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14. ABSTRACT

Post traumatic osteoarthritis (PTOA) is a debilitating disease that causes the breakdown of cartilage in articulating joints when triggered by an injury to the cartilage. It is a condition that represents 12% of all osteoarthritis (OA) cases, and it has a significant impact on soldiers and civilians who suffer from extensive pain and gradual degradative loss of joint function. There have been a number of attempts to create a biologic disease-modifying osteoarthritis drug (DMOAD) to either stop OA progression or reverse the disease entirely, but these drug candidates have failed in clinical trials due to poor delivery to cartilage and lowered access to cartilage matrix producing cells, which are critical for regeneration and recovery in cartilage. In order to achieve clinical success, the drug delivery challenges that caused the proposed drugs to fail must be resolved. Our labs demonstrated that biologic drugs can be directly conjugated to a positively charged, multivalent dendrimer nanocarrier that has been modified with biocompatible polymeric groups to yield a nanocarrier for biologic proteins without loss of bioactivity. The nanocarrier is successful in addressing the drug delivery challenges that caused OA biologic drugs to fail in clinical trials. These dendrimer-drug conjugates have been shown to create a tenfold increase in joint residence time compared to the free drug, from about 3 days to 30 days, and we have shown promising cartilage regeneration results in rat studies. This PRMRP grant will move this technology toward clinical translation by improving the procedure of attaching proteins to polymeric nanocarriers and investigating the best biologic for therapeutic efficacy using tissue regeneration as a primary and pain as a secondary endpoint. Work under this grant will also determine biodistribution, dosing, and ultimately efficacy in a large animal PTOA dog model to establish the promise of this approach and establish the data needed to move it forward for IND applications for clinical trials and ultimately to the clinic. The goal of the proposed research is to conduct essential translational research on this technology to evaluate and further develop the technology as a potential disease-modifying therapy for human PTOA. Our focus so far has been on the improvement of bioconjugation chemistry. Previous versions of our polymeric nanocarrier have utilized maleimide-thiol chemistry to attach bioactive proteins, namely insulin-like growth factor 1 (IGF-1), onto the carrier, resulting in only about 1 protein on every other carrier. This ratio of drug to nanocarrier indicates that there will be free cationic polymer without any therapeutic character in our mixture. To prevent this, we have implemented the use of azide-DBCO chemistry, allowing us to conjugate three proteins onto each nanocarrier, greatly reducing the likelihood of free polymers within the therapeutic mixture. In addition, this bioconjugation protocol has been adapted to an automated purification system, allowing for more uniform and replicable purifications of future conjugates. These results will help streamline the preclinical studies as well as the translational process. Once the retention of IGF-1 anabolic bioactivity is proven, the bioconjugation protocol will be applied to anti-catabolic interleukin-1 receptor antagonist. We anticipate the bioconjugation of this anti-catabolic protein to be similar in success to IGF-1. This improved bioconjugation protocol will then be implemented for all pre-clinical studies moving forward. We anticipate that as a result of this work, a minimally invasive intra-articular injection therapy for preventative and early stage OA will be advanced toward clinical translation that will prevent and ultimately reverse OA progression by promoting growth of healthy cartilage tissue to repair the joint. The potential impact of advancement of this technology on the outcomes of military patients includes increased productivity for much longer periods of their lives, and decreased military health care costs as a result of an accessible intra-articular treatment.

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Osteoarthritis following traumatic joint injury is a condition that can debilitate the lives of soldiers and veterans, making it difficult for them to function effectively while serving, and impacting their work and personal lives significantly on return from active duty. There is a need for regenerative therapy that can be administered quickly and early, within hours of an injury. Such therapy would ideally have disease-modifying properties that can arrest further damage of the cartilage and reverse degradation, preventing the onset of OA following trauma. The technology developed by our lab will provide a means of mediating gradual regeneration of healthy cartilage tissue and collagen matrix in the joint. The approach developed is cell-free and offers a unique early stage intervention to rescue damaged cartilage, without the risk of donor site morbidity or transmission of infection. We devised a nanoparticle that can penetrate the cartilage, act over multi-week timescales, and target the cells in the joint directly, stimulating regeneration and growth of chondrocytes and establishing a more rapid generation of new cartilage at the injury site, addressing OA before it has begun to become problematic. Another avenue of intervention is using anti-catabolic proteins, halting joint inflammation and degradation before it has begun. This capability would lead, in the worst case, to less severe OA symptoms appearing at a later point in life, and in the best case, to the full long-term remediation of joint damage and complete recovery, or lack of degradation, of the cartilage. By bringing the joint back to recovery, it is anticipated that OA can be fully eliminated when patients are treated early enough, thus greatly improving the quality of life for the 26% of soldiers who incur OA during service, and their families, and increasing the productivity of the affected military personnel.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

osteoarthritis, growth factor, drug delivery, cartilage repair, intra-articular injection, targeted nanoparticle, IGF, IL-1RA, anabolic, anti-catabolic corticosteroid, controlled release, layer-by-layer assembly, electrostatic complex, cartilage penetrating nanoparticle.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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 goal of the proposed research is to conduct essential translational research on this technology to evaluate and further develop the technology as a potential disease-modifying therapy for human post-traumatic osteoarthritis. Thus, the proposed research follows directly from the effort of the prior work and moves it toward potential clinical trials, but also extends the scope of prior work via the exploration of an additional drug candidate to use with our delivery technology. The addition of the second therapeutic candidate will validate our approach as a platform technology, enabling a broader set of potential therapeutic targets, as noted in the following proposed aims:

1) Exploration and comparison of disease-modifying biologics with anabolic and anti-catabolic mechanisms of action in OA Based on our success enhancing delivery and efficacy of IGF-1, a primarily anabolic therapeutic, with optimally PEGylated dendrimers, we seek to investigate if our dendrimer formulation can also improve the efficacy of an anti-catabolic biologic drug, and to determine whether the anabolic or anti-catabolic formulations should be moved forward for pre-clinical studies in Aims 2 and 3 . 1.1 We will first adapt our synthetic protocol to conjugate dendrimers with interleukin 1 receptor antagonist (IL-1RA), a clinical disease-modifying rheumatoid arthritis drug with published delivery challenges in human OA trials (6). 1.2 Once synthesis of a bioactive dendrimer-IL-1RA conjugate is successful, we will test the effects of dendrimer-IL-1RA vs. free IL-1RA and vehicle control in a cytokine injured cartilage model using ex vivo bovine and human cartilage explants. 1.3 We will then evaluate the delivery and efficacy of dendrimer-IL-1RA in the same rodent osteoarthritis model used to test dendrimer-IGF-1. Based on the in vitro and in vivo results, we will move forward with either IGF-1 or IL-1RA as the drug conjugated to our optimally PEGylated dendrimer nanocarrier.

2) Biodistribution, dose finding and immunogenicity studies of dendrimer-drug For the selected dendrimer-drug conjugate, we will identify the maximum tolerated dose in rats and characterize the accompanying toxicology to establish a dosing regimen to guide large animal studies. 2.1 We will first investigate the biodistribution of dendrimer-drug conjugates to identify any potential off-target tissues in which the dendrimer-drug conjugates may accumulate. 2.2 These data will indicate which organs to investigate in toxicology studies using histology and blood chemistry panels. Such toxicology biomarkers will be used to identify a maximum tolerated dose. 2.3 We will perform immunogenicity studies on single and repeat injections of dendrimer-drug conjugate to ascertain the risk of anti-PEG or anti-dendrimer antibody generation over the duration of chronic therapy.

3) Evaluation of improved delivery and efficacy of dendrimer-drug in a canine PTOA model 3.1 We will establish a surgically-induced model of post-traumatic osteoarthritis in canines by transection of the anterior cruciate ligament (ACLT) followed by 1 month of unrestricted movement to induce cartilage lesions. The animals will be administered dendrimer-drug conjugate intra-articularly into the affected knee joint. 3.2 We will evaluate the pharmacokinetics (PK) of dendrimer-drug in the injured canine joint using in vivo imaging of dendrimer-drug labeled with a radioactive or fluorescent tracer. Drug concentrations in synovial fluid aspirates will also be measured. 3.3 Pharmacodynamics (PD) will be observed by biomarker analysis of synovial fluid aspirates from longitudinal timepoints. 3.4 Finally, outcomes related to disease progression will be assessed throughout the study. MRI will be used to measure cartilage volume, which will be compared among treatment groups. We will examine terminal histopathology of cartilage, bone, and synovium at a specified endpoint of the study and score conditions of each tissue based on published research society (OARSI) guidelines. Routine veterinary assessment of joint pain and function will be performed throughout the study.

What was accomplished under these goals?

For this reporting period 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. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Activities and Significant Results and Outcomes by Task

Summary: Key advances over the past year are significant, and include optimization of the linker chemistry and bioconjugation efficacy (which was a liability in the original formulation), the first time incorporation of the anti-catabolic molecule IL-1RA onto our conjugates, and testing of this system for bioactivity in cartilage explants. The IGF-1 system was also tested in explants for bioactivity. In vivo pharmacokinetic studies have been initiated in rats for both conjugated constructs, and planning discussions for the dog studies with a contractor have also been started.

Protein Conjugation/Linker Chemistry

Aim 1 of the grant focuses on exploring the disease-modifying properties of dendrimer-conjugated anti-catabolic biologics, specifically interleukin 1 receptor antagonist (IL-1RA), versus ***Major Activities and Significant Results and Outcomes by Task*** anabolic biologics, insulin growth factor-1 (IGF-1). This aim required modifications of our bioconjugation protocol in order to increase bioconjugation efficiency and develop a more translational linker chemistry. Prior to our last annual update, we converted our thiol-maleimide chemistry to dibenzocyclooctyne (DBCO)-azide chemistry and successfully attached IGF-1 to PAMAM dendrimers with a ratio range of 1.6-2.7 proteins per dendrimer with batch-to-batch variability. This is a meaningful improvement over the original formulation which was less than 1.0 protein per dendrimer. The next step in developing the linker chemistry was to prove that IL-1RA can be conjugated to dendrimers in the same manner.

IL-1RA is significantly larger than IGF-1 (17 kDa vs. 7.6 kDa, respectively) and more costly. As a result, we were forced to experiment with IL-1RA at smaller scales than IGF-1. For example, when modifying IGF-1 with azide and AlexaFluor 647, a 100 nmol IGF-1 scale was used. When performing the same reaction with IL-1RA, a 25 nmol IL-1RA scale was used. As such, any loss due to unavoidable things such as syringe dead space, FPLC capillaries and columns, and filtering is detrimental to product recovery. Utilizing the same methods as IGF-1 with smaller scales, we were able to conjugate IL-1RA onto PEGylated dendrimers with a ratio range of 1.1-1.6 proteins per dendrimer with batch-to-batch variability and a protein recovery of about 20-30%.

We believe a source of the low yields and lower protein-to-dendrimer ratios is that the larger IL-1RA protein is shielding more PAMAM charges from interacting with the ion exchange column at higher protein ratios. The ion exchange purification of free protein from protein-dendrimer conjugates is our largest source of loss (~50-70%). At higher ratios of protein conjugation, the protein is able to shield more charges, which may prevent the dendrimer from adequately binding

to the negative charges of the column and the conjugates with higher ratios are being lost in the column with unbound protein. This would significantly reduce the yield and reduce the final ratio.

To increase yield and ratio, we are exploring ultrafiltration for separation of free protein from protein-dendrimer conjugates. The ultrafiltration would separate based on size, so the reduced charge from higher ratios would not be a problem. We are currently investigating this alternative purification method for IL-1RA conjugates.

Anabolic Bioactivity

The next step of Aim 1 is to determine the bioactivity of the proteins bound to dendrimers using the new DBCO-azide linker chemistry. We modify proteins with azide-containing PEG linkers and AlexaFluor 647 using N-hydroxysuccinimide-amine chemistry, which could potentially alter the accessibility and function of lysine residues of the proteins binding region. Additionally, we then attach these proteins to a PEGylated dendrimer, which could alter the orientation or accessibility of the protein, inhibiting the ability of the protein to bind to its extracellular receptor. In this aim, we are determining the bioactivity of the proteins using in vitro assays while examlning the protein activity in cartilage using ex vivo assays.

The first step with IGF-1 is to determine the anabolic bioactivity using an in vitro assay. To do this, we utilize an Invitrogen Click-it EdU flow cytometry assay, which assesses cell proliferation under different conditions. For this, we use NIH/3T3 mouse embryo fibroblast that we incubate in a low serum media (0.5% FBS) for 48 hours prior to treatment. This pretreatment forces the cells into a dormant state with very limited cell proliferation. They are then treated with the azide and fluorophore modified IGF-1, the IGF-1-dendrimer conjugate, native IGF-1, and no treatment, all normalized to 13 nM concentration of IGF-1. After 24 hours of treatment, 5-ethynyl-2'-deoxyuridine (EdU), which is a nucleoside analog of thymidine, is added to the culture flask and allowed to incubate for 2 hours.

If the cells in the culture flask are proliferating, EdU will be incorporated into the DNA during active DNA synthesis. EdU contains a click handle (alkyne) that can be labeled with an azide-containing fluorophore and studied using flow cytometry. In addition, the cells are labeled with DAPI, which allows the separation of cells into the cell cycle stages for analysis of the percent of cells in the S-phase, which we use as an indication of cell proliferation. Our samples are then compared to our positive (native IGF) and negative (no treatment) culture flasks to assess the level of IGF-1 bioactivity.

We found the percent of cells in S-phase had relatively large batch-to-batch variability. For example, native IGF-1 ranged from 14 to 21%, while IGF-1-dendrimer conjugates ranged from 21 to 37%. **When percent bioactivity is calculated for each experiment, IGF-1 dendrimer conjugates were consistently twice as bioactive as native IGF-1.** Percent bioactivity was calculated based on the following formula:

$$\%bioactivity = \frac{(\%Sphase_{sample} - \%Sphase_{negative})}{(\%Sphase_{positive} - \%Sphase_{negative})}$$

The IGF-1-dendrimer conjugates being twice as bioactive as native IGF-1 was surprising. A potential issue causing this was that IGF-1-dendrimers had unreacted DBCO molecules on the surface of the dendrimer. If the dendrimers were sticking to the surface of the cells, the azide-containing fluorophore that was supposed to be reacting with EdU may be reacting with the dendrimer-DBCO, increasing that fluorophore signal which looks like an increase in cells in S phase. To combat this, we synthesized an IGF-1-dendrimer conjugate with quenched DBCO, and added a free dendrimer-DBCO control (with no IGF-1 and active DBCO). The DBCO was quenched by adding an azide-terminated mPEG chain into the bioconjugation cocktail 48 hours after bioconjugation was started. Quenching of DBCO was confirmed via UV-vis with DBCO's absorbance at 309 nm. However, as can be seen in Figure 1, IGF-1-dendrimer conjugates with quenched DBCO exhibited the same increase in bioactivity over native IGF-1 while free dendrimer with unquenched DBCO exhibited no bioactivity (% of cells in S phase equal to no treatment). This proves that the increase in IGF-1 bioactivity is due to the conjugation to dendrimer and not a result of the dendrimer nanocarrier or methods. We believe this improvement in bioactivity is due to electrostatic interactions between dendrimer and the cell membrane or protein receptor,

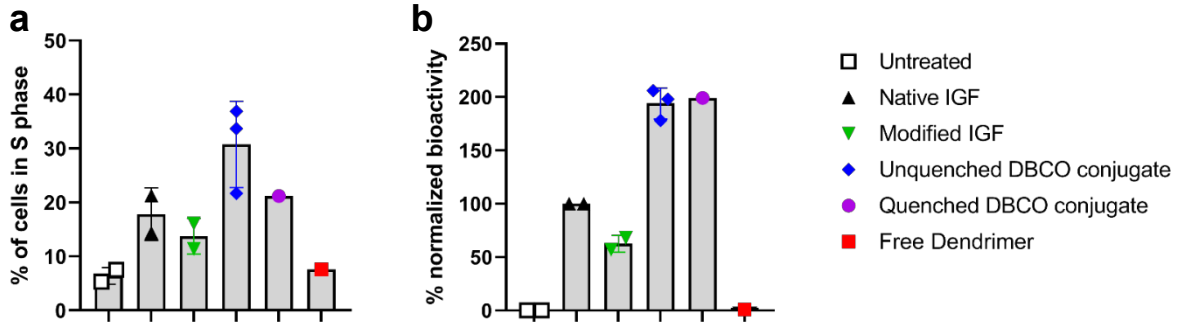

Figure 1 – In vitro bioactivity experiments of IGF-1 shows that dendrimer-bound IGF-1 is more bioactive than native IGF-1. (a) There is large variability when the raw data is displayed. (b) When the data is normalized to native IGF-1, IGF-1 bound to dendrimer with quenched and unquenched DBCO show two times the bioactivity, whereas free dendrimer shows no bioactivity.

bringing the protein in closer proximity to the receptor and improving bioactivity. This theory is described further in the next section.

Bioactivity in a cell monolayer, however, does not fully describe how the protein will perform in vivo. To try to probe this further before going in animals, we tested IGF-1 anabolic bioactivity ex vivo by probing the rate at which cells in a cartilage explant regrow the extracellular matrix in different conditions. Similar to the in vitro experiment, cartilage explants were incubated in a serum free media to force the cells into a dormant state. After 48 hours in serum free media, explants were incubated with radioactive sodium sulfate (^{35}S) and native IGF, IGF-dendrimer conjugates, or no treatment. At 24 and 48 hours post treatment, explants were removed, digested in proteinase K, analyzed for DNA content, and the radioactivity was measured. These data were used to calculate the rate of sulfated glycosaminoglycan (sGAG) synthesis of the cartilage explants, normalized to explant DNA content. A greater bioactivity will be related to a faster rate of sGAG synthesis.

As can be seen in Figure 2, both native IGF-1 and IGF-1-dendrimer conjugates had a statistically faster rate of radiolabeled sulfur incorporation into the cartilage explants. However, contrary to what was found in vitro, IGF-1-dendrimer conjugates did not exhibit an enhanced bioactivity based on explant sGAG synthesis over native IGF-1. Instead, the conjugate bioactivity was slightly lower. This is discussed further in the Anti-Catabolic Bioactivity section. In a separate experiment probing free dendrimer, we found the free dendrimer control did not increase the rate of sGAG synthesis, indicating the dendrimer does not contribute to bioactivity measurements. These data indicate that the bioactivity of the dendrimer bound IGF-1 has been conserved after bioconjugation.

Anti-Catabolic Bioactivity

In order to confirm the bioactivity of the conjugated IL-1RA we have used HEK293-NFκB (Luc) reporter cell line. If the IL-1 binds to the IL-1 receptor of this cell line, it will trigger subsequent NFκB signaling pathway, and firefly luciferase will be expressed. The amount of luciferase expressed will directly correlate to the IL-1 activity, which can be quantified by measuring the luminescence coming from the expressed luciferase. Anti-catabolic bioactivity of PAMAM-IL-1RA can be evaluated by looking at the inhibition of IL-1 activity. As controls, we have included free IL-1RA and free PAMAM.

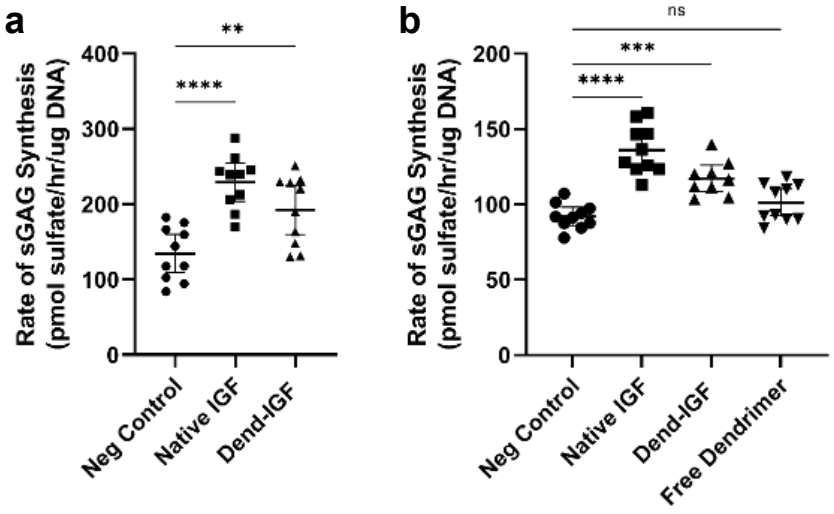

Figure 2 – Ex vivo bioactivity experiments of IGF-1 shows that dendrimer-bound IGF has comparable bioactivity as native IGF-1. (a) The initial set of experiments was verified by (b) a second set of experiments, which included free dendrimer that showed no bioactivity.

The PAMAM-IL-1RA did not only retain the bioactivity of the free IL-1RA, but the efficacy was boosted by the addition of PAMAM (Figure 3). **According to IC_{50}, PAMAM-IL-1RA was more than 30 times more efficacious than the free IL-1RA.** As mentioned in the Anabolic Bioactivity section, we found similar trends with IGF-1, where the dendrimer bound protein exhibited a greater bioactivity than the native protein. We believe this may be caused by either the positive charge of the dendrimer associating with the partially negative charge of the cell membranes, bringing the proteins in closer proximity to their receptors, or the positive charge associating with partial negative charges of the protein receptors, bringing the proteins in closer proximity. To probe this, we have included partially-acetylated-PAMAM-IL-1RA, which has significantly less positive charge compared to PAMAM-IL-1RA. As the charge of the PAMAM decreased, the efficacy decreased with it as seen by the partially-acetylated-PAMAM-IL-1RA formulation. One explanation is that the positively charged PAMAM is interacting with the negatively charged patch right outside the binding pocket of IL-1 receptor thereby making it easier for the IL-1RA to bind to the IL-1 receptor (Figure 4a). The reason why charge can boost the efficacy is because free IL-1RA mostly relies on hydrophobic interaction to bind to the receptor which is far weaker than charge-charge interaction. Therefore, introducing charge-charge interaction is expected to boost the interaction between IL-1RA and IL-1 receptor. A similar negatively charged patch is found adjacent to the binding region of type 1 IGF-1 receptor (Figure 4b). This will make the formulations even more effective as it will not only increase the retention time in the joint space by interacting with the negatively charge cartilage, but also potentially boost the protein activity on a molecular level by interacting more strongly with the respective receptors. This observation needs to be probed ex vivo before strong claims are made.

We have been collaborating with professor Grodzinsky's lab at the biological engineering department at MIT to study ex vivo IL-1RA bioactivity using cartilage degradation studies. We culture the cartilage explants in media and induce degradation by treating with interleukin-1 (IL-1). The effect of the formulations on inhibiting cartilage degradation will be evaluated by co-

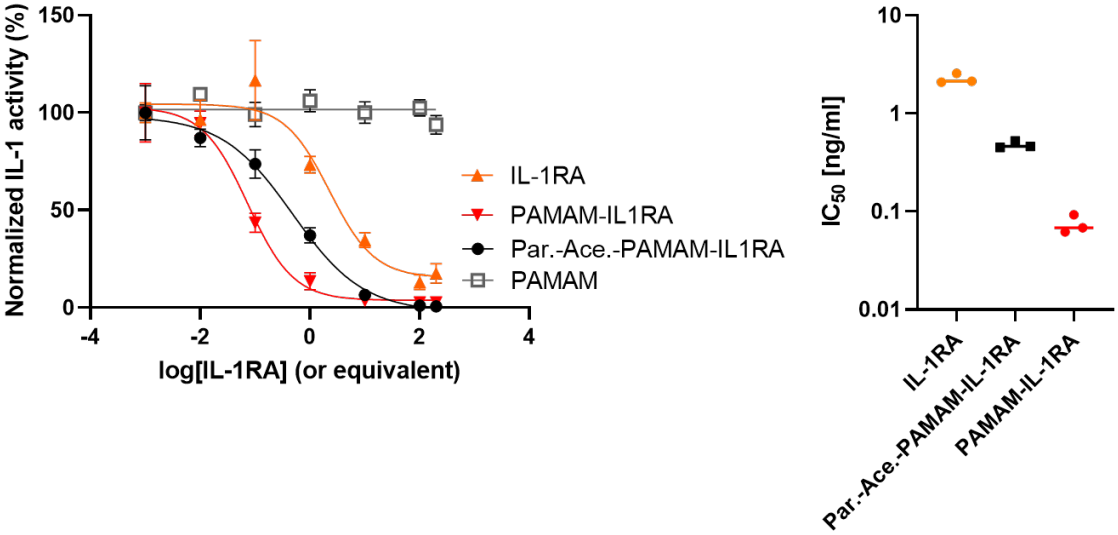

Figure 3 - Anti-catabolic activity of the conjugated IL-1RA. Positive charge of the PAMAM boosted the efficacy of IL-1RA compared to the free IL-1RA while PAMAM itself did not inhibit IL-1 activity.

treating IL-1RA or PAMAM-IL-1RA with IL-1. The concentration of glycosaminoglycan (GAG) released into the media as well as the remaining GAG content in the leftover explant will be measured using dimethylmethylene blue (DMMB) assay as an indicator of level of degradation

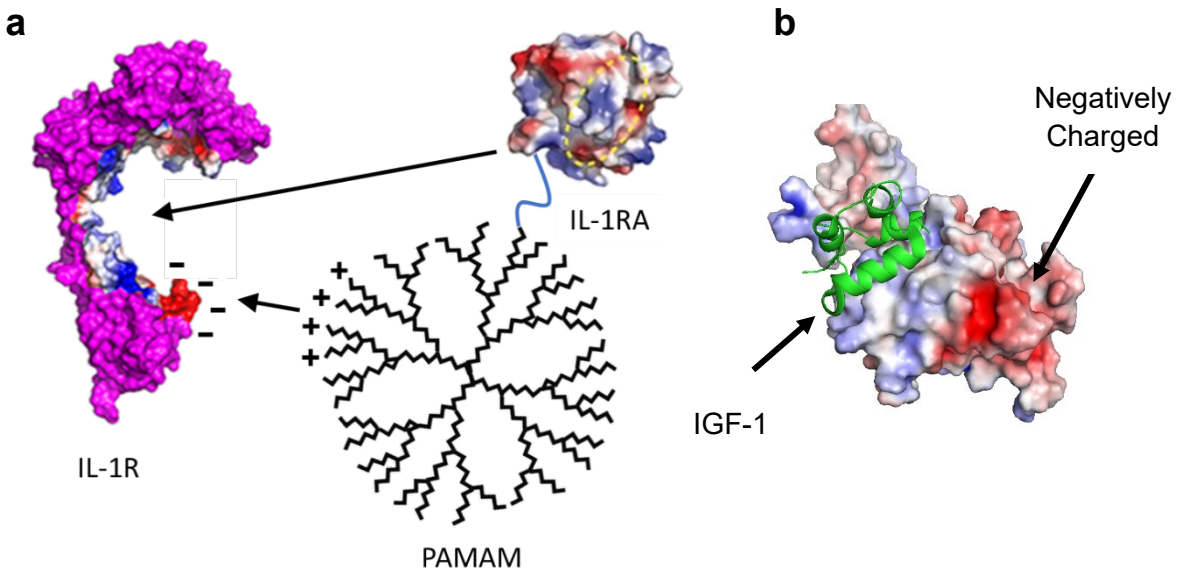

Figure 4 – (a) Proposed mechanism of PAMAM-IL-1RA. The positive charge of PAMAM will be able to interact with the negatively charged patch right outside the binding pocket of IL-1 receptor allowing it to bring IL-1RA to the binding pocket more effectively without hindering the binding. (b) A similar highly negatively charge patch is found adjacent to the binding region of site 1 of Type 1 Insulin-like Growth Factor Receptor.

(Figure 5). Supernatant is sampled every two days during the cartilage explant culture and the GAG content of the leftover explant is quantified on the last day by digesting the explant using proteinase K and measuring GAG content via DMMB assay.

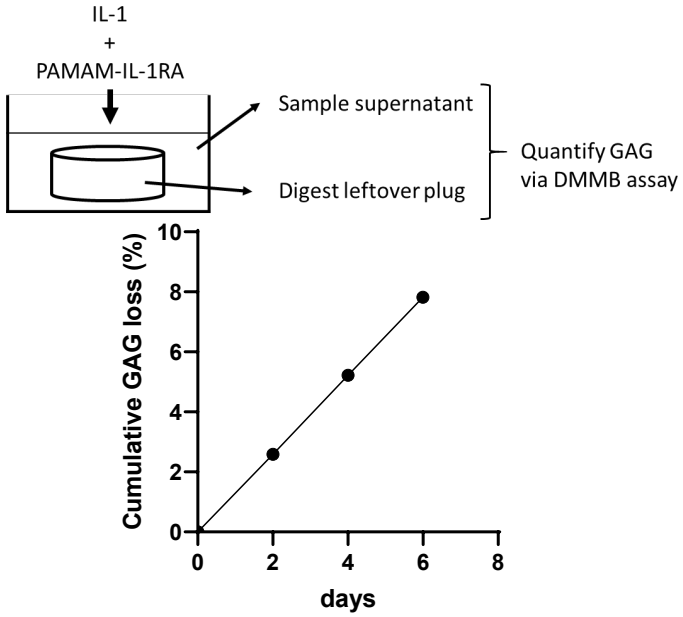

Figure 5 - Schematic of ex vivo cartilage degradation study and basal level of GAG loss quantified by DMMB assay. After treating the cartilage explant with IL-1 and the formulations, supernatant is sampled every two days and the GAG content of the leftover explant is quantified on the last day by digesting the explant and measuring GAG content via DMMB assay. The basal level of cumulative GAG loss linearly increased with time with very little variance.

We have established the DMMB assay and were able to successfully measure basal GAG loss (without the IL-1 treatment) which matched the previously reported literature (Figure 5). While we were in the process of optimizing the IL-1, IL-1RA, and PAMAM-IL-1RA treatment, the research specialist in the Grodzinsky Lab retired. Now we are in the process of relocating the bovine joint cartilage explant harvest station to the Koch Institute and we are waiting for the approval from the Environmental, Health, and Safety office. Once we have the approval, we will set up the harvesting station and continue on with the ex vivo experiments.

Pharmacokinetic Study

Evaluation of the formulations' ability to overcome the rapid clearance in the joint space was done by intra-articular injection of the formulations into the joint space of rats and by detecting the remaining proteins through IVIS imaging (Figure 6). The trend clearly indicated that all PAMAM conjugated formulations outperform their free protein counterparts. No signals were detected from all free protein injected joints as early as 24 hours after the injection, while all PAMAM conjugated formulations had detectible signals from at least one joint even at 14 days after injection. The fluorescence signals from PAMAM-IGF-1 and PAMAM-IL-1RA formulations were fit to a single exponential decay model, revealing half-lives of 2.3 days and 4.3 days, respectively, (Figure 7) which are similar to our preliminary results that showed a 4.2-day half-life. Half-lives of free proteins, IGF-1 and IL-1RA, were measured but are not included here; they will be reevaluated since we were not able to capture the initial rapid release. To better evaluate the half-lives of the free proteins, in our next trial, we will measure the fluorescence signals immediately after the injections now that we know how fast the free proteins are cleared, and we will increase the injected fluorescence concentration. Unfortunately, there were some uneven injections (one over-injection of PAMAM-IGF-1 and one failed injection of PAMAM-IL-1RA) due to faulty syringes and one PAMAM-IGF-1 injected rat died shortly after the injection due to an unknown cause, thus only one rat from that group was imaged. Due to these circumstances that led to inconsistent replicates across groups (uneven injection and loss of a rat) we will repeat this experiment to more concretely support the trends. In order to ensure the success of the next trial, we will practice intra-articular injection before beginning the next trial. In the current trial, we were forced to use

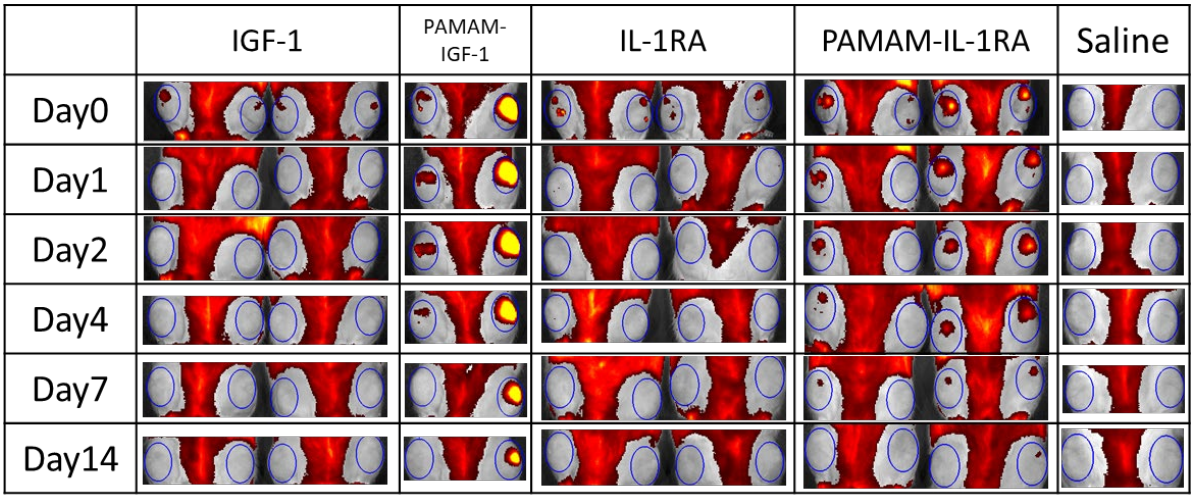

Figure 6 - Pharmacokinetics of formulations injected into the knee joint space of rats. Free proteins were cleared out from the joint space as early as 24 hours after injection as indicated by no signals detected from free proteins (IGF-1 and IL-1RA) on day 1 while all PAMAM conjugated formulations (PAMAM-IGF-1 and PAMAM-IL-1RA) showed signals from at least one joint after 14 days since injection.

slightly lower concentration then initially planned due to low yield of IL-1RA formulation. However, we will optimize the formulation synthesis, as discussed in the Protein Conjugation/Linker Chemistry section, for better yield so that we will be able to inject at the initially intended dose.

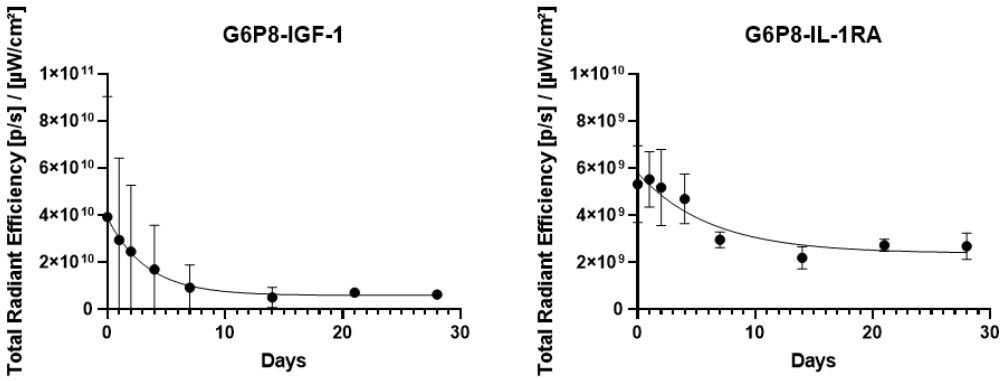

Figure 7 – Single exponential decay of the formulations. The PAMAM conjugated formulations were fitted to the single exponential decay model.

Planned Future Work

With an established team quickly gaining *in vivo* experience and expertise, we are also planning our future translational animal work in accordance with our proposed aims, both in house at MIT with a rat model and with our partner CRO with a dog model. The original pharmacokinetic study, though flawed, shows promise that dendrimer-bound IL-1RA has a greater residence time than free drug. However, we will first verify this trend by repeating the study with double the injection concentration (equal to our preliminary work) and after more practice with intra-articular injections. This second pharmacokinetic study is planned to begin in August 2022. Once verification of IL-1RA residence time is complete, our next step is to evaluate the efficacy of IL-1RA versus IGF-1 in a rat PTOA model. Training is ongoing to gain experience with the PTOA rat model (which involves a survival surgery) and all downstream tissue processing to evaluate efficacy, including joint harvesting, fixing, decalcifying, embedding, and histological staining. We plan to start the first cohort of animals for efficacy studies in the early Fall, followed quickly by the next cohorts. In addition to single protein treatments, we have also included a dual-delivery treatment group, where dendrimer-bound IL-1RA and IGF-1 will be contained in the same injection to investigate additive or synergistic effects of the two mechanisms. Using the data from this first couple of cohorts, we will identify the optimal mechanism of action (anti-catabolic versus anabolic, or both) based on pain reduction, reduced osteoarthritis lesions, reduced osteophyte volume, and reduced synovial fluid inflammation. Using the optimal treatment, we will then move forward to immunogenicity and biodistribution, followed quickly by dose finding studies by looking for the maximum tolerated dose.

Once the optimal treatment has been selected from efficacy studies, the rat studies (immunogenicity, biodistribution, dose finding) will be performed in parallel with a canine efficacy study performed by a CRO, specifically InterVivo. The canine efficacy study is a 72-day experiment that includes protein retention via fluorescence imaging, MRI images, plasma and synovial fluid collection, and ultimately the treated joints for histological analysis. We are currently

working to get approval from MIT's IACUC for the off-campus canine work through the CRO, which includes submitting a detailed animal protocol and ensuring the well-being of all animals involved. We are scheduling this work to begin as soon as the optimal treatment is identified from the rat efficacy studies.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

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

Nothing to report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Ongoing work indicates that the IL-1RA agonist can also be conjugated to the dendrimer construct to maintain activity, like the IGF-1, thus providing a platform technology for treatment relevant to anabolic and anti-catabolic therapies.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

As noted in earlier reports, COVID constraints that delayed lab activity and animal studies, and the difficulty in hiring a full time postdoctoral associate, have led to project delays. The program pace has increased significantly and progress is moving forward in a promising fashion, but we anticipate there could be a need for an NCE to complete all of our outlined tasks.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures
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.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Products include the IGF-1 and IL-1RA agonist dendrimer conjugates for treatment of post-traumatic osteoarthritis as outlined above.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) 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). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Paula T. Hammond
Project Role: PI, MIT
Nearest Person-Month Worked: .17 month(summer) each year (Academic salary covered by MIT)

Name: Alan Grodzinsky
Project Role: Co-I, MIT
Nearest Person-Month Worked: 0 month (summer) each year (Academic salary covered by MIT)

Name: Joon Ho Park
Project Role: Postdoctoral Associate, MIT – replacing Rami Chakroun
Nearest Person-Month Worked: 7 months this year (hired end of 12/2021)
Contribution to Project: IL-1RA agonist conjugation and rat model, coordination with dog model

Name: Rami Chakroun
Project Role: Postdoctoral Associate, MIT
Nearest Person-Month Worked: 0 months this past year *(left project to take a job at Moderna)*
Contribution to Project: Synthesis, bioconjugation, and bioactivity confirmation of dendrimer-IGF-1 conjugates.

Name: Brandon Johnston
Project Role: PhD Candidate, Graduate Research Assistant, MIT
Nearest Person-Month Worked: 0 months each year
Contribution to Project: Protein purification, conjugation and isolation of ILRA1 drug carrier-conjugate.

Name: Simone Douglas-Green
Project Role: Postdoctoral Associate, MIT
Nearest Person-Month Worked: 11.2 months - self-funded postdoctoral fellow (Ford Fellow)–
Contribution to Project: Design of cartilage transport measurements and key in vitro experiments to determine efficacy of nanoparticle release and the relationship between size and charge and protein corona in such systems, involved in design and integration of knowledge for in vivo studies. In vivo testing of the dendrimer-IGF-1 conjugates, including pharmacokinetics, surgical model induction.

Name: MayLin Howard
Project Role: PhD Candidate, Graduate Research Assistant, MIT
Nearest Person-Month Worked: 1 months each year
Contribution to Project: Assisted with preparation and training for in vivo testing in rat model.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.