IN SITU POLYMERIZING COLLAGEN FOR THE DEVELOPMENT OF
3D PRINTED TISSUE ENGINEERING SCAFFOLDS

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Master of Science

by
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JULY 2018
The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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presented by Christopher John Glover,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

To my parents, Stuart and Cindy Glover, for being the cornerstone of my success. Through all the late nights, you have always given me perspective.

Without you both, I simply would not have gotten this far.
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ABSTRACT

Natural materials have been processed and utilized as scaffold materials in the field of tissue engineering for many years. One natural material often utilized is collagen since it is the main structural protein in mammalian tissues and exhibits microstructures suitable for the survival and proliferation of many different cell lineages. However, a common challenge with fibrillized collagen is the difficulty associated with trying to process it into specific three-dimensional designs for the development of scaffolds aimed at regenerating particular tissue types. This project consists of utilizing a custom platform capable of 3D printing in situ polymerizing collagen into user-defined morphologies for the development of 3D collagen-based scaffolds. Various anti-inflammatory compounds such as gold nanoparticles and curcumin were also incorporated into the scaffolds post printing in order to further tailor the cellular responses to the scaffolds. Scanning electron microscopy and neutron activation analysis were performed to verify and quantify the attachment of the gold nanoparticles, respectively. Differential scanning calorimetry was utilized to examine and optimize the stability of the scaffolds after crosslinking. Lastly, water soluble tetrazolium salt and reactive oxygen species assays were performed to assess the biocompatibility of the scaffolds using L929 murine fibroblasts. The results exhibited the viability of the platform to become an effective technique to manufacture and process custom scaffolds for tissue engineering applications.
1. Literature Review

1.1 Introduction to 3D Printing and Bioprinting

3D printing is a manufacturing process in which a three-dimensional object is created one layer at a time. One layer of the object is one cross-sectional slice of its overall three-dimensional shape. Once a layer of the object is made, another layer is built upon that layer, and this process is repeated hundreds or even thousands of times until the object has been produced in its entirety. Chuck Hull, the father of 3D printing, was issued his first 3D printing patent in 1986 for the process he called stereolithography [1]. Since then, 3D printing has found its way into many different industries and has been used for a variety of applications including the construction of buildings, creating custom apparel, processing food, manufacturing firearms, developing airplanes, and advancing medicine. Virtually any physical object either already has or will soon be created via 3D printing techniques.

Stereolithography (SLA), which is one of now many methods of 3D printing, is the process by which a photopolymer is cured via ultraviolet light one layer upon another until a three-dimensional object is made. Other 3D printing methods include selective laser sintering (SLS), fused deposition modeling (FDM), polyjet printing, and inkjet-based printing. SLS utilizes an overhead laser above a bed of nylon powder to sinter the powder grains together to form a layer of the object. FDM heats a solid filament of polylactic acid or acrylonitrile butadiene styrene in an extrusion nozzle to deposit the molten material into the proper shape. Polyjet printing deposits acrylic photopolymer liquid resin into the
desired silhouette and binds it via ultraviolet light, similar to SLA. Finally, inkjet-based printing contains a bed of powder like gypsum and deposits a liquid binder from the print head to form a layer. These are just a few of the current 3D printing mechanisms as more variations of the technology are constantly being developed.

The drastic rise of 3D printing can be credited to its many advantages over traditional manufacturing processes. 3D printing is much more cost-effective than milling or cast/molding manufacturing, as it does not require nearly as many processing steps and does not require someone to handle the object as much. It is also much faster than traditional means for the same reasons and since 3D printers can run continuously until the object is made. Due to its superior speed, 3D printing is often referred to as “rapid prototyping” as it is a popular choice to create product prototypes. The object geometries achievable via 3D printing are also superior to traditional manufacturing, allowing for greater functionality and complexity of product. Lastly, it is much more feasible to create custom objects for clients with 3D printing, allowing companies to more accurately satisfy their clients’ needs particularly in clothing and medicinal applications. In summary, 3D printing allows for the rapid, cost effective production of more complex and custom products than those made by traditional manufacturing techniques.

As aforementioned, 3D printing can be applied to the medical field, in particular to create custom implants and biomaterial scaffolds. These scaffolds are simply materials that can be used to create new living tissues, the purpose of which being to replace injured or diseased tissues in the human body. Scaffolds
are either made or seeded with cells and incubated until the cells proliferate through the material, transforming it into a viable tissue. This tissue is then implanted into the site of the injured or diseased tissue. This subset of bioengineering that investigates the production of new, replacement tissues is aptly called “tissue engineering.” 3D printing that is used for tissue engineering applications is called “bioprinting” and the biological materials and/or cells that are being printed are therefore called “bioinks.” Various bioinks and bioprinting techniques will now be reviewed.

1.2 Bioinks

The most common bioink is a class of material called hydrogels. Hydrogels got their name from the amount of water they are able to house within their matrices; some hydrogels can store as much as 1000 times their dry weight in water without degrading [2]. Hydrogels are normally long polymer chains that are crosslinked, meaning they contain chemical bonds bridging their polymer chains together and creating complex three-dimensional structures. Like most materials, there exist natural and synthetic hydrogels, both of which are commonly used within bioengineering and gaining popularity as bioinks. Most natural hydrogels are harvested from animals, but a few are obtained from vegetation. Overall, the stigma within the field is that natural hydrogels can be safer and are more trustworthy since many of the compounds they degrade into are the same compounds found within the human body. Because of this, the body inherently possesses mechanisms to safely dispose of these molecules without causing harm to the cells. Synthetic materials are often scrutinized more
harshly because their degradation products are not necessarily found naturally within the body, which could pose a toxicity threat. Also, natural hydrogels often initiate more ideal cellular responses when implanted because they contain microstructures and chemical compounds similar those found in native human tissues. It takes more work to fine tune synthetic materials if one wants to induce those same responses seen from natural materials. For these reasons, most of the commonly used hydrogels are naturally-derived.

Naturally-derived hydrogels include chitosan, collagen type I, fibrin, gelatin, and hyaluronic acid. Chitosan is a linear polysaccharide and can be found in the rigid exoskeletal structures of various invertebrates like shrimp and crustaceans. It has thus far been used more as an agent for cellular encapsulation more than it has been used as the main backbone for 3D bioprinted scaffolds. This is because chitosan exhibits poor mechanical properties and can be difficult to print via different methods [3]. Collagen type 1 is the main structural protein found in mammalian tissues and is most often either derived from cow or pig tissues. As aforementioned, collagen is a natural hydrogel that often elicits favorable cellular responses and cellular attachment due to its abundance of ligands. These ligands bind to integrins, which are transmembrane receptors that aid in binding cellular membranes to surrounding tissue structures. The downside of collagen is its slow progression from a gelatinous state to a solid one, which makes it challenging to 3D print more complex structures. Fibrin is the fibrous protein product of the blood clotting cascade – the chemical chain reaction that begins with thrombin and fibrinogen
and ends with the formation of a clot. Fibrin itself is a soft compound that contains a matrix of filaments allowing for great mechanical strain without rupture [4]. Fibrinogen, fibrin’s precursor, is sensitive to shear forces but can be bioprinted at low concentrations via inkjet techniques [5]. Gelatin is a translucent derivative of collagen and is also derived from mammalian tissues. It is often utilized in both research settings and commercial industries for a wide array of applications. Like its predecessor, gelatin contains the arginine-glycine-aspartic acid (RGD) peptide and induces cellular adhesion as well as proliferation (cellular spreading), and differentiation (cells becoming other types of cells) [6]. Once crosslinked, gelatin can be bioprinted via extrusion methods. Hyaluronic acid is a glycosaminoglycan commonly found in the joints and connective tissues of mammals. Its gel-like composition makes it a natural lubricator which helps to minimize frictional forces in adjacent tissues. Similar to collagen, hyaluronic acid is slow to solidify and can also have poor mechanical properties [7]. Due to this, it is often mixed with other hydrogels in extrusion bioprinting to improve cellular responses and is not normally printed alone.

While there are not many synthetic hydrogels involved in bioprinting, the most common one is poly(ethylene glycol), or PEG. PEG is very commonly used in bioengineering as it is a biodegradable and biocompatible polymer that has been FDA approved for human implantation – which not many polymers have. PEG is a polyether compound that can be conjugated (or attached) with biomolecules like proteins and liposomes [8,9]. Like all hydrogels, it is hydrophilic meaning it likes water and readily absorbs it. Hydrophilicity is a very
important material characteristic for implantable scaffold materials as most of the human body's composition is water. Since it is not naturally derived, PEG lacks the ligands and bioactive molecules commonly found in collagen and gelatin that facilitate cellular adhesion and proliferation. To accommodate for this, PEG can be coated in fibronectin to improve these cellular responses [10]. The advantage of synthetic polymers is that their mechanical properties can be tuned by various chemical means such as additional crosslinking or incorporating other compounds. PEG is no exception to this and is often mechanically strengthened by the addition of diacrylate or methacrylate [11]. PEG can then be bioprinted by extrusion techniques or laser-based techniques.

The non-hydrogel class of bioinks includes decellularized matrix components and microcarriers. Most of living tissues is not made up of cells, but rather various proteins and biomolecules called the “extracellular matrix” or ECM. The ECM of animal tissues often mimics those of human tissues and can be used as the backbone of scaffolds. ECM is first harvested and decellularized – a process that removes all living animal cells from the ECM, leaving only that biomolecular backbone that is also found in humans. Once decellularized, the ECM can then be processed and digested to produce a gel-like substance that is able to be bioprinted. Similar to some hydrogels, ECM struggles to maintain its shape after printing and therefore is often extrusion printed with other materials like polycaprolactone. Microcarriers are microporous spherical structures made of natural or synthetic materials that help elicit favorable cellular responses and are normally incorporated into other materials for bioprinting [12]. Some of these
natural materials include gelatin and collagen, and the synthetic materials include glass and various plastics [13]. Microcarriers aid in cell adhesion, proliferation, and differentiation in part by providing large amounts of surface area upon which the cells thrive. Normally these microcarriers are mixed with hydrogels and extrusion printed.

Lastly, there is even a “scaffold-free” class of bioinks that only consist of cells and biological materials. These include cell pellets, tissue spheroids, and tissue strands. All three of these structures are very similar in composition and all three are primarily bioprinted via extrusion methods [14,15,16]. Cell pellets are simply agglomerations of cells that form when a cellular solution is centrifuged, since the cells separate and accumulate at the end of the centrifuge tubes. Tissue spheroids are similar in shape to cell pellets but also contain bits of ECM the cells manufacture when they are contained within some sort of housing like a hydrogel mold [17]. Tissue strands are an expansion off of tissue spheroids as they contain newly formed tissue only in a different shape. To produce tissue strands, cells are housed in cylindrical alginate tubes which are later chemically dissolved, leaving only the cells and their thin tissue fibers [18]. All three of these “scaffold-free” bioinks lack a strong backbone since they are primarily made of cells and small tissue structures. Nevertheless, they can aid in bioprinting other materials since the presence of cells helps to prepare the scaffold materials for implantation.
1.3 Bioprinting Techniques

There exist three main types of cellular bioprinting techniques: droplet based bioprinting, extrusion based bioprinting, and stereolithography based bioprinting. Each method varies slightly and has its own set of advantages and disadvantages when compared to the others. Each technique will now be reviewed.

The first type of bioprinting is droplet based bioprinting. The premise of droplet based bioprinting is that a cellular suspension is printed onto a pre-existing material substrate. The bioink may include hydrogels but the overall shape of the product is predetermined. There are four subsets of droplet based bioprinting: inkjet bioprinting, electrohydrodynamic jetting, pneumatic pressure assisted printing, and laser assisted bioprinting [19]. Inkjet bioprinters can often be made by adapting old inkjet printers, where the former ink cartridge now contains the bioink that is to be printed. This makes inkjet bioprinters one of the most readily available models and one of the cheapest. Inkjet bioprinters typically are able to produce patterns with high repeatability and low resolution (~50 um) [19]. Cell viability, or the number of cells in the bioink that survive the printing process, can be as high as 80% [20]. However, inkjet bioprinters normally are only suited for bioinks of low viscosities and low cell concentrations. The second subset is electrohydrodynamic jetting, which is very similar to a popular material synthesis method called electrospinning. A high voltage source is attached to the needle of the syringe that houses the bioink, and a syringe pump extrudes the bioink out of the needle onto a grounded surface.
size is controlled by the needle gauge, where the droplet diameter is typically about twice the size of the needle diameter [19]. Electrohydrodynamic jetting is capable of producing cell droplets on the microscale while still maintaining high cell viability.

The third subset of droplet based bioprinting is called pneumatic pressure assisted printing and involves the utilization of microelectromechanical valves that deposit the bioink. The droplet sizes are controlled both by the amount of pressure behind each valve and the length of time the valve is opened. Due to the usage of pneumatic pressure, bioinks of higher viscosities can be printed successfully but concerns about cell viability have been raised [19]. The bioinks that often accompany this technique usually require crosslinking post printing, which is another processing step to take into consideration. The fourth and final subset of droplet based bioprinting is laser assisted bioprinting. The three main components of this technique are a pulsing laser, a donor layer consisting of an energy absorbing material and the bioink, and the substrate that collects the droplets [19]. The laser pulses onto the absorbing material, typically a metal, and this creates high pressure bubbles that deposit bioink droplets onto the substrate [19]. Advantages of this technique include the ability to print highly viscous bioinks with large cell densities at high resolutions. Disadvantages include the high cost of the parts involved and the limited bioinks that are compatible with laser assisted bioprinting.

The second type of bioprinting is extrusion based bioprinting. In this technique, the bioink is housed within a cartridge in a dispensing system that
extrudes the bioink through a nozzle into cylindrical fibers. The position of these fibers is precisely controlled via an automation system that moves the extrusion nozzle, print bed, or both. The extrusion from the nozzle can be controlled either by pneumatic valves, mechanical piston force, or a solenoid (electromagnet) mechanism [21]. Because of the variety of extrusion methods, bioinks of different viscosities can be extruded depending on which method is being employed. Multiple cartridges can be utilized in customizable sequences to deposit a variety of bioinks at various points throughout the three-dimensional scaffold product. Extrusion based bioprinting is typically faster than droplet-based printing and can produce more geometrically complex scaffolds. It also is capable of printing a wider variety of bioinks. However, extrusion based bioprinting can suffer from lower resolutions and greater shear stresses.

The third type of bioprinting is stereolithography based bioprinting. Just like traditional stereolithography, a vat of light sensitive bioink lies under a light source that selectively illuminates precise points in the ink. Once exposed, those sections of bioink slowly harden while more liquid bioink is deposited on top of the hardening layer. This process is then repeated until a hardened three-dimensional scaffold is formed within the vat. The excess liquid bioink is then removed and can be reused in later builds. The light source is usually a laser that scans the selected areas of the bioink to be hardened, but a mask can be used that exposes an entire layer at once. The mask technique can be even faster than the laser since whole layers are cured simultaneously. Stereolithography based bioprinting has exhibited excellent resolutions as low as
5-10 um [22,23]. Overall it can produce more complex geometries than other methods, but the selection of photo-curable bioinks can be limited. However, the photo curing can diminish cell viability which is normally 40-80% [24,25]. Similar to the aforementioned laser assisted bioprinting, high costs can also be a downfall of stereolithography based bioprinting.
1.4 References


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2. Introduction to Research

2.1 Significance of Research

The goal of this research was to develop a 3D printing platform able to produce custom collagen-based scaffolds for tissue engineering applications. While no specific application was chosen for this project, the platform could potentially be utilized to construct replacement tissue for nearly any diseased or damaged tissue in the human body. Since collagen is the most abundant structural protein in mammalian tissues, it is an excellent choice to be utilized as the primary component of such versatile scaffolds. Nearly all tissues in the human body contain collagen including skin, muscle, nerves, vasculature, tendons, ligaments, and even bone. Skin, for example, is 80% collagen by mass [1]. Due to this abundance, collagen is extremely biocompatible and versatile. With the proper mechanical and chemical stimuli, stem cells seeded on collagen scaffolds have the potential to differentiate down a myriad of cell lineages and become nearly any tissue in the human body.

2.1.1 In Situ Polymerizing Collagen

The collagen utilized in this project was called in situ polymerizing collagen (IPC), which is a unique collagen bioink developed from Type I porcine collagen [1]. Via a process consisting of reverse dialysis, the porcine collagen is dissolved and resuspended, yielding a clear, viscous liquid solution. Ethylenediaminetetraacetic acid (EDTA) is incorporated into the collagen solution via dialysis in order to prevent spontaneous polymerization, maintaining a pre-fibrillized, suspended state. Once the EDTA molecules are displaced, either by
heat or dilution or physical agitation, the collagen monomers polymerize, forming banded collagen fibrils. This polymerization process results in a solid, opaque product. The IPC undergoes fibrillization when deposited into a bath of ddH₂O, making an ideal bioink. The water molecules displace the EDTA and induce polymerization of the collagen. This polymerization is typically complete within one minute, after which more IPC could be printed on top of what had polymerized. Depending on the print sequence and geometry, the next layer of IPC could even be successfully printed atop the previous collagen before it had polymerized, but the occurrence of this phenomenon was minimized as to avoid the disruption of pre-polymerized collagen.

2.1.2 Anti-Inflammatory Agents

Besides printing the collagen, this project also investigated chemically altering the scaffolds to enhance their durability, biocompatibility, and immunomodulatory abilities. For example, anti-inflammatory agents could be incorporated into the structure of the scaffolds to tailor and enhance the cellular responses. Since any surgical implantation of engineered tissue will cause some degree of inflammation, regardless of the location or type of tissue, anti-inflammatory agents are a judicious choice to incorporate into the scaffolds. There exists a wide array of anti-inflammatory agents to choose from, including both synthetically and naturally derived agents. We chose to investigate gold nanoparticles (AuNP) and curcumin.

Gold nanoparticles have gained significant interest within biomedical research due to their unique sizes and properties as well as their ease of
synthesis. Biomedical applications of gold nanoparticles include biosensors, gene therapy, tumor detection, and drug delivery systems [2-5]. One of the properties that makes AuNP a candidate for biomedical research is their anti-inflammatory effects on cells. For example, gold nanoparticles are being investigated to treat rheumatoid arthritis and chronic inflammation [6]. However, AuNP can also exhibit cytotoxic effects at various sizes [7]. It is therefore critical to continue experimentation with AuNP to ensure their safety and efficacy. The gold nanoparticles utilized in this study were synthetically derived and were purchased in a colloid form. The AuNP were 20 nm in size, and this size was chosen from prior research performed in the lab.

Curcumin is a natural plant-derived compound used for a variety of purposes including food preparation and medicine (Figure 2.1). One of the most common sources of curcumin is the root of turmeric, which has been utilized for medicinal purposes in Asia for nearly 4,000 years [8]. Turmeric has also made a significant impact on modern medicine as it has been cited in over 3,000 publications over the last 25 years [8]. In medicine, turmeric has been used to treat a myriad of conditions including rheumatoid arthritis, skin cancer, chicken pox, urinary tract infections, and conjunctivitis [9]. Most commonly it is ingested as an herbal remedy to combat digestive disorders. It is so multifaceted because of its anti-inflammatory, choleretic, carminative, anti-carcinogenic, and antimicrobial properties [10]. The turmeric root can be ground into a powder from which the curcumin is extracted. The curcumin used in this project was purchased as a powder and later dissolved. Curcumin and gold nanoparticles are
again just two anti-inflammatory agents of thousands of bioactive additives that could be incorporated into the scaffolds’ structures.

![Curcumin molecular structure]

**Figure 2.1** Curcumin is classified as a diarylheptanoid as it contains two aromatic rings bound by a seven-carbon chain.

2.2 Research Objectives

The purpose of this project was to develop and characterize 3D printed collagen-based scaffolds with and without crosslinked gold nanoparticles and curcumin. Experimental groups differ in their treatment post printing. Some samples were left uncrosslinked (just printed), some were crosslinked without AuNP or curcumin, and others were crosslinked with either 1X or 2X AuNP or curcumin. Characterization included examining the stabilities of the crosslinked scaffolds as well as verifying and quantifying the amount of AuNP and curcumin conjugated to the scaffolds. *In vitro* cellular assays determined the biocompatibility of the scaffolds and the anti-oxidant power of the anti-inflammatory agents. Between the characterization techniques and the cell assays, conclusions could then be drawn as to the viability of the 3D printed scaffolds and in particular, which scaffold treatment was optimal. These tasks were broken up into three distinct objectives, including:
Objective 1: To 3D print uniform and reproducible collagen-based scaffolds.

This objective will be reached by the experimentation and manipulation of different 3D printing parameters.

Objective 2: To examine the thermal properties of the crosslinked scaffolds.

Differential scanning calorimetry will be utilized to determine the denaturation temperatures of the crosslinked scaffolds and optimize the amount of crosslinker used.

Objective 3: To verify and quantify the presence of gold nanoparticles in the crosslinked scaffolds.

Scanning electron microscopy will verify the presence of AuNP while neutron activation analysis will quantify the AuNP.

Objective 4: To evaluate the cytotoxicity and anti-inflammatory capabilities of the gold nanoparticle and curcumin scaffolds.

Water soluble tetrazolium salt assays will examine the cytotoxicity and general biocompatibility of the scaffolds. Reactive oxygen species assays will determine the anti-inflammatory effects of the AuNP and curcumin scaffolds.
2.3 Research Design

The experiments performed to complete this project utilized six experimental groups, including uncrosslinked, crosslinked, AuNP, curcumin, 2X AuNP, and 2X curcumin. Each group of samples was first 3D printed and let polymerize overnight to ensure completion. All groups except the uncrosslinked group were then crosslinked and washed to remove any biproducts. Each group was then either utilized for characterization or sterilized in preparation for use in a cell study. Once sterilized, the groups could safely be used for the cell studies and any adverse effects accredited to the materials, not bacterial contamination. The uncrosslinked group exists to examine the effects of crosslinking alone; the AuNP and curcumin groups exist to determine the effects of each bioactive agent; the 2X AuNP and 2X curcumin groups exist to exacerbate those effects, for better or for worse.
2.4 References


3. Scaffold Production

3.1 3D Printing Process

3.1.1 Printer and Bioink

The 3D printer used for this project was a custom printer assembled from a computer numerical control (CNC) milling machine (Figure 3.1). The translational stages are controlled by 3 separate stepper motors accurate to movements of 0.1 microns or less. Each stepper motor controls movement in one spatial dimension: the X motor controls movement left and right, the Y motor controls movements to the front and back, and the Z motor controls movements up and down. The X and Y stages intersect each other to form the print bed, while the Z stage resides above them separately. A small vice centered on the

Figure 3.1 The 3D printer features a 3D printed holster to house the syringe pump and is seen here printing a circular grid pattern.
print bed was able to house a small petri dish, in which the *in situ* polymerizing collagen (IPC) was deposited. When a larger print was desired, an adapter was housed in the vice and a larger petri dish was set upon the adapter. In order to extrude the collagen, a syringe pump was mounted to the Z stage residing above the print bed. Room temperature double distilled water (ddH$_2$O) was put in the petri dish before starting each print. Printing the IPC into ddH$_2$O allowed the collagen to polymerize and solidify, yielding a product that could be handled and manipulated within reason.

The three stepper motors were connected to a control box which was itself connected to a computer. The programming fed to the computer’s software determined the sequence and magnitude of the stepper motors’ movements, which in turn determined the size and geometry of the printed product. This software was called Mach3 Mill (Figure 3.2). CNC milling machines and even

![Figure 3.2](image-url)
modern 3D printers are all controlled by G Code. Each line of G Code dictates one movement of the stepper motors, and it was G Code that was inputted to Mach3 Mill. An example of this G Code can be found in the white text box in the top left corner of the Mach3 Mill interface. The top center section of the interface outputs the real time coordinates of all three stepper motors in millimeters from the “zero.” The “zero” was also set using those buttons and is an arbitrary starting point that is used as a reference location by the motors during the print. The top right section of the Mach3 Mill interface depicts a print preview derived from the inputted G Code.

While there exist many G Code commands, the code needed for our prints was comprised of only five commands (Figure 3.3). “F” is the feed rate command, which established the speed of the stepper motors’ movements in the units cm/min. “G0” creates a rapid linear motion to the specified point

| G Code 8-8-16.3 - Notepad
| File Edit Format View Help |
|; Chris Glover 8-8-16 Version 3 |
|; G Code to create a Circular Grid |
|; 3 Layers, 10 mm radius |
| F 30.0000 |
| g0x 0.0000 |
| g0y 0.0000 |
| g0z 0.0000 |
| g1x 10.0000 |
| ; Layer 1 |
| g3x 14.841229 y 1.250000 r 10.0000 |
| g1x 5.158771 |
| g2x 3.385622 y 2.500000 r 10.0000 |
| g1x 16.61437 |
| g3x 17.80624 y 3.750000 r 10.0000 |
| g1x 2.193752 |
| g2x 1.339746 y 5.000000 r 10.0000 |
| g1x 18.66025 |
| g3x 19.27024 y 6.250000 r 10.0000 |
| g1x 0.729752 |
| g2x 0.317542 y 7.500000 r 10.0000 |
| g1x 19.68245 |

Figure 3.3 G Code could be easily written in Microsoft Notepad software and loaded into the Mach3 Mill software once ready for printing.
following the syntax “G0.” “G1” causes a linear motion just like “G0” but at the speed denoted by the feed rate. “G2” instigates a clockwise arc to the specified point using the specified radius following the command. “G3” also makes an arc following the same syntax except in a counter-clockwise direction. Finally, a semi-colon could be used to comment the code. Using these simple commands, any imaginable geometry could be printed, within the limitations of the collagen bioink.

3.1.2 Printing Parameters and Procedure

In order to fine-tune and perfect the printing process, several parameters were tracked and optimized. The first parameter was the pump rate, or the volumetric rate of extrusion of the IPC from the syringe into the ddH$_2$O bath. The greater this value, the more collagen deposited per feature and the larger the feature size. The next parameter was the Z height, or printing distance, defined by the distance from the needle tip to the surface of the petri dish. This parameter was found to be the most crucial parameter to control. Even a deviation of 0.1 mm could mean success or failure for the print. Another parameter was the needle gage. Plastic needles were utilized because metallic needles were found to occasionally contribute to premature polymerization of the IPC before deposition in the petri dish, causing clogs in the needle and disrupting the print. The larger the needle gage, the smaller the feature size created – as long as the feed rate was also increased, and the Z height decreased to compensate for the tear drop formation at the needle tip. Another important parameter was the feed rate, or the speed at which the stepper motors translated
The faster the feed rate, the smaller the feature sizes as each unit of collagen was stretched over a greater distance. The ideal values, or ranges of values, for these parameters were found from months of trial and error printing (Table 3.1). Other parameters that are important to track but do not have set values include the syringe size and style, the batch number of IPC, the solution in the petri dish (see next section for more details), and of course the G Code inputted.

**Table 3.1** After much trial and error, several ideal values and ranges were discovered for some of the printing parameters.

<table>
<thead>
<tr>
<th>Printing Parameter</th>
<th>Ideal Value/Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump Rate</td>
<td>0.5 mL / hr</td>
</tr>
<tr>
<td>Plastic Needle</td>
<td>20 Gage</td>
</tr>
<tr>
<td>Z Height</td>
<td>0.4 mm – 0.7 mm</td>
</tr>
<tr>
<td>Feed Rate</td>
<td>30 – 70 cm / min</td>
</tr>
</tbody>
</table>

The protocol for printing a collagen scaffold is straightforward. First, the control box and syringe pump are turned on and Mach3 Mill is opened on the computer. The appropriate pump rate is set on the syringe pump and the diameter is set to match the inner diameter of the syringe to be loaded. The G Code, which is written ahead of time, is loaded into the Mach3 Mill text window. The IPC is removed from the fridge and is carefully ejected into the desired syringe. A second syringe with needle is then used to remove any air bubbles within the volume of IPC in the loaded syringe. Once the bubbles have been minimized, the proper plastic needle is attached to the loaded syringe and the plunger is pushed until the collagen reaches the end of the needle. The IPC syringe with attached needle is then set in the syringe pump and fastened into place. A small petri dish is set in the vice on the print bed, or a large petri dish is
set in the adapter which is set in the vice.

Using the manual axis controls in Mach3 Mill, the needle tip is lowered onto the dish until the needle becomes bent. The minute manual adjustments are then opened in Mach3 Mill by pressing Tab, and these used to raise the needle until it is straight but still touching the petri dish. The needle is then raised to the appropriate Z height, or printing distance, and the Z axis is zeroed. The X and Y axes are then adjusted until the needle is at the appropriate starting point according to the G Code, at which point the X and Y axes are also zeroed. Double distilled water is then added to the petri dish, and both the syringe pump and G Code are initiated simultaneously. If the syringe pump senses high pressure, an alarm will sound, and the operator will need to manually resume the pump to continue the print by pressing the Start button twice. Once the G Code finishes, the syringe pump is

**Figure 3.4** A.) A circular grid pattern was printed with a 20 mm diameter, fibers 0.7 mm thick, and pores 0.67 mm wide. Each layer was 0.5 mm thick and 9 layers were printed. B.) A side view of the circular grid shows it is nearly half as tall as the petri dish is deep. C.) A series of 6 circles was printed, each 4 mm in diameter and 3 layers tall, to fit perfectly in the wells of a 96-well plate.
paused, and the print is complete. The collagen scaffold is left in water to finish polymerization and to prevent evaporation of the sample. Everything except loading the IPC syringe is then repeated for the next print.

The two most commonly printed shapes were a circular grid pattern used for optimizing the system and a series of circles used for cell assays (Figure 3.4). The most layers printed consecutively was twenty, past which point the print’s height would exceed the depth of the petri dish. Given a deeper water bath, more layers could be printed consecutively. The only limits to how wide or long a product could be printed were the dimensions of the dish. The best resolution ever obtained was fibers approximately 0.35 mm thick, which is estimated to be the best this system could achieve. This was obtained by increasing the feed rate to 75 cm/min and decreasing the Z height to 0.3 mm, but the fiber quality was inconsistent. Given a different system, finer resolution could be achieved.

3.1.3 Failed Printing Techniques

Numerous printing attempts were attempted, and some resulted in failure. In this section, some of the failed printing techniques are provided in detail. For example, the syringe pump originally was located on the side of the printer and tubing ran the collagen to the print bed. However, this setup failed because the IPC was viscous enough to cause the air in the tube to compress. This compression not only delayed the start of the print but also created inconsistent pressure behind the collagen during the print. This inconsistency ultimately yielded a product with varied collagen fiber diameters that was nonhomogeneous and not reproducible. Once this conclusion was reached, a holster for the syringe
pump was 3D printed and mounted to the Z axis stage. This change eradicated the need for tubing and the presence of air in the system.

Another method was to print ddH₂O into collagen instead of collagen into ddH₂O. If this method was successful, more complex geometries such as embedded features would have been possible as the unpolymerized collagen would have supported the weight of any polymerized collagen printed above it. This ultimately failed because in order to induce polymerization, there needed to be an ample volume of water in the dish to displace the EDTA molecules. Printing thin lines of water did not supply the IPC with enough dilution to wash away the EDTA and produce polymerized fibers. Utilizing greater amounts of water in this style of printing, the resolution would have been significantly worse than printing IPC into water and would not have been effective print method.

Since IPC can also be polymerized via heat, we also experimented with methods involving either a heated ddH₂O bath or a heated probe. It was hypothesized that printing into heated water would increase the rate of polymerization, allowing for printing consecutive layers atop the first sooner. We heated the water to as high as 50 °C and observed no significant increase in polymerization rate. A heated metal needle was also utilized as a heat probe to induce polymerization instead of ddH₂O. A needle was heated in a Bunsen burner flame until turning red hot and was then immersed into IPC. However, the needle almost immediately cooled off upon immersion and no polymerized fibers were formed.
Other aqueous solutions were also used in the petri dish to optimize the polymerization of IPC. Printing into PBS was hypothesized to enhance the rate of polymerization as the salt molecules could help displace the EDTA. However, no difference in polymerization rate was observed; the only difference was the polymerized collagen fibers seemed thicker after soaking in the PBS overnight compared to soaking in ddH$_2$O. The thickening effect decreased the resolution of the printed product and thus was not utilized. Additionally, printing directly into a crosslinking solution was also attempted, and these results are detailed in the Differential Scanning Calorimetry data of Chapter 5.

Lastly, a few different techniques were attempted to incorporate curcumin into the IPC. First, 10 mg of curcumin was manually mixed into about 1.4 mL of IPC before loading the IPC into the printing syringe in order to print IPC and curcumin simultaneously. After loading the mixture into the syringe, it was then centrifuged to remove any small air bubbles. While this yielded a very homogeneous bioink, the air bubbles were extremely difficult to remove and there was usually a couple of very small air bubbles in the syringe that caused the print to ultimately fail. Another technique that was attempted was to dissolve the 10 mg of curcumin in 100 µL of 1 N NaOH and mix that with the IPC prior to printing. However, the presence of the NaOH caused the IPC to polymerize and rapidly harden during mixing and thus was no longer printable. The technique that ultimately worked was to crosslink the curcumin to the collagen post printing, as is described in the next section.
3.2 Crosslinking Procedures

All of the 3D printed samples underwent crosslinking except the aptly named uncrosslinked samples. The purpose of crosslinking is to increase the durability and stability of the scaffolds as well as to allow the conjugation of bioactive agents like AuNP or curcumin. While there exist a variety of crosslinkers, two different ones were examined in this project. The first was 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, or EDC, which forms a peptide bond between carboxyl and amino groups (Figure 3.5) [1]. EDC is a zero length crosslinker, meaning the actual EDC molecule is not a part of the final crosslinked product. Unfortunately, the EDC crosslinking reaction can create caustic byproducts such as isourea, which is cytotoxic. For this reason, three washes of the samples are required post crosslinking to ensure those byproducts

![Figure 3.5](image.png)

**Figure 3.5** The EDC crosslinking mechanism forms an unstable intermediate that is stabilized by the presence of sulfo-NHS.
are thoroughly removed. One wash was one session of the samples sitting in their petri dishes in PBS on the orbital shaker at 75 RPM for about 12 hours (or overnight). EDC was only used for the first step of the project, which is why it was never used to conjugate curcumin to the scaffolds.

In order to achieve an efficient crosslinking procedure, EDC was eventually replaced by genipin as the ideal crosslinker. Genipin is a natural crosslinker that is extracted from the gardenia fruit. It does not create caustic byproducts like EDC and therefore only necessitates one wash post crosslinking instead of three. It also is a known anti-inflammatory compound, which is another reason it was chosen to replace EDC [2]. Genipin was used to conjugate both AuNP and curcumin to the scaffolds (Figure 3.6) [3]. Before beginning either crosslinking procedure, any superfluous printed structures were carefully separated from the bulk with a scalpel and removed. The water in the petri dishes was also removed so that the samples would be ready to be exposed to the crosslinking solution as soon as it was ready.

![Diagram of crosslinking process](image.png)

**Figure 3.6** The genipin crosslinker presumably reacts with amine moieties on the collagen fibers to form intramolecular bonds.
3.2.1 EDC Crosslinking Procedure

To make the EDC crosslinking solution, three different initial solutions were made first and were then mixed together. The first initial solution was 9.5 mL of acetone mixed with 9.5 mL of phosphate buffered saline (PBS). This acted as a solvent solution to adjust the volume for the proper molarity. The second initial solution was 11.52 mg of N-hydroxysulfosuccinimide, or sulfo-NHS, dissolved in 0.5 mL of dimethyl formamide (DMF). The third initial solution was 7.66 mg of EDC dissolved in 0.5 mL of 2-(N-morpholino)ethanesulfonic acid (MES). Both the sulfo-NHS and EDC solutions were kept on ice until the crosslinking solution was made to maintain the inactivity of the compounds.

If AuNP were to be included in the crosslinking process, then 25.2 mg of cysteamine was dissolved in 20 mL of ddH₂O. This was also kept on ice. The purpose of the cysteamine was to functionalize the AuNP by attaching an amino group to the nanoparticles. This would allow them to be covalently bound to the carboxyl groups of the collagen via peptide bonds formed by the EDC. Then 0.1 mL of 20 nm AuNP colloid was aliquoted into a microcentrifuge tube per AuNP sample to be crosslinked, plus one extra 0.1 mL of colloid. For example, if 4 samples were to be crosslinked with AuNP, then 0.5 mL AuNP colloid would be aliquoted into a microcentrifuge tube. If 2X AuNP was desired, then twice the needed volume would be aliquoted, spun down at 12,500 RPM for 7.5 minutes, and half its volume removed.

Once all the above solutions were prepared, crosslinking could begin. The three initial solutions were then combined to make a total 20 mL of 2 mM EDC.
solution. Each sample was then given 2.5 mL of the EDC solution except the AuNP samples which were instead given 2.3 mL of solution. This volume was just enough to cover the 3D printed collagen if the small petri dishes were used and if no more than 3 layers were printed. For the AuNP or 2X AuNP samples, the AuNP colloids in the microcentrifuge aliquots would receive an equal volume of cysteamine solution, vortexed for about 30 seconds, and 0.2 mL of that vortexed solution was added to each designated AuNP sample dish. This yielded a total of 2.5 mL of total crosslinking solution for each sample dish. In retrospect, the 2X AuNP aliquot should have received twice the amount of cysteamine solution, but this was corrected in the genipin protocol. Although the EDC crosslinking mechanism is complete within about 15 minutes, the samples were left overnight to finish crosslinking. It was found that setting the AuNP samples on an orbital shaker at 75 RPM improved the homogeneity of the AuNP spread. The next day, the solution was replaced with about 4 mL of PBS and let sit for about 12 hours (overnight) for the first wash. This was repeated twice for the second and third washes. After the third wash, the samples were ready for either characterization or sterilization (if they were intended for use in a cell assay).

3.2.2 Genipin Crosslinking Procedure

To make the genipin crosslinking solution, 13.56 mg of genipin was first dissolved using 120 µL of dimethyl sulfoxide (DMSO). DMSO is a caustic compound and was only used to help the genipin dissolve before adding solvent. Once mostly dissolved, 30 mL of PBS were added to the solution to make up the volume and yield a 2 mM genipin concentration. The ratio of genipin to PBS
could be increased or decreased proportionally according to how many samples there were to be crosslinked (each sample dish received 2.5 mL of genipin solution, regardless of if they had AuNP or curcumin added).

If AuNP were involved, then a cysteamine solution was needed just like the EDC protocol. However, significantly less cysteamine was used to conjugate the AuNP in the genipin protocol than the EDC protocol. A 1.5 mM solution of cysteamine was first made by dissolving 11.57 mg of cysteamine into 100 mL of ddH2O. This solution was retained and reused for different sessions of genipin crosslinking. AuNP colloid was aliquoted into a microcentrifuge tube the same way as before and spun down if 2X AuNP was needed. One µL of the cysteamine solution was then added per 100 µL of 1X AuNP colloid in the tube; 2 µL of the solution was added per every 100 µL of 2X AuNP colloid. The final concentration of cysteamine in colloid was 15 µM. 100 µL of the 1X AuNP solution or 100 µL of the 2X AuNP solution was added to each 1X AuNP sample dish or 2X AuNP sample dish, respectively.

If curcumin was utilized, then a ratio of 10 mg of curcumin to 100 µL of 1 N NaOH was used to dissolve the curcumin. Each sample dish that needed 1X curcumin received 100 µL of the solution, and each sample dish that needed 2X curcumin received 200 µL of the solution.

Once the genipin, AuNP, and curcumin solutions were prepared, 2.5 mL of the 2 mM genipin solution was added to each sample dish to be crosslinked. The samples that needed AuNP or curcumin then received the aforementioned amount of AuNP or curcumin solution. AuNP samples were set on the orbital
shaker at 75 RPM. Unlike the EDC reaction, the genipin reaction is slow which is why none of the solutions were set on ice. The genipin reaction is also unique because it will yield higher degrees of crosslinking the longer it interacts with the collagen. For this reason, the collagen was always exposed to the genipin solutions for about 4 hours, after which it was removed and replaced with about 4 mL of PBS. The samples then washed in PBS overnight, after which they were either ready for characterization or ready for sterilization prior to use in a cell assay.

3.3 Sterilization Procedure

Many different sterilization techniques exist to prepare materials for in vitro and in vivo testing applications including UV light exposure, peracetic acid immersion, ethylene oxide exposure, and radiation. Peracetic acid immersion was chosen for this project as it best conserves the collagen surface and microstructures [4]. More specifically, a pH-neutral 0.1% (by volume) peracetic acid solution in ddH₂O was prepared. Once the peracetic acid was diluted in ddH₂O, the pH was adjusted to 7 (with a tolerance of about 0.15) using 1 N NaOH. Under the sterile biological hood, the solution was then poured through a 0.22 um filter using a vacuum. The samples to be sterilized were left in their petri dishes but the PBS removed. About 4 mL of the sterile, pH-neutral, peracetic acid solution was then pipetted into each dish under the hood and set on the orbital shaker for 30 minutes at 75 RPM. The samples were then returned to the hood and the peracetic acid removed. Four mL of sterile PBS was then added to each dish to begin their first wash. Three total washes were performed on each
dish as described above except using *sterile* PBS under the hood. After the third wash was performed, the samples were always used for *in vitro* cell assays within the next day or two. Sterile samples were never stored any longer than two days prior to use because the petri dishes were not air tight, allowing for evaporation of the sterile PBS and the risk of bacterial contamination.
3.4 References


4. Methods of Characterization

4.1 Examining Thermal Properties

In order to examine the thermal properties of the 3D printed collagen-based scaffolds, differential scanning calorimetry (DSC) was utilized. DSC heats two hermetically sealed pans, one sample pan and one reference pan, to predetermined temperatures at constant rates. While heating, the amount of heat input required to maintain the constant temperature increase is recorded. At significant transformation points, the heat required will either increase or decrease depending on if the transformation is exothermic or endothermic, respectively. The data outputted is typically presented either as heat input over temperature or as temperature over time. DSC is capable of detecting a wide range of thermal transitions including curing, melting, crystallization, glass transitions, and decompositions [1]. The reference must not have any transformations within the predetermined temperature range in order to be used as a reliable control for the sample of interest. For this project, the observed thermal transition points were the denaturation temperatures of the uncrosslinked and crosslinked collagen materials.

A Q2000 differential scanning calorimeter (TA Instruments, New Castle, DE) was utilized for this study. The water was first removed from the petri dishes containing the samples to be tested, then the samples were cut and placed in the bottom of pre-weighed aluminum sample pans. These pans were then sealed and reweighed, and the sample masses were then calculated. These sample masses were kept consistent between pans. A pan with 2 µL of ddH₂O was used
as the reference. Modulated DSC was set to run between -5°C and 120°C. The temperature rate of increase was set to 3°C/min with modulation every 80 s ± 0.64°C. The denaturation temperatures were determined from the data using the integration function within Universal Analysis software.

4.2 Assessing the Attachment of Gold

4.2.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a characterization technique utilized to image a material surface on the microscale. An electron beam is emitted to the surface of the material, upon which elastic and inelastic collisions occur [1]. The elastic collisions deflect the electrons from the beam, which are then called backscattered electrons. The inelastic collisions cause electrons from the sample to break away from the surface, which are then called secondary electrons. The trajectories and intensities of both the backscattered electrons and the secondary electrons are detected and used to produce the image of the material surface. The intensities of the backscattered electrons in particular are used to discern between the presence of different elements in the sample. Traditional SEM is performed under a high vacuum and using a dry, conducting material. However, environmental SEM is performed with a lower vacuum and water vapor present and can be used to image damp, insulating materials [2]. Scanning microscopes are also usually capable of performing energy dispersive x-ray spectroscopy (EDS), which identifies elements by characteristic x-ray emission.
For this project, micrographs were acquired via the FEI Quanta 600 FEG Environmental Scanning Microscope located in the Electron Microscopy Core Facility at the University of Missouri. Since the environmental SEM was used, the collagen samples were in low vacuum without metallic spin coating. Magnification ranged from 75X to 20,000X and the electron beam was set to 10 kV. The backscattered electron detector was used to determine where AuNP were present, and EDS was performed to verify that those areas were in fact gold.

4.2.2 Neutron Activation Analysis

Neutron activation analysis (NAA) is a technique capable of identifying and precisely quantifying elements in a sample. The nuclei of the sample are forced to undergo neutron capture reactions, which turn the previously stable nuclei into radioactive isotopes [3]. These isotopes decay via the emissions of beta particles and gamma radiation unique to their elemental properties. A gamma-ray spectrometer detects this radiation and the data acquired is used to characterize and quantify which elements are present.

NAA was conducted at the University of Missouri Research Reactor Center. Once printed, the collagen samples were lyophilized, weighed, and secured within high-density polyethylene vials where they remained during the analysis. Samples were irradiated for two minutes, then allowed to decay for one to seven hours. Gamma radiation was measured for ten minutes via a Canberra High Purity Germanium detector. The detector has a relative efficiency of 33.7% and a full width half maximum resolution of 1.73 keV at 1.33 MeV. A Canberra digital signal processor, Model 9660A, was used in tandem with the detector and
a high voltage power supply. Analysis of the data was performed utilizing Canberra-VMS Genie 2000 software, and the quantities of gold were recorded in Microsoft Excel.

4.3 Investigating Biocompatibility

4.3.1 Cell Culture Protocol

All cell assays were conducted with L929 murine fibroblasts. The cells were passed once a week and those used in the assays were passed between two and twenty-eight times. During each weekly passage, a flask containing a 1/12 fraction of cells (amount of cell suspension to total media) was used. First the old media was discarded, and cellular debris was washed away with a 10 mL rinse of Dulbecco’s phosphate buffered saline (DPBS). In order to detach the cells from the flask, 1 mL of 0.25% trypsin-EDTA was added to the flask. The flask was then incubated for one minute, after which it was removed and gently tapped to the benchtop for another two minutes. To inactivate the trypsin, 10 mL of warm media was then added to the flask and was let sit for a few seconds. The mixture was then transferred into a 15 mL centrifuge tube and spun down at 1250 RPM for 7 minutes in a Z200A centrifuge (Hermle, Wehingen, DE). Afterwards, 9 mL of the supernatant was carefully removed as to avoid disrupting the pellet. Then 4 mL of fresh, warm media was added, and the pellet was resuspended. This created the final cell suspension that was diluted to 1/12 and 1/144 fractions in new, sterile flasks. The flasks were housed in an incubator set at 37°C and 5% CO₂. When beginning an assay, the only difference in protocol was only 2 mL of fresh media was added to the centrifuge tube for resuspension
of the pellet to ensure a high concentration of fibroblasts was achieved. Once resuspended, the cells were then counted and diluted to the appropriate concentration as stated in the assay protocols.

4.3.2 WST-1 Assay

A water-soluble tetrazolium salt (WST) assay is a technique utilized to determine the degree of cytotoxicity a scaffold material induces on a particular cell line. Different versions of the WST assay exist, and these variations are denoted by the number after the WST abbreviation. Common durations of WST assays are 3, 7, and 10-day studies; for this project, 3-day WST-1 assays were performed exclusively. WST-1 assays are able to measure cytotoxicity because the WST-1 reagent reacts with mitochondrial dehydrogenases to form formazan dye (Figure 4.1) [4]. This reaction creates an absorbance color change of intensity proportional to the mitochondrial activity of the cells. Since low cytotoxicity is desired, high intensity absorbance readings are positive results because they indicate healthy, active cells.

![Figure 4.1](image)

**Figure 4.1** When the WST-1 reagent is converted to formazan, a visible color change occurs.

Each WST-1 assay began by setting sterile 3D printed collagen scaffolds into separate wells of a 96-well plate with 150 µL of cell media. The fibroblasts were then harvested from a flask as previously described, counted, and diluted to
a concentration of $7.5 \times 10^3$ cells/mL. Then 250 µL of this cell suspension was added to each sample well and positive control well (wells with cells but no scaffolds). To prevent evaporation of the media, 250 µL of DPBS was transferred to each well surrounding the sample and control wells. This began the 3-day incubation, which allowed the fibroblasts to thoroughly interact with the scaffolds. Once incubated, 150 µL of cell media was removed from each well and 25 µL of the WST-1 reagent was added. The plate was then incubated once more for 4 hours. During this incubation, the WST-1 reagent permeated the fibroblasts' mitochondria was converted to formazan, inducing a color change. After the incubation, 100 µL of medial was removed from each well and transferred into a second 96-well plate, which was used for the measurement. An absorbance plate reader was then utilized to measure the absorbances of the wells at 450 nm with 655 nm reference readings.

4.3.3 Reactive Oxygen Species Assay

Reactive oxygen species (ROS) are oxidative chemicals such as hydrogen peroxide and super oxide anions whose presence in tissues is a known sign of the inflammatory response. Therefore, ROS assays used ROS as a metric for cellular inflammation caused by scaffold materials. The reagent involved is DCFH-DA, and it is broken down into DCFH by cellular esterases (Figure 4.2) [5]. From there, DCFH becomes the fluorescent molecule DCF in the presence of ROS. This fluorescence can then be measured as an indirect correlation to inflammation.
Each ROS assay began by harvesting the fibroblasts, counting them, and diluting them to a concentration of $4.42 \times 10^4$ cells/mL. 250 µL of this cell suspension was then pipetted into each desired well of a 96-well plate and incubated for 24 hours. After that time, the media was removed, and the wells were rinsed twice with 250 µL of DPBS. Then the concentrated DCFH-DA stock was diluted with DPBS and 150 µL of the 1X DCFH-DA/DPBS solution was put into each well. The plate was then incubated for 60 minutes to allow the reagent to infiltrate the fibroblasts. The solution was then removed, and the wells were again washed twice with 250 µL DPBS. One sterilized 3D printed collagen scaffold was then added to each well with 125 µL DPBS. Then the plate was again incubated but for 12 hours to allow the

![Diagram](image)

**Figure 4.2** One step at a time, the non-fluorescent DCFH-DA molecule is converted into fluorescent DCF.
fibroblasts to interact with the scaffolds. The assay was then terminated by adding 125 µL of 2X cell lysis buffer to each well. The plate was then incubated for 5 minutes and 100 µL of solution from each well was transferred to a second 96-well plate. DCF standards were then prepared by diluting the concentrated DCF stock in series, and 50 µL of each dilution was mixed with 50 µL of 2X cell lysis buffer in wells of the second well plate. The fluorescence of the second 96-well plate was then read at 530 nm emission and 480 nm excitation. Fluorescence precautions were taken throughout the assay anytime the reagent was involved, even if the reagent was not yet fluorescent.

4.4 Statistical Analysis

All experimental results were analyzed using a one-way analysis of variance (ANOVA) with pair-wise Tukey-Kramer post-tests. Analyses were performed via Prism 7 software (GraphPad Inc, La Jolla, CA) with a 95% confidence interval.
4.5 References


5. Results & Discussion

5.1 Examining the Thermal Properties

As aforementioned, crosslinking was performed on the scaffolds to increase the stability and durability of the collagen. Crosslinking is necessary because collagen alone is fragile, which is why working with scaffolds comprised entirely of collagen can be challenging. Greater degrees of crosslinking yield an increase in covalent bonds between collagen polymer chains; hence these bonds in turn yield more durable collagen. More covalent bonds present means that more energy is required to denature the materials. It is due to this logic that differential scanning calorimetry (DSC) could be used as a quantitative means of measuring how stable the collagen scaffolds products became after crosslinking. Higher denaturation temperatures correlated to more thoroughly crosslinked and stable scaffolds. Therefore, higher denaturation temperatures were desired.

The first DSC study involved the EDC crosslinker, and was meant to answer two questions. The first was which method of application yielded better degrees of crosslinking: printing the IPC into a crosslinking solution or crosslinking after the IPC was printed into water. The second question to be answered was simply which concentration of EDC produced the greatest degree of crosslinking. To answer these questions, EDC was dissolved in various concentrations (2 mM, 5 mM, 10 mM, and 20 mM) and each concentration was applied to the collagen either during printing or post-printing (Figure 5.1). These 8 sample groups were also compared to an uncrosslinked collagen control group.
Several conclusions can be drawn from this study. First of all, the EDC was able to successfully crosslink the 3D printed collagen regardless of the concentration or application method, as the average denaturation temperature of each group was greater than that of the control. Secondly, the post-print application groups (appearing green in Figure 5.1) exhibited higher denaturation temperatures than their during print counterparts (appearing in red) in 3 of the 4 different concentrations. One could therefore conclude that crosslinking post-print yields a greater degree of crosslinking (and more scaffold durability) than crosslinking while printing. Lastly, the 10 mM EDC post-print group and the 2 mM EDC post-print group exhibited the highest denaturation temperatures overall at 70.56 °C and 68.7 °C, respectively. Seeing as though the increase in

Figure 5.1 Each group of crosslinked collagen exhibited significantly higher average denaturation temperatures than the uncrosslinked control (purple) as denoted by the asterisks.
denaturation temperature was only about 2 °C from an additional 8 mM EDC, the 2 mM EDC group was decided to be the most viable concentration of crosslinker while maintaining an economical use of materials. In conclusion, the 2 mM EDC post-print crosslinking yielded the most efficient improvement in scaffold durability and was utilized for all following EDC experiments.

The second DSC study involved the genipin crosslinker and was meant to simply answer which concentration of genipin yielded the most stable scaffolds. Applying the genipin via different techniques was not attempted like in the previous study as it was assumed the post-print application would still yield greater stabilities. It was theorized that the pre-polymerized collagen monomers competed to both polymerize and crosslink simultaneously, which is why the post-print crosslinking proved more effective. Genipin during-print crosslinking was not attempted as to avoid inducing competition between polymerization and crosslinking. In the genipin study, the same 4 concentrations were used as the EDC study (2 mM, 5 mM, 10 mM, and 20 mM) with another uncrosslinked control group (Figure 5.2). It was again found that each crosslinked group was statistically significant than the control. While the 2 mM group was significantly different than the other crosslinked groups, it was again decided to use the 2 mM concentration in all following studies as to maintain an economical use of the genipin crosslinker.
5.2 Verifying and Quantifying the Gold

5.2.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was utilized to visually confirm the presence of gold nanoparticles (AuNP) on the surface of the crosslinked collagen scaffolds. Energy dispersive x-ray spectroscopy (EDS) was also used to verify that the particles found were in fact gold. These characterization techniques were made possible through the Electron Microscopy Core Facility at the University of Missouri. The low vacuum environmental SEM was utilized so that the samples could remain damp and nonconductive. The AuNP were 20 nm in size and both EDC and genipin were used to attach the nanoparticles to the scaffolds. The

Figure 5.2 In the DSC denaturation temperature determination, the 2 mM concentration yielded significantly enhanced stability and was chosen as the optimal crosslinker concentration.
EDC crosslinking proved a viable method of attaching the AuNP to the surfaces. The backscattered electron detector was able to detect various regions of fairly uniform spreading of the AuNP (Figure 5.3). Uniform distributions of the AuNP are desired as to produce a homogeneous product that will elicit a consistent response from the cells during the \textit{in vitro} assays. However, not all regions of the collagen demonstrated a uniform dispersion of AuNP from the EDC crosslinking. The cause of this was theorized to be the fact that the samples were let sit stagnant on the benchtop during the EDC crosslinking.

The genipin crosslinking was also found to be an effective method of attaching the AuNP to the collagen (Figure 5.4). Two different concentrations of AuNP, deemed “1X AuNP” and “2X AuNP”, were crosslinked to the collagen via genipin. During the genipin crosslinking, the samples were placed on an orbital shaker at 75 RPM in an effort to more uniformly distribute the AuNP. The scanning electron micrographs demonstrated that the orbital shaker was able to uniform distribution of AuNP. It was also found that the 2X AuNP samples did seemingly contain more AuNP than the 1X AuNP samples. EDS confirmed with both the EDC and genipin samples that the light particles in the SEM images were gold nanoparticles.
Figure 5.3 Backscattered electron images show an ideal spread of AuNP over A.) an area using 500X magnification and B.) the same area with a 2,000X magnification. Other images show an agglomeration of AuNP over C.) an area using 314X magnification and D.) the same area outlined in red at a 5,000X magnification.
Figure 5.4 Secondary and backscattered electron images of a region of 1X AuNP are given in A and B, respectively. The same are given of a 2X AuNP sample in C and D.
5.2.2 Neutron Activation Analysis

While SEM gives insight into how the gold nanoparticles are dispersed on the surface of the collagen, it only gives a qualitative, subjective view of how much gold is present. The data acquired by Neutron Activation Analysis (NAA) gives a quantitative measure of how much gold is conjugated to a crosslinked collagen sample. Both 1X AuNP and 2X AuNP samples were crosslinked with EDC and genipin, and all four sample types were analyzed via NAA. The goal was to determine if the 2X AuNP samples truly had twice the amount of gold as the 1X AuNP samples. The samples crosslinked via EDC exhibited an exact doubling of gold, from 0.008% by mass on the 1X AuNP samples to 0.016% by mass on the 2X AuNP samples (Figure 5.5). The samples crosslinked by genipin were just 0.003% by mass away from doubling exactly. The 1X AuNP samples had 0.014% gold by mass and the 2X AuNP samples had 0.025% gold by mass. While this was not exactly double, the difference from 1X AuNP to 2X AuNP was still very significant. All four of these sample types were later used in the in vitro cellular assays.

**Figure 5.5** A.) The mass percent of gold exactly doubled from the 1X AuNP to the 2X AuNP EDC samples. B.) The mass percent nearly doubled in the genipin sample analysis.
5.3 Evaluating Biocompatibility

5.3.1 WST-1 Assays

WST assays provide analysis as to whether a material will elicit a cytotoxic effect. All WST assays performed in this project were 3-day studies, meaning the scaffold materials and the fibroblasts interacted for 72 hours before the WST-1 reagent was added. Once introduced, the WST-1 reagent was incubated with the scaffolds and cells for four hours to ensure accurate absorbance values would be obtained. Since the presence of more metabolically active cells causes more of the WST-1 reagent to become formazan, and more formazan dye creates a greater color change, high absorbance values are desirable. If the absorbance values of the scaffolds are at least as high as the positive controls (wells with cells but no scaffolds), then the scaffolds are considered nontoxic.

![3-Day WST-1 Assay](image)

**Figure 5.6** The scaffolds crosslinked with EDC exhibited very significantly higher cell viability than the positive control, although the uncrosslinked group ranked the highest of them all.
The first WST assay performed involved 3D printed collagen scaffolds crosslinked by EDC (Figure 5.6). One of these groups was crosslinked with 1X AuNP, and a group of uncrosslinked printed collagen was also included. As is evident, all three scaffold groups exhibited an extreme lack of cytotoxicity as their absorbances were all significantly greater than that of the control group, ranging between 53% and 70% greater than the control. It is interesting to note that the more chemical changes were made to the collagen (crosslinking and crosslinking with AuNP), the lower the absorbance values became. Regardless, this first study showed excellent cell viability (lack of cytotoxicity) with the EDC crosslinked samples.

![3-Day WST-1 Assay](image)

**Figure 5.7** The scaffolds crosslinked with genipin exhibited lower cell viabilities than the positive control, with the curcumin group down to nearly half the viability of the control group.

The second WST assay involved 3D printed collagen scaffolds crosslinked by genipin (Figure 5.7). One of these groups was crosslinked with 1X AuNP, one of these groups was crosslinked with 1X curcumin, and another uncrosslinked
group was included. This time, every scaffold group exhibited lower cell viability than that of the positive control. The uncrosslinked group was not significantly different, but the crosslinked and the AuNP crosslinked groups were both significantly different at 89% and 88%, respectively. The curcumin crosslinked group demonstrated an alarming decrease of cell viability at 53%. Again, it is evident that the more chemical alterations that are performed on the collagen, the worse the cellular response. While the crosslinked and AuNP groups still exhibit acceptable levels of cell viability (considering crosslinking is necessary to produce a viable product), the curcumin group was unacceptable.

![3-Day WST-1 Assay](image)

**Figure 5.8** The scaffolds crosslinked with genipin and curcumin demonstrate a clear trend of decreasing cell viability with increasing concentrations of curcumin.

The third WST assay again involved 3D printed collagen scaffolds crosslinked by genipin, but this time with varied levels of curcumin each less than the previous 1X curcumin group (Figure 5.8). An uncrosslinked group was not
included in this study as the focus was on the crosslinked and curcumin groups. Again, a steady decline in cell viability was observed. The crosslinked group this time was not significantly different than the positive control, but all three of the curcumin groups were – each more significant than the last. It is obvious from this data that the incorporation of curcumin causes a drastic decrease in the metabolic function of the fibroblasts and elicits a negative effect on cell viability.

Overall, the WST studies showed that the EDC crosslinked scaffolds exhibit excellent cell viability, but the genipin crosslinked scaffolds decrease cell viability – drastically so in the case of the curcumin scaffolds.

5.3.2 Reactive Oxygen Species Assays

ROS assays help determine how much antioxidative and anti-inflammatory effects scaffold materials induce on cells. Since inflammation is an inevitable and unwanted consequence of implantation, it is crucial that scaffolds are designed to mitigate this negative response. Since ROS are correlated with the inflammatory response, ROS assays determine anti-inflammatory effects by indirectly measuring the levels of ROS in a sample. The reagent involved reacts with ROS and is converted to the fluorescent molecule DCF. This means lower fluorescent values are desirable because they are emitted from lower concentrations of DCF, and therefore lower levels of ROS and inflammation. ROS assays are particularly important to this project because the primary reason AuNP and curcumin are crosslinked to the 3D printed collagen is for their antioxidant and anti-inflammatory properties [1,2].
The first ROS assay involved 3D printed collagen scaffolds crosslinked by EDC (Figure 5.9). Two of the crosslinked groups also included the incorporation of 1X AuNP and 2X AuNP. The negative control in this and every ROS assay is groups with only cells and no scaffolds (the same as the positive controls of WST assays). As is clear, crosslinking with EDC significantly increased the amount of ROS in the system and including AuNP only exacerbated this increase in inflammation. Every group is significantly different than the others and the ROS only increase the more the collagen is altered. This study demonstrated that AuNP have the opposite effect as expected.

The second ROS assay involved 3D printed collagen scaffolds crosslinked by EDC in a myriad of ways (Figure 5.10). This study was an effort to troubleshoot the surprising negative outcome of the first study. It was theorized
that such negative results were obtained either because too many AuNP were used in the crosslinking process or too much MEA (cysteamine) was used relative to the amount of AuNP (causing a complete coating of the AuNP). To crosslink a 1X AuNP sample of 3D printed collagen, 100 µL of both AuNP colloid and MEA solution are used. Therefore, to test the above hypothesis, samples of collagen were crosslinked with 75, 50, and 25 µL of AuNP instead of 100 µL. Within each of those groups, 2/3 and 1/3 as much MEA was used to functionalize those AuNP. For example, groups crosslinked with 75 µL of AuNP were functionalized with either 50 µL or 25 µL of MEA. One group of crosslinked collagen was done so without using any MEA (75 µL AuNP 0 µL MEA) as to see whether the MEA was even necessary to crosslink AuNP to the collagen. NAA was also performed on these groups to see how much gold was truly present on these collagen samples (Figure 5.10). When analyzed together, these tests told

![Graph](image)

Figure 5.10 A.) NAA data and B.) ROS data consisting of the same sample groups. In multiple ways, AuNP were linked to increases in ROS, contrary to what was theorized.
us how much gold was present and what effect that gold had on ROS and inflammation.

The results obtained from the second EDC ROS assay were very negative. Every group demonstrated significantly higher levels of ROS than the control except for the 75 µL AuNP 50 µL MEA group. Within two of the three sets of AuNP (the 75 µL AuNP set and the 50 µL AuNP set) ROS increased with decreasing MEA. Since more of the AuNP surfaces are exposed with less MEA, this means the fibroblasts secreted more ROS with greater exposure to the bare AuNP surfaces. This is further exemplified by the NAA data. The increases and decreases in the ROS values nearly exactly copied the levels of gold actually found in the NAA study (all groups correlated except the 25 µL AuNP set). This further demonstrates that more ROS correlates to a greater amount of gold present on the collagen surfaces.

Figure 5.11 While genipin was not able to mitigate ROS and in fact drastically increased it, curcumin was able to eradicate the heightened levels to a point where it was no longer statistically significant.
The third ROS study involved 3D printed collagen scaffolds crosslinked by genipin (Figure 5.11). Uncrosslinked and crosslinked groups were included as well as groups crosslinked with 1X curcumin and 2X curcumin. Two positive control groups containing different concentrations of hydrogen peroxide, a known ROS, were also included as positive controls for comparison. The uncrosslinked group showed significantly higher ROS than the negative control, and the genipin crosslinked group proved to elicit the most ROS of any group by a very significant margin. This was particularly disappointing because one of the primary reasons we experimented with genipin crosslinking was for its supposed anti-inflammatory effects [3]. However, the 1X curcumin group was able to decrease the ROS, and the 2X curcumin group was able to mitigate the ROS so much so that it was no longer significantly different than the negative control. This result is extremely important as it exemplifies curcumin’s antioxidant and anti-

![Figure 5.12](image)

**Figure 5.12** Genipin crosslinking again was shown to drastically increase ROS. Unlike curcumin however, the AuNP were not able to significantly decrease the ROS.
inflammatory capabilities. The hydrogen peroxide positive controls exhibited comparable results to the curcumin groups because not enough hydrogen peroxide was used in retrospect.

The fourth ROS assay involved 3D printed collagen scaffolds again crosslinked by genipin (Figure 5.12). The same groups as the previous study were used except the 1X and 2X curcumin groups were replaced by the 1X and 2X AuNP groups. Every group exhibited significantly higher ROS than the negative control. The genipin crosslinked group nearly doubled the ROS as the uncrosslinked group, and this increase was only barely decreased by the additions of 1X AuNP and 2X AuNP. The 50 mM hydrogen peroxide group was consistent with the uncrosslinked group and the 100 mM hydrogen peroxide group was comparable to the crosslinked and AuNP groups. All of these results were negative.

In summary, EDC crosslinking increased ROS, and this was greatly exacerbated by the presence of AuNP – regardless of how many were used or how much MEA was used to conjugate them. Genipin crosslinking also increased ROS, but this effect was eradicated by the presence of curcumin. AuNP, on the other hand, were not able to decrease the ROS caused by genipin.
5.4 References


6. Conclusions

6.1 Review of Research Objectives

There are many challenges when it comes to tissue engineering, one of which being producing patient-specific products. 3D bioprinting has much potential to become the gold standard of biomaterial scaffold production as it is uniquely capable of efficiently manufacturing biocompatible materials in custom geometries meeting each patient’s individual needs. The goal of this project was to establish a platform capable of 3D printing collagen-based scaffolds and to characterize those scaffolds via a variety of techniques. Not only were uncrosslinked scaffolds produced, but two different classes of crosslinked scaffolds were also developed. These materials were further improved upon via the incorporation of gold nanoparticles and curcumin for enhanced cellular responses. It was hypothesized that crosslinking would enhance the stability of the scaffolds, while the incorporation of gold nanoparticles and curcumin would produce antioxidant and anti-inflammatory effects. From the beginning of the project, four objectives were identified, and they are listed on the next page.
Objective 1: To 3D print uniform and reproducible collagen-based scaffolds.

This objective was reached by the trial and error experimentation and manipulation of different 3D printing parameters.

Objective 2: To examine the thermal properties of the crosslinked scaffolds.

Differential scanning calorimetry was utilized to determine the denaturation temperatures of the crosslinked scaffolds and optimize the amount of crosslinker used.

Objective 3: To verify and quantify the presence of gold nanoparticles in the crosslinked scaffolds.

Scanning electron microscopy did verify the presence of AuNP while neutron activation analysis quantified the AuNP.

Objective 4: To evaluate the cytotoxicity and anti-inflammatory capabilities of the gold nanoparticle and curcumin scaffolds.

Water soluble tetrazolium salt assays examined the cytotoxicity and general biocompatibility of the scaffolds. Reactive oxygen species assays determined the anti-inflammatory effects of the AuNP and curcumin scaffolds.
6.2 Conclusions from Results

6.2.1 Thermal Stability of Scaffolds

Differential scanning calorimetry was able to confirm that crosslinking either with EDC or genipin increased the stability and durability of the 3D printed collagen-based scaffolds. By comparing the application of EDC crosslinking during printing versus post printing, it was found that crosslinking post printing yielded significantly greater stabilities than crosslinking during printing. It was also concluded that the 2 mM concentration of both EDC and genipin was the optimal crosslinking concentration as it produced competitively high stabilities while maintaining an economic use of materials.

6.2.2 Gold Nanoparticle Conjugation

Scanning electron microscopy verified that crosslinking gold nanoparticles to the surfaces of the 3D printed collagen-based scaffolds via EDC or genipin is successful. It was discovered that allowing the samples to stir while crosslinking created an improved, more uniform dispersion of the nanoparticles on the collagen surfaces. Energy dispersive x-ray spectroscopy was able to confirm that the particles in the micrographs were in fact gold.

Neutron activation analysis quantified the amount of gold actually bound to the surfaces of the collagen. The 2X gold nanoparticle samples were verified to contain approximately twice the amount of gold as the 1X gold nanoparticle samples using either the EDC or genipin crosslinker.
6.2.3 Biocompatibility of the Scaffolds

Water soluble tetrazolium salt assays exposed that 3D printed collagen-based scaffolds crosslinked with EDC exhibit superb cell viability, although this viability does decrease slightly with the addition of gold nanoparticles. The WST assays also revealed scaffolds crosslinked with genipin demonstrate decreased cell viabilities, which further decreased by including gold nanoparticles. The cell viabilities plummet with the incorporation of curcumin, calling into question the cytotoxicity of the compound.

The reactive oxygen species assays showed crosslinking the collagen scaffolds with EDC increases the degree of inflammation. This increase is spiked by the presence of gold nanoparticles and is even further exacerbated by decreasing the amount of MEA used to functionalize them. Crosslinking the collagen scaffolds with genipin also substantially increased the inflammatory response, but this increase can be eradicated by the inclusion of curcumin. Gold nanoparticles, however, were not able to significantly decrease ROS.

6.3 Proposed Future Investigations

As previously stated, collagen alone is a fragile material and even after crosslinking can deteriorate if over-handled. If this platform is to actually be utilized to produce implantable scaffolds, the durability of the collagen would need to be markedly improved. This could be accomplished by printing the IPC along with another material or by further post-print manipulation of the collagen other than simply crosslinking.
In order to 3D print realistic tissue scaffolds, the resolution of the printer would need to be improved as well as the range of geometries it is capable of producing. The resolution could be improved possibly by chemical manipulation of the IPC formula or by expanding the limitations of the 3D printer. Chemical manipulation could entail diluting the collagen concentration of the IPC to produce a less viscous solution. Expanding the printer’s capabilities could include using finer needles and a more powerful syringe pump. In order to widen the range of geometries attainable, a support material would be required. This support material would be printed in tandem with the IPC and somehow removed after printing – either by manual force or chemical dissolution.

Lastly, the anti-inflammatory capabilities of the printed products need to be enhanced. Curcumin proved to be an effective anti-inflammatory agent, but in this project was paired with genipin which drastically increased inflammation. If curcumin was crosslinked with EDC, it could lead to even lower levels of inflammation not seen in this project. The only concern would be if the EDC and curcumin pair would also decrease cell viability as was observed with the genipin and curcumin combination. Other anti-inflammatory agents could also be investigated such as green tea extract [1]. To demonstrate that the collagen products’ anti-inflammatory properties are comprehensive, other cell lines could be used in the in vitro assays like induced pluripotent stem cells [2]. If the ROS assays continue to lead to negative results, other assays could be used to demonstrate anti-inflammatory capabilities such as enzyme-linked immunosorbent assays [3].
6.4 References


Appendix A: Supplemental SEM Images

Scanning electron micrographs were acquired via the FEI Quanta 600 FEG Environmental Scanning Microscope located in the Electron Microscopy Core Facility at the University of Missouri. Since the environmental SEM was used, the collagen samples were in low vacuum without metallic spin coating. Magnification ranged from 75X to 20,000X and the electron beam was set to 10 kV.

A.1 1X 20 nm AuNP EDC crosslinked samples
A.2 1X 20 nm AuNP Genipin crosslinked samples
A.3 1X 20 nm AuNP Genipin crosslinked samples
A.4 1X 20 nm AuNP Genipin crosslinked samples
A.5 2X 20 nm AuNP Genipin crosslinked samples