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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

Note that this is a revised annual report. This revised report now includes coherent narrative relating progress to the work as outlined in the approved statement of work as well as the activity of the collaborating/partnering PI.

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, ¹⁸F), 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 metastatic 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 previously 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 to the animal model: the level of collagen remodeling produced by xenograft prostate tumor model may not have been high enough to be detected by CMP.

In this report period (2015-2016), we investigated i) the serum stability of collagen mimetic peptide (Yu lab), and ii) development and SPECT-CT imaging of CMP derivatives that have mild radiometal loading condition (Pomper lab). The serum stability test is 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.

Body

1. Serum Stability of Collagen Mimetic Peptide (Yu Lab)

Although understanding the pharmacokinetics of the CMPs is essential for their diagnostic applications, we know very little about the CHPs stability against proteinases in blood. This has become particularly important since our prior work on SPECT-CT imaging of PCa using CMP failed to give positive results and that there is now well accepted notion that the fluorescent dye conjugated to peptide via Cys-maleimide link can be transferred to serum protein. This made the following scenario possible which could explain the positive fluorescence signal but negative radioactive signal in the PCa animal model: the CMP degrades relatively quickly once injected into the animal (results in loss of radiometal from the peptide), but the fluorescent dye is transferred to serum protein and that the serum protein is accumulating at the tumor site. Therefore, it became clear to us that before any further development of CMP-based imaging agent, we first had to test the stability of the peptide in serum.

Without any strategic structural modification, peptides generally suffer from short lifetime in the blood stream because they are easily digested by the proteinases abundant in serum. It is well known that collagen and collagen mimetic peptides, which are in triple helical conformation, are resistant to most proteases except for collagen specific ones, such as MMPs and cathepsin K. In fact, most of the intravenously injected triple helical peptides are eliminated through glomerular filtration in the kidneys. Despite abundance of literature on the serum stability of triple helical CMPs, there is limited information on the serum stability for non-triple helical, *single strand* CMP. Our previous *in vivo* NIRF imaging results demonstrated that the binding of CMP in the targeted tissue is stable for days, suggesting high serum stability despite it being non-triple helical. Therefore, we set off to study the proteolytic stability of *monomeric* CMP in serum as the first step in assessing its pharmacokinetics. Such study will also benefit a number of applications based on collagen mimetic peptides in developing tissue scaffolds, drug delivery systems, and hydrogels. Here we investigated the serum stability of a series of monomeric CMP derivatives that vary in length, amino acid composition, and terminal modifications as determined by reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS). Our results indicate that the CMP peptides based on GPO repeats are stable in serum even in monomeric form, and that their stability can be further increased through N-terminal modification. Additionally, we found out that when a fluorescent dye is conjugated to CMP *via* a thioether link (formed by maleimide-thiol reaction), the dye can transfer from CMP to serum proteins (e.g. albumin) giving off-target distribution during *in vivo* imaging experiments. Finally, we suggest the most ideal CHP structure for targeting and imaging denatured collagens associated with prostate cancer and other diseases *in vivo*.

There are two main methods used for determining a proteolytic degradation profile of a compound in biological fluids: blotting analysis and direct stability assays. Blotting analysis is useful for investigating the action of a specific peptidase, while stability assays can provide a wide range of information, including the type of peptidase, enzyme specificity, and the degradation profile for a specific peptide sequence. It has been well demonstrated that the *in vivo* stability of peptides, which could help understand the peptide's pharmacokinetics, can be reliably modeled *in vitro* using either plasma or serum.

Our study confirmed that the triple helical collagen mimetic peptide, (GPO)₉, is highly stable in serum and that the single strand CMPs based on G-P-P/O repeats are generally resistant to proteolytic degradation when compared to peptide comprised of non-G-P-P/O sequence (Figure 1). The Gly, Pro, and Hyp, are hydrophilic and neutral amino acids which are all considered to be inert amino acids with respect to protein interactions. Typically, charged, hydrophobic or aromatic amino acids occupy the proteolytic location because they can interact with specific residues for cleavage site recognition. For example, thrombin and factor X which are abundant in blood specifically cleave at the carboxyl side of Arg, and hydrophobic and aromatic residues (such as Ala, Phe, Ile, Leu, Val) are generally found near the cleaved bond. It is the absence of such charged or hydrophobic residues that makes the CMP resistant to common serum proteases. This fact is most clearly reflected in our result where a peptide that was virtually indigestible became highly susceptible to digestion (completely digested in 5 hr) after only four Hyp→Lys substitutions [Figure 2A, CF-(GPO/K)₉]. Adding CF to the N-termini of CMP improved the serum stability, especially for those with short sequence (Figure 2 right).

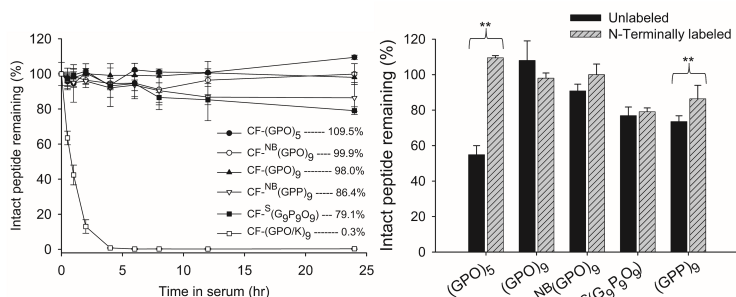


Figure 2. Stability profiles of peptides with N-terminal CF labels in mouse serum (left) and comparison of stability between peptides with and without N-terminal labeling (right). Amounts of intact peptides were determined from the areas under the HPLC peak after incubation in serum at 37 °C for indicated time intervals. All tests were run in triplicates. Error bars represent standard deviation. **p < 0.05 (Student's T-test), and differences in all other pairs are insignificant (p > 0.05).

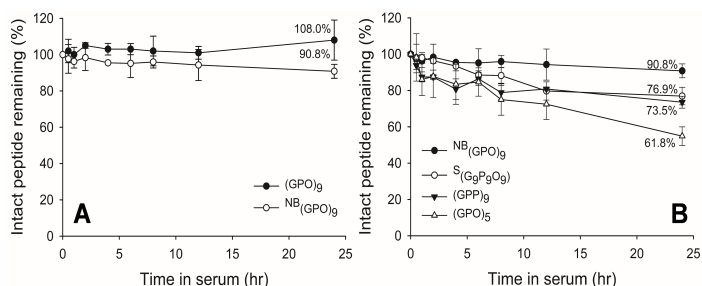


Figure 1. Stability profiles of unlabeled peptides in mouse serum. (A) Serum stability of triple helical (GPO)₉ and monomeric NB(GPO)₉. Both trimeric and monomeric CMPs exhibit high serum stability. (B) Serum stability of other unlabeled CHPs. (GPO)₅, (GPP)₉, and the scrambled control peptide S(G₉P₉O₉) all exhibit lower serum stability than NB(GPO)₉, with (GPO)₅ showing the lowest stability with 61.8% remaining after 24 hr. Amounts of intact peptides were determined from the areas under the HPLC peak after incubation in serum at 37 °C for indicated time intervals. Percentages of intact peptides remaining after 24 hr are shown. All tests were run in triplicates.

Although CMPs based on GPP and GPO triplets are resistant to common proteases, they are susceptible to a family of proteases specialized for hydrolyzing proline containing peptides. This family contains both endo- and exo-peptidases and their mechanisms have been fully documented in the literature (Figure 3). Some of the well-known proteases in this family are: prolyl endopeptidase (PE) which can hydrolyze the bond on the

carboxyl side of proline within the interior of a peptide, dipeptidyl peptidases II and IV (DPPs) which are exopeptidases that can cleave at N-terminal proline residues in blocks of two to three amino acids, and finally amino peptidase P (APP) and proline iminopeptidase (PIP), both of which can release a single N-terminal amino acid by cleaving either the amino side or carboxyl side of Pro, respectively. We were able to identify three structural features critical for the serum stability of CMP comprised of GPO and GPP sequences: (i) capping the N-terminus of the peptide with fluorophores or other moiety protects CHP from exopeptidase digestion, (ii) long peptide length protects the CHP against endopeptidase digestion, and (iii) the presence of hydroxyproline reduces endopeptidase activity.

The availability of intact CMP-dye conjugates in serum depends not only on resistance to proteinases but also on the chemistry used to link the peptide and the dye molecules. We found that the rapid decrease of serum concentration of Ac-C(IR800)-Ahx^{NB}(GPO)₉ was not due to peptide degradation but due to the unexpected transfer of IR800-maleimide group from CMP to serum proteins through a thiol-exchange reaction (Figures 4). As reported in recent years, this retro-Michael side reaction occurs readily in biological environments where excess thiol groups are present and is a major drawback of the popular thiol-maleimide chemistry, particularly in the preparation of antibody-drug conjugates (ADC). The NIRF-CHP linked by an amide bond, IR800-Ahx^{NB}(GPO)₉, was

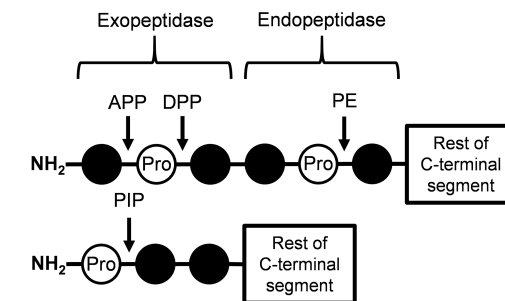


Figure 3. Schematic of cleavage positions for proline specific peptidases. This shows the general cleavage sites for proline specific peptidases on a peptide with a free N-terminus. DPPs have the highest cleavage efficiency when removing dipeptides from sequences as shown, however, they can also cleave tri- and tetra-peptides on the carboxyl side of proline residues at a reduced efficiency. PIP prefers to cleave a single proline residue off sequences but can cleave di- and tri-peptides on the carboxyl side of prolines at reduced efficiency as well. APP is responsible for the removal of a single residue on the amino side of proline. APP: aminopeptidase P, DPP: dipeptidyl peptidase, PE: prolidase, PIP: proline iminopeptidase.

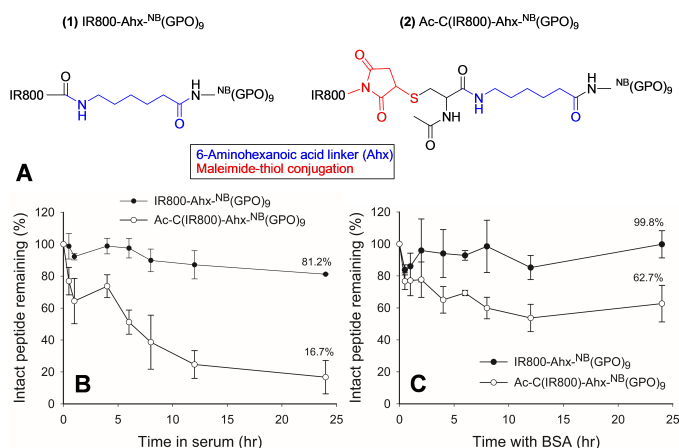


Figure 4. Serum stability of IR800-labeled CMPs. (A) The two different linker chemistries used to conjugate IR800-Ahx^{NB}(GPO)₉ and Ac-C(IR800)-Ahx^{NB}(GPO)₉. (B) Stability profiles of IR800-Ahx^{NB}(GPO)₉ and Ac-C(IR800)-Ahx^{NB}(GPO)₉ in mouse serum. (C) Percentages of intact IR800-Ahx^{NB}(GPO)₉ and Ac-C(IR800)-Ahx^{NB}(GPO)₉ remaining after incubation with BSA without serum. All tests were run in triplicates.

significantly more stable than the thioether linked version in serum, resulting in more efficient targeting of skeletal tissues and less off-target distribution *in vivo* (Figure 5). These results highlight that the NHS-mediated conjugation is more reliable and should be favored in preparation of future CMP theranostics.

In conclusion, we assessed the serum stability of multiple CMP derivatives and established how length, amino acid composition, N-terminal labeling, and linker chemistry contributed to the overall availability of monomeric CHPs in serum over time. We

demonstrated that, similar to triple-helical CMPs, monomeric CMPs comprised of repeating Gly-Pro-Hyp triplets are highly resistant to enzymatic degradation by many serum proteases and maintain elevated levels of stability despite their small size. The neutral and hydrophilic peptide sequence deters recognition by common serum enzymes and prevents non-specific binding to other biomolecules thereby extending CMP's availability in serum. Although CMPs avoid recognition by common serum enzymes, the specific class of enzymes known as proline-specific peptidases can act on CHPs with free N-termini, since a majority of them are exopeptidases. Therefore, CMP stability was increased by N-terminal modification with fluorescent dyes which capped the free N-terminus. As reported previously, the dye conjugated CMPs can target denatured collagens in the bones and cartilage of mice *in vivo* under normal conditions due to the high remodeling activity of the skeletal system. Our *in vivo* experiments indicated that although skeletal targeting behavior was observed in all NIRF-conjugated CMPs, there were subtle differences in bio-distribution due to differences in the linker chemistry and the structure of the dye molecules. Specifically, 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 CMP to serum proteins *via* thiol exchange reaction as supported by the emergence of albumin-dye conjugates during the serum stability assay. Based on all of our tests, we conclude that the most ideal NIRF-CMP for imaging denatured collagen *in vivo* is IR680-Ahx-(GPO)₉. CMP's offer high serum stability which is uncommon for conventional peptides that suffer from poor stability *in vivo*. CMP's serum stability provides strong foundation for transforming this peptides into an *in vivo* delivery vehicle for theranostic agents in managing a vast number of disease states that are characterized by excessive collagen remodeling, such as arthritis, cancer and fibrosis.

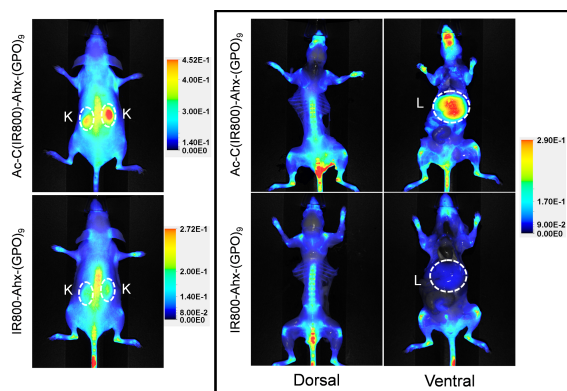


Figure 5. NIR fluorescence imaging showing the *in vivo* distribution of Ac-C(IR800)-Ahx-(GPO)₉ and IR800-Ahx-(GPO)₉ in mice 48 hr post tail vein injection. Both conjugates showed similar uptake in bones and joints due to the presence of denatured collagen produced during normal remodeling, while only Ac-C(IR800)-Ahx-(GPO)₉ showed high intensity signals in the kidneys and liver. The kidneys (K) and livers (L) are highlighted by dashed circles. The images in the box were taken after skin removal for clear display of signals from the skeleton and internal organs, and their fluorescence intensities were adjusted to the same scale for direct comparison. All *in vivo* experiments were performed three times with similar results.

2. PCa Imaging Using New CMP Structure with Mild Radiometal Loading Conditions (Pomper Lab)

We learned from the results above that our CMP comprised of ProHypGly sequence with N-terminal modification and with amide linked fluorescence dye has high serum stability and is a prime candidate for imaging invasive PCa. 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, in this funding period, 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 6: 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-CMP₉-CONH₂ and photo decaying (lower left box).

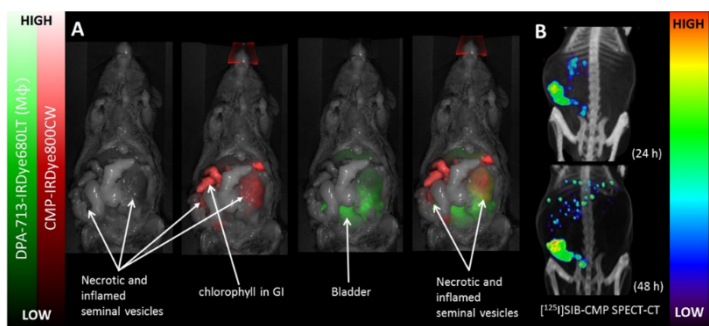
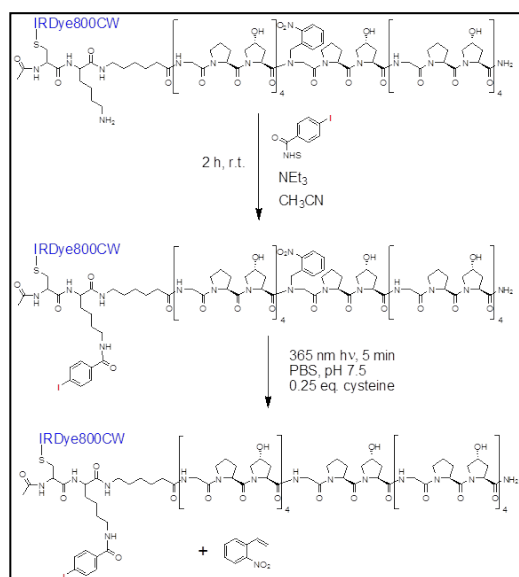
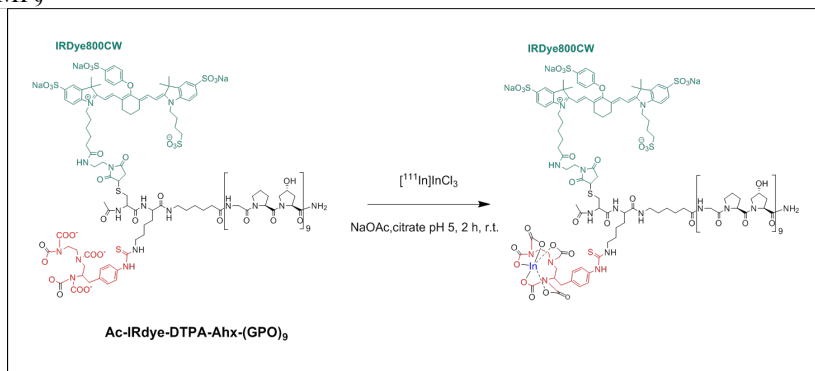


Figure 6. NIRF and SPECT-CT imaging in an F1 TRAMP mouse. A male TRAMP mouse devoid of prostate cancer but containing hypertrophied, inflamed seminal vesicles was scanned using CMP-800CW and an inflammation tracer, DPA-713-IRDye680LT using NIRF imaging. Both inflamed seminal vesicles are displaying CMP 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](lys₂)CHX-A-DTPA-(cys₁)IRDye800CW-CMP₉



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 7 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).

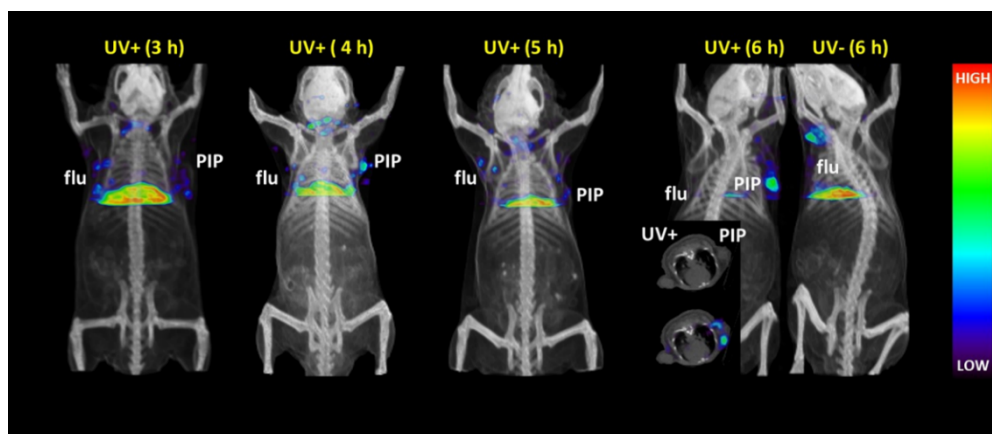


Figure 7. $[^{111}\text{In}](\text{CXH-A})-(\text{lys}_2)\text{-DTPA-CMP}_9(\text{cys}_1)\text{-IRDye800CW}$ SPECT-CT at 3-6 h post-injection. Five mice, each bearing a single PC-3 PIP (higher Δ collagen) and PC-3 flu (lower Δ collagen) tumor xenograft, were injected with radiolabeled CMP and imaged 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. 8). CMP distribution within the tumors was enriched at the edges where

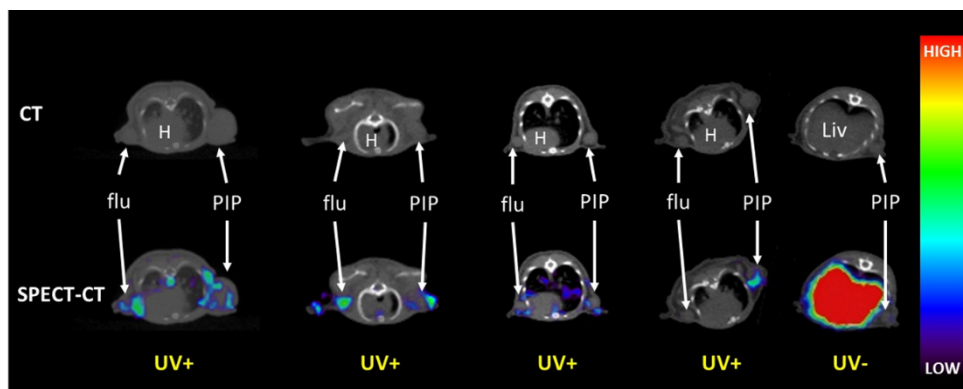


Figure 8. $[^{111}\text{In}](\text{CXH-A})-(\text{lys}_2)\text{-DTPA-CMP}_9(\text{cys}_1)\text{-IRDye800CW}$ SPECT-CT at 24 h post-injection. By 24 h post-injection, the same mice in figure 3 displayed stronger CMP uptake at the edges of the tumors, where the tumors are expanding. At this time point, 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.

growth is occurring. By 24 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 xenografts 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 μ m 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. 9, *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.

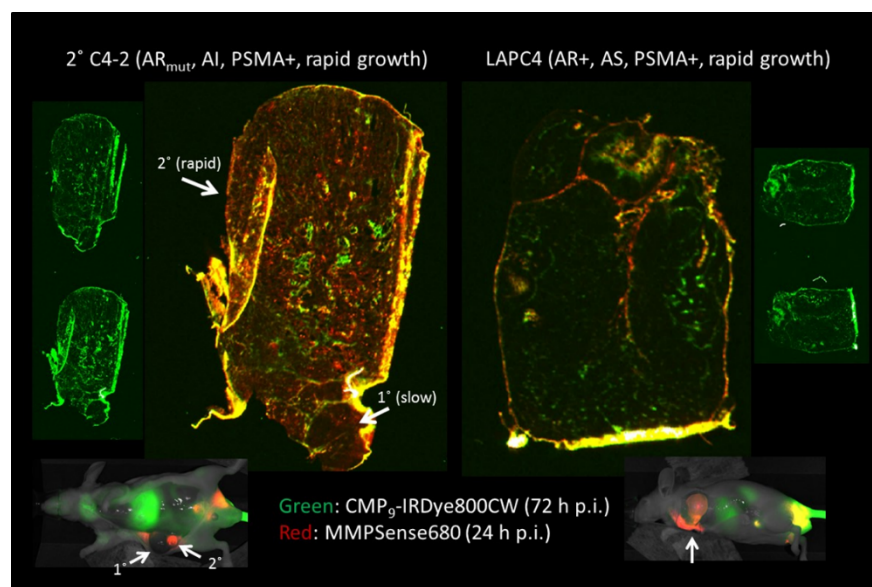


Figure 9. *CMP and MMPsense activity in C4-2 and LAPC4 xenografts.* Athymic nude mice bearing either a slow growing C4-2 xenograft with fast-growing secondary growth or a fast-growing LAPC4 xenograft were injected with 4 nmol of CMP-IRDye800CW and a single dose of MMPsense680. The mice were sacrificed 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.

Key Research Accomplishments (for this report year only)

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. Xenograft 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. [¹²⁵I]iodotyrosyl-CMP9 was visualized 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.

Reportable Outcomes (for this report year only)

- 1) Zitnay JL, +Li Y, Qin Z, San B-H, Depalle B, Reese SP, Buehler MJ, *Yu SM, and *Weiss JA (2017) Molecular level detection and localization of mechanical damage in collagen enabled by collagen hybridizing peptides. Nature Communications, *in press*. (* = co-corresponding authors) doi: 10.1038/ncomms14913.
- 2) Jeongmin Hwang, Boi Hoa San, Neill J. Turner, Lisa J. White, Denver M. Faulk, Stephen F. Badylak, Yang Li, S. Michael Yu (2017) Molecular Assessment of Collagen Denaturation in Decellularized Tissues using a Collagen Hybridizing Peptide. Acta Biomaterialia, 53, 268-278
- 3) Lucas L. Bennink, Daniel J. Smith, Catherine A. Foss, Martin G. Pomper, Yang Li, and S. Michael Yu (2017) High Serum Stability of Collagen Hybridizing Peptides, *in press*. doi: 10.1021/acs.molpharmaceut.7b00009.

Conclusion

During this reporting period, we learned from that our CMP comprised of ProHypGly sequence, and N-terminal modification and amide linked fluorescence dye has high serum stability. Based on all of our tests, we conclude that the most ideal NIRF-CMP for imaging denatured collagen *in vivo* is IR680-Ahx-(GPO)₉. We also demonstrated first successful imaging of PCa models via SPECT-CT. This was made possible by the use of new CMPs that allow mild metal loading condition. The signal was especially high at the edges of the tumor where tumors are expanding which suggest that CMP intensity correlates with invasiveness of the tumor. We also learned that CMP accumulates with higher density in rapidly growing tumors using fluorescence imaging although the fluorescence intensity likely reflects both collagen binding and accumulation of transconjugated dye-labeled serum proteins.

Future Plans

The PCa imaging in animal model was mainly conducted in the lab of Dr. Pomper at the Johns Hopkins University. The Pomper's project period has ended while Yu's lab still has NCE which was made possible by the recent relocation of Yu's lab (from Johns Hopkins to University of Utah). During the next funding cycle (until the end of the full project end date), Dr. Yu's research lab will investigate i) polymeric systems that display both PCa targeting CMP and metal loading chelator which is expected to dramatically improve the targeting capacity of the CMP. This will be made possible by developing new CMP structure that does not self-trimerize and deactivate. Dr. Yu's lab will also explore new animal model where cancer cells are directly injected into the bone (e.g. tibia). Such model was previously demonstrated to mimic the elevated level of ECM, in particular the collagen, remodeling activity of invasive tumors.