ABSTRACT

COMPUTATIONAL DESIGN AND OPTIMIZATION OF BONE TISSUE ENGINEERING SCAFFOLD TOPOLOGY

by Nicholas Peter Uth

Bone tissue engineering aims to help the body naturally regenerate critical sized defects (and non-unions) that would otherwise require bone grafts. Scaffolds are a vital tool in this field, as they act as temporary matrices for cellular adhesion and bear mechanical loads during patient recovery. However, there are many aspects to designing such scaffolds, and two of the most vital (compressive modulus and porosity) conflict. Commonly, studies that attempt to determine viable designs use “trial-and-error” methods, which are resource demanding and may not guarantee fully optimized results. In this study, we sought to generate a scaffold topology that satisfied the compressive modulus and porosity requirements for trabecular bone regeneration with COMSOL optimization software. To determine the validity of COMSOL’s optimized topology and predicted behavior, we constructed and tested 24 scaffolds via 3D bioplotting, an additive manufacturing technique, according to a physical I-optimal split-plot designed experiment. The results were used to generate response surface methodology models that could be used to optimize scaffold topology and predict the resultant compressive modulus and porosity.
COMPUTATIONAL DESIGN AND OPTIMIZATION OF BONE TISSUE ENGINEERING SCAFFOLD TOPOLOGY

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Geistlich Pharma AG (Wolhusen, Switzerland), TiGenix (Leuven, Belgium), ISTO
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Chapter 1

1.1 Introduction

1.1.1 Tissue Engineering (TE) & Bone Tissue

Bone tissue has a limited ability to self-repair, but it cannot regenerate if the damaged area is too large. Currently, there are limited methods of repairing critically damaged bone tissue. Typically, large-scale repair is done via grafts or replaced with synthetic materials [1]. Autografts, taken from the patient, are limited in size and require a second surgical site, whereas allografts, taken from a donor, carry the potential for disease transmission or immune response [2]. Synthetic materials also risk immune response from the patient and are subject to wear over time [1]. Annually, at least four million surgeries around the world utilize bone grafts or synthetic replacements [3], and this number can only grow as world populations do. Tissue engineering (TE) is an appealing alternative to these methods.

TE is a promising, multi-disciplinary field where replacement tissues are grown, instead of harvested, thus circumventing many issues that affect the aforementioned traditional methods [4, 5, 6]. As reported by Lacroix et al., in 2001 the market for tissue regeneration as a whole was estimated to be $25 billion worldwide [7], indicating both a medical demand and a financial prospect. In order to aid cells in proliferation and the filling of the damaged region, TE makes use of scaffolds [5, 8, 9]. A scaffold is a porous construct made out of biomaterials to act as a matrix and support structure. These structures need to be stiff (for load-bearing tissues), encourage cell adhesion, be porous, biodegradable, biocompatible, potentially be bioactive [6, 10], and be easily fabricated [4]. However, the stiffness and porosity of scaffolds are generally considered the two most important design goals for bone tissue scaffolds.

Bone tissue regrowth is believed to be connected to mechanical forces experienced by the regenerating cells (mechanoregulation effects) [13]. These mechanical forces determine how the cells differentiate into various tissues (bone, cartilage, or fibrous). The primary job of bone scaffolds is to support the tissue while transferring appropriate mechanical forces to the growing cells in order to induce proper differentiation [8, 14]. Increasing stiffness of bone tissue scaffolds can reduce the formation of fibrous tissues [8], which are notorious for causing failure in weight-bearing prostheses. However, excessive mechanical forces can cause apoptosis in regenerating tissues. As such, it is imperative that bone scaffold designs aim to mimic the stiffness of natural
bone as closely as possible. Ranges of scaffold properties can be targeted based on literature results for the tissues they are meant to replace. For instance, trabecular bone scaffolds can be designed based on values described in table 1.1 with a minimum of 5 MPa compressive strength [15].

Table 1.1: Mechanical properties of mature bone tissues, as described by literature search.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Tissue Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young’s Modulus (E)</td>
<td>50 - 100 MPa</td>
<td>(Trabecular bone)</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>2.23 - 25.9 GPa</td>
<td>(Trabecular bone)</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>20 GPa</td>
<td>(Trabecular bone)</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>6 GPa</td>
<td>(Cortical bone)</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>1.3 - 20 GPa</td>
<td>(Trabecular bone)</td>
<td>[20]</td>
</tr>
<tr>
<td>Poisson’s Ratio (ν)</td>
<td>0.3</td>
<td>(Cortical bone)</td>
<td>[18]</td>
</tr>
<tr>
<td>Porosity</td>
<td>&lt; 15 %</td>
<td>(Cortical bone)</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>&gt; 70 %</td>
<td>(Trabecular bone)</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>50 - 90 %</td>
<td>(Trabecular bone)</td>
<td>[22]</td>
</tr>
</tbody>
</table>

In addition to providing temporary mechanical support [9], scaffolds must also provide a surface for cell attachment [23]. Both surface chemistry and topography of the scaffolds play a role in cell adhesion and can be designed in such a way as to foster it [6, 7]. Ultimately, the scaffold’s porosity, a major feature of the topology, and the balance between it and scaffold stiffness is vital for new tissue formation. The two factors have a negative impact on each other; a large porosity will reduce stiffness, but is necessary for nutrient inflow, waste removal, and cell migration. Cells and vasculature need to be able to permeate the structure [4, 6, 23, 24], as vascularization will be hindered if the porosity is insufficient. Most cells, with the exception of cartilage-forming chondrocytes, cannot survive unless they are within 100 µm of vasculature due to oxygen diffusion limitations [4, 25, 26]. This 100µm distance has been used in previous research as a requirement for stem cell differentiation into bone [24, 26]. As such, cells will not be able to differentiate properly, if at all, within the central region of a low-porosity scaffold. For bone tissue, it is hypothesized that a pore size of 300 µm or greater is required for vascularization [10].

As cells regenerate at the surface of the scaffold, they may gradually close off access to the central regions [4]. This makes biodegradation a key material factor in designing scaffolds. The scaffold should degrade at the same rate as the tissue grows so that tissue replaces the scaffold and takes over mechanically [1, 4, 5]. Cell growth, vascularization, and fluid flow are
negatively affected if the scaffold degrades too slowly, but, if the scaffold degrades too quickly, scaffold stiffness will be reduced [6, 11].

Compressive testing can be used to find the bulk stress-strain behavior of scaffolds, which enables researchers to see how different topology design choices affect scaffold performance [7]. For instance, material choices and the geometry of the scaffolds both play a role in the compressive strength, and both can be adjusted to meet the mechanical requirements of the native tissue [15].

1.1.2 Biomaterials for Bone Tissue Engineering

It has been previously proposed that using materials found within the extracellular matrix (ECM) of bone would be ideal for bone scaffolds [27]. Bone has a cellular matrix that is composed of 30% (dry weight) collagen, 60-70% ceramics/hydroxyapatite [4, 6], and up to 10% water depending on the type and location of the bone [27].

Table 1.2: Summary of material types as TE scaffold biomaterials

<table>
<thead>
<tr>
<th>Material Type</th>
<th>Examples</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymers</td>
<td>PLLA, PCL, PLGA, Chitosan, Alginate</td>
<td>- Flexible &amp; easy to process, - Properties can be tailored by adjusting chemistry (copolymer ratios)</td>
<td>- Generally unsuitable for mechanical loading on their own, - Hydrolytic degradation releases acidic byproducts [4], which could trigger immune response and/or apoptosis in high concentrations</td>
</tr>
<tr>
<td>Ceramics</td>
<td>Hydroxyapatite, Tricalcium phosphate</td>
<td>- Mechanically strong, - Can be bioactive / biocompatible</td>
<td>- Difficult to process, - Brittle, - Degrade slowly</td>
</tr>
<tr>
<td>Metals</td>
<td>Titanium</td>
<td>- Mechanically strong</td>
<td>- Do not degrade, - Stress shielding if too stiff, - Can trigger immune response</td>
</tr>
</tbody>
</table>

1.1.3 Additive Manufacturing (AM)

Traditional scaffold production techniques typically work by producing a solid structure and removing portions of it to create pores. One such method, salt leeching, involves suspended salt particles in the material, which can then dissolved by immersing the scaffold in water, resulting in a porous structure [4]. In contrast, modern additive manufacturing (AM) uses 3D
computer-aided design (CAD) models that have been divided into multiple 2D layers to produce physical, geometrically-complex, objects quickly and accurately [4, 6, 12]. Also known as solid freeform fabrication (SFF) or rapid prototyping (RP), these methods produce scaffolds by building the geometry layer-by-layer [12].

Table 1.3: Summary of select traditional scaffold manufacturing techniques, as described in [4].

<table>
<thead>
<tr>
<th>General procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solvent-casting / particulate leaching</strong></td>
</tr>
<tr>
<td>Salts mixed with primary materials are dissolved in water to produce pores.</td>
</tr>
<tr>
<td><strong>Gas foaming</strong></td>
</tr>
<tr>
<td>CO₂ is bubbled through polymer before pressure is reduced to trap bubbles within</td>
</tr>
<tr>
<td>structure.</td>
</tr>
<tr>
<td><strong>Phase separation</strong></td>
</tr>
<tr>
<td>Polymer is dissolved in solvent, temperature is reduced to cause separation,</td>
</tr>
<tr>
<td>solvent is sublimed out to leave porous scaffold.</td>
</tr>
<tr>
<td><strong>Melt molding</strong></td>
</tr>
<tr>
<td>Polymer and gelatin are mixed in a mold and heated to induce polymer bonding.</td>
</tr>
<tr>
<td>Gel is leached out to leave pores.</td>
</tr>
<tr>
<td><strong>Solution casting</strong></td>
</tr>
<tr>
<td>Polymer is dissolved in chloroform and precipitated before being pressed into a</td>
</tr>
<tr>
<td>mold and heated.</td>
</tr>
<tr>
<td><strong>Freeze drying</strong></td>
</tr>
<tr>
<td>Polymer is dissolved in acid, frozen, and freeze-dried.</td>
</tr>
</tbody>
</table>

**Table 1.4: Summaries of select AM techniques.**

<table>
<thead>
<tr>
<th>General procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stereolithography</strong></td>
</tr>
<tr>
<td>Ultraviolet laser is used to cure a liquid polymer (layer-by-layer) into a</td>
</tr>
<tr>
<td>geometry dictated by CAD model [1, 4]</td>
</tr>
<tr>
<td><strong>Selective laser sintering (SLS)</strong></td>
</tr>
<tr>
<td>A laser is used to melt and fuse polymer powder on a surface [5].</td>
</tr>
<tr>
<td><strong>3D printing</strong></td>
</tr>
<tr>
<td>Polymer powder is sprayed with a binding agent in order to build layers according</td>
</tr>
<tr>
<td>to a CAD model. [4]</td>
</tr>
<tr>
<td>Alternatively, directly lays wax/polymer onto stage [1]</td>
</tr>
<tr>
<td><strong>3D (bio)plotter</strong></td>
</tr>
<tr>
<td>Polymer is forced through a nozzle, onto the stage or previous layers, by air</td>
</tr>
<tr>
<td>pressure [4]</td>
</tr>
</tbody>
</table>

Extrusion-based AM methods use temperature or solvents to process a polymer before pushing them through a nozzle [1]. Mechanical properties of the scaffolds produced by these methods can be tailored by adjusting the fiber diameter, spacing, and layer thickness [28]. Among extrusion-based AM techniques, the bioplotter is the ideal method if cells are to be placed simultaneously with the polymer [1]. Thanks to the temperature/solvent processing potential that bioplotters make use of, there are more materials that can be used by this method [1] compared to other AM techniques. However, the geometry that the bioplotter can produce is limited to layers of horizontal strands, which may be too smooth for cellular attachment [5]. Surface modification or use of materials that promote cellular attachment can overcome the latter issue.
Scaffold geometries produced by AM methods can be controlled to fine detail, which is a great advantage for cellular growth and mechanical strength [9, 15, 23, 29]. This is an improvement over traditional scaffold engineering techniques, which have very limited pore sizes, little interconnectivity for fluid flow [4, 15], small maximum sizes, potential for retaining organic solvents [4], little control over resulting topology [5], and are too mechanically weak for use in hard tissues and most soft tissues [1]. In addition, AM scaffolds have been shown to have more uniform distributions of cells after seeding and better nutrient flow throughout than traditional scaffold production techniques [12]. However, material choice in AM methods is comparatively limited [5].

It has been shown that scaffolds of a single material could fulfill mechanical requirements of a certain tissue when constructed with different geometries [7, 9]. By changing the geometry, such as the angle between strands in alternating layers [12], it is possible to tailor scaffold properties even if other factors, such as materials or porosity, are kept constant [1].

1.1.4 Computer-Aided Design (CAD) and Finite Element (FE) Simulation

The current methodology for the testing of factors and design of scaffolds is predominantly based on experimental trial-and-error [7, 9, 30], which is resource and time demanding [18]. The use of computer-aided design (CAD) in tissue engineering can speed up the design process of scaffolds and save time and resources by reducing the number of experimental tests needed [7, 8, 12]. Previous studies have suggested that simulations of tissue engineering scaffolds can reasonably predict experimental data, and may be a strong tool for a priori design [9].

When performing computational simulations, there is a need to balance computational speed and model accuracy. Simplifying the model improves speed, which is the purpose of using the computational method, but at potential cost of accuracy [8]. Currently, use of unit-cells, repeating subunits of the geometry, is the modeling method of choice for tissue engineering due to their less demanding size and the ease of use in multiscale studies [7]. It is also possible to produce custom models of scaffolds via CT or MRI technology [4]. Models can be cut into cross-sectional layers so that they can be built by AM technologies [4].

Computer simulations and finite element (FE) modeling have also been used to study cell growth and differentiation via mechanoregulatory effects [8]. Studies have examined scaffold structure and its effects on cellular differentiation in order to develop an optimal topology [8].
Specifically, these studies generally focused on porosity and/or sheer forces as a result of fluid flow and their relation to mechanical forces experienced by cells. While fluid flow simulation and the prediction of cellular differentiation in silico are beyond the scope of this research, these studies have shown that porosity, dissolution rate, and material stiffness are key factors to be targeted for maximizing osteogenesis in tissue scaffolds [8].

Topology optimization makes use of computational resources to simulate scaffolds of varied factors, changing the factors within specified ranges in order to find a scaffold with desired properties. Optimization strategies are needed to resolve conflicting factors, like porosity and mechanical strength [12]. Computational optimization methods have shown promise in designing scaffold geometries that balance such factors [29]. As reported by Hollister, computational optimization has been applied to scaffolds in order to maximize stiffness, meeting the (linear elastic) mechanical requirements of bone tissue, while holding porosity as a constraint [1, 9].

The primary limitation with computer simulation of tissue scaffolds has to do with microstructure. Actual scaffolds have a micro-topology (cracks, pores, material inconsistencies), which cannot be taken into account in most computational models. This limitation has been shown to cause simulations to over-predict the material properties of scaffolds to varying degrees [23, 31].

1.1.5 Statistical Approach & Designed Experiment (DE)

A response surface is a graphical representation of the relationship between design factors and the response of a system [32]. Response surface methodology (RSM) is a set of statistical and mathematical techniques that can be used to estimate a fitted regression model for the system for a given range of factor values (the design region) [15, 32, 33]. The resultant 2nd order model approximates the relationship between the factors and the response, which can then be optimized to estimate the factor value combination that produces an optimal response. If, as in the case of this study, there are two responses, a quadratic is fit to both and a compromise is worked out. In this case, we will choose factor levels that produce a maximum compressive modulus while maintaining an acceptable porosity.

As the goal of this study is to validate the prediction and optimization capabilities of a COMSOL FE model, an RSM analysis is an ideal baseline to compare the simulation results to. By performing RSM in the same design region and performing a constrained optimization on the
generated models (maximize compressive modulus, constrain porosity) in the same manner as the simulation, we can determine whether the simulation can reasonably optimize and/or predict scaffold behavior.

Part of developing a response surface is the experimental evaluation of factor effects on the response. By doing so using designed experiment (DE) methods, we can best evaluate the system. The use of a DE can greatly benefit the scaffold design process, as it confers certain advantages when compared to the traditional iterative design approach. The most relevant to this study are: (1) less experimental runs are required for similar precision, (2) we can estimate the effects of factor interactions, and (3) it is less likely to overlook optimal factor value combinations [32]. Thus, we adopted an I-optimal split-plot designed experiment. I-optimal designs minimize the average (integrated) prediction variance across the design region [34]. This type of design is highly suggested when developing models for prediction and/or RSM [34], which makes it desirable for validating the simulation.

Among the factors used in this study for scaffold design, percent ceramic composition poses an issue for designing the experiment. Changing this factor frequently would drain resources and potentially negatively affect the responses due to solvent evaporation between uses. In order to account for this, a split-plot design was used, which divides experimental runs in two ways: whole plots (WP) where the difficult-to-change factor is applied to all encapsulated runs, and split plots (SP), the individual experimental runs within a whole plot, where the remaining factors are applied. This is done to account for systems with factors that are large or difficult to change frequently [32, 35]. Such a design has two levels of randomization: WP scale, where the order of the WPs are randomized, and SP scale, where the trial runs within a given WP have their order randomized [32]. This maintains independence between the WPs, and between SPs within a given WP [35]. However, it also produces two levels of error, which need to be accounted for and analyzed when performing analysis of the results [32].

1.2 Statement of Problem

Bone tissue engineering has been seeking to develop biodegradable scaffolds that can support large mechanical loads and aid in osteochondral regeneration. However, the design methodology most commonly used is an iterative “trial-and-error” process, which is resource demanding. In order to circumvent this issue, there have been a few studies on the use of simulation/computational-analysis to test scaffold designs prior to experimental quantification.
Many of these studies have, unfortunately, found that their simulations or models tend to over-predict mechanical properties. We utilized simulation as a topology optimization tool that designed an “optimal” scaffold under a set of architectural conditions, and compared its design with an experimentally/statistically optimized scaffold.

1.3 Objectives & Hypothesis

- **Objective 1:** It will be possible to use COMSOL optimization module to determine the 'optimal' scaffold design. The optimized model will have a porosity greater than 50%, and, when steady-state loading is applied, have a compressive modulus similar to trabecular bone (≥10 MPa).

- **Objective 2:** Mechanical testing of physical scaffolds produced as described in the DE will generate a response surface that can be used to design an optimal scaffold. This optimal design (strand thickness, spacing, and %nHA) will produce scaffolds with a compressive modulus ≥ 10MPa and a porosity ≥ 50%.

- **Objective 3:** Comparison of the computational and statistical topologies will show agreement (values within 20%) between the two design strategies, which will validate the computational model as a primary design tool.

1.4 Outline of Thesis

Chapter 1 presents an introduction to the concepts relevant to this study. Chapter 2 describes the procedures used in this work.

Chapter 3 is a review paper published in the Journal of Biomedical Materials Research: Part A, which covers the topic of multiphasic osteochondral scaffolds. Such scaffolds are constructed out of multiple materials and/or contain multiple architectures in order to satisfy the regenerative needs of both bone and cartilage tissues. I provided the review on computational scaffold design and finite element analysis of the (bio)mechanical effects that take place within such scaffolds.

Chapter 4 presents the manuscript to be published based on the results of this research. Work for this study was performed by Nicholas Uth under the supervision of Dr. Amy Yousefi, Dr. Jens Mueller, and Dr. Byran Smucker.
1.5 References


Chapter 2

2.1 Scaffold Construction

2.1.1 Formula Preparation

The composite formula was prepared in two stages. First, 2.1g poly(D,L-lactide-co-glycolide) (PLGA) and a variable amount of nano-hydroxyapatite (nHA) (table 2.1) were mixed with 3.6mL of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) in one beaker, and 0.225g collagen was mixed with 3mL HFP in a second beaker. Immediately upon addition of HFP to the first beaker, the PLGA/nHA mixture was stirred for 120 seconds before being sealed with parafilm. Similarly, upon addition of HFP to the second beaker, the collagen was stirred for 90 seconds and then sealed. Both mixtures were given 23 hours to dissolve before being stirred a second time: PLGA/nHA for 180 seconds, and collagen for 60 seconds. The collagen mixture was then poured into the PLGA/nHA mixture. This final composite was then stirred for 300 seconds and sealed for an additional hour.

Table 2.1: amount of nHA used in formula according to desired percent ceramic composition. Percentage is based on the mass of mechanically relevant components (PLGA & nHA).

<table>
<thead>
<tr>
<th>% Ceramic</th>
<th>m nHA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>15%</td>
<td>0.372</td>
</tr>
<tr>
<td>30%</td>
<td>0.9</td>
</tr>
</tbody>
</table>

A 10 mL, low-temperature, syringe barrel was prepared with a plastic 410µm internal-diameter tapered needle (Nordson EFD 7018298, Blue, 22 gauge). The composite was smeared onto the internal walls of the barrel in order to minimize formation of air pockets that could damage the generated scaffolds. The composite was then pushed to the tip of the cartridge with a 10mL-compatible piston, immediately prior to entering the needle.

2.1.2 3D Model Setup

A 3D model of a 20mm x 20mm x 3mm box was used as a basic scaffold. The model was then partitioned into 10 layers with a thickness of 0.3mm each, and offset above the stage by 0.3mm. The offset ensured that the needle would not be in direct contact with the surface of the bioplotter stage while extruding the first layer of strands.
2.1.3 Bioplotter Setup

The cartridge containing the formula was inserted into the low temperature robot-head and connected to the gas flow port. A thin sheet of polypropylene (PP) was clamped down to the plotting stage at its four corners. This acted as a plotting surface and enabled transfer of the generated scaffolds to the fume hood immediately upon completion.

Within the bioplotter software, the plotting settings were defined according to the material in use. The defined parameters are shown in table 2.2. The pressure (P) used for extrusion was used as the sole means of controlling the diameter of the strands within the scaffold. As such, its value for any given scaffold was varied based on the strand diameter prescribed by the DE, and according to a calibrated relationship between pressure and strand diameter for each whole plot.

Table 2.2: Material defined parameters used in scaffold generation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (°C)</td>
<td>20</td>
</tr>
<tr>
<td>P (bar)</td>
<td>Variable</td>
</tr>
<tr>
<td>u (mm/s)</td>
<td>0.9</td>
</tr>
<tr>
<td>Pre-flow Delay (s)</td>
<td>0.10</td>
</tr>
<tr>
<td>Post-flow Delay (s)</td>
<td>0.15</td>
</tr>
<tr>
<td>Wait Between Layers (s)</td>
<td>5</td>
</tr>
<tr>
<td>Minimum Length (mm)</td>
<td>3</td>
</tr>
<tr>
<td>Platform Temperature (°C)</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.3: Dot printing parameters assigned under material properties.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>t (s)</td>
<td>5.7</td>
</tr>
<tr>
<td>P (bar)</td>
<td>2</td>
</tr>
<tr>
<td>Needle Z-offset (mm)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The material-settings were then applied to the low-temperature tool, which was then mounted to the bioplotter. The pressurized gas was then purged into the syringe barrel at 1bar in order to press the formula into the needle and dispel some minor air pockets. Once a small amount of composite had extruded from the needle, the purge flow was stopped, and the needle was cleaned using the machine’s plastic brush.

The needle was then calibrated via dot-printing to account for the location of the tip. Prior to beginning the calibration, the XY-offset would be reset to 0,0. A small amount of formula would then be extruded according to the parameters indicated in table 2.3 onto a colored-
background and photographed. A dark background was used for 15% and 30% ceramic composition formulas, and a light background was used for the 0% formula, due to its transparency. This was necessary for the bioplotter’s camera to properly detect the printed dot.

The internal structure of the scaffolds (the arrangement of strands within a given set of layers) was defined according to the values shown in table 2.4. All internal structures were set to use continuous strand patterns. The distance between strands used for any given scaffold was defined by the designed experiment.

<table>
<thead>
<tr>
<th>Layer(s)</th>
<th>Distance from Contour [mm]</th>
<th>Deposition Angle [°]</th>
<th>X-Shift [mm]</th>
<th>Y-Shift [mm]</th>
<th>Distance between strands [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 3, 5, 7, 9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Variable</td>
</tr>
<tr>
<td>2, 4, 6, 8, 10</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>Variable</td>
</tr>
</tbody>
</table>

Then the 3D model was imported into the software as a build project. The material settings and desired internal structure were then assigned to the project. In order to conserve materials and reduce construction time, contours were deactivated.

In order to minimize risk of carry-over effects between scaffolds, the bioplotter and associated software were fully restarted and recalibrated prior to construction of any given scaffold (either for DE or for calibration).

2.1.4 Construction of Scaffolds

Prior to constructing WP scaffolds, two calibration scaffolds were constructed to determine the relationship between plotting pressure and strand diameter. The first scaffold would be generated using 1 bar pressure, and the second would be generated using 1.2 bar. As these scaffolds were exclusively used for calibration purposes, they were reduced to six layers of 0.3 mm thickness each to preserve materials and reduce construction time.

After 10 minutes of drying time, the calibration scaffolds were placed onto slides and imaged using a Zeiss Axio Vert.A1 light microscope. The strand diameters were determined via the associated ZEN software’s annotation functions. From the results of the imaging, a linear relationship between extrusion pressure and strand diameter was determined for the whole plot to be produced. Preliminary tests indicated that the relationship between pressure and strand diameter is reasonably linear within the 0.3 to 0.5 mm strand diameter bounds of the experiment. After the pressure-diameter calibration was complete, the material pressure was adjusted and the bioplotter was setup to begin construction of DE scaffolds.
In order to determine the material properties (compressive modulus and density) of the three composite formulations for use in the simulation and porosity calculations, three non-porous blocks were fabricated using the 3D bioplotter (alternate settings used: \( P = 1.1 - 1.4 \) bar, center-to-center distance between strands = 420 – 480 \( \mu \)m, layer thickness = 400).

2.1.5 Scaffold Drying

Upon completion, scaffolds were placed in a fume hood for 24 hours to dry while exposed to the air. Afterwards, they were removed from their PP sheets and transferred into petri dishes lined with filter paper. This was a precaution taken to ensure that the HFP did not dissolve the PP sheets and contaminate the scaffolds. The petri dishes were left uncovered to ensure exposure to air, but were kept in the fume hood for 28 days for continued HFP evaporation.

2.1.6 TGA Detection of Solvent to Determine Drying Time

A porous scaffold was constructed within the parameters used for DE scaffolds so that an efficient drying time could be determined. Following the previously described drying procedure, the amount of HFP within the scaffold was tracked via thermogravimetric analysis (TGA) over time. 10-15 mg samples were taken from within the scaffold (strands near the edges were avoided due to the increased surface area, which would allow for more rapid local HFP evaporation) at multiple drying times: 7, 14, 21, 28, and 42 days. TGA was performed using a TA Instruments Q500-2063 device. The device was set to ramp the furnace temperature from initial conditions (23-29°C) to 800°C at a rate of 10°C/min. A platinum pan was zeroed (mass) and then filled with a sample taken from the aforementioned scaffold. The change in mass of the sample versus temperature was monitored; the region between 30°C and 200°C was of particular interest as it encompasses the possible range of temperatures where HFP evaporates out (initial boiling temp for raw HFP = 59 °C) before mass stabilizes. The relationship between mass loss in this region and scaffold drying time is shown in figure 2.1. It was decided that at least 21 days were the minimum required for scaffold drying under atmospheric conditions, but 28 days was used for experimental scaffolds to provide a degree of security. The improvement provided by drying 42 days could not justify the additional 14 days.
2.2 Scaffold Characterization

2.2.1 Sampling

Three samples were taken from scaffolds via biopsy punches with an internal diameter of 8mm. These samples were taken at the scaffold corners such that they were close to the edges of the scaffold, but did not encompass any of the edge strands, which could be prone to the effects of gravity due to exposed sides. These three circular samples were measured for diameter, height, and mass prior to any mechanical testing for calculation of scaffold porosities. The remaining quarter of the scaffold was left intact, so that there would be a maximum amount of material for electron microscopy.

2.2.2 Scanning Electron Microscopy (SEM)

To prepare a scaffold for SEM, the unsampled portion of the scaffold was cropped on all sides to remove edge strands. Since the metal cutter used in sampling can deform surrounding strands, regions close to where samples were taken were also cropped. The resulting box was then cut perpendicularly to the top layer of strands such that the resulting piece could be used to observe the scaffold cross section.

Adhesive was applied to an aluminum SEM peg, and the two pieces of the sample were mounted such that the larger piece provided a top-down view of the scaffold and the smaller piece demonstrated the cross-sectional view. A small amount of colloidal silver paint was then applied to the edges of the two pieces and to the aluminum peg. This grounds the samples and helps affix them to the peg. The paint was given 30 minutes to dry before being placed in a
Denton Desk II Sputter Unit and given a 20 nm gold layer to give it a conductive surface. Using a Zeiss Supra 35VP SEM, the scaffolds were imaged (working distance = 8 mm, EHT = 5 kV) and measured for strand diameter, strand thickness, and the distance between strands (edge-to-edge).

2.2.3 Calculating Scaffold Porosity

Initially, porosity was calculated based on the apparent and true volumes of three scaffold samples. Apparent volume was calculated as if a sample were a non-porous cylinder, and true volume was the ratio between the mass of the sample and the density of the material used. Due to HFP retention and formation of bubbles within the solid blocks used to determine the densities of the composites, it was necessary to change the approach used to calculate scaffold porosity.

As such, we combined geometric measurements from SEM: strand diameter (D), strand height (H), and edge-to-edge spacing (EtE) with an equation modified from Landers et al. (2002) [1]. The original equation estimated porosity based on the ratio between the volume of strands within the scaffold (simplified as perfect cylinders) and a solid cube with the same dimensions as the scaffold. The equation was modified to take into account that the scaffold strands produced in this study were elliptical. This modification simply replaced the term for area of a circle within the $V_{\text{scaffold}}$ equation for the area of an ellipse. The resultant equation is as follows:

$$P = 100 \times \left( 1 - \frac{V_{\text{scaffold}}}{V_{\text{cube}}} \right) = 100 \times \left( 1 - \frac{\pi}{4} \frac{(D)(H)}{(L)(D + EtE)} \right)$$

Where $L$ is the layer thickness, which was assumed to be equivalent to the value used for slicing the 3D model given to the 3D bioplotter (300 µm). Use of this modified equation improved the estimated porosity values for all constructed scaffolds compared to the original equation, but the degree of improvement varied based on how elliptical/circular the strand cross-sections were.

2.2.4 Determination of Instron Parameters

In order to select settings appropriate for compression tests of our scaffolds, we first examined three potential preload values (4.4 N, 0.88 N, 0.44 N) and then three potential strain rates (10 mm/min, 5 mm/min, 1 mm/min). Six test samples of scaffolds were generated at two different topologies (low strand diameter vs. high strand diameter) for each setting considered (a total of 12 scaffolds, 36 samples).
In the case of preload, compressive modulus of the scaffold was negatively impacted by increasing preloads. Since preloading causes the scaffold to be stressed prior to data recording, using higher preloads caused (normalized) stress data to begin at higher levels of strain, effectively misrepresenting the true mechanical strength of the scaffold. Thus, the smallest preload value of 0.44 N was selected for future tests.

Afterwards, strain rate was examined (at 0.44 N preload). Strain rate results conflicted: for low diameter topology the 10 mm/min rate produced the best modulus, but the high diameter topology suggested 5 mm/min. Since the software gathered stress-strain data at specific time intervals (2 samples/sec), there were less data points gathered within the 10% strain region at higher strain rates. This contributed to larger variations in the stress-strain curve. In order to prevent this from affecting future compression tests, the number of samples taken per second was improved to 10 samples/sec. Since a key assumption regarding the behavior of scaffolds is that they behave linear elastically at low strains, it was important to use a higher strain rate, which generates more linear results at low strains. However, in order to reduce potential complications caused by large strain intervals caused by time-dependent sampling, the strain rate selected was 5 mm/min.

2.2.5 Instron Stress-Relaxation

An Instron 3344 single tower device was equipped with a 100 N load cell and programmed to perform a stress relaxation test. After a 0.44 N preload was tripped, a scaffold sample would be compressed by 40% of its total height at a rate of 5 mm/min and then allowed to relax for 10 seconds afterwards. The stress-strain data gathered was analyzed at the 10% compression (linear) region. The point closest to 10% and four points on each side were used to generate a linear trend of compressive stress verses compressive strain. The slope of this trend was taken as the compressive modulus for the sample, and the average of the three samples’ moduli was used as the final compressive modulus for the whole scaffold.

2.3 COMSOL 4.4 Model

2.3.1 Parameterization

Due to the iterative nature of the optimization model, it was necessary to construct a flexible model. The features of the model related to its geometry were parameterized so that the geometry could be easily changed by the user or by the optimization module. The parameters used are described in table 2.5.
Table 2.5: List of parameters used in COMSOL and related expressions or initial values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Expression / Initial Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius (r)</td>
<td>$1.9 \times 10^{-4}$</td>
<td>m</td>
</tr>
<tr>
<td>Diameter (D)</td>
<td>$2r$</td>
<td>m</td>
</tr>
<tr>
<td>Edge-to-Edge Strand Spacing (ete)</td>
<td>$8 \times 10^{-4}$</td>
<td>m</td>
</tr>
<tr>
<td>Center-to-Center Strand Spacing (space)</td>
<td>$ete + D$</td>
<td>m</td>
</tr>
<tr>
<td>Strand Length (len)</td>
<td>$8 \times 10^{-3}$</td>
<td>m</td>
</tr>
<tr>
<td>Layer Overlap (ov)</td>
<td>71</td>
<td>%</td>
</tr>
<tr>
<td>Compressive Strain (targStrain)</td>
<td>0.1</td>
<td>mm/mm</td>
</tr>
<tr>
<td>Number of Layers (numL)</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>Number of Strands per Layer (numStr)</td>
<td>$\text{floor}(1 + ((\text{len} - D) / \text{space}))$</td>
<td>N/A</td>
</tr>
<tr>
<td>Scaffold Height (height)</td>
<td>$(\text{numL} - 1)D(ov)$</td>
<td>m</td>
</tr>
<tr>
<td>Top &amp; Bottom Layer Surface Area (xArea)</td>
<td>$(D)(\text{len})(\text{numStr})$</td>
<td>m$^2$</td>
</tr>
<tr>
<td>Theoretical Volume if Model Were a Solid Cylinder (theoVol)</td>
<td>$(\pi)(\text{len}/2)^2(\text{height})$</td>
<td>m$^3$</td>
</tr>
</tbody>
</table>

2.3.2 Geometry

The scaffold model was generated as a series of parallel and perpendicular cylinders. The first cylinder was placed along the Y-axis at zero height. A perpendicular cylinder was placed along the x-axis at a height of $(D \times \text{ov})$ to create a second layer with some overlap between layers. A series of array operations were then used to generate the rest of the model by duplicating the initial cylinders. Boxes and Boolean operations were then used to crop the top and bottom layers as well as the edges of the model that crossed the X and Y-axis of the model, so that they formed flat surfaces that boundary conditions could be applied to. The final geometry of the model is shown in figure 2.2.

Figure 2.2: Final geometry of scaffold model.
Two integral operations (one encompassing all domains, and one covering the top-most boundaries of the top layer of strands) were added to the geometry in order to help calculate porosity throughout the volume and surface area of the top layer where displacement would be applied. These geometry variables are shown in table 2.6.

Table 2.6: Geometry variables and their respective expressions.

<table>
<thead>
<tr>
<th>Expression / Initial Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Volume (modelVol)</td>
<td>m^3</td>
</tr>
<tr>
<td>Model Porosity (porosity)</td>
<td>%</td>
</tr>
<tr>
<td>Top Layer Surface Area of Model (surfArea)</td>
<td>m^2</td>
</tr>
</tbody>
</table>

A custom material was then generated and applied to the entire domain of the model. The material properties are shown in table 2.7.

Table 2.7: Relevant material properties used for solid mechanics and their respective values.

<table>
<thead>
<tr>
<th>Expression / Initial Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (rho)</td>
<td>kg/m^3</td>
</tr>
<tr>
<td>Poisson’s Ratio (nu)</td>
<td>N/A</td>
</tr>
<tr>
<td>Young’s Modulus (E)</td>
<td>Pa</td>
</tr>
</tbody>
</table>

The Young’s Modulus was dependent on the assumed percent composition of nHA. This value would be determined by Instron compression tests performed on non-porous samples of the three formula variations (E = 0.94MPa, 4.79MPa, 11.86MPa for 0%, 15%, 30% nHA respectively).

2.3.3 Solid Mechanics

In order to mimic the experimental assumption that scaffolds behave linear elastically at low strain, a linear elastic material behavior was first applied to all domains within the model. Afterwards, two symmetry conditions were applied to any boundaries that aligned with the X or Y-axis. The bottom surface boundaries of the model were set as fixed constraints, and an instantaneous prescribed Z-direction displacement equivalent to (-targStrain*height) was applied to the top surface boundaries. All remaining boundaries were subject to the default free boundary conditions.

2.3.4 Optimization

A boundary integral objective for optimization was defined under the model tab. The objective would be examined exclusively at the top surface boundaries where the displacement
took place. This mimicked the manner in which the Instron compression device would measure stress during mechanical testing. The objective expression targeted compressive modulus as the ratio between third principal stress and the prescribed compressive strain. Third principal stress was used for the stress term, as it assumes the maximum possible compressive (negative) stress experienced at the boundary.

Under the optimization module tab, a Nelder-Mead optimization method was used, as it is capable of solving a constrained optimization problem [2]. Tolerance, number of evaluations and number of simultaneous evaluations were left at the default values (0.01, 1000, and 1 respectively). Objective function solely referred to what had been defined under the model tab, and was to be maximized by the module. Radius and edge-to-edge spacing were used as control variables. Radius was initially 190\(\mu\)m, but was allowed to range between 150-230\(\mu\)m. The spacing began at 0.8mm, and could range from 0.6-1mm. Porosity was then constrained to be within 50-99% void space.
2.4 References


Chapter 3

Article 1

Current Strategies in Multiphasic Scaffold Design for Osteochondral Tissue Engineering: A Review

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Abstract

The repair of osteochondral defects requires a tissue engineering approach that aims at mimicking the physiological properties and structure of two different tissues (cartilage and bone) using specifically-designed scaffold-cell constructs. Biphasic and triphasic approaches utilize two or three different architectures, materials, or composites to produce a multilayered construct. This paper gives an overview of some of the current strategies in multiphasic/gradient-based scaffold architectures and compositions for tissue engineering of osteochondral defects. In addition, the application of finite element analysis (FEA) in scaffold design and simulation of in vitro and in vivo cell growth outcomes has been briefly covered. FEA-based approaches can potentially be coupled with computer-assisted fabrication systems for controlled deposition and additive manufacturing (AM) of the simulated patterns. Finally, a summary of the existing challenges associated with the repair of osteochondral defects as well as some recommendations for future directions have been brought up in the concluding section of this paper.
### 3.1 Introduction

Partial- and full-thickness cartilage lesions of the knee are common disorders affecting people of all ages. More than 500,000 procedures related to cartilage injury are performed each year in the US alone. Self-repair of hyaline cartilage is limited and the tissue that forms is usually a combination of hyaline and fibrocartilage, which does not perform as well as hyaline cartilage and can degrade over time. Current clinical strategies to repair cartilage include autologous chondrocyte implantation, microfracture and mosaicplasty. However, there is still uncertainty about the quality of the repaired tissue and its ability to restore long-term function.

Osteochondral defects affect articular cartilage as well as the underlying subchondral bone. These defects are often associated with mechanical instability of the joint, and therefore with the risk of inducing osteoarthritic degenerative changes. Hence the treatment of chondral and osteochondral lesions is of great interest to orthopedic surgeons. In addition to osteoarthritis (OA), there is an urgent need for more efficient treatment of focal osteochondral injuries arising from sports. A paradigm shift is taking place in orthopedic surgery from using synthetic implants and tissue grafts to a tissue engineering approach, which makes use of biodegradable scaffolds combined with biological molecules or cells to regenerate tissues. Tissue-engineering requires scaffolds that balance temporary mechanical function with architectural properties (pore shape, size and interconnectivity) to aid in biological delivery and tissue regeneration. In recent years, osteochondral tissue engineering has been the subject of considerable investigation. The repair of osteochondral defects requires a tissue engineering approach that aims at mimicking the physiological properties and structure of two different tissues (cartilage and bone) using specifically-designed scaffold-cell constructs. When monophasic scaffolds are used, the natural environment is not imitated well for new tissue formation. For such purpose, multiphasic and gradient-based scaffolds have been proposed.

In vivo studies have shown that the outcome of repairing articular cartilage defects with tissue-engineered osteochondral composites is better than that of tissue-engineered cartilage. For example, an osteochondral composite could be securely implanted by press-fitting into defect without additional fixation. In order to construct tissues with different cell types and/or gradients in mechanical properties, a successful osteochondral scaffold should ideally have two or more regions with different compositions and/or microstructures, including pore size and
porosity. An intermediate region between the cartilage- and bone-scaffolds would allow for smooth transition to avoid scaffold delamination while facilitating stress transfer.\textsuperscript{23,24}

This paper presents an overview of the most recent multiphasic/gradient-based scaffold architectures and compositions for the repair of osteochondral defects, with a focus on studies published since 2009. First, a brief introductory overview of the current clinical strategies for the treatment of osteochondral defects has been presented. The main body of the paper covers the most commonly used scaffold materials, growth factors and cell types in bone and cartilage tissue engineering, as well as an up-to-date review of the current osteochondral tissue engineering approaches using natural/synthetic gradient-based scaffolds and biological gradients. The application of finite element analysis (FEA) in scaffold design and the simulation of in vitro and in vivo cell growth have been briefly covered. A summary of existing challenges associated with repairing osteochondral defects and some recommendations for future directions have been brought up in the concluding section of this paper.

3.2 Clinical strategies for the treatment of osteochondral defects

In articulating joints, the osteochondral interface is the junction between the articular cartilage and the underlying bone. In a review paper, Heinegård and Saxne have discussed the role of the cartilage matrix in osteoarthritis.\textsuperscript{25} Figure 1A shows a healthy joint with normal articular cartilage composed of four distinct layers, in which the lower-most layer is in direct contact with the subchondral bone.\textsuperscript{25} The articular cartilage is organized into pericellular, territorial and interterritorial matrices, each located at a specific distance from the chondrocytes (Figure 1A, inset image).\textsuperscript{25} Figure 1B depicts an osteoarthritic joint showing cartilage destruction, thicker subchondral bone, and decreased trabecular volume. The cartilage compartments are altered even at early stages of disease and exhibit cloning and multiplication of cells.\textsuperscript{25} The change in the cartilage compartments can be verified through immunohistochemistry staining (Figure 1B, inset image, arrow heads).\textsuperscript{25} Partial loss of cartilage and alterations in the underlying bone often lead to discomfort, chronic pain, and a reduction of joint movement.\textsuperscript{25,26}

Degradation of articular cartilage can also result from traumatic or sport injuries and other inflammatory joint conditions.\textsuperscript{27} Surgical treatments have been extensively studied for osteochondral defects.\textsuperscript{28-30} The goal of surgical treatments is to prevent further cartilage deterioration and improve joint articulation by restoring the joint surface to as close to its
original condition as possible. Some of these treatments are illustrated in Figure 1C-1E. The process of autologous osteochondral transplantation involves the removal and transfer of osteochondral plugs from non-weight-bearing areas to the osteochondral defect (Figure 1C). Besides the limitations caused by donor site availability and morbidity, the space between cylindrical grafts may affect the quality of the repair and lead to poor integration of full thickness gaps. Autologous chondrocyte implantation (ACI) has provided a new alternative for the treatment of symptomatic osteochondral defects in young patients. In this method, cartilage is taken from a low-contact area, and then chondrocytes are harvested and cultured in vitro (Figure 1D). Injection of the chondrocytes into the defective area aids cellular adhesion and fills in the defect. Theoretically, ACI should produce hyaline-like cartilage rather than fibrocartilage, with subsequent improvement in clinical outcomes.

Marrow-stimulating techniques such as microfracture, drilling, and abrasion arthroplasty (debridement) have also been used for the treatment of osteochondral lesions. These techniques involve penetrating the articular cartilage down to subchondral bone, which allows marrow stromal cells, platelets and other factors to aid in the repair process (Figure 1E). Bleeding from the subchondral bone promoted by these techniques creates vascular communications to the bone marrow from which pluripotent mesenchymal stem cells (MSCs) are released. The advantages of microfracture over debridement and drilling are the preservation of the subchondral plate and avoiding thermal injury, respectively. In general, cartilage surrounding symptomatic lesions is fibrillated and non-functional. Surgical debridement involves the removal of loose and unstable cartilage to promote the formation of new tissue from the bony base of the debrided lesion. In clinical studies, a symptomatic improvement in approximately 50% of the treated patients, with therapeutic effects lasting for about 1 year, has been reported for debridement. Therefore, the lack of permanent long-term solution is one of the major limitations of this technique.

A comparison between individuals treated with ACI and microfracture (121 patients at 5 years) has shown that those with onset of symptoms of less than 3 years had better outcomes with chondrocyte implantation than microfracture, although functional outcomes were similar at 12 months and 18 months. A similar process, known as matrix-induced ACI (M-ACI), involves implantation of chondrocytes previously expanded in vitro under special culture conditions into a collagen matrix. Matrix and cells are subsequently fixed in place by fibrin glue and/or sutures. The technique has been reported to be a safe and clinically effective procedure.
leading to the formation of hyaline cartilage, although it requires a two-stage surgery and is cost-intensive.\textsuperscript{37}

### 3.3 Bone and cartilage tissue engineering

Tissue engineering applies the knowledge of biology, cell transplantation, materials science, and bioengineering to construct biological substitutes that can restore and maintain normal function in diseased or injured tissues.\textsuperscript{38-40} In this strategy, a biodegradable three-dimensional (3D) porous scaffold is often used as a matrix to support cell adhesion, to guide new tissue formation, and to restore organ function. Tissue engineering is a potential alternative for the treatment of osteochondral defects, as it can be effectively used to regenerate cartilage, bone and the cartilage-bone interface.\textsuperscript{41} Natural and synthetic polymeric biomaterials have been widely used for cartilage tissue engineering. It is well known that cell function on a scaffold is related to the chemical properties of the scaffold material, as the scaffold surface chemistry affects cell adhesion, morphology and activity.\textsuperscript{42} Incorporation of calcium phosphate ceramics, e.g., hydroxyapatite (HA), into polymeric biomaterials can result in matrices with improved mechanical strength and better osteoconductivity for bone tissue engineering.\textsuperscript{43-46} This section covers the various elements involved in scaffold-based bone and cartilage tissue engineering, including scaffold materials, growth factors and cell types. The following section gives an overview of multiphasic scaffold design and biological gradients considered in recent studies for osteochondral tissue engineering.

#### 3.3.1 Scaffold materials

Biomaterials used in tissue engineering can be categorized into four major groups: natural polymers, synthetic polymers, metallic materials, and inorganic materials such as ceramics and bioactive glasses. Based on the need, multicomponent systems are designed to generate composites of enhanced performance.\textsuperscript{47,48} Polymers are indispensable in present tissue engineering concepts. Natural polymers like glycosaminoglycan, collagen, starch, hyaluronic acid, chitosan, alginate, and biodegradable bacterial plastics such as poly(hydroxyalkanoates) (PHA) are excellent biomaterials that support cell adhesion and regeneration while offering biocompatibility. One of the major constraints of natural polymers is that their mechanical properties are weaker when compared to ceramics and metallic materials.\textsuperscript{49}
Natural polymers can be easily surface modified with RGD groups containing specific molecular recognition sites in the bulk of the polymer chain, which can support and enhance various cellular activities, including adhesion, cell-cell communication, and proliferation.\textsuperscript{50} For example, doping of gelatin in alginate scaffolds has been used in bone and cartilage tissue engineering. Ca-alginate scaffolds cross-linked with gelatin have been shown to enhance cell adhesion and proliferation of mesenchymal stem cells (MSCs), while promoting the differentiation of MSCs into osteogenic and chondrogenic cell lineages.\textsuperscript{51} Chitosan is another widely studied natural biomaterial for cartilage regeneration.\textsuperscript{52–54} Its chemical structure is similar to glycosaminoglycans (GAGs) found in the extracellular matrix (ECM) of cartilage. This biomimetic nature has shown an influence on the morphology, differentiation and function of chondrocytes.\textsuperscript{55,56}

Collagen is one of the most abundant proteins in animal tissues. Its primary function is to provide and maintain structural integrity of the ECM. Being a major component in the ECM of cartilage and bone, collagen is considered as an ideal biomaterial for bone (collagen type I) and cartilage (collagen type II) tissue engineering.\textsuperscript{57–59} Experimental studies have demonstrated that chondrocytes maintain their phenotype when cultured in 3D collagen gels.\textsuperscript{58} Collagen also plays a vital role in tissue repair and wound healing processes.\textsuperscript{58,60} Nevertheless, poor mechanical properties have limited the use of collagen in load-bearing applications.\textsuperscript{61} Composites of collagen and bioceramics have been shown to generate scaffolds with improved mechanical properties.\textsuperscript{61–63} Immunogenicity, large scale production and purification are major issues that limit the use of collagen in clinical settings.\textsuperscript{64}

Synthetic biodegradable polymers used in tissue engineering include polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), poly(L-lactic-co-glycolic acid) (PLGA), polydioxanone (PDO), poly(propylene fumarate) (PPF), polyorthoesters (POE), polyphosphazenes and polyanhydrides.\textsuperscript{65–67} The advantages that synthetic biodegradable polymers offer lie in their range of chemistries, ease of processing and controlled molecular weight distribution that can be tailored to the target application.\textsuperscript{50} Most synthetic polymers are hydrophobic, and therefore possess lower bioactivity than natural polymers. To overcome this drawback, blends of hydrophobic and hydrophilic polymers can be used to enhance hydrophilicity. Shafiee et al. used nanofibrous scaffolds made of a blend of polyvinyl alcohol (PVA)/PCL for cartilage tissue engineering.\textsuperscript{68} PVA was electrospun with PCL to enhance
hydrophilicity and support cell adhesion. Both in vitro and in vivo studies in rabbits suggested that PVA/PCL scaffolds supported the proliferation and chondrogenic differentiation of MSCs and enabled the regeneration of cartilage. Another strategy that can be adopted to enhance physico-chemical properties (e.g., hydrophilicity, bioactivity and elastic modulus) of synthetic polymers is to incorporate bioceramics into these matrices.69–71

Bioceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) are known to enhance and promote biomineralization, making them suitable for bone tissue engineering.72,73 When implanted, bioceramics can promote the formation of an apatite layer on their surface (similar to calcium deposition in bone) leading to their integration to the host bone. MSC-seeded porous HA scaffolds have shown good osteoconductive properties after implantation in mice, although various factors such as pore size and porosity of the scaffold may affect bone formation.74 In addition to being biologically active materials, bioceramic-based scaffolds exhibit suitable stiffness, although they are brittle and cannot resist mechanical stresses. Biodegradability of calcium phosphates can be controlled through Ca/P ratio, although compounds with Ca/P ratio of less than 1 are not suitable for biological implantation. This is due to the higher solubility and speed of hydrolysis with decreasing Ca/P ratio.75 Controlled degradation profiles can also be obtained by optimizing the porosity of the scaffolds. However, enhanced porosity will result in decreased mechanical properties.76

Bioactive glasses constitute another important class of bioceramics for bone regeneration, of which 45S5 Bioglass® is the most representative member.77 The composition of 45S5 Bioglass® includes 45wt% SiO₂, network modifiers of 24.5 wt% Na₂O and 24.5 wt% CaO, as well as 6 wt% P₂O₅ in order to simulate the Ca/P constituents of hydroxyapatite (HA).78 Xynos et al. seeded 45S5 Bioglass® substrates with human primary osteoblasts and evaluated them after 2, 6, and 12 days.79 The results showed the ability of 45S5 Bioglass® to stimulate cell cycling, enhance osteoblast turnover, and produce bone-like tissue in vitro in a relatively short period of time. In vivo studies have demonstrated that bioactive glasses bond with bone more rapidly than other bioceramics.80 However, it is difficult to produce porous bioactive glass scaffolds for bone regeneration from 45S5 Bioglass® because it crystallizes during sintering.80 Some recent reviews have elaborated on the application of bioactive glasses for bone tissue engineering.80–82

To improve the mechanical properties of bioceramic scaffolds, biodegradable polymers have been used as coating materials. O’Shea and Miao improved the mechanical properties of
porous HA/TCP scaffolds by coating them with PLGA. The coated scaffolds showed about a 10-fold increase in compressive strength when compared with control scaffolds, with a negligible compromise in porosity. In another study, 45S5 Bioglass®-based scaffolds coated with poly(D-L-lactic acid) (PDLLA) improved the compressive strength while retaining the bioactivity of 45S5 Bioglass®-based scaffolds. Some of the recent studies on polymer-coated inorganic scaffolds for bone tissue engineering, including HA, bioactive glass, titanium dioxide (TiO₂), alumina (Al₂O₃), and zirconia (ZrO₂), have been reviewed by Yunos et al.

### 3.3.2 Growth factors and cell types

A review paper by Martin et al. regrouped osteochondral studies conducted between 1999 and 2006 according to repair strategy (scaffold strategy vs. cell strategy). According to the authors, for small and confined osteochondral lesions it might be sufficient to use a cell-free approach with appropriate scaffolds (e.g., adequate biomechanical properties and the capacity to resorb/remodel). Although in the case of more extended injuries, the delivery of growth factors is necessary for local cell recruitment. The use of a cell-based approach becomes mandatory if the wound bed is further compromised. Therefore, in most practical cases the scaffolding material alone cannot initiate biological responses that could support the regeneration process.

For osteochondral tissue engineering, progenitor cells that can differentiate into several different lineages or tissue specific cells, such as chondrocytes and osteoblasts are used. In general, chondrocytes are often used for osteochondral constructs implanted in vivo and for further development in vitro. The major drawback when using chondrocytes is their limited number in native tissue and unstable expression of phenotype. Chondrocytes constitute less than 5% of cartilage volume. In addition, isolation of chondrocytes is a difficult process as it requires collagenase, which can harm the cells. Another source of concern about chondrocytes is that they lose their phenotypic expressions in culture environments. This phenomenon was reported by von der Mark et al. Fröhlich et al. studied this phenomenon by quantifying the extent of dedifferentiation using q-PCR on rabbit chondrocytes until passage four. The results indicated that there was a major decrease in aggrecan, collagen type II and type I gene expressions when comparing the freshly isolated chondrocytes to the passage one cells. In addition, the proliferation capacity decreased during cultivation and was accompanied by cell enlargement, which was particularly evident in the third and fourth passages. Yonenaga et al. addressed the difficulties in chondrocyte isolation and seeding through optimizing the
collagen concentration.\textsuperscript{98} They reported that the cell viability could be increased by up to 10 fold if the tissue was treated with graded doses of collagenase rather than a single concentration (e.g., 1.2\% for 4 h, 0.6\% for 6 h, and 0.3\% for 24 h).\textsuperscript{98}

Sheehy et al. compared the growth of porcine bone marrow mesenchymal stem cells (BM-MSCs) and chondrocytes by seeding the cells onto hydrogel scaffolds and culturing under static and dynamic (rotational) conditions. The scaffolds were analyzed by biochemical analysis, mechanical testing, histology and immunohistochemistry. Chondrocytes appeared to be superior to BM-MSCs in both culture conditions.\textsuperscript{99} Moreover, the formation of a more homogeneous tissue in chondrocyte-seeded constructs suggested that dynamic conditions that could be beneficial for chondrocytes might be suboptimal for BM-MSCs.\textsuperscript{99} In spite of all these advantages, many researchers prefer to work with MSCs because of their abundance, multipotency and rapid multiplication.\textsuperscript{100} Moreover, MSCs can be isolated from various tissues and cultured in chondrogenic, osteogenic or co-culture osteochondral media for clinical applications.\textsuperscript{101–105}

In bone and cartilage repair/regeneration, growth factors act as molecular cues that promote cellular maturation and differentiation in a guided manner.\textsuperscript{106} The growth factors essential for osteochondral repair are produced intrinsically by the body. However, to reach the goal of tissue regeneration, higher concentrations of these growth factors have to be incorporated locally into/onto the scaffolds. These growth factors are known to promote both in vitro and in vivo tissue regeneration. Major growth factors that contribute to osteochondral tissue engineering include insulin-like growth factor–1 (IGF-1), basic fibroblast growth factor (bFGF), and transforming growth factor-\(\beta\) 1 (TGF-\(\beta\) 1).\textsuperscript{107–110} These growth factors have demonstrated anabolic cellular effects and increased production of matrix molecules. Various growth factors along with their scaffold combination and cell type are listed in Table 1.\textsuperscript{111–117}

Cell-free approaches to osteochondral regeneration have also been investigated by a number of research groups. Filová et al. studied the effect of cell-free hyaluronate/collagen type I/fibrin composite scaffolds containing PVA nanofibers enriched with liposomes, bFGF and insulin on the regeneration of osteochondral defects.\textsuperscript{118} It was reported that the scaffolds were able to enhance the regeneration of osteochondral defects in minipigs. Cao et al. applied a bilayered construct with or without adipose-derived stem cells (ASCs) to repair full-thickness defects in the patellar groove of rabbits.\textsuperscript{119} Utilizing a score ranging between 0 (best) to 20
(worst), they reported that the semi-quantitative score of the cell-based group (4.2 ± 1.2) was significantly better than the cell-free group (13.8 ± 2.5). As mentioned earlier, the success of cell-free approaches depend on the defect size.\textsuperscript{9}

### 3.4 Osteochondral tissue engineering

Native tissues are anisotropic and inhomogeneous in nature, composed of different types of cells and extracellular matrices (ECMs) in specific spatial hierarchies.\textsuperscript{120} For example, articular cartilage consists of different zones with varying types and orientations of collagen fibers and collagen-binding proteins.\textsuperscript{25,121} The molecular organization of articular cartilage is shown in Figure 2A.\textsuperscript{25} The pericellular matrix is the zone where molecules that interact with cell surface receptors are located (e.g., hyaluronan binds the receptor CD44). Next to the pericellular matrix lies the territorial matrix, followed by the interterritorial matrix at the largest distance from the cell.\textsuperscript{25} The complex microstructure of cartilage enables proper dissipation of loads throughout the tissue. Similarly, bone has an anisotropic structure due to the spatial differences in the concentration and orientation of its mineral and organic constituents.\textsuperscript{121} The hierarchical structure of bone is shown in Figure 2B.\textsuperscript{122} During bone formation, collagen molecules assemble into fibrils, which are mineralized via the formation of apatite crystals.\textsuperscript{122}

The development of artificial micro- and nanostructures to replicate the complex features of biological tissues can lead to promising biomaterials for tissue engineering. For example, to replicate the extraordinary strength and durability of natural bone, the current trend is to design biomaterials that nearly mimic the structural organization of bone from the nanoscale upwards.\textsuperscript{123} Hence, some studies have aimed to produce functionalized scaffolds that can mimic the nanofibrous structure of the natural extracellular matrix (ECM) of biological tissues to enhance cell activation.\textsuperscript{124} In recent years, there has been an effort to combine additive manufacturing (AM) and electrospinning (ES) techniques to produce bimodal scaffolds, where micro- and nano-scale features can be combined.\textsuperscript{125}

Figure 2C shows the typical process steps for a combined AM-ES technique that makes use of PCL as a scaffold material.\textsuperscript{126} An AM system (e.g., melt-dispensing) is used to obtain the micro-sized PCL struts, whereas the ES system is used to generate the interlayered PCL micro/nanofibers. The system can potentially offer additional features, such as dispensing cell-laden hydrogel struts (e.g., alginate). The cross-sectional view of the scaffold in Figure 2C shows
the multiple features that can be generated during this process.\textsuperscript{126} The bimodal architecture of a 3D scaffold produced by the AM-ES technique is depicted in Figure 2D, showing a PCL microfibrous layer and an electrospun PCL/collagen nanofibrous matrix.\textsuperscript{124} These hierarchical constructs contain large pores enabling cell penetration, while the electrospun fibers effectively increase the surface area available for the adhesion of penetrating cells.\textsuperscript{125}

Nanostructured materials with surface properties promoting protein adsorption and favoring cell adhesion have a greater chance of stimulating new bone growth when compared to conventional materials (Figure 2E).\textsuperscript{123} This is one of the underlying mechanisms that make nanomaterials superior to conventional materials for tissue engineering applications.\textsuperscript{81,123,127} A significant volume of recent publications have been dedicated to understanding and controlling matter on the nanometer scale where unique phenomena enable new functional applications.\textsuperscript{128} Elaborating on the hierarchical approaches at macro-, micro-, and nanoscales goes beyond the scope of this review paper. Therefore, the emphasis is placed on macro/microscale features of multiphasic scaffolds, as well as a brief overview of additive manufacturing and computational modeling to optimize these constructs.

### 3.4.1 Multiphasic scaffold architectures

Given the district differences between the hierarchical structures of cartilage and bone, engineering multilayer scaffolds with controlled properties in each layer could allow the replication of the local microenvironment of the osteochondral tissue.\textsuperscript{120,129,130} In general, osteochondral tissue engineering strategies can be categorized into monophasic, biphasic, and triphasic depending on the cellular/biological or physical/chemical characteristics of the scaffold (Figure 3).\textsuperscript{18} Biphasic and triphasic approaches utilize two or three different architectures, materials, or composites to produce a multilayered construct. A single material can also be used to produce biphasic or triphasic constructs if significant variations in physical properties exist between the different layers.\textsuperscript{18} Recently, Martin et al.,\textsuperscript{9} Castro et al.,\textsuperscript{26} Nukavarapu and Dorcemus,\textsuperscript{41} and Keeney and Pandit\textsuperscript{131} have discussed the potential of multi-component scaffolds for osteochondral tissue engineering, while emphasizing the challenges in their use for clinical practice. Optimal scaffold design of such constructs is critical for cell attachment, survival and matrix production. Pore size, pore geometry, overall porosity and material used are all critical factors that influence cell biology.\textsuperscript{72}
To satisfy both the mechanical and biological requirements, a wide range of porous scaffold systems with gradient-based porosity and pores size have been developed. In addition, it has been hypothesized that mechanical properties of scaffolds should ideally match or be within the range of actual tissue properties. This could enable the scaffold to withstand the physiological loading without failure within the tissue defect, which would mean faster rehabilitation for the patient. Early force transmission through the repair site could stimulate the regenerated tissue with biomechanical properties closely matching those of surrounding native tissue. The rapid restoration of tissue biomechanical function remains an important challenge, emphasizing the need to replicate the structural and mechanical properties of the tissue using novel scaffold designs.

In the last decade, the advancement in additive manufacturing (AM) technology has led to the production of free-form porous scaffolds with custom-tailored architecture. Therefore, AM technology has become increasingly common in recent years mainly due to its ease of operation as well as its ability to translate a patient’s scanned image to a computer-aided design (CAD) model. Some of the enhanced features of current AM technology include the introduction of nano-sized features, as well as the tremendous potential the technology offers for producing functionally graded structures. Effective scaffold design optimization and subsequent fabrication using AM systems would allow meeting the mechanical requirements for faster restoration of tissue function.

Recent efforts in understanding scaffold architecture-property relationships include a study by Sudarmadji et al. on functionally graded scaffolds (FGS). The team developed a database to correlate the scaffold porosity and the corresponding compressive stiffness. The database included 13 different polyhedral units produced by selective laser-sintering that could be assembled into scaffold structures. The resulting porosity, compressive stiffness and yield strength of the scaffolds varied between 40–84%, 2.74–55.95 MPa and 0.17–5.03 MPa, respectively. This range of stiffness was reported to closely match the cancellous bone in the maxillofacial region. Nevertheless, in osteochondral tissue engineering the need to properly design the bone/cartilage interface adds to the complexity of the scaffold design strategy. This is mainly because the relationship between scaffold structural parameters and osteochondral tissue requirements is not well established.
Scaffolds with interconnected unidirectional channels are often used in bone and cartilage tissue engineering, since unidirectional channels may provide a path of least resistance and facilitate in vivo vascularization and the formation of new tissue. Moreover, it has been shown that scaffolds with orthogonal channels can exhibit a larger bone growth area than scaffolds with radially oriented channels. In extrusion-based techniques, a repeating pattern is often used to simplify the deposition process. More complex patterns can be obtained by changing the deposition angle between adjacent layers (Figure 4A), also known as honeycomb-like patterns. The use of space-filling curves has also been explored (Figure 4B). Due to the restricting features of extrusion-based techniques, non-intersecting continuous curves are particularly attractive. This includes fractal space-filling curves that can be generated using a simple pattern as a starting point, which then grow through the recursive application of a certain set of mathematical rules.

3.4.2 Multiphasic scaffold compositions

Biomaterial selection is challenging due to the need to satisfy the chemical, morphological, biological and surface requirements for a given application. Many of these properties remain unspecified until the final product is tested in vivo. The scaffold composition plays a vital role in providing a platform for cellular growth. Thus, there is still a constant search for ideal biomaterials. An in-depth understanding of the advantages and drawbacks of potential scaffold materials is required for rational biomaterial selection. A variety of scaffold systems for osteochondral tissue regeneration have been developed to meet the complex functional demands of cartilage and bone tissues, given the distinctive differences in their structural, chemical, and mechanical properties.

Multiphasic/gradient-based strategies that tailor the scaffold composition to the type of regenerated tissues are currently being sought. This involves both natural and synthetic materials, as well as extracellular matrix (ECM)-derived biomaterials. Figure 5 shows some osteochondral scaffold designs featuring cartilage- and bone-specific compartments. One strategy is to engineer two individual cartilage and bone scaffold layers, and then join the two separately fabricated scaffolds by suturing, glue, or simple press fitting. Swieszkowski et al. used biphasic constructs composed of fibrin/PCL or PCL/PCL-TCP phases (Figure 5A). These two phases were fabricated separately and seeded with an appropriate number of cells, and then cultured in chondrogenic and osteogenic media for cartilage and bone regeneration, respectively.
Finally, the two phases were integrated into one construct using fibrin glue. However, these methods are limited by the inferior integration between cartilage and bone tissues resulting in the eventual separation of the two tissues.

Interdiffusion of the two layers forming a biphasic osteochondral construct could serve as a means of integrating the chondral and bony phases. Grayson et al. used agarose gel for the cartilage phase of their osteochondral scaffolds, whereas decellularized bone was selected for the bone region (Figure 5B). Agarose was used due to its ability to yield good mechanical properties with immature chondrocytes. The rationale for selecting decellularized bone was to provide adequate mechanical properties, osteo-inductive architecture, and biochemical composition. The cell seeded bone scaffolds were overlaid allowing a penetration depth of 500 µm of agarose gel into the bone scaffold, followed by solidification of agarose at room temperature. It was reported that the interface formed in these biphasic constructs upon culturing in an osteochondral bioreactor was different from that of native issue. This study emphasized the need for interface design so as to recapitulate the native interface and investigate the heterogeneous cell-cell communication in this region.

Harley et al. fabricated a series of collagen type I/glycosaminoglycan/calcium phosphate (CGCaP) scaffolds by freeze-drying technique. The composition of the CGCaP suspension, the pore architecture, CaP phase chemistry, as well as the crosslinking density were independently controlled in this study. In addition, the team developed multiphasic osteochondral scaffolds from a mineralized CGCaP suspension and an unmineralized collagen type II/glycosaminoglycan (CG) suspension (Figure 5C). The interdiffusion between the layered suspensions before freeze drying enabled generating an interface zone between the two layers (liquid-liquid-phase cosynthesis). The study did not report cell seeding and growth for these scaffolds. Wang et al. used a similar interdiffusion step to produce biphasic scaffolds with a gradual interface. Articular cartilage extracellular matrix (ACECM) and hydroxyapatite (HA) were used for the two components, leading to a porous, oriented upper layer and a dense, mineralized lower layer (Figure 5D). It was reported that the difference in porosities and pore sizes between the two layers resulted in a low-permeable interface. The scaffolds seeded with rabbit chondrocytes revealed well-distributed cells in the non-mineralized zone, while showing only a few cells adhering to the interfacial zone. No cells entered into the mineralized component, suggesting a
cell-barrier layer at the interface.\textsuperscript{157} The team proposed further studies to evaluate the potential of these scaffolds for osteochondral tissue engineering.

Miyagi et al. proposed a combination of a $\beta$-TCP block with a scaffold-free sheet formed using MSCs for osteochondral regeneration.\textsuperscript{104} A similar approach using centrifuged chondrocyte cell sheets had been previously proposed by Niyama et al.\textsuperscript{105} It should be noted that the cell-sheet approach has some limitations due to technical challenges in stimulating the differentiation into two respective lineages (osteoblasts and chondrocytes), because only one type of culture medium can be used for a cell sheet.\textsuperscript{104}

A combination of additive manufacturing (AM) and electrospinning has been used to produce osteochondral scaffolds with multiscale features and varying compositions.\textsuperscript{158} Tuan et al. proposed biphasic scaffolds comprised of a PCL cartilage phase and a PCL – TCP matrix that served as the bone component.\textsuperscript{158} The scaffolds were built using the fused deposition modeling (FDM) process, seeded with MSCs via fibrin encapsulation, and patched with a PCL – collagen 20% electrospun mesh to prevent cell loss and facilitate the diffusion of nutrients from the synovial space. In vivo studies in a pig model indicated favorable outcomes in the cartilage region, with a reduced incidence of fibrocartilage and improved GAG content when compared to cell-free and mesh-free scaffolds. However, besides the implant design, the implantation site appeared to affect the in vivo outcomes (medial condyle vs. patellar groove).\textsuperscript{158}

When combined with appropriate growth factors (e.g., TGF-$\beta$1), alginate, agarose and chitosan have shown to help with maintaining the spherical morphology of chondrocytes and supporting chondrogenic differentiation and cartilage-specific matrix deposition.\textsuperscript{56,159} Jeon et al. developed multiphasic scaffolds comprised of 2% alginate hydrogel and a biphasic PCL scaffold (made by a combined FDM and electrospinning).\textsuperscript{159} To integrate the alginate and PCL components, alginate was partially de-cross-linked and press-fitted on top of the biphasic scaffold, which enabled alginate to partially infiltrate the pores of the PCL-FDM scaffolds, and then re-cross-linked. Histological analysis of the constructs implanted subcutaneously in rats showed that some alginate constructs had been separated from the PCL scaffolds possibly due to gradual weakening of the interface region. In another study, a biphasic osteochondral composite was developed by Liu and Jiang, combining a chondral phase composed of chitosan/collagen with a bone-ECM mimicking phase made of $\beta$-TCP (Figure 5E).\textsuperscript{160} A glue made of cross-linked 1.2 % (w/v) sodium alginate and CaCl$_2$ was used between the chitosan/collagen mixture and the

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sintered porous β-TCP scaffold, and then the set up was subjected to freeze-drying. The combination of biphasic scaffolds and a double-chamber bioreactor was found to promote cellular proliferation and trigger simultaneous chondrogenic and osteogenic differentiation of MSCs within the porous constructs.

Castro et al. have reviewed some other scaffold compositions for the treatment of osteochondral defects. Other recent multiphasic scaffold systems include bilayered chitosan–gelatin scaffolds, trilayered PEG-based hydrogel systems with varying ECM composition, and PCL/alginate scaffold systems. It should be mentioned that material selection for osteochondral regeneration strategies are highly contingent on the specific manufacturing technique employed, and on the way cells are used in each strategy (encapsulated within or seeded on the surface of the scaffold). Another recent review paper gives an overview of in vivo osteochondral repair studies that have been undertaken since 2009.

3.4.3 Bone-cartilage interface design

Natural articular joints are characterized by a strong, stable interface between cartilage and bone. The CaP content gradually decreases from ~75 wt % at the subchondral bone plate to zero in articular cartilage. In addition, collagen type II level decreases gradually from the superficial to the deep calcified zone of articular cartilage, whereas type X collagen and proteoglycan levels increase. These variations lead to an increase in compressive modulus from the superficial to the deep zone. The smooth compositional transition between vascular/mineralized bone and nonvascular/unmineralized cartilage has a major role in the stability of cartilage-bone interface. Therefore, it is important to reproduce the native architecture and function, as well as the interface zone of the osteochondral tissue. To date, the formation of a stable interface between cartilage and subchondral bone scaffolds remains a significant challenge. Some review papers have briefly covered the recent efforts in interface design for osteochondral scaffolds.

Nooeaid et al. developed triphasic scaffolds where the middle layer functioned as an adhesive at the transition zone. The subchondral bone scaffold was composed of porous 45S5 Bioglass® and alginate composites (Alg-c-BG), whereas freeze-dried alginate-based foams (Alg-foam) were used as the cartilage layer scaffolds. The two layers were integrated using an alginate/45S5 Bioglass® hybrid interface (Figure 5F). To generate the interface layer, 2 w/v%
solution of alginate/Bioglass® (1:3 by wt) in DI water was prepared. The solution was brushed on one side of the Alg-c-BG scaffold, and then the Alg-foam was placed on the adhesive coated side of the scaffold and pressed manually. Subsequent immersion in 0.5M CaCl₂·2H₂O for 24 h enabled crosslinking the Alg-foam and the interface. Delamination did not occur during normal handling for testing or upon immersion in a simulated body fluid (SBF) for 28 days.¹⁶⁴

Da et al. developed a compact layer made of PLGA/β-TCP as an interface zone between the cartilage and bone phases of their multilayer scaffolds (Figure 5G).¹⁶⁵ The cartilage phase was derived from bovine decellularized articular cartilage ECM. The bone phase was produced by additive manufacturing (AM), and was made of a PLGA/β-TCP skeleton wrapped with collagen Type I. To bond the two phases, the surface of the compact layer was dissolved by application of 1,4-dioxane. Then, the cartilage phase was pressed onto the dissolved compact layer, frozen for 2 h at −80°C, and subsequently lyophilized for 24 h. In vivo results in rabbits revealed superior GAG and collagen content in the compact layer-containing scaffolds compared to the control group.¹⁶⁵ The team suggested that the interface layer could potentially enhance the biomechanical properties of the biphasic scaffolds and the regenerated osteochondral tissue.

Cao et al. proposed layered scaffolds featuring three distinct regions.¹¹⁹ The upper chondral phase was composed of cross-linked collagen-chitosan, collagen gel and bovine bone morphogenetic proteins (bBMPs). The lower bony phase was composed of collagen-modified bovine cancellous bone, collagen gel and bBMPs. The two phases were separated by an air-dried collagen membrane. The constructs, with or without adipose-derived stem cells (ASCs), were applied to repair full-thickness defects in the patellar groove of rabbits. Implantation of the layered constructs alone did not enhance repair, whereas the contrasts combined with ASCs were found to enhance osteochondral regeneration.¹¹⁹ Qu et al. developed layered scaffolds composed of polyvinyl alcohol (PVA), gelatin, nano-hydroxyapatite (nHA), and polyamide6 (PA6).¹⁶⁶ The cartilage layer was made of porous PVA cryogel with pore diameter of 5–40 µm, 70% porosity and 71.6% water content. The bone layer was composed of porous nHA/PA6, with a pore diameter of 100–400 µm and 80% porosity. The interface was made of nonporous PVA. The scaffolds seeded with induced bone mesenchymal stem cells (BMSCs) and implanted at ectopic sites (rabbit muscle pouch) showed a potential to differentially support cartilage and bone tissue generation.¹⁶⁶ The team also reported that the subchondral bone layer was completely integrated with the cartilage layer.
Some studies have developed triphasic scaffolds featuring chemical and morphological gradients by stacking a highly mineralized composite layer made of HA (70%)/collagen (30%), resembling the subchondral bone layer, an intermediate layer with reduced mineralization (as tidemark), and an upper layer made of collagen. In a clinical study, twenty seven patients who were affected by osteochondritis dissecans (OCD) of the femoral condyles (average defect size $3.4 \pm 2.2 \text{ cm}^2$) were treated with the implantation of triphasic scaffolds (Figure 5H). The treatment results were analyzed using the cartilage standard evaluation form as proposed by the International Cartilage Repair Society (ICRS). A good clinical outcome at 2-year follow-up was reported, despite certain postoperative adverse events such as swelling and stiffness observed in some patients.

3.4.4 Biological gradients

In addition to physical gradients, biological gradients also play a vital role in tissue engineering. Since cartilage and bone have different biochemical, structural, and mechanical microenvironments, osteochondral scaffold designs that do not address such differences suffer from obvious limitations. Multiphasic designs do not necessarily replicate all such parameters; therefore, osteochondral constructs with tissue-specific designs may contribute to the generation of functional osteochondral constructs within a shorter timeframe. The ability to fabricate scaffolds containing systematic gradients in distribution of stimulators can enable simultaneous triggering of osteogenic and chondrogenic factors and provide additional means for mimicking the important gradients observed in native tissues. However, very few reports are available on gradient-based delivery systems of growth factors for osteochondral tissue engineering.

Wang et al. used PLGA and silk fibroin microspheres to investigate microsphere-mediated delivery of bone morphogenetic protein-2 (rhBMP-2) and insulin-like growth factor–1 (rhIGF-1) in polymer scaffolds and its impact on osteochondral differentiation of human bone marrow derived MSCs (hMSCs). The growth factors were incorporated in the scaffolds as a reverse gradient combining the two factors, as well as a single concentration gradient. Initially a cylindrical alginate gel was fabricated, and then microspheres were incorporated as gradients. Silk microspheres were found more efficient than PLGA microspheres in delivering rhBMP-2, probably due to sustained release of the growth factor, while less efficient in delivering rhIGF-1, which was attributed to loading efficiency. The shallow growth factor gradients induced non-gradient trends in hMSC osteochondral differentiation. Aqueous-derived silk porous scaffolds
were also used by the team to incorporate silk microspheres using the same gradient process. After culturing for 5 weeks in a medium containing osteogenic and chondrogenic components, hMSCs exhibited osteogenic and chondrogenic differentiation along the concentration gradients of rhBMP-2, but not along the rhIGF-1 gradient system. These results suggested that silk microspheres were more efficient in delivering rhBMP-2 than rhIGF-1 for hMSCs osteochondrogenesis.\textsuperscript{117}

A hydrogel composite consisting of oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) was used by Guo et al. for osteochondral regeneration.\textsuperscript{174} The top layer consisted of rabbit MSCs encapsulated in OPF with either blank MPs or TGF-\(\beta\) 3-loaded MPs. In the bottom layer, OPF hydrogel composites with blank MPs were used to encapsulate osteogenically precultured MSCs (0, 3, 6 and 12 days).\textsuperscript{174} After cell encapsulation, the bilayered composites were cultured in chondrogenic medium. The results indicated that TGF-\(\beta\) 3-loaded MPs could significantly enhance chondrogenic differentiation of MSCs in the chondrogenic layer. Osteogenically precultured cells maintained their osteoblastic phenotype in the osteogenic layer; however, TGF-\(\beta\) 3 showed an inhibitory effect on cell mineralization. In addition, encapsulated cells of different degrees of osteogenic differentiation were found to significantly affect the chondrogenic gene expression of co-cultured MSCs in both the presence and absence of TGF-\(\beta\)3.\textsuperscript{174}

Saha et al. used mulberry (Bombyx mori) and non-mulberry (Antheraea mylitta) silk fibroin scaffolds for osteochondral tissue engineering, with and without growth factors.\textsuperscript{175} Non-mulberry constructs seeded with hMSCs showed neo tissues containing chondrocyte-like cells after 4 to 8 weeks of in vitro culture, whereas mulberry constructs seeded with hMSCs formed bone-like nodules. The team also conducted cell-free growth-factor guided in vivo studies in order to determine the potential of these scaffolds to attract and differentiate endogenous progenitor cells. The constructs used for in vivo implantation were monophasic in composition, but were coated with TGF-\(\beta\)3 and BMP-2 in their respective cartilage and bone phases, before being assembled using fibrin glue (Figure 5I).\textsuperscript{175} The osteochondral defects in the patellar groove of the knee joints of Wistar rats were filled with mulberry or non-mulberry scaffold discs with or without growth factors.\textsuperscript{175} Excellent integration of the neo-tissue with the host tissue was reported in all constructs. Therefore, the team proposed the use of multi-layered combination of
mulberry and non-mulberry scaffolds, for bone and cartilage respectively, for cell-free osteochondral tissue engineering.\textsuperscript{175}

Additive manufacturing has been explored in recent years to generate biological gradients within tissue engineering scaffolds.\textsuperscript{126,163,176,177} In these studies, hydrogel systems have enabled encapsulating cells and growth factors in a multilayer fashion. Fedorovich et al. used a 3D fiber deposition (3DF) technique for the fabrication of cell-laden, heterogeneous hydrogel constructs as potential osteochondral grafts.\textsuperscript{177} The team encapsulated and printed fluorescently labeled human chondrocytes and osteogenic progenitors in alginate hydrogel, with different zones for both cell types. Changing the fiber spacing or angle of fiber deposition resulted in scaffolds with different porosities and elastic moduli. It was reported that distinctive ECM regions were formed in vitro and in vivo according to the anticipated tissue type. Some studies have made use of synthetic biomaterials such as PCL and PLGA to enhance the mechanical stability of biologically-graded constructs.\textsuperscript{126,163} In the bioprinting process shown in Figure 5J, the sequential dispensing is repeated to stack synthetic biomaterials and hydrogels, loaded with cells and growth factors, to build multiplayer constructs featuring chemical and biological gradients.\textsuperscript{163}

From cartilage/bone interface design perspective, microfluidic systems can offer opportunities for studying cell differentiation and interfacial construction in vitro.\textsuperscript{178,179} Figure 5K shows a microfluidic system for generating a gradient-based stem cell-laden hydrogel construct.\textsuperscript{178} The system enables different cell culture/differentiation media (OM: osteogenic medium, M: normal medium, CM: chondrogenic medium) to flow into the hydrogel slab, where distinct zones with specialized cell lineages and extracellular matrices can be formed.\textsuperscript{178} Shi et al. reported that after 25 days of culture using this microfluidic device, stem cells differentiated into osteoblasts and chondrocytes in their respective zones, while a biological gradient mimicking the bone-cartilage interface was observed in the middle zone of the hydrogel.\textsuperscript{178}

### 3.5 Computational scaffold design

Computational methods have been widely used in designing implants for tissue replacement. A recent FEA of implant design has suggested that both the size and material properties of implanted cartilage replacements (ICR) have a major role in the failure of the fibrin glue used to attach the implant to the native tissue.\textsuperscript{1} According to this study, increasing the compressive modulus (E) by 25%, with respect to that of native articular cartilage (AC), can
reduce the fibrin damage in both the osteochondral and chondral implants, whereas decreasing E by 25% may lead to a higher damage at the interface (Figure 6, A and B). This study also suggested that Poisson's ratio (v) of the ICR might affect the integrity of the fibrin adhesive. While the fibrin surrounding the osteochondral implant showed less damage at higher value of v and more damage at a lower values of v, the simulated trend was the opposite for the chondral implant (Figure 6, C and D). This was attributed to the important role of the collagen network in instantaneous lateral expansion of articular cartilage. Therefore, a less organized network of collagen fibers may result in a lower v. Similar results could be expected in tissue-engineered constructs, since they lack an organized collagen fiber distribution and have lower collagen content compared to native AC.

Finite element modeling tools have been used by many research teams to predict the modulus of 3D scaffolds produced by a variety of fabrication techniques. This is particularly important for multi-layer scaffolds used for regeneration of layered tissues consisting of cartilage and subchondral bone. In addition, scaffold design for tissue engineering involves many parameters that directly influence the rate of tissue regeneration throughout the scaffold microstructure. Investigating the effect of each specific scaffold parameter on tissue regeneration using in vitro and in vivo techniques can be costly and time consuming. Therefore, combining finite element modeling tools with mechano-biological models could potentially assist researchers in predicting the outcomes of tissue culture trials. Evaluating the effect of individual factors on cell migration, proliferation and angiogenesis may also help with generating experimentally testable hypotheses regarding optimal scaffold design, while allowing to predict the outcomes of tissue engineering based on in vitro/in vivo conditions. Multiple simulations can be performed in order to identify a topology that would perform well under physiological loading conditions while allowing tissue ingrowth. Then, additive manufacturing (AM) techniques can be used to produce prototypes of the optimized scaffold for experimental testing, enabling researchers to explore more innovative topologies and their resulting effects on mechanical strength and tissue regeneration.

Cahill et al. designed simple CAD models of two scaffold architectures using ABAQUS software. The models were used to estimate the effective and shear moduli of the scaffolds. Prototypes of the scaffolds were fabricated via selective laser sintering (SLS) and subjected to experimental testing. It was found that the FEA overpredicted the moduli of the scaffolds to
different extents in x-y-z directions. For polycaprolactone (PCL) scaffolds, the effective modulus was overestimated by 67%, assuming isotropic properties. For polyamide (PA) scaffolds, the effective modulus under compression was overpredicted by 81% in the x direction, whereas the moduli in the y and z directions were overestimated by 125% and 147%, respectively. The results called for a greater understanding of how the microstructure of the scaffolds, such as surface roughness and microporosity, affected the scaffold properties.\textsuperscript{190}

McIntosh et al. performed FEA in order to simulate the properties of three hydroxyapatite (HA) scaffolds of varied properties as they became integrated with surrounding healing bone.\textsuperscript{191} The scaffolds were produced using a directed deposition technique followed by sintering. It was found that shear modulus was affected by the geometry of the bone surrounding the scaffold. Whether the bone coated the scaffold or bridged across the pores affected the scaffold’s ability to resist shear forces. The instance where bone bridged the pores of the scaffold served to strengthen the system. However, the interaction with the bone geometry did not seem to affect the elastic modulus. Decreasing the elastic modulus of the material used for scaffold fabrication had a greater impact on the overall mechanical properties than did the scaffold porosity.\textsuperscript{191}

Melchels et al. generated CAD models of 3D scaffolds with varied architectures (cube, diamond, and gyroid). High-resolution stereolithography was used to fabricate the designed scaffolds made of poly (D,L-lactic acid) (PDLLA) or poly(D,L-lactide-co-ε-caprolactone) P(DLLA-co-CL).\textsuperscript{192} The bulk properties of solid materials made by stereolithography were measured and described mathematically using a constitutive model. The model was then implemented into ABAQUS finite element software, which allowed simulating the deformation characteristics of the porous scaffolds. The simulations suggested that the gyroid structure could provide evenly distributed mechanical stimuli to cells within the scaffold, which would be beneficial for cell growth and differentiation.\textsuperscript{192}

Olivares et al. used FEA to optimize the scaffold architecture so as to enhance cell differentiation.\textsuperscript{193} To this end, they examined the effect of scaffold microstructure and inlet fluid flow conditions on mechanical stimuli transferred to cells within the scaffold via scaffold deformation. The design variables included varied porosities (55%, 70%) for hexagonal and gyroid architectures, directions of load (longitudinal/transverse for hexagonal) and pore size gradients (radial/longitudinal for gyroid). The simulations suggested that pore size and porosity influenced tissue differentiation, whereas pore shape affected the movement of fluids within the
scaffold in addition to the mechanical load distribution. In that respect, the gyroid structure was superior to the hexagonal structure, as fluid flow was more easily distributed through the scaffold. However, the mechanical loading was more homogenous in the hexagonal structure. The simulated results of differentiations for a porosity of 70% under 0.1 mm/s of inlet fluid velocity showed how the tortuosity of the structures influenced the mechanical stimuli (Figure 7). Gyroid structures and the transversal fluid flow on hexagonal structures led to zones with a high percentage of cartilage phenotype differentiation, although they also had some regions with bone phenotype differentiation (shown in a darker color in Figure 7).

Sanz-Herrera et al. combined the macro-scale asymptotic homogenization theory with a micro-scale bone remodeling theory in an FEA performed in ABAQUS. Based on the simulation results, it appeared that a higher modulus for the scaffold led to improved cell differentiation. Higher porosity promoted bone formation due to increased mechanical stimuli, whereas larger pore sizes improved cell migration but also reduced specific surface area for cell adhesion. It was concluded that while the results of the simulations met expectations, multiple factors needed to be improved. For instance, they suggested that random-walk cell crawling be used to model cell migration instead of Fick’s Law. Checa and Prendergast used a mechano-biological model to simulate tissue formation and angiogenesis within a porous bone tissue engineering scaffold, while taking into account the individual cellular processes (e.g., migration and proliferation). The simulation results suggested that the seeding process and mechanical stimulation were key parameters when engineering large bone tissue volumes. Table 2 summarizes some of other recent studies on computational scaffold design.

Despite the recent advancements in computational methods applied to tissue engineering, simulation of the in vitro and in vivo outcomes for osteochondral tissue engineering is still premature. The existing theories of cartilage tissue as well as the experimental data available in literature may allow the development of a biphasic model of the tissue behavior. However, one should ideally consider the constitutive modeling of degenerated cartilage, cartilage growth, tissue differentiation models extended based on biphasic mechano-regulation theory, as well as appropriate cell migration/proliferation models and bone remodeling algorithms to properly simulate tissue differentiation during osteochondral defect repair.
3.6 Future directions and conclusions

Designing joint-scale osteochondral constructs is driven by consideration of biomedical need, as well as by the customization of size, maturity, and shape. Biological joint replacement can have a huge impact in joints afflicted with osteoarthritis and on the quality of life of patients, while addressing an unmet clinical need. Most current therapeutic tissue engineering treatments are intended primarily for relatively small defects, and are immature compared to native tissue (Figure 8). However, existing treatments using ACI, M-ACI, and small chondral and osteochondral constructs are incrementally shifting towards larger defects and more phenotypically stable and mature tissues. This is because biomechanically mature grafts could contribute to restoring the mechanical environment of the joint from a chronically abnormal state to a healthier state. It remains to be determined how mature joint-scale constructs should be at the time of implantation. Scientific investigation and engineering design should also look into the creation of complex tissue shapes, multi-tissue units, specialized tissue interfaces, and bioreactor systems for mechanical stimulation.

The local mechanical environment influences many critical steps during bone healing process. Corroborated mechanobiological models have the potential of improving our understanding of basic biology during bone regeneration, and could help to identify areas that need further investigation. In addition to sufficient nutrients and oxygen supply, appropriate biophysical stimuli are needed in bone scaffolds to favor appropriate tissue differentiation. As for cartilage regeneration, it has been hypothesized that the architecture of cell-seeded scaffolds can be manipulated in order to achieve collagen accumulation throughout the scaffold rather than preferentially in the construct periphery. Although the possibility of incorporating sophisticated designs into engineered tissues for clinical application is an open question, such designs may help to better understand basic chondrocyte mechanobiology. In light of this, many recent computational studies have focused on the role of scaffold design on mechanical properties, porosity and cell growth efficiency for tissue engineering of bone and cartilage.

The bone–cartilage interface in the osteochondral region resists remarkably high shear stresses under in vivo loading conditions and rarely fails. In particular, a stress concentration exists at the tidemark interface between the mineralized articular calcified cartilage (ACC) and the unmineralized hyaline articular cartilage (HAC). A better understanding of load transmission and mechanical properties across the osteochondral region would enable a more efficient
engineering of replacement materials. Additive manufacturing (AM) technologies have shown promise in developing scaffolds with optimal architectures for regeneration of multiple tissues within a single construct. Further advancements in fabrication methods would pave the way to creating biomimetic constructs satisfying the load-bearing requirements for osteochondral constructs and successful growth of various tissue types for the treatment of osteochondral defects. In addition, a better understanding of the osteochondral tissue requirements as well as scaffold architecture-property relationships could contribute to optimal design of the bone/cartilage interface zone by AM technologies. Despite the advancements in cartilage and bone tissue engineering, the true challenge in osteochondral repair lies in the comprehension of the bone-cartilage interface and its combined yet separate mechanical properties, structure, and biology.

Finally, in designing osteochondral grafts animal studies are considered to be an important validation step. The implantation site has shown to affect the in vivo outcomes of engineered osteochondral constructs. For example, subcutaneous environment differs considerably from the orthotopic environment. This includes the absence of mechanical cues, such as hydrostatic pressure and dynamic compression, which have been shown to influence the endochondral phenotype of MSCs and matrix production. Moreover, efficacy-driven guidelines could only be established from prospective, randomized clinical trials. This is primarily due to the highly different biochemical and biomechanical milieu in animal and human joints. It should be noted that young individuals affected by traumatic injuries or by osteochondritis dissecans are the main patient population targeted for the treatment with engineered osteochondral grafts. Therefore, future studies should look into the possibility of extending the same paradigm to the treatment of joint pathologies in the aging population.
3.7 Abbreviations

3D: Three-dimensional

3DF: 3D Fiber Deposition

AC: Articular Cartilage

ACC: Articular Calcified Cartilage

ACECM: Articular Cartilage Extracellular Matrix

ACI: Autologous Chondrocyte Implantation

Alg: Alginate

ASC: Adipose-derived Stem Cells

AM: Additive Manufacturing

bBMP: Bovine Bone Morphogenetic Protein

bFGF: Basic Fibroblast Growth Factor

BG: Bioglass®

BMDC: Bone-marrow-derived cell

BMP: Bone Morphogenetic Protein

rhBMP-2: Bone Morphogenetic Protein-2

BM-MSC: Bone Marrow Mesenchymal Stem Cell

BMSC: Bone Mesenchymal Stem Cell

CAD: Computer-Aided Design

CG: Collagen type II/Glycosaminoglycan

CGCaP: Collagen type I/Glycosaminoglycan/Calcium Phosphate

CM: Chondrogenic Medium

ECM: Extracellular Matrix
ES: Electrospinning
FEA: Finite Element Analysis
FDM: Fused Deposition Modeling
FGS: Functionally-Graded Scaffolds
GAG: Glycosaminoglycan
rhIGF-1: Insulin-like Growth Factor–1
hMSC: Human Mesenchymal Stem Cell
HA: Hydroxyapatite
HAC: Hyaline Articular Cartilage
ICR: Implanted Cartilage Replacement
ICRS: International Cartilage Repair Society
IGF-1: Insulin-like Growth Factor–1
μ-CT: Microcomputed Tomography
M: Normal Medium
Matrix-induced Autologous Chondrocyte Implantation (M-ACI)
MP: Microparticle
MSC: Mesenchymal Stem Cell
nHA: Nano-hydroxyapatite
OA: Osteoarthritis
OCD: Osteochondritis Dissecans
OM: Osteogenic Medium
OPF: Oligo(poly(ethylene glycol) fumarate)
PA: Polyamide
PA6: Polyamide 6
PCL: Polycaprolactone
PDO: Polydioxanone
PEG: Polyethylene glycol
PLA: Poly(lactic acid)
PLLA: Poly(L-lactic acid)
PLGA: Poly(lactic-co-glycolic acid)
PDLLA: Poly(D,L-lactic acid)
P(DLLA-co-CL): poly(D,L-lactide-co-ε-caprolactone)
PHA: Poly(hydroxyalkanoates)
PLCL: Poly(lactide-co-caprolactone)
POE: Polyorthoesters
PPF: Poly(propylene fumarate)
PVA: Polyvinyl alcohol
SBF: Simulated Body Fluid
SFF: Solid Free-form Fabrication
SLS: Selective Laser Sintering
TCP: Tricalcium Phosphate
TGF-β1: Transforming Growth Factor–β1
3.8 References


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<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Scaffolds</th>
<th>Experimental design</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Hyaluronan–gelatin composite sponge</td>
<td>In vitro using bone marrow mesenchymal progenitor cells</td>
<td>Enhanced type II collagen-rich extracellular matrix production by cells (Angele et al., 1999)¹¹¹</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Injectable oligo (poly(ethylene glycol) fumarate) and gelatin microparticles</td>
<td>In vitro using rabbit marrow mesenchymal stem cells</td>
<td>TGF-β3 significantly stimulated chondrogenic differentiation of MSCs (Guo et al., 2010)¹¹²</td>
</tr>
<tr>
<td>TGF-β1 + IGF-1</td>
<td>Gelatin – PEG scaffolds</td>
<td>Neo-surface repair, surface morphology, cartilage thickness, chondrocyte clustering, and the chondrocyte/glycosaminoglycan production were increased (Holland et al., 2007)¹¹³</td>
<td></td>
</tr>
<tr>
<td>TGF-β2 + BMP-7</td>
<td>Polycaprolactone</td>
<td>In vitro using rabbit osteochondral defect model</td>
<td>Improved differentiation of adipose stem cells to chondrogenic lineage (Im &amp; Lee, 2010)¹¹⁴</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Polycaprolactone</td>
<td>In vitro using primary chondrocytes</td>
<td>Promoted cartilage matrix production (Jeong et al., 2012)¹¹⁵</td>
</tr>
<tr>
<td>TGF-β1 + BMP-2</td>
<td>Poly(D,L-lactic-co-glycolic acid) microspheres</td>
<td>In vivo using rabbit knee defect model</td>
<td>Enhanced production of cartilage layer with high content of glycosaminoglycan content and integration with the surrounding cartilage and underlying bone (Mohan et al., 2011)¹¹⁶</td>
</tr>
<tr>
<td>rhBMP-2 + rhIGF-1</td>
<td>Polylactic-co-glycolic acid and silk fibroin microspheres in alginate gels</td>
<td>In vitro using human mesenchymal stem cells (hMSCs)</td>
<td>Osteogenic and chondrogenic differentiation were clearly observed (Wang et al., 2009)¹¹⁷</td>
</tr>
</tbody>
</table>
### TABLE II. Summary of some other recent studies on computational scaffold design.

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Methods</th>
<th>Results of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Byrne et al.</td>
<td>The study used a 3D FEA model of a poroelastic scaffold infiltrated by tissue, and simulated a vertical pressure to test the effects of porosity and dissolution rate of the scaffold on bone formation.</td>
<td>At low loading sites, high porosities and medium dissolution rate resulted in the greatest amount of bone. Lower porosities and dissolution rates were recommended under high loading.</td>
</tr>
<tr>
<td>Kelly &amp; Prendergast</td>
<td>A mechanoregulation model was used to simulate stem cell differentiation and growth within scaffolds exposed to strain and fluid flow. A homogenous linear poroelastic model and an inhomogenous model (chondral and bone phases) were used.</td>
<td>The simulations suggested that optimal stiffness and permeability could be estimated for a scaffold, which could contribute to promoting desired stem cell differentiation.</td>
</tr>
<tr>
<td>Khayyeryi et al.</td>
<td>A mechanoregulation model was used to determine stem cell differentiation over time when the scaffold stiffness was varied. Angiogenesis, cell differentiation, and cell migration were taken into account, although scaffold degradation was not.</td>
<td>Larger pore size was beneficial to bone growth and vascularization. Material stiffness from 1 to 1000 kPa did not contribute to tissue differentiation, whereas a stiffness greater than 10 MPa increased bone and cartilage formation.</td>
</tr>
<tr>
<td>Milan et al.</td>
<td>The scaffold architecture was converted into a 3D FEA model via micro-computed tomography (µCT). The model was used to simulate stress response to 5% compression at a 1 s⁻¹ strain rate.</td>
<td>The results suggested that applying 5%-compressive loading on the scaffolds generated a shear strain that stimulated osteogenesis (51% of the surface).</td>
</tr>
<tr>
<td>Sandino et al.</td>
<td>FEA model of scaffolds was used to simulate 0.5% and 1% compressive strain with varied states of stem cell preseeding. The study used previously validated mechanoregulation model to account for cell migration, differentiation, and angiogenesis.</td>
<td>Vascularization was predominant in external pores. Compressive strain of 0.5% produced favorable mechanical stimuli within 70% of the pore volume. Increasing the strain to 1% reduced osteogenesis.</td>
</tr>
</tbody>
</table>
FIGURE 1. (A) A healthy joint with normal articular cartilage. The inset image shows four distinct layers of articular cartilage as well as its pericellular, territorial and interterritorial matrices. \(^{25}\) (B) An osteoarthritic joint that shows partial loss of cartilage, subchondral bone thickening, as well as the alterations in cartilage matrices (inset image). \(^{25}\) Some of the surgical procedures for the treatment of osteochondral defects include: (C) autologous osteochondral transplantation, \(^{27}\) (D) autologous chondrocyte implantation, \(^{27}\) and (E) microfracture. \(^{27}\) Reproduced with permissions from Nature Publishing Group and Wiley Periodicals.

FIGURE 2. (A) The extracellular matrix surrounding chondrocytes in a healthy articular cartilage, which consists of pericellular, territorial, and interterritorial matrices. \(^{25}\) (B) The hierarchical structure of bone ranging from the macroscale skeleton to nanoscale collagen (green) and hydroxyapatite (red). \(^{122}\) (C) Schematic of the fabrication steps for cell-laden biomodal scaffolds produced by additive manufacturing and electrospinning. \(^{126}\) (D) Top view of a biomodal scaffold composed of microfibers and electrospun nanofibers. \(^{124}\) (E) Schematic of the mechanism by which nanomaterials may be superior to conventional materials for bone regeneration, through promoting protein adsorption and favoring cell adhesion. \(^{123}\) Reproduced with permissions from Nature Publishing Group, Royal Society of Chemistry and Elsevier Ltd.

FIGURE 3. Various osteochondral scaffold design approaches. \(^{18}\) Reproduced with permission from Wiley Periodicals.

FIGURE 4. Lay-down patterns with (A) honeycomb pores and (B) Hilbert recursive curve. \(^{138}\) Reproduced with permission from Elsevier Ltd.

FIGURE 5. Some multiphasic and gradient-based scaffolds for osteochondral tissue engineering: (A) A fibrin/PCL and a PCL/PCL-TCP scaffold; \(^{11}\) (B) an agarose/decellularized bone scaffold; \(^{154}\) (C) a biphasic scaffold composed of collagen type II -glycosaminoglycan (CG) and mineralized CG (CGCaP); \(^{156}\) (D) a scaffold made of articular cartilage ECM/hydroxyapatite (HA); \(^{157}\) (E) a chitosan-collagen/β-TCP scaffold; \(^{160}\) (F) A trilayered scaffold made of 45S5 Bioglass® and alginate; \(^{164}\) (G) A trilayered scaffold made of bovine decellularized articular cartilage ECM, PLGA/β-TCP wrapped with collagen type I, and a compact PLGA/β-TCP layer as an interface; \(^{165}\) (H) A trilayered scaffold made of HA and collagen with different compositions in each layer; \(^{168}\) (I) a trilayered silk fibroin scaffold loaded with different growth factors in each layer; \(^{175}\) (J) schematic of a bioprinting process that makes use of synthetic polymers and hydrogels encapsulating cells and growth factors; \(^{163}\) (K) schematic of a microfluidic device for generating a gradient-based stem cell-laden hydrogel slab (OM: osteogenic medium, M: normal medium, CM: chondrogenic medium). \(^{178}\) Reproduced with permissions from Elsevier Ltd., Wiley Periodicals, and IOP Publishing (* denotes Open Access).

FIGURE 6. (A) Simulation of damage distribution at the end of loading for osteochondral and chondral implants for different values of compressive modulus; (B) time-history of damage dissipation energy normalized by surface area of adhesive; (C,D) corresponding results for different values of Poisson’s ratio. \(^{1}\) Reproduced with permission from Elsevier Ltd.
FIGURE 7. Color map of the perfusion stimuli (0.1 mm/s) on surface areas for different scaffold architectures. (a,d,e) gyroid structures; (b,c) hexagonal structures. Reproduced with permission from Elsevier Ltd.

FIGURE 8. Developmental progression of biomimetic tissue engineering therapies for articular cartilage repair. Chondro-Gide®, ChondroCelect®, DeNovo® ET, and NeoCart® are products of Geistlich Pharma AG (Wolhusen, Switzerland), TiGenix (Leuven, Belgium), ISTO Technologies, Inc. (St. Louis, Missouri), and Histogenics Corporation (Waltham, Massachusetts), respectively. The image was reproduced with permission from Elsevier Ltd.
FIGURE 1
FIGURE 2
<table>
<thead>
<tr>
<th>Monophasic</th>
<th>Physical / Chemical</th>
<th>Cellular / Biological</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One material with one porosity and overall architecture</td>
<td>One cell type with no variation in overall biological environment</td>
</tr>
<tr>
<td>Biphasic</td>
<td>Two different materials or a material with two layers of significantly different porosity, interconnectivity, micro- or macro-architecture</td>
<td>Two different cell types (or one cell type with two different pre-differentiation) or two different biological environment created by the addition of growth factors or bioactive peptides</td>
</tr>
<tr>
<td>Triphasic</td>
<td>Three different materials or a material with three layers of significantly different porosity, interconnectivity, micro- or macro-architecture</td>
<td>Three different cell types (or one cell type with three different pre-differentiation) or three different biological environment created by the addition of growth factors or bioactive peptides</td>
</tr>
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**FIGURE 3**
FIGURE 4
FIGURE 5


FIGURE 6
FIGURE 7
Tissue Engineering Therapies for Articular Cartilage

FIGURE 8
Validation of Scaffold Design Optimization in Bone Tissue Engineering: Finite Element Modeling versus Designed Experiments

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(To be submitted to Biofabrication)
Validation of Scaffold Design Optimization in Bone Tissue Engineering: Finite Element Modeling versus Designed Experiments

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4.1 Abstract

This study sought to validate the use of a computational (finite element) optimization model in COMSOL Multiphysics for designing bone tissue scaffolds generated via 3D bioplotting. Scaffold topology was simplified to three factors: ceramic content, strand diameter, and strand spacing. These factors affect the ability of the scaffold to bear mechanical loads and how porous the structure can be while maintaining integrity. In this study, 24 scaffolds composed of a polymer-ceramic-collagen composite were then constructed according to an I-optimal designed experiment in order to generate experimental models of the factor-response relationships. Although the COMSOL and experimental models disagreed on the effect of strand spacing on compressive modulus, the topology suggested by COMSOL was found to nearly satisfy the requirements designated for bone tissue regeneration when analyzed by the experimental model.

Keywords: bone tissue engineering, optimization, simulation, additive manufacturing, 3D bioplotting, designed experiment, topology, COMSOL

4.2 Introduction

Bone tissue has a limited capacity for regeneration based on the extent of the damage and the bone involved [1]. In order to repair critical-sized defects and non-unions, either bone grafts or synthetic bone-graft substitutes are used to replace the tissue [2]. Grafts and substitutes are in such common use that, every year, at least four million operations worldwide make use of them
[3], and this number can only increase as global populations do. However, there are risks and limitations to these solutions. Bone-graft substitutes are subject to wear over time, immune response, or bone thinning due to stress shielding [2, 3]. Grafts taken from donors (allografts) risk immune response or disease transmission [2, 3], and grafts from the patient (autografts), while considered ideal, are limited in size/quantity and risk donor site complications/infection [2, 3]. Bone tissue-engineering (TE) aims to enable the patient’s body to regenerate the damaged tissue without the need for a donor or risk of spurring immunological action [4-6].

In order to regenerate damaged tissues, TE makes use of porous scaffolds made of biomaterials to act as a cellular matrix and support structure [6,7,8]. In designing scaffolds, the general consensus is that they should behave as similarly to the tissues they are meant to replace as possible [9, 10]. In the case of bone tissue, it is hypothesized that scaffolds need to have a large mechanical modulus and porosity based on the bone to be replaced (table 4-1), as mechanoregulatory effects are believed to be the key factor in bone tissue regrowth/cellular differentiation [8, 9, 11]. If the scaffold environment (mechanical forces transferred to the cells and vascularization) is unlike physiological conditions, there is a risk that mesenchymal stem cells (MSCs) differentiate into chondrocytes or fibroblasts, which grow cartilage and fibrous tissues respectively [12, 13]. However, stiffness and porosity directly conflict as design factors, which makes the design process critical.

Table 4.1: Mechanical properties of mature bone tissues, as described by literature.

<table>
<thead>
<tr>
<th>Young’s Modulus (E)</th>
<th>50 - 100 MPa (Trabecular bone)</th>
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<td>2.23 - 25.9 GPa (Trabecular bone)</td>
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<td>Porosity</td>
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<td>&gt; 70 % (Trabecular bone)</td>
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<td>50 - 90 % (Trabecular bone)</td>
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There are two key aspects to scaffold design: material choice and topology, of which both can be adjusted to produce scaffolds with properties akin to native tissue [21]. The material choices affect how readily the scaffold will biodegrade, and whether the scaffold will be bioactive [22]. While each class of biomaterial (polymers, metals, and ceramics) have their own
individual disadvantages that can restrict their applications in TE, composites can mitigate these limitations and it has been suggested that they can exhibit tissue-mimicking properties [4]. This study made use of a polymer-ceramic-protein composite, which combined the ease of use and controlled biodegradation rate of poly(D,L-lactide-co-glycolide) (PLGA), the mechanical strength and bioactivity of nano-Hydroxyapatite (nHA), and the cellular adhesiveness of collagen.

PLGA is a synthetic copolymer of poly(L-lactic) (PLLA) and polyglycolic acid (PGA), and has U.S. Food and Drug Administration (FDA) approval for some use in humans [4]. Its degradation rate has been shown to be customizable in the range of weeks to months based on the ratio of PLLA to PGA [4, 23, 24]. For instance, PLGA 85:15 has a reported degradation time of 5-6 weeks [24]. However, it has a low modulus even compared to its component polymers (PLGA 85:15 E = 2.0 GPa, PLLA E = 2.7 GPa, PGA E = 7.0 GPa [24]), so, on its own, PLGA is not reliable for trabecular bone regeneration [14]. On the other hand, nHA is a ceramic with a high modulus (E = 35-120 GPa for dense ceramics [25, 26]) that has been suggested to encourage osteogenesis [4]. In composite materials, nHA improves mechanical properties of scaffolds at low concentrations (tensile: ≤ 0.5 wt% [27], compressive: ≤ 20 wt% [28]), but proved detrimental at higher concentrations [27, 28]. Previous research has shown that nHA helps cells and proteins attach to scaffold surfaces when integrated into composite scaffold material [22]. To its detriment, nHA is difficult to process [14], brittle, and degrades slowly (adjustable via ratio of Ca/P [26]). Finally, collagen is another primary component of mammalian tissue matrices, and has been shown to support osteogenesis [4] and cellular attachment [5, 27, 29]. In addition, crosslinking has been shown to give some control over collagen’s mechanical properties and degradation [30]. The solvent 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) was used to process the composite, as it has previously been used in electrospinning studies that made use of PLGA-nHA-collagen composites [10, 27], and has been suggested to help electrospun collagen behave similarly to collagen in the natural bone matrix [31].

The scaffold topology also plays a role in scaffold modulus and is the primary determinant of porosity. The traditional approach to scaffold topology design and optimization is iterative; the experimental performance of a scaffold informs researchers how they can modify the topology in order to improve the performance of the next scaffold produced [13, 32]. Even in
cases where finite element analysis has been used for analysis, it has typically been post hoc in order to modify scaffolds that have already been fabricated and tested [33], or to examine how accurately a finite element model represents various designs [15, 34-37]. By using computer-aided design (CAD) and finite element analysis (FEA) as design tools, it may be possible to reduce the number of physical scaffolds that must be constructed and tested in order to determine optimal topologies. The resultant 3D models would be simple to produce and test via additive manufacturing (AM), which grant fine control over the topology of generated scaffolds [7, 21, 35, 36]. One such device, the 3D bioplotter (3DBP), constructs scaffolds by layering extruded strands of material. By adjusting the diameter and distance between extruded strands, it is possible to design various topologies with porosity and modulus in mind.

CAD has shown promise in scaffold design, but simulations tend to over-predict scaffold performance to varying degrees due to limitations in simulating micro-topologies (cracks, pores, material inconsistencies) [35, 37]. In order to validate CAD scaffold design, COMSOL Multiphysics software was used to optimize a 3DBP topology, which was compared to an optimized statistical model generated via designed experiment (DE) and response surface methodology (RSM). It was hypothesized that both the experimental and CAD models should suggest similar optimal topologies (±20% design factor value agreement) and the predicted resulting compressive moduli and porosities (±20% prediction agreement). In addition, both models were expected to yield an optimal topology within the design space while satisfying the minimum requirements for use in (trabecular) bone regeneration (compressive modulus ≥ 10 MPa [2], and porosity ≥ 50% [38]).

4.3 Materials & Methods

4.3.1 Materials

PLGA (Resomer LG 824 S) was purchased from Evonik Industries (Germany). Kensey Nash Corporation (USA) graciously provided type 1 collagen powder (PN 20003-04). nHA (nanopowder, < 200 nm particle size (BET), synthetic, product number 677418) and HFP (assay ≥99%, product number 105228) were purchased from Sigma-Aldrich Co. LLC (USA).

4.3.2 Designed Experiment (DE)

As the goal of this study is to validate the prediction and optimization capabilities of a COMSOL model, a response surface methodology (RSM) is an ideal baseline to compare the simulation results to. By performing RSM in the same design region, it is possible to generate
two 2nd order fitted regression models relating the effects (linear, quadratic, and interaction) of the factors to our responses [21, 39, 40]. Performing a constrained optimization on these models (maximize compressive modulus, constrain porosity) will estimate an optimal scaffold topology and the theoretical resultant compressive modulus and porosity, which can then be compared to those suggested by COMSOL in order to validate the simulation.

Part of developing a response surface is the experimental evaluation of factor effects on response. By using a designed experiment (DE), it is possible to efficiently evaluate the system and any potential interactions between factor effects. In addition, I-optimal designs minimize the average (integrated) prediction variance across the region defined by the factor levels [41], and are highly suggested when developing models for prediction and/or RSM [41].

Among the factors used for this scaffold design, ceramic composition cannot be changed easily between trial runs without wasting material or risking solvent evaporation. Split-plot designs can be used to account for such systems with factors that are difficult to change frequently [40, 42] by dividing the experimental runs at two levels: whole plots (WP), where the difficult to change factor is held constant across the WP’s encapsulated runs, and split plots (SP), the individual experimental runs within a whole plot, where the remaining factors are applied. Such a design has two levels of randomization: WP scale, where the WPs are arranged randomly, and SP scale, where the trial runs within a given WP are ordered randomly [40]. Validity of results will be maintained by completely shutting down and recalibrating the bioplotter in between trials, effectively resetting WP and SP factor settings, as prescribed in [42]. The final DE used for our RSM is shown in table 4.2.

<table>
<thead>
<tr>
<th>Whole Plots</th>
<th>Strand Diameter (µm) SP Factor</th>
<th>Strand Spacing (Edge-to-Edge) (µm) SP Factor</th>
<th>Composition (% nHA WP Factor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>380</td>
<td>1000</td>
<td>15</td>
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<tr>
<td>1</td>
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<td>3</td>
<td>300</td>
<td>600</td>
<td>0</td>
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</table>
### 4.3.3 Statistical Analysis

The DE in table 4.2 was executed and compressive modulus and porosity values were obtained (see table 4.3). For each response, the split-plot model from Jones and Nachtsheim [42] was fit using JMP software. Restricted maximum likelihood (REML), as described in [42], was used to estimate the variance components, which were then used to compute the fixed effects. In the analysis of both compressive modulus and porosity, the WP variance component was estimated as slightly negative. This is likely because (a) the experiment is relatively small and (b) there was little WP-to-WP variation. The prediction equations we used assumed the REML-based estimates, because the negative estimate was so inconsequential. Ordinary least squares was used to check the fixed effect estimates, and similar estimates were obtained. Significance of terms was determined roughly by evaluating the terms with relatively large ratios of estimate to standard error.

### 4.3.4 Scaffold Construction

**Formula Preparation:** A mixture of 2.1 g PLGA (824S resomer), a variable amount of nHA (0 g, 0.372 g, 0.9 g for 0%, 15%, and 30% respectively), and 3.6 mL of HFP was prepared. A second formula of 0.225 g type I collagen and 3 mL of HFP was also prepared. Both were sealed and allowed to homogenize for 23 hours before being combined. After an hour, the composite would then be transferred to a 10 mL bioplotter barrel.

**Solvent Detection & Drying Time:** In order to determine an efficient time frame for allowing the scaffolds to dry under solely atmospheric conditions, thermogravimetric analysis (TGA) was performed using a TA Instruments Q500-2063 device. The device was set to ramp
the furnace temperature from room temperature to 800 °C at a rate of 10 °C/min. The change in mass of a sample taken from a standard scaffold versus temperature was examined at multiple times: 7, 14, 21, 28, and 42 days.

**3D Bioplotter Setup & Scaffold Construction:** The 3D model used was a 20 mm x 20 mm x 3 mm box partitioned into 10 layers with a layer thickness of 0.3 mm, and offset above the stage by 0.3 mm. The 3D bioplotter (EnvisionTEC, Germany) settings were held constant (T = 20 °C, plotting speed = 0.9 mm/s, and atmospheric T = 20.6 °C), except for the extrusion pressure, which was varied (0.8 bar - 1.4 bar) to control the diameter of the strands within the scaffold. As such, its value for any given scaffold was varied depending on the strand diameter prescribed by the DE, according to a calibrated relationship between pressure and strand diameter for each whole plot. In addition, the distance between extruded strands was directly varied according to the DE. In order to minimize risk of carry-over effects between scaffolds, the bioplotter and associated software were fully restarted and recalibrated prior to construction of any given scaffold. Prior to constructing a WP, two calibration scaffolds were constructed with different extrusion pressures to approximate a linear relationship between plotting pressure and resultant strand diameter. A Zeiss Axio Vert.A1 light microscope was used to determine the strand diameters. Thus, a relationship between extrusion pressure and strand diameter was determined for that particular WP. The plotting settings were then adjusted and construction of DE scaffolds began. Upon completion, scaffolds were placed in a fume hood for 28 days to dry while exposed to the air.

**4.3.5 Scaffold Characterization**

Samples were taken from scaffolds via circular biopsy punches with an internal diameter of 8 mm. These samples were first measured for diameter, height, and mass.

**Scanning Electron Microscopy (SEM)**

Using a Denton Desk II Sputter Unit, a scaffold sample was sputter coated with a 20 nm layer of gold. A Zeiss Supra 35VP SEM was then used to image the scaffolds (EHT = 5 kV and 8 mm working distance) and measure for strand diameter, strand thickness, and the edge-to-edge distance between strands.

The porosity of the scaffold was estimated by following a geometric calculation modified from Landers et al. (2002) [43], where, instead of assuming a perfectly cylindrical strand geometry, the equation was modified to use elliptical strands.
\[ P = 100 \left( 1 - \frac{V_{\text{scaffold}}}{V_{\text{cube}}} \right) = 100 \left( 1 - \frac{\pi}{4} \frac{(D)(H)}{(L)(D + EtE)} \right) \]

D is the strand diameter, H is the strand thickness, L is the layer thickness (assumed equal to the 3D model’s layer thickness: 300 \( \mu \)m), and EtE is the edge-to-edge strand spacing.

**Instron Stress-Relaxation**

An Instron 3344 single tower compression device was equipped with a 100 N load cell and programmed to perform a stress relaxation test. After a 0.44 N preload, the sample was compressed up to 40% of its total height at a rate of 5 mm/min.

**4.3.6 COMSOL 4.4 Model**

**Parameterization:** Due to the iterative nature of the optimization process, it was necessary to construct a flexible model. The features of the model related to its geometry were parameterized so that the model would automatically update when the topology was changed.

**Geometry:** The scaffold model (Fig. 4.1) was generated as a series of parallel and perpendicular cylinders. Boolean operations were then used to crop the top and bottom layers of the model such that they formed flat surfaces that boundary conditions could be applied to. The outer edges of the model were cropped into a curve in order to mimic the circular DE scaffold samples. A custom material (\( v = 0.49 \), E based on the assumed %nHA) was then generated and applied to the entire domain of the model. The Young’s Modulus was determined by Instron compression tests performed on non-porous samples of the three composite variations (E = 0.94 MPa, 4.79 MPa, and 11.86 MPa for 0%, 15%, and 30% nHA respectively).

![Figure 4.1: Final geometry of scaffold model.](image-url)
Solid Mechanics: First, linear elastic material behavior was applied to the entire domain. Symmetry conditions were then applied to the boundaries that aligned with the X or Y-axis. The bottom surface boundaries of the model were set as fixed constraints, and an instantaneous prescribed Z-direction displacement equivalent to the total height of the scaffold times the percent of compressive strain desired (10%) was applied to the top surface boundaries.

Optimization: A boundary integral objective for optimization was defined and examined at the top surface boundaries where the displacement took place. The objective expression examined compressive modulus as a ratio between third principal stress and the prescribed compressive strain. Third principal stress (sp3) was used as the stress term, as it assumes the maximum possible compressive stress experienced at the boundary region. A Nelder-Mead optimization method was then used to maximize the objective function. Radius and edge-to-edge spacing were used as control variables. Radius was initially 190µm, but was allowed to range between 150-230 µm. The spacing began at 0.8 mm, and could range from 0.6-1 mm. Porosity was then constrained within 50-99% void space.

4.4 Results

4.4.1 Scaffold Characterization

The SEM evaluated the strand diameters and strand spacing for the scaffolds using the top-down perspective, while the cross-sectional view was used to determine the thickness of the strands in order to estimate the porosity of the scaffold. The estimated porosities of all scaffolds satisfied our 50% minimum requirement for use in bone tissue regeneration. At the lowest spacing and highest diameter, the porosities were approximately 56-58% void space. The best scaffold designs for porosity, high spacing and low diameter, were able to reach over 75%. Based on this, even if the optimal design for compressive modulus were to fall in the denser area of the design region, the topology would still satisfy the minimum porosity. Based on the factor-porosity scatter plot (Fig. 4.4, bottom 3 plots), the strand diameter and strand spacing have visible effects on porosity.
Figure 4.2: SEM image of scaffold 2-2 in top-down view (left) and scaffold 1-3 in cross-section (right).

Figure 4.3 demonstrates three examples of the stress-strain behavior for scaffolds between 0% and 40% strain. Although the system is non-linear at larger strain values, the stress-strain relationship around the 10% strain region behaved reasonably linearly. Compressive modulus was thus determined by taking the slope of the trend comprised of the point closest to 10% strain and the 4 adjacent points above and below it. The 30% nHA scaffolds had the highest compressive moduli, ranging from 5.69 MPa to 9.07 MPa. Plotting factor values versus the compressive modulus (Fig. 4.4, top 3 plots) shows that %nHA had the largest consistent effect on modulus. Diameter has a smaller positive impact.

Figure 4.3: Stress-strain behavior of the first three scaffolds in WP 3. Material behavior is reasonably linear at low compressive strains (~10% region).
<table>
<thead>
<tr>
<th>WP</th>
<th>Scaffold</th>
<th>Avg Diameter (µm)</th>
<th>Avg Spacing (µm)</th>
<th>Porosity (%)</th>
<th>Avg Comp. Modulus (MPa)</th>
<th>SD</th>
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Table 4.3: Experimentally determined characteristics of the DE scaffolds.
4.4.2 DE Optimization

The experimental model for compressive modulus is comprised of: a quadratic effect due to %nHA, a linear effect caused by strand diameter, and an interaction term where the two have a multiplicative effect on the response. The interaction term, although not as influential as the quadratic effect of %nHA, indicates that the positive effect caused by either factor is diminished if the other factor is at a small value. Conceptually, if a 30% nHA formula were utilized, the use of thin strands would reduce the amount of material present, mitigating the effect of the material choice. Similarly, high diameter strands are less effective if they are composed of material with low mechanical strength. Strand spacing was determined to have a negligible effect on compressive modulus, so it was not included in the model.

In comparison, the porosity model is solely composed of the architectural factors: strand diameter and strand spacing. Unlike the compressive modulus model, the strand spacing factor has the greatest effect (quadratic) on porosity, and strand diameter has a linear effect. A key determinant of porosity for this type of scaffold topology is the number of strands that can be plotted within the 20 mm space, thus the factor that most affects the number of strands per layer will have the greatest effect on porosity. Since strand diameter varied across a smaller range of
space than strand spacing ($\partial_{\text{diam}} = 160 \, \mu m$, $\partial_{\text{spacing}} = 400 \, \mu m$) it had less capacity to affect the number of strands in the design than strand spacing.

The accuracy of the models was determined by comparing the experimental responses to the predicted responses when identical scaffold topologies are given to the models (Fig. 4.6). The compressive modulus model and experimental results show general agreement ($R^2=0.87$), but the porosity model accurately predicts the behavior of the physical scaffolds ($R^2=0.96$).

![Figure 4.5: Optimization profile for the system. Solid lines indicate the effects of each factor on a given response; dotted lines indicate the values selected for each factor and the total result on the responses.](image)

Compressive Modulus = $2.9401 + 2.7612(n) + 1.8625(n^2) + 1.3894(d) + 0.7101(n)(d)$

Porosity = $75.6456 - 5.9465(d) + 3.0693(s) - 2.3130(s^2)$

$$n = \left(\frac{(%nHA) - 0.15}{0.15}\right)$$

$$d = \left(\frac{\text{(Strand Diameter)} - 380}{80}\right)$$

$$s = \left(\frac{\text{(Edge to Edge Strand Spacing)} - 800}{200}\right)$$
Performing a constrained optimization on the system suggests a scaffold design of 30% nHA:PLGA, 460 µm strand diameter, and 923 µm strand spacing. For nHA composition, selecting the highest level is ideal, as it has the largest effect on compressive modulus of all factors with no detrimental effect on porosity. Selecting strand spacing is the inverse scenario: choosing a high spacing has a positive effect on porosity without any detrimental effect on compressive modulus. Strand diameter is the only factor that affects both responses, according to this model. It is possible to choose the maximum diameter for its positive effect on compressive modulus as, regardless of the loss of porosity, the response is still within acceptable bounds. The experimental models predict that, for this optimized design, the resultant scaffold would have a compressive modulus of 9.66 MPa, and a porosity of 70.7%. This porosity is highly desirable, but the modulus is barely insufficient for bone regeneration.

**4.4.3 COMSOL Optimization**

Optimizations were run at three levels of Young’s Modulus (E) corresponding to the three %nHA levels. All three optimizations iterated through the same topologies, and finished at a topology of 460µm strand diameter and 601.47 µm strand spacing. As the architectural factors were identical, the three simulations agreed upon a resultant porosity 54.26%. The predicted compressive moduli, however, varied according to the assumed ceramic composition, improving as %nHA did. At the highest level, the predicted compressive modulus was 5.67MPa.
Table 4.4: Abridged results of COMSOL optimization. The module began at the same diameter and strand spacing, and searched in the same order for 30 iterations.

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Strand Diameter (µm)</th>
<th>Strand Spacing (µm)</th>
<th>Porosity (%)</th>
<th>Compressive Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0% nHA</td>
</tr>
<tr>
<td>1</td>
<td>380.0</td>
<td>800.0</td>
<td>65.2300</td>
<td>0.3192</td>
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<td>5</td>
<td>416.0</td>
<td>710.0</td>
<td>60.8517</td>
<td>0.3638</td>
</tr>
<tr>
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<td>431.3</td>
<td>659.1</td>
<td>58.9022</td>
<td>0.3924</td>
</tr>
<tr>
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<td>454.0</td>
<td>604.8</td>
<td>54.7021</td>
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</tr>
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<td>457.4</td>
<td>600.4</td>
<td>54.3462</td>
<td>0.4461</td>
</tr>
<tr>
<td>25</td>
<td>458.7</td>
<td>601.1</td>
<td>54.3111</td>
<td>0.4479</td>
</tr>
<tr>
<td>30</td>
<td>460.0</td>
<td>601.5</td>
<td>54.2641</td>
<td>0.4495</td>
</tr>
</tbody>
</table>

Figure 4.7: Surface plot of the stress experienced at the top surface of the scaffold model. Peaks of stress are in alignment with the strands of the previous layer.

4.4.4 Methodology Comparison

The two models suggest using the highest values for strand diameter and ceramic content. However, the experimental model found the effects of strand spacing on modulus to be insignificant, while the simulation made a distinct effort to improve modulus by reducing the spacing and increasing the number of strands per layer as a result. That said, the spacing only has an effect on porosity in the experimental model, so it would be feasible to use the topology suggested by COMSOL because the reduction in porosity would still fall within the acceptable bounds according to the experimental model.
4.5 Discussion

Computational over-prediction of scaffold performance has been partly attributed to the presence of a micro-topology on the surface of scaffolds that has not been accounted for in simulation models, and it has been suggested that the architecture of the scaffold affects the degree of the impact [35, 37]. However, the COMSOL model presented here under-predicted compressive modulus of the scaffolds. It is possible that this could also be caused by the scaffold micro-topology. In comparison to other AM methods, such as sintering, scaffolds produced by 3D bioplotting have distinctly smooth surfaces [6]. Thus, it is possible that the lack of a micro-topology contributed to COMSOL’s under-prediction of scaffold compressive modulus.

Both the experimental and computational model suggested the maximum %nHA value (30%) in order to improve compressive modulus of the scaffolds. However, Shuai et al. (2013) found that, for a PLGA+nHA composite, nHA concentrations above 20 wt% caused compressive modulus to decrease [28]. As such, while the lack of a maximum peak within our design region may suggest examining a larger %nHA, it may not produce an improvement in compressive modulus. Both models also suggested maximizing the strand diameter of scaffolds, and agreed that the factor has a positive effect on compressive modulus and a negative effect on porosity. This behavior is consistent with past simulation studies, such as the one performed by Giannitelli et al. (2014) who also found that increasing strand diameter of their model, while holding all other aspects of the topology constant, caused an increase in stiffness and reduced porosity [7].

However, the two models conflict on the optimal strand spacing value. The experimental model nearly maximized spacing because it found no statistically relevant effect on the compressive modulus, but COMSOL minimized spacing in order to improve the modulus. The negative impact porosity has on compressive modulus is well established [7, 20, 45], as such any factor that affects porosity, such as strand spacing, should affect modulus as well. In addition, Giannitelli et al. (2014) examined the effect of strand spacing on their simulation model and found that (even when porosity was held constant) larger strand spacing values reduced scaffold stiffness [7]. The cause of this disagreement between our two models may be indicated by figure 4.8, which outlines the compressive modulus predicted by COMSOL as it iterated across the design region. At iteration 10, COMSOL increased porosity by increasing strand spacing and decreasing diameter. This resulted in a decrease in compressive modulus, however the degree of impact was dependent on the material assumed. The 30% material (with the largest Young’s
modulus) suffered the largest loss in compressive modulus, whereas the 0% nHA simulation was less affected. It may be possible that the relationship between ceramic concentration and strand spacing affected the statistical relevance of the relationship between strand spacing and compressive modulus.

![Figure 4.8: Compressive modulus of COMSOL scaffold model across iterations of the optimization process.](image)

It has been recommended that bone tissue scaffolds have a minimum pore size of 300µm [20]. Although, it has been suggested by Fisher et al. (2002) that pore sizes up to 800µm perform similarly to 300µm pores in vivo [44]. As such, there is osteogenic support for the use of the strand spacing suggested by COMSOL, but not for the spacing suggested by the experimental model. In addition, the use of such a large spacing may cause a significant lack of surface area available for cellular attachment. Thus, it may be more beneficial to consider a smaller strand spacing than the experimental model suggests. Such a decision will still produce scaffolds with acceptable porosities because the experimental model indicates that all strand spacing values within the design region result in porosities greater than 50%.

### 4.6 Conclusions

A 3D bioplotter was used to produce 24 bone tissue scaffolds according to a split-plot designed experiment, and mathematical models were generated relating ceramic content and strand diameter to compressive modulus, and strand diameter and spacing to porosity. An optimized design was generated from these models and used to determine the usefulness of an optimized design given by the COMSOL optimization module. The two agreed that high ceramic content and large strand diameters were optimal factor levels, but, while the experimental model
found compressive modulus unaffected by spacing, COMSOL found that a minimum strand spacing improved the modulus. In addition, the simulation under-predicted modulus and porosity in all cases. However, COMSOL’s suggested design, when examined through the experimental model, surpasses the porosity minimum and nearly satisfies the modulus minimum. Expanding on topology optimization models has a great potential in improving the efficiency of scaffold design studies, even in cases where the property predictions are over/under-predicted. The experimental model was able to determine optimal factor values within the design region, but the trends found had no peaks within the bounds. Expanding on the design region in future experiments could reveal an improved topology that may allow PLGA-nHA-Collagen scaffolds to meet the mechanical requirements for bone TE.

4.7 Acknowledgements

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4.8 References


Chapter 5

5.1 Future Work

Presence of HFP in completed scaffolds has a negative effect on the mechanical strength and would be hazardous to patients if implanted. Although volatile enough to evaporate under environmental conditions, TGA showed that there were still reductions in mass loss between 28 and 42 days of drying, suggesting incomplete extraction. Exploring alternate drying techniques would be highly desirable before progressing with this composite material. For instance, although heating would extract HFP efficiently, it would also denature the collagen present, potentially reducing its effectiveness in cellular adhesion. Alternatively, vacuum drying or solvent replacement techniques may be considered in order to preserve the protein. Vacuum drying would create conditions where the HFP would more readily evaporate at lower temperatures, but may harm the scaffold’s structural integrity by forming pockets of gaseous HFP within the strands. Solvent replacement would need to make use of collagen-safe solvents than would not affect the structural integrity of the PLGA or nHA.

One manner of enhancing compressive modulus without sacrificing porosity, as established in this study, is via material choice. PLGA is a soft polymer in comparison to others, such as poly(L-lactic acid) (PLLA). Examining a similar scaffold design region with PLLA (or other mechanically strong polymer) and nHA composite could improve compressive modulus far above 10MPa, but may also affect biodegradation rate and flexibility of processing. Polymers that do not readily dissolve in collagen-friendly solvents may need to be heated in order to be fluid enough for use in 3D bioplotting, which would denature the collagen. Thus, in addition to examining alternative materials, it may be necessary to research alternative processing approaches as well.

Given that the two models suggest factor values at the extreme ends of the design region, there is reason to look beyond the defined boundaries of this study. nHA content had a significant effect on compressive modulus and the trend found did not have an obvious peak within the 0-30% range. Examining larger nHA concentrations may find such a peak, but previous studies have shown that large quantities of nHA can interfere with mechanical properties [1]. In addition, large concentrations will have a significant impact on the brittleness and biodegradation rate of the produced scaffolds. Strand spacing was found to have a peak
around 935\(\mu\)m, so examining a region above that value may not produce improvements. In addition, large strand spacing reduces strand quantity and surface area available for cellular attachment, which would reduce healing effectiveness \textit{in vivo}. However, there may be benefit to compressive modulus while designing within a lower spacing region. This expanded region is limited by a minimum strand spacing (edge-to-edge) of 300\(\mu\)m, as this is the minimum acceptable pore size for capillary formation within the scaffold [2]. No peak was found for strand diameter, but, by following the trend predicted by the model beyond the design region, it may be possible to find curvature for this factor. In addition, looking at a larger range for this factor may help compensate for any negative effects caused by the other two factors.

Here we considered compressive modulus exclusively, but this is not a reasonable simplification for some types of bone, such as those found in the arm. Tensile modulus of the scaffolds, based on their topology, is also a necessary consideration in the further development of bone tissue scaffolds.

\textbf{5.2 Conclusion}

This study sought to show that computer simulations could realistically design scaffolds for bone tissue regeneration and predict their mechanical properties. Current development of scaffold designs is primarily done using resource demanding methods, so the use of optimization/simulation software would improve research speed and costs. We found that, while the simulation under-predicted physical properties, COMSOL generated a reasonable design. Though not in full agreement with the experimental design, the COMSOL-optimized design gave an acceptable porosity and nearly met the compressive modulus requirement when analyzed with the experimental model. Thus, computational optimization of scaffold topologies, at this stage, could reasonably be used as a tool to determine high-interest design regions, which could be explored more efficiently than trial-and-error searches.
5.3 Resources
