note that this protection seen in this example is extraordinary. The conditions for this digestion assay were extremely harsh (low pH, high enzyme concentration), and yet the peptide was protected from proteolysis when displayed as a HDBP. This is a promising result, too, as most peptide therapeutics must be systemically administered via injection and are not orally bioavailable due to the harsh conditions of the stomach.¹⁻⁶ Thus, HDBPs offer a protection to these conditions that could help render these peptides orally bioavailable.

3.4 Proteolytic Stability of Peptides Displayed as HDBPs to Thrombin

Thrombin, unlike trypsin or pepsin, requires a multi amino acid recognition sequence instead of a single amino acid. The sequence GALVPRGS was chosen for this study as it is known to be readily cleaved by bovine thrombin and is widely used in molecular biology.⁵⁵ To also increase the hydrophilicity of the peptide, a water soluble sequence was added to the C-terminus of the peptide. The complete sequence that was polymerized is NorGGALVPRGSGERDG (Figure 3.8). The thrombin monomer was subjected to standard polymerization conditions targeting M:I ratios of 10:1, 20:1, and 30:1. Upon complete monomer consumption, the polymers were terminated then characterized by batch mode static light scattering (SLS) (Figure 3.9, Table 3.4).

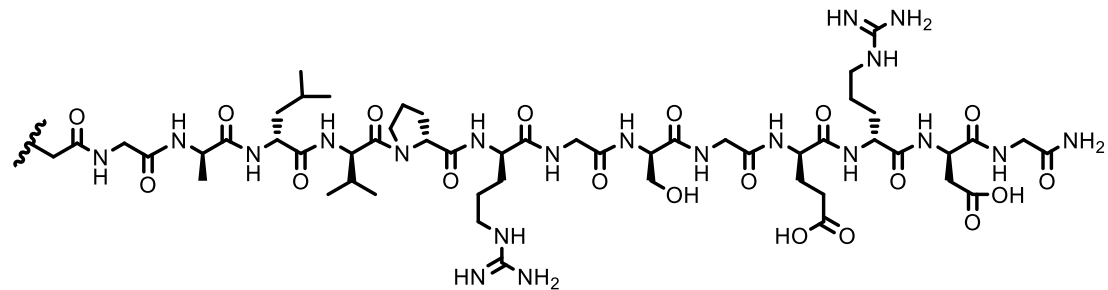


Figure 3.8 Polymerization scheme for thrombin peptide where $m=10$ (TR1), $m=20$ (TR2), and $n=30$ (TR3).

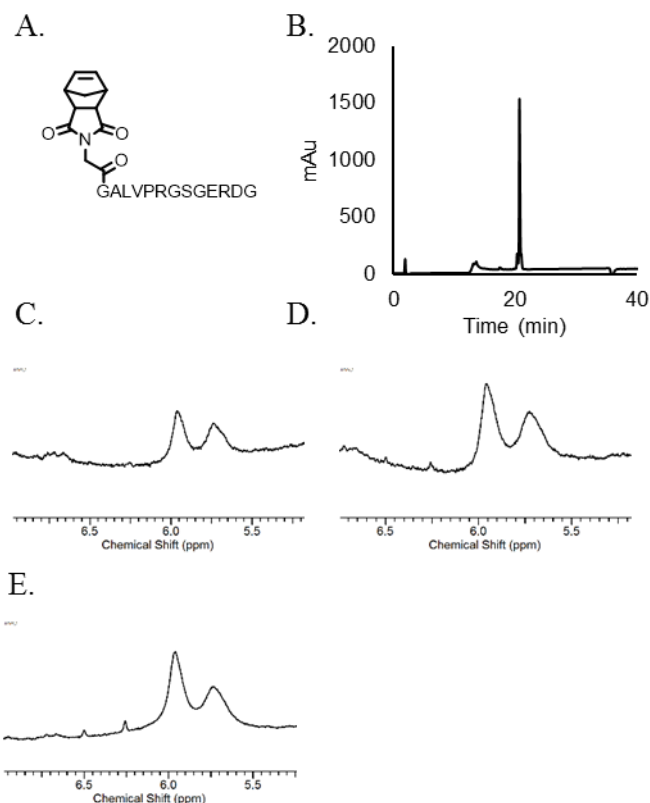


Figure 3.9 Peptide purification and polymerization characterization. A. Structure of Peptide monomer. B. RP-HPLC of purified peptide. C. ^1H NMR of polymer with a DP 10. D. ^1H NMR of polymer with a DP 20. E. ^1H NMR of polymer with a DP 30.

Table 3.4 Characterization of Thrombin peptide polymers by batch mode SLS.

Polymer	DP	M_n
TR1	11	16,800
TR2	23	33,300
TR3	30	43,900

The thrombin peptide and polymers were subjected to thrombin treatment and analyzed by RP-HPLC. The peptide monomer was readily cleaved to 100% over 12 hours, however when displayed as a HDBP, the peptides were only cleaved to around 50%, showing protection to proteolysis. Note that the polymers were not completely resistant to proteolysis, however there did exist some protection.

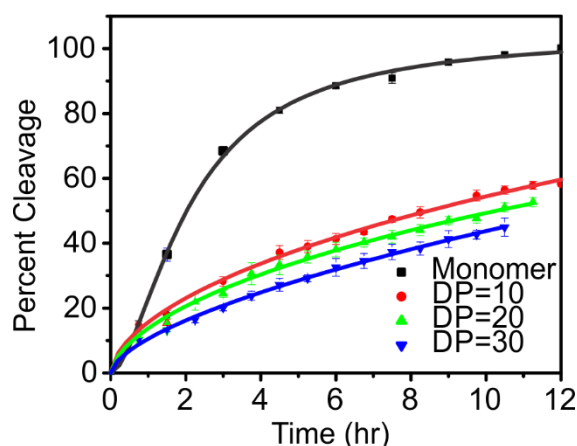


Figure 3.10 Thrombin cleavage of monomer and polymers.

With these data, protection from proteolysis is seen even in cases where the enzyme is more specific and recognizes longer sequences than just a single amino acid. This protection also seems to be dependent on the degree of polymerization with the larger polymers showing more resistance. Larger degrees of polymerization could not be tested in this case because the polymer could not reach high degrees of polymerization. Though the peptide does not show complete protection from proteolysis, it is protected to a greater extent than the peptide monomer which could lead to improved outcomes *in vivo*.

3.5 Conclusion and Future Outlook

Herein, protection of peptides displayed on HDBPs from proteolysis by pepsin, trypsin and thrombin was demonstrated. Each of these enzymes has unique sequence preferences and each operate under different conditions. In all cases, the peptide monomers were readily cleaved by the enzymes, whereas peptides displayed as HDBPs showed resistance. Resistance to proteolysis is dependent on the cleavage site location in that when the cleavage site is spaced further from the backbone, the peptide is more susceptible to

proteolysis. Resistance is also dependent on degree of polymerization in that larger molecular weight polymers showed a greater resistance.

It is believed that this resistance to proteolysis is a generalizable trend across a variety of proteases. Other work conducted in the laboratory has shown protection to substrates for matrix metalloproteinases.⁵⁶ Furthermore, a set of bioactive cell penetrating peptides were protected from proteolysis and maintained their bioactivity when packaged as HDBPs, even in the presence of a protease. Furthermore, it was computationally determined that the protection from proteolysis is likely due to a dense packaging of the peptide. A variety of polymers were synthesized in which the density of the peptide brush was varied by blending in a short oligoethyleneglycol spacer between peptide units in a controlled fashion, and it was found that decreasing the density of the peptide brush resulted in an increase in proteolysis.

Though the work described here, along with the other reports in the research group, show promise in the protection of peptides by displaying them as HDBP,⁵⁶⁻⁵⁸ there still needs to be further investigation of the limits of this protection in more biologically relevant media, such as serum. Furthermore, the protection from proteolysis should be investigated to see if it is generalizable across all polymeric platforms where high density brush formation is possible, especially polymers with biodegradable backbones which is necessary for translation of these materials *in vivo*. These key pieces of data are necessary to ensure utility of this strategy *in vivo*.

3.6 Methods

For general methods, see section 2.5.1. For peptide synthesis protocol, see section 2.5.2. For polymer synthesis, see section 2.5.4. For standard SEC-MALS conditions, see section 2.5.5.

3.6.1 Enzymatic Studies with Trypsin.

Dry polymer was dissolved in Tris buffer to yield a 2 mM solution. Next, 20 μ L of Trypsin (1 mM), 20 μ L of monomer or polymer (2 mM), 20 μ L Tris buffer (250 mM Tris pH 7.2, 1500 mM NaCl, 500 mM CaCl₂) and 140 μ L of water were added to a HPLC vial and loaded in the autosampler carousel and a sample immediately taken for injection at 1 min. Subsequently, the reaction was monitored via RP-HPLC (10-80% buffer B) with an injection every 45 minutes following the initial injection.

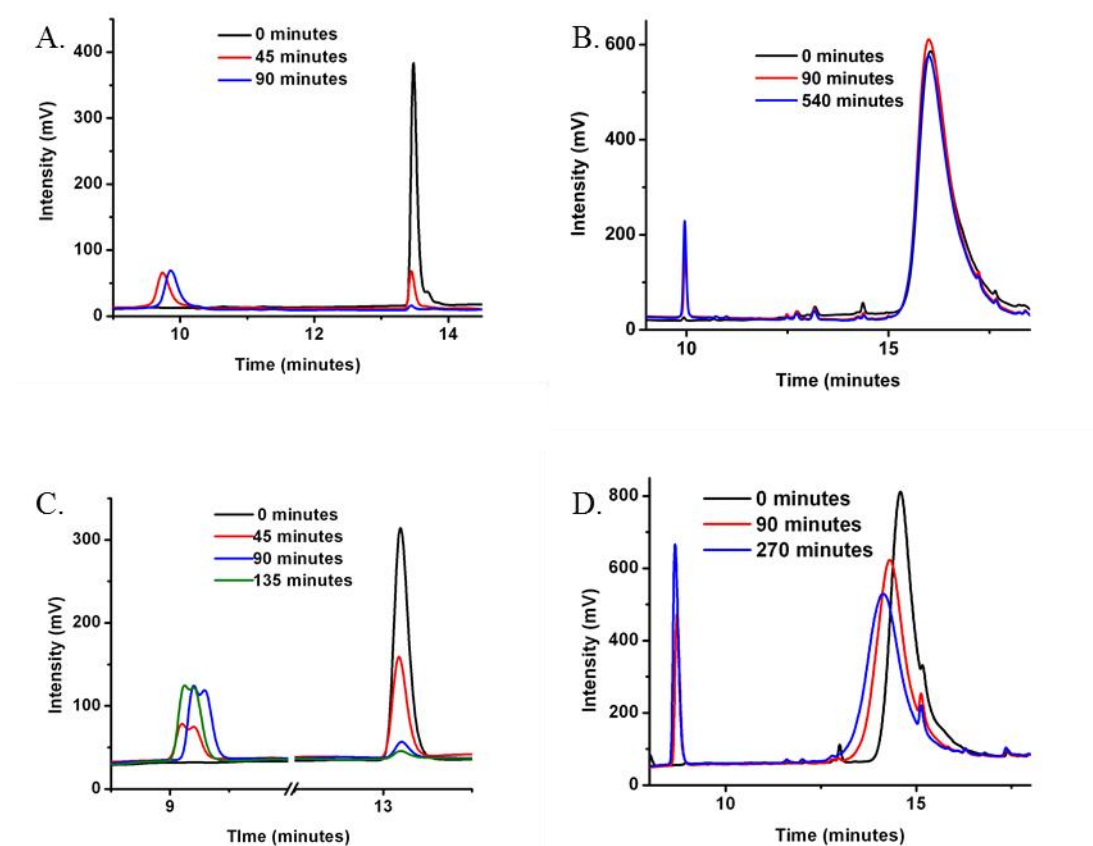


Figure 3.11 HPLC traces of trypsin cleavage studies. A. Trypsin cleavage of NorG-KQLISGSGS monomer. B. Trypsin cleavage of NorG-KQLISSGSGS polymer. C. Trypsin cleavage of NorG-SGSGKQLIS monomer. D. Trypsin cleavage of NorG-SGSGKQLIS polymer.

To quantify the cleavage products by HPLC, standard curves of the expected cleavage products of the two peptide (QLISGSGS and QLIS) were made. Purified peptides were dissolved to a final concentration of 1 mM in 10% buffer B (ACN in 0.1% TFA), and serial dilutions were made (500, 200, 100, 60, 40, 20 μ M). These dilutions were analyzed by RP-HPLC by relating the peak area to concentration.

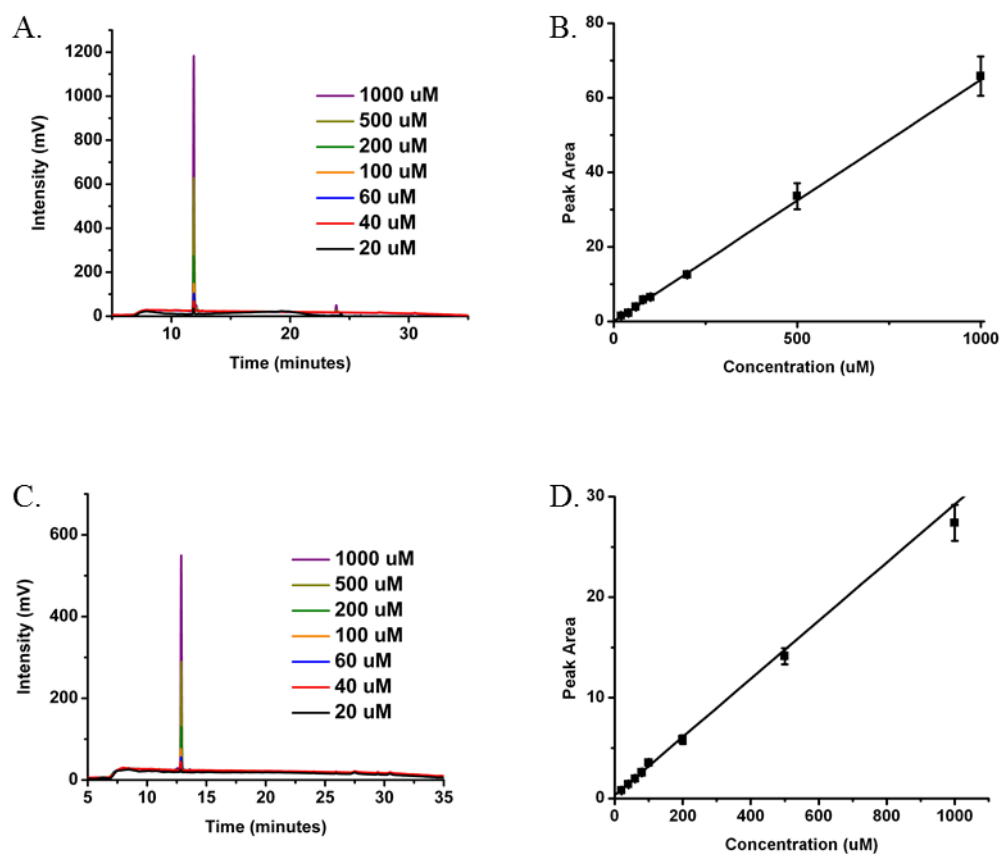


Figure 3.12 HPLC traces and standard curves for cleavage fragment A-B. KQLISGSGS and C-D. KQLIS.

3.6.2 Enzymatic Studies with Pepsin.

For the pepsin digestion assay, FaSSGF buffer was prepared by dissolving 1g NaCL in 500 mL (34 mM), and adjusting the pH to 1.6. Then 30 mg of FaSSGF powder (Biorelevant media, London) as added.

For the assay, a 10X solutions of peptide/polymer and pepsin were made. Peptide and polymer solutions were prepared by dissolving in water (pH 1.6) (6000 μ M with respect to peptide). Pepsin solution was made by suspending pepsin in water (pH 1.3) (500 μ M). 1x of peptide or polymer were added to 1.2 mL of FaSSGF buffer and heated to 37 degrees Celsius. 1x of pepsin was added and 100 μ L aliquots were removed at timepoints (0, 5, 10, 30, 60, 120, 180 min) and quenched with 40 μ L of 160 mM solution of NaHCO₃ to raise the pH to 8, irreversibly denaturing pepsin, the heated to 95 degrees Celsius for 1 minute. Samples were analyzed by RP-HPLC using a gradient from 1-60% buffer B (0.1% TFA in ACN).

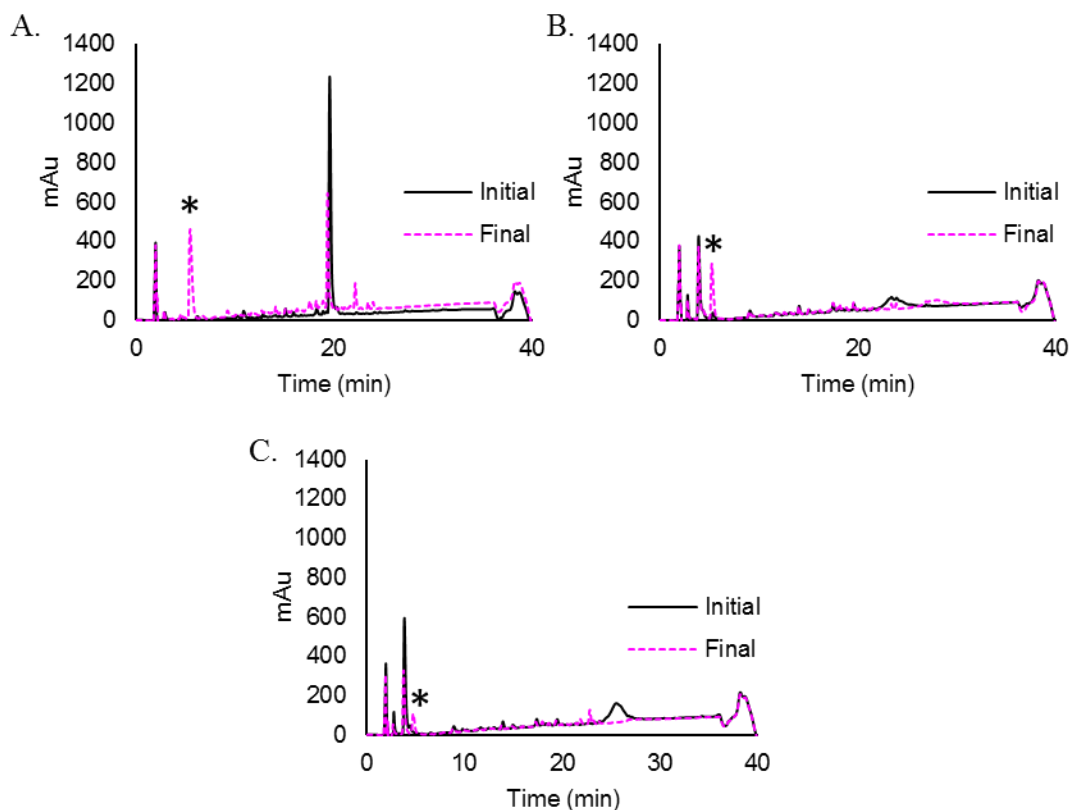


Figure 3.13 Raw HPLC trace of Pepsin peptide and polymers before and after (180 min) addition of Pepsin. A. Peptide. B. Polymer dp 15. C. Polymer DP 60. The cleavage product is denoted with an asterisk (*).

3.6.3 Enzyme Studies with Thrombin.

Peptides and polymers were dissolved in PBS buffer (2.2 mM with respect to peptide). Thrombin (10 units) was added to each sample and an HPLC trace was immediately obtained followed by subsequent HPLC injections every 45 minutes. Percent cleavage was determined by analyzing the peak area of the cleavage products.

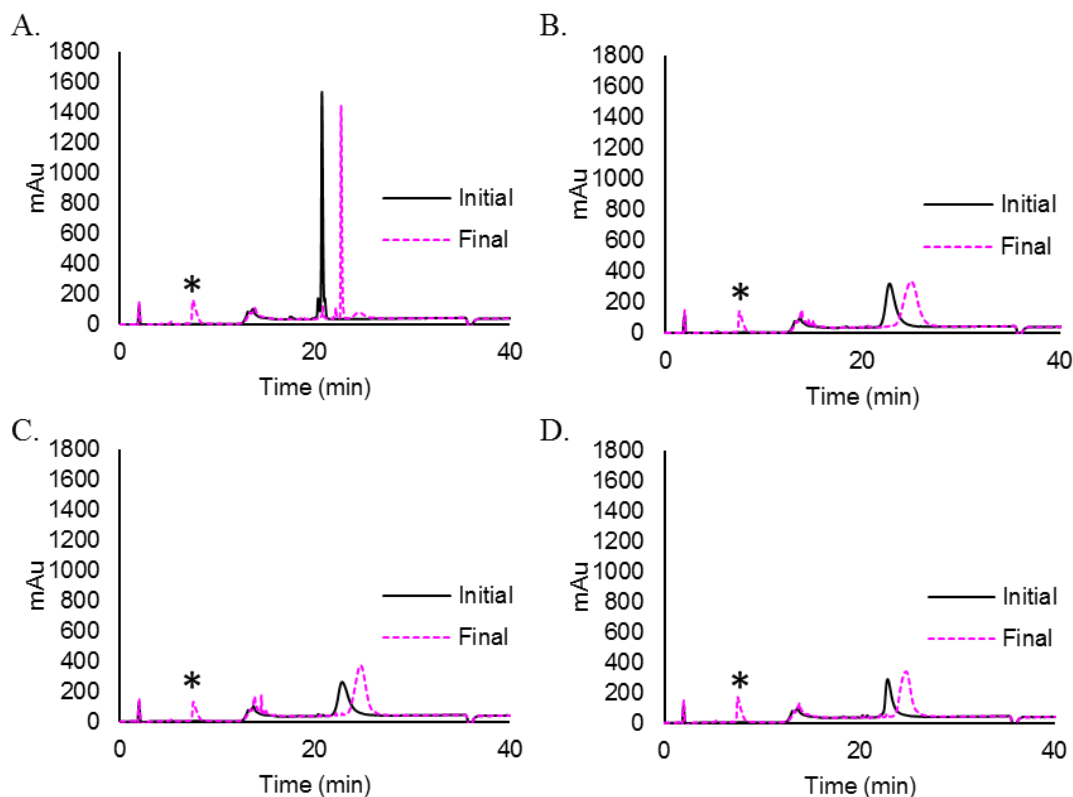


Figure 3.14 Raw HPLC traces of monomers and polymers treated with thrombin. A. Peptide monomer. B. Peptide polymer at DP 10. C. Peptide polymer at DP of 20. D. Peptide polymer at DP 30.

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High Density Brush Polymers Resist Proteolysis and Retain Biactivity.” *J. Am. Chem. Sci.*, 2014, 136 (43), 15422-15437. Copyright 2014 American Chemical Society. Permission to use copyrighted images and data in the manuscript was also obtained from Angela P. Blum, Jian Yin, Dustin T. Crystal, Anthony M. Rush, Michael E. Hahn, and Nathan C. Gianneschi. The dissertation author is the second author on this manuscript.

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Chapter 4

Activating Peptides for Cellular

Internalization by Preparing Peptides as High

Density Brush Polymers

4.1 Introduction

Many therapeutic targets of peptides reside in the cytosol of cells, yet most peptide therapeutics lack the ability to cross cellular membranes. Typically, for materials to cross the cellular membrane, they need to be hydrophilic enough for solubility in the cytosol, yet lipophilic enough to favor membrane translocation. Many peptides simply cannot cross cellular membranes due to the lack of lipophilicity. Yet, a special class of peptides known as cell penetrating peptides (CPP) can cross cellular membranes via active and passive diffusion without the need of lipophilicity. Over the years, a variety of CPPs have been identified, and are either comprised of multiple cationic amino acids such as lysine (Lys, K) or arginine (Arg, R), or an amphipathic alpha helical structure. Furthermore, using the properties that endow CPPs the ability to internalize into cells, numerous synthetic CPPs have been developed that show higher internalization efficiencies and can improve cytosolic access.¹

Amphipathic alpha helical CPPs are designed to alternate between hydrophobic and hydrophilic amino acids in a specific manner and form alpha helices where one face is hydrophobic, and the other is hydrophilic. An alpha helix contains 3.6 residues per turn,

therefore to form an amphipathic alpha helix, a hydrophilic residue should be incorporated at positions i , $i+3/4$, $i+7$, and so on.² For example, a synthetic amphipathic alpha helical peptide known as model amphipathic peptide (MAP) has the sequence KLALKLAKLAKAALKLA. MAP has amphipathic alpha helical character, and this allows for internalization of the peptide.^{3,4} MAP has been used to internalize a variety of bioactive molecules like protein cargo such as cytochrome *c*,⁵ and siRNA.⁶ Several other designed versions of MAP have been synthesized and show similar behavior of internalization. For example, the GALA peptide (sequence: WEAALAEALAEALAEHLAEALAEALAEALAA) was designed to mimic viral fusion proteins where at a low pH, it forms an alpha helix that can lyse membranes.^{7,8} This peptide can be used to destabilize membranes of endosomes and lysosomes allowing for more efficient delivery of cargo to the cellular cytosol. GALA is used to enhance the transfection efficiency of oligonucleotides.⁹ This is an improvement over traditional methods where oligonucleotides are complexed with polycationic carriers and internalize via endocytosis, accumulating in the endosomes and lysosomes and unable to escape. This lowers the transfection efficiency of the oligonucleotides. However, when GALA is the appended carrier, it forms an amphipathic alpha helical structure in the acidic endosomes and lysosomes, causing escape of the carrier into the cytosol and more efficient transfection.⁸ Variants of GALA that replace the glutamic acid (E) residues with lysine (K) residues (known as the KALA peptide) are also used to transfect DNA into cells.¹⁰ This peptide serves two purposes: complexing with DNA due to its polycationic nature, and endosomal escape due to its amphipathic alpha helical character.¹⁰

The most commonly used CPPs are cationic peptides containing multiple Arg and Lys residues. These peptides are derived from the internalization domain of the trans-activator of transcription (Tat) protein encoded by HIV. In the late 1980s, researchers discovered that Tat protein was able to efficiently cross the plasma membrane and localize in

the nucleus of cells.¹¹⁻¹⁶ Further studies of this protein led to the identification of the region responsible for cellular uptake, residues 49-57 known as the Tat peptide (sequence: RKKRRQRRR).¹⁷⁻²² Wender and coworkers determined that the cationic residues, more specifically Arg, enabled the cell penetration of these peptide fragments due to the reduction in internalization when Arg was replaced with Ala, a non-cationic amino acid.²³ This led to the discovery of Arg9 (sequence: RRRRRRRRR) which internalized into cells to an even greater extent than the Tat peptide. This work was corroborated by others, and it was found that it was the guanidinium present in the side chain of arginine that was responsible for uptake, and not the charge as other positively charged polypeptides (polylysine, polyhistidine) did not internalize as efficiently.²⁴⁻²⁶ When appended to cargo, Tat peptide and oligoarginine are able to carry a wide variety of cargo into cells including proteins, peptides, and small molecule drugs.^{2,21,27-36} Furthermore, many have incorporated guanidinium, the functional group on the side chain of Arg that enables cellular internalization, into a variety of molecules to give them the ability to internalize into cells.³⁷⁻⁴³

The mechanism of uptake of cationic CPPs is highly debated.⁴⁴ Some researchers have found that Tat and other Arg-rich peptides internalize after binding to cell surface heparin sulfate proteoglycans,⁴⁵⁻⁵⁰ yet others have found that internalization does not depend on HS proteoglycans.⁵¹ Once the CPP is associated with the cellular membranes, the mechanism of its internalization is unclear. It was first thought that Tat internalized in a non-energy dependent pathway due to cytosolic presence when incubated with cells at low temperatures.²⁰ However, this result was later found to be a result of fixation that led to artificial internalization.^{19,52} Groups now believe that it is an energy dependent endocytic mechanism, with evidence supporting micropinocytosis,^{53,54} clathrin mediated,⁵⁵ and/or caveolae mediated endocytosis.⁵⁶ For the CPP Arg8, there is some evidence suggesting the

peptide internalizes passively, yet there also is evidence of active endocytosis due to the presence of Arg8 in endocytic structures.^{53,57,58}

CPPs have been widely used in the delivery of therapeutic peptides and other bioactive cargo to cells *in vitro*,^{33–36} but more recently CPPs have been utilized *in vivo* for delivery of cargo. One of the first *in vivo* uses involved conjugation of Tat to β -galactosidase fusion protein followed by administration of the molecule via intraperitoneal injection (IP).⁵⁹ In this study, it was found that the protein-CPP conjugate was delivered to almost all tissues including the brain. Since this first report, CPPs have been used to deliver a wide variety of cargo to many different diseased tissues.^{27,34,36} For example, a Tat- conjugate to p53 peptide suppressed tumor growth post IP injection.^{60,61} Many pharmaceutical companies have also started investigating CPP drug conjugates (Kai Pharmaceuticals, Avi Biopharma, and Traversa Inc).⁶² PsorBan® designed by CellGate is in clinical trials for the topical treatment of psoriasis where cyclosporine is conjugated to polyarginine.⁶²

In summary, cell penetrating peptides have shown promise in numerous applications *in vitro* and *in vivo*, however CPP-peptide conjugates are still linear arrangements of peptides that can be susceptible to proteolytic degradation as discussed in Chapter 3. A more general strategy that can potentially protect a peptide from proteolysis while enabling the therapeutic to internalize into cells could have the potential to revolutionize the way peptides are packaged and delivered. The use of high density brush polymers (HDBP) for activating peptides for cellular delivery is described in this chapter.

4.2 Cellular Internalization of Peptides Displayed as High Density Brush Polymers

The strategy to activate HDBP for cellular delivery takes inspiration from a ROMP-based polymer developed by Kiessling and coworkers, where guanidinium units are appended to a preformed polymer to enable cellular internalization.^{38,39} Our strategy utilizes peptide-based monomers with a single Arg (R) appended to either the *N*- or the *C*- terminus. We hypothesized that the presence of a single R in a non-cell penetrating peptide sequence and subsequent polymerization would result in a cell penetrating polymer due to multiple Arg groups present in the polymer (Figure 4.1).

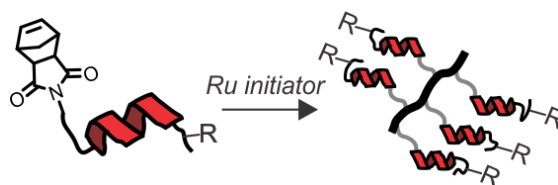


Figure 4.1 Scheme of incorporation of arginine into HDBPs.

To test this hypothesis, a series of HDBPs containing a non-cell penetrating peptide were prepared. The sequence GSGSG was chosen as it was previously shown to not internalize into cells as a peptide or HDBP.⁶³ One or two R were appended to either the *N*- or *C*-terminus of the peptide and then polymerized *via* ROMP (Figure 4.2). A similar set of peptides, but with 1 or 2 Lys (K) at the *C*- terminus, were also prepared and polymerized *via* ROMP (Figure 4.2). In order to ensure solubility of all polymers, a short block of oligoethylene glycol (OEG) was polymerized after the peptide (Figure 4.2). All peptide polymers were end labeled with a fluorescein chain transfer agent to allow for cellular uptake quantification via fluorescence in flow cytometry (Figure 4.2). Each peptide was also synthesized and labeled with fluorescein as a monomeric peptide control.

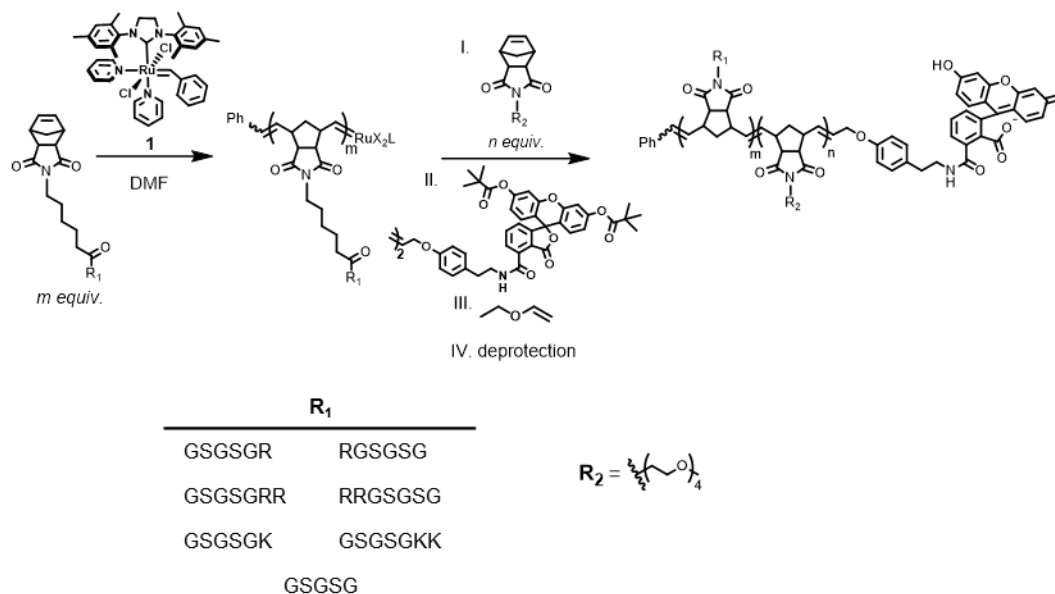


Figure 4.2 Scheme for synthesis of polymers containing peptides with cationic amino acids.

To determine if incorporation of a cationic amino acid into the peptide sequence, followed by subsequent polymerization into a HDBP could enable cellular internalization of a peptide, HeLa cells were treated with monomeric or polymeric peptides for 30 minutes, and then analyzed by flow cytometry analysis to quantify the fluorescence signatures of cells compared to the vehicle control (Figure 4.3). Prior to analysis, cells were washed with heparan sulfate to ensure that any fluorescence captured by flow cytometry is that of internalized peptides/polymers and not of peptides/polymers adhered to the cellular surface.⁶⁴ In all cases, the monomeric peptide controls showed fluorescence counts that were indistinguishable from the vehicle control (PBS) (Figure 4.3). Peptides that contained at least one R in the peptide sequence and polymerized to a degree of polymerization (DP) of 60 were able to penetrate cells as seen by the high fluorescence in the cells. These fluorescence signatures were similar to that of Tat, the canonical CPP (Figure 4.3).

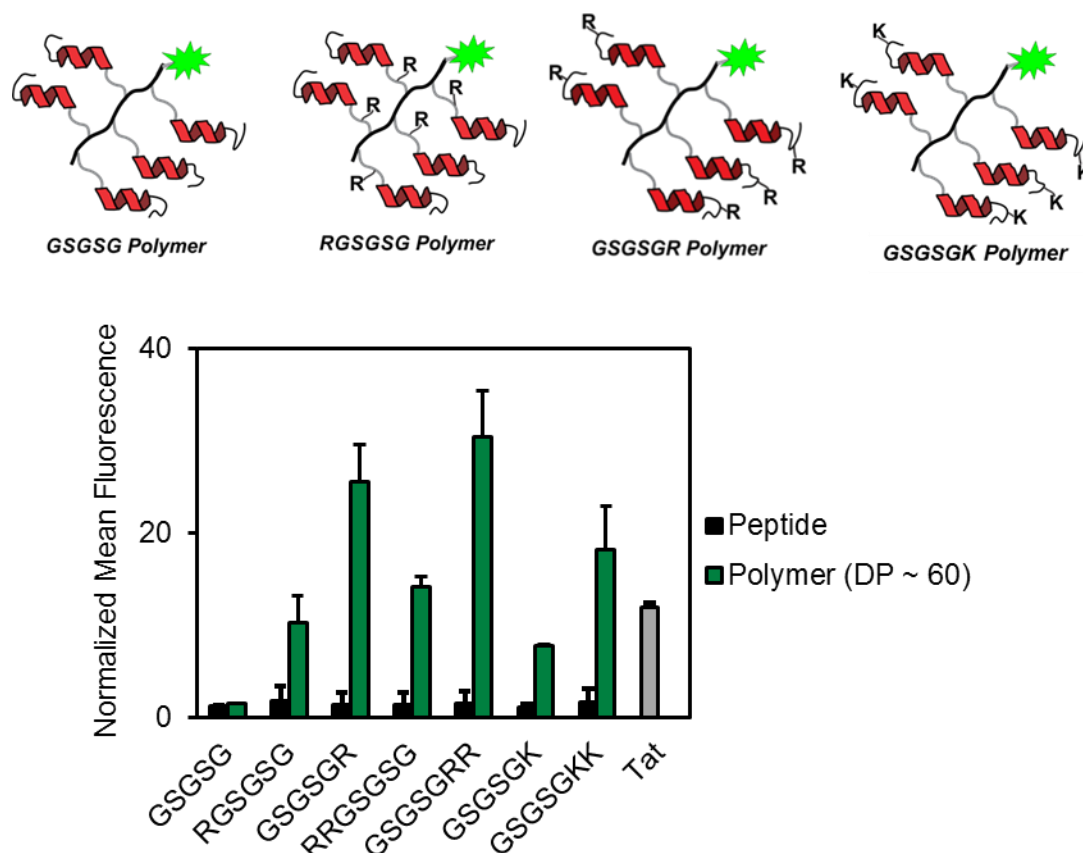


Figure 4.3 Flow cytometry data showing fluorescence signatures of cells treated with peptide polymers at a DP of 60 and the monomeric peptide counterparts. Data is normalized to the vehicle control, which is assigned to a value of 1.

Polymeric peptides in which R is appended to the C-terminus showed greater uptake than those with R appended to the N-terminus (Figure 4.3). This is likely due to the R being buried near the backbone of the peptide polymers when present on the N-termini, preventing solvent access and therefore interactions with the cellular membrane. Moreover, when two R residues are incorporated into the peptide sequence, the polymers exhibit greater fluorescence signatures likely due to the increased number of guanidinium units (Figure 4.3). Furthermore, peptides containing the cationic amino acid lysine (K) were also able to internalize into cells, despite the poor internalization of polylysine oligoamino acids compared to their polyarginine

counterparts.^{24,26,65} In all cases, peptides displayed as HDBPs containing any cationic amino acid (K, R) internalized to a much greater extent than polymeric GSGSG, indicating that the presence of any cationic amino acid is sufficient to allow for peptide brush polymers to internalize into cells (Figure 4.3). The internalization of cationic polymeric peptides was also found to be dependent on concentration of material, as well as on the DP. Higher concentrations and larger DPs showed greater fluorescent signatures in HeLa cells.⁶⁶

Cells were next analyzed using live cell confocal microscopy to confirm that the fluorescence signatures found via flow cytometry were representative of internalized peptide materials and not simply materials associated with the cellular membrane. Confocal analysis was performed on live cells to rule out artificial internalization reported in previous studies when cells were fixed.^{19,52} Cells were analyzed via Z stack analysis to investigate if materials were present throughout the cells or simply located on the cellular membranes. Cells treated with monomeric peptides exhibited minimal fluorescence signals; however, cells treated with polymeric peptides with a single R or K showed fluorescence signals throughout the cells, and not simply on the cellular membrane (Figure 4.4). The fluorescence signals seen in the cells treated with polymeric peptide materials was a mixture of diffuse and punctate fluorescence signal suggesting that the materials were localized both in endocytotic compartments (endosomes/lysosomes) as well as in the cellular cytosol. Cells treated with polymeric GSGSG showed minimal fluorescence, similar to the peptide monomer controls further confirming that the presence of a single cationic amino acid is both necessary and sufficient for internalization of polymeric peptides (Figure 4.4).

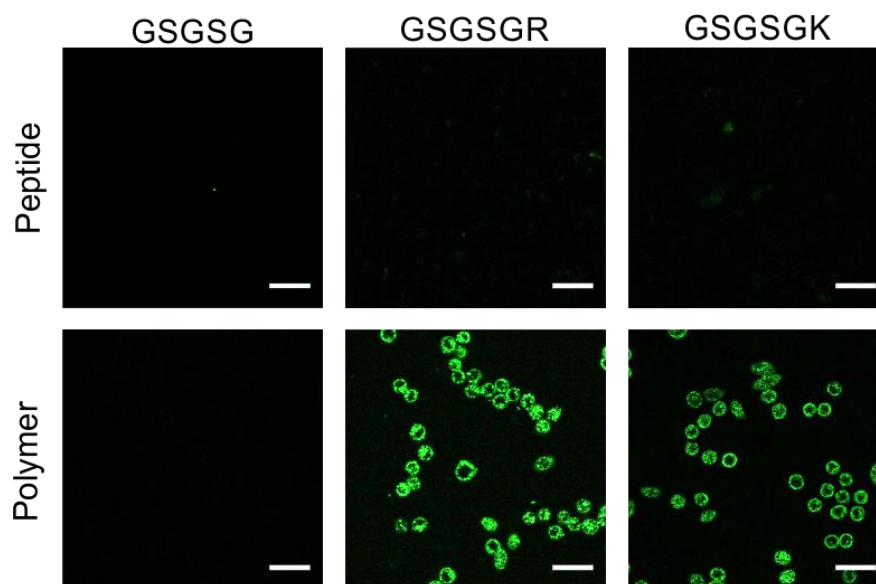


Figure 4.4 Z-stack confocal analysis of HeLa cells treated with fluorescently labeled monomeric and polymeric GSGSG, GSGSGR, and GSGSGK. Scale bars are 50 μm .

In order to better understand the localization of peptide materials after internalization, cells were stained with nuclear and lysosomal trackers and investigated for colocalization with fluorescent polymeric materials. To this end, CHO-K1 cells were treated with GSGSGRR polymers at a DP of 30 and stained with a nuclear stain (Hoechst 33342) and a lysosomal stain (LysoTracker Red) followed by analysis via live cell confocal microscopy (Figure 4.5). When cells were imaged using the 40x objective, there was significant colocalization with the lysosomes as indicated with an orange color suggesting that most of the polymeric material resided in the lysosomes (Figure 4.5A). However, when imaged at 100x, colocalization with the lysosomes was still present, however, cells also showed dim but diffuse green signal, suggesting cytosolic localization of the materials (Figure 4.5B). With these data, it is believed that the polymeric material is able to access the cytosol, however it seems that localization in the endocytosis vesicles is dominant.

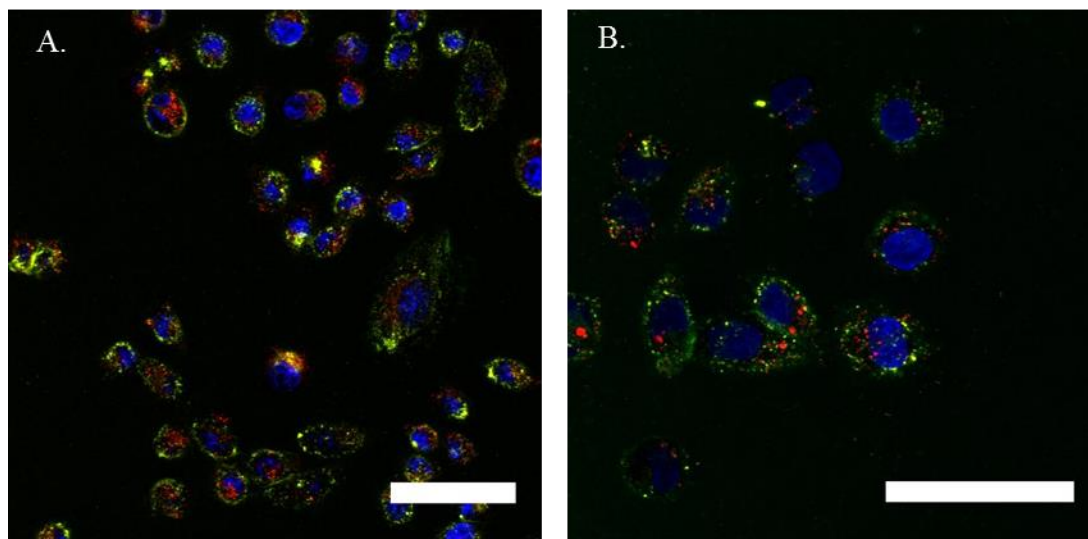


Figure 4.5 Live cell confocal microscopy of CHO-K1 cells treated with polymeric GSGSGRR for 30 minutes. The nucleus is stained with DAPI and false colored blue. The lysosomes are stained with lysotracker red and false stained with red. The polymeric peptides are labeled with fluorescein and false labeled green. Scale bars are 50 μm . A) Z stack analysis at 40x. B) Z stack analysis at 100x. Scale bars are 50 μm .

To ensure that peptides displayed as HDBPs were a viable option for delivering therapeutic peptides, the cytotoxicity of the materials was tested. HeLa cells were incubated for 3 days at various concentrations of GSGSGRR and GSGSGSKK HDBPs (DP=60) ranging from 1-100 μM . These polymeric materials were chosen as they exhibited the most significant cellular uptake. Note that concentrations in the uptake studies ranged from 2.5-12 μM , and significant uptake was seen at these concentrations. Across all concentrations tested, the polymeric materials showed no cytotoxicity (Figure 4.6). Peptides prepared as HDBPs are therefore a potential safe scaffold for the delivery of peptide therapeutics at the concentrations tested herein.

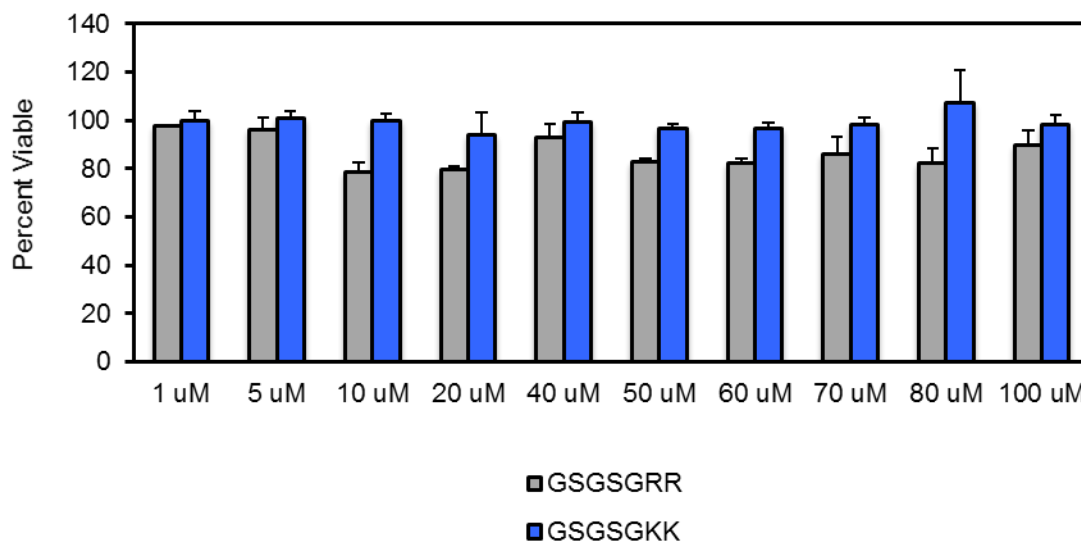


Figure 4.6 Cell viability of HeLa cells treated with GSGSGRR and GSGSGKK polymers to a DP of 60.

With these data, we believe that preparing peptides as HDBPs is a new method for activating peptides for cellular delivery, while protecting peptides from proteolysis (see Chapter 3). This strategy offers a solution to two of the key problems that limit the clinical utility of most peptide therapeutics. Moreover, the strategy is simple, and potentially broadly applicable for the packaging and delivery of peptide therapeutics, and the only requirement for successful internalization is a cationic peptide sequence.

4.3 Mechanism of Cellular Uptake of High Density Peptide Brush Polymers

The mechanism of the internalization of cationic polymeric peptides was next investigated. Many guanidinium based molecular transporters internalize into cells in a heparan sulfate (HS) proteoglycan process. This is due to the fact that the guanidinium group present on arginine is able to favorably hydrogen bond with the sulfated proteoglycans and

other anionic components of cellular membranes such as carboxylates and phosphates. To test if the GSGSGRR polymers also internalized in a similar fashion, the polymers were incubated with CHO-K1 wild type cells, and PGSA-745 cells. PGSA-745 cells are derived from CHO-K1 cells, however these cells lack glycosaminoglycans due to a defect in xylosyltransferase, a necessary enzyme for sugar transfer in glycosaminoglycan synthesis.

Homopolymers of the peptide GSGSGRR were prepared to DPs of 15, 30, and 60 and end labeled with a fluorescein chain transfer agent to have a handle to quantify internalization via flow cytometry. Polymers were added to CHO-K1 and PGSA-745 cells at concentrations 0.1, 0.5, and 1 μ M (Figure 4.7). Similar to previous results, as the degree of polymerization or concentration increases, the internalization into the wild type cell also increased (Figure 4.7). Most interestingly, cells lacking HS proteoglycans showed significant reduction in internalization when compared to the wild type counterpart (Figure 4.7). This suggests that the polymers interact with HS proteoglycans and that this interaction aids in internalization. In most cases however, there is still some background uptake in the cells lacking the HS proteoglycans suggesting there might also be other mechanisms that allow the polymers to internalize or that proteoglycan synthesis is perturbed, but not completely turned off in these cells.

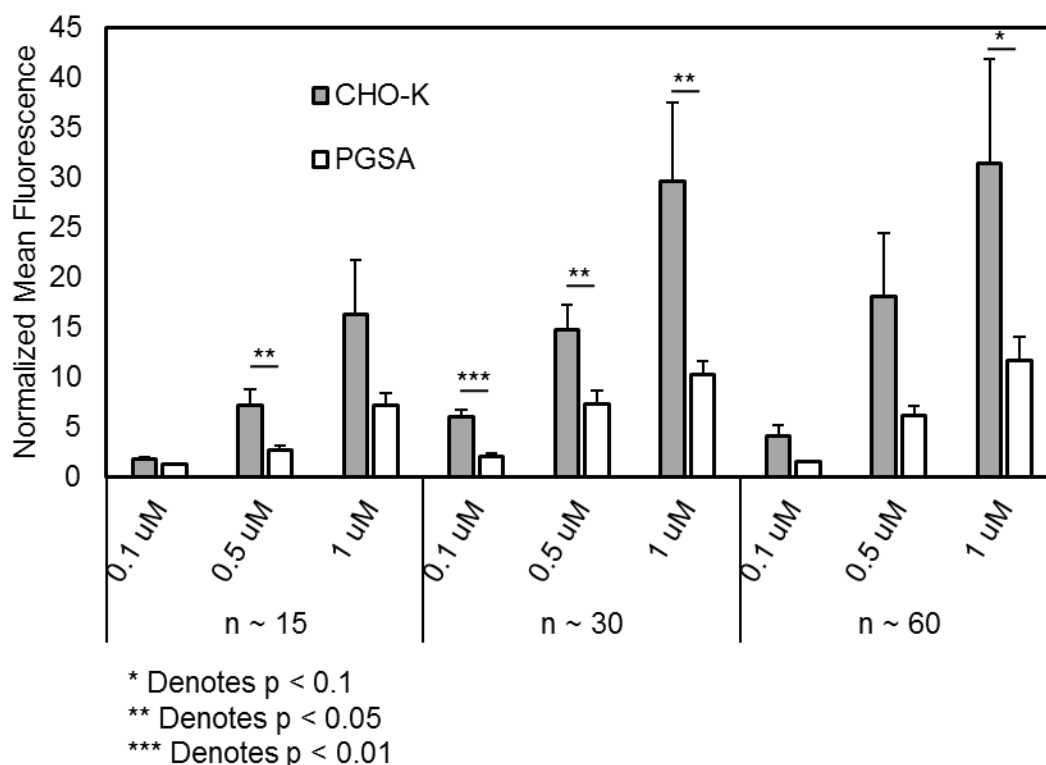


Figure 4.7 Treatment of GH0-K and PGSA cells with GSGSGRR homopolymers.

Next, the mechanism of internalization was investigated by treating cells with various inhibitors of internalization. HeLa cells were pretreated with inhibitors Dynasore, and methyl-beta-cyclodextran (M β CD), which inhibited clathrin-mediated endocytosis and caveolae-mediated endocytosis respectively, or incubated at 4 degrees Celsius to arrest all active transport across the membrane.⁶⁷ After treatment with the inhibitors, cells were then incubated with polymer materials with the peptide sequence GSGSGRR or GSGSGKK at DP 60, then analyzed by flow cytometry to measure the fluorescence intensities of the cells. Cells pretreated with all inhibitors and at low temperature showed significant decrease in fluorescence inside of the cells suggesting that the polymeric materials could not internalize as efficiently (Figure 4.8). These data suggest that the polymer materials internalize via active endocytosis pathways, and not simply via passive diffusion.

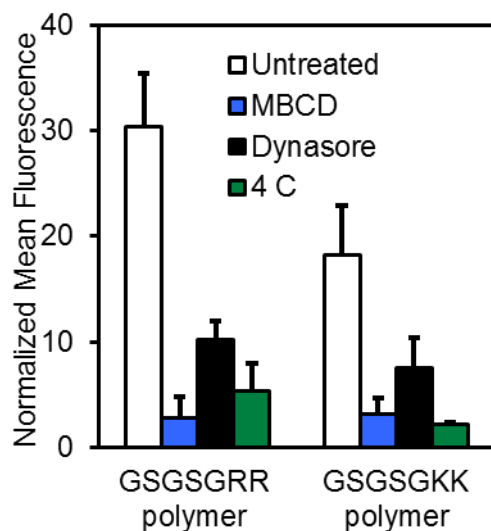


Figure 4.8 Mechanism of cellular uptake of peptide HDBPs using pharmacological inhibitors and low temperature incubation in HeLa cells.

4.4 Cellular Delivery of a Therapeutic Peptide

Described above is a strategy using *graft through* ROMP to prepare HDBPs where the peptides are protected from proteolysis and able to internalize into cells. To test if this strategy is viable for delivery of a therapeutic peptide, KLA HDBPs were investigated to determine if the material could internalize, and maintain bioactivity. KLA was originally developed as an antimicrobial peptide due to its cationic alpha helical structure that was able to lyse bacterial cell walls.⁶⁸ Since mitochondria have lipid membranes similar to bacteria, a group of researchers hypothesized that delivery of the KLA peptide to the cytosol of mammalian cells would cause mitochondrial lysis leading to cellular apoptosis.⁶⁸ However, despite having multiple copies of lysine (K) present in the sequence (KLA: KLAKLAKKLAKLAK), the peptide is unable to internalize into cells.⁶⁹ Previous groups have conjugated KLA to a CPP,^{68,70} prepared it as a multimer,⁶⁹ or appended the peptide to a

Figure 4.9 Polymerization scheme of KLA peptide into high density brush polymers via graft through ROMP.

HeLa cells were treated with monomeric and polymeric KLA peptides and quantified by flow cytometry to determine the internalization efficiencies of the materials tested. The monomeric KLA did not efficiently enter cells (normalized mean fluorescence = 3.8), however polymeric KLA to a DP of 5 showed fluorescence signatures similar to the canonical CPP Tat (normalized mean fluorescence = 11.9) (Figure 4.10). If prepared to higher DPs, the polymers showed an increase in internalization efficiencies showing that when prepared as a HDBPs, KLA is able to robustly internalize into cells.

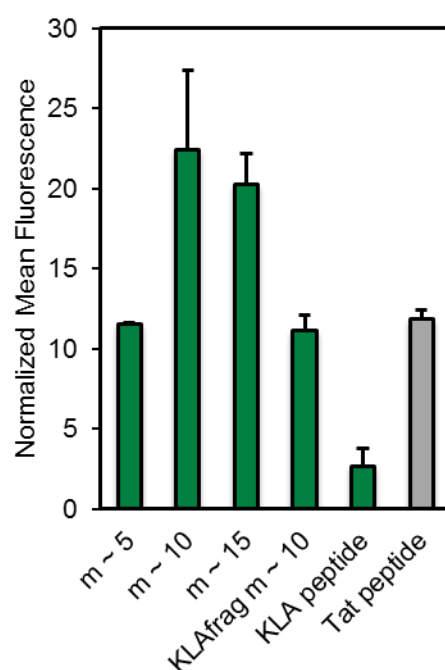


Figure 4.10 Cellular internalization of monomeric and polymeric KLA, KLA frag, and Tat peptide. Materials were incubated with HeLa cells at 2.5 μ M with respect to fluorophore. All materials were normalized to the vehicle control, which is assigned a value of 1.

To confirm the flow cytometry results, cells treated with monomeric and polymeric KLA were analyzed by live cell confocal microscopy (Figure 4.11). Monomeric KLA showed minimal fluorescence signals in the cells, however, polymeric KLA (DP = 10) showed fluorescence signatures throughout the cells and not on the cellular membrane,

suggesting the polymers were internalized and not simply associated with the membrane. The polymeric KLA also showed a mixture of diffuse and punctate fluorescence, signifying that the KLA is located both in endocytotic compartments and in the cellular cytosol. In order for KLA to function, it needs to access the cellular cytosol so that it can target mitochondria. Hence, the presence of diffuse fluorescence indicates that KLA might still be able to function when prepared as a HDBP.

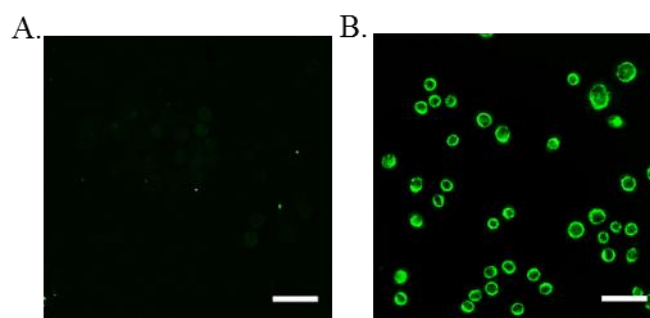


Figure 4.11 Live cell confocal microscopy of cells treated with A. monomeric KLA, and B. polymeric KLA. Scale bars are 50 μm .

Since the KLA peptide causes cellular apoptosis via a mitochondrial dependent process, cells treated with KLA polymers were interrogated for cell death via mitochondrial dependent apoptosis. To do so, it was first investigated whether polymeric KLA was cytotoxic using a cell viability assay. Cells were incubated for 3 days with various concentrations of polymeric KLA ranging from 1-100 μM where concentration is with respect to peptide and not polymer. Cells were also treated with monomeric KLA at the same concentrations. Cells treated with monomeric KLA showed no cytotoxicity across all concentrations tested; however, cells treated with polymeric KLA showed a dose dependent response across all degrees of polymerization (IC_{50} for DP 5, 10, 15 is 12.5, 25, and 30 μM respectively) (Figure 4.12). Interestingly, the polymer of lowest molecular weight was

cytotoxic at lower concentrations than the higher molecular weight polymers. It has been shown that large molecular weight structures have a difficulty escaping the endosomal compartments; therefore, the lower molecular weight polymer might be able to escape these compartments more readily and gain greater access to the cytosol and mitochondria.

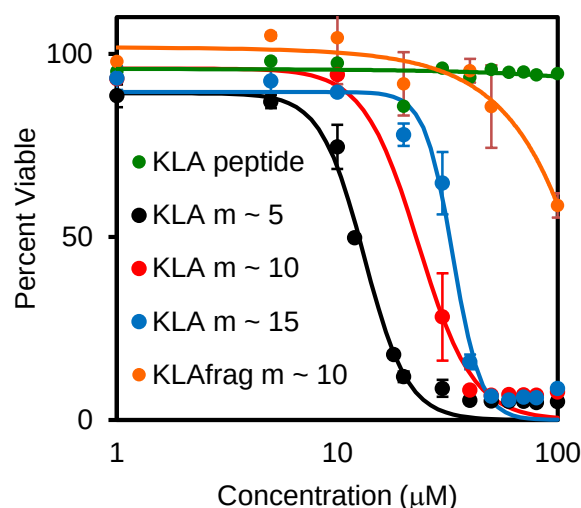


Figure 4.12 Cytotoxicity of monomeric and polymeric KLA, and polymeric KLA_{frag}.

To test if the full KLA sequence was necessary for cytotoxicity, a variant of the polymeric KLA was synthesized that only consisted of the first 8 amino acids in the repeat (KLAKLAK). This peptide polymer was termed KLA_{frag}, and was similar to that of KLA except that it lacked the alpha helical character (Figure 4.14). When cells were treated with various concentrations of polymeric KLA_{frag} at a DP of 10 the polymer was able to internalize into cells similar to KLA DP 5 (Figure 4.10), however this polymer showed no-dose dependent cytotoxicity. Note that polymeric KLA_{frag} DP10 has the same KLA content as polymeric KLA DP5. This suggests that the cytotoxicity seen in this study might be dependent on the presence of the alpha helix, and not simply the result of having multiple copies of lysine.

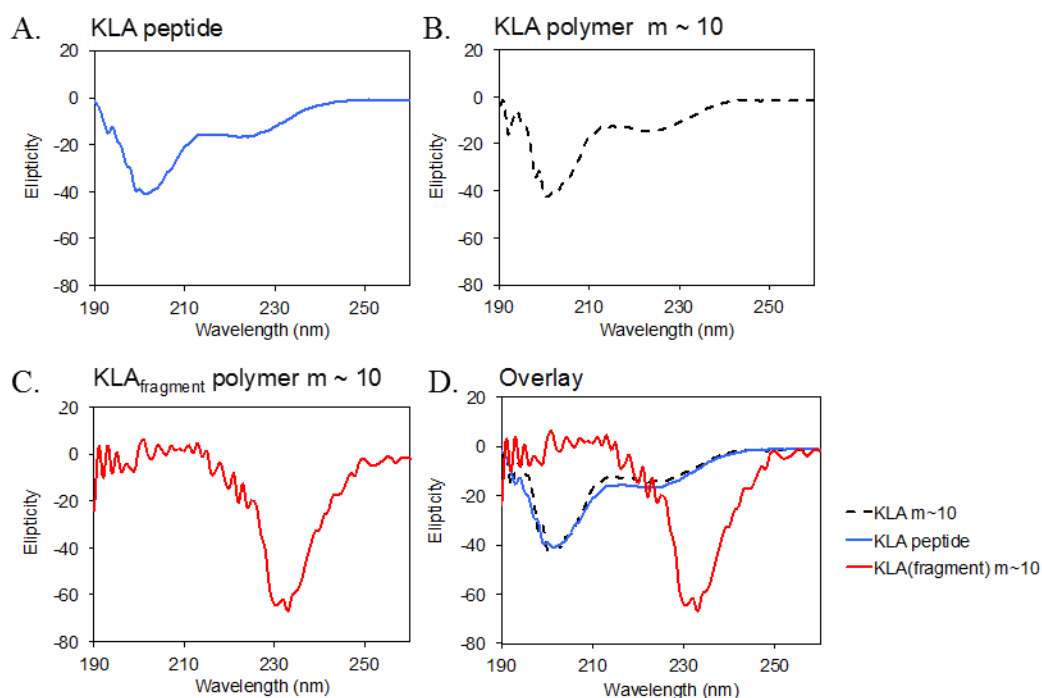


Figure 4.13 UV-Vis circular dichroism of KLA peptide and polymer ($m \sim 10$). Spectra of A. KLA peptide, B. KLA polymer, C. KLA_{frag} polymer and D. an overlay of all three. Notice that the spectra for the KLA peptide and polymer overlay nicely and show slight alpha helical character whereas the spectra for the fragment shows only random character.

Next, cells treated with polymeric KLA were studied to determine if the cytotoxicity was a result of mitochondrial dependent apoptosis. First, the mitochondria depolarization was investigated using the JC-1 dye. In mitochondrial dependent apoptosis, the mitochondria first become depolarized, reducing the mitochondrial membrane potential ($\Delta\Psi_M$). The JC-1 dye fluorescence properties are known to be dependent upon mitochondrial membrane potential ($\Delta\Psi_M$), such that in normal healthy mitochondria with a high $\Delta\Psi_M$, the dye forms J aggregates in the mitochondrial membrane that are red fluorescence. In contrast, when cells are undergoing apoptosis and the mitochondria have a low $\Delta\Psi_M$, the JC-1 dye can no longer form the J aggregates reducing the red fluorescence.⁷² This dye can therefore be used to determine how healthy the mitochondria are post incubation with polymeric KLA.

Cells were treated with either polymeric KLA (DP 5), or controls for 30 minutes prior to staining with the JC-1 dye. For a positive control, the small molecule carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as this small molecule is known to depolarize mitochondria. Cells were then analyzed by flow cytometry to measure the extent of red fluorescence in the cells. When compared to the vehicle control, cells treated with CCCP showed a significant reduction in red fluorescence (Figure 4.14). When cells were treated with polymeric KLA, about 41.3% of cells showed signs of disrupted mitochondria, suggesting that the polymeric KLA causes some sort of mitochondrial depolarization (4.14). Furthermore, this mitochondrial disruption is dependent on the alpha helical structure of the peptide because cells treated with KLA_{frag} showed no signs of disrupted mitochondria. These data further suggest that polymeric KLA causes cell death via a mitochondrial dependent process.

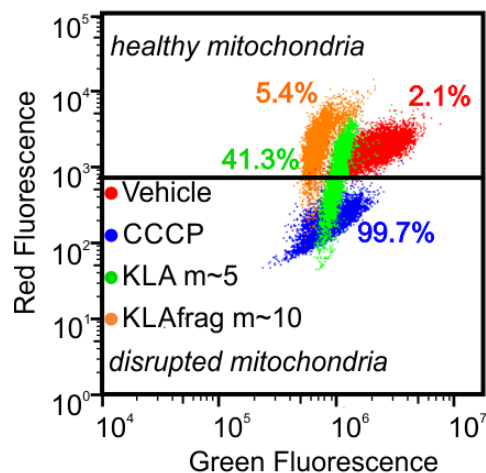


Figure 4.14 Mitochondrial membrane potential of HeLa cells treated with polymeric KLA and KLA frag.

To further investigate if KLA causes apoptosis and not simply necrosis, cells were treated with polymeric KLA and stained using propidium iodide (PI) and annexin V. PI is a

membrane impermeable dye that intercalates between DNA bases. Once intercalated, its fluorescence increases 20-30 fold. This dye can be used to label cells undergoing necrosis because necrotic cells have leaky membranes and cytosolic DNA. If a cell is undergoing necrosis, PI can transverse through the leaky cell membrane, intercalate between the DNA and fluoresce. PI is known to not stain apoptotic cells. Annexin V is a protein that binds to phosphatidyl serine.⁷³ In healthy cells, PS is expressed on the inside of the cellular membranes, but when cells begin to undergo apoptosis, PS is translocated to the outer leaflet, enabling annexin V to bind to cells.⁷⁴ Annexin V is labeled with GFP for green fluorescence detection; therefore, if cells are labeled with annexin V, it is likely they are undergoing apoptosis.

HeLa cells were treated with polymeric KLA to a DP of 10 and then stained with PI and annexin V, and then analyzed by flow cytometry (Figure 4.15). In these experiments, staurosporine was used as a positive control for apoptosis⁷⁵, and DMSO was used as a positive control for inducing cell death *via* both necrosis and apoptosis. Cells treated with polymeric KLA showed an increase in green fluorescence compared to the vehicle control in a manner similar to that of the staurosporine positive control suggesting that cells were undergoing apoptosis due to annexin V binding. Cells treated with KLA, staurosporine and the vehicle control did not show a fluorescence change when treated with PI, however, DMSO showed an increase in PI fluorescence. These data suggest that the polymeric KLA materials causes apoptosis but not necrosis.

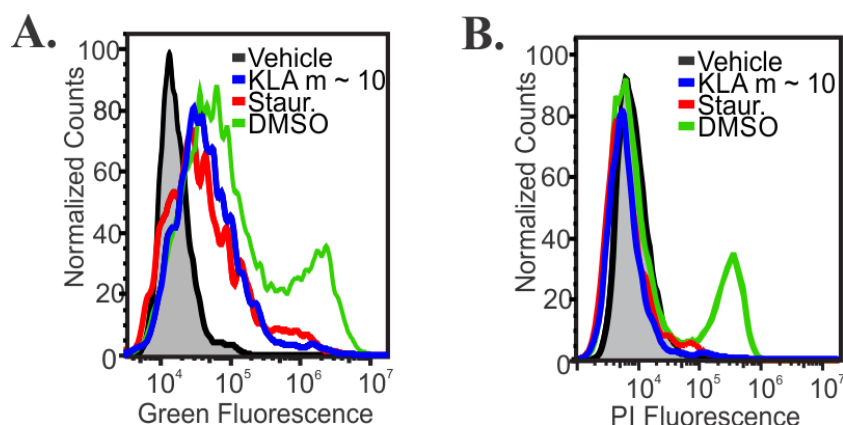


Figure 4.15 Annexin V and PI staining of cells treated with polymeric KLA. A. Cells stained with Annexin V, a marker for apoptosis. An increase in green fluorescence is indicative of apoptosis as seen in cells treated with a positive control for apoptosis, staurosporine (Staur.). B. Cells stained with PI, a marker for necrosis. An increase in PI fluorescence is indicative of necrosis as seen in cells treated with DMSO.

Finally, cells treated with polymeric KLA were investigated for apoptosis markers of caspase 3/7, which are upregulated in cells undergoing apoptosis.⁷⁶ Cells treated with polymeric KLA were investigated using a fluorogenic substrate for caspase 3/7, and thus an increase in fluorescence compared to the vehicle control is indicative of an upregulation of caspase 3/7. Indeed, HeLa cells treated with polymeric KLA followed by the fluorogenic substrate showed an increase in fluorescence compared to the vehicle treated cells; an indication of an increased expression in caspase activity, and therefore apoptosis (Figure 4.16).

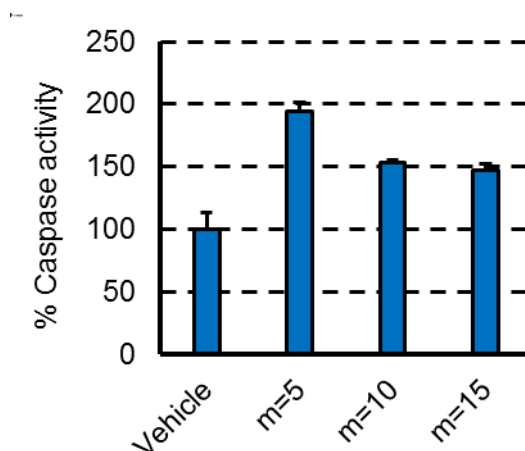


Figure 4.16 Caspase activity of cells treated with polymeric KLA. In all cases, polymeric KLA treated cells showed an increased fluorescence, indicating an increase in caspase activity. This suggests that the cells are undergoing apoptosis.

In conclusion, packaging the cationic therapeutic peptide KLA into a high density brush polymer activates the peptide for cellular internalization. KLA is able to maintain therapeutic function, namely inducing mitochondrial dependent apoptosis. Furthermore, studies also show that preparing KLA as a high density brush polymer protects the peptide from proteolysis.⁶⁶

4.5 Conclusion and Future Outlook

The *in vivo* utility of peptide therapeutics is thwarted due to the inability to cross biological membranes. Preparing peptides as HDBPs activates peptides for delivery to therapeutic targets inside of cells. This strategy also offers protection from proteolysis and has the potential to revolutionize the way peptides are packaged and delivered. Further investigation of peptide HDBPs is necessary to elucidate if *in vivo* delivery of peptide therapeutics is possible.

First, to date, KLA is the only therapeutic peptide investigated to determine if biological function is maintained when prepared as a HDBP. Other types of therapeutic peptides as HDBPs should therefore be investigated to determine therapeutic scope. For example, therapeutic peptides can act as receptor binding agonists or antagonists and preparing these peptides as HDBPs could reduce receptor binding of the peptide. Other types of peptide therapeutics are those that modulate enzyme activity through inhibition. An HDBP of a peptide is resistant to proteolysis due to steric exclusion (Chapter 3), and this might also prevent enzyme inhibitor peptides from working. Of course, if these types of therapeutic peptides fail to work on the HDBP scaffold, there are strategies to incorporate the peptide *via* a cleavable linker that can be cleaved at the site of interest, releasing high payloads of the therapeutic peptide.

Second, further understanding of the mechanism of internalization and fate of peptide materials is needed for the proper packaging and delivery of therapeutics. Polymeric materials seem to reside largely in the endosome, with some cytosolic access and this access seems to be dependent on the size of the polymeric material. This raises many questions: Are the cytosolic polymers there because of passive diffusion through the membrane? Are polymeric materials able to escape the endosomes and lysosomes? How much of the material is escaping the endosome? Is the ratio of polymer in cytosol versus endosome dependent on charge, shape, or size of the nanomaterial or the identity of the peptide? Answering these questions will enable modulation of the polymeric material to allow for greater delivery of therapeutic peptides across all biological membranes.

Third, peptide HDBPs might be able to cross other biological barriers such as the blood brain barrier and intestinal wall. The holy grail of peptide therapeutics is preparing a peptide so that it is orally bioavailable. It has been shown that other CPP peptides have the ability to penetrate the small intestine and the BBB, so HDBPs might make excellent

candidates for crossing biological barriers.^{77,78} Furthermore, peptides displayed as HDBPs are resistant to proteolysis, and therefore might be able to evade digestion by pepsin, trypsin and chymotrypsin, which are present in gram quantities in the stomach and lumen of the small intestine and rapidly digest peptides.

Fourth, many peptide therapeutics often suffer from rapid renal and hepatic clearance due to their small size. Typically, material larger than 50 kD can evade filtration by the kidneys.^{79,80} Furthermore, half-lives of peptides are often quite short due to rapid degradation by proteolysis. By preparing peptides as HDBPs, the molecular weights are dramatically increased compared to the peptide monomer counterparts, and peptides are protected from proteolysis. It is therefore plausible that peptide HDBPs will have longer circulation half-lives' compared to their monomeric counterparts.

Finally, for the best utility *in vivo* a biodegradable polymeric scaffold should be used. The current scaffold consists of an unsaturated polynorbornene backbone that is not biodegradable. In other work with these materials, they accumulate in the liver and other satellite organs, and remain in these organs upto 30 days. For a more translatable system, HDBPs should be prepared using a biodegradable scaffold such as a polyester, polycarbonate, or polycarbamate, so that any material accumulated off target can be cleared from the system.

4.6 Methods

For general methods, see section 2.5.1. For standard SEC-MALS conditions, see section 2.5.5.

4.6.1 Peptide Synthesis

Peptides were synthesized using standard Fmoc-chemistry solid phase peptide synthesis procedures on an AAPPTec Focus XC automated synthesizer. If possible, peptide

monomers were synthesized to contain amino acid side chain protecting groups by the use of the highly acid-sensitive Sieber Amide resin, which allows for cleavage of the peptide from the resin without removal of the protecting groups. Protected peptides that were not soluble in DMF when protected were prepared protecting group-free using Rink Amide MBHA resin. Standard Fmoc SPPS procedures can be found in section 2.5.2.

For the synthesis of fluorescently labeled monomeric peptide controls, peptides were synthesized using standard Fmoc-SPPS procedures. The *N*-terminus of the peptide was then conjugated to a Boc-Lys(Fmoc)-OH amino acid. The Fmoc group was removed with 20% 4-methyl-piperidine in DMF, followed by amide coupling to 5/6-carboxy fluorescein using standard amide coupling where fluorescein:HBTU:DIPEA was 3:2.9:6. Peptides were cleaved from resin using a cleavage cocktail. For peptides on Seiber amide resin, a cocktail of 2%TFA in DCM was used. For peptides on Rink Amide MBHA resin, a cocktail of 95:2.5:2.5 of TFA:TIPS:H₂O was used. Peptides were precipitated with cold ether, dissolved in ACN:H₂O and purified by RP-HPLC.

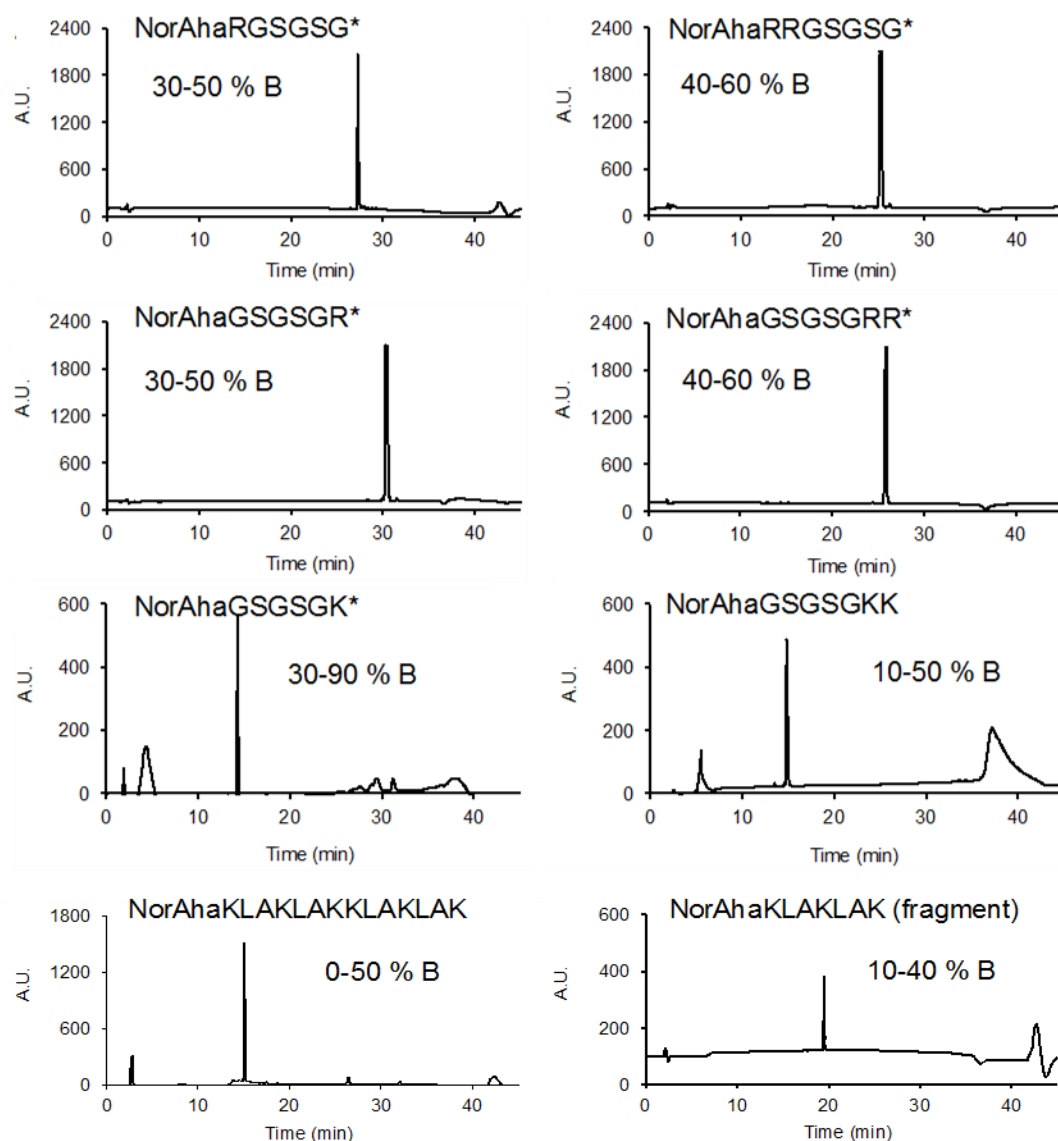


Figure 4.17 RP-HPLC chromatograms of purified peptide monomers. NorAha = *N*-(hexanoic acid)-*cis*-5-norbornene-*exo*-dicarboximide; NorGly = *N*-(glycine)-*cis*-5-norbornene-*exo*-dicarboximide. * indicates the peptide was synthesized with protecting groups. The gradient used on HPLC is indicated where buffer B is ACN with 0.1% TFA and buffer A is water with 0.1% TFA. The identity of each peak was confirmed by ESI MS as shown in Table S1.

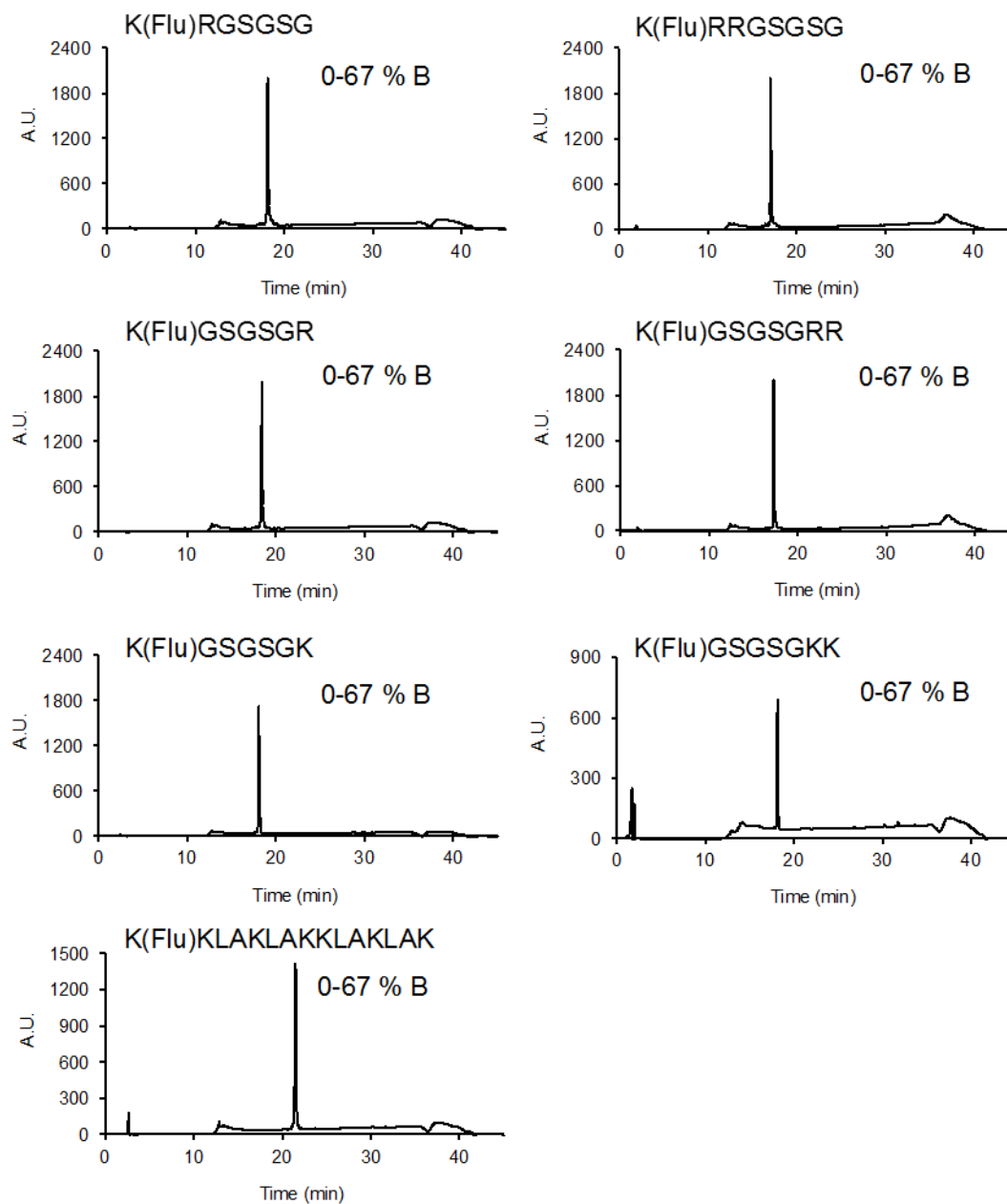


Figure 4.18 RP-HPLC chromatograms for purified fluorescent monomeric peptide controls. The gradient used on HPLC is indicated where buffer B is ACN with 0.1% TFA and buffer A is water with 0.1% TFA. The identity of each peak was confirmed by ESI MS as shown in Table S2.

Table 4.1 Molecular masses determined by ESI-MS for each peptide monomer.

Peptide Monomer	Calculated Mass	Obtained Mass (ESI)
NorAhaR(Pbf)GS(OtBu)GS(OtBu)G-NH ₂	1142	1143
NorAhaR(Pbf)R(Pbf)GS(OtBu)GS(OtBu)G-NH ₂	1550	1552
NorAhaGS(OtBu)GS(OtBu)GR(Pbf)-NH ₂	1142	1143
NorAhaGS(OtBu)GS(OtBu)GR(Pbf)R(Pbf)-NH ₂	1550	1552
NorAhaGS(OtBu)GS(OtBu)GK(Boc)-NH ₂	964	963
NorAhaGSGSGKK-NH ₂	879	878
NorAhaKLAKLAKKLAKLAK-NH ₂ (full length)	1781	1782
NorAhaKLAKLAK-NH ₂ (fragment)	1029	1027

Table 4.2 Molecular masses for monomeric fluorescent peptides. Note that Flu indicates 5/6-carboxyfluorescein conjugated to the lysine side chain at the *N* terminus of the peptide.

Peptide Control	Calculated Mass	Obtained Mass (ESI)
K(Flu)RGSGSG-NH ₂	1005	1004
K(Flu)RRGSGSG-NH ₂	1161	1160
K(Flu)GSGSGR-NH ₂	1005	1004
K(Flu)GSGSGRR-NH ₂	1161	1160
K(Flu)GSGSGK-NH ₂	977	978
K(Flu)GSGSGKK-NH ₂	1106	1105
K(Flu)KLAKLAKKLAKLAK-NH ₂	2011	2010

4.6.2 Polymerizations

All polymerizations were carried out in a glove box under N₂ atmosphere. A typical protocol used to generate a polymer with DP = 8 involved mixing the monomer (0.0125 mmol, 8 equiv., 25 mM) with the catalyst initiator (0.00156 mmol, 1 equiv., 3.1 mM) in dry DMF_{d7} (0.5 mL). For each peptide monomer that has not been polymerized and reported previously in the literature, the polymerization was followed by ¹H NMR to determine the time for complete monomer consumption as indicated by the disappearance of the chemical shift around δ 6.3 and the appearance of a broad doublet between δ 5.5 and 6.0. To track cellular uptake, each polymer was end-labeled with a copy of fluorescein by treatment with a chain transfer agent (1.5 equiv.) for 2 hrs as described previously,⁸¹ followed by termination with ethyl vinyl ether (10 equiv.). Block copolymers were prepared by first polymerizing the peptide monomer to completion prior to adding and polymerizing the OEG. The block

copolymers were prepared in this manner to obtain an accurate estimation of the DP or m of the functional peptide block (using a dn/dc of 0.179 in DMF) since the peptide and OEG blocks have different dn/dc values in DMF. The accessory, water-solubilizing OEG block ($dn/dc = 0.11$ in DMF) was then polymerized second and ratios of the dn/dc values of the two blocks were used to calculate values for this block (i.e., the dn/dc for $m = 8, 15, 30$ and 60 was 0.131, 0.141, 0.151 and 0.161 based on the ratio of peptide:OEG used). Following completion of the second block, the resulting polymers were end-labeled with the fluorescein chain transfer agent and the active catalyst was then terminated with ethyl vinyl ether as described above. Polymerization reactions of peptides intended for use in cytotoxicity studies were split in half prior to addition of the fluorescein end label. One half was then labeled with the fluorescein chain transfer agent and terminated as described (for use in cell-uptake studies) and the second half was terminated directly with ethyl vinyl ether (for use in toxicity studies). Fluorescein-labeled polymers were treated with NH_4OH (aq) for 30 min to remove the pivolate protecting group, as described previously.⁸¹ The resulting polymers were then directly characterized by SEC-MALS to obtain the polymer molecular weight (M_n), dispersity and degree of polymerization (DP).

All polymers were then precipitated with cold ether and collected by centrifugation. For the side-chain protected peptide monomers, the resulting powder was dissolved in 2 mL of a mixture of TFA:H₂O:TIPS (95:2.5:2.5) and stirred for 4 hours at room temperature. The product was precipitated with cold ether, collected by centrifugation and dried. In preparation for in vitro cell studies, all polymers were washed (3×) with cold ether (to remove the Ru catalyst) and then dissolved in DPBS and dialyzed in dialysis cups with a molecular weight cut-off value of 3500 with 3 buffer changes in an effort to remove any residual monomer or catalyst.

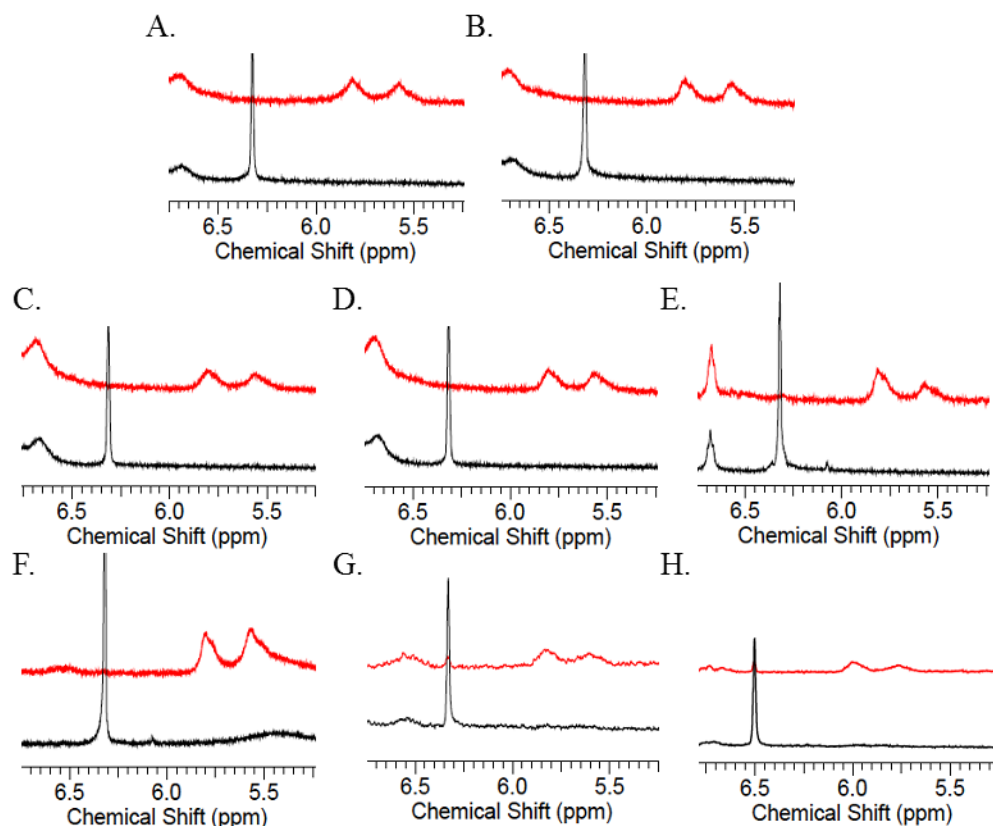


Figure 4.19 ^1H NMR of the polymerization of peptide monomers. Shown in black is the peptide monomer spectra prior to addition of the catalyst. Shown in red is the spectra of the peptide monomer after the addition of the catalyst. Note that the disappearance of the singlet shift at δ 6.3 and appearance of a broad doublet shift between 5.5 and 6.0 is indicative of complete monomer consumption. A. GSGSGR. B. RGS GSG. C. RRGSGSG. D. GSGSGRR. E. GSGSGK. F. GSGSGKK. G. KLA full. H. KLA fragment.

Table 4.3 SEC-MALS characterization for polymeric materials. ^a Polymer name used in the text. ^b The IUPAC name of the polymer with the actual block sizes of the copolymer. ^c Number average molecular weight, except for the KLA, and GSGSGKK polymers, where the weight average molecular weight is given. ^d Disperisty of each block. ^e Experimentally determined DP with the theoretical DP, based on M:I, given in parentheses. DP values were determined by SEC-MALS.

Polymer Name ^a	Polymer IUPAC ^b	First Block (Peptide Block)			Second Block (OEG Block)		
		M _n ^c	M _w /M _n ^d	DP ^e (“m”)	M _n ^c	M _w /M _n ^d	DP ^e
GSGSG, m ~ 8	GSGSG ₁₁ - <i>b</i> -OEG ₁₈	6,560	1.012	11 (8)	12,800	1.12	18 (20)
GSGSG, m ~ 15	GSGSG ₁₆ - <i>b</i> -OEG ₂₁	9,800	1.108	16 (15)	17,000	1.054	21 (20)
GSGSG, m ~ 30	GSGSG ₂₈ - <i>b</i> -OEG ₁₆	17,700	1.125	28 (30)	23,500	1.075	16 (20)
GSGSG, m ~ 60	GSGSG ₅₆ - <i>b</i> -OEG ₂₈	35,000	1.274	56 (60)	45,100	1.052	28 (20)
RGSGSG, m ~ 8	RGSGSG ₁₀ - <i>b</i> -OEG ₂₁	11,600	1.005	10 (8)	19,300	1.015	21 (20)
RGSGSG, m ~ 15	RGSGSG ₁₅ - <i>b</i> -OEG ₁₆	16,500	1.09	15 (15)	22,230	1.06	16 (20)
RGSGSG, m ~ 30	RGSGSG ₂₉ - <i>b</i> -OEG ₂₇	33,100	1.007	29 (30)	42,800	1.014	27 (20)
RGSGSG, m ~ 60	RGSGSG ₆₈ - <i>b</i> -OEG ₂₆	78,000	1.143	68 (60)	90,300	1.142	26 (20)
GSGSGR, m ~ 8	GSGSGR ₁₁ - <i>b</i> -OEG ₂₂	12,130	1.08	11 (8)	20,000	1.034	22 (20)
GSGSGR, m ~ 15	GSGSGR ₁₈ - <i>b</i> -OEG ₁₈	20,000	1.034	18 (15)	26,300	1.085	18 (20)
GSGSGR, m ~ 30	GSGSGR ₂₆ - <i>b</i> -OEG ₁₄	30,100	1.053	26 (30)	35,000	1.025	14 (20)
GSGSGR, m ~ 60	GSGSGR ₇₁ - <i>b</i> -OEG ₂₆	81,100	1.151	71 (60)	90,300	1.142	26 (20)
RRGSGSG, m ~ 8	RRGSGSG ₉ - <i>b</i> -OEG ₁₆	14,500	1.018	9 (8)	20,000	1.018	16 (20)
RRGSGSG, m ~ 15	RRGSGSG ₁₅ - <i>b</i> -OEG ₂₈	23,800	1.018	15 (15)	33,700	1.019	28 (20)
RRGSGSG, m ~ 30	RRGSGSG ₃₁ - <i>b</i> -OEG ₁₄	48,500	1.006	31 (30)	53,400	1.007	14(20)
RRGSGSG, m ~ 60	RRGSGSG ₅₂ - <i>b</i> -OEG ₂₉	80,700	1.001	52 (60)	90,800	1.003	29 (20)
GSGSGRR, m ~ 8	GSGSGRR ₈ - <i>b</i> -OEG ₁₉	12,500	1.052	8 (8)	19,200	1.014	19 (20)
GSGSGRR, m ~ 15	GSGSGRR ₁₈ - <i>b</i> -OEG ₂₃	28,500	1.056	18 (15)	36700	1.078	23(20)
GSGSGRR, m ~ 30	GSGSGRR ₃₀ - <i>b</i> -OEG ₁₉	47,000	1.27	30 (30)	48,200	1.041	19 (20)
GSGSGRR, m ~ 60	GSGSGRR ₅₇ - <i>b</i> -OEG ₁₃	89,000	1.103	57 (60)	93,600	1.053	13 (20)
GSGSGK, m ~ 8	GSGSGK ₁₀ - <i>b</i> -OEG ₂₃	9,650	1.047	10 (8)	17,900	1.044	23 (20)
GSGSGK, m ~ 15	GSGSGK ₁₃ - <i>b</i> -OEG ₁₆	12,100	1.022	13 (15)	17,800	1.16	16 (20)
GSGSGK, m ~ 30	GSGSGK ₂₆ - <i>b</i> -OEG ₁₇	24,800	1.056	26 (30)	30,700	1.042	17 (20)
GSGSGK, m ~ 60	GSGSGK ₅₃ - <i>b</i> -OEG ₂₀	50,800	1.114	53 (60)	57,900	1.165	20 (20)
GSGSGKK, m ~ 8	GSGSGKK ₈ - <i>b</i> -OEG ₂₂	7,170	n.d.	8 (8)	14,900	n.d.	22 (20)
GSGSGKK, m ~ 15	GSGSGKK ₁₆ - <i>b</i> -OEG ₁₇	13,700	n.d.	16 (15)	19,800	n.d.	17 (20)
GSGSGKK, m ~ 30	GSGSGKK ₃₃ - <i>b</i> -OEG ₁₉	29,000	n.d.	33 (30)	35,600	n.d.	19 (20)
GSGSGKK, m ~ 60	GSGSGKK ₆₁ - <i>b</i> -OEG ₃₀	54,000	n.d.	61 (60)	64,500	n.d.	30 (20)
KLA full length, m ~ 5	KLA ₆	10,300	n.d.	6 (5)	-	-	-
KLA full length, m ~ 10	KLA ₁₁	20,200	n.d.	11(10)	-	-	-
KLA full length, m ~ 15	KLA ₁₄	25,000	n.d.	14 (15)	-	-	-
KLA fragment, m ~ 10	KLA(fragment) ₁₁	11,200	n.d.	11(10)	-	-	-

4.6.3 Cell Culture

HeLa cells were purchased from ATCC (CCL-2) and cultured at 37 degrees Celsius under 5% carbon dioxide in Dulbecco's Modified Eagle Medium containing phenol red supplemented with 10% fetal bovine serum, 1x non-essential amino acids, 1x sodium pyruvate, 1x L-glutamine and 1x penicillin/streptomycin. Cells were grown in T75 culture flasks and subcultured every 3-4 days when reached ~80% confluency.

CHO-K1 (CCL-61) and PGSA-745 (CRL-2242) were obtained from ATCC and cultured at 37 degrees Celsius with 5% carbon dioxide in F12K medium supplemented with 10% fetal bovine serum. Cells were cultured in T75 flasks and subcultured every 3-4 days when reached ~80% confluency.

4.6.4 Analysis of Cellular Uptake in HeLa, CHO-K1 and PGSA-745

Cells were plated at a density of 90,000 cells per well of a 24-well plate 18 hrs prior to treatment with the material of interest. Materials dissolved in Dulbecco's Phosphate Buffered Saline (DPBS without Ca^{2+} or Mg^{2+}) at 10 $\times$ the desired concentration (where concentration is with respect to fluorophore concentration to ensure proper comparison of each molecular transporter) were added to the wells and the plates were incubated for 30 min at 37 °C. The medium was then removed and the cells were washed 2 $\times$ with DPBS and then incubated 3 $\times$ for five minutes each with heparin (0.5 mg/mL in DPBS) to remove any membrane-bound, non-internalized material as described by Dowdy,⁶⁴ and finally rinsed again with DPBS. The cells were then trypsinized (0.25 % trypsin in DPBS) for 10 min, cold medium was added, and the cells were transferred to Eppendorf tubes. The suspended cells were centrifuged to pellets and then resuspended in a minimal amount of cold DPBS (~60 μL). Flow cytometry data (10,000 events on three separate cultures per condition) was then

acquired. For cellular uptake, the fluorescence was normalized to the vehicle control by taking the fluorescence of the population divided by the fluorescence of the vehicle control.

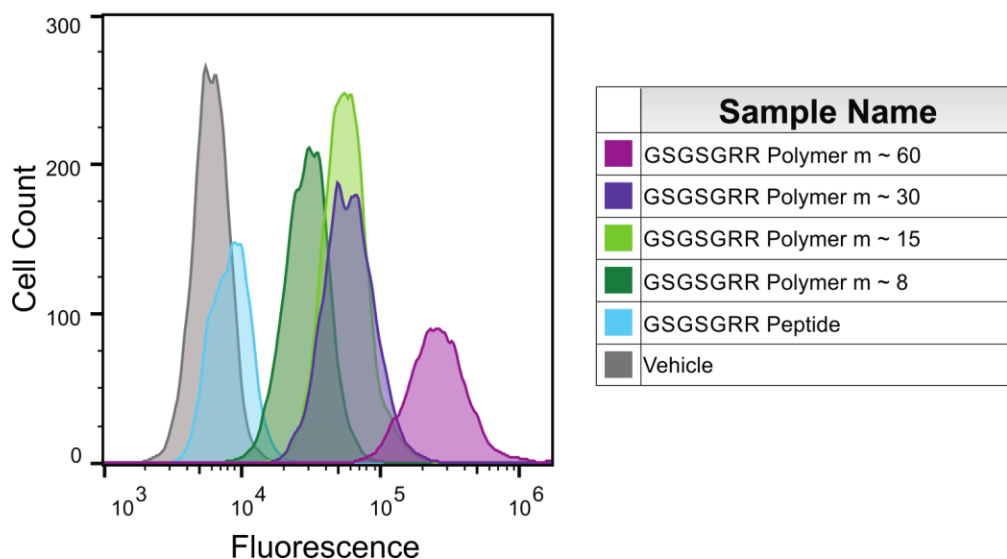


Figure 4.20 Example histogram from flow cytometry analysis of monomeric and polymeric GSGSGRR. Healthy populations were gated identically and referenced to the vehicle control. 10,000 counts were acquired.

4.6.5 Mechanistic Studies by Flow Cytometry

For mechanistic studies, cells were plated and treated as described in the previous flow cytometry experiments, except here cells were preincubated with the indicated compound for 30 minutes at 37 °C prior to addition of the cell-penetrating material. The following concentrations were used: 80 μ M dynasore and 9.5 mM M β CD. For studies at reduced temperature, cells were incubated at 4 °C for 30 min prior to and during incubation with the compound of interest. For the reduced temperature studies only, all subsequent washes and manipulations were also done with ice-cooled media and other materials. Data is reported as the normalized mean fluorescence, which is the mean fluorescence yielded by the material divided by the mean fluorescence from the vehicle control. Each measurement was preformed 3 $\times$ on at least three separate subcultures.

4.6.6 Live Cell Confocal Microscopy

For live cell confocal microscopy of uptake of GSGSG and KLA peptides and polymers, HeLa cells were plated on glass-bottom 24-well plates at a cell density of 90,000 cells per well 18 hrs prior to treatment with the compound of interest. The medium was removed and then replaced with medium lacking phenol red to minimize background fluorescence. Materials dissolved in DPBS (at 10× the desired concentration, 2.5 μ M, where concentration is with respect to fluorophore) were added to the wells and the plates were incubated for 30 min at 37 °C. The medium was then removed and the cells were rinsed 2× with DPBS, and then fresh medium (phenol red- free) was added to each well. Live cells were imaged on an Olympus FV1000 confocal microscope with a chamber at 37 ° C under 5% CO₂ *via* a Z-stack analysis (set to 1 μ m slices) using identical instrument settings for each image and a 40× or 100x objective.

For colocalization studies, CHO-K1 cells were plated on glass-bottom 24-well plates at a cell density of 90,000 cells per well 18 hrs prior to treatment with the compound of interest. Materials dissolved in DPBS (at 10× the desired concentration, 0.5 μ M, where concentration is with respect to fluorophore) were added to the wells and the plates were incubated for 30 min at 37 °C. The medium was then removed and the cells were rinsed 2× with DPBS, and then fresh media was added to each well. Cells were then stained using Image-IT™ Live lysosomal and nuclear labeling kit. Cells were first stained with Hoechst 33342 dye for 5 minutes, then washed with DPBS. Cells were then stained with LysoTracker Red for 1 minute. Live cells were imaged on an Olympus FV1000 confocal microscope with a chamber at 37 ° C under 5% CO₂ *via* a Z-stack analysis (set to 1 μ m slices) using identical instrument settings for each image and a 40× or 100x objective.

4.6.7 Cell Viability Assay

The cytotoxicity of materials was assessed using the CellTiter-Blue® assay, which measures the metabolic reduction of resazurin to resorufin *via* fluorescence. For these studies, HeLa cells were plated at a density of 3,500 cells per well of a 96-well plate 18 hrs prior to treatment. Materials dissolved in DPBS at 10× the desired concentrations were added to the wells along with a 10% DMSO positive control. Cells were incubated for 72 hrs at 37 °C. Note that concentration for all toxicity measurements is with respect to peptide concentration to ensure that all peptides and polymers are fairly compared with respect to their therapeutic components. There is also no fluorophore present on the polymers or peptides used in these experiments to minimize potential background signals in the fluorescent measurements. The medium was removed and 80 µL of fresh medium lacking phenol red was added. To this was added 20 µL of the CellTiter-Blue® reagent and the cells were then incubated for 2 hrs prior to measuring fluorescence using 560 nm excitation and 590 nm emission. The fluorescence measurements were corrected for background fluorescence from the CellTiter-Blue® reagent by subtracting the fluorescence reading of wells treated with the reagent in the absence of cells. Fluorescence values were then referenced as a percentage of the value obtained for the DPBS vehicle control.

4.6.8 Circular Dichroism

UV-Vis circular dichroism (CD) was used to evaluate whether the secondary structure of KLA, a mixture of random coil and alpha helix, is maintained upon polymerization. The peptide and polymer were dissolved in DPBS to a final concentration of 100 µM (with respect to peptide concentration). CD spectra were measured using an Aviv 215 spectrometer and each sample was measured from 190 to 260 nm with a slit width of 1

nm, scanning at 1 nm intervals with a 1s integration time. Measurements were taken 3× at 25°C and then averaged to give the spectra in Figure 4.13.

4.6.9 JC-1 Mitochondrial Integrity Assay

The mitochondrial membrane potential was measured using the MitoProbe® JC-1 assay kit, which measures the membrane potential using the JC-1 dye. The JC-1 dye forms J aggregates in healthy mitochondria that are red fluorescent, but cannot form the J aggregates when the mitochondria are disrupted, leading to a decrease of red fluorescence and an increase of green fluorescent J monomers in the cytosol. For these studies, HeLa cells were plated at a density of 90,000 cells per well of a 24-well plate 18 hrs prior to treatment. Materials dissolved in DPBS at 10× the desired concentrations (where concentration is with respect to peptide and no fluorophore is present) were added to the wells and incubated for 30 minutes. The materials were removed and the cells were washed with DPBS, then medium was added to the cells followed by incubation with 10 µL of a 200 µM solution of JC-1 dye to give a final concentration of 2 µM. To one set of wells, 2 µL of 50 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to give a final concentration of 50 µM. The small molecule CCCP is used as a positive control because it can associate with and depolarize mitochondrial membranes. The cells were incubated for 30 more minutes. The medium was then removed and the cells were washed 2× with DPBS and then incubated 3× for five minutes each with heparin (0.5 mg/mL in DPBS), and finally rinsed again with DPBS. The cells were then trypsinized (0.25 % trypsin in DPBS) for 10 min, cold medium was added, and the cells were transferred to Eppendorfs. The suspended cells were then centrifuged to pellets and then resuspended in a minimal amount of cold DPBS. Flow cytometry data (10,000 events on three separate cultures per condition) was then acquired.

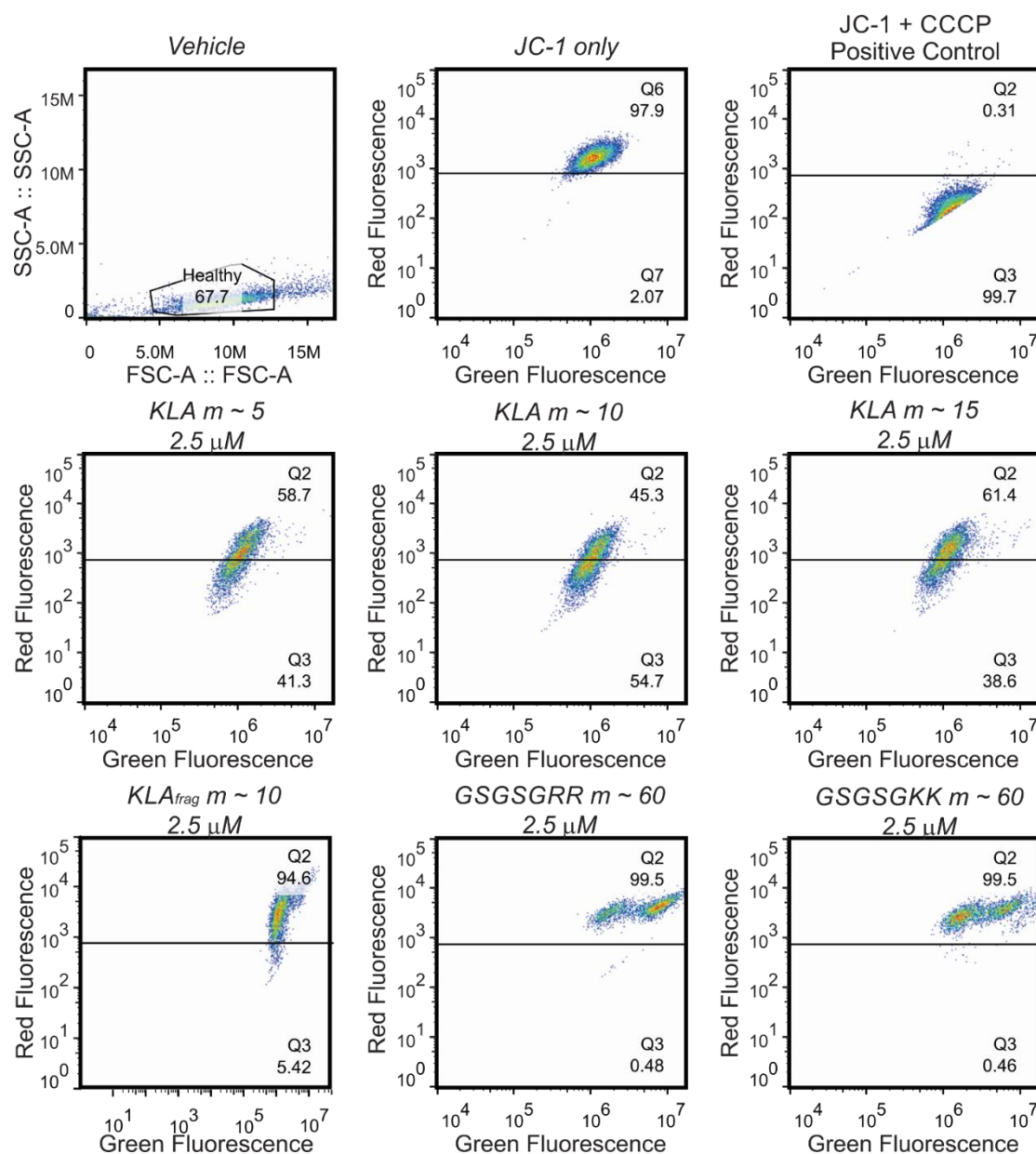


Figure 4.21 JC-1 assay for mitochondrial integrity. The samples were run on flow cytometry and each sample was gated using the vehicle control (DPBS). The quadrants were chosen based on where cells treated with CCCP (Q#) and cells that were untreated reside (Q2).

4.6.10 Apoptosis and Necrosis Assay

Apoptotic and necrotic cells were identified using the CFTM488A Annexin V and PI Apoptosis Kit, in which Annexin V (green) binds to apoptotic cells and PI (red) stains

necrotic cells. For these studies, HeLa cells were plated at a density of 420,000 cells per well of a 6-well plate 18 hrs prior to treatment. KLA, $m \sim 10$, was dissolved in DPBS to a concentration of 2000 μM , where concentration is with respect to peptide and no fluorophore is present. Staurosporin, a small molecule that is known to cause apoptosis, was dissolved at 1000 $\times$ (desired concentration on cells is 1 μM) in DMSO. Staurosporin and the vehicle control were added to the wells 24 hrs prior to analysis, and KLA ($m \sim 10$) and 10% DMSO control were added 5 hr prior to analysis. After incubation, materials were removed and the cells were washed 2 $\times$ with DPBS followed by 3 $\times$ 5 minute incubations with heparin (0.5 mg/mL in DPBS), and finally rinsed once with DPBS. The cells were then trypsinized (0.25% trypsin in DPBS) for 10 min, cold medium was added and the cells were transferred to Eppendorf tubes. The suspended cells were centrifuged to pellets and then resuspended in 100 μL Annexin V binding buffer. Then 5 μL of Annexin V was added to each Eppendorf, followed by 1 μL of a working solution of PI. The cells were then incubated in a 37 ° C water bath for 15 minutes in the dark. After incubation, flow cytometry data was acquired monitoring the FITC channel (for Annexin V staining) and the PI channel.

4.6.11 Caspase 3/7 Assay

The expression of caspase enzymes, markers of apoptotic cells, in cells treated with KLA materials was assessed using the Apo-One® assay, which measures the expression of caspase 3/7, using a fluorogenic substrate for those enzymes. For these studies, HeLa cells were plated at a density of 5,000 cells per well of a 96-well plate 18 hrs prior to treatment. Materials dissolved in DPBS at 10 $\times$ the desired concentration (10 μM , where concentration is with respect to peptide and no fluorophore is present) were added to the wells. Cells were incubated for 30 minutes at 37 °C. The medium was removed the cells were washed 3 $\times$ with DPBS. Then 100 μL of the Apo-One® reagent was added to the cells and to wells that

contain no cells to get a baseline reading. The plate was mixed for 30 seconds using a shaker followed by incubation at 37 °C. After 3 hrs of incubation, the fluorescence was measured using an excitation of 499 nm and emission at 521 nm. The baseline fluorescence was subtracted from the fluorescence values for the wells, and fluorescence values were then referenced as a percentage of the value obtained for the DPBS vehicle control.

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Chapter 5 Optimization and Application of

MMP-Targeted Near Infrared Labeled

Nanoparticles

5.1 Introduction

The goal of nanomedicine is to safely package and deliver a therapeutic or imaging agent to the site of interest in a higher concentration than exist in healthy tissues and organs. To do so, nanomaterials need to be targeted either passively or actively to diseased tissue sites. Traditionally, the enhanced permeability and retention (EPR) effect was used to target types of diseases such as cancer, myocardial infarction, and hind limb ischemia.^{1,2} This effect results from the enhanced permeability through leaky vasculature, and enhanced retention of materials due to poor lymphatic drainage. Active targeting strategies are more favorable for tissue targeting due to the poor translatability of nanomaterials relying on the EPR effect.²

Typically, accumulation of nanomaterials in diseased tissue relies on targeting disease-specific factors such as overexpressed receptors. A commonly used targeting agent is the peptide RGD, which binds to integrins that are overexpressed in many cancerous tissues.³ Other times, a nanomaterial can accumulate specifically in diseased tissue and deliver a payload in response to specific stimuli (pH, heat, light, enzymatic processing, etc).⁴ One common stimulus used is enzymatic processing to direct accumulation or release a payload.

Diseased tissues often overexpress a variety of enzymes to enable disease progression. Matrix metalloproteinases (MMPs) are extracellular enzymes that help with ECM processing and tissue remodeling and are often upregulated in cancer, myocardial

infarction, peripheral artery disease, inflammatory bowel disease, rheumatoid arthritis, and many other diseases based on inflammation.⁵⁻⁹ Therefore, targeting nanomaterials to MMPs can direct specific accumulation. For example, Tsien and coworkers have utilized an activatable cell penetrating peptide (ACPP) that can internalize into cancer cells in response to MMP 2/9 cleavage.¹⁰ ACPPs are small peptides with a polyarginine sequence (Arg9), followed by a MMP recognition sequence, then a polyglutamic acid sequence (Glu9). When intact, ACPPs form a hairpin and the Arg9 cannot internalize into cells. However, the MMP recognition sequence is cleaved when in the presence of MMPs in tumor tissue, unmasking the Arg9 due to a release of the Glu9, resulting in Arg9 internalization. Many times, Arg9 can be conjugated to cargo and deliver the payload to cancer cells upon internalization. ACPPs have been successfully used to internalize imaging agents and nanoparticles into tumors as well as allowing for fluorescence-guided surgery.¹¹

Similarly, Fe₃O₄ nanoparticles can specifically internalize into tumor tissue through an MMP stimulus. A cell internalization domain was masked by a PEG polymer conjugated through an MMP2 cleavable sequence and loaded onto the nanoparticles. The PEG units allowed for favorable *in vivo* properties, but when the particle encountered MMP2 in the tumor tissue, the peptide was cleaved, unmasking the cellular internalization domain, and thereby allowing the particles to be taken up by cells. This was shown to improve both fluorescence and T2 MRI imaging in tumor-bearing mice.¹²

An alternative approach utilized an enzyme triggered assembly of in tumor tissue for improved T2 MRI imaging. This strategy utilized Fe₃O₄ particles labeled with either biotin or neutravidin, which aggregated together when combined. The biotin and neutravidin moieties were sterically blocked from interacting with each other through conjugation of PEG polymers to the nanoparticles through an MMP cleavable peptide sequence. When the PEG is

present, the particles cannot aggregate, but post release of the PEG due to MMP cleavage of the peptide in tumor tissue, the particles can then aggregate.¹³

An approach similar to the above from our lab involves an enzyme-directed assembly of nanoparticles in response to MMPs. Our approach utilizes amphiphilic block copolymers synthesized *via* ring opening metathesis polymerization (ROMP) that self-assemble into spherical nanoparticles.^{14,15} The hydrophilic block is comprised of a peptide substrate for MMP-2/9. When the nanoparticles are incubated with MMP-2/9, the peptide is cleaved causing a change in the hydrophilicity of the peptide block resulting in a morphology switch to a micronscale aggregate.^{14,16} Particles can be labeled with fluorescein or rhodamine dyes, and upon aggregation due to MMP cleavage a fluorescence resonance energy transfer (FRET) signal can be detected *in vitro*, and these particles have been shown to label diseased tissue in animal models of cancer and myocardial infarction (diseases where MMPs are upregulated).^{16,17} Particles can also be synthesized with a hydrophobic block consisting of taxol, a chemotherapeutic, allowing for drug delivery to tumor tissue.¹⁸

Though these first reports show promise, the preparation of the material relied on a *grafting to* approach where the peptide was conjugated post polymerization to a preformed polymer bearing NHS activated esters. This necessitated the use of excess peptide material, as well as purification due to the incomplete conjugation of the peptide to the polymer.^{15,17} Furthermore, these original reports utilized dyes that have poor utility for *in vivo* imaging when compared to the near infrared dyes (NIR).¹⁹ Therefore, a second generation of this particle system was developed that was synthesized in a *graft through* polymerization, and labeled with NIR fluorescent dyes.

Particles and other materials are favorable for biomedicine applications because of the ease of tailoring their size, shape, and surface chemistries; however, many of these properties can cause poor *in vivo* performance such as short circulation times due to strong

opsonization, resulting in macrophage uptake, liver accumulation, and clearance from the body.^{20–27} In general, to improve circulation, a “stealthy” particle that is not opsonized is ideal. Traditionally, PEG was used as a stealth agent, and high density of PEG coated nanomaterials showed decreased protein absorption and longer circulation times.^{28–32} The incorporation of PEG in nanomaterials has raised some concerns such as its immunogenicity, as well as the lack of biodegradability, therefore groups have investigated the use of other stealth agents as PEG substitutes.^{32–34}

Charge can also alter the fate of nanomaterials *in vivo*. Highly positively charged materials have been shown to potentially be hemolytic. Both highly positive and negatively charged materials can be strongly opsonized, taken up by macrophages and removed from circulation.³⁵ Zwitterionic particles, however, were shown to not opsonize as strongly, were not taken up by macrophages as readily, and exhibit longer half-lives *in vivo*.^{24,36–39}

Herein, nanomaterials were developed for *in vivo* application using *graft through* ROMP, which enabled a more defined polymer system compared to our previous reports, and labeled with NIR fluorescent dyes to allow for better *in vivo* imaging. Post IV administration of NIR-labeled material in tumor-bearing mice and a mouse model of PAD revealed that the majority of the particles were residing in the liver and had poor accumulation in the tissue of interest. For this reason, a new NIR dye monomer was synthesized that contained zwitterionic character, and these new particles showed reduced liver accumulation.⁴⁰

5.2 Synthesis of NIR-Labeled Peptide Polymer Amphiphiles that Self-Assemble into MMP Responsive Particles

To prepare NIR-labeled particles, a peptide polymer amphiphile (PPA) must be synthesized that contains a hydrophobic block, and a hydrophilic block consisting of the

MMP responsive peptide, and a NIR label. Polymers then must be self-assembled into particles that are stable and responsive to enzymes.

5.2.1 Synthesis and Polymerization of MMP Peptide Monomer

Previous synthesis of the PPA required the use of a *grafting to* approach where the peptide was conjugated to the polymer at reactive NHS groups post polymerization.¹⁵ The peptides often did not undergo complete conjugation, leaving free carboxylic acid groups. Though the number of peptides conjugated could be determined, their precise location was unclear and the conjugation reaction was unpredictable. A *graft through* approach would negate the need for post polymerization conjugation and would result in a more controlled and easily characterized polymeric material.

To prepare PPAs through *graft through* ROMP, a peptide sequence that could undergo polymerization was needed. The original reports used the peptide sequence GPLGLAGGWGERDGS where PLGLAG is the MMP recognition sequence and GERDGS is a sequence used to increase the hydrophilicity.¹⁵ This peptide, when prepared as a norbornyl monomer (N-(peptide)-*cis*-5-norbornene-*exo*-dicarboximide), could not be directly polymerized by ROMP due to unfavorable gelation properties after addition of the peptide monomer. Instead, the peptide sequence GPLGLAGGFGSGERDG was chosen (Figure 5.1). This sequence differed from the first in that tryptophan (W) was replaced with a phenylalanine (F), and the C-terminal serine was moved to the interior of the sequence.

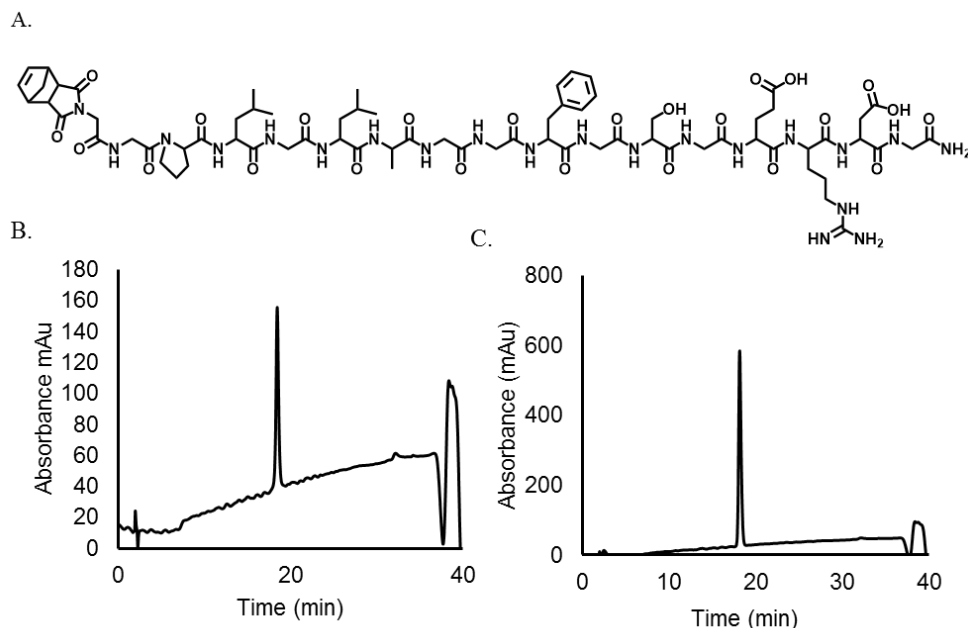


Figure 5.1 Preparation of Norbornyl-MMP substrate. A. Structure of the norbornyl peptide monomer. The sequence is NorG-GPLGLAGGFGSGERDG. B. Purified HPLC trace of the peptide monomer prepared with L -amino acids. Peptide was confirmed by ESI-MS. Ex: 1647.76 Found:1648. C. Peptide monomer prepared with D -amino acids. ESI-MS. Ex: 1647.76 Found:1648.

PPAs using the new norbornyl-MMP peptide monomer were prepared by ROMP to determine if the new MMP peptide monomer could be incorporated into PPAs (Figure 5.2). The polymer targeted was phenyl₂₅-*b*-peptide₂₅ (*b* denotes block and the subscript number denotes the block length). First, N-(Benzene)-*cis*-5-norbornene-*exo*-dicarboximide (Nor-Ph)⁴¹ was polymerized and followed by ^1H NMR (Figure 5.2). Upon complete consumption of Nor-Ph, a small aliquot was removed and quenched with ethyl vinyl ether (EVE) and analyzed by SEC-MALS to determine polymer molecular weight and dispersity (Figure 5.2). N-(GPLGLAGGFGSGERDG)-*cis*-5-norbornene-*exo*-dicarboximide (Nor-Pep) was then added and monitored by ^1H NMR. Nor-Pep was polymerized to completion, and no unfavorable gelation resulted (Figure 5.2). The polymer was then quenched with EVE and

analyzed by SEC MALS. The resulting polymers reached the targeted molecular weights with low dispersities and no unfavorable gelation properties.

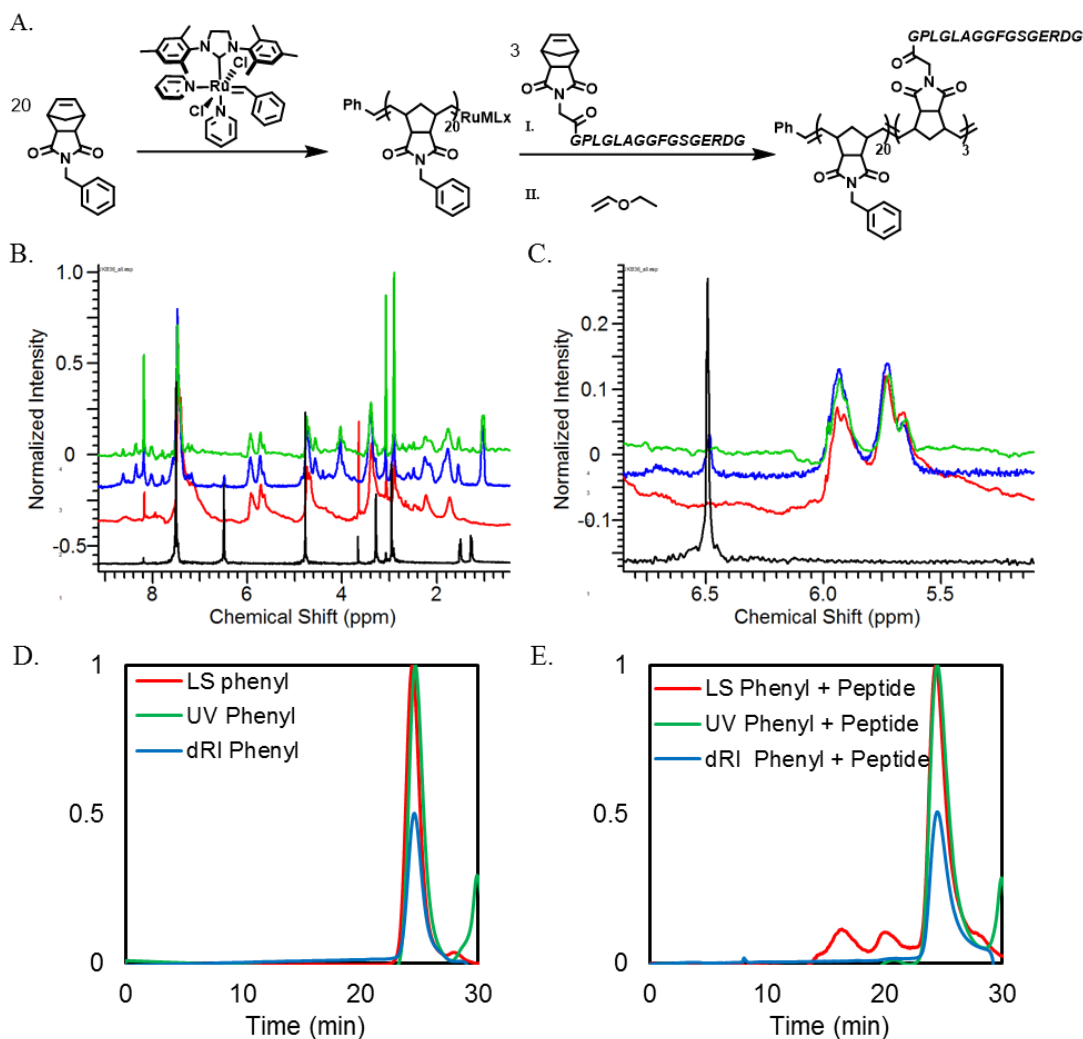


Figure 5.2 Preparation of PPAs via *graft through* ROMP. A. Scheme for the preparation of PPAs. First, a norbornyl-phenyl (20 equiv) was mixed with the ruthenium based initiator and followed by NMR. After complete consumption, the norbornyl-peptide monomer (3 equiv) was added. After complete consumption, the polymerization was quenched with ethyl vinyl ether (EVE). B. Full ^1H NMR spectrum of the polymerization. In black is the initial NMR of the norbornyl-phenyl. In red is the NMR after complete consumption of the norbornyl-phenyl monomer. In blue is the NMR post addition of the norbornyl-peptide monomer. In green is the NMR after the complete consumption of the norbornyl-peptide monomer. C. A zoom on of the NMR showing the norbornene olefin peaks for the monomers around 6.25 ppm and the polymers between 5.5 and 6.0 ppm. D. SEC-MALS trace for the phenyl block. E. SEC-MALS trace for the full polymer.

With polymers in hand, micellar nanoparticles were prepared through self-assembly. This process begins with dissolving the polymer in an organic solvent that solubilizes both blocks, followed by a slow addition of aqueous solvent to cause a hydrophobic collapse of the material.⁴² For determining the best conditions to form spherical micelles in high yield, the selection of organic solvent must be determined.

First, the organic solvent choice was investigated. For polymers containing peptides, dimethylformamide (DMF), or dimethylsulfoxide (DMSO) can be used as both blocks are readily soluble in these solvents. Polymers were dissolved in DMF or DMSO and self-assembled by slowly adding Dulbecco's phosphate-buffered saline (DPBS) followed by dialysis to remove all organic solvent. Particle morphologies were measured using dynamic light scattering (DLS) to determine the hydrodynamic diameter.

Based on the DLS data, self-assembly using DMF as a cosolvent lead to spherical, micellar nanoparticles with minimal larger aggregation. Before filtering, particles around 29 nm and 250 nm were present, but after filtering only a single distribution was present (Figure 5.3). In contrast, particles formed from DMSO showed >95% of the material was larger than the targeted diameter of ~20nm prior to filtering. After filtering, the aggregates were removed and the particles showed a distribution around 20 nm (Figure 5.3). Though both cosolvents led to spherical micelles around 20 nm after filtering, particles formed from DMF had a higher percentage of material residing in the favorable diameter range (~20nm) when compared to DMSO prior to filtering (18% and 3% respectively) suggesting that using DMF as a cosolvent will lead to a higher yield of particle material in the 20 nm range.

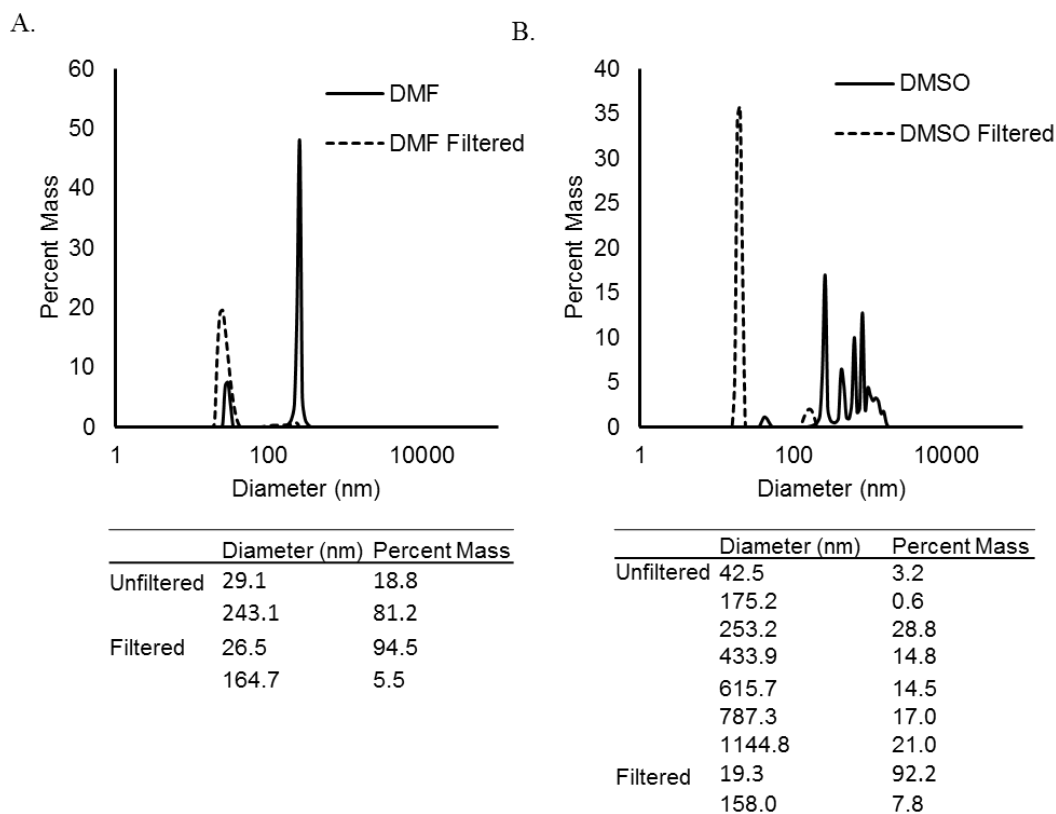


Figure 5.3 DLS characterization of particles formed from A. DMF, and B. DMSO cosolvents.

As this work was being performed, a report from the lab was published showing peptides can be protected from proteolysis when using *graft through* ROMP.^{43,44} In this report, it was found that decreasing the peptide density with small hydrophilic spacers can increase the rate of polymerization. For micellar nanoparticles bearing peptides, proteolysis is actually necessary. The density of the peptide in the hydrophilic brush of PPAs was investigated by preparing two different PPAs, one where the hydrophilic block was a homopolymer of the peptide sequence, and the other was blend copolymer of the peptide sequence with a carboxylic acid spacer. In order to synthesize a true blend copolymer as the hydrophilic block, the rates of polymerization of each monomer needed to be determined. Monomers exhibit varying rates of polymerization, and therefore cannot be added together to

afford a blend copolymer. By calculating the rates, the addition of each of the monomers can be controlled so that a blend copolymer was synthesized.

To better mimic the original reports, N-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide (Nor-Gly) was chosen as the spacer between Nor-Pep. The rate of polymerization was determined by monitoring the disappearance of the monomeric olefin by ^1H NMR until complete consumption. The normalized integrals of the monomer and polymer olefin were converted to percent conversion then to concentration of monomer. This was used to determine the number of monomers polymerized per unit time, and initial rates were determined by plotting $\ln([M_0]/[M_t])$ as a function of time. Nor-Gly was polymerized at a rate of 1 monomer per 0.20 minutes (Figure 5.4), whereas Nor-Pep was polymerized at a rate of 1 monomer per 2.01 minutes (Figure 5.5).

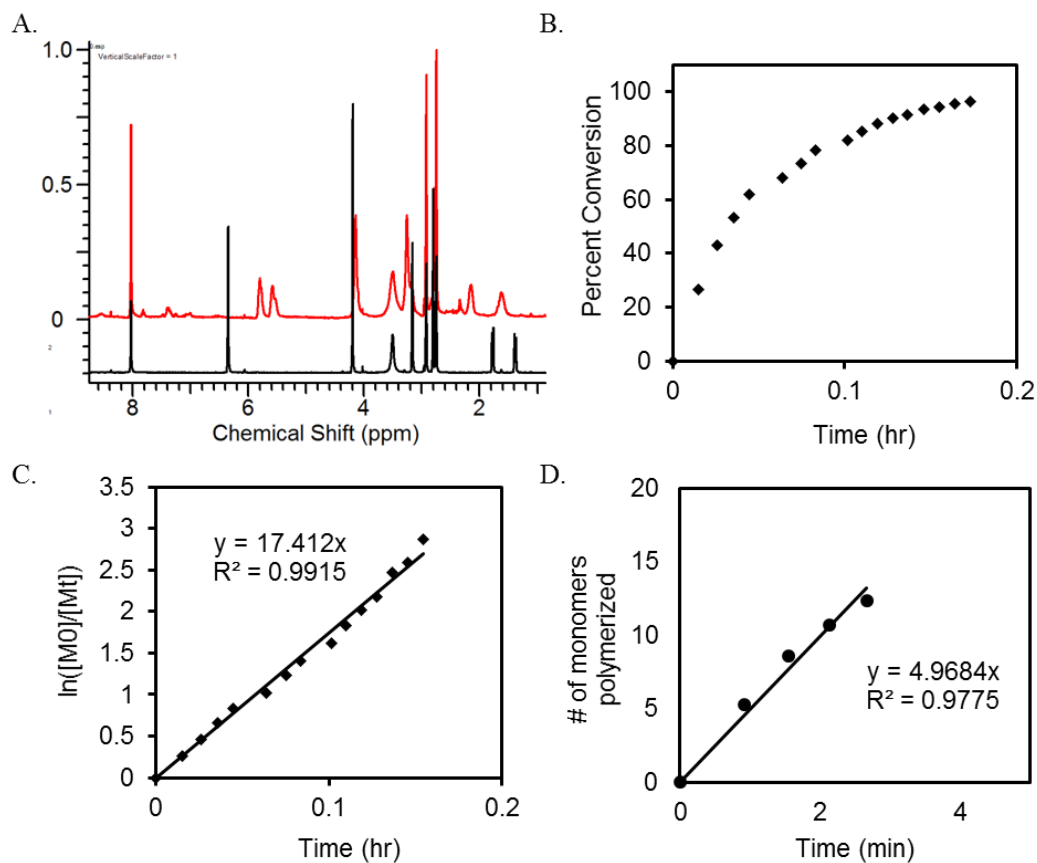


Figure 5.4 Rate of polymerization of Nor-Gly. A. ^1H NMR before (black) and after (red) complete monomer consumption. B. Percent conversion of the monomer over time. C. Initial rate of monomer vs time. D. Number of monomers polymerized vs. time.

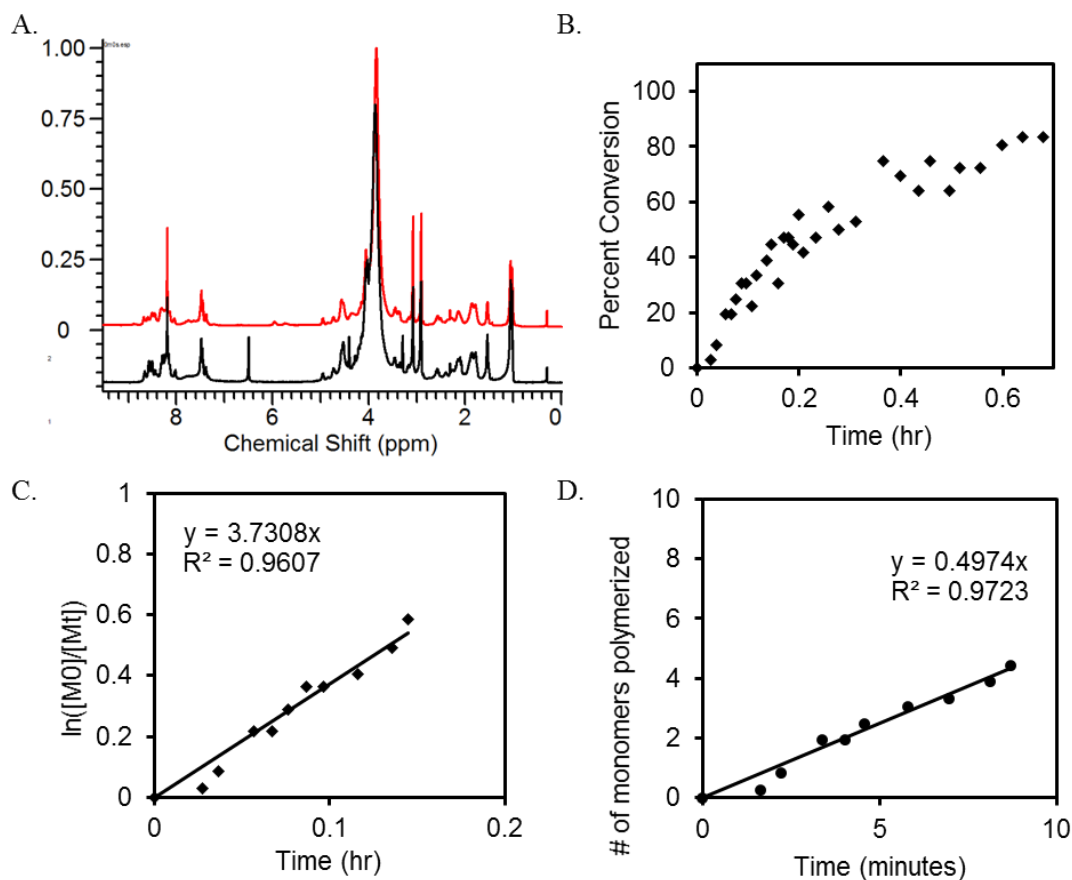


Figure 5.5 Rate of polymerization of Nor-Pep. A. ^1H NMR before (black) and after (red) complete monomer consumption. B. Percent conversion of the monomer over time. C. Initial rate of monomer vs time. D. Number of monomers polymerized vs. time.

To make the blend copolymer of Nor-Pep (3 equiv.) and Nor-Gly (4 equiv.), the Nor-Pep is added to the polymerization after complete monomer consumption of Nor-Ph (20 equiv.) by the ruthenium initiator (1 equiv.), then every 2.01 minutes an aliquot of Nor-Gly was added. The polymer was terminated with ethyl vinyl ether and analyzed by SEC-MALS to determine molecular weight and dispersity index (Figure 5.6). The IUPAC name of the resulting polymer is identified as Phenyl₂₅-*b*-peptide₃-*co*-glycine₄.

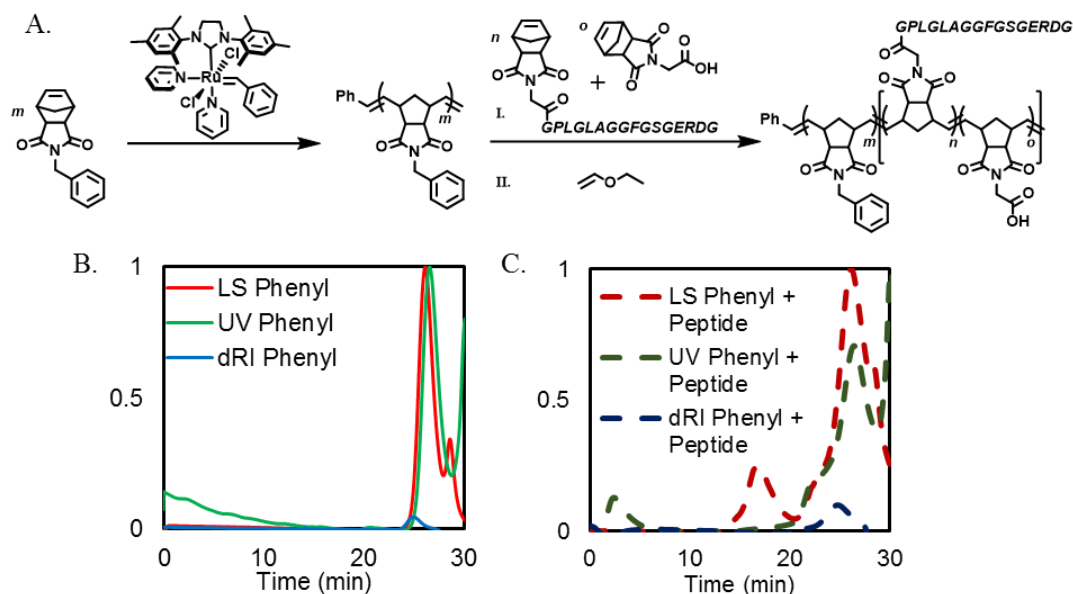


Figure 5.6 Preparation of peptide polymer amphiphile containing a blend copolymer as the hydrophilic block. A. Scheme for the polymerization. Nor-Ph is first polymerized using the ruthenium initiator. After complete monomer consumption, Nor-Pep and Nor-Gly are polymerized into a blend copolymer. After complete monomer consumption, ethyl vinyl ether is added to terminate the polymerization. B. SEC-MALS traces for the phenyl block. C. SEC-MALS traces for the phenyl and the peptide and glycine blend copolymer.

To determine if the blend copolymer enabled an increase in cleavage efficiency of the particles compared to the block copolymer, each particle was incubated with thermolysin and analyzed by RP-HPLC (Figure 5.7). The blend copolymer reached ~35 percent cleavage over 14 hours whereas the block copolymer reached ~25 percent. Based on these results, a blend copolymer should be used so that when the particles encounter MMPs present in the tissue, they have the greatest change of cleaving and aggregating.

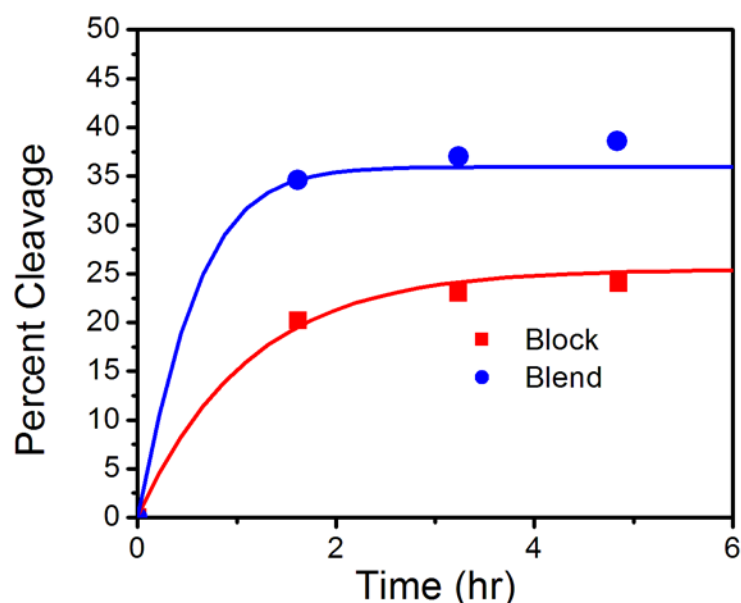


Figure 5.7 Proteolysis of block or blend particles by thermolysin.

In conclusion, PPAs were synthesized *via* graft-through ROMP and aggregate in response to enzymatic treatment. Phenyl₂₅-*b*-peptide₃-*co*-glycine₄ was shown to have a higher cleavage efficiency than phenyl₂₅-*b*-peptide₃ likely due to the decreased density of the peptide block, allowing the enzyme to access the peptide more freely.

5.2.2 Preparation of Norbornyl NIR Dye Monomers and Incorporation of Monomers into PPAs

Using the ruthenium based ROMP initiator, there are 3 common ways to fluorescently label the polymeric materials; the initiator can be labeled with a chain transfer agent (CTA) bearing a fluorescent group to end label the polymer material during initiation, a norbornyl monomer bearing a fluorescent group can be incorporated into the polymer material, or chain transfer agent can be added to the living polymer to end label the polymer material and release the catalyst (Figure 5.8).^{45,46}

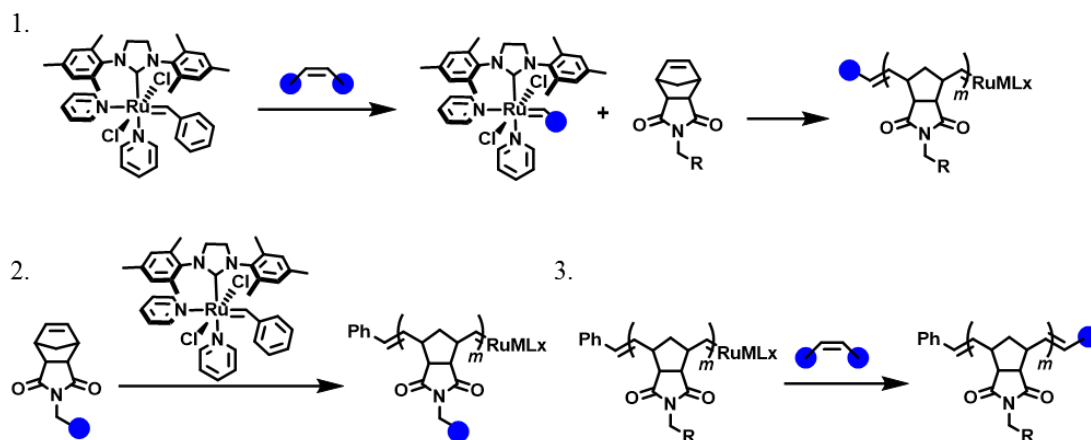


Figure 5.8 Ways to label ROMP based polymers.

When incorporating a label into a polymer, it is desired to only incorporate one label per polymer chain through either using a labeled initiator (Figure 5.8 1), or a chain transfer agent (Figure 5.8 3), or only adding 1 monomer (Figure 5.8 2). When modifying the initiator with a fluorophore, the initiation kinetics can be lowered resulting in different initiation rates among the catalysts leading to polydispersed material. A CTA to end label a polymer is desired, however half of the dye material is wasted labeling the catalyst due to the symmetric nature of the CTA. Groups have developed a nonsymmetric chain transfer agent that employs a substituted vinyl ether to end label polymers (Figure 5.9).⁴⁶ This type of chain transfer agent is beneficial due to the monosubstituted nature, as well as forming a Fisher Carbene and deactivating the catalyst post termination. Typically for these types of terminations to occur, upwards of 10-30 equivalents of CTA are necessary.^{47,48} In efforts to minimize the amount of fluorescent dye needed, and to ensure labeling of the polymer materials, a norbornyl-dye monomer was utilized reasoning that only 1 equivalent of dye is necessary to label the polymer compared to potentially 4-30 equivalents if using chain transfer agents.

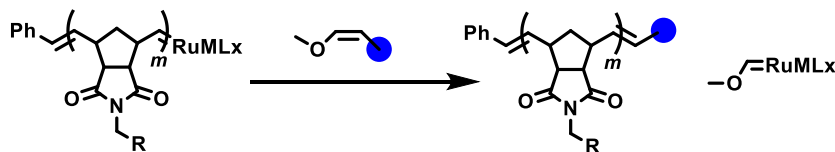


Figure 5.9 Substituted vinyl ether termination of ruthenium polymerization.

NIR dye monomer was synthesized by amide bond coupling between *N*-(aminoethyl)-*exo*-norborn-5-ene-2,3-dicarboxylic imide⁴⁹ (Nor-Amine) and carboxylic acid bearing polymethine cyanine dye (Figure 5.10). Two polymethine cyanine dye analogs were synthesized, a cy 5.5 and a cy7. These dyes were chosen as they are known FRET pairs with each other, therefore if cy 5.5 labeled and cy 7 labeled particles were aggregated together in response to MMP, a FRET signal would be generated.

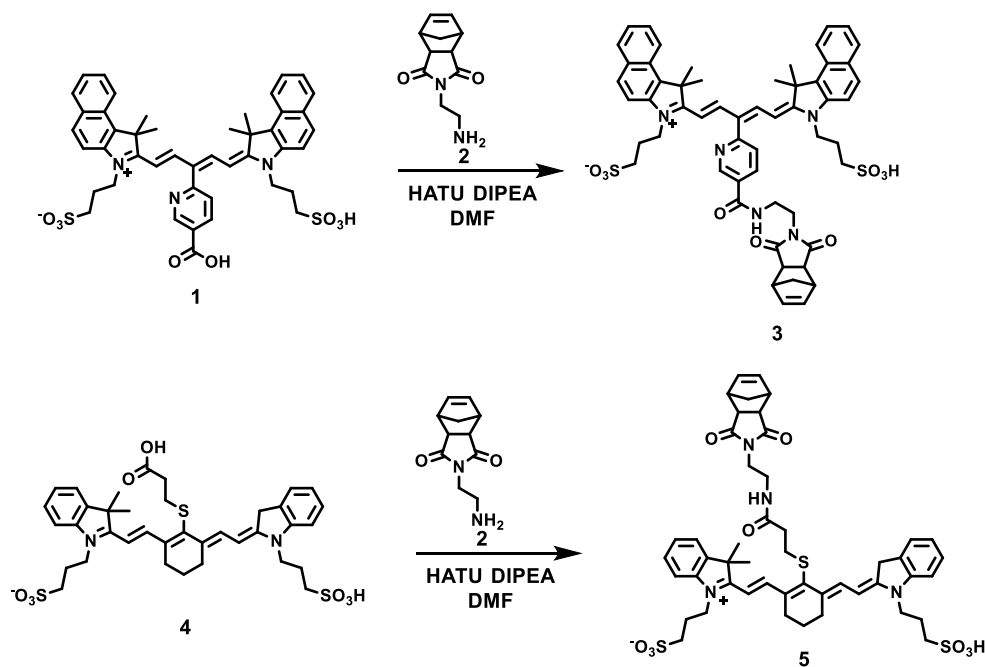


Figure 5.10 Synthetic scheme for the synthesis of NIR dye norbornene monomer of cy 5.5 and cy 7. NIR dye **1** and **4** is couple to Nor-Amine (**2**) using standard peptide coupling agents for afford NIR dye monomers **3** and **5**.

To ascertain if the NIR dye monomer (Nor-Cy5.5/Nor-Cy7) could be incorporated into ROMP based polymers using the ruthenium based initiator, the polymer phenyl₂₅-*b*-pep₄-*b*-Cy5.5₁ and phenyl₂₅-*b*-pep₄-*b*-Cy7₁ was synthesized and followed by ¹H NMR (Figure 5.11). After Nor-Cy5.5/Nor-Cy7 was added to the polymerization, an olefin proton around 6.5 ppm was present, indicating the presence of Nor-Cy5.5/Nor-Cy7 monomers. After 1 hour, the proton peak around 6.5 ppm was no longer present, suggesting that the Nor-Cy5.5/Nor-Cy7 was incorporated into the polymer (Figure 5.11). Each block, except for the dye block was characterized by SEC/MALS. Once the polymer is labeled with the dye, it cannot be analyzed by MALS due to the absorbance of the laser by the NIR dyes, but SEC and UV absorbance can still be employed. To this end, an SEC UV trace was acquired for phenyl₂₅-*b*-pep₃ polymer monitoring at 280nm and 600nm for phenyl and the dye respectively. The unlabeled polymer showed absorbance in the UV at 280 nm, but no absorbance at 600 nm. When phenyl₂₅-*b*-pep₄-*b*-Cy5.5₁ or phenyl₂₅-*b*-pep₄-*b*-Cy7₁ was analyzed by SEC UV at 600 nm, a clear absorbance band was present at a similar elution time to the unlabeled polymer (Figure 5.11). Together with the NMR data, the Nor-Cy5.5 and Nor-Cy7 can be incorporated into polymers synthesized via ROMP.

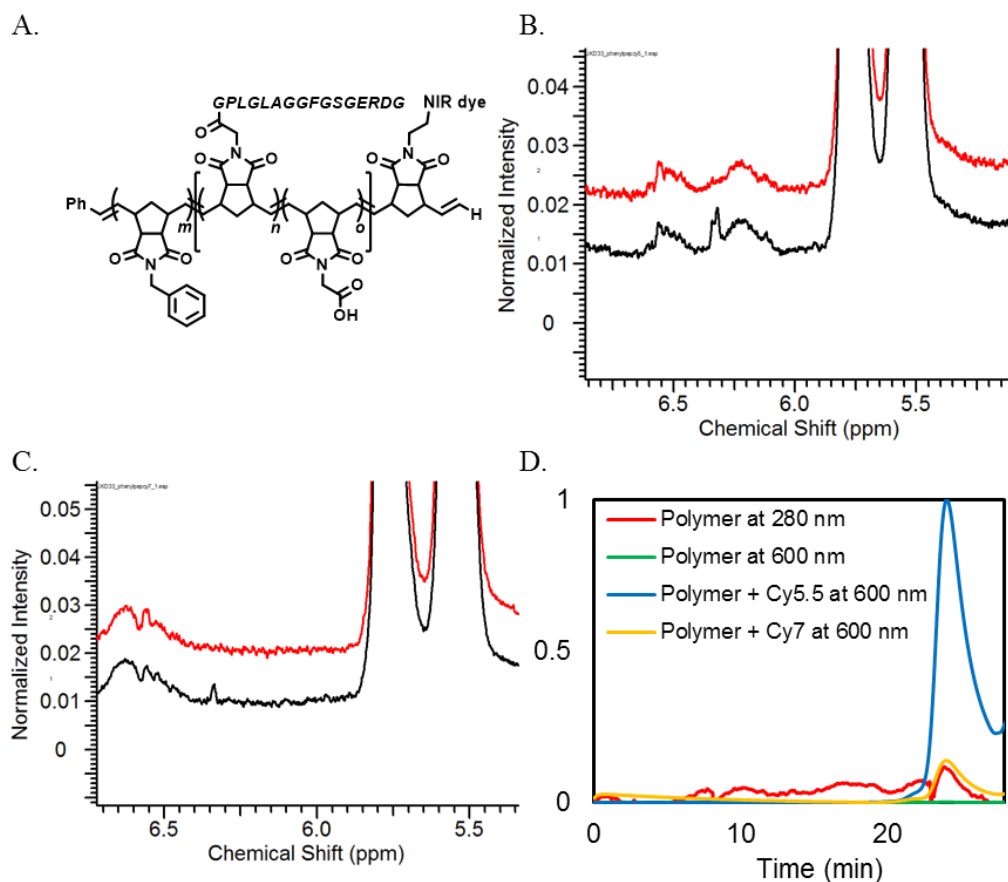


Figure 5.11 Incorporation of Nor-Cy5.5 into ROMP derived polymers. A. Chemical structure phenyl₂₅-b-pep₄-b-Cy5.5₁. B. ¹H NMR showing the spectrum immediately after the addition of Nor-Cy5.5 (black) and 1 hour after the addition of Nor-Cy5.5 (red). Note that the small chemical shift around 6.3 ppm corresponds to the olefin protons on the Nor-Cy5.5 monomer. After 1 hour, the chemical shift has disappeared indicating the consumption of Nor-Cy5.5. C. ¹H NMR showing the spectrum immediately after the addition of Nor-Cy7 (black) and 1 hour after the addition of Nor-Cy7 (red). Note that the small chemical shift around 6.3 ppm corresponds to the olefin protons on the Nor-Cy7 monomer. After 1 hour, the chemical shift has disappeared indicating the consumption of Nor-Cy7. D. SEC UV trace of phenyl₂₅-b-pep₄ monitored at 280nm to track the phenyl, and at 600nm to track the dye. Since no dye is present on the polymer, there is no signal when tracked at 600nm. When phenyl₂₅-b-pep₄-b-Cy5.5₁ and When phenyl₂₅-b-pep₄-b-Cy7₁ is analyzed by SEC UV at 600nm, a clear absorbance peak is present.

Polymethine cyanine dyes such as cy 5.5 and cy 7 can self-quench if positioned in close proximity.^{50–52} Labeling each polymer in the particle could potentially lead to self-quenching and a reduced fluorescence signal. Particles were self-assembled using different ratios of labeled and unlabeled polymers to determine the optimal ratio for maximum

fluorescence and minimal self-quenching (Figure 5.12). For particles self-assembled from phenyl₂₅-*b*-pep₄-*b*-Cy5.5₁ (labeled) and phenyl₂₅-*b*-pep₄ (unlabeled), the fluorescence was minimal when only labeled polymers were incorporated into particles indicating the dye was self-quenching, but as the particles were diluted out to 3:1 labeled:unlabeled, the fluorescence increased, and reached a maximum at 1:1 labeled:unlabeled. For particles self-assembled from phenyl₂₅-*b*-pep₄-*b*-Cy7₁ (labeled) and phenyl₂₅-*b*-pep₄ (unlabeled), substantial self-quenching was identified even for particles at a ratio of 1:1 labeled:unlabeled.

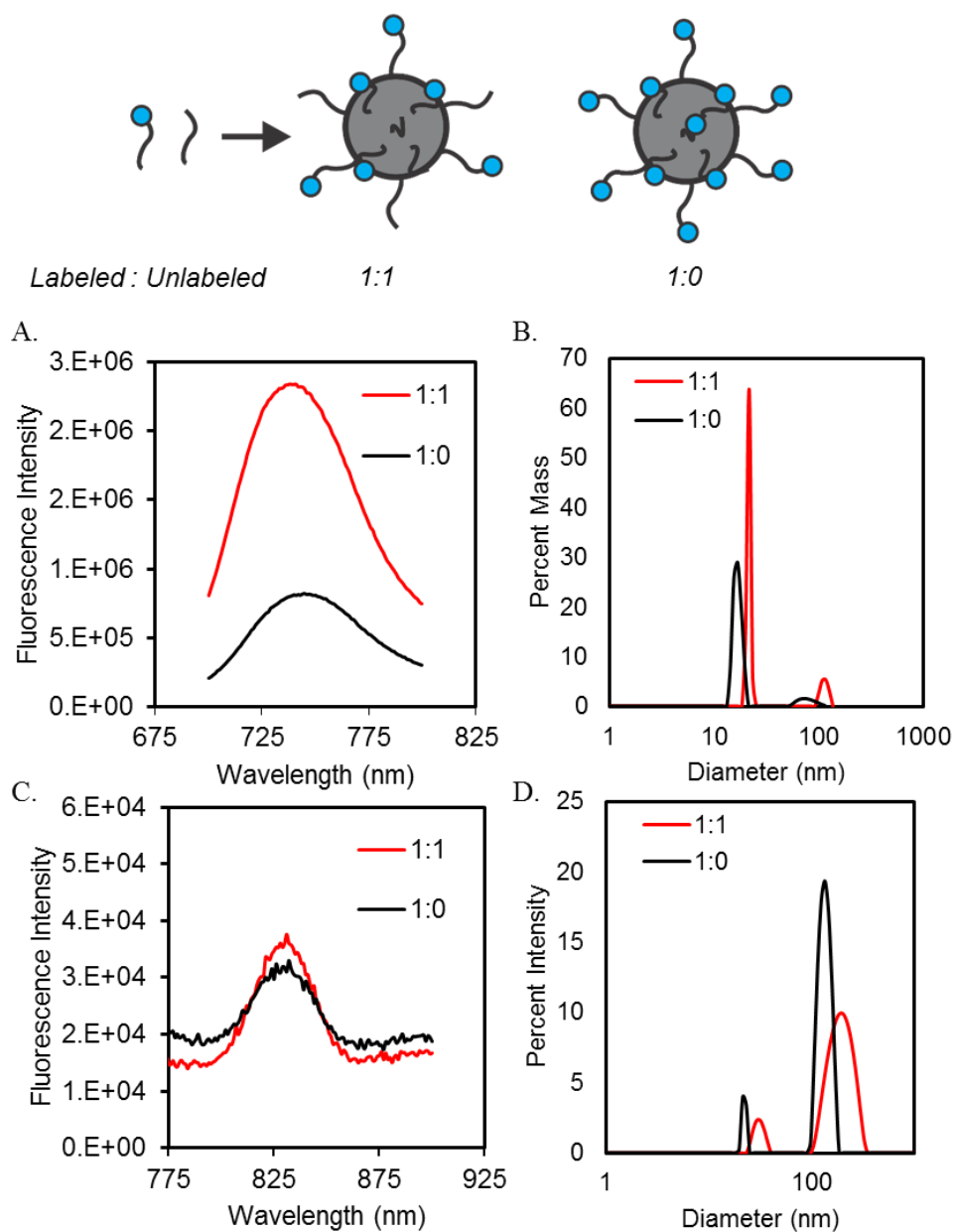


Figure 5.12 Determining the optimal ratio of labeled to unlabeled PPAs in the self-assembled particle. A. Fluorescence of particles labeled with cy5.5 that were self-assembled using ratio of 1:1, 3:1 and 1:0 labeled to unlabeled polymers. B. DLS of self-assembled particles labeled with cy5.5. C. Fluorescence of particles labeled with cy7 that were self-assembled using ratios of 1:0, 1:1, 1:5, 1:10, and 1:20 labeled to unlabeled polymers. D. DLS of self-assembled particles labeled with cy7.

Particles labeled with cy 7 showed unfavorable fluorescent properties compared to cy 5, however these properties could be improved if undergoing FRET, therefore a FRET active micelle was prepared where phenyl₂₅-*b*-pep₄-*b*-Cy5.5₁ and phenyl₂₅-*b*-pep₄-*b*-Cy7₁ PPAs were self-assembled into micelles in a 1:1 ratio. The FRET active micelle showed minimal fluorescence, especially when compared to a micelle formed from a 1:1 ratio of labeled to unlabeled cy 5.5 polymers (Figure 5.13). For this reason, using both cy 7 and cy 5.5 labeled micelles were not further investigated for *in vivo* imaging. Instead, cy 5.5 labeled micelles were utilized.

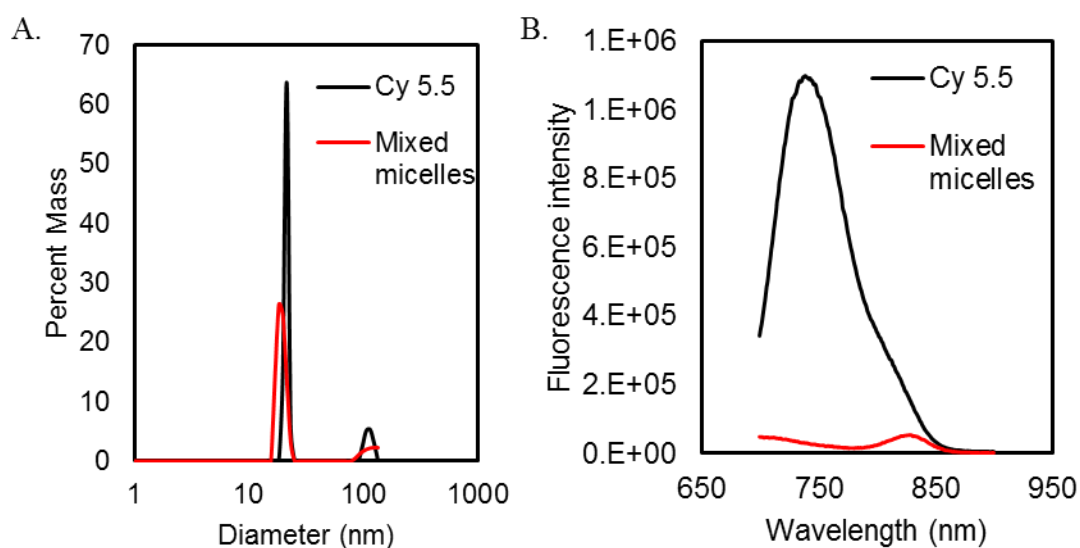


Figure 5.13 Characterization of FRET in mixed micelles labeled with Cy 5.5 and Cy 7. A. DLS showing diameter of particles. B. Fluorescence intensities of the emissions of the micelles.

NIR dye norborneyl monomers were successfully incorporated into PPAs synthesized *via* ROMP. The resulting PPAs could self-assemble into fluorescent nanoparticles. Particles derived from cy5.5 labeled PPAs showed favorable fluorescence when a ratio of 1:1 labeled:unlabeled polymer was used in the self-assembly. Particles derived from Cy7 labeled

PPAs showed minimal fluorescence, and therefore were not developed further for *in vivo* studies.

5.2.3 Preparation of NIR Labeled Micellar Nanoparticles for *In Vivo* Studies

NIR-labeled micellar nanoparticles were synthesized from PPAs prepared using *grafting through* ROMP. PPAs phenyl₂₅-*b*-_Lpep-co-gly-*b*-Cy5.5_{0.5} and phenyl₂₅-*b*-_Dpep-co-gly-*b*-Cy5.5_{0.5} were prepared as described above where _Lpep refers to the peptide prepared with _L-amino acids (GPLGLAGGFGSGERDG) and _Dpep refers to the peptide prepared with _D-amino acids (gplglaggfgsgerdg). PPAs were characterized by SEC-MALS prior to the addition of the NIR dye to determine molecular weight and polydispersity (Figure 5.14). PPAs were then self-assembled into micellar nanoparticles to afford **M_L^{5.5}** and **M_D^{5.5}**. The resulting particles were characterized by DLS and cryo TEM (Figure 5.14).

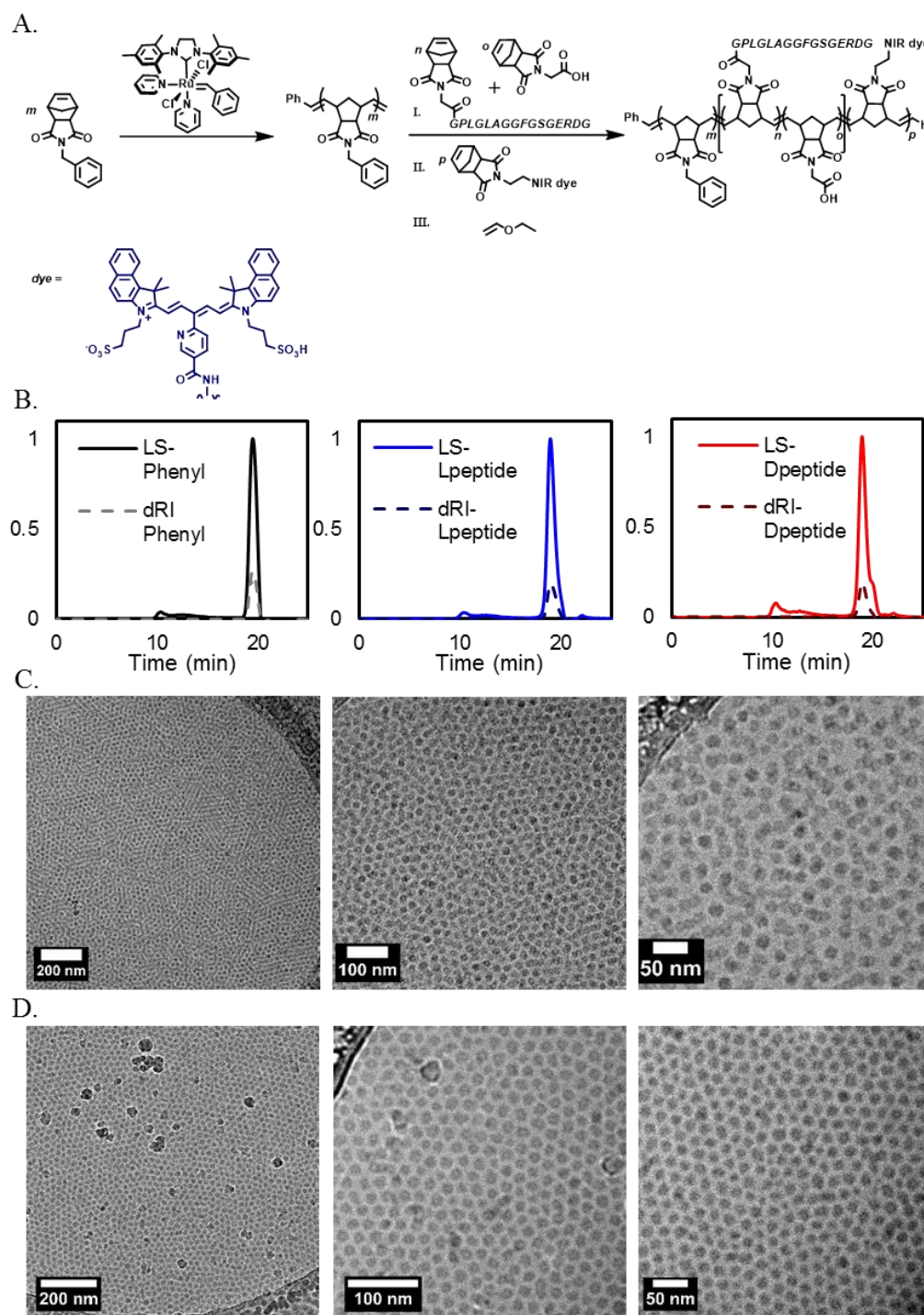


Figure 5.14 Polymer synthesis and particle characterization. A. Scheme for polymer synthesis. B. SEC MALS traces for each block. C. CryoEM of particles formed from phenyl₂₅-b-Lpeptide₃-co-glycine₄-b-Cy_{5.50.4}. D. CryoEM of particles formed from phenyl₂₅-b-Dpeptide₃-co-glycine₄-b-Cy_{5.50.4}. CryoEM images provided by Mollie Touve

PPAs were then self-assembled into micellar nanoparticles to afford $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$. The resulting particles were characterized by DLS and cryo TEM (Figure 5.14).

5.3 Tumor Targeting of NIR-Labeled Micellar Nanoparticles

NIR-labeled particles were synthesized as described above and investigated for tumor targeting ability in mice inoculated with Cal-27 cancer cells, a head and neck squamous cell carcinoma (HNSCC). These types of cancer are known to have upregulation of MMP activity.⁵³ Previously, MMP targeted micellar nanoparticles were shown to accumulate in tumor tissue after systemic administration; however, these particles utilized a dye that performs poorly *in vivo* due to the absorbance by the tissue.¹⁵ Particles labeled with a NIR dye would allow for better *in vivo* imaging, and for quantification of particle concentrations in the satellite organs to better determine the tumor targeting ability.

$\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ were intravenously (IV) administered to mice bearing Cal-27 tumors and imaged at 30 minutes, 1, 2, 4, and 24 hours and 7 days (Figure 5.15 and 5.16). After the final time point, animals were imaged with the skin removed to eliminate the background fluorescence from the skin (Figure 5.17). Images shown are representative images from the groups. Tumor targeting of $\mathbf{M_L^{5.5}}$ seemed to be variable between groups, in that the 24-hour group shows apparent fluorescence in the tumors around 1 hour (Figure 5.15, top row), whereas the 7-day group seemed to have no apparent fluorescence in the tumors until 24 hours (Figure 5.16 top row). The variability in fluorescence could be the result of differences in the tumors between groups. For example, the 7-day group had smaller tumors that were deeper in the muscle compared to the 24-hour group and with these data, it is hard to conclude if the particles were able to target the tumor tissue. Due to the variability, no conclusions could be drawn from the $\mathbf{M_D^{5.5}}$ group as well. Animals who received $\mathbf{M_D^{5.5}}$ seemed to have minimal tumor accumulation, but it was difficult to determine if this was a

result of the variability in tumor size, or a lack of accumulation due to being non-responsive to enzymes.

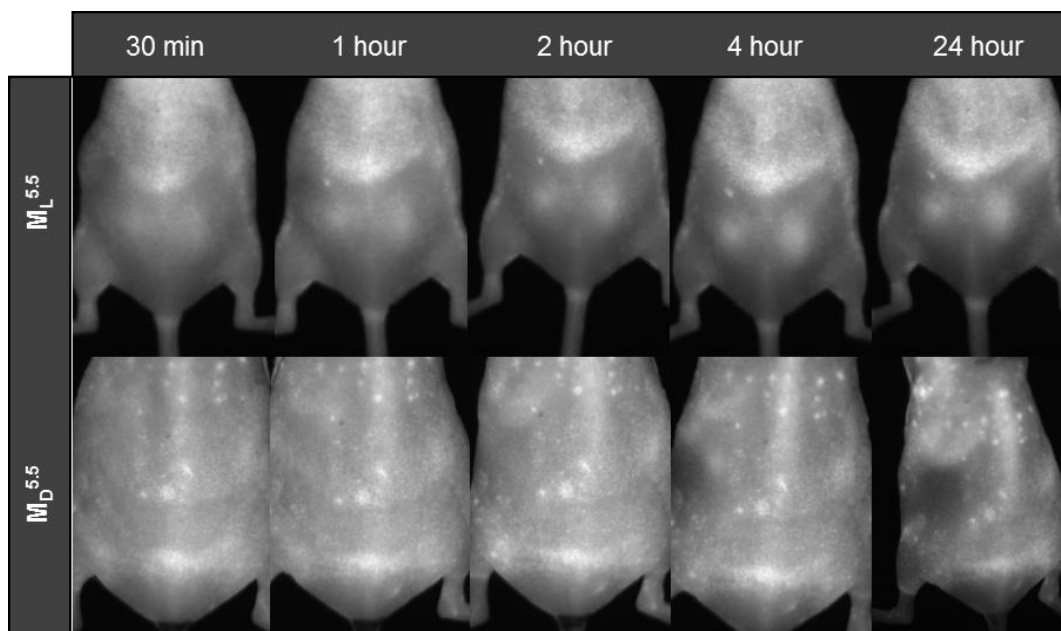


Figure 5.15 Live whole animal images of mice with Cal-27 tumors after intravenous injection. The top row corresponds to the mice who received the $M_L^{5.5}$ (responsive) particles, and the bottom row corresponds to the mice that received the $M_D^{5.5}$ (non-responsive particles). This cohort of mice was sacrificed 24 hours post IV injection.

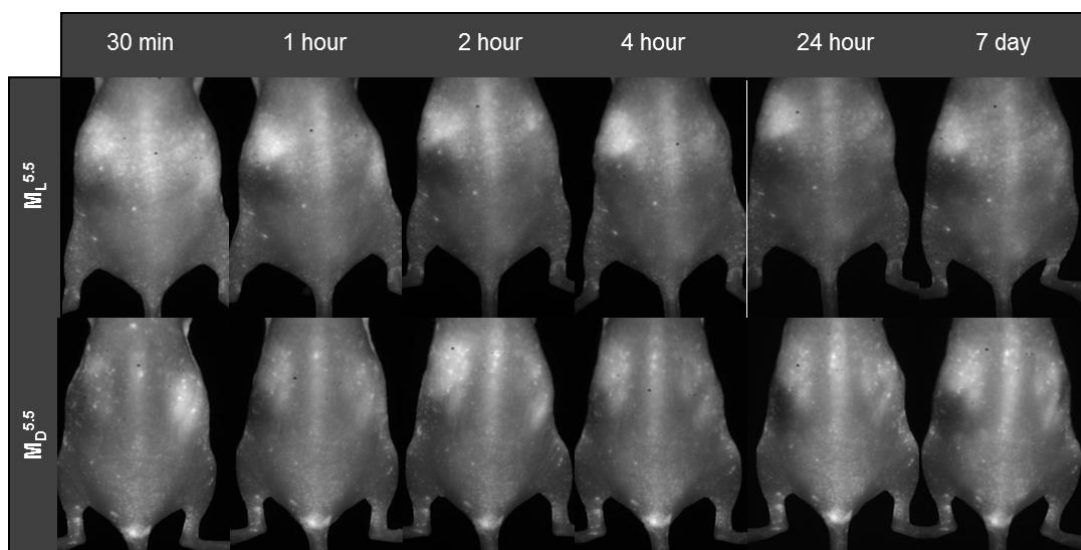


Figure 5.16 Live whole animal images of mice with Cal027 tumors after intravenous injection. The top row corresponds to the mice who received the $M_L^{5.5}$ (responsive) particles, and the bottom row corresponds to the mice that received the $M_D^{5.5}$ (non-responsive particles). This cohort of mice was sacrificed 7 days post IV injection.

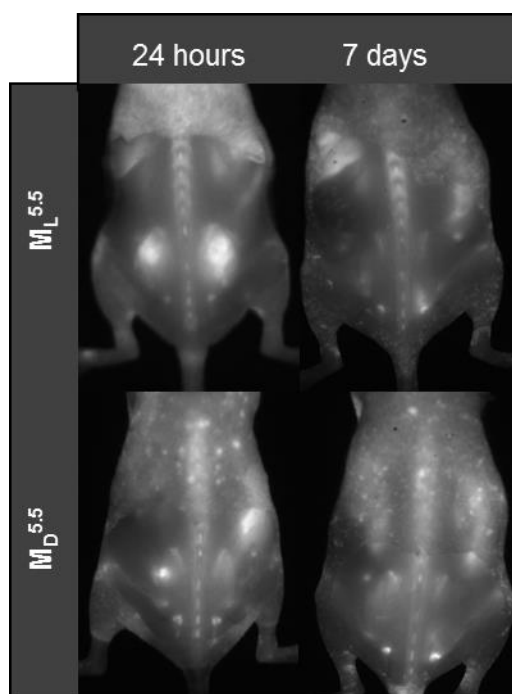


Figure 5.17 Whole animal images of mice at terminal time point with the skin removed to eliminate background fluorescence from the skin.

Analysis of tumor targeting was inconclusive using whole animal fluorescence imaging, therefore quantification of the particle concentration in *ex vivo* tissue homogenates

were performed. Satellite organs (liver, kidney and muscle) were digested and fluorescence was measured, and converted into concentration of particles in the tissue (Figure 5.18). With these data, the standard uptake value (SUV) was determined where $\text{SUV} = \text{Molality in tissue/molality injected}$. The SUVs indicate that the liver has a high particle accumulation, especially when compared to the other organs. There also appears to be some particles present in the kidneys, and minimal particles present in the muscle. These data suggest that the particles are being cleared through hepatic clearance, yet after 7 days, particles are still present in the liver. This is most likely a result of a non-biodegradable polymer platform used. When looking at the tumor SUV data, there is no difference between $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ in tumor targeting. Note that the particles aggregate in response to MMP 2/9 present in the tumor tissue, and therefore the $\mathbf{M_L^{5.5}}$ should be aggregated and retained due to being enzyme responsive, and $\mathbf{M_D^{5.5}}$ should not accumulate due to being non-responsive. In fact, the SUVs show that $\mathbf{M_D^{5.5}}$ is present in the tumor tissue, suggesting that there is another mechanism of accumulation happening such as the EPR effect.

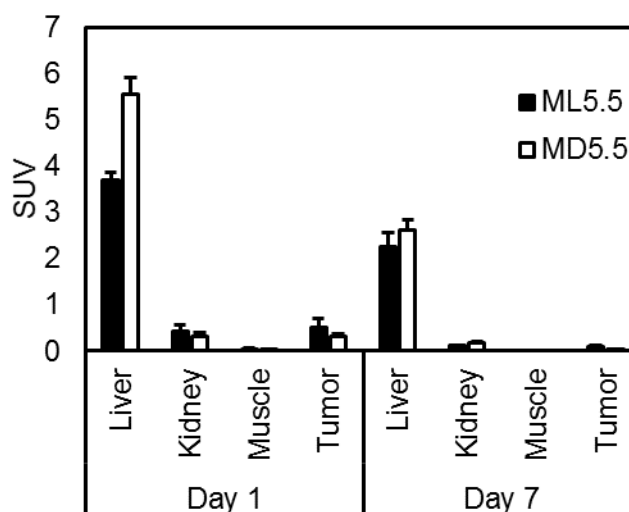
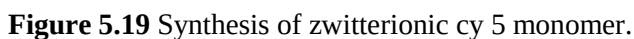


Figure 5.18 Standard uptake values of organs.

Based on the SUV data, the majority of administered particles reside in the liver. For particles to be most useful in labeling tumors, there needs to be a decrease in the hepatic clearance. Furthermore, having a significant portion of the particles in the liver is suggestive that particles are being opsonized and engulfed by macrophages. This likely results from the multiple negative charges present in the particles. A more zwitterionic “stealthy” particle could therefore mitigate these issues, reducing macrophage uptake and potentially an increased tumor accumulation.

When investigating the particle composition, there are three areas that can be modified to manipulate the charge of the particle. The peptide sequence used in the hydrophilic block (GPLGLAGGFGSGERDG) has two amino acids that are negatively charged (Glu, E; and Asp, D) at a neutral pH, and one amino acid that is positively charged (Arg, R), therefore the overall charge of the peptide should be -1 at a neutral pH. The hydrophilic spacer between each of the peptides is a carboxylic acid that is also -1 at a neutral pH, and finally the cy 5.5 dye used has an overall -1 charge. Each of these units can be changed to incorporate either a zwitterionic group, or a neutral group; however, modifying the polymer so that it is completely zwitterionic could lead to difficulty in obtaining a spherical, stable morphology. For example, when uncharged amphiphilic block copolymers are self-assembled (phenyl_m-*b*-peg_n or phenyl_m-*b*-ethanol_n), complex morphologies can be obtained due to the lack of charge repulsion in the corona.⁴² When similar polymers that contain a carboxylic acid group are self-assembled, only small, spherical micelles are obtained, most likely due to the charge repulsion that is now present in the corona of the particle.⁴² This charge repulsion might be necessary to maintain the shape of the micelle, therefore it is not advantageous to create a completely zwitterionic polymer.

To ensure particle morphology is not altered, yet incorporate zwitterionic character to the particle, a new NIR dye was synthesized that has a zwitterionic surface charge and has



Phenyl₂₅-*b*-peptide₃-*co*-glycine₄-*b*-Cy 5^{ZW}_{0.4} were prepared *via* ROMP. Note that an olefin proton is present at 6.5 ppm after the addition of NorCy5^{ZW} and after 1 hour of polymerization the olefin is no longer present indicating the NIR dye has been incorporated into the ROMP polymer (Figure 5.20). The resulting polymers were dialyzed into buffer to form spherical nanoparticles. The particles could not be observed by DLS due to the absorption of the laser by the cy5 dye. Particles were analyzed by cryo EM and found to be spherical in a similar size to the cy5.5 labeled particles (Figure 5.21).

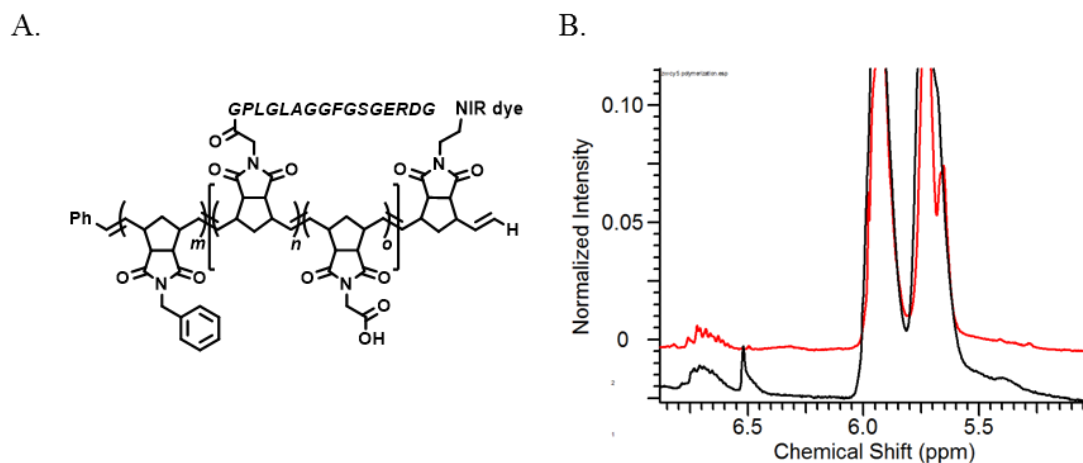


Figure 5.20 Incorporation of Nor-ZWcy5 into ROMP derived polymers. A. Chemical structure phenyl₂₅-b-pep₃-co-glycine₄-b-ZWcy5₁. B. ¹H NMR showing the spectrum immediately after the addition of Nor-ZWcy5 (black) and 1 hour after the addition of Nor-ZWcy5 (red). Note that the small chemical shift around 6.3 ppm corresponds to the olefin protons on the Nor-ZWcy5 monomer. After 1 hour, the chemical shift has disappeared indicating the consumption of Nor-ZWcy5.

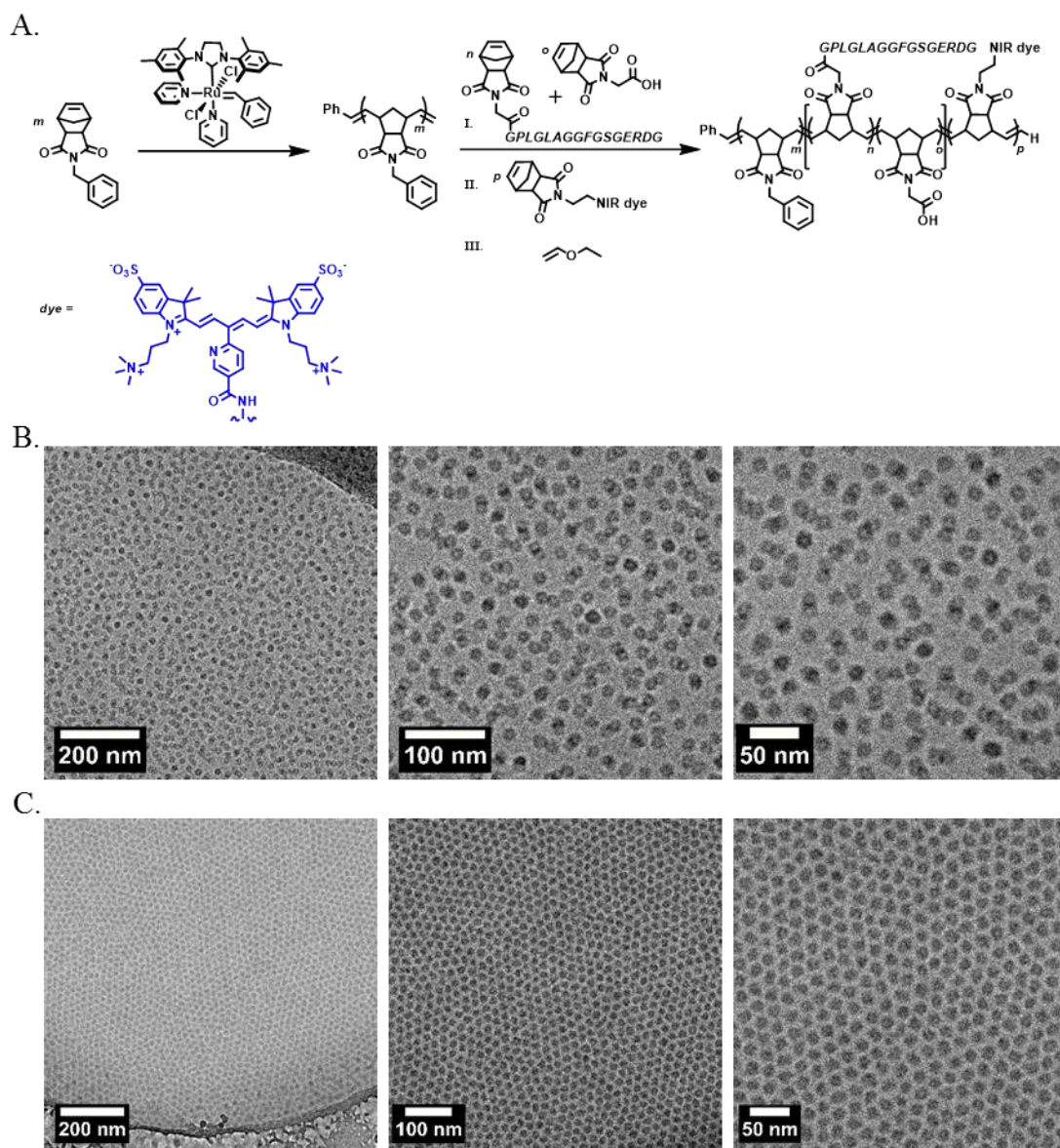


Figure 5.21 Zwitterionic polymer synthesis and particle characterization. A. Scheme for polymer synthesis. B. Particles formed from phenyl₂₅-*b*-L-peptide₃-co-glycine₄-*b*-ZWcy_{50.4}. C. Particles formed from phenyl₂₅-*b*-D-peptide₃-co-glycine₄-*b*-ZWcy_{50.4}.

To determine if a zwitterionic dye could reduce the liver uptake and increase tumor uptake, tumor-bearing mice were injected with $\mathbf{M_L^{ZW5}}$ and imaged at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 7 days (Figure 5.22 and 5.23). Animals were sacrificed at

24 hours or 7 days, imaged with the skin off (Figure 5.24) and the organs were harvested for SUV calculations (Figure 5.25).

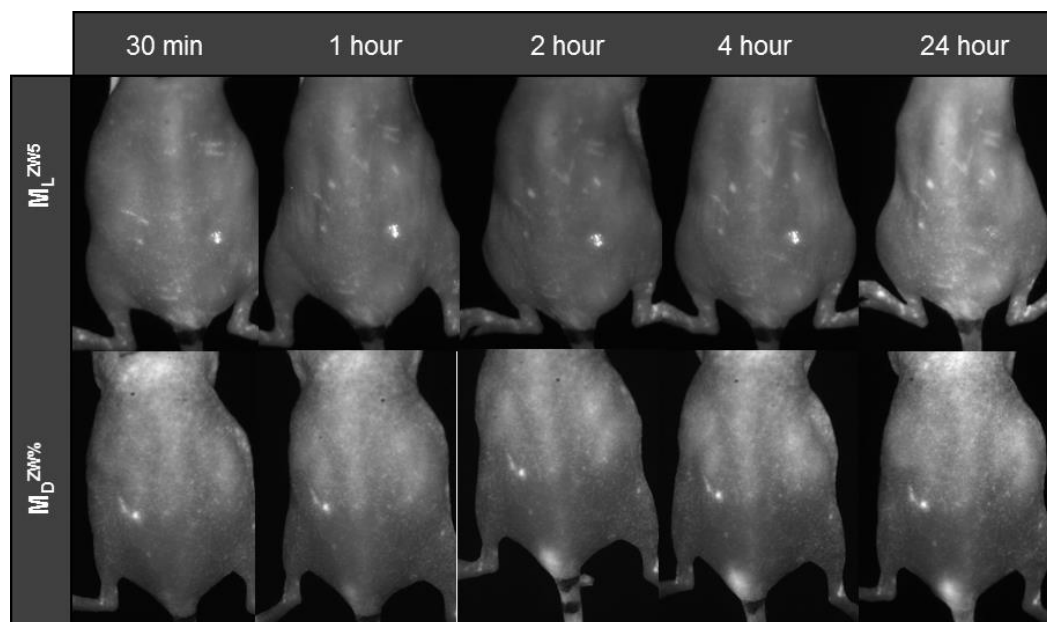


Figure 5.22 Live whole animal images of mice with Cal027 tumors after intravenous injection. The top row corresponds to the mice who received the M_L^{ZW5} (responsive) particles, and the bottom row corresponds to the mice that received the M_D^{ZW5} (non-responsive particles). This cohort of mice was sacrificed 1 day post IV injection.

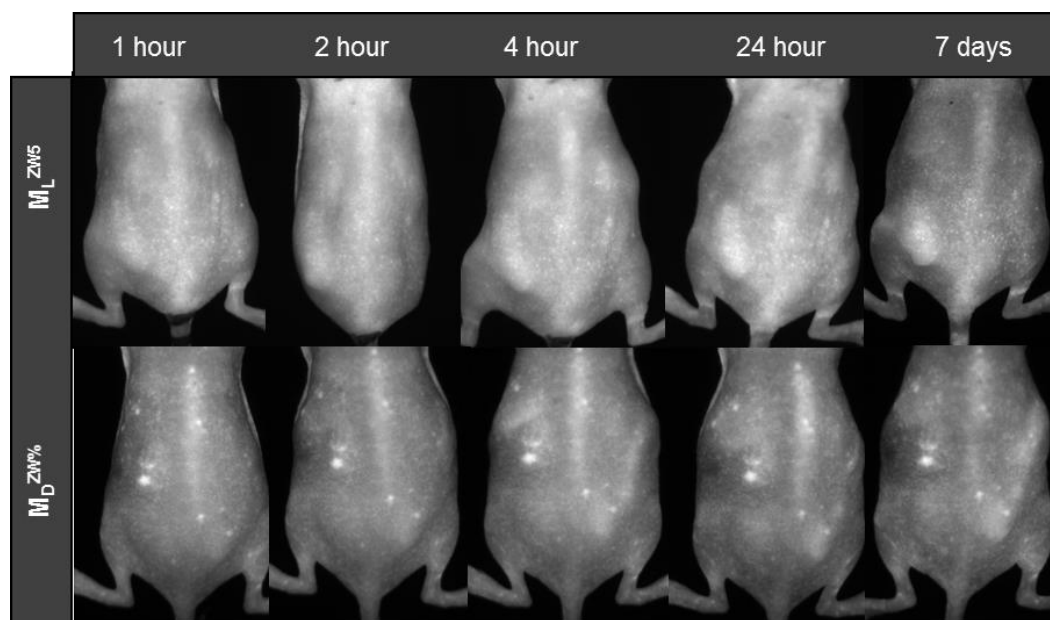


Figure 5.23 Live whole animal images of mice with Cal027 tumors after intravenous injection. The top row corresponds to the mice who received the M_L^{ZW5} (responsive) particles, and the bottom row corresponds to the mice that received the M_D^{ZW5} (non-responsive particles). This cohort of mice was sacrificed 7 days post IV injection.

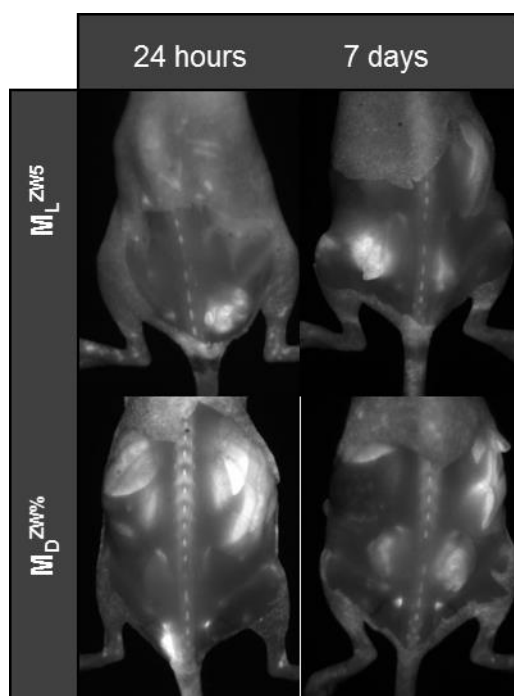


Figure 5.24 Skin off images of animals given M_L^{ZW5} and M_D^{ZW5} .

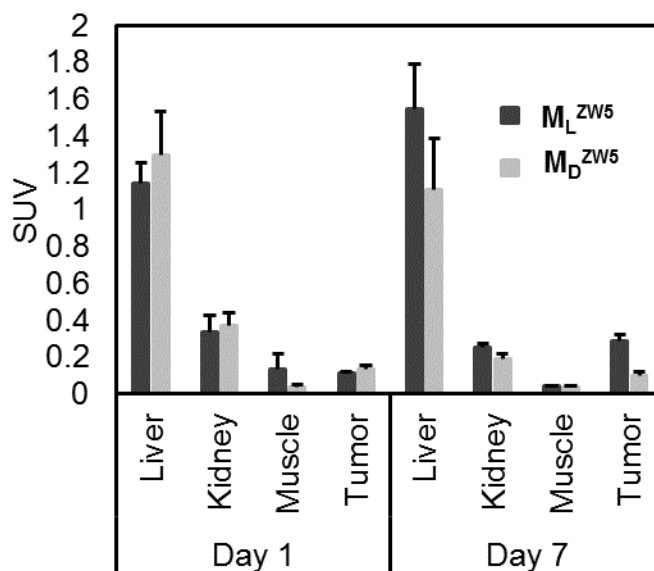


Figure 5.25 Standard uptake values of organs.

Looking at the standard uptake values, it is apparent that the liver accumulation of the zwitterionic particles is lower than those treated with the negatively charged particles. The SUV of the liver in mice treated with $M_L^{5.5}$ and $M_L^{5.5}$ was between 3.8 and 5.5 after 1 day, and 2.5 after 7 days. For M_L^{ZW5} and M_D^{ZW5} the liver SUVs were between 1 and 1.6 after 1 day, and 1.2 and 1.8 after 7 days, much lower than the negatively charged particles. Furthermore, the SUV of tumors at 7 days show that the responsive particles did accumulate to a greater extent than the non-responsive particles, suggesting that the mechanism of accumulation is an enzyme directed event.

Based on the SUVs, the tumor accumulation of M_L^{ZW5} increased from day 1 to day 7, which could suggest that the particles are circulating longer than 1 day. To determine if the zwitterionic particles were circulating longer than the negatively charged particles, the half-lives of circulations were determined for M_L^{ZW5} and $M_L^{5.5}$ (Figure 5.26), however there was no difference in the half-lives of the particles.

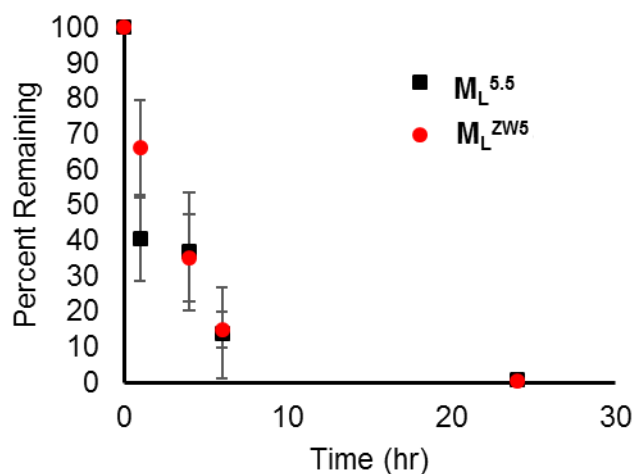


Figure 5.26 Blood circulation half-life.

Though the zwitterionic particles are not circulating longer, they still exhibit more favorable biodistribution, namely reduction in liver accumulation. This is a promising result, but before more conclusions can be drawn, a full targeting study must be performed where tumor volume is more controlled as well as the tumor location. This will allow for better comparison between the negative particles and zwitterionic particles in terms of accumulation in tumor tissue.

5.4 Targeting MMPs in Hind Limb Ischemia

Cardiovascular disease remains one of the leading causes of mortality in the US. Peripheral artery disease (PAD), a cardiovascular disease resulting from atherosclerosis, leads to lower limb pain and can progress to critical limb ischemia (CLI), often necessitating amputation. Currently, the only option for patients is revascularization therapy, which is relatively ineffective as the rates of amputation have not decreased significantly in the last 30 years.⁵⁴ Therapeutic angiogenesis was introduced in the field in the 1990s as a new way to

treat PAD and has shown success in preclinical trials.⁵⁵ There recently has shown promise in improving outcomes by upregulating angiogenesis through directly injecting a hydrogel like network displaying a VEGF mimicking peptide. This led to increased proliferation and vascularization in mice with critical limb ischemia (CLI).⁵⁶ These materials necessitated direct injections at the site of ischemia, which is not as therapeutically translatable, and to further advance the use of therapeutic angiogenesis, it is necessary to use a targeted delivery system. Using MMP-targeted particles could be utilized to deliver pro-angiogenic growth factors to the ischemic muscle to help aid in regeneration.

Before delivering a therapeutic, the MMP responsive particles were first investigated to see if targeting ischemia is possible. To determine if MMP responsive particles can accumulate in ischemic tissue, a rat model of PAD was generated and $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ were administered via IV injection and monitored by whole animal *in vivo* fluorescence. The model has one limb that is ischemic (right gracilis) and another limb that is healthy as a control (left gracilis). To determine how well particles can target the ischemic muscle, the fluorescence of the ischemic muscle was compared to the healthy muscle (Figure 5.28).

$\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ were IV administered to rats 5 days post ischemia surgery and imaged. A third group of animals received a sham surgery, where the entire surgery was performed except for inducing ischemia. These rats were given $\mathbf{M_L^{5.5}}$ particles, and this would allow us to determine if particle accumulation was a result of the increased inflammation from the surgery, or ischemia itself. Representative images of rats given $\mathbf{M_L^{5.5}}$ particles are shown in Figure 5.27, where the limb on the left is ischemic and the limb on the right is healthy. Notice that there is particle accumulation in the healthy limb. This accumulation is in the joints, where a type of MMP is present that can also cleave our particles.

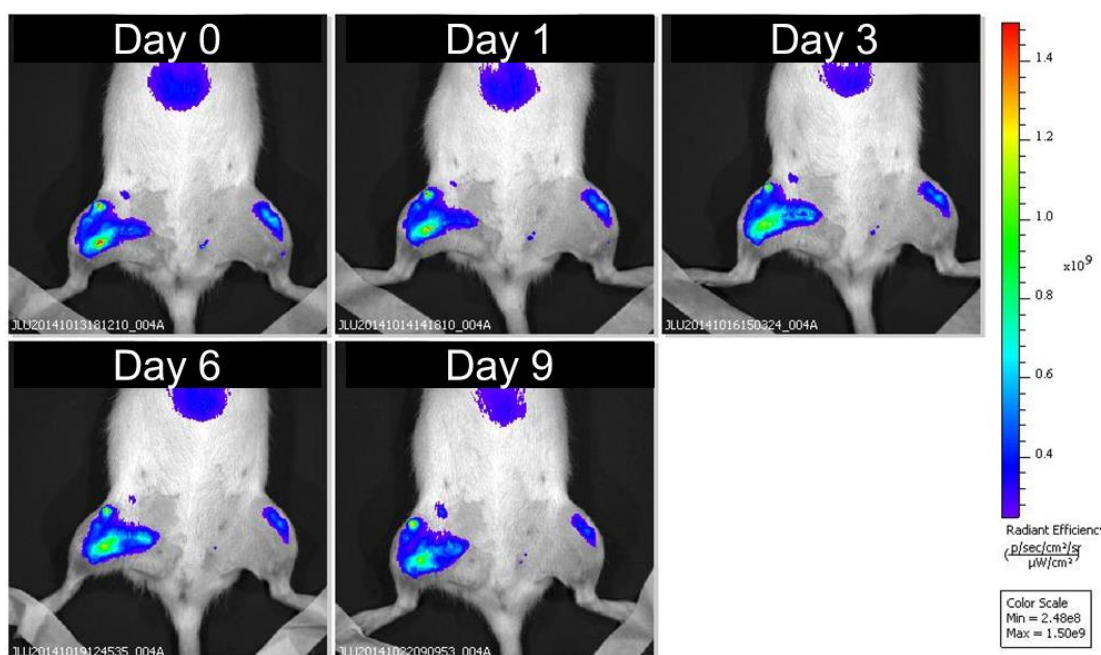


Figure 5.27 Whole animal *in vivo* imaging of animals with an ischemic limb (right leg, on the left). Animals were injected with $\mathbf{M_L^{5.5}}$ 5 days post ischemia surgery. Images taken by Jessica Ungerleider.

To determine ischemic tissue targeting, SUV ratios of the ischemic to healthy tissue fluorescence are compared between $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ (Figure 5.28). These ratios show that both particles target the ischemic tissue, even though the $\mathbf{M_D^{5.5}}$ should be nonresponsive to enzyme, and therefore not aggregate and target. At 28 days, the data suggests that the $\mathbf{M_L^{5.5}}$ are accumulating to a greater extent than the $\mathbf{M_D^{5.5}}$.

Based on SUV analysis of the satellite organs (Figure 5.29), the majority of the particles reside in the liver and spleen suggesting hepatic clearance of the materials. Over time, the SUV of these organs did decrease, but the particles were never completely eliminated. The SUV of the ischemic muscle (R Gracilis) is minimal compared to the other organs, likely because the particles are negatively charged and eliminated from the system rapidly. At 28 days, there is a trend that the $\mathbf{M_L^{5.5}}$ particles are still present in the ischemic limb compared to the $\mathbf{M_D^{5.5}}$ particles and the sham animals. What might be happening is that

all particles are accumulating into the ischemic limb due to the EPR effect, but only the $\mathbf{M_L^{5.5}}$ particles are able to undergo a morphology switch and stick around longer than the $\mathbf{M_D^{5.5}}$ particles. Nonetheless, the zwitterionic particles were tested in this animal model due to the large accumulation of the $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ particles in the liver.

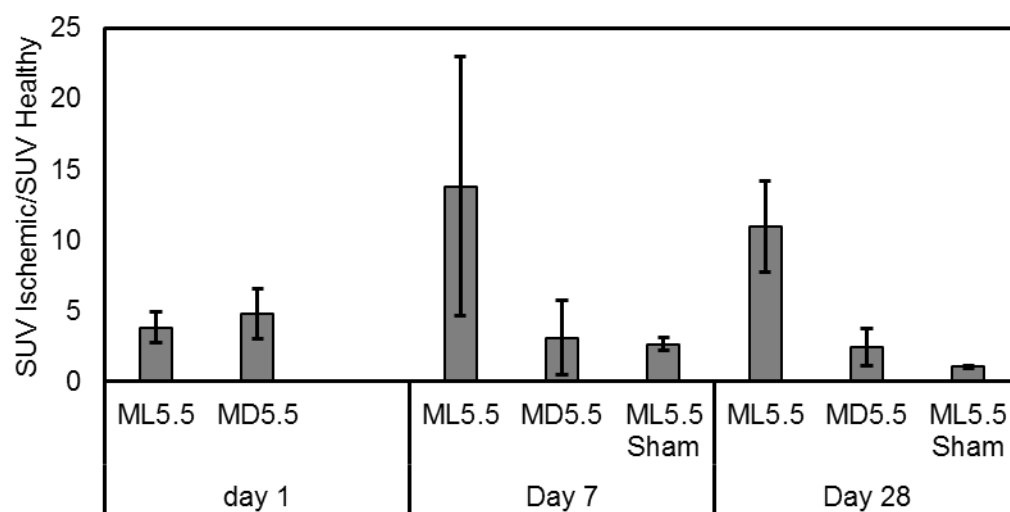


Figure 5.28 Comparing the targeting ability of $\mathbf{M_L^{5.5}}$ and $\mathbf{M_D^{5.5}}$ via SUV analysis of the *ex vivo* organs. N = 2

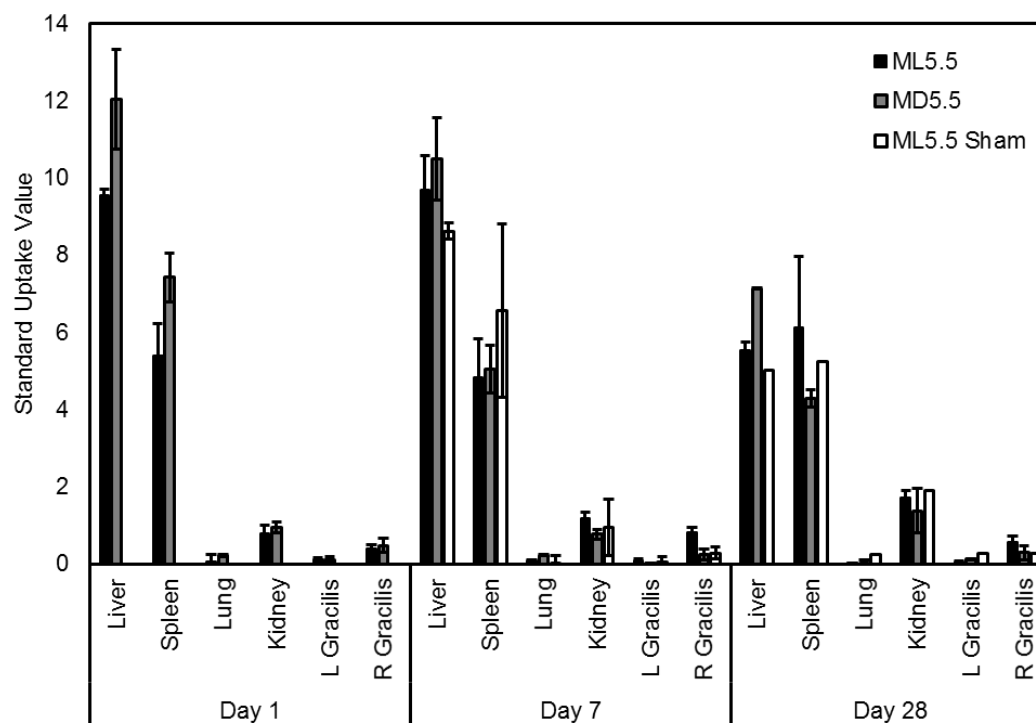


Figure 5.29 Standard uptake values of satellite organs of animals treated with $M_L^{5.5}$ and $M_D^{5.5}$. N = 2

Zwitterionic particles were tested in this animal model as well to see if a reduction in liver accumulation could be achieved similar to the cancer animal model. The ratio of ischemic to healthy calculated from SUV showed that neither responsive particle targeted better than the non-responsive particle, but the M_L^{ZW5} had a higher ratio than the $M_L^{5.5}$ suggesting the zwitterionic might have a better targeting ability (Figure 5.30).

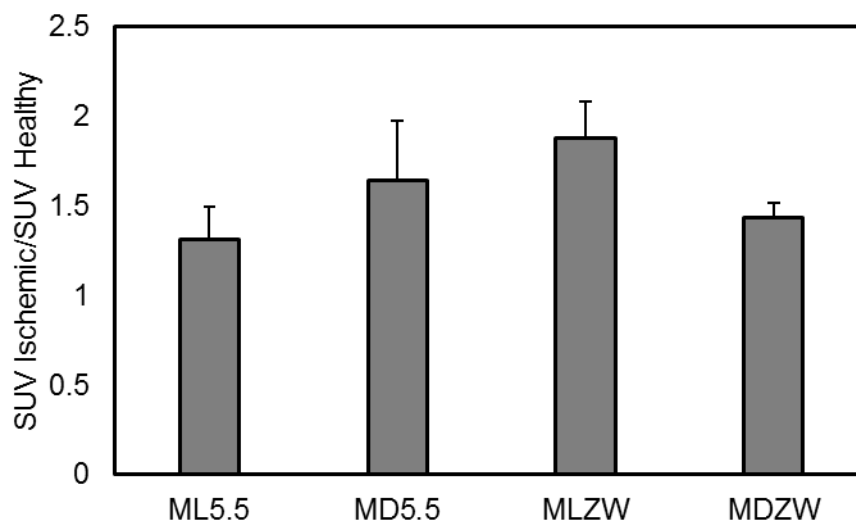


Figure 5.30 Comparing the targeting ability of negatively charged particles to zwitterionic particles. N = 4

The standard uptake values were calculated for the satellite tissues (Figure 5.31). The zwitterionic particles showed lower SUVs in the liver similar to the cancer studies, as well as greater accumulation in the muscles. However, both the responsive particles and nonresponsive particles had high SUVs in the ischemic tissue which does not confirm that the particles are accumulating based on MMP directed assembly. One issue with using Rats as the animal model is that the rats rip their sutures which can cause an increase in inflammation. For this reason, future animal studies are being conducted in a mouse model of peripheral artery disease to eliminate suture ripping.

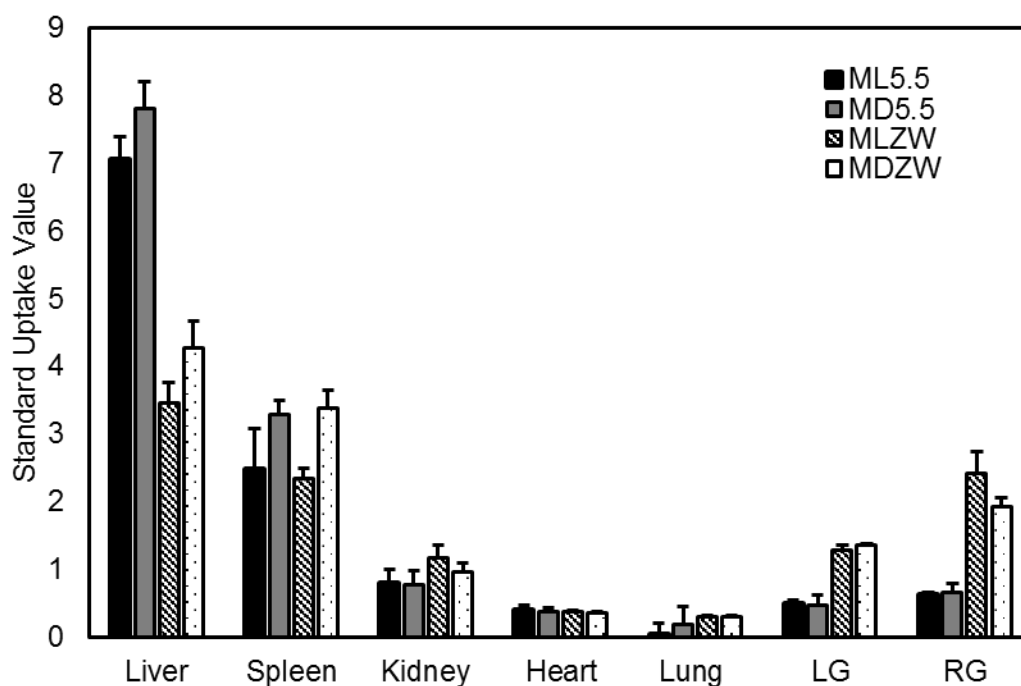


Figure 5.31 Standard uptake values of satellite organs. Note that LG is the left gracilis which is healthy and RG is the right gracilis which is ischemic. (n=4)

Ischemic gracilis (RG) from animals treated with M_L^{ZW5} and M_D^{ZW5} were sectioned, stained and imaged to visualize the particle accumulation (Figure 5.32). Both M_L^{ZW5} and M_D^{ZW5} were accumulated throughout the muscle tissue, but there was no significant difference of the fluorescence from the particles in these two tissues, confirming the results from the *in vivo* fluorescence data and SUV data.

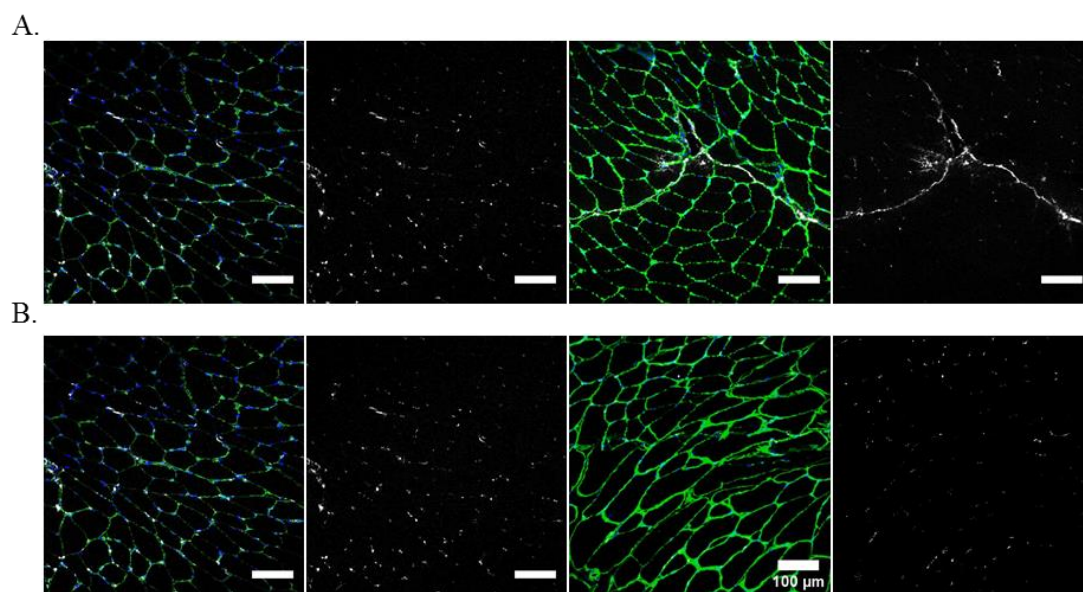


Figure 5.32 Confocal imaging of ischemic gracilis tissue sections. A. Animals treated with M_L^{ZW5} and B. animals treated with M_D^{ZW5} . Note that the muscle tissue is stained green, the nuclei are stained blue, and the particles are false colored white.

With these data, particles might be a good candidate for targeting ischemic tissue, however the targeting mechanism might not be fully understood due to the fact that the non-responsive particles are targeting almost as well as the responsive particles. Control particles are made with a peptide that is completely comprised of D amino acids. This might not be the best control because the peptide cannot be cleaved by any enzyme present in the blood, or other tissues. The L amino acid particles have multiple sites where enzymes in off target organs and blood can cleave and induce early aggregation. Instead, either a scrambled MMP sequence is desired, or a peptide where only the MMP recognition sequence is made of D amino acids, and the remaining peptide is L amino acid based. Based on these results, it is however advantageous to utilize the zwitterionic dye as more favorable biodistribution properties are obtained.

5.5 Conclusions and Future Directions

In conclusion, MMP responsive particles were generated *via graft through* ROMP and NIR dyes were successfully incorporated into the polymeric material. Polymers were successfully self-assembled into small, spherical nanomaterials that exhibited enzyme responsive aggregation *in vitro*. Furthermore, particles could be monitored *in vivo* using whole animal imaging, and biodistribution could be calculated to determine standard uptake values. When a zwitterionic dye is incorporated into the particle, there is a lower accumulation or clearance of the particles through the liver.

Though the particles behave like predicted *in vitro*, the same results were not recapitulated *in vivo* in two different animal models of diseases where MMPs are upregulated. This could be the result of many issues facing nanomaterials *in vivo* such as strong opsonization, instability in blood, and rapid macrophage uptake. In order for particles to be more effective *in vivo*, I believe more *in vitro* optimization and testing are necessary to fully understand how these particles behave *in vivo*.

One of the key issues with nanomaterials is opsonization, and I believe the particles should be further investigated to determine what proteins are opsonizing to the corona of the particle, and how tightly these proteins are bound. If there is a strong opsonization of proteins to the corona, these proteins could be blocking MMPs from enzymatically processing the particle shell, inhibiting the accumulation of these particles. Incorporation of zwitterionic groups, as well as PEG has been shown to reduce opsonization, therefore polymers can be synthesized that incorporate these moieties to create a more stealthy particle. For PEG to elicit stealth like properties, large molecular weights must be utilized, and this is not advantageous because this will most likely result in a PEG shell blocking the peptide sequence from MMP recognition.

The polymer could be synthesized with more zwitterionic character by incorporating a zwitterionic spacer between the peptide units. Moreover, the solubilizing peptide sequence located at the end of the peptide (GPLGLAG**GFGSGERDG**) could be replaced with a zwitterionic molecule, such as phosphatidyl choline. The advantages are twofold; this would create a zwitterionic, hydrophilic portion of the polymer, and remove excess peptide sequence that might be cleaved *in vivo* by other enzymes present in blood and tissue.

The MMP recognition sequence can further be optimized to use a more responsive sequence. The peptide sequence used in these studies is GPLGLAG, however in newer studies done by Tsien and colleagues use GPLGC(me)AG as it is more responsive to MMP-2/9.^{10,11} A more responsive sequence will enable faster cleavage and potentially faster aggregation *in vivo*. Furthermore, instead of incorporating 3 peptides per polymer, only a single peptide is necessary. This would decrease the density of the peptide, also allowing an increased in cleavage efficiency and faster aggregation.

All in all, NIR-labeled polymeric materials show promise for being used to target disease tissue, but more investigation and optimization *in vitro* is necessary improve targeting of the materials, as well as thoroughly understand the mechanism of accumulation.

5.6 Methods

For general methods see section 2.5.1.

5.6.1 Peptide Synthesis

Peptides were synthesized using standard Fmoc-chemistry solid phase peptide synthesis procedures on an AAPPTec Focus XC automated synthesizer. Peptides were prepared protecting group-free using Rink Amide MBHA resin. Standard Fmoc SPPS procedures can be found in section 2.5.2. Peptides were cleaved from resin using

TFA:TIPS:H₂O 95:2.5:2.5 and precipitated with cold ether, collected and purified by RPHPLC.

5.6.2 NIR Dye Monomer Synthesis

Cy 5.5 Monomer Synthesis

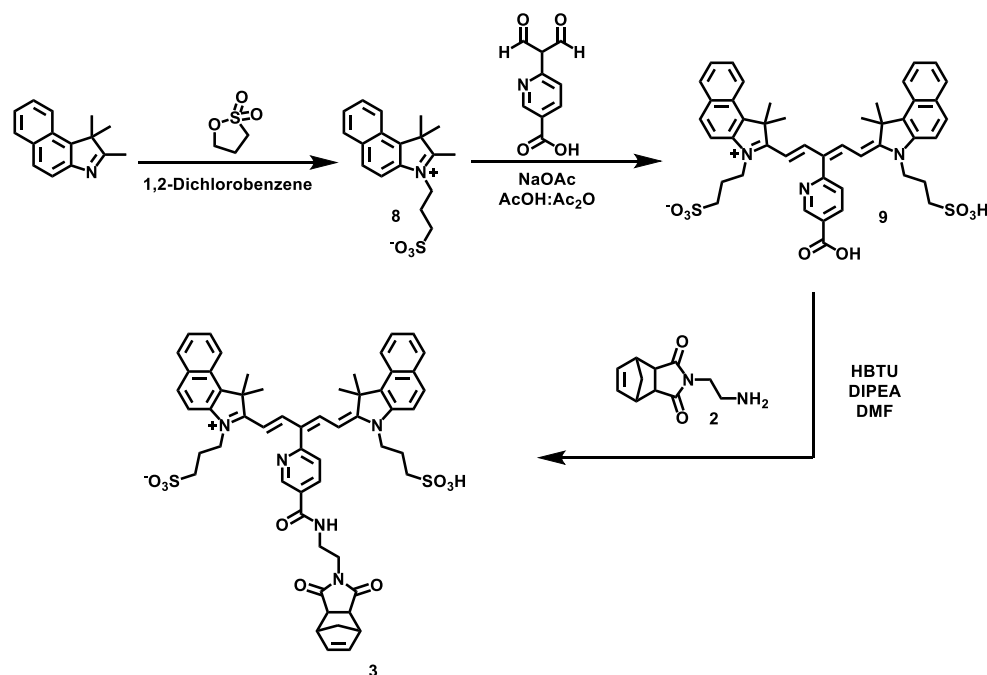


Figure 5.33 Synthetic scheme for the synthesis of cy 5.5 monomer 3.

8⁵⁷ has been synthesized previously.

3-(2-((1E,3Z,5E)-3-(5-carboxypyridin-2-yl)-5-(1,1-dimethyl-3-(3-sulfopropyl)-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-ium-3-yl)propane-1-sulfonate (9)

8 (1.60 g, 4.9 mmol), 6-(1,3-dioxopropan-2-yl)nicotinic acid (0.43 g, 2.2 mmol), and sodium acetate (1.30 g, 16.1 mmol) was dissolved in 125 mL of a 1:1 mixture of acetic anhydride and acetic acid and heated to reflux for 5 h. Once complete, the solvent was removed by rotary evaporation and the blue solid was dissolved in water with 0.1% TFA and

purified by reverse phase HPLC using a gradient from 10-60% buffer B where buffer A is water with 0.1% TFA and buffer B is acetonitrile with 0.1% TFA. The pure fractions were collected and lyophilized. Yield: 1.04 g, 56%. ¹H NMR (DMSO-d₆): δ = 9.32 (s, 1H), 8.74 (d, 1H), 8.70 (d, 2H), 8.25 (d, 2H), 8.07 (t, 4H), 7.90 (d, 1H), 7.83 (d, 2H), 7.69 (t, 2H), 7.53 (t, 2H), 6.04 (b, 2H), 4.32 (t, 4H), 2.53 (t, 4H), 2.00 (m, 4H), 1.99 (s, 12H). HRMS (ESI-TOF): m/z (%) [M-H] - calcd for C₄₅H₄₅N₃O₈S₂: 818.2648, found: 818.4485.

3-(2-((1E,3Z,5E)-5-(1,1-dimethyl-3-(3-sulfopropyl)-1,3-dihydro-2Hbenzo[e]indol-2-ylidene)-3-(5-((2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisindol-2-yl)ethyl)carbonyl)pyridin-2-yl)penta-1,3-dien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-yl)propane-1-sulfonate (3)

9 (100 mg, 0.13 mmol), HATU (47 mg, 0.13 mmol) and DIPEA (97 mg, 0.75 mmol) were added to 2 mL of anhydrous DMF. After 10 min, **2**-(2-aminoethyl)-3a,4,7,7a-tetrahydro-1H-4,7-methanoisindole-1,3(2H)-dione (**2**) (115 mg, 0.37 mmol) was added. After 45 min, cold diethyl ether was added to the reaction and the precipitate was collected and dried under vacuum. The dry blue powder was dissolved in buffer A (water with 0.5% TFA) and purified on reverse phase HPLC (gradient 20-60% buffer B, ACN with 0.1% TFA). The pure fractions were collected and lyophilized. Yield: 77 mg, 62%. ¹H NMR (DMF-d₇): δ = 9.66 (t, 1H), 9.51 (s, 1H), 9.15 (d, 2H), 8.94 (d, 1H), 8.48 (d, 2H), 8.31 (t, 4H), 8.15 (t, 2H), 7.87 (t, 2H), 7.73 (t, 2H), 6.48 (s, 2H), 6.41 (b, 2H), 4.65 (t, 4H), 3.93 (t, 2H), 3.79 (t, 2H), 3.28 (s, 2H), 3.15 (m, 2H), 2.37 (t, 4H), 2.22 (s, 12H), 1.59 (m, 2H). [M-H] - calcd for C₅₆H₅₇N₅O₉S₂: 1006.3598, found: 1006.4623

Cy 7 Monomer Synthesis

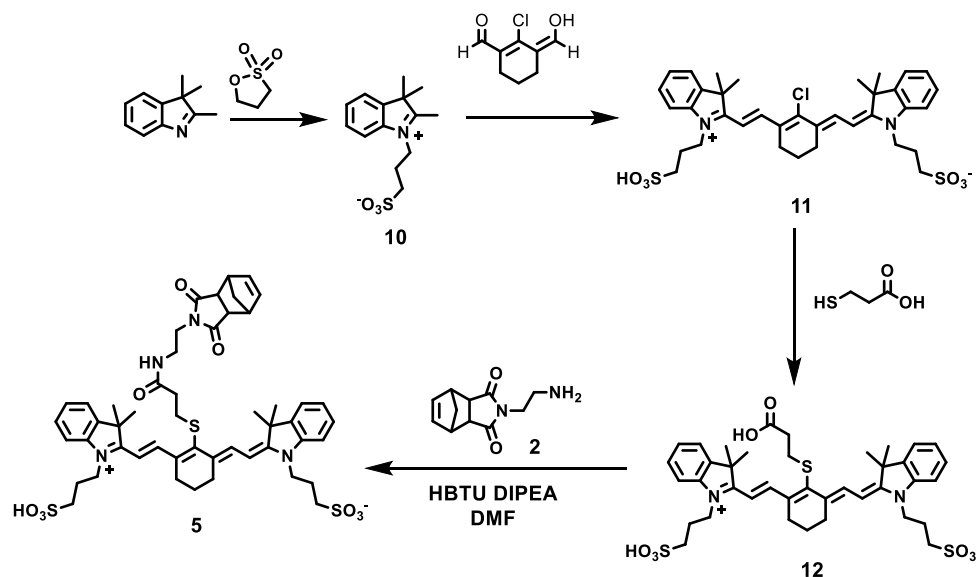


Figure 5.34 Synthetic scheme for cy 7 monomer.

10⁵⁸ has been reported previously.

3-((E)-2-((E)-2-(2-chloro-3-((E)-2-(3,3-dimethyl-1-(3-sulfopropyl)-3H-indol-1-ium-2-yl)vinyl)cyclohex-2-en-1-ylidene)ethylidene)-3,3-dimethylindolin-1-yl)propane-1-sulfonate (11)

10 (28.45 mmol, 2.2 equiv), the Vilsmeier-Haack reagent (13.9 mmol, 1 equiv) and a 7:3 mixture of n-butanol in benzene (500 mL) is added to a roundbottom and heated to reflux for 2 days. The solvent was removed by rotary evaporation, and the green solid was dissolved in 30% ACN in H₂O and purified by RPHPLC using the gradient 30%-60% buffer B (0.1%TFA in ACN) in buffer A (0.1%TFA in H₂O). ¹H NMR (DMSO-d₆): δ = 8.27 (d, 2H), 7.63 (d, 2H), 7.50 (d, 2H), 7.42 (t, 2H), 7.27 (t, 2H), 6.39 (d, 2H), 4.22 (t, 4H), 2.73 (t, 4H), 2.58 (t, 4H), 2.04 (t, 4H), 1.80 (t, 2H), 1.67 (s, 12H). [M-H]⁻ - calcd for C₃₆H₄₃ClN₂O₆S₂: 698.23, found: 699.26.

3-((E)-2-((E)-2-(2-((2-carboxyethyl)thio)-3-((E)-2-(3,3-dimethyl-1-(3-sulfopropyl)-3H-indol-1-ium-2-yl)vinyl)cyclohex-2-en-1-ylidene)ethylidene)-3,3-dimethylindolin-1-yl)propane-1-sulfonate (12)

11 (0.028 mmol, 1 equiv), and mercaptapropionic acid (0.055 mmol, 2 equiv) were added to a round bottom with 1 mL DMF and stirred for 18 hours. Diethyl ether was added to the reaction, and the precipitate was collected, dissolved in 10% buffer B and purified by RP-HPLC using the gradient 10-90% buffer B. ¹H NMR (DMF-d₇): δ = 8.85 (d, 2H), 7.62 (t, 4H), 7.45 (t, 2H), 7.29 (t, 2H), 6.65 (d, 2H), 4.51 (t, 4H), 3.11 (t, 2H), 2.84 (t, 4H), 2.67 (t, 2H), 2.22 (t, 4H), 1.85 (t, 2H), 1.78 (s, 12H). [M-H] - calcd for C₃₉H₄₈N₂O₈S₃: 768.26, found: 767.16.

3-((E)-2-((E)-2-(3-((E)-2-(3,3-dimethyl-1-(3-sulfopropyl)-3H-indol-1-ium-2-yl)vinyl)-2-((3-((2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisoindol-2-yl)ethyl)amino)-3-oxopropyl)thio)cyclohex-2-en-1-ylidene)ethylidene)-3,3-dimethylindolin-1-yl)propane-1-sulfonate (5)

12 (0.097 mmol, 1equiv), HBTU (0.097 mmol, 1equiv), and DIPEA (0.58 mmol, 6 equiv) were mixed in DMF, then **2** (.2929 mmol, 3 equiv) was added and mixed for 3 hours. Ether was added to the reaction and the precipitate was collected, dissolved in 15% buffer B and purified by RP-HPLC from 15-80% B. ¹H NMR (DMF-d₇): δ = 8.87 (d, 2H), 8.13 (d, 1H), 7.67 (t, 4H), 7.48 (t, 2H), 7.30 (t, 2H), 6.71 (d, 2H), 6.28 (s, 2H), 4.57 (t, 4H), 3.50 (t, 2H), 3.31 (t, 2H), 3.10 (t, 2H), 2.78 (t, 2H), 2.49 (t, 2H), 2.24 (t, 4H), 1.85 (t, 2H), 1.79 (s, 12H), 1.50 (d, 1H), 1.39 (d, 1H).). [M-H] - calcd for C₅₀H₆₀N₄O₉S₃: 956.35, found: 956.58.

Zwitterionic Cy 5 Synthesis

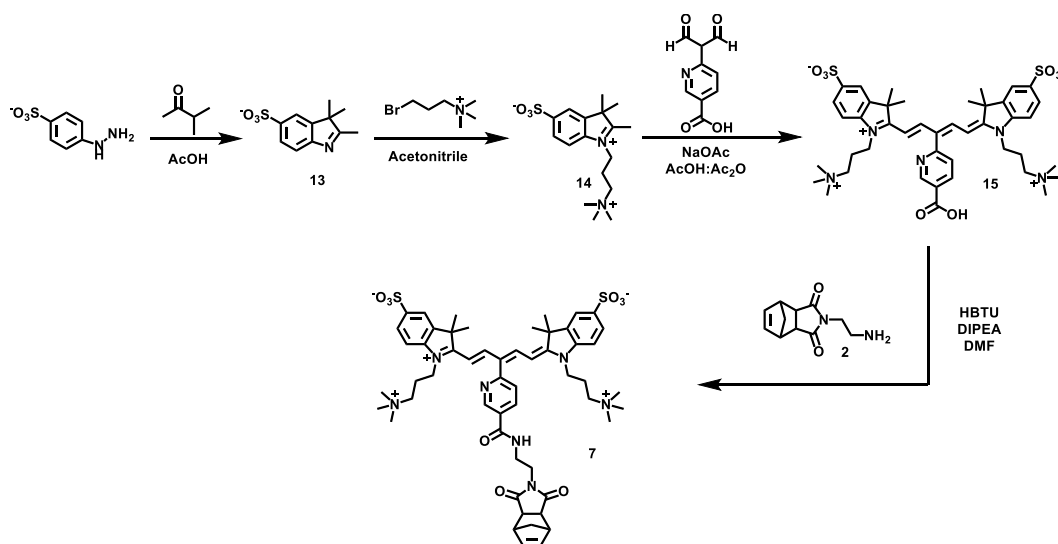


Figure 5.35 Synthetic scheme for zwitterionic cy 5 monomer.

13 and **14** have been synthesized previously.⁴⁰

2-((1E,3Z)-3-(5-carboxypyridin-2-yl)-5-((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate (15)

14 (1.97 mmol, 2.2 equiv), 6-(1,3-dioxopropan-2-yl)nicotinic acid (0.895 mmol, 1 equiv), and sodium acetate (6.265 mmol, 7 equiv) was dissolved in 4 mL of a 1:1 mixture of acetic anhydride and acetic acid and heated to reflux for 18 hr. Once complete, the solvent was removed by rotary evaporation and the blue solid was dissolved in water with 0.1% TFA and purified by reverse phase HPLC using a gradient from 10-60% buffer B where buffer A is water with 0.1% TFA and buffer B is acetonitrile with 0.1% TFA. MS: Calcd for $C_{47}H_{56}N_5O_8S_2^+$ m/z 834.3565, found m/z . 832.43.

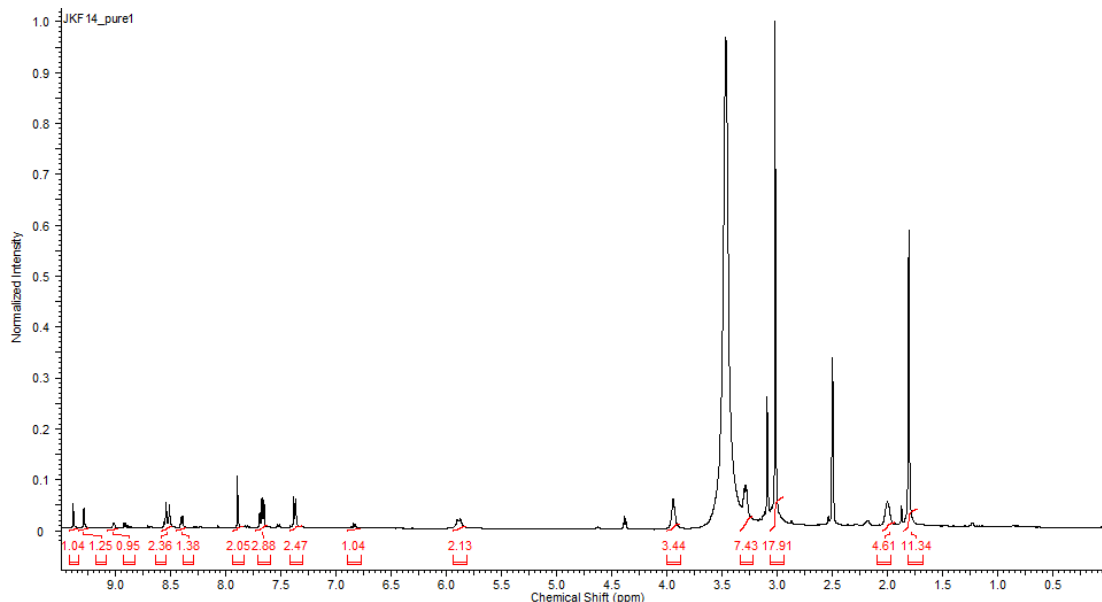


Figure 5.36 ^1H NMR of 15.

2-((1E,3Z)-5-((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)-3-(5-((2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisindol-2-yl)ethyl)carbamoyl)pyridin-2-yl)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate (7)

15 (.0179 mmol, 1equiv) was activated with HBTU (0.0179 mmol, 1 equiv.) and DIPEA (.1076 mmol, 6 equiv.) in DMF for 10 minutes. Following activation, **2** (.0538 mmol, 3 equiv.) was added and the reaction was stirred at room temperature for 2 hours. The product was precipitated with the addition of cold ether and purified by reverse phase HPLC using a gradient 10%-60% buffer B (Acetonitrile with 0.1% TFA). The pure fractions were lyophilized to obtain a blue powder. MS: Calcd for $\text{C}_{54}\text{H}_{68}\text{N}_7\text{O}_9\text{S}_2^+$ m/z 1022.4541, found 1022.43 m/z

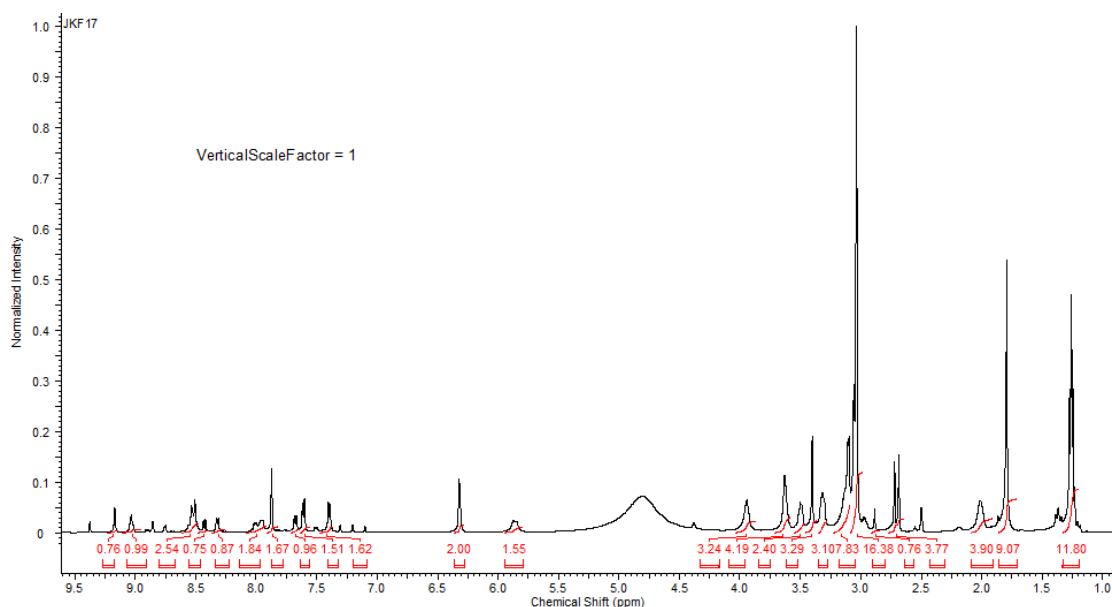


Figure 5.37 ^1H NMR of 7.

5.6.3 Polymerizations

All polymerizations were carried out in a glove box under N_2 atmosphere. A typical protocol used to generate the polymer $\text{phenyl}_{25}\text{-}b\text{-peptide}_3$ involved mixing the phenyl monomer (0.04 mmol, 20 equiv.) with the catalyst initiator (0.002 mmol, 1 equiv) in dry DMF d_7 . After 20 minutes, the peptide monomer (0.006 mmol, 3 equiv) was added to the polymerization. After 30 minutes, polymers that were not labeled with NIR dye were quenched with ethyl vinyl ether (10 equiv.). For NIR labeled polymers, NIR dye monomer (0.0008 mmol, 0.4 equiv.) was added for 1 hour, then termination ethyl vinyl ether. Before each addition of subsequent blocks, a small aliquot (~20 μL) was removed from the polymerization reaction, quenched with ethyl vinyl ether and analyzed by SEC MALS to determine molecular weight and dispersity.

A typical protocol used to generate the polymer phenyl₂₅-*b*-peptide₃-co-glycine₄ involved mixing the phenyl monomer (0.04 mmol, 20 equiv.) with the catalyst initiator (0.002 mmol, 1 equiv) in dry DMF_{d7}. After 20 minutes, the peptide monomer (0.006 mmol, 3 equiv) was added to the polymerization. To make the blend copolymer, 1 equiv of glycine (0.008 mmol, 4 equiv) was added to the polymerization every 2 minutes, which is the time necessary to polymerize one peptide monomer (see method section 5.7.). After final addition of glycine, polymers that were not labeled with NIR dye were quenched with ethyl vinyl ether (10 equiv.). For NIR labeled polymers, NIR dye monomer (0.0008 mmol, 0.4 equiv.) was added for 1 hour, then termination ethyl vinyl ether. Before each addition of subsequent blocks, a small aliquot (~20 uL) was removed from the polymerization reaction, quenched with ethyl vinyl ether and analyzed by SEC MALS to determine molecular weight and dispersity.

All polymers were then precipitated with cold ether and collected by centrifugation. Polymers were then dissolved in a 1:1 mixture of H₂O:ACN and lyophilized.

5.6.4 Polymerization Kinetics

The kinetics of polymerization were determined for Nor-Peptide monomer and the Nor-Glycine monomer by monitoring the polymerization by ¹H NMR. A standard protocol for the experiment involved dissolving the monomer (0.0275 mmol, 20 equiv.) in DMF_{d7} and transferring to an NMR tube under N₂ atmosphere. An initial NMR spectrum was acquired. The NMR tube was removed from the NMR, and the catalyst was added (0.0014 mmol, 1 equiv), followed by immediately returning the NMR tube to the instrument and acquiring spectrum. The polymerization was monitored until complete monomer consumption.

To determine the kinetics of polymerization,⁴³ the integrals of the monomer and polymer at each spectrum were determined. A corrected integral for each was calculated by

subtracting the background integration. For example, the background of the monomer was determined using the final NMR spectrum where no monomer is present. This background was subtracted from each integral. The background of the polymer integral was determined by the initial NMR and subtracted from the polymer integral at all timepoints. The percent conversion was then determined using the polymer and monomer integrals (percent conversion = (polymer/(monomer + polymer))*100. The number of monomers polymerized was determined by dividing polymer concentration by initial monomer concentration and multiplying by the DP targeted.

5.6.5 Particle Formation

Polymers were dissolved in dried organic solvent (DMF or DMSO) at a concentration of 2 mg/mL and then DPBS was added at a rate of 1 mL/hr with stirring until a final concentration of 1 mg/mL was reached. The mixture was then transferred to 10000 MWCO dialysis tubing and dialyzed into DPBS with 3 buffer changes. For *in vivo* studies, particles were then sterile filtered using 0.22 um filters and then concentrated using steril Millipore centrifugal units with 10000MWCO until particle concentration was 1.6 mM with respect to NIR dye.

5.6.6 CryoTEM

Quantifoil R2/2 400 mesh holey carbon TEM grids were cleaned and made hydrophilic using air plasma from a glow discharge system (2 minutes, 20 mA). 3 µL of a 10 mg/mL sample was pipetted onto each grid and blotted for approximately 3 seconds to make a thin film of liquid on the surface of the grid. Immediately afterwards, the grid was directly plunged into liquid ethane and transferred into liquid nitrogen. Cryo-TEM imaging was performed on a FEI Tecnai G2 Sphera operated at 200 keV. Low electron dose imaging procedures were used in order to prevent beam damage of the samples.

5.6.7 *In Vitro* Enzyme Assay with Thermolysin

100 mM of particles with respect to peptide were incubated with 1 mM thermolysin in DPBS buffer and analyzed by RP-HPLC for 14 hours. An initial RP-HPLC was taken, followed by addition of thermolysin, then subsequent HPLC analysis every 1.5 hrs.

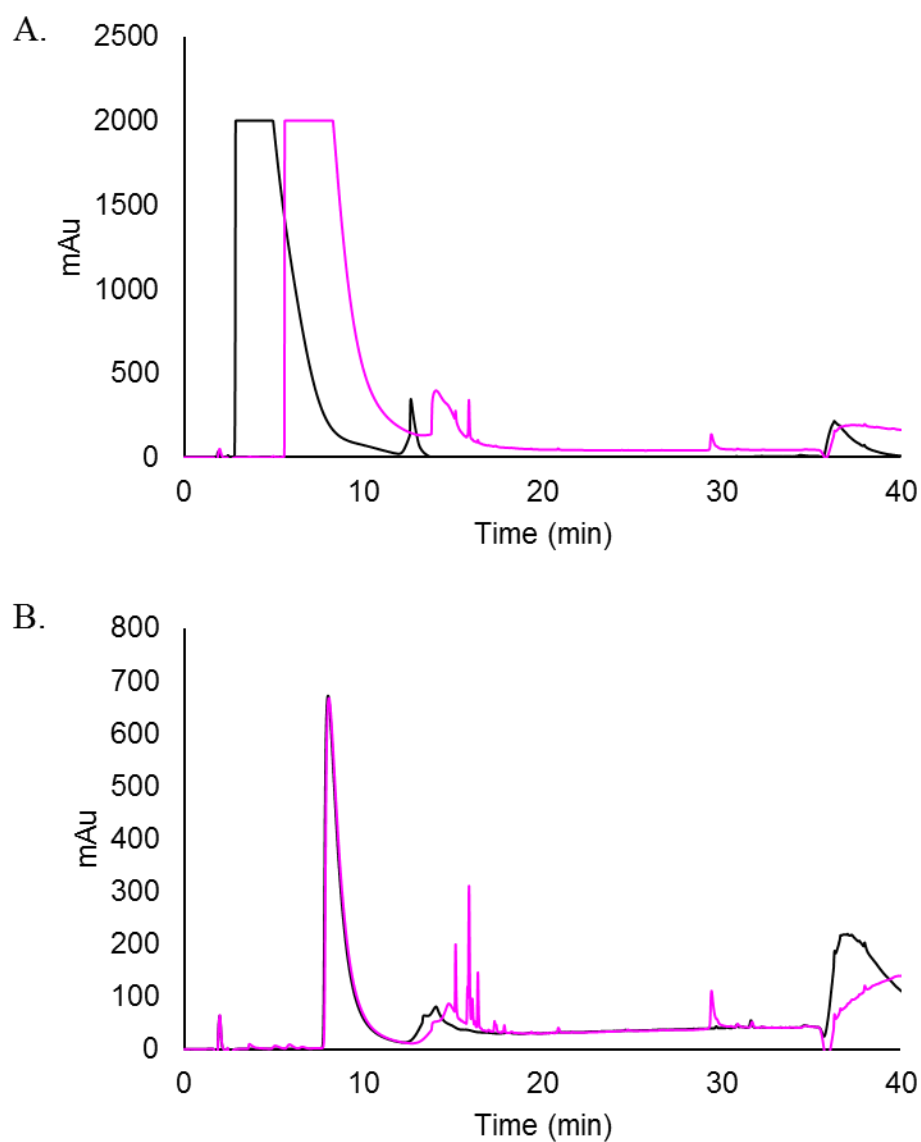


Figure 5.38 Raw RP-HPLC chromatograms of A. Block particles, or B. Blend particles treated with thermolysin. Black is the initial trace and pink is after 14 hours.

5.6.8 *In Vivo* Tumor Targeting Studies

All animal experiments were done in Dr. Quyen Nguyen's laboratory. Susie Johnson generated all of the animal models and performed the injections.

Nu/nu mice were injected with Cal27 cancer cell line which is known to overexpress MMPs. Mice were innoculated with 5 million cells per flank and tumors were allowed to grow for 2-3 weeks. Mice were injected with 160 nmol of particles with respect to dye and imaged at 30, 60, 120, and 240 minutes. Animals that were sacrificed at 24 hours were imaged at 24 hours, then anesthetized with ketamine midazolam and a final image was taken with the skin removed to remove excess background. Animals were then sacrificed and liver, kidney muscle and one tumor were harvested and frozen. The other tumor was embedded in OTC for tissue sectioning. Animals that were sacrificed at 7 days were imaged at 24 hours, then again at 7 days followed by a skin off image and organs were harvested as detailed above. Animal imaging was done on a Maestro with filtersets 640/47 excitation and 700LP for emissions for cy 5.5 and 607/36 excitation and 685/40 emission.

5.6.9 *In Vivo* PAD Imaging

All PAD animal studies were performed in Dr. Karen Christman's group. Dr. Rebecca Braden performed all the ischemia surgeries. Jessica Ungerleider performed all injections, imaging, and harvesting.

Female Sprague Dawley rats underwent hindlimb ischemia surgery (day -5). Animals were anesthetized, and the surgical site on the right limb was shaved and cleaned with betadine and isopropanol along with 1% lidocaine delivery subcutaneously along the incision site. An incision along the limb was performed and the femoral artery and vein were dissected away from the femoral nerve and ligated. A 2 cm segment was removed. The

incision through the skin was closed. Initial Perfusion measurements were taken to determine degree of ischemia.

At day 0, animals were injected with 400 nmol of particles with respect to dye via an intravenous injection. Animals were then imaged using In Vivo Imaging System (IVIS) with the filter sets 675nm/720nm excitation and emission for cy 5.5 labeled particles, and 640nm/680nm excitation and emission for zwcy 5 labeled particles with 2.5 second exposure time. At final timepoints, animals were euthanized and the liver, spleen, kidney, heart, lung, left gracilis, and right gracilis were harvested for tissue sectioning and standard uptake value analysis.

5.6.10 Standard Uptake Values

50-90 mgs of organ (liver, kidney, muscle, tumor) were added to 9uL/mg of digestion buffer (0.25 mg/mL proteinase K, 0.1 mg/mL DNase, 150 mM NaCl, 10 mM tris pH 8, 0.2%SDS) and homogenized using a tissue tearer. The solution was transferred to an Eppendorf and sonicated for 10 s then heated for 18hrs at 50°C. Samples were then sonicated for 2 seconds, vortexed for 10 seconds, then transferred to a 96 well plate and fluorescence was measured using a plate reader. The fluorescence was compared to a standard curve made using homogenized tissue from an untreated mouse with particles various concentrations of particles added. Standard uptake values were calculated using the following equation:

$$SUV = \frac{\text{mmol of partile in tissue/mg of tissue}}{\text{mmol injected/mg of animal}}$$

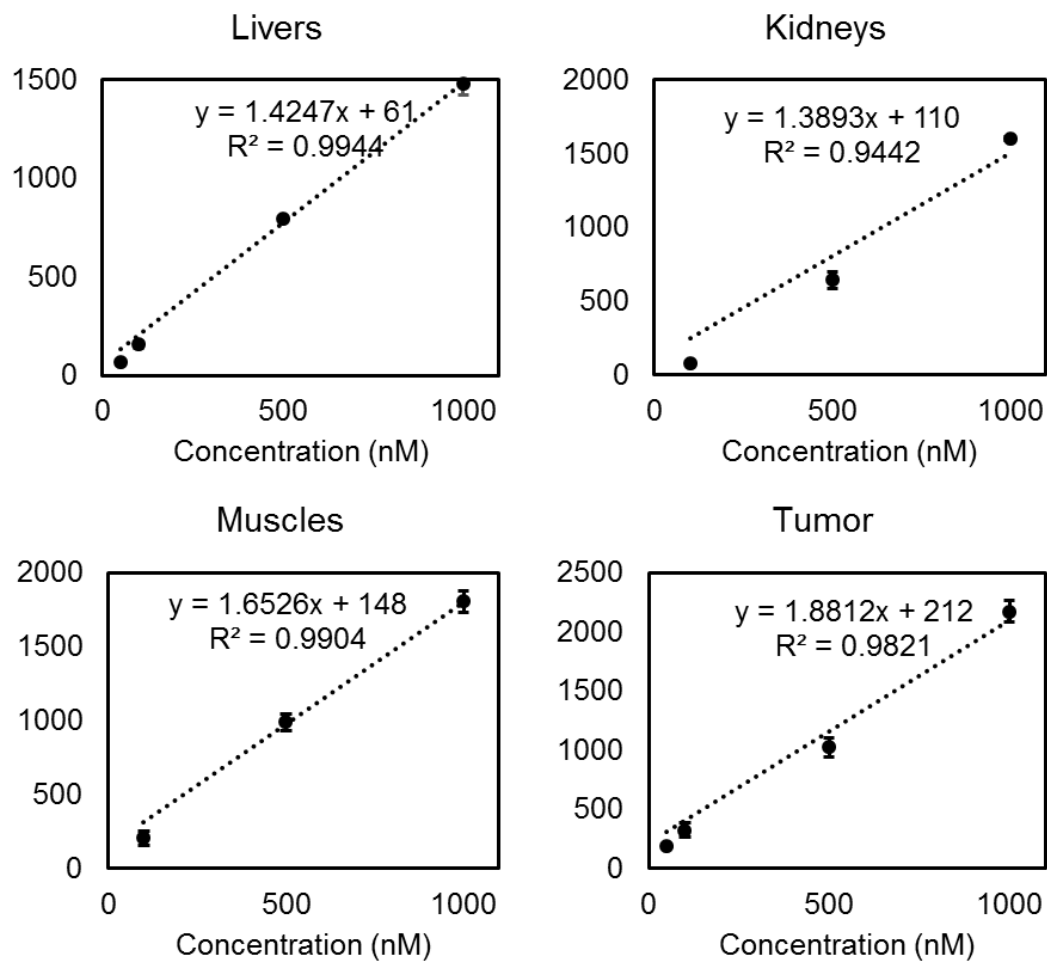


Figure 5.39 Standard curves for cy 5.5 labeled particle concentration in each tissue from nu/nu mice.

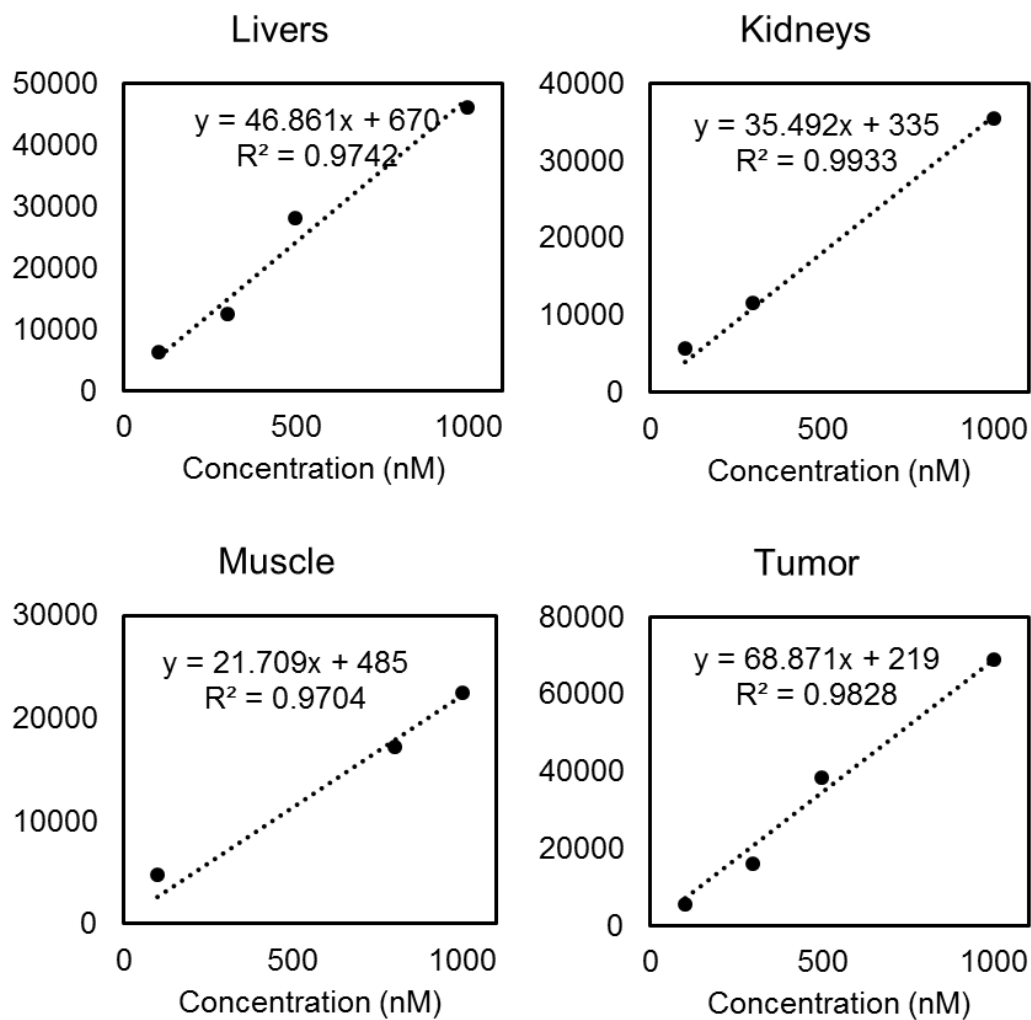


Figure 5.40 Standard curves for zw cy 5 labeled particle concentration in each tissue from nu/nu mice.

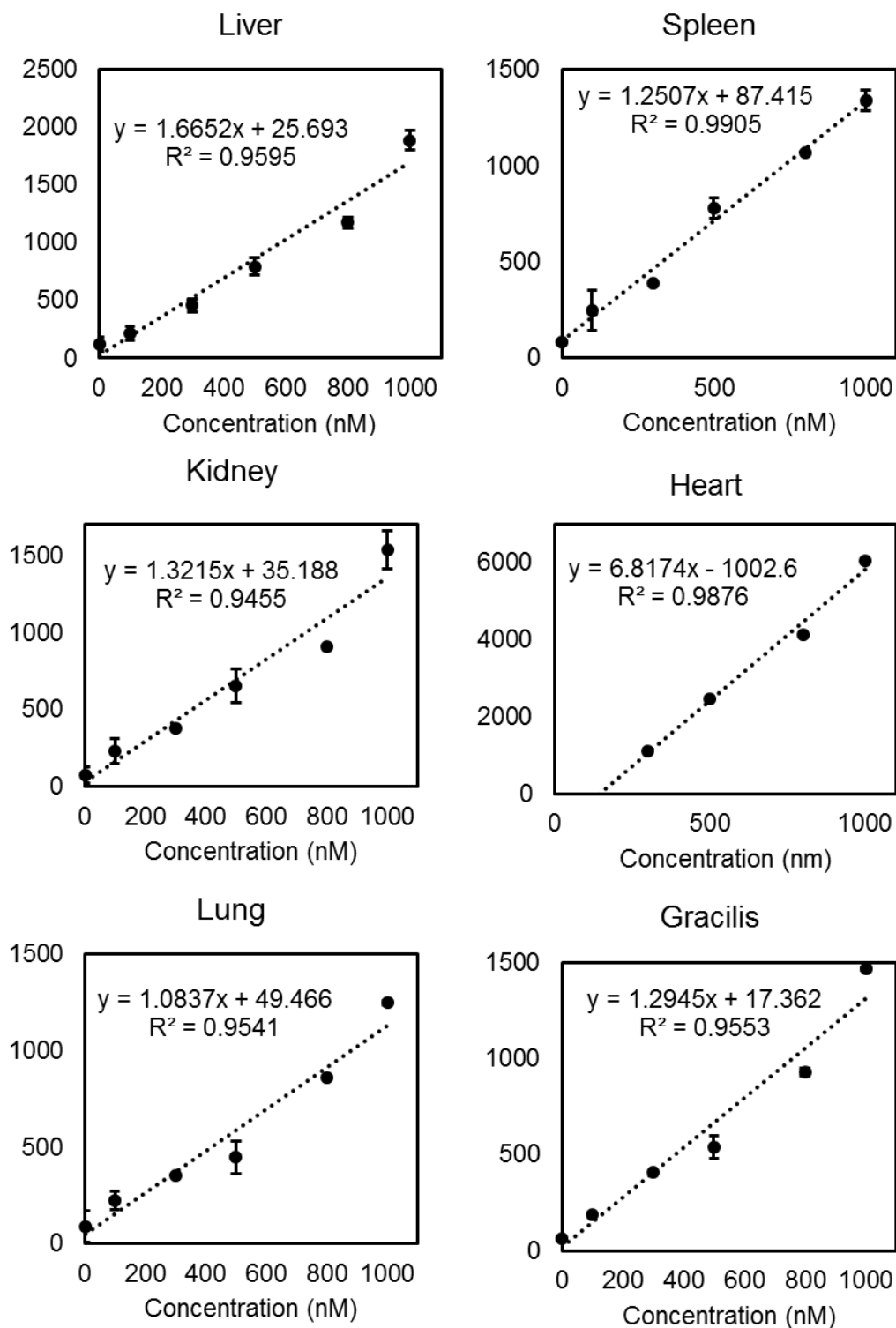


Figure 5.41 Standard curves for cy 5.5 labeled particle concentration in each tissue from Sprague Dawley rats.

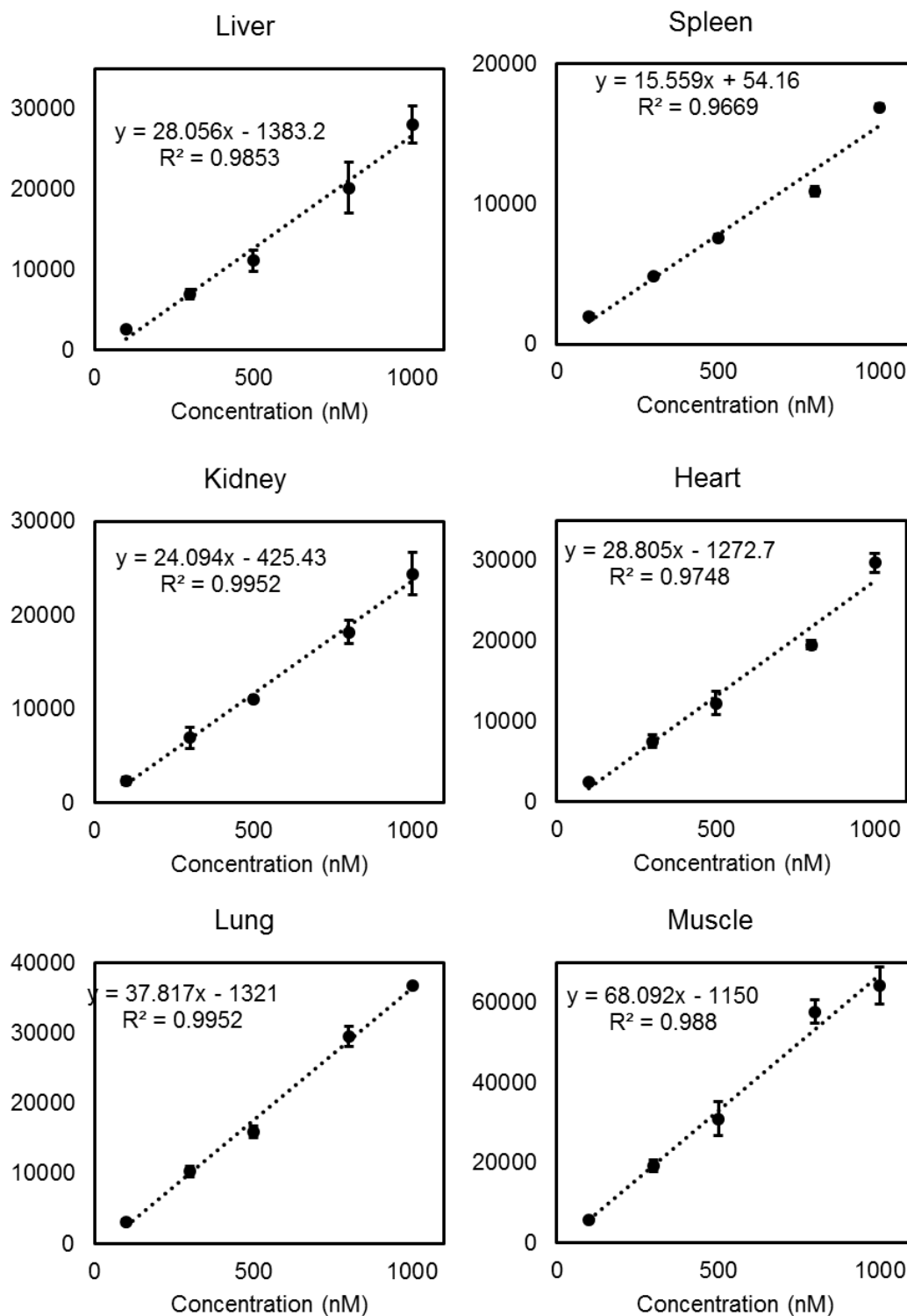


Figure 5.42 Standard curves for zw cy 5 labeled particle concentration in each tissue from Sprague Dawley rats.

5.6.11 Half-life Circulation Time

Healthy nu/nu mice were injected with 160 nmol of $\mathbf{M_L}$ - and $\mathbf{M_{Lzw}}$ and blood was collected at 2, 60, 240, 360 minutes and 24 hours in heparinized hematocrit tubes after a tail nick and imaged using the Maestro with the filtersets 640/47 excitation and 700LP for emissions for cy 5.5 and 607/36 excitation and 685/40 emission. Images were analyzed in image J for fluorescence intensities at 3 different areas on the tube and averaged followed by background subtraction. The intensity of the blood at the 2 minute timepoint was taken as the maximum signal in the blood, and every other timepoint was normalized to the original to determine percent remaining.

5.7 Acknowledgements

The synthesis of a Cy 5 dye in Chapter 5 is adapted from Maria T. Proetto, Christopher R. Anderton, Dehong Hu, Craig J. Szymanski, Zihua Zhu, Joseph P. Patterson, Jacquelin K. Kammeyer, Lizanne G. Nilewski, Anthony M. Rush, Nia C. Bell, James E. Evans, Galya Orr, Stephen B. Howell, and Nathan C. Gianneschi., Cellular Delivery of Nanoparticles Revealed with Combined Optical and Isotopic Nanoscopy. *ACS Nano*, 2016, 10 (4), 4046-4054. Copyright 2016 American Chemical Society. Permission to use copyrighted images and data in the manuscript was also obtained from Maria T. Proetto, Christopher R. Anderton, Dehong Hu, Craig J. Szymanski, Zihua Zhu, Joseph P. Patterson, Lizanne G. Nilewski, Anthony M. Rush, Nia C. Bell, James E. Evans, Galya Orr, Stephen B. Howell, and Nathan C. Gianneschi. The dissertation author is seventh author on the manuscript. The data in chapter 5, in part, is unpublished work and may be published in a manuscript authored by Jacquelin K. Kammeyer, Jessica Ungerleider, William L. Pistel II, Rebecca Braden, Karen Christman, and Nathan C. Gianneschi. Permission to use the data in

this manuscript was given by Jessica Ungerleider, William L. Pistel II, Karen Christman, Rebecca Braden and Nathan C. Gianneschi.

5.8 References

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