Award Number:	W81XWH-20-1-0343
Project Title:	Hybrid Bone-Tendon Grafts for Enhanced Tendon Healing
Principal Investigator Name:	Yunzhi Peter Yang
CONTRACTING Organization	Stanford University, 240 Pasteur Drive, BMI 258, Palo Alto, CA 94304
Report Date:	JUNE 2021
REPORT TYPE	ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
JUNE 2021	ANNUAL	1JUN2020 - 31MAY2021

4. TITLE AND SUBTITLE

Hybrid Bone-Tendon Grafts for Enhanced Tendon Healing

5a. CONTRACT NUMBER
W81XWH-20-1-0343

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Yunzhi Peter Yang

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Stanford University
240 Pasteur Drive, BMI 258
Palo Alto, CA 94304

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: This proposal addresses the Surgical Care Focus Area of Soft Tissue Trauma. Over 200,000 rotator cuff surgeries are performed annually in the United States alone with an estimated cost of $3.44 billion USD. Rotator cuff injuries in the active military present unique challenges because traumatic or repetitive overhead injuries are inherent with the job description. Re-tear rates after rotator cuff repair ranges from 20% to 94% due to a combination of factors, including age, tissue quality, tear size, fatty infiltration of the associated rotator cuff muscles, and biological healing response at the bone-tendon interface. Commercially available tissue augmentation grafts had been attempted in the past, with unpredictable results. While the underlying reasons for this lack of efficacy vary, materials and devices that mimic the features of native tissues are expected to improve clinical outcomes. Research efforts have focused primarily on the development of materials to sustain physiological loading and the administration of biochemical cues to direct healing. However, there has been a paucity of literature in re-establishing native bone to tendon interface and multi-tissue continuity. More specifically, no previous studies have simultaneously demonstrated synthetic or native tissue derived tendon grafts with approximation of bone and tendon mechanical properties, spatial control of musculoskeletal differentiation, and physicochemical features for structural continuity and integrity. Rotator cuff injury in active service members continues to be a clinically significant problem.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	**19b. TELEPHONE NUMBER** *(include area code)*

1. **Accomplishments:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.

The major goals of the project are two folds: **The first goal** is to investigate if the soft-rigid hybrid graft can be established as a model system to study growth factor presentations to guide stem cells' differentiation to form bone-tendon interface. **The second goal** is to investigate if the discoveries in hybrid grafts from the previous goal could lead to a therapy in bone tendon injury repair. The three tasks are listed below:

Task 1. Develop and characterize bioactive graft in vitro (1-12months)

Task 1. Develop and characterize bioactive graft in vitro	1-12
1a. Obtain IACUC approval	1 (accomplished)
1b. Obtain ACURO approval	1-3 (accomplished)
1c. Measure mechanical properties	1-6 (accomplished)
1d. Evaluate cell dose response to proteins in medium	1-8 (on-going iteration)
1e. Optimize protein loading onto grafts	7-12 (on-going iteration)
1f. Evaluate growth factor release and retention profiles from grafts	8-12 (on-going iteration)
1g. Evaluate cell dose response in grafts	8-12 (on-going iteration)

Task 2: Assess effect of bioactive grafts on multi-tissue healing and rotator cuff repair in rats (13-24months)
Task 3: Examine the clinical applicability of bioactive graft in a large pre-clinical sheep rotator cuff model (25-36 months)

What was accomplished under these goals?
For this annual reporting period only describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided.

We have made significant progresses according to our project design and timeline.

First, we optimized the gel formulation to enable cell encapsulation for 3D prints and to improve mechanical properties. The current formula and crosslinking parameters are listed below: 10% GelMa, 2% PEGDMA, 2.5% alginate, 0.5% PI, 2%, 3% and 5% Laponite. The curing parameters for dual crosslinking mechanisms: UV cure for 5 minutes, and Calcium chloride. The mechanical properties are shown in **Figure 1**. The strain of new hydrogels will allow for mechanical conditioning of tissue engineered tendon grafts within physiologically relevant ranges (5-16%, mostly 4-7%).

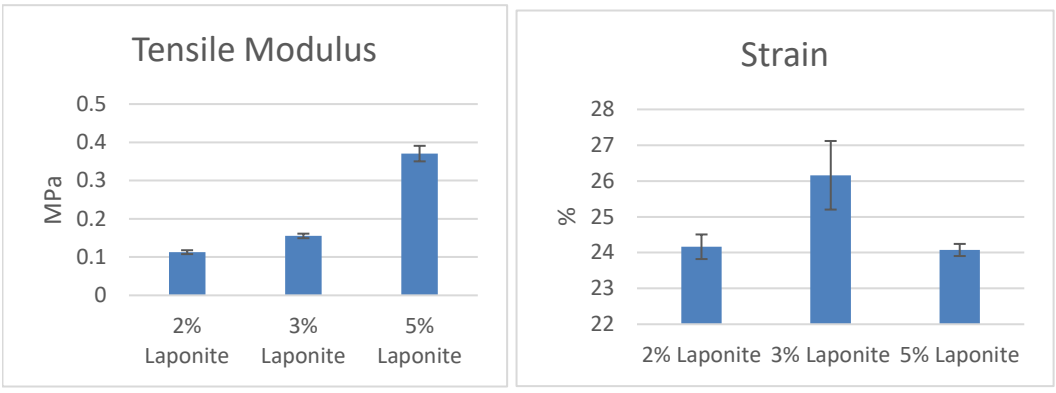

Figure 1. Mechanical properties of gel formulation

Second, we improved hydrogel printing quality by introducing a temperature control unit to allow for better printability due to the temperature sensitivity nature of GelMA component. Specifically, a heating pad was wrapped around the 3cc syringe during printing and the temperature was adjusted to 37 ℃. After extrusion the temperature dropped to room temperature, allowing for better structural integrity of the print. **Figure 2** shows representative images of 3D printed hydrogel scaffolds before and after a temperature control unit. Compared to the previous struts (**Figure 2a**), the clear sharp edge of hydrogel scaffolds can be observed using our improved method (**Figure 2b**). We ere able to maintain a resolution of m after la ers of printin mm t ic Both improved formulation of hydrogel and printing method will significantly improve producibility and quality control of engineered grafts, paving the way for biological discovery.

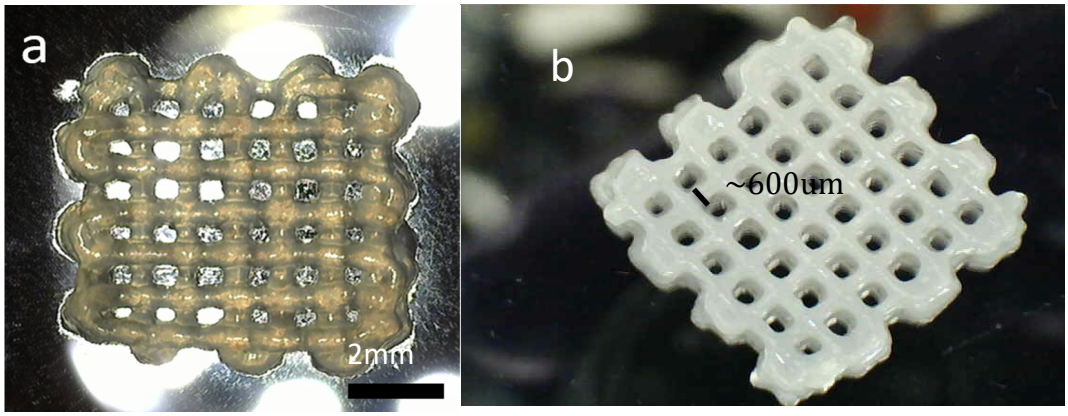

Figure 2. Representative images of 3D printed hydrogel scaffolds using our in house IMHAP hybprinter. (a) previous formulation without a temperature control unit; (b) current formulation with a temperature control unit.

Third, we also characterized cell viability of cell laden hydrogels using live/dead staining and cell proliferation. **Figure 3** shows dead/live staining of cell laden hydrogels using two formation methods. We fabricated hydrogel samples with both molding and printing approaches. For molding, the mixture was sandwiched between two glass slides it an m-thick spacer before it was exposed to a 3.5 mW/cm^2, 365 nm UV light for 5 min. After UV crosslinking, the gel membrane was further crosslinked by 1% $CaCl_2$ in PBS, and then cut into disks with a 6 mm-diameter biopsy punch. For printing, the mixture was loaded into a 3cc syringe and extruded into mesh pattern with our IMHAP system, followed by the same crosslinking protocol. Both molded and printed samples were then transferred into 24 well plates and cultured in DMEM medium for 0, 1, 4 days before they were analyzed. For viability analysis, LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen™) was used following standard protocol. After staining and washing, the samples were analyzed under a Keyence microscope (**Figure 3**). It can be seen that cell viability is above 95% for all samples during incubation. Also, we further validate the cell viability using dsDNA assay of cell laden hydrogels. As shown in **Figure 4**, there is no decrease in dsNDA amount between Day 0 and 1, and there was a slightly increase in dsDNA amount between Day 1 and 4. This further validate non cellular toxicity or highly cell viability of our hydrogels and printing method. We will continue to incubate cells within hydrogels for a longer term and expect to significant increase in dsDNA amount over time. Note, there is a subtle balance in hydrogel formulation for cell viability and mechanical properties.

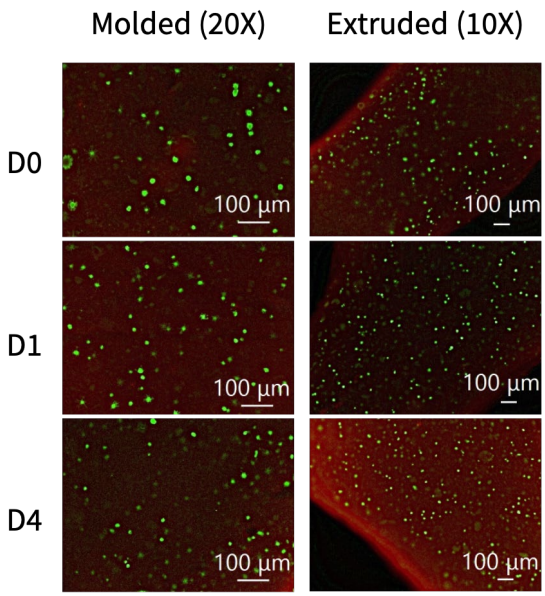

Figure 3 Live/dead staining of cells in hydrogels.

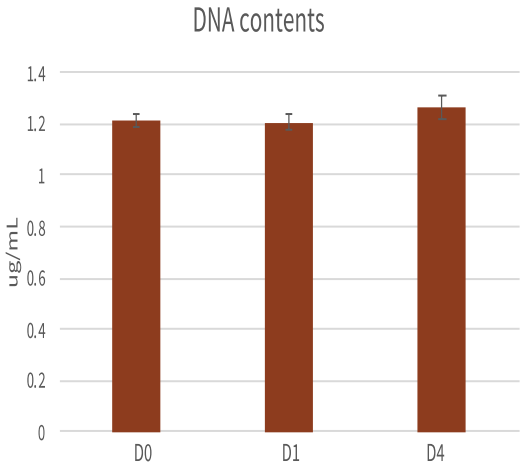

Figure 4 Cell growth in hydrogels

Fourth, we characterized cell response to localized growth factors, and observed cell differentiation effects induced by growth factor immobilization. **Figure 5** shows both growth factors (GDF-7) in medium and immobilized onto grafts induced tenogenic cell differentiation evidenced by increased tendon-associated markers, Collagen type I (COL 1), Tenascin C (TNC) and Tenomodulin (TNMD) after 6 days culture, suggesting the effective growth factor doses and immobilization method.

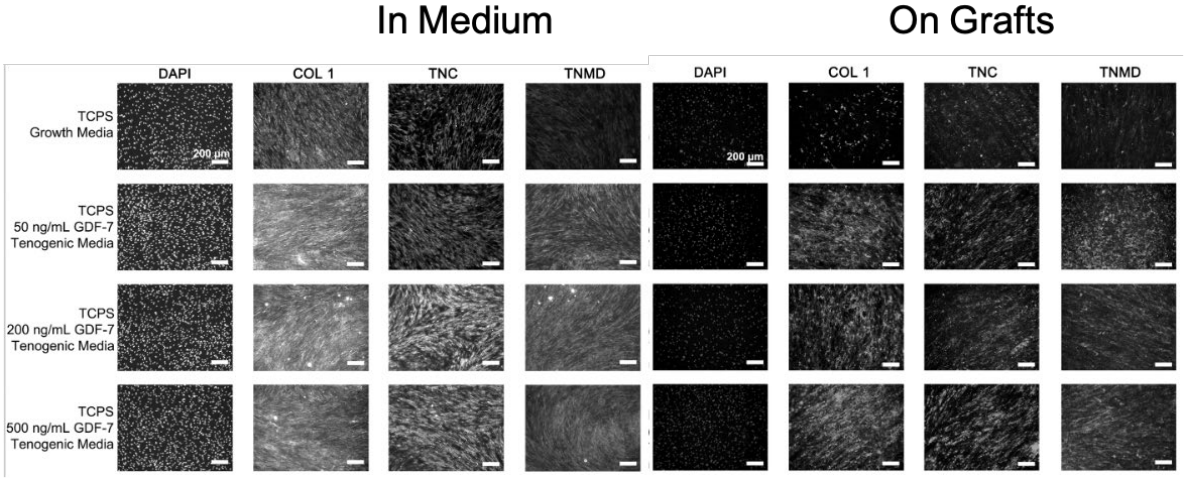

Figure 5. Effect of GDF-7 on hMSC tendon-like differentiation *in vitro.* GDF-7 mediated hMSC tendon-like differentiation *in vitro*. (**A**) hMSCs cultured on TCPS with 50, 200, or 500 ng/mL GDF-7-containing tenogenic media showed increased expression of COL 1, TNC and TNMD relative to growth media control after 6 days culture (n = 3 independent experiments). (**B**) hMSCs cultured on polymer graft with 50, 200, or 500 ng/mL GDF-7-containing tenogenic media showed increased expression of COL 1, TNC, and TNMD relative to growth media control after 6 days culture (n = 3). Scale bars as indicated.

Finally, we printed proteins onto the 3D printed grafts and generated gradient of proteins for cell response under appropriate mechanical conditions. **Figure 6** shows the GFP tagged protein onto the hydrogel struts. As shown in **Figure 5**, different concentrations of proteins can be printed onto the designated spots and the GFP-tagged proteins still can be seen even after two washes, suggesting stable immobilization. **In summary, in the past year, we optimized hydrogel formulation for high cell viability, robust mechanical properties and stable immobilization of protein for signaling molecule gradation. All the progresses pave the way for identifying growth factor combination and gradients in a highly reproducible, high throughput way.**

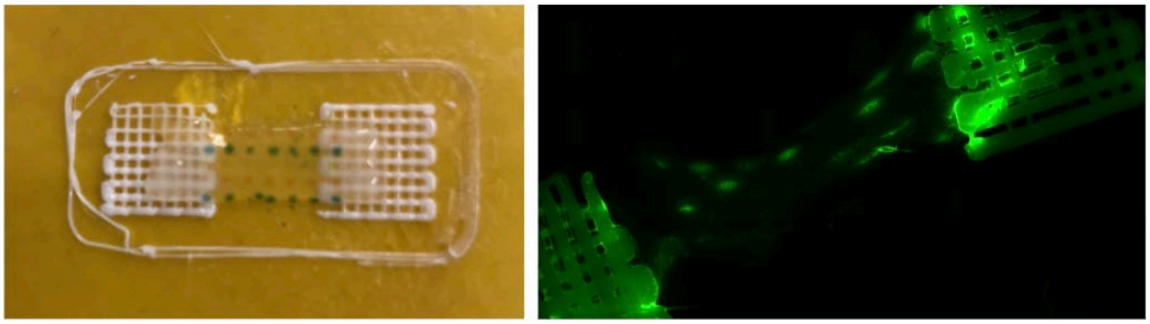

Figure 6. Representative image of graded GFP tagged protein on gel-plastic tendon graft prototype.1%, .5%, and 1% concentrations with amounts increasing linearly; time 0, wash 1x with PBS, wash 2x to determine physical absorption and binding.

Describe the Regulatory Protocol and Activity Status (if applicable).

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals and objectives?

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will perform the research activities as we planned.

Task 1. Develop and characterize bioactive graft in vitro	1-12
1e. Optimize protein loading onto grafts	7-12
1f. Evaluate growth factor release and retention profiles from grafts	8-12
1g. Evaluate cell dose response in grafts	8-12
Task 2: Assess effect of bioactive grafts on multi-tissue healing and rotator cuff repair in rats (180 rats)	13-24
2a. Prepare grafts for implantation	10-12
2b. Implantation for in vivo dose response using a subcutaneous model	13-15
2c. Evaluation of dose response from a subcutaneous model	15-19
2d. Establish and optimize a rotator cuff model	11-16
2e. Optimize graft for tendon repair	17-20
2f. Evaluation of bioactive grafts using a rotator cuff model	18-26

We have demonstrated the effectiveness of protein loading and cell responses using our new formulation and improved fabrication method. We will carry on the experiments meanwhile keeping solving the challenges we encounter by iteration of experiment design and dose response

2. Products:

List any products resulting from the project during the reporting period. If there are no products to report for the current quarter, state "Nothing to report."

Nothing to report

3. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Provide the following information for: (1) Project Directors (PDs)/ PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

Name:	*Yunzhi Peter Yang*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*yuyang (commons ID)*
Nearest person month worked:	*1.2*
Contribution to Project:	*Manage and guide the project*

Name:	*Carolyn Kim*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Kim is working on graft development and characterization and protein immobilization and gradation.*

Name:	*Sien Lin*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.6*
Contribution to Project:	*Dr. Lin performed in vivo work in the area of chronic rotator cuff injury rodent animal models.*

4. Changes/Problems: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

a. Actual Problems or delays and actions to resolve them

Provide a description of current problems or issues that may impede performance or progress of this project along with proposed corrective action. Also describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

For an award that includes the recruitment of human subjects for clinical research or a clinical trial, discuss any problems or barriers encountered, if applicable, and what has been done to mitigate those issues. Discussion may highlight enrollment problems, retention problems, and actions taken to increase enrollment and/or improve retention.

As we reported in our previous report, our lab moved to a new building on Stanford campus by the end of December. The moving caused disruption of some experiments. In the past six months, all equipment including in house printing platform, cell culture and evaluation equipment, such as Milli Q water supply, incubators, biosafety cabinets, microscope, cryotanks, and surgical room were finally established. We expect no more challenges in experimental facility and are confident to perform the experiments as planned.

b. Anticipated Problems/Issues

Provide a description of anticipated problems or issues that have a potential to impede performance or progress. Also provide course of actions planned to mitigate problems or to take should the problem materialize.

N/A

5. Special Reporting Requirements:

Quad Charts: N/A