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14. ABSTRACT
In order to find out why our CMP based imaging agent produced weak signals in vivo, we performed to serum stability test. Here, we report the serum stability of a series of monomeric CMP derivatives and establish how peptide length, amino acid composition, terminal modification, and linker chemistry influence their availability in serum. We show that monomeric CMPs comprised of the collagen-like Gly-Pro-Hyp motif are resistant to common serum proteinases and that their stability can be further increased by simple N-terminal labeling which negates CMP's susceptibility to proline-specific exopeptidases. When fluorescent dyes are conjugated to CMP *via* maleimide-thiol reaction, the dye can transfer from CMP onto serum proteins (e.g. albumin) resulting in an unexpected drop in signal during serum stability assays and off-target accumulation during *in vivo* tests. This work is the crucial first step toward understanding the pharmacokinetic behavior of CMPs which can facilitate the development of CHP-based diagnostics.

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Introduction

Prostate cancer (PCa) is the leading cancer in the U.S. population and the second leading cause of cancer death in men. One of the most pressing issues in PCa management is the need to predict, at the time of diagnosis, which tumors will remain indolent and which will progress rapidly. The ability to fulfill that goal would eliminate the prostate-specific antigen (PSA)-mediated over-detection and over-treatment of clinically insignificant disease. PCa tends to undergo definitive treatment despite the side effects of bowel, bladder and/or sexual dysfunction. The ability to predict the "bad actors" among diagnosed tumors would provide rationale for expectant management, which may be appropriate and obviate serious morbidity. There is an emerging concept of using non-cellular solid state compartment as a source for therapeutic targets and for selective imaging of micro-metastasis. In particular, collagens in metastatic tumors have been one of the major targets for this purpose since hallmark of malignant tumor progression involves proteolytic remodeling of the ECM which results in unique structural and biochemical state of stromal collagens. Traditionally, these collagens were targeted by antibodies (monoclonal antibody raised against denatured collagen); however antibodies have poor pharmacokinetics for *in vivo* imaging. Recently, small collagen mimetic peptide (CMP) that mimics the amino acid sequence and three dimensional structure of collagen was shown to have specific binding affinity to type I collagen fibers. Although exact mechanism of binding is not known fully, evidences are accumulating that support the idea that the CMP is binding to partially denatured domains of natural collagen by triple helical hybridization. We proposed to use CMP as a collagen targeting agents that will allow imaging of PCa. Since CMP binds to unstructured collagen domains more readily, we had expected it to exhibit selective affinity to metastatic PCa tumors known to contain processed and denatured collagens. *This was the first attempt that the remodeled ECM of tumor microenvironment was targeted for cancer imaging which is an entirely new way to image PCa with a potential to revolutionize the cancer community.*

Specific aims of the work originally approved at the start of the project were:

1. Synthetic optimization of collagen mimetic peptides for high binding affinity to denatured collagens and collagens undergoing remodeling which simulate the microenvironment of metastatic tumors. We will focus on previously studied neutral CMP, $(ProYaaGly)_x$, as well as new charged CMPs known to exhibit high collagen/gelatin binding affinity and test their ability to specifically target digested collagens with unfolded and partially denatured collagen triple helices.
2. Demonstration of *ex vivo* and *in vivo* targeting/imaging of malignant PCa mediated by CMP hybridization. CMPs selected from aim 1 will be labeled with various imaging agents (e.g. IR dye, radioiodine, ^{18}F), and its pharmacokinetics and ability to target malignant PCs will be studied in mouse models.
3. Determination of tumor associated collagen signatures (TACS) for PCa's malignancy level by CMP-mediated imaging methods. Human PCa lines with known malignancy profiles, metastatic bone models and PCa microarray will be used to correlate the TACS with stromal metastic potential. We plan to develop TACS as a potential biomarker for predicting PCa progression in patients.

During the year 1 and 2 of the grant period, we have successfully completed Aims 1 and 2 of the stated work. We have demonstrated that the collagen mimetic peptide (CMP, also known as collagen hybridizing peptide, CHP) in the form of ProHypGly has the most potential to image invasive prostate cancer due to the absence of non-specific affinity and high propensity to hybridize with denatured collagen strand (Aim 1). We have also investigated numerous CMP derivatives in targeting/imaging malignant prostate cancer (PCa). We were able to image PCa using near-IR fluorescence dye (Aim 2).

Despite our continuous efforts to develop CMP based SPECT-CT imaging agents for prostate cancer models, nearly all the radiolabeled CMPs developed during this study did not result in expected in *vivo* targeting of cancer micro-environment. We believe that such failure may have been caused by either i) peptide degrading after tail vein injection or ii) dehalogenation and unstable loading/ release of the radioactive metal from the peptide during or prior to tail vein injection. We also found out that part of this problem could have been due the animal model: the level of collagen remodeling produced by xenograf prostate tumor model may not have been high enough to be detected by CMP.

During the later part of the program, we investigated i) the serum stability of collagen mimetic peptide (Yu and Pomper lab), and ii) development and SPECT-CT imaging of CMP dervatives that have mild radiometal loading condition (Pomper lab). The serum stability test was the crucial first step toward understanding the pharmacokinetic behavior of CMPs which can facilitate the development of CMP-based diagnostics, and the new radiolabeling loading strategy could reduce the loss of radiometal and help realize the SPECT imaging of invasive PCa in animal models. The serum stability study clearly showed that the peptide is stable in serum and can be used faithfully as drug or imaging delivery vehicle (published in *Molecular Pharmaceutics*). The new radiolabeling condition has not produced any positive results and we are now testing more severe disease model. At this point, we started using "collagen hybridizing peptide (CHP)" to describe our peptide (instead of CMP), because we realized that we are not developing peptide that mimic collagen but rather we are working on non-triple helical peptide that can hybridize to collagen strands.

While we were struggling with developing radioactive CMPs, we also explored CHP in histological study of other pathologic conditions such as osteoarthritis and fibrosis, as well as mechanical damage of the collagen rich tissues (such as tendon). These recent work were published in high profile journals including *Nature Communications*, and *ACS Nano*. Most importantly, we collaborated with Dr. Stephen Weiss (University of Michigan) who is the world expert on tumor invasion and studied cancer cell invasion in transgenic mouse in regard to MT-MMP expression. This work was submitted to *Nature Cell Biology*, and we are excited to report that we jusrt received a request to submit a revise manuscript.

Finally, we tried to improve the collagen binding affinity of the CHP by producing nanofibers that are decorated with dense layer of CHPs. Although we have yet to test these nanofibers with PC animal models, the nanofibers have demonstrated strong affinity to denatured collagen in the bone when used in vivo (with NIRF imaging) and that we are hopeful that the new CHP-based nanofiber

will help us finally realize the SPECT imaging of PCs.

Overall, we achieved most of the goals set in aims 1 and 2, and have achieved limited goals set in aims 3. Although we need more work to realize the radio-label based detection system based on CHP technology, we were able to probe mechanisms of cancer invasion using our CHP and also learned *in vivo* behavior and other associated disease targets that would pave the way for clinical translation of the peptide in prostate cancer detection. Seven high impact papers directly resulted from this research activity and in six addition publications were published which were supported in part by this research fund.

Body

Summary of the main outcomes of the proposed aims are presented. Details can be found in the published papers which are cited here and attached to this document.

Updated AIM 1
Develop CHPs and Demonstrate CHP-mediated targeting of tumor environment and other pathologic condition characterized by collagen degradation.

1-1. Degradation of the extracellular matrix (ECM) is one of the fundamental factors contributing to a variety of life-threatening or disabling pathological conditions. However, a thorough understanding of the degradation mechanism and development of new ECM-targeting diagnostics are severely hindered by a lack of technologies for direct interrogation of the ECM structures at the molecular level. Previously we demonstrated that the collagen hybridizing peptide [CHP, sequence: $(GPO)_9$, O: hydroxyproline] can specifically recognize the degraded and unfolded collagen chains through triple helix formation. Here in this work, we showed that fluorescently labeled CHP robustly visualizes the pericellular matrix turnover caused by proteolytic migration of cancer cells within 3D collagen culture, without the use of synthetic fluorogenic matrices or genetically modified cells. To facilitate *in vivo* imaging, we modified the CHP sequence by replacing each proline with a (2S,4S)-4-fluoroproline (f) residue which interferes with the peptide's inherent propensity to self-assemble into homo-triple helices. We showed that the new CHP, $(GfO)_9$, tagged with a near-infrared fluorophore, enables *in vivo* imaging and semi-quantitative assessment of osteolytic bone lesions in mouse models of multiple myeloma. Compared to conventional techniques (e.g., micro-CT), CHP-based imaging is simple and versatile *in vitro* and *in vivo*. Therefore, we envision CHP's applications in broad biomedical contexts ranging from studies of ECM biology and drug efficiency to development of clinical molecular imaging. Manuscript describing this work is currently under review in the journal "*Biomaterials.*"

1-2. Invasiveness of cancer cells are known to correlate with the level of ECM remodeling since such remodeling is pre-requisite for tumor growth. Metastasizing breast carcinoma cells in particular have been hypothesized to mobilize tissue-invasive activity by co-opting the proteolytic systems employed by normal mammary epithelial cells undergoing branching morphogenesis. However, the critical effectors underlying morphogenesis remain unidentified and their role in

breast cancer invasion remain to be established. In this work, we used CHP, along with other methods to identified the membrane-anchored matrix metalloproteinase, *Mmp14*/MT1-MMP, but not the closely related proteinase, *Mmp15*/MT2-MMP, as the dominant proteolytic effector of both branching morphogenesis and carcinoma cell invasion *in vivo*. Unexpectedly, however, epithelial cell- specific targeting of *Mmp14*/MT1-MMP in the normal mammary gland fails to impair branching, whereas deleting the proteinase in carcinoma cells abrogates invasion, preserves matrix architecture and completely blocks metastasis. By contrast, in the normal mammary gland, extracellular matrix remodeling and morphogenesis are ablated only when *Mmp14*/MT1-MMP expression is specifically deleted from the periductal stroma. Together, these findings uncover the overlapping, but divergent strategies that underlie developmental versus neoplastic matrix remodeling programs. This work was submitted to *Nature Cell Biology* and we just received a request to submit a revised version of the manuscript.

1-3. In this work published in ACS Nano (2017), we showed that CHP can be used to image degraded collagen and inform tissue remodeling activity in various tissues: labeled with 5-carboxyfluorescein (5-FAM) and biotin, CHPs enabled direct localization and quantification of collagen degradation in isolated tissues within pathologic states ranging from osteoarthritis and myocardial infarction to glomerulonephritis and pulmonary fibrosis, as well as in normal tissues during developmental programs associated with embryonic bone formation and skin aging. The results demonstrated the general correlation between the level of collagen remodeling and the amount of denatured collagen in tissue, and show that the CHP probes can be used across species and collagen types, providing a versatile tool for not only pathology and developmental biology research but also histology-based disease diagnosis, staging and therapeutic screening. This study lays the foundation for further testing CHP as a targeting moiety for theranostic delivery in various animal models.

Updated AIM 2

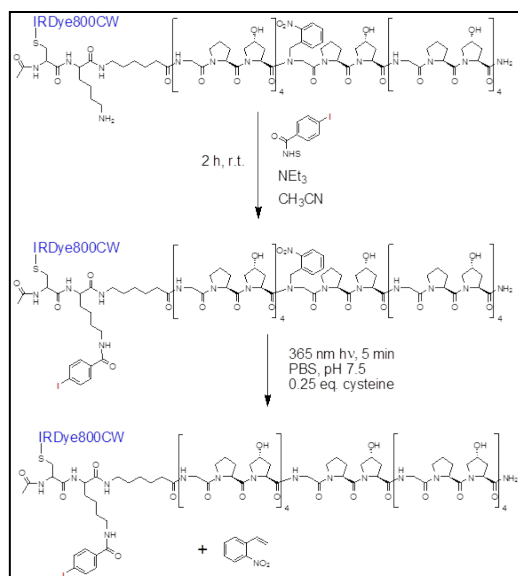
Demonstrate in vivo imaging of invasive prostate cancer and other tumors using near-IR fluorescence and SPECT-CT. We attempted PCa imaging using New CMP Structure with mild radiometal loading conditions (Pomper Lab). Because we were unable to acquire satisfactory SPECT-CT result, we turned our attention to serum stability of the CHP which is part of the PK and BD studies of necessary for translational application.

2-1. Since radiometal labeling work was not successful and since non-of this work was published, we are including full report (with data) for this part of the project.

Our prior work of SPECT imaging of invasive PCa in animal model was unsuccessful because the radiometal either became unbound during peptide preparation or during in vivo delivery. Therefore, we focused on two dual-labeled CMP analog that allow for gentle radiolabeling conditions and we tested it *in vivo* for its pharmacokinetic profile in a transgenic and subcutaneous model of PCa. We first tried a follow up SPECT-CT imaging experiment using a dual labeled CMP with [^{125}I]SIB and IRDye800CW in a transgenic F1 FVB/TRAMP mouse with severely hypertrophied seminal vesicles. The radiolabeling of this analog is shown in Scheme 1. In healthy mice, this analog rapidly washed out (progress report, year 2). In mice with enlarging,

diseased seminal vesicles, this dual labeled CMP bound to the enlarged vesicles and little else as seen by NIRF imaging. CMP binding overlapped with an inflammation probe that was co-injected (Figure 1: DPA-713-IRDye680LT, green), suggesting the CMP was binding to inflammation-induced tissue remodeling. SPECT-CT imaging of the same animal on the same days as NIRF imaging revealed rapid washout of the CMP from everything except tissue in the lower right quadrant. The SPECT signal remains the same through 24 and 48 hours post-injection, suggesting stable radiotracer accumulation although the SPECT signal does not match the observed fluorescence signal in both inflamed seminal vesicles. This particular analog did not produce matching NIRF and SPECT signal distributions in this model. This supports earlier results showing *in vivo* lability of the radioiodine label despite using the *p*-iodobenzoyl group and the adjacent bulky dye

Scheme 1. *Conjugation of [^{125}I]SIB to Ac-C(IRDye800CW)-K-CMP$_9$-CONH$_2$ and photo decaging (lower left box).*



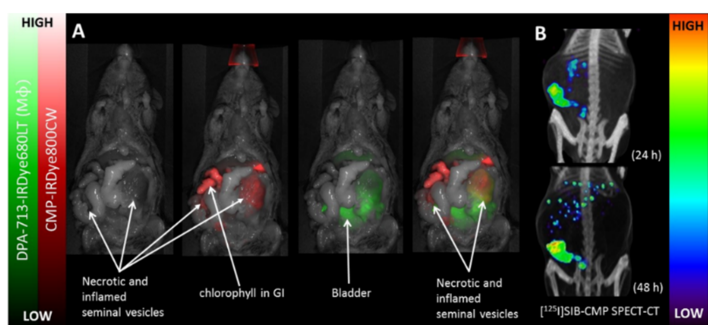
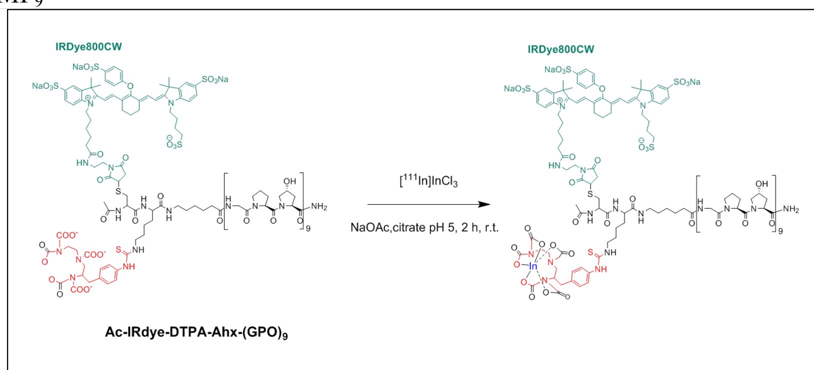


Figure 1. *NIRF and SPECT-CT imaging in an F1 TRAMP mouse.* A male TRAMP mouse devoid of prostate cancer but containing hypertrophied, inflamed seminal vesicles was scanned using CMP-800CW and an inflammation tracer, DPA-713-IRDye680LT using NIRF imaging. Both inflamed seminal vesicles are displaying CMP uptake (red) co-localizing with the inflammation marker in green. SPECT-CT imaging of the same animal with [^{125}I]SIB-CMP shows intense radiotracer uptake at both 24 and 48 h post-injection in the lower right quadrant, possibly representing GI clearance.

Scheme 2. Radiolabeling of [^{111}In](lys$_2$)CHX-A-DTPA-(cys$_1$)IRDye800CW-CMP$_9$



In an effort to move away from radioiodine and what appears to be *in vivo* dehalogenase activity, we next synthesized and radiolabeled a dual labeled CMP instilled with both IRDye800CW and CHX-A-DTPA, which chelates In-111 under gentle conditions. Scheme 2 depicts the structure and labeling conditions.

We then tested this analog in a subcutaneous model of PCa in which mice (n = 4) bore one each of a PC-3 PIP tumor with generally higher collagen remodeling and one PC-3 flu tumor with less. Figure 2 shows the SPECT-CT images of CMP distribution in the first 6 hours after injection. One mouse was injected with still-caged (inactive) CMP while the remaining four mice were injected with UV-activated labeled CMP. Scant tumor accumulation was observed in the mice until 6 hours after injection. At that time, the PC-3 PIP tumor retained radiotracer signal at the edges of the tumor while the PC-3 flu tumor displayed very little (3D projection and inset). The mouse injected with still-caged CMP displayed very little tumor uptake although had some uptake in the neck. All mice displayed high radiotracer uptake in the liver and kidneys, as it also appears using NIRF detection (data not shown).

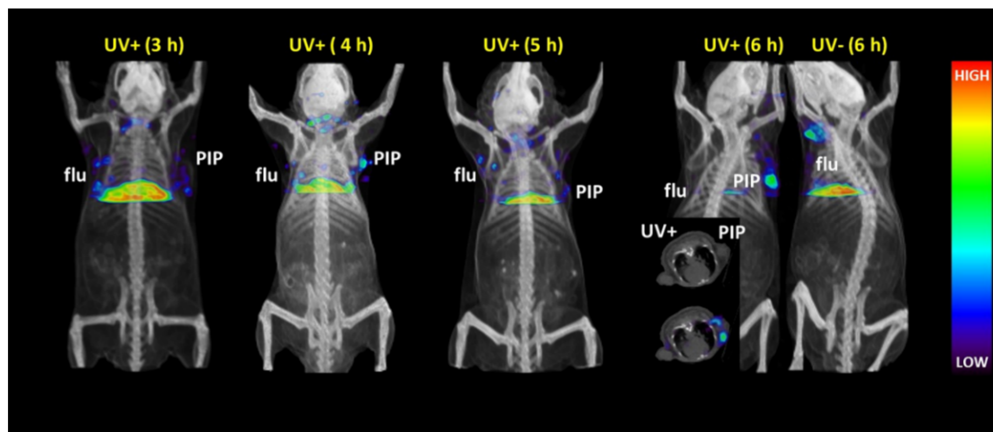


Figure 2. *[^{111}In](CXH-A)-(lys$_2$)-DTPA-CMP$_9$-(cys$_1$)-IRDye800CW SPECT-CT at 3-6 h post-injection.* Five mice, each bearing a single PC-3 PIP (higher Δ collagen) and PC-3 flu (lower Δ collagen) tumor xenograft, were injected with radiolabeled CMP and imaged by SPECT-CT at the indicated times. PIP tumor uptake of CMP was favored in most mice with the 6 h time point showing clear accumulation of de-caged CMP while still-caged CMP displayed almost no uptake. Liver and renal uptake dominated however (not shown), making tumor uptake appear relatively weak.

At 24 h post-radiotracer injection, higher tumor uptake was apparent in all of the mice except the mouse injected with still-caged (inactive) CMP (Fig. 3). CMP distribution within the tumors was enriched at the edges where growth is occurring. By 24

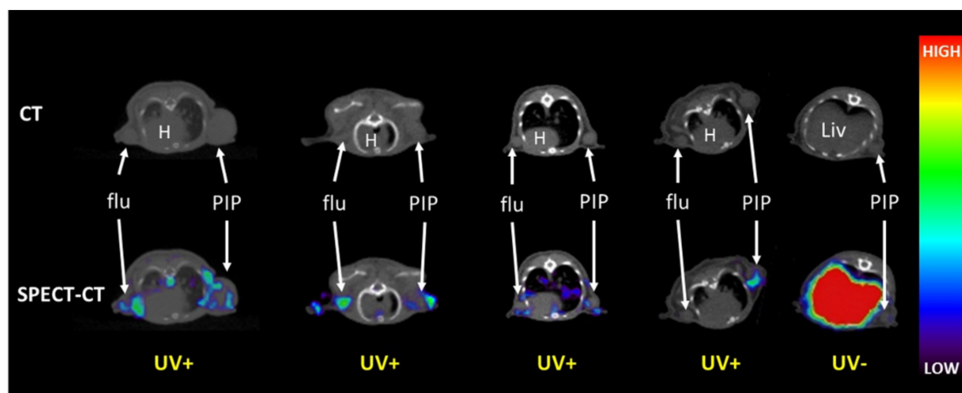


Figure 3. [^{111}In](CXH-A)-(lys$_2$)-DTPA-CMP$_9$-(cys$_1$)-IRDye800CW SPECT-CT at 24 h post-injection. By 24 h post-injection, the same mice in figure 3 displayed stronger CMP uptake at the edges of the tumors, where the tumors are expanding. At this time point, both PC-3 PIP and PC-3 flu tumors are taking up the CMP probe except in the mouse receiving still-caged CMP. "H" represents heart and "Liv" represents liver.

h, PC-3 flu tumors were also taking up labeled CMP, except in one mouse. Liver and renal uptake of CMP persisted, even in the still-caged CMP mouse, indicating non-specific metabolic excretion in these tissues. This is undesirable and will prompt the pursuit of dye-free CMP analogs as the dye is targeting this excretion pathway.

We finished probing the library of PCa xenogratfs to reflect androgen receptor sensitivity status, expression of the biomarker PSMA and speed at which the tumors were growing. Mice bearing an LAPC4 (AR+, androgen sensitive, PSMA moderate, moderately rapid growth rate) or a C4-2 (AR_{mut}, androgen insensitive, PSMA low, slow growth rate of primary with fast growth rate of local secondary) were injected with a fluorescent-only CMP-IRDye800CW analog followed by MMPSense680 (Perkin Elmer) and the tumors were harvested 72 h (CMP), 24 h (MMPSense) later. Frozen sections of 20 µm thickness were made and scanned using a LI-COR Odyssey scanner. The scans revealed very low CMP uptake in the slow growing primary C4-2 tumor while the fast growing local secondary offshoots displayed CMP binding throughout (Fig. 4, *in vivo* inset and section). MMPSense680 probe showed high MMPase activity in the secondary offshoots but not in the large primary tumor, providing rationale for extracellular matrix remodeling in the secondary tumors where CMP binding is observed.

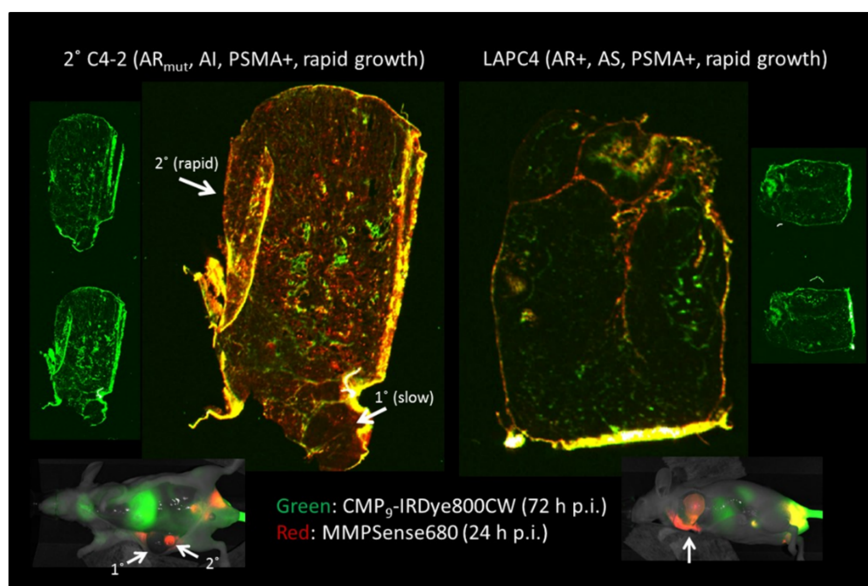


Figure 4. *CMP and MMPSense activity in C4-2 and LAPC4 xenografts*. Athymic nude mice bearing either a slow growing C4-2 xenograft with fast-growing secondary growth or a fast-growing LAPC4 xenograft were injected with 4 nmol of CMP-IRDye800CW and a single dose of MMPSense680. The mice were sacrificed and imaged at 72 h post-CMP and 24 h post-MMPSense. The tumors were then collected, sectioned and imaged to reveal the pattern of CMP distribution relative to MMP activity. Fast-growing tumor tissue took up and retained CMP probe while slow-growing primary C4-2 tissue took up very little.

LAPC4 xenografts display a moderately rapid rate of growth and were found to contain a somewhat lower amount of CMP uptake compared with the fast-growing C4-2 secondary tumors. MMPSense uptake was lower than in the secondary C4-2 growths and took on a focal branching pattern, which is also seen in the CMP distribution (green inset). Overall, these uptake patterns in LAPC4 and C4-2 are consistent with the patterns observed for tumor growth rate in the rest of the xenograft library.

We achieved what appeared to be a biologically stable dual-modality CMP but it suffers from high liver and kidney uptake. We have determined that CMP binding does clearly distinguish between tumor and benign enlarged lymph nodes in our mouse model library of xenograft lines.

Additionally, CMP uptake appears to distinguish between rapidly growing and slowly growing xenografts while MMPSense probe does not.

As mentioned in the serum stability part of the report, we have recently determined that CMPs labeled through maleimide linkage with IRDye800CW are especially prone to reverse Michael transconjugation of the dye onto serum protein thiols *in vivo*. This is the reason why CMP conjugates targeted different tissues *in vivo* based on the dye present. Instead of visualizing the remodeling of collagen I in vivo, CMP-IRDye800CW conjugates were largely reporting on the distribution of transconjugated serum proteins within the body and PCa tumors.

In conclusion, the chemistry conditions for the conjugation or radiometallation of IRDy-labelled CMPs appear to greatly affect the integrity of the dye and/or the nitrobenzoyl photo cage group. Deviations away from pH 6-8 and heating are to be avoided. Experiments using CMP9-IRDye800CW to map collagen remodeling signatures within mice bearing a range of selected subcutaneous prostate cancer xenografts resulted in the observation of a trend in which CMP-800 accumulates with higher density in rapidly growing tumors. This trend was also observed in similarly prepared mice bearing subcutaneous xenografts of pancreatic cancer origin. We finished probing the existing prostate cancer library and have confirmed the trend of CMP binding to growth kinetics to be conserved. Unfortunately, that observed signature within tumors reflects both collagen binding and accumulation of transconjugated dye-labeled serum proteins.

The concurrent binding of MMPSense™, reporting on the enzymatic activities of MMPs 2, 3, 9 and 13, to each of the tumor models described above revealed no trend in discerning tumor growth kinetics, propensity to metastasize and equally bound to tumor xenografts and benign inflamed lymph nodes while CMP-800 bound to tumors but not benign inflamed lymph nodes.

The goal to probe a frozen library of human PCa sections was not performed due to the finding that CMP-IRDye800CW cannot be used in vivo due to serum protein side chemistry.

2-2. Collagen hybridizing peptides (CHPs) have a great potential for use in targeted drug delivery, diagnostics, and regenerative medicine due to their ability to specifically bind to denatured collagens associated with many pathologic conditions. Since peptides generally suffer from poor enzymatic stability resulting in rapid degradation and elimination *in vivo*, CHP's serum stability is a critical parameter that may dictate its pharmacokinetic behavior. Here, we reported the serum stability of a series of monomeric CHP derivatives and established how peptide length, amino acid composition, terminal modification, and linker chemistry influence their availability in serum. We showed that monomeric CHPs comprised of the collagen-like Gly-Pro-Hyp motif are resistant to common serum proteinases and that their stability can be further increased by simple N-terminal labeling which negates CHP's susceptibility to proline-specific exopeptidases. When fluorescent dyes were conjugated to CHP *via* maleimide-thiol reaction, the dye could transfer from CHP onto serum proteins (e.g. albumin) resulting in an unexpected drop in signal during serum stability assays and off-target accumulation during *in vivo* tests. This work was the crucial first step toward understanding the pharmacokinetic behavior of CHPs which can facilitate the development of CHP-

based theranostics. This work was published in Molecular Pharmaceutics (2017).

AIM 3 (updated)
One of the reasons for poor SPECT-CT result may have been due to low binding affinity of the CHP. In order to improve the binding affinity, we prepared a fibrous form of CHP by conjugating beta-sheep peptide motif to CHP. We also explored using our peptide to detect mechanical damage to collagen in tendon and artery.

3-1. CHP have been demonstrated as a powerful vehicle for targeting denatured collagen produced by disease or injury. Conjugation of β-sheet peptide motif to the CHP resulted in self-assembly of nonaggregating β-sheet nanofibers with precise structure. Due to the molecular architecture of the nanofibers which puts high density of hydrophilic CHPs on the nanofiber surface at fixed distance, the nanofibers exhibited high water solubility, without any signs of intramolecular triple helix formation or fiber-fiber aggregation. Other molecules that were flanked with the triple helical forming GlyProHyp repeats can readily bind to the nanofibers by triple helical folding, allowing facile display of bioactive molecules at high density. In addition, the multivalency of CHPs allowed the nanofibers to bind to denatured collagen in vitro and in vivo with extraordinary affinity, particularly without preactivation that unravels the CHP homotrimers. The length of the nanofibers could be tuned from micrometers down to 100 nm by simple heat treatment, and when injected intravenously into mice, the small nanofibers could specifically target denatured collagen in the skeletal tissues with little target-associated signals in the skin and other organs. The CHP nanofibers can be a useful tool for detecting and capturing dColl, understanding how ECM remodelling impacts disease progression, and development of new delivery systems that target such diseases.

3-2. Mechanical injury to connective tissue causes changes in collagen structure and material behaviour, but the role and mechanisms of molecular damage have not been established. In the case of mechanical subfailure damage, no apparent macroscale damage can be detected, yet this damage initiates and potentiates in pathological processes. Here, we utilized collagen hybridizing peptide (CHP), which binds unfolded collagen by triple helix formation, to detect molecular level subfailure damage to collagen in mechanically stretched rat tail tendon fascicle. Our results directly revealed that collagen triple helix unfolding occurs during tensile loading of collagenous tissues and thus is an important damage mechanism. Steered molecular dynamics simulations suggested that a likely mechanism for triple helix unfolding is intermolecular shearing of collagen α-chains. Our results elucidated a probable molecular failure mechanism associated with subfailure injuries, and demonstrated the potential of CHP targeting for diagnosis, treatment and monitoring of tissue disease and injury.

3-3. In addition to the above work on mechanical damage to tendon, we also investigated CHP localizing to the mechanically damaged collagens in blood vessels. It is well established that overstretch of arteries alters their mechanics and compromises their function. However, the underlying structural mechanisms behind these changes are poorly understood. Utilizing a recently developed collagen hybridizing peptide (CHP), we demonstrated that a single mechanical overstretch of an artery produces molecular-level unfolding of collagen. In addition, imaging and quantification of CHP binding revealed that overstretch produces damage (unfolding) among fibers aligned with the direction of loading, that damage increases with overstretch severity, and that the

onset of this damage is closely associated with tissue yielding. These findings held true for both axial and circumferential loading directions. Our results were the first to identify stretch-induced molecular damage to collagen in blood vessels. Furthermore, our approach is advantageous over existing methods of collagen damage detection as it is non-destructive, readily visualized, and objectively quantified. This work opens the door to revealing additional structure-function relationships in arteries. We anticipate that this approach can be used to better understand arterial damage in clinically relevant settings such as angioplasty and vascular trauma. Furthermore, CHP can be a tool for the development of microstructurally-based constitutive models and experimentally validated computational models of arterial damage and damage propagation across physical scales.

Key Research Accomplishments (for full grant period)

Denatured collagen imaging by CMPs

1. We showed that fluorescently labeled CHP robustly visualizes the pericellular matrix turnover caused by proteolytic migration of cancer cells within 3D collagen culture, without the use of synthetic fluorogenic matrices or genetically modified cells.
2. We showed that the new CHP, $(GfO)_9$, tagged with a near-infrared fluorophore, enables *in vivo* imaging and semi-quantitative assessment of osteolytic bone lesions in mouse models of multiple myeloma.
3. CHPs enabled direct localization and quantification of collagen degradation in isolated tissues within pathologic states ranging from osteoarthritis and myocardial infarction to glomerulonephritis and pulmonary fibrosis, as well as in normal tissues during developmental programs associated with embryonic bone formation and skin aging.

Serum stability of CMPs

1. The neutral and hydrophilic peptide sequence of CMP deters recognition by common serum enzymes and prevents non-specific binding to other biomolecules thereby extending CHP's availability in serum.
2. CHP stability was increased by N-terminal modification with fluorescent dyes which capped the free N-terminus.
3. Ac-C(IR800)-Ahx-$^{NB}(GPO)_9$ exhibited noticeable off target signal, particularly in the liver, which is believed to be caused by transfer of IR800 dye from CHP to serum proteins *via* thiol exchange reaction.
4. Based on all of our tests, we conclude that the most ideal NIRF-CHP for imaging denatured collagen *in vivo* is IR680-Ahx-$(GPO)_9$.
5. Xenograf tumor model does not create extensive collagen remodeling and is not suited for tumor imaging by denatured collagen binding.

In vivo PCa imaging using new CMP

1. Five new CMP analogs were synthesized, radiolabeled and evaluated *in vivo*.
2. A library of six prostate cancer xenograft lines were evaluated *in vivo* and *ex vivo* for CMP-IRDye800CW binding and MMPSense-680 binding.
3. Three additional pancreatic cancer xenograft lines were tested to confirm CMP binding trends observed for xenograft growth kinetics.
4. [^{125}I]iodotyrosyl-CMP9 was viualized by SPECT-CT and specifically accumulates in skeleton
4. The dual-labeled CHX-A-DTPA-IRDye800CW-CMP is stable *in vivo* and is retained within growing prostate tumor xenografts.

Next generation CMPs and targeting mechanical damage in collagen

1. Conjugation of β-sheet peptide motif to the CHP resulted in self-assembly of nonaggregating β-sheet nanofibers with precise structure. Due to the molecular architecture of the nanofibers which puts high density of hydrophilic CHPs on the nanofiber surface at fixed distance, the nanofibers exhibited high water solubility, without any signs of intramolecular triple helix formation or fiber-fiber aggregation.
2. Using CHPs, we demonstrated for the first time that mechanical damage in collagen-rich, load-bearing tissue such as tendon and blood vessel is partly caused to denaturation of collagen triple helix.

Reportable Outcomes (for full grant period)

Journal Publication

1. Lucas Bennink, B. J. Kim, B. H. San, I. Shin, D. Yoon, Y. Li and S. M. Yu (2018) “Visualizing collagen proteolysis by peptide hybridization: from 3D cell culture to in vivo imaging” *submitted to Biomaterials (Manuscript ID: BIOMAT-S-18-01706)*

2. T. Y. Feinberg, H. Zheng, R. Liu, M. S. Wicha, S. M. Yu, and S. J. Weiss (2018) “Divergent Tissue-Remodeling Strategies Differentiate Branching Morphogenesis from Neoplastic Invasion Program” *submitted to Nature Cell Biology (Manuscript ID: NCB-W36752)*

3. M.I. Converse, J.T. Ingram, R.G. Walther, Y. Li, S.M. Yu, and K.L. Monson (2018) "Molecular level detection and characterization of stretch-induced collagen unfolding in cerebral arteries" *Acta Biomaterialia*, 67, 307.

4. San BH, Hwang J, Sampath S, Li Y, Bennink, LL, and Yu SM (2017) "Self-assembled water-soluble nanofibers displaying collagen hybridizing peptides" *J. Am. Chem. Soc.,* 139, 16640-49.

5. Jared L. Zitnay, Yang Li, Boi-Hoa San, Shawn P. Reese, S. Michael Yu*, and Jeffrey A. Weiss* (2017) "Molecular level detection of mechanical damage in collagen fibers assisted by collagen hybridizing peptide" *Nature Communication*, 8, 14913 [*Co-corresponding authors].

6. Hwang J, Huang Y, Burwell TJ, Peterson NC, Connor J, Weiss SJ, Yu SM, and Li Y (2017) "*In situ* imaging of tissue remodeling with collagen hybridizing peptides" *ACS Nano*, 11, 9825-35.

7. Bennink LL, Smith DJ, Foss CA, Pomper MG, Li Y, and Yu SM (2017) "High serum stability of collagen hybridizing peptides" *Molecular Pharmaceutics*, 14, 1906–1915.

8. Hwang J, San BH, Turner NJ, White LJ, Faulk DM, Badylak SF, Li Y, and Yu SM (2017) "Molecular assessment of collagen denaturation in decellularized tissues using a collagen hybridizing peptide" *Acta Biomaterialia*, 53, 268–278.

9. Boi-Hoa San, Yang Li, and S. Michael Yu (2016) "Nanoparticle assembly and gelatin binding mediated by triple helical collagen mimetic peptide" *ACS Applied Materials and Interfaces*, 8, 19907–19915.

10. Hendra Wahyudi, Amanda A. Reynold, and S. Michael Yu (2016) "Collagen Targeting for Diagnostic Imaging and Therapy" *Journal of Controlled Release*, *240, 323-331.*

11. Chan TR, Stahl PJ, Li Y, and Yu SM (2015) "Collagen-gelatin mixtures as wound model, and substrates for VEGF-mimetic peptide binding and endothelial cell activation" *Acta Biomaterialia* 15, 164-172.

12. Li Y, San BH, Kessler JL, Kim JH, Xu Q, Hanes J, and Yu SM (2015) "Non-covalent photo-patterning of gelatin matrices using caged collagen mimetic peptides" *Macromolecular Bioscience* 15, 52-62.

13. Santos JL, Li Y, Culver HS, Luo H, Yu SM*, and Herrera-Alonso M*(2014) "Conducting polymer nanoparticles decorated with collagen mimetic peptides for collagen targeting" *Chem. Commun*. 50, 15045-15048 [*Co-corresponding authors].

14. P. J. Stahl, T. R. Chan, Y.-I. Shen, G. Sun, S. Gerecht, and S. M. Yu (2014) "Capillary Network-Like Organization of Endothelial Cells in PEGDA Scaffolds Encoded with Angiogenic Signals *via* Triple Helical Hybridization" *Adv. Funct. Mater, 24, 3213-3225*

15. Y. Li, C. A. Foss, M. G. Pomper, and S. M. Yu (2013) "Imaging denatured collagen strands *in vivo* and *ex vivo via* photo-triggered hybridization of caged collagen mimetic peptides" *J. Vis. Exp. (JoVE)*, 83, e51052, doi:10.3791/51052.

16. Y. Li, and S. M. Yu, (2013) "Targeting and mimicking collagens *via* triple helical peptide assembly. *Curr. Opin. Chem. Biol*." 17, 968.

17. Y. Li, D. Ho, H. Meng, T. R. Chan, B. An, H. Yu, B. Brodsky, A. S. Jun, and S. M. Yu (2013) Direct detection of collagenous proteins by fluorescently labeled collagen mimetic peptides. *Bioconjugate. Chemistry.* 24, 9.

Book Chapter

1. Y. Li and S.M. Yu, "In situ detection of degraded and denatured collagen via peptide hybridization," *Methods in Molecular Biology/Collagen* Eds I. Sagi, N. Afratis: Springer, 2018, *in press.*
2. H. Wahyudi, B.H. San, and S.M. Yu, "Targeting disease-related proteins *via* secondary protein structure-formation." *Bio-inspired Materials from Mother Nature*, Ed. S.W. Lee: World Scientific Publishing, 2018, *in press*.

Patents

(P-1) J. Kessler, M.S. Yu, Y. Li. "Dimeric collagen hybridizing peptides and methods of using" US20180000960A1, *pending.*

(P-2) Yu, Michael S., Y. Li, H. Wahyudi. "Modified collagen hybridizing peptides and uses thereof" US20170112940A1, *pending.*

Invited Presentations

(1) "In Situ Detection of Degraded Collagen via Triple Helical Hybridization: From Histology to In Vivo Imaging" Korean Academy of Science & Technology Workshop (KAST), Salt Lake City, UT, June 2018.

(2) "In Situ Detection of Degraded and Denatured Collagen via Triple Helical Hybridization: From Histology and In Vivo Imaging" Korea Institute of Science & Technology (KIST), Seoul, Korea, Nov. 2017.

(3) "Detecting Enzymatic and Mechanical Stress-Induced Unfolding of Collagen by Triple Helical Peptide Hybridization" Dept of Mechanical Engineering, Korea University, Seoul, Korea, Nov 2017.

(4) "In Situ Detection of Degraded and Denatured Collagen via Triple Helical Hybridization: From Histology and In Vivo Imaging" Korea Research Institute of Chemical Technology (KRICT), Daejun, Korea, Nov. 2017.

(5) "Targeting Collagen Remodeling Activity by Peptide Hybridization: Prospects for New Diagnostics and Therapeutics" Thursday Investigator Meeting, Huntsman Cancer Institute, Salt Lake City, Oct 2017.

(6) "In Situ Detection of Degraded and Denatured Collagen via Triple Helical Hybridization: From Histology and In Vivo Imaging" **Gordon Research Conference** on Collagen, New London, NH, July 2017.

(7) "Collagen Hybridizing Peptide: Targeted Binding, Self-Assembly and Biomedical Application" American Chemical Society National Meeting, San Francisco CA, March 2017.

(8) "3Helix collagen targeting technology" Translational Medicine Symposium, University of Utah, Salt Lake City, Feb, 2017

(9) "Collagen Hybridizing Peptide: Self-Assembly and Denatured Collagen Targeting" World Biomaterials Congress, Montreal Canada, 2016.

(10) "Collagen Hybridizing Peptide: Self-Assembly and Denatured Collagen Targeting" Materials Research Society, Phoenix, AZ, March 2016.

(11) "Targeting Collagen Remodeling Activity by Triple Helical Peptide Hybridization: Prospects for New Diagnostics and Therapeutics" School of Pharmacy, University of Utah, Salt Lake City, March 2016.

(12) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics"
Department of Chemical & Biomolecular Engineering, Seoul National University, Seoul, Korea, November 2015.

(13) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics"
Department of Biomedical Engineering, Korea University, Seoul, Korea, November 2015.

(14) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics"
Department of Biomedical Engineering, POSTECH, Pohang, Korea, November 2015.

(15) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics"
Department of Biomedical Engineering, Hanyang University, Seoul, Korea, November 2015.

(16) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics" US-Korea Conference, Altanta, GA, August 2015.

(17) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics" Canadian Chemistry Conference, Ottawa, Canada, June 2015.

(18) "Targeting Collagen Strands by Peptide Hybridization: from Histology to Tissue Engineering and Theranostics" 17th International Drug Delivery Symposium, Salt Lake City, UT, June 2015.

(19) "Nanoparticle assembly and gelatin binding mediated by collagen mimetic peptide hybridization" American Chemical Society National Meeting, Denver, Co, March 2015.

(20) "Targeting Collagens Strand by Triple Helical Hybridization" American Chemical Society National Meeting, Denver, Co, March 2015.

(21) "Targeting Collagens Strand by Triple Helical Hybridization" University of Utah, Department of Biochemistry, Salt Lake City, UT, January 2015.

(22) "Targeting Collagens Strand by Triple Helical Hybridization" US-Korea Conference, San Francisco, CA, August 2014.

(23) "Targeting Collagens Strand by Triple Helical Hybridization" KIST, Seoul, Korea, July 2014.

(24) "Helical Proteins for Materials Application: from piezoelectric nanofibers to collagen hybridization" SPE ANTEC, Las Vegas, NV, April, 2014.

(25) "Targeting Collagens Strand by Triple Helical Hybridization" American Chemical Society National Meeting, Dallas, TX, March 2014.

(26) "Targeting Collagens Strand by Triple Helical Hybridization" American Chemical Society National Meeting, Dallas, TX, March 2014 (confirmed invited speaker for Poly Symposium).

(27) "Targeting Collagens Strand by Triple Helical Hybridization" University of Utah, Department of Bioengineering, Salt Lake City, UT, April 2013.

(28) "Targeting Collagens Strand by Triple Helical Hybridization" SUNY at Buffalo, Department of Biomedical Engineering, Buffalo, NY, March 2013.

(29) "Targeting Collagens Strand by Triple Helical Hybridization" University of Connecticut, Department of Biomedical Engineering, February 2013.

(30) "Targeting Collagens Strand by Triple Helical Hybridization" University of Delaware, Department of Biomedical Engineering, January 2013.

Conclusion

This research program resulted in publication of over 17 high impact publication (this include two papers under review), 2 book chapters, 2 patent applications, and 30 invited talks. We believe that it was a highly successful program in terms of advancing the science in regards to understanding how collagen remodeling is related to cancer progression and the program opened up new possibilities for prostate cancer imaging and therapy. We understand that our high hope for using CMP (aka CHP) to gauge the level of invasiveness, particularly with the use of radioactive imaging, was met with challenges. However with our understanding of stable serum stability of CHP, the ability to improve the binding affinity using multi-ligand effects (e.g. nanofibers) and our demonstration of CHP binding to many different types of diseases, the future of CHP in cancer detection and other major debilitating human disease is brighter than ever.