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14. ABSTRACT

Vascularized composite allotransplantation is now a valid therapeutic option. However, the debilitating side effects of current immunosuppressive therapy counterbalance its benefits. We have discovered that pharmacological inhibition of inflammatory cytokines, through the Jak3/1 inhibitor Tofacitinib (Tofa), is effective in synergizing with the biologic CTLA4-Ig to promote long-term transplant survival. To translate this combination strategy into a clinical application, we propose a novel two-component delivery platform that combines two technologies: injectable peptide hydrogels, and lipid nanoparticles (LNp). The goal of this proposal is to optimize such a platform to tune the localized delivery of Tofa-loaded LNp to the activity of rejection-associated proteases and demonstrate the confined effect on the rejection response. Major finding from this first year of investigation are: 1) injectable peptide hydrogels can be designed to be protease sensitive; 2) certain LNp formulations are compatible with encapsulation within hydrogels. These composites represent a new class of immune-modulating implantable material.

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

   Vascularized Composite Allotransplantation has become a viable approach for functional restoration of limb loss. However, the toxic and debilitating side effects of the currently implemented multi-drug immunosuppression used to protect transplanted limbs from rejection counterbalances its benefits and prevents widespread use. To solve this problem, we propose to embrace cutting edge advances in biomaterial design via a collaboration among three Principal Investigators with complementary expertise. The goal of our study is to optimize and demonstrate the efficacy of a novel drug delivery platform designed to suppress the rejection response in a localized and tunable fashion via a regimen that is permissive of immunomodulatory mechanisms. We propose to use a "Russian dolls" approach where one material, lipid nanoparticles carrying Tofacitinib, is enclosed into a second biomaterial, a peptide hydrogel, that can be injected in any tissue. Part of the research is to design a hydrogel to be degradable by the effector mechanisms of the rejection response. By injecting this bio-construct subcutaneously in the vascularized composite allograft, lipid nanoparticles will be released in response to the intensity of the rejection response and will deliver Tofacitinib locally and selectively to immune cells where the rejection response is initiated. By using our unique mouse model of hind limb transplantation, we plan to demonstrate that combining this delivery platform with the systemic administration of the biologic CTLA4-Ig, will render a regulated and localized synergism that will be effective in modulating the rejection response. Ultimately, we plan to also accrue preliminary data in our pre-clinical large animal model to support further studies of the clinical translatability of our proposed strategy.

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

   Transplant Rejection; Costimulation Blockade; Peptide Hydrogel; Lipid Nanoparticle; Localized Drug Delivery; Lymphatics; Matrix Metallo-Proteases; Tofacitinib; CTLA4-Ig

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.

   <u>Research-Specific Tasks</u>
   Major Task 1: Hydrogel Design and enzyme-based studies of proteolytic degradation [Months 1-10] Percentage of completion: 60%

   Major Task 2: Optimize Tofa-LNp tropism and drug release profile

[Months 1-12] Percentage of completion: 60%

Major Task 3: In vivo study of targeted release by Hydro(Tofa-LNp) composites
[Months 6-18] Percentage of completion: 10%

Major Task 4: Assess localized synergism of Hydro(Tofa-LNp) with CTLA4-Ig to promote mouse hind limb survival
[Months 12-24] Percentage of completion: 0%

Major Task 5: Assess the transplant-associated biodistribution of LNp from hydrogel
[Months 16-28] Percentage of completion: 0%

Major Task 6: Determine protective impact of Hydro(Tofa-LNp) + CTLA4-Ig on survival of ischemic limbs
[Months 24-32] Percentage of completion: 0%

Major Task 7: Determine graft survival extension conferred by combined use of Hydro(Tofa-LNp) + CTLA4-Ig in swine VCA model
[Months 28-36] Percentage of completion: 0%

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.

Research-Specific Tasks

Major Task 1: Hydrogel Design and enzyme-based studies of proteolytic degradation

Site 3 NCI
Subtask 1: *Assessment of MMP-specific hydrogel degradation in cell free enzymatic assays*

Over this first year of the award, we have designed, synthesized, and purified 6 self-assembling peptides and characterized the rheological properties of their gels. We assessed peptide susceptibility to MMP12 degradation as well as measured degradation kinetics for the self-assembled peptide hydrogels in solution.

The sequences of the peptides are provided in **Table 1** with MMP cleavage sites underlined and indicated by red arrow). Peptide HPLC chromatograms showing purity and corresponding mass spectra, confirming the primary sequence of each peptide, are provided in **Figure 1**, **Figure 2**, and **Figure 3**.

Active site	Name/short name	sequence	MW(g/mol)
ELR	Mmp_seq6/mmp(6)	IKVKIKVKV^{D}PPTE↓LRVKIKV	2315.0
	Mmp_seq7/mmp(7)	IKVKIE↓LRV^{D}PPTKIKVKIKV	2329.2
PLGVR	Mmp_seq9/mmp(9)	IKVKIKVKV^{D}PPLG↓VRVKIKV	2241.2
	norV_mmp_seq9/mmp(9) norV	(norV)K(norV)K(norV)KVKV^{D}PPLG↓VR(norV)K(norV)K(norV)	2198.9
PLGLFAR	V_R&D	VKVKVKVKV^{D}PPLG↓LFARVKV	2203.8
	Viv_R&D	VKIKVKIKV^{D}PPLG↓LFARIKV	2246.1

Table 1. Sequences and calculated molecular weights of hydrogel-forming peptides. Peptides susceptible to the enzymatic action of MMP-12 are highlighted in yellow.

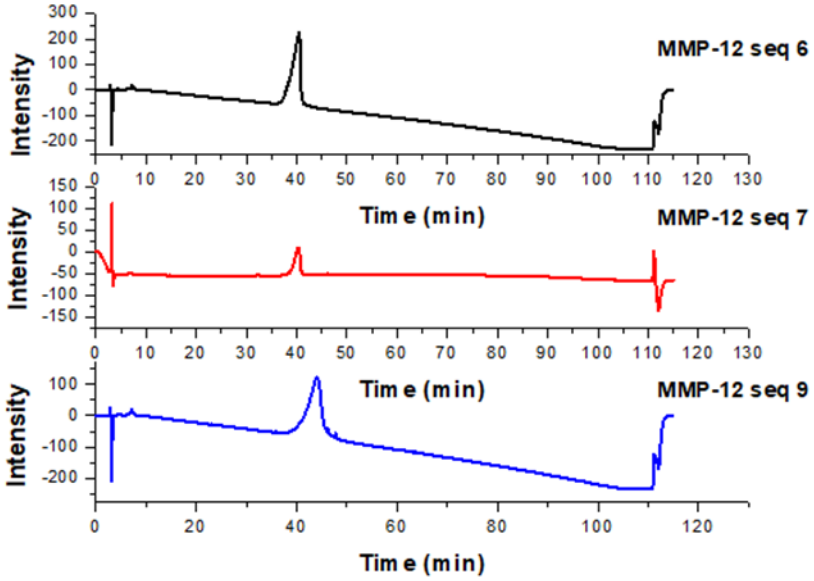


Figure 1. Reverse phase HPLC chromatogram of MMP-12_seq 6, MMP-12_seq7, and MMP-12_seq9. Gradient is 0-100 solvent B over 100 minutes employing a Vydac C18 analytical column. Solvent A = 100 % water, 0.1 % TFA; solvent B = 90 % ACN in water, 0.1 % TFA.

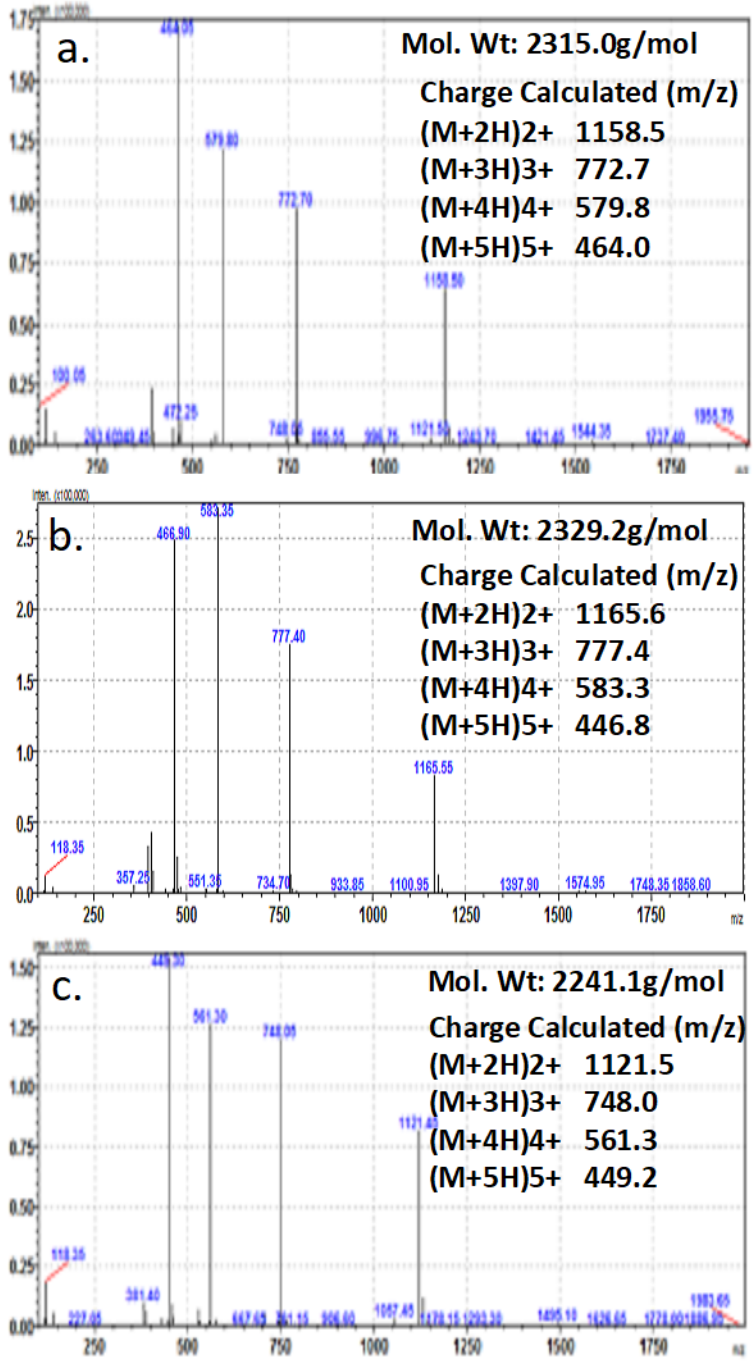


Figure 2. LCMS-derived mass spectra of a. MMP-12_seq 6, b. MMP-12_seq7, and c. MMP-12_seq9 collected on an LC-2018 Smimadzu mass spectrometer.

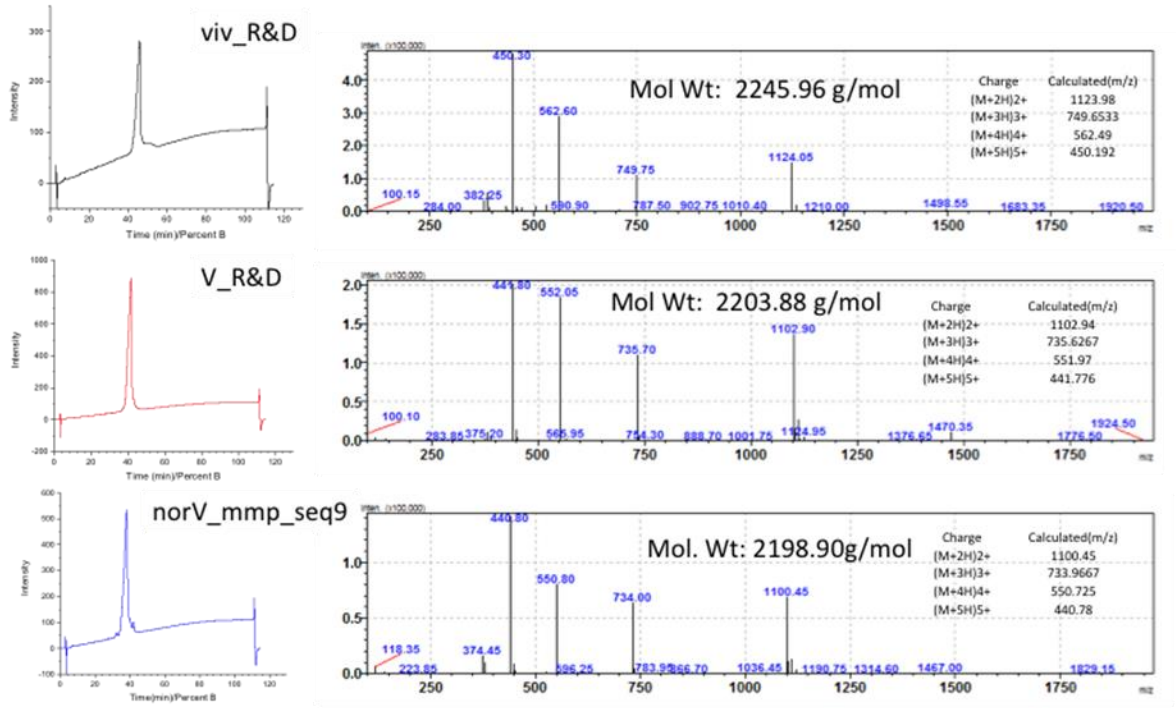


Figure 3. HPLC Chromatograms and MS spectra of viv_R&D, V_R&D, norV_mmp_seq9.

Soluble peptide degradation in solution

In this subtask, we assess the MMP-based proteolytic susceptibility of the peptides used to form the gels to first gain an understanding of the susceptibility of the soluble peptide alone before initiating the more laborious gel studies. We used a commercially available MMP12 enzyme. As can be seen in **Figure 4**, MMP_seq6/mmp(6) and MMP_seq7/mmp(7) are not cleaved by the enzyme over days. However, MMP_seq9/mmp(9) is partially degraded slowly over days and will be suitable to carry forward in gel-based degradation studies.

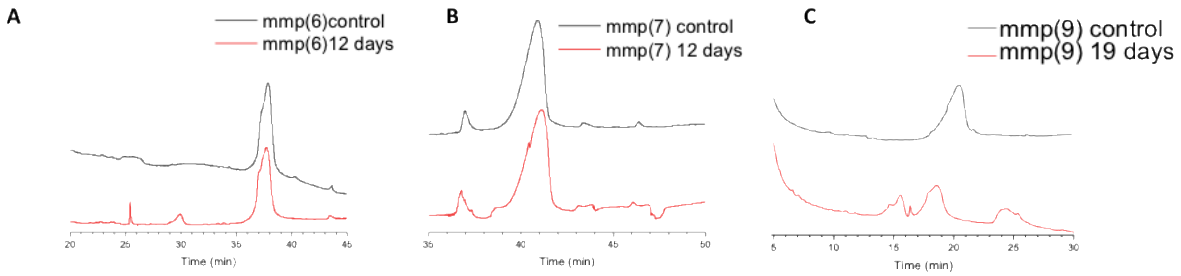


Figure 4. MMP12 degradation of indicated peptides as measured by HPLC. Only the mmp(9) peptide is degraded.

Although we obtained one peptide (MMP_seq9/mmp(9)) suitable for future development, we wanted to have several peptide gels to take forward to have a broad range of materials with respect to degradation times (slow/medium/fast degrading). As such, we designed three additional sequences (i.e. norV_mmp_seq9/mmp(9)norV, V_R&D, Viv_R&D) in which the MMP12 cleavage site has been reengineered by the following approach. We first synthesized short peptide sequences comprising residues of known substrates of MMP12 and assessed their susceptibility to enzyme-mediated cleavage. **Figure 5** shows the sequences of the short peptides as well as HPLC chromatograms in the presence and absence (control) of MMP12. All three peptides are, in fact, cleaved by the enzyme. However, our previous studies showed that full

length peptides containing the -ELR- sequence were not susceptible and that full length peptides containing the –PLGVR- sequence were cleaved slowly. Thus, for the new full length peptides, we incorporated the -PLGLFAR- sequence. In addition, we further modified the hydrophobicity of the new gel-forming peptides.

- hELR PTELRVKIVK-NH_2
- hPLGVR PLGVRVKIKV-NH_2
- hR&D PLGLFARVKV-NH_2

Peptide concentration: 20uM
MMP-12: 0.8ug
Total Volume: 400uL

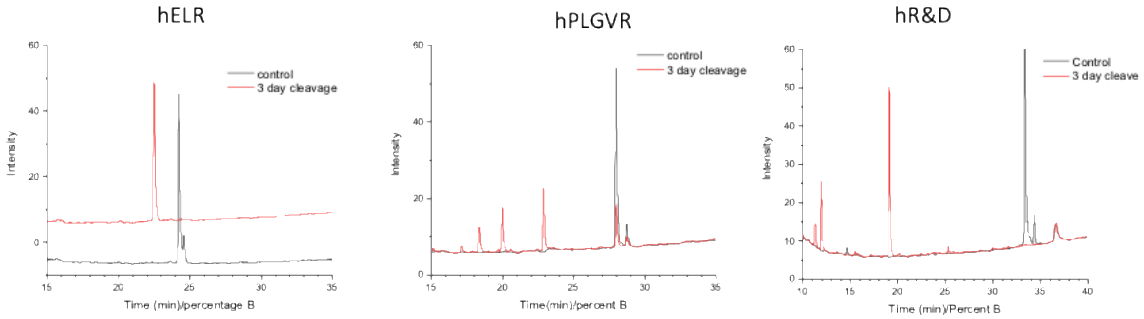


Figure 5. Enzyme susceptibility of short model peptides comprising MMP12 substrate residues monitored by HPLC.

We next studied the MMP12-mediated cleavage of the three new full length peptides. Gratifyingly, **Figure 6** shows that all three peptides are cleaved by the enzyme to varying degrees over days. In conclusion, solution-based assays show that four of the six peptides are susceptible to the enzymatic action of MMP-12 (highlighted yellow in **Table 1**).

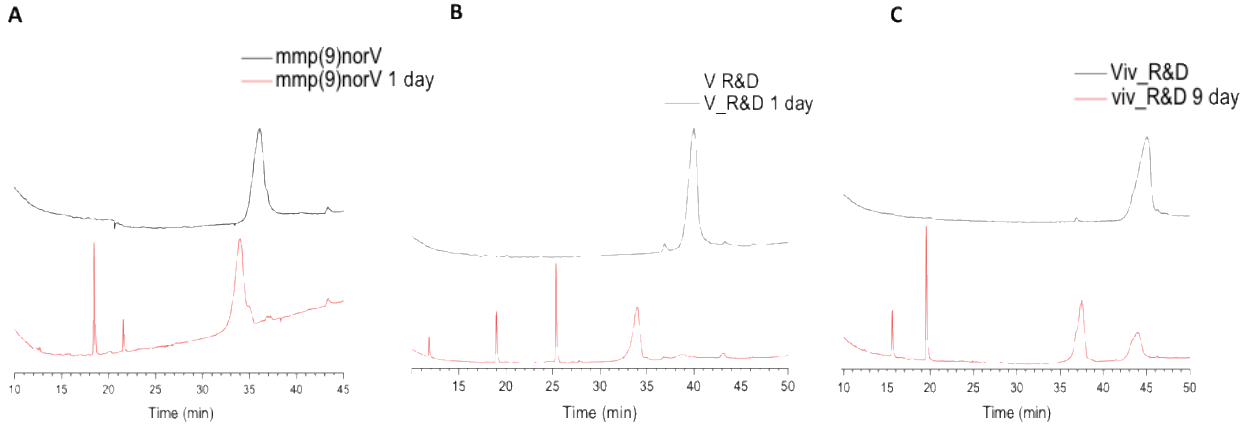


Figure 6. MMP12 degradation of indicated full-length peptides as measured by HPLC.

Degradation kinetics of self-assembled peptide hydrogels

We then quantitated in vitro MMP12-mediated degradation kinetics of self-assembled peptide fibrils in solution (**Figure 7**). We found that it is possible to generate two classes of self-assembling peptides, one that is degraded quickly (within 3d) and the second that is degraded slowly (>20d). From a peptide design perspective, the incorporation of isoleucine into the peptide's primary sequence decreases the rate of MPP-12 proteolysis (**Table 1**, **Figure 7**). This solution-based assay reports on enzymatic susceptibility in vitro over 21d. We later assessed resorption rates *in vivo* (*vide infra*) in **Major Task 3.**

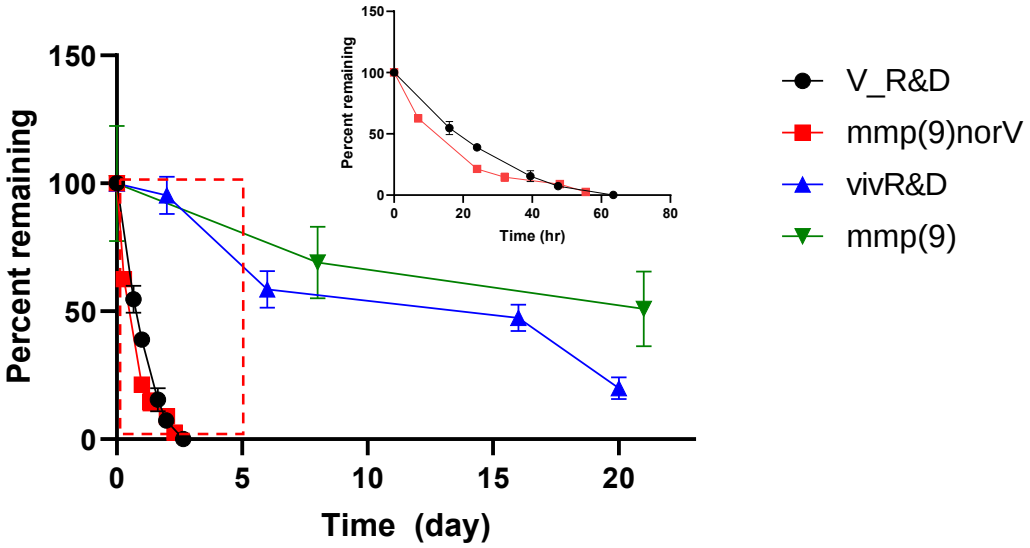


Figure 7. Solution-based in vitro MMP-12 degradation of self-assembled peptide fibrils over 21d.

Rheology properties of self-assembled peptide hydrogels

We went on to form gels with a total of four full-length peptides using our self-assembly protocol and defined their mechanical properties by time-sweep oscillatory rheology. **Figure 8** shows that each peptide self-assembles to form semi-rigid viscoelastic gels capable of undergoing repeated cycles of shear-thin/recovery. This study shows that all gels can be delivered to tissue by simple syringe injection as proposed.

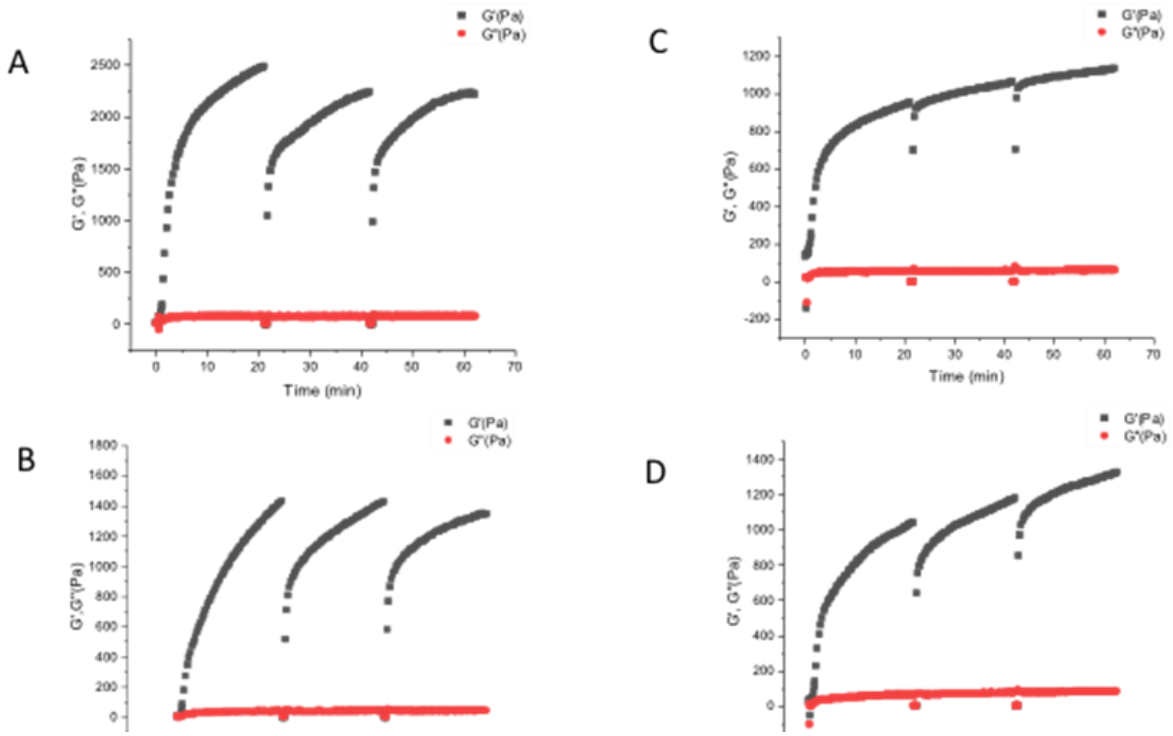


Figure 8. Dynamic oscillatory rheology of 1wt% gel of(A)mmp_seq9, (B) norV_mmp_seq9, (c) vivR&D, (D)V_R&D in 50mM BTP 150mM NaCl at pH 7.4 and 37ºC.

Site 1 JHU

Subtask 2: *Assessment of hydrogel susceptibility to degradation by supernatant of macrophages and related Tofa release.*

***In vitro* macrophage activity and MMP production**

To complement the previous experiments based on the use of recombinant MMP12 (and obtain data that could better represent the *in vivo* conditions found during transplant rejection), we tested if *in vitro* generated mouse bone marrow-derived macrophages would release active MMPs once stimulated. To this end, macrophages were derived from mouse bone marrow cells using standard published protocols based on culturing with M-CSF. At the end of the culture, maturation of the macrophages was induced via exposure to either LPS or Poly(I:C) (two well characterized Toll Like Receptors ligands). Supernatant from cell culture plates was collected 18 hours after stimulation and the MMPs production were detected using a fluorogenic probe (Mca-K-P-L-G-Dpa-A-R-NH2, Fluorogenic Peptide Substrate IX from R&D systems). In a first experiment, the fluorogenic probe was combined with a titration of the supernatants (using unstimulated macrophages as control supernatant) and the fluorescence of the solution at different incubation time points was detected via a fluorometer (SoftMax Pro analysis software). However, the fluorescence intensity from supernatants had no difference between stimulated and unstimulated groups, and the intensity of signal did not correspond to the titration level (data not shown).

We then tested our detection system by using a solution of recombinant mouse MMP12 enzyme. In this case, we had no trouble observing an increasing level of fluorescence generated by a titration of the recombinant enzyme. Based on this result, we suspected that our *in vitro* stimulation of macrophages would possibly not induce a significant production of MMPs. To test this, we measured intracellular mRNA levels of a set of MMPs before and after stimulation of the macrophages. As shown in **Figure 9**, quantitative real time PCR showed that mRNA levels of MMP-1b, 3, 7, 9 ,12 and 13 increase with the maturation status. Interesting, this experiment indicated a different dynamic in mRNA accumulation, with MMP 1b and 13 peaking after 4 hours, while MMP-7, 9 and 12 mRNA levels were higher after a longer maturation process and in the presence of Poly(I:C).

Having confirmed at the gene expression level the stimulation of the production of MMPs by in vitro activated macrophages, we tested the presence of enzymatic activity in the supernatant with a modified protocol of the R&D fluorogenic probe. In this case, the supernatants were pre-incubated with a solution that promotes the full activation of MMPs. Again, unfortunately, the fluorescent intensity in stimulated group had no difference comparing to that of the unstimulated group.

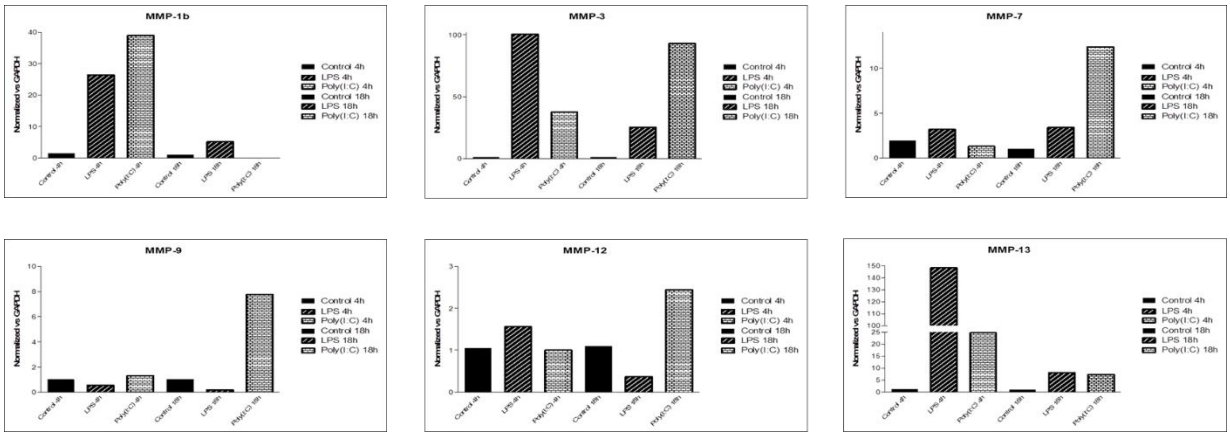


Figure 9. Expression of MMPs mRNA in BM derived macrophages. BM-derived-macrophages were left untreated or matured for 4 or 18h with either LPS or Poly(I:C). Cells were then lysed and mRNA levels of MMP-1b, 3, 7, 9, 12 and 13 were measured by qPCR. ΔΔCT method was used to calculate their relative expression, normalizing them to GAPDH. The graph bars show the fold change between immature and matured macrophages in the different conditions.

***In vivo* MMP activity in mouse hind limb transplant model**

Because of the issues encountered in assessing MMP activity in the supernatants of mouse macrophage cultures, we decided to focus on demonstrating the presence of MMP activity during hind limb rejection and possibly determine the most relevant MMP involved. To demonstrate the presence of MMP activity *in vivo*, we implemented the "activatable" probe MMPSense 750 Fast (Perkin Elmer) that causes the localized release of a fluorophore when degraded by MMPs (and detectable vie the IVIS whole animal imaging system). We performed 4 mouse hind limb transplants (the allogeneic combination BALB/c into C57BL/6 mice) assigning 2 recipients to the sequential observation of post-operative day (POD)-5 and 11 and 2 recipients to observation of POD7 and POD13 (as the probe requires 5-6 days for complete clearance). All four animals were subjected to administration of CTLA4-Ig, as per our proposed treatment strategy. In addition, we repeated the experiment with syngeneic hind limb transplants, where BALB/c hind limb was transplanted into BALB/c mouse. In principle, the syngeneic model allows to detect the extent and timing of MMP activity associated with the surgical insult and with wound healing, but not with graft rejection (absent in this model). In allogeneic settings, the transplanted limbs showed a significant fluorescent signal, associated with MMP enzymatic activity, mostly in the muscular component of the graft (**Figure 10**). The MMP activity was low on POD 5 and 7 and increases on POD 11 and 13 with a wide distribution throughout the graft. As anticipated, the syngeneic model also revealed MMP activity in the graft, with a predominant focus on the suture site (**Figure 11**). In comparison to the allogeneic combination, the syngeneic model showed a more scattered and lower MMP activity. With this preliminary screening, we noted that the results of the syngeneic model are complicated by the observation that some of the mice inflicted bite wounds in the graft, events that caused transplant unrelated MMP activity. These observations warrant additional syngeneic model experiments, where precautions will be taken to minimize biting of the graft. Furthermore, we plan to investigate the specific composition of MMP involved in allogeneic and syngeneic models and identify if significant differences exists between these conditions. This information will be pivotal in guiding the optimization of hydrogel susceptibility to degradation in response to transplant rejection.

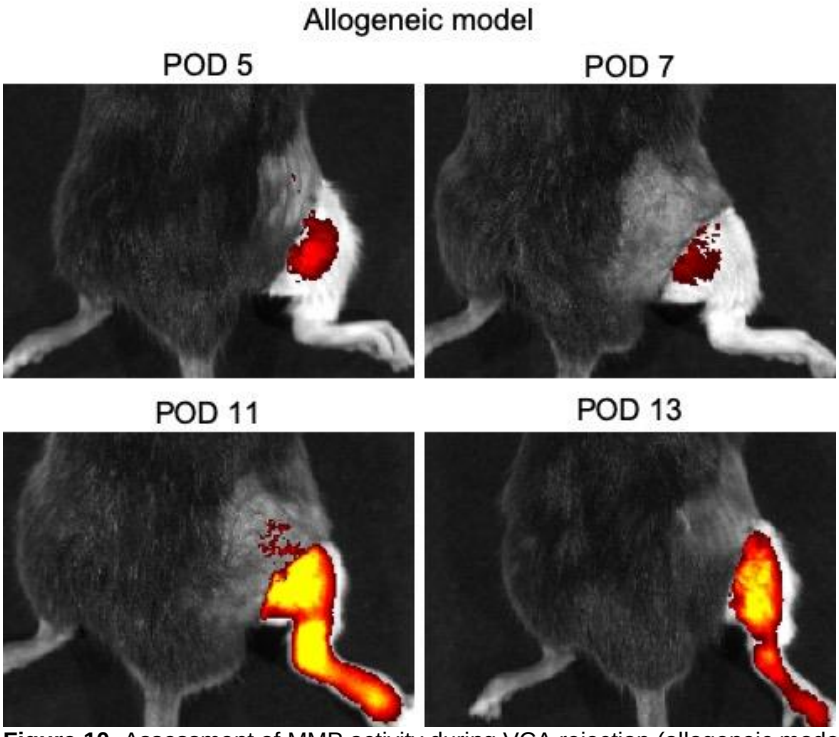


Figure 10. Assessment of MMP activity during VCA rejection (allogeneic model). Representative images of MMP activity detection via "MMPSense 750 Fast" probe into B6 mice recipient of BALB/c hind limb (and subjected to CTLA4-Ig on PODs 0, 2, 4, 6) at the indicated post-operative days.

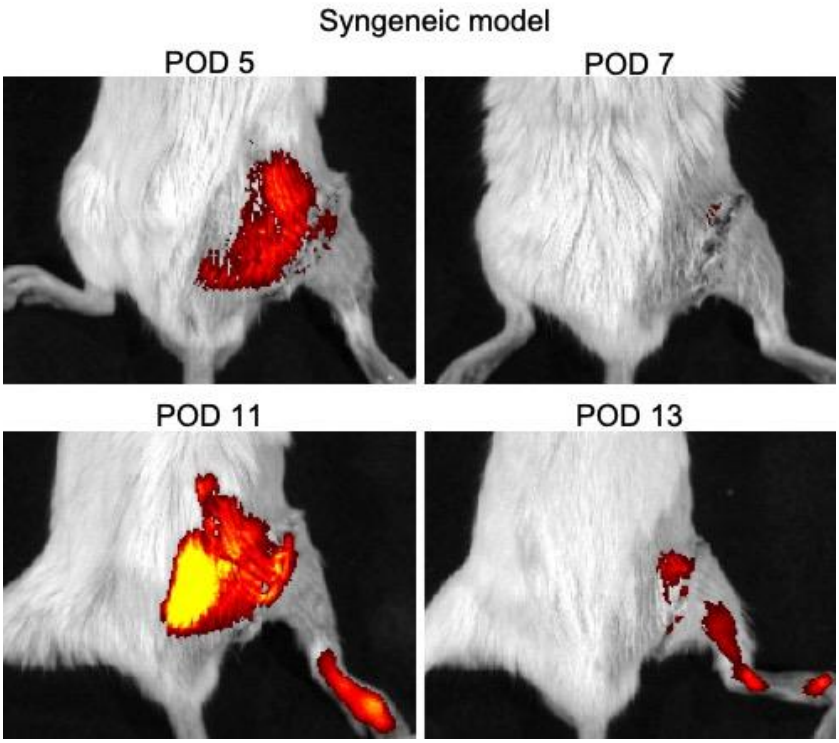


Figure 11. Assessment of MMP activity associated to transplantation but in absence of rejection. Representative images of MMP activity detection via "MMPSense 750 Fast" probe into BALB/c mice recipient of BALB/c hind at the indicated post-operative days.

Major Task 2: Optimize Tofa-LNp tropism and drug release profile

Site 2 JHU/APL

Subtask 1: *Preparation of altered formulations of Tofa-LNp and characterization of physical and thermal properties*

NLC (44/14) particle characterization

We explored altered formulations of LNp that will be well-suited to incorporation of Tofacitinib (Tofa) and for inclusion in the hydrogel material. These nanostructured lipid carrier (NLC) formulations contain both solid and liquid lipid components and are hypothesized to exhibit a higher drug loading potential in comparison with our previous solid lipid nanoparticle (SLN) formulations. NLCs were formulated using mixtures of tetracosane, tocopherol, and Gelucire 44/14 and were prepared using the phase inversion temperature method (PIT). The PIT method keeps the composition constant while the temperature is changed and is well-suited to the synthesis of small particles, characterized by low polydispersity. **Table 2** summarizes the processing conditions for the control and Tofa-loaded NLC formulation. The loading concentration of the Tofa-NLCs was 6 mg/mL. Further characterization of the Tofa-NLCs showed a melting point of 43.4˚C and a latent heat of melting of 1.31 J/g.

Table 2. Summary of the Synthesis Processing Conditions, Particle Size and Polydispersity

Sample	Solid Lipid	Liquid Lipid	Surfactant	Sol. to Liq. Lipid Ratio	Lipid to Surfactant Ratio	Processing Temperature (°C)	Particle Size (nm)	PDI
Control	Tetracosane (C24)	Tocopherol	Gelucire 44/14	50:50	1:2	90	83 ± 7	0.22 ± 0.07
Tofa	Tetracosane (C24)	Tocopherol	Gelucire 44/14	50:50	1:2	90	118 ± 70	0.18 ± 0.13

HPLC method for Tofa quantification

We developed a high performance liquid chromatography (HPLC) method for detection of Tofa. Methods development included system suitability analysis and calibration. The mobile phase for analysis was 1-octanesulfonic acid with 0.1% phosphoric acid (H_3PO_4) and acetonitrile (75:25, v/v). The analysis was performed isocratically with a flow rate of 0.25 mL/min. The detection wavelength was set to 290 nm. Each volume of accurately weighed Tofa standard sample was diluted to volume with diluent to obtain a standard curve. The R^2 value for the best fit line for the calibration curve had a value of 1.000 (**Figure 12**). The system proved to be suitable for the analysis with a %RSD value of replicate injections of the 40 µg/mL standard solution of 0.41. Each accurately measured aliquot of the separated NLC solutions, containing Tofa, was diluted to volume with diluent and run on HPLC using the exact parameters as above. All samples were injected using a 20 µL sample loop. Tofa eluted at approximately 2.04 minutes for all samples and standards injected. The complete run time for all chromatographic runs was 6.0 minutes.

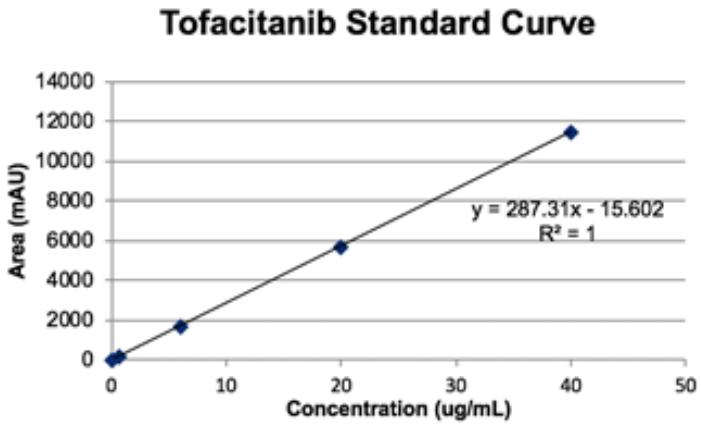


Figure 12. Tofacitinib system suitability.

Tofa encapsulation efficiency and release profile for NLC (44/14)

High performance liquid chromatography (HPLC) was utilized to determine the encapsulation efficiency for Tofa, in comparison with previous synthesis methods (solid lipid nanoparticles, SLN, see ***Table 3.*** *Encapsulation efficiency measurements* **Table 3**). Briefly, the determination of the Tofa-NLC drug loading, or encapsulation efficiency (EE%), was calculated following separation of the organic and aqueous phases using a spin-X UF concentrator followed by centrifugation. Following this separation, the final volumes for both phases were analyzed using HPLC to quantify the amount of Tofa in each phase. Freshly prepared Tofa-NLCs were analyzed in parallel to quantify the total amount of Tofa in each starting preparation. Encapsulation efficiency was calculated by dividing the mass of Tofa in the organic phase by the total mass of Tofa and multiplying by 100. Three independent batches were prepared and tested by this method. The Tofa-NLC (44/14) formulations were found to exhibit a slightly higher encapsulation efficiency in comparison with previous particle formulations (**Table 3**).

Table 3. Encapsulation efficiency measurements.

Formulation	NLC Encapsulation Efficiency	SLN Encapsulation Efficiency
1	79	72
2	78	73
3	79	75
Average	79 +/- 1	73 +/- 1

The release kinetics of Tofa-NLC (44/14) were determined following dialysis of a 200 µL sample of each freshly prepared Tofa-NLC (44/14) sample into a 5 mL volume of phosphate buffered saline (37°C, shaking) for a given time period. Three technical replicates of each sample were prepared and analyzed over a 72-hour time course. At specific time points, an aliquot of the buffer was collected for subsequent HPLC analysis. This experiment revealed a biphasic drug release pattern (**Figure 13**) where a burst release occurred in the first 8 hours and was then sustained at a low level over approximately 30 hours. Under these experimental conditions, the maximum drug release achieved was 90%.

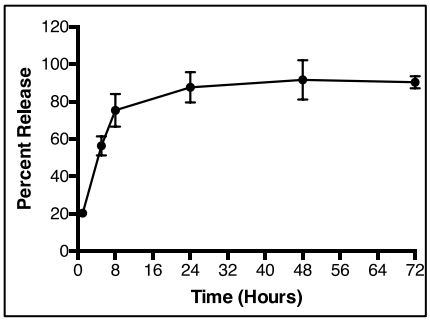


Figure 13. Tofa release profile from Tofa-NLC (44/14).

Additional NLC formulations

Based on the efforts to stabilize the Tofa-NLC (44/14) in the peptide hydrogels, the current Tofa-NLC (44/14) formulation was found to require slight modifications (please see **Major Task 3** update). Substitution of a percentage of the Gelucire 44/14 surfactant has resulted in several stable nanoemulsions, which exhibit suitable particle size and PDI, and have been shown not to impede gelation of the peptide hydrogel. Two additional surfactants were successfully incorporated into the NLC platform and shown to allow stable gelation using Dr. Scheider's protocol. Based on these results, as well as particle size measurements, several preparations were chosen for further characterization. Four test preparations were synthesized in triplicate, using different ratios of Gelucire 48/16 and 50/13 (**Table 4**, blue rows).

Table 4. NLC Properties. Altered NLC formulations were previously characterized by DLS to determine particle size and PDI. Rows highlighted in blue indicate the formulations that were further tested for Tofa encapsulation efficiency.

Sample	Gelucire	Surfactant1:Surfactant2	Lipid:Surfactant	Particle Size (nm)	PDI
1	44/14, 48/16	100:0	1:2	333	0.726
2	44/14, 48/16	75:25	1:2	30	0.26
3	44/14, 48/16	50:50	1:2	33	0.319
4	44/14, 48/16	25:75	1:2	33	0.279
5	44/14, 50/13	0:100	1:2	158	0.484
6	44/14, 50/13	75:25	1:2	28	0.207
7	44/14, 50/13	50:50	1:2	68	0.225
8	44/14, 50/13	25:75	1:2	60	0.553

Tofa encapsulation was determined by HPLC and found to range from 76-82 EE% across all samples (**Table 5**). Additionally, a drug release experiment was conducted in which samples were taken at multiple time points over the course of three days. HPLC analysis of these samples is currently in progress and results are expected to inform *in vivo* administration and dosing protocols in the upcoming year.

Table 5. Encapsulation Efficiency. Tofacitinib encapsulation resulting from varied NLC surfactant composition.

Sample	NLC Surfactant Composition	Encapsulation Efficiency (EE%)
44/14 Control	Gelucire 44/14 **Control**	82 ± 2
2	Gelucire 44/14:Gelucire 48/16 **75:25**	78 ± 6
3	Gelucire 44/14:Gelucire 48/16 **50:50**	81 ± 3
4	Gelucire 44/14:Gelucire 48/16 **25:75**	76 ± 11
6	Gelucire 44/14:Gelucire 50/13 **75:25**	83 ± 10
SLN Control	Solid Lipid Nanoparticle	72 ± 11

Site 2 JHU/APL & Site 1 JHU

Subtask 2: Assessment of new LNp formulations biodistribution (DilC-LNp), cell tropism (DilC-LNp), and functional release of Tofa (Tofa-LNp).

Cytotoxicity measurements for NLC formulations

With the initial optimization of Tofa-NLC (44/14) formulation (focused on obtaining particles with size and properties that would maintain the desired unique properties of *in vivo* distribution and uptake of LNp, **Subtask 1**), we performed a first screening of the toxicity of this formulation on bone marrow-derived mouse dendritic cells. As indicated in **Figure 14**, different formulations of LNp had different effects on cells viability following 24-hour incubation. In comparison to the original SLN formulation, NLC (44/14) and Tofa-NLC (44/14) showed a very promising low toxicity profile that supported their implementation in both our in vitro and in vivo studies.

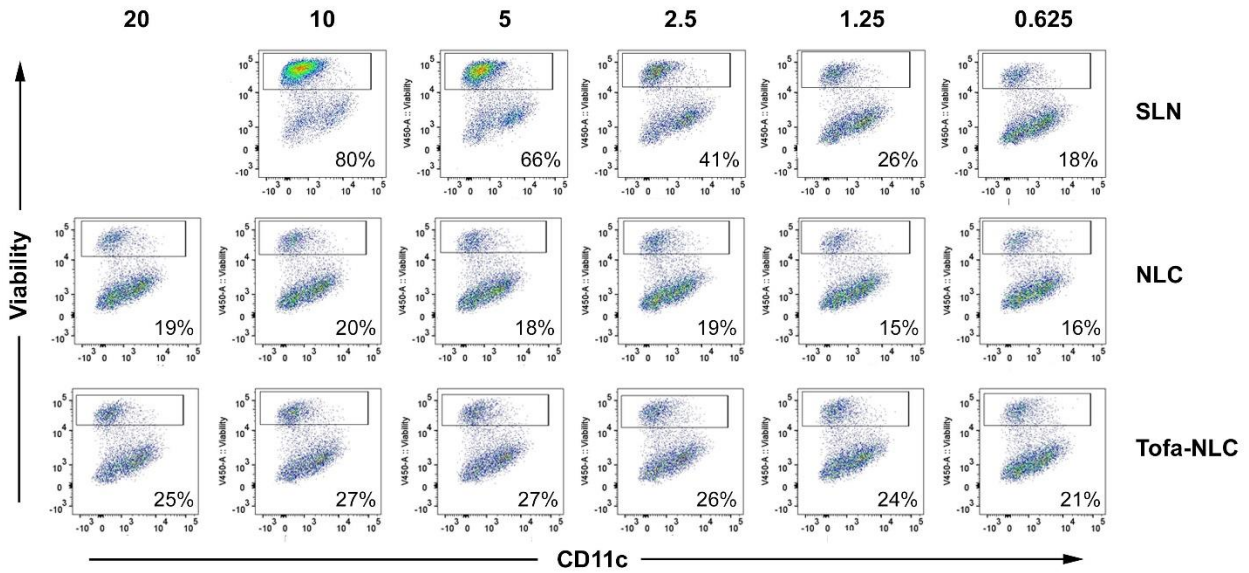


Figure 14. Screening of toxicity profile of NLC (44/14) formulations. Graph depicts the impact of different LNp formulations on the viability of mouse bone marrow-derived dendritic cells. DC were cultured for 24 hours with titrations (numbers on top of each column, expressed as µg/ml of LNp concentration) of different formulations of LNp (indicated on the right). Numbers within each plot indicate the percentage of dying cells for the specific condition.

We performed additional measurements of the cellular toxicity associated with the new NLC formulations (50/13 and 48/16), that were identified to be better compatible with hydrogel gelation, and compared them to the original control NLC (44/14). NLCs were incubated with bone marrow-derived dendritic cells for 24 hours at different concentrations, followed by cell viability measurement by flow cytometry. Results of two independent experiments are shown in **Figure 15**. Both experiments (performed in slightly different conditions – see figure legend for details) indicated that percentages of dead cells (%FVD +) after NLC treatments are similar to baseline percentages in untreated controls. The NLC variants do not appear to be toxic to bone-marrow derived dendritic cells at the concentration range of 0.5-200 µg/mL, indicating that the new formulations remained highly bio-compatible as the initial formulation studied.

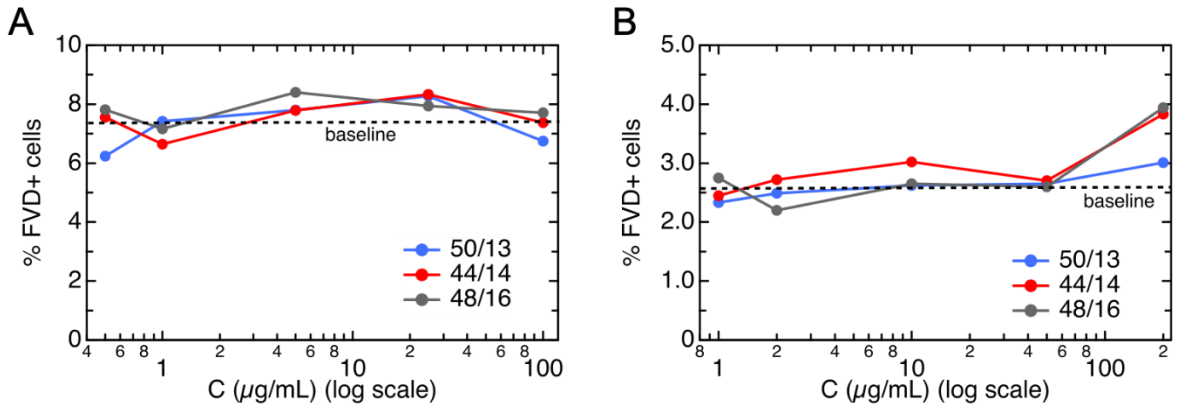


Figure 15. Screening of toxicity profile of NLC formulations 44/14, 50/13, and 48/16. NLCs were incubated with bone marrow-derived dendritic cells for 24 hours and dead cells were detected by the uptake of FVD dye (FVD+ cells) and quantified by flow cytometry. Dashed line represents the percentage of dead cells in untreated control. Data are shown for two independent experiments: (A) NLCs were incubated with approximately $5x10^5$ cells per condition. DCs were first differentiated from bone marrow and then aliquoted into a 24-well plate for the experiment. (B) NLCs were incubated with approximately 10^5 cells/condition. DCs were differentiated directly in 24-well plates, which is expected to decrease DC maturation and baseline counts of dead cells. Data represent mean.

DilC-NLC (44/14) production

In preparation for *in vivo* biodistribution experiments, we synthesized NLC loaded with a fluorescent dye (1,1',3,3,3',3'-Hexamethylindotricarbocyanine iodide, HLDI, DilC), according to our previously determined methods. In this formulation, the dye replaces Tofa. Briefly, the dye was dissolved into ethanol prior to co-melting with tetracosane and tocopherol at 90˚C. The resulting mixture was stirred using a vortex prior to addition of the surfactant (Gelucire 44/14). The mixture was again co-melted at 90˚C and stirred. Water was added next, and the mixture was then heated until two phases were visible. Controlled cooling of the sample while stirring resulted in the transparent nanoemulsion.

Cell uptake of DilC-NLC (44/14)

Though new formulations of lipid nanoparticles were being designed by our collaborators at APL to address the issue of hydrogel stability, we continued the characterization of the interaction of immune cells with Tofa-NLC (44/14). Having confirmed the low toxicity of NLC, we used DilC-NLC (44/14) (prepared at Site 2) to study the incorporation kinetic of these particles by mouse bone marrow-derived dendritic cells (BMDC). As shown in **Figure 16**, DilC-NLC (44/14) are rapidly incorporated by BMDC with a function that is proportional to their concentration and to the length of exposure.

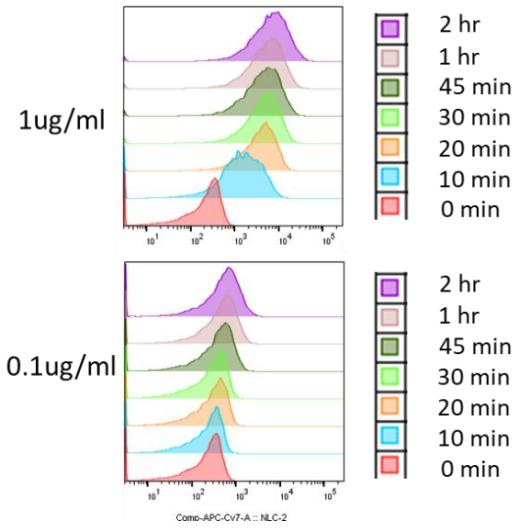


Figure 16. Incorporation kinetic of NLC by mouse BMDC. Representative results of the incorporation of DilC-NLC (44/14) (added at either 0.1 or 1 µg/ml) by mouse dendritic cells exposed for the time indicated. Incorporation is measured as increase of fluorescence in comparison to baseline (time 0).

However, those experiments could not indicate if the association of dendritic cells to particle fluorescence was due to a “superficial coating” of the cells or an actual internalization. We then performed a similar analysis but using confocal microscopy to determine the level of cellular internalization of DilC-NLC (44/14) at different time points. As shown in **Figure 17**, particle fluorescence can be detected very early (10 min exposure) in intracellular compartments of exposed DC. These results confirm that NLC can deliver their cargo intracellularly.

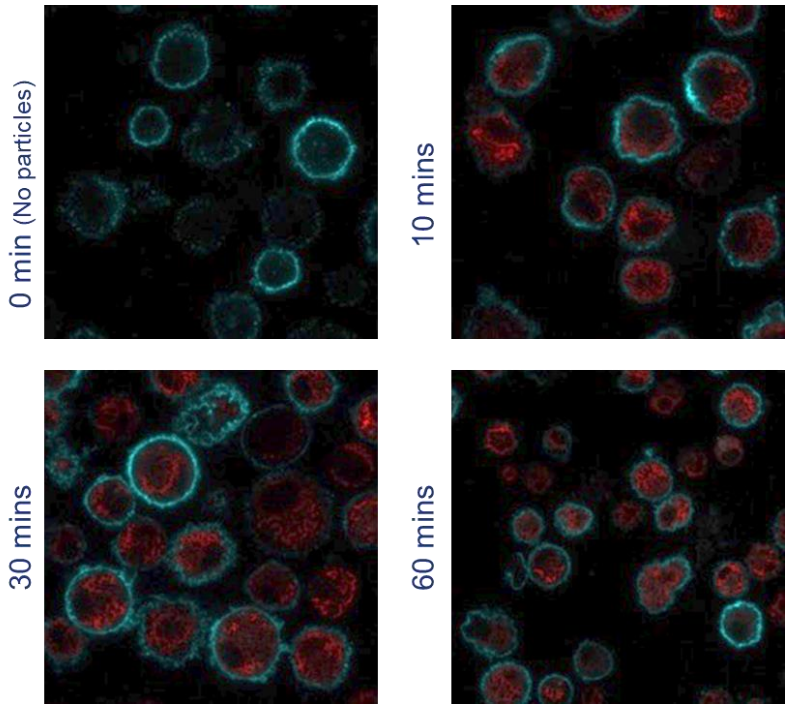


Figure 17. Lipid nanoparticles internalization by dendritic cells. Representative images of confocal microscopy imaging of mouse bone marrow derived dendritic cells incubated with DilC-NLC (44/14) (red) for the indicated times and then stained for CD11c (cyan).

Spectral characteristics of DilC dye and DilC-NLC

The Patrone and Schneider laboratories worked to jointly determine the spectral characteristics of the DilC dye and DilC-NLC, as a means to measure hydrogel particle release kinetics. Two excitation wavelengths (587 and 675 nm) were tested for direct comparison to both fluorescence spectra from literature[1] as well as previous *in vivo* fluorescence measurements taken using the In Vivo Imaging System (IVIS) (**Figure 18**). This system will be used by Dr. Raimondi's laboratory for whole animal imaging following Hydrol(Tofa-LNp) administration. The fluorescence emission was observed to shift to a longer wavelength with increasing sharpness as the dye or dye-NLC concentration increased. This phenomenon is likely due to dye aggregation in solution. Moving forward, it will be important to standardize our analytical methods when using the dye as a proxy for Tofa, in order to directly compare results of dye/particle release kinetics.

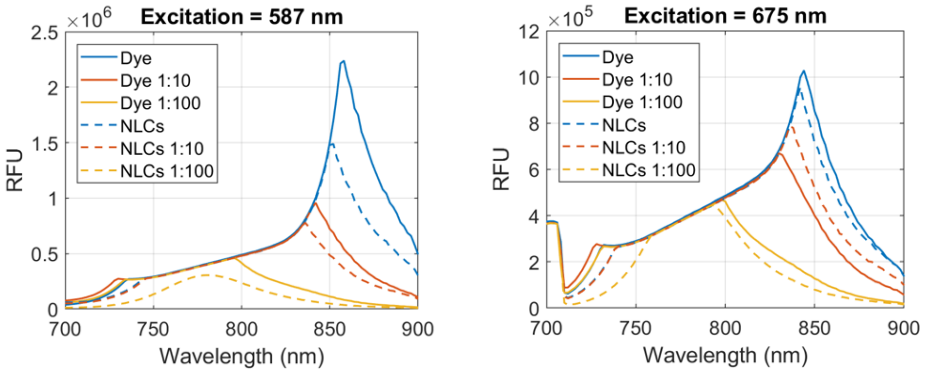


Figure 18. Emission spectra of free dye and dye-loaded NLC. Free dye was dissolved in ethanol at a concentration of 0.35 mg/ml and serially diluted in water (1:10 and 1:100, pH = 7.4) to more closely match the NLC solution. Fluorescence measurements were taken using a Tecan microplate reader, using a single excitation wavelength, either 587 nm (left), or 675 nm (right) and an emissions wavelength range of 700-900 nm.

[1] Ivri, J., Z. Burshtein, and E. Miron. "Characteristics of 1, 1′, 3, 3, 3′, 3′-hexamethylindotricarbocyanine iodide as a tunable dye laser in the near infrared." *Applied optics* 30.18 (1991): 2484-2488.

Major Task 3: Optimize in vivo targeted drug release by Hydro(Tofa-LNp) composite

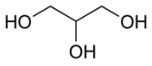
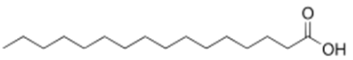
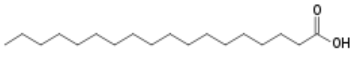
Subtask 1: Formulation of protease-sensitive Hydro(Tofa-LNp) composite and characterization

Site 2 JHU/APL

Hydrogel formation with Tofa-NLC (44/14)
To begin optimizing the Hydro(Tofa-LNp) for *in vivo* experiments, Tofa-NLC (44/14) formulations were tested for their influence on hydrogel gelation (in comparison with previous Tofa-SLNs). Tofa-NLCs (44/14) were freshly prepared and sent to our NCI collaborators (Site 3) for incorporation into hydrogels. This NLC formulation appeared to limit gelation, and it was hypothesized that this may be due to the different components in the formulation in comparison to SLN, and possibly excess surfactant. As a first attempt to improve the hydrogel gelation in the presence of the Tofa-NLC (44/14), we employed two different methods to remove excess surfactant molecules: dialysis and size exclusion. Samples were analyzed by dynamic light scattering (DLS) following both purification methods in order to confirm that processing did not remove or destroy the particles. Unfortunately, these initial purification steps were not sufficient to improve hydrogel stability (data not shown).

Alternate NLC formulations and their characterization
To achieve synthesis of stable Hydro(Tofa-LNp) for *in vivo* experiments, the Tofa-NLC formulation was modified with the goal of altering the surfactant components without significantly impacting particle physicochemical properties. The surfactant originally used to form Tofa-NLC (44/14) is a mixture of mono, di-, and triglycerides with PEG esters of fatty acids (Gelucire 44/14), however this surfactant appears to be impeding gelation of the peptide hydrogel. Gelucire comprises a family of surfactants with a range of hydrophilic lipophilic balance (HLB) values and melting temperatures, allowing additional flexibility to the components of the NLC formulation without dramatic changes. We identified two additional surfactants, Gelucire 48/16 and Gelucire 50/13, that are also non-ionic surfactants and have a similar HBL as Gelucire 44/14 (**Table 6**), making them suitable for side-by-side comparison in the NLC formulation.

Table 6. Gelucire Products. All three formulations have a similar HLB and have been used in drug delivery formulations to extend release.

Name	Composition	Hydrophilic Lipophilic Balance	Structure
Gelucire 44/14	PEG-esters, a small glyceride fraction and free PEG	11	
Gelucire 48/16	PEG-32 (MW 1500) esters of palmitic (C_{16}) and stearic (C_{18}) acids	12	
Gelucire 50/13	mono, di- and triglycerides and PEG-32 (MW 1500) mono- and diesters of palmitic (C_{16}) and stearic (C_{18}) acids	11	

All three Gelucire products were evaluated for their ability to form stable nanoemulsions and result in small particle size with low polydispersity (PDI, **Figure 19**). During testing of these three products, the surfactant ratios were varied. The particle size and PDI were measured using dynamic light scattering (DLS) (**Table 7**). Two promising formulations (**Table 7**, blue rows) were sent to our Dr. Schneider's laboratory to determine their compatibility with the peptide hydrogels.

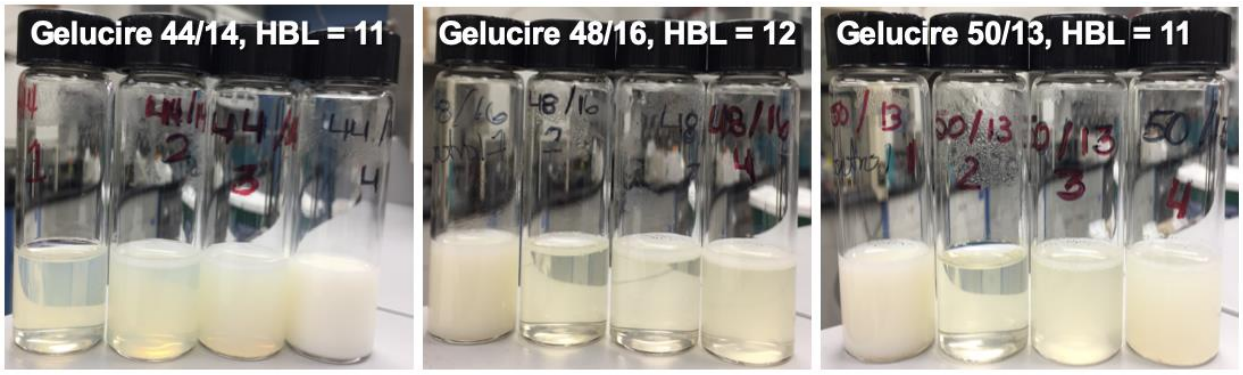


Figure 19. NLC Formulations. Four formulations were prepared, using different Gelucire products. The transparency of the solution correlates with a smaller particle size and lower PDI.

Table 7. NLC Properties. Altered NLC formulations were characterized by DLS to determine particle size and PDI. Rows highlighted in blue indicate the formulations that resulted in stable hydrogel formation.

Sample	Gelucire	Surfactant1:Surfactant2	Lipid:Surfactant	Particle Size (nm)	PDI
1	44/14	0.5	1:2	64	0.242
2	44/14	0.375	1:2	107	0.126
3	44/14	0.250	1:2	94	0.085
4	44/14	0.125	1:2	201	0/194
1	44/14, 48/16	100:0	1:2	333	0.726
2	44/14, 48/16	75:25	1:2	30	0.26
3	44/14, 48/16	50:50	1:2	33	0.319
4	44/14, 48/16	25:75	1:2	33	0.279
1	44/14, 50/13	0:100	1:2	158	0.484
2	44/14, 50/13	75:25	1:2	28	0.207
3	44/14, 50/13	50:50	1:2	68	0.225
4	44/14, 50/13	25:75	1:2	60	0.553

Site 3 NCI

Hydrogel stability with alternate NLC formulations

We performed preliminary ex-vivo hydrogel formulations experiments in which the stability of the peptide-based hydrogels was assessed as a function of nanoparticle composition. Our target material is one in which Tofa-loaded nanoparticles are encapsulated in peptide gels. Our initial formulation experiments indicated that the original 44/14_1 nanoparticles caused some of the peptide gels in **Table 1**, as well other previously designed gels that we had access from other projects, to undergo an undesirable gel-to-solution phase state change (**Table 8**). Gels were prepared by dissolving solid peptide in H_2O (2 wt%) and chilling the resulting solution on ice. Then, 25 µL of nanoparticle stock solution, 25 µL 4xHBS buffer, and 50 µL peptide solutions were mixed and allowed to gel at 37°C. Non-compatible nanoparticles caused the gels to undergo the phase change over days. Nanoparticle 44/14_1 was incompatible with all peptide gels tested. As a result, additional nanoparticles were prepared by Dr. Patrone's lab via adjusting lipid/surfactant composition and encapsulated into independent peptide gels to screen for gel-compatible nanoparticles . Gratifyingly, we found that 48/16_2 and 50/13_2 particles to be compatible to the first two peptide gels tested. Thus, we plan to use these particles for future studies.

	44/14_1	44/14_2	44/14_3	48/16_2	48/16_3	50/13_2
mmp(6)	unstable	unstable	unstable	stable	unstable	stable
mmp(7)	unstable	N/A	N/A	stable	unstable	stable
mmp(9)	unstable	N/A	N/A	N/A	N/A	N/A
max-1	unstable	N/A	N/A	N/A	N/A	N/A
max-8	unstable	N/A	N/A	N/A	N/A	N/A
HLT-2	unstable	N/A	N/A	N/A	N/A	N/A
Ac-VES3	unstable	N/A	N/A	N/A	N/A	N/A

Table 8. Peptide-gel stability as a function of nanoparticle composition

***In vivo* resorption of peptide hydrogels**

We next assessed the *in vivo* resorption potential of peptide hydrogels alone and gels containing LNp (48/16). This experiment uses athymic nude mice having only innate immune systems. Peptide hydrogels were prepared by mixing equal volumes of 2wt% peptide solution with 2xHBS buffer. Hydrogels containing LNp were prepared by mixing 2wt%, 4xHBS, 0.44 mg/mL LNp (48/16) in a volume ratio of 2:1:1. Then, 0.2 mL of each hydrogel was injected subcutaneously into each mouse (n=3). Percent volume of material remaining over time was measured by ultrasound. **Figure 20** shows that hydrogels with and without LNp nanoparticles persist in the animal for about 1 month. Previous studies in our group indicate that the main mechanism of bioresorption is macrophage-mediated phagocytosis. The experiment in **Figure 20** defines the baseline resident time from which MMP-mediated degradation can hasten material degradation.

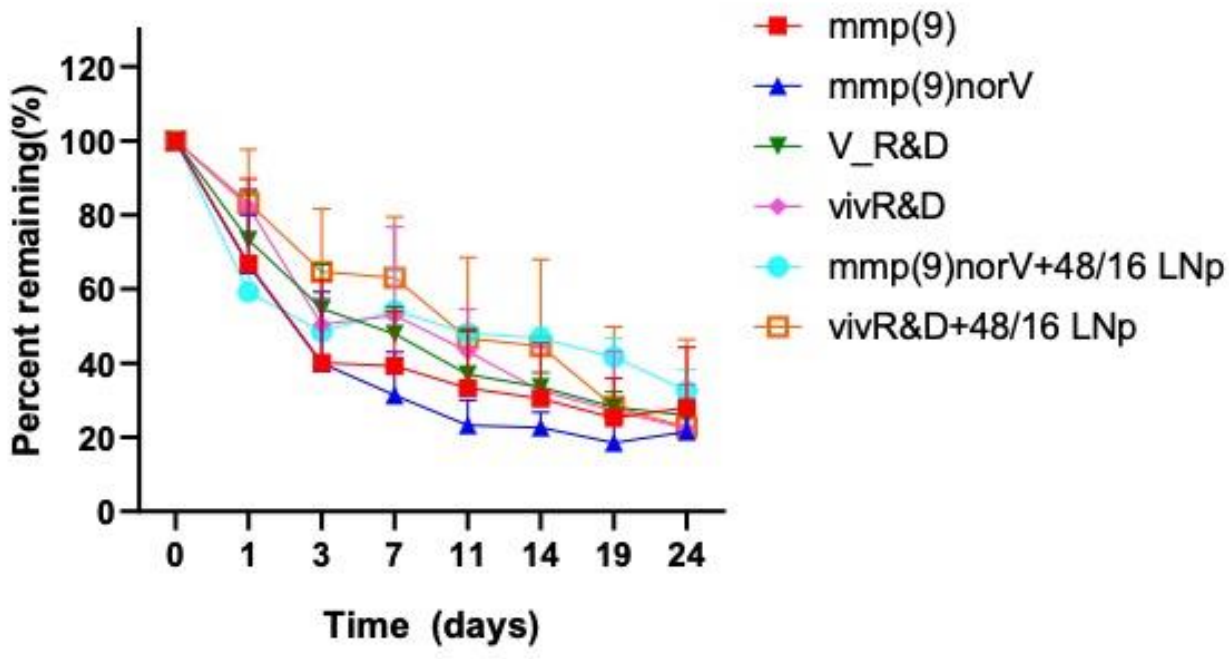


Figure 20. *In vivo* resorption of peptide hydrogels with/without LNp (48/16).

Figure 21 shows example primary data for one mouse that had been injected with the mmp(9)norV gel, from which the data in **Figure 21** was generated. Ultrasound images for the other gels are of similar quality.

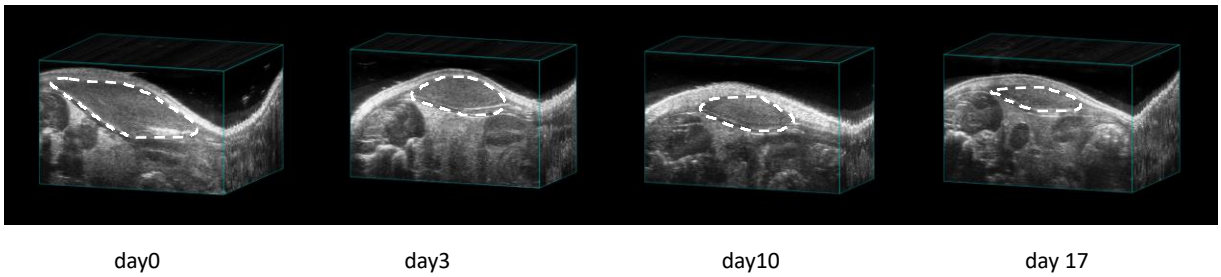


Figure 21. Representative primary ultrasound data measuring bioresorption of an subcutaneously implanted mmp(9)norV hydrogel over time.

We are now beginning to optimize the final Tofa-containing Hydro(Tofa-LNp) composite material as well as a new composite that not only contains Tofa within the nanoparticle but also contains microcrystalline Tofa deposits within the hydrogel matrix to provide further control over Tofa release kinetics.

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.

Site 1 JHU
On August 2019, a new postdoctoral fellow (Dr. Alexander Komin) was hired by the laboratory to focus on the execution of the experiments proposed for Site 1 (JHU). This project provided the opportunity for the PI and the senior postdoc of the laboratory (Dr. Marcos Iglesias) to train Dr. Komin in the execution of proper immunological techniques as well as experimental design and planning, data interpretation, and troubleshooting. This training will continue in the next quarters to promote the independent activity of Dr. Komin.

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.

In light of the results obtained, the following activities are planned for the next reporting period:

Site 1 JHU

1. Continue the assessment of in vivo MMP activity in allogeneic transplanted hind limbs via live animal imaging and PCR-mediated detection of specific MMP mRNA transcripts.
2. Test the ability of the new Tofa-LNp formulations to prevent the maturation of stimulated mouse dendritic cells as indicator of the effective release of Tofa.
3. Initiate the assessment of the in vivo distribution of DilC-LNp when injected alone or as part of hydrogel formulations (conventional and MMP-sensitive) via live animal imaging.

Site 2 JHU/APL

1. Complete the release profile analysis of the modified NLC formulations to determine the impact of additional surfactants.
2. Characterize the stability of the DilC dye and DilC-NLC under different conditions and prepare formulations for initial in vivo tracking experiments.
3. Test the tunability of the overall release profile of the Hydro(Tofa-LNp) system.

Site 3 NCI

1. Develop formulations containing Tofa and study the release of Tofa and LNp from the peptide hydrogel matrix in detail.
2. Formulate different Tofa-containing composites to produce a range of composites that can deliver Tofa with differing ex-vivo release profiles.

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).

Over the past year, we have made significant progress in the design and synthesis of a novel two-component therapeutic delivery platform intended to be easily translatable to the clinic, to impact how immunosuppression is delivered and actuated. Many elements related to the dynamic interactions between lipid nanoparticles and peptide hydrogels were undefined at the start of this project. The results obtained during the first year of this award have delineated a few impactful concepts:

- The nanostructured lipid carrier (NLC) formulations developed over the past year were found to be well-suited to the delivery of tofacitinib, with a promising low toxicity profile and an encapsulation efficiency greater than 75%, higher than that of our previous solid lipid nanoparticle (SLN) formulations.
- The drug release profile measured in vitro revealed a burst release that occurred in the first 8 hours but was then sustained at a low level over approximately 30 hrs.
- We have engineered a family of different gels whose proteolytic degradation is dependent on MMP activity, which should offer control over release rates in response to immune surveillance of the graft.
- We made considerable progress is optimizing both the hydrogel and nanoparticle composition to ensure product stability. Resulting hydrogel-nanoparticle composites represent a new class of immune-modulating implantable material.

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:*

   Nothing to Report

   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.

   Nothing to Report

   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.

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.

  Nothing to Report

- **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.)*

Site 1 JHU

Name:	*Giorgio Raimondi*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Raimondi led and oversaw the activity of the whole team. He facilitated project planning, execution, and troubleshooting with his team.*

Name:	*Marcos Iglesias*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Iglesias was involved in the design and execution of the experiments for the detection of MMP (mRNA and enzymatic activity) by macrophages.*

Name:	*Byoung Chol Oh*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Oh assisted in the design of in vivo MMP detection, performed the indicated mouse hind limb transplants, and he was responsible for monitoring the status of the animals.*

Name:	*Gerald Brandacher*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Brandacher contributed to the design of the in vivo experiments for detection of MMP activity during VCA rejection and provided support in performing the murine hind limb transplant model.*

Name:	*Alexander Komin*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Komin has been trained in the execution of all the in vivo and ex vivo experiment proposed and performed some of the NLC toxicity assays.*

Site 2 JHU/APL

Name:	*Julia Patrone*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*N/A*

Nearest person month worked: *1*
Contribution to Project: *Dr. Patrone has been involved in the design and characterization of biocompatible nanoparticles within this study, as well as coordination with the other PIs.*

Name: *Xiomara Calderon-Colon*
Project Role: *Senior Research Scientist*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *2*
Contribution to Project: *Dr. Calderon-Colon has been involved in the nanoparticle formulation and materials characterization.*

Name: *Olivia Tiburzi*
Project Role: *Associate Scientist*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *1*
Contribution to Project: *Ms. Tiburzi has been has been involved in nanoparticle synthesis and materials characterization.*

Name: *Jill La Favors*
Project Role: *Analytical Chemist*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *1*
Contribution to Project: *Ms. La Favors has been leading the HPLC methods design, validation, and analysis for characterization of the nanoparticles.*

Site 3 NCI

Name: *Joel Schneider*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): *NA*
Nearest person month worked: *1*
Contribution to Project: *Dr. Schneider is directing the research of Dr. Liang, the postdoctoral fellow carrying out the experiment outlined in the statement of work.*

Name: *Chen Liang*
Project Role: *Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *12*
Contribution to Project: *Dr. Liang has been working under the supervision of Dr. Schneider to execute the experiments to synthesize peptides that will be used in the formulation of the peptide-based hydrogel material.*

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.*

Nothing to Report

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://ers.amedd.army.mil*** *for each unique award.*

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

Please, see attachment

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.*

Nothing to Report

A Biocompatible Therapeutic Platform for Precise Regulation of Vascularized Composite Allotransplant Rejection via Enhanced Costimulation Blockage

Log No. RT170075P2

Award No. W81XWH1810793

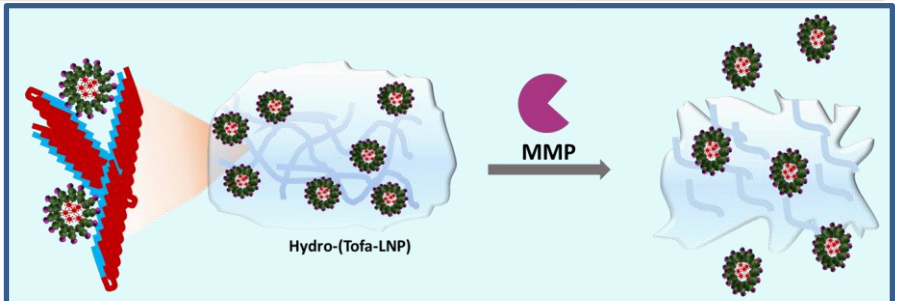
PI: Joel Schneider **Org:** The Geneva Foundation **Award Amount:** $409,563

Study/Product Aim(s)

- Aim 1: Optimize the release and delivery of Tofa to maximize the local control of alloreactivity
- Aim 2: Demonstrate localized regulation of alloreactivity by Hydro(Tofa-LNp) and promotion of VCA survival
- Aim 3: Pre-clinical testing of translatability of Hydro(Tofa-LNp) based ECoB approach.

Approach

Here we propose a novel delivery system in which Tofacitinib-loaded Lipid Nanoparticles (LNp) are encapsulated into hydrogels that can be injected into transplanted tissues. Once implanted, the gel continuously and locally releases LNp that affect the local cell populations as well as rapidly accumulate in the draining lymphoid tissue where they deliver their cargo to immune cells involved in rejection. Moreover, such an approach will be further optimized by engineering the hydrogel to be susceptible to degradation by proteases released by the alloresponse. This would tune the release of Tofa where and when it is most needed and will realize a highly selective synergism with systemic CTLA4-Ig administration actuating an enhanced and localized immunomodulation while minimizing side effects.



Hydro(Tofa-LNp) design. Representation of the planned hydrogel for the localized release of anti-inflammatory drugs that is also tailored to the strength of the immune response to a composite allotransplant

Accomplishment: Generated and characterized hydrogel formulations with different MMP-sensitivity. Identified new formulations of NLC compatible with hydrogel formation and optimal biocompatibility; their characterization is being completed. We have successfully implemented an in vivo system to detect MMP enzymatic activity and obtained preliminary indication of the actuation of rejection-specific activity that, once fully characterized, will inform more advanced hydrogel design.

Timeline and Cost

Activities	Y1	Y2	Y3
Regulatory Tasks			
Specific Aim 1			
Specific Aim 2			
Specific Aim 3			
Estimated Budget ($K)	**$121K**	**$128K**	**$161K**

Updated: 10/29/2019

Goals/Milestones

CY18 Goal – Regulatory tasks

☑ Obtain ACUC and ACURO approvals of animal protocols

CY19 Goals

☑ Fabrication of hydrogel formulations with heightened susceptibility to MMP-mediated degradation

☐ Synthesis of Tofa-LNp with optimal tropism and drug release profile.

CY20 Goals

☐ Fabrication of Hydro(Tofa-LNp) composite for localized in vivo release

☐ Verify synergism of Hydro(Tofa-LNp) with CTLA4-Ig in promoting mouse hind limb survival

CY21 Goals

☐ Assess impact of Hydro(Tofa-LNp) + CTLA4-Ig on survival of ischemic limbs

☐ Collect preliminary pre-clinical data on use of Hydro(Tofa-LNp) + Abatacept in swine VCA model

Comments/Challenges/Issues/Concerns

- Nothing to report.

Budget Expenditure to Date

Projected Expenditure: $121,157

Actual Expenditure: $121,157.33