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Synthesis, optimization, and degradation of a fish glue/PLGA polymer.

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Synthesis, Optimization, and Degradation of a Fish Glue/PLGA Polymer

By

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Synthesis, Optimization, and Degradation of a Fish Glue/PLGA Polymer

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ABSTRACT

Biomaterials used for tissue engineering, specifically polymers, come from either naturally occurring, like gelatin or collagen, or synthetic sources, such as polyesters. Often times, it is advantageous to combine biomaterials in order to maximize certain material properties, such as cellular adhesion and degradation. The purpose of this study is to provide direction for the synthesis, characterization, and a combination of a combination biomaterial between fish glue (FG), a natural source polymer, and poly-lactic-co-glycolic acid (PLGA), a synthetically made polyester. First, FG is characterized for molecular weight via Gel Permeation Chromatography, GPC. Two combination methods to mix FG/PLGA solutions are then compared. Finally, FG/PLGA mixtures of varying relative concentrations are subjected to degradation via treatment with phosphate buffered saline (PBS) and topographical analysis is performed. Degradation rate analysis showed that the samples tested lost approximately 70% of their mass after 12 hours of PBS treatment. Topographical analysis of FG/PLGA provided evidence indicating that in the later stages of degradation, as the FG is completely dissolved, small particles of PLGA remain. The work discussed in the proceeding document confirms the mixing of FG/PLGA, provides direction for an optimal mixing method and for characterization of FG, and documents a degradation and topographical analysis for small set of FG/PLGA mixes.

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I. INTRODUCTION

1.1 Definition of the Problem

A major area of concern in Tissue Engineering (TE) involves material selection; specifically, it is necessary to select a material which will provide a substrate for cellular attachment and proliferation and degrade in a desirable timeframe. The focus of the work presented in this study is to develop a material that will degrade but maintain structural integrity for 3-14 days to enable deposition and proliferation of capillary cells to form functional 3D capillaries. The problem to be addressed by this thesis work is to identify the optimal mixing method to combine PLGA and Fish Glue, to characterize fish glue as a material used in TE as its main area of use is by artists, to identify the degradation rate of a PLGA/Fish Glue polymer in an environment similar to that of the human body, and to topographically visualize the physical features of a PLGA/Fish Glue polymer while it is subjected to a physiologically analogous environment.

1.2 Brief History

Biomaterials used for TE can come from various sources but can be grouped into two categories, synthetic or naturally occurring. In the field of TE, capillary synthesis is dependent upon using a proper biomaterial as a viable tissue scaffold. Naturally occurring materials, such as collagen, gelatin, or silk, can degrade too quickly or are too cumbersome to manipulate in a repeatable fashion. Synthetic materials like poly lactic acid (PLA), poly glycolic acid (PGA), poly co-lactic glycolic acid (PLGA) degrade too

slowly and can cause adverse reactions from the native tissues. Therefore, a material

which combines the cellular growth benefits of a naturally occurring polymer, like gelatin

or FG, and the repeatable processing and manipulation ability of PLGA would be

advantageous for capillary synthesis.

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1.3 Purpose of Research

The primary purpose of this research is to characterize the degradation of differing

concentrations of PLGA and Fish Glue in a physiologically analogous environment;

effectively, characterizing the hydrolysis rate of different relative concentrations of the

two polymers in question. The secondary purpose was to determine the best mixing

method for FG and PLGA.

II.   LITERATURE REVIEW

2.1 Overview

In the field of TE, capillary synthesis, specifically the ability to create a 3D network of microvascular tissue, is necessary for organ synthesis due to the need for nutrient, gas, and waste exchange provided by a capillary bed. In order to create a complex capillary system, a cellular scaffold must be used to support the capillary cell growth and proliferation. Such scaffolds can be comprised of many various synthetic, such as PLA, PGA, PLGA, or biologically derived materials, like collagen, gelatin, chitosan, or silk. The following section contains a brief literature review of research related to Fish glue, a gelatinous biologically derived material, and PLGA, a commonly used synthetic polymer in TE.   Finally, multiple approaches to mixing polymers are outlined as well as their efficacy.

2.1.1   Fish Glue

Fish glue (FG) is a natural polymer commonly used as an adhesive made by the heating of fish scales and bones and other various parts in water until dissolution, this heating can be performed at atmospheric pressure or at an elevated temperature to speed up the process. FG has been used as a material by artists for millennia because it is inexpensive and possesses the ability to be re-worked (Petukhova 2000). Even after a bond is made, FG maintains the property of being water soluble enabling an artist to easily make changes through wetting. In the field of Food Science, FG is a commonly used material;

therefore, many characteristics and properties of fish gelatins have been analyzed including amino acid content and its dependence on fish species (Carbini et al. 1996). Even though FG is an established tool for artists, its use as a biomaterial has not been published to date. Fish gelatin has been shown to have a lower amount of hydroxyproline and proline imino acids than mammalian gelatins; moreover, colder water fish species proved to have the lower imino acid content than warmer water fish species (Gudmundsson 2002). Degradation of the collagen present in the fish scales and bones occurs during this heating process, the extent of degradation is generally unknown. Carbini et al. a curie-point pyrolysis-gas chromatography/mass spectroscopy study of fish glue versus other proteinaceous binders: milk casein, egg yolk, egg albumin, bone glue, skin glue, and rabbit glue (Carbini et al. 1996). Amino acid analysis, SDS-PAGE electrophoresis, and FTIR spectroscopy has been used as a means of analysis to determine the makeup of extracted fish collagen(J.H. Muyonga, Cole, and Duodu 2004)(Zhang, Xu, and Wang 2011). FTIR spectrum were compared between gelatin and collagen extracted from fish(J.H Muyonga, Cole, and Duodu 2004). Varying species of fish gelatin were compared through molecular weight determination via HPLC/GPC, isoelectric point determination, SDS-PAGE electrophoresis, viscosity, circular dichroism, and rheological measurements (Gudmundsson 2002)(Turnay and Ferna 2002). However, a comparative study of fish glue to both gelatin and collagen has not been performed.

2.1.2   Poly co-lactic glycolic acid

PLGA is a polyester synthesized through the polymerization of glycolic acid and lactic acid monomers. PLGA has many uses and applications as a biomaterial: drug delivery in pharmaceuticals, bone grafts, sutures, and as cellular scaffolds. The degradation rates of PGA, PLA, and PLGA have been highly studied; furthermore, studies have shown that the degradation time of PLGA changes with the relative ratio of lactic to glycolic acid monomers. The higher the ratio of lactic acid to glycolic acid then degradation time is longer. This phenomena is due to the fact that PGA is highly crystalline and has a faster degradation rate as compared to PLA, 2 months versus 12 months. (Park 1995) (Ulery, Nair, and Laurencin 2011) (Makadia and Siegel 2011) (Grayson et al. 2004)

2.1.3   Polymer mixing methods

Two groups discuss success in mixing fish gelatin and PLLA dissolved in 88% formic acid or 90% acetic acid in distilled water and dichloromethane, respectively, through gently stirring gelatin (An 2010) (Gu 2009). With the use of aqueous solvents, collagen and poly acrylic acid have been shown create complexes by means of shaking followed by centrifuging (Barbani 1999). Composite films of collagen and poly(ε-caprolactone) have been fabricated by first treating freeze-dried collagen with PCL dissolved in dichloromethane then allowing the solvent to evaporate (Coombes 2002). Gu et al. outline the creation of a PLGA/gelatin composite through first dissolving PLGA in chloroform, then dispersing gelatin microparticles into the solution via ultrasonic wave energy, chloroform was then completely removed via evaporation followed by vacuum drying. The resulting scaffold was then treated with water dissolved gelatin; finally, the

scaffold was twice cross-linked via treatment with 1-ethyl-3-(3-dimethyl-amino- propyl)-carbodiimide (EDAC) and freeze-dried (Gu 2009). PLGA and collagen, of an unknown source, have been dissolved in hexafluoroisopropanol and successfully mixed with magnetic stir bar (Jose 2009). PLGA, of an unknown relative ratio, and gelatin, from an unknown source, have been reported to be mixed through dissolution of both materials in dichloromethane, treatment with an ultrasonic wave, followed by solvent evaporation (Li 2007). Li et al. make no mention or measurement of mixing; however, they do present degradation profiles of differing relative ratios as well as SEM images to illustrate the microstructure and level of mixing. No previous research has been found that illustrates the successful mixing of FG and PLGA.

III. INSTRUMENTATION AND EQUIPMENT

The following section identifies the equipment and instrumentation used for this study. In addition, a description of each piece of equipment and instrumentation is provided. A description of how each piece of equipment and the instrumentation were implemented in the study is also provided.

3.1 Sonication Instrumentation

The instrument used for ultrasonic mixing for this study was a FS60 Ultrasonic Cleaner (Thermo Fisher Scientific, Inc., Waltham, MA; FIGURE 1). This ultrasonic cleaner consists of a 6 quart stainless steel tank which is bonded to a piezoelectric transducer. To operate, the tank was filled with water until the water level was 1 inch from the top of the tank. A glass vial filled with the desired amount of materials to be mixed was placed in a plastic holding tray, which floated on the top of the water. The cleaner was activated by turning the timing knob clockwise to 60 minutes. When the cleaner is activated, the piezoelectric transducer vibrates at a frequency of 40 kHz causing air bubbles to form, grow, and collapse within the water in the tank, which indirectly mixed solutions in the glass vial.

FIGURE 1—Ultrasonic Cleaner used to indirectly mix solutions

3.2 Mechanical Mixing Instrumentation

An overhead stirrer (RZR 2021, Heidolph Instruments, Schwabach, Germany;

FIGURE 2) was used to directly mechanically mix the solutions. The overhead stirrer

allowed the user to change the stirring rate, in rotations per minute, to a desired setting

and operated continuously for a desired amount of time. To set up, a steel wire,

approximately 2.5 cm in length with a bend of 15 degrees in the middle of the wire, was

fastened in the Jacob's chuck of the overhead stirrer. Before mixing, the stirrer was

turned on and the mixing speed knob was toggled until the desired mixing rate of 100

rpm appeared in the LCD display. A vial containing the desired solution to be mixed

was positioned below the stirrer so that the bottom end of the steel wire was

approximately 1 mm from the bottom of the vial.

FIGURE 2—Heidolph overhead stirrer used for mechanical mixing of solutions.

3.3 UV-VIS Spectrophotometer

A UV/Vis Spectrophotometer (DU 530 Life Science, Beckman Coulter Corp., Brea, CA; FIGURE 3) was used in this study to measure absorbance of light at varying wavelengths through samples.   Briefly, the spectrophotometer operates by emitting a precise wavelength of light onto a sample cuvette and measures the amount of light which passes through the sample. Absorbance is then calculated by the spectrophotometer by performing a base ten logarithm of the quotient of the incident and transmitted light intensities, respectively.   To operate, the system was blanked by placing a cuvette of distilled water in the specimen holder and pressing the blank command button on the system. Each sample used for absorbance analysis was diluted to a 1% concentration in distilled water. An absorbance spectra was obtained for each sample between the

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wavelengths of 190 nanometers and 350 nanometers using the absorbance sweep function

of the spectrophotometer with a 1 nanometer step setting.

FIGURE 3—Spectrophotometer used for absorbance measurements.

3.4 Spin Coating Instrumentation

A spin coater (Model WS-400B-6NPP/LITE, Laurell Technologies Corporation, North

Wales, PA; FIGURE 4) was utilized for depositing a thin layer of polymer solution onto a

glass cover slide. Briefly, a spin coater works by rotating a substrate, that has been

deposited with an excess amount of liquid material, at a high speed causing the material

to spread evenly across the substrate due to centrifugal force. For this study, spin coated

samples were prepared by depositing a 25 μL volume of the desired polymer mixture

onto the center of a glass microscope cover slide (12-5545-80 12 CIR.-1 Glass

Microscope slide, Thermo Fisher Scientific, Waltham, MA). Immediately after the

polymer has been deposited, the glass microscope cover slide was rotated at a speed of

3000 rpm for 15 seconds.

FIGURE 4—Laurell Technologies spin coater used for cover slide coating.

3.5 Scanning Electron Microscope

In order to topographically analyze the spin coated samples, a scanning electron

microscope (SEM), LEO Supra 35 (Carl Zeiss; Oberkochen, Germany) was employed in

order to visualize the surfaces at the resolution needed for analysis. Imaging samples

using an SEM involves sending electrons directly to the entire surface of a specimen.

The electrons subsequently bounce off of the surface of the specimen and back to a

detector which enables the SEM to construct an image of the specimen.

3.6 Sputter Coating Instrumentation

Since the specimen imaged for this study are polymeric in nature and would thus absorb the electrons projected by the SEM, the specimen must be first sputter coated with a thin film of gold to enable SEM imaging. The sputter coating was performed using a PS2 Coating Unit (International Instruments; Santa Clara, CA). Briefly, a small layer of gold is deposited on the samples surface through delivering gold particles to the surface. The gold particles are delivered through release from a gold target by plasma which is created through applying an electric field to inert gas.

3.7 Data Processing

UV-VIS data was first obtained by means of the COM port connection from the spectrometer to the computer. All of the .txt files from all of the samples were compiled into one single Excel File (Microsoft Corp., city, state). The degradation rates were computed from the data attained in the Excel spreadsheets. Further data analysis will be described in detail in the proceeding section.

IV.   PROCEDURE

The methods used to conduct this study are described in the following section. First, the initial processing steps of the raw materials prior to mixing are described including their development. Next, the procedures for ultrasonic and mechanical mixing are outlined followed by the procedure to determine the optimal mixing method. Finally, the in-vitro degradation rate quantification and method of FG/PLGA polymer mixtures with differing relative concentrations is described.

4.1 Material Preparation

Prior to the combination of the polymers used in this study, both raw materials were subjected to pre-mixing protocols. The material preparation described in this section was performed before all mixing, testing, or use occurred of either polymer. First, PLGA dissolution will be discussed followed by FG heating.

4.1.1   Poly-lactic-co-glycolic acid dissolution

Poly-co-lactic-glycolic [75:25] acid (PLGA) procured from Lactel Absorbable Polymers Company (Pelham, AL) in solid form was dissolved in chlorobenzene. Berry et al. identified chlorobenzene as the optimal solvent for the direct-write fiber drawing method with PLGA due to its ability to dissolve PLGA at room temperature [10]. Berry et al. also determined that the ideal concentration of PLGA in chlorobenzene was between 29-30% (%w PLGA/total %w) for the successful fabrication of fibers, with the greatest amount of success occurring when the concentration is on the higher end of the range. Therefore, a 30% concentration of PLGA to chlorobenzene was used in this study.

To create PLGA solutions, a glass vial, with screw cap removed, was first placed onto a scale and tared. The desired amount of PLGA was placed in the vial and the weight of chlorobenzene was calculated based upon the measured PLGA weight. The chlorobenzene was then added directly into the vial in drop-wise fashion by use of a glass transfer pipette. Once the desired amount of chlorobenzene was added, the cap was then replaced and wrapped with parafilm to prevent solvent evaporation. Complete PLGA dissolution at room temperature generally took 24-48 hours before use.

4.1.2 Fish Glue Heating

Throughout this study, Norland Products Inc. (Cranbury, NJ) High Tack Fish Glue was heated prior to mixing. The heating process was developed to optimize the fiber drawing ability of FG by increasing the viscosity through the removal of excess water present. First, a direct heating method, via a glass beaker with a specified amount of FG was placed on a hot plate. This method was quickly abandoned due to the viscosity of FG and non-uniformity in the heating of the FG was observed, even with stirring. For more uniform heating, a double-boiler method was developed, which involved placing a beaker of FG inside a beaker of water on a hot plate. However, extreme variations in FG/PLGA polymer drawing ability and degradation rates were observed. During initial work, it was observed that the source of variation in the drawing ability and degradation rate of the FG/PLGA polymer could be due to the heating process. During the heating process, the water boils in the outside double-boiler beaker expelling water vapor. It was hypothesized that this vapor could then subsequently

condense in the beaker of FG, thereby, retarding the efforts of water removal. To remove this potential source of variability, the water bath was replaced with a sand bath.

The sand bath procedure consisted of heating the external 250 mL beaker filled with sand to 100ºC.. Once the sand bath reached the desired temperature, a 50 mL beaker filled with 15 mL of FG and a magnetic stir bar was placed in the sand bath. The sand bath was maintained at a temperature of 100ºC and the FG beaker was heated to 100ºC for 10 minutes with constant stirring. After ten minutes, the beaker of FG was removed from the sand bath and the FG was transferred to a plastic centrifuge tube. Before the tube was capped for storage or use, the FG was allowed to return to room temperature.

4.2   Fish Glue Solvent Concentration Determination

Norland High Tack Fish Glue is comprised of Gelatin, Water, and Phenol, as stated on the product's labeling. The exact amount of each component is not specified in the packaging and we were unable to obtain these specifications from Norland Products. To quantify the percentage of solute (gelatin and phenol) versus solvent (water) concentration of stock FG and heated FG the following procedure was used. First, a 15 mL sample of heated FG was prepared using the sand bath heating method described in the previous section. The following steps were performed for five replicates of both a stock FG sample group and a heated FG sample group. A glass microscope cover slide was weighed and recorded. A 25 μL sample of FG was deposited via pipette onto the center of the cover glass. The cover glass with fish glue was immediately returned to the scale and the weight was recorded (Note: this step was performed as quickly as possible

to measure full weight before evaporation commenced). All samples were placed in a laminar flow hood for 24 hours and allowed to dry at room temperature and humidity. After drying, each glass cover slide was weighed to determine the final dry weight.

4.3   Gel Permeation Chromatography Measurements

Samples of Type I collagen from calf skin, Type II collagen from chicken, Type IV collagen from human, cold water fish gelatin, and bovine gelatin were obtained from Sigma-Aldrich Canada. Norland High Take Fish Glue was obtained from Norland Products Inc, Cranbury, NJ. Gel Permeation Chromatography (GPC) was performed to determine the molecular weight of FG through regression analysis of by using collagen and gelatin samples with known molecular weights using a Waters Ultrahydrogel 250 column with a mobile phase of 20% acetonitrile in 0.1 M sodium nitrate with a 0.3 ml/min flow rate using a Waters Binary Pump. Column temperature and pressure were maintained at 30°C and 285 mm Hg, respectively. Each sample was dissolved in the mobile phase at a concentration of 0.7 mg/mL. Samples were detected using a Waters Refractive Index RI detector and a Waters UV/Vis detector at a detection wavelength of 280 nm for a 60 minute elution time.

4.4   Mixing Method Determination

The ability to mix PLGA with FG in a reliable and repeatable manner is necessary for its potential use as a biomaterial. In this section, the procedures for each of the two mixing methods tested are first described. Then, the test method to determine level of mixing is described.

4.4.1 Ultrasonic Mixing

In preliminary investigations, the initial mixing method explored consisted of placing PLGA and FG into a glass vial at their desired relative concentration (%w FG/total %w). The vial was then capped, wrapped with parafilm, and placed in the ultrasonic cleaner. After placement, the ultrasonic cleaner was activated for a full cycle time of 60 minutes. Each vial mixed was be subjected to four cycles, total mixing time of 4 hours. This 4 hour mixing time was qualitatively determined to be ideal prior to this thesis. After each cycle, the water in the cleaner was replaced to ensure excess water heating did not occur. Following the final cycle, the glass vial was removed from the ultrasonic cleaner.

4.4.2 Mechanical Mixing

A second mixing method was developed during the course of this thesis. Briefly, FG and PLGA were combined in a glass vial at their desired relative concentrations. The glass vial was then positioned under the Heidolph overhead stirrer to the point that the bottom tip of the steel mixing wire was approximately 1 mm from the bottom of the vial. After placement, the mixer was turned on, placed at a rotation setting of 100 rpm and mixed for different amounts of time (30 minutes, 1 hour, 2 hours and 4 hours). Once the desired amount of time was reached, the vial was removed from the mechanical mixer, capped, and was ready for use.

4.4.3 Mixing Method Testing

When mixing is initiated, the polymers are in a distinct suspension due to the insolubility of gelatin, the polymer component of FG, with chlorobenzene. However after mixing is induced, chlorobenzene soluble PLGA begins to mix into the water phase, containing FG. Visually this occurs with a color change from caramel to off-white of the water soluble phase containing FG. Due to this optical change, the level of mixing was determined through UV-VIS absorbance spectra measurements. For testing, three samples of each of the following solutions were created: FG, PLGA, 4 hour ultrasonic mixing, 4 hour mechanical mixing, 2 hour mechanical mixing, 1 hour mechanical mixing, and 30 minute mechanical mixing. FG and PLGA samples were created as standards; each sample was created as described in the Material Preparation section. The absorbance spectra for all samples were measured with a distilled water blank. To prevent signal saturation, all samples were diluted serially from their vial to a 1% concentration in distilled water in the specimen cuvette. Absorbance measurements were taken at wavelengths varying from 190-350 nm at 1 nm increments. Analysis was performed at wavelengths of 260 and 280 due to their importance with DNA and protein spectrophotometry.

4.5   Degradation Characterization

This section outlines the determination of the degradation rate for differing FG/PLGA solutions with respect to their relative weight concentrations. A spin coating method of sample fabrication was chosen over the direct-write method due to its ease of use, speed of fabrication, and ability to produce samples of similar properties to direct-write fibers. First, the surface area to volume calculation is explained. Then, the test

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procedure is outlined. Finally, the method for image analysis of a representative group is stated.

4.5.1 Surface Area to Volume Calculation

Due to the fact that hydrolysis, the degradation mechanism for PLGA, is increased with an increase in the surface area to volume ratio (SA/V) of the scaffold, the spin-coated samples surface area to volume ratios must maintain consistency with direct-write fibers. In order to maintain a surface area to volume ratio consistent with the application of fibers, the following equations and calculations were used. A characteristic PLGA direct-write fiber was modeled as a perfect solid cylinder. To calculate the SA/V ratio of the fiber, the top and bottom faces of the cylinder were neglected because they are connected to a supportive frame. Equation 1 shows the Surface Area calculation and Equation 2 depicts the Volume calculation of a fiber. Dividing Equation 1 by Equation 2 yields the SA/V ratio shown in Equation 3. For the spin coating application:

$$Surface\ Area = 2\pi rh \tag{1}$$

$$Volume = \pi r^2 h \tag{2}$$

$$\frac{SA}{V} = \frac{2}{r} \tag{3}$$

The substrate used for spin coating was a circular glass covers slide. Equation 4 shows the simple surface is calculation of the circle. To calculate the SA/V ratio for the cover slide shown as Equation 5, Equation 4 was divided by an unknown Volume (Vu).

$$Surface\ Area = \pi R^2 \tag{4}$$

$$\frac{SA}{V} = \frac{\pi R^2}{Vu} \tag{5}$$

Equilibrating the Equation 3 with Equation 5 and solving for Vu yields Equation 6.

$$Vu = \frac{\pi R^2 r}{2} \tag{6}$$

Assuming that the presence of FG in PLGA will not drastically change fiber diameter, the radius of the representative fiber, r = 8.435, was gleaned from a diameter measurement of a 75:25 PLGA fiber at 30% in chlorobenzene by Berry et al. of 16.87 μm. The glass cover slides used as a substrate for spin coating had a radius, R, of 12.5 mm. Solving for Equation 6 with the previously stated values of R and r and converting from meters to liters, yields the unknown Vu of 2.1 μL. The actual injected volume added for spin coating was 20 μL which was the calculated Vu plus a safety factor to

account for polymer loss during the spin process as well as volume loss during the drying process.

4.5.2   Degradation Rate Measurements

Degradation measurements were taken from samples of mixed FG/PLGA polymers at percent relative concentrations of 65/35, 70/30, 75/25, and 80/20 (%w FG/%w PLGA). Samples were prepared using the methods discussed above. Briefly, for each sample, PLGA was dissolved in chlorobenzene at a 30% concentration in a glass vial. Then, an excess of heated FG was heated to be used for all mixtures. Finally, FG was added to the PLGA at one of the desired concentrations mentioned previously and mixed mechanically at 100 rpm for 1 hour. The amount of time used for mechanical mixing was determined through data analysis of the mixing test previously described.

After mixing, 24 spin-coated specimens were created from each of the four different mixtures. Six time points of 12 hours, 1 day, 2 days, 6 days, 10 days, and 14 days were used and for each time point three replicates were fabricated. To create a specimen, a clean glass cover slide was secured on the spin coater. Then, a 25 μL volume of the desired polymer mixture was deposited in the center of the cover slide. The sample was then spun within the spin coater for 15 seconds at a speed of 3000 rpm. After spinning, the cover slide was removed from the spin coater and allowed to dry for 24 hours.

Day 0 of the study began with each dry specimen being weighed to obtain the initial weight, W_o. Replicates of the same time point and FG/PLGA concentration were

placed in a petri dish and 1.5 mL of 1X Phosphate Buffered Saline (PBS), was added.

Then, all specimens were placed in an incubator at 37ºC.   Every 2 days, old PBS was

removed and replaced with fresh PBS. At each time point, specimens labeled with the

corresponding time point were removed from the incubator, aspirated with PBS, and

allowed to dry for 24 hours. Once a specimen was dried, the final weight was measured,

W_i.

4.3.3 Degradation Topographical Analysis

Scanning Electron Microscopy imaging was used to qualitatively determine

topographical changes in the surface of the FG/PLGA polymer mixture as the material

degraded. A single replicate from each time point (including t=0) of the 65/35 FG/PLGA

mixture group was reserved after the Degradation Rate Analysis procedure described in

the previous section for SEM analysis. Each cover slide was placed on the SEM sample

plate using doubled sided carbon conductive tape. Extreme care was taken during this

process to mount each specimen flatly as well as to maintain time point numerical order

for specimen labeling during SEM imaging. The sample plate was then sputter coated

with a thin layer of conductive gold using the PS2 Coating Unit. To sputter coat, the

sample plate was placed inside the sputter chamber and the vacuum pump was activated

to establish a pressure of 0.1 torr. A 1 kilovolt electric field was used to create plasma

which bombards a gold target freeing gold atoms which then attach in layer form to the

sample.   The sputter coating process was carried out for 90 seconds. After sputter

coating, the sample plate was placed in the SEM sample carousel. The SEM chamber

was closed, sealed, and a vacuum was established. An electron beam voltage of 5 kV was established and the secondary electron detector was turned on. Images were captured of each time point specimen at similar zoom settings, with scale bars of approximately 2µm,   20 µm, and 100µm. Clear images were obtained through manipulation of wobble, stigmation, brightness, and contrast settings.

V.   RESULTS AND DISCUSSION

5.1 Fish Glue Concentration Results

The percentage of water versus solid components in FG was determined for stock FG and heat treated fish glue. Five specimen of each heat treatment weighing approximately 40 mg were allowed to sit over night to dry. Full results of the recorded measurements can be found in Appendix 1. The final weights proved that the heating process was indeed evacuating liquid from the stock fish glue yielding a more concentrated final product. This is shown by a Mann Whitney Median test between the two samples. A two sample student's t-test would have been preferred; however, as FIGURE 5 shows below, the No-heat sample's FG concentration percentage was not normally distributed (p –value = 0.007 in test for normality).   However, the heated group of FG was shown to have a larger span in percent solid concentration which could be due to variability in the heating process. Figure 5 also shows that the stock fish glue has a solid concentration of 56.87% while the heated samples have a concentration of 52.70%.   FIGURE 6 illustrates the details of the Mann-Whitney test, which suggests that the Heated FG group's concentration is 4 percent higher this implies that the heating process causes a sample of stock fish glue to lose approximately 4 percent of its mass.
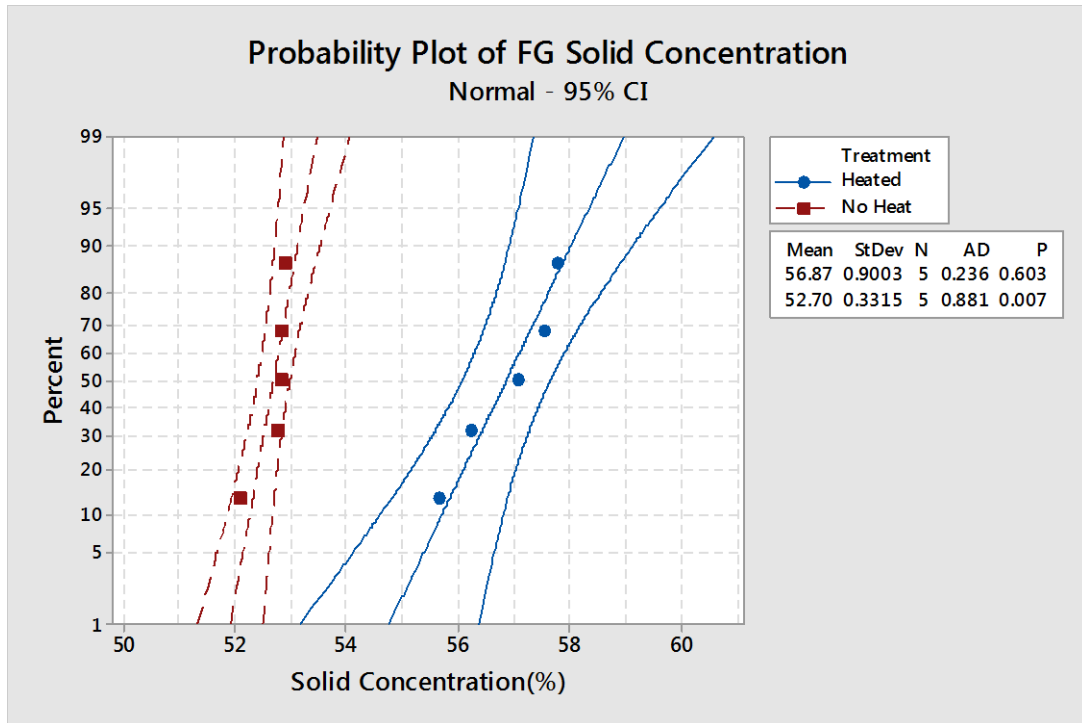
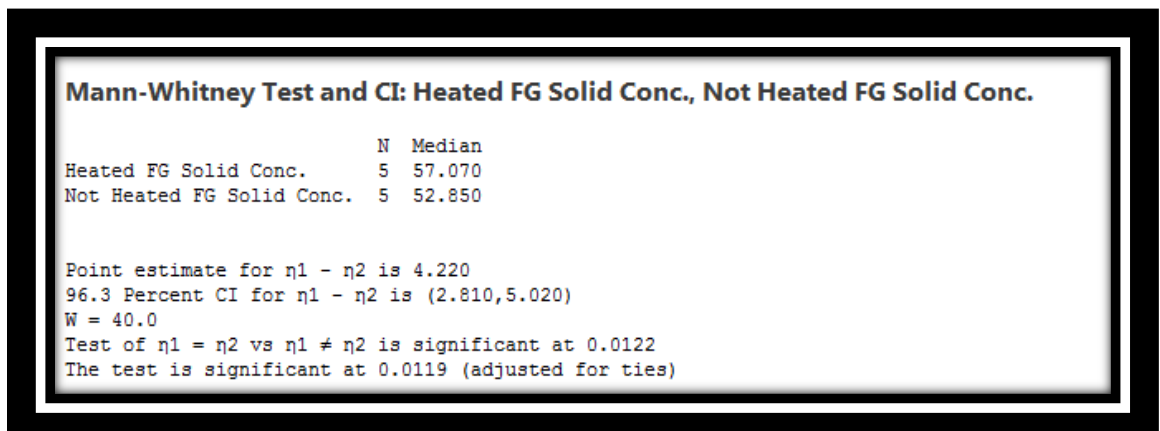FIGURE 5—Probability plot of Heated and No Heat % Solid Conc.

FIGURE 6—Mann-Whitney Test of Heated vs No Heat % Solid Conc.

5.2 Gel Permeation Chromatography Results

Refractive Index (RI), a measurement made from visible light, and 280 nm spectra were successfully measured for each sample of FG and the standard animal gelatins and collagens. FIGURE 7 and FIGURE 8 illustrate the RI and 280 nm spectra of FG, respectively. FIGURE 9 and FIGURE 10 illustrate the RI and 280 nm spectra of FG and all animal gelatins and collagens, respectively. All standards molecular weight and elution time until first peak for RI and 280 are shown in TABLE 1. Standard curves of molecular weight versus elution were generated for each spectra (RI and 280) along with a linear fit shown in FIGURE 11 and FIGURE 12. Higher molecular weight samples were seen to have a shorter elution time for a peak with the RI sensor as compared to lower molecular weight samples; whereas, the elution time for a higher molecular weight sample was longer than a lower molecular weight sample with the 280 nm sensor. This discrepancy could be due to a couple of factors: the sensors are in series of each other, potentially causing a time lag and a constituent of a sample could cause a signal with one sensor and not the other, i.e. the 280 nm sample could cause excitation of a constituent producing a signal that would not be seen with the RI sensor. Molecular weight values for the standards were gleaned from the Extracellular Matrix Factsbook (Ayad et al. 1998). The linear fit from each detection method was used to determine the molecular weight of FG based on elution time. The curve for the RI detector and 280 nm detector yielded a molecular weight prediction for FG of 176,500 Da and 165,200 Da, respectively. The variable prediction of molecular weight could be due to technical difficulties with the Waters GPC pump, a gradient chromatography was not able to be

run. Having the ability to run a gradient chromatography would allow the elution times

of the molecular weights to gain more resolution, thus increasing the accuracy of the

standard curve.
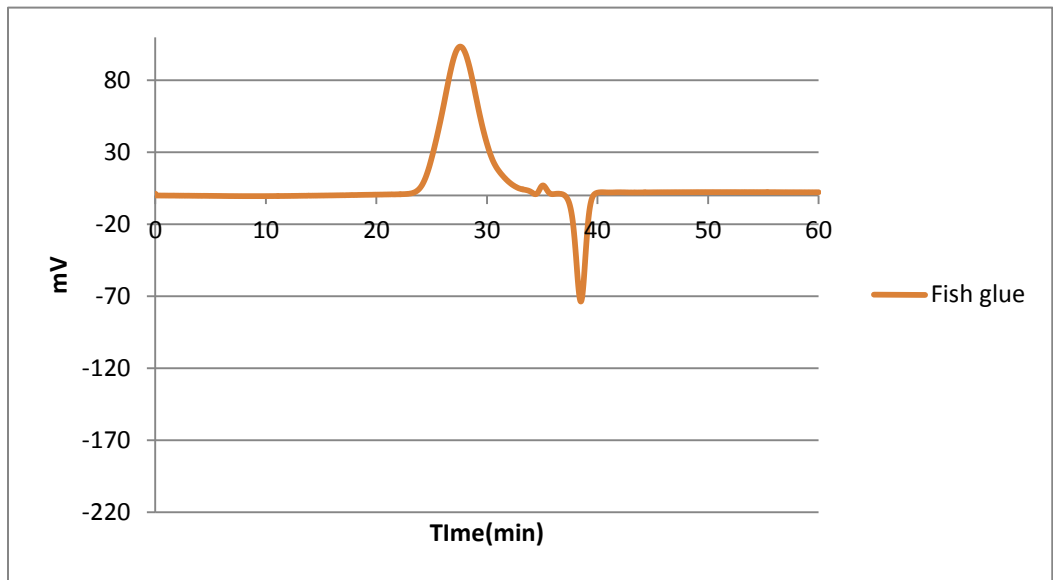
FIGURE 7—Fish glue refractive index spectra.
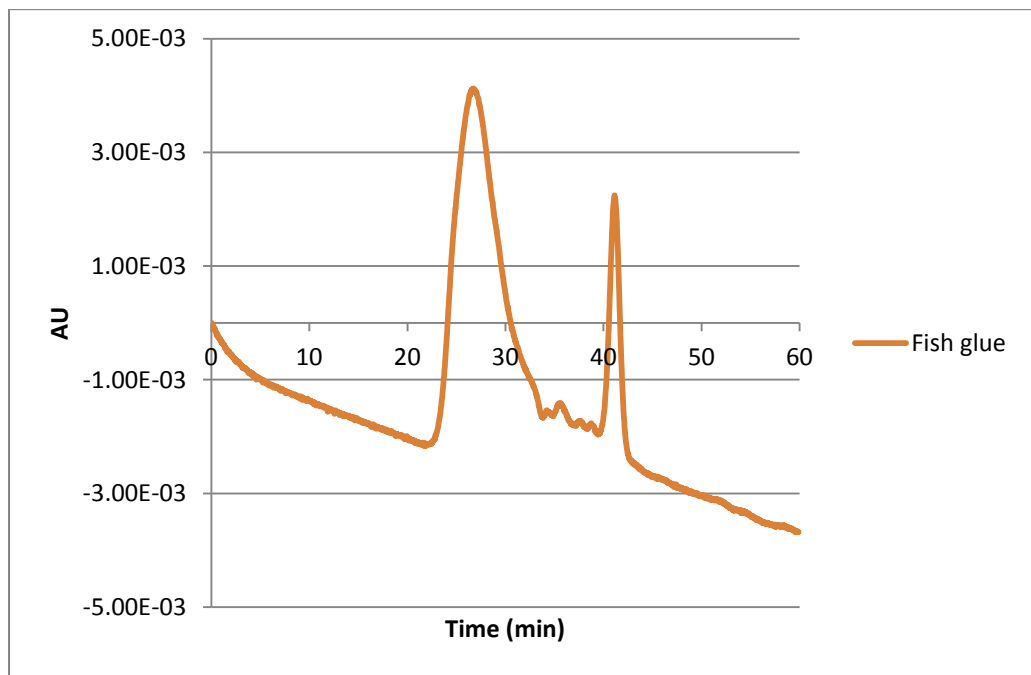
FIGURE 8—Fish glue 280 nm spectra.
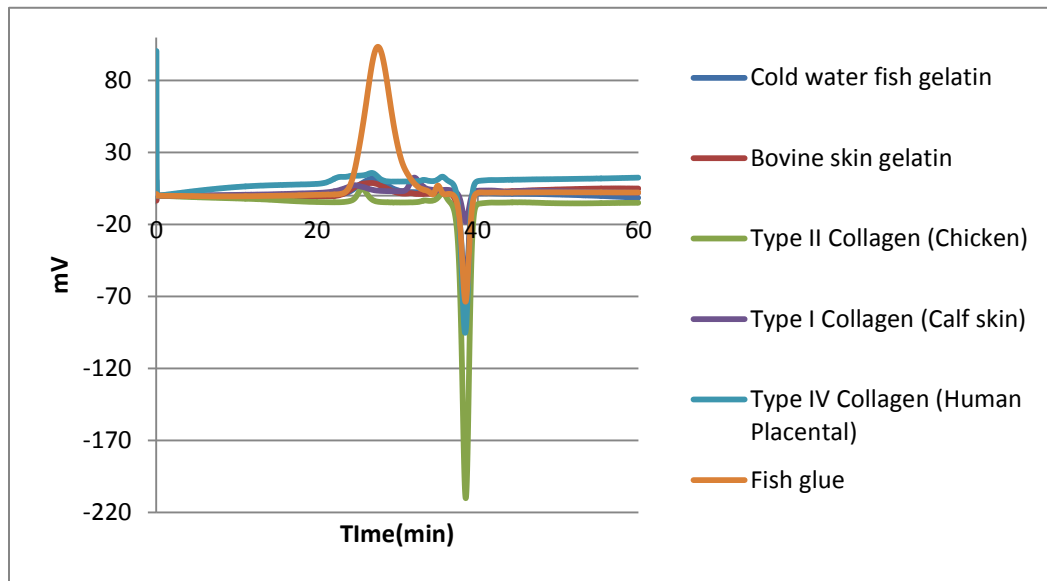
FIGURE 9—All sample RI spectra.
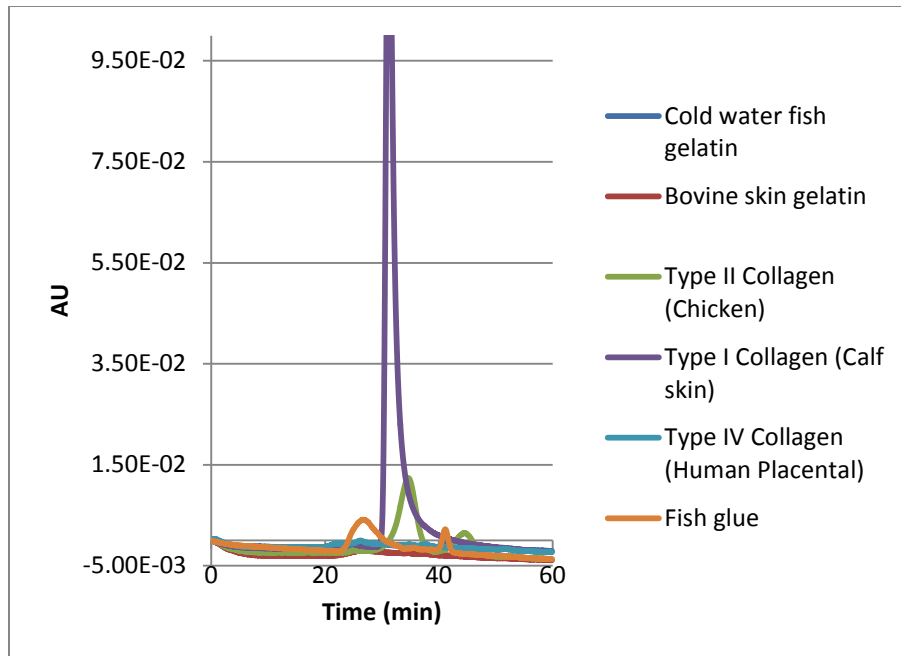
FIGURE 10—All sample 280 nm spectra.

TABLE 1—Standard Elution times and Molecular weights.

Standard	Elution Time (min) RI	Elution Time (min) 280	Molecular Weight(Da)
Cold water fish gelatin	27.2	26.8	169500
Bovine skin gelatin	26.9	26.5	171000
Type II Collagen (Chicken)	25.6	34.7	141800
Type I Collagen (Calf skin)	24.7	31.2	138700
Type IV Collagen (Human Placental)	26.8	26.2	161700
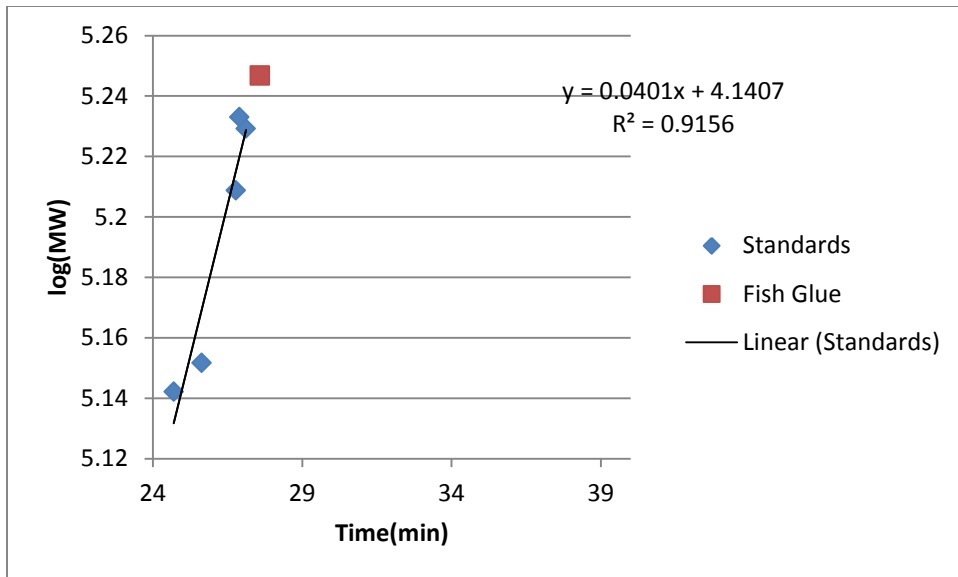
FIGURE 11—Standard Curve of RI elution time and MW.
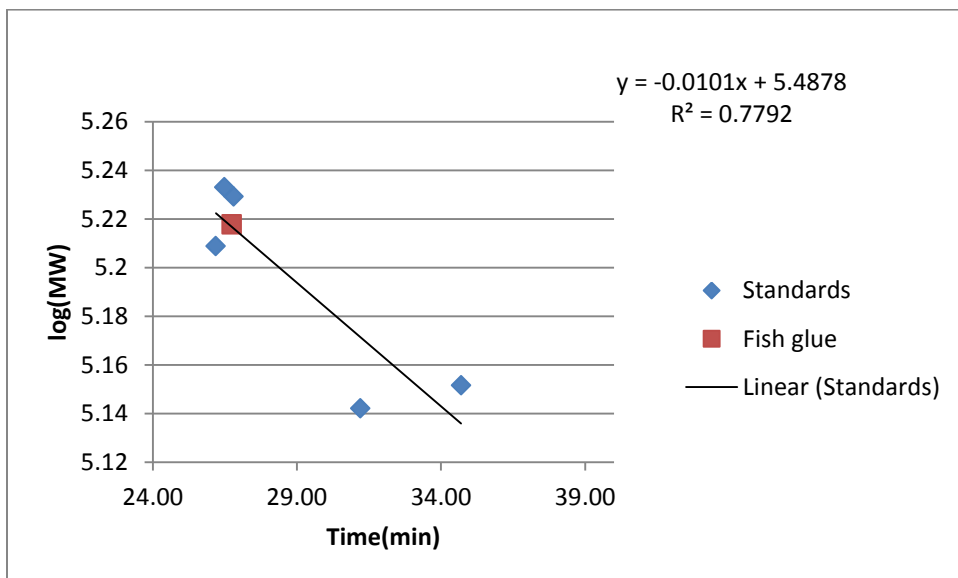
FIGURE 12—Standard Curve of 280 nm elution time and MW.

5.3 Mixing Method Results

Absorbance spectra were successfully obtained for all samples of different mixing methods and time. The full results of UV-Vis spectra and graphs can be found in Appendix 3. Spectral analysis showed that as the amount of mechanical mixing time increased, the amount of light absorbance for a mixture also increased, potentially indicating that as more PLGA mixes into the FG, it increases its ability to absorb light, as was shown at 260 nm and 280 nm. Full results can be found in Appendix 2. FIGURE 13 and **FIGURE 14** provide an illustration of the average spectra of FG and PLGA at varying mechanical mixing times and a 4 hour sonication. The mechanical mix times of 30, 1 hour, 2 hours, and 4 hours provides a time sequence of mixing levels. The two sample student's t-test between mechanical and sonication at 260 nm proved significant (p = 0.001) meaning that the absorbance measurements for mechanical mixing were greater than that of sonication as shown in **FIGURE 15**. However, **FIGURE 16** and **FIGURE 17** show that the two sample t-tests between sonication and mechanical mixed groups at 280 nm as well as the ratio 260:280, which approximately gives nucleic acid to protein ratio, both prove no statistical differences between mixing groups. Statistically nothing can be proven as to the amount of time necessary to properly mix FG and PLGA mechanically, no tests showed a statistical difference between any mixing times in terms of absorbance at 280 and 260 nm. However, a difference in mixture variation can be shown graphically as seen in FIGURE 18 and FIGURE 19, which show the mean absorbance at 280 nm and 260 nm, respectively; furthermore, it can be seen that a 1 hour mixing time could potentially be optimal because it is a relatively short amount of time

among the choices and provides a lower amount of variation against the other groups.

Limitations of this testing include: variability of serial dilution causing variability in

measured absorbance, not performing a blank of chlorobenzene, and only having three

replicates per sample.
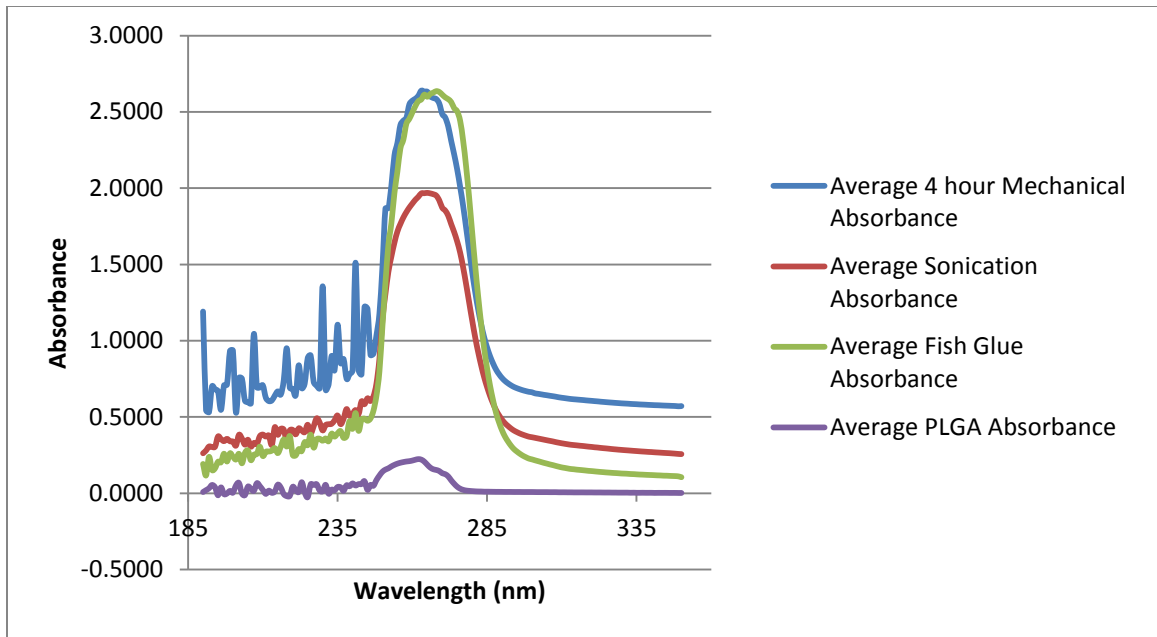
FIGURE 13—Absorbance spectra of FG, PLGA, 4 hr Sonication, 4 hr Mechanical mix.
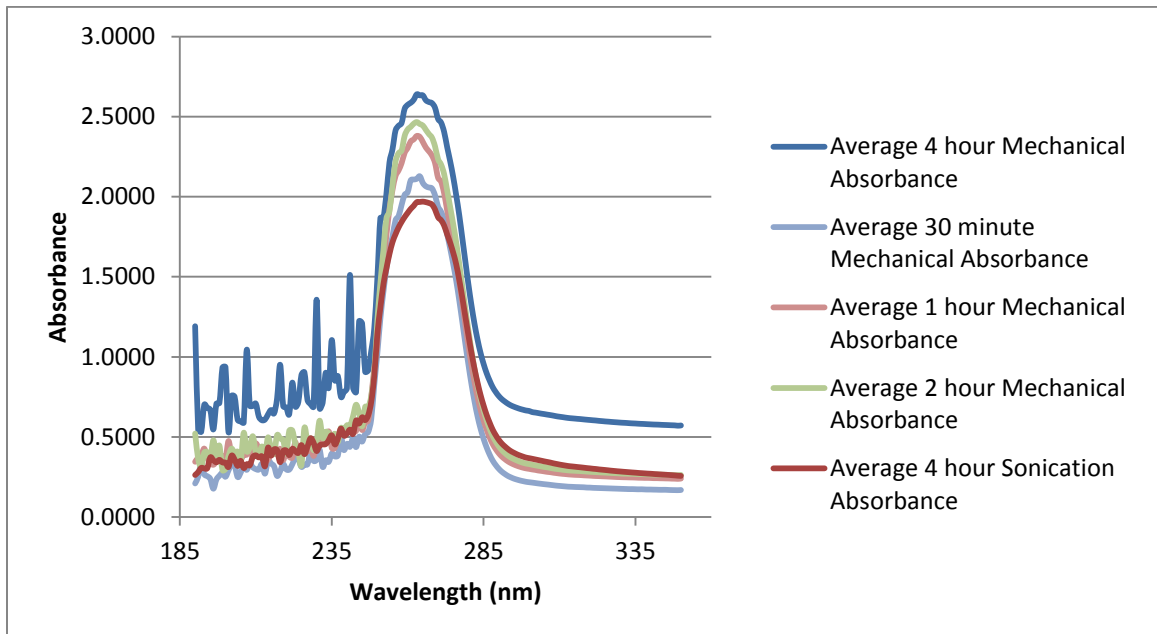
FIGURE 14—Absorbance spectra of timed mechanical mixes and 4 hr Sonication.

```
Two-Sample T-Test and CI: Absorbance at 260, Mixing Type

Two-sample T for Absorbance at 260

Mixing Type  N   Mean  StDev  SE Mean
Mechanical   9  2.574  0.396     0.13
Sonication   9  1.899  0.303     0.10


Difference = μ (Mechanical) - μ (Sonication)
Estimate for difference:  0.675
95% CI for difference:  (0.318, 1.031)
T-Test of difference = 0 (vs ≠): T-Value = 4.06  P-Value = 0.001  DF = 14
```
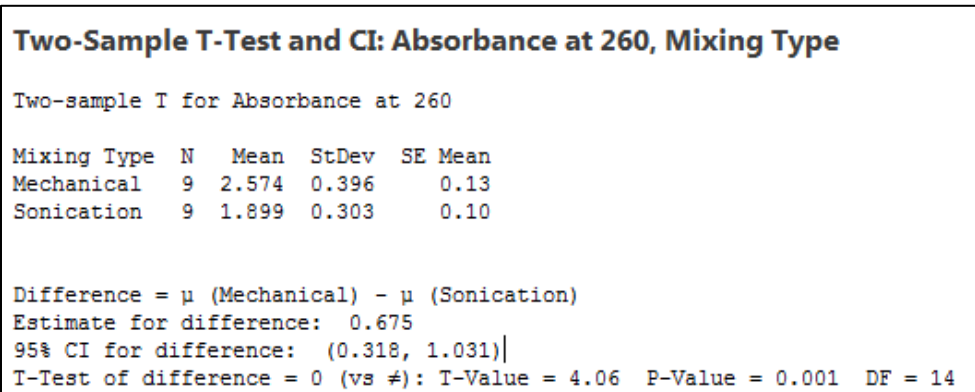
FIGURE 15—Two Sample T-test of Absorbance at 260 of mixing types

```
Two-Sample T-Test and CI: Absorbance at 280, Mixing Type

Two-sample T for Absorbance at 280

Mixing Type  N   Mean  StDev  SE Mean
Mechanical   9  1.453  0.431     0.14
Sonication   9  1.135  0.229    0.076


Difference = μ (Mechanical) - μ (Sonication)
Estimate for difference:  0.318
95% CI for difference:  (-0.037, 0.672)
T-Test of difference = 0 (vs ≠): T-Value = 1.95  P-Value = 0.074  DF = 12
```
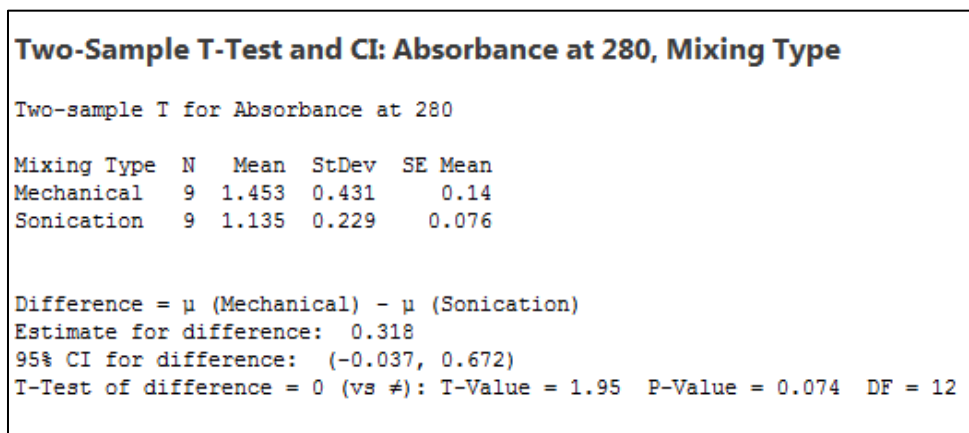
FIGURE 16-- Two Sample T-test of Absorbance at 280 of mixing types

```
Two-Sample T-Test and CI: 260:280, Mixing Type

Two-sample T for 260:280

Mixing Type  N   Mean  StDev  SE Mean
Mechanical   9  1.842  0.275    0.092
Sonication   9  1.688  0.153    0.051


Difference = µ (Mechanical) - µ (Sonication)
Estimate for difference:  0.154
95% CI for difference:  (-0.074, 0.383)
T-Test of difference = 0 (vs ≠): T-Value = 1.47  P-Value = 0.167  DF = 12
```

FIGURE 17- Two Sample T-test of Absorbance at 260:280 of mixing types
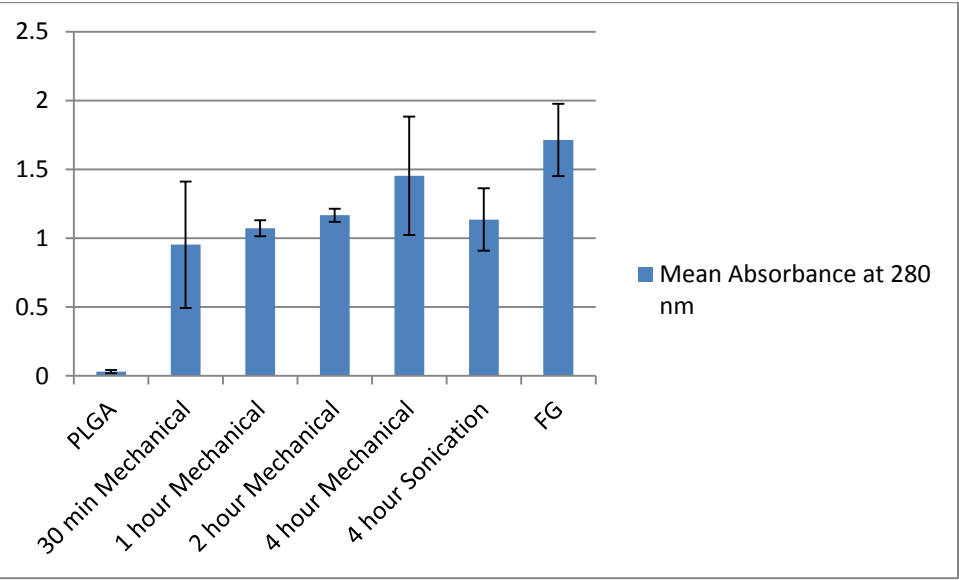
FIGURE 18—Mean absorbance measurements at 280 nm with standard deviation bars
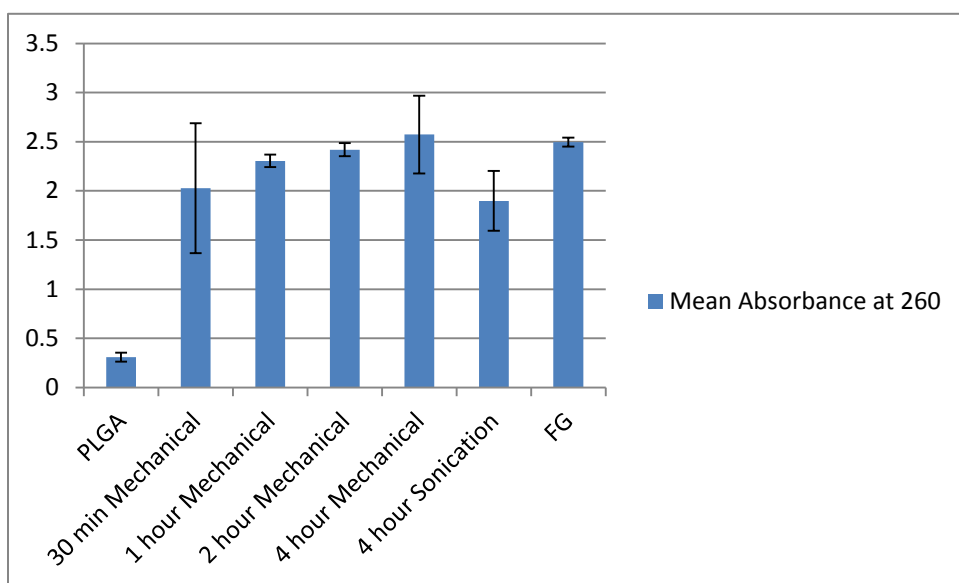
FIGURE 19—Mean absorbance measurements at 260 nm with standard deviation bars

5.4 Degradation Study Results and Analysis

5.4.1   Degradation Rate Results

Three replicates of each varying concentration were successfully weighed at 12 hours, 1 day, 2 days, 6 days, 10 days, and 14 days as well as at day zero. The percent weight remaining was calculated for each measured specimen based off of its initial weight. Full data can be found in Appendix 3. The measured results show that all samples lose at least 70% of their weight after twelve hours and all samples losing at least 90 percent of their weight by day 2. All samples showed a leveling off around 5% weight remaining at day 14. It is hypothesized that the initial loss of mass is mainly comprised of FG as FG is more readily soluble in water than PLGA; whereas, PLGA dissolves via hydrolysis. No

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findings statistically could be reported to show differences in degradation amounts between the varying mixture concentrations; however, **FIGURE 20** provides an illustration of the degradation of the different concentration mixtures of FG/PLGA. Limitations that could have influenced the results obtained include: only having the replicates per time point per concentration combination, not weighing the aspirate liquid to further measure percent loss per time point per specimen, and not measuring standard PLGA and FG only specimen degradation rates.
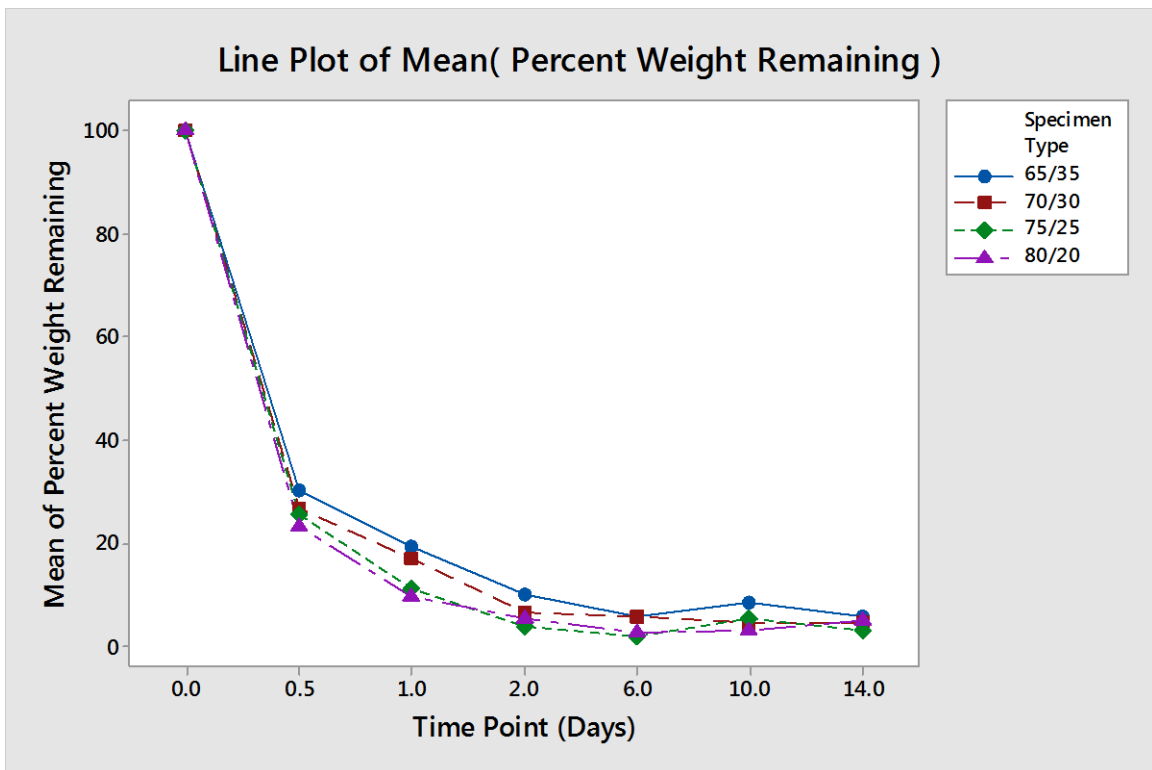
FIGURE 20—Percent Weight remaining by days of different concentrated mixtures.

5.4.2 Topographical Analysis

Due to the large number of specimen and the amount of time necessary to properly SEM image each specimen, the 65/35 concentration was chosen as the representative group to be imaged for this topographical analysis. Full SEM images from each time point can be found in Appendix 4. FIGURE 21 and FIGURE 22 show selected SEM images at time points 5 and 6, respectively. From these images and comparison with the earlier time points, it is potentially shown that a colloid-like mixture has likely been made of FG and PLGA where the PLGA appears as circular artifacts in the later time points as seen in time points 5 and 6.
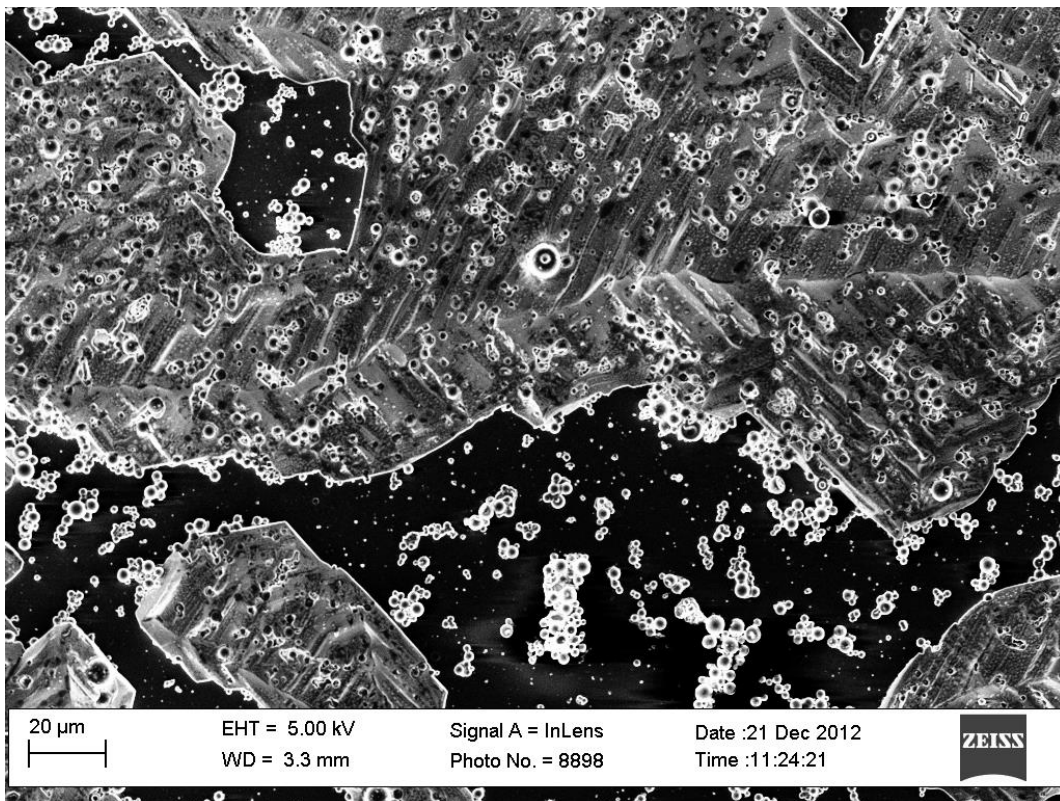
FIGURE 21—SEM image of 65/35 Time point 5
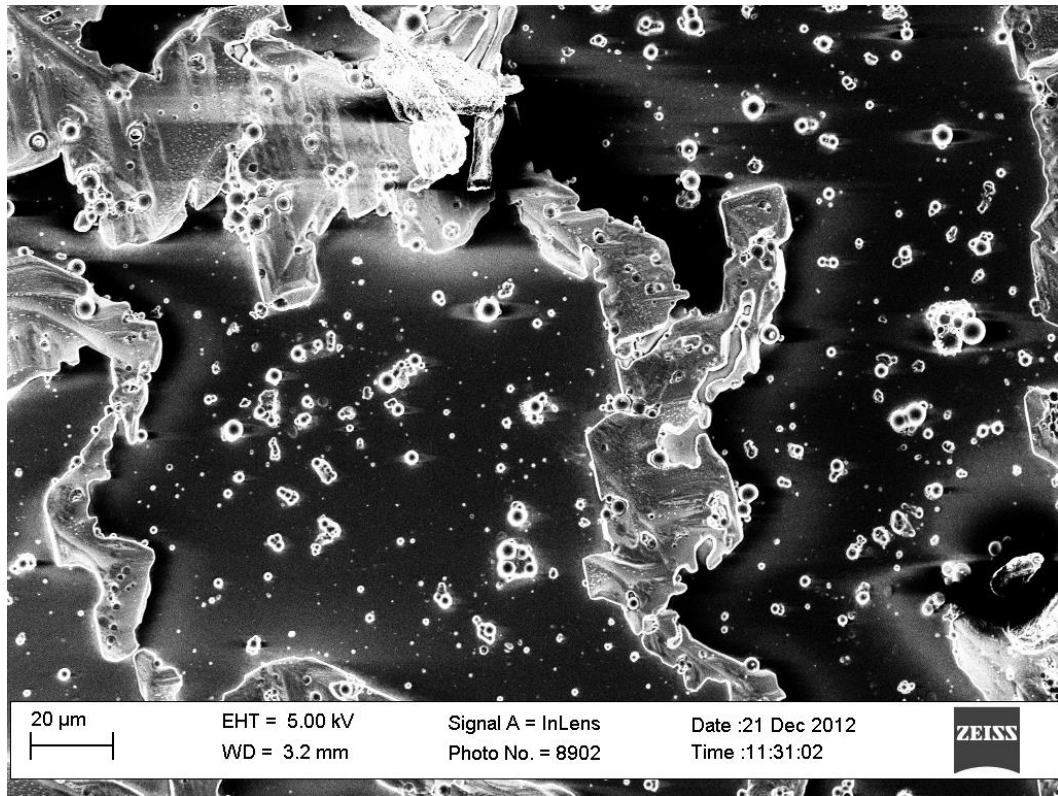
FIGURE 22—SEM image of 65/35 Time point 6

VI.   CONCLUSIONS

FG/PLGA polymer mixtures were shown to be mixed more thoroughly through mechanical mixing via an overhead stirrer versus sonication. Due to issues of heating, water ingress, and variability of mixing outcomes, as seen through absorbance measurements, with sonication, mechanical mixing was determined to be a superior choice for FG/PGLA mixing.   Further, degradation rates of varying relative concentrations of FG/PLGA were determined in a physiologic environment through treatment with PBS. Through this work, it has been proven that FG loses approximately 4% of its mass in the form of water when submitted to a heating process as mentioned previously in this document. Through GPC testing, it has also been shown that the molecular weight of FG is around 165,000 Da which is very similar to cold water fish gelatin. The mixing of fish glue appears to be more thorough and with less variation at 1 hour of mechanical mixing time as compared to 30 minutes, 2 hours and 4 hours. The degradation rate of FG/PLGA mixtures at varying concentrations was successfully measured showing a 70% drop within the first 12 hours of the exposure in a physiologically equivalent environment to the human body.

VII.   RECOMMENDATIONS

If further research is to be pursued with a FG/PLGA mixture as a biomaterial for fiber fabrication, more studies need to be performed in fiber creation, cell growth and proliferation, and fiber degradation. First, an optimal relative concentration of FG/PLGA needs to be established with regards to fiber creation. The output of fiber diameter and ease of creation needs to be measured for varying relative concentrations of FG and PLGA. Once this is established, the ability for cells to grow and proliferate needs to be measured, imaged, and documented. Finally, the degradation rate of the fiber needs to be measured as cells proliferate and grow around it, this test can also be done with or without attempting to provide fluid flow within the fabricated capillary's lumen.

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CURRRICULUM VITA

Born on January 2nd, 1989, in Edgewood, Kentucky to Philip Joseph and Mary Lynn Schworer, Adam Thomas Schworer has two older siblings: Andrew and Alex. During his undergraduate years, Adam spent multiple semesters on the Dean's list while serving as a Student Government Senator, performing Undergraduate Research under Dr. Robert Keynton, highlighted with part authorship credit on a manuscript published in the Journal of Biomaterials, and being active in his fraternity, Sigma Chi, most memorably as External Social Chair. Adam worked for three Co-op terms with Ethicon Endo Surgery Inc. a Johnson and Johnson Company in their Research and Development division. There, Adam gained experience on all stages of the New Product Development process for semi-disposable surgical instruments. Excelling in his first two co-op terms, Adam was appointed the Co-op Coordinator, by a committee of full-time employees, for his third term where he oversaw all Co-op related committees and activities. During his Graduate career, Adam studied the physiological processes of the body, cardiovascular dynamics, artificial organs, and the synthesis and characterization of biodegradable polymers. After completion of his Master's of Engineering in Bioengineering, Adam worked as an Associate Research and Development Engineer with Ethicon Inc a Johnson and Johnson Company in Blue Ash, Ohio for 18 months before returning to his Master's work. Adam looks forward to the prospect of a fruitful and fulfilling career in the development of technologies as he hopes to continues his education in law school.

APPENDICES

Appendix 1: Fish Glue Weight Analysis Raw Data

Specimen #	Treatment	Slide Weight(mg)	Wet Fish Glue on Slide Weight (mg)	Wet FG weight (mg)	Dry Fish Glue on Slide Weight (mg)	Dry FG weight (mg)	% Dry FG
1	Heated	41.08	70.15	29.07	57.67	16.59	57.07%
2	Heated	40.43	69.25	28.82	56.47	16.04	55.66%
3	Heated	40.96	68.47	27.51	56.86	15.9	57.80%
4	Heated	40.73	68.93	28.2	56.59	15.86	56.24%
5	Heated	40.73	71.17	30.44	58.25	17.52	57.56%
6	No Heat	41.01	66.27	25.26	54.36	13.35	52.85%
7	No Heat	40.62	68.08	27.46	54.93	14.31	52.11%
8	No Heat	41.83	69.73	27.9	56.59	14.76	52.90%
9	No Heat	40.84	63.86	23.02	52.99	12.15	52.78%
10	No Heat	41.18	68.75	27.57	55.75	14.57	52.85%

Appendix 2: Mixing Tests

Mixing Type	Vial Number	Replicate	Absorbance at 280	Absorbance at 260	280/260
Mechanical	1	1	1.702	2.849	0.597402597
Mechanical	1	2	1.292	2.413	0.535433071
Mechanical	1	3	1.122	2.225	0.504269663
Mechanical	2	1	0.931	2.083	0.446951512
Mechanical	2	2	1.123	2.33	0.481974249
Mechanical	2	3	1.056	2.225	0.474606742
Mechanical	3	1	1.984	3.144	0.631043257
Mechanical	3	2	1.987	2.893	0.68683028
Mechanical	3	3	1.879	3	0.626333333
Sonication	1	1	0.941	1.59	0.591823899
Sonication	1	2	0.915	1.54	0.594155844
Sonication	1	3	0.94	1.567	0.599872368
Sonication	2	1	1.211	1.998	0.606106106
Sonication	2	2	1.058	1.839	0.57531267
Sonication	2	3	1.11	2.243	0.494872938
Sonication	3	1	1.531	2.279	0.671785871
Sonication	3	2	1.476	2.236	0.660107335
Sonication	3	3	1.033	1.798	0.574527253
FG	1	1	1.57	2.478	0.633575464
FG	1	2	2.017	2.548	0.791601256
FG	1	3	1.554	2.462	0.631194151
PLGA	1	1	0.017	0.331	0.051359517
PLGA	1	2	0.035	0.256	0.13671875
PLGA	1	3	0.038	0.341	0.11143695
30 min	1	1	1.321	2.494	0.529671211
30 min	1	2	1.098	2.318	0.473684211
30 min	1	3	0.439	1.271	0.345397325
1 hr	1	1	1.131	2.373	0.476611884
1 hr	1	2	1.016	2.248	0.451957295
1 hr	1	3	1.067	2.293	0.465329263
2 hr	1	1	1.22	2.497	0.488586304
2 hr	1	2	1.139	2.381	0.478370433
2 hr	1	3	1.139	2.381	0.478370433

Appendix 3: Degradation Weight Analysis

Specimen Number	Specimen Type	Time Point (Days)	Replicate	Cover Glass Weight	Initial Fish Glue + Coverglass Weight	Final Fish Glue + Coverglass Weight	Initial Fish Glue Weight	Final Fish Glue Weight	Percent Weight Remaining
49	65/35	0.5	1	40.98	46.54	43.31	5.56	2.33	41.91
50	65/35	0.5	2	40.91	47.32	42.34	6.41	1.43	22.31
51	65/35	0.5	3	41.02	45.18	42.12	4.16	1.1	26.44
55	70/30	0.5	1	41.12	48.87	42.58	7.75	1.46	18.84
56	70/30	0.5	2	40.87	47.79	43.36	6.92	2.49	35.98
57	70/30	0.5	3	40.85	46.92	42.4	6.07	1.55	25.54
61	75/25	0.5	1	40.78	52.21	44.51	11.43	3.73	32.63
62	75/25	0.5	2	40.87	47.34	42.34	6.47	1.47	22.72
63	75/25	0.5	3	40.68	46.98	41.98	6.3	1.3	20.63
67	80/20	0.5	1	41.31	51.38	43.05	10.07	1.74	17.28
68	80/20	0.5	2	40.77	50.13	43.86	9.36	3.09	33.01
69	80/20	0.5	3	40.67	48.23	42.12	7.56	1.45	19.18
52	65/35	1	1	41.28	47.18	42.1	5.9	0.82	13.90
53	65/35	1	2	40.47	44.31	41.21	3.84	0.74	19.27
54	65/35	1	3	40.91	45.73	42.12	4.82	1.21	25.10
58	70/30	1	1	41.17	48.34	42.9	7.17	1.73	24.13
59	70/30	1	2	41.53	52.1	43.18	10.57	1.65	15.61
60	70/30	1	3	41.33	49.32	42.2	7.99	0.87	10.89
64	75/25	1	1	40.9	47.92	41.86	7.02	0.96	13.68
65	75/25	1	2	41.02	48.28	41.98	7.26	0.96	13.22
66	75/25	1	3	41.04	51.76	41.72	10.72	0.68	6.34
70	80/20	1	1	41.12	47.87	42.19	6.75	1.07	15.85
71	80/20	1	2	40.99	50.87	41.72	9.88	0.73	7.39
72	80/20	1	3	41.43	51.03	41.99	9.6	0.56	5.83
1	65/35	2	1	40.82	46.64	41.49	5.82	0.67	11.51
2	65/35	2	2	40.91	46.12	41.22	5.21	0.31	5.95
3	65/35	2	3	41.14	45.95	41.72	4.81	0.58	12.06
13	70/30	2	1	40.64	49.38	41.2	8.74	0.56	6.41
14	70/30	2	2	41.2	49.4	41.89	8.2	0.69	8.41
15	70/30	2	3	40.45	49.04	40.84	8.59	0.39	4.54
25	75/25	2	1	41.11	51.69	41.39	10.58	0.28	2.65

26 75/25	2	2	40.5	50.89	41.18	10.39	0.68	6.54
27 75/25	2	3	41.18	51.3	41.37	10.12	0.19	1.88
37 80/20	2	1	40.91	45.47	41.23	4.56	0.32	7.02
38 80/20	2	2	40.81	44.88	41.08	4.07	0.27	6.63
39 80/20	2	3	41.39	46.24	41.52	4.85	0.13	2.68
4 65/35	6	1	41.48	49.69	41.93	8.21	0.45	5.48
5 65/35	6	2	40.65	49.29	41.24	8.64	0.59	6.83
6 65/35	6	3	41.12	46.92	41.42	5.8	0.3	5.17
16 70/30	6	1	40.92	49.37	41.57	8.45	0.65	7.69
17 70/30	6	2	40.79	49.39	41.3	8.6	0.51	5.93
18 70/30	6	3	41.44	49.77	41.7	8.33	0.26	3.12
28 75/25	6	1	40.62	50.23	40.82	9.61	0.2	2.08
29 75/25	6	2	41.38	49.44	41.53	8.06	0.15	1.86
30 75/25	6	3	40.98	51.37	41.16	10.39	0.18	1.73
40 80/20	6	1	40.98	45.11	41.13	4.13	0.15	3.63
41 80/20	6	2	40.95	45.33	41.08	4.38	0.13	2.97
42 80/20	6	3	41	46.91	41.07	5.91	0.07	1.18
7 65/35	10	1	40.76	47.05	41.49	6.29	0.73	11.61
8 65/35	10	2	40.98	46.56	41.32	5.58	0.34	6.09
9 65/35	10	3	40.63	45.09	40.99	4.46	0.36	8.07
19 70/30	10	1	40.97	49.18	41.23	8.21	0.26	3.17
20 70/30	10	2	40.58	48.68	40.95	8.1	0.37	4.57
21 70/30	10	3	40.67	46.67	41.04	6	0.37	6.17
31 75/25	10	1	41.1	49.21	41.46	8.11	0.36	4.44
32 75/25	10	2	40.82	50.77	41.42	9.95	0.6	6.03
33 75/25	10	3	40.96	50.55	41.45	9.59	0.49	5.11
43 80/20	10	1	40.75	46.2	40.91	5.45	0.16	2.94
44 80/20	10	2	40.62	45.81	40.77	5.19	0.15	2.89
45 80/20	10	3	40.61	44.8	40.75	4.19	0.14	3.34
10 65/35	14	1	40.98	46.77	41.13	5.79	0.15	2.59
11 65/35	14	2	41.03	46.82	41.38	5.79	0.35	6.04
12 65/35	14	3	40.77	47.43	41.35	6.66	0.58	8.71
22 70/30	14	1	40.73	49.43	40.96	8.7	0.23	2.64
23 70/30	14	2	40.74	50.18	41.12	9.44	0.38	4.03
24 70/30	14	3	40.69	48.49	41.23	7.8	0.54	6.92
34 75/25	14	1	40.46	49.12	40.83	8.66	0.37	4.27
35 75/25	14	2	40.91	51.01	41.13	10.1	0.22	2.18
36 75/25	14	3	41.54	53.52	41.91	11.98	0.37	3.09

46	80/20	14	1	40.51	44.69	40.78	4.18	0.27	6.46
47	80/20	14	2	40.58	44.47	40.68	3.89	0.1	2.57
48	80/20	14	3	40.84	46.16	41.13	5.32	0.29	5.45

Appendix 4: SEM images of mixtures

6535_TP0_1

6535_TP0_2

3 μm	EHT = 5.00 kV	Signal A = InLens	Date :21 Dec 2012	ZEISS
	WD = 4.4 mm	Photo No. = 8871	Time :10:28:11	

6535_TP0_3

100 μm	EHT = 5.00 kV	Signal A = InLens	Date :21 Dec 2012	ZEISS
	WD = 4.4 mm	Photo No. = 8872	Time :10:29:31	

6535_TP0_4

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 4.4 mm | Photo No. = 8873 | Time :10:32:05 | |

6535_TP1_1

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.0 mm | Photo No. = 8874 | Time :10:35:07 | |

6535_TP1_2

| 100 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.0 mm | Photo No. = 8875 | Time :10:39:54 | |

6535_TP1_3

6535_TP1_4

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.0 mm | Photo No. = 8877 | Time :10:42:08 | |

6535_TP1_5

100 µm   EHT = 5.00 kV   Signal A = InLens   Date :21 Dec 2012   ZEISS
WD = 2.8 mm   Photo No. = 8878   Time :10:44:26

6535_TP1_6

6535_TP2_1

100 µm   EHT = 5.00 kV   Signal A = InLens   Date :21 Dec 2012   ZEISS
WD = 3.3 mm   Photo No. = 8880   Time :10:50:01

6535_TP2_2

| 20 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8881 | Time :10:51:51 | |

6535_TP2_3

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8882 | Time :10:53:12 | |

6535_TP2_4

| 2 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8883 | Time :10:56:00 | |

6535_TP2_5

| 1 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8884 | Time :10:59:46 | |

6535_TP3_1

100 μm   EHT = 5.00 kV   Signal A = InLens   Date :21 Dec 2012
   WD = 3.0 mm   Photo No. = 8885   Time :11:03:28   ZEISS

6535_TP3_2

| 20 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| WD = 3.1 mm | Photo No. = 8886 | Time :11:05:10 | |

6535_TP3_3

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.1 mm | Photo No. = 8887 | Time :11:06:17 | |

6535_TP3_4

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.1 mm | Photo No. = 8888 | Time :11:07:16 | |

6535_TP4_1

| 100 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.5 mm | Photo No. = 8889 | Time :11:10:46 | |

6535_TP4_2

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.6 mm | Photo No. = 8890 | Time :11:11:57 | |

6535_TP4_3

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.6 mm | Photo No. = 8891 | Time :11:13:12 | |

6535_TP4_4

| 2 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.6 mm | Photo No. = 8892 | Time :11:14:52 | |

6535_TP5_1

| 100 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8893 | Time :11:18:13 | |

6535_TP5_2

6535_TP5_3

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8895 | Time :11:20:36 | |

6535_TP5_4

2 μm   EHT = 5.00 kV   Signal A = InLens   Date :21 Dec 2012
        WD = 3.3 mm   Photo No. = 8896   Time :11:22:03

6535_TP5_5

6535_TP5_6

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8898 | Time :11:24:21 | |

6535_TP5_7

Scale bar: 100 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS
WD = 3.1 mm | Photo No. = 8899 | Time :11:25:38

6535_TP5_8

| 20 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8900 | Time :11:26:33 | |

6535_TP6_1

| 100 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.3 mm | Photo No. = 8901 | Time :11:29:55 | |

6535_TP6_2

6535_TP6_3

| 20 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.2 mm | Photo No. = 8903 | Time :11:31:17 | |

6535_TP6_4

| 100 µm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.2 mm | Photo No. = 8904 | Time :11:32:31 | |

6535_TP6_5

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.2 mm | Photo No. = 8905 | Time :11:33:39 | |

6535_TP6_6

| 2 μm | EHT = 5.00 kV | Signal A = InLens | Date :21 Dec 2012 | ZEISS |
| | WD = 3.2 mm | Photo No. = 8906 | Time :11:33:56 | |