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PRINCIPAL INVESTIGATOR: Phillip Messersmith, Ph.D.

CONTRACTING ORGANIZATION: University of California, Berkeley

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Phillip B. Messersmith

E-Mail: philm@berkeley.edu

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The Regents of the University of California
c/o Sponsored Projects Office
1608 Fourth Street, Suite 220
Mail Code 5940
University of California, Berkeley, CA 94710-1749

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the proposed studies, we are attempting to leverage our experience in soft and hard tissue regeneration induced by the HIF1α-stabilizing drug 1,4-DPCA in a drug delivery system (PEG-DPCA nanogel) towards therapies for hand and nerve injuries and digit regeneration. Here, we are exploring the effect of this drug on 1) digit regrowth post-amputation and nerve growth, 2) the role of peripheral re-innervation in rat forelimbs on injury restoration, and 3) optimization of drug potency and delivery in these systems. Our progress during this first year includes studies on surgically amputated digits in mice using Micro-CT analysis and immunohistochemistry showing changes with drug therapy as early as day 7. Significant changes at the digit cut site and formation of a boney callus are observed. Optimal drug dosing experiments for the rat forelimb nerve repair experiments were accomplished. Finally, we made progress in developing a new carrier system for 1,4-DPCA, making it more potent and easier to deliver. During this coming year, we will explore both earlier and later timepoints post digit amputation, begin extensive studies in forelimb nerve regrowth and effects on digit function, and further develop the 1,4-DPCA drug delivery system and modification of 1,4 DPCA compounds.

15. SUBJECT TERMS

Blastema, Bony callus, Digit amputation, HIF-1α, Micro-CT, Marrow canal, Neurofilament, Nerve regeneration, PEG-DPCA-nanogel, Sprague Dawley rats, Swiss Webster mice

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1. **INTRODUCTION**

The current studies address a biomedical issue of importance to warfighters and the general patient population, namely we are attempting to leverage our experience in soft tissue and hard tissue regeneration induced by a small molecule HIF-1α stabilizing-drug therapy towards treatments for hand injuries and digit regeneration. In this proposal, we are invoking a classical regenerative response rarely observed in mammals, but which is common in lower species such as newts and salamanders that can readily regrow lost limbs. This proposal specifically focuses on the development of novel therapies to repair neurosensory damage, maintain the distal end organ interface, or regenerate the neuromuscular junction for re-innervation of end organs during peripheral nerve regeneration using a rat model and a digit amputation model in mice in an attempt to restore macroanatomic and functional digit restoration through a regenerative process.

2. **KEYWORDS**

Blastema, Bony callus, Digit amputation, HIF-1α, Micro-CT, Marrow canal, Neurofilament, Nerve regeneration, PEG-DPCA-nanogel, Sprague Dawley rats, Swiss Webster mice

3. **ACCOMPLISHMENTS**

What were the major goals of the project?

There are 4 major goals of this project as stated in the SOW:

1. Determine the effect of drug/gel on nerve regeneration at terminal sites in the rodent digit after amputation
2. Healing across the nerve ends after transection in forelimb, and tracking nerve recovery in both mouse and rat
3. Synthesis and Characterization of 1,4-DPCA-PEG Conjugates
4. Synthesis and Characterization of 1,4-DPCA-Peptoid Conjugates

What was accomplished under these goals?

Aim 1. Nerve regeneration at sites in the proximal digit post amputation.

Experiment #1. We initiated pilot studies of drug-induced digit regeneration and nerve regrowth in 2-month-old B6/129 HIF1α-luc reporter male mice which could show local concentrations of HIF-1α. The second phalanx of the 3rd digit from the hind-paw was surgically amputated midway between the proximal and distal joints under isoflurane and buprenorphine administered every 8 hours. (see **Fig 1**).

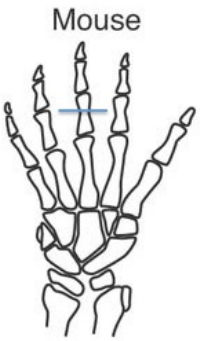


Figure 1

In our first group of mice, at one week (7 days) post-amputation, we examined the histology of the digit with and without the drug 1,4-DPCA (**Fig 2**).

I. Histological Changes after Drug Administration

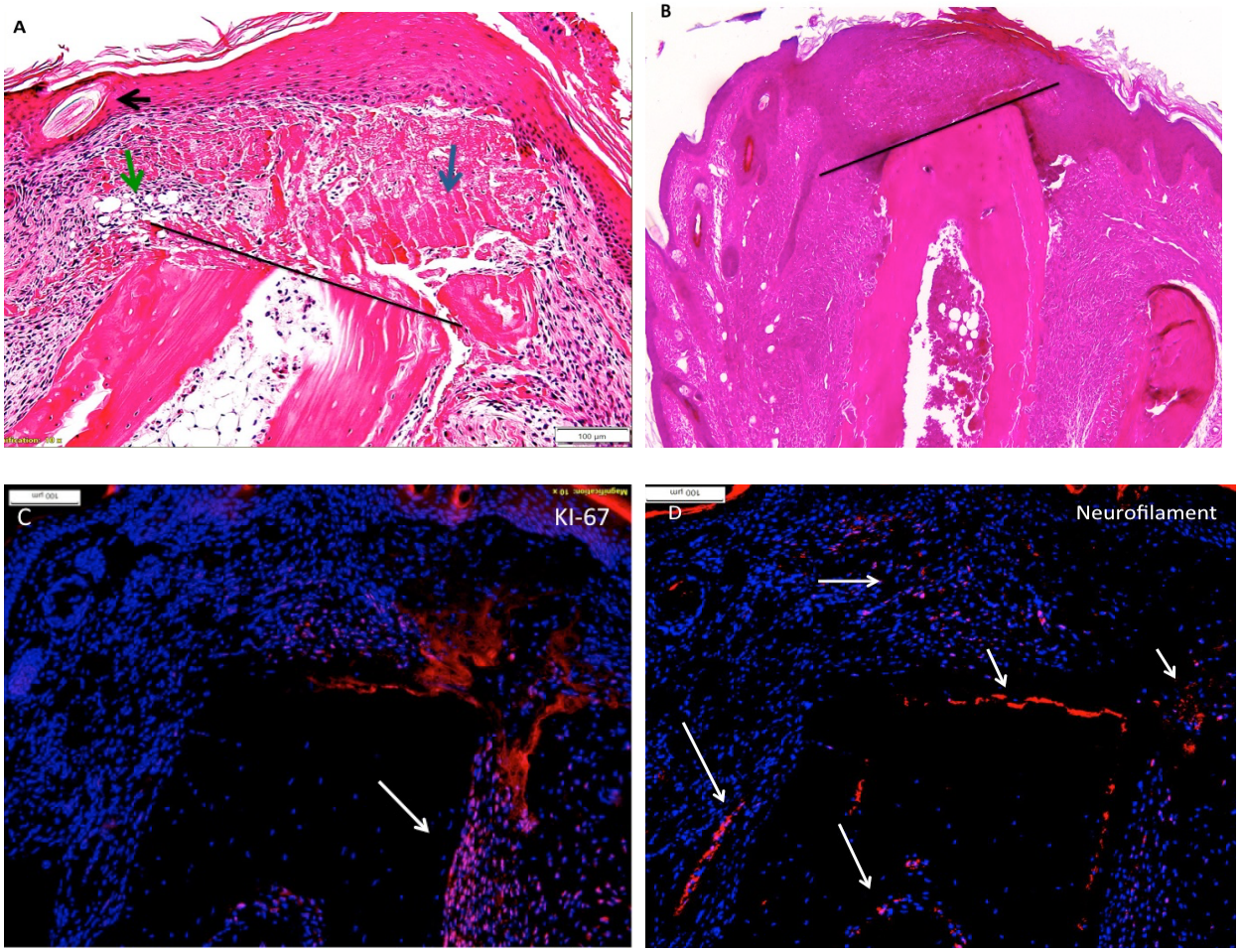


Figure 2. Histology and immunohistochemistry. Digits were removed, fixed, decalcified, embedded and 5 micron sections cut. The two upper digit sections were stained with H&E and represent (A) a mouse given 1,4-DPCA on the day of amputation (day 0) (upper left) and (B) a mouse not treated with drug on the upper right. The upper sections show a black line indicating where the digit had been cut. In (A), new tissue shows a new hair follicle (black arrow), adipocytes (green arrow), and islands of tissue that could be bone (blue arrow). In (B), the control 7-day amputated digit shows no new growth above the cut site except a thickened epidermal layer. Below is seen immune-histochemical staining using anti-KI-67 (C) to show proliferation on the side of the bone (white arrow) and epidermis (not shown) and using anti-neurofilament (D) (white arrows) with staining that looks like nerve cross-sections above the amputation site, a potential nerve fiber on the left side of bone and at the cut site.

The difference between the H&Es of the treated vs untreated digits on day 7 is most striking. The treated digit shows significant activity, with new tissue and space between tissues and what appears to be a de-differentiation of the bone, forming new islands of bone (blue arrow), highly nucleated tissue indicating proliferation, the appearance of a new hair follicle (black arrow), and presumptive adipocytes (green arrow). In fact, this digit looks like it is forming a blastema. The digit from the untreated mouse shows compacted tissue with no activity above the bone and a reformed and thickened epidermis which may have a region of clotted blood. The immuno-histochemistry shown in both lower images is the treated digit and shows areas of staining with KI-67. Considering the number of nuclei seen, we would expect to see more KI-67 positive nuclei. However, KI-67 is temporal and stains dividing cells, and much of the proliferation could have occurred earlier than day 7. We will thus use Brdu in the future which captures cell division and is cumulative. Also, the presence of nerves is seen by neurofilament staining (D). We will further explore this with other markers of nerve such as NeuN.

II. Bone changes after amputation using Micro-CT Scanning

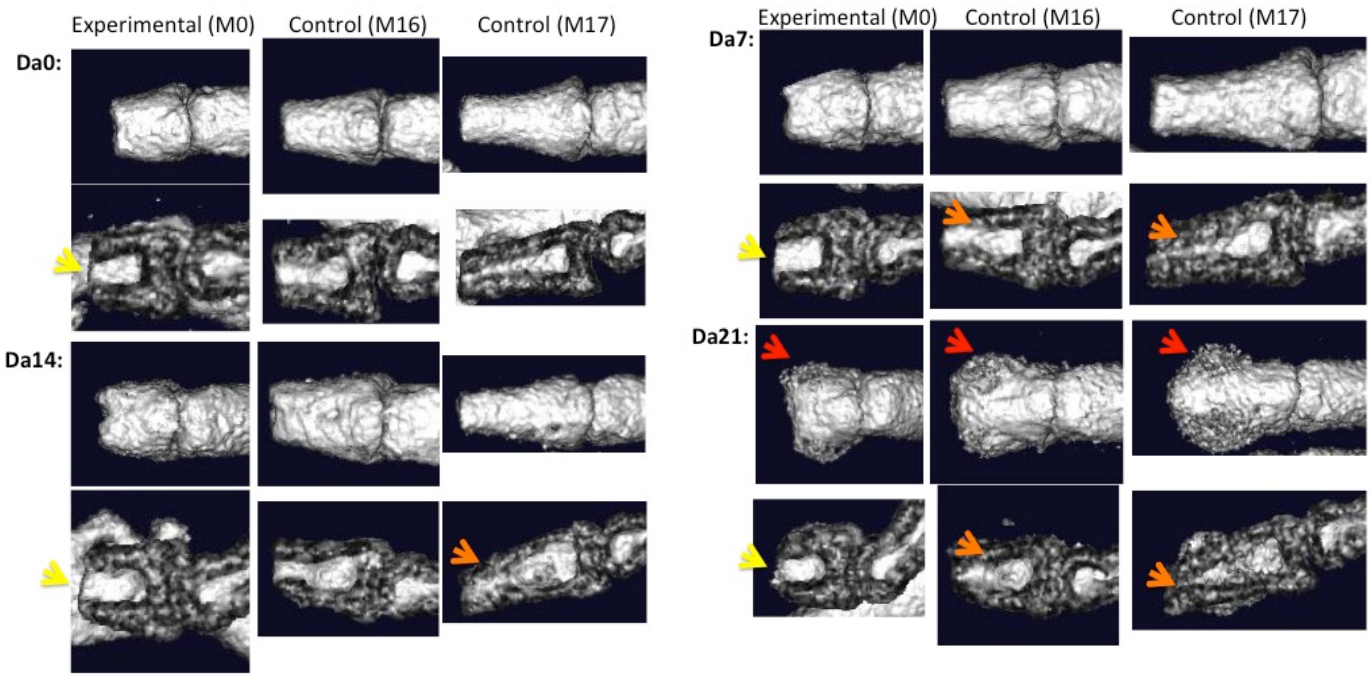


Figure 3: Micro-CT images of digits post amputation with and without drug treatment. Scans of amputated digits are shown for two control mice not receiving any drug compared to a mouse receiving drug on day 0 and 8 (the second mouse died from anesthesia early in the experiment). For days 0, 7,14, and 21, scans of the outside of the digit are shown on the top and below that are cross-sections of the bone. Observing the cross-sections, it can be seen that the marrow canal remains open in the experimental (yellow arrows), whereas the controls begin to close as early as day 7 (orange arrows). It appears that a bony callus forms at the cut phalange between days 14 to 21 and forms closer to the cut end in the experimental than the controls (red arrows), which perhaps leads or contributes to the growth of the bone.

We carried out Micro-CT scanning of all mice for up to 30 days. In **Figure 3**, two control mice (no drug) and one experimental mouse (given drug) are shown. These were B6/129 Hif-Luc mice. Unfortunately, the second experimental mouse succumbed to an anesthesia overdose. From this small pilot study, we observed multiple differences between the experimental and control animals.

1. Closing off the Marrow Canal
The control mice showed that over time, the marrow canal became constricted and then closed off as seen in the cross-sections in **Fig 3** (orange arrows). Furthermore, in the controls, a bony plug at the end was observed (data not shown). The experimental mouse, however, did not show constriction of the marrow canal (yellow arrows) and a bony plug did not form.

2. Formation of a Bony Callus: An unusual structure on the amputated phalange appeared on day 21. As seen in the day 21 images, a knoblike radio-dense structure surrounds the region near the cut phalange and is observed in both the experimental and controls. It is interesting that this resembles a bony callus that typically forms with bone fractures. Its formation may be beginning sometime between days 14 and 21. As will be seen more clearly in the next experiment (**Fig 6**), the difference in location of the “callus” may be significant between the experimental and control.

3. Changes in Bone Length: Measurements of bone length from Micro-CT images were carried out. Only the (single) experimental mouse that had received 1,4-DPCA showed any change in bone length on days 14 and 21 (**Fig 4**).

4. Changes in Bone Density: Measurements in bone density were also carried out. Again, only in the (single) experimental mouse given drug did we observe that the bone density increased above background (**Fig 5**).

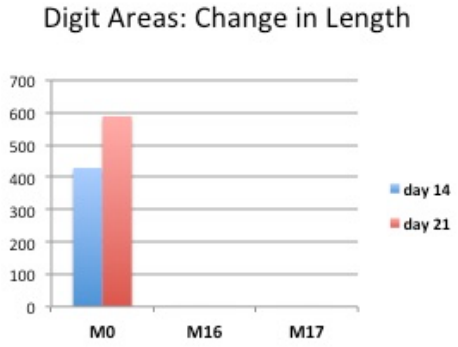


Figure 4. Change in Digit Length.

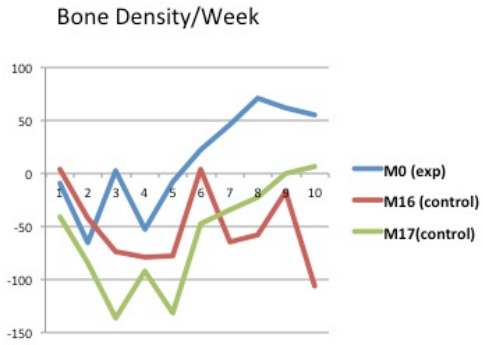


Figure 5. Change in Bone Density.

Experiment #2: To give us time to further breed the HIF-luc mice, we used a second strain, the Swiss Webster mouse. We also used female mice in these experiments, as we have previously used for jaw regeneration experiments with very successful results. We examined differences in the number of drug injections and the time between injections.

We analyzed the digits by Micro-CT for 35 days to again look for changes in length and bone volume. In this preliminary experiment, we examined four groups of 2 mice given drug on days 0 and 8 (A); on days 0,8, and 21 (B, C); and no drug controls (D). The data quartet is presented per mouse for days 0, 7,14, and 21. In **Fig 6**, are shown 3 experimental mice (A, B, and C) and one control (D). In these CT scans, one can see the formation of the radiodense region or “callus” seen previously. In this experiment, the callus is seen as early as day 14 and may have formed between days 7 and 14, earlier than in the previous experiment. Again, this is true for both the experimentals and controls. This could be a strain difference or male versus female difference. However, here we see that the formation of the callus changes in location in drug treated versus controls. Thus, the callus is more central to the amputated digit in the control but is located near the cut end in the mice getting drug. This location could very well add to the length increase in the digit and be of biological importance for regeneration.

In **Fig 7** is shown changes in bone length, with a small reduction in length in the controls compared to the experimentals, wherein the experimentals (given drug on days 0,8, and 21) gain about 4 to 10% in length and the controls lose between 3 to 10% (**Fig 7**, left panels). Bone volume changes showed variations that were not readily interpretable given the variability seen (**Fig 7**, right panels).

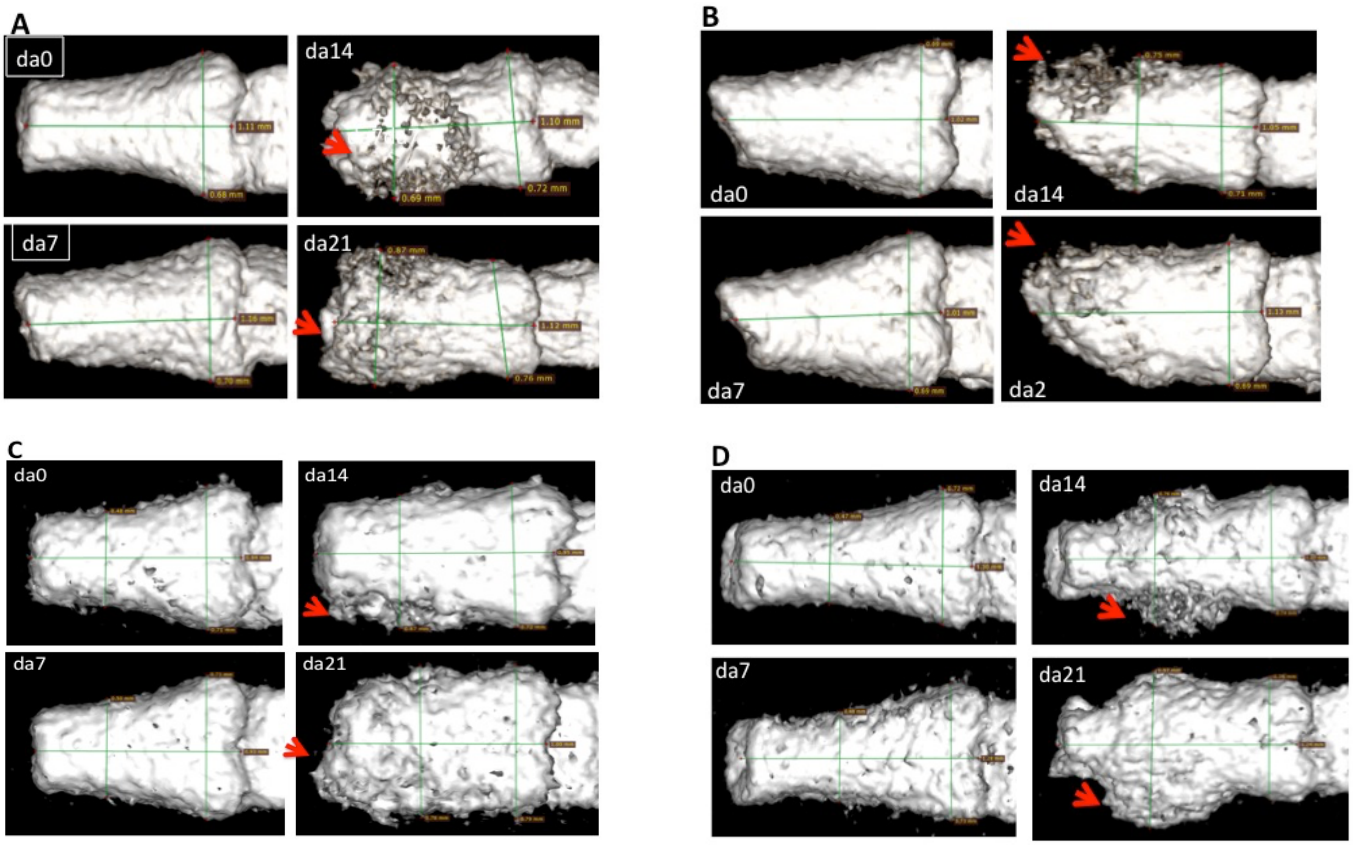


Figure 6: Analysis of Bony Callus and Growth. In the first quartet of pictures from a mouse that received drug on days 0 and 8 (A), Micro-CT scans of da0, da7, da14, and da21 show that the bony callus forms sometime between day 7 and 14 and continues to day 21. In B) and C) are mice receiving the drug on days 0, 8, and 21. The bottom panel (D) is from a control mouse receiving no drug.

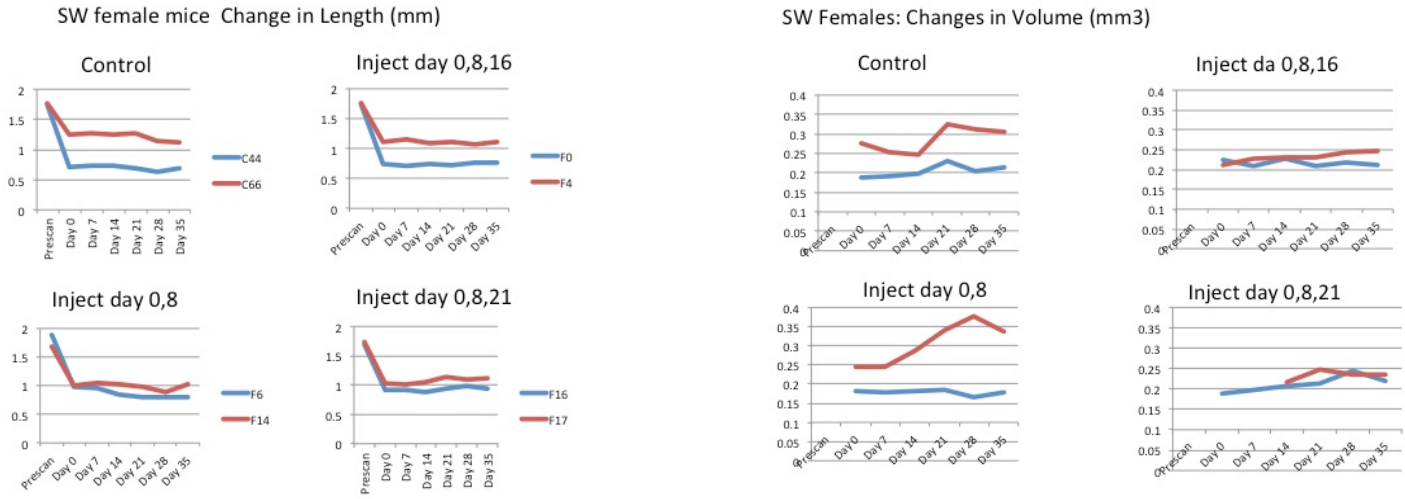


Figure 7: Analysis of Bone Growth and Bone Volume.
The graphs on the left and right represent two mice for each drug variation and the determination of the change in length over a 35-day period and the change in volume, respectively.

Aim 2. Healing across co-apted nerve ends after transection and tracking nerve recovery.
It has become clear from classic regeneration studies that nerve innervation is critical to achieve complete structural and functional regeneration in digits. In these nerve growth studies across co-apted severed nerves the effect of drug treatment to restore digit mobility will be tested.

Since the first nerve growth experiments will be carried out in the male Sprague Dawley (SD) rat in the Giladi laboratory, and the drug will be given systemically, we first determined the best dose for ear hole closure in the rat. We tested different hole sizes and found that a 3 mm hole was far better than a 2 mm hole in the rat because tissue swelling confounded the 2 mm results.

The best dose in the mouse was found to be 25-50 ul of PEG-DPCA nanogel given on day 0 and day 8. For the rat, we started with 50ul of drug given 3 times, every 5 days and found that this was no different than the no drug control. We then tested 100, 200, and 300ul drug given either 2x or 3x and found that 2x 200ul drug schedule was optimal (**Fig 8**). However, we did not achieve full ear hole closure at any time using a 3mm hole punch. This dose, 200 ul, will be given on day 0 after nerve repair and on day 8.

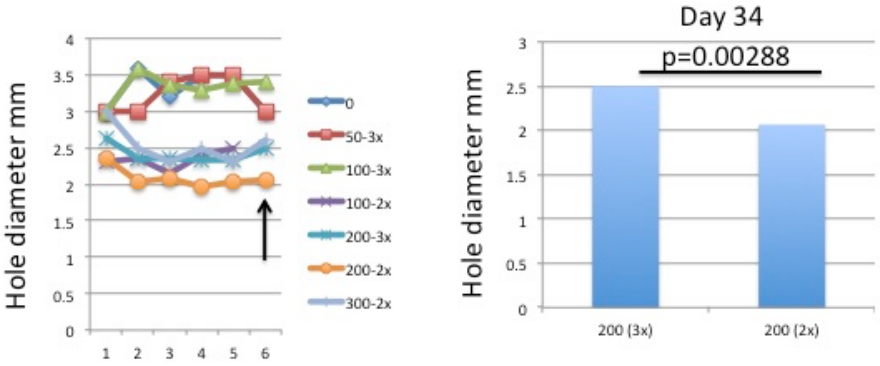


Figure 8. Drug Dosing and Ear Hole Closure in the Sprague Dawley Rat.

1,4-DPCA hydrogel (Ref.1) will be injected subcutaneously in rats pre-surgery.

Five and twelve weeks after surgery, tissue sections from the surgical site will be recovered. The sections will be cut into 3 parts: 1) the nerve/muscle junction which will be stained with bungertoxin, neurofilament (NF), and laminin; we will look for muscle atrophy and perform quantitative NMJ analysis; 2) the down-stream nerve stained with KI-67 (proliferation), S100 (glial cells and neurons), and Osmium (myelin sheath); and 3) the co-aptation site which will be stained for scar formation with trichrome and PSR.

For these studies, the tissue collected will be sent to Philadelphia for the Heber-Katz laboratory to carry out histochemistry and immunohistochemistry for nerve growth, muscle degeneration, and scar formation.

The Giladi laboratory has been consulting with Dr. Tuffaha of the Brandacher laboratory (Ref.2) who will also guide us through the histological analysis.

References:

1. Cheng J, Amin D, Latona J, Heber-Katz E, Messersmith PB. 2019. Supramolecular Polymer Hydrogels for Drug-Induced Tissue Regeneration. ACS Nano. 13(5):5493-5501. PMID: 31067407

2. Tuffaha SH …Brandacher G. 2016. Growth Hormone Therapy Accelerates Axonal Regeneration, Promotes Motor Reinnervation, and Reduces Muscle Atrophy following Peripheral Nerve Injury. Plast. Reconstr. Surg. 137: 1771.

The Following Describes Activity in the Messersmith Laboratory at University of California, Berkeley

Aim 3. Optimization of nerve regrowth induced by drug/hydrogel

This aim has the dual purpose of supplying PEG-DPCA nanogel for use in aims 1 and 2, and optimization of the DPCA delivery system.

Synthesis of PEG-DPCA Nanogel for Aims 1 and 2

Important features of the PEG-DPCA constructs used in the nanogel include the use of the biocompatible polymer, coupling of DPCA to polymer via an ester for hydrolysis and release in-vivo, and high drug loading. Our current approach (**Figure 9A**) satisfies all of these requirements through the use of the biocompatible polymer poly(ethylene glycol) (PEG), a tris linker allowing for three DPCA per endgroup, each of which is coupled to the polymer via an ester. Synthesis of these PEG-DPCA conjugates involves conversion of hydroxyl terminated Peg to carboxylic acid terminated PEG using TEMPO, followed by coupling of CDI-activated DPCA. Using this approach, we synthesized two conjugates based on 700 Da and 8 kDa PEGs (**Figure 9B**). The 700 Da PEG was derivatized at only one end of the polymer (**P7D3**), whereas the 8 kDa Peg was derivatized at both ends to yield a telechelic polymer (**P80D6**). Both **P7D3** and **P80D6** were synthesized in multigram quantities, mixed together in a mole ratio 53:47 (P7D3:P80D6) and hydrated to produce a gel with shear-thinning behavior. Structural investigations revealed the presence of nanofibers as a consequence of PEG-DPCA self-assembly to form DPCA-rich nanofiber cores due to the hydrophobicity of 1,4-DPCA. This nanogel formulation was supplied to Dr. Heber-Katz for use in the experiments described in Aims 1 and 2 (see above).

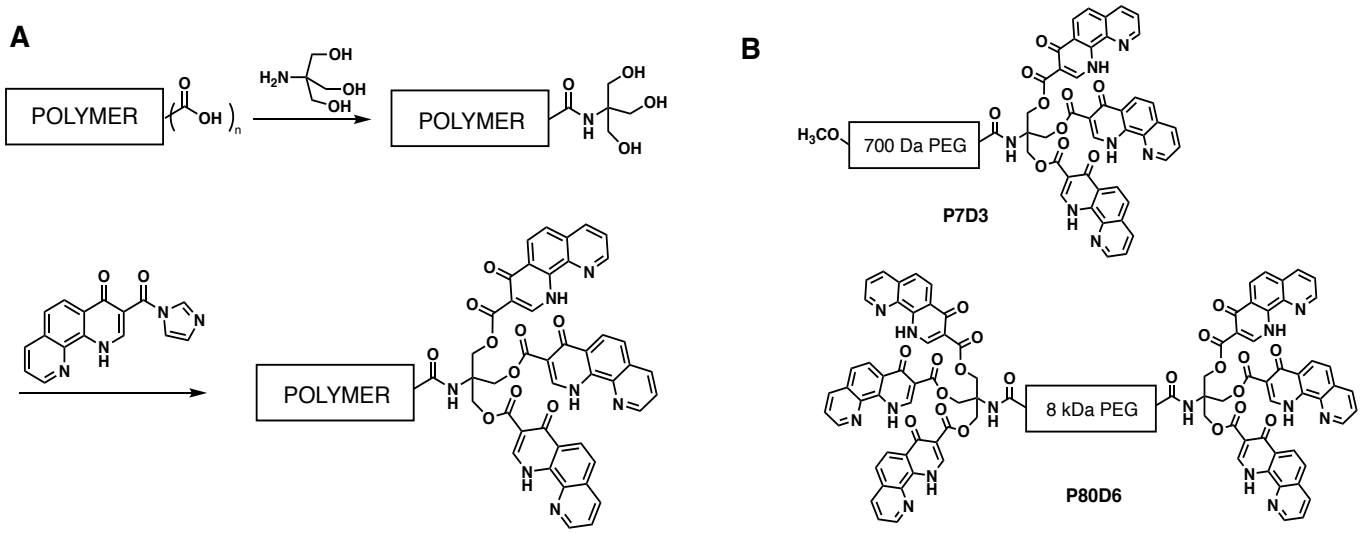


Figure 9. Scheme for synthesis of PEG with multiple DPCA molecules conjugated to the terminal hydroxyl of linear PEG. **A**. A trifunctional linker was used to increase the drug loading. **B**. Chemical structures of **P7D3** and **P80D6**.

Optimization of PEG-DPCA Synthesis

Further detailed characterization of **P7D3** by mass spectrometry revealed sub-quantitative conjugation of 1,4-DPCA to the PEG (i.e. slightly <3 1,4-DPCA per PEG). As a result of this discovery, an ongoing effort is devoted to improving our synthetic approach to **P7D3** and **P80D6** (**Figure 10**). Work in progress has shown that reaction temperature, reagent concentrations and reaction times have a significant impact on coupling efficiency.

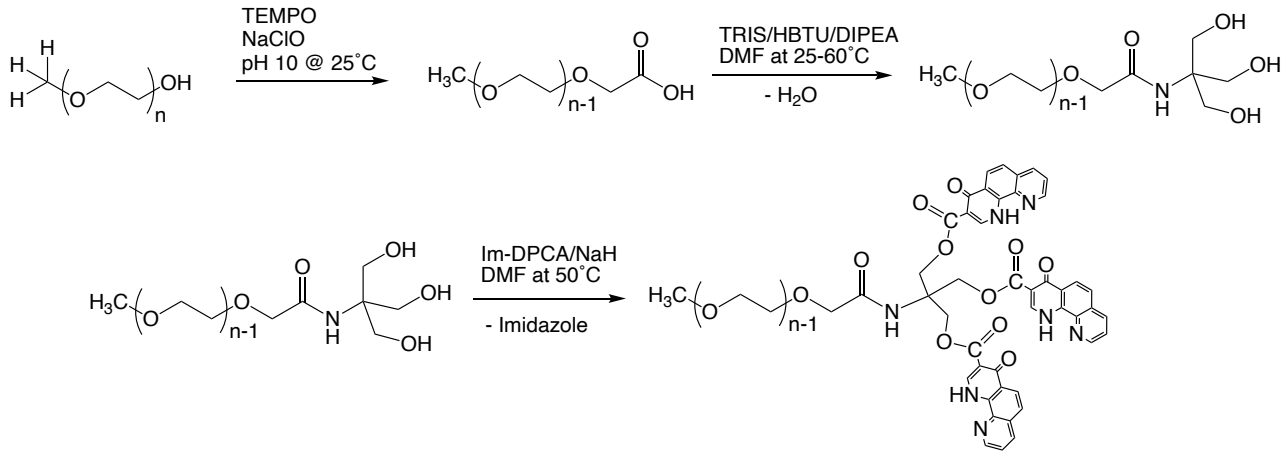
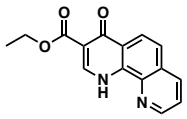


Figure 10. Optimized synthetic approach for PEG-DPCA. Compared to our previous published strategy, we have optimized the reaction concentrations, temperatures and reaction times in an effort to increase coupling efficiency.

Synthesis of PLGA Polymer Microparticles for 1,4-DPCA Delivery

Another goal of this aim is to develop poly(lactic-co-glycolic acid) (PLGA) polymer microparticles for sustained release of 1,4-DPCA. We originally proposed to use an oil-in-water emulsion technique in which a solution of PLGA and DPCA is dispersed in water followed by evaporation of the organic solvent to yield drug-entrapped PLGA microparticles. An unexpected discovery was that PLGA and 1,4-DPCA were not mutually soluble in any organic solvents, rendering this approach impossible. To solve this problem, we synthesized the ethyl ester derivative of 1,4-DPCA, 1,4-DPCE (**Figure 11**), which hydrolyzes in the presence of water to yield the active drug 1,4-DPCA.



1,4-DPCE

Figure 11. Chemical structure of 1,4-DPCE.

DPCE loaded PLGA particles were created by a single emulsion technique. 5-10kDa PLGA polymer was dissolved in dichloromethane (DCM) at a concentration of 1.25% (w/v), and DPCE was added to the organic phase at a concentration of 0.1% (w/v). Polyvinyl alcohol was dissolved in Milli-Q water to yield a 0.2% (w/v) solution which will be referred as the aqueous phase. The organic solution was added dropwise to the aqueous phase in which the final volume ratio was 0.5 of organic phase to aqueous phase. This suspension was homogenized by ultrasonication and left to stir overnight for evaporation of the organic solvent. The resulting suspension was centrifuged at 5000 rpm three times for 30 minutes with water washes in between each centrifugation step. The resulting particles were characterized by SEM (**Figure 12**), revealing an average particle size of 273 +/- 10 nm.

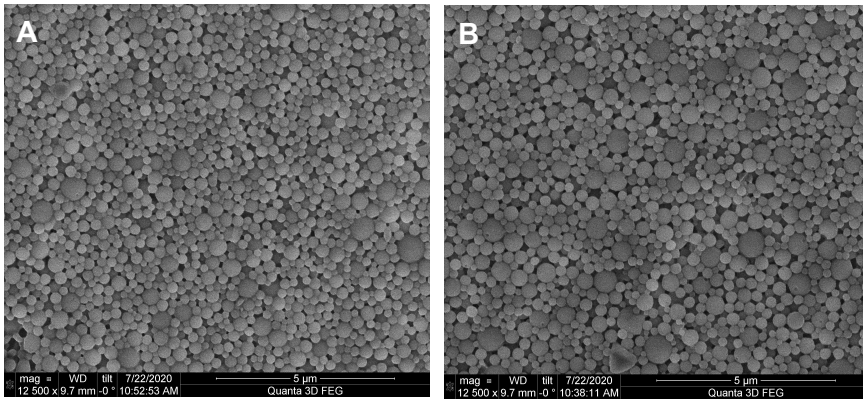


Figure 12. SEM characterization of PLGA (A) and DPCE-entrapped PLGA (B) microparticles.

Release of DPCE from PLGA microparticles was characterized by placing microparticles inside a 3500 MWCO dialysis membrane. The dialysis membrane and outer compartment were filled with Milli-Q water at a final volume ratio of 0.0375. At specific time intervals, small volumes of the outer compartment were collected and the amount of DPCE present was measured by HPLC analysis. As can be seen in **Figure 13**, DPCE was gradually released over approximately 24 hours using this method. In the future we plan to optimize the microparticle formulation to achieve more extended release of entrapped drug.

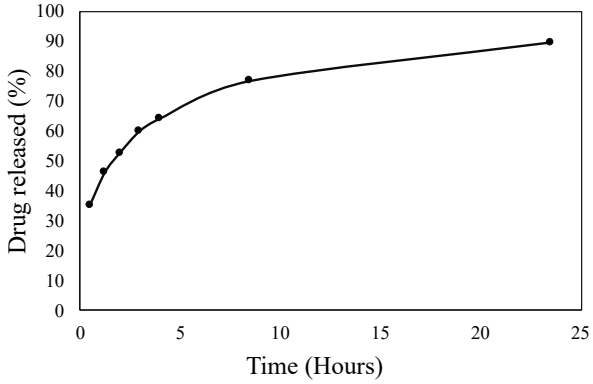


Figure 13. In-vitro release of 1,4-DPCE from PLGA microparticles.

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Initial results were written up and submitted to the MHSRS meeting for presentation. It was accepted for a poster presentation. However, the meeting was canceled due to the COVID-19 situation. The abstract, however, was published online.

What do you plan to do during the next reporting period to accomplish the goals?
For our first goal, we noted that mice 7 days post digit amputation, and after being given drug, showed by H&E staining what appears to be a blastema with proliferating cells, new tissue such as hair follicles, adipocytes, and tissue resembling bone. This is compared to mice receiving no drug which showed compacted tissue and little activity. We noted nerve growth at day 7 by anti- neurofilament immunohistochemistry. Using Micro-CT analysis, we noted the formation of a boney callus in all amputated digits but in those receiving drug has this structure at the tip of the cut bone, potentially adding to the length. During this coming year, we will explore earlier timepoints, multiple markers of nerve growth and vascular changes, and compare local versus systemic drug application.

For our second goal, we will carry out forelimb nerve resection experiments in rats and look for hand grasping effects weekly and extensive histological studies to determine changes in nerve growth and muscle degeneration at 5 weeks and 12 weeks.

For the 3rd and 4th goals, ongoing experiments will perfect drug release over a longer period of time. These studies will be done in vitro and in vivo. As an initial test system, we will use the ear hole closure model in mice.

4. **IMPACT**

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. **CHANGES/PROBLEMS**

Changes in approach and reasons for change
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
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
A review manuscript has been submitted and is currently under peer review, acknowledging support from this grant:

K. DeFrates, E. Heber-Katz, P.B. Messersmith, "*Achieving Regeneration in Mammals through Manipulation of Cellular Oxygen-Sensing Pathways*", submitted.

Professor Messersmith gave the following presentation in which support from this grant was acknowledged: "*Supramolecular polymer prodrugs for drug-induced tissue regeneration*", presented at the Advanced Science Research Center, CUNY, New York, NY, November 25, 2019.

Also, it was noted above that an abstract was submitted to the MHSRS meeting and it was accepted for a poster presentation. However, the meeting was canceled due to the COVID-19 situation. The abstract, however, was published online.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Phillip B. Messersmith, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	Dr. Messersmith oversees all technical, budgetary and reporting aspects of this proposal. Dr. Messersmith also directly supervises other personnel working on the project.
Funding Support:	N/A

Name:	Kyueui Lee, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	8
Contribution to Project:	Dr. Lee participated in characterization of polymer conjugates of 1,4-DPCA.
Funding Support:	The rest of Dr. Lee's support came from project BASF #041901.

Name:	Joakim Engstrom, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Dr. Engstrom participated in synthesis of 1,4-DPCA and polymer conjugates of 1,4-DPCA.
Funding Support:	The rest of Dr. Engstrom's support came from faculty discretionary funds

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Dr. Messersmith reports the following changes in other support.

Completed Support:
1. Grant "Enhanced Adhesion and Cohesion in Bioinspired Polyphenol Polymers" (BASF #041901) has closed.
2. Grant "The Factory: Any Microbe, Any Molecule (Zymergen, Inc. /DOD Advanced Research Projects Agency, 4209)" has closed.
3. Grant "Bioinspired Polymers for Fetal Membrane Pre-Sealing (NIH R01EB022031)" is in no-cost extension.

New Support:
1. Grant "Enhanced Adhesion and Cohesion in Bioinspired Polyphenol Polymers" (BASF #041901) has closed.
2. Grant "The Factory: Any Microbe, Any Molecule (Zymergen, Inc. /DOD Advanced Research Projects Agency, 4209)" has closed.
3. Grant "Bioinspired Polymers for Fetal Membrane Pre-Sealing (NIH R01EB022031)" is in no-cost extension.

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.*

Organization Name:	Lankenau Institute for Medical Research
Location of Organization:	100 East Lancaster Avenue, Wynnewood, PA 19096
Partner's contribution to the project:	
Financial Support:	
In-kind support:	
Facilities:	
Collaboration:	Dr. Heber-Katz is the originating PI on thi grant.
Personnel exchanges:	
Other:	

8. APPENDICES

NA