ABSTRACT

NARAYANAN, LOKESH KARTHIK. Manufacturability Considerations in Biofabrication – Functional Mapping, Risk Assessment and Dielectric Impedance Spectroscopy-based Non-destructive Quality Evaluation (Under the direction of Dr. Rohan Shirwaiker).

Biofabrication processes enable the creation of engineered tissues containing living cells that can restore, maintain, or improve lost function of damaged tissue. For engineered tissues to become mainstream clinical alternatives, biofabrication processes must be appropriately scaled up and scaled out. To aid this translation, manufacturing aspects such as the definition of process endpoints, quality by design, and process validation must be considered during the product development phase along with development of effective process monitoring tools to achieve reproducible biological characteristics. Current state-of-the-art methods for measuring critical quality attributes (CQA) of engineered tissues, such as number of viable cells and cellular metabolic activity, are often destructive in nature and not applicable in a scalable manufacturing setting. Towards addressing these shortcomings, this dissertation focuses on systematic functional modeling and risk assessment of engineered tissue manufacturing (ETM) systems, and investigation of dielectric impedance spectroscopy (DIS) as a new non-destructive quality monitoring method for bioprinting-based and scaffold-based biofabrication. The specific objectives of this dissertation include:

- Systems-level mapping of engineered tissue manufacturing (ETM) system, including the functional decomposition of biofabrication processes and risk assessment of the identified functions.
- Investigation of a non-destructive quality monitoring method based on DIS for bioprinting, and characterization of the relationships between the CQA of bioprinted constructs and the process parameters through correlation with monitored DIS parameters.
Investigation of DIS monitoring of cell-seeded scaffolds and characterization of the relationships between scaffold design and culture conditions, CQA, and the DIS permittivity spectra.

To address these objectives, systems-level maps of bioprinting-based and scaffold-based ETM systems were created using integrated definition language 0 (IDEF0) and functional black box modeling to define the functional requirements and the flow of inputs and outputs of different processes within the systems. Risk assessments were performed on the specific process functions using failure modes and effects analysis (FMEA), and potential controls to mitigate the risks were identified. Towards non-destructive quality monitoring, the relationships between DIS parameters (permittivity change - Δε, cole-cole slope factor - α, and critical polarization frequency - fc), process parameters, and CQA of bioprinted cellular constructs and cell-seeded 3D-printed scaffolds were characterized. Overall, results show that Δε and α were dependent on the type and the number of viable cells. The Δε, α, and fc were also correlated with the changes in cellular metabolic activity and viability due to the variations in critical bioprinting parameters. In the context of bioprinting, and additive manufacturing process, the DIS signal attenuation and superposition phenomena due to addition of new layers was also modeled. Finally, the DIS monitoring of cellular metabolic activity and viability across anatomically biomodeled geometry and addition of layers over 7 days of in vitro culture was demonstrated. Comprehensively, the findings from this dissertation support the utilization of DIS to non-destructively monitor biofabrication processes and engineered tissues.
Manufacturability Considerations in Biofabrication – Functional Mapping, Risk Assessment and Dielectric Impedance Spectroscopy-based Non-destructive Quality Evaluation

by
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DEDICATION

To my entire Karur family, who have been my support system through thick and thin.
BIOGRAPHY

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CHAPTER 1: INTRODUCTION

1.1. Background

Humans are susceptible to congenital anomalies, tissue or organ damage due to injuries/trauma and chronic diseases that disrupt homeostasis. Currently, the treatment strategies for such conditions involve the use of pharmaceuticals and/or surgical procedures involving medical devices, implants and autologous/allogenic tissue/organ transplants. Implants made of polymers, metals or ceramics are primarily used to replace or augment the function of the tissue or organs that provide structural stability such as in the skeletal and muscular system. Although these implants, which stay inert inside the host body, satisfy the functional requirements, there can be a considerable mismatch between their structural, mechanical and biological properties when compared to the host tissue. For example, the compressive strength of titanium alloys typically used in orthopedic implants lies between 450-1850 MPa [1], whereas the compressive strength of cortical bone lies between 100-230 MPa [2]; this causes “stress shielding” and reduction in density of the bone, potentially resulting in eventual implant failure [3]. In the cases involving an organ or system failure in the endocrine, digestive, circulatory or excretory systems, organs transplants are used. The organs transplants are often allografts, and there is often high demand for those transplants. According to the organ procurement and transplant network (OPTN) data, as of 4/1/2019, there were approximately 124,606 registered patients waiting for a transplant, whereas the number of transplants performed until that date is staggering behind at 6,100 [4]. Over the last decade, the gap between the number of patients in need of transplants and the number of donors has continued to widen. Another major issue is the risk of rejection of a transplanted organ by the recipient’s body [5].
Owing to the limitations with respect to implants and transplants, the need to investigate alternatives such as tissue engineered replacements has become essential. Tissue engineering and regenerative medicine (TERM) strategies focus on the creation of living tissue system substitutes as opposed to non-biological devices. Tissue engineering is defined as “The application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function” [6].

Different types of methodologies are used in TERM based treatment. Overall, the strategies in TERM can be classified into three categories [7]:

1. Implantation of isolated cells or cell substitutes into the subject (cell therapy)
2. Delivery of tissue formation-inducing substances (e.g., growth factors) into the subject
3. Implantation of biologically active products combining biological components (cells, growth factors etc.) and biomaterial matrices (e.g., alginate, collagen, polycaprolactone (PCL), decellularized extracellular matrix (ECM), polylactic acid (PLA)).

The first two strategies have been used increasingly in medical treatment over the last decade. The third strategy focusing on engineered tissues and organs is being widely researched and would alleviate the need for organ transplants. In addition to therapeutics, engineered tissues and organs are also being increasingly used in drug testing, disease modeling and precision medicine applications [8]. There have been successful reports on the fabrication of engineered tissue such as cartilage [9], skin [10], trachea [11], bladders [12] in a laboratory environment. The processes that enable fabrication of such tissues and organs are collectively known as biofabrication processes.
1.2. Biofabrication

During the advent of these TERM based treatments, the US Defense Advanced Research Projects Agency (DARPA) defined biofabrication as “the use of biological materials and mechanisms for construction” to describe the methods used to create high-resolution 3D structures that mimic biological growth mechanisms [13]. Most recently, the increased use of fabrication techniques like additive manufacturing, bioprinting, micro/nano and bio patterning/assembly, the definition was appended in a featured article in the Biofabrication journal as “the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes” [14]. In general, biofabrication encompasses a broad range of physical, chemical, biological, and/or engineering processes that enable fabrication of complex biological products containing living cells, molecules, extracellular matrices, and engineered biomaterials. Examples of biofabrication process strategies include porous scaffold-based biofabrication, embedding/molding technology, directed tissue self-assembly technology, bioprinting etc.

Scaffold-based biofabrication is currently one of the most popular tissue engineering approach. Scaffolds are extracellular substrates that provide the proliferating cells with a three-dimensional template to organize into a regulated functional unit, subsequently enabling generation of ECM. Scaffolds are designed as porous 3D structures mimicking the ECM organization of the native tissue and can be made of decellularized ECM or biomaterials. These scaffolds are typically seeded with cells and cultured in vitro prior to transplantation. This lab-grown tissue is expected to resume tissue growth and maturation post-implantation to
replace/repair/restore its expected in vivo function. The methods of fabricating scaffolds can be broadly classified into traditional methods (e.g., solvent casting/particulate leaching, freeze-drying, phase inversion, electrospinning) and additive manufacturing (AM) processes (e.g., stereolithography (SLA) [15,16], selective laser sintering (SLS) [17,18], fused deposition modeling (FDM) [19,20], 3D bioplotting (3DB) [21–23]). In recent years, there has been a transition towards AM methods because they offer better control over 3D porous architecture, better reproducibility, and faster fabrication efficiency when compared to traditional processes. There are some acellular scaffold-based wound healing products such as Puraply® [24], Pelnac™ [25], and Terudermis [26] made with animal collagen using traditional methods already available in markets. Other commercial products like Apligraf® [27] and Dermagraft® [28] also contain living human fibroblasts that aid in wound healing. Apart from applications in skin regeneration, there are other scaffold-based products in human and animal trials for bone and cartilage repair such as electrospun grafts [29,30] and AM grafts [31–33]. Scaffold-based biofabrication has especially shown a lot of potential in osteoinduction and osteoconduction [34,35].

Another traditional biofabrication approach is embedding or molding (casting) technology, and it involves compacting a mixture of hydrogels and living cells into a pre-shaped mold [36]. This method was popular before the advent of bioprinting. Embedding technology has demonstrated promising results in cases of biofabrication of tissue engineered vascular grafts and heart valves [37,38]. One of the limitations of this method is the low seeding density and challenges associated with remodeling the construct post-ECM generation by the embedded cells [39]. Another method that is widely used in tissue engineering of vascular networks is directed tissue self-assembly or often referred to as organ printing. This technique is similar to the principle of additive manufacturing, employing self-assembling tissue spheroids to build 3D
tissue constructs [40,41]. The efficiency of the process relies on the extent of tissue fusion of closely placed tissue spheroids. The method is still in the early stages of development and investigation of performance in vivo has been limited.

Currently, one of the most actively researched biofabrication approach is 3D bioprinting. The popularity of this approach can be attributed to its ability to create tissue constructs with patient specific geometry and high spatial control, in addition to offering good resolution. Bioprinting can be defined as the positioning of bioinks (an appropriate mixture of biomaterials and living cells) in a prescribed layer-by-layer stacking organization to fabricate engineered tissues and organs [42]. The success of bioprinting is governed to a large extent by the bioink properties. The bioink must provide a conducive environment for the cells to resume normal metabolic functions and proliferation while providing mechanical properties that can enable processing.

The different methods of 3D bioprinting include extrusion-based, inkjet-based, stereolithography-based and laser-assisted bioprinting among others. Among these methods, owing to its simplicity, extrusion-based bioprinting is very popular. It involves dispensing of bioink (viscosity range of 30-36 x 10^7 mPas [43]) in the shape of the construct, layer by layer, and strengthening (fixation or gelation) it using various methods such as chemical, heat or photo crosslinking. Extrusion-based bioprinting has been widely researched for bone and cartilage tissue engineering applications. There are several published studies evaluating the efficacy of the process and the biomaterials used in relation to the tissue generation in both in-vitro and animal models. Currently, there is a lot of interest bioprinting heterocellular tissue models such as liver, kidney, heart, and tumor 3D models with two or more cell types for drug testing and high-throughput screening [44,45]. In cancer research, these models provide notable advantages over
the traditional 2D models, in studying the interactions between the tumor cells and the metabolic activity of the healthy cells or tissue [46].

Stereolithography is another bioprinting method that has been widely researched due to its ability to fabricate constructs with precise control and its versatility [47,48]. It is based on the principle of photopolymerization of light-sensitive polymers with a directed light source controlled using digital mirrors. The use of the method has waned after reports of the UV light source causing irreversible damage to the DNA of the cells [49]. Another method that has gained a substantial interest of late is laser-assisted bioprinting (LAB), based on the principle of laser-induced forward transfer (LIFT) [50]. LAB employs focused laser pulses on the absorbing layer of the ribbon to generate a high-pressure bubble that propels cell-containing materials toward the collector substrate, building 3D constructs in layers. LAB process has been demonstrated to fabricate skin constructs [51] and vascular tubules [52]. The in vivo performance of tissue constructs fabricated via LAB has not been investigated extensively.

1.3. Motivation and Research Objectives

Biofabrication field is expanding rapidly with several established and newer processes under various stages of development and application. As the field evolves, significant efforts are being focused on minimizing or eliminating shortcomings associated with spatial resolution, shape fidelity, geometric reproducibility, and enabling the hierarchical arrangement of vascular networks including circulatory, lymphatic and neural networks among others. From the manufacturing perspective, one limitation that needs further attention is better methods for monitoring the functional efficacy of cellular constructs during or after processing.
Eventually, for tissue engineered products to become mainstream clinical alternatives, biofabrication processes must be appropriately scaled up and scaled out. To enable this translation, current process development efforts must be complemented with the development of effective monitoring and process control tools to achieve a repeatable biological quality (potency) in each manufacturing batch. The biological attributes such as quantity of cells, cell phenotype, and stage of cell differentiation or tissue maturation must be maintained across different samples of the batch. Traditionally, in the TERM field, the manufacturing scale-up/scale-out efforts are considered only after clinical effectiveness of the product is verified. However, once the processing protocols are established for clinical trials and the results are favorable, it is arduous to make changes to the manufacturing approach. As such, even small changes in the process would require new validation of the process performance as dictated by the regulatory requirements. To avoid this, manufacturing aspects such as the definition of process endpoints, quality by design and process validation must be considered during the product development phases. The process endpoints must define the acceptable range of the biological quality attributes. Quality by design can help identify the critical quality attributes (CQA) that affect the product performance, and process validation can help understand how the identified critical biological attributes are affected by the process parameters.

Lean manufacturing and Six Sigma approaches and tools for process mapping, systematic decomposition, and risk assessment can be utilized to determine manufacturing and translation concerns in biofabrication. These can help provide an understanding of all the processing steps right from the isolation of cells to the in-vitro culture of the engineered tissue, their functions, inputs and outputs, as well as the relationships between these steps and product performance.
Furthermore, these can be used to identify indicators for implementing potential process endpoints where the quality could be evaluated. Traditionally, the biological quality of the engineered tissue constructs at the endpoints are evaluated using destructive methods. In current practice, an identical sample is fabricated with the same initial attributes such as cell number, geometry, and biomaterial and process parameters [53,54]. One of the samples is then sacrificed to evaluate the quality characteristics, which would serve as the measure of the actual product. This is not an ideal approach. Development of better non-destructive testing strategies can make the operations easier and less expensive, most importantly from the perspective of regulatory agencies such as the food and drug administration (FDA) and European commission (EC), which require quality assurance and testing at every stage of manufacturing.

This dissertation will focus on systematic mapping and determination of manufacturing-related challenges in biofabrication of tissues. Specifically, the bioink-based bioprinting and scaffold-based biofabrication processes will be modeled to understand the functions of different process steps and their interactions. Further, a new non-destructive quality assessment methodology using dielectric impedance spectroscopy (DIS) will be investigated. The objectives and the specific tasks (ST) for the dissertation are presented below:

**Objective 1: Functional mapping and risk assessment of bioprinting and scaffold-based 3D biofabrication processes**

**ST1.1:** Create a system-level map of the biofabrication process using integrated definition (IDEF) modeling.
ST1.2: Create a detailed process and sub-system level functional decomposition of the steps in biofabrication.

ST1.3: Identify failure modes associated with sub-system level functions and the potential causes and effects for each mode.

**Objective 2: Non-destructive quality assessment in bioprinting-based biofabrication**

ST2.1: Develop a DIS system setup and approach to evaluate CQA of biofabricated 3D constructs, and understand the relationship between DIS parameters and two primary CQA (cell number, cell viability).

ST2.2: Investigate the effects of extrusion-bioprinting process parameters on cell viability of 3D cellular constructs and its correlation with DIS parameters.

ST2.3: Evaluate the correlation between cell viability and DIS parameters of extrusion-bioprinted constructs over time and scale.

**Objective 3: Non-destructive quality assessment in scaffold-based biofabrication**

ST3.1: Investigate the effect of changes in the scaffold design on CQA (cell viability, cell number) of additive manufactured scaffolds and its correlation with DIS parameters.

ST3.2: Investigate the effect of changes in the culture conditions (seeding density) on CQA (cell viability) of 3D-biopotted scaffolds and its correlation with DIS parameters.

The major contributions of this dissertation will include:

- Detailed process maps of 3D biofabrication processes including potential failure modes of the steps involved.

- A label-free, non-destructive method to evaluate CQA of biofabricated constructs using DIS.
• Relationships between extrusion-based bioprinting process parameters and DIS parameters that could lead to closed-loop control of extrusion-based bioprinting in future.

1.4. Chapter Summary

TERM strategies have facilitated the creation of engineered tissues that could serve as clinical alternatives for a wide variety of medical procedures. The biofabrication processes that enable manufacturing of such tissue have evolved to provide the structural fidelity and resolution required for clinical applications, but more work is needed to improve the ability to recapitulate biological functions as well as to streamline the processes and improve reproducibility. For a successful translation of these processes, the manufacturing aspects such as quality monitoring and process validation should be considered during product development. This dissertation will focus on the creation of a systematic map and risk assessment of the steps and functions involved in biofabrication processes, and investigating a new non-destructive quality monitoring method using DIS. The following chapters (Ch. 2-4) are organized by the three specific objectives. Each chapter will include an introduction to the specific objective, materials and methods, results and discussion, and a concluding summary. The dissertation concludes in Ch. 5 with an overall summary and future directions.
CHAPTER 2: FUNCTIONAL MAPPING AND RISK ASSESSMENT OF 3D BIOFABRICATION PROCESSES

A brief overview of the complexity involved in fabricating engineered tissue was discussed in Chapter 1. Engineered tissue manufacturing (ETM) system consists of several interconnected processing steps starting with sourcing of cells from a biopsy of patient tissue up until shipping of matured tissue. There are several inputs in the form of material, energy and information associated with each processing step that can affect the functional outcomes of the manufactured tissues. Furthermore, the same input may be utilized by multiple processing steps, and the output of one process might affect other processes downstream. As such, the variability and errors in one step can significantly affect the output of the ETM system. This chapter focuses on Objective#1 of the dissertation which entails functional mapping and risk assessment of the ETM system, with a primary focus on the processing steps involved in biofabrication.

Section 2.1 explains the systems-level mapping of the ETM system. Section 2.2 presents the functional decomposition of biofabrication processes, followed by risk assessment in Section 2.3.

2.1. Systems level mapping of engineered tissue manufacturing

At the highest level, ETM can be categorized into a series of interconnected processes as presented in Figure 1. ETM starts with the sourcing of cells used in the engineered tissue. The cells are obtained from a human host through a tissue biopsy. The cells are extracted from the tissue sample and allowed to proliferate (multiple orders of magnitude) through in vitro culturing. The culturing process is continued until the required number of cells is achieved, then
the cells are harvested from the culture vessels. The cell suspension resulting from the harvesting procedure is reduced to manageable volumes required for biofabrication. If the therapy is autologous, the 3D model required for biofabrication is created, replicating the patient organ or tissue geometry, by processing the patient’s CT or MRI scans. Then, depending on the type of biofabrication approach, the cells are either encapsulated into bioinks and bioprinted into constructs or are seeded onto polymer-based scaffolds fabricated based on the tissue 3D model.

The cellular constructs/scaffolds are then cultured *in vitro* to allow cells to proliferate and generate ECM. At some point in time during its maturation, the construct is either implanted into the patient or used for other applications including pharmaceutical testing. There are several steps and functions associated with each processing step in Figure 1. In this dissertation at the highest level, integrated definition language 0 (IDEF0) is used to define and characterize the inputs, outputs, functions and interrelationships between different processes in the ETM system.
Figure 1: Overview of different processes used in engineered tissue manufacturing (ETM)
IDEF0 is a graphical modeling language that was a product of the U.S. Air Force program for integrated computer-aided manufacturing (ICAM) [55]. The ICAM program sought to increase manufacturing productivity through systematic application of computer technology. There were a series of techniques developed by ICAM including IDEF0, IDEF1, and IDEF2, commonly referred to as IDEF. Later, IDEF1 and IDEF2 were integrated into IDEFX, a semantic data modeling technique. IDEF0 is used to create a "function model". A function model is a structured axiomatic representation of the functions, activities or processes within the modeled system or subject area. IDEF0 can be used to analyze the functions the system performs and to record the mechanisms by which those functions are achieved. The result of applying IDEF0 to a system is a model that consists of a hierarchical series of diagrams and text cross-referenced to each other. The two primary modeling components are functions (represented on a diagram by boxes) and objects that inter-relate those functions (represented by arrows). This modeling feature is very applicable to model the interconnected steps in ETM. The syntax used in the IDEF0 modeling is presented in Figure 2.

![Figure 2: IDEF0 syntax](image)
A function node is an activity that transforms inputs into outputs under constraints using mechanisms. The inputs, outputs, constraints, and mechanisms are directed information flows that connect functions in IDEF0 models. IDEF0 model is a hierarchical structure, starting from A-0 level, continuing to A0, A1, and A11 levels [56]. The A-0 diagram is a single function box diagram that bounds the context for the entire model and forms the basis, for further decomposition. The A0 level is the topmost level in this model's hierarchy. A0 level defines all the process systems and their interrelationships. A0 level will provide a map of how the inputs and outputs of the processes are interconnected. The A1 level is a process decomposition of each process system listed in A0 level. The A11 level further details on the process nodes defined in the A1 level.

In the context of this dissertation, the A-0 level explaining the biofabrication process and the A0 level functional decomposition for bioprinting-based and scaffold-based biofabrication processes and subsequent A1 levels for the A0 level models were developed. The A-0 level IDEF0 model is presented in Figure 3. This model was developed from the perspective of manufacturer to understand how the model’s inputs and outputs are related, and their effect on process functions.
**Context**
Engineered tissue manufacturing includes a broad range of interconnected processes that enable fabrication of biological substitutes to restore, maintain, or improve function lost function. The process scope begins at cell sourcing and ends with in-vitro culture of the manufactured tissue.

**Viewpoint**
The viewpoint is based on the perspective of the manufacturer and not the other stakeholders.

**Purpose**
The purpose is to define and understand the processes, functions and activities involved in the manufacturing of engineered tissue.

**Figure 3:** A-0 level of IDEF Modelling of ETM

Going a level down the hierarchy of the IDEF0 model, A0 level mapping of the bioprinting-based ETM is presented in Figure 4 and scaffold-based ETM is presented in Figure 5. In Figure 4 and Figure 5, the dotted box represents the process steps directly pertaining to biofabrication. The functional decomposition of these steps will be discussed in detail in the following section. The scaffold-based ETM discussed in this dissertation only pertains to additive manufactured scaffolds. As seen in the figure, most of the early steps involving obtaining the appropriate number of cells and generation of CAD models of the tissue are common to the two approaches. The cells used in the construct are either obtained from the patient in case of autologous treatment or from a donor for allogenic treatment. The A0 level of IDEF0 model (Figure 4 and Figure 5) only provides an overview of the different processes involved in the system. Each function node (labeled as A1, A2, B1, B2 and so forth) represents an individual process within the ETM system. In the A0 level when two processes occur simultaneously and is not dependent on each other, they are given a different letter prefix. For example, CAD and CAM modelling must occur simultaneously with cell expansion process to
accomplish bioprinting. Hence in Figure 4, CAD and CAM process has a function label as B1. Each function node presented in the A0 level model has been further elaborated using individual A1 level IDEF0 models where the mechanisms by which the function transforms input and the constraints for the process have been presented.

For example, the function node “Cell Isolation” in the A0 model is described in detail using A1 level model in Figure 6. A small biopsy is obtained by the surgeon and sent to the manufacturer for extraction of the cells. The manufacturer does not have control over the biopsy process, hence, this model starts with a quality check. The tissue sample obtained from a biopsy is minced and subjected to digestive enzymes that dissolve the ECM, leaving the cells unharmed. The cells are then subject to wash cycles to remove the enzymes and are centrifuged to obtain cell pellets which is the end product of the “Cell Isolation” function. The cells are then proliferated to the numbers required for ETM in the next process, “Cell Expansion”, as presented in Figure 7. The extracted cells are mixed with culture media and are cultured in flasks or in bioreactors. Depending on the method, the cells may have to be passaged one or more times until the desired number of cells is obtained. Thereafter, the cells are harvested from the culture vessels by using dissociation solutions such as trypsin. The detached cells with the dissociation agent are neutralized by adding culture media. This cell suspension is then centrifuged to obtain cell pellets that would be used in either bioink preparation in case of bioprinting or cell suspension preparation to seed scaffolds. Other functions presented in A0 level of IDEF0 model have been presented as individual A1 level IDEF0 models in Appendix I for bioprinting-based ETM and Appendix II for scaffold-based ETM.
The constraints presented in the A1 level model can be used for defining the process endpoints for each of the individual processes. Defining process endpoints is critical towards translation of these processes, and the A1 level model provides the criteria for that definition. A manufacturer can set the level of these endpoints based on the tissue being manufactured. For example, in “Cell Isolation” A1 model, in the function node “incoming tissue quality check”, a manufacturer can set the tolerance limit for acceptable biomarker level and reject the sample when it fails to meet it before subjecting to subsequent processes that may be wasteful.

The IDEF models presented in this chapter were designed to operate with autologous sourcing of cells for manufacturing of engineered tissue. In case of allogenic sourcing of cells, additional processing steps including ones pertaining to immunoisolation and immunosuppression (e.g., microencapsulation of cells [57–59], addition of immunosuppressive drugs [60,61]) within the manufactured tissue would have to be included in the A1 levels of the respective processes.
Figure 4: A0 level of IDEF0 model for bioprinting-based ETM
Figure 5: A0 level of IDEF0 model for scaffold-based ETM
Figure 6: A1 level IDEF0 model for the function “Cell Isolation” for bioprinting-based ETM and scaffold-based ETM
Figure 7: A1 level IDEF0 model for the function “Cell Expansion” for bioprinting-based ETM and scaffold-based ETM
2.2. Functional decomposition model of biofabrication processes

Functional decomposition refers to a description of a set of steps that are required to satisfy the overall function of a device, system, or process, broken down into smaller functions and supporting functions. Functional decomposition is usually accomplished through thoughtful analysis and discussions of the device/system/process resulting in a chart that describes the device/system/process functions and their interrelationships. The first step in this method is identifying the overall function of the process. The overall function must be broken down into supporting functions. Then, the different embodiments of components and sub-components of the product or the process that are capable of meeting the functions must be identified. These steps will enable the creation of a map with different pathways directed towards the fulfillment of product or process requirements. This method focuses on the functional requirements that affect the product or process performance and is independent of the process type or design. In this section, a function-structure diagram, commonly referred to as black box model [62], is used for functional decomposition of biofabrication processes. The syntax of the nodes in a black box diagram is presented in Figure 8.

![Figure 8: Syntax: black box model](image)
As shown in **Figure 8**, the black box is a set of functions that change or transform, connect or disconnect, increase or decrease, store or retrieve, conduct or block the flow of energy, material or information to achieve the desired outcome. Within the black box, the overall function of the product or a process is decomposed into several supporting functions that are connected through the flow of energy, material and information.

Black box models of the bioprinting-based biofabrication and scaffold-based biofabrication are presented in **Figure 9** and **Figure 10**, respectively. The schematic flow of the functions in the black box models follows the syntax defined in **Figure 8**. The material, energy, and information inputs to the process are presented in orange, yellow and green boxes, respectively. The supporting sub-functions are presented in blue boxes within the black box which is represented with black dashed lines, and the auxiliary functions are presented outside the black box. The auxiliary functions are external functions whose outputs have an effect on the overall function but cannot be controlled in the domain of the main function.

Black box models provide information on the flow of material, energy and information between different supporting functions within the overall function of bioprinting-based or scaffold-based biofabrication. It presents the logical sequence of operations based on the outcome of the previous step. In bioprinting-based biofabrication (**Figure 9**), the overall function of the black box model is to fabricate engineered tissue through extrusion based bioprinting of cellular bioinks. To accomplish this overall function, simultaneous inputs from different sets of processes are required. For example, to start bioprinting a layer of the construct, bioink containing cells and toolpath for depositing the bioink are needed. Hence, two sets of processes – “loading the bioink into bioprinter” and “CAD modeling” (generation of toolpath) must have their outputs ready simultaneously. The black box model also provides information on
the logical flow of steps while bioprinting including the crosslinking process and the action that needs to be taken on completion of print cycle.
**Bioprinting parameters**

Bioink (cells + hydrogel)

Maintain bioink at specified temperature

Cross link layer

Culture bioprinted constructs

Culture bioprinted constructs

Engineered tissue

**Supporting Sub-Functions**

- Print container
- CAD model
- Cross linker
- Culture medium

**Auxiliary Functions**

- Patient data
- Cell suspension
- Hydrogel

**Preparation of bioink**

**CAD modeling**

**Load bioink into bioprinter**

**Deposit layer**

**Cross link layer**

**Electrical energy**

**Thermal energy**

**Translational energy**

**Figure 9:** Black box model of bioprinting-based biofabrication
Figure 10: Black box model of scaffold-based biofabrication
A brief description of functions within the black box has been presented in section 2.3. For the functions within the black box, there can be different methods or embodiments through which the functions could be achieved. For example, the function “extrusion of polymer or deposit bioink” can be achieved through methodologies or energies such as direct pneumatic, screw-driven or pneumatic plunger-driven extrusion as presented in Figure 11. Similarly, Figure 12 presents different methods through which the toolpath can be translated from the CAD geometry. The positioning systems used in 3D printers can offer control in 3, 5 or 6 axis. There can be different combinations of position control through which the movements can be achieved; Figure 12 further presents the combinations for a 3-axis system.

![Diagram of crosslinking mechanisms](image)

Figure 13 presents the embodiments for crosslinking mechanisms for both scaffold-based and bioprinting-based methods.
Figure 11: Embodiments of "Extrude polymer/ deposit bioink"

Figure 12: Embodiments of "Translation of toolpath to extrusion geometry"
In this dissertation, the overall function of bioprinting-based biofabrication has been restricted to extrusion-based bioprinting. However, the modeling concepts and the final models themselves can be extended to other bioprinting processes such as SLA, LAB and inkjet bioprinting relatively easily. The risk assessments for each of the functions in the black boxes presented above are discussed in the following section.

2.3. Risk assessment

Engineered tissue, like any medical product, have risks that need to be mitigated during the manufacturing process. The term risk is defined as the probable rate of occurrence of a hazard causing harm and the degree of severity of the harm [63]. According to FDA, the risk
 factors for a medical device fall into these four major categories – design, human factors engineering, manufacturing of quality control and quality assurance, materials toxicity and material degradation [64]. For engineered tissue, there is an additional category related to immune reaction to the cells in the construct. Hence, the tolerance of risk management for engineered tissue is much stringent than other non-biologic medical devices. It is important to identify the risks and have a management plan to mitigate them. In this section, as a first step towards developing a potential risk management plan for biofabrication, the risks associated with the manufacturing of engineered tissues have been identified and assessed.

There are several tools such as hazard/risk analysis [65], failure modes and effects analysis (FMEA) [66], fault tree analysis (FTA) [67], and cause-and-effect diagrams [68] that can be used to conduct a risk assessment. In this dissertation, FMEA was chosen as it operates on core principle of taking preventative action to reduce risk. FMEA is a systematic, proactive method aimed to identify and assess the causes and effects of potential failures in a system, thereby preventing them from happening beforehand [69]. Typically, FMEA is performed by completing the following 5 steps: selection of processes to be assessed, construction of a multidisciplinary team, collection and classification of risk scores from each process, conducting a risk analysis, and implementation of remedial actions and reanalysis to see if those actions are effective [63]. The multidisciplinary team performs brainstorming to assign a risk priority number (RPN) to each process of potential failure. RPN is the product of scores representing the 3 aspects of a risk: severity (S) (how severe is the consequence if the process fails?), occurrence (O) (how frequently can a process fail?), and detection (D) (how easily can a failure be detected if the process fails).
Currently, ETM is a lab-based process and there are no published literature or data that can warrant an estimation of an RPN number. Hence in this dissertation, the columns O, C, D and RPN have been excluded. An FMEA was performed based on discussions with our laboratory team who have experience with ETM projects. For the functions or process steps identified in section 2.2, a brief explanation of the function is given in the “Requirement” column, then potential failure modes of each function are presented in the next column. For the identified failure modes, possible effects of failure and potential causes or mechanisms for that failure are then presented in the subsequent columns. The effects of failure are categorized based on the stakeholder that would likely be affected, either the manufacturer (prefixed by M) or the customer (prefixed by C). For the identified effects of failure, possible control or mitigation strategies are presented in the last column. The controls are categorized to be either detection-based (prefixed by D) or prevention-based (prefixed by P). FMEA of bioprinting-based and extrusion-printed scaffold-based biofabrication is presented in Table 1 and Table 2 respectively.
### Table 1: Failure modes and effects of functions in bioprinting-based biofabrication.

<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>Load Bioink into Bioprinter</td>
<td>Contain the prepared volume of bioink within the specified bioprinter reservoir in a sterile environment.</td>
<td>• Cartridge does not retain the entire volume of the added bioink</td>
<td>• M: Unable to complete bioprinting of the construct.</td>
<td>• Print cartridge is leaky or faulty</td>
<td>• D: Leak test for bioprinter reservoir prior to bioprinting</td>
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<td></td>
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<td></td>
<td>• Print container does not properly fit into print head</td>
<td>• M: Unable to perform bioprinting</td>
<td>• Incorrect print container used</td>
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<td></td>
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<td></td>
<td>• Insufficient volume for bioprinting.</td>
<td>• M: Unable to complete bioprinting of the construct.</td>
<td>• Incorrect volume added to the reservoir</td>
<td>• P: Poke-yoke the design of the container so that only correct type of container can be used.</td>
</tr>
<tr>
<td>20</td>
<td>Maintain the bioink @37°C</td>
<td>Heat the print cartridge to the specified temperature and hold the temperature (@ 37°C to ensure the physiological conditions are maintained)</td>
<td>• Inactivation or reduced activity of the cells.</td>
<td>• M: Loss of viable cell population, Change in phenotype of the cells</td>
<td>• The temperature of the print cartridge not set to the specified temperature</td>
<td>• D: Check for the temperature of the print head in the software prior to bioprinting and change setting in to the specified temperature</td>
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<td>Sno</td>
<td>Process Step</td>
<td>Requirement</td>
<td>Potential failure mode</td>
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<td></td>
<td>Generate toolpath</td>
<td>Transforming the geometric data of the model into toolpath required to fabricate the structure by defining the position of the structure on the print container and assigning</td>
<td>• Change in physical and chemical properties of the bioink</td>
<td>• M: Change of viscosity in bioink - unable to extrude the bioink to specifications. • M: Visual appearance of bioink altered. • M&amp;C: Breakage of chemical bonds in bioink resulting in a environment non-conducive to the cells</td>
<td>• Faulty heating system</td>
<td>• D: Check for the temperature of the print head in the software prior to bioprinting. • P: Follow regular maintenance and calibration schedule for heating element and sensors.</td>
</tr>
<tr>
<td>40</td>
<td>Generate toolpath</td>
<td>Toolpath cannot be generated</td>
<td>• Toolpath cannot be generated</td>
<td>• M: Cannot accomplish bioprinting</td>
<td></td>
<td>• D: Simulate and verify toolpath before bioprinting</td>
</tr>
<tr>
<td></td>
<td>Generate toolpath</td>
<td>Incorrect toolpath is generated</td>
<td>• Incorrect toolpath is generated</td>
<td>• M: Construct of incorrect geometry is generated.</td>
<td>• The geometrical data obtained is inaccurate or corrupt</td>
<td>• P: Check and obtain complete geometrical model data as specified</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
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<tbody>
<tr>
<td>50</td>
<td>Deposit layer</td>
<td>Extrude and deposit the bioink (with functional living cells) in form of continuous strands with uniform required area of crosssection along the toolpath as defined by the CAD model geometry.</td>
<td>• Bioink not ejected from the print reservoir</td>
<td>• M: Mechanical collision of the print head with the machine components</td>
<td>• Incorrect layer height or other geometric parameters were assigned during toolpath generation</td>
<td>• P: Check and assign the correct type of print container</td>
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<td></td>
<td></td>
<td></td>
<td>• M: Cannot create construct</td>
<td>• M: Cannot complete bioprinting the complete model</td>
<td>• Incorrect definition of print envelope</td>
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<td></td>
<td></td>
<td></td>
<td>• Bioink is too viscous</td>
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<td>• P: Verify the bioink viscosity before start of print cycle</td>
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<td>Sno</td>
<td>Process Step</td>
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<td>Potential failure mode</td>
<td>Potential Effects of Failure</td>
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<td></td>
<td>• Ejected strands do not have an uniform cross-section (thinner or thicker) as per previously defined toolpath.</td>
<td>• Incorrect Extrusion Pressure</td>
<td>• D: Test and validate the bioprinting parameters before starting the print cycle.</td>
<td>• D: Test and validate the bioprinting parameters before starting the print cycle.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• M: Poor shape fidelity</td>
<td>• P: Follow regular maintenance and calibration schedule for pneumatic system and sensors.</td>
<td>• P: Follow regular maintenance and calibration schedule for pneumatic system and sensors.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• M: Constructs with undesired voids</td>
<td>• P: Check and replace the sensor if faulty</td>
<td>• P: Check and replace the sensor if faulty</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• M: Construct collapse during build.</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts of the compressor</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts of the compressor</td>
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<td></td>
<td>• C: Construct has incorrect mechanical strength</td>
<td>• D: Test and validate the bioprinting parameters before starting the print cycle.</td>
<td>• D: Test and validate the bioprinting parameters before starting the print cycle.</td>
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<td>• C: Construct does not mature correctly</td>
<td>• P: Follow the print protocols for the bioink</td>
<td>• P: Follow the print protocols for the bioink</td>
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<td>• Bioink properties deteriorate overtime in the bioprinter reservoir</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
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<td>• Leaks in the pressure delivery system (leaks in tubes, O rings and connectors)</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
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<td>Sno</td>
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<td>• The print container not retained in the specified location</td>
<td>• D: Check for the current position of the print container and take action to move to specified location if needed</td>
<td>• D: Check for the current position of the print container, if the container is move pause the print cycle, take action to move to specified location and restart the print</td>
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<td>• The print container's position has been altered during the print cycle</td>
<td>• D: Check for the current position of the print container, if the container is move pause the print cycle, take action to move to specified location and restart the print</td>
<td>• D: Check for the current position of the print container, if the container is move pause the print cycle, take action to move to specified location and restart the print</td>
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<td>• The print cartridge was not completely secured in the print head</td>
<td>• P: Check if the cartridge is secured firmly by locking it to the complete extent before start of print cycle</td>
<td>• P: Check if the cartridge is secured firmly by locking it to the complete extent before start of print cycle</td>
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<td></td>
<td>• Nozzle height offset not completed successfully</td>
<td>• P: Follow protocol to complete the nozzle height offset procedure</td>
<td>• P: Follow protocol to complete the nozzle height offset procedure</td>
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<td>Sno</td>
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<td></td>
<td>*P: Check of the composition (% constituents) of bioink in the quality control sheets of the bioink preparation step and take recommended action</td>
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<td></td>
<td>*P: Follow protocols for the crosslinking methods and expose to the described crosslinking agent for the specified duration</td>
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<td>*P: Check for the type of print container and take action to replace it specified container if needed</td>
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<td></td>
<td>*P: Test and validate the adhesion between the bioink and the substrate</td>
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<td>• The ejected strands merge previously deposited strands.</td>
<td>• The viscosity of the bioink does not match specification</td>
<td>• D: Check of the composition (% constituents) of bioink in the quality control sheets of the bioink preparation step and take recommended action. • P: Stop and restart the print with right bioink</td>
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<td></td>
<td>• Incorrect Extrusion Pressure</td>
<td>• D: Test and validate the bioprinting parameters before starting the print cycle. • P: Follow regular maintenance and calibration schedule for pneumatic system and sensors.</td>
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<td></td>
<td>• Faulty heating system</td>
<td>• D: Check for the temperature of the print head in the software prior to bioprinting. • P: Follow regular maintenance and calibration schedule for heating element and sensors.</td>
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<td>• M: Poor shape fidelity • M: Constructs with undesired voids • M: Construct collapse during build.</td>
<td>• C: Construct has incorrect mechanical strength • C: Construct does not mature correctly</td>
<td>• D: Check of the composition (% constituents) of bioink in the quality control sheets of the bioink preparation step and take recommended action. • P: Stop and restart the print with right bioink</td>
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<td>• Deposited bioink does not stick to the previous layer (substrate if first layer) and moves/floats</td>
<td>• M: Poor shape fidelity</td>
<td>• The viscosity of the bioink does not match specification</td>
<td>• D: Check of the composition (% constituents) of bioink in the quality control sheets of the bioink preparation step and take recommended action.</td>
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<td></td>
<td></td>
<td></td>
<td>• C: Construct has incorrect mechanical strength</td>
<td>• M: Constructs with undesired voids</td>
<td>• Incorrect crosslinking agents or methods</td>
<td>• P: Stop and restart the print with right bioink</td>
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<td></td>
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<td>• C: Construct does not mature correctly</td>
<td>• M: Construct collapse during build.</td>
<td>• Incorrect toolpath</td>
<td>• D: Simulate and verify toolpath before print cycle</td>
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<td>• Inactivation or reduced activity of the cells.</td>
<td>• M: Loss of viable cell population, Change in phenotype of the cells</td>
<td>• Incorrect material composition</td>
<td>• P: Check of the composition (% constituents) of bioink in the quality control sheets of the bioink preparation step and take recommended action.</td>
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<td>Incorrect Extrusion Pressure</td>
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<td>D: Test and validate the bioprinting parameters before starting the print cycle.</td>
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<td>P: Follow regular maintenance and calibration schedule for pneumatic system and sensors.</td>
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<td>P: Check and replace the sensor if faulty</td>
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<td>Faulty heating system</td>
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<td>D: Check for the temperature of the print head in the software prior to bioprinting.</td>
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<td>P: Follow regular maintenance and calibration schedule for heating element and sensors.</td>
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<td>Contamination</td>
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<td>P: Follow sterile handling methods as described in the protocols.</td>
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<td></td>
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<td></td>
<td>Incorrect crosslinking agents or methods</td>
<td></td>
<td>P: Follow protocols for the crosslinking methods and expose to the described crosslink agent for the specified duration</td>
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<td>60</td>
<td>Crosslink layer</td>
<td>Enhance the mechanical/physical/biological/chemical properties of the deposited bioink utilizing the crosslinking medium of specified composition/properties for the specified exposure time while maintaining the cellular functionality.</td>
<td>• Deposited bioink is not completely crosslinked • Incorrect crosslinking (Bioink swells/ shrinks more than the required geometry)</td>
<td>• M: Poor shape fidelity • M: Construct collapse during build • C: Construct has incorrect mechanical strength • C: Construct does not mature correctly</td>
<td>• Exposure to incorrect crosslinking agents or methods (wavelength, voltage, amperage, composition, concentration, volume, exposure time)</td>
<td>• D: Test and validate the crosslinking protocols before starting the print cycle • P: Follow protocols for the crosslinking methods and expose to the described crosslinking agent for the specified duration</td>
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<td>• Inactivation or reduced activity of the cells.</td>
<td>• M: Loss of viable cell population, Change in phenotype of the cells</td>
<td>• Exposure to incorrect crosslinking agents or methods (wavelength, voltage, amperage, composition, concentration, volume, exposure time)</td>
<td>• D: Test and validate the crosslinking protocols before starting the print cycle • P: Check for specifications (pH, composition, wavelength, intensity, voltage) before starting the print cycle. • P: Follow protocols for the crosslinking methods and expose to the described crosslinking agent for the specified duration</td>
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<td>• Contamination</td>
<td>• P: Follow sterile handling methods as described in the protocols.</td>
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<tr>
<td>70</td>
<td>In vitro - Culture of Bioprinted constructs</td>
<td>Bioprinted construct containing viable cells are cultured in a laboratory setting for a predefined time, to allow the cells to mature and resume their regular function. (cells continue to proliferate and generate ECM, forming tissue)</td>
<td>• Inactivation or reduced activity of the cells.</td>
<td>• M: Loss of viable cell population, Change in phenotype of the cells • C: Construct does not mature correctly • M: Constructs do not mature within the predefined time. (lower proliferation rate of the cells)</td>
<td>• Contamination • Optimal culture conditions not maintained (37°C , 5% CO2, humidity, pH) • Incorrect media composition and conditions (material composition, expiration date, concentration, temperature)</td>
<td>• P: Follow sterile handling methods as described in the protocols. • D: Check for media turbidity or growth of contaminants (bacteria, fungus, virus) through optical; methods as described in the protocols • D: Checking for incubator warning signals and taking recommended actions • P: Check expiry date, composition of the culture media, color of the pH indicator and warm the media to 37°C before use.</td>
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|     |              |             | • Construct degrades (dissolves, breaks, loses strength) | • M: Construct collapse during maturation  
• C: Construct has incorrect mechanical strength  
• C: Construct does not mature correctly | • Insufficient volume of culture media (poor diffusion into constructs) | • P: Add necessary volume of media at specified intervals as described in the protocols (account for the cell proliferation rate).  
• P: Using rocking, shaking or perfusion mechanism to improve diffusion of media into constructs |
|     |              |             |                        | • Cells reaching their senescence | | Cells with lower passage numbers as described in the SOP should be used. |
|     |              |             |                        | • Incorrect media composition and culture conditions (material composition, pH, temperature) | | • P: Check composition of the culture media prior to use.  
• P: Use correct culture conditions (intensity of rocking/shaking, perfusion flow rate) |
|     |              |             |                        | • Decrosslinking of the bioink | | • D: Test and validate the stability of crosslinked bioink through pilot tests  
• P: Follow protocols and maintain specified conditions (regular media change, temperature, luminescence, |
Table 2: Failure modes and effects of functions in scaffold-based biofabrication

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| 10  | Preparation of polymer melt   | Mix the solid polymer (powder, pellets or flakes) with a solvent to make a solution with a viscosity that enables extrusion and load it into the printer. (If the polymer used is not in form of a solution, and is thermoplastic, it must be heated above its glass transition temperature to enable extrusion.) | • Polymer not completely dissolved | • M: Cannot accomplish extrusion  
• M: Discontinuous extruded filaments | • Incorrect methods or protocols used for preparation of polymer solution (material composition, concentration, temperature, mixing time) | • P: Follow specified protocol and methods of polymer preparation. |
|     |                               |                                                                              |                        |                                                                                                |                                                                                           |                                                                         |
|     |                               |                                                                              | • Polymer not completely melted | • M: Cannot accomplish extrusion  
• M: Discontinuous extruded filaments | • Incorrect temperature and duration of heating | • P: Check and maintain the temperature for the duration specified in the protocol. |
|     |                               |                                                                              |                        |                                                                                                |                                                                                           |                                                                         |
|     |                               |                                                                              |                        |                                                                                                |                                                                                           | • D: Check for the temperature of the to printing. |
|     |                               |                                                                              |                        |                                                                                                | • P: Follow regular maintenance and calibration schedule for heating element and sensors. |                                                                         |
Table 2 (continued)

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<tbody>
<tr>
<td>20</td>
<td>Store cell suspension</td>
<td>Store the prepared cell suspension in a sterile container prior to seeding on to the scaffold and hold the temperature (@ 37°C) to ensure</td>
<td>• Inactivation or reduced activity of the cells.</td>
<td>• M: Loss of viable cell population</td>
<td>• Contamination</td>
<td>• P: Follow sterile handling methods as described in the protocols.</td>
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<tr>
<td>30</td>
<td>Generate toolpath</td>
<td>Transforming the geometric data of the model into toolpath required to fabricate the structure by defining the position of the structure on the print container and assigning layer height and internal build geometry</td>
<td>• Toolpath cannot be generated</td>
<td>• M: Cannot accomplish bioprinting</td>
<td>• The geometrical data obtained is incomplete or the file is corrupt (STL files have voids)</td>
<td>• D: Simulate and verify toolpath before bioprinting</td>
</tr>
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<td></td>
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<td></td>
<td>• Incorrect toolpath is generated</td>
<td>• M: Scaffold of incorrect geometry is generated</td>
<td>• Incorrect layer height or other geometric parameters were assigned during toolpath generation</td>
<td>• P: Check and obtain complete geometrical model data as specified</td>
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<td></td>
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<td></td>
<td>• Unable to position the model in the print container</td>
<td>• M: Cannot complete printing the complete model</td>
<td>• Incorrect definition of print envelope</td>
<td>• P: Check and assign the correct type of print container</td>
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| 40  | Extrude layer| Extrude the polymer in the toolpath defined by the CAD model | • Polymer melt not ejected from the print reservoir | • M: Cannot print scaffold | • Clogging of the nozzle by external particulates or aggregates in the polymer melt | • P: Test and validate that the nozzle size is suitable for extruding the polymer.  
• P: Perform a purge before printing of each layer, clean the nozzle and the reservoir before every print cycle.  
• P: Regular maintenance of the pneumatic system.  
• P: Verify the polymer melt viscosity before start of print cycle.  
• D: Test and validate the printing parameters before starting the print cycle.  
• P: Follow regular maintenance and calibration schedule for pneumatic system and sensors.  
• P: Check and replace the sensor if faulty. |
|     |              |             | • Ejected polymer strands do not have an uniform cross-section (thinner or thicker) as per previously defined toolpath. | • M: Poor shape fidelity  
• M: Scaffold with undesired voids  
• M: Scaffold collapse during build.  
• C: Scaffold has incorrect mechanical strength | • Incorrect Extrusion Pressure |
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<td></td>
<td>• Compressor unable to attain the specified pressure</td>
<td>• D: Test and validate the printing parameters before starting the print cycle.</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts of the compressor</td>
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<td>• Polymer melt properties deteriorate overtime in the reservoir</td>
<td>• P: Follow the print protocols for the polymer (pressure, temperature, heating cycle time)</td>
<td>• D: Test and validate the printing parameters before starting the print cycle.</td>
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<td>• Leaks in the pressure delivery system (leaks in tubes, O rings and connectors)</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
<td>• P: Regular maintenance and replacement of malfunctioning parts (tubes, O rings and connectors)</td>
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<td>• The print container not retained in the specified location</td>
<td>• D: Check for the current position of the print container and take action to move to specified location if needed</td>
<td>• D: Check for the current position of the print container and take action to move to specified location if needed</td>
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<td>• The print container's position has been altered during the print cycle</td>
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<td>• D: Check for the current position of the print container, if the container is move pause the print cycle, take action to move to specified location and restart the print</td>
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<td></td>
<td>• The print cartridge was not completely secured in the print head</td>
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<td>• P: Check if the cartridge is secured firmly by locking it to the complete extent before start of print cycle</td>
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<td></td>
<td>• Nozzle height offset not completed successfully</td>
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<td>• P: Follow protocol to complete the nozzle height offset procedure</td>
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<td>• Incorrect polymer melt composition</td>
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<td>• D: Perform a test print to confirm the correct height offset and start the actual print cycle</td>
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<td>• P: Check for the polymer melt properties before starting the print cycle.</td>
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<td>• The ejected strands merge previously deposited strands.</td>
<td>• Incorrect type of print container used</td>
<td>• P: Check for the type of print container and take action to replace it with the specified container if needed</td>
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<td>• Incorrect characteristics of print surface</td>
<td>• D: Perform a test print (one layer) before printing the scaffold.</td>
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<td>• The viscosity of the polymer melt does not match specification</td>
<td>• D: Check of the consistency of the polymer melt by printing a test layer and take recommended action.</td>
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<td></td>
<td>• Incorrect Extrusion Pressure</td>
<td>• D: Test and validate the printing parameters before starting the print cycle.</td>
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<td>• P: Follow regular maintenance</td>
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<td>• Faulty heating system</td>
<td>• D: Check for the temperature of the print head in the software prior to printing.</td>
<td>• P: Follow regular maintenance and calibration schedule for heating element and sensors.</td>
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<td></td>
<td></td>
<td></td>
<td>• Deposited polymer does not stick to the previous layer (substrate if first layer) and moves</td>
<td>• M: Poor shape fidelity • M: Scaffold with undesired voids • M: Scaffold collapse during build. • C: Scaffold has incorrect mechanical strength</td>
<td>• D: Check of the consistency of the polymer melt by printing a test layer and take recommended action. • P: Stop and restart the print with the specified polymer melt</td>
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<td></td>
<td>• Incorrect toolpath</td>
<td>• The viscosity of the polymer melt does not match specification</td>
<td>• D: Simulate and verify toolpath before print cycle</td>
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<td>50</td>
<td>Seed scaffold</td>
<td>The sterile scaffold is placed in a fixture or the container as described in the protocol and cell suspension is added over it. The scaffolds are then left undisturbed for the specified duration to allow the cells to adhere to the scaffold. (methods of seeding may differ such as static and dynamic seeding)</td>
<td>• Scaffold not retained in the container during addition of cell suspension (scaffold moves or floats)</td>
<td>• Incorrect seeding fixture/container</td>
<td>• M &amp; C: Scaffolds do not mature correctly • M &amp; C: Non-Uniform cell distribution • M: Loss of viable cell population</td>
<td>• P: Follow protocols for the seeding methods (use specified container)</td>
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Table 2 (continued)

<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• D: Check for structural defects in the scaffold post-printing and use scaffolds that passed the test</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• P: Follow protocols while printing scaffold and take recommended action during printing when a defect is identified</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Incorrect volume of cell suspension or cell suspension prepared with incorrect specifications</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• P: Check for specifications of cell suspension (pH, cell concentration, volume) before seeding.</td>
<td></td>
</tr>
<tr>
<td>Sno</td>
<td>Process Step</td>
<td>Requirement</td>
<td>Potential failure mode</td>
<td>Potential Effects of Failure</td>
<td>Potential Causes of failure</td>
<td>Controls</td>
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</tr>
</tbody>
</table>
|     |              |             | • Inactivation or reduced activity of the cells. | • M & C: Scaffolds do not mature correctly  
• M & C: Non-Uniform cell distribution  
• M: Loss of viable cell population  
• M: Change in phenotype of the cells | • Incorrect seeding methods | • P: Follow protocols for the seeding and ensure that the scaffolds are left undisturbed in the specified environmental conditions (humidity, temperature, CO2) for the specified duration |
<p>|     |              |             |                        |                            | • Contamination | • P: Follow sterile handling methods as described in the protocols. |</p>
<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
</tr>
</thead>
</table>
| 60  | Invitro - Culture of scaffold constructs | Bioprinted construct containing viable cells are cultured in a laboratory setting for a predefined time, to allow the cells to mature and resume their regular function. (cells continue to proliferate and generate ECM thus forming tissue) The scaffold containing cells are cultured in the laboratory for a predefined time, to allow the cells to stabilize and continue their regular function. The cells continue to proliferate and generate ECM, forming tissue. | • Inactivation or reduced activity of the cells. | • M & C: Scaffolds do not mature correctly  
• M & C: Non-Uniform cell distribution  
• M: Loss of viable cell population  
• M: Change in phenotype of the cells  
• M: Constructs do not mature within the pre-defined time. (lower proliferation rate of the cells) | • Contamination  
• Optimal culture conditions not maintained (37°C, 5% Co2, humidity, pH)  
• Incorrect media composition and conditions (material composition, expiration date, concentration, temperature) | • P: Follow sterile handling methods as described in the protocols  
• P: Ensure the sterility of scaffolds prior to seeding  
• D: Test and validate the sterility of scaffolds through pilot test before seeding.  
• D: Check for media turbidity or growth of contaminants (bacteria, fungus, virus) through optical methods as described in the protocols  
• D: Checking for incubator warning signals and taking recommended actions  
• P: Check expiry date, composition of the culture media, color of the pH indicator and warm the media to 37°C before use. |
Table 2 (continued)

<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Incorrect seeding density</td>
<td>• D: Test and validate the seeding density required through pilot testing.</td>
<td>• P: Prepare and use cell suspension with the specified cell concentration.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Insufficient volume of culture media (poor diffusion into constructs)</td>
<td>• P: Add necessary volume of media at specified intervals as described in the protocols (account for the cell proliferation rate).</td>
<td>• P: Using rocking, shaking or perfusion mechanism to improve diffusion of media into constructs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Cells reaching their senescence</td>
<td></td>
<td>Cells with lower passage numbers as described in the SOP should be used.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Sno</th>
<th>Process Step</th>
<th>Requirement</th>
<th>Potential failure mode</th>
<th>Potential Effects of Failure</th>
<th>Potential Causes of failure</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Scaffold degrades</td>
<td>• M: Scaffold collapse during maturation</td>
<td>• Incorrect media composition and culture conditions (material composition, pH, temperature)</td>
<td>• P: Check composition of the culture media prior to use. • P: Use correct culture conditions (intensity of rocking/shaking, perfusion flow rate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(dissolves, breaks, loses strength)</td>
<td>• C: Scaffold has incorrect mechanical strength</td>
<td>• C: Scaffold does not mature correctly</td>
<td>• D: Test and validate the chemical stability of printed scaffold through pilot tests • P: Follow protocols and maintain specified conditions (regular media change, temperature, luminescence)</td>
</tr>
</tbody>
</table>
The potential failure modes and effects presented in the above tables are the ones that we have identified based on our lab experience, and the list may not be exhaustive. During a production run for making these tissues, there can be multiple other ways or modes by which a process step could fail. These FMEA should be considered as exploratory analyses to determine the possible modes of failure and preventative actions that can be taken to mitigate the risks associated with major common failures. A more thorough analysis can be performed when specific data on production of engineered tissue is accessible. FMEA described in this dissertation can be used as a framework to perform the risk analysis and a RPN number can be also be estimated in the future by assigning the severity, occurrence and detection numbers for the process steps based on the collected data.

2.4. Chapter Summary

Engineered tissue manufacturing is a complex process with many interconnected steps, each serving a different function. Within ETM, biofabrication is a set of processes serving different functions that enables creation of the physical tissue constructs. The IDEF0 model developed in this chapter provides a system-level mapping of ETM, highlighting all the processes involved, their interconnectivities, and also the criteria for defining process endpoints. The black box models focusing on the core biofabrication processes further enunciate the relationships between various process steps/functions and the flow of material, energy and information. Finally, the FMEA elaborates on the end requirement of these processes and identifies potential failure modes and strategies to mitigate such failures. Thus, using the models presented in this chapter can aid a manufacturer to scale up/out a lab based ETM system. FMEA presented in this chapter is based on the process steps which are presented as supporting
functions in the black box models. The supporting functions in turn are function nodes in the A1 level of IDEF0 model. Taken together the analysis presented in this chapter presents a hierarchical systems level model of both bioprinting-based and scaffold-based ETM systems.
CHAPTER 3: NON-DESTRUCTIVE QUALITY ASSESSMENT OF CONSTRUCTS AND MONITORING OF BIOPRINTING-BASED BIOFABRICATION

3.1. Introduction

The importance of developing quality monitoring tools for biofabrication processes towards acceleration of the scale-up/scale-out translation was briefly discussed in section 1.3. In-process and non-destructive monitoring modalities can enhance biofabrication process control and performance validation driving further innovation in manufacturing of cellular and tissue constructs. Such process control tools provide a stronger emphasis on the link between the tissue construct design and process development to ensure effective control of all critical quality attributes (CQA). In-process measurement tools help monitor the state of the biofabricated process and can assist in active control of the process to maintain a desired state. Biofabrication processes must include sensing mechanisms for the raw input materials – both living and non-living components, while enhancing the reliability of CQA measurement tools, preferably in real-time, and ensure that all process end-points are met to guarantee consistent quality of the output biofabricated product.

Examples of CQA end-points associated with living components of biofabricated tissue constructs include the viable cell volume (VCV), distribution of encapsulated cells within the biofabricated construct, and cellular state (e.g. differentiation potential of stem cells or assessing damage to cellular membranes) immediately post-fabrication. Other long-term end points include cellular proliferation rate within the construct and cellular/tissue functional characteristics. Such end-points are significantly affected by biofabrication process parameters such as extrusion
pressure, processing temperature, and processing time as well as bioreactor culture conditions for long-term culture among other factors [70–74]. Currently, the primary method available to monitor constructs in real-time during biofabrication processes is via live video feed or by capturing optical images at regular intervals during the process [75,76]. Identified deviations are captured into a process model and process parameters are adjusted appropriately to help achieve desired dimensional characteristics. Although feature dimensions play an important role in the functionality characteristics of a biofabricated construct, it is equally essential to monitor the living component of the construct – the cells, due to the inherent variability associated with biological components and its criticality to meeting the quality attributes of the construct design and functionality. Without the ability to assess or monitor the ‘state’ of the cellular components, all biofabrication processes will be limited in their translational ability, and it will not be possible to integrate them into a fully automated closed-loop controlled production system.

Currently, offline methods are used to assess the quality of bioprinted constructs, i.e. performed after the biofabrication process is completed. These methods primarily include histological and biochemical assays to evaluate viability and proliferation (e.g., LIVE/DEAD®, alamarBlue® L-Lactate®, MTT®), permeability (e.g., CultureCoat®, Caco-2), cell differentiation (e.g., Alizarin red staining), reproductive assays and morphological assays among others. These assays are fairly well established and standardized across literature [77–79]. However, these methods are also slow and expensive to use and are primarily meant for 2D flat plate cultures. From a biofabrication perspective, the limitation of these methods is that they are inherently destructive; constructs have to be fabricated first, then stained and sectioned. Not only is it time consuming and labor intensive, but the biological characteristics of the construct including cell morphology and function can also be potentially altered during the sectioning and
assaying process. As a result, these techniques cannot be used as real-time metrology tools to assess construct characteristics during the biofabrication process. New methods that are capable of evaluating the in-process state of cells and construct CQA are critical to the advancement of the manufacturing science of living biological systems.

There have been some recent efforts on the development of non-destructive quality assessment methods. For example, molecular probes have been investigated to detect biomarkers within the growing structures of cells or in the secreted extracellular matrix which can then be correlated with relevant CQA [80,81]. Another example is the use of light-emitting fiber-optics within bioreactors to quantify cell density by measuring the amount of scattered light [82]. An alternative mode of measuring key changes in the cellular constituents of a biofabricated construct is by assessing changes in the dielectric properties of cells within the construct post-fabrication. Dielectric impedance spectroscopy (DIS) involves measuring relative permittivity resulting from the dielectric response of cells to an alternating electric field applied across a range of frequencies. Indeed the dielectric properties of various types of cells have been studied in the past. DIS has been primarily used to monitor biomass of cell cultures, particularly in the bioreactor-based culture of mammalian cells, bacteria and insect cells for the production of recombinant proteins and viral vectors [83,84]. It has also been utilized in large vats to monitor the yeast-based fermentation of beer and wine [85,86]. Furthermore, DIS has been used in a few novel 3D applications such as in distinguishing temperature induced cell morphology changes in bioreactors [87] and identifying cancerous and healthy tissue [88].

This dissertation chapter focuses on the label-free and non-destructive DIS approach which can offer distinct advantages in assessing quality of 3D constructs during and after biofabrication. The chapter is designed to address the specific tasks presented in Objective 2 of
this dissertation. The DIS principle and theory, and a preliminary computational model to verify the dielectric behavior of cells encapsulated within a hydrogel are discussed in rest of this section. The material and methods used in the experimental studies to investigate the ability of DIS to monitor CQA of biofabricated constructs and characterize the relationships between the CQA and DIS parameters are described in section 3.2. The results of these studies are presented and discussed in section 3.3. Parts of the studies presented in this chapter have been previously been published in two conference proceedings [89,90] and a journal paper [91].

3.1.1. Dielectric properties of cells

In general, dielectric property of a material refers to its permittivity and conductivity constants in the presence of an alternating electric field. Cells comprise a permeable membrane which provides structural integrity and enables essential cellular functions such as osmosis. This non-conductive phospholipid bilayer membrane is surrounded by a conductive extracellular medium and intracellular cytoplasm, which makes living cells behave as electrical capacitors [92]. Molecules in the medium and cytoplasm can possess both positive and negative charges that are free floating as ions within the fluids. In the presence of an external applied alternating electric field, the ions within the medium and cytoplasm are displaced. In healthy cells with intact membranes, the cell membrane circumscribes cytoplasmic ions leading to a buildup of opposing charges on either side of the thin phospholipid bilayer. An intact cell membrane provides a separation between the accumulated positive and negative charge causing the cell to behave like a capacitor (Figure 14), with the permittivity dependent on the frequency of the alternating electric field [20]. This phenomenon is known as the Maxwell-Wagner effect [18,21]. Such interfacial polarization is not observed in non-viable cells which are often characterized by
a ruptured membrane resulting from necrosis or apoptosis [22]. In general, non-viable cells, cell debris, gas bubbles and other media components together have a negligible contribution to the permittivity [23,24], particularly within the frequency range in which cells are polarized. This distinction in permittivity characteristics of viable cells and damaged cells can be utilized to characterize the CQA of biofabricated constructs.

![Diagram](image)

**Figure 14:** An illustration of dielectric impedance spectroscopy principle

### 3.1.2 Theory of dielectric impedance spectroscopy

The dielectric properties of the cells described in the previous section are dependent on the frequency of the alternating electric field. The impedance characteristics including resistivity, permittivity and inductivity change in response to the applied frequency. In general impedance spectroscopy refers to the small-signal measurement of the linear electrical response of a material of interest and subsequent analysis of that response to yield information about the physiochemical properties of the system [93]. When the relative permittivity of cells is measured across a spectrum of frequencies, three notable dispersions are generated as presented in **Figure 15**. The \( \alpha \)-dispersion occurs at low frequencies in which intracellular and extracellular ions have adequate time to accumulate on either sides of the cell membrane resulting in higher interfacial polarization [94]. In this frequency region, there is a lack of appropriate dielectric
sensitivity necessary for quality monitoring. On the other end of the spectrum, $\gamma$-dispersion occurs wherein ions do not have enough time to polarize at the high frequencies resulting in low permittivity readings [95]. This region is primarily indicative of the permittivity of the media environment and is not suitable for quality monitoring. Between the $\alpha$-dispersion where the cell membrane is highly polarized and $\gamma$-dispersion where it is not, there is a steady decrease in permittivity with increasing frequency. This region of interest, usually characterized by an inverse sigmoid shape, is referred to as the $\beta$-dispersion. The $\beta$-dispersion is characterized by three important parameters – delta permittivity ($\Delta\varepsilon$), critical frequency ($f_c$) and Cole-Cole alpha ($\alpha$).

![Illustrative plot of permittivity of a cell mass as a function of input frequency](image)

**Figure 15:** An illustrative plot of permittivity of a cell mass as a function of input frequency

The difference between the permittivity in the high permittivity-low frequency region and low permittivity-high frequency region of the $\beta$-dispersion is referred to as the $\Delta\varepsilon$. The $\Delta\varepsilon$ is proportional to the total volume of viable cells as well as the mean radius of the cells present in the measurement volume \( \text{(Equation 1)} \) [96].
\[ \Delta \varepsilon = \frac{9 \cdot P \cdot r \cdot C_m}{4 \varepsilon_0} \]  

Equation 1

Where \( P \) is the volumetric fraction of viable cells (i.e., volume of material bounded by the cell membrane per unit measurement volume), \( r \) is the radius of nominally spherical cells, \( C_m \) is the cell membrane capacitance per unit area, and \( \varepsilon_0 \) is the permittivity of free space (8.854 x \( 10^{-12} \) F/m). The frequency corresponding to the midpoint of the \( \beta \)-dispersion slope is referred to as the critical frequency \( (f_c) \) \([87,97]\). The \( f_c \) is inversely proportional to the mean cell radius (Equation 2) and is expected to be characteristic of a given cell type regardless of the total viable cell volume \([98,99]\).

\[ f_c = \frac{1}{2\pi \cdot r \cdot C_m \left( \frac{1}{\sigma_i} + \frac{1}{2\sigma_0} \right)} \]  

Equation 2

`Where \( \sigma_i' \) is the internal cytoplasmic conductivity of the cell and \( \sigma_0' \) is extracellular conductivity. \( C_m, \sigma_i', \) and \( \sigma_0' \) are constants for a given cell type. The permittivity, determined at a frequency- \( f_c \), can be used as a measure of cell viability. The slope of the \( \beta \)-dispersion is referred to as the Cole-Cole \( \alpha \). The dimensionless \( \alpha \) corresponds to \( \tau \) (relaxation time) and the number of dipoles formed during the interfacial polarization across the cell membrane and has been shown to be related to cell size distribution within the measured volume \([87,100]\).

3.1.3. Finite element modelling

A simplified 2D multiphysics simulation (AC/DC Module, COMSOL, Stockholm, Sweden) was created to verify the dielectric response of cells encapsulated within alginate
constructs. The basic model was setup to capture the setup in later experimental studies. A cross-section of the simulation setup in the YZ plane is presented in Figure 16 (a). The electric signals were applied to platinum electrodes which were modeled as rectangles with dimensions and positions reflecting those of the actual DIS probe. Human adipose-derived stem cells (hASC) were used as the representative cell type in the modeling. Each cell was designed as two concentric circles (outer Ø = 22 μm and inner Ø = 21.9 μm) [101] to capture the double shell properties of hASC, and a total of 1500 cells were encapsulated within the alginate construct.

The electrodes and the cellular construct were enclosed within a rectangular envelope that served as the volume containing Hank’s balanced salt solution (HBSS). Permittivity and conductivity of all entities in the model were assigned values as published in the literature [35]. An electric field was generated by sending 1μA of current through the electrodes swept with frequencies between 50 - 20,000 kHz, and the permittivity of the model at those frequencies were obtained.

Figure 16 (b) and 16 (c) show the electric flux lines and the permittivity spectra after the alginate construct was introduced without and with 1500 cells, respectively. It is evident that the pattern of the electric field was altered and dispersion observed only in the presence of a cellular construct. This indicates that other entities in the model did not contribute to the permittivity dispersion at the measured frequency range. In the magnified inset in Figure 16 (c), it can be seen that the electric field lines passed through the construct and through some cells. This phenomenon is the result of the higher conductivity of the cell cytoplasm relative to HBSS. A trend of increasing $\alpha$ and $\Delta\varepsilon$ with an increase in the number of encapsulated cells was also observed. The observations from this preliminary 2D computational model in the context of evaluating 3D cellular constructs are in agreement with the Maxwell-Wagner effect and the theory of polarization discussed in Section 3.1.2.
3.2. Materials and Methods

The experiments in this chapter were designed to investigate and characterize the relationship between DIS parameters and CQA of 3D constructs under different scenarios via nine separate studies. In the first two studies, a DIS annular probe (Ø 12 mm; ABER, Aberystwyth, UK) was used, and for the latter seven studies, a DIS flush probe (Ø25 mm; ABER Instruments Ltd.). The annular probe was procured first and was used in feasibility studies that were used to establish experimental testing protocols. In an annular probe, the electrodes are designed as rings on the periphery of the cylinder of the probe. The electric field radiates from the cylindrical surface of the probe, perpendicular to the probe’s long axis. To make measurements of the 3D constructs, the probe has to be tilted at an angle directing the electric field towards the constructs. Although the annular probe was useful in assessing the basic DIS
characteristics of biofabricated constructs, it became evident early on that this setup would not lend well to real-time measurements during bioprinting in future. Hence, the decision was made to switch to the flush probe for rest of the studies. In the flush probe, the electrodes are located on the flat bottom of the cylindrical probe. The electric field envelopes a spherical volume of a radius of 40 mm below the electrodes, and the probe can make measurements when the construct is located within this electric field sphere. This mode of measurement can be beneficial to make measurements during bioprinting, between layers as the bioink is being deposited.

An overview of the experimental studies is presented in Figure 17. Studies 1-3 were designed to address the ST#2.1 of developing the DIS system setup and approach and assessing the relationship between CQA of bioink-based 3D constructs and DIS parameters. In Study 1 the relationship between permittivity measured at a single frequency of the electric field and concentration of cells within 3D alginate constructs was studied. In Study 2, the ability of DIS to determine and distinguish the β-dispersion characteristics between 3D alginate constructs with two different encapsulated cell types – hASC and osteosarcoma (MG63) – at two concentrations was investigated. In Study 3, the relationship between DIS parameters and % viability of hASC encapsulated in cast alginate 3D constructs with different total cell concentrations was mapped.

Studies 4-5 were performed to investigate how the changes in extrusion-bioprinting parameters affected the impedance spectral parameters in response to underlying changes in CQA of bioprinted constructs, addressing ST#2.2. In particular, the effect of non-optimal 3D-bioprinting parameters (high extrusion pressure and high print-head temperature) on the $f_c$ response of encapsulated cells was characterized in Study 4. In addition to the pressure and temperature, the effect of 3D-bioprinting processing time on the viability of hASC encapsulated
within alginate inside the print-head and the resulting DIS parameters from constructs bioprinted over time was assessed in Study 5.

Finally, studies 6-9 were designed to assess the correlation between cell viability and DIS parameters of extrusion-bioprinted constructs over time and scale as per ST#2.3. In Study 6, the ability of DIS to provide localized readouts of CQA in a bioprinted knee meniscus-shaped construct along the XY plane over 2 days of culture was assessed. In Study 7, the effect of layering in bioprinting on the CQA due to DIS signal superposition and attenuation was investigated. In Study 8, the effect of DIS signal attenuation due to the relative XYZ position of the construct with respect to the probe was studied in single layered constructs. Finally, in Study 9, the ability of utilizing DIS to map the CQA of a bioprinted meniscus-shaped construct along it’s semi-lunar geometry (XY plane) across layers (along Z) in culture over time was demonstrated.
Figure 17: Overview of the experimental studies to address Objective#2 (2.1 - 2.3)
<table>
<thead>
<tr>
<th>Objective 2.1</th>
<th>Study 1: Relationship between cell concentration and Corresponding cell permittivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG63 (1x10^6 cells/mL)</td>
</tr>
<tr>
<td></td>
<td>MG63 (2.5x10^6 cells/mL)</td>
</tr>
<tr>
<td></td>
<td>MG63 (4x10^6 cells/mL)</td>
</tr>
<tr>
<td></td>
<td>MG63 (6.5x10^6 cells/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study 2: Relationship Between cell type, cell concentration and Corresponding β-Dispersion Spectral Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>hASC (2x10^6 cells/mL)</td>
</tr>
<tr>
<td>hASC (5x10^6 cells/mL)</td>
</tr>
<tr>
<td>MG63 (2x10^6 cells/mL)</td>
</tr>
<tr>
<td>MG63 (5x10^6 cells/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study 3: Relationship Between Cell Viability Ratio and Corresponding β-Dispersion Spectral Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>hASC (5x10^6 cells) 100% viable</td>
</tr>
<tr>
<td>hASC (5x10^6 cells) 85% viable</td>
</tr>
<tr>
<td>hASC (5x10^6 cells) 50% viable</td>
</tr>
<tr>
<td>hASC (2.5x10^6 cells) 100% viable</td>
</tr>
<tr>
<td>hASC (4.25x10^6 cells) 100% viable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Objective 2.2</th>
<th>Study 4: Effects of 3D Bioprinting Process Parameters on CQA of 3D Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>Constructs bioprinted with optimal parameters</td>
</tr>
<tr>
<td>50°C</td>
<td>Constructs bioprinted with non-optimal parameters</td>
</tr>
<tr>
<td>hASC (5x10^6 cells)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study 5: Effects of Fabrication Processing Time on CQA of 3D Bioprinted Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C hASC (5x10^6 cells)</td>
</tr>
<tr>
<td>37°C hASC (5x10^6 cells)</td>
</tr>
<tr>
<td>37°C hASC (5x10^6 cells)</td>
</tr>
</tbody>
</table>

**Time**

- **Time = 0 hrs**
- **Time = 3 hrs**
- **Time = 6 hrs**
<table>
<thead>
<tr>
<th>Objective 2.3</th>
<th>Study 6: Monitoring Anatomical 3D Bioprinted Constructs in Culture Over Time and localized readouts of CQA in XY plane.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1.png" alt="Image" /> Meniscus construct bioprinted with optimal parameters hASC (2x10^6 cells) DIS of a bioprinted meniscus construct at 3 different zones and monitored over 2 days in culture</td>
</tr>
<tr>
<td>Study 7: Monitoring 3D Bioprinted Constructs in Culture Over Time with Layer by Layer measurements upon addition of Layers along the Z axis.</td>
<td>DIS of each layer after bioprinting. Study the superimposition of DIS signals up to three layers. Monitor the readings over 7 days in culture.</td>
</tr>
<tr>
<td></td>
<td><img src="image2.png" alt="Image" /> 1 Layer - hASC (5x10^6 cells) 2 Layers hASC (10x10^6 cells) 3 Layers hASC (15x10^6 cells)</td>
</tr>
<tr>
<td>Study 8: Effect of DIS signal attenuation on CQA readouts of a single Layer Bioprinted construct.</td>
<td>Study the DIS signal attenuation of one layered bioprinted construct by measuring at different distances in X, Y and Z directions.</td>
</tr>
<tr>
<td></td>
<td><img src="image3.png" alt="Image" /> 1 Layer - hASC (5x10^6 cells) Z 2, 4, 6, 8 mm in Z axis Z' X' Y' X Y 0, 10 mm in X &amp; Y axis</td>
</tr>
<tr>
<td>Study 9 Monitoring Anatomical 3D Bioprinted Constructs in Culture Over Time and Layer by Layer measurements with addition of Layers along the Z axis and localized readouts of CQA in XY plane.</td>
<td>DIS of each layer after bioprinting. Study the superimposition of DIS signals up to three layers. Monitor the readings over 7 days in culture.</td>
</tr>
<tr>
<td></td>
<td><img src="image4.png" alt="Image" /> &amp; DIS of a bioprinted meniscus construct at 3 different zones and monitored over 7 days in culture</td>
</tr>
</tbody>
</table>
3.2.1. Cell expansion

For studies 1-3, MG63 (ATCC® CRL-1427) were cultured in Eagle's Minimum Essential Medium (EMEM, no Ca no Mg; ATCC, Manassas, VA) with 10% heat-inactivated fetal bovine serum (FBS), and hASC (StemPro® R7788115, Thermo Fisher Scientific, Waltham, MA) in MesenPro RS basal medium with growth supplement and 1% L-Glutamine (Thermo Fisher Scientific), both in T75 cell culture flasks at 37°C (5% CO₂). The cells were passaged when confluency was approximately 70%. Trypan blue viability assay (Life Technologies, Grand Island, NY) and a hemacytometer were used to count the number of viable cells during cell passaging, and during the preparation of individual cell-encapsulated constructs.

For studies 4-9, owing to the large number of cells required for the experiments, the hASC were cultured in stacked flasks. The hASC were cultured with MesenPro RS basal medium containing growth supplement and 1% L-Glutamine in a Corning® CellBIND® Surface HYPERFlask® (10-layer, 1720 cm² surface area; Corning, NY) at 37°C (5% CO₂). The flask was primed with 560 mL of media and cultured up until a cell confluency of 80% was achieved. The cells were harvested by adding 60 mL of 0.25% trypsin EDTA (Thermo Fisher Scientific) after two washes with 100 mL of phosphate-buffered saline (PBS). The trypsinised cell suspension was neutralized with media and centrifuged to obtain cell pellets.

3.2.2. Bioink preparation

Batches of 30 mL 2% w/v alginate solution were prepared by mixing 0.6 g of sterilized sodium alginate powder (WillPowder, Miami Beach, FL) with 29.4 mL of sterilized PBS and sonicated in an ultrasonic water bath at 60 Hz for 2 hours [102]. For Study 1, the cell-encapsulated bioinks with four test concentrations (1, 2.5, 4, and 6.5 x 10⁶ cells/mL) were
prepared by mixing 1 mL of the alginate hydrogel with the appropriate MG63 cell pellet obtained after centrifuging. For Study 2, the same method was used to prepare the bioinks, with cell pellets of MG63 and hASC (1 and 5 x 10^6 cells/mL) obtained after centrifuging. For study 3, the bioinks were prepared with varying combinations of hASC number and % cell viability (n = 5 per group) as shown in Table 3. For bioinks with 50% and 85% cell viabilities (groups B and D, respectively), the appropriate proportion of cells were rendered non-viable through heat inactivation before mixing with alginate solution. The bioinks for studies 4 and 5 were prepared by suspending 5 x 10^6 hASC in 2 mL of 2% alginate solution. 20 x 10^6 hASC were suspended in 5 mL of 2% alginate solution for the bioink in Study 6. Bioinks for Study 7 were prepared by mixing 2% w/v alginate hydrogel with hASC at a concentration of 3.7 x 10^6 cells/mL. This concentration was determined based on the estimated volume of bioink required to print individual layers containing a constant number of 5 x 10^6 hASC. The volume of bioink prepared for one, two and three layered constructs was 1.35, 2.7 and 4.1 mL, respectively. 1.35 mL of bioink containing 3.7 x 10^6 hASC/mL was prepared for Study 8 to print each one layered construct, and 7 mL of bioink containing 5 x 10^6 hASC/mL was prepared for Study 9.

Table 3: Bioink formulations with varying number of hASC and cell viability for Study 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Total number of hASC</th>
<th>Number of viable hASC</th>
<th>Corresponding cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>5 x 10^6</td>
<td>5 x 10^6</td>
<td>100%</td>
</tr>
<tr>
<td>Group B</td>
<td>5 x 10^6</td>
<td>2.5 x 10^6</td>
<td>50%</td>
</tr>
<tr>
<td>Group C</td>
<td>2.5 x 10^6</td>
<td>2.5 x 10^6</td>
<td>100%</td>
</tr>
<tr>
<td>Group D</td>
<td>5 x 10^6</td>
<td>4.25 x 10^6</td>
<td>85%</td>
</tr>
<tr>
<td>Group E</td>
<td>4.25 x 10^6</td>
<td>4.25 x 10^6</td>
<td>100%</td>
</tr>
</tbody>
</table>
3.2.3. Casting of 3D cellular constructs for studies 1, 2 and 3

For studies 1 and 2 in which cell concentration was the factor, the following method was used to make the 3D cellular constructs. For each concentration, 3D constructs (n = 6 per group) were cast from 1 mL of the prepared bioink by depositing 500 µL bioink in each well of a 24-well plate and crosslinking with 500 µL of 2% CaCl2 for 5 minutes, resulting in a Ø12 X 5 mm thick construct. To improve the degree of crosslinking, the constructs were immersed into a 3 mL bath of 2% CaCl2, then gently swirled in 3 mL of deionized (DI) water prior to evaluation.

For Study 3 in which the cell number and viability of the cells were factors, the 3D constructs were fabricated by casting 500 µL bioink in wells of a twelve-well culture plate and crosslinking with 500 µL of 2% CaCl2 for 5 minutes, resulting in Ø18 X 3 mm thick constructs (n = 3 per group). The constructs were then immersed in wells containing 1 mL of 2% CaCl2 to improve their degree of crosslinking and strength. Finally, these constructs were washed in wells containing 3 mL of deionized (DI) water prior to DIS evaluation.

3.2.4. 3D Bioprinting of cellular constructs for studies 4-5

For Study 4, cuboids of 20 x 20 x 3 mm were 3D bioprinted with optimal and non-optimal parameters (Table 4; n = 3 per group) to obtain ideal (i.e., high cell viability) and non-ideal (i.e., low cell viability) constructs, respectively. The cuboid was modeled as a STL file in MagicsRP (Materialise NV, Leuven, Belgium) and sliced with appropriate layer height in BioplotterRP (EnvisionTEC, Gladbeck, Germany). The sliced file was processed in Visual Machines (EnvisionTEC), and appropriate set of bioprinting process parameters (Table 4) were assigned. 2 mL of bioink was filled into the stainless steel cartridge and bioprinted on a 3D-Bioplotter (Manufacturer series, EnvisionTEC). Each layer of the bioprinted construct was partially crosslinked by micro-pipetting sterile 1% CaCl2 solution over the printed construct. The
entire 2 mL bioink was bioprinted to complete each ideal and non-ideal construct. Post printing, each construct was immersed in 3 mL of 2% CaCl$_2$ in a well of a 6-well plate for 5 minutes to improve the degree of crosslinking. Finally, the constructs were washed in wells containing 3 mL of DI water prior to DIS evaluation.

**Table 4**: 3D Bioprinting process parameters for fabricating ideal and non-ideal constructs for studies 4 and 5.

<table>
<thead>
<tr>
<th></th>
<th>Non-optimal Parameters (Study 3)</th>
<th>Optimal Parameters (Studies 3 and 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of hASC</td>
<td>5 x 10$^6$</td>
<td>5 x 10$^6$</td>
</tr>
<tr>
<td>Nozzle diameter</td>
<td>0.2 mm</td>
<td>0.3 mm</td>
</tr>
<tr>
<td>Extrusion pressure</td>
<td>0.12 N/mm$^2$</td>
<td>0.03 N/mm$^2$</td>
</tr>
<tr>
<td>Print-head temperature</td>
<td>50°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Print-head speed</td>
<td>18 mm/sec</td>
<td>12 mm/sec</td>
</tr>
<tr>
<td>Laydown orientation</td>
<td>0°/ 90°</td>
<td>0°/ 90°</td>
</tr>
<tr>
<td>Needle height offset</td>
<td>0.5 mm</td>
<td>0.2 mm</td>
</tr>
<tr>
<td>Number of layers</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

For Study 5, the same 3D model (20 x 20 x 3 mm) was bioprinted using only the optimal parameters (**Table 4**) out of the entire 2 mL of bioink containing 5 x 10$^6$ cells at 37°C. The first sample was bioprinted immediately (time 0), and the second and third samples were printed after 3 hours and 6 hours, respectively. Each sample was evaluated via DIS immediately after it was bioprinted and crosslinked.
To assess the cell viability through offline fluorescent labeling methods and to help compare spectral signal characteristics, all constructs were subjected to the LIVE/DEAD® assay (Life Technologies, Carlsbad, CA) following DIS measurements. Each construct was placed in 1 mL PBS containing 0.5 µl calcein AM and 2 µl EthD-I and incubated for 10 minutes and imaged using a fluorescence microscope (Leica DM5500B, Leica Microsystems, Wetzlar, Germany). For each construct, images were obtained from five randomly selected sections. All images were binarized and quantified using “Analyze Particles” feature in ImageJ [103].

3.2.5. Digital modeling and 3D Bioprinting of knee meniscus for study 6

3D model of a human medial knee meniscus was constructed from MRI scan of the right knee joint of an unidentified patient. The MRI DICOM files were processed in Mimics Research (v18, Materialise NV, Belgium) to create the STL file. The model was sliced with a layer height of 1 mm, and positioned on the platform using BioplotterRP. The file was then processed for bioprinting and assigned optimal process parameters (Table 4) in Visual Machines. Using the 3D-Bioplotter, 5 mL of the bioink containing 20 x 10^6 hASC was bioprinted following the meniscus STL geometry in a standard petri dish with a total of 6 layers, with 1% CaCl\(_2\) solution micro-pipetted for crosslinking after every two layers. The completed construct was then immersed in 15 mL of 2% CaCl\(_2\) solution for 10 minutes to improve the crosslinking. Finally, the construct was cultured (37°C, 5% CO\(_2\)) in a petri dish in 15 mL of Mesenpro containing 10% alamarBlue (aB) reagent (Thermo Fisher Scientific) to assess hASC proliferation over 2 days, with the first reading taken 4 hours after bioprinting completion. Using the same procedure, 5 mL of acellular 2% alginate solution was printed to serve as a control for the aB assay. Each media change for the cellular meniscus and acellular control contained 10% v/v of the aB reagent. At each time point, three 1mL samples were pipetted from each petri dish into a
standard 24-well plate, and the absorbance was measured using a microplate reader (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths of 570 nm and 600 nm, respectively. The absorbance data was converted to and is reported as % aB reduction.

### 3.2.6. 3D Bioprinting of cellular constructs for studies 7 and 8

Study 7 was designed to evaluate the effect of layer addition on the measured CQA and corresponding IDS parameters. Three types of constructs (one layered, two layered and three layered) (n = 3 per group) were bioprinted using BioAssemblyBot™ (AdvancedSolutions Life Sciences, Louisville, KY). The constructs were modeled as a cuboidal (20 x 20 x 4.5 mm) STL file in TSIM® software (Advanced Solutions Life Sciences). The layer height was set to 1.5 mm and post-processing was performed to assign other printing parameters. Each layer of the construct was experimentally designed to have dimensions of 20 x 20 x 1.5 mm containing 5 x 10^6 hASC. One, two and three layered constructs (n = 3 per group) were bioprinted using an extrusion pressure of 0.02 N/mm^2 at 37°C through a nozzle with 0.26 mm internal diameter at a nozzle traverse speed of 10 mm/s. The constructs were crosslinked after deposition of each layer by pipetting 1 mL of 1% CaCl_2 solution. Post printing, each construct was immersed in 3 mL of 2% CaCl_2 in a six-well plate for 5 minutes to improve the degree of crosslinking. The first set of DIS readings (Day 1) were taken immediately after. The constructs were then cultured (37°C, 5% CO_2) in a six-well plate with Mesenpro (in 4, 5 and 6 mL for one, two, and three layered constructs, respectively) containing 10% alamarBlue (aB) reagent (Thermo Fisher Scientific) to assess hASC proliferation over 7 days, with the first reading taken 4 hours post-bioprinting. The constructs were subject to DIS measurement on alternate days for 7 days in culture. On each reading day, the aB assay was performed on the constructs following the procedure described in section 3.2.4 immediately following the DIS assessment. For each layered group, an acellular
construct of the same geometry was used as a control for the aB assay. For Study 8, one layered constructs (n = 3) were bioprinted following the same procedure as discussed above.

3.2.7. 3D Bioprinting of knee meniscus for study 9

The 3D model of a human knee meniscus as described in section 3.2.5 was bioprinted using the BioAssemblyBot™. The layer height of the model was set to 1.5 mm, and it was bioprinted with the same parameters as provided in section 3.2.6. One, two and three layered meniscus constructs were bioprinted with bioink containing 5 x 10^6 hASC/mL. The meniscus constructs were cross-linked after each layer using 1 mL of 1% CaCl_2 and were then further cross-linked by immersing into 5 mL of 2% CaCl_2 post-bioprinting. The constructs were cultured (37°C, 5% CO_2) in a 60 mm petridish with 4, 6 and 8 mL of Mesenpro containing 10% alamarBlue (aB) reagent (Thermo Fisher Scientific) for one, two, and three layered constructs, respectively, to assess hASC proliferation over 7 days. The constructs were subject to DIS measurements on alternate days starting Day 1 for 7 days. On each reading day, an aB assay was performed on the constructs right after the DIS measurements using the procedure described in section 3.2.4. For each group, an acellular construct bioprinted with the same geometry was used as a control for the aB assay.

3.2.8. DIS (relative permittivity) setup and assessment methodology using an annular dis probe for studies 1-2

The permittivity spectra of constructs were measured using an annular probe (Ø 12mm; ABER, Aberystwyth, UK) as illustrated in Figure 18. Test samples were exposed to the electric field established by the two sets of platinum electrodes on the probe. The changes in electric
field due to the capacitance of cells were amplified and processed into digital signals which are recorded by the probe system software as permittivity and conductivity readings.

![Diagram of DIS experimental setup for annular probe]

**Figure 18:** Illustration of the DIS experimental setup for annular probe

During assessment, the constructs were located on a custom 3D-printed nylon fixture to eliminate any positional error during repeated measurement trials. The nylon fixture, with a 1 mm deep cylindrical recess to accommodate the construct, was designed to fit inside a 100 mL standard low form glass beaker (Pyrex, Corning, NY). During each measurement, the construct was gently introduced into the beaker filled with 75 mL of HBSS (Lonza) and located on the fixture. The probe was then inserted into the beaker and was set to an orientation angle of 65.1° with respect to the horizontal plane using a standing fixture. This angle ensured that the construct remained within the electric field of the probe. Prior to introducing each construct, the total capacitance of the setup was measured and zeroed. The measured capacitance was scaled by the
probe constant to reflect the permittivity of the constructs. Therefore, the reported permittivity values are relative to the setup conditions in each experimental run.

For Study 1, capacitance and conductivity of the setup with MG63-alginate construct within HBSS was measured at a frequency of 580 kHz that is the default recommended value for mammalian cell culture (ABER). As mentioned earlier, the reported permittivity values are relative to the setup conditions in each experimental run, while the reported conductivity values are absolute. Relative permittivity and absolute conductivity data was recorded every 4 seconds over a period of 15 minutes, and average values were reported for each construct during each experimental run.

For Study 2, a frequency scan ranging from 50 kHz to 20,000 kHz was performed on each construct (n = 6), and the resulting capacitance readings were used to characterize the β-dispersion of the constructs. The permittivity values at different frequencies are reported as an average of the readings over a 15-minute interval. The β-dispersion curve was constructed by plotting the average permittivity at different frequencies, and Δε, f_c, and α were determined from this plot.

### 3.2.8. DIS (relative permittivity) setup and assessment methodology using flush probe for studies 3-9

The dielectric permittivity spectra of the constructs were measured using a DIS flush probe for studies 3, 4, 5, 6, 7 and 9 (Ø25 mm; ABER Instruments Ltd.) as illustrated in Figure 19. Test samples were exposed to the electric field established by the two sets of platinum electrodes on the bottom of the probe, and changes in electric field due to the capacitance of cells
were amplified, processed and recorded as permittivity readings across the default frequency scan (50 – 20,000 kHz) using FUTURA SCADA (ABER).

**Figure 19:** Measurement setup for the assessment of relative permittivity of bioprinted 3D constructs

During each experiment in studies 3-5 and 7, the construct was gently placed underneath the probe (electrodes) inside a glass beaker (Pyrex, Corning, NY) containing 125 mL of HBSS such that the probe’s bottom face was 2 mm above the construct’s top surface. For Study 6, measurements were taken at three different locations along the meniscus construct’s semilunar profile. In each experiment, the total capacitance of the setup was measured and zeroed prior to introducing the construct. The measured capacitance was normalized using the probe constant to obtain the permittivity of the constructs.

For Study 8, DIS measurements were made on the constructs at different distances in X, Y and Z directions as presented in **Figure 20.** The construct was positioned at 4 different points on XY plane (x = 0, y = 0; x = 10 mm, y = 0; x = 10 mm, y = 10 mm; x = 0, y = 10 mm), such that the center of top face of the construct was at the specified coordinates from the center of the bottom face of the probe. DIS measurements were made at four different Z height offsets (2, 4, 6, 8 mm) of probe bottom face from the top surface of the construct for all four points on the XY
plane. In Study 9, DIS measurements were made on one, two and three layered meniscus constructs at three different zones along the construct’s semilunar profile.

Figure 20: DIS measurement positions at different X, Y and Z distances of the construct center from the probe center for Study 8.

A standard frequency scan of 50 - 20,000 kHz was performed on each construct, and data from the eleven preset frequencies between 150 - 2500 kHz (174, 224, 287, 368, 473, 607, 779, 1000, 1284, 1648 and 2115 kHz), as recommended for mammalian cell culture, was used for β-dispersion characterization [94,104]. β-dispersion curves of the constructs were graphed by plotting the relative permittivity against log scale frequency. The permittivity values at different frequencies are reported as an average of the permittivity readings over a 15-minute measurement interval. The $\Delta \varepsilon$, $f_c$, and $\alpha$ values were determined from these β-dispersion curves.
The $\Delta \varepsilon$ was calculated as the difference in relative permittivity between the low-frequency high-plateau and high-frequency low-plateau regions of the curve. The $f_c$ was determined by fitting a fifth-degree polynomial to the decline region of the curve and solving for the frequency at a relative permittivity value of $\Delta \varepsilon/2$. $\alpha$ was calculated as the slope of the decline region of the curve.

### 3.2.9. Experimental Design

In Study 1, a two-way analysis of variance (ANOVA) was performed on the measured permittivity values with cell concentration and day of the experiment as factors at a significance level $\alpha = 0.05$ to determine the statistical significance (JMP Pro, Cary, NC). Tukey’s HSD post hoc tests were then performed ($\alpha = 0.05$) to determine significant differences between constructs with different cell concentrations.

In Study 2, a two-way ANOVA was performed on the $\beta$-dispersion parameters ($\Delta \varepsilon$, $f_c$ and $\alpha$) with cell type and cell concentration as factors ($\alpha = 0.05$). Following ANOVA, Tukey’s HSD post hoc tests were performed ($\alpha = 0.05$) on $\Delta \varepsilon$ and $\alpha$ values to determine significant differences between constructs with different cell type-cell concentration combinations.

In Study 3, a one-way ANOVA and Tukey’s HSD post hoc tests were performed on $\Delta \varepsilon$ and $\alpha$ with cell number-cell viability groups (Table 3) as an independent variable. A one-way ANOVA and Tukey’s HSD post hoc tests were performed on $\Delta \varepsilon$ and $\alpha$ with a given set of bioprinting parameters (Table 4) as the independent variable for Study 4.

In Study 7, a two-way ANOVA was performed on the $\Delta \varepsilon$ with number of layers in the construct and day of DIS measurement as factors ($\alpha = 0.05$).
In Study 8, a three-way mixed level ANOVA was performed on the $\Delta \varepsilon$ with the aforementioned X-Y-Z coordinates as factors ($\alpha = 0.05$).

3.3. Results and Discussion

3.3.1. Study 1: Relationship between MG63 cell concentration in alginate hydrogel constructs and corresponding permittivity

Study 1 was designed to evaluate if the changes in permittivity of 3D hydrogel constructs with different concentrations of cells can be measured at a single frequency. The average relative permittivity of the MG63 alginate constructs recorded for the four cell concentration levels is represented in Figure 21. Prior to the experiments, the relative permittivity of alginate constructs without cells were measured and found to have no effect on permittivity. The relative permittivity increased with the increase in cell concentration, and the relationship was linear ($R^2 = 0.986$). The results corroborate the fundamental theory that the permittivity increases with increase in cell number [105].

Two-way ANOVA results demonstrate that the cell concentration had a statistically significant effect ($p < 0.001$) on the permittivity signals, whereas the day of experiment and its interaction with cell concentration were not significant. The lack of statistical significance associated with the experimental time point indicates the measurement reproducibility and confirms that the permittivity was not affected by the setup or experimental variability. Post hoc Tukey HSD analysis show that the permittivity values were significantly different ($p < 0.001$) for all pairwise comparisons of the four cell concentration levels. This confirms that the dielectric signatures of 3D alginate constructs with different encapsulated-MG63 cell concentrations are distinct, and can be detected by DIS. Such a DIS strategy can be used to monitor the proliferation of cells encapsulated in 3D hydrogel constructs.
Figure 21: Average permittivity of MG63-alginate hydrogel constructs measured across different cell concentrations. Error bars denote standard deviation.

In addition to permittivity of the constructs, the conductivity of the media containing the construct was also measured, and the differences in media conductivity were not significant between different cell concentrations. The average conductivity across all experiments was 9.937 ± 0.391 mS/cm. The conductivity of the medium is dependent on its ionic composition which can be potentially affected by changes in temperature [106] and excretion of charged metabolites by cells [107,108]. The variations in conductivity were negligible because the temperature of the setup was constant at room temperature and relatively quick measurements meant that the metabolite concentrations in the media barely altered over the 15 minute measurement interval.
3.3.2. Study 2: Relationship between cell type and cell concentration and corresponding β-dispersion characteristics

Study 2 was designed to evaluate the effect of cell concentration and cell type on DIS parameters. Constructs of all four combinations of cell type and concentration were subject to relative permittivity measurements at 25 different frequencies between 50-20,000 kHz. The average relative permittivity of the constructs was plotted against their corresponding measurement frequency to obtain the β-dispersion curves shown in Figure 22. The average values of β-dispersion parameters determined from these plots are reported in Table 5. It can be observed that these experimentally-determined curves closely resemble the inverted sigmoid shape of the typical β-dispersion curve reported in the literature, characterized by two plateaus separated by a steep frequency dependent region [95].

![Figure 22](image)

**Figure 22:** Characteristic β-dispersion Plots obtained from alginate constructs with all cell type-cell concentration combinations. β-dispersion characteristics of 5 x 10⁶ hASC are presented in the plot as an example.
As usual, $\Delta \varepsilon$ was calculated as the difference in permittivity between the low-frequency high-plateau and high-frequency low-plateau regions. The $f_c$ was determined by fitting a sixth degree polynomial to the recorded permittivity data in the decline phase of the $\beta$-dispersion curve and solving for the frequency at a relative permittivity value of $\Delta \varepsilon/2$. As an example, the polynomial equation fitted for the $\beta$-dispersion curve of $5 \times 10^6$ hASC is given below (Equation 3).

$$\text{Relative Permittivity} = (2E-18)x^6 - (1E-14)x^5 + (3E-11)x^4 - (5E-08)x^3 + (4E-05)x^2 - 0.0206x + 4.5341$$  \hspace{1cm} \text{Equation 3}

Where, $x = \log$ (frequency). $\alpha$ was calculated as the slope of the steep region of the $\beta$-dispersion curve.

### Table 5: $\beta$-dispersion parameters of constructs with MG63 and hASC

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>Cell type</th>
<th>$\Delta \varepsilon$ (pF/cm)</th>
<th>$f_c$ (kHz)</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x $10^6$/mL</td>
<td>MG63</td>
<td>0.346</td>
<td>441.86</td>
<td>-0.358</td>
</tr>
<tr>
<td>5 x $10^6$/mL</td>
<td>MG63</td>
<td>0.877</td>
<td>435.86</td>
<td>-1.007</td>
</tr>
<tr>
<td>1 x $10^6$/mL</td>
<td>hASC</td>
<td>0.358</td>
<td>313.01</td>
<td>-0.455</td>
</tr>
<tr>
<td>5 x $10^6$/mL</td>
<td>hASC</td>
<td>1.923</td>
<td>300.67</td>
<td>-2.260</td>
</tr>
</tbody>
</table>

Results from two-way ANOVA show that the cell type, cell concentration and their interaction had a statistically significant effect ($p < 0.05$) on $\Delta \varepsilon$ (Figure 23) and $\alpha$. The differences in $f_c$, however, were not significant. Post hoc Tukey HSD analysis show that the $\Delta \varepsilon$ and $\alpha$ were significantly different ($p < 0.05$) for all pairwise comparisons with the exception of 1 x $10^6$ MG63 versus 1 x $10^6$ hASC. The lack of statistical significance, which indicates the inability of the method to distinguish between the two cell types at 1 x $10^6$/mL concentration, can be attributed to the sensitivity threshold of the DIS probe. This is corroborated by observations.
from Figure 22 wherein the $\beta$-dispersion curves of constructs of both cell types with a concentration of $1 \times 10^6$ cells/mL were very similar over the scan frequency range. The $\beta$-dispersion characteristics of any biological sample subject to DIS depend on the physiological state of the cells and cell anatomy [109]. For instance, $\Delta\varepsilon$ is proportional to the total viable volume of cells as well as the mean radius of the cells present in the measurement volume (Equation 1) [109].

![Figure 23: $\Delta\varepsilon$ of constructs with all cell type-cell concentration combinations. Error bars denote standard deviation, * represent statistically significant difference ($p < 0.05$).](image)

Figure 23 clearly shows that the $\Delta\varepsilon$ was higher at higher cell concentration, and also higher for hASC ($\varnothing = 22 \, \mu m$) compared to MG63 ($\varnothing = 14 \, \mu m$). Hence, if DIS is used to monitor constructs in culture, an increase in $\Delta\varepsilon$ over time will signify cellular proliferation within the construct. On the contrary, $f_c$ of a $\beta$-dispersion curve is inversely proportional to the mean radius (Equation 1) [110]. The average $f_c$ of MG63 cells was approximately 440 kHz whereas $f_c$ of hASC was 310 kHz. Although not statistically significant, likely due to the relatively large
variations in $f_c$ values calculated from individual $\beta$-dispersion curves that can be attributed to the sensitivity of the DIS probe and the experimental setup, this trend follows the theoretical relationship that $f_c$ decreases with increasing cell radius.

3.3.3. Study 3: Relationship between cell viability ratio and corresponding $\beta$-dispersion spectral parameters

Study 3 was designed to evaluate if only viable living cells contributed to the DIS signals. Five groups of cast constructs containing hASC (Table 3), each with a different combination of cell number and cell viability, were subject to DIS evaluation. In theory, the characteristic DIS spectra result from the polarization of only healthy viable cells. Accordingly, these five combinations of cell number and cell viability were chosen to test the null hypothesis that the permittivity ($\Delta\varepsilon$) of constructs containing the same number of viable cells (and corresponding cell volumes, $P$, in Equation 1) are not significantly different even when their %viability (resulting from a different total number of viable and non-viable cells) is different. Groups A and B contained the same total number of cells, but 100% of the cells in group were viable compared to only 50% viable cells in Group B. Similarly, Groups A and D contained the same total number of cells, but only 85% of cells in Group D were viable. In theory, $\Delta\varepsilon$ of Group A should be higher than those of Groups B and D, and Group B should have the lowest $\Delta\varepsilon$ among the three. In contrast, Groups B and C contained different total number of cells, but the total number of viable cells in both was the same. The same was true about Groups D and E which contained different total number of cells but the same number of viable cells. In theory, the $\Delta\varepsilon$ of each of Groups B and C and Groups D and E should not be significantly different. The $\beta$-dispersion plots and corresponding $\Delta\varepsilon$ and $\alpha$ of the five groups are presented in Figure 24.
Figure 24. Comparison of β-dispersion parameters for constructs with varying hASC viabilities. Error bars denote standard deviation, * represents statistically significant difference (p < 0.05) between groups.

Results of one-way ANOVA show that the bioink group (cell number and cell viability combination) had a significant effect (p < 0.05) on both ∆ε and α. Individual comparisons of groups via Tukey’s HSD post-hoc tests showed that ∆ε of Groups A and B as well as of Groups B and D were significantly different (p < 0.05), but those of Groups B and C as well as of Groups D and E were not. This result corresponds with the theoretical relationships that were discussed above and confirms that ∆ε depends only on the viable number of cells and not the total number of cells in the construct. Although ∆ε of group A was 7% higher than that of Group D, the difference was not statistically significant. This lack of statistical significance can be attributed, in part, to the sensitivity of the probe in its current design and inherent variability associated with the manual fabrication and cell counting procedures used to make and assess the constructs. The critical frequency, $f_c$, of the constructs was also evaluated and was found to not be significantly different between the groups. This observation corresponds to the theory that $f_c$ of constructs remains the same for a given cell type irrespective of the volume of viable cells.
These results confirm the ability to detect changes in $\Delta \varepsilon$ and $\alpha$ corresponding to underlying changes in cell viability, keeping in mind the resolution of the current DIS probe and system wherein differences in cell viability of 15% or less cannot be detected with statistical significance. Taken together, results of studies 2 and 3 demonstrate the ability to non-destructively monitor the proliferation and viability of cells within maturing 3D constructs in culture by tracking $\Delta \varepsilon$ and $\alpha$ over time via DIS.

3.3.4. Study 4: Effects of 3D Bioprinting process parameters on CQA of 3D constructs and their correlation with $\beta$-dispersion spectral parameters

The effects of 3D bioprinting process parameters on cellular quality attributes have been widely studied, and it is known that variations in critical process parameters can adversely affect these attributes [111,112]. Study 4 was designed as a simulated scenario to investigate the relationships between DIS parameters and CQA of constructs when the 3D bioprinting process is operated with or without optimal process parameters. From a practical perspective, the results would demonstrate whether monitoring DIS parameters of the bioprinted construct could help identify any process deviations from preset optimal conditions.

Representative Live/Dead images of the constructs (20 x 20 x 3 mm, 5 x 10^6 hASC) bioprinted with optimal and non-optimal process parameters (Table 4) are presented in Figure 25(a) and 25(b) respectively. The constructs in the optimal parameters group had higher cell viability ($87 \pm 1\%$) than constructs in the non-optimal parameters group ($50 \pm 1\%$). These viability results are in accordance with extrusion-based bioprinting literature wherein increase in extrusion pressure has been demonstrated to adversely affect cell viability; viability dropped from 85% to 50% as the pressure was increased from 0.03 to 0.27 N/mm^2 [111]. This decrease is
attributed to irreversible cell injury caused by high pressure which leads to cell necrosis and apoptosis. For the non-optimal group in this study, primarily, the 0.12 N/mm² extrusion pressure, which is above the recommended range of 0.01 - 0.10 N/mm² [112], coupled with extrusion temperature of 50°C, which is higher than 37°C recommended for cells, would have caused significant irreversible cell injury leading to cell death and manifesting as lower % viability.

Figure 25: (a) Representative Live/Dead image of constructs bioprinted with optimal process parameters. (b) Live/Dead images with non-optimal process parameters. Scale bar is 250µm. (c) DIS parameters and (d) β-dispersion curve of 3D bioprinted constructs with optimal and non-optimal process parameters as given in Table 4. Error bars denote standard deviation, *represents statistically significant difference (p < 0.05) between groups. Note that the destructive Live/Dead assay was performed after completing DIS evaluations.
The DIS parameters and β-dispersion plots of the two groups are presented in Figure 25(c) and 25(d) and correspond with the cell viability results of Live/Dead analysis. Unlike the plot of the optimal bioprinting parameters group, the plot of the non-optimal parameters group did not resemble the inverse sigmoid shape which is characteristic of a β-dispersion [83,99,113,114]. These discrepancies in cell viability between the two groups are also reflected in the DIS parameters (Figure 25 (c)); the $\Delta \varepsilon$, $\alpha$, and $f_c$ of constructs in the two groups were significantly different ($p < 0.05$). The $\Delta \varepsilon$ of the optimal group was 70% higher than that of the non-optimal group, and it signifies the decrease in volume of viable cells in the measured volume as per Equation 1. For the non-optimal group, the incongruous shape of the β-dispersion plot and the higher $f_c$ compared to the optimal group is an indication of adverse changes in cell morphology during the bioprinting process [111,112,115]. From Equation 2, it is known that $f_c$ increases with decreasing cell radius, and it has been demonstrated that the shear stresses experienced by the cells at higher extrusion pressures compress the cells [111], which corroborate our findings here.

These results demonstrate the ability to use DIS monitoring to identify deviations in process parameters that would result in constructs with inferior CQA. Extrusion bioprinting was used as a model process in this study, but the DIS monitoring method can be extended to other 3D biofabrication processes as well. For example, in laser-assisted bioprinting [116–118], cell viability has been shown to be affected by the kinetic forces experienced by the cells due to the laser-induced jet dynamics [117]. DIS can be used to monitor and map the effects of changing jet dynamics on the viability of cells in the bioink.
3.3.5. Study 5: Effects of bioprinting processing time on CQA of 3D constructs and their correlation with β-dispersion spectral parameters

One of the important factors to consider while 3D bioprinting is the time required for fabrication of the constructs [73,119]. This is especially relevant in extrusion-based processes which may require a substantial amount of time to dispense the bioink due to limitations with the extrusion flow rate compared to other processes. The flow rate, primarily controlled by the extrusion pressure during bioprinting, depends on the viscosity of the bioink and the flow and shear stresses that the cells can endure. The viscosity of bioinks must be within a range that allows extrusion at lower pressures (less than 0.1 N/mm$^2$) [112] and enables the construct to maintain shape fidelity post-printing. Depending on the bioink properties, bioprinting process parameters, and size and geometry of the construct, the cells encapsulated in the bioinks may often remain stored in the printing reservoir or cartridge for more than 2 hours [73,119]. The conditions in the reservoir may not be conducive to the bioactivity of the cells over longer time periods. For example, high cell concentration in the bioink can make the cells more prone to hypoxia and media deprivation within the limited nutrient volume of the material reservoir.

In this study, the ability to capture the changes in cell viability due to long processing times using DIS was evaluated. Constructs were bioprinted immediately after loading the 3D-Bioplotter cartridge or after 3 hour or 6 hour intervals with the bioink stored in the cartridge at 37°C. Results of Live/Dead assay showed the hASC viability to be 90%, 70%, and 64% after intervals of 0 hours, 3 hours, and 6 hours, respectively. The β-dispersion plots and DIS parameters from the three constructs are presented in Figure 26. The β-dispersion plots of all three constructs followed the inverse sigmoid shape, and corresponding to the Live/Dead results, Δε and α decreased with increasing process intervals, signifying a decrease in volume of viable
cells (Equation 1). These results further demonstrate the ability of the DIS measurement approach to conduct quality assessment of the bioprinted constructs.

![Diagram](image)

**Figure 26:** β-dispersion curves of bioprinted constructs evaluated immediately and after 3 hour and 6 hour delay

3.3.6. Study 6: Monitoring anatomical 3D bioprinted constructs in culture over time and determining localized readouts of β-dispersion characteristics of the construct in XY plane.

In this study, the ability to use DIS to monitor the changes in cellular attributes in an anatomically relevant human medial knee meniscus construct was investigated. To capture any variations in initial hASC distribution and track changes in proliferation over time along the crescent-shaped geometry, DIS measurements were taken at three different zones of the construct immediately after bioprinting and after 24 hours and 48 hours in culture. Localized variations in cell distribution and cell viability in constructs without vascular networks due to proximity of different regions to the culture media, poor nutrient diffusion and hypoxia have been reported in literature [72,73,120,121]. Cell clustering and agglomerations caused during
bioink preparation and the bioprinting process itself also contribute to such localized variations which were intended to be captured using DIS.

The bioprinted meniscus construct and DIS evaluation setup are shown in Figure 27. The % aB reduction measured for the entire construct and Δε obtained from β-dispersion curves of each of the three zones are plotted in Figure 27 (e). The aB results indicate an increase in proliferation from day 1 to day 2 and a drop from day 2 to day 3. The same trend was observed in Δε consistently across the three zones. Furthermore, at each time point, the trend in Δε across the zones was the same, with the highest Δε reported in zone 2 and the lowest Δε in zone 3, indicating localized variations in cell distribution. That the trend in Δε across the zones was the same also indicates that all regions of constructs were equally affected by cell culture conditions. The correlation between %aB reduction and Δε demonstrates that DIS can be used to monitor the CQA of 3D bioprinted constructs in culture. The biggest advantage of DIS is that the quality attributes can be measured non-destructively over time (unlike sectioning in Live/Dead assay) and more granularly across multiple regions of constructs relatively quickly (unlike aB assay wherein only a single reading can be obtained for an entire construct irrespective of its size and geometric complexity).
Figure 27: (a) Schematic of the zones of DIS measurement of the bioprinted meniscus construct. (b) DIS evaluation of 3D bioprinted meniscus at zone 1. (c) 3D bioprinted meniscus containing $20 \times 10^6$ hASC (d) 3D bioprinted meniscus construct without cells used for alamarBlue control (e) $\Delta \varepsilon$ of different zones of meniscus over 2 days and % aB reduction of the meniscus construct

3.3.7. Study 7: Effect of Layer Addition on $\Delta \varepsilon$ over 7 Days in culture

In any bioprinting process, 3D constructs are fabricated by addition of layers. In studies 4-5, DIS measurements were made post-fabrication after all the layers of the construct were
bioprinted and crosslinked. In addition, the entire construct was located within the sphere of measurement of the DIS probe in these studies. Study 6 explored an approach to assess constructs of anatomical shapes with sizes beyond the measurement volume. But if the DIS probe is to be used for in-process measurements during bioprinting, the measurements will also have to be made after deposition of every layer. Accordingly, Study 7 was designed to investigate the effect of addition of new bioprinted layers on the measured $\beta$-dispersion characteristics, primarily $\Delta \varepsilon$.

In the layer-wise measurement scenario, if measurements were to be made after deposition of second layer, the probe will still pick up signals from the layer beneath it. Therefore the DIS signals measured for a two layered construct is a resultant sum of signals from individual layers, as illustrated in Figure 28. This phenomenon is referred to as superposition and is the net response caused by two or more stimuli; it is a sum of the responses that would have been caused by each stimulus individually [122]. This interference can be constructive or destructive depending on the magnitude and direction of the signal source. In a layer-wise measurement scenario, the interference is constructive. If $x_1$ and $x_2$ are signals from layers 1 and 2 respectively, then the resultant $\Delta \varepsilon$ of superimposed signal is given by Equation 3 [123].

$$\Delta \varepsilon (x_1 + x_2) = \Delta \varepsilon (x_1) + \Delta \varepsilon (x_2)$$

**Equation 3**

![Figure 28: An illustration of DIS signal superposition](attachment:image.png)
In conjunction with superposition, another phenomenon based on the relative strength of the electric field with respect to the distance of the construct from the electrodes contributes to the resultant signal. In section 3.1.3, it was discussed how the measurements made with DIS are reflective of the volume of viable cells that the electric field encompasses. The strength of the electric field measured is dependent on the proximity of the test sample to the electrodes. This phenomenon of reduction in signal strength as the distance between the electrodes and sample increases is referred to as signal attenuation (Figure 29). In general, for an electromagnetic source, attenuation is dependent on the attenuation coefficient of the medium the length of electromagnetic wave propagation and its frequency [124].

**Figure 29:** An illustration of DIS signal attenuation

DIS signals behave like any electromagnetic wave in a medium of a known dielectric constant following exponential decrease in the strength (amplitude) as the distance of wave propagation increases. DIS signal attenuation based relative loss factor ($\alpha_{\text{loss}}$) in dB/mm for the experimental setup can thus be given by a modified equation of power loss function as given in Equation 4 [125], where $L$ is the distance of wave propagation in mm which in this experimental scenario is the distance between the sample and the electrodes, $\Delta \varepsilon_0$ is the relative permittivity at the initial position of the construct and $\Delta \varepsilon_1$ is the relative permittivity at the altered position.
\[ \alpha_{\text{loss}} = \frac{1}{L} \log \left( \frac{\Delta \varepsilon_0}{\Delta \varepsilon_1} \right) \]  

Equation 4

In Study 7, DIS measurements were made on one, two and three layered bioprinted constructs with each layer containing \(5 \times 10^6\) hASC. The constructs were subject to DIS measurements post-crosslinking and on alternate days over 7 days in culture. The results of mean \(\Delta \varepsilon\) and the corresponding % aB reduction on Days 1, 3, 5 and 7 are presented in Figure 30. The \(\Delta \varepsilon\) was proportional to the number of layers across different days in culture. Results from the two-way analysis (ANOVA) show that both the number of layers in the construct and the day of measurement had a significant effect on \(\Delta \varepsilon\) (p < 0.05).

Figure 30: \(\Delta \varepsilon\) and % aB reduction of one, two and three layered constructs over 7 days in culture. Error bars denote standard deviation.
The general trend in % ab reduction across days correlated with the trend $\Delta \varepsilon$ for all three types of constructs. In one layered construct $\Delta \varepsilon$ decreased at Day 3 but there was an increase on Day 5 and Day 7, and % ab reduction also followed the similar trend. In contrast, for two layered and three layered constructs, $\Delta \varepsilon$ and % aB reduction decreased over time. In the case of one layered construct, there were $5 \times 10^6$ hASCs within the construct cultured with 4 mL of media. The initial drop could be due to the change in environment for the cells, transitioning from optimal adherent culture dish to encapsulation in a hydrogel. Over time, the cells adapted to the new environment and resumed normal function as indicated by the increases in $\Delta \varepsilon$ and % aB reduction on Days 5 and 7, which point to an increase in viable cell volume (Equation 1). In contrast, the one possible reason in the drop in $\Delta \varepsilon$ and % aB reduction could be hypoxia and poor cellular metabolite exchange arising as a result of high cell density in the constructs cultured in static conditions without constant media flow [72,73,120,121]. The amount of media for the two layered and three layered constructs containing $10 \times 10^6$ hASC and $15 \times 10^6$ hASC, 5 mL and 6 mL respectively, may not have been sufficient to allow cells to resume their normal function over time, resulting in the drop in cellular activity and subsequent loss of VCV after Day 1.

Figure 31 shows $\Delta \varepsilon$ on Day 1 (immediately post-bioprinting and cross-linking) for the single and multi-layered constructs. It can be seen that the increase in $\Delta \varepsilon$ with addition of layers is not linear (exact multiples of cell number), as discussed in section 3.3.3. Although the number of hASC in two layered and three layered constructs were twice ($10 \times 10^6$) and thrice ($15 \times 10^6$) the number of cells in one layered constructs ($5 \times 10^6$), $\Delta \varepsilon$ increased only by a factor of 1.54 and 2.24, respectively. This trend can be attributed to a combination of DIS signal superposition and attenuation. With addition of layers (cells), there was an increase in $\Delta \varepsilon$ as a result of
superposition of DIS signal, but this was offset by attenuation of DIS signal as the distance between the bottom layers and the electrodes increased. In Study 8, the isolated effect of signal attenuation on the measured $\Delta \varepsilon$ will be investigated.

**Figure 31:** $\Delta \varepsilon$ of one, two and three layered constructs on Day 1. Error bars denote standard deviation.

### 3.3.8. Study 8: Effect of DIS signal attenuation on assessed $\Delta \varepsilon$

In Study 8, the effect of DIS signal attenuation with increasing offsets in X, Y and Z directions between the probe and single layered bioprinted construct (20 x 20 x 1.5 mm) containing 5 x 10^6 hASC were characterized. The construct was positioned at four different points on XY plane ($x = 0$, $y = 0$; $x = 10$ mm, $y = 0$; $x = 10$ mm, $y = 10$ mm; $x = 0$, $y = 10$ mm) and DIS measurement were made at four different Z height offsets ($z = 2, 4, 6, 8$ mm). X and Y offsets were varied by moving the constructs such that the center of the top surface of the constructs were at the specified distances from the center of the bottom face of the probe. In a pilot study, DIS measurements were also made at X and Y offset of 20 mm, but the measured DIS spectra was very weak ($\Delta \varepsilon < 1 \times 10^{-3}$ pF/cm), and hence, X or Y offsets of 20 mm or greater are not being reported.

The $\Delta \varepsilon$ of the constructs at the different XYZ coordinates are presented in
Figure 32. It can be observed that $\Delta \varepsilon$ decreased as the offsets increased in all three directions. For example, at $z = 2 \text{ mm}$, $\Delta \varepsilon$ decreased significantly as the construct was moved 10 mm farther from the center of the probe in X (from 12.4 pF/cm to 0.07 pF/cm) and Y directions (from 12.4 pF/cm to 0.56 pF/cm). That there was no discernable dispersion in the DIS spectra at X and offsets $\geq 20 \text{ mm}$ in pilot experiments, and the observation above prove that the measurement volume around the probe’s bottom surface as discussed in section 3.3.6 is smaller than the manufacturer specified measurement volume that is sphere shaped with a diameter of 30 mm.
Figure 32: $\Delta \varepsilon$ of one layered bioprinted construct containing $5 \times 10^6$ hASC at different $X$, $Y$ and $Z$ distances from the center of the bottom face of the probe. Error bars denote standard deviation.

Results from the three-way ANOVA show that offsets in $X$, $Y$ and $Z$ had a significant effect on $\Delta \varepsilon$ ($p < 0.05$). The average relative loss factor ($\alpha_{\text{loss}}$) in $X$, $Y$ and $Z$ direction calculated using Equation 5 were 1.26, 1.33 and 0.29 dB/mm, respectively. It is evident that the attenuation was significantly greater in $X$ and $Y$ directions than $Z$ direction. Hence, for optimum
measurement of DIS spectra, the geometrical extents of the construct relative to the center of the bottom face of the probe should be considered. This observation was utilized in Study 9, as DIS measurements were made on three localized regions of 20 mm diameter in one, two and three layered human-meniscus shaped constructs.

At the position of $x = 0$, $y = 0$, $\Delta \varepsilon$ of one layered construct containing $5 \times 10^6$ hASC measured at different $Z$ height offsets is presented in Figure 33. An empirical model of the attenuation loss was created by fitting a third degree polynomial regression model ($R^2 = 1$) and is presented in Equation 5.

\begin{equation}
\Delta \varepsilon = -0.2991x^2 + 1.2021x + 11.697
\end{equation}

Figure 33: $\Delta \varepsilon$ of one layered construct containing $5 \times 10^6$ hASC at different $Z$ height offsets
Where \( z \) is the Z height offset between the top surface of the one layered bioprinted construct containing \( 5 \times 10^6 \) hASC positioned at \( x = 0, y = 0 \) and the bottom surface of the probe. Using this empirical model, \( \Delta \varepsilon \) of the construct at other Z height offsets can be predicted. To test the validity of this empirical model, a new construct of the same geometry containing \( 5 \times 10^6 \) hASC was bioprinted and was subject to DIS measurement at Z height of 5 mm from the center of the top surface of the construct with the construct positioned at \( x = 0 \) & \( y = 0 \). The experimentally measured \( \Delta \varepsilon \) (9.61 pF/cm) was within 6% of the \( \Delta \varepsilon \) estimated from Equation 5 (10.22 pF/cm). Models similar to the empirical model presented can be used to predict the \( \Delta \varepsilon \) at different Z heights of measurement from the bioprinted construct.

### 3.3.9. Study 9: Effect of Layer addition and biomodel Geometry on \( \Delta \varepsilon \) across 7 Days in culture

In this study, an anatomical model of human knee meniscus was bioprinted with a layer height of 1.5 mm. One, two and three layered meniscus constructs were bioprinted and subject to three zonal measurements (Ø 20 mm) as described in section 3.2.8 on days 0, 2, 4 and 6 in culture. An alamarBlue assay was performed on the whole construct in culture as a conventional measure for cell proliferation. This study combines the methods used in Study 6 and Study 8 by reporting measurements across various zones the XY plane and along the Z direction due to the addition of layers. \( \Delta \varepsilon \) calculated from the measured DIS spectra for individual zones of the single and multi-layered constructs over 7 days in culture are presented in Figure 34.
Figure 34: $\Delta \varepsilon$ of one, two and three layered constructs across different zones for 7 days in culture.

Results show that the $\Delta \varepsilon$ increased with addition of layers and also with time in culture. Individual differences in $\Delta \varepsilon$ within each zone of the three groups were also observed, indicating differences in VCV within the constructs post-bioprinting. Within each zone also, the $\Delta \varepsilon$ varied as the constructs matured. For example for one-layered constructs, $\Delta \varepsilon$ of Zone 3 was lowest on Day 1 and highest on Day 7. This is an indication that the VCV within the constructs increase or decrease based on the culture conditions. Bioprinted constructs of anatomical geometries can have localized regions of poor cellular activity due to various factors such as media deprivation, poor diffusion of nutrients, hypoxia etc. For example, there can be parts of a construct which raised above media level in the culture dish causing poor diffusion of media and subsequent reduced cellular activity in that exposed region. By monitoring $\Delta \varepsilon$ across the construct geometry over time, the cellular proliferation within different zones of construct can be determined. This presents and advantage over methods such as alamarBlue assay which will provide a reading for
the entire construct but cannot map the zone-based variations. Due to the aforementioned reason, % aB reduction data for the entire construct of the three groups is presented in Figure 35 along with the sum of $\Delta \varepsilon$ across three different zones (representing a comprehensive $\Delta \varepsilon$ for the construct as a whole).

Figure 35: Sum of $\Delta \varepsilon$ across three zones and % ab reduction of one, two and three layered constructs across 7 days in culture

It can be observed that in the one-layered and three-layered meniscus construct, the trend in % aB reduction correlated with the changes in $\Delta \varepsilon$. The values for both $\Delta \varepsilon$ and % ab reduction first decreased from Day 1 to Day 3 and increased thereafter until Day 7, signifying that the VCV represented by $\Delta \varepsilon$, increased as the construct matured. In the two-layered construct, there was a minor discrepancy in the trends between $\Delta \varepsilon$ with % aB reduction. Although $\Delta \varepsilon$ increased from Day 3 to Day 5, % aB reduction decreased during that period. This difference could have
been a result of the difference in the principles that these methods are based upon. \( \Delta \varepsilon \) is a measure of VCV while \( \% \) aB reduction is a primary measure of cellular metabolic activity [126,127] which can be correlated to cellular proliferation in many scenarios. However, this could have been a scenario where the cells proliferated contributing to the increase in VCV but the overall metabolic activity of the cells decreased due to culture conditions.

Overall \( \Delta \varepsilon \) of all three groups at Day 7 was significantly higher than Day 1, signifying that the cells proliferated as constructs matured in culture. With more number of zonal measurements, a 3D graph of the CQA at different points can be created. As the cells proliferate and constructs mature in culture, the trend in proliferation can be studied at any region of interest in the construct over time. This method zonal monitoring demonstrated in this study can be used as a basic testing protocol that can be applied to monitor the quality of anatomically relevant 3D bioprinted constructs over time.

This chapter describes one of the first known measurement approaches for non-destructively characterizing the CQA of 3D bioprinted constructs. Studies 1-9 taken together, demonstrate how DIS can be used to non-destructively evaluate cell proliferation and cell viability in 3D constructs in culture over time. For layer-by-layer biofabrication, DIS can be used for in-process monitoring wherein CQA of each layer are measured and qualified immediately after a layer or a portion of it is printed, as presented in studies 7 and 8. If the DIS parameters and corresponding underlying cellular attributes are deemed to be within pre-defined control limits, the process can continue onto the next layer. If the attributes in a particular layer are outside acceptable control limits, the process can be terminated. In the absence of such in-process monitoring methods, significant effort and resources would have to be expended on creating and culturing a defective construct that would eventually be rejected in downstream
manufacturing operations. Another potential approach in the latter situation would be to track the β-dispersion parameters during the fabrication process and then make in-process fabrication control parameter adjustments (such as pressure, temperature etc.) to keep CQA within control limits. Information about the effect of superposition and signal attenuation as the distance between probe and construct increase as new layers are added was also presented.

3.7. Chapter Summary

In this chapter, we have experimentally characterized the relationship between β-dispersion parameters and CQA of 3D constructs under various scenarios. In Study 1, the feasibility of using DIS to measure the dielectric signatures of MG63 cells encapsulated in alginate hydrogel constructs was investigated, and the results show that Δε was significantly affected by cell concentration. In Study 2, the ability of DIS to distinguish between the β-dispersion characteristics of 3D alginate constructs with MG63 and hASC in two different concentrations was assessed, and the results show that the Δε and α were significantly affected by both cell type and cell concentration. In Study 3, the mapping of DIS parameters in response to the changes in the number of cells and % viability of hASC encapsulated in alginate 3D constructs was presented, and the results confirm that Δε is proportional to VCV (living cells only) and not affected by total number of cells in the construct. In studies 4-6, the effect of changes in 3D bioprinting parameters on the DIS parameters in response to underlying changes in CQA attributes of bioprinted constructs was investigated. In particular, the effect of non-optimal 3D bioprinting parameters (high extrusion pressure and high print-head temperature) on β-dispersion parameters (Δε, fc and α) was characterized in Study 4. The effect of 3D-bioprinting processing time on the hASC encapsulated within un-crosslinked sodium alginate inside the print-head and the resulting β-dispersion parameters from constructs bioprinted over different
time points was assessed in Study 5. Results showed that changes in hASC proliferation and viability in response to changes in critical bioprinting parameters and the processing time significantly affected $\Delta c$, $a$, and $f_c$. In Studies 6 and 8, a potential application of DIS to monitor cell proliferation in a 3D bioprinted knee meniscus construct and localized assessment of CQA in XY plane was demonstrated. In studies 7 and 8 the use of DIS to characterize CQA of bioprinted constructs layer by layer and the effect of DIS signal superposition and attenuation were investigated. Overall, the results from this chapter support the ability and the approach to use DIS as a label free, non-destructive method to evaluate CQA of biofabricated constructs, a necessary step to enable the scale to up/scale out and translation biofabrication processes.
CHAPTER 4: NON-DESTRUCTIVE QUALITY MONITORING OF

SCAFFOLD-BASED BIOFABRICATION

4.1. Introduction

DIS based non-destructive evaluation of cellular constructs can be applied to other biofabrication strategies in addition to bioprinting. Scaffold-based biofabrication approaches are being used widely for manufacture of engineered tissue including bone and cartilage among others [128,129] [130,131], and non-destructive evaluation of CQA through DIS can reduce the dependence on destructive assays. Scaffolds are created out of biomaterials as 3D porous structures tailored to provide a template for tissue growth and support cell adhesion, ECM deposition and biodegradation at a controllable rate approximating the rate of tissue regeneration [132].

Scaffold features such as the geometry, porous architecture, mechanical strength and surface properties have been shown to influence cell adhesion, migration, signalling, proliferation, and tissue formation [133]. The porous architecture of a scaffold translates into the space available for cells to migrate and develop vasculature, and it also facilitates transportation of nutrients and metabolic wastes. For additively manufactured (AM) scaffolds, strands are the fundamental building blocks; scaffolds are built layer by layer, and strands constitute each layer. The porous architecture of AM scaffolds is governed by the strand dimensions, lay orientation, and inter-strand separation. Strand dimensions and inter-stand separation affect the scaffold mechanical properties as well as biological aspects such as the distribution of cells and nutrient exchange once they are seeded [134,135]. Hence, the scaffold design is governed by the tissue being engineered and the type of cells used. For example, the average pore size for scaffolds
used in bone tissue engineering is 300 μm [136] whereas the scaffold pore size for muscles cells ranges from 63-150 μm [137]. With the increase in utilization of stem cells for engineered tissues, the microenvironments that the scaffolds provide have been altered to allow cell differentiation into vascular, osteogenic and chondrogenic pathways [130,138].

Referring to the ETM system map in Figure 1, the seeding and culturing post-scaffold fabrication also impact the engineered tissue characteristics. In addition to scaffold design, factors such as seeding density and culture conditions have been shown to have an effect on the CQA of the scaffold. For example, Troy et al. have shown that seeding density of bone marrow stromal cells on scaffolds had an effect on the cartilage production [139]. Bean et al. have demonstrated how the strand thickness and seeding density influenced the differentiation of stems cells to chondrocytes producing cartilage [140].

The advancements in scaffold fabrication have enabled precise control over the scaffold geometry, architecture and material properties providing biomechanical cues necessary for the supporting cells. As such, scaffold-based treatments for osteochondral defects [141,142] and vascular defects [143,144] are already in clinical trials. However, the biological performance of the cells and the tissue formation during in-vitro culture is still predominantly determined through destructive labeling methods such trichome staining [145,146] and H&E staining [145,147] among others. These methods are often accomplished by fixing and sectioning of the scaffold, thus destroying parts of the scaffold. Therefore, use of a non-destructive method like DIS could enhance the monitoring of scaffold-based tissue constructs, similar to monitoring of bioprinted constructs as presented in Chapter 3.

This chapter focuses on objective 3 of the dissertation – the ability of DIS to evaluate the CQA of scaffold-based constructs and study their relationship to the scaffold design/culture
conditions and permittivity spectra. An overview of the studies conducted in the chapter is presented in **Figure 36**. Study 1 was designed to assess the effect of scaffold fabrication process – 3D-Biplotting (3DB) and 3D-Melt blowing (3DMB) – on the CQA of the cellular scaffolds and its relationship to DIS parameters (ST#3.1). In Study 2, the effect of 3DB scaffold design (inter-strand separation) on cellular constructs’ CQA and its correlation with DIS parameters was investigated (ST#3.1). Study 3 focused on investigating the effect of seeding density on CQA of 3DB scaffolds through DIS monitoring (ST#3.2).

<table>
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<td><strong>Study 1</strong></td>
<td>DIS of seeded scaffolds monitored over 7 days in culture</td>
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<tr>
<td></td>
<td>3D-Biplotted (3DB) scaffold – 3DB1</td>
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<tr>
<td></td>
<td>3D-Melt Blown (3DMB) scaffold – 3DMB1</td>
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<td></td>
<td>Both scaffolds Ø 20 mm, 3 mm thick</td>
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</table>

**Figure 36**: Overview of the experimental studies to address Objective#3
4.2. Materials and Methods

4.2.1. Cell expansion

Human ASC were cultured with MesenPro RS basal medium containing growth supplement and 1% L-Glutamine (Thermo Fisher Scientific) in a Corning® CellBIND® Surface HYPERFlask® (10-layer, 1720 cm² surface area; Corning, NY) at 37°C (5% CO₂). The flask was primed with 560 mL of Mesenpro RS culture media and cultured up until a cell confluency of 80% was achieved. The cells were harvested by adding 60 mL of TrypLE™ Select Enzyme (Thermo Fisher Scientific) after two washes with 100 mL of phosphate-buffered saline (PBS). The dissociated cell suspension was neutralized with media and centrifuged to obtain cell pellets.

4.2.2. Scaffold fabrication

3DB scaffolds were fabricated on a 3D-Bioplotter (Manufacturer Series, EnvisionTEC GmbH) with PCL pellets (Mw: 43–50 kDa, Polysciences, Inc., Warrington, PA) as the raw material. The base CAD model was created in SOLIDWORKS 2016 (Dassault Systems, SolidWorks Corporation, Waltham, MA) as a cylinder (Ø 20 mm, 3 mm thick). The STL file of the model was sliced into layers of 240 µm thickness and positioned on the bioplotting stage using BIOPLOTTERRP software (EnvisionTEC GmbH). The file was then preprocessed in VISUALMACHINES software (EnvisionTEC GmbH) to assign an alternating 0°/90° strand lay down pattern and set the processing parameters. The high-temperature print head was set to 120°C with a preheat interval of 30 minutes before the initiation of the fabrication cycle. A 300 µm nozzle (inner Ø) with 0.5 N/mm² extrusion pressure and 1.4 mm/s nozzle speed was used for fabrication. The inter-strand separation was set to 300 µm and 600 µm for 3DB1 and 3DB2,
respectively. The strand thickness and inter-strand separation were characterized using a digital microscope (KH-7700, Hirox, Hackensack, NJ) with previously established protocols [23].

3DMB scaffolds were fabricated by extruding PCL melt heated to a temperature of 120°C through a fiber fabrication die (Biax Fiber Film, WI) comprising of nine nozzles arranged in a 3x3 grid. Each nozzle consisted of a fine capillary (Ø 0.38 mm) through which the polymer melt was extruded at a rate of 0.08 g/capillary/minute and an annular coaxial gas outlet through which a jet of attenuating high temperature and pressure air exits, fed by an industrial air process heater (Backer Hotwatt, MA). As fibers emitted from the die, they were collected on a high-speed rotating mandrel positioned in space relative to the nozzles by an industrial robotic 6-axis arm (Denso, Japan) for a specified duration.

3DMB scaffolds of two different porous architectures – 3DMB1 and 3DMB2 were fabricated by collecting fibers for 15 minutes and 10 minutes respectively at a die-to-collector distance of 250 mm, positioned so fibers deposited 15 mm towards the leading edge of the mandrel from the central axis of rotation at 600 m/min, with attenuating air throughput of 120 and 110 liters per minute, respectively. Resultant fiber mats were both approximately 3 mm in thickness. Ø20 mm samples of 3DMB fiber mats were cut using a machined punch to obtain the scaffolds.

4.2.3. Scaffold treatment and sterilization

To enhance hydrophilicity and improve cellular adhesion during the seeding process, all scaffolds were subjected to alkaline hydrolysis surface modification. Scaffolds were submerged in solutions of 2.5M NaOH for 30 minutes at 37°C under continuous agitation. After this treatment, scaffolds were rinsed thoroughly with DI water, sterilized for 30 minutes in a bath of
70% ethanol, and rinsed thoroughly with sterile Dulbecco’s PBS then dried within a biosafety cabinet prior to seeding.

4.2.4. Seeding of scaffolds

Sterile-treated scaffolds were placed in a twelve-well culture plate (Thermo Fisher Scientific). The scaffolds were seeded with harvested hASC cells suspended in 1 mL of Mesenpro RS media at different concentrations for different studies as listed in Table 6.

<table>
<thead>
<tr>
<th>Study</th>
<th>Scaffold types</th>
<th>Seeding concentration per scaffold side (hASC/mL)</th>
<th>Total number of hASC seeded on the scaffold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>3DB1, 3DMB1</td>
<td>1x10⁶</td>
<td>2x10⁶</td>
</tr>
<tr>
<td>Study 2</td>
<td>3DB1, 3DB2</td>
<td>1x10⁶</td>
<td>2x10⁶</td>
</tr>
<tr>
<td>Study 3</td>
<td>3DB2</td>
<td>1.25 x10⁶, 2.5 x10⁶</td>
<td>2.5 x10⁶, 5 x10⁶</td>
</tr>
</tbody>
</table>

Four scaffolds of each type were used for each experiment; three scaffolds (n = 3) were seeded and used as treatment samples, and one acellular scaffold served as control for aB assay. Each scaffold was seeded by pipetting the 1 mL of cell suspension on top of the scaffold housed inside a well. The scaffold with the pipetted cell suspension was cultured in an incubator (37°C, 5% CO₂) for 2 hours, and additional 2 mL of Mesenpro RS media was added. After 24 hours of incubation, the scaffolds were flipped and the process was repeated to seed the other side. After 24 hours of incubation, the scaffolds were moved from the twelve-well plate to a six-well plate.
(Thermo Fisher Scientific) and cultured in 4 mL of Mesenpro RS for 7 days, with a media change interval of 24 hours. The seeding efficiency of the scaffold was calculated by counting cells left behind in the wells of the twelve-well plate after the seeded scaffold was removed. The cells in the twelve-well plate were dislodged by adding 1 mL TrypLE and counted using a hemocytometer. For the 3DB2 scaffolds seeded with 5 x 10^6 hASC in Study 3, 6 mL of culture media was added to well after the aB assay instead of 4 mL used for Studies 1 and 2.

### 4.2.5. DIS and alamarBlue reduction monitoring

The dielectric permittivity spectra of the seeded scaffolds were measured using a DIS flush probe following the procedure described in section 3.2.8. The β-dispersion curves of the scaffolds were plotted, and the Δε values were determined. The measurements were made on days 1, 3, 5 and 7 of culture. On each measurement day, aB assay was also performed after DIS evaluation. Each media change for the three seeded scaffolds and one control contained 10% v/v of the aB reagent on the day of measurement, irrespective of treatment condition. For each time point reading, three 1 mL samples were pipetted from each sample into a twenty four-well plate, and the absorbance was measured using a microplate reader (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths of 570 nm and 600 nm, respectively. The absorbance data was converted to and is reported as % aB reduction.

Cell viability in the scaffolds was assessed using LIVE/DEAD® assay (Life Technologies, Carlsbad, CA) following aB measurement on Day 7. Each scaffold was placed in 1 mL PBS containing 0.5 µl calcein AM and 2 µl EthD-I and incubated for 10 minutes and imaged using a fluorescence microscope (Revolve, Echo, San Diego, CA).
4.2.6. Experimental design

In Study 1, a two-way ANOVA was performed to assess the effects of scaffold type (3DB or 3DMB) and day of DIS measurement on $\Delta \varepsilon$ ($\alpha = 0.05$). In Study 2, a two-way ANOVA was performed on $\Delta \varepsilon$ of the 3DB scaffolds with inter-strand separation and day of DIS measurement as factors ($\alpha = 0.05$). In Study 3, a two-way ANOVA was performed on $\Delta \varepsilon$ of the 3DB scaffolds with cell seeding density and day of DIS measurement as factors ($\alpha = 0.05$).

4.3. Results and Discussion

4.3.1. Study 1: Effect of scaffold fabrication process on $\Delta \varepsilon$ over 7 Days in culture

Several studies have characterized the differences in cellular CQA due to differences in scaffold porous architecture arising as a result of differences in the scaffold fabrication process [148–150]. This study was designed to monitor and compare the CQA of 3DB and 3DMB scaffolds with same external geometry (Ø 20 mm x 3 mm, cylinder) and same initial seeding density ($2 \times 10^6$ hASC). The porous architecture of the 3DB and 3DMB scaffolds was different due the difference in the fabrication process principles. The differences in CQA arising due to this difference in porous architecture were monitored over 7 days in culture by measurement of DIS parameters and % aB reduction on alternate days.

A representative image of both type of scaffolds along with their porous architecture are presented in Figure 37. The strand width and inter-strand separation of 3DB1 scaffolds were 315 ± 7 µm and 285 ± 5 µm, respectively, and the resulting mean porosity determined using a previously established calculation method [23] was 41%. The microstructure of 3DMB1 scaffolds was captured using a scanning electron microscope (SEM) due to the difficulties in imaging them with an inverted light microscope owing to the poor light penetration through the
irregular shaped pores. The strand width of 3DMB1 scaffolds was $28 \pm 21 \mu m$, and the resulting porosity was 65%.

**Figure 37**: Representative images of (a) 3D-bioplotted scaffold – 3DB1, (b) Microscopic structure of 3DB1, (c) 3D-melt blown scaffold – 3DMB1, (d) SEM image of 3DMB1 at 2650X.

$\Delta \varepsilon$ calculated from the $\beta$-dispersion plots of the scaffold along with % aB reduction for the 3DB1 and 3DMB1 scaffolds are presented in **Figure 38**. It can be observed that $\Delta \varepsilon$ of both 3DB and 3DMB scaffolds increased initially until Day 5, signifying that the cells seeded on the scaffolds proliferated (Equation 1). Even though the external geometry and seeding density of
the two types of scaffold were the same, there were differences in the measured $\Delta \varepsilon$ due to the differences in dielectric properties of the cells arising as a result of differences cell attachment to the scaffolds [151–153]. Results from the two-way ANOVA shows that both type of scaffold fabrication process and day of measurement had a statistically significant effect on $\Delta \varepsilon$ ($p < 0.05$).

![Graphs and images](image)

**Figure 38:** (a) $\Delta \varepsilon$ and % aB reduction of 3DB1 scaffolds over 7 days. (b) $\Delta \varepsilon$ and % aB reduction of 3DMB1 scaffolds over 7 days. Insets shows a representative micro-structure of respective scaffolds. (c) Representative Live/Dead image of 3DB1 on Day 7. (c) Representative Live/Dead image of 3DMB1 on Day 7. Error bars denote standard deviation.
The trend in $\Delta \varepsilon$ corresponded to the trend in % aB reduction for 3DB1 scaffolds, but for 3DMB1 scaffolds there was a drop in % aB reduction on Day 3 and Day 5 while the $\Delta \varepsilon$ increased. To understand whether this decrease of % aB reduction on Day 5 of 3DMB scaffolds was a result of an experimental error or due to the differences in dielectric properties arising as a result of interaction between the scaffold’s porous architecture and culture conditions, another set of 3DMB scaffolds with slightly different porous architecture (3DMB2) were subjected to the same experimental testing. The strand width of 3DMB2 scaffolds was $28 \pm 22 \mu m$ and the resulting porosity was 60%. Fiber morphologies of the two 3DMB scaffolds were qualitatively different. 3DMB1 comprised more film-like fiber layers due to the use of higher air throughput. The increased volume of air resulted in a decrease in the cooling rate of the produced fibers, and an increase in the interlayer lamination. In contrast, 3DMB2 scaffolds were fabricated using a lower air throughput, thereby increasing the rate of cooling of the deposited fibers and resulting in less interlayer lamination. To compensate for this phenomenon and to still achieve a 3 mm thick fiber mat, 3DMB1 scaffold fiber mats were collected for a longer period of time than 3DMB2. The resultant porous architecture of 3DMB1 scaffolds was denser than 3DMB2, given that the scaffold dimensions were equal. The results of $\Delta \varepsilon$ and % aB reduction for the 3DMB2 scaffolds are presented in Figure 39.
Figure 39: $\Delta \varepsilon$ and % aB reduction of 3DMB2 scaffolds over 7 days. Inset shows a representative SEM image of 3DMB2 scaffold. Error bars denote standard deviation.

Trends in % aB reduction of 3DMB2 scaffolds were similar to those of 3DMB1. On days 3 and 5, $\Delta \varepsilon$ increased while % aB reduction decreased. This provides evidence that this phenomenon is a result of interaction of scaffold design and culture conditions or aB measurement method. During the 4 hour incubation period of scaffolds for aB assay, there could have been a lower rate of diffusion of the media containing aB reagent to the insides of the scaffold owing to its relatively smaller pore sizes for the 3DMB scaffolds. 3DMB scaffolds had a closed pore interconnectivity as opposed to the open structure design of 3DB scaffolds as seen in Figure 37. aB assay is based on the reduction of aB reagent during oxidation of the cells [154,155]. The uptake of the reagent by the cells depend on the availability of reagent containing media and proximity to them. As the scaffolds were cultured in a stationary well plate, time of diffusion of the aB reagent containing media into parts of 3DMB scaffolds not in direct contact of the media could have been relatively lower compared to 3DB and as a result the % aB
reduction could have decreased. As such, the % aB reduction may not accurately represent the holistic cellular metabolic of the 3DMB scaffolds. This conjecture will need to be validated in future studies.

4.3.2. Study 2: Effect of scaffold design (inter-strand separation) on $\Delta\varepsilon$ over 7 Days in culture

Study 2 was designed to investigate the effect of changes in porous geometry of 3DB scaffolds on CQA and its correlation with $\Delta\varepsilon$ through DIS monitoring. 3D-bioplotted scaffolds (3DB1 and 3DB2) were seeded with $2\times10^6$ hASC and subject to DIS monitoring and aB assay over 7 days in culture. The strand width and inter-strand separation of 3DB2 scaffolds were $314 \pm 5 \mu m$ and $591 \pm 12 \mu m$, respectively, and the resulting mean porosity was 65%. $\Delta\varepsilon$ and % aB reduction for the 3DB1 and 3DB2 scaffolds are presented in Figure 40.
Figure 40: (a) A representative microscopic image of 3DB1 scaffold. (b) A representative microscopic image of 3DB2 scaffold. (c) \( \Delta \varepsilon \) and % aB reduction of 3DB1 and 3DB2 scaffolds over 7 days. Error bars denote standard deviation.

\( \Delta \varepsilon \) of both types of 3DB scaffolds increased from Day 1 until Day 5 signifying cell proliferation within the scaffolds (Equation 1). The trends in \( \Delta \varepsilon \) correlated with the trends in %
aB reduction for both 3DB1 and 3DB2. Results from the two-way ANOVA show that only the day of measurement had a statistically significant effect on $\Delta \varepsilon$ ($p < 0.05$). Inter-strand separation did not have a significant effect on the monitored $\Delta \varepsilon$. This deduction indicates that the CQA of the hASC-seeded scaffolds were not dependent on change in porous architecture of the 3DB scaffolds within the chosen levels of inter-strand separation (300 µm and 600 µm) and strand width (300 µm) dimensions. However, other cell types could perform differently, and other design attributes such as strand width and strand lay down pattern might affect CQA of cellular scaffolds, and those interactions can be studied in future.

### 4.3.3. Study 3: Effect of seeding density on $\Delta \varepsilon$ over 7 Days in culture

Study 3 was designed to investigate the effect of seeding density on CQA of 3D-bioplotted scaffolds over 7 days and if that change can be evaluated through $\Delta \varepsilon$ monitoring. Two groups of 3DB2 scaffolds, each seeded with either $2.5 \times 10^6$ or $5 \times 10^6$ hASC, were subject to DIS monitoring and aB assay over 7 days in culture. $\Delta \varepsilon$ and % aB reduction outcomes of the two scaffold groups are presented in Figure 41.
Figure 41: (a) $\Delta \varepsilon$ and % aB reduction of 3DB2 scaffolds with $2.5 \times 10^6$ hASC and $5 \times 10^6$ hASC over 7 days. (c) Representative Live/Dead image of 3DB2 scaffold seeded with $2.5 \times 10^6$ hASC on Day 7. (c) Representative Live/Dead image of 3DB2 scaffold seeded with $5 \times 10^6$ hASC on Day 7. Error bars denote standard deviation.

$\Delta \varepsilon$ of both groups decreased from Day 1 until Day 3 and increased starting Day 5. The initial drop could be due to the change in environment for the cells, transitioning from optimal
adherent culture dish to the strands of the scaffold, and higher seeding density compared to
studies 1 and 2. Over time, the cells adapted to the new environment and resumed normal
function as indicated by the increases in $\Delta \varepsilon$ and $\% \Delta B$ reduction on Days 5 and 7, signifying cell
proliferation within the scaffolds. The trends in $\Delta \varepsilon$ correlated with the trends in $\% \Delta B$ reduction.

Results from the two-way ANOVA show that both the day of measurement and seeding
density had a statistically significant effect on $\Delta \varepsilon$ ($p < 0.05$). This indicates that the CQA of the
cellular scaffolds were dependent on the seeding density, similar to the results published in
literature. For example, Yassin et al. have studied the effect of seeding density ($1 \times 10^6$
cells/scaffold and $2 \times 10^6$ cells/scaffold) and demonstrated that the higher seeding density
resulted in denser osteogenic tissue formation [156]. Hadidi et al. have studied the effect of
different seeding densities on fibrocartilage formation and have shown that at a critical seeding
density of $5 \times 10^6$ cells/scaffold, there was a higher cartilage formation [157]. While higher
seeding density can promote denser tissue formation, detrimental phenomena such as media
deprivation and hypoxia can also occur leading to a loss of VCV and change in cell phenotype.
Hence it is important to study the effect of seeding density on the CQA of scaffold constructs.
With DIS monitoring, the changes in CQA as the scaffolds mature can be monitored non-
destructively.

4.4. Chapter Summary

Scaffold-based biofabrication is one of the well-established methods for manufacturing of
engineered tissue. It is important to monitor the CQA of cellular scaffolds to evaluate the quality
of tissue formation. In Study 1, the differences in CQA of 3DB and 3DMB scaffolds were
monitored using DIS over 7 days in culture. $\Delta \varepsilon$ monitoring results showed that CQA of the
scaffolds with different micro-architectural characteristics resulting from different scaffold fabrication processes was significantly different. In Study 2, the effect of scaffold porous architecture of 3DB scaffolds on CQA were monitored using DIS over 7 days in culture. \( \Delta \varepsilon \) monitoring show that the change in porous geometry at the chosen inter-strand separation levels (300µm and 600µm) did not have a significant effect on CQA of the cellular scaffolds. In Study 3, the effect of cell seeding density on CQA of 3D-bioplotted scaffolds was monitored using DIS. \( \Delta \varepsilon \) monitoring results show that cell seeding density had a significant effect on CQA of the scaffolds. Results from both studies 2 and 3, showed that the trends in \( \Delta \varepsilon \) of the scaffolds seeded with hASC correlated with the trends observed in %aB reduction, providing evidence that DIS monitoring can be used in CQA evaluation of maturing cell-seeded scaffolds in in-vitro culture.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

This chapter presents a summary of the research presented in this dissertation and the potential directions for future research.

5.1. Summary

TERM strategies focus on the creation of living tissue substitutes as opposed to non-biological alternatives. These engineered tissue substitutes can potentially be used in clinical treatment, drug testing and disease modelling. The processes that enable fabrication of such tissues and organs are collectively known as biofabrication. Biofabrication encompasses a broad range of physical, chemical, biological, and/or engineering processes including approaches such as scaffold-based biofabrication, embedding/molding technology, bioprinting etc. The biofabrication field is evolving rapidly, and significant progress has been made on minimizing or eliminating shortcomings associated with spatial resolution, shape fidelity, and manufacturing repeatability with respect to geometry among other aspects. For effective transition of these engineered tissues to clinical treatment, the biofabrication processes must be appropriately scaled up and/or scaled out. To aid this translation, the manufacturing aspects such as the definition of process endpoints, quality by design and process validation must be considered during the product development phase. Further, current process development efforts must be complemented with the development of effective process monitoring and control tools to achieve a repeatable biological quality in each manufacturing batch. Towards addressing these shortcomings, this dissertation has focused on the systematic mapping and risk assessment of biofabrication processes and investigated the use of DIS as a non-destructive quality assessment and monitoring method for biofabrication.
Chapter 2 presented the functional mapping and risk assessment of 3D biofabrication processes (Objective 1). Systems level IDEF0 maps of ETM (ST#1.1) and functionally decomposed black box models of biofabrication (ST#1.2) were presented. The A-0 level IDEF0 model of ETM highlighted the overall relationship between inputs and outputs of the process from a manufacturing perspective. The A0 level models of scaffold-based and bioprinting-based ETM helped understand the flow of inputs and outputs between different steps within the process. Further, the A1 level models provides a detailed breakdown of mechanisms and constraints within which the different process steps operate. The black box models of bioprinting-based and scaffold-based biofabrication mapped the different processes supporting the overall function and the flow pathways of material, energy and information between those functions. For the functions identified in the black box model, risk assessment was performed using FMEA and potential control strategies were presented (ST#1.3).

Addressing Objective 2, the DIS system setup and approach for non-destructive evaluation of CQA of bioprinted 3D constructs was presented in Chapter 3. The relationship between the CQA and DIS parameters were investigated through nine separate studies. Results of studies 1-3 designed to map the relationships between CQA and DIS parameters (ST#2.1) show that the relative permittivity measured at a single frequency was significantly affected by the concentration of MG63 cell encapsulated within alginate constructs. Results of Study 2 show that that β-dispersion characteristics (Δε and α) were significantly affected by the cell type (MG63 and hASC) and cell concentration within the constructs. Results from Study 3 showed that the Δε and α were only affected by the number of viable cells and not the dead cells within the constructs. Results of Studies 4 and 5 (ST#2.2) showed that changes in hASC proliferation and viability in response to changes in critical bioprinting parameters (extrusion pressure,
temperature, processing time) significantly affected $\Delta \varepsilon$, $\alpha$, and $fc$. Studies 6-9 were designed to address the task of evaluating the correlation between cell viability and DIS parameters of bioprinted constructs over time and scale (ST#2.3). Study 6 demonstrated the DIS monitoring of hASC distribution post-bioprinting and changes in proliferation across the cross-section of a bioprinted medial knee meniscus construct over time. The phenomenon of DIS signal superposition and signal attenuation occurring as a result of DIS monitoring of layered constructs were characterized in studies 7-8. In Study 9, application of DIS to monitor cell proliferation in a 3D bioprinted knee meniscus construct and localized readouts of CQA in XY plane and across layers was demonstrated. The trends in $\Delta \varepsilon$ over time were in agreement with the aB assay results for the whole construct providing evidence for utilization of DIS in monitoring CQA of bioprinted constructs over time.

**Chapter 4** focused on the non-destructive DIS evaluation of hASC-seeded scaffold-based constructs (Objective 3). Results of Studies 1 and 2, designed to evaluate the effect of scaffold design on CQA and its correlation with DIS parameters (ST#3.1), show that trends and magnitudes of $\Delta \varepsilon$ of 3DB and 3DMB scaffolds were different. Furthermore, differences in interstrand separation and resulting porosity of 3DB scaffolds did not affect their $\Delta \varepsilon$. Results of Study 3 show that the seeding density had a significant effect on the monitored $\Delta \varepsilon$ (ST#3.2). The findings from Chapter 4 support the application of DIS monitoring to evaluate the CQA of cell-seeded scaffolds over time as they mature.

5.2. Research Contributions

This dissertation provides a systems level model of an ETM system for bioprinting-based and scaffold-based biofabrication. It also sets the groundwork for application of DIS as a non-
destructive quality monitoring method for biofabrication processes. Main contributions are as follows:

- Systems level IDEF0 models of bioprinting-based and scaffold-based biofabrication processes that provide the mapping of relationships between different processes within the system, and definition of their inputs, outputs, specific functions, constraints and mechanisms within which the processes operate.

- Functionally decomposed black box models of bioprinting and scaffold fabrication processes that define the system and sub-system level functions and the flow of material, energy and information through these functions.

- FMEA (without characterization of the risk priority number) highlighting risks and mitigation strategies for all functions identified in the black box models of bioprinting and scaffold fabrication processes.

- Mapping between DIS parameters (Δε, α, and fc) and CQA (viable cell number, cell type) of 3D cellular constructs.

- Demonstration of the ability to monitor bioprinting process parameter deviations from optimal setting through correlation with measured DIS parameters.

- Models to estimate DIS signal attenuation and superposition due to addition of bioprinted layers.

- Demonstration of DIS monitoring of bioprinted constructs’ CQA across biomodel geometry, layers and time in in-vitro culture.

- Application of DIS to monitor CQA of cell-seeded scaffold constructs and characterize the effect of scaffold design and culture conditions on CQA through correlation with DIS
parameters. These findings show that DIS monitoring can be applied to monitor the scaffolds in *in-vitro* culture.

### 5.3. Future Research Directions

**Computational modelling:** The COMSOL model presented in Chapter 3 is a simplified 2D model of the experimental setup and provides a basic framework for simulation of DIS of cellular constructs. This model was primarily created to verify if the DIS spectra of cells encapsulated with hydrogel constructs can be measured. But, the DIS parameters estimated from this 2D model did not correspond to the experimental results owing to the simple setup and assumptions about the dielectric properties of the model constituents. In future, the computational model can be refined by use of experimentally determined dielectric constants for cells, crosslinked hydrogel, and setting up 3D models of the construct and the electrodes.

**Inclusion of resistance in measurement:** In theory, it may be possible to use DIS to track changes in cell morphologies, particularly cellular aggregation and cellular state changes. Monitoring changes in the dielectric properties of the cells can be more comprehensive by reading both, resistance and capacitance signals, which are two essential electrical signal markers of the cells within the construct impeding the flow of AC current when placed in an electric field. The approach investigated in this dissertation focuses on the capacitance (hence relative permittivity) of the cells within the construct. In future, building upon this approach to include resistance measurements as well can provide more effective insight into the cell-cell barrier resistance when cellular spheroids form within the construct and to measure the overall impedance characteristics of the 3D cellular constructs.
Investigation of differentiation state of cells within constructs: Several tissue engineering and regenerative medicine applications use stem cells that are induced to differentiate into application-specific cell types as the construct matures in culture [120,158,159]. In such applications, DIS parameters can be potentially used to detect differentiation state of the cells. While an increase in $\Delta \varepsilon$ over time indicates cellular proliferation, a change in $f_c$ without a corresponding change in $\Delta \varepsilon$ will indicate underlying changes in physiological state of cells such as those observed during stem cell differentiation. Deduction of the cellular physiological state including stem cell differentiation based on $\beta$-dispersion characteristics, albeit in monolayer cultures, has been reported in literature [126,127], and can be investigated in the context of DIS for 3D biofabrication in future.

Refinement of DIS system design: The DIS probe used in this study is primarily intended for use in large bioreactors and fermenters used in brewing industries [83,85,86,98]. The number of cells in suspension in these applications typically exceeds $100 \times 10^6$, and the existing probe design and DIS system resolution has been optimized to account for such large cell numbers. The inability to distinguish between constructs with small differences in the total VCV as reported in sections 3.3.2 and 3.3.3 resulted, in part, from these sensitivity and resolution issues. In future, the DIS probe and system design will need to be optimized specific to 3D constructs. The following two approaches can be investigated to achieve this. First, the distance between the measurement electrodes at the bottom surface of the probe can be reduced. In the present design, the measurement volume encompasses a sphere of $\varnothing 20$ mm underneath the probe. This measurement volume is dictated by the distance between the electrodes. Reducing the distance between the electrodes will make the measurement volume smaller enabling more precise localized CQA measurements over a 3D construct. Second, the resolution of the $\beta$-dispersion
curves can be improved by increasing the number of frequencies at which the permittivity is measured within the 150 - 2500 kHz frequency range that is relevant to mammalian cells. At present, a frequency scan lasts for 30 seconds, permittivity readings are obtained across 25 discrete frequencies between Adjust the frequency range of 50 - 20,000 kHz, and only eleven frequencies are relevant. In addition to a more focused scan range, increasing the number of measurement frequencies within the range (from the current eleven) can help improve the β-dispersion curve resolution. During the production scale fabrication of engineered tissue constructs, only one particular frequency may be needed to query the CQA of the bioprinted construct. This is assuming that prior studies have been conducted to determine the appropriate frequency at which the CQA must be measured. Similar studies have been conducted in biopharmaceutical production, and the selection of the frequency is highly dependent on the cell types involved [160,161]. Together, these changes can make the system more robust for quality monitoring of 3D bioprinted constructs. Although DIS evaluation of extrusion-bioprinted and scaffold constructs was demonstrated in this dissertation, the DIS monitoring methodology itself is independent of the biofabrication process. This measurement approach can be easily extended to evaluate constructs made by other processes including vat polymerization [162,163], laser-assisted bioprinting [116,117], inkjet bioprinting [159,164] among others.
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APPENDICES
### Appendix I: IDEF0 models for bioprinting-based etm

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<td>Figure I5</td>
<td>A1 level IDEF0 model for the function “Culturing” for bioprinting-based ETM</td>
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Figure I: A1 level IDEF0 model for the function “Harvest Cells” for bioprinting-based ETM and scaffold-based ETM
Figure I 2: A1 level IDEF0 model for the function “Bioink Preparation” for bioprinting-based ETM
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**Appendix II: IDEF0 models for scaffold-based etm**

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<td>Figure II 4</td>
<td>A1 level IDEF0 model for the function “Cell suspension Preparation” for scaffold-based ETM</td>
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<tr>
<td>Figure II 5</td>
<td>A1 level IDEF0 model for the function “Scaffold Seeding” for scaffold-based ETM</td>
</tr>
<tr>
<td>Figure II 6</td>
<td>A1 level IDEF0 model for the function “Culturing” for scaffold-based ETM</td>
</tr>
</tbody>
</table>


Figure II 1: A1 level IDEF0 model for the function “Polymer Processing” for scaffold-based ETM
Figure II 2: A1 level IDEF0 model for the function “Printing Scaffold” for scaffold-based ETM
Figure II 3: A1 level IDEF0 model for the function “Scaffold Sterilization” for scaffold-based ETM
Figure II 4: A1 level IDEF0 model for the function “Cell suspension Preparation” for scaffold-based ETM
Figure II 5: A1 level IDEF0 model for the function “Scaffold Seeding” for scaffold-based ETM
Figure II 6: A1 level IDEF0 model for the function “Culturing” for scaffold-based ETM