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Design and Application of Matrix Metalloproteinase-9-Responsive Peptide Nanostructures

Jiye Son
The Graduate Center, City University of New York

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DESIGN AND APPLICATION OF
MATRIX METALLOPROTEINASE-9-RESPONSIVE PEPTIDE NANOSTRUCTURES

by

JIYE SON

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2019
Design and Application of
Matrix Metalloproteinase-9-Responsive Peptide Nanostructures

by

Jiye Son

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

__________________________________________                           ___________________________________________
Date                                                                          Prof. María Contel
                                                                                       Chair of Examining Committee

__________________________________________                           ___________________________________________
Date                                                                          Prof. Rein V. Ulijn
                                                                                       Co-chair of Examining Committee

__________________________________________                           ___________________________________________
Date                                                                          Prof. Brian Gibney
                                                                                       Executive Officer

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Prof. Daniel Heller
Prof. Guillermo Gerona-Navarro

THE CITY UNIVERSITY OF NEW YORK
This dissertation is dedicated to my Haeyeun Unni and Halmuni,
the strong women who raised me and taught me the value of hard-work and resilience.
ABSTRACT

Design and Application of Matrix Metalloproteinase-9-Responsive Peptide Nanostructures

by

Jiye Son

Advisor: Prof. María Contel
Co-advisor: Prof. Rein V. Ulijn

Matrix metalloproteinase (MMP)-responsive materials have been investigated since the late 1990’s as scaffolds for tissue engineering and since then, have evolved into sophisticated nanomaterials for cancer-targeting therapy. In this thesis titled, “Design and Application of Matrix Metalloproteinase-9- Responsive Peptide Nanostructures,” we aim to answer the following key questions: can MMP-responsive nanomaterials improve the efficacy of anti-cancer treatments? How can we achieve specificity towards MMPs using nanomaterials? Finally, what are the advantages in using peptides as building blocks to create MMP-responsive nanostructures? Each chapter in the thesis will address one or more of the key questions and draw conclusions at the end.
ACKNOWLEDGEMENTS

I would like to thank my PhD advisors, Prof. Rein V. Ulijn and Prof. María Contel, for their tremendous support and kindness over the past five years. This unique collaboration introduced me to two wonderful mentors and two amazing research groups at the CUNY Advanced Science Research Center and at Brooklyn College, whom I am extremely grateful for.

Rein, thank you for inspiring me with your vision and passion for science. When nothing was working in lab and I was frustrated out of my mind, you always helped me rebuild my confidence and reminded me of how ‘cool’ my research is. You have created a welcoming and scientifically stimulating environment in the group and at the ASRC, where I felt proud and grateful to be part of the team. Thank you for giving me the opportunity to learn how to write a great story, present ‘power statements,’ and think and act like a PhD scientist.

I would also like to thank María, who truly cares about the growth and well-being of her students. María, thank you for always looking out for me—mentally, physically, financially, and professionally. It is rare to find a PI or any other supervisor who can open up their hearts and welcome me like family, while still being very strict to make sure everyone does their job! Thank you for helping me grow as a person and graduate the program as a doctor.

I am also thankful for my committee advisors, Prof. Daniel Heller and Prof. Guillermo Gerona-Navarro, who challenged me to step-up beyond my own expectations and dive deeper into scientific research. Special thanks to Prof. Heller who co-supervised me as a Predoctoral Tow Fellow, which helped me to complete my PhD without financial burden. I’d also like to thank Mr. Leonard Tow for the generous financial support I received through the Tow Foundation Graduate Fellowship from the MSKCC Center for Molecular Imaging and Nanotechnology. I’d like to thank
the CUNY Science Scholarship and the Ph.D. Program in Chemistry, The Graduate Center of The City University of New York, for accepting me into the program and giving me the opportunity to make this small, but a proud contribution, to the scientific community.

Finally, I would like to thank my family, friends, and all the wonderful people I met during the program who made this, what could have been a dreadfully hard experience, into the most special and rewarding five years of my life. Thank you to my friends at ASRC, starting from the OG’s when the building was empty: Meghan, Jacob, Daniela, to when the bullpen started filling up: Mohit, Ayala, Nadeesha, Roxy, Francesco, Freddy, to now when we’re running out of desk space: Mona, Daniela, Richard, Scott, Ankit, Silvio, Rob, and thank you to my friends from Brooklyn College: Jacob, Flavia, Natalia, Kirill, Mike, Lina, Yaron, Virginia, you guys have all helped me graduate with sanity! I am grateful for the unwavering support from more special people in my life, Xing, Lily, Lisa, Noory Oppa, Heeyeun, Abba, my Hambu Chris, and my Buddha Haeyeun Unni, I love you all and thank you for loving me back.
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Chapter 1

Overview

1.1. Introduction to thesis

Matrix metalloproteinase (MMP)-responsive materials have been investigated since the late 1990’s as scaffolds for tissue engineering and since then, have evolved into sophisticated nanomaterials popularly used for cancer-targeting therapy. In this thesis titled, “Design and application of matrix metalloproteinase-9-responsive peptide nanostructures,” we aim to answer the following key questions: can MMP-responsive nanomaterials improve the efficacy of anti-cancer treatments? How can we achieve specificity towards MMPs using nanomaterials? Finally, what are the advantages in using peptides as building blocks to create MMP-responsive nanostructures? Each chapter in the thesis will address one or more of the key questions and draw conclusions at the end.

Chapter 2 will analyze literature examples to examine if MMP specificity can be better achieved using nanomaterials. The advantages and shortcomings of MMP-responsive nanomaterials in improving the efficacy of anti-cancer treatments will also be discussed. Chapter 3 describes the synthesis and characterization of short peptides as building blocks to form MMP-responsive nanostructures. A systematic design of the peptide sequence will be used to evaluate important parameters in achieving MMP specificity and to regulate nanostructure morphology and response kinetics. Chapter 4 will address a common challenge that reduces the reproducibility in peptide self-assembly (through residual TFA), and how to rectify it. The biocompatibility and bioapplication of MMP-responsive nanostructures will be examined in Chapter 5. Finally, the last chapter will summarize the findings of this thesis and discuss future prospects.
1.2. Hypothesis and specific aims

Nanomedicines take advantage of particle size (1-500nm) and surface properties of nanoscale materials to accumulate selectively in the tumor microenvironment. Examples of FDA-approved nanomedicines include Doxil (pegylated liposomal doxorubicin), Abraxane (albumin bound paclitaxel), and dozens more in clinical phase III trials. In addition to passive targeting, nanomedicine can be designed to actively target tumor cells using various endogenous stimuli such as pH, antigens, integrins, and enzymes that are characteristic of cancer cells. However, it is still a challenge to design nanostructures that can respond to cancer cell specific stimuli. Part of the challenge is the identification and realization of appropriate materials that can be fine-tuned to have the desired chemical and physical properties. Peptides have made a significant impact in biomedical applications due to their inherent biocompatibility and versatility in forming stable supramolecular nanostructures with desired functionalities, as well as their potential to be fully biodegradable (or metabolizable).

Using systematic peptide design, my hypothesis is that the morphology, size, and charge of nanostructures can be customized to respond to MMP-9 with controlled response kinetics and mode of action for biological applications. The specific aims of my research project are:

**Aim 1:** Systematic design and characterization of peptide nanostructures

**Aim 2:** Control of MMP-9 responsive kinetics and mode of action

**Aim 3:** Biocompatibility and application of peptide nanostructures using metallodrugs

The primary sequence of peptide nanostructures is the key to achieving MMP specificity, attaining desired morphology and functionalities, and co-assembly with payloads to form stable nanocarriers.
Chapter 2

Literature analysis of MMP-Responsive Nanomaterials for Targeted Therapy

Summary

Matrix metalloproteinases (MMP) are enzymes that degrade extracellular proteins and multiple diseases, including cancer progression, are linked to their irregular overexpression and activity. Inhibiting the enzymes using small molecules has been a challenge due to the similar substrate binding sites of the MMPs and multiple roles they play during stages of cancer. Rather than inhibiting them, MMP-responsive nanomaterials take advantage of the enzyme overexpression and utilize MMP activity to trigger events that cause desired changes in the nanomaterials. The nanomaterials can act as drug-loaded nanocarriers and respond to MMP action to undergo different modes of responses including particle/micelle size shrinking, aggregation, and morphology switch from biocompatible nanomaterials to cytotoxic nanofibers. By analyzing over 40 different systems described in the literature, we have formulated guiding principles in designing MMP-responsive nanomaterials to achieve MMP specificity. The key components include modification of the cleavable substrate and the supramolecular architecture and electrostatic charge of the nanomaterial. Finally, we review how the different modes of response effect the rate of payload release and the efficacy of the treatments in biological systems.
2.1. Introduction

Matrix metalloproteinases (MMP) are a family of enzymes that are responsible for degrading components of the extracellular matrix and regulating extracellular cell signals for normal cell behaviors such as turnover of tissues, angiogenesis, wound healing, etc. Thus, irregular expression and activation of MMPs have been linked to multiple diseases including arthritis, cardiovascular diseases, and cancer progression. Specifically, the upregulation of MMPs in many types of cancer have been studied in cell culture, animal models, and in patient-derived samples. Therefore, MMPs have become a popular target for anti-cancer therapy, either directly by inhibiting MMP activity using small molecules, collagen peptidomimetics, etc. or indirectly by inhibiting MMP gene expression or MMP interaction with other proteins. However, many of these strategies have failed during clinical trials (Batimastat, Marimastat, CGS 27023A, Tanomastat, etc.) and MMPs remain as unattained, highly desirable targets. Major reasons for the failure include non-specific inhibition of the 21 different MMPs with similar substrate pockets and the timing of the MMP inhibition during cancer progression, which led to broad inhibition of MMPs, including the ones that have anti-cancer effects at specific stages of cancer. While this lack of specificity has led the big pharmaceutical companies to focus less on investigating MMP inhibitors, researchers continued to develop new and improved strategies to target MMPs to address these issues, including the use of MMP-responsive nanomaterials.

A relatively new strategy in targeting MMP is to exploit the innate activity of the proteinases, instead of inhibiting it, to trigger anti-cancer effects using nanomaterials to release payloads (small or bio-molecule) or form cytotoxic nanofibers. This strategy could be more advantageous because the desired anti-cancer effect will, in theory, only be triggered in areas where MMP activity is upregulated during specific stages of cancer progression, which allows
temporal and spatial control of the nanomaterial response. In addition, the approach incorporates MMP substrates, and with good understanding of MMP specificity it can be optimized to be selective towards specific MMPs.

The first MMP-responsive material was described by Jeffrey Hubbell in 1999, pertaining the production of polyethylene glycol (PEG)-based polymer hydrogels that contained crosslinks that could be degraded by MMP to enable cell migration. Specifically, the PEG-based polymers were crosslinked with MMP-1 cleavable peptide sequence and RGD motif to form a hydrogel that could be used as a 3D tissue scaffold, in which entrapped fibroblasts can proteolytically degrade the gel and invade outward. This MMP-responsive hydrogel was subsequentially modified to non-covalently incorporate bone morphogenetic proteins and implanted into defected rat cranium in vivo. Within 4 weeks, localized invasion of fibroblasts and complete bone regeneration of the defect was observed. Following Hubbell’s example, numerous types of MMP-degradable hydrogels have been described for stem cell and tissue engineering, and expanded the field to investigate nanomaterials to target MMP related diseases, including cancer.

Since these early reports, MMP-responsive materials have been developed for other applications, including targeted delivery of small and biomolecular therapeutic agents, imaging, phototherapy, as well as sensors to detect and quantify MMP levels in vitro and in vivo. Using MMP-responsive materials, an ideal system could selectively engage with a specific MMP target at the tumor site and respond at a pre-defined rate (from few hours to multiple days) to have spatial and temporal control of the desired effect. This chapter will discuss the importance of targeting MMPs in anti-cancer therapy and analyze literature on MMP-responsive nanomaterials for anti-cancer therapy. We will review different modes of MMP-triggered response that the nanomaterials can have and evaluate which properties of the nanomaterial such as composition, size, shape,
charge, etc. can influence MMP targeting by directly comparing the rate of peptide hydrolysis, or by indirect comparison in the rate of nanomaterial response such as changes in physical properties or release of payloads. Finally, we will analyze how both the mode and rate of MMP-response in nanomaterials can affect the targeting activity of the nanomaterial in vitro and in vivo, and ultimately draw conclusions on guiding principles to help create MMP targeting systems that are customized to specific needs and functionalities.

### 2.2. Structure and functions of matrix metalloproteinases

Matrix metalloproteinases, also called matrixins, are a family of zinc dependent endopeptidases that degrade the extracellular matrix and communicate extracellular cell signals. There is a total of 23 known MMPs found in humans which are either secreted from the cells or are bound to the surface membrane (called membrane type-MMP, MT-MMP).\(^{17}\) Traditionally, MMPs were grouped by their substrates, such as collagenases or gelatinases, but upon discovery of many more substrates they are now grouped together according to their domain structures represented in Figure 1 and are sequentially numbered. Regulation of MMP activity and expression at the transcription and translational levels are necessary and crucial for maintaining normal biological processes.\(^{18}\)

All MMPs are synthesized as inactive pro-peptides or zymogens and are activated extracellulary (except for furin-activated and secreted MMPs) when required through a “cysteine switch” process which breaks the Cys-Zn\(^{2+}\) interaction between the cysteine in the pro-peptide domain and the Zn\(^{2+}\) in the catalytic domain of the enzyme. Once activated, MMP activity is regulated by a class of proteins called tissue inhibitors of metalloproteinases (TIMPs) that can form a complex with the catalytic domain of MMP and inhibit MMP activity. Thus, the up- or down-
regulation of MMP expression and activity have been linked with multiple diseases, including a wide variety of cancers. Detailed descriptions of the structures and functions of MMPs have been published widely by Hideaki Nagasse,\textsuperscript{17,18} and Table 1 summarizes the 23 human MMPs with their common names, domain structure, substrates, and biological roles. The isoelectric points (pI) of the MMPs are also listed which is relevant in designing electrostatically charged nanomaterials to match the enzyme’s charge (will be discussed in Section 2.3.3.).\textsuperscript{19} The membrane types are generally more basic, with pI values ranging from 5.96 to 9.70, compared to the secreted MMPs which range from 5.26 to 7.73 (except MMP-12 which has pI of 8.75).

2.2.1. MMP overexpression in cancer

The role of MMPs in cancer progression has been studied since the 1980’s and has been mainly associated with one of the six hallmarks of cancer, tissue invasion and metastasis.\textsuperscript{20} Since then many other cancer development processes have been associated to MMPs, including cancer cell invasion, proliferation, apoptosis, tumor angiogenesis and vasculogenesis, cell adhesion, migration, and epithelia to mesenchymal transitions, as well as escaping the immune surveillance.\textsuperscript{3,10} The regulation of MMPs is a complex biological process, and both down- or up-regulation of MMPs have been observed based on subtypes\textsuperscript{8} and stages\textsuperscript{9} of cancer. Of the different MMPs, MMP-2, -7, and -9 have been most widely studied for their overexpression during cancer progression.\textsuperscript{7} Table 2 lists concentrations of MMPs measured in various cancer cell lines, mice models, and patient derived samples quantified using ELISA or other fluorimetric assays. The concentrations vary by cancer types and samples, but in general they range around ng/ml for MMP-2 and -9, and µg/ml for MMP-7.
**Figure 2.1.** Simplified representation of domain structures that are found in 5 secreted and 3 membrane type MMPs. The pre domain guides the enzyme towards the endoplasmic reticulum, the pro domain keeps the catalytic domain inactive until needed, and the homopexin domain is connected to the catalytic domain by a hinge to mediate interaction with other proteins. Fibronectin-like domains can bind to collagen, furin domains can bind to serine proteinases to activate the enzyme, and some contain Vit = vitronectin like inserts. Membrane types contain TM = transmembrane domain and CT = cytoplasmic domain, or GPI = glycosylphosphatidylinositol anchoring domain, or SA = signal anchor, Cys = cysteine array, and immunoglobulin-like anchors. Adapted from references 10 and 18.
Table 2.1. List of 23 human MMPs and their biological details, substrates, and roles. Adapted from references 10, 18, and 19.

<table>
<thead>
<tr>
<th>Human MMPs</th>
<th>Other names</th>
<th>Location</th>
<th>Domain structure class</th>
<th>pi*</th>
<th>Substrates</th>
<th>Biological effects mediated by substrate cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase 1; interstitial/ fibroblast/ tissue collagenase</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>6.47</td>
<td>Type I collagen, Fibronectin, IGFBP-3, IL-1β degradation, Monocyte chemoattractant protein-3, Protease activated receptor 1</td>
<td>Keratinocyte migration and reepithelialization; apoptosis (amnion epithelial cells)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A; 72-kDa gelatinase; 72-kDa type IV collagenase; neutrophil gelatinase</td>
<td>Secreted</td>
<td>Gelatin-binding</td>
<td>5.26</td>
<td>Chondroitinsulphate proteoglycan, Fibronectin, BM-40 (SPARC/Osteonectin), Laminin 5γ2 chain, IL-1β degradation, Monocyte chemoattractant protein-3, Decorin, Big endothelin, Adrenomedullin, Stromal cell-derived factor 1α, Fibronectin, Basement membrane</td>
<td>Neurite outgrowth, Cell migration, Increased bioavailability of IGF1 and cell proliferation, Enhanced collagen affinity, Epithelial cell migration, Anti-inflammatory, Anti-inflammatory, Increased bioavailability of TGF-β, Generation of vasoconstrictor, Conversion of vasodilator to vasoconstrictor, Neuronal apoptosis leading to neurodegeneration</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin 1; transin 1; proteoglycanase; procollagenase activating protein</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>5.77</td>
<td>E-cadherin, Plasminogen, Perlecan, BM-40 (SPARC/Osteonectin), Monocyte chemoattractant protein-3, Decorin, E-cadherin</td>
<td>Cell migration, Mammary epithelial cell apoptosis and alveolar formation, Epithelial-mesenchymal conversion, Generation of angiotatin-like fragment, Release of bFGF, Increased bioavailability of IGF1 and cell proliferation, Enhanced collagen affinity, Anti-inflammatory, Increased bioavailability of TGF-β, Disrupted cell aggregation and increased cell invasion</td>
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<tr>
<td>MMP-7</td>
<td>Matrilysin 1; matrin; PUMP1; small uterine metalloproteinase</td>
<td>Secreted</td>
<td>Minimal domain</td>
<td>7.73</td>
<td>Fibronectin, IGFBP-3, BM-40 (SPARC/Osteonectin), Decorin, E-cadherin, Fas ligand, Pro-TNFα, RANK ligand, Heparin-binding EGF</td>
<td>Adipocyte differentiation, Increased bioavailability of IGF1 and cell proliferation, Enhanced collagen affinity, Increased bioavailability of TGF-β, Disrupted cell aggregation and increased cell invasion, Fas-receptor mediated apoptosis, Pro-inflammatory, Osteoclast activation, Vasocnstriction and cell growth</td>
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<td>MMP-8</td>
<td>Collagenase 2; neutrophil/ PMN/ granulocyte collagenase</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>6.38</td>
<td>Type I collagen</td>
<td>Apoptosis (amnion epithelial cells)</td>
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</table>

*pl = isoelectric point
<table>
<thead>
<tr>
<th>Human MMPs</th>
<th>Other names</th>
<th>Location</th>
<th>Domain structure class</th>
<th>pi*</th>
<th>Substrates</th>
<th>Biological effects mediated by substrate cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Gelatinase B; 92-kDa gelatinase; 92-kDa type IV collagenase</td>
<td>Secreted</td>
<td>Gelatin-binding</td>
<td>5.69</td>
<td>BM-40 (SPARC/Osteonectin), ICAM-1, IL-1β, IL-2Rα, precursor of TGFβ, Collagen IV, Galactin-3</td>
<td>Enhanced collagen affinity, Tumor cell resistance, Anti-inflammatory, Reduced IL-2 response, Bioavailability of TGFβ, Thymic neovascularization, Hypertrophic chondrocytes apoptosis and recruitment of osteoclast</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin 2; trasin 2</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
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<td></td>
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<td>MMP-11</td>
<td>Stromelysin 3</td>
<td>Secreted</td>
<td>Furin-activated and secreted</td>
<td>6.38</td>
<td></td>
<td></td>
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<tr>
<td>MMP-12</td>
<td>Macrophage elastase/metalloelastase; Metalloelastase</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>8.75</td>
<td>Plasminogen</td>
<td></td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>5.32</td>
<td>Type I collagen, Perlecain, Monocyte chemoattractant protein-3</td>
<td>Osteoclast activation, Release of IGF, Anti-inflammatory</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP; MT-MMP1</td>
<td>Membrane type</td>
<td>Transmembrane</td>
<td>7.63</td>
<td>CD44, Type I collagen, Lamminin 5γ2 chain, Monocyte chemoattractant protein-3, Cell surface tissue transglutaminase, Transmembrane mucin 1</td>
<td>Cell migration, Kidney tubulogenesis, Epithelial cell migration, Anti-inflammatory, Reduced cell adhesion and spreading, Embryo attachment to uterine epithelia</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP; MT-MMP2</td>
<td>Membrane type</td>
<td>Transmembrane</td>
<td>7.03</td>
<td></td>
<td>Cell surface tissue transglutaminase, Reduced cell adhesion and spreading</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP; MT-MMP3</td>
<td>Membrane type</td>
<td>Transmembrane</td>
<td>8.72</td>
<td></td>
<td>Cell surface tissue transglutaminase, Reduced cell adhesion and spreading</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP; MT-MMP4</td>
<td>Membrane type</td>
<td>GPI-anchored</td>
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<tr>
<td>MMP-19</td>
<td>RAS1-1; MMP-18</td>
<td>Secreted</td>
<td>Simple hemopexin domain</td>
<td>7.22</td>
<td>IGFBP-3, Lamminin 5γ2 chain</td>
<td>Increased bioavailability of IGF1 and cell proliferation, Epithelial cell migration</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
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<td>Simple hemopexin domain</td>
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<td>MMP-21</td>
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<td>Vitronectin-like insert</td>
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<tr>
<td>MMP-23</td>
<td>Cysteine array MMP (CA-MMP); Femalyisin; MIFR</td>
<td>Membrane type</td>
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<td>MMP-24</td>
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<td>Membrane type</td>
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<tr>
<td>MMP-25</td>
<td>MT6-MMP; MT-MMP6; leukolysin</td>
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<tr>
<td>MMP-26</td>
<td>Matrilysin 2; endometase</td>
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<td>Minimal domain</td>
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<td>MMP-27</td>
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<td>8.83</td>
<td></td>
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<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Secreted</td>
<td>Furin-activated and secreted</td>
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</table>

* pi* = isoelectric point
Table 2.2. Concentrations of MMP-2, 5, 21-25, 7, 26 and -9 5, 25, 27-29 in samples from non-cancerous and 10 different types of cancers.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Source</th>
<th>Type</th>
<th>Sample</th>
<th>MMP</th>
<th>Concentration</th>
<th>Assay</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-cancerous</td>
<td>Healthy normal (non-cancerous) control population</td>
<td>Plasma</td>
<td>MMP-2</td>
<td>75.4 ± 5.7 ng/ml</td>
<td>ELISA (Oncogene Research Products, Cambridge, MA)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal colonic tissue</td>
<td>MMP-2</td>
<td>4.2 ± 0.4 ng/ml</td>
<td>5</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>MMP-9</td>
<td>36.3 ± 13.2 ng/ml</td>
<td>27</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Normal colonic tissue</td>
<td>MMP-9</td>
<td>19.7 ± 2.5 ng/ml</td>
<td>ELISA (Oncogene Research Products, Cambridge, MA)</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>0.1 µg/ml</td>
<td>Fluorimetric (Sensolyte 520, AnaSpec, Fremont, CA)</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>0.2 µg/ml</td>
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<tr>
<td>Human cell line</td>
<td>Primary human pancreatic epithelial cells</td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>300 pg/ml</td>
<td>ELISA</td>
<td>22</td>
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<tr>
<td></td>
<td>Dermal microvascular endothelial cells</td>
<td>Cell culture media</td>
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<td>2 ng/mg</td>
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<td>14 ng/mg</td>
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<td>Liver tissue</td>
<td>MMP-2</td>
<td>1 ng/mg</td>
<td>ELISA</td>
<td>22</td>
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<tr>
<td></td>
<td></td>
<td>Spleen tissue</td>
<td>MMP-2</td>
<td>2 ng/mg</td>
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<tr>
<td></td>
<td></td>
<td>Lung tissue</td>
<td>MMP-2</td>
<td>4 ng/mg</td>
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<td></td>
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<td>Kidney tissue</td>
<td>MMP-2</td>
<td>9 ng/mg</td>
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<tr>
<td></td>
<td></td>
<td>Tumor extract</td>
<td>MMP-2</td>
<td>1 ng/mL at 6 h</td>
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<tr>
<td>Lung cancer</td>
<td>Human cell line AS49</td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>1 ng/mL at 12 h</td>
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<tr>
<td></td>
<td>Mice model (non-small cell lung cancer)</td>
<td>Heart tissue</td>
<td>MMP-2</td>
<td>2 ng/mL at 24 h</td>
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<td></td>
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<td></td>
<td></td>
<td>Liver tissue</td>
<td>MMP-2</td>
<td>3 ng/mL at 24 h</td>
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<tr>
<td></td>
<td></td>
<td>Spleen tissue</td>
<td>MMP-2</td>
<td>4 ng/mL at 36 h</td>
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<tr>
<td></td>
<td></td>
<td>Kidney tissue</td>
<td>MMP-2</td>
<td>1 ng/mL at 6 h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Tumor extract</td>
<td>MMP-2</td>
<td>1 ng/mL at 12 h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-9</td>
<td>18.5 ± 4.1 ng/ml</td>
<td>Fluorimetric (Sensolyte, Plus 520 MMP-9 assay kit, AnaSpec, Fremont, CA)</td>
<td>28</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-9</td>
<td>18.5 ± 4.1 ng/ml</td>
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<td></td>
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<tr>
<td>Breast cancer</td>
<td>SKBR3</td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>2.2 µg/ml</td>
<td>Fluorimetric (Sensolyte 520, AnaSpec, Fremont, CA)</td>
<td>26</td>
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</tr>
<tr>
<td></td>
<td>MCF-7</td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>1.8 µg/ml</td>
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<tr>
<td></td>
<td>Human cell line MDA-MB-231Luc-D3H2LN</td>
<td>Cell culture media (serum starved)</td>
<td>MMP-9</td>
<td>18.5 ± 4.1 ng/ml</td>
<td>Fluorimetric (Sensolyte, Plus 520 MMP-9 assay kit, AnaSpec, Fremont, CA)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell culture media (serum starved and PMA activated)</td>
<td>MMP-9</td>
<td>18.5 ± 4.1 ng/ml</td>
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<td></td>
<td>Mouse cell line 4T1</td>
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<td>MMP-2</td>
<td>1 ng/mL at 6 h</td>
<td>ELISA (Aviva Systems Biology, Catalog#: OKEH00176)</td>
<td>24</td>
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<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>2 ng/mL at 12 h</td>
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<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>3 ng/mL at 24 h</td>
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<td></td>
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<td></td>
<td></td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>4 ng/mL at 36 h</td>
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### Table 2.2. (continued)

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<th>Cancer</th>
<th>Source</th>
<th>Type</th>
<th>Sample</th>
<th>MMP</th>
<th>Concentration</th>
<th>Assay</th>
<th>Ref</th>
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<tbody>
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<td>Cervical cancer</td>
<td>Human cell line</td>
<td>HeLa</td>
<td>Cell culture media</td>
<td>MMP-7</td>
<td>2.0 μg/ml</td>
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<td>Prostate cancer</td>
<td>Human cell line</td>
<td>DU-145</td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>463 ± 144 pg/ml</td>
<td>ELISA (Life Technologies)</td>
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<td>Bladder carcinoma</td>
<td>Mice model</td>
<td>RT-112</td>
<td>Tumor extract</td>
<td>MMP-2</td>
<td>1.5 ± 1.1 ng/ml</td>
<td>ELISA</td>
<td>25</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>MMP-9</td>
<td>0.1 ± 0.01 ng/ml</td>
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<tr>
<td>Colorectal cancer</td>
<td>Colorectal cancer patient</td>
<td>Plasma</td>
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<td>MMP-2</td>
<td>568.9 ± 44.8 ng/ml</td>
<td>ELISA (Oncogene Research Products, Cambridge, MA)</td>
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<td>MMP-9</td>
<td>56.5 ± 7.9 ng/ml</td>
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<td>Colorectal tissue</td>
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<td>MMP-2</td>
<td>180.4 ± 99.3 ng/mg</td>
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<td>MMP-9</td>
<td>483.4 ± 73.9 ng/mg</td>
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<tr>
<td>Pancreatic cancer</td>
<td>Human cell line</td>
<td>MIA PaCa-2</td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>372 pmol/min/μg</td>
<td>Gelatin zymography assay</td>
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<td>MMP-9</td>
<td>224 pmol/min/μg</td>
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<td>MMP-7</td>
<td>8 ± 4 pg/ml</td>
<td>Fluorimetric (Sensolyte 520, AnaSpec, Fremont, CA)</td>
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<td>MMP-9</td>
<td>1.7 μg/ml</td>
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<td>Skin carcinoma</td>
<td>Human cell line</td>
<td>A431</td>
<td>Cell culture media</td>
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<td>126 ± 23 pg/ml</td>
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<td>Fibrosarcoma</td>
<td>Mice model</td>
<td>HT1080</td>
<td>Tumor extract</td>
<td>MMP-2</td>
<td>17.0 ± 6.3 ng/ml</td>
<td>ELISA</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MMP-9</td>
<td>5.2 ± 1.7 ng/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human cell line</td>
<td>HT1080</td>
<td>Cell culture media</td>
<td>MMP-2</td>
<td>30 ng/ml at 6 h</td>
<td>ELISA (Aviva Systems Biology, Catalog#: OKEH00176)</td>
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<td>45 ng/ml at 12 h</td>
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<td></td>
<td></td>
<td>60 ng/ml at 24 h</td>
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<tr>
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<td></td>
<td>63 ng/ml at 36 h</td>
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<tr>
<td>Glioblastoma</td>
<td>Mice model</td>
<td>U-87</td>
<td>Tumor extract</td>
<td>MMP-2</td>
<td>16.1 ± 12.4 ng/ml</td>
<td>ELISA</td>
<td>25</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>MMP-9</td>
<td>5.0 ± 1.0 ng/ml</td>
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</tbody>
</table>
2.3. Achieving MMP specificity using nanomaterials

MMP-responsive nanomaterials take advantage of the inherent overexpression and activity of MMPs produced by cells to trigger a desired response in the material. These responsive nanomaterials are made up of various building blocks, such as peptides,28,30–35 lipids,22,23,26,29,36–41 polymers,14,24,42–54 naturally derived,55–59 and inorganic27,60–62 materials that have different chemical and physical properties. The versatility of MMP-responsive nanomaterials allow them to be used in any biological applications that involve overexpression and activity of MMPs, including regenerative medicine,30,31,55 targeted delivery of therapeutic21–24,27–29,32,36–41,46–48,50,52–54,56–59,62 or imaging agents,51,60,63–65 phototherapy,61,66 and diagnostic sensors.67–70

The one shared key component in MMP-responsive nanomaterials is a peptide sequence that can be recognized and hydrolyzed by MMP and cause a physical or chemical change in the nanomaterial. This peptide sequence is often used as a linker to covalently link the building blocks together or may be a structural part of the material itself, in the case of self-assembled peptides. The MMP-cleavable sequence is typically at least 6 amino acids long in order to be recognized by the binding site of the enzyme and is modified on the C- and N-terminus to be imbedded or attached onto the building block. Because MMPs are highly specific enzymes, the design of MMP-responsive materials needs to be considered thoroughly in many aspects beyond the cleavable substrate, such as the properties and functions of the nanomaterial.

For MMPs to cleave the substrate, the enzyme must first interact with the nanomaterial and have access to the substrate before it can bind to it. Based on a simplified estimate of spherical proteins with similar molecular weight of MMPs, a 100 nm nanoparticle is about 30-50 times larger than the enzyme (radius of proteins with 10-100 kDa can be calculated 2.4–3.05 nm).71 Thus the interaction of the enzyme with the nanomaterial is completely different from its interaction
with a monomeric peptide substrate. Therefore, both the properties of the nanomaterial and the primary sequence of the cleavable substrate must be designed to be compatible with the targeted MMP.

To analyze which features of the MMP-responsive materials are most relevant, we have surveyed the literature and analyzed 41 different systems that have been published after 2010 and the early pioneering work published by Hubbell. Table 3 lists the different material compositions, forms, shapes, sizes (storage modulus for hydrogels), ζ-potentials, and primary sequences of the cleavable substrate and compares them to the rate of conversion, that is the rate in which the substrate in the nanomaterial is hydrolyzed by MMP. In Table 3, the rate of conversion is considered to be measured directly if the authors have provided evidence showing that the peptide substrate was cleaved over time by chromatogram or gel electrophoresis. It is considered to be an indirect measurement if the authors reported changes in the nanomaterial as a response to the MMP action over time, such as changes in size, rheology or weight % for gels, ζ-potential, or drug release profiles. This distinction is important because the rate and degree of the response in the nanomaterial does not always correlate with the rate or the amount of substrate cleaved by MMP. Lastly, measurements are considered to be relevant if the authors only showed the performance of the nanomaterial in vitro or in vivo. In addition, while most of the conversion rates are measured by incubating MMP with the nanomaterial, some rates are measured on the isolated cleavable substrate, which is not comparable since the substrate is in an entirely different molecular context (size, surface properties, etc.) when packaged into the nanomaterial. The molecular context of cleavable-substrate is an important factor to distinguish in comparing the different MMP-responsive materials.
Through analyzing the rates of conversion with the properties of the nanomaterials, we have identified 3 key factors that can enhance the nanomaterial and enzyme interaction and specificity towards the targeted MMP as illustrated in Figure 2. First and foremost, the primary sequence of the substrate must be specific for the enzyme to recognize it and subtle changes in the amino acids or terminal modification of the primary sequence can alter the cleavage site. Second, the morphology of the nanostructure must allow MMPs to access the substrate, and the supramolecular organization of the substrate can also target specific MMPs. Lastly, by either matching or mis-matching electrostatic interactions, the short-range interaction between the nanoparticle and the enzyme can be tuned.
Figure 2.2. Three key factors to achieve MMP specificity using nanomaterials. (1) Modification of the primary sequence can shift the cleavage site of the peptide and slow down the rate of hydrolysis.\textsuperscript{77,78} Based on the supramolecular structure\textsuperscript{31,33,56} (2) and electrostatic charge\textsuperscript{31,36} (3) of the nanomaterial, specificity towards different MMPs can be achieved: β-sheet fibers can be cleaved by MMP-13 (green) but not by MMP-9 (pink), and cationic nanoparticles are readily cleaved by more negatively charged MMP-2 (red) in comparison to the neutral charged MMP-7 (blue).
Table 2.3. List of MMP-responsive hydrogels and nanomaterials categorized by building block material.

<table>
<thead>
<tr>
<th>Material</th>
<th>Form</th>
<th>Morphology</th>
<th>Size (nm)/gel storage mod</th>
<th>ζ-potential (mV)</th>
<th>MMP cleavable substrate</th>
<th>MMP hydrolysis</th>
<th>MMP</th>
<th>Application (Ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>self-assembled MMP cleavable peptide</td>
<td>anti-parallel β-sheet fibers</td>
<td>300 bundle</td>
<td>+11.5 ± 0.8</td>
<td>FFAKLAGGLGKK</td>
<td>Direct (HPLC): 1 mM peptide + 100 ng/mL MMP</td>
<td>MMP-9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>250 bundle</td>
<td>-34.9 ± 1.1</td>
<td>FFAKLAGGLGKK</td>
<td>0% conversion at 96 h</td>
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<tr>
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<td></td>
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<td>10 diameter</td>
<td>+15.4 ± 0.3</td>
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<td>90% conversion at 96 h</td>
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<td>5 diameter</td>
<td>+8.2 ± 2.8</td>
<td>FFGAGLAGGLGKK</td>
<td>7.5% conversion at 96 h</td>
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<td></td>
<td></td>
<td></td>
<td>10 diameter</td>
<td>-30.3 ± 1.4</td>
<td>FFGAGLAGGLGKK</td>
<td>0% conversion at 96 h</td>
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<td></td>
<td>30 bundle</td>
<td>+1.3 ± 0.6</td>
<td>FFGAGLAGGLGKK</td>
<td>10% conversion at 96 h</td>
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<td>10 bundle</td>
<td>+7.5 ± 1.8</td>
<td>FFGAGLAGGLGKK</td>
<td>0% conversion at 96 h</td>
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<td>Worm-like micelle</td>
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<td>+4.1 ± 1.0</td>
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<td>100% conversion at 96 h</td>
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<td>500 bundle</td>
<td>-41.2 ± 2.3</td>
<td>FFGAGLAGGLGKK</td>
<td>2.5% conversion at 96 h</td>
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<td>100 bundle</td>
<td>+1.1 ± 1.5</td>
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<td>90% conversion at 96 h</td>
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<td>100 bundle</td>
<td>-28.2 ± 0.7</td>
<td>FFGAGLAGGLGKK</td>
<td>0% conversion at 96 h</td>
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<td>Spherical micelle</td>
<td>167 ± 5</td>
<td>Phenylacetyl-GFFGLD GLD GDD</td>
<td>Direct (HPLC): 5 mM peptide + 50 ng/mL MMP</td>
<td>~100% conversion at 96 h</td>
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<td>200</td>
<td>GFFGLD GLD GDD</td>
<td>Direct (HPCL): 2.5 mM peptide + 50 ng/mL MMP</td>
<td>~60% conversion at 96 h</td>
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<td>Crosslinked with MMP cleavable substrate</td>
<td>α-helical filament</td>
<td>25 diameter</td>
<td>acetyl-KKY-GPQSL JAGQ-YKK-NH2, acetyl-KKY-IPVS- LKGG- YKK-NH2</td>
<td>Observed degradation at 6 h</td>
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<td></td>
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<td>self-assembled MMP cleavable peptide</td>
<td>β-hair pin hydrogel</td>
<td>820 Pa (G')</td>
<td>IKVKKIKKVK(d)-PPTG-FKKVKKVNHH2</td>
<td>Indirect gel degrade (rheology): 1% wt peptide</td>
<td>~65% conversion at 14 d (80 nM MMP-13)</td>
<td>MMP-2</td>
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<td>1900 Pa (G')</td>
<td>IKVKKIKKVK(d)-PPTG-LKVKKVNHH2</td>
<td>~58% conversion at 14 d (80 nM MMP-13)</td>
<td>0% conversion at 14 d (40 or 400 nM MMP-3)</td>
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<td>2800 Pa (G')</td>
<td>IKVKKIKKVK(d)-PPTG-LKVKKVNHH2</td>
<td>~44% conversion at 14 d (80 nM MMP-13)</td>
<td>0% conversion at 14 d (40 or 400 nM MMP-3)</td>
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<td>350 Pa (G')</td>
<td>IKVKKIKKVK(d)-PPTG-AVKKKVNHH2</td>
<td>~32% conversion at 14 d (80 nM MMP-13)</td>
<td>0% conversion at 14 d (40 or 400 nM MMP-3)</td>
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<td>β-sheet fiber hydrogel</td>
<td>175 Pa (G')</td>
<td>KK-SLSLRLRG-SLSSLK</td>
<td>Indirect gel degrade (wt%): 10 mg/mL peptide + 100 ng MMP</td>
<td>~100% conversion in 14 d</td>
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<td>1000 Pa (G')</td>
<td>Acetyl-III-LKG-NH2</td>
<td>Indirect gel degrade (rheology): 8 mM peptide + 100 ng/mL MMP</td>
<td>~60% conversion at 15 d</td>
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<td>1000 Pa (G')</td>
<td>Acetyl-III-LKG-NH2</td>
<td>~0% conversion at 15 d</td>
<td>MMP-2</td>
<td>Anti cancer peptide delivery (In vitro) 32</td>
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Table 2.3. (continued)

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<thead>
<tr>
<th>Material</th>
<th>Form</th>
<th>Morphology</th>
<th>Size (nm)/ Gel storage mod</th>
<th>ζ-potential (mV)</th>
<th>MMP cleavable substrate</th>
<th>MMP hydrolysis (Direct/indirect/relevant)</th>
<th>MMP</th>
<th>Application</th>
<th>Ref</th>
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<tr>
<td>Lipid micelles and liposomes</td>
<td>Spherical micelle</td>
<td>~20</td>
<td>C_{18}-GGHGPGQ_micelle</td>
<td>Direct: 0.2% wt lipopeptide + 2 μg/ml MMP ~50% conversion at 1 h</td>
<td>MMP-7</td>
<td>cytotoxic fibers</td>
<td>26</td>
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<td></td>
<td>Spherical vesicle</td>
<td>65.4 ± 2.3</td>
<td>-22.1 ± 0.3</td>
<td>SDK-C_{18}-GGHGPGQ-micelle</td>
<td>Substrate only (TOF-SIMS): 100 μM substrate + 0.2 μg/μl MMP ~100% conversion at 3h</td>
<td>MMP-2</td>
<td>Drug delivery (in vitro &amp; in vivo)</td>
<td>23</td>
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<td>Liposome with MMP cleavable mAb conjugate</td>
<td>Spherical</td>
<td>86 ± 18</td>
<td>POPE-GPOGIAAGQ-R-PEG</td>
<td>Indirect size change and drug release</td>
<td>MMP-9</td>
<td>Drug delivery (in vitro &amp; in vivo)</td>
<td>29</td>
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<td>Spherical</td>
<td>207</td>
<td>-10.23</td>
<td>DOPE-GPLG-mAb</td>
<td>Substrate only (HPLC): 1 mg/mL polymer + 10 ng/μL MMP ~100% conversion at 24 h</td>
<td>MMP-2</td>
<td>Drug delivery (In vitro)</td>
<td>36</td>
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<tr>
<td>Lipid and PEG co-assembly</td>
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<td>22.5 ± 2.7</td>
<td>PEG-GPLG-IAQQ-PTX</td>
<td>Substrate only (HPLC): 2.5 mg/mL polymer + 5 ng/μL MMP ~100% conversion overnight</td>
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<td>Drug delivery (In vitro &amp; in vivo)</td>
<td>22</td>
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<td></td>
<td>Spherical</td>
<td>16.5 ± 5.1</td>
<td>+26.8 ± 2.4</td>
<td>PEG-GPLG-IAQQ-PEI-PE</td>
<td>Substrate only (HPLC): 1 mg/mL polymer + 5 ng/μL MMP ~100% conversion overnight</td>
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<td>Spherical</td>
<td>69.5±10.6</td>
<td>+8.2 ± 3.4</td>
<td>PEG-GPLG-IAQQ-TAT-DOX</td>
<td>Substrate only (HPLC): 1 mg/mL polymer + 6 ng/μL MMP ~100% conversion overnight</td>
<td>MMP-2</td>
<td>Drug delivery (In vitro)</td>
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<td></td>
<td>Spherical</td>
<td>33.0 ± 1.2</td>
<td>close to 0</td>
<td>PEG-GPLG-IAQQ-PEI-PE</td>
<td>Indirect size change and drug release</td>
<td>Collagenase IV</td>
<td>Drug delivery (In vitro)</td>
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<td>Spherical</td>
<td>90.0 ± 0.4</td>
<td>+1.9 ± 0.2</td>
<td>PEG-GPLG-IAQQ-TAT-PEG-PE</td>
<td>Substrate only (HPLC): 2 mg/mL polymer + 10 ng/μL MMP ~70% conversion at 12 h</td>
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<td>Drug delivery (In vitro)</td>
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<td>Oval</td>
<td>186.17 ± 5.56</td>
<td>-7.02 ± 2.63</td>
<td>PEG-GPLG-IAQQ-PE</td>
<td>Indirect size, ζ-potential change and drug release</td>
<td>MMP-2</td>
<td>Drug delivery (In vitro &amp; in vivo)</td>
<td>41</td>
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<td>Polymer micelles and crosslinked polymers</td>
<td>Hydrogel</td>
<td>Acr-(APG↓L)-PEG-(APG↓L)-Acr</td>
<td>Indirect gel degrade (weight): 0.35g gel + 2 mg/mL collagenase ~100% conversion in 110 h</td>
<td>Collagenase</td>
<td>Design</td>
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<td></td>
<td>Soluble</td>
<td>Acr-GPQG↓GWQ-NH₂</td>
<td>k_{cat}/K_{m} determined using fluorometric assay and Michaelis-Menten analysis: 870 ± 170 (M^{-1}s^{-1})</td>
<td>MMP-1</td>
<td>cell migration (in vitro &amp; in vivo)</td>
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<td>Ac-GCRO-GPQG↓GWQ-DRCG-NH₂</td>
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<td>Ac-GCRO-GPQG↓GWQ-DRCG-NH₂</td>
<td>k_{cat}/K_{m}: 400 ± 80 (M^{-1}s^{-1})</td>
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<td>Hydrogel</td>
<td>PEG-GPQG↓GWQ-DRCG-NH₂</td>
<td>k_{cat}/K_{m}: 2130 ± 430 (M^{-1}s^{-1})</td>
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<td></td>
<td>PEG-GPQG↓GWQ-DRCG-NH₂</td>
<td>k_{cat}/K_{m}: 970 ± 190 (M^{-1}s^{-1})</td>
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<td>polymer-Ac-FKGGGPQG↓GWQ-ERCG-NH₂-polymer</td>
<td>Indirect gel degrade (weight): 40 nM MMP ~100% conversion in 3 h</td>
<td>MMP-1</td>
<td>cell migration (In vitro)</td>
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Table 2.3. (continued)

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<th>Material</th>
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<th>Morphology</th>
<th>Size (nm)</th>
<th>ζ-potential (mV)</th>
<th>MMP cleavable substrate</th>
<th>MMP hydrolysis (Direct/Indirect/relevant)</th>
<th>MMP</th>
<th>Application</th>
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<td>Block copolymer linked with MMP cleavable substrate</td>
<td>Spherical</td>
<td>24</td>
<td>Polymer-LRRASKGKPLG↓LAG</td>
<td>Indirect size change (DLS): 20 μM polymer + 100 μM MMPs ~10% conversion at 24 h</td>
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<td>MMP-9</td>
<td>Design</td>
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<td>33</td>
<td>Polymer-KKPLG↓LAGLRRASLGG</td>
<td>Direct (HPLC): 120 μM polymer + 100 μM MMP-9 ~21% conversion at 24 h Indirect size change (DLS): 20 μM polymer + 100 μM MMPs ~100% conversion at 24 h</td>
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<td>MMP-2</td>
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<td>20</td>
<td>polymer-GPLG↓LAGGWGERDGS-probe</td>
<td>Indirect size change (DLS): 1 μM polymer + 100 nM MMP-9 ~100% conversion at 4 h</td>
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<td>polymer(PTX)-polymer(GPLG↓LAGGERDGG)</td>
<td>Indirect size change (DLS): 500 μM polymer + 100 nM MMPs ~95% conversion at 4 h</td>
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<td>MMP-12</td>
<td>Drug delivery (in vitro)</td>
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<td>Polymer micelles and crosslinked polymers</td>
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<td>43.5</td>
<td>PEG-GPLG↓VRGDP-PBLA</td>
<td>Direct (HPLC): 5.52mg/2mL polymer + 1 μg/mL MMP 75% conversion after 24 h</td>
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<td>MMP-2</td>
<td>Drug delivery (in vitro)</td>
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<td>76.2</td>
<td>PEG-GPLG↓VRGDP-PDLLA</td>
<td>Direct (HPLC): 1 μg/mL MMP 80% conversion after 25 h</td>
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<td>78.2 ± 5.8</td>
<td>PEG-PLG↓LAG(R)2-PCL</td>
<td>Direct: 5mg/mL polymer + 2 μg/mL of MMP 100% conversion at 1h</td>
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<td>MMP-2</td>
<td>siRNA delivery (in vitro &amp; in vivo)</td>
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<td>68.1 ± 5.6</td>
<td>PEG-LAL↓GPG(R)2-PCL</td>
<td>0% conversion at 1h</td>
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<td>133.3 ± 12</td>
<td>polymer-GPGV↓UGK-polymer</td>
<td>Direct (GPC chromatography): 25% wt polymer +250 μg/mL MMP 100% conversion by 24 h</td>
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<td>Drug delivery (design)</td>
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<td>121</td>
<td>(F)20-PGL↓LAG-VS(F)20 GGGGRRR</td>
<td>Relevant in vitro drug release</td>
<td>n/a</td>
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<td>Drug delivery (in vitro &amp; in vivo)</td>
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<td>PAMAM dendrimer with MMP cleavable surface</td>
<td>Spherical</td>
<td>5.4</td>
<td>PLG↓LAG</td>
<td>Relevant in vivo fluorescence</td>
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<td>Imaging (in vivo)</td>
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<tr>
<td></td>
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<td>~200</td>
<td>PLG↓LAG</td>
<td>Indirect size change (DLS): 4 mg/mL polymer + 0.5 mg/mL MMP ~20% conversion after 6 h</td>
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<td>Drug delivery (in vitro &amp; in vivo)</td>
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<td>20-45</td>
<td>KLGPKA</td>
<td>Indirect payload release: 0.65 mg gelatin + 50 U/mL collagenase 60% payload release at 30 min</td>
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<td>Collagenase*</td>
<td>Protein delivery (in vitro)</td>
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<td>PLA based</td>
<td>Spherical</td>
<td>112.47 ± 3.21</td>
<td>linker-PVGLIG</td>
<td>Relevant in plasma drug release</td>
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<td>n/a</td>
<td>Drug delivery (in vitro &amp; in vivo)</td>
<td>54</td>
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## Table 2.3. (continued)

<table>
<thead>
<tr>
<th>Material</th>
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<th>Morphology</th>
<th>Size (nm)/ Gel storage mod</th>
<th>z-potential (mV)</th>
<th>MMP cleavable substrate</th>
<th>MMP hydrolysis (Direct/Indirect/relevant)</th>
<th>MMP</th>
<th>Application</th>
<th>Ref</th>
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<tr>
<td>Protein based</td>
<td>Silk elastin protein polymer</td>
<td>Soluble monomer</td>
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<td>silk-(GPQGIFGQ)-elastin-lysine elastin-silk</td>
<td>Direct (SDS-PAGE): 1 mg/mL polymer + 40 nM MMP ~100% conversion in 20 min</td>
<td>MMP-2</td>
<td>Gene delivery (in vivo)</td>
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<td>Hydrogel</td>
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<td></td>
<td>silk-elastin (GPQGIFGQ)-lysine elastin-silk</td>
<td>~100% conversion in 30 min</td>
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<td>silk(GPQGIFGQ)-elastin-lysine elastin-silk</td>
<td>~100% conversion in 30 min</td>
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<td>MMP responsive alginate</td>
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<td>120 Pa (G')</td>
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<td>GGYGPVG↓LIGGK</td>
<td>Relevant in vivo cell migration</td>
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<td>Cell implantation (in vivo)</td>
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<td>Crosslinked gelatin</td>
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<td>Gelatin</td>
<td>Relevant in vitro and vivo fluorescent imaging</td>
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<td>Drug delivery (in vitro &amp; in vivo)</td>
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<td>Co-assembled gelatin</td>
<td>4.7 ± 0.6</td>
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<td>Gelatin B</td>
<td>Indirect: 0.65 mg gelatin + 2.84 µg MMP ~60% payload release at 24 h</td>
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<td>Drug delivery (in vitro)</td>
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<td>Dendrimer in collagen gel</td>
<td>Triple helix</td>
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<td>Type-IV and type-I collagen</td>
<td>Relevant in vitro and vivo biological activity</td>
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<tr>
<td>Inorganic NPs</td>
<td>Au surface modified MMP cleavable substrate linked to Cy5.5</td>
<td>Rod</td>
<td>40 (l) x 20 (w)</td>
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<td>Cy5.5-GPLGVRGC-AuNR</td>
<td>Indirect (fluorescence): 7.8x increase at 1 h (6.75 nM MMP-2) 6.6x increase at 2 h (MMP-3) 8.2x increase at 2 h (MMP-9) 6.6x increase at 2 h (MMP-13) 3.7x increase at 2 h (MMP-7)</td>
<td>MMP-2</td>
<td>Phototherapy (in vivo)</td>
<td>61</td>
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<td>Gelatin with quantum dot surface</td>
<td>Spherical</td>
<td>97.9 ± 2.1</td>
<td>-6.29 ± 0.22</td>
<td>Gelatin type A</td>
<td>Indirect (fluoresce): 0.1 mg polymer + 230 ng MMP ~85% conversion at 12 h</td>
<td>MMP-2</td>
<td>Imaging (in vivo)</td>
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<td>Magnetic iron oxide particles</td>
<td>Spherical and ellipsoid</td>
<td>186.5 ± 15.8</td>
<td>-3.37</td>
<td>acrylate-PEG-GGPGQG↓LWGGQK-PEG-acrylate</td>
<td>0.1 mg/mL + collagenase type I enzyme 0.1 mg/mL</td>
<td>collagenase type I</td>
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<td>CdSe/ZnS quantum dots</td>
<td>12.8 ± 3.6</td>
<td>-8.4</td>
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<td>GGPGVRGC-NH2</td>
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<td>MMP-9</td>
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2.3.1. Sequence specificity

The amino acid residues in the MMP-cleavable substrate are labeled by the number of positions starting from the scissile bond (bond between P1 and P1’) and are labeled P1 through P6 towards the N terminus, and P1’ through P6’ towards the C terminus. Understanding substrate specificity of this family of enzymes can elucidate the functions of specific MMPs, and help to design more specific inhibitors. According to MEROPS database, over 7,600 MMP-cleavable substrates have been observed and recorded. In addition to natural substrates, many engineered sequences have been identified using various high throughput techniques such as phage display, oriented peptide library method, and proteomic identification of protease cleavage site (PICS).

Although MMPs are highly specific enzymes, the general consensus sequence found in the database are similar among MMPs, with Pro in P1, Gly in P3, and Leu in P1’ being the hallmark of MMP substrates. This could be partly due to a bias towards working with sequences that has already been tested and are copied by other researchers. A thorough examination by Overall et al. in 2015, revealed that of 112 P1-P1’ cleavages reported for MMP-1, -2, -3, -7, -8, -9, -12, -13, and -14 in MEROPS database, only 79 cleavages were unique, and only 5 sequences contained both Pro in P1 and Leu in P1’.

The authors investigated the substrate specificity of MMPs using FRET-based peptides by conjugating a fluorophore, Mca (7-methoxycoumarin-4-acetic acid N-succinimidyl ester), on the N terminus, and a quencher, Dpa (dipicrylamine), on the C terminal end of P1’ residue (Figure 2A). The MMP cleavage of the peptides was analyzed by measuring the recovered fluorescence of Mca over time (Figure 2B). In comparison to the general consensus PLG↓L peptide, PLN↓L was cleaved 2.5 times faster by MMP-1 and PAG↓L was cleaved 2 times faster by MMP-2 and 0.25 times faster by MMP-3 (Figure 2B). Clearly, beyond the general consensus sequence, the primary sequence can be tailored to target specific MMPs.
Figure 2.3. Investigation of substrate specificity of MMPs using FRET-based peptides. A) Chemical structure of the FRET-based peptides (Mca fluorophore and Dpa quencher). B) Recovered fluorescence of Mca over time. Adapted from ref 77.

However, MMP-responsive nanomaterials require the enzyme to interact with the insoluble nanomaterial and not just the free peptide molecules. MMP-cleavable substrates are typically covalently modified on the C and the N terminus of the peptides to act as linkers for block-co-polymers or they are used to attach functional moieties on the surface of the nanomaterial, like targeting ligands. This modification of the peptide can have significant impact on the enzyme recognition and hydrolysis of the cleavage site. The Bing Xu group has observed that covalent modifications with hydrophobic residues of peptides PLGLRSK and PLSLRSK on the N terminus...
can shift the cleavage site and change the rate of hydrolysis by MMP-9. In this study the two sequences were systematically modified by adding one or two phenylalanine residues on the N terminus followed by capping the terminus with acetyl, fluorenylmethyloxycarbonyl (Fmoc), pyrene, or naphthalene group. They observed that the cleavage site of unmodified peptide (PLGL↓RSK) shifted to PL↓GLRSK with the addition of one or two Phe residues, both with and without the capping groups, and the rate of hydrolysis decreased with increasing hydrophobicity of the capping group (100% conversion for uncapped peptides, 60% conversion for naphthalene capped peptides, and 30% conversion for Fmoc capped peptides in 72 h). This shows that even a small modification of length and N terminal substitution can lead to different products and conversion rates. Therefore, certainly more than the primary sequence of the substrate needs to be considered in designing MMP-responsive materials.

2.3.2. Supramolecular specificity

The family of MMPs can essentially degrade all components of the extracellular matrix, ranging from highly ordered proteins such as helical collagen fibers to smaller proteins such as fibronectin or gelatin (Table 1). Traditionally, MMPs were named after their common substrates for example, MMP-1, -8, and -13 are also called collagenase 1, 2, and 3, respectively, and MMP-2 and -9 are called gelatinase A and B, respectively. As the names suggest, MMPs have different roles in tissue remodeling, which require them to have specific interaction with their substrates. For instance, MMP-2 and -9 has been reported to bind to native type I collagen but cannot cleave it until it is in the denatured gelatin form, whereas MMP-1 can easily digest the collagen fiber. Therefore, in addition to the primary sequence, the topology or the supramolecular structure of the substrate must also be appropriate for the targeted MMP.
Figure 2.4. MMP-13-responsive β-hairpin peptide hydrogels. A) Cartoon representation and B) amino acid sequence of self-assembling β-hairpin nanofiber hydrogels. C) The β-hairpin peptide fibers are hydrolyzed by MMP-13 and not by MMP-3. The rate of hydrogel degradation is more dependent on the rigidity of the gel than the substrate sequence. Adapted from reference 31.

This structural specificity was demonstrated by Schneider et al. using MMP-13 responsive β-hairpin peptide hydrogels (Figure 4A). These peptides contain two proline residues in the middle of repeating IKV units to create a bend for the β-hairpin structure, and the second proline was used to insert the MMP-cleavable substrate PTG↓X, where X = Leu, Ile, Phe, and Ala (Figure 4B). When β-hairpin peptide hydrogels were incubated with 80 nM of MMP-13 for 14 days, 32-65% hydrogel degradation was observed, whereas no degradation was observed when incubated with 40 or 400 nM of MMP-3 for 14 days (Figure 4C). Based on the sequence, both MMPs should be able to cleave PTG↓X, however, the densely packed β-hairpin peptide fibers in the hydrogel was only degraded by MMP-13 that naturally digests collagen fibers and not by MMP-3 which
digests sheet-like structures such as the collagen IV, perlecan, etc. in the basement membrane (Table 1). In addition, the rate of conversion in MMP-13 degradation of the hydrogel was also dependent on the structural topology of the gel. According to the sequence specificity, the expected rate of PTG↓X hydrolysis should have followed X = Leu > Ile, Phe > Ala. However, the observed order of degradation was X = Phe (65%) > Leu (58%) > Ile (44%) > Ala (32%), due to the low rigidity and larger pores of the Phe containing hydrogel which allows MMP-13 to penetrate gel faster (Figure 4C). This study shows that the morphology of the peptide fiber can be designed to target specific MMPs and the degree of crosslinks in the fiber network can be manipulated to control the rate of hydrogel degradation.

A study by Ghandehari et al. demonstrated that the location of MMP-cleavable substrate in the nanostructure can also control the rate of degradation (Figure 5A). In this study, silk elastin polymers (silk-elastin-lysine elastin-silk motif) were modified with MMP-2 cleavable substrate (GPQGIFGQ) imbedded in 1) between the silk and elastin blocks, 2) within the elastin block, or 3) within the silk block (Figure 5C). When a low concentration of 1 mg/ml of the MMP-responsive silk polymer was incubated in the soluble form with 40 nM of MMP-2, the three modified polymers were all digested 100% within 20-30 min. However, when 4% weight silk polymer hydrogel was incubated with 40 nM of MMP-2 for 14 days, different amount of digested and undigested polymers was released from the hydrogel (Figure 5B). Higher ratio of digested vs. undigested released polymer was observed when the MMP-cleavable substrate was placed between the silk and the elastin blocks, followed by the substrate imbedded within the elastin block, and the substrate imbedded within the silk block had the lowest ratio of digested polymer. This is because the silk block (GAGAGS) is part of the major structural component, and the substrate is more accessible in the block junctions and in the elastin block (GVGVP).
Figure 2.5. MMP-2-responsive silk elastin polymer hydrogels. A) Cartoon representation and B) amino acid sequence of self-assembling silk elastin nanofiber hydrogels. C) The placement of the MMP-2 cleavable sequence within the silk elastin polymer dictates the rate of hydrogel degradation. Adapted from ref 56.

The two previous examples by Schneider and Ghandehari groups altered the amino acid sequence and the placement of the cleavable substrate in the hydrogel to manipulate the rate of gel degradation and MMP specificity. Next, we discuss an example that will be discussed in detail in Chapter 3 to demonstrate that the morphology and the accessibility of the substrate in peptide nanostructures that do not gel can also be manipulated to control the rate of MMP hydrolysis. 33 Twelve amphiphilic peptides with diphenylalanine hydrophobic end and either lysine or aspartic acid containing hydrophilic end were systematically modified using MMP-9 cleavable PXG↓LXG
or AXG↓LXG motif in the center. The peptides were self-assembled at 1 mM and incubated with 100 ng/ml of MMP-9 for 96 hours. We observed that among PXG↓LXG containing peptides, spherical micelles (100% in 48 h) were cleaved faster or at the rate as worm-like micelles (90% at 96 h). In AXG↓LXG containing peptides, the higher rigidity of the peptide backbone contributed to higher ordered assemblies which slowed down the enzyme cleavage (from 60% to 10% in 96 h), and MMP-9 was unable to digest peptides that formed anti-parallel β-sheet fibers.

Using nanomaterials allow incorporation of more features, in addition to substrate specificity, to target specific MMPs and manipulate the rate of enzyme cleavage. By changing the amino acid sequence or the placement of MMP-cleavable substrate, the supramolecular structure and properties of the materials can be modified and play a large role in MMP specificity.

2.3.2. Electrostatic specificity

Electrostatic specificity may be one of the simplest ways to target enzymes but is often overlooked in meeting MMP specificity. The substrate binding pockets of MMPs are suited for hydrophobic interactions and the main focus has been on aromatic and aliphatic residues of the cleavable substrate. However, as emphasized in this literature analysis chapter, the short-range interaction of the MMP and the nanomaterial is as important as the sequence of the cleavable substrate. The electrostatic charge of the nanomaterials can be readily tuned to enhance or block enzyme engagement and can also be used to target specific MMPs. The isoelectric points (pI) of MMPs were computed by Rani et al. using ExPASy’s ProtParam tool and range broadly from 5.26 to 9.94 (Table 1). In addition, the pI of MMPs can differ by isoform and charge variants. Using 2D isoelectric focusing, Riccio et al. measured pI of 4.1-4.6 for several charge variants of 92 kDa pro-MMP-9 and 82 kDa activated MMP-9, and 6 different charge variants of 65 kDa MMP-9 with
pI ranging from 4.82-5.15. Using this information, the nanomaterials be designed to have same or opposite charge to repel or recruit specific MMPs, respectively, and to control the rate of enzyme cleavage.

In our aforementioned work (Chapter 3), the 12 different MMP-9 responsive self-assembling peptides were systematically modified to have either PXG↓LXG or AXG↓LXG motif and positive or negative charge using lysine or aspartic acid residues, respectively. As expected, the cationic peptides were readily cleaved by MMP-9 whereas the analogous anionic peptide had very low conversion (2.5% in 96 h) or were not cleaved at all. This was observed for both spherical and worm-like micelle peptide nanostructures. This electrostatic (mis) matching of charges between nanomaterial and MMPs is a simple yet powerful technique to enhance or prevent enzyme cleavage.

In addition to the rate of MMP cleavage, electrostatic specificity can be utilized to target a specific MMP. Ahn et al. have designed a FRET-like system using gold nanorod particles coated with an MMP-cleavable substrate (GPLGVRGC) conjugated to Cy 5.5, a positively charged near-infrared dye. The cleavage of the substrate was monitored by recovered fluorescence of Cy 5.5. dye. After 2-hour incubation period with different MMPs, the fluorescence was recovered 8.2 times with MMP-9, 7.8 times incubated with MMP-2, 6.6 times with either MMP-3 or -13, and 3.7 times with MMP-7. The authors recognized this selectivity but did not comment on the reasons underpinning it. Our speculation is that the electrostatic interaction of the modified cationic gold nanorods and the MMPs were favorable for anionic MMP-2, -3 -9, and -13 which have pI of 5.26 - 5.77. On the other hand, the pI of MMP-7 is 7.73, which makes it almost neutral at physiological pH and least likely to be recruited by the cationic gold nanorod.
Using (mis) matching electrostatic interactions between the enzyme and the nanostructures, the enzyme cleavage rate and specificity can be controlled. In Table 3, there are nanomaterials with negative zeta potentials that are cleaved by MMP-2 characterized by Torchillini and Zhu groups, however, the rate of conversion was measured on the isolated cleavable substrate and not on the anionic nanomaterial, or was indirectly measured based on payload release. Therefore, these results cannot be quantitatively compared because the rate of substrate cleavage is different when isolated vs. as part of the nanomaterial (as discussed previously in section 2.3.2.) and does not equal to the rate of payload release. Before comparing the rate of payload release to the rate of nanomaterial response, we will first discuss the typical types of MMP-responsive changes that nanomaterials have.

2.4. MMP-triggered modes of response for targeted anti-cancer therapy

Inhibition of MMPs using small molecular compounds or macro biomolecules for anti-cancer therapy has failed in the past mainly due to the similar substrate binding site of multiple MMPs as well as the varying expression of MMPs during various stages of cancer development.\textsuperscript{11-13} Instead of inhibiting the MMP action, MMP-responsive nanomaterials are designed to take advantage of the inherent overexpression and activity of MMPs to trigger a response in the nanomaterial which allows spatial and temporal control of the anti-cancer activity. As discussed in the previous Section 2.2., nanomaterials provide a versatile platform to tune the specificity towards an individual MMP using modification of the MMP-cleavable substrate and the morphology and charge of the nanomaterials.

In this section, 12 different MMP-responsive nanomaterials which were used for anti-cancer therapy, are described have been categorized into 4 different modes of responses: 1) particle
shrinkage, 2) particle aggregation, 3) nanofiber formation, and 4) de-PEGylation of the nanomaterial (Figure 6 and Table 4). Following the nanomaterial’s response, the anti-cancer activity is achieved either by releasing therapeutic small molecules from bio-compatible nanocarriers, or by switching morphology into nanofibers which can themselves have a therapeutic effect. As we review the different categories, we will analyze how the different modes of response can affect the rate of payload release and influence the bio-activities in vitro and/or in vivo.

**Figure 2.6.** MMP-triggered modes of response for anti-cancer therapy. As a response to MMP hydrolysis of the substrate in the nanomaterial, the nanomaterial can 1) shrink in size,\textsuperscript{27,52,59,60} 2) aggregate,\textsuperscript{39,46,48} 3) switch into cytotoxic nanofibers,\textsuperscript{26,28} or 4) reveal a functional moiety in the nanomaterial without changing in size.\textsuperscript{40,41,47}
Table 2.4. MMP-triggered modes of response for anti-cancer therapy.

<table>
<thead>
<tr>
<th>Mode of response</th>
<th>Pre-MMP cleavage nanomaterial</th>
<th>Post-MMP cleavage nanomaterial</th>
<th>Payload</th>
<th>Rate of MMP triggered nanomaterial response</th>
<th>Rate of MMP triggered payload release</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle shrinking</strong></td>
<td>209.9 ± 24.3 nm PEG coated iron oxide particles</td>
<td>30.86 ± 5.03 nm Iron oxide particles</td>
<td>Doxorubicin</td>
<td>Conversion in 1 d</td>
<td>60% release in 4 d (+ collagenase) 36% release in 4 d (- collagenase)</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>~ 200 nm Hyaluronic acid coated dendrimer</td>
<td>~ 10 nm Dendrimer</td>
<td>Doxorubicin</td>
<td>~20% size conversion at 4 h</td>
<td>N/A</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>193.1 nm Dendrimer on gelatin surface</td>
<td>34.4 nm (Synthesized dendrimer)</td>
<td>Doxorubicin</td>
<td>Not enzymatically produced</td>
<td>N/A</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>97.9 ± 2.1 nm QD on gelatin surface</td>
<td>~ 10 nm Quantum dot</td>
<td>QD particles</td>
<td>90% conversion in 12 h</td>
<td>90% in 12 h</td>
<td>60</td>
</tr>
<tr>
<td><strong>Aggregation</strong></td>
<td>78.2 ± 5.8 nm Block-co-polymer</td>
<td>Over 1000 nm Aggregated polymer</td>
<td>siRNA</td>
<td>Conversion in 24 h</td>
<td>N/A</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>100 nm Block-co-polymer</td>
<td>400 - 1000 nm Aggregated polymer</td>
<td>Paclitaxel</td>
<td>~95% size conversion in 4 h</td>
<td>N/A</td>
<td>46</td>
</tr>
<tr>
<td><strong>Nanofiber formation</strong></td>
<td>33.0 ± 1.2 PEGylated liposome</td>
<td>818.3 ± 104.7 nm Destabilized liposome</td>
<td>Paclitaxel</td>
<td>Conversion in 2 h</td>
<td>55% release in 4 h (+ collagenase) 30% release in 4 h (- collagenase)</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>~ 20 nm Spherical lipopeptide micelle</td>
<td>Peptide nanofiber</td>
<td>None</td>
<td>~50% conversion at 1 h</td>
<td>N/A</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>~ 200 nm Spherical peptide micelle</td>
<td>Peptide nanofiber</td>
<td>Doxorubicin</td>
<td>~ 60% conversion in 96 h</td>
<td>N/A</td>
<td>28</td>
</tr>
<tr>
<td><strong>De-PEGylation</strong></td>
<td>62.6 ± 5.1 nm PEGylated polymer micelle</td>
<td>65.2 ± 8.8 nm Polymer micelle</td>
<td>Paclitaxel</td>
<td>~ 0% size conversion at 25 h</td>
<td>80% release in 72 h (+ MMP-2) 80% release in 72 h (- MMP-2)</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>93.4 ± 0.4 nm PEGylated liposome</td>
<td>102.4 ± 6.2 nm Liposome</td>
<td>Paclitaxel</td>
<td>Conversion in 2 h</td>
<td>~ 50% at 24 h (+ collagenase) ~ 50% at 24 h (- collagenase)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>186.17 ± 5.56 nm -15.05 ± 1.84 mV PEGylated liposome</td>
<td>215.45 ± 7.85 nm 4.25 ± 1.89 mV Liposome</td>
<td>Dasatinib</td>
<td>Conversion in 1 h</td>
<td>~ 40% release in 6 h (+ collagenase) ~ 40% release in 6 h (- collagenase)</td>
<td>41</td>
</tr>
</tbody>
</table>
2.4.1. Size shrinking

Nanomaterials that are 100-200 nm in size can have enhanced accumulation in the tumor microenvironment, but this accumulation is limited to the periphery edge of the tumor. It has been proposed that by using nanomaterials that can shrink into smaller particles or release small particles in response to MMP cleavage, it allows the shrunken 10-40 nm particles to penetrate the interstitial tumor space. Fukumura et al. have used this strategy and decorated the surface of gelatin nanoparticles with quantum dots to form 98 nm nanoparticles (Figure 7A). After incubating the particles with MMP-2, 90% quantum dots (10 nm) were released from the gelatin particles in 12 hours (Figure 7B). When the MMP-responsive gelatin nanoparticles and comparable non-responsive silica particles decorated with quantum dots were directly injected into tumors in vivo, the quantum dots released from the gelatin particle had diffused 300 µm from the injection site, whereas the quantum dots on the silica particle remained concentrated in the initial injection location (Figure 7C). This example demonstrated a proof-of-concept that the MMP-triggered release of quantum dots allowed deeper penetration into the solid tumor mass, which could potentially be replaced by 10 nm nanocarrier of cancer therapeutics.

In another system, Kizilel et al. developed magnetic iron oxide particles coated with MMP-cleavable acrylate-PEG polymer loaded with doxorubicin (Figure 8A). These 210 nm particles shrunk into 31 nm particles after 1 day of incubation with collagenase (bacterial equivalent of MMP) (Figure 8B). Interestingly, this size shrinkage did not trigger an increase in doxorubicin (DOX) release until day 3. At day 4, 60% of doxorubicin was released from the particles that were incubated with collagenase, in comparison to the 36% released without collagenase. Our speculation is that although the nanoparticle’s size was responsive to the enzymatic cleavage of the polymer coating, the rate of payload release is not simultaneously triggered by cleavage of the
enzyme substrate, likely because the drugs are still embedded in the polymer coating and continues to degrade in solution over time. The DOX-loaded MMP-responsive nanoparticles were internalized by HeLa cells 11 times more than non-responsive or bare iron oxide particles \textit{in vitro} (Figure 8C). In addition, the drug-loaded nanoparticle displayed time-dependent activity in which it was less cytotoxic to HeLa cells than free doxorubicin at 24 and 48 h, but more cytotoxic at 72 h incubation, in agreement with the release rate of DOX from the nanoparticle. The two examples by Kizilel et al. and Fukumura et al. demonstrated that MMP-triggered shrunken nanoparticles can have enhanced internalization by cells \textit{in vitro} and penetration in solid tumors \textit{in vivo}, in comparison to non-responsive (not shrunken) nanoparticles.

\textbf{Figure 2.7.} MMP-2-responsive size shrinking gelatin nanoparticles. (A) Cartoon representation and (B) experimental measurement of 10 nm quantum dots being released from 100 nm gelatin nanoparticles upon incubation with MMP-2. (C) The quantum dots from MMP-responsive gelatin particles were able to penetrate throughout the solid tumor \textit{in vivo}, whereas the quantum dots from non-responsive silica particles stayed localized. Scale bar 100 µm. Adapted from ref 60.
**Figure 2.8.** MMP-responsive size shrinking magnetic iron oxide nanoparticles. (A) Cartoon representation and (B) experimental measurement of 200 nm polymer coated particles shrinking to 30 nm after incubation with collagenase. (C) Doxorubicin from dox-loaded MMP-responsive nanoparticles were internalized 11 times more than non-responsive particles. Scale bar 10 µm. Adapted from ref 27.

### 2.4.2. Induced aggregation

Nanomaterials can increase the accumulation in the tumor site as discussed in the previous section and can also increase retention of the material by preventing it from diffusing back into the bloodstream. In this strategy, 20–80 nm particles enter the periphery of the tumor and aggregate upon MMP action to stay immobilized around the tumor tissue. Gianneschi *et al.* have developed paclitaxel conjugated block-co-polymers using MMP-cleavable substrate (GPLG↓LAGGERDG) as linkers to form 20 nm spherical particles (Figure 9A-B).\(^{46}\) When the substrate was cleaved by MMP-12, the amphiphilic balance of the polymer is altered, 95% of particles aggregated into 400-1000 nm in 4 hours (Figure 9C). When treated *in vivo*, the tumor growth inhibition of 15 mg/kg paclitaxel in the MMP-responsive nanomaterial was slightly lower than that of free paclitaxel.
(Figure 9D), however, the systematic toxicity was much lower, and the maximum tolerated dose was 15 times higher than that of free paclitaxel (Figure 9E).

**Figure 2.9.** MMP-12-induced aggregating polymer nanoparticles. (A) Cartoon representation and TEM images of (B) 20 nm particles aggregating into (C) 400-1000 nm aggregates upon MMP action. (D) Inhibition of tumor growth by paclitaxel-loaded MMP-responsive nanoparticle was lower than free paclitaxel but, (E) the systematic toxicity much lower than free paclitaxel. Adapted from ref 46.

MMP-induced aggregating micelles were developed by Zhu et al. using liposomes conjugated to PEG via a MMP-2 cleavable linker (GPLGIAGQ) to encapsulate paclitaxel. After 2 hour incubation period with collagenase, the initial 33 nm spherical liposomes aggregated into 818 nm. In this system, hydrolysis of MMP-2 cleavable linker separates the lipid and the PEG building blocks causing the post-cleavage hydrophobic lipid to aggregate while the released hydrophilic PEG is solubilized. After 4-hour incubation period, 55% of the payload was released, in comparison to 30% released without collagenase. When tested *in vitro*, the IC₅₀ of paclitaxel
loaded MMP-responsive liposomes were ten folds lower than free drug in paclitaxel-resistant cell lines, MDA-MB-231 and NCI/ADR-RES (5-6 µM vs. 45-34 µM) due to increased uptake of the drugs, but was higher in drug sensitive cell line, A549 (0.042 µM vs. 0.008 µM). In this system, the destabilization of the micelles induced increased paclitaxel release and was more cytotoxic to paclitaxel-resistant cells in vitro.

2.4.3. Cytotoxic amyloid-like nanofibers

Enzymatically triggered formation of cytotoxic nanofibers is an emerging field in anti-cancer therapy. This mode of response is extensively researched by Bing Xu’s group that use phosphorylated precursors to form cytotoxic fibers upon dephosphorylation by alkaline phosphatases overexpressed in cancer cells. Likewise, MMP-responsive precursors can also form cytotoxic nanofibers upon MMP-cleavage. Maruyama et al. designed lipopeptides that can be cleaved by MMP-7 and form nanofibers with high gelation propensity as a response (Figure 10A). The amphiphilic lipopeptides form 20 nm spherical micelles and when incubated with MMP-7, the lipid portion of the cleaved product self-assembles into fibers within 1 hour (Figure 10B). These fibers caused selective toxicity to cells in vitro, that correlated with the amount of MMP-7 produced by the cancer cells and were non-toxic to normal cells with low concentrations of MMP-7 (Figure 2.10.C).
Figure 2.10. MMP-7-triggered formation of cytotoxic lipopeptide nanofibers. (A) Cartoon representation and molecular structure of the lipopeptide that switch morphology from (B) spherical micelles to nanofibers upon MMP-7 hydrolysis (TEM images, scale bar left 50 µm; right 100 µm). (C) The cytotoxicity of the nanofibers increases in cell lines that have high concentrations of MMP-7. Adapted from ref 26.

Our group has also demonstrated that MMP-9-triggered formation of nanofibers has anti-cancer properties in vitro and in vivo.\textsuperscript{28} In this system, 200 nm spherical peptide micelles were used to co-assemble with doxorubicin and cleaved by MMP-9 to form cytotoxic nanofibers (Figure 11A). In 96 hours, 60% of the peptide (GFFLGL↓DD) was cleaved by MMP-9 and formed bundles of fiber (Figure 11B). The co-assembled doxorubicin was more cytotoxic in vitro and inhibited tumor growth more than free doxorubicin in vivo. Remarkably, the peptide alone displayed similar tumor growth inhibition effects as doxorubicin alone (Figure 11C).
2.4.4. Activation by de-PEGylation

Polyethylene glycol or PEG is a hydrophilic polymer block often used as a coating to solvate nanoparticles. In biological applications, it is popularly used as a “stealth” material because macrophages are less likely to detect nanomaterials that are coated in PEG. In previous examples, removal of PEG groups caused a change in the amphiphilic balance of the building blocks and caused the material to aggregate or revealed smaller inorganic particles beneath the PEG layer. In this category, unlike the example discussed above, the removal of PEG groups from the nanomaterials does not cause a dramatic change in the size of the nanomaterials. On the contrary, these materials are designed to maintain their shapes and sizes after the PEG cleavage. This allows
the nanomaterials to maintain their pre-designed features while revealing the functional ligands that were shielded by PEG.

Zhu et al. have developed MMP-responsive polymer lipid conjugate building blocks using PEG and phosphoethanolamine (PE) conjugated by MMP-cleavable linker (GPLG↓IAGQ) and/or a trans-activating transcriptional activator (TAT) peptide that can form paclitaxel-loaded micelles. Two paclitaxel-loaded MMP-responsive micelles were made using PEG$_{2k}$-GPLG↓IAGQ-TAT-PEG$_{1k}$-PE or PEG$_{2k}$-GPLG↓IAGQ-TAT-PE and incubated with 50 µg/ml collagenase IV. In 2 hours, micelles made of PEG$_{2k}$-GPLG↓IAGQ-TAT-PE aggregated from 58 nm to 998 nm, whereas micelles made of PEG$_{2k}$-GPLG↓IAGQ-TAT-PEG$_{1k}$-PE slightly increased in size from 93 nm to 102 nm. In addition, the non-aggregating micelles did not show a significant increase in drug release (about 50% release with and without collagenase), whereas the aggregated micelles showed significant increase in paclitaxel release (80% in comparison to 50% without collagenase). In the first micelle, the cleavage of PEG$_{2k}$-GPLG exposes the cell penetrating TAT peptide on the surface while maintaining the structural integrity of the micelle due to the additional PEG$_{1k}$ group in the remaining building block, IAGQ-TAT-PEG$_{1k}$-PE. In the second micelle, only IAGQ-TAT-PE is left, which disrupts the amphiphilic balance and the structural integrity of the micelle, resulting in increased release of paclitaxel and aggregation of the post-enzymatic product. The TAT activated and paclitaxel-loaded micelles were able to penetrate and reduce the size of NCI/ADR-RES (ovarian tumor) spheroids significantly more than the aggregating counter micelle and were retained longer in tumors of mice in vivo.

Ge et al. developed block-co-polymer with poly-D, L lactide and PEG linked by MMP-2 cleavable linker (PEG$_{113}$-GPLG↓VRGDG-PDLLA$_{100}$) that can encapsulate paclitaxel and form 62 nm spherical micelles. When the particles were incubated with 1 µg/ml of MMP-2, 80% of PEG$_{113}$
was cleaved in 25 hours observed by HPLC. Although the PEG\textsubscript{113} layer was removed, the size of the particle remained the same at 65 nm. In addition, the MMP-2 cleavage of PEG did not trigger release of paclitaxel. At 72 hours, 80\% of paclitaxel was released from the micelle with and without incubation with MMP-2. When the PEG\textsubscript{113} is cleaved from this micelle, the PDLA\textsubscript{100} core remains conjugated to VRGDG which is surprisingly sufficiently hydrophilic to keep the particles from aggregating. The drugs remain within the core of the micelle and are unaffected by the MMP-2 triggered cleavage of PEG\textsubscript{113}. The paclitaxel-loaded MMP-activated polymer micelle was more cytotoxic to 4T1 cells (breast tumor) \textit{in vitro} and was able to inhibit tumor growth significantly in H22 tumor (hepatic carcinoma) bearing mice in comparison to free paclitaxel \textit{in vivo}. The two examples by Ge and Zhu, and one more in Table 4, show that de-PEGylation of these micelles does not cause morphology change in the nanoparticle nor induce drug release and the anti-cancer efficacy of the treatments are increased.

2.5. \textbf{Summary and future directions}

In this chapter, we analyzed MMP-responsive nanomaterials and drew conclusions on guiding principles to design nanomaterials with specificity and functionality. First, the hydrolysis rate of MMP-cleavable peptide can be tuned by modifying the peptide substrate or the morphology and electrostatic charge of the nanomaterial that hosts it. Specificity towards a specific MMP can also be tuned by these methods. Second, the rate of payload release can be controlled by choosing the mode of response in the nanomaterial after MMP action. In general, the rate of MMP-induced payload release is delayed from the nanomaterial response. Third, the mode of response can have different advantages in the biological context. Size shrinking particles can penetrate deeper into solid tumors and increase uptake intracellularly, cytotoxic nanofibers can achieve highly selective
toxicity in vitro and enhance the efficacy of drugs in vivo, and activated nanoparticles can reveal targeting ligands without inducing payload release or morphological change. The two examples of aggregating particles did not always increase the efficacy of the drugs, however, the overall systematic toxicity was lowered in vivo, and increased efficacy was observed in drug-resistant cell lines. Currently, the efficacy of anti-cancer treatments is measured by the ability to kill cells and reduce tumor size. However, as observed in this chapter, MMP-responsive systems do not always increase the toxicity nor tumor reducing ability of the payloads. Instead, the system achieves more selective and targeted therapy which could potentially reduce adverse side effects and transform current chemotherapy into safer treatment options.

2.6. References

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(33) Son, J.; Kalafatovic, D.; Kumar, M.; Yoo, B.; Cornejo, M. A.; Contel, M.; Ulijn, R. V. Customizing Morphology, Size, and Response Kinetics of Matrix Metalloproteinase-Responsive


Chapter 3
Customizing Morphology, Size, and Response Kinetics of Matrix Metalloproteinase-Responsive Nanostructures by Systematic Peptide Design

Summary

Over-expression and activation of matrix metalloproteinase-9 (MMP-9) is associated with multiple diseases, and can serve as a stimulus to activate nanomaterials for sensing and controlled release. In order to achieve autonomous therapeutics with improved space-time targeting capabilities, several features need to be considered beyond the introduction of an enzyme-cleavable linker into a nanostructure. We introduce guiding principles for a customizable platform using supramolecular peptide nanostructures with three modular components to achieve tunable kinetics and morphology changes upon MMP-9 exposure. This approach enables: (1) fine-tuning of kinetics through introduction of ordered/disordered structures, (2) a 12-fold variation in hydrolysis rates achieved by electrostatic (mis) matching of particle and enzyme charge, and (3) selection of enzymatic reaction products that are either cell-killing nanofibers or that disintegrate. These guiding principles, which can be rationalized and involve exchange of just a few amino acids, enable systematic customization of enzyme-responsive peptide nanostructures for general use in performance optimization of enzyme-responsive materials.
3.1. Introduction

Nanomaterials impact biomedicine by taking advantage of their inherent chemical and physical properties to achieve increased circulation lifetime and selective bio-distribution \textit{in vivo}, and dictate cellular uptake mechanisms,\textsuperscript{1} all of which cannot be attained by molecular drugs alone. Another beneficial feature of nanomaterials is the ability to incorporate stimuli-responsive functionalities to enhance selectivity in targeting diseased cells, and manipulate drug release profiles.\textsuperscript{2–5} In particular, an inherent biological stimulus such as (over-) expression of enzymes, can serve as a marker for diseased cells, as well as a trigger to facilitate desired changes in the nanostructure.\textsuperscript{6} Understanding the relationship between properties of nanomaterials, such as size, shape, and charge, with their bio-distribution or cellular uptake patterns have allowed researchers to develop refined systems to target specific organs and cells. For example, Discher \textit{et al.} described that the increase in length of flexible filomicelles up to 8 μm increased the circulation lifetime \textit{in vivo} in mice, in comparison to shorter filomicelles and spherical vesicles.\textsuperscript{7} Likewise, a thorough investigation is necessary in order to develop design rules for nanomaterials that can engage with the enzymatic stimuli with varying degrees of affinity in order to predetermine response kinetics for the desired enzyme-responsive action (\textit{i.e.} disassembly, morphology switch, \textit{etc.}).\textsuperscript{6}

For instance, an over-expression and activation of matrix metalloproteinase-9 (MMP-9), an enzyme that is crucial to normal behavior of cells such as degradation of extracellular matrix,\textsuperscript{8} is associated with multiple diseases including cancer metastasis,\textsuperscript{9} cardiovascular diseases,\textsuperscript{10} arthritis,\textsuperscript{11} \textit{etc.} Since the introduction of polymeric MMP-responsive materials by Hubbell \textit{et al.},\textsuperscript{12,13} there have been numerous strategies to exploit this highly disease-relevant enzyme for biomedical applications.\textsuperscript{14–21} One of the challenges in designing MMP-9 responsive nanomaterials is optimizing a cleavable segment that meets the enzyme specificity,\textsuperscript{22} and is concurrently compatible
with the nanoparticle system. Another, largely overlooked but important aspect of particle design is regulating the susceptibility of the nanoparticle to the enzyme stimuli through the manipulation of electrostatic properties of nanoparticles to attract or repel enzymes of opposite or same charge,23 and to control the degree of supramolecular organization of the nanoparticle to increase or limit enzyme access to the particle, and ultimately influence the observed reaction and response kinetics.24

A further design aspect is the morphology of the particle pre- and post-cleavage; there is increasing evidence that enzymatically triggered formation of nanofibers on tumor cells can cause cytotoxic effects,14,25 and Xu et al. have extensively studied this mechanism to overcome drug-resistance in cancer cells,26,27 while disintegrating particles may be beneficial for controlled drug release.19,20 The anti-cancer activity of the nanofibers depends on the kinetics of fiber formation which in turn depend on the interaction between the enzyme and the precursor, as well as the self-assembling ability of the post-enzymatic product.28 By employing these strategies, the response behavior of the enzyme-responsive nanomaterials can be optimized to achieve selective and controlled rates of drug release, and to introduce additional therapeutic functionalities.

Thus, by using rational design of peptide sequences, we present here a modular platform to customize surface charge, supramolecular organization, and enzyme specificity of peptide nanostructures. We demonstrate the significance of these properties in showing that simple, few amino acid replacements can systematically control enzyme engagement and susceptibility to the enzymatic action. This further dictates the response kinetics and the action of the nanostructures (degradation or β-sheet formation) to ultimately influence cells’ fate.
3.2. Rational design of peptide sequences

For this study, we designed self-assembling peptide amphiphiles\textsuperscript{29} that form stable nanostructures under physiological conditions, and undergo morphological change or degradation upon MMP-9 hydrolysis of the peptides. This study is based on our previous work which demonstrated that peptide micelles can encapsulate doxorubicin and transform into fibrous drug depots upon MMP-9 action.\textsuperscript{15,30} Thus, we created a modular system in which the peptide sequences contain three segments: (1) cationic or anionic hydrophilic segment to modulate enzyme engagement, (2) MMP-9 cleavable segment with ordered or disordered regions to influence enzyme kinetics and predetermine self-assembly or dis-assembly of post-cleavage product and, and (3) hydrophobic segment to drive self-assembly of precursor and of post-enzymatic self-assembling product (with potential to bind hydrophobic drugs)\textsuperscript{15} (Figure 1).

To demonstrate the ability to electrostatically recruit MMP-9 (pI = 5.7, net negative charge in physiological pH)\textsuperscript{31} to the peptide particles, we designed a hydrophilic segment in the peptide amphiphiles with cationic or anionic C termini. Each peptide was given a positive (1-3 AK/PK) or negative (1-3 AD/PD) charge using two lysine or two aspartic acid residues, thus creating 12 sequences (Table 1). In addition, these surface charges increase solubility of the peptide nanostructures and can be customized to potentially influence cellular uptake and bio-distribution.

To achieve MMP-9 specificity, and to program the distinct morphologies of the peptide nanostructures and the resulting enzymatic products, we designed the cleavable segment based on data from the MEROPS database,\textsuperscript{32} which suggests PX\textsubscript{1}G↓LX\textsubscript{2}G, where ↓ represents the scissile bond, and X\textsubscript{1} and X\textsubscript{2} represent positions where there are no significant preferences for a single amino acid. The amino acid positions are designated starting from the scissile bond (red dash line in Table 1) and are labeled P\textsubscript{1} through P\textsubscript{6} towards the N terminal, and P\textsubscript{1}’ and P\textsubscript{5}’ towards the C
terminal. Peptides 1-3 PK/PD contain Pro in $P_3$ which is prevalent in substrates of most MMPs, however, Pro is known to disrupt assembly of secondary structures like $\alpha$-helices and $\beta$-sheets in proteins. Therefore, in order to promote fiber formation of the post-cleavage enzymatic products, we substituted Pro for Ala in $P_3$ in 1-3 AK/AD which is also found in natural substrates of MMP-9. In addition, we varied $P_2$ with small aliphatic residues to observe differences in enzyme specificity, and inserted Gly in $P_4$ to change the self-assembling behavior of peptides and observe the consequent changes in enzyme kinetics.

Table 3.1. Single letter code of 12 peptides before MMP-hydrolysis where the red dash line indicates expected cleavage site, and the post-enzymatic products ($P_5$-$P_1$ or $P_6$-$P_1$).
Figure 3.1. Cartoon of sequence dependent peptide nanostructures. (A) Chemical structure and (B) cartoon of self-assembling peptide amphiphiles that respond to MMP-9 action. Positive (blue) or negative (red) charges on the nanoparticle electrostatically recruit or repel MMP-9 to influence enzyme kinetics. Self-assembling (purple) and MMP-9 cleavable segments (gray and/or orange) dictate the susceptibility of the nanostructures to MMP-9 hydrolysis by forming ordered/disordered structures, and control the fiber formation or disassembly of the post-enzymatic products. Red dash line indicates the scissile bond.

Lastly, to introduce the hydrophobic segment of our amphiphilic peptides, we used the well-known self-assembling sequence diphenylalanine\textsuperscript{35,36} on the first two positions of the N termini to drive self-assembly of nanostructures prior to enzyme action via hydrophobic and aromatic interactions, and to form nanofibers in a subset of sequences after enzyme action (1-3 AK/AD). These 12 sequences represent a modular design in which the rate and morphology of the enzymatic response can be customized for desired applications.
3.3. Characterization of the pre-enzymatic peptide nanostructures

The 12 pre-enzymatic (1-3 AK/AD and 1-3 PK/PD) and 6 post-enzymatic (1-3 A and 1-3 P) peptide sequences were synthesized using Fmoc-based solid phase peptide synthesis (SPPS), purified on a preparatory high-performance liquid chromatography (HPLC) using C18 column, lyophilized in water, and identified by high resolution liquid chromatography-mass spectrometry (HRMS) and 1H nuclear magnetic resonance (NMR) spectroscopy. The self-assembly of peptide nanostructures was investigated using CAC, FTIR, and ζ-potential, and finally the morphology of the nanostructures was imaged using AFM and TEM.

The CAC values for the 12 precursor peptides were determined using pyrene as a solvachromatic fluorophore in PBS (pH 7.4) and are listed in Table 2. In general, the cationic peptides had the lowest CAC of 0.3 mM and 0.4 mM for 1-2 PK and 1-2 AK, respectively, except for 3 PK and 3 AK which aggregated at 0.7 mM and 0.8 mM, respectively. While the CAC of PK peptides were lower than its analogous AK peptides, no clear trend was observed for the anionic peptides. On the contrary to the cationic peptides, which had the highest CAC for 3 AK and 3 PK, the counter peptides 3 AD and 3 PD had the lowest CAC of 0.5 mM among the anionic peptides. According to clogP values predicted using Chemdraw, the cationic peptides are more hydrophobic (higher clogP) than the anionic peptides and have overall lower CAC than anionic peptides. In addition, the cationic peptides show a positive correlation of larger CAC values for lower cLogP values (more hydrophilic peptides) (Figure 2). However, for the anionic peptides larger CAC values were observed for smaller cLogP values (more hydrophobic peptides) with exception of 1 PD.
Table 3.2. Critical aggregation concentrations of peptides determined using pyrene (Figure S25-26) and log of partition coefficient (c_{octanol}/c_{water}) as predicted by Chemdraw.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CAC (mM)*</th>
<th>cLogP**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1AK</td>
<td>0.4</td>
<td>-1.5256</td>
</tr>
<tr>
<td>1AD</td>
<td>0.6</td>
<td>-7.4872</td>
</tr>
<tr>
<td>1PK</td>
<td>0.3</td>
<td>-0.865</td>
</tr>
<tr>
<td>1PD</td>
<td>0.5</td>
<td>-6.82659</td>
</tr>
<tr>
<td>2AK</td>
<td>0.4</td>
<td>-1.8346</td>
</tr>
<tr>
<td>2AD</td>
<td>0.6</td>
<td>-7.7962</td>
</tr>
<tr>
<td>2PK</td>
<td>0.3</td>
<td>-1.174</td>
</tr>
<tr>
<td>2PD</td>
<td>0.7</td>
<td>-7.1436</td>
</tr>
<tr>
<td>3AK</td>
<td>0.6</td>
<td>-3.2916</td>
</tr>
<tr>
<td>3AD</td>
<td>0.5</td>
<td>-9.2532</td>
</tr>
<tr>
<td>3PK</td>
<td>0.5</td>
<td>-2.631</td>
</tr>
<tr>
<td>3PD</td>
<td>0.5</td>
<td>-8.5926</td>
</tr>
</tbody>
</table>

*CAC: critical aggregation concentration

**cLogP: log of partition coefficient (c_{octanol}/c_{water}) as predicted by Chemdraw

Figure 3.2. CAC vs. clogP to compare the trends in hydrophobicity and aggregation propensity.
The ζ-potentials of the peptide assemblies confirmed the presence of the expected positive or negative charges and the average ζ-potential values for the 12 precursor peptide nanostructures are listed in Table 3. Higher zeta potentials were observed for the anionic peptides which have negatively charged Asp residues on the free carboxyl termini of the peptides, in comparison to the cationic peptides in which the positive charge of Lys residues is negated by the free C termini. We note that measurements were made at high concentrations (5 mM) due to the limited light scattering properties of the peptide particles that may have contributed to aggregation which results in overall lower zeta potential values.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge pH 7.4</th>
<th>Mean (mV) ± Std. dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1AK</td>
<td>+2</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>1AD</td>
<td>-2</td>
<td>-34.9 ± 1.1</td>
</tr>
<tr>
<td>1PK</td>
<td>+2</td>
<td>15.4 ± 0.3</td>
</tr>
<tr>
<td>1PD</td>
<td>-2</td>
<td>-38.0 ± 1.7</td>
</tr>
<tr>
<td>2AK</td>
<td>+2</td>
<td>8.2 ± 2.8</td>
</tr>
<tr>
<td>2AD</td>
<td>-2</td>
<td>-30.3 ± 1.4</td>
</tr>
<tr>
<td>2PK</td>
<td>+2</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>2PD</td>
<td>-2</td>
<td>-41.2 ± 2.3</td>
</tr>
<tr>
<td>3AK</td>
<td>+2</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>3AD</td>
<td>-2</td>
<td>-7.5 ± 1.8</td>
</tr>
<tr>
<td>3PK</td>
<td>+2</td>
<td>1.1 ± 1.5</td>
</tr>
<tr>
<td>3PD</td>
<td>-2</td>
<td>-28.2 ± 0.7</td>
</tr>
</tbody>
</table>

Analysis of Fourier-transform infrared spectroscopy (FTIR) spectra reveal that the self-assembly of most of the peptide sequences are not majorly driven by highly ordered hydrogen bonding of the peptide backbones. Instead, the major peaks in the amide I region absorb between 1640-1650 cm⁻¹, indicative of disordered hydrogen bonds (shaded green in Figure 3C-D).³⁷ Thus, the major contribution in the self-assembly of 1-3 PK/PD and 2-3 AK/AD is driven by the formation of diphenylalanine hydrophobic core solubilized by the hydrophilic lysine or aspartic acid tail.³⁸
Figure 3.3. AFM images and FTIR spectra of self-assembled peptide nanostructures. A-B) 1 AK/AD form large fibers organized in anti-parallel β-sheet arrangement, 2-3 AK/AD and 1 PK/PD form elongated worm-like micelles, and 2-3 PK/PD form spherical micelles. Scale bar 500 nm. Additional AFM images can be found in Figure S33-34. C-D) FTIR spectra of the peptides in amide I region. Red shade highlights the carboxylate groups (1580-1590 cm$^{-1}$) which can red shift to lower wavenumber (1541 cm$^{-1}$ for 1 AD) upon cation complexation, blue shade highlights parallel β-sheet (near 1620 cm$^{-1}$) and anti-parallel β-sheet (additional peak at 1688 cm$^{-1}$) hydrogen bonding of the peptide backbones, and the green shade highlights disordered hydrogen bonds (1640-1650 cm$^{-1}$). Peaks are listed in Table S1.
Atomic force microscopy (AFM) images show that AK/AD sequences form one-dimensional nanostructures and PK/PD sequences form spherical nanostructures with the exception of 1 PK/PD (Figure 3A-B). Analysis of Fourier-transform infrared spectroscopy (FTIR) spectra reveals that the self-assembly of most of the peptide sequences are not majorly driven by highly ordered hydrogen bonding of the peptide backbones. Instead, the major peaks in the amide I region absorb between 1640-1650 cm⁻¹, indicative of disordered hydrogen bonds (shaded green in Figure 3C-D).³⁷ Thus, the major contribution in the formation of these spherical micelles (2-3 PK/PD) or worm-like micelles (1 PK/PD and 2-3 AK/AD) are driven by the formation of diphenylalanine hydrophobic core solubilized by the hydrophilic lysine or aspartic acid tail.³⁸ In contrast, 1 AK/AD show prominent characteristics of anti-parallel β-sheet arrangement of the peptide backbone which absorbs at 1620 and 1687 cm⁻¹ for 1 AK and at 1623 and 1696 cm⁻¹ for 1 AD (shaded blue in Figure 3C-D). In addition, the carboxylate peak, which absorbs in 1580-1590 cm⁻¹ (shaded red in Figure 3C-D), is red shifted to 1541 cm⁻¹ in 1 AD. This is indicative of cation complexation which suggests intermolecular salt bridge formation between the C and N terminal (or aspartic acid residues next to the C terminal) that contributes to the long-range order of the peptide assembly.³⁹ We speculate that these highly ordered nanofibers are caused by the assembly of linear and rigid backbone of 1 AK/AD in P₀-P₁ (FFALG), and this is clearly evident in 1 PK/PD in which a single amino acid substitution of Ala to Pro in P₃ (FFPLG) creates a kink in the peptide backbone and significantly disrupts the anti-parallel β-sheet formation. Furthermore, the addition of Gly in P₄ (FFGAX₁G) in 2-3 AK/AD adds flexibility to the peptide backbone⁴⁰ which hinders long range order of the peptide assembly and thus forms smaller nanofibers.
3.4. Controlling enzyme kinetics

Next, we measured the rate of MMP-9 cleavage of the 12 peptides. Lyophilized peptides were dissolved in phosphate-buffered saline (PBS) supplemented with 1 mM CaCl₂ and 55 μM ZnCl₂ to be compatible with MMP-9 (a metalloproteinase with zinc and calcium dependent catalytic domain), the pH adjusted to 7.4, and sonicated for 10 mins to achieve 1 mM of peptide solutions. 100 ng/mL MMP-9 was incubated with peptides at 37°C and the reaction was monitored up to 96 h using LC-MS to identify and quantify the enzymatic products by calculating the area of the product peak over the initial peptide peak (Figure S35-46).

3.4.1. Electrostatic recruitment or repulsion of MMP-9

Comparing the P₁↓P₁’ enzymatic products, the cationic peptides (Figure 4A) were preferentially cleaved by (anionic) MMP-9. For example, 2 PK had a 12-fold higher conversion in comparison to 2 PD (Figure 4B). This stark difference resulting from preferential hydrolysis of nanostructures by oppositely charged enzymes has also been observed by Wooley et al.²⁴ using polymeric micelles.

Interestingly, the major cleavage site for the cationic peptides was between G↓L in P₁↓P₁’ as anticipated, whereas the major cleavage site for the anionic peptides was between F↓F in P₆↓P₅ with inconsistent results between two separate trials (Table S2). It has been reported that MMP-9 cleavage site in peptides of similar length can shift to P₂↓P₁ or P₁↓P₂,⁴¹ but this drastic shift to P₆↓P₅ suggests that the anionic peptides do not meet MMP-9 specificity. By electrostatic (mis) matching, we are able to recruit or repel MMP-9 to trigger the desired response in the nanomaterial.
Figure 3.4. 1 mM of (A) 1-3 AK/PK and (B) 1-3 AD/PD incubated with 100 ng/mL of MMP-9 at 37°C. Peptides with Pro in P₃ are marked grey, and Ala with orange. Average % conversion of peptides to the post-enzymatic products (P₁→P₁’) from two separate trials. 1-3 PK showed the highest conversion and further degradation of the post-enzymatic product was observed for 2 PK (blue dash line). Over 50% of 2 AK was cleaved, while less than 10% of 3 AK and 1-2 PD was converted, and the rest were not responsive to MMP-9.

3.4.2. Increasing or limiting accessibility to enzymatic hydrolysis

In addition to the dramatically different conversion rates (and cleavage sites) resulting from electrostatic interactions, we can further fine-tune the rate of enzymatic hydrolysis by achieving enzyme specificity and by controlling the degree of order in the supramolecular peptide nanostructures. Of the cationic peptides, sequences with Pro in P₃ (1-3 PK) were almost completely digested by MMP-9 within 96 h. In particular, 1 PK, which forms the smallest micelles of the three, was completely converted to the enzymatic product, FFPLG, in 48 h, and continued to be further hydrolyzed between F↓F (dash blue line in Figure 4A). This demonstrates the biodegradability of peptide nanoparticles, which may be useful for applications where (di)phenylalanine generated amyloid-like toxic fibrils are of concern. Overall, 1-3 PK were cleaved
to completion at a similar rate due to the preferred Pro residue in P₃, and the subtle differences in the peptide sequences did not affect the enzymatic hydrolysis rates.

In the case of 1-3 AK, significant differences were observed due to the degree of supramolecular organization of the nanostructures. 3 AK, which has the highest CAC value of 0.8 mM, had a low conversion (below 10% by 96 h), whereas, 2 AK with a lower CAC value of 0.4 mM had the highest conversion (up to 60% by 96 h), and no cleavage was observed for 1 AK (CAC value 0.4 mM). These data suggest that the kinetics of the enzyme hydrolysis are determined by the supramolecular order of the peptide assemblies rather than the hydrolysis of unassembled peptide monomers. The slight difference of introducing a glycine residue in the sequences of 2 AK and 1 AK (between diphenylalanine and alanine) contributes to a major change in the morphology of the two nanostructures. 2 AK, which has a more flexible peptide backbone due to the insertion of Gly residue in P₄, forms disordered hydrogen bonds (Figure 3C) that leads to the formation of smaller fibrils (Figure 3A and Figure S33) and is susceptible to MMP-9 hydrolysis. However, without the Gly residue, the rigid peptide backbone of 1 AK in P₆-P₁ arranges in an anti-parallel β-sheet configuration (Figure 3C) and form fibers that are microns in length (Figure 3A and Figure S33). These highly organized fibers are less susceptible to enzyme degradation, especially for the endopeptidase MMP-9, which has been observed to bind to type I fibril collagen, a large extracellular matrix component, but cannot digest it until it is in the denatured, gelatin form. Concluding from this set of 12 peptides, it is clear that we can dramatically control the rate and specificity of MMP-9 hydrolysis through systematic exchange of amino acids.
3.5. Characterization of post-enzymatic peptide nanostructures

The hydrophobic N terminal fragments of the resulting enzymatic products (herein referred as 1-3 A/P) by SPPS and characterized using HRMS, \(^1\)H NMR, AFM, and FTIR. Controlling the morphology of the post-enzymatic products is important in designing MMP-responsive nanomaterials. For instance, enzyme-triggered fibers which form on or near the cell surface of cancer cells that produce the enzyme stimuli are known to be cytotoxic to the cells,\(^{44}\) and disassembling nanocarriers can be used for burst release of payloads. Distinct differences can be observed by peptides with Ala in P\(_3\) (1-3 A) which formed ordered structures, and peptides with Pro in P\(_3\) (1-3 P) which was expected not to self-assemble due to the Pro disrupting formation of ordered hydrogen bonds. FTIR spectra of the non-assembling peptides (Figure 5B gray lines) show absorptions at 1641, 1643 and 1653 cm\(^{-1}\) for 1 P, 2 P and 3 P, respectively, which is observed for amides of peptide backbones with disordered hydrogen bonds. A mixture of parallel \(\beta\)-sheet (1628 cm\(^{-1}\)) and disordered (1643 cm\(^{-1}\)) hydrogen bonds were observed for 2 A. In contrast, 1 A absorbs at 1627 and 1687 cm\(^{-1}\), and 3 A absorbs at 1624 and 1688 cm\(^{-1}\), which are distinct characteristic peaks of anti-parallel \(\beta\)-sheet hydrogen bonds (Figure 5B orange lines). AFM images (Figure 5A) show that 1-3 P form random aggregates. In comparison, 1-3 A form fibers.
Figure 3.5. AFM and FTIR of synthesized post-enzymatic products. (A) AFM image of 1-3 A (top panel, left to right) and 1-3 P (bottom panel, left to right). Scale bar 500 nm. (B) FTIR spectra of 1-3 A in orange and 1-3 P in gray lines.

3.5.1. Enzymatically formed fibers have higher order assembly

The morphology of the enzymatic products of 2 AK and 2 PK incubated with MMP-9 was monitored over time by FTIR and AFM. In addition to identifying and quantifying the post-enzymatic products of peptides after incubation with MMP-9 (Figure 4), the morphology of the reaction product was also monitored over time for 2 AK and 2 PK by FTIR and AFM. Figure 6B shows that the disordered region of 2 AK (black line) disappears by 24 h and a sharp peak is observed in the 1620 cm\(^{-1}\) region indicative of ordered β-sheet hydrogen bonds (purple line). By 48 h, an additional peak at 1680 cm\(^{-1}\) region can be observed which indicates the peptides are arranged in an anti-parallel β-sheet manner (pink line). In comparison, the synthesized 2 A, although identical in chemical composition (FFGALG), has a completely different absorbance and less order than the MMP-9 induced assembly. The AFM image of 2 AK after 24 h incubation with MMP-9 shows that enzymatically formed 2 A also forms fibers (Figure 5A). On the other hand,
the FTIR of **2 PK** does not change after incubation with MMP-9 or in comparison to the synthesized **2 P** (Figure 6D). However, the AFM images of **2 PK** prior to incubation with MMP-9 show formation of uniformly spherical micelles about 50 nm in diameter (Figure 3A). The synthesized **2 P** and the enzymatically formed **2 P** both show small random aggregates, which confirms the lack of ordered hydrogen bond absorbance by FTIR.

![AFM images of 2 AK and 2 PK](image)

**Figure 3.6.** AFM images of **2 AK** at 24hr (A) and **2 PK** at 24hr (B), scale bar 500nm. (C) FTIR absorbance of **2 AK**: 0 h, 24 h, 48h (0%, 24%, and 24% enzymatic products, respectively) and **2 A** for comparison. (D) FTIR absorbance of **2 PK**: 0 h, 24 h, 48h (0%, 86%, and 91% enzymatic products, respectively) and **2 P** for comparison.

### 3.6. Biocompatibility of peptide nanostructures *in vitro*

Moving forward, we chose to test the biocompatibility of the two peptides that were active against MMP-9, **2 AK** and **3 AK**, and their Pro analogous peptides **2 PK** and **3 PK**. We chose a human clear cell renal cell carcinoma line, Caki-1, because tissues from patients with this type of kidney cancer show significantly higher expression of MMP-9 mRNA, with increasingly higher expression in advanced tumor stages. As a control, we chose a non-cancerous human lung
fibroblast cell line, IMR90, which was reported to have negligible expression of MMP-9 mRNA.\(^{46}\) 1 mM of the peptides were incubated with Caki-1 and IMR90 cells at 37°C for 72hrs and the cell viability was determined using the Presto Blue assay (Figure 3.7. A-B). The peptides are non-toxic to both cell lines with cell viabilities over 90%, except for **3 AK** which forms β-sheet fiber post-cleavage and decreased the cell viability of Caki-1 cells to 66%, despite the observed low conversion (Figure 4A).

**Figure 3.7.** Biocompatibility of peptide nanostructures. Cell viability of human renal cancer cell line, Caki-1, with high expression of MMP-9 (A), and non-cancerous human fibroblast, IMR90, with normal expression of the enzyme (B), incubated with 1 mM peptides for 72 hrs. The peptides are non-toxic to both cell lines, except for **3 AK** which decreased the cell viability of Caki-1 to 66%. (C) FTIR spectra of the post-cleavage peptides show that only **3 A** forms anti-parallel β-sheet peptide fibers which causes the cytotoxic effects in Caki-1 only. (D) TEM images of **3 AK** and **2 PK** show formation of worm-like and spherical micelles, respectively, and the post-
enzymatic products, $3\ A$ and $2\ P$ which form rigid fibers or random aggregates, respectively. Scale bar 100nm.

### 3.6.1. Nanofibers induce selective toxicity in cancer cells

In order to understand the effects of the post-cleavage products \textit{in vitro}, we examined the hydrophobic N terminal fragments of the resulting enzymatic products characterized in section 4.5. In comparison to $2\ P$ and $3\ P$, which form random aggregates, $2\ A$ formed short fibers and $3\ A$ formed large fiber bundles (Figure 5A). TEM images (Figure 7D) show that the precursor $2\ PK$ forms spherical particles which, when converted to $2\ P$, disassemble and remain as random aggregates. In comparison, precursor $3\ AK$ forms flexible fibers and the post-enzymatic product, $3\ A$, forms ordered rigid $\beta$-sheet fibers. Enzyme-triggered fibers which form on or near the cell surface of cancer cells that produce the enzyme stimuli are known to be cytotoxic to the cells.\textsuperscript{44} Likewise, the observed decrease in viability of Caki-1 cells treated with $3\ AK$ is most likely due to the formation of toxic anti-parallel $\beta$-sheet nanofibers ($3\ A$) that are selectively triggered to form by the overexpressed MMP-9 in the diseased cells.\textsuperscript{44} This effect is not observed in the control cell line, IMR90, where overexpression of MMP-9 is not expected.

### 3.7. Conclusion

In conclusion, we have created a modular system of self-assembling peptide nanostructures to customize surface charge and supramolecular order to control enzyme kinetics and response action. These peptide nanostructures are bio-degradable and non-toxic, and after enzyme action, the non-assembling products remain non-toxic while the $\beta$-sheet nanofiber forming products can selectively kill cancer cells. The kinetics of nanoparticles is important in biomedical applications in which the rate of enzyme responsiveness should be regulated (\textit{i.e.} predetermined drug release.
profile, degradation rate of hydrogels, etc.). In addition, the morphology of the nanoparticles should be logically designed to achieve optimal function of the precursors (i.e. manipulation of cellular uptake, bio-distribution, etc.) and of the post-enzymatic product (i.e. biodegradability or therapeutic nanofiber/drug depots, etc.).

3.8. Acknowledgements

I would like to thank the individuals who contributed to the work in this chapter: Dr. Daniela Kalafatovic for help in peptide design and synthesis, Dr. Barney Yoo for help in LC-MS, Dr. Mohit Kumar for help with TEM images, Mike A. Cornejo for performing the biological experiments, Dr. María Contel for supervision and Dr. Rein V. Ulijn for conceptualizing and supervising the project as well as help in writing the chapter.

3.9. Methods

Solid Phase Peptide Synthesis (SPPS). Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and pre-loaded Wang resins were purchased from Bachem. Peptides were synthesized on CEM Liberty Blue microwave assisted solid phase peptide synthesizer using ~1:5 resin to amino acid ratio and excess of diisopropylcarbodiimide (DIC), Oxyma (Ethyl(hydroxyimino)cyanoacetate), and 20% piperidine in dimethylformamide (DMF). The complete peptide-loaded resins were washed three times in dichloromethane, followed by three washes in diethyl ether on a filtration column. The peptides were cleaved from the resins and side chain protecting groups were removed by reacting with TFA cocktail (95% trifluoroacetic acid, 2.5% triisopropyl silane, and 2.5% water) for 2 h. The cleaved peptides were recovered by
removing TFA cocktail, followed by precipitation in cold diethyl ether. Peptides were washed three times in cold diethyl ether, using centrifugation to collect the precipitated crude peptide.

**Preparatory High-Performance Liquid Chromatography (HPLC).** Crude peptides were dissolved in 50% acetonitrile in water containing 0.1% TFA and purified through a preparatory C\textsubscript{18} column on the Thermo Scientific Dionex Ultimate 3000. Acetonitrile was removed from the collected fragments on a rotary evaporator before lyophilization in MQ water or 10 mM HCl to remove residual TFA salts.

**TFA removal.** Removing residual TFA salts is crucial for investigation of self-assembled peptide nanostructures. Chapter 4 will go over the issues that arise when residual TFA salts are ignored and a detailed procedure on how to remove them will be provided.

**High Resolution Mass Spectrometry (HRMS).** HRMS data were obtained on an Agilent 6550 QToF, with a dual sprayer ESI source, coupled to an Agilent 1290 Infinity LC system. Samples were analyzed by FIA (flow injection analysis) using a mobile phase of 50% acetonitrile in water (0.1% formic acid) with a flow rate of 0.4 mL/min.

**\textsuperscript{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy.** Spectra were recorded on a Bruker AV400 at 400 MHz. Chemical shifts (\(\delta\)) are given in ppm using D\textsubscript{2}O as solvent.

**Critical Aggregation Concentration (CAC).** The critical aggregation concentrations of the precursor peptides were determined using pyrene as a fluorescent probe (Figure 2 and Table 2). Briefly, 1 mM of peptides were prepared in PBS and the pH was adjusted to 7.4 using 0.5 M NaOH or 0.5 M HCl prior to serial dilution in PBS with thorough vortexing. Peptide solutions were incubated at 50°C for 15 minutes, then 2 \(\mu\)L of stock pyrene solution (100 \(\mu\)M in methanol) was added to 100 \(\mu\)L of each peptide solution, gently mixed, and incubated for 5 minutes at 50°C, then finally cooled down to room temperature to co-assemble the peptides with pyrene molecules.
Pyrene emission spectra were measured from 350-450 nm ($\lambda_{ex} = 310$ nm) in a micro fluorescence cuvette (3 mm path length) on Jasco FP-8500 Spectrofluorometer (measurement parameter: 20 nm excitation and 1nm emission bandwidth, 0.2 sec response, medium sensitivity, 0.2 cm$^{-1}$ data interval, at 200 nm/min). The CAC was determined by plotting the ratio between intensities of the 3$^{rd}$ to 1$^{st}$ peak of the pyrene emission spectra (Figure S25-26). Increasing peptide concentrations were measured in 0.1 mM increments until the slope of the plot had changed, and simultaneously the 3$^{rd}$ peak shifted from 382.4 nm to 384.4 nm and the 1$^{st}$ peak shifted from 371.8 nm to 373.4 nm.

$\zeta$-potential. Measurements were made using an Anton Paar Litesizer 500 Particle Analyzer. 5 mM of peptide samples were prepared in 2% PBS and the pH was adjusted to 7.4 using dilute NaOH and HCl. 50 $\mu$L of samples were pipetted into Univette low volume cuvette and three series of measurements were taken at 25$^\circ$C using Smoluchowski approximation.

Fourier Transform Infrared Spectroscopy (FTIR). Absorbance spectra were taken from 4000 cm$^{-1}$ to 800 cm$^{-1}$ with 64 scans at 4 cm$^{-1}$ resolution on the Bruker Vertex 70 spectrometer. 20 mM peptide solutions were prepared in deuterated phosphate buffer (pH = 8), pH was adjusted to 7.4 using 0.5 M NaOH or 0.5 M HCl and sonicated for 10 min. 5 $\mu$L of sample solutions were drop casted between two CaF$_2$ cells with PTFE spacers (12 $\mu$m thickness x 13 mm diameter). For analysis, deuterated phosphate buffer absorbance spectrum was subtracted from the sample absorbance and spectra were normalized from 1560 to 1655 cm$^{-1}$.

Atomic Force Microscopy (AFM). Images were taken on Bruker Dimension FastScan using FASTSCAN-B tip on fast scan mode. 1 mM of peptide solution was prepared in phosphate buffer (pH 7.4), sonicated for 10 min and drop casted on freshly cleaved mica and allowed to dry for 48 h before imaging.
Transmission Electron Microscopy (TEM). TEM images were taken on FEI Titan Halo 80-300 microscope. 1 mM of peptide solution was prepared in 10 mM phosphate buffer (pH 7.4) sonicated for 10 min and 5 µL of the solution was drop casted on a carbon film grid (400 mesh, copper) and dried completely. To the dry grid, 5 µL of MilliQ water was drop casted and quickly blotted to wash away the phosphate salts and dried completely. Finally, 5 µL of methylamine vanadate based negative stain (NanoVan® by Nanoprobes) was drop casted, blotted away, and dried completely.

Cell lines. Human clear cell renal cell carcinoma line Caki-1 was newly obtained for these studies from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and cultured in Roswell Park Memorial Institute (RPMI-1640) (Mediatech Inc., Manassas, VA) media containing 10% Fetal Bovine Serum, certified, heat inactivated, US origin (FBS) (Gibco, Life Technologies, US), 1% Minimum Essential Media (MEM) nonessential amino acids (NEAA, Mediatech) and 1% penicillin–streptomycin (PenStrep, Mediatech). IMR90 (human fetal lung fibroblast) cells were purchased from ATCC (Manassas, Virginia, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% FBS, 1% NEAA and 1% Penicillin Streptomycin.

Cell viability assay. Human fetal lung fibroblast (IMR90) and human clear cell renal cell carcinoma cells (Caki-1) were seeded in 96-well flat bottom microplate (BioLite Microwell Plate, Fisher Scientific, Waltham, MA). For IMR90 we used 6.0 X 10^3 cell per well and for Caki-1 we used 5.6 X 10^3 cells per well in 90µL of complete phenol red free cell culture media. The cells were allowed to grow for 24 hours at 37°C and 5% CO₂ in a humidified incubator. 10mM peptide solutions were prepared in phosphate buffer saline, pH adjusted to 7.4 using NaOH or HCl and sonicated for 10 minutes. Then 10µL of each sample was added into wells containing 90µL of media (in triplicate). Following the administration of the peptides, cells were incubated for 72
hours at 37°C under 5% CO₂. After each period of incubation, Presto Blue (Life Technologies, Carlsbad, CA) was used as an indicator of cellular toxicity; 11μL of presto blue was added to each well and incubated for 1 hours at 37°C under 5% CO₂. The 96-wells plate was then analyzed using a multi-mode plate-reader BioTek Microplate Reader (BioTek U.S., Winooski, VT) at 530nm and 590nm wavelength. The percentage of surviving cells was calculated as a normalized ratio of the fluorescence intensity between cells treated with media and phosphate buffer saline alone.

3.10. References


(11) S Burrage, P.; Mix, K.; Brinckerhoff, C. Matrix Metalloproteinases: Role in Arthritis; 2006; Vol. 11.


3.11. Supplementary Information

Figure S1. HR-MS spectra of peptides 1 AK/AD.

1AK

1AD

1PK

1PD
Figure S2. HR-MS spectra of peptides 1 PK/PD.

Figure S3. HR-MS spectra of peptides 2 AK/AD.

Figure S4. HR-MS spectra of peptides 2 PK/PD.
Figure S5. HR-MS spectra of peptides 3 AK/AD.

Figure S6. HR-MS spectra of peptides 3 PK/PD.
Figure S7. HR-MS spectra of peptides 1 A/P.

Figure S8. HR-MS spectra of peptides 2 A/P.
Figure S9. HR-MS spectra of peptides 3 A/P.

Figure S10. $^1$H NMR spectra of 1 AK.
Figure S11. $^1$H NMR spectra of 1 AD.

Figure S12. $^1$H NMR spectra of 1 PK.
Figure S13. $^1$H NMR spectra of 1 PD.

Figure S14. $^1$H NMR spectra of 2 AK.
**Figure S15.** $^1$H NMR spectra of 2 AD.

**Figure S16.** $^1$H NMR spectra of 2 PK.
Figure S17. $^1$H NMR spectra of 2 PD.

Figure S18. $^1$H NMR spectra of 3 AK.
Figure S19. $^1$H NMR spectra of 3 AD.

Figure S20. $^1$H NMR spectra of 3 PK.
Figure S21. $^1$H NMR spectra of 3 PD.

Figure S23. $^1$H NMR spectra of 1 A.
Figure S24. $^1$H NMR spectra of 1 P.

Figure S23. $^1$H NMR spectra of 2 A.
Figure S24. $^1$H NMR spectra of 2 P.

Figure S23. $^1$H NMR spectra of 3 A.
Figure S24. $^1$H NMR spectra of 3 P.

Figure S25. CAC of 1-3 AK and 1-3 PK.
Figure S26. CAC of 1-3 AD and 1-3 PD.

Figure S27. Triplicate measurements of ζ-potentials of 1 AK/AD.
Figure S28. Triplicate measurements of ζ-potentials of 1 PK/PD.

Figure S29. Triplicate measurements of ζ-potentials of 2 AK/AD.
Figure S30. Triplicate measurements of ζ-potentials of 2 PK/PD.

Figure S31. Triplicate measurements of ζ-potentials of 3 AK/AD.
Figure S32. Triplicate measurements of $\zeta$-potentials of 3 PK/PD.
Figure S33. AFM images of 1-3AK/PK. Scale bar 500 nm.

Figure S34. AFM images of 1-3AD/PD. Scale bar 500 nm.
Table S1. FTIR absorbance of 1-3 AK/AD, 1-3 PK/PD, and 1-3 A/P in the amide I region.

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Table S2. The enzymatic products were identified and quantified for two separate trials. Column ‘# K/D’ lists the MW of the 12 peptides and column ‘P6-P1↓’ lists the MW of the N termini portion of the enzymatic product (and observed major products for the anionic peptides).

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Figure S35. LCMS spectra of 1 AK in A) first and B) second separate trials.

Figure S36. LCMS spectra of 1 AD in A) first and B) second separate trials.

Figure S37. LCMS spectra of 1 PK in A) first and B) second separate trials.
Figure S38. LCMS spectra of 1 PD in A) first and B) second separate trials.

Figure S39. LCMS spectra of 2 AK in A) first and B) second separate trials.

Figure S40. LCMS spectra of 2 AD in A) first and B) second separate trials.
Figure S41. LCMS spectra of 2 PK in A) first and B) second separate trials.

Figure S42. LCMS spectra of 2 PD in A) first and B) second separate trials.

Figure S43. LCMS spectra of 3 AK in A) first and B) second separate trials.
Figure S44. LCMS spectra of 3 AD in A) first and B) second separate trials.

Figure S45. LCMS spectra of 3 PK in A) first and B) second separate trials.

Figure S46. LCMS spectra of 3 PD in A) first and B) second separate trials.
Chapter 4
Residual Trifluoroacetate Salt Adducts Substantially Alters Self-Assembly of Peptide Nanostructures

Summary

Trifluoroacetic acid (TFA), as the reagent of post synthesis cleavage of peptides from solid phase, is commonly present in peptide products and is often ignored as an inert impurity. We demonstrate that TFA forms stable adducts with amine groups of the peptides in water. These adducts remain in place during aqueous solution and they can inhibit or otherwise influence the self-assembly of peptide nanostructures by disrupting the intermolecular ionic and hydrogen bonds. Using a peptide sequence which self-assembles to form nanostructures that are responsive to MMP-9, we characterized and compared the self-assembly behavior of the peptide with different concentrations of trifluoroacetate (TFAc) salts using $^{19}$F NMR, FTIR, AFM, TEM, critical aggregation concentration assay, and $\zeta$-potential. We show that TFAc dramatically alters the morphology and properties of peptide nanostructures, which can lead to inconsistent characterization and biological study results between batches of peptides containing different concentrations TFA.
4.1. Introduction

Self-assembling peptides are versatile in their chemical composition and provide an excellent platform to design stable nanostructures such as micelles used in drug delivery or fibers that form hydrogels for 3D cell culture.¹ Using commercially available amino acids, any desired peptide sequence can be readily synthesized due to the well-established solid phase peptide synthesis (SPPS) procedure developed by Merrifield, who won the Nobel Prize in Chemistry in 1984.² Most common methods of SPPS use trifluoroacetic acid (TFA) in the final step to remove the peptide product from the solid resin and to cleave acid labile bonds such as the protecting groups on the side chains of the amino acids (e.g., lysine-Boc).³ However, the disadvantage of using TFA is that it is difficult to remove due to the stability of the adducts formed. As a result, residual trifluoroacetate (TFAc) often remains in the final peptide products, even in commercial preparations that are sold as TFA free. In academic studies on peptide self-assembly, traces of TFA are often ignored or they can lead to misinterpretation as part of the peptide characterization.

The impact of TFA is especially profound when the peptides are used to create nanostructures by supramolecular self-assembly. It can change the molecular properties of the peptide mainly by forming adducts with amine groups of the peptides that play key roles in formation of hydrogen bonds or electrostatic interactions critical for self-assembly. Directly or indirectly, the blocking of amine groups may impact the driving forces of peptide self-assembly including electrostatic, hydrogen bonding, and cation-pi interactions. Specifically, the TFAc ion can block positively charged functional groups from interacting with negatively charged residues, as well as preventing formation of salt bridges between C and N termini that stabilize self-assembled peptides.
Previously, our group has characterized Fmoc-FF peptide fibers co-assembled with Fmoc-RGD that form hydrogels and can be used as 3D-scaffolds for anchorage-dependent cells. Fmoc-FF self-assembles into β-sheets and showed a broad negative peak at 218 nm in CD and strong absorbance at 1630 and 1690 cm\(^{-1}\) in FTIR spectra. Fmoc-RGD showed weak signals in both analysis and a broad peak at 1655 cm\(^{-1}\) in FTIR, indicative of random coil arrangements. Thus, by incorporating less or more % of Fmoc-RGD to Fmoc-FF fibers, the stiffness of the hydrogel was controlled. Shortly after, Hamley et al. reported contradicting findings in which Fmoc-RGD could self-assemble into β-sheets and form stable hydrogels by itself. However, as acknowledged by the authors, this Fmoc-RGD contained high amounts of TFA, as clearly shown by the large 1673 cm\(^{-1}\) absorbance peak detected in FTIR. In contrast, the Fmoc-RGD used in our study did not absorb at 1672 cm\(^{-1}\) in FTIR analysis. The authors compared the similar hydrogel dynamic elastic shear moduli of their Fmoc-RGD hydrogel to their previously studied Fmoc-KLV(Boc) hydrogel, emphasizing that this Lys residue is capped by a Boc protecting group, whereas Fmoc-RGD contains uncapped Arg residue. In this case, the authors failed to realize that the high concentrations of TFA acted like a protecting group on the Arg residue and formed hydrogels similar to Fmoc-KLV(Boc), which resulted in completely different morphology from the previously reported TFA free Fmoc-RGD.

Here we use a model peptide sequence with 3 amine groups, FFGALGLKGK, which was previously described in Chapter 4 to form matrix metalloproteinase-9 (MMP-9) responsive nanocarrier for biomedical applications. Full description of peptide FFGALGLKGK can be found in Chapter 4 as peptide 2 AK. We report that residual TFAc salts in the peptide product significantly changes the self-assembly behavior of peptides using \(^{19}\)F NMR, FTIR, AFM, TEM, and show how this implication can contribute to false ζ-potential, critical aggregation
concentration, and morphology. Lastly, we demonstrate how the changes in the nanostructure properties can also affect biological studies, such as a delay in enzyme hydrolysis rates, and ultimately cause irreproducibility in experiments.

4.2. Method development for washing TFA

A self-assembling peptide sequence, FFGALGLKGK, that was previously characterized (Chapter 4) was used to demonstrate the significance of TFAc salt presence in self-assembly. During our investigation, a surprising amount of residual TFA was found in batches of peptide products. Peptides were synthesized using standard Fmoc-based SPPS, purified by prepHPLC on a C18 column using increasing gradient of acetonitrile with 0.1% TFA or 0.1% acetic acid against water with 0.1% TFA or 0.1% acetic acid, respectively. Purified peptides were lyophilized in water 1-3 times before being characterized by LC-MS (Figure S3 in Chapter 4), $^1$H NMR (Figure S14 in Chapter 4), $^{19}$F NMR, and FTIR. Lyophilized peptides were dissolved in D$_2$O and TFA concentration was quantified using $^{19}$F NMR.

![Diagram of proposed trifluoroacetate salt adducts](image)

**Figure 4.1.** (A) Chemical structure of the proposed trifluoroacetate salt adducts formed with the amine groups of peptide FFGALGLKGK. (B) $^{19}$F NMR spectra of 1mM peptide in D$_2$O, and 1mM TFA solution in D$_2$O used to integrate the peak intensities.
For peptides purified on the prepHPLC with 0.1% TFA containing solvents, 3.04 mM of TFA was found per 1 mM of peptide, suggesting that TFA forms molar equivalent TFAc salts with the 3 amine groups of the peptide (Figure 1). For peptides that were purified using 0.1% acetic acid containing solvents, 0.39 mM of TFA was found per 1 mM of peptide, indicating that acetic acid can displace some of the TFAc salts. However, acetate salts could also alter self-assembly of peptides and quantification of acetate salt adducts formed with the peptide would require $^1$H NMR which is more challenging to analyze than $^{19}$F NMR. Therefore, we continued this investigation with peptides that were purified on prepHPLC with 0.1% TFA containing solvents. The 1:1 molar ratio of amine to TFA was also observed in peptide GPKGLRGD that contains 3 amine groups, and 2.91 mM TFA per 1 mM peptide was observed (data not shown).

4.2.1. Washing 20 mM peptide in 10 mM HCl

The TFAc were removed based on a modified protocol using HCl and verified by $^{19}$F NMR and FTIR. 6 10 mM HCl solution was chosen to assure peptide stability and personal safety while handling large volumes of acidic solution. 6 Briefly, lyophilized peptide was dissolved in aqueous 10 mM HCl solution to prepare a 20 mM peptide solution (about 20 mg/mL). The solution was vortexed thoroughly at room temperature to allow protonation of the TFAc salts followed by removal of solvent by vaporization or lyophilization to complete the first round of TFA removal or “wash”. The procedure was repeated 1-4 times and peptides were subsequently dissolved in D$_2$O for $^{19}$F NMR and FTIR analysis. The unknown TFA concentration was quantified using $^{19}$F NMR by integrating the area under the peak measured against 1 mM of TFA in D$_2$O (Figure 2A). After 4 rounds of washes, 113% molar equivalent TFA still remained which was also observed in FTIR by the absorbance of TFA carbonyl peak at 1672 cm$^{-1}$ (Figure 2B). These results indicated
that TFAc-amine salt adducts form strong ionic bonds which could not be displaced by water or dilute HCl.

Figure 4.2. 20 mM peptide washed 0-4 times with 10 mM HCl solution. One round of wash indicates dissolving peptides in 10 mM HCl aqueous solution followed by removal of the solvent. (A) $^{19}$F NMR spectra of 1 mM peptide dissolved in D$_2$O show % of TFA per peptide molar ratio. (B) Normalized FTIR spectra of 20 mM peptide dissolved in deuterated buffer (pH 7.4) show the absorbance of carbonyl peak of TFA at 1672 cm$^{-1}$.

4.2.2. Washing 1 mM peptide in 10 mM HCl

In order to displace the TFAc with chloride ion, a large excess of HCl to peptide-TFA ratio was required. Therefore, the peptide concentration was decreased to 1 mM (about 1 mg/mL) instead of increasing the HCl concentration to avoid destabilization of the peptide and corrosion of lab equipment. The $^{19}$F NMR spectra show significant decrease in TFA concentration after one wash, from over 300% molar ratio to 9.3%, and after 3 rounds of washes only 1.6% TFA remained (Figure 3A). On the contrary, the TFA carbonyl peak at 1672 cm$^{-1}$ was undetectable on FTIR after the 1$^{st}$ wash (Figure 3B). The sensitivity range of each technique should be considered, with 22.6
mM TFA being the lower limit for FTIR (Figure 2B, 4th wash) and 0.0003 mM TFA for $^{19}$F NMR (data not shown).

**Figure 4.3.** 1 mM peptide washed 0-3 times with 10 mM HCl solution. One round of wash indicates dissolving peptides in 10 mM HCl aqueous solution followed by removal of the solvent. (A) $^{19}$F NMR spectra of 1 mM peptide dissolved in D$_2$O show % of TFA per peptide molar ratio. (B) Normalized FTIR spectra of 20 mM peptide dissolved in deuterated buffer (pH 7.4) show the absorbance of carbonyl peak of TFA at 1672 cm$^{-1}$.

### 4.3. TFAc adducts alter peptide self-assembly

Peptides were washed using 1 mM peptide in 10 mM HCl method and the TFA concentration was quantified by $^{19}$F NMR. The washed and unwashed peptides were characterized using FTIR, analysis of CAC, and MD simulations to study the self-assembly behavior, TEM and AFM to compare morphology, and $\zeta$-potential analysis to measure the surface charge potential. Finally, the two peptides were incubated with MMP-9 to study the rate of enzyme hydrolysis.
4.3.1. Disruption of intermolecular peptide bonds

The FTIR spectrum of unwashed peptide shows the characteristic TFA carbonyl peak at 1672 cm\(^{-1}\) ([Figure 3](#)) which disappears after the first wash. This peak directly overlaps with the absorbance of peptide amide I band, which originates mainly from the C=O stretching of the peptide and typically consists of a series of overlapping components bands, making it difficult to accurately analyze the secondary structure without manipulating the data to subtract the TFA contribution.\(^7\) However, we found that the presence of the TFAc physically changes the supramolecular organization of the peptides, therefore, deriving the structure conformation information by subtracting the TFA absorbance signal would be inappropriate for our system.

In order to resolve the individual components of the amide I band profile, curve fitting analysis was performed and the best fit was achieved using the Levenberg Maquardt algorithm. The spectral analysis of the unwashed peptide shows absorbance at 1678 and 1646 cm\(^{-1}\) (Figure 4A), whereas the washed peptides show peaks at 1666 and 1632 cm\(^{-1}\) which can be identified as β-sheets, a strong peak centered at 1652 cm\(^{-1}\) that is exhibited by α-helices, and another peak at 1642 cm\(^{-1}\) that can be attributed to random coil arrangement of the peptide (Figure 4B). The complete absence of 1672 cm\(^{-1}\) that is attributed to the TFA band confirms that TFA removal was successful. The curve fitting analysis suggests that the TFAc interacts with the cationic amine groups which hinders salt bridge formation and alters peptide back bone interactions and changes the self-assembly behavior. Consequently, it was important for us to chemically remove the TFAc in order to characterize the peptide nanostructures with minimal contribution from TFAc.
Curve fitted FTIR of amide I region of unwashed (A) and washed (B) peptides after subtracting the D$_2$O background. (A) Unwashed peptides absorb at 1678 and 1646 cm$^{-1}$ and (B) washed peptides absorb at 1666, 1652, 1642, 1632 cm$^{-1}$, demonstrating that the hydrogen bonding pattern of the peptides are altered by presence of TFAc salts.

4.3.2. Induced aggregation of peptide nanostructures

Both TEM and AFM techniques show that the peptide with minimal amount of TFA self-assembles into individual fibers rather than bundles of fibers. For peptides containing 0.03% TFA, discrete nanofibers about 5 nm in diameter were found evenly across the TEM grid (Figure 5A). For peptides with 1.6% TFA, uniform sample of individual fibers were observed on the mica for AFM (Figure 6A). On the contrary, peptides with 113% TFA formed indistinguishable fiber-like aggregates (Figure 6B). Unwashed peptides containing 304% TFA dried in a fractal-like pattern on the TEM grid (Figure 5B). This fractal pattern was also observed in AFM images of different peptide sequences which caused an overall major challenge in imaging dried samples of peptide nanostructures. The aggregation of the peptide structures is likely due to TFA which forms ionic bonds with the positively charged lysine residues and prevents charge repulsion of the peptide structures.
Figure 4.5. (A) TEM images of 1 mM peptide containing 0.03% TFA self-assembled into individual fibers of ~5 nm diameter. Scale bar 200 nm. (B) The same peptide with 304% TFA forms aggregates and dried in a fractal pattern. Scale bar 1 µm.

Figure 4.6. (A) AFM images of 1 mM peptide containing 1.6% TFA also self-assembles into discreet fibers. Scale bar 500 nm. (B) Peptides with 113% TFA forms aggregated fibers. Scale bar 200 nm.

Computational simulation using MARTINI\textsuperscript{8,9} coarse grain model agrees with the TEM and AFM images and predicts that the peptide self-assembles into a network of thin, individual fibers, each 3.5 nm in diameter, instead of forming thicker fiber bundles (Figure 7). The cross section of the fiber shows the hydrophobic Phe residues (orange) are buried in the core of the fiber. The cationic Lys residues (blue) decorate the fiber surface, thereby facing the water (Figure 7B). The chloride ions that are represented in yellow form weak ionic bonds with Lys in water and the fibers are still able to repel each other. However, if those anions are replaced with TFA, which can form stable salt adducts with Lys residues in water, the positive charges are screened causing the fibers to aggregate (Figure 6B).
Figure 4.7. 500 ns simulation of (A-B) 30 mM and (C) 60 mM FFGALGLKGK in 12.5 nm$^3$ water box neutralized with 60 molecules of chloride, at 298°K and 1 bar. Phe residues are colored purple, Lys residues are blue, Gly residues are grey, Ala and Leu residues are orange and chloride salts are colored yellow.

4.3.3. Decrease in positive charges and increase in hydrophobicity

To confirm the decrease in positive charges of the peptide, $\zeta$-potential analysis of the unwashed and washed peptides was performed (Table 1). The $\zeta$-potential was negative for peptides containing 40-300% TFA. The negative $\zeta$-potential is likely due to the free carboxylic group of the peptide which gives the peptide molecule a net charge of -1 if all of the amine groups are blocked by TFAc (in the case of 304% TFA) and becomes more positive as TFAc ions get displaced by chloride ions during the 10 mM HCl washes. Finally, the $\zeta$-potential of the washed peptides increases to 2.2 ± 0.5 mV which is comparable for the reported $\zeta$-potential of 8.2 ± 2.8 mV (peptide 2 AK in Chapter 3).
Table 4.1. ζ-potential of 5 mM peptides in 2% PBS (pH 7.4) at 25°C.

<table>
<thead>
<tr>
<th>TFA (mM)</th>
<th>Mean ± Std. dev (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>0.30</td>
<td>-5.2 ± 0.5</td>
</tr>
<tr>
<td>3.04</td>
<td>-12.6 ± 0.3</td>
</tr>
</tbody>
</table>

Next, we investigated the CAC of the unwashed and washed peptides using pyrene as a solvatochromatic fluorophore. The 3:1 ratio which represents the hydrophobicity of the pyrene’s environment is overall higher for the unwashed peptides with 304% TFA. This is likely due to the TFAc adduct on the amine group of the N terminus which makes the diphenylalanine hydrophobic core more lipophilic. Unexpectedly, the CAC of unwashed peptide increases to 0.5 mM in comparison to the true value of 0.4 mM. Although the peptide is overall more hydrophobic with TFAc adducts, the amphiphilicity of the peptide decreases when the hydrophobic TFAc binds to the hydrophilic side. This change in the amphiphilic balance most likely causes the CAC to increase, since now the main driving force is hydrophobic aggregation.

![Graph showing CAC of washed and unwashed peptides](image)

Figure 4.8. CAC of washed peptide (304% TFA) and unwashed peptide (0.03%TFA) determined using pyrene in PBS (pH 7.4).
4.3.4. Delays MMP-9-responsiveness

Lastly, we compared the MMP-9 hydrolysis rate of washed and unwashed peptide. Previously, we have demonstrated that self-assembled nanostructures and the enzyme of interest should be oppositely charged in order to optimize enzyme engagement through short-range electrostatic interaction, and that highly ordered structures can hinder the enzyme’s accessibility to the substrate. Here we observed that the hydrolysis of unwashed peptide is delayed compared to the washed peptide (Figure 9).

![Conversion vs. Incubation Time for Washed and Unwashed Peptide](image)

**Figure 4.9.** 1 mM of washed peptide (1.6% TFA) and unwashed peptide (304% TFA) incubated with 100 ng/mL MMP-9 in PBS (pH 7.4, supplemented with 1 mM CaCl$_2$ and 55 µM ZnCl$_2$) at 37°C.

4.4. Conclusions

We have demonstrated that the presence of residual TFAc significantly alters properties of peptide nanostructures. TFA forms strong ionic bonds with the amine groups of the peptides in 1:1 molar ratio, which cannot be removed by lyophilization in water. This leads to large molar excess of TFA per peptide molecule after synthesis, since many peptides contain at least one amine group
on the N-terminus. In our system, the presence of TFA leads to a decrease in intermolecular hydrogen and electrostatic interactions, which prevented fiber formation, increased the CAC, rendered the $\zeta$-potential negative, and delayed the hydrolysis of the peptide by MMP-9. Although removing TFA can be a tedious and arduous task, having unknown concentrations of TFA in different batches of peptides will lead to different self-assembly behavior and inevitably produce irreproducible results as exemplified here and in literature.\textsuperscript{4,5} Especially in biomedical applications, reproducibility of both morphology and size is crucial for translating the system for potential use in clinical and commercial application. In addition, the residual TFA may cause unanticipated cytotoxicity in biological assays. It is also worth noting that even commercial peptides which costs extra for TFA removal were still found to contain TFA (data not shown). Therefore, quantifying the amount of TFA present in a peptide batch by $^{19}$F-NMR and, if necessary, removing residual TFA from the final peptide product should be implemented in the general protocol for peptide synthesis. At the very least, the concentration of TFA should be consistent in different peptide batches in order to reproduce the desired experimental results. Finally, we encourage the readers to follow the protocol outlined in Supporting Information for a successful removal of TFA.

4.5. Acknowledgements

I would like to thank the individuals who contributed to the work in this chapter: Roxana Piotrowska for performing the curve fitting analysis of the FITR spectra, Hailin (Richard) Huang for conducting the $\zeta$-potential and MMP cleavage experiments, Dr. Ankit Jain for help with TEM imaging, Dr. James Aramini for performing $^{19}$F NMR analysis, Daniela Kroiss for performing the
molecular dynamic simulations, Dr. María Contel for supervision, and Dr. Rein V. Ulijn for conceptualizing and supervising the project as well as help in writing the chapter.

4.6. Methods

**Solid Phase Peptide Synthesis (SPPS).** Fluorenylmethyloxy carbonyl (Fmoc)-protected amino acids and pre-loaded Wang resins were purchased from Bachem. Peptides were synthesized on CEM Liberty Blue microwave assisted solid phase peptide synthesizer using ~1:5 resin to amino acid ratio and excess of diisopropylcarbodiimide (DIC), Oxyma (Ethyl(hydroxyimino)cyanoacetate), and 20% piperidine in dimethylformamide (DMF). The complete peptide-loaded resins were washed three times in dichloromethane, followed by three washes in diethyl ether on a filtration column. The peptides were cleaved from the resins and side chain protecting groups were removed by reacting with TFA cocktail (95% trifluoroacetic acid, 2.5% triisopropyl silane, and 2.5% water) for 2 h. The cleaved peptides were recovered by removing TFA cocktail, followed by precipitation in cold diethyl ether. Peptides were washed three times in cold diethyl ether, using centrifugation to collect the precipitated crude peptide.

**Preparatory High-Performance Liquid Chromatography (HPLC).** Crude peptides were dissolved in 5% acetonitrile in water containing 0.1% TFA or 0.1% acetic acid and purified through a preparatory C_{18} column on the Thermo Scientific Dionex Ultimate 3000 using 5-50% acetonitrile (+0.1% TFA or acetic acid) gradient in water (+0.1% TFA or acetic acid).

**^{19}F NMR.** Sample solutions were prepared by dissolving peptide powder into D_{2}O. Peptide powder was prepared by washing 1 mM peptide 0-3 times with 10 mM HCl, or by washing 20 mM peptide 0-4 times with 10 mM HCl. 500µL of sample solutions (1 or 0.5 mM peptide) were placed in 5mm NMR tubes. ^{19}F NMR experiments were performed on a Bruker AVANCE HD III
700 MHz spectrometer equipped with a 5-mm QCI-F cryoprobe at a frequency of 658.79 MHz. All $^{19}$F spectra were acquired were acquired locked and at 25 °C. Typical 1D $^{19}$F NMR acquisition parameters were as follows: 8.0 ms pulse length (60° flip angle), 100 ppm sweep width (65,789 Hz) centered at -80 ppm, 0.50 sec acquisition time, 2 sec relaxation delay time, and 256 scans (11 min). 1D $^{19}$F NMR spectra were processed with 5 Hz exponential line broadening and visualized using TopSpin 3.5.

**Computational Simulation.** The MARTINI force field\textsuperscript{8,9} was applied to model peptide self-assembly using the GROMACS molecular dynamics package.\textsuperscript{10} This force field utilizes a 4-to-1 atom:CG-bead mapping to represent protein backbone and side chains (and a 2-3:1 atom:CG-bead mapping for ring systems). A cubic box with 30 peptide molecules, placed randomly with a minimum distance of 3 Å between them, was solvated in standard MARTINI CG water (4 water molecules per bead) to a final concentration of approximately 30 mM. The resulting net charge was neutralized by adding 60 molecules of chloride to the box. A Berendsen thermostat and barostat were used to keep the temperature at 298K and pressure at 1 bar, respectively.\textsuperscript{11} The box was energy minimized using the steepest descent integrator and then equilibrated for 6.25x10\textsuperscript{6} time steps of 20 fs. The total simulation time equates 125 ns, but due to the smoothness of the CG potentials, this roughly equates to an effective 500 ns of atomistic simulation time.\textsuperscript{12}

**Fourier-Transform Infrared Spectroscopy (FTIR).** Sample solutions were prepared by dissolving peptide powder into D\textsubscript{2}O using vortex followed by sonication at room temperature for 10 min. Peptide powder was prepared by washing 1 mM peptide 0-3times with 10 mM HCl, or by washing 20 mM peptide 0-4 times with 10mM HCl. 10 μL of sample solutions (20 mM peptide) were drop casted between two CaF\textsubscript{2} cells with PTFE spacers (12 μm thickness x 13 mm diameter). Absorbance spectra were taken from 4000 cm\textsuperscript{-1} to 800 cm\textsuperscript{-1} with 64 scans at 4 cm\textsuperscript{-1} resolution on
the Bruker Vertex 70 spectrometer. For analysis in Figures 2-3, D2O absorbance spectra was subtracted from the sample absorbance and graphed from 1550 to 1750 cm$^{-1}$. For analysis in Figure 4, curve fitting analysis was performed to resolve the individual components of the amide I band profile. Input parameters such as the estimated number of components bands, their positions and widths were selected. To increase the digital resolution spectrum was post zerofilled by an additional factor of 8. The best fit was achieved with the Levenberg Maquardt algorithm with which yielded the lowest residual RMS error value of 0.000148.

**Transmission Electron Microscopy (TEM).** TEM images were taken on FEI Titan Halo 80-300 microscope. 1 mM of peptide solution was prepared in 10 mM phosphate buffer (pH 7.4) sonicated for 10 min and 5 µL of the solution was drop casted on a carbon film grid (400 mesh, copper) and dried completely. To the dry grid, 5 µL of MilliQ water was drop casted and quickly blotted to wash away the phosphate salts and dried completely. Finally, 5 µL of methylamine vanadate based negative stain (NanoVan® by Nanoprobes) was drop casted, blotted away, and dried completely.

**Atomic Force Microscopy (AFM).** Sample solutions were prepared by dissolving peptide powder into 10 mM phosphate buffer (pH 7.4) using vortex followed by sonication at room temperature for 10 min. Peptide powder was prepared by washing 1 mM peptide 3 times with 10 mM HCl (Figure 6A) or 20 mM peptide 4 times with 10 mM HCl (Figure 6B). 5 µL of sample solutions were drop casted on freshly cleaved mica and allowed to dry for 48 h before imaging. AFM images were taken on Bruker Dimension FastScan using FASTSCAN-B tip on fast scan mode.
4.7. References


Chapter 5

MMP-9-Responsive Peptide Nanocarriers for Encapsulation of Metallodrugs

5.1. Introduction

Despite the plethora of cancer treatments available, which include surgery, radiation, chemotherapeutics, targeted therapies, and immunotherapy, there will be an estimate of 0.6 million cancer deaths in US in 2019.\(^1\) In search for novel cancer chemotherapeutics, both organic and metal-based compounds are being developed and investigated. In the field of metallodrugs, the most widely used drug is cisplatin that has been used for treatment of cancer since the 1970’s. Cisplatin and the follow up drugs, carboplatin and oxaliplatin, have a long history of use in treatment of ovarian, testicular, bladder, head and neck, and non-small cell lung cancers for the past 40 years.\(^2\) Although popular in clinical use, their effectiveness is still hindered by many problems including poor solubility, quick clearance, a limited spectrum of activity, acquired or intrinsic resistance, and a lack of selectivity leading to high nephrotoxicity and crippling adverse side effects in patients.\(^2\) To overcome these shortcomings, new platinum(IV) pro-drugs such as ormaplatin, iproplatin, and satraplatin have undergone clinical trials but have not yet been approved by the US FDA.\(^3\)

Additionally, it is important to explore metallodrugs (denominated as unconventional) based on metals other than platinum which display modes of action\(^4\) that are different from cisplatin,\(^5–7\) as well as their effects in the immune system.\(^8\) Current efforts have also been focused on developing agents that can be photoactivated\(^9\) or delivery systems that may improve their pharmacological profiles.\(^10\) Examples of unconventional metallodrugs in phase I/II clinical trials include a ruthenium-based compound, NAMI-A,\(^3\) for treatment of colorectal cancer, and a
photodynamic compound (TLD-1433) that was studied successfully in Phase Ib clinical trials for non-muscle invasive bladder cancer and has now entered Phase II clinical trials. More recently, copper(II)-casiopeina and copper(I) (HydroCuP) compounds have entered Phase I clinical trials, and a gold based drug, auranofin, is being investigated to be repurposed for the treatment of leukemia, small lung and ovarian cancer. However, for all small molecule-based drugs, it is imperative that they reach the tumor sites and enter the cancer cells. In order to improve on the efficacy and selectivity of molecular drugs, formulations using nanomaterials have become increasingly popular.

Nanomedicine takes advantage of its particle size (1-500nm) and surface properties to accumulate selectively in the tumor microenvironment through leaky blood vessels that causes the enhanced permeability and retention effect. This passive targeting mechanism is used by Lipoplastin, a liposomal formulation of cisplatin in phase III clinical trial, to prolong circulation lifetime and to increase tumor uptake of cisplatin. In addition to passive targeting, nanomedicine can be designed to actively target tumor cells using various endogenous stimuli such as pH, antibodies, integrins, and enzymes that are characteristic of cancer cells. Of the endogenous stimuli, the up-regulation of matrix metalloproteinases (MMPs) have been observed in almost all types of cancers, including ovarian cancer, and the roles of the enzymes have been associated to cancer invasion and metastasis. More details in passive and active targeting nanomaterials can be found in Chapter 2, and MMP-responsive nanomaterials can be found in Chapter 3. Therefore, MMP-9 is a suitable cancer specific bio-target and the enzyme action can be exploited to produce a desirable response, such as disassembly in the presence of MMP-9 to achieve site specific release of payloads.
Using previously described MMP-9-responsive peptide nanostructures in Chapter 5, we have encapsulated unconventional metal-based compounds with high anti-cancer properties to create a novel metallodrug-loaded nanocarriers. This system will allow payloads to be selectively released in the tumor microenvironment where MMP-9 is overexpressed, while controlling the rate of drug release by controlling the kinetics of MMP-9 hydrolysis of the peptide nanocarriers. Upon MMP-9 cleavage, the nanocarriers will disassemble to release the payloads and biodegrade into amino acids (Figure 1).

5.2. Design of metallodrug-loaded nanocarriers

Our group at Brooklyn College is focused on studying metal-based compounds for kidney cancer for which renal cell carcinoma (RCC) constitutes the most common type. Currently there are no efficient treatment options for advanced stage and metastatic RCC, since pharmacological interventions such as chemotherapy, targeted therapy or immunotherapy are very limited and only improve survival rates in periods of 9 to 20 months. Our group has developed a number of metal-based compounds that have demonstrated enormous potential as chemotherapeutics against renal, colorectal and prostate cancers (including mechanistic and \textit{in vivo} studies). These compounds include bimetallic compounds that incorporate the gold(I) compounds containing phosphanes\textsuperscript{21–24} or N-heterocyclic carbene ligands\textsuperscript{25–27} and a second metallic fragment being titanocenes or ruthenium(II)-arene based complexes (Ti-Au and Ru-Au compounds). Gold(I) compounds containing either phosphanes\textsuperscript{28} or N-heterocyclic carbenes\textsuperscript{29,30} are emerging as derivatives with enormous potential as chemotherapeutic agents.

For this project, we chose two gold(I) compounds that contain N-heterocyclic carbenes that were found to have relevant anti-cancer properties in many different cell lines (Figure 1). The
compounds are ([AuX(NHC)]) where: NHC = 1,3-dibenzyl-4,5-diphenylimidazol-2-ylidene NHC-Bn; X = Cl A; 1,3-diethyl-4,5-diphenylimidazol-2-ylidene NHC-Et; X = Br B).\textsuperscript{31,32} These compounds contain 4,5-diarylimidazoles cores with well-known pharmacological properties.\textsuperscript{33} Compound A (described by Tacke \textit{et al.}) and a variation with the 2’,3’,4’,6’-tetra-O-acetyl-β-D-glucopyranosyl-1’-thiolate showed very good activity against a wide range of human cancer cell lines from the NCI 60 cell line panel, and relevant tumor growth inhibition \textit{in vivo} for a human clear cell renal carcinoma Caki-1 xenograft mice model.\textsuperscript{31,34,35} Compound B, described by Gust \textit{et al.} in 2011, was found to be cytotoxic (low or sub-micromolar range) in breast and colon cancer cell lines.\textsuperscript{32}

\textbf{Figure 5.1.} Cartoon representation of cancer targeted release of metallodrugs (compounds A and B) from biodegradable MMP-9-responsive peptide nanocarriers. Chemical structures of N-heterocyclic carbene containing gold(I) based compounds with relevant anti-cancer properties.\textsuperscript{31,32,35}
In addition, modifications of compounds A and B showed improved anti-cancer activities.

We have coordinated the [Au(NHC)]⁺ fragments of compounds A and B to [Ti(Cp)2M] fragments through a dual linker (-OC(O)-p-C₆H₄-S-) containing both a carboxylate and a thiolate group.⁴⁷

The new compounds have been efficacious in vitro against human clear-cell renal carcinoma Caki-1 and human prostate PC3 cells while being apoptotic and inhibiting migration, inhibition of thioredoxin reductase (TrRx) and vascular endothelial growth factor (VEGF) in prostate PC3 cancer cell lines.⁴⁷ In another example, an antibody drug conjugate (ADC) of compound A with an engineered antibody (Thiomab LC-V2050) via cysteine conjugation, showed moderate improvement of anti-proliferative activity in HER2 positive breast cancer cell line, in comparison to the non-conjugated drug.²¹

**Figure 5.2.** Chemical structures of peptide 1 PK and 1 PD, and cartoon representations and pre and post MMP-9 hydrolysis of peptide nanocarriers. 80% of 1 PK is cleaved by MMP-9 in 48 h whereas less than 10% of 1 PD is cleaved in 96 h (described in Chapter 3).
Although compounds A and B show potential anti-cancer activity, both drugs are highly insoluble in water and DMSO which makes it difficult to treat cancer cells in vitro or to administer them as solutions in vivo. Therefore, we sought out to encapsulate compounds A and B in MMP-9-responsive peptide nanocarriers to solubilize the compounds in physiological conditions which could potentially increase the efficacy of the drugs (Figure 1). As examined in Chapter 3, tumor tissues from patients with renal cell carcinoma show significantly higher expression of MMP-9 mRNA, with increasingly higher expression in advanced tumor stages. We hypothesize that by encapsulating the metallodrugs, which are highly active against this type of cancer, we can control the spatial and temporal release of the compounds based on MMP-9 activity, and increase the selectivity of these compounds.

For the nanocarriers, we chose self-assembling peptides 1 PK and 1 PD, previously described in Chapter 3, which form ~200 nm long bundles of worm-like micelles with a hydrophobic core and a cationic or anionic hydrophilic tail (Figure 2). Full characterization of the peptide nanostructures can be found in Chapter 3. The anionic nanostructure, 1 PD, was found to have slow response to MMP-9, due to the electrostatic repulsion between the anionic nanostructure and the negatively charged MMP-9. On the contrary, the analogous cationic nanostructure, 1 PK, readily engages with MMP-9 and is cleaved to become the enzymatic product, 1 P, which no longer forms a self-assembled nanostructure (Figure 3). Using 1 PK and 1 PD to encapsulated compounds A and B, we can investigate how the rate of MMP-9 responsiveness could effect the performance of the compounds in the biological setting.
5.3. Encapsulation of metallodrugs

The encapsulation efficiency of compounds A and B by peptide nanocarrier 1 PD was investigated. Metal based drugs are advantageous as cytotoxic payloads in nanomedicine because the metal element can be precisely quantified by inductively-coupled plasma optical emission spectrometry (ICP-OES) or atomic absorption spectrometry (AAS). To quantify the amount of compounds A and B, samples were digested in concentrated nitric acid prior to analysis.

5.3.1. Digestion of gold-based compounds

The concentrations of the compounds A and B were analyzed using ICP-OES at 242.8 nm and 267.6 nm and measured against calibration curve of 0.01 - 1 mg/L Au standard prepared in aqueous 5% HNO₃. In order to detect the elemental gold, compounds A and B were digested in concentrated nitric acid prior to analysis. First, a stock solution of 0.05 mM compound B was prepared in acetonitrile and was refluxed in concentrated nitric acid for 15 min, 30 min, or 60 min at 100°C. The digested samples were diluted 200 times in 10% HCl (aq) solution to achieve final concentrations within the range of calibration curve in a total of 5% HNO₃ and 10% HCl aqueous solution. The measured mg/l values were calculated into mM and multiplied by the dilution factor to compare to the stock solution (Table 1). Higher concentrations of Au was detected with longer refluxing times. After 1 h, 0.045 mM of Au was detected in comparison to the actual 0.05 mM that should have been measured. A second stock solution of 0.075 mM compound B was prepared in acetonitrile and refluxed for 2 h or heated until dryness. The refluxed sample was diluted in 10% HCl (aq) and the dry samples were dissolved in 5% HNO₃ and 10% HCl aqueous solution and diluted 100 times. For both methods, the detected Au was near 0.075 mM, which suggests that 2 h or longer is sufficient time to digest all of compound B. Likewise, the accurate amount of
compound A was also detected when digested until dryness. Moving forward, we decided to digest compounds A and B by heating it until dryness in order to minimize errors from losing volume during reflux that will lead to incorrect dilution factor.

Table 5.1. ICP-OES analysis of 0.05 mM or 0.075 mM compound B refluxed in concentrated nitric acid at 100°C over time or heated until dryness. The reported concentrations of Au were measured at 267.6 nm and 242.8 nm, and multiplied by the dilution factor.

<table>
<thead>
<tr>
<th>Detected Au (mM)</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>Dry</th>
</tr>
</thead>
<tbody>
<tr>
<td>267.595 nm</td>
<td>0.016</td>
<td>0.027</td>
<td>0.031</td>
<td>0.037</td>
<td>0.074</td>
<td>0.074</td>
</tr>
<tr>
<td>242.795 nm</td>
<td>0.014</td>
<td>0.034</td>
<td>0.039</td>
<td>0.045</td>
<td>0.075</td>
<td>0.075</td>
</tr>
<tr>
<td>Actual Au (mM)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.075</td>
<td>0.075</td>
</tr>
</tbody>
</table>

5.3.2. Encapsulation efficacy of peptide nanocarriers

Next, the encapsulation efficiency of the compounds in peptide nanocarriers was investigated following the procedure described in Figure 3. Briefly, peptides were dissolved in 10 mM phosphate buffer and the pH was adjusted to 7.4 using 0.5 mM HCl or NaOH to make 2 mM peptide solution. To the peptide solution, stock drug solution (prepared in acetonitrile) was added for a final concentration of 1 mM peptide and 100, 10, 1, or 0.1 µM of drug in 1:1 ratio of buffer and acetonitrile. The mixture was vortexed and sonicated for 20 min and dried to a film then suspended in phosphate buffer and sonicated for 20 min. The non-encapsulated, hydrophobic drug was separated from the suspended drug-loaded peptide nanocarriers by centrifugation and the supernatant was collected. The Au content in the supernatant was digested in concentrated nitric acid as described above, and analyzed by ICP-OES or AAS. The encapsulation efficiency of compounds A and B by 1 PD was similar for lower concentrations. Interestingly, 91% and 100%
of 10 µM compounds A and B were encapsulated, respectively, whereas only 23% and 24% of 1 µM compounds A and B were encapsulated. At 100 µM, 61% of compound A and 20% of compound B was encapsulated by 1 PD, likely due to the higher hydrophobicity of compound A.

**Figure 5.3.** Encapsulation of metallodrugs in peptide nanocarriers. Step 1) Prepare 2 mM peptide stock solution in 10 mM phosphate buffer and adjust the pH to 7.4 using 0.5 mM HCl or NaOH. Prepare 200, 20, 2, 0.2 µM drug stock concentrations in acetonitrile. Step 2) In 1.5 mL centrifugal tubes, combine 100 µL of aqueous peptide solution with 100 µL of the drug stock solution for a final concentration of 1 mM peptide and 100, 10, 1, 0.1 µM of drug in 1:1 ratio of buffer and acetonitrile. Vortex the mixture and sonicate for 20 min. Step 3) Remove solvents completely under pressure. Step 4) Add 200 µL of 10 mM phosphate buffer to the dried film to suspend the peptide-drug mixture. Gently vortex the aqueous mixture and sonicate for 20 min. Step 5) Separate the non-encapsulated, hydrophobic drug from the suspended peptide-drug nanocarriers by centrifugation (10,000 rpm for 10 sec). Collect 200 µL of supernatant for analysis and characterization.
Table 5.2. Encapsulation efficiency of 1 mM 1 PD with compounds A and B.

<table>
<thead>
<tr>
<th>Drug (µM)</th>
<th>Compound A</th>
<th>Compound B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Final</td>
<td>61.4</td>
<td>9.1</td>
</tr>
<tr>
<td>EE (%)*</td>
<td>61%</td>
<td>91%</td>
</tr>
</tbody>
</table>

*EE: encapsulation efficiency = (final/initial) x 100%

5.4. Molecular dynamic simulations

In order to investigate the interactions between the metallodrugs and the peptides, dynamic simulations were performed. The structures of compounds A and B were initially optimized by using a computational approach based on the framework of the density functional theory (DFT), which has been widely exploited in studying the properties and reactivity of metallodrugs.\textsuperscript{37–40} DFT methods encompass a wide range of approximations that leads to the so-called density functionals, usually defined as pure, hybrid and range-separated hybrid depending on the theoretical treatment of the electron cloud.\textsuperscript{41} Herein, we have used PBE0,\textsuperscript{42} that have previously demonstrated to provide reliable results for structural and reactive properties gold(I)-based organometallic compounds.\textsuperscript{43,44}
Figure 5.4. Optimized structures for compounds A and B at the selected DFT level of theory (PBE0-D3/def2-SVP). Molecules are represented in ball and stick model. Color scheme: hydrogen in white, carbons in grey, nitrogen in blue, chloride in green, bromide in red and gold in yellow.

Next, we assessed the interaction of the metallodrugs with peptides 1 PK/PD and 2 PK/PD for comparison. Peptides were built up with the Protein Preparation Wizard module implemented in Maestro\(^45\) and protonation states of all side chains were defined at pH 7 by using PROPKA 3.1.\(^46\) As illustrated in Figure 2, (1) the resulting peptides are located at the vicinity of the metallodrug (3.5 angstroms of distance). Aiming to mimic the conditions in solution and accurately correct solvent effects, (2) the model system is embedded in box filled with water molecules. Sodium cations or chlorides anions were added to counterbalance the total electric charge in of the peptides. Additional sodium and chloride ions were added as well to reproduce the physiological NaCl salt concentration of 0.15 M, so that our simulations can be extrapolated to real in vivo conditions.
Figure 5.5. We illustrate the main steps of the MD workflow: (1) the peptide-compound B model is built up; (2) the systems is embedded in a water box; (3) MD simulations run for 100 ns, where the evolution of atoms are monitored by plotting the root-mean-square deviation (RMSD); (4) the equilibrated peptide-compound pair is extracted from the trajectory for further quantum mechanical calculations.

Four systems are simulated: compound B with the peptides 1 PK, 1 PD, 2 PK, and 2 PD (peptide sequences can be found in Table 3). In MD trajectories, the root-mean-square deviation (RMSD) of atomic positions can be used to assess the average distance between atoms during the simulation, that is, the stability of the system. As shown in Figure 7, the structure of compound B is fairly stable during the whole simulation (red line), with a change of less than 1 Å only form the very beginning of the four simulations. A dissimilar behavior is observed for peptides. A close
inspection of Figure 7 reveals that the associated RMSD to peptides significantly increase during the first ns of the simulation, which quickly raised up to 10-12 Å. This is the logical consequence of the larger flexibility of peptides. However, such oscillations are attenuated after 2-5 ns. Indeed, although blue lines demonstrated that the peptides are more flexible during the whole simulations, the RMSD for peptides are restricted to 6±2 Å in the last part of the trajectory.

**Figure 5.6.** Root-mean-square deviation (RMSD) of compound B with Peptides 1 PK/PD and 2 PK/PD in angstroms. RMSD for the compound are plotted in red, and for peptides in blue.

Our computational study is completed by extracting the compound B-peptide pairs from the trajectory to compute their interaction energies at DFT level (Table 3). It is worth stressing that compound B and 2 PK remains unbound at the end of the MD simulation, and consequently we cannot compute the pairwise energy.
Table 5.3. Calculated interaction energy of compound B-peptide pairs.

<table>
<thead>
<tr>
<th>Peptide name and sequence</th>
<th>Interaction energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PK FFPLGLAGKK</td>
<td>-8.32</td>
</tr>
<tr>
<td>1 PD FFPLGLAGDD</td>
<td>-13.04</td>
</tr>
<tr>
<td>2 PK FFGPLGLKGG</td>
<td>unbound pair</td>
</tr>
<tr>
<td>2 PD FFGPLGLDGK</td>
<td>-11.71</td>
</tr>
</tbody>
</table>

5.5. Conclusion

In conclusion, we have demonstrated a proof-of-concept in which peptide nanostructures can be used to encapsulate metallodrugs. The highest concentration of compounds A and B encapsulated by 1 PD are only about 60 and 20 µM. According to the computational studies, 1 PD should have the highest stability with compound B and we can expect the drug loading capacity of 1 PK and 2 PD to be lower and weakest with 2 PK. However, the IC50 values of these compounds are well below 20 µM and higher drug concentrations are not necessary. In addition, the compounds are completely soluble within the nanocarrier which could lead to even smaller IC50 values since the unencapsulated drugs are highly hydrophobic and precipitates easily.

5.6. Acknowledgements

I would like to thank the individuals who contributed to the work in this chapter: Yaron Marciano and Lina Alhanshali for help in drug encapsulation and AAS analysis, Dr. Ali Younes for ICP-OES analysis, Dr. José Cerón for the performing the computational simulations, Dr. Rein V. Ulijn for supervision, and Dr. María Contel for conceptualizing and supervising the project as well as help in writing the chapter.
5.7. Methods

Atomic absorption spectroscopy (AAS). Digested samples were measured on a Perkin Elmer Analyst 800 using a transversely heated graphite furnace (THGA) system at 242.8 nm.

Inductively coupled-plasma optical emission spectroscopy (ICP-OES). Digested samples were measured Perkin Elmer-Optima 7000DV ICP-OES at 242.795 nm and 267.595 nm.

5.8. References

(1) Cancer Facts & Figures 2019 | American Cancer Society


(5) Metal-Based Anticancer Agents; 2019.


Chapter 6
Conclusion

6.1. Summary

In this thesis, we have successfully demonstrated that peptide building blocks can be systematically modified to design MMP-responsive nanostructures. This approach allows control of the morphology and charge of the nanostructures with simple changes in the amino acid sequence, including the MMP-cleavable P$_6$-P$_6'$ positions. Through the systemic study discussed in Chapter 3, we validated parameters other than the P$_6$-P$_6'$ sequence which affect the rate of MMP-responsiveness, such as the morphology and electrostatic charge of the nanostructures which can help achieve MMP specificity. In Chapter 2, we analyzed over 40 different systems described in the literature and found multiple examples that confirm our conclusion. In addition, the systematic approach to peptide design allows us to control the morphology of the post-enzymatically cleaved product, or the mode of response. We demonstrated that amyloid-like fiber forming products causes selective cytotoxicity to cells that overexpress MMPs and disintegrating products are nontoxic and continues to degrade into amino acids. As discussed in Chapter 2, the modes of MMP-responsiveness can influence multiple factors such as the rate of payload release and its efficacy. Lastly, we demonstrated that peptide nanostructures can be used to encapsulate anti-cancer organometallic compounds that are otherwise too hydrophobic to be administered in vitro or in vivo. During this investigation, it is imperative that the self-assembly of the peptide nanostructures are reproducible each time, which can be significantly altered by trace amounts of TFA salts that are left from peptide synthesis but are often neglected. Through a number of techniques, we demonstrated that peptide self-assembly is significantly altered by TFA which could explain the vastly different self-assembly behavior of a same peptide molecule reported in literature.
In summary, we have addressed the questions asked in the beginning of Chapter 1 through experimental results and literature analysis. As demonstrated in Chapters 2 and 3, specificity towards MMPs can be achieved by nanomaterials by 1) using optimized P_{6}-P_{6}’ sequence for the target MMP, 2) designing morphologies that are similar to the native substrate of the target MMP, and 3) by mis-matching the short-range electrostatic interaction of the MMP and nanomaterial. The three criteria can be readily modified in peptide nanostructures by small changes in amino acids, unlike other materials which require a different building block (ie. negatively charged phosphatidylglycerol vs. neutral phosphatidylethanolamine).

Literature analysis showed that MMP-responsive nanomaterials can improve the efficacy of anti-cancer treatments based on the modes of response. The efficacy of the drugs loaded in MMP-responsive materials are not always improved by increasing toxicity or tumor reducing ability, but by making the treatment more selective and causing less systematic damage. We demonstrated that the modes of response can also be controlled using peptide nanostructures to form cytotoxic fibers or non-toxic products. In addition, we showed proof-of-concept in which peptide nanostructures can be used to encapsulate metallodrugs which can potentially increase the efficacy of these drugs by making them more soluble and selective towards cells that overexpress MMP.

6.2. Future work

The work of MMP-responsive peptide nanostructures will carry on testing if the efficacy of the metallodrugs can be improved in vitro. Currently, we are characterizing drug-loaded peptide nanocarriers to study the stability of the nanocarrier and the rate of MMP-responsiveness and drug release. Our plan is to incorporate other sequences such as AK which can have additional
therapeutic effects. The *in vitro* studies will be conducted in cell lines with high and low expression of MMP-9 which will be quantified by ELISA. In addition, the stability of the peptides will be tested in human plasma which can help to predict the half-life of the drug-loaded peptide nanocarriers when administered *in vivo*. This work will continue as a joint-collaboration between the Contel group at Brooklyn College and the Ulijn group at ASRC.

Due to the versatility of the peptide nanostructures, and the highly disease relevant MMP target, many collaborative interests have sprung out from this research. For example, MMPs are expressed in mice with multiple sclerosis that break down the blood-brain-barrier. By treating the mice with one of AK peptides loaded with therapeutic payloads, the fibers can localize at the site of MMP overexpression and build up the blood-brain-barrier and simultaneously release the therapeutic agents. Other research groups have shown interest in modifying the terminal Phe to Tyr residue which can be used to conjugate radioactive iodine (\(^{131}\)I) for diagnostic imaging. Another research group is interested in using the MMP-response peptides as capsules to encapsulate growth factors that can simulate cell differentiation. The possible applications of these MMP-responsive peptide nanostructures are enormous, and while they may not be successful for every application, the design principle behind the peptides can be applied to all MMP-responsive nanomaterials.
I. Publications and invention disclosure


MMP-9 Responsive Nanoparticles (Patent pending).

II. Fellowships and awards

2017-2019: Tow Foundation Graduate Fellowship from the MSKCC Center for Molecular Imaging and Nanotechnology

2016: Predoctoral Fellowship from National Institutes of Health G-SOAR Program

2014-2019: CUNY Science Scholarship

2018: Doctoral Student Research Grant Award, Round 13

2017: Brooklyn College Chemistry Department Student Award

2016: Doctoral Student Research Grant Award, Round 11

2015: CUNY Conference Presentation Support Award
III. Oral and poster conference presentations


IV. Completed workshops

**Martini** Workshop (Coarse Grain Forcefield for Biomolecules), University of Groningen, Groningen, The Netherlands, 2017.

**Blogging- Social Media** Workshop, Decker Design (deckerdesign.com), New York, NY, 2017.


**Data Science Training**, Software Carpentry (software-carpentry.org), Bethesda, MD, 2016.