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14. ABSTRACT

Deployment-related injuries to the central nervous system (CNS) have increased in frequency since World War II. The increasingly frequent use of improvised explosive devices over the past decade has dramatically increased the rate of these injuries. Unfortunately, rapid diagnosis of these injuries during the window of opportunity for significant CNS regeneration remains problematic. Further, no treatments have yet been developed to the point of clinical applicability which successfully regenerate CNS tissues. We have therefore developed and will use a unique and novel animal model of traumatic optic neuropathy (TON) which allows ready access to the central nervous system for studies on CNS regeneration.

15. SUBJECT TERMS
Nerve injury modeling; traumatic optic neuropathy; diagnosis; neural regeneration

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TABLE OF CONTENTS

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

   Traumatic optic neuropathy (TON) is a blinding injury to the optic nerve. While it is known to occur in a significant number of military and civilian injuries each year, no early diagnostic criteria or treatments are currently available. This critical gap is due, at least in part, to the lack of a suitable animal model. We have therefore developed a non-surgical model of TON, as well as several methods which may allow early diagnosis. Phase I of this study will establish those diagnostic criteria, while Phase II will use them to quantify changes in optic nerve structure and function following one of several candidate treatments.

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

   Nerve injury modeling; traumatic optic neuropathy; diagnosis; neural regeneration

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.

Major Task 1: Develop injury diagnostics	Completion Date
Milestone 1: ACURO approval	June 2019
Milestone 2: Co-author manuscript on diagnostic methods for TON	Mar 2023
Major Task 2: Develop vehicles for delivery of neuroprotective agents	
Milestone #3: Co-author manuscript on hydrogel "cast" for drug delivery and mechanical assistance for neuroprotection	July 2022
Major Task 3: Characterization of optic nerve rescue by proposed treatments in vivo	
Milestone #4: Co-author manuscript on changes in optic track following treatment	N/A

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

Major Task 1: Develop injury diagnostics

We have significant evidence that photopic negative responses (PhNRs) are diagnostic for TON as little as 24 hours after injury. Other electrophysiological biomarkers, including flash electroretinograms (FERGs), flash oscillatory potentials (FOPs), and flash visual evoked potentials (FVEPs) appear unchanged at this early time point. Since PhNR primarily originates in the retinal ganglion cells (RGCs) and RGCs are those thought to be injured in TON, this finding is exciting for several reasons. First, it implies that our animal model isolates injury to the RGCs and is therefore a specific model of TON rather than a non-specific retinal injury model. Second, PhNR measurements may be diagnostic for TON. These measurements may be readily performed in

human subjects and, by comparing the amplitude in one eye to the other, a deficit will be at least suggestive of TON.

Methods

Visual Electrophysiology

Rats received intraperitoneal injection of ketamine (0.8 mL/kg ketamine; West-Ward; Eatontown, NJ) and xylazine (0.375 mL/kg xylazine; Akorn). Once pain reflex was absent, whiskers were cut back, and the eyes were dilated with 1% Tropicamide. The rat was then placed on a temperature-controlled electrophysiology system (Celeris Testing System with Espion V6 software; Diagnosys LLC; Lowell, MA; Figure 1). A platinum subdermal needle electrode (Natus Manufacturing Limited; Gort, Co., Galway Ireland) was placed in the tail while another was placed at bregma as ground and active electrodes respectfully. One 6 mm gold surface cup electrode (Natus) was placed on top of the tongue to serve as a reference. The eyes of the animal were numbed with 0.5% Tetracaine Hydrochloride Ophthalmic Solution (Bausch & Lomb Inc.; Tampa, FL). The eyes were then lubricated with Systane gel (Alcon Laboratories Inc.; Fort Worth, Texas) or GentleTears gel (Alcon). The eye gel lubricant was then used on the cup of the Diagnosys flash stimulators. The stimulators were aligned in the center of axis of vision and then placed lightly on the corneal surface. FVEPs, FERGs, FOPs, and flickers were obtained in light adapted conditions with a 200 cd.s/m^2 flash. Three PhNR responses were recorded in light adapted conditions using the following stimuli: 200 cd.s/m^2 white flash with a 20 cd.s/m^2 green background, 200 cd.s/m^2 white flash with a 40 cd.s/m^2 green background, and a 200 cd.s/m^2 green flash with a 40 cd.s/m^2 green background. PhNR values were taken from measuring baseline to trough of the resultant wave. After testing was complete, the stimulators were removed from the eyes and cleaned with saline. The electrodes were removed from the animal, and the animal's eyes were cleaned with saline to remove excess eye lubricant. The animal was then injected with an IP dose of Antipamizole (0.4 mL/kg per kg of body weight) to reverse the xylazine and ease the recovery of the animal. This process was utilized to establish baseline values for each animal prior to injury, then repeated 24 hours after injury to determine the diagnostic value of electrophysiology measurements. Finally, measurements were repeated 7 days after injury prior to humane euthanasia and tissue harvesting for histology.

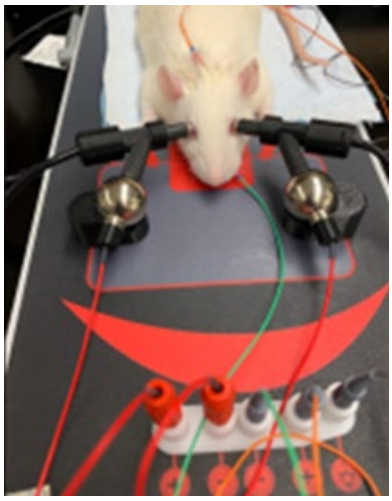

Figure 1: Rat connected to the Celeris for photopic ERG collection. The stimulators are positioned on the corneas of the animal while subdermal needle electrodes are placed over the visual cortex (active) and in the tail (ground). A reference disk electrode is placed in the mouth of the animal on the tongue.

Injury Model

The injury was induced by utilizing our custom torsional injury device (Figure 2). Our device features a motor rigidly mounted on a rat stereotaxic. This allows for precise positioning of the motor with respect to the eye's axis of vision. The stereotaxic serves to immobilize the animal's head to ensure accurate rotation. A fixation hook is then secured to the motor and inserted into the orbit of the animal, hooking onto the ocular muscles. The user then inputs the desired degrees of rotation and the microcontroller and motor execute the motion. This device allows for

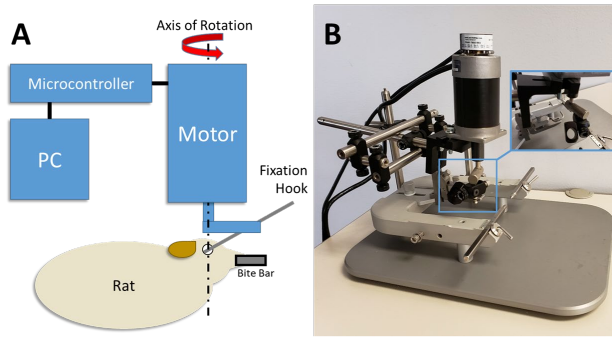

Figure 2: Schematic of custom injury device (A). Image of stereotaxic with motorized unit (B).

independent variation of the amplitude, rate, and direction of rotation to either eye of the rat through a custom user interface. The right eyes (OD) of all animals were injured utilizing the custom torsional injury-inducing device on D0. The left eyes (OS) of all animals did not receive the injury event. Importantly, our novel injury method is a non-surgical technique in which an indirect injury can be administered. Before injury event, animals were anesthetized using the same method described previously. Once pain reflex was absent, rodents were transferred to the custom injury device in which their head was stabilized in the rat stereotaxic. They were placed on a heating pad to maintain body temperature. The motorized instrument then rotated the eye with a peak amplitude of 42.3°±4.5° (mean ± standard deviation) with peak velocity 3034°/s±256°/s. After injury, animals received an IP dose of Antipamizole to reverse anesthesia and facilitate recovery.

Histology and Immunohistochemistry

Rats were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde, and the brain and optic nerve were dissected and incubated overnight at 4°C in 4% paraformaldehyde. Samples were transferred into PBS and shipped to Vanderbilt University Medical Center where the optic nerves and chiasm were dissected away from the brain and cryo-preserved in 20% sucrose in PBS for 2 hrs at 4°C followed by 30% sucrose in PBS overnight at 4°C. The optic nerves were then embedded in OCT (Fisher Scientific, Waltham, MA) and longitudinally sectioned on a Microm HM550 cryostat (Fisher Scientific) at 10 µm thickness. For immunohistochemistry, sections were incubated in PBS to remove the OCT and then incubated in 1:20 normal donkey serum in PBS plus Triton-X-100 (PBT) at room temperature for 2 hrs. Sections were then incubated with specific antibodies as follows: anti-β-tubulin (1:1000; MAB5564; Millipore, Burlington, MA) and anti-glial fibrillary acidic protein (GFAP; 1:50; Z0334; DAKO, Santa Clara, CA) in PBT overnight at 4°C, rinsed with PBS, and incubated in donkey anti-mouse Alexa 488 and donkey anti-rabbit Alexa 594 (1:200; Fisher Scientific) in PBT overnight at 4°C. Finally, the sections were rinsed, mounted in Vectashield plus DAPI (Vector laboratories, Burlingame CA) and imaged on a wide-field fluorescence microscope (Nikon Eclipse, Melville, NY) using consistent settings.

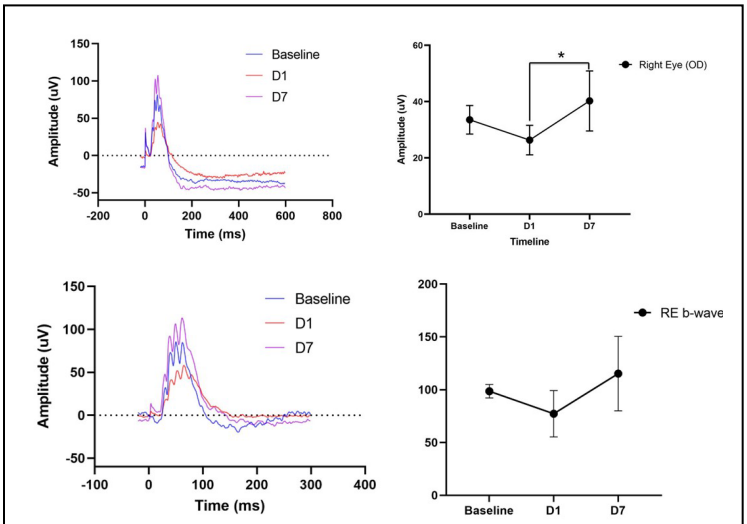

Figure 3: Above: Representative of the mean PhNR waveform amplitudes and averages at three different time points in the injured eye. A significant difference was detected at D7 as compared to D1. Below: Representative of the mean fERG waveform depicting b-wave amplitudes and averages at three different time points in the injured eye. The b-wave is the highest peak in the positive portion of the fERG waveform.

Results

Of the myriad electrophysiologic tests performed, only PhNR and FERG were altered in the acute aftermath of injury: both lower in the injured eye 24 hours after injury, then elevated 7 days after injury (Figure 3; Table 1).

PhNR responses in the injured eyes of these animals were smaller in magnitude than those of the contralateral eye 24 hours after injury (p=0.002; n=19; Figure 4). PhNRs in the injured eye had smaller amplitude and longer latencies relative to baseline both one day after injury. This suggests that asymmetry in the PhNR response is diagnostic for TON, and that the eye having a smaller PhNR is the afflicted eye.

Table 1: D1 PhNR Amplitudes in Microvolts

Animal #	Ipsilateral	Contralateral

1	33.77	33.73
2	22.96	34.17
3	23.65	39.30
4	19.38	39.23
5	28.90	31.63
6	37.91	38.59
7	17.07	24.65
8	26.16	29.45
9	20.50	26.76
10	19.42	28.54
11	19.52	33.04
12	12.42	63.68
13	13.95	19.36
14	22.91	23.56
15	28.24	32.52
16	32.81	34.11
17	27.40	27.34
18	25.33	27.54
19	21.50	27.82

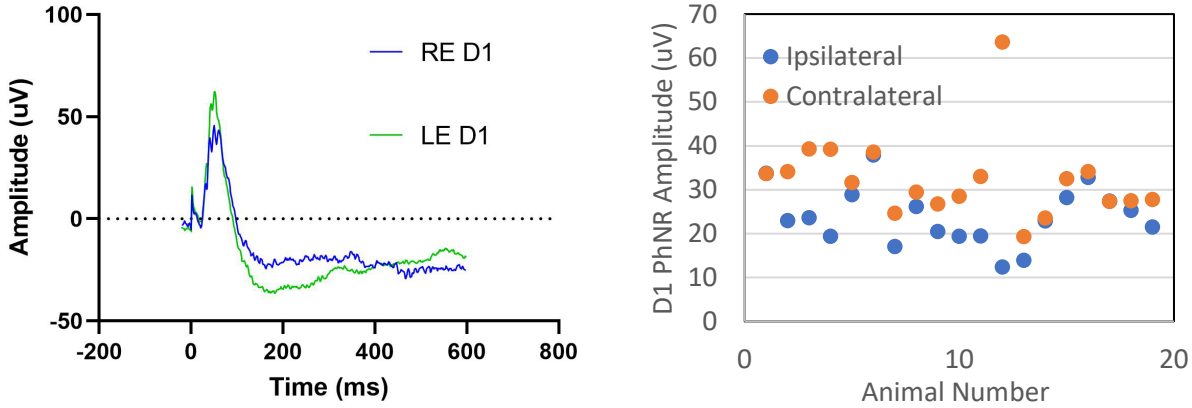

Figure 4: PhNRs were asymmetric between injured and contralateral eyes 24 hours after injury. Left: Representative D1 PhNR signals for ipsilateral (blue) and contralateral (green) eyes 24 hours after injury. Right: Scatter plot of D1 PhNR amplitudes for injured and contralateral eyes for each animal.

Immunohistochemical analysis found decreased labeling intensity for both β-tubulin and GFAP in injured nerves relative to sham (Figure 5). Changes were observed bilaterally, though not symmetrically, suggesting that the biomechanical mechanism of injury employed must transmit stresses through the optic chiasm. GFAP labeling was far more prevalent in the optic nerve than in the retina (Figure 6), reinforcing the conclusion that the injury model is indeed TON rather than a non-specific injury.

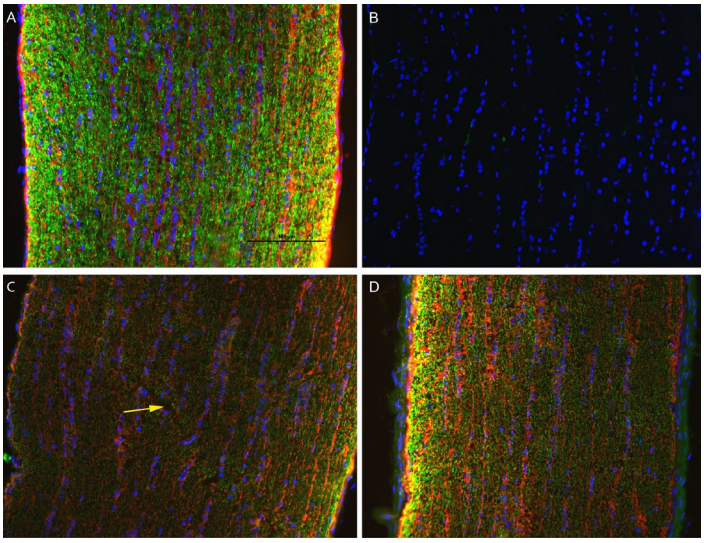

Figure 6: DAPI (blue), β-tubulin (green), GFAP (red). A: uninjured B: uninjured, no primary antibody C: Left D:Right Optic Nerves of injured animals.

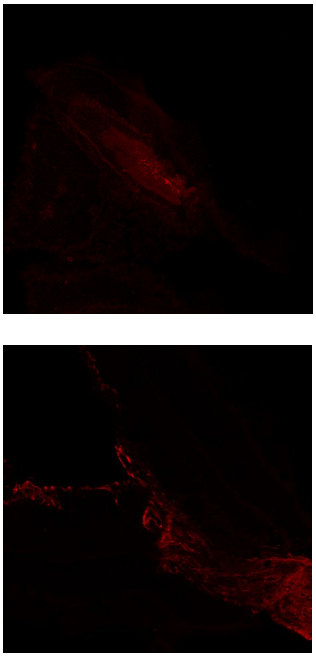

Figure 5: Above: GFAP labeling in the optic nerve from a sham animal. Below: Retina (at left) and optic nerve (at right) of the injured eye from an experimental animal.

Major Task 2: Develop vehicles for delivery of neuroprotective agents

Nine (9) hydrogel formulations were prepared and qualitatively evaluated regarding their ability to form solid, homogeneous hydrogels. Based on this initial screen, an additional 12 formulations were then prepared and characterized for gelation time, swelling behavior, biocompatibility, rheological properties, biodegradation, pH history, and methylene blue (MB) release characteristics. The most promising formulations were then further evaluated to determine the ability of such treatments to maintain cellular viability in an oxidative stress model. These findings form the basis of a manuscript which has been published in the *Journal of Biomedical Materials Research Part A* (impact factor: 4.854).

Methods

Materials

Sodium alginate (Protanal PH 1033) was provided by FMC Biopolymer (Philadelphia, Pennsylvania). Methylene blue (MB) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Sigma-Aldrich (Saint Louis, Missouri). Calcium carbonate (CaCO3) was purchased from ChemProducts (Tualatin, Oregon). D-(+)-glucono-1,5-lactone (GDL) was purchased from Alfa Aesar (Haverhill, Massachusetts). Colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay were purchased from Fisher Scientific Inc. (Hampton, NH). Human retinal pigment endothelial cells (ARPE-19, ATCC CRL-2302) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco's Modified Eagle's/Nutrient Mixture F-12 Ham's Medium (DMEM/F-12), phenol-free DMEM, fetal calf serum (FCS), penicillin-streptomycin (PS), trypsin, dimethyl sulfoxide (DMSO) and hydrogen peroxide (H2O2) were all purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Hydrogel Synthesis

Sodium alginate hydrogels were synthesized based on methods reported in literature with modifications. 21 total hydrogel formulations were prepared and evaluated using design of experiments to modify alginate, CaCO3 and GDL concentrations. The first 9 formulations were selected based on their consistency in forming solid homogeneous hydrogels (Table 2). They served as the models for pH testing in preliminary experiments.

Table 2: Composition of hydrogel formulations.

Formulation ID	Ca^{2+}:Alginate Monomer (mol:mol)	GDL:$CaCO_3$ (mol:mol)	[$CaCO_3$] (g/L)	[GDL] (g/L)
1	0.469	2.493	1.600	7.100
2	0.733	1.596	2.500	7.100
3	0.997	1.173	3.400	7.100
4	0.469	3.301	1.600	9.400
5	0.733	2.113	2.500	9.400
6	0.997	1.553	3.400	9.400
7	0.469	4.109	1.600	11.700
8	0.733	2.630	2.500	11.700
9	0.997	1.933	3.400	11.700
10	0.500	0.125	0.400	0.400
11	0.500	0.250	0.900	0.800
12	0.500	0.500	1.700	1.500
13	0.500	1.000	3.400	3.000
14	1.000	0.125	0.400	0.800
15	1.000	0.250	0.900	1.500
16	1.000	0.500	1.700	3.000
17	1.000	1.000	3.400	6.100
18	1.500	0.125	0.400	1.100
19	1.500	0.250	0.900	2.300
20	1.500	0.500	1.700	4.600
21	1.500	1.000	3.400	9.100

Briefly, sodium alginate (0.63% - 1.85% final w/v in gel) was dissolved in DI H2O by vortexing for 30 seconds and heating in 37°C water for 24 hours. Aqueous 1 mg/mL MB was added to a final concentration of 0.05 mg/mL, followed by the addition of CaCO3 and vortexed. As gelation is initiated rapidly following addition of GDL, the solution was quickly transferred to a mold or onto the rheometer stage following subsequent mixing of all components.

To prepare the remaining hydrogel formulations (10 – 21), GDL:CaCO3 molar ratio concentrations were based off of the original 9 formulations that exhibited a neutral pH of 7.0 ± 1.0 (Figure 2, Table 2). These GDL:CaCO3 ratios ranged from 0.125 – 1.00. The Ca2+:alginate monomer molar concentrations were also evaluated as a factor and varied from 0.5 – 1.5. The ratios were selected to assess the influence of alginate and crosslinker concentrations on drug release, cytotoxicity and viscoelastic properties. Hydrogel formulations 10, 16 and 21 were selected as low, medium and high concentration hydrogels due to their GDL:CaCO3 molar ratios; 0.125, 0.500 and 1.00, respectively. They were analyzed further in cytotoxicity and ROS experimentation.

Hydrogel pH

The pH values of alginate hydrogel formulations 1 – 9 were evaluated using a calibrated pH probe (Mettler Toledo, InLab Expert Pro-ISM, Columbus, OH) for 72 hours to evaluate pH evolution and determine the final compositions of hydrogel formulations 10 – 21. The final pH was reported as the equilibrium pH (pH_E).

Hydrogel Cytotoxicity

The biocompatibility of representative low, medium and high concentration hydrogels (formulations 10, 16, 21) were evaluated using the MTS assay. ARPE-19 cells were first seeded at 5×10^3 cells per well in a 96 well-plate and incubated for 24 hours in 200 µL base media (DMEM/F12, 10% FBS, 1% PS). 1 mL hydrogels were formed in 15 mL conical tubes and allowed to completely gel for 72 hours before 60 – minute UV light exposure, ensuring sterility. The hydrogels were then immersed in 1 mL base media for 24 hours before media collection. The cells were incubated in 200 µL samples for 48 hours prior to performing the MTS assay. A positive control of base media, negative control of 1:9 dimethyl sulfoxide (DMSO): growth media and blank of phenol-free DMEM were used to validate the assay. After incubation, the hydrogel-soaked media was removed and each well washed three times with 200 µL DPBS. Following, 180 µL of phenol-free growth media and 20 µL MTS reagent was added to each well and allowed to incubate for 1

hour. Optical density (OD) of the MTS-treated media was measured at 490 nm using a BioTek Elx808 plate reader (Winooski, VT).

ROS Scavenging

A DCFH-DA assay was used to evaluate the ability of MB to scavenge ROS in cell culture based on the methods of Ludmila et al. (2005) [27]. ARPE-19 cells were seeded on a 96 well-plate at a density of 2×10^4 cells per well in DMEM/F12 media supplemented with 10% FBS and 1% PS and incubated for 24 hours. MB at concentrations of 0 mg/L (positive control), 0.05 mg/L, 0.25 mg/L, 0.50 mg/L, 1.0 mg/L and 2.0 mg/L and a positive control of H_2O_2 and a negative control of DPBS were added to the wells and incubated for 24 hours. Following incubation, the media was removed and 100 µL of DCFH-DA solution was added to each well and incubated for 1 – 2 hours. The cells were washed with DPBS once and the excitation and emission wavelengths; 485 nm and 535 nm, respectively, were measured using a microplate reader.

To further confirm the ROS scavenging ability of MB, 1 mL hydrogels (formulations 10, 16, 21) were formed in 15 mL conical tubes and allowed to gel for 72 hours. Following gelation, the hydrogels were exposed to UV light for one hour to sterilize. The hydrogels were then immersed in 1 mL base media for 24 hours before media collection. ARPE-19 cells were seeded on a 96 well-plate with 2×10^4 cells per well in base growth media and allowed to grow for 24 hours. The culture media was removed and the hydrogel – soaked medium was added to the wells. Hydrogen peroxide (10 µL, 600 µM final concentration) was added to test wells while DPBS was added to the other wells as a negative control. Additionally, hydrogel formulation 16 (medium concentration hydrogel) without MB was included as a negative control. Cells were incubated for 24 hours. Following incubation, the media was removed and 100 µL of DCFH-DA solution was added to each well and incubated for 1 – 2 hours. The cells were thoroughly washed with PBS, and the excitation and emission wavelengths were measured at 485 nm and 535 nm, respectively.

Gelation Kinetics and Mechanical Properties

Oscillatory shear rheology was used to characterize the gelation kinetics, strain amplitude response, and frequency response of alginate hydrogel formulations 10 – 21. The rheometer used was a Malvern Panalytical Kinexus Ultra+ (Malvern, United Kingdom) with a 20 mm titanium parallel plate upper geometry (PU20 SW1511 TI) and aluminum lower geometry (PLC61 S3722 AL). For all rheological tests, the gap height between the lower and upper geometries, the temperature and sample size were kept constant at 1 mm, 37°C and 375 µL, respectively.

To measure the gelation kinetics of alginate hydrogels, the alginate solution was dispensed as a liquid directly onto the lower geometry of the rheometer immediately following the addition and mixing of GDL. A constant frequency and strain amplitude of 1 Hz and 1% respectively (within linear viscoelastic region), were applied to the sample with its resulting shear stress measured every 5 seconds for 2 hours. The gelation time was defined as the time which gelation had terminated and was determined from the constant frequency and strain test as the first timepoint where complex shear modulus (G*) did not increase by more than 1% of the average of the 10 previously collected measurements. A frequency sweep test immediately followed the gelation test, evaluating the frequency response of the hydrogel. Here, a constant strain amplitude of 1% was applied to the sample while frequency increased from 1 Hz to 100 Hz. The stiffness of the hydrogels is reported as the value of G* at 1 Hz from frequency sweep tests.

Representative low, medium, and high concentration $CaCO_3$ and GDL hydrogels (formulations 10, 16, and 21, respectively) were additionally subjected to an amplitude sweep test to evaluate strain amplitude response. A constant frequency of 1 Hz was applied to the sample while the strain amplitude increased from 0.1% to 100%, and resulting stress was measured.

Hydrogel Swelling and MB Release

1 mL samples of hydrogel formulations 10 – 21 were cast in pre-weighed 15 mL conical tubes and weighed. Formulations 1 – 9 were not included as preliminary studies determined only GDL:$CaCO_3$ ratios of 0.25 – 1.0 were relevant for our studies due to the determined crosslinking maximum between Ca^{2+} ions and alginate.

Hydrogels were then immersed in 1 mL DPBS modified without calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) at 37°C and at regular intervals (0, 1, 3, 7, and 14 days), DPBS was removed and the mass of the hydrogels was recorded. Results were calculated according to the following equation:

$$Q = \frac{M_S - M_D}{M_D} \times 100\%$$

Here, Q is the swelling ratio, M_s is the mass of the formed hydrogel following incubation in DPBS at 37°C and excess water removal and M_D is the mass of the 1 mL alginate solution placed in the tube.

The release kinetics of MB were evaluated using the same formulations (10 – 21) evaluated for swelling. 1 mL hydrogels (10 – 21) loaded with 1 mg/mL MB were created. Following immersion in DPBS and incubation at 37°C, 1 mL DPBS was removed at the given intervals (0, 1, 3, 7, and 14 days). 100 µL samples of the DPBS were placed in a 96 well-plate and absorbance measured. The concentration of MB remaining in hydrogels following DPBS incubation was then determined using a standard concentration-absorbance curve measured at 630 nm using a plate reader (BioTekElx808).

Hydrogel Degradation

1 mL hydrogel solutions based on formulations 10 – 21 were cast in pre-weighed 15 mL conical tubes and weighed. After incubation at 37°C for 72 hours, excess water was removed from tube and hydrogels were weighed again to determine weight following incubation. Hydrogels were immersed in 10 mL 1X DPBS with $MgCl_2$ and $CaCl_2$ at 37°C for 0, 1, 3, 7 or 14 days. At each timepoint, the DPBS was removed, the hydrogels were frozen at -80°C for 24 hours and lyophilized for 24 hours. Hydrogel degradation was reported as the percentage change in the mass of dry components used to create the hydrogel to the dried hydrogel mass after freezing and lyophilization.

Statistical Analysis

Data analysis was performed using two-tailed student t-test. Statistical significance was defined as $p < 0.05$. All values and data points are reported as the average ± standard deviation.

Results

The pH of hydrogel formulations 1 – 9 was recorded for 72 hours (Figure 7A), with all formulations initially at ~6 pH. Formulations demonstrated clear pH value groupings based on GDL:CaCO3 molar ratios. Formulations in the lower group (1, 4, 5, 7, 8) had ratios greater than two, whereas the upper group (2, 3, 6, 9) had ratios less than two. The GDL:CaCO3 molar ratio was plotted against pH_E over 72 hours (Figure 7B). Higher variability within the groupings is observed in the lower group with pH values ranging from ~3.5 to ~4.5; however, the variability may be explained by the

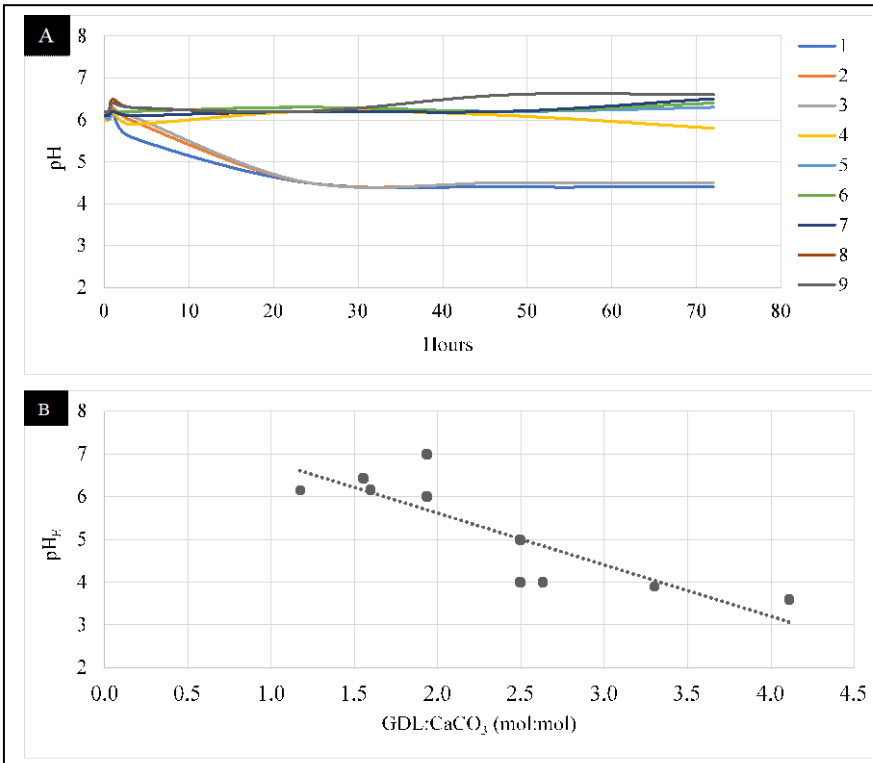

Figure 7: Characterization of pH of hydrogel formulations 1 – 9. (A) Evolution of hydrogel pH over 72 hours. Formulations exhibit clear groupings of pH values. (B) Plot of hydrogel equilibrium pH (pHE) reached after 72 hours of gelation. There is a linear and inverse relationship between GDL:CaCO3 and pHE (R2 = 0.8; p < 0.0001).

larger range of GDL:CaCO3 ratios. Greater molar concentration of GDL and CaCO3 caused lower pH. Therefore, formulations with close to neutral pH were evaluated in subsequent studies.

As shown in Table 2, alginate hydrogels 10 – 21 were prepared by varying $CaCO_3$ and GDL concentrations. Time sweep rheology analysis (Figure 8, Table 3) found that different concentrations of the hydrogel components had an observable influence on complex shear modulus (G*). As the concentrations of both $CaCO_3$ and GDL increased, the complex shear modulus also increased. Increasing GDL content significantly increased G* more than the addition of $CaCO_3$. Additionally, complex modulus was dependent on GDL:$CaCO_3$ ratios. Lower ratios corresponded lowered moduli and vice versa. All gelation times for formulations excluding 11 and 14 were significantly different from each other ($p < 0.05$). Gelling time was found to be tunable, decreasing with higher concentrations of both GDL and $CaCO_3$. All hydrogels exhibited a storage modulus significantly greater than their loss moduli and had a G* of at least 35 Pa at 1 Hz.

The observed influence of the hydrogel composition on G* is detailed in Figure 9. When the Ca^{2+}:alginate and GDL:$CaCO_3$ were 0.500 – 1.000 mol:mol and 0.125 – 0.250 mol:mol respectively, G* gradually increased with time and their respective gelation times were among the lowest (formulations 10, 11, 12, 14, 15, 18), ranging from 1517 – 2803 seconds, or 20 – 48 minutes. When the Ca^{2+}:alginate and GDL:$CaCO_3$ were 1.000 – 1.500 mol:mol and 0.250 – 0.500 mol:mol respectively, G* increased more rapidly with time and their respective gelation times ranged from 1055 – 1400 seconds i.e. 17 – 23 minutes

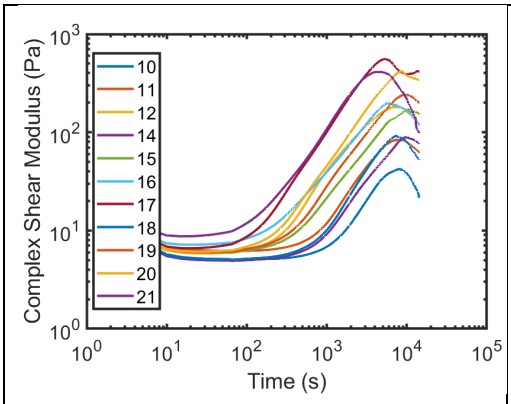

Figure 9: Gelation characterization of hydrogel formulations. Time sweep results of hydrogel formulations 10 – 21, excluding 13. Formulations had observable groupings of low and high GDL:CaCO3 ratio hydrogels. Gelation times ranged from 707 ± 59 to 2803 ± 40 seconds.

Table 3: Stiffness and Gelation Time

Formulation	Gelation Time (s)	G* 1 Hz (Pa)
10	2803 ± 40	36 ± 16
11	2025 ± 120	60 ± 21
12	1223 ± 91	125 ± 48
14	2270 ± 26	60 ± 26
15	1517 ± 99	113 ± 53
16	1275 ± 248	247 ± 151
17	707 ± 59	559 ± 34
18	2190 ± 42	70 ± 34
19	1400 ± 72	187 ± 92
20	1055 ± 78	312 ± 134
21	660 ± 198	225 ± 111

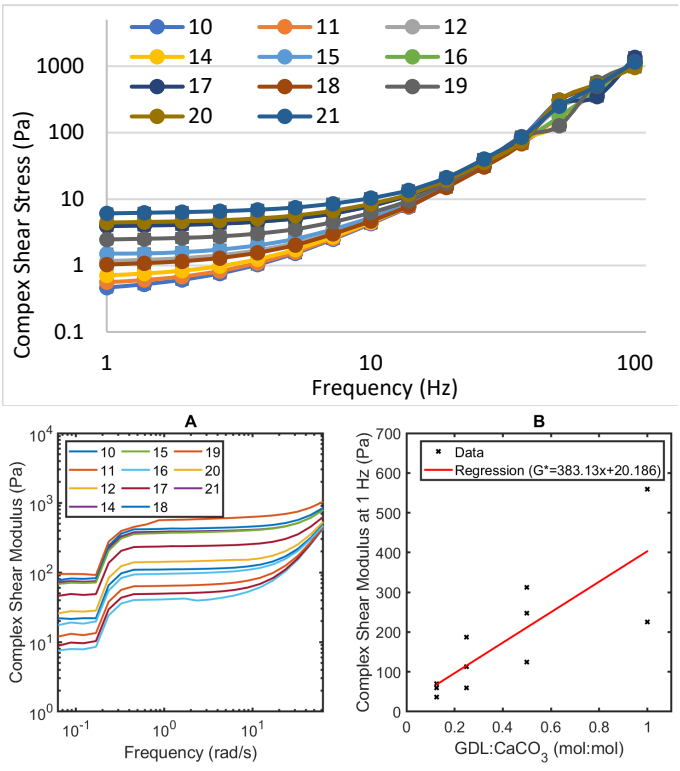

Figure 8: Frequency sweep results of hydrogel formulations. (A) Frequency sweep data from hydrogel formulations 10-21 (except 13). There is a positive exponential relationship between increasing frequency and complex shear stress. (B) Complex shear stress (G) as a function of concentration ratio ($CaCO_3$:GDL) from hydrogel formulations 10–21 at low frequencies. GDL:$CaCO_3$ ratios significantly influence G*, with higher ratios contributing to high complex shear stresses.*

(formulations 16, 19, 20). Lastly, when Ca^{2+}:alginate and GDL:$CaCO_3$ mol concentrations were 0.500 – 1.000 and 0.125 – 0.250 mol:mol respectively, G* dramatically increased within a short period of time and their respective gelation times were among the fastest, averaging around 660 – 707 seconds or 11 minutes to gel completely (formulations 17, 21).

Immediately following the gelation test, a frequency sweep was run on each hydrogel sample in triplicate with the result reported as the average ± standard deviation (n = 3). Figure 9 details the viscoelastic properties of the alginate hydrogels.

Similar to the grouping for the gelation test, there was grouping observed between low, medium and high concentration alginate hydrogels. The lower crosslinker concentration hydrogels (GDL:$CaCO_3$ ratios of 0.125 and 0.250) corresponded with softer hydrogels and lower complex shear stress whereas higher concentration hydrogels (GDL:$CaCO_3$ ratios of 0.5 and 1.0) were stiffer and therefore had a higher complex shear stresses. The data show that by varying the components of the gels, a significant influence on complex shear modulus and gelation time were observed.

The strain amplitude response of low, medium, and high concentration hydrogels of both $CaCO_3$ and GDL (10, 16, 21) were evaluated via amplitude sweep. Low (formulation 10) and high (formulation 21) concentration hydrogels and medium (16) and high concentration hydrogels were significantly different from each other ($p = 0.0021$ and 0.0006, respectively). All hydrogels contained a linear viscoelastic (LVE) region response to dynamic shear stress, originating at ~1% strain and ending at ~20% (Figure 10). Maximum shear stress varied among the hydrogels. Formulation 21 demonstrates a sharp increase in complex shear strain from followed by a decrease around 20% complex shear strain, indicative of "fracturing."

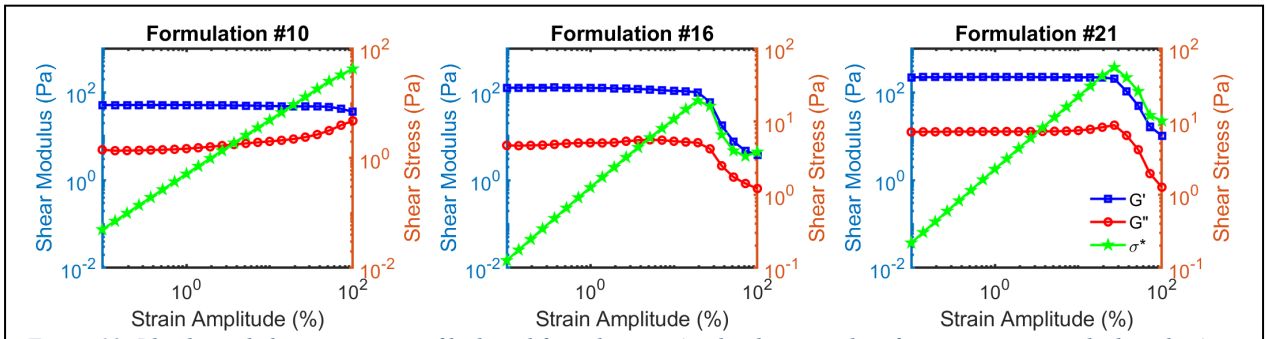

Figure 10: Rheological characterization of hydrogel formulations. Amplitude sweep data from representative hydrogels. A linear viscoelastic region of stiffness response corresponding to 1 Hz dynamic shear is observed up to 1% strain.

Swelling and MB Release of Alginate Hydrogels

The swelling and MB release profile of the hydrogels was recorded *in vitro* over a period of 14 days (Figure 11). The degree of equilibrium swelling varied among hydrogels, ranging from 0 – 150%. Formulations 12 and 21 had the lowest and highest swelling percentage, respectively, correlating to low and high GDL:$CaCO_3$ ratios. The degree of swelling varied is indicative of the components within the hydrogels. Low to medium concentration (of both $CaCO_3$ and GDL) hydrogels had degrees of swelling reported around 100-120%, whereas high concentration hydrogels had swelling above 120%.

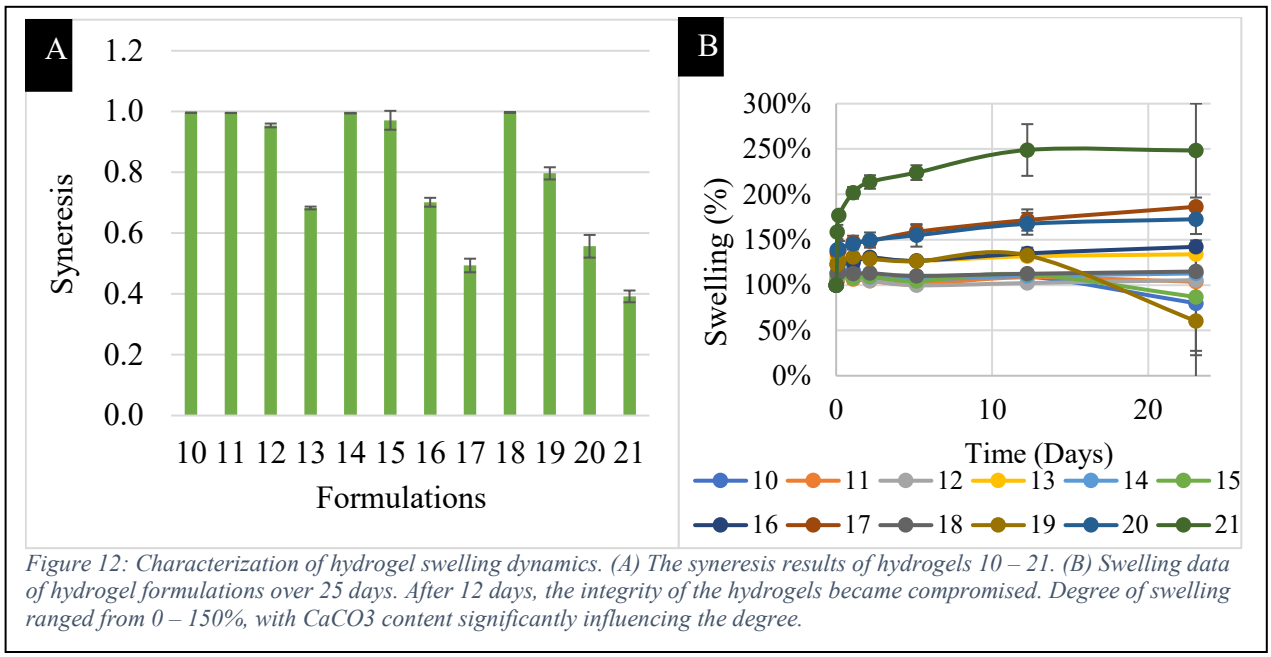

Figure 12: Characterization of hydrogel swelling dynamics. (A) The syneresis results of hydrogels 10 – 21. (B) Swelling data of hydrogel formulations over 25 days. After 12 days, the integrity of the hydrogels became compromised. Degree of swelling ranged from 0 – 150%, with CaCO3 content significantly influencing the degree.

MB release from the hydrogel formulations is further detailed in Figure 12. Among all hydrogels, an initial burst release was observed within the first 5 days, with over 50% MB released. Following the initial burst, a slower and more sustained release followed until the hydrogels disintegrated. Lower concentration hydrogels had the most cumulative MB release (~90%) by 12 days, the point at which the alginate hydrogels were mostly dissolved and released remaining MB.

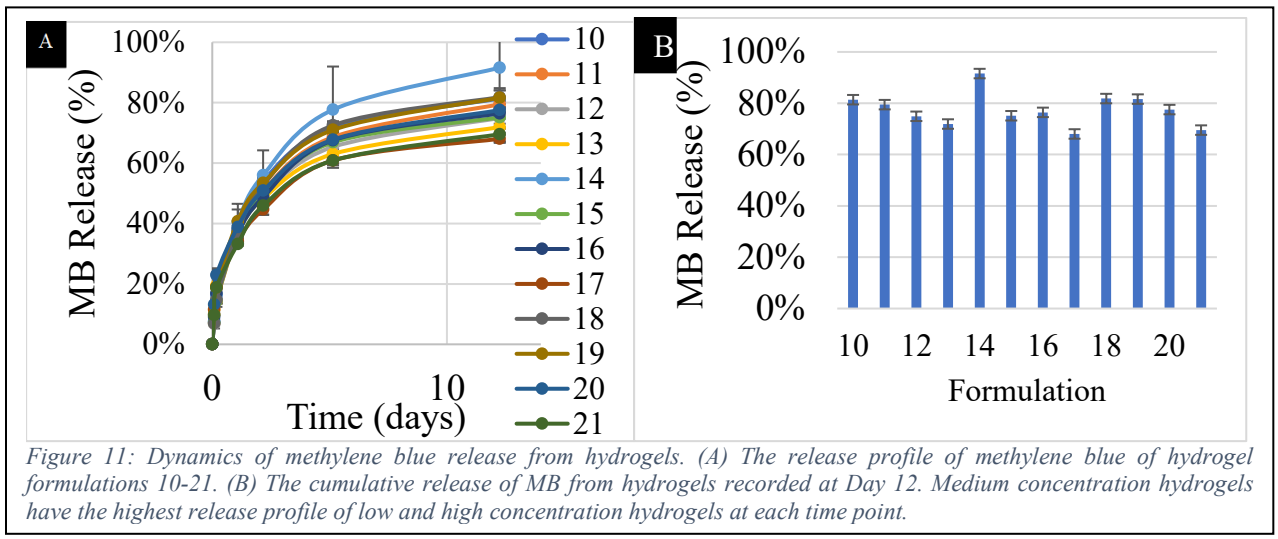

Figure 11: Dynamics of methylene blue release from hydrogels. (A) The release profile of methylene blue of hydrogel formulations 10-21. (B) The cumulative release of MB from hydrogels recorded at Day 12. Medium concentration hydrogels have the highest release profile of low and high concentration hydrogels at each time point.

Cytotoxicity of Alginate Hydrogels

A fundamental requirement for injection is minimal cytotoxicity. To this end, following synthesis of representative hydrogels, we studied their biocompatibility using a human retinal cell line (Figure 13). ARPE-19 cells were incubated with the representative hydrogels for 24 hours and assessed via MTS assay. Formulations 10 and 21, demonstrated excellent cellular viability at over 95%, whereas formulation 16, had viability of 70% of following one-day exposure with the alginate hydrogels. Alginate hydrogels have proven to be biocompatible in various studies and our results using the different crosslinker further support biocompatibility as well as their potential for injection.

MB as a ROS Scavenger

Scavenging of ROS by MB was evaluated through *in vitro* testing based on published methods. ARPE-19 cells were first incubated with MB concentrations of 0, 0.05, 0.25, 0.50, 1.0 and 2.0 mg/mL for 24 hours and then treated with H_2O_2 for 24 hours. ROS levels/activity was characterized by the appearance of highly fluorescent compound DCF in the DCFH-DA assay. There was an observable decrease in fluorescence of the cells corresponding to increased MB concentrations. We confirmed that ROS levels decreased significantly with concentrations of 0.500, 1.00 and 2.00 g/L ($p < 0.05$) (Figure 15). These results suggest the potential of using MB as ROS scavengers for TON treatment.

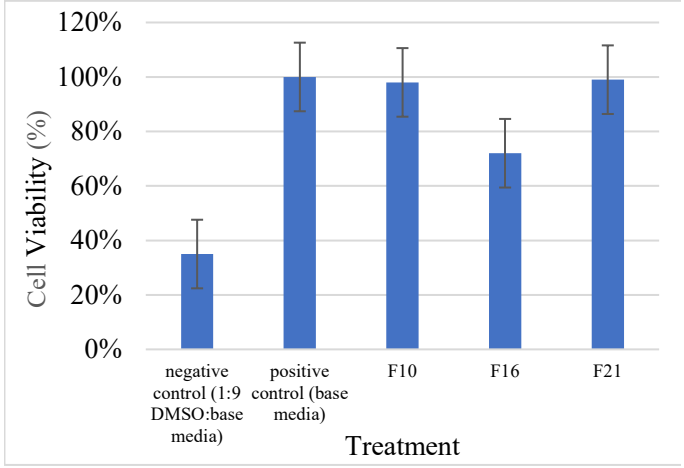

Figure 13: Cytotoxicity of Representative Hydrogels. Cellular viability as measured by optical density (OD) of the MTS reagent product following exposure to alginate hydrogels. The low, medium, and high concentration hydrogels that were evaluated maintained a cell viability of at least 70% that of the positive control base media (DMEM/F12, 10% FBS, 1% PS).

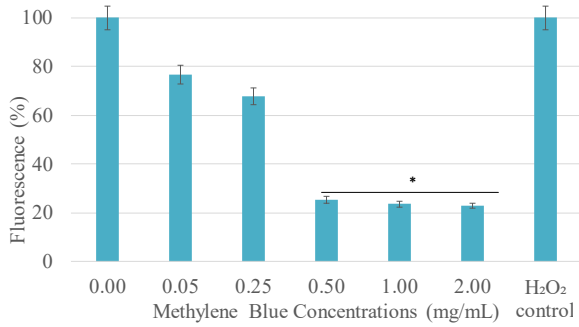

*Figure 15: Methylene blue inhibition of oxidative stress. ROS activity measured by DCF fluorescence in ARPE-19 cells induced by 600 µM H2O2. Increased concentrations of methylene blue contributed to higher cell survival during prolonged exposure to H_2O_2. Data (n = 5) is presented as mean ± standard deviation. Results were normalized against H2O2 control. Higher fluorescence is indicative of greater DCF presence, more ROS activity and lowered cell survivability. Differences in the fluorescence of MB concentrations of 0.5, 1.0 and 2.0 were found to be statistically significant (*p < 0.05).*

alginate hydrogels (10, 21) achieved ARPE-19 survival of over 60% when exposed to the highly cytotoxic H_2O_2. Medium concentration hydrogels (16) maintained cell survival of ~35% (with MB); however, survival was lowered to ~10% when cells were exposed to the hydrogels without MB. The presence of MB was found to significantly influence cell survival when loaded into hydrogel formulation 16 ($p < 0.01$) as survival increased from ~10% without MB to 35% with MB.

The ability to scavenge ROS was confirmed with MB (Figure 14). Additional studies were performed with alginate to further confirm MB's ROS scavenging ability while loaded into a hydrogel. All hydrogels were loaded with 1.0 g/L MB, except the negative control 16, which as loaded without MB. ARPE-19 cells were incubated with hydrogel formulations 10, 16, 16 without MB and 21 for 24 hours. Following incubation, the hydrogels and cells were exposed to H_2O_2 for 24 hours with resulting DCF fluorescence measured. Hydrogels 10 and 21 displayed higher degrees of cell survival compared to 16, yielding similar results to our cytotoxicity study. Low and high concentration

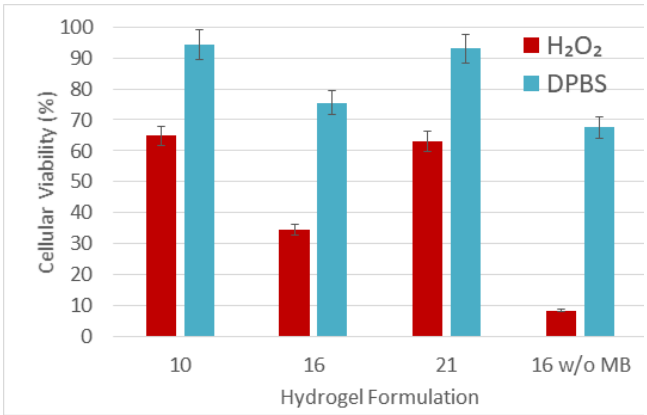

Figure 14: Hydrogel inhibition of oxidative stress. Cell survival was maintained at over 50% for formulations 10 and 21. Cell survival did decrease following exposure to H2O2. Differences between 10 and 21 were found to not be statistically significant ($p > 0.05$). Differences between formulation 16 with and without MB was found to be statistically significant ($p < 0.01$).

Naturally derived biomaterials can be advantageous for drug delivery applications as their components can be broken down and removed by the body. Biodegradation of alginate can be more challenging than other biomaterials as it degrades by ion exchange. The *in vitro* degradation of alginate hydrogels was studied for two weeks with the mass of the initial and final mass recorded. Table 4 summarizes the degradation results of hydrogel formulations 10 – 21. The masses of hydrogel formulations 10 – 21 were recorded over 14 days and weighed at 0, 1, 3, 7, and 14-day timepoints following lyophilization.

Swelling rates varied significantly on Days 0 and 14 based on hydrogel composition. Higher concentration hydrogels displayed the highest degree of swelling on Day 0; however, on Day 14, low concentration hydrogels displayed the highest degree of swelling. The average mass of the hydrogels (mg) following 0, 1, 3, 7, and 14 days were 8.0 ± 1.2, 12.7 ± 1.0, 12.0 ± 0.76, 12.3 ± 2.4, and 18.3 ± 5.4, respectively. On days 1, 3 and 7 of incubation, the degree of swelling as well as the average mass did not differ significantly from previous time points. Low concentration hydrogels among all time points displayed the lowest degree of swelling over time whereas higher concentration hydrogels had the highest degree of swelling, as expected. $CaCO_3$ values were found to influence swelling over time with the lowest GDL:$CaCO_3$ ratio swelling most rapidly. The ratio of Ca^{2+}:alginate was the primary driver of hydrogel swelling with the highest ratios swelling the most overall. Hydrogels prepared with Ca^{2+}:alginate ratio of 0.50 were at approximately equilibrium swelling when formed.

Table 4: Hydrogel Degradation Results

Formulation	Day 0 (mg)	Day 1 (mg)	Day 3 (mg)	Day 7 (mg)	Day 14 (mg)
10	6.4 ± 0.5	12.2 ± 0.2	11.5 ± 0.8	10.3 ± 1.4	24.9 ± 2.2
11	6.4 ± 0.5	13.5 ± 1.0	12.2 ± 0.3	10.5 ± 0.7	24.9 ± 11.0
12	8.0 ± 0.5	12.8 ± 0.5	11.7 ± 0.3	10.6 ± 0.9	13.7 ± 1.4
13	9.6 ± 0.2	14. ± 2.9	12.2 ± 0.8	12.0 ± 0.8	21.3 ± 6.0
14	6.7 ± 0.6	13.8 ± 1.8	13.2 ± 0.3	11.1 ± 0.4	27.7 ± 10.7
15	7.7 ± 0.2	12.6 ± 2.3	11.4 ± 1.1	10.5 ± 0.4	11.8 ± 0.6
16	8.5 ± 0.2	12.3 ± 1.3	11.0 ± 0.1	11.4 ± 0.7	13.8 ± 2.7
17	9.9 ± 0.3	11.4 ± 0.6	11.8 ± 3.2	11.4 ± 0.7	16.1 ± 1.9
18	7.2 ± 0.5	13.7 ± 0.5	12.3 ± 1.1	12.9 ± 7.5	14.7 ± 5.4
19	7.8 ± 0.3	12.8 ± 0.5	11.1 ± 0.9	17.6 ± 3.0	14.0 ± 1.3
20	8.5 ± 0.5	12.9 ± 2.	12.4 ± 2.6	13.4 ± 1.1	15.5 ± 3.6
21	9.3 ± 0.8	10.6 ± 0.9	13.4 ± 3.4	16.3 ± 0.9	20.9 ± 8.8

The differences between the hydrogels among timepoints were not significant until Day 14 ($p < 0.05$), with observable groupings between the low, medium and high concentration hydrogels. Low and high concentration hydrogels displayed the highest degrees of swelling in this timepoint (Day 14). Day 0 differed significantly among all timepoints ($p < 0.001$); Day 1 differed significantly from Day 14 ($p = 0.0020$) and Day 7 differed significantly from Day 14 ($p = 0.0021$).

Major Task 3: Characterization of optic nerve rescue by proposed treatments in vivo

No progress to report.

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.

Graduate Research Assistant Annie Ryan has received extensive training from Dr. Julie Racine with regard to rodent visual electrophysiology; development of protocols relevant for diagnosing TON in our model system; and data analysis procedures. Annie has demonstrated her ability to conduct each aspect of the experiments in rats, producing the preliminary results described above. She has presented these methods and results at multiple conferences, including the Association for Research in Vision and Ophthalmology (ARVO).

Multiple undergraduate students have also volunteered to assist Annie on this project: Samuel Duckworth, Emma Lally, Eve Howard, Michelle Mosko, and Stephanie Small have all helped care for animals during these challenging experiments. This was the first research experience for each of these students and has significantly contributed to an interest in scientific careers for several of them.

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.

Dissemination of results was achieved locally, nationally, and internationally via presentation of grand rounds, seminars, conference presentations, and archival journal publications.

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

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

Nothing to Report.

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

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

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

The animal model and diagnostic criterion described herein are likely to be used by our research group and others to evaluate candidate therapeutics for the treatment of TON. Since TON is currently untreatable, this will likely have the long-term outcome of significantly improving outcomes for individuals suffering from TON.

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.*

The findings of this study offer the first quantitative diagnostic criterion for TON, a common injury in both battlefield and civilian settings. Once adopted, this criterion will enable improved decision making regarding treatment approaches for individuals suffering from acute TON. Furthermore, the rigorous characterization of a specific TON animal model will significantly catalyze development of therapeutic approaches for the treatment of TON.

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

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

Several significant setbacks occurred (as detailed below) limited the time and resources available to complete several key aspects of the original Statement of Work, including some subtasks of Major Task 1 (magnetic resonance imaging, matrix-assisted laser desorption-ionization imaging mass spectrometry, and single-cell RNA sequencing) as well as all of Major Task 3. We instead focused on using electrophysiological techniques to diagnose TON as these may be more accessible, portable, and therefore pertinent to defense needs.

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.

Prior setbacks related to grant transfer, ACURO approval, and the COVID-19 pandemic have significantly impacted this project's timeline and budget. We have therefore focused our efforts on Major Tasks 1 (developing diagnostic criteria for TON) and 2 (developing a drug-eluting cast for TON). We have sought and obtained additional funding related to Major Task 3 (determination of safety and efficacy of candidate TON treatments) which exploit the animal model and diagnostic capabilities described herein.

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.

Nearly ten months passed after submission before ACURO approval was finally granted. We made use of this time to improve training and protocols, as well as perform computational studies, but the personnel expenses incurred during this time were significant and not matched by experimental outputs as agreed in the Statement of Work.

Approval was finally obtained in late February, 2020. We immediately purchased animals and planned to commence experiments, but the laboratory was closed in March, 2020 due to the COVID-19 pandemic. Our research assistant assigned to experimental work contracted long COVID immediately after the laboratory reopened and eventually left the group without completing any experiments. We immediately replaced him with a graduate research assistant who has conducted all animal experiments described above after a considerable training period. Together, these setbacks significantly limited the resources available for conducting additional experiments as described in the original Statement of Work.

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).*

- Maxwell, C.J., Soltisz, A.M., Rich, W.W., Choi, A., Reilly, M.A., Swindle-Reilly, K.E., Alginate Hydrogels as Injectable Drug Delivery Vehicles for Optic Neuropathy Treatment. *Journal of Biomedical Materials Research A*, 2022, 110:1621-1635.
- Ryan, A.K., Rich, W., Reilly, M.A., Oxidative Stress in the Brain and Retina after Traumatic Injury and Neurodegenerative Disorders. *Frontiers in Neuroscience*, 2023, 17:1021152.
- Ryan, A.K., Asemota, B.I., Rodriguez, L., Sponsel, W.E., Racine, J., Rex, T., Glickman, R.D., Reilly, M.A., Torsional Indirect Traumatic Optic Neuropathy (TITON): A Physiologically Relevant Animal Model of Traumatic Optic Neuropathy. Submitted.

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).*

- *Ryan, A.K., Rich, W.W., Jansen, P.A., Allyn, M.M., Swindle-Reilly, K.E. Reilly, M.A., Oxidative Stress in the Eye, in: Molecular Basis of Oxidative Stress: Chemistry, Mechanisms, and Disease Pathogenesis, 2nd edition; Frederick A. Villamena, ed., Wiley, in press.*

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.*

Conference Papers

- *Jones, K.R., Reilly, M.A., Glickman, R.D., Identification and localization of trauma related biomarkers using matrix assisted laser desorption/ionization imaging mass spectrometry. SPIE Proceedings, Photonics West, San Francisco, California, January-February 2017.*

Conference Presentations

- *Ryan, A.K., Landreth, C., Artis, E., Cheung, R., Rex, T.S., Racine, J., Reilly, M.A., An Electrophysiological Technique for the Diagnosis of TON in a Small Animal Model. Association for Research in Vision and Ophthalmology, Annual Meeting, New Orleans, Louisiana, April 2023.*
- *Ryan, A.K., Racine, J., Reilly, M.A., Diagnosing Traumatic Optic Neuropathy Using Visual Electrophysiology, Vision Injury Research Forum, Virtual, April 2023.*
- *Reilly, M.A., Ryan, A.K., Racine, J., Diagnosing Traumatic Optic Neuropathy Using Visual Electrophysiology. Ohio State Neuroimaging Symposium, Annual Meeting, Columbus, Ohio, October 2022.*
- *Reilly, M.A., Biomechanical Engineering Approaches in Presbyopia and Ocular Trauma. Biomedical Engineering Society, Annual Meeting, San Antonio, Texas, October 2022.*

- *Ryan, A.K., Racine, J., Reilly, M.A., An Electrophysiological Technique for the Diagnosis of TON in a Small Animal Model. Biomedical Engineering Society, Annual Meeting, San Antonio, Texas, October 2022.*
- *Ryan, A.K., Racine, J., Reilly, M.A., Diagnostic Electrophysiology in a Small Animal Model of Traumatic Optic Neuropathy (TON). Association for Research in Vision and Ophthalmology, Annual Meeting, Denver, Colorado, May 2022.*
- *Swindle-Reilly, K.E., Maxwell, C.J., Soltisz, A.M., Choi, A., Rich, W., Reilly, M.A., Injectable Alginate Hydrogels for Traumatic Optic Neuropathy, Association for Research in Vision and Ophthalmology, Annual Meeting, Virtual, May 2021.*
- *Soltisz, A.M., Ruzga, M.N., Reilly, M.A., Swindle-Reilly, K.E., Spatial Variations in Optic Nerve Mechanical Properties, Association for Research in Vision and Ophthalmology, Annual Meeting, Vancouver, British Columbia, April 2019.*
- *Soltisz, A.M., Thobe, S.M., Ruzga, M.N., Reilly, M.A., Swindle-Reilly, K.E., Evaluation of Semi-Interpenetrating Network for Treating Traumatic Optic Neuropathy, Society for Biomaterials, Annual Meeting, Seattle, Washington, April 2019.*
- *Higuita-Castro, N., Wier, C., Moore, J., Sunyecz, A., Cho, R., Reilly, M.A., Sen, C.K., Kolb, S., Gallego-Perez, D., In Vivo Non-Viral Delivery of Gene and Cell Therapies to Mangled Nerves, Military Health System Research Symposium, Annual Meeting, Kissimmee, Florida, August 2018.*
- *Swindle-Reilly, K.E., Thobe, S., Jiang, P., Soltisz, A.M., Tram, N.K., Reilly, M.A., Development of Therapeutic Hydrogels for Traumatic Optic Neuropathy, Society for Biomaterials, Annual Meeting, Atlanta, Georgia, April 2018.*
- *Glickman, R., Gray, W., Reilly, M.A., Sponsel, W.E., Emerging Threats with Ocular Trauma Challenges Panel, Association for Research in Vision and Ophthalmology, Annual Meeting, Baltimore, Maryland, May 2017. Invited*
- *Jones, K.R., Reilly, M.A., Glickman, R.D., Identification and Localization of Trauma-Related Biomarkers using Matrix Assisted Laser Desorption/Ionization Imaging Mass Spectrometry, SPIE Biomedical Optics, Annual Meeting, San Francisco, California, January-February 2017.*
- *Swindle-Reilly, K.E., Tram, N.K., Reilly, M.A., Jones, K.R., Glickman, R.D., Development of Hydrogel Therapeutic Delivery System for Traumatic Optic Neuropathy, Biomedical Engineering Society, Annual Meeting, Minneapolis, Minnesota, October 2016.*
- *Jones, K., Glickman, R.D., Reilly, M.A., Torsional Indirect Traumatic Optic Neuropathy (TITON): Identifying Biomarkers of Trauma using Matrix Assisted Laser Desorption/ Ionization (MALDI), Association for Research in Vision and Ophthalmology, Annual Meeting, Seattle, Washington, May 2016.*
- *Swindle-Reilly, K.E., Asemota, B.I., Rodriguez, L., Jones, K.R., Glickman, R.D., Reilly, M.A., Development of Animal Model and Hydrogel Delivery System to Treat Traumatic Optic Neuropathy. Translational to Clinical (T2C) Regenerative Medicine Wound Care Conference, March, 2016.*

Other Presentations

- Biomechanical Analysis of the Eye as a Result of Aging and Trauma, School of Optometry, Indiana University, Bloomington, Indiana, January 2020.
- Engineering Approaches in Presbyopia and Ocular Trauma, Skaggs Institute of Molecular Medicine, The Scripps Research Institute, San Diego, California, June 2018.
- Biomechanics of Presbyopia and Ocular Trauma, Auckland Bioengineering Institute, University of Auckland, Auckland, New Zealand, April 2018.
- Pathophysiology of Presbyopia and Ocular Trauma, New Zealand National Eye Center Seminar Series, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, March 2018.
- Ocular Biomechanics of Aging and Trauma, Department of Biological Sciences, University of Delaware, Newark, Delaware, February 2018.
- Biomechanics of Presbyopia, Cataract, and Ocular Trauma, Department of Biomedical Engineering, University of Akron, Akron, Ohio, February 2018.
- Bio-chemo-mechanics of Presbyopia, Cataract, and Ocular Trauma, Department of Bioengineering, University of Maryland, College Park, Maryland, December 2016.
- Biomechanics of Presbyopia and Ocular Trauma, College of Optometry, Ohio State University, Columbus, Ohio, May 2016.
- Biomechanics of Presbyopia and Ocular Trauma, Grand Rounds, Havener Eye Institute, Ohio State University, Columbus, Ohio, May 2016.

- **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.

  We developed technology which allows for extended release of methylene blue, a candidate therapeutic for TON. This hydrogel-based drug delivery platform may be readily adapted to release other small molecules which prove efficacious for treatment of TON. This work was published in the *Journal of Biomedical Materials Research Part A*.

- **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.*

  We have developed two novel computational mechanics models relevant to understanding the pathogenesis of traumatic optic neuropathy, as well as evaluation of mechanical aids to treatment. These finite element models allow thorough investigation of biomechanical mechanisms and, when supplemented with experimental data (as described above) may be useful for the study of additional diseases involving the optic nerve (e.g. glaucoma).
  - Computational chemo-mechanical finite element model of hydrogel nerve cast
  - Computational mechanical finite element model of ocular rotation

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

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

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

Name: Matthew Reilly
Project Role: PI
Researcher Identifier (e.g. ORCID ID): ORCID 0000-0001-8029-0084
Nearest person month worked: 16
Contribution to Project: Dr. Reilly is the project PI and has overseen all aspects of the project. He has also constructed mechanical models of ocular rotation and hydrogel casting.

Name: Katelyn Swindle-Reilly
Project Role: co-PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-1739-0263
Nearest person month worked: 6
Contribution to Project: Dr. Swindle-Reilly has coordinated the hydrogel development, has attended meetings related to the research project, and has managed students performing experiments.

Name: Julie Racine
Project Role: co-PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-4409-0936
Nearest person month worked: 4
Contribution to Project: Dr. Racine has developed visual electrophysiology protocols, trained graduate students and employees to implement them, and analyzed electrophysiological data.

Name: Courtney Maxwell
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12
Contribution to Project: Ms. Maxwell has performed experiments with hydrogels, drug release, swelling, pH, rheological and biocompatibility testing. She has been investigating optic

nerve biomechanical testing and is first author of a manuscript describing the hydrogel cast.

Name: Danny Mackessy
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 9
Contribution to Project: Mr. Mackessy learned to design and conduct electrophysiology experiments in collaboration with Dr. Racine.

Name: Wade Rich
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 18
Contribution to Project: Mr. Rich has been evaluating porcine optic nerve samples for cell separation techniques. He has also contributed to the development of biomechanical testing protocols for the optic nerve.

Name: Bharat Kumar
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 4
Contribution to Project: Dr. Kumar investigated the most appropriate staining protocols for accurate identification of protein biomarkers.

Name: Annie Ryan
Project Role: Graduate Research Assistant
Researcher Identifier (e.g. ORCID ID): 0000-0003-3645-8649
Nearest person month worked: 36
Contribution to Project: Ms. Ryan has conducted electrophysiology experiments, injury modeling, immunohistochemistry, and data analysis.

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.*

Organization Name: Nationwide Children's Hospital
Location of Organization: Columbus, OH
Partner's contribution to the project: Collaboration Dr. Julie Racine, a visual electrophysiologist at Nationwide Children's Hospital, has been instrumental in training personnel for visual electrophysiology measurements and protocol development. She regularly comes to Ohio State for hands-on training and collaborative efforts and contributes extensively to data analysis.

Organization Name: Vanderbilt University
Location of Organization: Nashville, TN
Partner's contribution to the project: Collaboration Dr. Tonia Rex is a professor of ophthalmology at Vanderbilt. Her laboratory trained Annie Ryan to conduct immunohistochemistry analysis for samples.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*