ABSTRACT

HUEBNER DOS REIS, PEDRO. 3D-Bioplotted Scaffold for Autograft Substitution in Osteochondral Plug Transfer Approaches: Design and Process Characterization. (Under the direction of Dr. Rohan A. Shirwaiker).

Osteoarthritis and related injuries to the osteochondral tissue are characterized by strong pain, deformity, and functional disability of affected sites. The tissue’s uniquely complex structure and healing mechanisms challenge the ability of current treatment alternatives to promote full regeneration and recover function. Osteochondral plug transfer approaches, such as the chondral-osseous autograft transplantation technique (COR), have demonstrated to perform favorably, offering positive long-term results. However, limitations of the COR technique include donor site morbidity, limited graft availability, and possible damages to the graft during handling and implantation. Recently, scaffold-based tissue engineering (TE) approaches have been investigated as alternatives to native tissue grafts in the regeneration of functional tissues. Additive manufacturing processes, such as 3D-bioplotting (3DB), have evolved to become capable processes in the fabrication of osteochondral scaffolds, but additional research focusing on the characterization of associated material-process-structure interrelationships is required in the pursuit of clinical translation.

The focus of this dissertation is on the definition and characterization of design and process-related aspects in the fabrication of 3DB osteochondral scaffolds for TE applications. Here, a multiphasic, multi-material scaffold featuring zones matching those of the osteochondral tissue is designed based on established functional requirements for biomimicry and implantability. The 3DB process is characterized for the extrusion of mineralized composites used in select zones of the osteochondral scaffold. Specific objectives include:
• Establish a set of relevant scaffold attributes that represent functional requirements for implantability and biomimicry. These include the compressive elastic modulus of distinct zones across the osteochondral tissue, and specific design requirements to match the size and implantation characteristics of osteochondral plug grafts.

• Characterize the material-process-structure interrelationships associated with the 3DB process and the fabrication of osteochondral scaffolds, including the development and validation of models for predicting design features and scaffold characteristics of interest.

• Investigate a set of function-related aspects associated with the developed scaffold design, including size characteristics and their effect on implantation and primary stability forces, implantation success rates, as well as the biological cues provided by its multiphasic, multi-material structure. Finally, provide an assessment of risks and potential failure modes associated with the developed scaffold design.

From a product engineering perspective, this research undertakes a holistic approach to address current challenges in the design and fabrication of 3DB osteochondral scaffolds. Primary contributions include the development of testing protocols and experimental modeling approaches that can be readily adapted for a range of other applications in TE. Furthermore, select models here proposed lay the groundwork for smarter manufacturing control systems and support the development of algorithms based on the response surface modeling of relevant relationships between 3DB process parameters and outcomes, as well as design parameters and scaffold properties of interest. Lastly, functional assessment of the proposed 3DB osteochondral scaffold design featuring select biomimetic characteristics
provides preliminary evidence of stem cell biocompatibility and successful implantability in a clinical-type setting.
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3D-Bioplotted Scaffold for Autograft Substitution in Osteochondral Plug Transfer
Approaches: Design and Process Characterization

by
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DEDICATION

To Raven and my incredible family, whose support and encouragement made me get this far.
BIOGRAPHY

Pedro Huebner received his B.S. degree in Production Engineering from the Pontifical Catholic University of Parana, Brazil, in 2013. Shortly after, he joined the Ph.D. program in Industrial and Systems Engineering at the Edward P. Fitts Department of Industrial and Systems Engineering, and received his en route Master in Industrial Engineering degree in 2015. He is an active member of the Comparative Medicine Institute (CMI) and the Center for Additive Manufacturing and Logistics (CAMAL) at North Carolina State University. Over the years, he has worked in companies from diverse markets, including ERP consulting, customer support in the energy industry, and process engineering at a large-scale cosmetics production plant. More recently, he has held the position of teaching assistant for both graduate- and undergraduate-level courses in the fields of systems analysis and optimization, production engineering, and manufacturing engineering, and served as the instructor on record for a core undergraduate course in manufacturing engineering. He has also served as a Graduate Student Representative in the University Library Committee at NC State, as President of the Industrial and Systems Engineering Graduate Student Association, and Chair of the IISE Manufacturing and Design Division Student Committee. He is a recipient of the Edward A. Shook Mentor Award (2018), the IISE Gilbreth Memorial Fellowship (2017), the ISE Outstanding Teaching Assistant Award (2017), and the NCSU Mentored Teaching Assistant Award (2017). He will join the School of Industrial and Systems Engineering at the University of Oklahoma as an Assistant Professor stating Fall 2018.
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Finally, I would like to thank my family, especially my beautiful mom Emerly, for their continued and endless love and support. Not any less important, I would like to thank my husband Raven, the love of my life, for always standing by my side and constantly reminding me that everything is worth it when we are following our dreams.
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<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>3DB</td>
<td>Three dimensional bioplotting; Three dimensional biopotted</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>AM</td>
<td>Additive manufacturing</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>CAD</td>
<td>Computer aided design</td>
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<tr>
<td>CAE</td>
<td>Computer-aided engineering</td>
</tr>
<tr>
<td>CAM</td>
<td>Computer aided manufacturing</td>
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<tr>
<td>COR</td>
<td>Chondral-osseous autograft transplantation technique</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (also presented as kDa)</td>
</tr>
<tr>
<td>dECM</td>
<td>Decellularized extracellular matrix</td>
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<td>DoE</td>
<td>Design of experiment</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FDM</td>
<td>Fused deposition modelling</td>
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<td>FMEA</td>
<td>Failure modes and effects analysis</td>
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<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>hASC</td>
<td>Human adipose-derived stem cells</td>
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<tr>
<td>ln</td>
<td>Natural logarithm</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
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<td>O</td>
<td>Objective</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>OTE</td>
<td>Osteochondral tissue engineering</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal (also presented as kPa, MPa, GPa)</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
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<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<td>PGA</td>
<td>poly(glycolic acid)</td>
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<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
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<td>Pre-market approval</td>
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<td>Second</td>
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<tr>
<td>SLA</td>
<td>Stereolithography</td>
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<tr>
<td>SLS</td>
<td>Selective laser sintering</td>
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<tr>
<td>SQRT</td>
<td>Square root</td>
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<td>ST</td>
<td>Specific task</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>TCP</td>
<td>Tri-calcium phosphate</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering; Tissue engineered</td>
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<tr>
<td>TERM</td>
<td>Tissue Engineering and Regenerative Medicine</td>
</tr>
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<td>μm</td>
<td>Micron</td>
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CHAPTER 1 – INTRODUCTION

The multidisciplinary nature of the TERM field is characterized by the active contributions of several distinct scientific areas, as represented in Figure 1. Although the origin of the term “tissue engineering” dates back to 1987,1 the level of complexity introduced by a merge between engineering and life sciences still holds TERM far from mainstream. Recent studies utilizing advanced manufacturing techniques such as 3D printing have demonstrated the feasibility of creating tissue engineered medical products for clinical treatment of diseases and traumatic injuries, medical diagnostics, and *ex vivo* models for research.2 Examples of such applications include multilayered skin, bone, vascular grafts, tracheal splints, heart tissue, cartilaginous structures, and organs-on-a-chip.2–4 However, advanced-manufacturing-related aspects applied to TERM have been underexplored, and further understanding and characterization of those is necessary to enable future advancements in the field, as well as accelerating bench-to-bedside translation of promising solutions.5,6

Figure 1 – Disciplines playing a role in TERM. The role of Manufacturing Engineering is central to this dissertation.
In their current state, many of the processes involved in the design and fabrication of TERM products lack several requirements desired in a scaled-up manufacturing scenario. Work is still necessary to enable highly automated processes to reproducibly manufacture products with consistent critical design features and quality attributes with little to no human intervention. While keeping these motivations in mind, this dissertation discusses the current state of established clinical techniques adopted in the treatment of osteoarthritis, a common degenerative disease of joint tissues, and investigates important functional design and manufacturability aspects of a TERM-based alternative approach.

1.1 **Clinical Motivation: Osteoarthritis and Related Injuries**

Osteoarthritis (OA), the most common joint disease worldwide, is characterized by the degeneration of the articular cartilage that covers and protects the ends of bones against friction. Sites commonly affected by the disorder include the joints in arms and hands, legs and feet, and the spine. Severe cases may induce the rubbing of opposing bones in the joint and permanent changes in their structure, including the formation of spurs or cavities. Traumatic injuries, hereditary factors, and the effects of aging can directly contribute to the development of OA. The disease is generally associated with strong pain, discomfort, deformity, and functional disability, affecting, as a result, the quality of life of diagnosed individuals. Statistics show that about one in every two adults may develop symptomatic knee OA by the age of 85, with an increased probability for obese persons (two every three). In younger populations, OA is typically a consequence of repetitive stresses or trauma of the knee joint. Palmieri-Smith *et al.* observe that OA in being increasingly associated with posttraumatic injuries, accounting for approximately 5.5 million US cases.
every year, leading that age group into high risk of developing the disease before the age of 40.11 Curl et al. reported that about 19% of chondral injuries detected in a sample of knee arthroscopies were of the most severe grade, in which bone is exposed.12

In anatomy, the term “osteochoondral tissue” is used to refer to the junction between the articular cartilage (-chondral) and the underlying bone (osteo-) present in joint interfaces. In a simple manner, the osteochondral unit can be divided into three primary zones: (1) the articular cartilage above a thin tidemark, (2) the transitional interface immediately below the tidemark including calcified cartilage and subchondral bone, and (3) the underlying cancellous bone, as illustrated in Figure 2 (adapted from Kerr, 1999).13 As suggested by such organization, higher levels of tissue calcification are observed at larger depths.14–16 The articular cartilage component itself (zone 1) is formed by several subzones featuring specific collagen network organizations.17 The tidemark, a thin layer composed by a dense network of collagen fibers, provides structural integrity at the interface between zones 1 and 2.18

![Diagram of osteochondral tissue zones](image)

**Figure 2 – Histological representation of the osteochondral tissue and the several zones across its depth.**
When damaged, the chondral component of the osteochondral complex offers low potential for self-regeneration due to limited blood supply and innervation. The subchondral bone, on the other hand, hosts mesenchymal stem cells and a network of blood vessels which are readily available to trigger a healing process upon an injury-related inflammatory response. Due to its unique characteristics, the subchondral bone is considered a prominent agent in the regenerative process, suggesting that favorable viability levels of its own can positively contribute to the viability of the overlying cartilage layers.

1.2 Current Treatments for OA

Commonly adopted treatment strategies for OA lesions include both non-surgical (conservative) or surgical (operative) therapies, which can take place over long periods of time, and may offer partial recovery. Operative treatment techniques are chosen on a case-by-case basis, with factors such as surface area/depth of the lesion and surgeon experience/preference being taken into account. Conventional approaches include debridement, marrow tapping, abrasion, subchondral drilling, microfracturing, and osteochondral auto/allografting. These methods are usually associated with a fibrocartilaginous ingrowth with unpredictable results that may not restore tissue function in a satisfactorily manner. A newer generation of operative treatments include autologous cell implantation, use of growth factors, and gene therapies, but, despite the evidence of positive early outcomes, follow up studies have shown limited long-term improvement of targeted tissues. Akhavan et al. developed a generalized treatment algorithm for approaching osteochondral defects in the knee joint, presented in Figure 3.
As noted in the figure, autografting (for small lesions, in the form of an osteochondral plug) and allografting (for large lesions, in the form of bulk osteochondral grafts) are both well-accepted techniques for providing restorative and reconstructive potential of the damaged tissue, respectively. The chondral-osseous autograft transplantation technique (COR® Precision Targeting System, Depuy-Mitek, Westwood, MA)\textsuperscript{23} enables a relatively simple procedure for harvesting autografts from healthy, low-load-bearing areas of the joint and transferring them into osteoarthritic diseased areas. For about a decade, the COR system technique has demonstrated favorable clinical results and has been fairly well utilized by clinicians for addressing injury-related symptoms.\textsuperscript{24}

However, due to the numerous aspects factoring in the success of plug transfer procedures, a number of potential shortcomings have been associated with them:\textsuperscript{19,25}

- Donor site morbidity;
- Disease transmission or infection;
- Immunologic rejection;
- Cartilage degeneration due to excessive implantation pressures;
- Asymmetric graft;
- Loose/proud graft;
- Graft reabsorption;
- Separation of cartilage from subchondral bone during harvesting;
- Limited graft availability.

Despite the associated risks, autograft plug transfer approaches have shown an over-90% rate of good to excellent long-term results, although donor site morbidity remains an unaddressed issue.\textsuperscript{26} While pursuing a solution, tissue engineering strategies are being
investigated as alternatives to overcome the limitations of current surgical options. The following section discusses the evolution of engineered osteochondral tissues as an alternative to replace the utilization of native tissue grafts.

1.3 Scaffold-based TE Approaches

The field of TE is characterized by a diverse pool of possible strategies to achieve tissue regeneration, including purely cell-, biomolecule-, hydrogel-, or scaffold-based strategies. The latter, and most frequently applied, scaffold-based strategy focuses on developing approaches that attempt to provide cells with specific 3D environments that match or closely resemble the characteristics of native extracellular matrix (ECM). Such 3D environments are referred to as “scaffolds”.27 The ECM of human tissues allows anchorage-dependent cells to reside and develop their particular functions while dictating the mechanical properties of the structures that they form. In addition, it provides bioactive cues (chemical and physical) for cells and serves as a reservoir of growth factors that stimulate tissue formation and development. Therefore, scaffolds are used in TE to offer similar functions while providing a degradable environment to allow vascularization and remodeling of the targeted areas during the regenerative process.28 Sundelacruz & Kaplan (2009) have proposed a series of biologically informed scaffold design considerations for tissue engineering, presented in Table 1.29
Table 1 – Scaffold design requirements in TE.

<table>
<thead>
<tr>
<th>Scaffold Design Considerations</th>
<th>Biological Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choice of biomaterial and biocompatibility</td>
<td>– Support cell proliferation and differentiation&lt;br&gt;– Suitable for implantation in vivo</td>
</tr>
<tr>
<td>Geometry and architecture</td>
<td>– Support 3D tissue growth&lt;br&gt;– Control morphology of the growing tissue&lt;br&gt;– Support cell proliferation&lt;br&gt;– Favor cell differentiation into particular lineage</td>
</tr>
<tr>
<td>Porosity</td>
<td>– Support cell differentiation&lt;br&gt;– Support cell recruitment&lt;br&gt;– Support cell aggregation&lt;br&gt;– Support vascularization</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td>– Support mechanical loading</td>
</tr>
<tr>
<td>Degradation rate</td>
<td>– Permit new tissue ingrowth&lt;br&gt;– Permit remodeling of the ECM&lt;br&gt;– Match healing rate of the new tissue</td>
</tr>
<tr>
<td>Biochemical stimuli</td>
<td>– Incorporate appropriate growth factors and cytokines for enhanced cell function</td>
</tr>
</tbody>
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Current research in bone and cartilage TE focuses mostly on the utilization of scaffolds that combine living cells and porous biomaterials organized in 2D or 3D structures that provide a template for tissue growth. These scaffolds are commonly built with biodegradable polymers, which allow a desired level of flexibility in processing and the ability to alter their chemical properties.30

The choice of biomaterials in scaffold fabrication is a function of diverse factors relevant to their processability and contributions to the regenerative process of the tissues they mimic. Natural or biodegradable synthetic polymers have been widely investigated, and the later are of particular interest due to their ease of processing, controlled biodegradation rates, surface characteristics, mechanical properties, and biocompatibility.2,31 Many biodegradable synthetic polymers play a significant role in a range of TERM applications, notably poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ethylene glycol) (PEG), and
poly(ɛ-caprolactone) (also referred to as polycaprolactone, PCL).\textsuperscript{31-33} The later, PCL, a biodegradable aliphatic polyester, has been shown to be favorable for bone and cartilage scaffold fabrication. It is fully biocompatible and presents desirable degradation rates, similar to those of new bone and cartilage formation.\textsuperscript{34} Also, select properties of this class of synthetic biopolymers can be tailored by copolymerizing it with other biopolymers – i.e. PLA-PCL copolymers express larger percent mass loss over time (faster degradation) and tunable mechanical properties depending on chemistry,\textsuperscript{35} similarly for PCL-PEG copolymers which also demonstrate much improved hydrophilicity levels.\textsuperscript{36} In TERM-specific applications, PCL-based scaffolds are suitable for stem cell utilization, allowing cooperative differentiation of vascular, osteogenic, and chondrogenic cells within the microenvironment they provide.\textsuperscript{37,38} Porous, biocompatible scaffolds fabricated by traditional processes such as electrospinning, freeze-drying, gas foaming, and solvent casting (particulate leaching) have been extensively investigated for both bone and cartilage tissues.\textsuperscript{39-41}

TE-based approaches for the regeneration of viable, full thickness osteochondral tissue are still in developmental stages. Biomimetic considerations play a key role when defining optimal scaffold design, composition, and functional characteristics. As a matter of fact, the nature of the osteochondral complex has driven the utilization of biphasic (or even multiphasic) scaffolds.\textsuperscript{17} Several studies have utilized different materials and/or internal microarchitectures across the thickness of these scaffolds, while also investigating the behavior of different cell types seeded onto them (osteoblasts, chondrocytes, and marrow- or adipose-derived mesenchymal stem cells).\textsuperscript{42-47} Typically, the cartilage and bone phases are fabricated separately and then assembled/joined together.
Chen et al. (2006) introduced a scaffold design in which collagen was used as a base material in both phases, but included an additional poly(lactide-co-glycolide) (PLGA) component in the bone phase. PLGA “sponges” were prefabricated by particulate-leaching using sodium chloride as a porogen (particulates diameters ranging from 355 to 425 µm). The biphasic structure was achieved by adding, lyophilizing, and crosslinking bovine collagen within and above the prefabricated PLGA sponges. Cylindrical samples were seeded and cultured with canine bone mesenchymal stem cells for one week, and posteriorly implanted into canine osteochondral defects for *in vivo* analysis. After 3 months, layer-specific cartilage and bone tissues were formed, showing a smooth surface and good integration with the surrounding tissue, highlighting the potential of such approach for osteochondral tissue regeneration.42 Kon et al. (2010) evaluated the safety and efficacy of a triphasic scaffold in a pilot human clinical trial. Their design consisted of a cartilaginous phase (type I collagen), an intermediate or “tidemark” phase (60% type I collagen and 40% HA), and finally a bone phase (mineralized blend of 30% of type I collagen and 70% of HA). The three phases were separately fabricated by freeze drying and then knitted together. A total of 28 patients were surgically implanted with these scaffolds and evaluated after 6, 12, and 24 months. Significant improvements were observed in all follow up evaluations, and histological analysis of biopsies after two years revealed a complete adsorption of the biomaterial and presence of osteochondral-like tissue.45 Lien et al. (2009) introduced a quadriphasic scaffold design in which a porous calcium polyphosphate phase represented the bone, followed by a narrow separation phase of beta tricalcium phosphate (TCP), and two distinct cartilage phases representing, in order, the calcified cartilage with a porous ceramic, and the remainder of the articular cartilage with a porous gelatin phase. Each phase was
fabricated separately by particulate-leaching, spin coating, or freeze drying to achieve desired
design features and porosities. Although a full-thickness osteochondral scaffold design was
used, *in vitro* cell response assays focused on characterizing the formation of cartilage tissue.
Seeded rat joint chondrocytes were able to attach to the surface of the scaffolds in 1 week
and proliferate through most of it in 2 weeks. At the end of 4 weeks, cell number had
increased by four-fold and the glycosaminoglycan content had increased by 20 times,
indicating the formation of cartilage neotissue.46 More recently, Mellor *et al.* (2015)
proposed an approach based on stacking several layers of electrospun sheets of polylactic
acid (PLA) nanofibers with gradually varying levels of TCP (0 or 20% by weight) seeded
with human adipose-derived stem cells (hASC). Their results have shown that, when cultured
in chondrogenic differentiation media, stem cells differentiated locally and induced the
formation of bone-like tissue in layers containing TCP, and cartilage-like tissue in layers
without TCP.47 This particular approach is notable for utilizing a scaffold that features
appropriate thickness for implantation into an osteochondral defect and induces site-specific
differentiation of stem cells. However, although electrospun matrices are good emulators of
the ECM nanostructure, they also are, at the macroscopic level, notably fragile, lacking
desired mechanical properties.48 Standard implantation techniques in orthopedic research will
likely require scaffolds capable to offer better handling characteristics.

In conclusion, the aforementioned studies so far have demonstrated the feasibility of
multi-material, multi-geometry scaffolds for osteochondral applications. However, the
fabrication methods and scaffold handling characteristics are not yet suitable for clinical
translation. The traditional fabrication methods used, including electrospinning, particulate
leaching, and freeze drying offer limited control over the geometry and composition of the
scaffolds, as well as limited reproducibility and control over pore sizes, shapes, and structural organization. More importantly, these approaches require phases with different materials and design specifications to be independently fabricated and subsequently joined together. From a manufacturing perspective, these processes are unable to yield quality products targeting precisely defined design and functional parameters, and are evidently unsuitable to be included in highly controllable and reproducible fabrication protocols. As an attempt to overcome these drawbacks, 3D printing processes are now being investigated for multi-phasic scaffold fabrication.

1.4 Additive Manufacturing and 3D-Bioplotting

Recent advances in manufacturing technologies have enabled the design and fabrication of scaffolds with geometries and features that could not be easily achieved with traditional processes. Additive manufacturing (AM), commonly referred to as 3D printing, represents a family of digitally-driven processes in which constructs are created layer-by-layer using different direct-writing principles. In the context of TE, the synergistic use of computer-aided design and manufacturing (CAD/CAM) techniques, 3D printing processes, appropriate biomaterials and cell delivery methods enables the reproducible fabrication of scaffolds with tailored architectures to match mechanical and biological characteristics of interest.49

Several different 3D printing processes, including robotic assisted deposition (robocasting),50,51 stereolithography (SLA),52,53 selective laser sintering (SLS),54,55 fused deposition modeling (FDM),56,57 and 3D-bioplotting,58–60 are being explored in various TE applications. Some of these processes allow a one-step fabrication of integrated
heterogeneous scaffolds. In the context of osteochondral TE, these scaffolds are composed of two (or more) distinct phases for the cartilage and bone regions (and phases within them) built sequentially in a single 3D-printing cycle, eliminating the need to assemble the separate sections together prior to implantation. In this diverse pool of additive manufacturing processes, 3D-bioplotting (3DB) is of particular interest due to its ability to process multi-material and multi-geometry scaffold designs in a highly controllable and reproducible manner.

3DB, an extrusion-deposition-based process, offers precise control over the micro-architecture and material composition of produced scaffolds, and is able to create anatomically relevant geometries with relative ease. Additionally, 3DB is relevant for its ability to incorporate both living and non-living biomaterials (e.g., biodegradable polymers, polymer-ceramic composites, decellularized ECM hydrogels, growth factors, cells etc.) within fabricated structures. Some of the basic 3DB process and setup parameters in 3DB are presented below:

- Print-head travel speed (X-Y);
- Extrusion pressure;
- Extrusion temperature;
- Nozzle diameter;
- Pre/post-flow delay;
- Strand lay-down pattern;
- Layer offset;
- Distance between strand axes (X-Y);
- Layer thickness (Z);
- Platform height offset.

By controlling these parameters, 3DB allows the creation of scaffolds with controllable design features including strand width, interstrand spacing, layer-by-layer strand pattern, pore size, and pore interconnectivity. These design features are critical because they directly affect the mechanical properties of the resulting structure, the cell migration,
adhesion, differentiation and ECM formation abilities, as well as the transport of nutrients and waste in and out of the scaffold.62

3DB scaffolds have been independently tested for bone and cartilage tissue engineering, and have been found to sustain desirable levels of cell activity and potential for tissue formation. For example, in bone TE, Yilgor et al. (2008) studied the effects of PCL (Mₜₜ = 37 kDa) scaffolds plotted in four different lay-down patterns, including 0°/90° and 0°/120°/240° with no offsets between layers in the same direction, and 0°/90° and 0°/120°/240° with offsets (so that pores were not through), on rat bone marrow-derived MSC. In vitro testing demonstrated higher cell proliferation on the two designs with the offset layers after 21 days in culture. However, the 0°/120°/240° non-offset design demonstrated the highest alkaline phosphatase (ALP) activity which indicates the degree of differentiation of MSCs into osteoblastic cells.63 Shor et al. (2007) demonstrated that fetal bovine osteoblasts seeded onto PCL(Mₜₜ = 44 kDa)-HA (75-25% by weight) scaffolds featuring 450 µm strand width, 0°/90° strand lay-down pattern, and 60%-70% porosities were able to show higher ALP activity. Consequently, they produced more mineralized matrix than those seeded onto pure PCL scaffolds during a 21-day incubation period.64 Lee and Kim (2011) seeded human osteoblast cells into collagen-coated 3D plotted scaffolds made with a composite of PCL (Mₜₜ = 80 kDa)-TCP (60-40% by weight). Scaffolds extruded through a 460 µm nozzle with 300 µm strand separation in a 0°/90° lay-down pattern demonstrated favorable osteoblastic cell behavior, encouraging initial cell attachment and an increase in viable cell count after 7 days.65 In a cartilage TE-related study, Park et al. (2009) fabricated 3D plotted porous PCL (Mₜₜ = 80 kDa) scaffolds with distinct strand diameter (200, 250, 330 µm) and separation (300, 500, 700 µm). A dynamic mechanical testing
In vitro culturing of seeded chondrocytes demonstrated that cells were able to spread over the surface of the scaffolds after 4 weeks and form a dense cellular network completely filling the pores after 8 weeks. The authors’ results suggest that 3DB PCL scaffolds could be designed to serve as biological substitutes offering compressive moduli and overall ECM characteristics comparable to some cartilaginous tissues. Lee et al. (2014) characterized the performance of protein releasing 3DB PCL scaffolds with human MSC, in vitro. Results demonstrated a favorable formation of cartilaginous matrix throughout the surface of the scaffolds, including zone-specific collagen type I and II matrixes, sustained over a period of 42 days. They also assessed anatomically relevant scaffolds featuring varying strand widths (100 - 200 µm) and separation (50 - 500 µm) in a sheep model. Results showed that 12 weeks after implantation, the scaffolds were fully integrated with native tissue without major signs of damage to surrounding areas.

For OTE, 3DB scaffolds have shown to be a promising element in tissue regeneration. Luo et al. (2013) tested a novel organic-inorganic biphasic scaffold fabricated by 3DB. In their design, a bottom phase of calcium phosphate cement was plotted intercalated with a highly concentrated alginate paste, followed by an upper phase of alginate only. Scaffolds printed in a 0°/90° lay-down pattern using a 610 µm nozzle and seeded with hMSC were cultured for 7 days. The results were promising, with desirable levels of cell viability and population size being observed within the hybrid structures, suggesting a favorable design for future osteochondral research.
1.5 **3D-Bioplasted Osteochondral Scaffolds: Proof-of-Concept**

In scaffold-based TE, it is important for scaffolds to mimic the physical, chemical, and mechanical characteristics of native tissue ECM as closely as possible. This aspect is of particular interest for osteochondral applications, wherein the tissue characteristics vary significantly along the depth of the tissue.

A recent study by our group (in collaboration with Drs. Elizabeth Loboa and Jeffrey Spang) has demonstrated that a biomimetic osteochondral scaffold, which included multiscale and multi-material features, was able to demonstrate site-specific osteochondral tissue characteristics following *in vitro* culture with hASC. We first assessed the *in vitro* potential of single-phase 3DB scaffolds with PCL-decelularized cartilage matrix (dECM), or a composite of PCL-TCP (80-20% by weight). Respectively, these scaffolds were intended to provide the microenvironment necessary to trigger the differentiation of stem cells into cartilage and bone progenitors after several weeks of culture. In both cases, the observed results were promising. PCL-dECM scaffolds offered a more uniform cell proliferation and higher levels of cell differentiation (chondrogenesis, indicated by Safranin-O staining and RNA expression) when compared to purely PCL scaffolds. Furthermore, PCL-TCP scaffolds demonstrated reasonable calcium release levels in culture, which triggered osteogenic differentiation of seeded hASC, indicated by Alizarin Red staining and enzymatic Alkaline Phosphatase tests.

The study also investigated hASC and ECM responses in an integrated heterogeneous 3DB scaffolds featuring both cartilage and bone phases, as defined above. A prefabricated electrospun sheet of PCL separated these two phases, mimicking the function of the tidemark layer (see Section 1.1). These “triphasic” scaffolds were cultured *in vitro* for 28 days, then
fixed and characterized through standard staining protocols (Alizarin Red, Alcian Blue, and Safranin-O). Results suggested site-specific metabolic activity of cells within each phase, separated by the electrospun tidemark, demonstrating a desired osteochondral-like organization.

The aforementioned study serves as the proof-of-concept for 3DB biomimetic osteochondral scaffolds. The following section defines an adapted design incorporating some of the fundamental scaffold design characteristics identified from this study and the clinical COR system-based autograft transfer procedure. The experimental assessment of such scaffold design represents a first step in the conceptualization of a functionally viable product fabricated using advanced manufacturing techniques in a reproducible manner.

1.6 3D-Bioplotted Hybrid Osteochondral Scaffold Design: A Closer Look

This dissertation conceptualizes a hybrid 3DB osteochondral scaffold design that, upon further optimization of its design and fabrication aspects, could offer the potential to eliminate the utilization of native tissue grafts in plug transfer procedures and address some of its related shortcomings. Figure 4 describes the overall design of such scaffold. Note the assimilation of the hybrid multi-material structure setup (B) to that of native osteochondral tissue (A).
In brief, an upper PCL-only phase (I) is intended for developing cartilage tissue, separated by a thin electrospun layer (II) to represent the tidemark, and two underlying layers for increasingly mineralized tissues (III and IV). The diameter of such plug is intended to be approximately 8 mm dictated by the standard size of the chosen COR kit (C). The full thickness is equally divided into three phases, each 2 mm thick. As discussed in Section 1.5, and supported by the proof-of-concept data,70,71 this scaffold design is expected to induce site-specific osteogenic and chondrogenic differentiation of hASC under specific culturing conditions.

Recent TERM literature on the osteogenic differentiation of stem cells has given relative focus to the effects of added HA or TCP to the composition of bioactive scaffolds as means of providing the necessary biochemical cues for cell differentiation. HA is a calcium
phosphate-based ceramic that can be found in either natural or synthetic forms. It is closest to human bone mineral content and is known for having very slow resorbability rates, likely to persist for years post implantation. On the other hand, TCP is a calcium salt that, when added to scaffolds, has shown to be highly reactive with water to form an HA group that undertakes faster dissolution rates under physiological conditions. The choice between HA and TCP will be based on the application, considering factors such as desired scaffold mechanical properties, resorbability, and biological properties. Huang et al. (2018) compared PCL-TCP against PCL-HA 3D printed scaffolds at concentrations of 90-10% and 80-20% by weight for both groups. Their findings demonstrated that PCL-TCP scaffolds had larger compressive moduli than their PCL-HA counterparts but, while both groups presented equality satisfactory levels of seeded hASCs viability (cultured for 14 days), the PCL-HA group offered higher levels of overall cell proliferation at the end of culture. In the scope of this dissertation, TCP concentrations of 10% and 20% for the transitional and osseous zones of the osteochondral scaffold design, respectively, have been chosen, having substantial literary evidence of osteogenic stem cell differentiation at those levels. The lower, 10% TCP concentration in the transitional zone is intended to account for the presence of calcified cartilage interfacing with the subchondral bone. These concentrations also provide desirable mechanical properties for PCL-TCP scaffolds. For instance, Dávila et al. (2016) found that a TCP concentration of 30% decreased the scaffold stiffness compared to 20%. Park et al. (2015) tested 50% and 70% concentrations, and found that their compressive strength decreased significantly and were even lower than pure PCL. Therefore, concentrations up to 20% provide a satisfactory trade-off between the ability of the material to provide cues for cell differentiation and offer desirable mechanical properties.
From a clinical translation perspective, another advantage of this scaffold is its ability to be implanted using the clinically relevant COR procedure. For instance, as represented in Figure 5, the steps including graft harvest and transfer would be eliminated (represented in red) and replaced with alternatives (represented in yellow). By doing so, issues such as donor site morbidity and autograft failure would be eliminated. From a manufacturing perspective, several aspects related to the design and fabrication of hybrid scaffold designs featuring specific material compositions have yet to be studied and characterized. The following section identifies process- and design-specific requirements that support the bench-to-bedside translation of this category of products that will be the focus of this dissertation.

Figure 5 – The COR Procedure (A) and a proposed alternative (B) in which no graft harvest is involved.
1.7 Motivation and Research Objectives

In the global market of medical products, regulatory agencies govern minimum safety and efficacy standards applied to the products they regulate and oversee. Intrinsically, these performance measures are directly affected by the choice of design features, materials, and processing parameters of choice during the fabrication of those products, which must, in effect, be well planned, executed, tested, and documented. In the relatively young TERM field, some preliminary work has been done to characterize material-process-structure-function interrelationships in osteochondral TE solutions, however, additional work is necessary to design safe and effective products suitable for scaled-up manufacturing. One of the critical challenges from a clinical application perspective lies in creating a scaffold that can mimic the multi-phasic nature of the osteochondral complex which performs well during and post-implantation. Currently, published literature offers supporting evidence that 3DB is a feasible process for fabricating highly reproducible TE scaffolds. Further research is necessary to define basic functional requirements and optimal design parameters of a well-performing full-thickness 3DB osteochondral scaffold.

Developing a TE-based alternative to a clinically relevant technique, such as the COR procedure, requires the definition of implantability- and biomimicry-related functional requirements. In terms of the implantation and primary stability forces of osteochondral plugs, some preliminary work has been reported, but so far, it lacks validation and interpretation of the results from an engineering standpoint. Furthermore, the literature provides substantial characterization of the 3DB process for the fabrication of pure PCL structures, but there is limited information about mineralized PCL-TCP composites to be used as agents in the regeneration of calcified tissue zones. Therefore, a comprehensive study
motivated by these opportunities is hereby proposed, paving the way to an alternative and feasible approach in the field of osteochondral TE. Ultimately, following the characterization of materials and processes involved, a proposed scaffold design with focus on implantability and biomimicry will be tested and discussed.

**Figure 6** illustrates the interrelationships between four defining nodes in product engineering: material, process, structure, and function. Choices made at each node have the potential to introduce effects in all the others. From a functional perspective (central node), understanding and characterizing such potential interactions facilitates the development of well-performing products. In the scope of TE scaffolds, the same interrelationships are present, and some of the relevant ones are investigated as part of this dissertation.

![Figure 6](image.png)

*Figure 6 – Graphical representation of Material-Process-Structure-Function interrelationships accounted for during the stages of product engineering.*

This dissertation focuses on understanding the material-process-structure-function interrelationships (**Figure 6**) relevant during the design, fabrication, and usage stages of the multi-phasic osteochondral scaffold design discussed earlier. It begins by establishing a set of
main functional requirements for implantability and biomimicry, then characterizing the 3DB process and resulting 3DB structure characteristics, and finally developing a full-thickness osteochondral scaffold design. Three objectives (O) and associated specific tasks (ST) are defined as follows:

- **O1**: Establish a set of relevant scaffold attributes that represent functional requirements for implantability and biomimicry: These include the compressive elastic modulus of distinct zones across the osteochondral tissue, and specific design requirements to match the size and implantation characteristics of osteochondral plug grafts. O1 is associated with the Function node in Figure 6. Specific tasks include:
  
  - **ST1.1**: Establish appropriate testing protocols and define target compressive elastic moduli of osteochondral grafts at different tissue depths (superficial, transitional, and osseous zones, as represented in Figure 2) from samples harvested using the 8-mm COR kit.
  
  - **ST1.2**: Dimensionally characterize osteochondral grafts and implantation sites produced with the 8-mm COR kit and define target dimensional aspects of the fit between osteochondral implants and their implantation sites.
  
  - **ST1.3**: Define target implantation and primary stability forces between osteochondral grafts and their implantation sites produced with the 8-mm COR kit in an ex vivo model.

- **O2**: Characterize the material-process-structure interrelationships associated with the 3DB process and the fabrication of osteochondral scaffolds, including the development and validation of models for predicting design features and scaffold
characteristics of interest. **O2** is associated with the interrelationships represented in dark red color in **Figure 6**. Specific tasks include:

- **ST2.1**: Conduct screening studies to define **feasible 3DB processing parameter ranges** (bounds for temperature, pressure, speed, and nozzle diameter) for PCL-TCP composites (weight ratios 90-10% and 80-20%).

- **ST2.2**: Model the **effects of varying levels of controllable 3DB parameters** (defined in ST2.1) on the **width of deposited strands** of multi-layer structures of PCL-TCP composites using statistical design of experiments (DoE). Utilize the data to construct a mathematical model capable of predicting scaffold strand width based on a set of processing parameters.

- **ST2.3**: Model the **effects of different scaffold design features** (material, strand width and interstrand spacing) on the **compressive elastic modulus** of multi-layer structures using statistical DoE. Utilize the data to construct a mathematical model that predicts these outcomes based on a set of scaffold design parameters.

- **ST2.4**: Validate the predictive models developed in ST2.2 and ST2.3, based on the requirements of a scaffold design expected to closely resemble the functional characteristics of osteochondral grafts as defined in O1.

- **O3**: Investigate a set of function-related aspects associated with the developed scaffold design, including size characteristics and their effect on implantation and primary stability forces, implantation success rates, as well as the biological cues provided by its multiphasic, multi-material structure. Finally, provide an assessment of risks and failure modes associated with the developed scaffold design. O3 is
associated with the *Structure-Function* interrelationship and the *Function* node itself in Figure 6. Relevant specific tasks include:

- **ST3.1**: Assess the biological *in vitro* behavior of stem cell populations seeded and cultured within scaffolds designed to match the compressive elastic properties of native osteochondral tissue. Investigate cell viability and metabolic activity, as well as site-specific stem cell differentiation induced by osteogenic factors within scaffold zones.

- **ST3.2**: Investigate scaffold *macro-geometries* to maximize the ease of implantation and implantation success rates in a simulated surgical-type setting based on surgeon’s feedback, then benchmark the implantation and primary stability force characteristics of the best-performing scaffold design against the clinical gold standard.

- **ST3.3**: Conduct risk assessment of the design, fabrication, and use of 3D-bioplotted osteochondral scaffolds by means of failure modes and root cause analyses, focusing on implant functional performance and translational aspects of manufacturing.
CHAPTER 2 – DEFINITION OF RELEVANT SCAFFOLD ATTRIBUTES

2.1 Chapter Introduction

In order to restore normal joint biomechanics in articulating surfaces of the knee, engineered tissue replacements need to target specific functional properties of their native counterparts.86 Within the scope of this dissertation, the experimental definition of a set of functional requirements for implantability and biomimicry will provide target characteristics to be matched by a scaffold-based TE alternative. This chapter describes the definition of the following requirements: (1) target **compressive elastic moduli** of osteochondral grafts at different tissue depths (superficial, transitional, and osseous zones); (2) target **dimensional aspects of the fit** between osteochondral implants and their implantation sites; and (3) the target **implantation and primary stability forces** between osteochondral grafts and their implantation sites produced with the 8-mm COR kit.

The mechanical characterization of bone and cartilage has been extensively discussed in the literature.87,88 A wide range of sample preparation approaches and testing protocols have been reported, including those focusing on the individual zones of the osteochondral complex.89 According to experts in the biomedical field, the varying morphology and physiology across these zones dictate the unique biomechanical properties of the osteochondral tissue, and matching these characteristics with tissue engineered alternatives remains one of the major challenges in proposing clinically relevant solutions.90 For the purposes of this dissertation, as defined in Section 1.6, the thickness of the osteochondral grafts produced with the COR kit can be divided in three major zones: the articular cartilage (zone 1, superficial), the calcified cartilage and subchondral bone (zone 2, transitional), and
the cancellous bone (zone 3, osseous). In humans, the compressive elastic moduli across these different zones have been reported to fall within the ranges of 0.07-1.23 MPa, 1150-19800 MPa, and 1147-4590 MPa, respectively. Sources of variability in these results likely include the choice of testing protocols and specimen preparation. Thus, in order to establish the first functional requirement of TE scaffolds intended to replace osteochondral autografts in plug transfer techniques, the compressive elastic modulus of each of the three aforementioned zones will be experimentally determined from grafts harvested using the 8-mm COR kit.

Furthermore, the fit and biomechanical characteristics between grafts and their respective implantation sites are of equal importance. Bowland et al. (2015) identify two types of graft settling characteristics following implantation: bottomed or unbotteded grafts; see Figure 7, adapted from Bowland et al. (2015).

![Figure 7 – Diagrams of osteochondral grafts and their implantation sites. (A) Bottomed grafts, and (B) unbotteded grafts, in which the depth of the defect is larger than the length of the graft.](image-url)
Bottomed grafts occur when the graft thickness and implantation site depth are equal, and unbotted occur when the implantation site is deeper than the graft length and a void exists underneath it. In both cases, the external surface of the graft must be leveled with the surface surrounding the implantation site. If the graft happens to be longer than the depth of the implantation site, it will protrude out, which is undesired due to increased contact pressures. In practice, the challenges associated with producing grafts and implantation sites of equal sizes during surgery are evident and, for that reason, unbotted grafts are preferred as they minimize the risk of protruding grafts. In that case, the resistance to motion arising from the interference fit between grafts and implantation sites is determinant of the graft stability following surgery. This dissertation defines, through metrology, the fit characteristics between grafts and implantation sites produced with the 8-mm COR kit. The forces necessary to implant or to shift these grafts after implantation are also experimentally characterized. Published studies provide some background with respect to these functional characteristics, but none has directly tested grafts matching the geometrical characteristics discussed previously (Table 2).

All biological experimentation requiring harvest and testing of native tissue will be performed on ex vivo porcine specimens. Although the utilization of an animal model is not optimal, swine models are deemed ideal for preclinical model systems and have been extensively used in the development of medical skill education and the development of new devices and therapeutic strategies. Notable advantages include close resemblance to select human musculoskeletal characteristics (including the structure of the osteochondral complex and joint loading mechanics), their large size which is ideal for surgery-based studies,
and the lower cost associated with larger availability of specimens and testing facilities that do not require certification for testing of human samples.

Table 2 – Implantation and primary stability forces of press-fit osteochondral grafts.

<table>
<thead>
<tr>
<th>Test</th>
<th>Sample Source</th>
<th>Toolkit</th>
<th>Graft Geometry (diameter x length)</th>
<th>Result (Force in N)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation</td>
<td>Femur of 6-month old pigs</td>
<td>MosaicPlasty System</td>
<td>4.5 x 15 mm</td>
<td>43.5 (average)</td>
<td>Kordás et al. (2006)²²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.5 x 15 mm</td>
<td>76.2 (average)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyurethane foam block</td>
<td>COR System</td>
<td>6 x 15 mm</td>
<td>132.6 (maximum)</td>
<td>Barber et al. (2008)⁸⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 x 15 mm</td>
<td>175.9 (maximum)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human distal femur</td>
<td>OATS</td>
<td>6 x 8 mm</td>
<td>143 (maximum)</td>
<td>Kock et al. (2006)⁹⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 x 12 mm</td>
<td>150 (maximum)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 x 16 mm</td>
<td>138 (maximum)</td>
<td></td>
</tr>
<tr>
<td>Primary Stability</td>
<td>Femur of 6-month old pigs</td>
<td>MosaicPlasty System</td>
<td>4.5 x 15 mm</td>
<td>69.4 - 92.5 (push in)</td>
<td>Kordás et al. (2006)²²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.5 x 15 mm</td>
<td>118.1 - 122.2 (push in)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Femur of adult pigs</td>
<td>OATS</td>
<td>8 x 15 mm</td>
<td>41 (pull out)</td>
<td>Duchow et al. (2000)³³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 x 15 mm</td>
<td>92 (pull out)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human distal femur</td>
<td>OATS</td>
<td>6 x 8 mm</td>
<td>135 (push in)</td>
<td>Kock et al. (2006)⁹⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 x 12 mm</td>
<td>145 (push in)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 x 16 mm</td>
<td>153 (push in)</td>
<td></td>
</tr>
</tbody>
</table>

This chapter addresses O1 and its associated tasks defined in Section 1.7. Materials and Methods are described in Section 2.2, and Results and Discussion in Section 2.3. In order, the subjects of interest discussed are:

(2.2.1 & 2.3.1) Characterization of Compressive Elastic Modulus of Osteochondral Grafts;
(2.2.2 & 2.3.2) Dimensional Characterization of Osteochondral Grafts and Implantation Sites;

(2.2.3 & 2.3.3) Determination of Implantation and Primary Stability Forces.

A chapter summary is presented in Section 2.4.

2.2 Materials and Methods

2.2.1 Characterization of Compressive Elastic Modulus of Osteochondral Grafts

Hind legs from 3- to 6-month-old porcine specimens were sourced from a local butcher (Nahunta Pork Center, Pikeville, NC). Whole-leg samples were kept under refrigeration or frozen for no longer than 48 hours following slaughter until the time of processing. If frozen, a thawing process of 24 hours at room temperature was followed prior to tissue harvest. In all samples, the knee joints were dissected to expose the femoral condyle and facets under the knee patella.

Grafts (n = 12) were harvested following standard steps in the COR procedure using the 8-mm harvester. All grafts selected for mechanical characterization presented axial symmetry and featured osteochondral zone divisions parallel to the surface of harvest. To assess the stiffness of the tissue at different depths (Figure 8), samples were sectioned into three different zones using a custom-made tool, resulting in test specimens shaped as discs of approximately ∅ 8 x 2 mm. Following the platen technique, unconfined compression tests in phosphate buffered saline (PBS) were performed at constant axial strain rates in a universal testing machine (ATS 1620, Applied Test Systems Inc., Butler, PA) set up with a 1 kN load cell. Synovial fluid was applied to the specimen-anvil interfaces to minimize the effects of friction. All samples were subjected to 20 preconditioning cycles to a strain of
0.005 at a rate of 0.002 s\(^{-1}\) to reach a viscoelastic steady state.\(^{101-103}\) Zone-specific testing parameters are presented in Table 3. The maximum strain levels were deliberately set to levels significantly higher than those of native bone and cartilage under natural conditions as suggested by the literature.\(^{101,104-106}\)

Figure 8 – Sectioning zones for mechanical characterization at different tissue depths. Zone 1: superficial, zone 2: transitional, and zone 3: osseous.

Table 3 – Unconfined compression testing parameters.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Sample Size</th>
<th>Strain Rate (s(^{-1}))</th>
<th>Max. Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone 1</td>
<td>12</td>
<td>0.01</td>
<td>0.30</td>
</tr>
<tr>
<td>Zone 2</td>
<td>12</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>Zone 3</td>
<td>12</td>
<td>0.01</td>
<td>0.15</td>
</tr>
</tbody>
</table>

From the resulting stress-strain responses, two measures of interest were obtained: the compressive modulus (derived from the slope of the linear region of the stress-strain
response) and the maximum stress (at any point during the test). Results were compared statistically using Student’s t-tests (JMP Pro 12, SAS Institute Inc., Cary, NC).

### 2.2.2 Dimensional Characterization of Osteochondral Grafts and Implantation Sites

Hind legs with the same characteristics as those in Section 2.2.1 were sourced and pre-processed by isolating the femur and exposing the femoral condyle and facets under the patella.

- **Plug grafts:** Following standard steps in the COR procedure for harvesting full-thickness osteochondral plugs, five grafts were harvested per leg (n = 4 legs) using the harvester tool part of the 8-mm COR kit. For each harvest, the tool was inserted perpendicular to the surface of the lateral or medial facets in the patellar surface of the femur and tapped down to the 10-mm mark, at which point the graft was extracted. Harvest sites were spaced at least 2 mm apart. In total, 20 plugs were extracted.

- **Implantation sites:** Similarly, following standard steps in the COR procedure for preparing recipient sites, five defects were produced per leg (n = 6 legs) using the drill guide and the drill bit from the 8-mm COR kit attached to a power drill. The defect sites were spaced apart 2 mm or more and distributed across the lateral and medial facets in the patellar surface of the femur. In total, 30 osteochondral defects were produced.

To characterize the dimensional aspects of the fit between grafts and their implantation sites, the diameters of both were characterized via metrology. For each of the plug grafts, five measurements of their external diameter were sampled across different
locations along the thickness of the specimen using an electronic digital caliper (Model 01407A, Neiko Tools USA). Similarly, immediately following the preparation of the implantation sites, five measurements of their internal diameters were taken using the internal feature measuring jaws of the electronic caliper.

JMP Pro was used for statistical evaluation of both datasets (assumption of normality via the Shapiro-Wilk and presence of outliers via Grubbs’ test). Lastly, the powers of both tests were estimated from the experimental variability and sample size. Significance levels were set to $\alpha = 0.05$.

### 2.2.3 Determination of Implantation and Primary Stability Forces

Hind legs with the same characteristics of those in Section 2.2.1 were sourced and pre-processed by isolating the femur and exposing the femoral condyle and facets under the patella. In each femur (n = 2), three grafts and three defects were produced using the COR kit along the lateral and medial facets in the patellar surface of the femur. Grafts were harvested to a thickness of 6 mm and defects produced to a depth of 8 mm. Each bone specimen was held by a custom-made fixture that allowed the surface of the implantation sites to be positioned facing upwards and horizontally. A universal testing machine (ATS 1620, Applied Test Systems Inc., Butler, PA) set up with a 1 kN load cell was used to apply compressive forces onto the surface of grafts sat over randomly assigned implantation sites, as represented in Figure 9. Thereafter, two distinct tests were performed:

- **Implantation force test**: Using an indenter of corresponding diameter, grafts were pushed into their respective implantation sites at a constant displacement rate of 1.5 mm/min (cross-head speed). Forces perpendicular to the top surface of the graft were
recorded as the implantation was performed (Figure 9A-B). The test was interrupted when the graft was leveled with the articular cartilage surface around the implantation site and forces were released (Figure 9C). The implantation force for each sample is defined as the peak force (in N) observed in each force curve (n = 12 curves).

![Diagram of implantation forces](image)

**Figure 9 – Graphical representation of the process for measuring implantation forces. A, B, and C represent different time points during the execution of the test.**

- **Primary stability force test:** Continuing with the setup from the previous test, the displacement rate was resumed so that the indenter was reengaged with the graft surface (Figure 10A). Forces were recorded as the graft was pushed deeper into the defect until it eventually hit the bottom of the implantation site (Figure 10B-C). At this point, the forces were released and the cross-head returned to its initial position. The primary stability force is defined as the critical friction force required to shift the graft deeper into the defect after it has been implanted and rest in a stable condition.
All statistical analyses were performed using JMP Pro. Resulting datasets were tested for normality (Shapiro-Wilk test) and evaluated for the presence of outliers (Grubbs’ test). Furthermore, to investigate for the presence of any correlation between implantation and primary stability forces, the correlation coefficient R was calculated. Lastly, the power of the test was determined to evaluate if a larger sample size is necessary assuming true means that do not differ plus or minus one standard deviation from the experimental means. All significance levels were set to $\alpha = 0.05$.

2.3 Results and Discussion

2.3.1 Characterization of Compressive Elastic Modulus of Osteochondral Grafts

Means, standard deviations, and coefficients of variation of the elastic compressive modulus of samples ($n = 12$) are reported in Table 4. Box plots illustrating the data
distribution are presented in Figure 11 and representative stress vs. strain curves for each tested zone are presented in Figure 12.

<table>
<thead>
<tr>
<th>Table 4 – Summary of compression test results.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compressive Elastic Modulus (MPa)</td>
</tr>
<tr>
<td>Zone 1</td>
</tr>
<tr>
<td>MEAN</td>
</tr>
<tr>
<td>ST.DEV.</td>
</tr>
<tr>
<td>CV</td>
</tr>
</tbody>
</table>

Assuming that the true means of the compressive elastic moduli for all tissue zones do not deviate more than one standard deviation unit from the respective experimentally obtained means, the power of the test is, for a sample size of $n = 12$, equal to 0.8828. Therefore, no further experimentation is necessary.
Using JMP 12, the resulting distribution of experimentally obtained modulus values was tested for normality with the Shapiro-Wilk test, which did not reject the $H_0$ hypothesis of normally distributed data ($p = 0.2342$). Grubbs’ test did not indicate the presence of outliers ($G = 2.4393 < G' = 2.9906$), thus, the entire dataset was tested via a one-way ANOVA. Interestingly, no statistically significant differences between the mean compressive elastic moduli of any of the three tissue zones were detected ($p = 0.7307$).

The compressive elastic modulus determined for Zone 1, consisting purely of non-calcified articular cartilage, is consistent with the range of values available in the literature, with reported results ranging from a few hundred KPa to over 100 MPa.\textsuperscript{89,100,107} It is suggested that the wide range of values reported arise due to the viscoelastic, time-dependent behavior of articular cartilage when subjected to loads and deformation, which affect the results captured by some testing protocols, including the platen technique.\textsuperscript{108} Furthermore, specimen-specific characteristics (animal model, age, health, etc.) and harvest zone may also affect the results.\textsuperscript{107} However, for Zones 2 and 3, composed primarily of highly calcified
tissue including the subchondral bone plate and the underlying cancellous bone, the results
diverged from what is reported in literature. Commonly, the elastic compressive modulus
reported for bone samples may surpass the GPa mark, with values in the range of 250 MPa to
38 GPa for several bone types.101,109–111 As mentioned in Section 2.1, bone types found in the
osteochondral complex have been reported to have compressive elastic modulus ranging
from 1150-19800 MPa,89 which is still higher than the means found in this experiment. The
underestimation of these values may arise from the testing protocol used, as suggested by
Helgason et al. (2008), wherein the platen technique may be subject to machine compliance
and structural end-effects at small deformations of the bone specimen. Also, the geometry of
the test specimens (in this case, possessing a thickness-to-diameter a ratio of about 1:4) may
have resulted in the estimation of lower modulus values. As suggested by a number of
studies, the thickness-to-diameter ratio adopted during mechanical characterization of tissue
specimens may have a significant effect on the estimation of their elastic modulus.99,112,113 A
larger ratio ranging from 1:1 to 2:1, where all the external dimensions are larger than 4-5
mm, is usually recommended.99,114 However, in an attempt to characterize the mechanical
properties of the aforementioned zones of osteochondral grafts harvested with the 8-mm
COR kit, such specimen geometry characteristics cannot be achieved.

Based on the results obtained, it is suggested that the information presented in the
literature is not directly relatable to the results obtained experimentally due to differences in
the testing protocols. In order to increase confidence in the obtained compressive modulus
for the three tissue zones, the sample size could be doubled, potentially bringing the power of
the test up to 0.9967, which requires relatively little effort. No changes to the resting protocol
are recommended, because, in its current structure, the same protocol can be applied to the
characterization of the compressive elastic modulus of 3DB porous scaffolds, as suggested in a comprehensive study presented by Nyberg et al. (2017)\textsuperscript{115}, which will be performed as discussed in Section 3.4.

\subsection{2.3.2 Dimensional Characterization of Osteochondral Grafts and Implantation Sites}

Five diameter measurements were taken from each of twenty osteochondral plug grafts yielding a sample size of 100 measurements. Figure 13 presents an example of a harvested graft (A) and the measuring approach (B). The resulting diameter distribution was tested for normality using the Shapiro-Wilk test. The H\textsubscript{0} hypothesis of normally distributed data was not rejected with p = 0.5720 (normal quantile plot presented in Figure 14). Grubbs’ test did not indicate the presence of outliers in the dataset with G = 2.7232 (for this experiment, any G > 3.3840 would reject the H\textsubscript{0} hypothesis that there are no outliers in the data). Hence, the mean and standard deviation of the diameter of osteochondral plugs harvested with the 8-mm COR kit was determined to be 8.17 ± 0.04 mm (CV = 0.4%). Assuming that the true mean of the diameter of osteochondral grafts does not deviate more than one standard deviation unit from the experimental mean, the power of the test was determined to be equal to 0.9999. Assuming an even smaller effect size (one-half unit of the standard deviation), the power of the test remains at a high level of 0.9936. Therefore, no further experimentation is necessary.
Following preparation of the 30 implantation sites according to the standard COR procedure (Figure 15A-B), each implantation site was measured by sampling five different diameters. Figure 15C provides an augmented view of a defect site.
The Shapiro-Wilk test did not reject the H₀ hypothesis of normally distributed data (p = 0.9288). Moreover, Grubbs’ test did not reject the H₀ hypothesis of no outliers in the dataset (G = 3.1128 > 3.5170). The associated normal quantile plot is presented in Figure 16. Therefore, all measurements were used to compute a mean and standard deviation of 8.01 ± 0.07 mm (CV = 0.8%) for the diameter of defect sites produced with the 8-mm COR kit. Considering an effect size of one standard deviation unit, the power of the test approaches 1.0. For an even smaller effect size (one-half unit of the standard deviation), the power of the test remains at a high level of 0.9999. Similarly, no further experimentation is necessary.

The results presented above indicate the clear presence of an interference fit between autografts and implantation sites. By directly comparing means, it can be established that the diameter of grafts is 0.16 mm larger than the diameter of implantation sites (an increase of about 1.99%). Following implantation, such interference is determinant of the ability of grafts to resist motion and remain stable, which is of particular relevance in unbottomed implantation scenarios. The information produced in this section defines a target initial diameter of 3DB osteochondral scaffolds fabricated with focus on matching the
implantability and stability of grafts. Evidently, at this point, there is no empirical evidence that grafts and scaffolds with matching diameters behave the same way with respect to implantation characteristics – such aspects will be investigated in the following chapters, with scaffold diameter being subject to further adjustment.

Figure 16 – Normal quantile plot of implantation site diameter measurements.

2.3.3 Determination of Implantation and Primary Stability Forces

Force curves representative of both tests are presented in Figure 17. For implantation forces, in Figure 17A, two different zones are observed. Zone I represents the portion of the test at which only compression of the tissue occurs, with no graft movement. Zone II represents the part at which the graft started to sink into the defect and was gradually implanted. At this latter zone, the steady increase in forces is expected since the surface area
in contact between the graft and the internal wall of the defect increases as the graft moves deeper.

While determining the primary stability forces, three distinct zones in the force curves were observed. Zone I represents the elastic deformation of the tissue with no graft shift, which only starts to happen in Zone II. At this point, a steady force curve with little to no slope was captured as expected, justified by the constant contact surface area between the graft and the walls of the defect. Lastly, in Zone III, the graft hits the bottom of the defect and forces increase exponentially, at which point the test was interrupted. Primary stability forces were determined from the mean force values in Zone II. For both curves, their overall appearance resembles those presented in previous studies.82–84,96

Figure 17 – Representative plots of force vs. displacement from implantation (A) and primary stability forces (B) for native osteochondral tissue grafts harvested with the 8-mm COR kit.

Results for implantation and primary stability forces are presented in Table 5. Means ± standard deviations were 81.5 ± 16.4 N and 68.1 ± 14.7 N for both measures, respectively. Coefficients of variation ranged from 20 to 22%.
Table 5 – Implantation and primary stability forces results for osteochondral native tissue grafts.

<table>
<thead>
<tr>
<th></th>
<th>Implantation Force (N)</th>
<th>Primary Stability Force (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>81.5</td>
<td>68.1</td>
</tr>
<tr>
<td>ST.DEV.</td>
<td>16.4</td>
<td>14.7</td>
</tr>
<tr>
<td>CV</td>
<td>0.20</td>
<td>0.22</td>
</tr>
</tbody>
</table>

The Shapiro-Wilk test suggested that the assumption of normality is valid for both datasets: \( p = 0.8030 \) and \( p = 0.8362 \), respectively, for implantation and primary stability forces, where any \( p < 0.05 \) would reject the \( H_0 \) hypothesis that the data is normally distributed. To investigate for the presence of outliers, Grubb’s test has shown that no outliers were part of either dataset with a 0.05 significance level: \( G = 1.7480 \) and \( G = 2.0547 \), respectively, for implantation and primary stability forces, where any \( G > 2.4117 \) would reject the \( H_0 \) hypothesis that there are no outliers in the data. Therefore, no values were excluded from future analyses. **Table 6** presents, for both experiments, a power analysis if \( \alpha = 0.05 \). Results have shown that, for both tests, the power levels are higher than a desired level of 0.8. No further experimentation is deemed necessary. Lastly, the correlation coefficient between both datasets was found to be \( r = 0.2818 \) (**Figure 18**), consequently rejecting the \( H_0 \) hypothesis of correlated data with \( p = 0.3748 \).

**Table 6 – Power of the test for implantation and primary stability force experiments.**

<table>
<thead>
<tr>
<th>Test</th>
<th>St. Dev.</th>
<th>Sample Size</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation</td>
<td>16.4</td>
<td>12</td>
<td>0.8829</td>
</tr>
<tr>
<td>Primary Stability</td>
<td>14.7</td>
<td>12</td>
<td>0.8829</td>
</tr>
</tbody>
</table>
Figure 18 – Correlation plots between implantation and primary stability forces.

For both measures, the results observed were as expected. There is substantial evidence that the resistance to motion (i.e. the force measured by the load cell) is primarily dictated by the contact interface between the external lateral surface of grafts and the internal walls of their respective implantation sites, a phenomenon that is also supported by interference fit identified in Section 2.3.2. As it can be seen in the representative force curves presented above, the resistance to motion arose as a function of how deep the grafts were in the defects during implantation, and such relationship was not observed while shifting the grafts deeper into the defects when their entire length were beneath the surface of the joint (or the articular cartilage). Interestingly, no convincing evidence of correlation between those two measures was identified. This could be an effect of a sample size not large enough to detect such relationship with statistical significance. In conclusion, this section has provided
a pair of measures to be matched by 3DB osteochondral scaffolds focusing on providing implantation characteristics similar to those offered by the clinically relevant COR procedure.

2.4 Chapter Summary

This chapter described the experimental approach employed to establish a set of functional requirements of grafts used in plug transfer techniques. Statistically relevant experiments defined the compressive mechanical properties of osteochondral grafts, the dimensional fit characteristics between these grafts and their implantation sites, and, finally, the implantation and primary stability forces during and post-implantation in a relevant ex vivo animal model.

The compressive elastic modulus of articular cartilage (zone 1, superficial), the calcified cartilage and subchondral bone plate (zone 2, transitional), and the cancellous bone (zone 3, osseous) were determined to be $39.3 \pm 9.8$, $37.1 \pm 11.2$, and $41.6 \pm 18.5$ MPa, respectively. The average diameter of grafts and implantation sites produced with the 8-mm COR kit were $8.17 \pm 0.04$ mm and $8.01 \pm 0.07$ mm, respectively, evidencing the presence of an interference fit. Lastly, the maximum implantation and average primary stability forces were found to be $81.5 \pm 16.4$ N and $68.1 \pm 14.7$ N, respectively.

These findings establish a set of functional requirements for implantability and biomimicry defined in Section 1.7, Objective 1 and its associated specific tasks. They will be revisited later in design process of a hybrid osteochondral scaffold design and taken as characteristics to be matched or closely resembled.
CHAPTER 3 – MATERIAL-PROCESS-STRUCTURE INTERRELATIONSHIPS

3.1 Chapter Introduction

Since its emergence, additive manufacturing and its related technologies have undergone significant development, but the same is not valid for the rate at which products taking advantage of these technologies have been introduced in the market. Guo et al. (2013) list a series of research needs that, if addressed, could potentially induce higher acceptance of additive manufactured products across different industries. From that list, some items are highlighted:6

- Process-structure-property relationships modeled and integrated with computer-aided design, engineering, and manufacturing tools for each material and process.
- Closed-loop adaptive control systems, the control algorithms of which based on predictive models of system response to process changes.
- A better understanding of the basic physics and chemistry of AM processes that capture complexities in the multiple interacting physical phenomena inherent in most AM processes.

Characterizing the effects of different materials and processing parameters on resulting structures enable the development of predictive models for design features of interest, as well as functional and mechanical properties, in various application domains. Many studies have characterized material-process-structure interrelationships for a range of AM processes and associated materials giving focus to resulting structural and mechanical properties of fabricated parts.116–118 Furthermore, narrowing down to extrusion-deposition-based AM processes, characterization studies commonly focus on the properties of the
extrudate as an effect of material composition and processing parameters like temperature, pressure, and feed, as well as the resulting characteristics of the fabricated parts. Lee et al. (2010) characterized the dimensional and mechanical properties of PCL ($M_w = 80$ kDa) strands produced in a modified 3D plotting system with varying nozzle speeds and oscillating frequencies. Also, the structural morphology and compressive modulus of full-3D scaffolds were determined for different nozzle diameters (200, 250, or 330 μm) and interstrand distances (300, 500, or 700 μm). They found that the denser scaffold architecture (200 μm nozzle and 300 μm spacing) possessed the highest compressive modulus of about 10 MPa.119

Durgun et al. (2014) investigated the effects of raster angles (0°, 30°, 45°, 60°, and 90°) and build orientation (horizontal, vertical, and perpendicular) on the mechanical and surface properties of ABS parts fabricated by FDM. Results suggest that optimal part properties were achieved at a raster angle of 0° and horizontal build orientation.120 Hwang et al. (2015) evaluated the tensile strength and thermal conductivity of FDM parts fabricated from a composite of ABS, copper and iron particles. Varying levels of metal powder composing the mix (10% - 50% by weight), as well as the extrusion temperature (190°C - 220°C) and fill density (20% - 80%) were tested. Results have shown that lower powder concentration and extrusion temperatures yielded parts with higher tensile strength, but fill density decreased the strength at lower levels.121

On TERM-focused applications of extrusion-deposition-based processes, Sheshadri et al. (2015) evaluated the effects of extrusion temperature (80, 90, and 100°C) and nozzle diameter (300 μm and 400 μm) on the structure and compressive mechanical properties of PCL (45 kDa) porous scaffolds. Their results demonstrated that significantly larger strand diameter and compressive strength were present in scaffolds fabricated with a larger nozzle.
diameter or higher temperatures. The data also served as input for of predictive equations for those two measures given a set of processing parameters.\textsuperscript{58} Likewise, Narayanan (2017) characterized process-structure interactions of 3DB PCL including the effects of process parameters (extrusion pressure, print head speed, strand lay orientation, and strand length) on structural (strand width) and mechanical (tensile) properties of fabricated structures. Their results have shown that, of the process parameters tested, only extrusion pressure and print head speed had significant effects on the width of deposited strands. Tensile elastic modulus, yield strength and fracture strength were significantly affected by the strand lay orientation. They also created a predictive response surface model for the width of strands, and a finite element model to estimate the mechanical properties of porous PCL structures.\textsuperscript{122}

In the TERM field, process characterization is a subject of particular importance. Biofabrication processes that take advantage of AM technologies must demonstrate repeatability and yield parts with a desired level of consistency. Considering the scope of this dissertation, the literature provides limited comprehensive modeling of the material-process-structure interrelationships present in the extrusion-deposition-based processing of mineralized PCL composites, which are the base materials utilized for zones 2 and 3 of the scaffold design proposed in Section 1.6. Therefore, such study framework is proposed in this chapter to complement the available literature on the topic. A factor-screening pilot experiment aims to identify the feasible range of processing parameter levels (temperature, speed, pressure, and nozzle diameter) for the PCL-TCP composites discussed in Section 1.6. Then, a design of experiments study will characterize the effect of these processing parameter levels on the width of extruded strands, which serve as the building blocks for true 3D structures and significantly affect their properties. Furthermore, a second design of
experiments will characterize the compressive elastic modulus of true 3D scaffolds featuring varying design characteristics. Response surface models fit to the collected data will allow for the prediction of scaffold characteristics given a choice of processing parameters. The completion of these tasks contributes directly to the fulfillment of the research needs identified by Nannan et al. (2013), discussed at the beginning of this chapter.

Lastly, in a continued effort to approximate the hybrid 3DB scaffold design to its native counterparts, the aforementioned predictive models will be validated by setting the 3DB process parameters in a way to fabricate scaffolds with size and compressive elastic moduli that match or closely resemble those defined in Chapter 2.

The sections that follow in this chapter are organized as defined below:

(3.2) Screening Experiment for 3DB Parameters of PCL-TCP Composites;
(3.3) Modelling of the Effects of 3DB Parameters on PCL-TCP Strand Widths;
(3.4) Modelling of the Effects of Design Parameters on the Compressive Elastic Modulus of PCL-TCP 3DB Scaffolds;
(3.5) Validation of Predictive Models and Proposal of a Scaffold Design.

A chapter summary is presented in Section 3.6.

### 3.2 Screening Experiment for 3DB Parameters of PCL-TCP Composites

#### 3.2.1 Materials and Methods

To establish feasible processing parameter ranges for PCL-TCP composites in 3D-Bioplotting, a factor-screening pilot study was performed. The extrudability of both 80-20% and 90-10% weight fractions of the PCL-TCP composite were evaluated at different combinations of extrusion temperatures (°C), pressures (N/mm²), speeds (mm/s), and nozzle
internal diameter (mm). The rationale behind the selection of the PCL-TCP composite at the
described concentrations was discussed in Section 1.6 – it is important to highlight that
these concentrations provide a satisfactory trade-off between the ability of the material to
provide cues for cell differentiation and offer desirable compressive mechanical properties,
approximating those of cancellous bone. The processing parameters have been chosen
because they are hypothesized to have a direct effect on the width of deposited strands and
can be easily controlled in the 3DB process. The extrusion temperature has a direct effect on
the viscosity of polymer melts, therefore varying such property likely affects the melt flow
rate when all other parameters are constant. Extrusion pressure also controls the flow rate of
polymer by “pushing” the melt as it travels through the nozzle. The nozzle diameter, as it gets
smaller, may increase the shear forces suffered by the polymer melt and decrease the flow
rate. Lastly, the print head travel speed also has an effect on the strand width due to its direct
relationship to the polymer melt extrusion rate – if one is significantly larger than the other,
deposited strands are likely to experience thinning/thickening.

Material preparation: The composite materials were prepared by mixing PCL pellets
(M_w = 45 kDa, Polysciences, Inc., Warrington, PA) and β-TCP powder (Riedel-de Haën,
Seelze, Germany) in their respective weight ratios in a glass beaker up to a total of 20 g
(composite preparation batch size) of the mix. The beaker was then transferred to a 120°C
hot plate. Upon initial melting of the polymer pellets, the mixture was stirred vigorously with
a metal rod until no distinguishable phases of PCL or TCP were identifiable, characterizing a
homogeneous mix. The mix was transferred to and spread across a polished aluminum
surface at room temperature where it solidified into a thin sheet. Lastly, the solidified sheets
were cut into small pellets no larger than 3 mm. Until utilization, materials were stored in a dehumidified chamber.

**3D-Bioplotter setup:** 2 g of material was loaded into a clean hot temperature extrusion cartridge of a 3D-Bioplotter (4th generation manufacturer series, EnvisionTEC GmbH, Gladbeck, Germany). The composite was allowed to melt and stabilize at the selected temperature level during a pre-heat interval of 20 minutes, followed by a 30-second purge at maximum pressure (0.6 N/mm²) to ensure removal of air trapped inside the extrusion nozzle. The machine was then subjected to its standard calibration protocol to determine nozzle and platform height offsets, at which point the setup was complete.

**Experimental algorithm:** To evaluate the extrudability potential of each process parameter combination, the single layer spiral pattern illustrated in Figure 19 defines a multidirectional toolpath featuring movements in the X and Y axes for a total length of 350 mm.

![Figure 19 – Single-layer pattern used in the screening experiments. All dimensions in mm](image-url)
The initial extrusion temperature was set equal to that used for extrusion of PCL 45 kDa (120°C, according to our lab protocols) and maximum obtainable pressure (0.6 N/mm²). The algorithm (Figure 20) starts by attempting extrusion at lower pressures without changes in the temperature. If extrusion becomes not possible, the temperature is raised to the next level. The same protocol was applied to three different extrusion nozzle internal diameters (0.2, 0.3, and 0.4 mm) and tested across four nozzle speeds (in order, 2.0, 1.5, 1.0, and 0.5 mm/s). Three replicates were performed for each parameter combination. To minimize the effects of repeated temperature change cycles, a new batch of material was used whenever the algorithm called for a change in temperature. Table 7 presents a summary of the controlled 3D-bioplotting parameters used in the screening experiments.

![Diagram showing the algorithm used to determine feasible 3DB processing parameters.](Figure 20)
Table 7 – 3D-Bioplotting processing parameters and their levels tested during screening experiments.

<table>
<thead>
<tr>
<th>Processing Parameter</th>
<th>Unit</th>
<th>Levels</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrusion Temperature</td>
<td>°C</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>Extrusion Pressure</td>
<td>N/mm(^2)</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Print Head Speed</td>
<td>mm/s</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Extrusion Nozzle Diameter</td>
<td>mm</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Parameter feasibility criteria: Each parameter combination was deemed feasible if the conditions below were satisfied upon visual inspection:

- Strands are continuous and complete, meaning that no visible interruptions exist along the deposited structure and that the entire intended length has been produced from the start to end points.
− Strands are linear and attached to the substrate on which they are deposited, meaning that no “pulling” occurred and the deposited structure is visually equivalent to the virtual model.
− Strands have uniform diameter, meaning that no visually significant variations (detected by qualitative inspection) along the length of the deposited structure exist; Additionally, to avoid selection of parameters that may potentially introduce changes in the material properties due to polymer degradation, the color of deposited structures was evaluated over time. A darker tone would indicate that the extrusion temperature was too high, affecting the material in an undesired way. Consequently, that temperature was deemed unfeasible for processing.

Final classification: Each replicate of a parameter combination was classified into one of the following groups:

− ✓ Feasible parameter combination with no printing defects. Occurs when all of the criteria above are satisfied.
− ○ Unfeasible parameter combination with significant printing defects. Occurs when at least one of the criteria above is not satisfied due to an observance of minor printing defects.
− × Unfeasible parameter combination with major printing defects. Occurs when one or more of the criteria above is not satisfied due to the occurrence of major and consistent printing defects or apparent material degradation.

Out of three replicates, if any happened to be classified as ○ or ×, that parameter combination was deemed to be unfeasible. Likewise, feasibility was confirmed when all replicates received a ✓. Ultimately, these screening experiments served to determine
workable ranges of extrusion temperatures, pressures, and speeds for three commonly used nozzle sizes in 3D-Bioplotting. The information acquired serves as an input for the upcoming DoE.

3.2.2 Results and Discussion

Specific combinations of 3D-Bioplotting parameters were tested to evaluate for the extrudability potential of structurally viable strands. A single-layer squared spiral pattern was modeled, which required material to be deposited along four independent directions of movement of the print head in the X and Y axes: (1,0), (0,1), (-1,0), and (0,-1).

A summary of the classification results is presented from Table 8 to Table 13. Each parameter combination is marked with either ✓, ○, or × symbols according to the classification rules defined in Section 3.2.1. If no classification is present, that specific parameter combination was not tested as defined by the experimental algorithm.

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.4 mm</th>
<th>0.6 N/mm²</th>
<th>0.5 N/mm²</th>
<th>0.4 N/mm²</th>
<th>0.3 N/mm²</th>
<th>0.2 N/mm²</th>
<th>0.1 N/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 9 – Classification results for PCL-TCP 80-20 with a 0.3 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.3 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 N/mm²</td>
</tr>
<tr>
<td>0.5 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 10 – Classification results for PCL-TCP 80-20 with a 0.2 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.2 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 N/mm²</td>
</tr>
<tr>
<td>0.5 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td>✓</td>
</tr>
<tr>
<td>120°C</td>
<td>✓</td>
</tr>
<tr>
<td>130°C</td>
<td>✓</td>
</tr>
<tr>
<td>140°C</td>
<td>✓</td>
</tr>
</tbody>
</table>
### Table 11 – Classification results for PCL-TCP 90-10 with a 0.4 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 90-10</th>
<th>0.4 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 N/mm²</td>
</tr>
<tr>
<td>0.5 mm/s</td>
<td>120°C ✓ ✓ ✓ ✓ ✓ ✓</td>
</tr>
<tr>
<td></td>
<td>1.0 mm/s</td>
</tr>
<tr>
<td></td>
<td>1.5 mm/s</td>
</tr>
<tr>
<td></td>
<td>2.0 mm/s</td>
</tr>
</tbody>
</table>

### Table 12 – Classification results for PCL-TCP 90-10 with a 0.3 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 90-10</th>
<th>0.3 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 N/mm²</td>
</tr>
<tr>
<td>0.5 mm/s</td>
<td>120°C ✓ ✓ ✓ ✓ ✓ ✓</td>
</tr>
<tr>
<td></td>
<td>1.0 mm/s</td>
</tr>
<tr>
<td></td>
<td>1.5 mm/s</td>
</tr>
<tr>
<td></td>
<td>2.0 mm/s</td>
</tr>
</tbody>
</table>
Representative pictures from each parameter combination were taken using a digital camera set up to capture a top-down view of the resulting 3D-bioplot pattern. Figure 21 presents the most commonly observed structural defects, which can be divided in three categories: (A) discontinuous strand, (B) localized thinning of strand, and (C) generalized strand pulling and consequent detachment from the printing platform. Localized defects are indicated with red arrows.
Figure 21 – Defects in the 3D-Bioplotting of single layer PCL-TCP structures. (A) Discontinuous strand, (B) localized thinning of strand, and (C) generalized strand pulling and consequent detachment from the printing platform.

Overall, the resulting effects of each parameter on the quality of 3D-bioplotted structures are summarized below:

- Temperature: higher levels yield better extrudability due to the reduced viscosity of the polymer melt and lower shear resistance as the melt passes through the nozzle.
- Pressure: higher levels yield better extrudability due to a higher rate of material flow.
- Speed: lower levels yield better extrudability due to a decreased chance of the speed surpassing the material deposition rate.
- Nozzle diameter: larger diameters yield better extrudability due to lower shear resistance as the melt passes through the nozzle.

With respect to the material composition, the 80-20 PCL-TCP composite demonstrated overall worse extrudability when compared to the 90-10 composite extruded with the same choice of 3DB parameters. This result is reasonable since TCP, a dense
ceramic powder, is expected to significantly change the rheological properties of the melt at higher concentrations. For illustration purposes, Figure 22 presents a set of photographs representing the results obtained in the 3DB of the 80-20 PCL-TCP composite with a 0.3 mm nozzle at a speed of 2.0 mm/s. The color coding around the borders of each photograph corresponds to the classification received by those structures, as defined previously.

Few exceptions to the behaviors outlined above were observed. For instance, as indicated in Table 8 to Table 13, an overall increase in the temperature of the 80-20 PCL-TCP composite extruded through a 0.4 mm nozzle at 2 mm/s did not yield better extrudability. This effect could be due to a possible interaction between high speeds and temperatures for that particular material composition, which has reduced the ability of the material to attach well to the print platform.

The determination of feasible parameter combinations in the extrusion of PCL-TCP composites through this set of screening experiments determines the factor levels to be part of a design of experiments study defined in Section 3.3.
3.3 Modelling the Effects of 3DB Parameters on PCL-TCP Strand Widths

Strands are the “building blocks” of 3DB constructs. Hence, a comprehensive characterization of associated material-process-structure interactions is necessary in the fabrication of scaffolds for tissue engineering. From the set of feasible processing parameter combinations determined by the experiments described in Section 3.2, a more extensive
study, involving the fabrication of multi-layer 3D structures and the characterization strand widths, a primary geometrical characteristic of 3DB strands, is proposed.

### 3.3.1 Materials and Methods

Cylindrical scaffolds (dia. 8 mm x 2mm) were 3D-Bioplotted following material preparation and machine setup protocols previously discussed in Section 3.2.1. The choice of scaffold diameter and thickness refers back to the approximate dimensions of the distinct zones of osteochondral tissue grafts harvested with the 8-mm COR kit (recall Figure 8). Following fabrication, scaffolds were inspected under a digital microscope and one pair of measurements of strand widths was taken at randomly determined locations within each quadrant of the circular structure, totaling eight total measurements per scaffold sample. A summary of experimental parameters is presented in Table 14.

Each set of experimental results were analyzed using JMP Pro 12. If necessary, data transformations were applied to account for the assumptions of ANOVA models and achieve better goodness-of-fit (measured by R-squared values) between the resulting multiple-linear regression models and the raw data. Significance levels were set to $\alpha = 0.05$. Because of the substantially large number of data points in both experimental designs, the assumption of normality was assessed by visual inspection of each distribution. The previously referenced Shapiro–Wilk and other normality tests are known to be sensitive to extremely large sample sizes and generally reject their null hypothesis no matter how closely a sampled distribution resembles the normal.
Table 14 – Parameters for the experimental design of strand diameter.

<table>
<thead>
<tr>
<th>Parameter(s)</th>
<th>Value(s)</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material Composition</td>
<td>PCL-TCP 80-20</td>
<td>Two distinct composite biomaterials featuring varying levels of mineralized content. Each composition represents an independent design of experiments through which the effects of predictor variables were investigated.</td>
</tr>
<tr>
<td></td>
<td>PCL-TCP 90-10</td>
<td></td>
</tr>
<tr>
<td>Extrusion Temperature</td>
<td>Defined from screening experiments. Section 3.2.</td>
<td>These parameters represent the set of independent variables for the experiment. It is hypothesized that they will have a direct effect on the diameter of deposited strands. Only feasible parameter combinations will be tested as established by the parameter screening experiments presented in Section 3.2.</td>
</tr>
<tr>
<td>Extrusion Pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Print Head Speed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extrusion Nozzle Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strand Width</td>
<td>Response variable represented in μm (micrometers) and determined from the width of scaffold strands measured across several random locations of a top-down view under a microscope.</td>
<td></td>
</tr>
<tr>
<td>Scaffold Dimensions</td>
<td>Ø 8 x 2 mm</td>
<td>Resembles the diameter of osteochondral grafts extracted with the 8 mm COR kit and the thickness of distinct zones of the osteochondral complex. Quantity of deposited strands should be enough to offer several regions for characterization under a microscope.</td>
</tr>
<tr>
<td>Strand Lay-down Pattern</td>
<td>0°-120°-240°</td>
<td>Provides “triangular” pore microarchitectures (top-down view) which has been widely used in the literature and is known to provide good structural stability.</td>
</tr>
<tr>
<td>Strand Inter-axial Separation</td>
<td>1000 μm</td>
<td>Expected to prevent adjacent strands from merging (if too thick), allowing determination of their width.</td>
</tr>
<tr>
<td>Layer Height</td>
<td>80% of Nozzle Diameter</td>
<td>Offers appropriate bond between adjacent layers. It is recommended by the 3D-Bioplotter’s manufacturer.</td>
</tr>
<tr>
<td>Sample Size (specimens)</td>
<td>3</td>
<td>Three scaffolds will be fabricated from each feasible 3DP parameter combination and material composition.</td>
</tr>
<tr>
<td>Sample Size (measurements)</td>
<td>8</td>
<td>Eight measurements of the diameter of deposited strands will be taken from microscopic top-down images of fabricated specimens.</td>
</tr>
</tbody>
</table>
3.3.2 Results and Discussion

As defined by the screening experiments, both models are represented by incomplete factorial designs due to the reduced number of feasible parameter combinations from a theoretical full factorial design. As it was reasonably expected, this situation was further aggravated when translating from single-layer “2D” material deposition, as in the experimental screening phase, to multi-layer 3D deposition required to fabricate scaffolds of relevant shape and size. Table 15 to Table 20 present a summary of results with the actual mean values for strand widths (micrometers, μm) in green cells, where the fabrication of 3D scaffolds was feasible, and ‘FAIL’ labels in red cells where the parameter combinations were previously determined to be feasible by the screening experiments, but fabrication of 3D scaffolds was not.

Table 15 – Results of strand width characterization for PCL-TCP 80-20 with a 0.4 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.4 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mm/s</td>
<td></td>
</tr>
<tr>
<td>120°C 0.4 N/mm²</td>
<td>414</td>
</tr>
<tr>
<td>130°C 0.5 N/mm²</td>
<td>476</td>
</tr>
<tr>
<td>140°C 0.6 N/mm²</td>
<td>759</td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td></td>
</tr>
<tr>
<td>120°C 0.6 N/mm²</td>
<td>FAIL</td>
</tr>
<tr>
<td>130°C 0.5 N/mm²</td>
<td>381</td>
</tr>
<tr>
<td>140°C 0.4 N/mm²</td>
<td>509</td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td></td>
</tr>
<tr>
<td>120°C 0.4 N/mm²</td>
<td>FAIL</td>
</tr>
<tr>
<td>130°C 0.3 N/mm²</td>
<td>308</td>
</tr>
<tr>
<td>140°C 0.2 N/mm²</td>
<td>403</td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td></td>
</tr>
<tr>
<td>120°C 0.4 N/mm²</td>
<td>FAIL</td>
</tr>
<tr>
<td>130°C 0.1 N/mm²</td>
<td>278</td>
</tr>
<tr>
<td>140°C 0.1 N/mm²</td>
<td>316</td>
</tr>
</tbody>
</table>
Table 16 – Results of strand width characterization for PCL-TCP 80-20 with a 0.3 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.6 N/mm²</th>
<th>0.5 N/mm²</th>
<th>0.4 N/mm²</th>
<th>0.3 N/mm²</th>
<th>0.2 N/mm²</th>
<th>0.1 N/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>359</td>
<td>313</td>
<td>282</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>373</td>
<td>339</td>
<td>267</td>
<td>249</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>526</td>
<td>416</td>
<td>375</td>
<td>316</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>253</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>253</td>
<td>234</td>
<td>228</td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>320</td>
<td>279</td>
<td>FAIL</td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>227</td>
<td>202</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>249</td>
<td>224</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 17 – Results of strand width characterization for PCL-TCP 80-20 with a 0.2 mm nozzle

<table>
<thead>
<tr>
<th>PCL-TCP 80-20</th>
<th>0.6 N/mm²</th>
<th>0.5 N/mm²</th>
<th>0.4 N/mm²</th>
<th>0.3 N/mm²</th>
<th>0.2 N/mm²</th>
<th>0.1 N/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>137</td>
<td></td>
<td></td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>141</td>
<td></td>
<td></td>
<td>FAIL</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>173</td>
<td></td>
<td></td>
<td>FAIL</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>1.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 mm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 18 – Results of strand width characterization for PCL-TCP 90-10 with a 0.4 mm nozzle

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Velocity</th>
<th>0.6 N/mm²</th>
<th>0.5 N/mm²</th>
<th>0.4 N/mm²</th>
<th>0.3 N/mm²</th>
<th>0.2 N/mm²</th>
<th>0.1 N/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>120°C</td>
<td>0.5 mm/s</td>
<td>766</td>
<td>638</td>
<td>500</td>
<td>443</td>
<td>372</td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>0.5 mm/s</td>
<td>FAIL</td>
<td>826</td>
<td>607</td>
<td>498</td>
<td>366</td>
<td>270</td>
</tr>
<tr>
<td>140°C</td>
<td>0.5 mm/s</td>
<td>FAIL</td>
<td>904</td>
<td>798</td>
<td>635</td>
<td>499</td>
<td>337</td>
</tr>
<tr>
<td>120°C</td>
<td>1.0 mm/s</td>
<td>424</td>
<td>393</td>
<td>335</td>
<td>255</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>1.0 mm/s</td>
<td>501</td>
<td>447</td>
<td>385</td>
<td>325</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>1.0 mm/s</td>
<td>856</td>
<td>642</td>
<td>493</td>
<td>412</td>
<td>305</td>
<td>FAIL</td>
</tr>
<tr>
<td>120°C</td>
<td>1.5 mm/s</td>
<td>343</td>
<td>298</td>
<td>271</td>
<td>217</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>1.5 mm/s</td>
<td>403</td>
<td>331</td>
<td>286</td>
<td>260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>1.5 mm/s</td>
<td>541</td>
<td>498</td>
<td>401</td>
<td>328</td>
<td>271</td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>2.0 mm/s</td>
<td>283</td>
<td>239</td>
<td>FAIL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>2.0 mm/s</td>
<td>323</td>
<td>285</td>
<td>267</td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>2.0 mm/s</td>
<td>409</td>
<td>360</td>
<td>329</td>
<td>262</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 19 – Results of strand width characterization for PCL-TCP 90-10 with a 0.3 mm nozzle

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Velocity</th>
<th>0.6 N/mm²</th>
<th>0.5 N/mm²</th>
<th>0.4 N/mm²</th>
<th>0.3 N/mm²</th>
<th>0.2 N/mm²</th>
<th>0.1 N/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>120°C</td>
<td>0.5 mm/s</td>
<td>471</td>
<td>418</td>
<td>343</td>
<td>285</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>0.5 mm/s</td>
<td>499</td>
<td>430</td>
<td>384</td>
<td>327</td>
<td>270</td>
<td>FAIL</td>
</tr>
<tr>
<td>140°C</td>
<td>0.5 mm/s</td>
<td>810</td>
<td>539</td>
<td>447</td>
<td>346</td>
<td>273</td>
<td>207</td>
</tr>
<tr>
<td>120°C</td>
<td>1.0 mm/s</td>
<td>305</td>
<td>264</td>
<td>229</td>
<td>199</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>1.0 mm/s</td>
<td>339</td>
<td>296</td>
<td>261</td>
<td>234</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>1.0 mm/s</td>
<td>375</td>
<td>336</td>
<td>267</td>
<td>215</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>1.5 mm/s</td>
<td>235</td>
<td>203</td>
<td>FAIL</td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>1.5 mm/s</td>
<td>279</td>
<td>260</td>
<td>215</td>
<td>194</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>1.5 mm/s</td>
<td>271</td>
<td>247</td>
<td>218</td>
<td>186</td>
<td>FAIL</td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>2.0 mm/s</td>
<td>189</td>
<td>FAIL</td>
<td>FAIL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130°C</td>
<td>2.0 mm/s</td>
<td>259</td>
<td>219</td>
<td>FAIL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140°C</td>
<td>2.0 mm/s</td>
<td>218</td>
<td>199</td>
<td>195</td>
<td>FAIL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Separate statistical analysis protocols were followed for each material as a result of the significantly distinct experimental design spaces in each case. For PCL-TCP 80-20 scaffolds, the set of feasible parameter combinations was not enough to estimate higher-order factor interactions (wherein their estimation either failed or became biased), thus only main effects of Extrusion Nozzle Diameter, Pressure, Temperature, and Print Head Speed were tested. The initial data set had strong positive skewness and was therefore transformed by taking the natural-log of each response value to achieve a distribution that better resembles and approaches the normal distribution, as illustrated in Figure 23. Hence, the statistical model was applied to the transformed dataset. All factors were significant with \( p < 0.0001 \).

**Equation 1** presents, in generic form, the resulting multiple-linear regression model with one dependent variable \( y \), and four predictor variables \( x_i \), and their associated \( \beta_i \) coefficients.

**Equation 2** presents the regression model following substitutions. At this stage, a graphical representation of the model’s residuals in the form of a histogram resembles the bell shape of a normal distribution, with mean of zero and overall constant variance, despite small negative
skewness. Lastly, Equation 3 presents the ultimate form of the regression equation, back-transforming the response variable to its original form so that results are estimated in μm (micrometers), as opposed to ln(μm). For the proposed model, the goodness-of-fit parameter R-square is equal to 0.8451, suggesting a decent fit to the experimental data for strand width. As an attempt to improve fit, transformation of predictor variables were attempted (SQRT, natural logarithm, square), but none yielded practically better R-square values to the model. Therefore, the model presented in Equation 3 is deemed final.

\[
\ln y = \beta_0 + \beta_1 \times x_1 + \beta_2 \times x_2 + \beta_3 \times x_3 + \beta_4 \times x_4 + \epsilon
\]

Equation 1
On the other hand, for the strand widths of PCL-TCP 90-10 scaffolds, which has a larger quantity of feasible parameter combinations and, therefore, allows for a more robust statistical model, all main effects and their higher-order interactions could be estimated. Hence, the incomplete factorial design was modeled into a multi-linear regression model including all main and interaction effects. In this case, the original response data set was also highly positively skewed, and was transformed by taking the natural logarithm of all response values so that its histogram resembled the bell shape typical of a normal distribution, as illustrated in Figure 24. No other transformation technique (i.e. square-root, reciprocal, Box-Cox, etc.), yielded better visual results.

\[
\ln \text{Width} = 2.5730616 + \left(0.2 \Rightarrow -0.7236634\right) \times \text{Nozzle} \\
+ 0.0204012 \times \text{Temperature} + 1.267593 \\
\times \text{Pressure} + (-0.509454) \times \text{Speed} \tag{Equation 2}
\]

\[
\text{Width} = \exp \left[ 2.5730616 + \left(0.2 \Rightarrow -0.7236634\right) \times \text{Nozzle} \\
+ 0.0204012 \times \text{Temperature} + 1.267593 \\
\times \text{Pressure} + (-0.509454) \times \text{Speed} \right] \tag{Equation 3}
\]
Figure 24 – Histogram and normal quantile plot of natural-log transformed response values of PCL-TCP 90-10 strand widths

The significance of all factors, from first to fourth order, are presented in Table 21. In this model, the plot of the residuals in the form of a histogram resembles the bell shape of a normal distribution, with mean of zero and overall constant variance, despite small negative skewness. The interaction of fourth-order was not significant. Second and third-order interactions were only significant if Extrusion Nozzle was present, with one exception, while all main factors were significant.
Table 21 – Effect tests of all factors and their higher-order interactions for the PCL-TCP 90-10 strand width characterization experiment

<table>
<thead>
<tr>
<th>Factor / Factor Interaction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nozzle</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Temperature</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pressure</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Speed</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Nozzle*Temperature</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Nozzle*Pressure</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Nozzle*Speed</td>
<td>0.002</td>
</tr>
<tr>
<td>Temperature*Pressure</td>
<td>0.0764</td>
</tr>
<tr>
<td>Temperature*Speed</td>
<td>0.5744</td>
</tr>
<tr>
<td>Pressure*Speed</td>
<td>0.291</td>
</tr>
<tr>
<td>Nozzle<em>Temperature</em>Pressure</td>
<td>0.0191</td>
</tr>
<tr>
<td>Nozzle<em>Temperature</em>Speed</td>
<td>0.0004</td>
</tr>
<tr>
<td>Nozzle<em>Pressure</em>Speed</td>
<td>0.4087</td>
</tr>
<tr>
<td>Temperature<em>Pressure</em>Speed</td>
<td>0.5062</td>
</tr>
<tr>
<td>Nozzle<em>Temperature</em>Pressure*Speed</td>
<td>0.7281</td>
</tr>
</tbody>
</table>

To increase confidence in the estimation of main effects and lower-order interactions, and supported by the fact that most third- and fourth-degree interactions were not statistically significant, the statistical model was simplified wherein only main factors and second-degree interactions were estimated. In this case, all factors and factor combinations become significant with $p < 0.05$, as presented in Table 22. Equation 4 presents the generic form of the regression equation, with one response variable $y$, four predictor variables $x_i$, and the $\beta_i$ coefficients of main factors and their second-order interactions. Equation 5 presents the regression model following substitutions. For that model, the plot of residuals resembles the characteristic bell shape of the normal distribution, with mean of zero and constant variance,
despite small negative skewness. The goodness-of-fit parameter R-square for this model is 0.9223. No transformation of predictor variables practically improved this measure. Lastly, **Equation 6** presents the back-transformed model, which allows for the estimation of a strand width $y$ in micrometers given a set of 3DB processing parameters.

<table>
<thead>
<tr>
<th>Factor / Factor Interaction</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nozzle</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Temperature</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Pressure</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Speed</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Nozzle*Temperature</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Nozzle*Pressure</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Nozzle*Speed</td>
<td>0.0002</td>
</tr>
<tr>
<td>Temperature*Pressure</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Temperature*Speed</td>
<td>0.0182</td>
</tr>
<tr>
<td>Pressure*Speed</td>
<td>$&lt; 0.0001$</td>
</tr>
</tbody>
</table>

$$\ln y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_1 x_2 + \beta_6 x_1 x_3 + \beta_7 x_1 x_4 + \beta_8 x_2 x_3 + \beta_9 x_2 x_4 + \beta_{10} x_3 x_4 + \varepsilon$$  

**Equation 4**
$$\ln \text{Width} = 3.5002423 + \left( \begin{array}{c}
0.2 \Rightarrow (-0.6027911) \\
0.3 \Rightarrow 0.0645504 \\
0.4 \Rightarrow 0.5381806
\end{array} \right) + 0.0152856$$

* Temperature + 1.5800826 * Pressure + (-0.5759043) 
* Speed

$$\left( \begin{array}{c}
0.2 \Rightarrow (\text{Temperature} - 131.46789) * (-0.0005984) \\
0.3 \Rightarrow (\text{Temperature} - 131.46789) * (-0.0057108) \\
0.4 \Rightarrow (\text{Temperature} - 131.46789) * 0.00630922
\end{array} \right) + 0.0274341 * (\text{Temperature} - 131.46789)$$

Equation 5

$$\text{Width} = \exp \left[ 3.5002423 + \left( \begin{array}{c}
0.2 \Rightarrow (-0.6027911) \\
0.3 \Rightarrow 0.0645504 \\
0.4 \Rightarrow 0.5381806
\end{array} \right) + 0.0152856 \right]$$

* Temperature + 1.5800826 * Pressure + (-0.5759043) 
* Speed

$$\left( \begin{array}{c}
0.2 \Rightarrow (\text{Temperature} - 131.46789) * (-0.0005984) \\
0.3 \Rightarrow (\text{Temperature} - 131.46789) * (-0.0057108) \\
0.4 \Rightarrow (\text{Temperature} - 131.46789) * 0.00630922
\end{array} \right) + 0.0274341 * (\text{Temperature} - 131.46789)$$

Equation 6

$$\left( \begin{array}{c}
0.2 \Rightarrow (\text{Pressure} - 0.4357798) * (-0.4227741) \\
0.3 \Rightarrow (\text{Pressure} - 0.4357798) * 0.09727148 \\
0.4 \Rightarrow (\text{Pressure} - 0.4357798) * 0.32550269
\end{array} \right) + (\text{Speed} - 1.0596331) * (\text{Pressure} - 0.4357798)$$

$$+ (\text{Speed} - 1.0596331)$$
For both materials, each model demonstrated results that matched what was initially hypothesized from the discussion presented in Section 3.2. Here, the effects of each tested 3DB processing parameter were consistent and in accordance to similar preceding studies. For the internal diameter of the extrusion nozzle, both models identified a significant direct proportionality effect, which related larger strand widths with larger nozzle diameters. This is a result of the increased melt flow as an effect of decreased pressure drops along the nozzle canal. The effect of pressure and temperature were also significant and of direct proportionality, wherein higher pressures and temperatures resulted in higher strand widths. Generally corresponding effects can be found in the literature. An increase in pressure is directly associated with an increase in melt flow rate and, for some materials (PCL included), shear thinning and consequent drop in viscosity. Temperature, alternatively, primarily affects the material’s viscosity and, as a secondary consequence, the melt flow rate. Lastly, the effects of speed were significant and inversely proportional to the resulting width of strands.

Lastly, it is also relevant to mention that the results discussed in this section pertain to a specific TCP powder particle size distribution and the specific geometry of the extrusion nozzle. Both are defining characteristics of the experiments here performed, and were maintained constant during every experimental replicate. In the literature, Bohnera and Baroudb (2005) found the effects of TCP particle size on the extrudability of phosphate pastes to be significant, wherein composites featuring smaller ceramic particles demonstrated improved extrudability. Here, despite not being directly characterized, the mean particle size of the TCP powder used in the PCL-TCP blends was approximately 11 µm as reported elsewhere for a batch from the same supplier. Therefore, the models presented in this
section apply to the processing of raw materials with similar characteristics. Regarding the geometry of the nozzles used for melt extrusion, it has been previously demonstrated that pressure drops affecting the flow of polymer melt are influenced by specific nozzle geometries, sizes, and interactions with the flow characteristics of the extrudate. Here, traditional Luer Lock nozzles featuring a cylindrical channel geometry (as opposed to the common conical alternative) with length of approximately 2 mm and no angle at the tip were used for extrusion. The effects of nozzle geometry on the pneumatic extrudability of PCL-TCL melts should be further explored as means to determine the most suitable option in terms of improved melt flow and reduced clogging rates.

3.4 Modelling the Effects of Design Parameters on the Compressive Elastic Modulus of PCL-TCP 3DB Scaffolds

Here, a distinct set of experimental studies serves as an extension of Section 3.3, in which the resulting strand width was a response variable. Now, in combination with another controllable scaffold design parameter (inter-axial separation between strands), strand width becomes an independent (predictor) variable. Hence, the established goal is to model the effects of those scaffold microarchitectural characteristics on the elastic compressive modulus of 3DB scaffolds.

3.4.1 Materials and Methods

Cylindrical scaffolds (dia. 8 mm x 2mm) were 3D-Bioplotted following material preparation and machine setup protocols previously discussed in Section 3.2.1. The choice of sample geometry, dimensions, and overall testing protocols was established in analogy to the protocols for mechanical characterization of the native osteochondral tissue zones described
in Section 2.2.1. Table 23 summarizes the choice of design and experimental parameters to be used in this study. Note the inclusion of pure PCL (100% by weight) in addition to the two typical mineralized PCL-TCP blends. For the three materials, all levels of strand widths and inter-axial separations were combined to form distinct scaffold microarchitectures, resulting in three full factorial designs of experiments for a comprehensive estimation of the compressive elastic moduli of osteochondral zone-specific 3DB scaffolds.

As mentioned, the testing protocols were equivalent to those followed in Section 2.2.1 while attempting to match the testing conditions applied to native osteochondral tissue samples. In brief, testing was performed following the platen technique, and unconfined compression followed at a constant axial strain rate of 0.01 s⁻¹ in a universal testing machine set up with a 1 kN load cell. Similarly, petroleum jelly was applied to the specimen-anvil interfaces to minimize the effects of friction. Here, testing did not occur under liquid medium as the scaffolds are not directly susceptible to the effects of dehydration. All samples were subjected to 20 preconditioning cycles to a strain of 0.005 at a rate of 0.002 s⁻¹ to reach a viscoelastic steady state. Zone-specific maximum strains were equivalent to those presented in Table 3 for each material composition: Zone 1 (PCL 100); Zone 2 (PCL-TCP 90-10); and Zone 3 (PCL-TCP 80-20). Similar testing protocols for PCL-TCP composites have been presented in the literature.⁶⁸,⁷⁹,¹¹⁵
Table 23 – Parameters for the experimental design for mechanical properties.

<table>
<thead>
<tr>
<th>Parameter(s)</th>
<th>Value(s)</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material Composition</td>
<td>PCL-TCP 80-20</td>
<td>Same as in Table 14. Each material defines an independent design of experiments from which the effects of each predictor variables were investigated.</td>
</tr>
<tr>
<td></td>
<td>PCL-TCP 90-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCL 100</td>
<td></td>
</tr>
<tr>
<td>Strand Width</td>
<td>200 μm</td>
<td>A set of strand diameters expected to provide a wide range of structural/mechanical properties. This range of values is common in the literature.</td>
</tr>
<tr>
<td></td>
<td>300 μm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 μm</td>
<td></td>
</tr>
<tr>
<td>Strand Inter-Axial</td>
<td>500 μm</td>
<td>Controls the pore size in association with the strand widths. These values should generate interstrand separation values ranging from 100 μm to 800 μm.</td>
</tr>
<tr>
<td>Separation</td>
<td>660 μm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>830 μm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 μm</td>
<td></td>
</tr>
<tr>
<td>Compressive Elastic Modulus</td>
<td><strong>Response variable</strong> measured in MPa. Determined from the slope of the elastic zone in the stress-strain curves acquired by uniaxial compression at constant strain rate.</td>
<td></td>
</tr>
<tr>
<td>Scaffold Dimensions</td>
<td>Ø 8 x 2 mm</td>
<td>Same as in Table 14.</td>
</tr>
<tr>
<td>Strand Lay-down Pattern</td>
<td>0°-120°-240°</td>
<td>Same as in Table 14.</td>
</tr>
<tr>
<td>Layer Height</td>
<td>80% of Nozzle Diameter</td>
<td>Same as in Table 14.</td>
</tr>
<tr>
<td>Sample Size</td>
<td>3</td>
<td>Three scaffolds will be fabricated from each design parameter combination.</td>
</tr>
</tbody>
</table>

Statistical analyses and regression modelling were performed using JMP Pro 12.

Assumption of normality of response distributions were tested via the Shapiro-Wilk test. If necessary, data transformations were applied to account for the assumptions of ANOVA models and achieve better goodness-of-fit (measured by R-squared values) between the resulting multiple-linear regression models and the raw data. Significance levels were set to $\alpha = 0.05$. 
3.4.2 Results and Discussion

A summary of results for each material and scaffold design parameters is presented in Table 24. Overall trends were consistent across each design parameter, wherein higher moduli values were characteristic of scaffolds with larger strand width and lower inter-axial separation, as opposed to lower moduli when strand width is lower and separation is higher. Such phenomenon was anticipated as the porosity of 3DB structures is directly affected by microarchitectural design changes and, in consequence, so is their structural integrity and response to mechanical stimuli.

Table 24 – Compressive elastic modulus in MPa for each combination of scaffold design parameter and material composition

<table>
<thead>
<tr>
<th></th>
<th>Strand Inter-axial Separation</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500 μm</td>
<td>660 μm</td>
<td>830 μm</td>
<td>1000 μm</td>
<td></td>
</tr>
<tr>
<td><strong>PCL-TCP 80-20</strong></td>
<td>200 μm</td>
<td>36.3</td>
<td>18.0</td>
<td>13.8</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>300 μm</td>
<td>59.7</td>
<td>41.7</td>
<td>17.9</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>400 μm</td>
<td>87.7</td>
<td>56.1</td>
<td>20.3</td>
<td>17.8</td>
</tr>
<tr>
<td><strong>PCL-TCP 90-10</strong></td>
<td>200 μm</td>
<td>43.1</td>
<td>32.9</td>
<td>16.6</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>300 μm</td>
<td>70.5</td>
<td>51.1</td>
<td>21.5</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>400 μm</td>
<td>102.8</td>
<td>75.1</td>
<td>30.6</td>
<td>22.1</td>
</tr>
<tr>
<td><strong>PCL 100</strong></td>
<td>200 μm</td>
<td>43.4</td>
<td>32.4</td>
<td>25.0</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>300 μm</td>
<td>58.4</td>
<td>40.7</td>
<td>30.8</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>400 μm</td>
<td>87.3</td>
<td>64.1</td>
<td>41.4</td>
<td>24.2</td>
</tr>
</tbody>
</table>
For **PCL-TCP 80-20** scaffolds, the original response dataset failed the assumption of normality with a rejected null hypothesis via the Shapiro-Wilk test \((p = 0.0003)\). A fitted multiple linear model produces residuals that were normally distributed \((p = 0.1123)\) but had a plot of residuals vs. predicted values that suggested inconstant variances, violating the assumption of homoscedasticity. Therefore, dataset transformations were applied to the response dataset wherein the natural-log transformation demonstrated to better approach the normal distribution \((p = 0.4056)\). A preliminary model included both main factors and their respective second-order interaction. The residuals for this fitted model were normally distributed \((p = 0.3478)\). Results have shown that both main factors were statistically significant \((p < 0.0001)\) but their interaction was not \((p = 0.6767)\). Therefore, the interaction was removed and the final model includes only the two main factors, which were both statistically significant. Figure 25 illustrates the distribution of natural-log transformed response values used in the final model. No transformations of predictor variables generated a better fit of the model measured by the goodness-of-fit parameter R-square, which was ultimately estimated to be equal to 0.9286. The model’s residuals were normally distributed \((p = 0.2359)\) and their variance was suggested to be constant following visual inspection.

**Equation 7** presents the regression equation in its generic form and **Equation 8** with the respective substitutions for the response variable \(y\), the predictors \(x_i\), and their \(\beta_i\) coefficients. Lastly, **Equation 9** presents the back-transformed response so that the moduli values can be calculated in the scale of MPa, as opposed to ln(MPa) in the transformed dataset.
Figure 25 – Histogram and normal quantile plot of the natural-log transformed response dataset used in the statistical analysis of the compressive elastic modulus of PCL-TCP 80-20 scaffolds

\[
\ln y = \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \epsilon \quad \text{Equation 7}
\]

\[
\ln \text{Modulus} = 4.4678157 + 0.0045388 \cdot \text{Width} + (-0.0035741) \cdot \text{Separation} \quad \text{Equation 8}
\]

\[
\text{Modulus} = \exp(4.4678157 + 0.0045388 \cdot \text{Width} + (-0.0035741) \cdot \text{Separation}) \quad \text{Equation 9}
\]

Graphical representation of the model presents visual evidence that, within the experimental ranges for strand width and inter-axial separation, the predictive equation closely follows the overall distribution of the raw data. Figure 26 plots the natural-log-transformed dataset, where experimentally defined moduli values are presented as blue dots and the color mesh represents a plot of Equation 8 above. Figure 27, on the other hand, plots the back-transformed dataset and its corresponding predictive equation in MPa. Strand width and separation axes are scaled in μm.
Figure 26 – Plot of ln-transformed modulus [ln(MPa)] of PCL-TCP 80-20 scaffolds for a range of strand widths and inter-axial separations. Blue dots represent experimental data and the color mesh represents the regression model.
The overall model characteristics were similar for PCL-TCP 90-10. The original response dataset also failed the assumption of normality (p = 0.0033) and a preliminary multiple linear model yielded residuals that were borderline normally distributed (p = 1072) but had inconstant variance. Hence, the response dataset was natural-log transformed and did not fail the assumption of normality (p = 0.2851, plotted in Figure 28), which is also valid for the residuals of a multiple linear model fit for both first-order factors and their interaction (p = 0.1458). In this model, both main factors were statistically significant (p < 0.0001) but
their interaction was not \( (p = 0.3258) \). Therefore, the final model includes only both main factors and yields normally distributed residuals \( (p = 0.1405) \) with constant variance. The regression model fit the ln-transformed experimental data with an R-square value of 0.9579. No transformation of predictor variables yielded a practically better fit, so the model is considered final. **Equation 10** presents the regression equation in its generic form and **Equation 11** with the respective substitutions for the response variable \( y \), the predictors \( x_i \), and their \( \beta_i \) coefficients. Lastly, **Equation 12** presents the back-transformed response in MPa values.

![Histogram and normal quantile plot](image)

**Figure 28** – Histogram and normal quantile plot of the natural-log transformed response dataset used in the statistical analysis of the compressive elastic modulus of PCL-TCP 90-10 scaffolds
\begin{align*}
\ln y &= \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \epsilon & \text{Equation 10} \\
\ln \text{Modulus} &= 4.8512646 + 0.0043063 \cdot \text{Width} + (-0.003641) \cdot \text{Separation} & \text{Equation 11} \\
\text{Modulus} &= \exp(4.8512646 + 0.0043063 \cdot \text{Width} + (-0.003641) \cdot \text{Separation}) & \text{Equation 12}
\end{align*}

Graphical representation of the model also suggests that the regression follows the data in close proximity. \textbf{Figure 29} brings a plot of the natural-log transformed dataset (blue dots) against its corresponding statistical regression model (color mesh), while \textbf{Figure 30} presents the back-transformed model (color mesh) against experimental raw data in MPa (blue dots). Strand width and separation axes are scaled in μm.
Figure 29 – Plot of ln-transformed modulus [ln(MPa)] of PCL-TCP 90-10 scaffolds for a range of strand widths and inter-axial separations. Blue dots represent experimental data and the color mesh represents the regression model.
Lastly, for **PCL 100** scaffolds, slight differences were implemented in the modelling protocol as an effect of the unique mechanical response of pure PCL compared to the two aforementioned mineralized blends. Here, the raw experimental dataset did not fail the assumption of normality when tested with the Shapiro-Wilk test ($p = 0.0951$, plotted in Figure 31). Furthermore, a preliminary model including both main factors and their interaction yielded a distribution of residuals that did not fail the assumption of being normally distributed ($p = 8799$) and did not show evidence of inconstant variance. The model
was centered around the means of the interaction terms for better estimation of the interaction effect. The model is considered final with a goodness-of-fit R-square rating of 0.9726. No transformation of the response dataset was necessary and no transformation of any predictor variable yielded a practically better fit. Equation 13 presents the regression model in its generic form and Equation 14 with the respective substitutions for the response variable $y$, the predictors $x_i$ (including their means for centering polynomials), and each respective $\beta_i$ coefficient. Figure 32 plots the original dataset against the predictive statistical regression model illustrated with the color mesh in MPa. Strand width and separation axes are scaled in $\mu$m. The plot provides visual proof that the regression equation follows the raw data with close proximity within the range of values for width and separation tested during the experiments.
The experimental models developed and presented here provide a comprehensive characterization of the effects of varying scaffold materials and microarchitectural design parameters on the compressive elastic modulus of 3DB structures. Furthermore, the statistical models include:

\[ y = \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \beta_3 \cdot (x_1 - \bar{x}_1) \cdot (x_2 - \bar{x}_2) + \epsilon \]  

*Equation 13*

**Modulus**

\[ = 69.50199 + 0.1392471 \cdot Width + (-0.097208) \cdot Separation \\
+ (-0.000265) \cdot (Width - 300) \cdot (Separation - 747.5) \]

*Equation 14*

Figure 32 – Plot of compressive elastic modulus (MPa) of PCL 100 scaffolds. Blue dots represent experimental data and the color mesh represents the regression model.
tools and techniques utilized in the development of regression models enable a direct prediction of scaffold compressive mechanical response for a range of scaffold material compositions, strand width, and inter-axial separation. As proposed, this set of preliminary models mathematically characterizes relevant interrelationships between important scaffold characteristics and lay the groundwork for future development of machine control algorithms that target improved process autonomy and adaptation to process or other environmental changes. Within the realms of osteochondral TE, this section’s primary contributions go hand in hand with current technological requirements in the fabrication of integrated heterogeneous constructs. As previously emphasized, the accurate control of defining geometric and positional characteristics of scaffold strands (the building blocks of 3DB structures) allows, in consequence, for precise control of a scaffold’s compressive mechanical response – particularly relevant in the pursuit of biomimetic characteristics by integrated heterogeneous constructs in TE.

Recalling the compressive elastic modulus of the distinct zones of osteochondral tissue, as presented in Section 2.3.1, evidence suggests that, for each of those zones, there is a corresponding 3DB scaffold design with similar compressive response characteristics. **Zone 1**, defined as the superficial articular cartilage layer and matched by a pure PCL scaffold (labeled PCL 100) had a modulus of 39.3 ± 9.8 MPa under uniaxial compression at a constant strain rate. The mean value is encompassed by the two extremes found in the range of compressive elastic modulus of PCL 100 scaffolds, with a minimum of 4.9 ± 1.6 MPa and maximum of 87.9 ± 3.8 MPa (see Table 24 for more details). As for **Zone 2**, defined as the intermediate transitional zone between calcified cartilage and the subchondral bone plate, which is matched by a PCL matrix scaffold featuring 10% by weight of TCP (labeled PCL-
TCP 90-10), a compressive elastic modulus of 37.1 ± 11.2 MPa was experimentally observed. Similarly, that value is encompassed by the low and high extremes of moduli values observed for PCL-TCP 90-10 scaffolds, respectively 7.0 ± 0.2 and 102.8 ± 4.9 MPa (Table 24). The same is valid for Zone 3, the deep cancellous bone, matched by a PCL matrix scaffold with 20% TCP by weight (labeled PCL-TCP 80-20), which experimentally revealed a compressive elastic modulus of 41.6 ± 18.5 MPa and scaffolds ranging from 5.6 ± 2.7 to 72.7 ± 30.4 MPa (Table 24).

In conclusion, this section provided empiric evidence that scaffolds can have their microarchitecture designed in a way to achieve compressive elastic moduli matching those of the distinct zones of the osteochondral tissue (a functional requirement for biomimicry, as defined in Section 2.3.1). In addition, the development of predictive models for relevant material-structure-function relationships is noted as a useful engineering resource, which can be later integrated with computer-aided design, engineering, and manufacturing tools for the materials and processes here involved. With these results on hand, the following section verifies the validity of each of the aforementioned predictive regression models, including those presented in Section 3.3, and concludes this chapter.

3.5 Validation of Predictive Models and Proposal of a Scaffold Design

Here, two sets of validation experiments were followed to provide reasonable assurance that the predictive multiple linear regression models proposed in Sections 3.3 and 3.4 are able to determine theoretical values that match those observed in practice.
3.5.1 Materials and Methods

General description:

A “back-tracking” approach was followed to determine required scaffold microarchitectural design attributes from a set of target compressive elastic moduli, as defined for each zone of the osteochondral tissue (Section 2.3.1). Once a feasible combination of strand width and inter-axial separation was obtained via the developed regression models from Section 3.4, a following step utilized the required strand width value as the response in the regression models proposed in Section 3.3. Ultimately, a set of 3DB processing parameters was determined and scaffolds were manufactured using them. Post fabrication inspection of three samples in each situation had their results compared against the output of each respective regression equation. Figure 33 describes the procedure in more detail.

Ultimately, 95% confidence intervals (CI) for the predicted response of each regression models were compared against 95% CI for the experimentally obtained data. The absence of statistically significant differences between predicted vs. actual values was defined when their respective CIs did not overlap. All statistical analyses were performed using JMP Pro 12.
Validation of PCL 100 scaffold compressive elastic modulus model:

As determined in Section 2.3.1, the compressive elastic modulus of the articular cartilage component of the osteochondral tissue (labeled Zone 1), as tested, was found to be 39.3 ± 9.8 MPa. The mean value of 39.3 MPa is then taken as the desired response Modulus in Equation 14. As a result, there is a theoretically infinite range of possible combinations between strand width and inter-axial separation that would result in $Modulus = 39.3$, represented by the intersection of the [0, 0, 39.3] plane and the plot of Equation 14. For simplicity, and justified by the fact that a predictive model for PCL 100 strand width was not in the scope of this dissertation, a strand width of 300 μm was chosen and the corresponding strand separation was determined using Equation 14. This particular choice of strand width is based from evidence presented in Table 24, where the combination of [width, separation]
= [300, 660] yielded a mean modulus closest to 39.3 MPa. Thus, substituting Modulus for 39.3 and Width for 300 in Equation 14, the resulting Separation value that upholds the equality is 740. Therefore, three PCL 100 scaffolds featuring strands with a width of 300 μm and inter-axial separation of 740 μm were manufactured and tested under uniaxial compression following previously defined protocols (Section 3.4.1).

Validation of PCL-TCP 90-10 scaffold compressive elastic modulus model:

Section 2.3.1 defined a compressive elastic modulus of 37.1 ± 11.2 MPa for the transitional zone of the native osteochondral tissue (labeled Zone 2). Hence, that value is taken as the response Modulus in Equation 12. From Table 24, the only tested inter-axial separation value that encompasses the target modulus for a range of strand widths is 660 μm, therefore chosen as input Separation in Equation 12. To maintain equality, a strand width of 271 μm is required. Therefore, three PCL-TCP 90-10 scaffolds featuring strands with a target width of 271 μm and inter-axial separation of 660 μm were fabricated and tested for their compressive elastic response as previously described.

Validation of PCL-TCP 90-10 strand width model:

As defined above, a strand width of 271 μm is required to yield desired compressive elastic properties of PCL-TCP 90-10 scaffolds for a fixed inter-axial strand separation. Upon inspecting Table 18 through Table 20, the following 3DB parameter combinations yielded PCL-TCP 90-10 strand widths near the target value of 271 (± 5%) μm:

- [Nozzle Diameter (mm), Temperature (°C), Pressure (N/mm²), Speed (mm/s)]
  - [0.3, 120, 0.5, 1.0]
  - [0.3, 130, 0.2, 0.5]
The *Nozzle Diameter* is set to a fixed level corresponding to that with the most occurrences (0.3 mm) in the list above. Within that subset, the *Temperature* is similarly set to the one with the most occurrences (130 °C). Likewise, within that second subset, the *Pressure* is also set to the one with the most occurrences (0.6 N/mm²), leaving only the input *Speed* to be defined by *Equation 6* in order to maintain its equality. Upon doing so, the model predicts that the following 3DB parameter combination yields strands with a width of 271 μm:

- Nozzle = 0.3 mm, Temperature = 130 °C, Pressure = 0.6 N/mm², Speed = 1.54 mm/s.

Therefore, three PCL-TCP 90-10 scaffolds were manufactured with the 3D-Bioploter set to the parameters above and the resulting widths were characterized following previously defined protocols (*Section 3.3.1*).
Validation of PCL-TCP 80-20 scaffold compressive elastic modulus model:

Similarly, a compressive elastic modulus of $41.6 \pm 18.5$ MPa was experimentally determined for the osseous zone of the native osteochondral tissue (labeled Zone 3). The mean value 41.6 MPa is taken as the desired response Modulus in Equation 9. Fixed separations of either 500 or 660 μm encompass that target value for a range of strand widths, as shown in Table 24. For consistency with the PCL-TCP 90-10 model, the same inter-axial separation of 660 μm was chosen. So, substituting Separation in Equation 9 with 660 and Modulus with 41.6 requires a Width value of 356 to maintain equality. Therefore, three PCL-TCP 80-20 scaffolds featuring strands with a target width of 356 μm and inter-axial separation of 660 μm were fabricated and tested for their compressive elastic response as previously described.

Validation of PCL-TCP 80-20 strand width model:

As defined above, a strand width of 356 μm is required to yield desired compressive elastic properties of PCL-TCP 80-20 scaffolds for a fixed inter-axial strand separation. Following inspection of Table 15 through Table 17, it was found that the following 3DB parameter combinations yielded PCL-TCP 80-20 strand widths near the target value of 356 (± 5%) μm:

- [Nozzle Diameter (mm), Temperature (°C), Pressure (N/mm²), Speed (mm/s)]
  - [0.3, 120, 0.6, 0.5]
  - [0.3, 130, 0.6, 0.5]*
  - [0.3, 130, 0.5, 0.5]
  - [0.4, 120, 0.5, 0.5]
  - [0.4, 140, 0.5, 1.5]
For consistency, Nozzle Diameter, Temperature, and Pressure were set to the same levels as before (0.3 mm, 130 °C, and 0.6 N/mm²), which is also justified by a corresponding parameter combination presented above. Therefore, to maintain the equality of Equation 3, Speed must be set to 0.61 mm/s and, as a result, the following set of parameters is defined for a predicted strand width of 356 μm:

- Nozzle = 0.3 mm, Temperature = 130 °C, Pressure = 0.6 N/mm², Speed = 0.61 mm/s.

Therefore, three PCL-TCP 80-20 scaffolds were fabricated with the 3D-Bioploter set to the parameters above and the resulting widths were characterized following the protocols previously mentioned.

3.5.2 Results and Discussion

Table 25 presents a summary of the results obtained from each validation experiment previously described. 95% confidence intervals from both predicted and actual values are presented and compared.
Table 25 – Summary of model validation results including predicted and actual responses, confidence intervals, and differences

<table>
<thead>
<tr>
<th>Model</th>
<th>PREDICTED</th>
<th>ACTUAL</th>
<th>Difference P vs. A</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL 100 Compressive Elastic Modulus (MPa)</td>
<td>39.3 (38.0, 40.7)</td>
<td>36.4 (33.6, 39.3)</td>
<td>-7.4%</td>
</tr>
<tr>
<td>PCL-TCP 90-10 Compressive Elastic Modulus (MPa)</td>
<td>37.1 (34.8, 39.7)</td>
<td>39.9 (22.8, 57.0)</td>
<td>+7.5%</td>
</tr>
<tr>
<td>PCL-TCP 90-10 Strand Width (μm)</td>
<td>271 (268, 275)</td>
<td>278 (274, 281)</td>
<td>+2.6%</td>
</tr>
<tr>
<td>PCL-TCP 80-20 Compressive Elastic Modulus (MPa)</td>
<td>41.6 (37.6, 45.7)</td>
<td>40.0 (32.8, 47.1)</td>
<td>-4.1%</td>
</tr>
<tr>
<td>PCL-TCP 80-20 Strand Width (μm)</td>
<td>356 (350, 362)</td>
<td>344 (335, 352)</td>
<td>-3.3%</td>
</tr>
</tbody>
</table>

Direct comparison of experimental means against their respective predicted values demonstrated that no absolute relative deviation larger than 7.5% was observed for the compressive elastic modulus models and 3.3% for the strand width models. Larger deviations were observed for the compressive elastic modulus models, a group which also expressed wider confidence intervals experimentally – an indicator of the inherent variability in that particular mechanical property of 3DB scaffolds. Statistically, 95% confidence intervals overlap in all pairs of predicted vs. actual tested values indicating no statistically significant differences. Therefore, the predictive models proposed in Sections 3.3 and 3.4 were demonstrated to be capable tools for mathematically modeling relevant material-process-structure interrelationships in the 3D-bioplotting-based fabrication of osteochondral scaffolds.
In this dissertation, for practical reasons, the moduli values chosen for validation (see Section 3.5.1) happened to be near the central region of the range of values obtained empirically. In future, as means of assessing the sensitivity of the proposed models across different regions of the experimental design spaces, lower or higher response values could be arbitrarily chosen in models for the compressive elastic moduli (i.e. less than 10 MPa or more than 80 MPa for models presented in Section 3.4) and strand width (i.e. in the neighborhood of 100 or 400 µm for models presented in Section 3.3).

3.6 Chapter Summary

This chapter described a set of modelling approaches for select properties of 3DB scaffolds fabricated using PCL and PCL-TCP mineralized composites. Process parameter screening experiments (Section 3.2) defined ranges of 3DB parameter combinations that allow for feasible strand deposition in the fabrication of 3D plotted structures. Distinct levels of extrusion nozzle internal diameter, temperature, pressure, and print head travel speed were investigated following an algorithm for both PCL-TCP 80-20 and PCL-TCP 90-10 composites. Here, PCL 100 was not accounted for as the literature presents substantial characterization of its processing behavior in 3DB. From the resulting set of feasible parameter combinations, which enabled the deposition of strands free of defects, a larger DoE targeted the characterization of each 3DB parameter effect on the width of deposited strands for both mineralized blends (Section 2.3). Similarly, a subsequent set of experiments included the width of strands as a predictor variable alongside the inter-axial separation between adjacent strands as part of DoEs modelling the effects of these two microarchitectural scaffold characteristics on the compressive elastic modulus of 3DB.
scaffolds, this time accounting for all relevant material compositions (Section 3.4). A total of five mathematical models were proposed considering statistically significant effects observed experimentally for strand width and scaffold compressive elastic modulus. These models were finally validated using target properties of the distinct zones of the osteochondral tissue, as discussed in Chapters 1 and 2, as means of demonstrating that 3DB scaffolds fabricated with select material compositions and microarchitectural characteristics present the potential to match or closely resemble select native tissue properties.

Primary contributions include the identification of feasible 3DB processing parameters for PCL-TCP composites and a comprehensive characterization of how they affect the quality and structural properties of fabricated scaffolds for osteochondral TE. The studies developed in this chapter provide significant contributions to each of the research needs identified and discussed in Section 3.1, including the characterization of material-structure-function interrelationships and predictive models that pave the way for the development of closed-loop control systems in extrusion-deposition-based additive manufacturing processes and, as a result, enabling better process automation and in-process quality control.
CHAPTER 4 – TRANSLATIONAL ASPECTS

4.1 Chapter Introduction

Characterizing structure-function interrelationships relevant to the safety and effectiveness of medical products is an imperative step during the stages of product development. Within the scope of TERM, a highly regulated market calls for a systematic demonstration of a product’s performance of its intended functions. In osteochondral tissue engineering, particularly the scaffold-based branch, the evaluation process is multifaceted and reliant on a relevant set of scientifically sourced evidences that establish reasonable assurance of a product’s safety and effectiveness. From a clinical translational perspective, preliminary assessment of a 3DB scaffold for osteochondral tissue engineering purposes should focus on the implant’s ability to offer a biologically viable microenvironment for cells to reside and develop their functions.

Polycaprolactone and many of its blends have been tested as a material for bone and cartilage implants. Public records of PCL-based products having successfully gone through the regulatory pathway for commercialization are also available and studies have reported favorable biocompatibility levels of PCL-TCP composites. Despite some preliminary work available in the literature (recall Sections 1.5 and 1.6), the promise of a multiphasic, multi-material scaffold that precisely controls site-specific regeneration of bone and cartilage tissue once implanted is yet to be demonstrated. The in vitro assessment of a 3DB osteochondral scaffold featuring the shape, scale, and properties of the design proposed in this dissertation is yet to be performed. For that purpose, established histological assays such as Live/Dead, alamarBlue, and Alizarin Red, can provide qualitative or
quantitative evidence of cell viability, metabolic activity, and response to differentiation factors. The Live/Dead assay stains cells with two distinct fluorescent dyes and, when imaged with a microscope setup with appropriate color filters, allows for direct visualization of living, healthy cells in a uniform green color, or dead and dying cells typically appearing red.\textsuperscript{137} The alamarBlue assay allows for an assessment of cell metabolic activity by exposing cultures to a reagent that changes its fluorescence levels according to the number and health of cells. A plate reader is necessary to quantify the change in fluorescence, which can be correlated to the metabolic activity of the cell population.\textsuperscript{138} The Alizarin Red is a histochemical dye that highlights calcium deposits in the culture, providing evidence of osteogenic activity in the culture.\textsuperscript{139} Ultimately, \textit{in vitro} characterization of the aforementioned scaffold design should provide reasonable evidence that the product is suitable for \textit{in vivo} animal testing and, therefore, one step closer to human clinical trials.

Furthermore, from a product usability perspective, the ability of 3DB osteochondral scaffolds to be appropriately handled and implanted in a typical surgical scenario requires specific design considerations that extend the product development process. Here, based on functional design requirements established in \textbf{Chapter 2} and inspired by current “gold standards” in the treatment of small osteochondral injuries (i.e. the COR technique), select macro-characteristics of the scaffold design, such as its diameter and shape features that facilitate implantation, are assessed as means of maximizing implant performance in a clinical-type setting. Based on a series of iterative trial-and-error trials of distinct scaffold designs, a best-performing case is compared against native tissue osteochondral grafts. Lastly, as a result of the learnings and experiences obtained with this dissertation, risk assessment and management tools were employed as means to provide the literature with
known or hypothesized issues that should be addressed in future design iterations of the 3DB osteochondral scaffold here discussed. In the literature, instances of the Ishikawa (fishbone, cause-and-effect) diagrams being used as a tool to investigate potential issues associated with the design and fabrication of medical implants have been reported.\textsuperscript{140,141} Similarly, failure modes and effects analysis (FMEA) can serve as proactive technique for error detection and reduction, and its utilization has been reported for patient safety,\textsuperscript{142} medical product development,\textsuperscript{143,144} and biomanufacturing.\textsuperscript{145} Both of these root-cause analysis tools offer opportunities for risk assessment and management in osteochondral scaffold design and manufacturing and, therefore, are herein explored.

This chapter addresses \textbf{Objective 3} and its associated tasks defined in Section 1.7. The sections that follow in this chapter are organized as defined below:

\begin{itemize}
  \item[(4.2)] Assessment of Biological \textit{In Vitro} Response;
  \item[(4.3)] Assessment of the Effects of Bulk Design Parameters on the Implantation Performance of 3DB Osteochondral Scaffolds;
  \item[(4.4)] Risk Assessment and Management.
\end{itemize}

A chapter summary is presented in \textbf{Section 4.5}.

\section*{4.2 Assessment of Biological \textit{In Vitro} Response}

This section evaluates the biological response of hASCs seeded and cultured within scaffolds with design attributes providing biomimetic mechanical properties matching those of native tissue as previously established. Cell viability after 14 and 28 days, metabolic every 3 days, and calcium deposition after 14 and 28 days were assessed and quantified via established protocols detailed as follows.
4.2.1 Materials and Methods

Scaffold Preparation and Cell Culture Protocols:

Scaffolds featuring characteristics matching each of the osteochondral tissue zones were fabricated as previously discussed. Additionally, a set of full-thickness multiphasic osteochondral scaffolds featuring the three zones in a single integrated construct were also fabricated. Preceding all assays, sterilization of all scaffolds was performed by submersion in 70% ethanol for 20 minutes under vigorous shaking, twice, followed by two washes and storage in PBS until seeding. Sterility was verified by two-day incubation of one acellular scaffold of each group in culture media.

Cell expansion began by plating a cryopreserved 3-million hASC population (passage 1, Cat. #R7788115, ThermoFisher Scientific) onto seven T-75 culture flasks, cultured in stem-cell optimized culture media (MesenPRO RS™ Medium, Life Technologies Corporation, Grand Island, NY) with 1% v/v added of L-glutamine and streptomycin. Following a complete media change after three days in culture, cells were washed with Hank’s Balanced Salt Solution and harvested by trypsin digestion when average flask confluence was in the range of 80 - 90% after the sixth day in culture. Cells were suspended in appropriate media aliquots and seeded onto scaffolds for each assay.

Figure 34 illustrates the overall cell seeding, culture, and assay procedures. In detail, sterilized PCL 100, PCL-TCP 90-10, and PCL-TCP 80-20 scaffolds were individually placed onto the wells of 48-well culture plates for higher cell adherence to scaffold strands. 1 ml of media containing a suspension of 100,000 cells was added to the wells containing each scaffold. Cells were allowed to adhere to surfaces overnight followed by each scaffold being transferred to the larger wells of 24-well culture plates for larger media volume allowance.
during culture. For the multiphasic scaffold group, a two-step seeding protocol was followed. Firstly, a 200,000-cell suspension in 1 ml of media was added to wells containing each multiphasic scaffold with their mineralized zones (PCL-TCP) facing up. A 12-hour incubation period allowed cells to settle and adhere to the structure. Secondly, media was aspirated and each multiphasic scaffold was flipped over having their non-mineralized zone (PCL) facing up, followed by seeding with another 100,000 cell suspension in 1 ml of media. Again, overnight incubation allowed for cell adherence, followed by scaffolds being transferred to the larger wells of 24-well culture plates for prolonged culture at 37°C and controlled atmosphere containing 5% CO₂. A complete media change was performed every three days (1 ml for single phase scaffolds and 1.5 ml for multiphasic scaffolds). To prevent media exhaustion and allow complete exposure of nutrients to cells residing within the internal volumes of scaffolds, the entire 28-day culture was performed dynamically by introducing slight media motion using an orbital shaker in continuous operation at 60 RPM.
Qualitative Assessment of Cell Viability:

For visualization of cell viability, the LIVE/DEAD assay (LIVE/DEAD® Viability/Cytotoxicity Kit, Molecular Probes, Inc., Eugene, OR) was performed at culture days 14 and 28. All three single-phase designs were submitted for viability assessment at
both time points (n = 2 scaffolds/design/time point), totaling 12 scaffolds. At each time point, assayed scaffolds had their culture media aspirated and were washed twice in PBS followed by 30-minute incubation (37°C, 5% CO₂) in 3 ml aliquots of the staining solution (prepared as a dilution of 40 µL of EthD-1 and 10 µL of calcein AM in 20 ml of PBS – proportions suggested by the kit supplier). Visualization of cells was performed via fluorescence microscopy using the appropriate light wavelength filter for each live or dead cell condition. Images were taken perpendicular to and facing the top and bottom flat surfaces of each scaffold.

Quantitative Assessment of Cell Metabolic Activity:

For quantification of cell metabolic activity, the alamarBlue non-destructive assay (alamarBlue® Cell Viability Assay Protocol, Invitrogen Corporation, Carlsbad, CA) was performed at culture day one and then every three days (preceding media changes). All three single-phase designs were submitted for metabolic activity quantification assessment (n = 3/design) and the same number of acellular negative controls were cultured under equal conditions. At each assay time point, samples had their culture media aspirated and replaced by 1 ml of media solution containing 10% v/v of the alamarBlue dye. Cultures were then incubated (37°C, 5% CO₂) for 4 hours followed by three 100 µL aliquots of media sampled into the wells of 96-well culture plates for light absorbance readings through 570 nm and 600 nm light wavelength filters using a microplate reader (Tecan™ GENios® Microplate Reader, Tecan Group Ltd., Männedorf, Switzerland). The percent reduction of the alamarBlue dye, an indicator of cell metabolic activity during the incubation period, was calculated using

**Equation 15.** Having completed the assay, the media containing alamarBlue was aspirated
from cultures and replaced with fresh, dye-free media. A two-way full factorial ANOVA model was fit as means of statistically comparing the results obtained for each scaffold design and time point using JMP Pro 12. Significance was set to $\alpha = 0.95$. Tukey HSD post-hoc tests were performed to evaluate differences between groups in significant factors or factor interactions.

$$\% \text{ aB Reduction} = \frac{(\varepsilon_{\text{OX}})_{\lambda_2}A_{\lambda_1} - (\varepsilon_{\text{OX}})_{\lambda_1}A_{\lambda_2}}{(\varepsilon_{\text{RED}})_{\lambda_1}A'_{\lambda_2} - (\varepsilon_{\text{RED}})_{\lambda_2}A'_{\lambda_1}} \times 100 \quad \text{Equation 15}$$

Where:

- $\lambda_1 = 570$ nm, wavelength
- $\lambda_2 = 600$ nm, wavelength
- $(\varepsilon_{\text{OX}})_{\lambda_1} = 80586$ M$^{-1}$cm$^{-1}$, molar extinction coefficient (570 nm)
- $(\varepsilon_{\text{OX}})_{\lambda_2} = 117216$ M$^{-1}$cm$^{-1}$, molar extinction coefficient (600 nm)
- $(\varepsilon_{\text{RED}})_{\lambda_1} = 155677$ M$^{-1}$cm$^{-1}$, molar extinction coefficient (570 nm)
- $(\varepsilon_{\text{RED}})_{\lambda_2} = 14652$ M$^{-1}$cm$^{-1}$, molar extinction coefficient (600 nm)
- $A_{\lambda_1} =$ observed absorbance reading for test well (570 nm)
- $A_{\lambda_2} =$ observed absorbance reading for test well (600 nm)
- $A'_{\lambda_1} =$ observed absorbance reading for negative control well (570 nm)
- $A'_{\lambda_2} =$ observed absorbance reading for negative control well (600 nm)

Quantitative Assessment of Calcium Deposition:

For quantification of the calcium content deposited by cells over time, the Alizarin Red S stain (ARS, Sigma-Aldrich Corporation, St. Louis, MO) was used as a calcium staining agent applied to all three scaffold groups cultured over 14 or 28 days ($n = 3$/design) and corresponding acellular negative controls. The quantification protocol performed here is based on a protocol presented in Yu et al. (2016). In brief, after 14 and 28 culture days, scaffolds were removed from culture and fixed for 30 minutes in 10% buffered formalin under orbital agitation at 120 RPM. Followed by two washes in PBS, all samples were stained for 2 minutes in ARS solution (2 g ARS powder dilution in 100 ml deionized water).
Samples were then washed twice in PBS for dye removal and allowed to air dry overnight. Imaging was performed the following day using a dissection microscope (EZ4 educational stereomicroscope, Leica Microsystems Inc., Buffalo Grove, IL) under consistent image-taking conditions. Once all figures were taken, single-phase scaffolds were submerged in individual 1 ml aliquots of 10% v/v acetic acid solution for 45 minutes under orbital agitation at 120 RPM. For each scaffold unit, three 150 µL aliquots of the acetic acid solution were sampled into the wells of 96-well culture plates for light absorbance readings through a 405 nm light wavelength filter using a microplate reader (Tecan™ GENios® Microplate Reader). The percent light absorbance is reported – higher values indicate higher calcium content dissolution within the acid. A three-way full factorial ANOVA model was fit to compare the results obtained for each scaffold design, time point, and experimental group using JMP Pro 12. Significance was set to $\alpha = 0.95$. Tukey HSD post-hoc tests were performed to evaluate differences between groups in significant factors or factor interactions.

Multiphasic scaffolds were also stained with ARS following the protocols described above. They were imaged for qualitative visualization of the calcium content throughout the distinct zones after 14 and 28 days.

### 4.2.2 Results and Discussion

**Qualitative Assessment of Cell Viability:**

Across all scaffold designs, large cell populations remained viable after 14 and 28 days as indicated by fluorescence microscopy. Representative images of the 14-day time point are shown in Figure 35, and the 28-day time point in Figure 36. Living cells are shown in green and dead cells are shown in red – each image pair displays distinct fluorescence...
images taken at the exact same location. Dense cell clusters are visible throughout most of
the scaffolds surface, including groups of cells that began to bridge the gap between adjacent
strands and form a dense matrix partially filling the interstrand regions (pores) of the
scaffolds. This phenomenon is already observed in the earlier time point and has continued to
progress throughout the later time point. Visually, overall cell viability did not change across
scaffold designs or time points, where roughly the same amount of live and dead cells could
be identified at the time of imaging.
Figure 35 – Representative fluorescence images of cell cultures assayed via LIVE/DEAD at the 14-day time point. Each row presents a pair of images taken at the exact same field of view but with distinct light filters for visualization of each cell condition. Scale bar = 300 μm; valid for all images.
Figure 36 – Representative fluorescence images of cell cultures assayed via LIVE/DEAD at the 28-day time point.

Each row presents a pair of images taken at the exact same field of view but with distinct light filters for visualization of each cell condition. Scale bar = 300 µm; valid for all images.
Quantitative Assessment of Cell Metabolic Activity:

The quantification of alamarBlue percent reduction over time provided direct assessment of the metabolic activity of cell populations residing and proliferating within each scaffold. Although the alamarBlue assay is well regarded in the literature as a method to estimate cell proliferation levels, the interpretation here is restricted to metabolic activity because of the potential differentiation of hASCs in contact with TCP over time, which is prone to confound the results of alamarBlue reduction for purposes of estimating cell proliferation. Having said that, Figure 37 presents a plot of the results for each scaffold design and time point. Bars represent the mean percent reduction of the alamarBlue dye after a 4-hour incubation period. Error bars indicate the standard deviations around each mean.

Results indicate that cell populations had a rapid increase of their metabolic activity that peaked around days 10 to 13. Following that peak, a constant plateau is observed with the dye reduction hovering at similar levels for several days, until it reaches a noticeably lower level at the last time point, suggesting that extended culture times may be detrimental to the overall viability of cells in such culture conditions.
Statistical analyses have shown that significant effects from both scaffold design and time are present in the alamarBlue percent reduction results. The normality of data was verified with a Shapiro-Wilk score of \( p = 0.2692 \). Figure 38 plots the alamarBlue reduction dataset and its respective normal quantile plot.

A 2-way full factorial ANOVA model was fit and identified significant effects for scaffold design \( (p < 0.0001) \), time \( (p < 0.0001) \), and their interaction \( (p < 0.0023) \). Post-hoc Tukey HSD tests for scaffold design did not identify statistically significant differences between the PCL-TCP 90-10 and PCL-TCP 80-20 scaffold groups, but both were significantly different from the PCL 100 group. Post-hoc tests for time identified several differences across groups, which are summarized in Table 26.
<table>
<thead>
<tr>
<th>Time Point</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 13</td>
<td>A</td>
</tr>
<tr>
<td>Day 10</td>
<td>A, B</td>
</tr>
<tr>
<td>Day 19</td>
<td>A, B</td>
</tr>
<tr>
<td>Day 07</td>
<td>A, B</td>
</tr>
<tr>
<td>Day 25</td>
<td>A, B</td>
</tr>
<tr>
<td>Day 22</td>
<td>B, C</td>
</tr>
<tr>
<td>Day 16</td>
<td>B, C</td>
</tr>
<tr>
<td>Day 04</td>
<td>C, D</td>
</tr>
<tr>
<td>Day 28</td>
<td>D</td>
</tr>
<tr>
<td>Day 01</td>
<td>E</td>
</tr>
</tbody>
</table>

However, the presence of a statistically significant interaction effect suggests that hASCs demonstrate distinct metabolic activity trends over time when residing across distinct scaffold groups, which is potentially an effect of the induced differentiation of those cells in contact with TCP. Therefore, final statistical inferences must be drawn from the within-group post-hoc comparisons for the interaction effect presented in Table 27. In this case, statistically significant differences between consecutive time points for each scaffold group were identified as follows:

- **PCL 100**: no consecutive days were significantly different from each other
- **PCL-TCP 90-10**: [Day 01, Day 04], [Day 25, Day 28]
- **PCL-TCP 80-20**: [Day 04, Day 07]

These results suggest that the most abrupt changes in cell metabolic activity happened at the beginning and at the end of the 28-day culture period. These trends can also be observed graphically in Figure 37. In the literature, the reduction of the alamarBlue dye exposed to eukaryotic cell cultures has expressed a similar behavior characterized by an
initial rapid increase, then a peak, and then drop in quantified values over time.\textsuperscript{75,147,148} The drop at the end of the culture period may be attributed to possible effects of over-confluent cell clusters, media exhaustion for populations of large size, or hindered nutrient and waste flow as scaffold pores are filled with matrix. Therefore, under the cell seeding and culturing protocols outlined in this set of experiments, it is suggested that \textit{in vitro} culture does not continue further than the 28\textsuperscript{th} day (or, perhaps, the 25\textsuperscript{th} day) as means to obtain desirably higher levels of cell metabolic activity in the occasion of implantation of the cell-seeded osteochondral construct.
Table 27 – Tukey HSD post-hoc group comparisons for the interaction effect between scaffold group and time point for the percent alamarBlue reduction results

<table>
<thead>
<tr>
<th>Scaffold Group</th>
<th>Time Point</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 10</td>
<td>A</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 13</td>
<td>A</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 25</td>
<td>A B</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 13</td>
<td>A B C</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 07</td>
<td>A B C D</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 07</td>
<td>A B C D</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 13</td>
<td>A B C D E</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 19</td>
<td>A B C D E</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 19</td>
<td>A B C D E F</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 10</td>
<td>A B C D E F</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 19</td>
<td>A B C D E F G</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 22</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 16</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 16</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 25</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 25</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 22</td>
<td>A B C D E F G H</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 10</td>
<td>B C D E F G H</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 07</td>
<td>C D E F G H I</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 04</td>
<td>D E F G H I</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 22</td>
<td>E F G H I</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 28</td>
<td>E F G H I</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 04</td>
<td>E F G H I J</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 16</td>
<td>E F G H I J</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 04</td>
<td>F G H I J K</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 28</td>
<td>G H I J K</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 28</td>
<td>H I J K</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Day 01</td>
<td>I J K</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Day 01</td>
<td>J K</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Day 01</td>
<td>K</td>
</tr>
</tbody>
</table>
Quantitative Assessment of Calcium Deposition

Representative images of Alizarin Red S stained PCL 100, PCL-TCP 90-10, and PCL-TCP 80-20 samples are shown in Figure 39, Figure 40, and Figure 41, respectively. Visually, few inferences can be made. Imaging ARS-stained 3D scaffolds is challenging due to suboptimal lighting conditions compared to monolayer cultures in transparent substrates that can be easily illuminated from a source opposite to the microscope viewpoint, which highlights the characteristic red color of the dye. Nonetheless, suggestive differences can be observed between select groups. Note the strong background staining of acellular controls containing TCP, which highlights their role in preventing misinterpretation of quantitative results later on.

Figure 39 – PCL 100 samples stained with Alizarin Red S for calcium deposition quantification. Scale bar = 700 µm; valid for all images.
Figure 40– PCL-TCP 90-10 samples stained with Alizarin Red S for calcium deposition quantification. Scale bar = 700 µm; valid for all images.

Figure 41– PCL-TCP 80-20 samples stained with Alizarin Red S for calcium deposition quantification. Scale bar = 700 µm; valid for all images.
Optometric quantification of the dissolved Alizarin Red S dye in 10% acetic acid allowed for an indirect assessment of the amount of calcium deposited by cells after 14 and 28 days in culture. The higher the light absorbance within each sampled acid aliquot, the higher the amount of dye present in it, which may be correlated back to larger calcium content within the scaffold-cell unit exposed to the acid. Figure 42 presents a summary of the results obtained including means and one-standard deviation intervals around each mean. Note the overall increase of light absorbance of each cell-seeded group against its corresponding control group, suggesting higher calcium content. The PCL-TCP 80-20 scaffold group expressed the highest amount of dissolved dye for the cell-seeded group. Over time, the highest increase occurred for the cell-seeded PCL-TCP 90-10 group, suggesting a delayed calcium deposition effect for cells residing whiting the scaffold with less TCP. A larger amount of calcium deposition was detected at an earlier stage and in higher strength for the PCL-TCP 80-20 group. The increase in absorbance numbers for the control PCL 100 scaffolds is not immediately obvious but could very well be an effect of the extended exposure of the PCL matrix to the complex chemistry of media constituents. Although not exposed to a calcified matrix, cells in the PCL 100 group seemed to stain in higher strength over time. This effect could be due to a known staining of Alizarin Red S to elements other than calcium\textsuperscript{149} (although in less significant strength) and spontaneous mineralization of certain stem cell lineages over time, particularly when cultured with 5\% CO\textsubscript{2}.\textsuperscript{139,150}
Figure 42 – Percent light absorbance results for each scaffold group, experimental group, and time point. Color bars represent each mean per group, black bars represent one standard deviation unit from the mean.

Statistical analyses required the raw dataset to be transformed via Box-Cox due to high skewness of the overall distribution of results. The transformation parameter $\lambda = -1.136$ was used and a 3-way full factorial ANOVA was then fit to the transformed data. Despite the asymmetry and lack of resemblance to the typical bell shape of the normal distribution of the transformed data set (as seen in Figure 43), the 3-way full factorial ANOVA model produced normally distributed residuals ($p = 0.1080$) with constant variance, therefore deeming the model as appropriate for statistical inferences. Table 28 summarizes the test results for statistically significant effects.
Figure 43 – Histogram and normal quantile plot of the response dataset used in the statistical analysis of the 405 nm light absorbance experiment.

Table 28 – Effect tests of all factors and their higher-order interactions for the 405 nm light absorbance dataset.

<table>
<thead>
<tr>
<th>Factor / Factor Interaction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold Design Group</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Experimental Group</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Time Point</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Scaffold Design Group* Experimental Group</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Scaffold Design Group*Time Point</td>
<td>0.4984</td>
</tr>
<tr>
<td>Experimental Group*Time Point</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Scaffold Design Group* Experimental Group*Time Point</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

The results presented above suggest that statistically significant differences were present between the amounts of calcium deposited by cells cultured within each scaffold group over time, with corresponding significant differences against each design’s negative controls. Due to the complexity of this model, Tukey HSD post-hoc analyses are presented
solely for the highest level interaction factor (Table 29). Results show that most cell-seeded scaffolds expressed higher amounts of calcium at both time points compared to negative controls. For a same hASC seeded scaffold group, PCL-TCP 80-20 was the only that did not express significant differences over time, but yet had the highest absorbance results overall, suggesting an early activity of calcium deposition by cells between seeding day and the first time point (Day 14). The only negative control group to surpass a cell-seeded group was PCL-TCP 80-20 at Day 28 versus PCL 100 at 14 days. It is hypothesized that such effect is a consequence of the ‘background staining’ of the TCP matrix within the acellular PCL-TCP scaffolds. Other results go along expectations, with generally higher amounts of calcium detected in scaffolds featuring higher TCP concentration, stem cells, and cultured for longer periods of time.

Table 29 – Tukey HSD post-hoc group comparisons for the highest degree interaction effect between scaffold group, experimental group, and time point for the 405 nm light absorbance experiment.

<table>
<thead>
<tr>
<th>Scaffold Group</th>
<th>Experimental Group</th>
<th>Time Point</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-TCP 80-20</td>
<td>hASC Seeded</td>
<td>28 days</td>
<td>A</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>hASC Seeded</td>
<td>14 days</td>
<td>A  B</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>hASC Seeded</td>
<td>28 days</td>
<td>B</td>
</tr>
<tr>
<td>PCL 100</td>
<td>hASC Seeded</td>
<td>28 days</td>
<td>C</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>hASC Seeded</td>
<td>14 days</td>
<td>C</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Control</td>
<td>28 days</td>
<td>D</td>
</tr>
<tr>
<td>PCL 100</td>
<td>hASC Seeded</td>
<td>14 days</td>
<td>D</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Control</td>
<td>28 days</td>
<td>E</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Control</td>
<td>28 days</td>
<td>E</td>
</tr>
<tr>
<td>PCL-TCP 80-20</td>
<td>Control</td>
<td>14 days</td>
<td>E  F</td>
</tr>
<tr>
<td>PCL-TCP 90-10</td>
<td>Control</td>
<td>14 days</td>
<td>F</td>
</tr>
<tr>
<td>PCL 100</td>
<td>Control</td>
<td>14 days</td>
<td>G</td>
</tr>
</tbody>
</table>
Finally, for illustration purposes of the site-specific behavior of stem cells cultured within a single heterogeneous construct featuring distinct mineralization levels across its thickness, Figure 44 displays representative images of multi-phasic scaffolds cultured for 14 and 28 days in same conditions as the single-phase scaffolds discussed above. Despite the strong background staining of the mineralized scaffold matrices, cell seeded samples expressed a much stronger staining of the overall structures. Direct image analysis does not allow for a meaningful conclusion of whether the three zones of the osteochondral scaffold induced distinct levels of calcium deposition when cultured in the same wells. It does, however, provide observational evidence that calcium deposition has occurred across all zones. Better assessment protocols must be developed as means to enable direct assessment of the site-specific behavior of seeded stem cells.
Figure 44 – Multiphasic samples stained with Alizarin Red S for visualization of calcium deposition. Scale bar = 2 mm; valid for all images.

Going forward, the *in vitro* characteristics of the proposed PCL and PCL-TCP scaffold zones could be potentially improved via surface treatment following scaffold fabrication. Surface treatment has been demonstrated to be a viable way of achieving higher seeding efficiency of cells onto and into porous scaffolds and providing additional growth and/or differentiation factors to cells residing within the 3D structure. Treatment approaches include surface modification by plasma deposition,\textsuperscript{151–153} collagen coating,\textsuperscript{65,154,155} alkaline treatment,\textsuperscript{156} precalcification,\textsuperscript{157} and others. Particularly in plasma-based techniques, the modified surface chemical composition and morphology leads to improved hydrophilicity of
the scaffold surfaces. That allows a more homogeneous and fluid distribution of the cell-media concoction during the seeding process throughout the scaffold microarchitecture and, as a consequence, a denser population of viable cells attached to strands at day culture day 0. For instance, Domingos et al. (2013) reported a significant increase in the surface area of surface-treated PCL scaffolds covered by cells when compared to non-treated controls.\textsuperscript{151} Similarly, Jeon et al. (2014) verified corresponding effects, in which plasma-treated PCL scaffolds demonstrated significantly higher cell densities and spread of cells the day after seeding when compared to non-treaded samples.\textsuperscript{158} Therefore, future research in scaffold-based osteochondral TE must consider the potential contributions of surface treatment methodologies to improve the \textit{in vitro} behavior of cells seeded and cultured within heterogeneous proposed multi-material scaffold designs.

4.3 Assessment of the Effects of Bulk Design Parameters on the Implantation Performance of 3DB Osteochondral Scaffolds

In this section, the effects of macro-design characteristics of 3DB scaffolds were characterized as means to pursue better implantation performance in simulated surgical trials. With procedures led by a research partner and sports medicine surgeon from the Department of Orthopaedics at the University of North Carolina at Chapel Hill, Dr. Jeffrey T. Spang, several scaffold diameters were tested and evaluated for their ease of positioning and implantation, and rate of implantation success. Secondarily, additional design attributes such as non-uniform diameters and chamfers were tested to further improve procedural success rates. At last, for the best-performing scaffold design, mechanical characteristics pertaining
to the implantation procedure (implantation and primary stability forces) were measured and compared against those of osteochondral native tissue grafts presented in Section 2.3.3.

4.3.1 Materials and Methods

Hind legs from 3- to 6-month-old porcine specimens were sourced from a local butcher (Nahunta Pork Center, Pikeville, NC) and kept under refrigeration for no longer than 24 hours or frozen for nor longer than 48 hours following slaughter until the time of processing. If frozen, samples were allowed to thaw at room temperature overnight. In all samples, the knee joints were dissected to expose the femoral condyle and facets under the knee patella. Following standard steps in the COR procedure for preparing recipient sites, five defects were produced per leg using the drill guide and the drill bit from the 8-mm COR kit attached to a cordless power drill. The defect sites were spaced apart 2 mm or more and distributed across the lateral and medial facets in the patellar surface of the femur, avoiding the central groove.

Multiphasic osteochondral scaffolds were manufactured as previously described. In brief, the mineralized component (PCL-TCP) was 3D-bioplotted first, then immediately overlaid with a pre-cut electrospun sheet of nanofibrous PCL, over which finally the non-mineralized component (pure PCL) was finally 3D-bioplotted, resulting in the hybrid structure central to this study. An iterative trial-and-error approach was followed to define which combination of overall scaffold diameter and design attributes allowed an orthopedic surgeon to implant 3DB osteochondral scaffolds easily and successfully. A starting diameter of 8.2 mm was chosen for initial assessment based on the results presented in Section 2.3.3,
where the diameter of native osteochondral plugs harvested with the standard 8 mm COR kit was experimentally defined. Improvements were made based on two main criteria:

- **Implantability**: 5-point Likert-type scale to classify aspects such as overall ease of implantation, scaffold positioning and insertion into defect, and the occurrence of any undesired events during each implantation attempt.

- **Success rate**: 2-point binary scale (success / fail) to classify whether the scaffold was implanted exactly as intended, and it did not collapse, delaminate, or was implanted at an incorrect angle or position.

An iterative process took place and continued in search of a scaffold design that maximized both criteria. All proposed designs were tested multiple times (n = 5) and their corresponding characteristics are presented in the upcoming Results and Discussion section (4.3.2). The best performing design was finally tested for implantation and primary stability force characteristics during and post scaffold implantation. In brief, similar to the protocol outlined in **Section 2.2.3**, a universal testing machine (ATS 1620) was set up with a 1 kN load cell and used to apply compressive forces onto the surface of 3DB multiphasic scaffolds and insert them into randomly assigned recipient sites prepared with the 8-mm COR kit. For **implantation forces**, scaffolds were pushed into their respective implantation sites at a constant displacement rate of 1.5 mm/min (cross-head speed). Forces perpendicular to the top surface of the scaffolds were recorded. The test was interrupted and forces were released when the top surface of the scaffold was leveled with the articular cartilage surface around the implantation site. The implantation force for each sample is defined as the peak force (in N) observed in each force curve (n = 12 curves). For **primary stability forces**, the displacement rate was resumed so that the indenter was reengaged with the scaffold surface.
Forces were recorded as the scaffold was pushed deeper into the defect until it eventually reached the bottom of the recipient site. At this point, the forces were released and the cross-head returned to its initial position. The primary stability force is defined as the critical friction force required to shift the graft deeper into the defect after it has been implanted and rest in a stable condition. Direct comparison via t-tests of the 95% confidence intervals for both measures (α = 0.05) observed for native tissue grafts and multiphasic scaffolds were compared using JMP Pro 12.

4.3.2 Results and Discussion

Figure 45 illustrates (A) the implantation procedure and brings representative examples of (B) scaffold implantation success and (C) failure. The iterative process to determine a suitable scaffold design for best implantability is described in Table 30.

Figure 45 – Representative photographs representing the scaffold implantation procedure (A), scaffolds that were successfully implanted by the surgeon (B), and a scaffold that failed implantation (C) and collapsed during the process.
Table 30 – Description of iterative process followed to determine a scaffold design that allows for easier implantability and higher implantation success rate by an orthopedic surgeon

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Attribute of Interest</th>
<th>Attribute Value</th>
<th>Implantability Score</th>
<th>Success Rate</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diameter</td>
<td>8.2 mm</td>
<td>1.5 (poor)</td>
<td>50% (poor)</td>
<td>decrease diameter to 7.9 mm.</td>
</tr>
<tr>
<td>2</td>
<td>Diameter</td>
<td>7.9 mm</td>
<td>3.5 (average)</td>
<td>80% (good)</td>
<td>decrease diameter to 7.6 mm.</td>
</tr>
<tr>
<td>3</td>
<td>Diameter</td>
<td>7.6 mm</td>
<td>2.5 (poor)</td>
<td>100% (excellent)</td>
<td>retest (validate) diameter of 7.9 mm.</td>
</tr>
<tr>
<td>4</td>
<td>Diameter</td>
<td>7.9 mm</td>
<td>3.7 (average)</td>
<td>80% (good)</td>
<td>increase diameter to 8.0 mm.</td>
</tr>
<tr>
<td>5</td>
<td>Diameter</td>
<td>8.0 mm</td>
<td>2.8 (poor)</td>
<td>60% (poor)</td>
<td>test uneven diameters of 7.9 mm (PCL) and 7.8 mm (PCL-TCP).</td>
</tr>
<tr>
<td>6</td>
<td>Diameter</td>
<td>7.9 / 7.8 mm</td>
<td>3.9 (average)</td>
<td>80% (good)</td>
<td>test uniform diameter of 7.9 mm with chamfer for seating.</td>
</tr>
<tr>
<td>7</td>
<td>Chamfer</td>
<td>1 mm / 30°</td>
<td>4.5 (good)</td>
<td>100% (excellent)</td>
<td>retest (validate) diameter of 7.9 mm with 1 mm / 30° chamfer.</td>
</tr>
<tr>
<td>8</td>
<td>Chamfer</td>
<td>1 mm / 30°</td>
<td>4.5 (good)</td>
<td>100% (excellent)</td>
<td>Stop. Design is final.</td>
</tr>
</tbody>
</table>

The best-performing scaffold design featured a mean diameter of 7.9 mm and a 30-degree, 1-mm high chamfer at the bottom of its mineralized content which allowed for easier seating onto the recipient site at the time of implantation. Such design feature was necessary because the original 8-mm COR toolkit does not provide the necessary tools to mount scaffolds onto the implantation guide as it does for native tissue grafts. Therefore, the
implantation process of scaffolds relied on manual alignment of each implant with a pair of surgical forceps prior to implantation, a step which was significantly facilitated following the addition of the chamfer.

Sample force vs. displacement curves for implantation and primary stability forces for the optimized scaffold design are presented in Figure 46, with corresponding overall results in Table 31. A matching sample size of $n = 12$ was adopted to maintain consistency with the native tissue graft characterization results presented in Section 2.3.3. Note that the force curves have similarly distinct zones as observed for tissue grafts. For implantation, a first zone represents initial elastic compression of the scaffold without shift into the defect, which only begins to happen after a specific force is overcome, entering the second zone with continuously increasing forces until implantation is completed. For primary stability, the same characteristic initial compression followed by a long plateau is observed, followed by implant bottoming and exponentially increasing forces until the test is interrupted. It is worth noticing how the curves presented here, in Figure 46, compare to the curves presented in Figure 17. The considerably irregular lateral surfaces of the scaffolds, an effect of the pattern chosen for strand deposition by the 3D-Bioplotter, causes curves equally irregular, characterized by sudden, localized changes in slope as strand edges rub against the porous cartilage and bone interfaces of the recipient sites. Despite these artifacts in the force curves, the overall trends can be easily identified and associated to the case of native osteochondral tissue grafts.
Figure 46 – Representative plots of force vs. displacement from implantation (A) and primary stability forces (B) of 3DB multiphasic scaffolds with an optimized diameter of 7.9 mm.

Table 31 – Implantation and primary stability force results for 3DB multiphasic scaffolds with an optimized diameter of 7.9 mm.

<table>
<thead>
<tr>
<th></th>
<th>Implantation Force (N)</th>
<th>Primary Stability Force (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MEAN</strong></td>
<td>33.9</td>
<td>23.4</td>
</tr>
<tr>
<td><strong>ST.DEV.</strong></td>
<td>12.2</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>CV</strong></td>
<td>0.36</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Statistical comparisons via one-way analysis (t-test) in JMP revealed that, for both implantation and primary stability forces, the mechanical behavior observed for scaffolds is significantly different ($p < 0.0001$) than that observed for native osteochondral tissue grafts as determined in Chapter 2. Figure 47 illustrates all four distributions. For implantation forces, the overall mean of native tissue grafts is 2.4 times higher than that of scaffolds. For primary stability forces, the factor multiplication factor is approximately 2.9. This difference can be directly attributed to the smaller average diameter of scaffolds (nominal of 7.9 mm), defined based on criteria for implantability. When compared against the mean diameter of osteochondral grafts harvested with the 8-mm COR kit, scaffolds have a diameter 0.3 mm
smaller. Such decrease in size reduces the interference between the interfacing surfaces of the scaffolds and their implantation sites. Less interference causes less friction, which directly justifies the overall lower forces observed.

Figure 47 – Comparisons between native osteochondral tissue grafts and 3DB scaffold implantation and primary stability forces

A drawback of the results presented in this section is defined by the significantly different implantation characteristics between the proposed scaffold design and native tissue grafts. In other words, a scaffold design that has been optimized for better implantability based on surgeon’s feedback did not match critical functional requirements of osteochondral implants in plug transfer approaches. Despite the lower implantation forces not representing a problem per se (they are, in fact, preferred), significantly lower primary stability forces represent an issue because, post implantation, the scaffolds may be more prone to dislocation and reside in suboptimal depths within the osteochondral defects. Future work must account for this pitfall and approach the scaffold design issue from different perspectives. A few potential options to address the issue can be considered:
If the scaffold diameter is increased so that primary stability forces become higher, a scaffold-specific implantation toolkit must be designed. Current plug transfer toolkits are optimized for native tissue handling and lack a fail-proof way to provide guided implantation of 3DB scaffolds. More on this topic is discussed in Section 5.3.

If the scaffold diameter remains the same so that implantability characteristics remain unchanged, the surgeon must prepare recipient sites with a depth exactly the same to the thickness of scaffolds. As mentioned in Section 2.1, bottomed implants will not sink post-implantation, but achieving a very specific desired depth is a challenging practice during surgery.

In future, the effects of scaffold diameter and design features should be investigated not only from implantability and mechanical stability perspectives, but also for healing potential post-implantation. It is reasonable to anticipate unwanted damage at the interface of the scaffold and native osteochondral complex within the recipient sites if localized friction forces resulting from the scaffold rubbing against the recipient sites are too high. Any damage to the tissue, particularly the superficial articular cartilage, should be avoided as means of preventing the onset of degenerative processes, which are notably difficult to revert. Hence, a design of experiments, conceivably in an in vivo model, to investigate the effects of varying strand width and scaffold diameters on the healing characteristics of target tissues in the medium to long terms is suggested.

4.4 Risk Assessment and Management

As a last contribution presented in this dissertation, root-cause analysis techniques such as cause-and-effect diagrams, failure modes and effects analysis, and fault tree diagrams
are presented as means of assessing opportunities for further improvement of the 3DB osteochondral scaffold design here discussed and potentially other similar medical products from implantability, biomimicry, and implant performance perspectives. Evidence collected from several sources, including relevant literature (discussed in Chapter 1, and Sections 2.1, 3.1, and 4.1), practice sessions with specialists, and the author’s own experiences during this dissertation have served as inputs for those quality tools. This section introduces fundamental concepts of risk-mitigation tools into the design and fabrication processes of 3D tissue engineering products which, to date, do not have a strong presence in academic literature.

The inherently complex nature of the multi-material, multiscale scaffold design discussed in this dissertation is prone to failures resulting from a multitude of potential causes. Figure 48 presents a high-level map of the engineering process including all the focus areas investigated in this report. The cause-and-effect diagram presented in Figure 49 captures these potential causes and links several aspects that directly or indirectly affect the performance of a 3DB scaffold for osteochondral tissue regeneration. Colored dots correlate cause groups with key process steps in Figure 48 that were identified to introduce significant variability in the performance outcomes of a 3DB osteochondral scaffold. The 5 M’s of lean manufacturing\textsuperscript{161} served as a base for defining the main cause groups listed below:

- **Materials**: labeled as “Scaffold Materials”
- **Machines**: labeled as “3D-Bioplotting Equipment”
- **Manpower**: labeled as “Human Factors”
- **Methods**: split into two groups, labeled as:
  - “Scaffold Engineering and Fabrication”
  - “Surgical Implantation Process”
• **Measurements**: labeled as “Inspection and Quality Control”

The development of a cause-and-effect diagram allowed for a better conceptualization and understanding of potential sources of failure modes in the system, including aspects related to both product and process. It encompasses several steps of the 3DB osteochondral scaffold lifecycle, from design conceptualization to scaffold manufacturing and, ultimately, implantation as a functional medical product.

![Figure 48 – High-level process map of scaffold engineering from design conceptualization to fabrication and implantation](image)

- **Step I**: Define Scaffold Design
  - I.a Characterization of clinical ‘gold standard’
  - I.b Definition of biomimicry design criteria
  - I.c Definition of implantability design criteria
  - I.d Testing and definition of optimized design

- **Step II**: Fabricate Scaffold
  - II.a Characterization of relevant material-process-structure interrelationships
  - II.b Development of fabrication process plan
  - II.c Preparation of materials for fabrication
  - II.d Setup of machine and supporting equipment
  - II.e 3D-printing (fabrication) of scaffold
  - II.f Inspection of scaffold for defects
  - II.g Sterilization of scaffold

- **Step III**: Embed Scaffold with Cells
  - III.a Cell harvesting and isolation
  - III.b Cell expansion
  - III.c Scaffold seeding
  - III.d *In vitro* culture of seeded scaffold

- **Step IV**: Implant Scaffold
  - IV.a Preparation of patient
  - IV.b Preparation of recipient site
  - IV.c Implantation of scaffold
  - IV.d Finalization of surgery
Figure 49 – Cause-and-effect diagram displaying potential causes with direct effects on 3DB scaffold performance
Further extending the discussion of potential causes for poor 3DB scaffold performance, Figure 50 presents a fault tree analysis\textsuperscript{162} diagram tracking back potential issues that can affect desired outcomes. Beginning with the effect (or event) “Degenerated tissue following scaffold implantation”, potential failures are linked via logical connectors to their immediate causes, continuing down to the desired level of detail or until no further causes can be identified. In the first diagram, events highlighted in teal are considered significant issues in the performance of 3DB scaffolds and were extended in further detail via additional fault tree analysis diagrams presented in Figure 51 to Figure 56, respectively for root-cause analyses of:

- **Scaffold delaminated prior to implantation** (Figure 51); referring to the catastrophic failure of the multi-phasic scaffold before surgical implantation that, if undetected, could lead into future problems and suboptimal implant performance.
- **Scaffold delaminated during implantation** (Figure 52); referring to the same structural failure above, however induced during the medical procedure performed to implant the scaffold and during any preceding fabrication step.
- **Scaffold too loose during implantation** (Figure 53); referring to the suboptimal loose fit between the 3DB scaffold and its respective implantation site causing it to lack the required friction to be implanted successfully and remain securely in place.
- **Scaffold too tight during implantation** (Figure 54): referring to the suboptimal tight fit between the 3DB scaffold and its respective implantation site causing it to potentially structurally collapse or damage the tissue surrounding the implantation site.
• Scaffold too loose post-implantation (**Figure 55**); referring to same suboptimal fit but only after an admittedly successful implantation surgery has been completed.

• Microbiological contamination (**Figure 56**); referring to the undesirable introduction of microbiological contaminants pre-scaffold implantation.

Lastly, as means of providing a more holistic overview of potential failure modes associated with the development and use of a 3DB osteochondral scaffold, **Appendix A** presents a detailed list of potential failure modes and their respective effects following careful review of the design and fabrication processes discussed in this report. Information was taken from potential causes raised by the cause-and-effect previously discussed (**Figure 49**) and its relationships with the process map presented in **Figure 48**. It is important to note that most of the listed items are not restricted to the materials, methods, and goals discussed in this report as they highlight potential risks and failure modes with presence across many subject areas within the field of TERM.

Future work should tackle the prioritization of recurring high-impact issues as means of advancing process scalability and the clinical translation of early-stage TERM products. Performing risk assessment and failure analyses via the tools here illustrated, as well as others, is typically an unspoken requirement in many manufacturing industries – medical products included. From a translational perspective, having a solid understanding of the issue itself is hardly enough. As means of improving product safety, effectiveness, and establishing full control over the manufacturing process, the investigation of root causes and prioritization of relevant action plans becomes an imperative practice. In the context of this dissertation, this section contributed with a preliminary assessment of these issues in the design, fabrication, and function of 3DB osteochondral scaffolds.
Figure 50 – Fault tree analysis diagram for ‘Poor tissue regeneration following scaffold implantation’
Figure 51 – Fault tree analysis diagram for ‘Scaffold delaminated prior to implantation’
Figure 52 – Fault tree analysis diagram for ‘Scaffold delaminated during implantation’
Figure 53 – Fault tree analysis diagram for ‘Scaffold too loose during implantation’
Figure 54 – Fault tree analysis diagram for ‘Scaffold too tight during implantation’
Figure 55 – Fault tree analysis diagram for ‘Scaffold too loose post-implantation’
Figure 56 – Fault tree analysis diagram for ‘Microbiological contamination’
4.5 Chapter Summary

This chapter first investigated the *in vitro* response of hASCs seeded and cultured within scaffolds designed to resemble the composition, architecture, and mechanical characteristics of native osteochondral tissue and its distinct zones. Following the definition of required scaffold microarchitectural design parameters such as strand inter-axial separation and strand thickness, batches of PCL 100, PCL-TCP 90-10, PCL-TCP 80-20 and multiphasic scaffolds were 3D-bioplotted, sterilized, seeded, and cultured for up to 28 days. The LIVE/DEAD qualitative cell assay was performed for visual assessment of overall cell population viability at days 14 and 28. Results have shown that cell clusters began to form and bridge the gap between adjacent scaffold strands, forming a dense matrix partially filling the interstrand regions of scaffolds. Overall cell viability did not change across scaffold designs or time points, where roughly the same amount of live and dead cells could be identified, confirming sustained cell viability throughout the entire culture interval. The alamarBlue quantitative cell assay was performed to assess the metabolic activity of cell populations periodically during the entire culture interval. Results have shown a fast initial increase in the metabolic activity of cells within all scaffold designs, which peaked around day 10 to 13 and followed a steady plateau until eventually demonstrating a slight drop at day 28. Statistically, PCL 100 differs from both PCL-TCP mineralized scaffold designs, which did not differ from each other. Also, PCL-TCP 90-10 expressed statistically significant differences between consecutive days in culture only at the start and end of culture, while PCL-TCP 80-20 only at the end, and PCL 100 not expressing any differences at all. These results suggest that scaffolds are able to maintain metabolically active hASC populations for several weeks following the protocols here described, and present preliminary evidence that
cultures may be hindered by extended culture intervals longer than 28 days, possibly as an
effect of over-confluent cell clusters, media exhaustion for populations of large size, and
hindered nutrient and waste flow as scaffold pores are filled with matrix. Lastly, the Alizarin
Red S assay allowed for quantification of the calcium content present within the surface of
the scaffold-cell units after 14 and 28 days. Calcium dissolution followed by colorimetric
light absorbance quantification indicated that cell-seeded scaffolds with higher TCP content
had significantly higher amounts of calcium aggregated to their surface, which was also
higher at 28 days compared to 14 days. These results suggest that the culture of hASCs
within a mineralized scaffold matrix containing TCP is affected by the signals provided by
that calcified matrix. The detected mineralization of cell populations over time suggested that
a differentiation pathway into bone progenitor cells may have been followed by those
cultures in TCP scaffolds. This is particularly important in the engineering of osteochondral
tissue as the presence of different cell phenotypes and ECM characteristics throughout the
thickness of a single-but-heterogeneous scaffold is a functional requirement from a
biomimetic perspective. A last qualitative assessment of multiphasic scaffolds stained with
ARS suggested that calcium deposition has occurred across all zones of interest.

Following favorable evidence of sustained cell viability in vitro, the 3DB
osteochondral scaffolds were submitted to an additional set of design optimizations based on
select implantability criteria. Here, following simulated surgeries by means of an ex vivo
porcine model, distinct scaffold diameters and design characteristics were tested to maximize
implantation performance. Measures such as ease of implantation and implantation success
rate were used to grade iterations of distinct scaffold designs, ultimately defining that a
scaffold with a mean diameter of 7.9 mm and a 1-mm 30° chamfer at the bottom of the
mineralized zone allowed for near-fail-proof implantation. The proposed scaffold design was tested for its implantation and primary stability forces following protocols established in Chapter 2 for native tissue grafts harvested with the 8-mm COR kit. It was demonstrated that grafts can be implanted with forces 2.4 times higher than that of scaffolds, and shift post-implantation with primary stability forces 2.9 larger. Both datasets were statistically significantly different from each other. The overall lower forces for scaffolds could be anticipated as an effect of the smaller mean diameter of the cylindrical structure. Although not an immediate red flag with respect to implantation forces, action should be taken to increase the primary stability forces necessary to displace the scaffold once implanted. The lack of true interference causes friction that can be easily overcome post-implantation, enabling easier displacement and scaffolds eventually residing in suboptimal depths within the osteochondral defects.

Lastly, root-cause analysis techniques such as cause-and-effect diagrams, failure mode analysis, and fault tree diagrams were presented as means of identifying opportunities for further improvement of the 3DB osteochondral scaffold design discussed in this report. This initiative enables already identified issues in the engineering process of 3DB osteochondral scaffolds to be prioritized in future development iterations and product versions.
5.1 Research Summary

When injury is caused to the osteochondral tissue due to trauma or disease, it possesses limited regenerative capacity due to its complex structure and healing mechanisms. Clinically accepted treatment techniques present results that are inconsistent and suboptimal in most cases. Of them, the COR technique has shown to be a viable alternative in which a healthy, full-thickness chondral-osseous replacement plug is harvested and transferred into a diseased or injured area. However, autografting tissue is associated with donor site morbidity, limited availability, and possible damages to the tissue graft during handling.

The promise of scaffold-based TERM has been widely explored for several applications, including the regeneration of the osteochondral tissue. From a translational perspective, the fabrication of osteochondral scaffolds in a reproducible, scalable, and cost-effective manner remains unachieved as it has been challenged by the nature of the manufacturing processes involved and the inherently complex tissue function. AM processes such as 3DB have recently grown in interest and their potential for scaffold fabrication follows under investigation. Relevant interrelationships between material, process, structure, and function in the design, fabrication, and utilization of osteochondral scaffolds have not yet been characterized in a comprehensive manner. Motivated by this opportunity, this dissertation researches a multiphasic, multi-material 3DB scaffold as a potential alternative to the autologous, full-thickness plug utilized in the traditional COR procedure for osteochondral tissue regeneration. A holistic approach was taken to characterize relevant aspects ranging from the design, fabrication and utilization as a functional medical product.
In Chapter 1, a comprehensive literature review defined the role of manufacturing engineers in the field of TERM and identified opportunities for tackling the limitations of currently established clinical techniques for treating damages to the osteochondral tissue as an effect of injury or disease. A prominent additive manufacturing processes, 3D-bioplotting, was identified as a capable manufacturing choice in the fabrication of TERM-based biodegradable scaffolds featuring relevant material compositions and design attributes that enable them to mimic select characteristics of native tissue. Research objectives and specific tasks were defined based on research needs related to the definition of functional design requirements, process modelling, and preliminary testing of a proposed heterogeneous scaffold design.

In Chapter 2, all tasks related to the definition of functional requirements defined by Objective 1 have been addressed. The compressive elastic moduli of the three zones of native tissue autografts harvested from the femoral condyle of porcine specimens (ST1.1) were characterized by uniaxial compression at a constant strain rates. Results were as follows: 39.3 ± 9.8 MPa (zone 1, articular cartilage), 37.1 ± 11.2 MPa (zone 2, transition between calcified cartilage and cortical bone), and 41.6 ± 18.5 MPa (zone 3, cancellous bone). Furthermore, the dimensions of the autologous grafts and implantation sites were characterized (ST1.2) and found to be 8.17 ± 0.04 mm, and 8.01 ± 0.07 mm, respectively, providing evidence of an interference fit that is critical for implant stability. Lastly, the forces necessary to implant osteochondral grafts into defects, as well as the forces to shift them once in their final implanted position (referred to as primary stability forces) were determined (ST1.3) to be 81.5 ± 16.4 N and 68.1 ± 14.7 N, respectively. The primary stability forces were significantly lower than the implantation forces, meaning that more pressure is
necessary to implant osteochondral grafts than to shift them post-implantation. These results serve as functional requirements to be considered in parts of Objectives 2 and 3 during the design process of an osteochondral scaffold designed for enhanced implantability and biomimicry.

In Chapter 3, all tasks proposed as part of Objective 2 were completed, with strong focus given to process characterization and overall assessment of relevant material-process-structure interrelationships in the 3DB of PCL-TCP osteochondral scaffolds. Screening experiments characterizing the extrudability potential of mineralized PCL composites were performed (ST2.1) and generated, as a result, a comprehensive mapping of 3DB process parameters including extrusion temperature, pressure, nozzle diameter, and print head speed. Single-layer PCL-TCP structures (80-20 or 90-10 ratios) were plotted and checked for defects. Feasible processing parameter levels in 3DB for the two compositions served as a design space for a more extensive empirical model development (ST2.2) relating the 3DB process parameters and structural and mechanical outcomes. Two models were proposed and later validated for their accuracy in predicting the width of PCL-TCP strands extruded via 3DB. The resulting PCL-TCP 80-20 model fit the experimentally obtained data with an R-square value of 0.8451. For PCL-TCP 90-10, the goodness-of-fit measure was 0.9223.

Similar modelling techniques were followed to characterize the effects of strand width, now a predictor variable, and strand inter-axial separation on the compressive elastic modulus of 3DB scaffolds (ST2.3). In this case, three distinct full factorial experimental designs were proposed for PCL-TCP 80-20, PCL-TCP 90-10, and PCL 100 scaffolds. Goodness-of-fit R-square values were 0.9286, 0.9579, and 0.9726, respectively. Validation experiments provided evidence that the proposed models were capable tools for characterizing relevant
material-process-structure interrelationships in the design and 3D-bioplotting of osteochondral scaffolds (ST2.4).

In Chapter 4, all tasks proposed as part of Objective 3 were completed, starting with the assessment of the biological in vitro response of hASCs seeded and cultured within scaffolds of osteochondral zone-specific composition and compressive mechanical properties (ST.3.1) for 28 days. Assayed designs included PCL 100, PCL-TCP 90-10, PCL-TCP 80-20, and a combined multiphasic design resembling the organization of the osteochondral complex. The LIVE/DEAD assay provided evidence that cell cultures remained mostly viable throughout the entire culture interval and formed dense clusters and matrices bridging the gap between adjacent strands in all tested scaffold designs. The alamarBlue assay suggested that cell cultures sustained steady metabolic activity levels throughout most of the culture period. Results indicate that scaffolds should not be cultured for more than 25 days prior to implantation under the culture protocols followed. Furthermore, the Alizarin Red S assay suggested that significant differences in the mineralization of cell cultures can be obtained as an effect of the concentration of TCP in the scaffold’s material. Multiphasic scaffolds stained with ARS suggested that calcium deposition has occurred across all zones of interest. Shifting the focus to the implantational characteristics of multiphasic scaffolds in simulated surgical procedures, the diameter of 3DB osteochondral scaffolds was iteratively optimized (ST3.2) based on 5-scale ratings of ease of implantation and binary ratings of implantation success from feedback provided by a surgeon familiar with plug transfer approaches. The best performing scaffold design featured a mean diameter of 7.9 mm and a 30-degree, 1-mm high chamfer at the bottom of its mineralized content which allowed for easier seating onto the recipient site at the time of implantation. A benchmark against native
tissue autografts prepared and implanted with the COR procedure indicated that implantation and primary stability forces of osteochondral scaffolds are significantly lower due to the smaller diameter necessary for successful surgical procedures. Finally, root-cause analysis tools (cause-and-effect diagram, identification of failure modes, fault tree diagrams) were presented (ST.3.3) as means to promote and prioritize future work on issues presently identified.

5.2 Primary Research Contributions

This dissertation provides a systematic characterization of implantation and function-related aspects of a widely accepted clinical treatment for lesions to the osteochondral tissue in large joints, the COR procedure. Furthermore, it sets the groundwork for a TE graft substitute designed to relevant biomimicry and implantability aspects, fabricated in a reproducible manner, and suitable for scaled up production. Main contributions are as follows:

- The characterization of select functional requirements from implantability and biomimicry perspectives provides a basic framework for the design and fabrication of TE scaffolds to replace autograft substitutes. The established methodologies and experimental protocols (e.g., for assessment of mechanical properties and micro-architecture and macro-geometry) can be extended to other similar applications, including osteochondral plug transfer techniques involving grafts of different diameters, depths, multiple graft implantation, and even other tissue types.

- The characterization of material-process-structure interrelationships of 3DB PCL-TCP scaffolds contributes to the literature by providing predictive equations for
variables of interest, such as strand width and compressive elastic modulus of scaffolds, based on a choice of main 3DB process parameters and scaffold design attributes. These models can be used in the development of enhanced engineering tools (CAD, CAE, CAM) for TE applications and closed-loop control systems that allow machines to adapt to process changes. The materials and processes involved can also be utilized for TE of other musculoskeletal tissues.

- The confirmation of sustained *in vitro* hASC viability, metabolic activity and osteogenic function when cultured within 3DB scaffolds featuring microarchitectures adjusted to mimic zone-specific compressive mechanical properties of the osteochondral tissue. These findings provide a proof-of-concept regarding the potential of controlling site-specific behavior of cells residing within the multiphasic, multi-material structure of additively manufactured scaffolds, which is critical given the transitional interface between bone and cartilage that makes the osteochondral tissue unique.

- The utilization of root-cause analysis tools enabled a preliminary assessment of potential risks associated with the design and fabrication processes, as well as the utilization of the proposed 3DB osteochondral scaffold in clinical-type settings. The information provided by these tools is critical in prioritizing actions against high-impact risks and failure modes, which facilitates the pursuit of process scalability and the clinical translation of early-stage TERM products.
5.3 Ongoing and Future Research

Assessing the *in vivo* performance: The characterization of relevant macro- and micro-architectural design attributes represents an early step towards the development of functionally well-performing scaffolds in OTE. Preliminary *in vitro* results have been reported in this dissertation but, from clinical and regulatory perspectives, these are not sufficient to establish a solid understanding of the scaffold’s functional characteristics. As it is the case for any biologically active medical product, applicable regulations require that, before any human clinical trials are administered, substantial evidence of safety and effectiveness of a developing product must be demonstrated via relevant and comparable *in vivo* animal models. In view of this requirement, the 3D Tissue Manufacturing Research Team at NC State (of which the author has been a member since 2014) partnered with members from the UNC-NCSU Joint Department of Biomedical Engineering, the UNC Department of Orthopaedics, and the University of Missouri to investigate the *in vivo* regenerative characteristics of multiphasic PCL-TCP 3DB osteochondral scaffolds implanted in 6-month old mini pigs over 4 months. The study aims to compare distinct implant types and designs, including native tissue autografts (following the COR procedure), acellular 3DB osteochondral scaffolds, cell-seeded 3DB osteochondral scaffolds, and 3DB scaffolds without an electrospun tidemark layer separating cartilage and bone zones. Open-wound negative controls are also included. **Figure 57** illustrates the procedure led by an orthopedic surgeon (Dr. Jeffrey T. Spang) and one assistant (the author) (A) and a close-up of a scaffold following successful implantation (B).
Implanted scaffolds, despite having a simplified composition (no PCL-TCP 90-10 component was included), took advantage of the design improvements proposed by Section 4.3 of this dissertation. Cell-seeded groups were cultured following seeding and culturing protocols similar to those presented in Section 4.2 (with the exception of media motion and cell seeding density). Upon completion of all intended characterization assays after 120 days of implantation-to-harvest interval, results will provide a direct assessment of distinct performance measures pertaining to the regeneration of the osteochondral tissue in vivo. Periodical CT scans (Figure 58A) and biomodeling methods (Figure 58B) will allow for a quantification of bone volumetric ingrowth and estimation of the mechanical properties of nascent bone tissue as an effect of its observed mineralized content. Histology of explanted regenerated tissue (Figure 58C) will allow for an assessment of tissue characteristics, primarily its architecture (i.e. distinct cartilage vs. bone zones), as well as cellular and ECM composition. This author has contributed to the scaffold fabrication, surgical implantation,
and biomodeling development protocols, but the results and analyses of the *in vivo* study are outside the scope of this dissertation.

![Figure 58 – Data sources used in the assessment of the regenerative performance of implants.](image)

Figure 58 – Data sources used in the assessment of the regenerative performance of implants. (A) Sample of a CT scan at the earliest time point showing two defects produced in the knee joint. (B) A 3D STL model generated from CT scans. (C) Neotissue formed after 120 days within implanted scaffolds.

**Developing better implantation tools:** Currently, there are no customized tool kits that offer appropriate handling of scaffolds during the implantation procedure. The trials described in this report were limited to a minimally assisted and purely manual process of retrieving scaffolds from their transport container and positioning over their recipient sites preceding implantation. The use of standard surgical tools for these purposes is suboptimal and contributes to many of the failure modes and causes identified in Section 4.4. The lack of a ‘delivery guide’, as present in the COR kit for donor tissue autografts, hinders the ability of the surgeon to maintain constant and appropriate scaffold alignment during implantation. Also, lateral shear forces can be easily introduced to the unconfined scaffold ultimately causing it to delaminate and catastrophically fail. Furthermore, the development of a
customized set of scaffold implantation tools could potentially enable the successful implantation of scaffolds featuring larger diameters, which will likely address the issue of undesirably low primary stability forces identified in Section 4.3 with the current scaffold design.

**Designing more resilient scaffolds:** The presence of a nanofibrous electrospun layer of PCL between 3DB scaffold zones has shown to be a major issue concerning the sustained physical integrity of multiphasic scaffolds during fabrication, handling, and implantation. The electrospun matrix has virtually no tensile resistance to being deformed in a direction perpendicular to that in which its fibers were spun. Therefore, 3DB scaffold zones attached to the opposing sites of an electrospun sheet will be held together by minimal forces, even if their surface has been thermally bonded with the matching surface of the electrospun sheet. That severely poor resilience characteristic is a primary issue to be considered in future development iterations of this category of osteochondral implants. From a clinical translation perspective, such a fragile implant is likely not going to be brought forward into a scaled-up manufacturing scenario. A better alternative should be determined for the current electrospun sheet embodiment of the tidemark.

**Researching and developing better machines and machine control systems:** As means of introducing further autonomy to in-process quality control, which is currently dependent on continuous observation, judgement, and actions taken by a human operator, extrusion-deposition-based machines such as the 3D-Bioplotter must be able to detect process changes via a variety of sensing channels and autonomously adapt processing parameters when perceived necessary. Response surface models such as those proposed in Section 3.3 represent a resourceful relationship between select process parameters and specific outcomes.
of interest (i.e. scaffold width). Machine control algorithms can be developed to ‘understand’ the information supplied by those models and determine, if necessary, exactly how the process needs to change as means of reducing variability in scaffold width (or any other modeled outcome) and overall scaffold quality.

**Computational modelling of scaffold mechanical response to loads:** The empirical development of models relating scaffold design characteristics with its compressive mechanical properties (as performed in Section 3.4) could be complemented by using finite element analysis (FEA) models with specialized software such as ANSYS, Autodesk Nastran In-CAD, COSMOS/M, COMSOL, and many others. FEA models validated using experimental data can enable a more rapid assessment of the mechanical response for a given virtual model with select attributed parameters (meshes, material properties, loads, constraints), and can be easily adapted to simulate and assess the response of different materials and structures submitted to a variety of loading conditions.

**Extensive in vitro assessment:** A limitation encountered in Section 4.2 of this report is attributed to the inability of the Alizarin Red S assay to readily detect site-specific differentiation of hASCs seeded and cultured within the multiphasic osteochondral scaffold design. The strong background staining of strands in the mineralized scaffold zones hindered visual assessment of the staining strength of cells residing within the distinct PCL-TCP zones. Future tests must consider a dye that is not likely to stain the TCP content of scaffolds, or histochemical histology must be dropped altogether replaced by another method of assessing the mineralized content of cell populations. Furthermore, improvements to the cell culture protocols themselves are necessary as means to achieve:

- Better scaffold seeding efficiency;
• Uniform proliferation of cell populations covering the entire surface of strands;
• Higher cell viability levels maintained for longer culture intervals (i.e. > 28 days);
• Assessment of osteochondral-zone-relevant tissue characteristics (cell phenotypes, gene expression, collagenous spatial organization, etc.);
• Non-destructive quality assessment of relevant implant characteristics post-culture.
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APPENDIX
## Appendix A – List of Failure Modes and Effects

<table>
<thead>
<tr>
<th>STEP</th>
<th>POTENTIAL FAILURE MODE</th>
<th>POTENTIAL EFFECTS OF FAILURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.a</td>
<td>Methodology used to characterize relevant attributes of clinical gold standards are inadequate or irrelevant.</td>
<td>- Misconception and/or misinterpretation of design goals for scaffold performance (biomimicry and implantability).</td>
</tr>
</tbody>
</table>
| I.a  | Instruments and/or equipment used to characterize relevant attributes of clinical gold standards are inaccurate and/or imprecise. | - Incorrect definition of relevant quantitative characteristics of scaffolds.  
- Scaffold design and corresponding fabrication process plan become irrelevant and will need to be reworked.                                                                                                                                                        |
| I.b  | Incorrect choice of relevant scaffold design attributes for biomimicry and implantability. | - Proposed scaffold design will not perform according to critical design goals.  
- Scaffold will hinder cellular or tissular development, healing, and regeneration.  
- Scaffold cannot be implanted successfully.                                                                                                                                                                         |
| I.c  | Methodology used to test biomimetic and implantational performance of proposed designs are inadequate or irrelevant. | - Irrelevant assessment of scaffold performance.  
- Unknown measures pertinent to relevant performance attributes.  
- Hindered biomimetic and implantational performance of proposed scaffold design.                                                                                                                                 |
| I.d  | Instruments and/or equipment used to test biomimetic and implantational performance of proposed designs are inaccurate and/or imprecise. | - Incorrect assessment of scaffold performance.  
- Scaffold will hinder cellular or tissular development, healing, and regeneration.  
- Scaffold cannot be implanted successfully.                                                                                                                                                                         |
| I.d  | Poor choice of manufacturing materials.                                                   | - Materials cannot be processed using equipment of choice.  
- Materials will not offer desired biomimetic characteristics in scaffolds.  
- Materials will hinder cellular or tissular development, healing, and regeneration.                                                                                                                                 |
| II.a | Poor choice of manufacturing processes.                                                   | - Inability to repeatably fabricate three-dimensional scaffolds according to relevant design attributes and goals.  
- Waste of time and resources with rework.                                                                                                                                                                          |
| II.a | Methodology used to characterize relevant material-process-structure interrelationships are inadequate or irrelevant. | - Misconception and/or misinterpretation of relevant interrelationships.  
- Resulting process models do not accurately characterize empirical evidence.  
- Uselessness of resulting process models.                                                                                                                                                                           |
| II.a | Instruments and/or equipment used to characterize relevant material-process-structure interrelationships are inaccurate and/or imprecise. | - Incorrect modeling of relevant interrelationships.  
- Resulting process models do not accurately characterize empirical evidence.  
- Uselessness of resulting process models.                                                                                                                                                                           |
| II.b | Process plan does not include or describe all necessary fabrication steps. | - Scaffold will be fabricated with missing or out-of-specification features.  
- Fabrication will require undocumented post-processing operations.  
- Variability introduced to process outcomes.  
- Hindered biomimetic and implantational performance of scaffolds. |
| --- | --- | --- |
| II.b | Process plan describes fabrication steps that could be replaced or eliminated. | - Waste of time and resources.  
- Variability introduced to process outcomes.  
- Hindered biomimetic and implantational performance of scaffolds. |
| II.c | Incorrect material composition. | - Materials cannot be processed using equipment of choice.  
- Affected quality outcome of fabricated scaffolds.  
- Introduction of toxic agents to cells and consequent poor tissue development.  
- Scaffolds must be scrapped. Time and resources wasted with remanufacturing.  
- Unexpected immunologic response post-implantation (inflammation, infection, etc.). |
| II.c | Incorrect ratio of materials in blend/mix/composite. | - Blend/mix/composite cannot be processed using equipment of choice.  
- Affected quality outcome of fabricated scaffolds.  
- Scaffolds must be scrapped. Time and resources wasted with remanufacturing.  
- Undesired cellular response to growth and differentiation factors present in the blend/mix/composite. |
| II.c | Contaminants present in materials for fabrication. | - Introduction of toxic agents to cells and consequent poor tissue development.  
- Inability to complete *in vitro* culture of cell-embedded scaffolds.  
- Scaffolds must be scrapped. Time and resources wasted with remanufacturing. |
### II.d

**Incorrect material loaded into machine.**
- Issues with affected extrudability for a predefined set of processing parameters.
- Resulting melt flow will increase or decrease as an effect of change in viscosity.
- Scaffold strands may become too thick while significantly reducing pore size and mechanical properties.
- Scaffold strands may become too thin while affecting structural integrity and mechanical properties.
- Unexpected immunologic response post-implantation (inflammation, infection, etc.).
- Undesired cellular response to growth and differentiation factors present in the blend/mix/composite.

**Incorrect choice of extrusion temperature.** *(specific to 3DB process)*
- Increase (temperature too high) or decrease (temperature too low) in melt flow due to change in material viscosity.
- Thinning (temperature too low) or thickening (temperature too high) of scaffold strands and consequential change in porosity, structural integrity, and mechanical properties.
- Early material molecular degradation and consequential loss in mechanical strength.

**Incorrect choice of extrusion pressure.** *(specific to 3DB process)*
- Increase (pressure too high) or decrease (pressure too low) in melt flow.
- Thinning (pressure too low) or thickening (pressure too high) of scaffold strands and consequential change in porosity, structural integrity, and mechanical properties.

**Incorrect choice of print head travel speed.** *(specific to 3DB process)*
- Thinning (speed too high) or thickening (speed too low) of scaffold strands and consequential change in porosity, structural integrity, and mechanical properties.

**Incorrect choice of extrusion nozzle.** *(specific to 3DB process)*
- Affected melt flow and consequential strand thinning or thickening.
- Early material degradation due to excessive shear during extrusion.

**Incorrect choice of inter-axial separation of scaffold strands.** *(specific to 3DB process)*
- Undesirable changes in scaffold porosity, structural integrity, and mechanical properties.
- Lack of a biomimetic environment for cells.
| II.d           | Poor machine Z-axis calibration (specific to 3DB process)                                                                 | - Lack of adherence of extruded strand to deposition platform or preceding scaffold phase(s) during printing if Z-offset is too high.  
- Collision of extrusion nozzle with printing platform or preceding scaffold phase(s) if Z-offset is too low. |
|               | Poor machine X-Y axes calibration (specific to 3DB process)                                                            | - Incorrect positioning of upper scaffold phase(s) to preceding phase(s) if X or Y offsets are incorrect. |
|               | Insufficient material preheat interval (specific to 3DB process)                                                         | - Material temperature too low at the start of deposition.  
- Reduced melt flow and consequential strand thinning. |
|               | Extrusion cartridge loaded with incorrect amount of material for fabrication. (specific to 3DB process)                  | - Unable to complete an entire fabrication run (if not enough material).  
- Material will sit for too long at elevated temperatures and start to degrade (if too much material).  
- Change in viscosity as an effect of molecular degradation.  
- Affected melt flow over time and inconsistent strand width throughout the scaffold. |
| II.d           | Strand width appear out of specification.                                                                             | - Undesirable changes in scaffold porosity, structural integrity, and mechanical properties.  
- Lack of a biomimetic environment for cells. |
| II.e           | Lack of adherence of scaffold to deposition platform during printing. (specific to 3DB process)                         | - Excessive warping of scaffold.  
- Shape infidelity.  
- Affected microarchitectural characteristics. |
| II.e           | Lack of adherence of scaffold phase to preceding phase(s) (specific to 3DB process)                                   | - Scaffold is likely to more easily delaminate and structurally fail during handling.  
- Printing process will fail if no adherence occurs at all, requiring rework. |
| II.e           | Extrusion nozzle is clogged (specific to 3DB process)                                                                   | - No material deposition will happen.  
- Decreased melt flow and rate of material deposition.  
- Thinner strands and consequential undesirable changes in scaffold porosity, structural integrity, and mechanical properties.  
- Waste of time as an effect of prolonged fabrication times to account for reduced rate of material deposition. |
| II.f | Instruments and/or equipment used to inspect scaffolds are inaccurate and/or imprecise. | - Quality inspection is unreliable.  
- Good scaffolds may fail inspection.  
- Bad scaffolds may pass inspection. |
| II.f | Microarchitecture of scaffold is detected out of specification. | - Scaffold should be scrapped.  
- Waste of time and resources with rework.  
- Waste of time and resources with rework. |
| II.f | Bulk geometry and shape of scaffold is detected out of specification. | - Scaffold should be scrapped.  
- Waste of time and resources with rework.  
- Waste of time and resources with rework. |
| II.f | A scaffold out of specification is incorrectly classified as being within specification. | - Lack of a biomimetic environment for cells to proliferate and develop their intended metabolic activity.  
- Scaffold will hinder cellular or tissular development, healing, and regeneration.  
- Negatively affected ease of implantation and rate of implantation success. |
| II.g | Sterilization process is not effective. | - Microbiological contamination during *in vitro* culture. |
| II.g | Sterilization process introduces toxic agents to scaffolds. | - Reduced cell viability during *in vitro* culture.  
- Unexpected immunologic response post-implantation (inflammation, infection, etc.). |
| III.a | Incorrect donor tissue type. | - Inability to isolate desired cell type for expansion and scaffold seeding. |
| III.a | Incorrect cell type isolated. | - Lack of cell potency.  
- Hindered tissue regeneration potential.  
- Adverse immunologic response to scaffold implantation. |
| III.a | Excessive stress caused to cells during harvest and isolation process. | - Poor cell viability after plating.  
- Extended expansion period required to reach seeding number.  
- Larger number of cell divisions required and consequential loss in potency and metabolic activity.  
- Waste of time and resources. |
| III.a | Incomplete cell isolation and incorporation of contaminant agents to cell population. | - Inability to complete *in vitro* culture of cell-embedded scaffolds.  
- Hindered tissue regeneration potential.  
- Adverse immunologic response to scaffold implantation. |
| III.b | Infrequent media changes. | - Reduced cell viability.  
- Extended expansion period required to reach seeding number.  
- Waste of time and resources. |
| III.b | Excessive exposure to digesting enzymes when passaging. | - Poor cell viability after replating.  
- Extended expansion period required to reach seeding number.  
- Waste of time and resources. |
| III.b | Over-confluent cultures. | - Loss of cell potency.  
|       |                        | - Decrease in cell viability. |
| III.b | Contamination of cultures under expansion. | - Inability to complete *in vitro* culture of cell-embedded scaffolds.  
|       |                        | - Waste of time and resources. |
| III.c | Poor seeding efficiency. | - Extended culture period required to reach desired cell density at the time of implantation.  
|       | Contamination of scaffolds during seeding. | - Inability to complete *in vitro* culture of cell-embedded scaffolds and follow through with implantation.  
|       |                        | - Waste of time and resources. |
| III.d | Poor cell viability. | - Hindered tissue regeneration potential.  
| III.d | Poor cell proliferation and metabolic activity. | - Hindered tissue regeneration potential.  
| III.d | Lack of cell differentiation. | - Inexistence of distinct cell phenotypes required to promote regeneration of specific tissues or tissue characteristics.  
| III.d | Contamination of scaffolds under culture. | - Inability to complete *in vitro* culture of cell-embedded scaffolds and follow through with implantation.  
|       |                        | - Waste of time and resources. |
| IV.a  | Inadequate anesthesia administered to patient. | - Patient may be under pain.  
|       |                        | - Patient may involuntarily move via sudden spasms during procedure.  
|       |                        | - Improper diameter of implantation site if movements occur during drilling.  
|       |                        | - Scaffold structural failure if movements occur during implantation.  
|       |                        | - Scaffold implanted at incorrect depth if movements occur during implantation.  
| IV.a  | Inadequate sterilization of incision site. | - Contaminants may be brought into surgical area and scaffold implantation site.  
|       |                        | - Unexpected immunologic response post-implantation (inflammation, infection, etc.).  
|       |                        | - Hindered cellular or tissular development, healing, and regeneration. |
| IV.b  | Implantation site drilled too deep. | - Excessive removal of tissue that could provide faster healing response.  
| IV.b  | Implantation site drilled too shallow. | - Scaffold will be left proud and likely need to be removed.  
|       |                        | - If not removed, excessive concentration of stresses on scaffold surface may affect its microarchitecture and hinder tissue development.  
<p>|       |                        | - Articular cartilage tissue on opposing joint surface will be damaged. |</p>
<table>
<thead>
<tr>
<th>IV.b</th>
<th>Implantation site drilled too wide.</th>
<th>- Suboptimal fit between the implant and its respective implantation site causing it to lack the required friction to be implanted successfully and remain securely in place.</th>
</tr>
</thead>
</table>
| IV.b | Surroundings of implantation side were inadvertently damaged during drilling. | - Poor healing and integration of joint tissue to newly implanted scaffold.  
- Triggering of degenerative process of the articular cartilage surrounding the site. |
| IV.c | Excessive compressive forces applied to scaffold during implantation. | - Catastrophic implant failure.  
- Implant needs replacement.  
- Cell viability may be affected.  
- Permanent changes in microarchitecture.  
- Hindered cellular or tissular development, healing, and regeneration. |
| IV.c | Excessive lateral shear forces applied to scaffold during implantation. | - Catastrophic implant failure (delamination).  
- Hindered cellular or tissular development, healing, and regeneration.  
- Implant needs replacement. |
| IV.c | Improper handling of implant. | - Implant may fail undetectably if not held and transported with care.  
- Implant needs replacement. |