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14. ABSTRACT

The major goal of the proposed work is to develop new PCa imaging methods based on the collagen mimetic peptide (CMP)- mediated structural interrogation of the collagens associated with invasive tumor. In this proposal, we propose to use collagen mimetic peptide (CMP) as a collagen targeting agents that will allow imaging of invasive PCa. Since CMP binds to unstructured collagens more readily, it is expected to exhibit selective affinity to metastatic PCa tumors known to contain processed and denatured collagens. The motivating hypothesis is that the CMP's ability to bind to collagen/denatured collagen can be used to image PCa *in vivo* as well as to determine the level of PCa malignancy.

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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[1]. 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[2-5]. 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[6,7]. Traditionally, these collagens were targeted by antibodies (monoclonal antibody raised against denatured collagen); however antibodies have poor pharmacokinetics for *in vivo* imaging[2]. 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[8-10]. 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[11]. We propose to use CMP as a collagen targeting agents that will allow imaging of PCa. Since CMP binds to unstructured collagen domains more readily, it is expected to exhibit selective affinity to metastatic PCa tumors known to contain processed and denatured collagens. *This is the first time that the remodeled ECM of tumor microenvironment is targeted for cancer imaging which is an entirely new way to image PCa with a potential to revolutionize the cancer community.*

During the periods prior to this past funding cycle, we made the following major progress.

1. Demonstration of CMP mediated *in vivo* targeting of tissues undergoing normal (e.g. skeleton) and pathological (arthritis) remodeling.
2. Demonstration of the use of CMP as collagen staining agent in SDS-PAGE gel and tissue sections.
3. Synthesis and *in vivo* targeting of dual modality CMPs, which has both fluorescent and radioactive.

In the past funding cycle (9/30/2014-9/29/2015) we made progress in the following three major areas.

1. Determination of the serum stability of collagen mimetic peptide.
2. Demonstration of the CMP binding to mechanically damaged collagens (e.g. tendon injury).
3. Synthesis and *in vivo* targeting of polymer-conjugated CMPs to prostate cancer tumors (DU145-PC).

Summary of these three progresses made during the past funding cycle is presented below.

BODY

1. Determination of serum stability of collagen mimetic peptide (CMP).

The use of CMP for imaging PCa *in vivo* necessitates that the peptide is stable when injected into the blood circulation so that it can reach the tumor site before being degraded by proteases. To date, we had little information about CMP's stability to degradation in serum. Our recent unsuccessful results in imaging PCa using CMP prompted us to investigate CMP's serum stability. As you can see below, our results indicated that CMP's are highly stable peptide which exhibits extremely low degradation in serum. This instructed us to keep using CMP for tumor targeting. Manuscript on this work is currently being put together and is expected to be submitted in January 2016 to the journal *Molecular Pharmaceutics*.

It has been reported that *in vivo* stability of peptides can be reliably modeled *in vitro* using either plasma or serum[12]. The serum stability protocol was modified from Takaki Kiode et al. and Stein I. Aspmo[13], with adaptations to use commercially available mouse serum instead of fresh rat plasma. In physiological conditions, there are active enzymes and proteases that can degrade peptides. Two general categories of enzymes are: exopeptidases (or exoproteases) which cleave peptide from the end of its sequence, and endopeptidases (or endoproteases) which cleave peptides in the interior of their sequence. The specific peptidases such as prolinase could potentially cleave CMPs since it contain repeats of Pro. Both exo- and endopeptidases act on peptides in a highly specific manner and their efficiencies are affected by the peptide length, composition, and sequence[14-17]. Here we investigated the serum stability for a series of CMPs using mouse serum. The results show that C-terminal amidation has little effect on enzymatic resistance or degradation in serum; however, N-terminal modification (e.g. acetylation) increases resistance to enzymatic degradation. As can be seen in Figure 1, we observed that $(GPO)_9$ in a triple helix formation did not appreciably degrade after 24 hours in serum. This result was similar to the findings of Takaki Kiode et al. This stability was not mirrored in monomeric CHPs, which could be attributed to the fact that natural collagen, once folded into a triple helix, resists degradation by non-matrix metalloproteinases. The monomeric CHPs showed that composition, sequence order, and length did have an impact on the overall stability of the CHP. Length, composition and the inclusion of charged residues significantly reduced stability, which is corroborated by the low percentage of the $(GPO)_5$, the $(GPP)_9$, and the $(GPO)_4$-PK sequences remaining when compared to the monomeric $^{NB}(GPO)_9$ sequence.

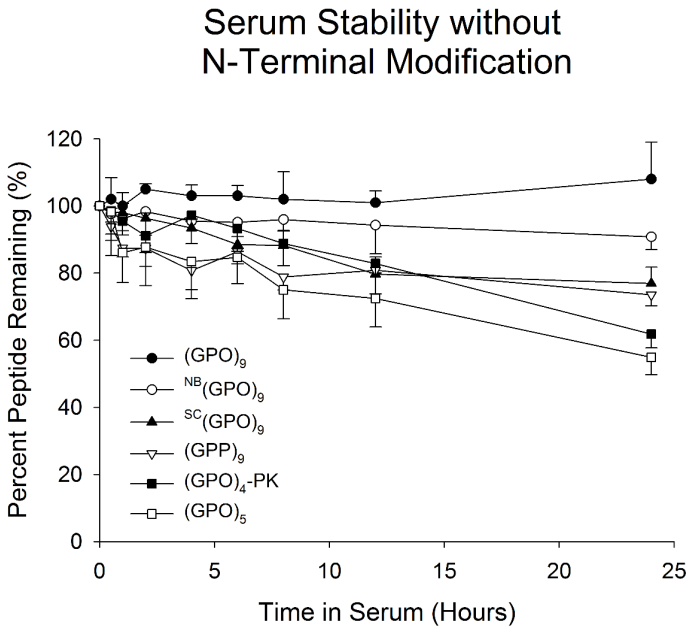

Figure 1. Serum Stability Profile for Peptides without N-terminal Modification. This graph shows 24 hr stability. It is important to note that $(GPO)_9$ is in a triple helical form and resists degradation from all non-MMP enzymes. The other peptides are all in monomeric form and are subject to degradation from many enzymes. Each experiment was run in triplicate, the data shown here represents their average degradation with their individual standard deviations.

Figure 2 shows the stability profiles of the N-terminally modified peptide sequences. The most notable difference is the increase in the stability of the $(GPO)_5$ sequence from Figure 1 to the stability of CF-$(GPO)_5$ seen in Figure 2. The stability of the triple helical CF-$(GPO)_9$ sequence did not change much from its non-modified counterpart which was expected due to the intrinsic ability of the triple helix to resist degradation without any modification. The stability of the scrambled CF-$G_9P_9O_9$ increased slightly, though not a statistically significant amount. The stability of all other peptides was increased by a statistically significant amount as determined by Student's T-Test (p-value). With the addition of the CF group on the N-terminus we see less degradation of the peptides when compared to the peptides without the N-terminal modification (Figure 1). The exception is the extremely fast degradation of the CF-$(GPK)_9$ sequence which has a half-life of about 1-hour. We were able to assess the serum stability of multiple CHP derivatives, which consisted of a repeating peptide motif of Glycine-Proline-Hydroxyproline [$(GPO)_n$, where O = hydroxyproline and n = 5 or 9]. We reiterated the enzymatic stability that the triple helical structure imparts to CHPs, and also evaluated the stability of monomeric CHP derivatives. More interestingly, we were able to show:

- Monomeric CHPs containing the $(GPO)_n$ sequence are resistant to endopeptidase activity, but are subject to a low level of N-terminal exopeptidase activity.
- Degradation of the $(GPO)_n$ sequence by exopeptidase activity can be suppressed by N-terminal modification.
- With N-terminal modification, monomeric CHPs composed of $(GPO)_n$ repeats have high serum stability which is comparable to triple helical CHPs.

After 24 hours of incubation in serum one can see the positive effect that N-terminal modification had on stability. With the exception of $(GPO)_9$, which was in triple helical formation, all of the peptides exhibited increased stability after N-terminal modification. We have established the importance of peptide composition and peptide sequence on resisting degradation, which was corroborated by the literatures on specificity and cleavage restrictions of proline specific enzymes. This work will lead to improved peptide design for targeted drug delivery, tissue engineering scaffolds, and diagnostic applications.

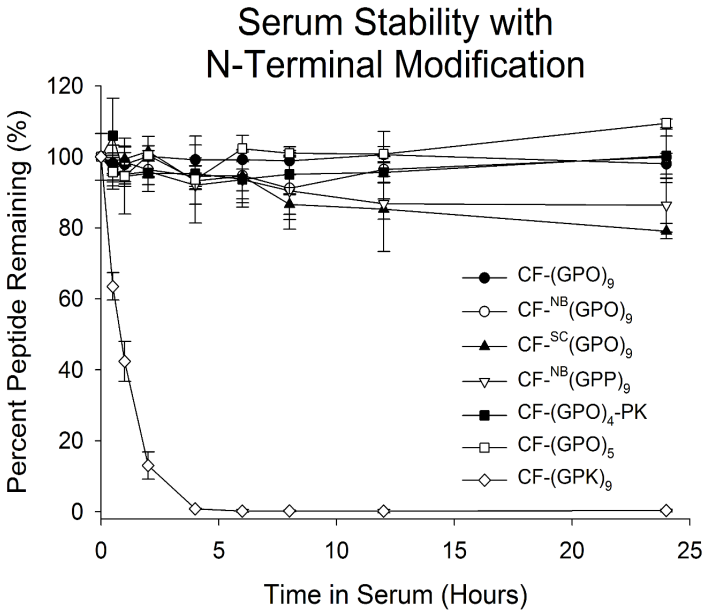

Figure 2. Serum Stability Profile for Peptides with N-Terminal Modification. The CF-(GPO) $_9$ sequence is in a triple helix, this shows how the triple helix resists degradation with or without modification. All of the other peptides shown are in monomeric form, and after the modification each peptide has increased resistance to degradation and thus higher stability. All peptides were run as triplicates, the data shown here represents their average degradation with their individual standard deviations.

2. Demonstration of the CMP binding to mechanically damaged collagens (e.g. tendon injury) Considering the ability of CMP to hybridize with the unfolded collagen triple helix, we hypothesized that carboxyfluorescein (CF) labeled CMP (CF-CMP) could be used to detect, localize, and quantify molecular level damage to collagen caused by mechanical loading (Fig. 3). This was not the original goal of the proposed project; however musculoskeletal injuries are the number one medical issue for the DOD when it comes to encounters and evacuation from the battlefield[21]. Therefore understanding the mechanism of tendon injury that could help prevent and even promote healing would be of prime interest to DOD. This work produced one of the most

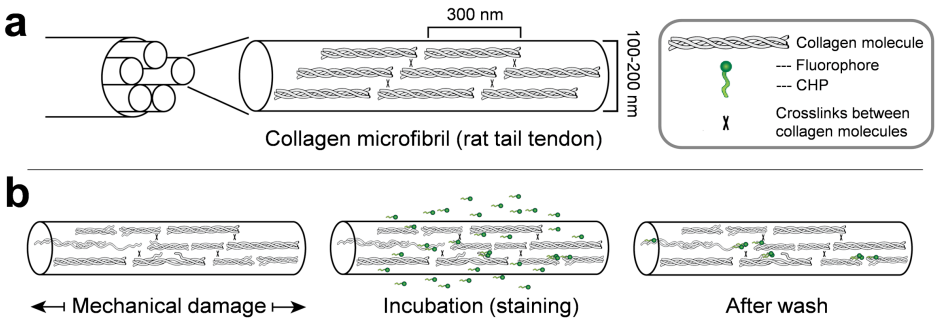

Figure 3. Collagen mechanical damage and CMP binding. (a) Simple schematic of triple-helical collagen organization in intact tendon microfibrils, based on the Hodge-Petruska model. (b) Intact rat tail tendon fascicles were stretched in uniaxial tension to initiate mechanical damage within the tissue structure. Tested tendon fascicles were incubated with single strand CF-CMP to allow binding to denatured molecular collagen and washed to remove unbound CMP.

exciting results in the past 3 years: mechanical damage to tendon occurs by collagen molecule (triple helix) unraveling. **A manuscript on this work is currently being reviewed in the journal *Nature Communication* (manuscript passed the initial screening by the editor and was sent out to reviewers).**

We used CF-CMP to detect damage due to collagen unfolding during subfailure loading for a series of mechanically stretched rat tail tendon fascicles. We showed that the triple helix mediated hybridization process effectively reports the location and level of molecular damage of the collagens in tendon, which provides unprecedented insights into the mechanism of mechanical damage to fibrous collagens in loading bearing tissues (Figure 4). Steered molecular dynamics simulations allowed us to investigate the molecular response of the collagen triple helix under loading with atomic resolution, enabling identification of the dominating molecular mechanism of permanent mechanical damage to fibrous collagens in load bearing tissues (Figure 5). CHP binding provides an exciting and unprecedented tool for detection and quantification of mechanical damage to collagen in connective tissues, and offers the potential for translational application to diagnosis, staging, and treatment of numerous connective tissue disorders and diseases. Standard antibody staining methods that rely on epitopes of a few amino acid residues do not provide sufficient binding sensitivity to distinguish damaged collagen molecules from intact molecules. Unlike antibodies that are specific to different types of collagens, CHP targets any denatured collagen strands with triple-helical propensity, which is shared by all types of collagens.[18,19] Thus, CHP targeting could potentially be used for *in vivo* detection of collagen damage associated with traumatic injuries, for the initial detection and monitoring of diseases such as osteoarthritis and tendinosis, as well as detection of mechanical damage to collagenous tissues caused by extreme mechanical conditions such as tendon fatigue before tissue failure under cyclic loading. Since CHP can be readily conjugated to bioactive molecules, it could also serve as a targeting moiety to deliver therapeutic agents to sites of injury. These translational applications provide exciting topics for further research and development.

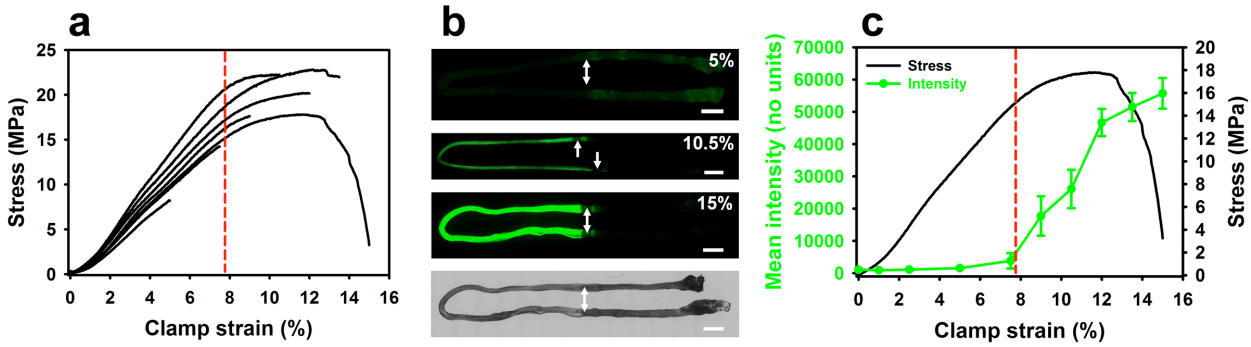

Figure 4. Fluorescence intensity and stress in tail tendon fascicles at incremental strains. (a) Average stress-strain curves for each strain group (n = 3 each curve). Behavior encompassed the range of linear behavior to tissue failure and the red dotted line indicates the approximate transition strain from linear to damage behavior, which also correlated with onset of CF-CMP intensity. (b) Representative whole sample fluorescence images of 5, 10.5, and 15% strain samples and brightfield image of the 15% strain sample, clearly showing an increase in CF-CMP staining with increased strain. In these images, fascicles have been folded to show the stretched section on the left half of the image and the clamped sections on the right half of the image. Arrows indicate approximate location of clamping. (c) Mean pixel intensity and average stress vs. strain plot for the 15% strain group (n = 3). Red line corresponds to the same red line in Fig. 4a, and indicates the approximate transition strain from linear to damage behavior for all tested samples. The apparent shift of this transition in the plot is due to sample variation. Scale bars 2 mm.

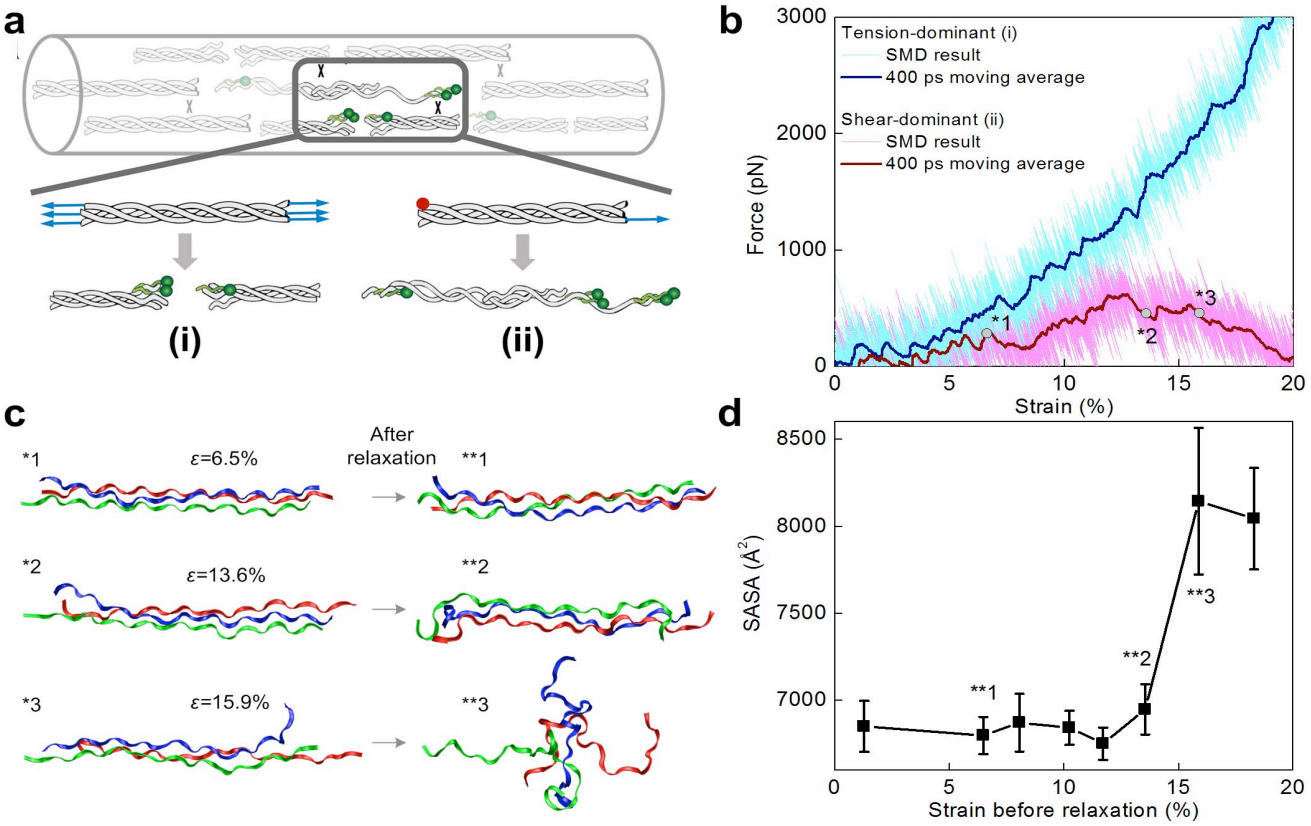

Figure 5. Steered MD simulations of tensile strain applied to collagen triple helix. We investigated two possible mechanisms for damage to the collagen triple helix under tension that would allow CMP binding: pull-out of a single α-chain, referred to as the shear-dominant case, and peptide backbone break of one or more α-chains, referred to as the tension-dominant case. These cases were investigated using steered MD simulations. (a) Schematic of the two possible loading mechanisms that were investigated in the numerical simulations: the tension-dominant case (a-i) and the shear-dominant case (a-ii). (b) The force-strain curve of the collagen molecule under tension-dominant and shear-dominant loading. The thicker curves are results of a moving averaging window with width of 400 ps. (c) Simulation snapshots taken before and after each relaxation simulation for the shear-dominant case. Starting conformations were obtained after a specific amount of strain (ε) in the shear-dominant test as indicated in panel b. Water molecules and ions are not shown for clarity. (d) SASA, a measure of triple helix unfolding, as a function of applied strain for the shear-dominant case. Structural changes to the triple helix started to take place at 13.6% strain, and by 15.9% nearly maximum SASA was reached. Mean ± standard deviation, computed during the last 20 ns of relaxation for each simulation.

3. Synthesis and *in vivo* targeting of polymer-conjugated CMPs to Prostate cancer tumors (DU145-PC).

In the progress report for prior funding period, we listed our many failed attempts at SPECT imaging of PCa tumor using new CMP structures. Even to date, we have not been able to obtain any positive SPECT imaging data using simple CMP architectures despite our efforts in designing over 5 different types of radiolabeled CMP. This prompted us to explore an entirely new direction in CMP-based tumor targeting, namely the

polymeric targeting system which displays multiple CMPs as well as radionuclei, all linked to a single hydrophilic polymer. At the University of Utah, Dr. Hamid Ghandehari has developed a polymeric drug delivery system based on hydroxypropylmethyacrylamide (HPMA) which was previously used to target anti-cancer drugs to tumors.[20] The polymeric system offers multiple advantages. First, the polymer can protect peptide from being digested by enzymes and immune cells. Second, since multiple CMPs are attached to single polymer molecule, the polymer-CMP can bind to tumor more effectively due to multi ligand effect. Third, polymer backbone provides versatile platform for displaying various imaging modalities in addition to CMP. Using previously reported chemistry, this polymer was modified to display several CMPs as well as DTPA which can chelate to radioactive metals (Figure 6). When this new compound was loaded with ^{111}In and used to image prostate cancer tumor (xenograph, DU145-PC), we detected for the first time a clear SPECT signal from the target tumors (Figure 7). The polymer was incubated $^{111}InCl_3$ at 60°C for 1 hr in sodium acetate (pH 5). After cooling room temperature, the polymer was treated with EDTA (0.05 M) to capture free ^{111}In ions and further purified by PD-10 column. This compound was heated to 70°C, quenched to room temperature and immediately injected (tail vein catheter) into the mice bearing DU145 prostate cancer tumor. Approximately 200-350 μCi was injected for each mouse. SPECT-CT was taken 1, 24, 96 hr post injection (PI). At 1 hr PI, both targeted and control (same polymer with scrambled CMP sequence) experiments showed similar high uptake at the tumor site. By 96 PI (Figure 7), intensity from the control experiments was almost gone while the targeted image showed clear intensity from the tumor site. This is the first positive result of PCa tumor imaging by SPECT-CT enable by CMP and we are currently optimizing the polymer structure and metal loading condition for enhanced targeting. We believe that this new polymer system will finally allow imaging of invasive PCa as we had originally proposed.

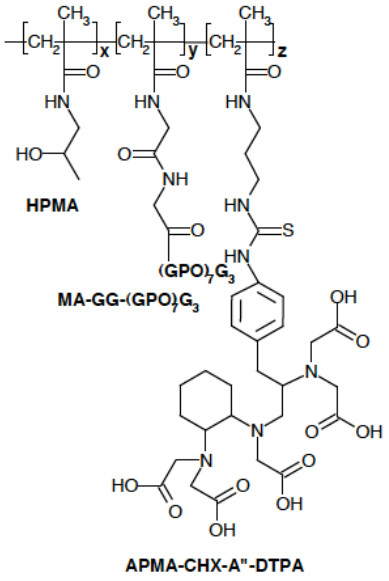

Figure 6. Structure of HPMA displaying CMP and DTPA.

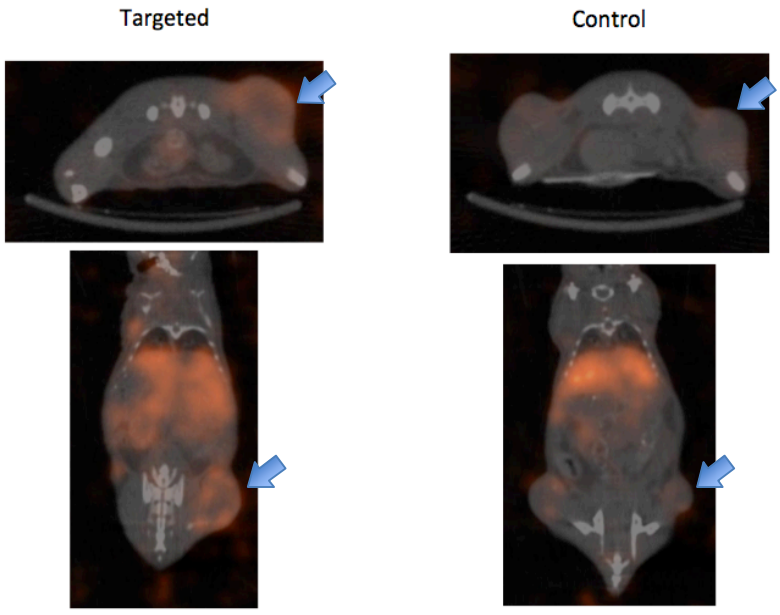

Figure 7. SPECT-CT of tumor (DU-145 PC) bearing mice imaged using HPMA displaying CMP and loaded with ^{111}In. Mice was injected with 200-350 μCi of polymer via tail vein catheter and imaged 96 post injection. Targeted image clearly show high level of radioactivity (compared to control image probed by scrambled CMP) at the tumor site which is marked by arrow head (Buckway and Ghandehari).

KEY RESEARCH ACCOMPLISHMENTS

During the past funding period (year 1), we have made major progress in the following areas.

1. Determination of the serum stability of collagen mimetic peptide.
2. Demonstration of the CMP binding to mechanically damaged collagens (e.g. tendon injury).

3. Synthesis and *in vivo* targeting of polymer-conjugated CMPs to prostate cancer tumors (DU145-PC).

During the past funding cycle (1 year), these research activities resulted in publication of 6 high impact research papers (3 published and 3 under review), 9 invited presentations in major institutions.

REPORTABLE OUTCOMES

Journal publications for the past funding cycle.

Published

1. 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.

2. 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.

3. 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.

Under review

1. Jared L. Zitnay, Yang Li, Boi-Hoa San, Shawn P. Reese, S. Michael Yu, and Jeffrey A. Weiss "Molecular level detection of mechanical damage in collagen fibers assisted by collagen hybridizing peptide" *Nature Communication*, submitted.

2. Boi-Hoa San, Yang Li, and S. Michael Yu "Nanoparticle assembly and gelatin binding mediated by triple helical collagen mimetic peptide" *ACS Nano*, submitted.

3. Hendra Wahyudi, Amanda A. Reynold, and S. Michael Yu "Collagen Targeting for Diagnostic Imaging and Therapy" *Journal of Controlled Release*, submitted.

Invited presentation during last funding cycle

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

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

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

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

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

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

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

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

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

CONCLUSION

We made a significant progress in terms of understanding the serum stability of the CMP. We also made a ground breaking discover that the CMP can be used to detect mechanical damage to collagen in tendon which could be used for diagnostic and therapeutics of musculoskeletal injury which are of prime interests to DOD. Most importantly, for the first time, we succeeded in prostate cancer tumor imaging by SPECT-CT enabled by CMP conjugated to polymer backbone. This was the result of our continuing efforts of designing and testing over 6 different types of CMP derivatives. The results were published in 3 peer-reviewed journals and 3 additional manuscripts are currently under review. Nine invited talks were given during the past funding cycle.

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