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THE DEVELOPMENT OF A NOVEL 3D BIOPRINTING STRATEGY TO DRIVE VASCULARISATION, AND ITS APPLICATION FOR BONE TISSUE ENGINEERING

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A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of Doctor in Philosophy

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Declaration

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Jessica Nulty
Summary

One of the major challenges facing the field of tissue engineering today is vascularisation. Without a system in place to circulate oxygen, nutrients and metabolites, the size of many tissue engineered constructs is restricted, as embedded cells must rely on the diffusion of these factors for their survival. This has limited the clinical translation of engineered tissues and organs, with the majority of successful cases being limited to cases which are inherently less reliant on vasculature, such as cartilage, as well as relatively thin tissues such as skin and bladder. The inclusion of a vascular network within tissue engineered constructs could overcome this constraint, thereby enabling the scaling up of such tissues to previously unattainable, clinically relevant sizes. There are multiple strategies being investigated in the field to promote vascularisation of engineered tissues, including the incorporation of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), to accelerate host vessel invasion into constructs; the inclusion of endothelial cells (with or without supporting cells) to encourage the de novo formation of microvessels within the constructs; and the direct patterning of luminised channels within engineered constructs. 3D bioprinting is an emerging technology which offers the prospect of revolutionising the field of tissue engineering. The development of a 3D bioprinting strategy to engineer vascularised tissues of scale would be a significant advance, bringing the field closer to its goal of biofabricating whole organs and functional tissues.

One branch of tissue engineering which has a considerable need for more effective vascularisation strategies is the field of bone tissue engineering. Bone tissue engineering endeavours to produce bone substitutes for treating critically sized bone defects. These types of defects are too severe for the body’s innate healing capacity to repair and require surgical intervention. The current “gold standard” treatment for critical sized defects is the implantation of an autologous bone graft, which is harvested from elsewhere in the patient’s
body. This approach, however, has numerous drawbacks including donor site morbidity and a limitation in the quantity of available tissue to harvest. For bone tissue engineering to provide a viable alternative to autologous bone grafts, new approaches to produce large, viable bone tissues must be identified.

The overall aim of this thesis was to develop a 3D bioprinting strategy to vascularise tissue engineered constructs. This thesis sought to achieve this through preforming microvessels in vitro within a 3D printed construct prior to its implantation in vivo, a process known as ‘prevascularisation’. The first phase of this thesis focused on identifying a suitable biomaterial formulation which could support endothelial sprouting and be 3D bioprinted. This type of biomaterial is known as a bioink. After determining printability, the optimum cell combinations and culture conditions to produce stable microvessels in vitro and in vivo were established. The next phase of this thesis applied this prevascularisation strategy to multiple bone tissue engineering strategies to investigate whether prevascularisation could enhance in vivo vascularisation of these constructs and whether this could enhance bone formation.

The first bone tissue engineering strategy investigated involved engineering a 3D bioprinted cartilage template. Upon implantation in vivo, this cartilage template underwent endochondral ossification (the replacement of cartilage with bone), thereby replicating the process by which long bones are formed during embryonic development. Prevascularising this bioprinted cartilage template, using the strategy designed in the first phase of this thesis, enhanced vascularisation of the engineered graft in vivo.

A strategy to fabricate micron-scaled hypertrophic cartilage tissues (termed μTissues), and then combining them together to form scaled-up constructs, was then investigated. Theses engineered constructs also underwent endochondral ossification in vivo, forming a bone-like tissue. Prevascularising these μTissues prior to implantation significantly
accelerated bone formation. Furthermore, these µTissues are compatible with 3D bioprinting technologies and could be integrated with other modular tissue engineering strategies.

Finally, this thesis investigated the effect which prevascularisation had on bone healing in critical sized femoral defects. Prevascularisation of a 3D printed scaffold (using the bioink developed in the first phase of the thesis) led to significantly higher levels of vascularisation in the defect site and more rapid bone regeneration.

To conclude, this thesis demonstrates a novel 3D bioprinting technique to improve the vascularisation of tissue engineered constructs and further demonstrates how this method can be incorporated into multiple bone tissue engineering strategies to improve vascularisation and enhance bone formation.
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## Nomenclature

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>% (v/v)</td>
<td>Percentage volume per volume</td>
</tr>
<tr>
<td>% (w/v)</td>
<td>Percentage weight per volume</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AV</td>
<td>Arterio-venous</td>
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<tr>
<td>BMP2</td>
<td>Bone morphogenic protein 2</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone marrow-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer aided design</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DLP</td>
<td>Digital light processing</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECFC</td>
<td>Endothelial colony forming cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDM</td>
<td>Fused deposition modelling</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GelMA</td>
<td>Gelatin Methacryloyl</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>hBMSC</td>
<td>Human bone marrow-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hg</td>
<td>High glucose</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>IVB</td>
<td>In vivo bioreactor</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>nHA</td>
<td>Nano-hydroxyapatite</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure oxygen</td>
</tr>
<tr>
<td>PV</td>
<td>Prevascularising/Prevascularised</td>
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<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine 1-phosphate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>sGAG</td>
<td>Sulphated glycosaminoglycan</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma irradiated</td>
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<tr>
<td>μCT</td>
<td>Micro-computed tomography</td>
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<td>μTissue</td>
<td>Micro-tissue</td>
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Chapter 1

Introduction

1.1 Vascularisation in Tissue Engineering

Tissue engineering is a multidisciplinary field which endeavours to develop biological substitutes which are capable of replacing or repairing damaged tissues and organs. This goal is achieved through the integration of cells, biomolecules and biomaterials using the principles of engineering (Shafiee and Atala, 2017). While tissue engineering has made great strides towards achieving its goals, it is limited, in many cases, by its inability to produce tissues of clinically relevant dimensions. This limitation is due to difficulties in providing adequate nutrient transfer and waste removal when scaling up which can result in necrosis at the tissue core.

When attempting to scale up tissue engineered constructs in the laboratory, bioreactors can be utilised to ensure that sufficient levels of nutrients reach all cells within a construct successfully (Zhao et al., 2016). However, after implantation, a circulatory system must be in place in order to overcome mass transport limitations. In vivo, most cells are found within a 100-200 μm distance from the nearest capillary (Carmeliet and Jain, 2000). These challenges have resulted in a greater interest in recent years in the development of embedded vascular networks within tissue engineered constructs. There are multiple approaches currently being investigated to achieve this goal, including scaffold functionalisation through the incorporation of different angiogenic factors, cell-based approaches involving the incorporation of co-cultures of endothelial cells with supporting cells capable of the
establishment of primitive vascular networks within constructs as well as other more sophisticated approaches using immersing bioprinting techniques.

1.2 Vascularisation and Bone Regeneration

Traditional bone tissue engineering has focused on engineering tissues which have with similar mechanical integrity and structure as bone, often overlooking the importance of vasculature and frequently relying of the porosity of the scaffold material to facilitate nutrient diffusion throughout the construct (Mercado-Pagán et al., 2015). This has resulted in the failure of many regenerative strategies due to the limited capacity of these porous constructs to obtain the necessary substrate molecules and to remove the accumulating metabolic by-products which can result in decreased cell viability (O’Brien, 2011). In recent years, there has been a shift in bone tissue engineering approaches and with a greater recognition of the importance of actively encouraging vascularisation in contrast to previous approaches which tended to rely on the passive vascularisation of these constructs upon implantation. As well as enhancing nutrient diffusion throughout constructs, the establishment of a vascular network has also been shown to enhance bone formation (Correia et al., 2011).

While the exact role that vasculature plays during this process has not been fully elucidated, it is widely accepted that vascularisation plays a key role in osteogenesis (Saran, Gemini Piperni and Chatterjee, 2014). There are two distinct modes of ossification by which bone is formed: intramembranous ossification and endochondral ossification. Despite their differences, both processes require the establishment of a vascular network prior to osteogenesis (Kanczler and Oreffo, 2008). For example, during endochondral bone healing, an intermediary cartilaginous callus gradually becomes vascularised and is subsequently replaced by bone. Endothelial cells have been shown to play a crucial role in this process by secreting the matrix-degrading protease-9 (MMP-9) to facilitate vascularisation of the cartilage matrix (Colnot et al., 2003; Wang et al., 2013). It has been suggested that
endothelial cells may play a role in enabling mineralisation of the cartilage matrix by secreting bone morphogenetic proteins (BMPs) (Yu et al., 2010; Matsubara et al., 2012; Bahney et al., 2014) and in regulating the deposition of collagen I (Ben Shoham et al., 2016).

Recent studies have identified a possible new role for vasculature in bone repair, suggesting that the vasculature which is present during bone repair may have a signalling role in regulating the transformation of chondrocytes to osteoblasts. Hu et al. propose that the paracrine factors secreted from the vascular endothelial cells may prompt the chondrocyte to osteoblast transformation by the activation of pluripotent stem cell programs thereby initiating cell division and/or stimulating the bone phenotype (Hu et al., 2017).

The incorporation of a vascular network within bone tissue engineered constructs may have the added role of dictating the architecture of new bone (Mikos et al., 2006). Arnold Caplan described vasculature as the “orientor of osteogenesis” in vivo, attributing the vessels of the periosteum to providing the negative template for newly apposed layers of bone (Caplan, 1991).

1.3 Objectives of Thesis

Three-dimensional (3D) Bioprinting has the potential to transform the field of TE, enabling the production of large-scale, anatomically accurate constructs with the capacity to regenerate target tissues and organs. 3D Bioprinting uses computer-controlled hardware to accurately deposit cells, biomolecules and supporting biomaterials (often termed ‘bioinks’) in 3D space. However, this technology will never move beyond the bioprinting of small ‘proof-of-concept’ tissues unless strategies can be identified to pre-vascularise these constructs to ensure their survival in vivo and ultimately to facilitate functional tissue regeneration.

The overall goal of this thesis is to identify an approach for prevascularising tissue engineered constructs which is compatible with 3D bioprinting technologies. This platform will then be
used to test the hypothesis that the inclusion of a primitive vascular network into a Tissue engineered construct prior to implantation will result in improved vascularisation \textit{in vivo} and therefore prolonged implant survival and improved tissue formation. To realise this goal, this thesis will address the following objectives:

Objective 1: Identification of an endothelial cell-compatible bioink which can support the formation of a primitive microvascular network \textit{in vitro}. Specifically, this thesis will compare alginate, Gelatin-Methacryloyl (GelMA) and fibrin-based bioinks for their printability and their ability to facilitate the sprouting of human umbilical vein endothelial cells (HUVECs) \textit{in vitro}.

Objective 2: Identify the appropriate cell combinations and culture conditions to produce stable microvessels in 3D bioprinted constructs \textit{in vitro} and \textit{in vivo}.

Objective 3: To leverage this bioprinting strategy to prevascularise engineered hypertrophic cartilage and investigate whether the presence of a vascular network can improve the osteogenic potential of such bioprinted constructs following \textit{in vivo} implantation.

Objective 4: Investigate whether 3D bioprinted prevascularised constructs can accelerate large bone defect healing in the rat femur.

The hypothesis of this thesis is that prevascularisation of a 3D bioprinted implant will enhance vascularisation of the treated defect \textit{in vivo} and subsequently accelerate bone regeneration.
Chapter 2

Literature Review

2.1 Introduction

The loss or failure of an organ or tissue is one of the most frequent and devastating sequelae in medicine today. Organ transplantation is often the only effective medical treatment available to patients with end-stage vital organ failure. However, there is a global shortage of donor organs relative to patients in need of a transplantation (Johnson et al., 2008). Developing an adequate supply of organs and tissues cannot be achieved through donations alone and huge efforts have been made to engineer laboratory-grown tissues. Although relatively still in its infancy, the field of tissue engineering offers hope for bridging the gap between organ shortage and growing transplantation needs (Ozbolat and Yin Yu, 2013).

Tissue engineering is a multidisciplinary field which combines the principles of cell biology, materials science and engineering to promote the regeneration of functional living tissues and organs. The classical tissue engineering paradigm is to seed cells onto a three-dimensional supportive matrix and, using signalling molecules, stimulate the cells to proliferate, differentiate and ultimately produce functional substitutes for damaged tissues. Although significant progress has been made in the field of tissue engineering to date, many of the successes are limited to thin or avascular tissues such as skin, bladder and cartilage (Oberpenning et al., 1999; Supp and Boyce, 2005; Dewan et al., 2014; Huang, Hu and Athanasiou, 2016) This limitation is primarily due to the immense challenge of adequate nutrient supply with larger tissues.
For tissue engineering constructs to become a clinically viable alternative to donor material in the replacement or repair of tissues and organs, large-scale tissue constructs must be engineered. Large, complex and metabolically dense tissue requires a circulatory system to provide all the nutrients and metabolites necessary for healthy metabolism and to remove any waste by-products. Cell survival is so dependent on a circulatory system that it is the first functioning organ system to develop in the growing vertebrate embryo (Lucitti et al., 2007). When the embryo is merely a few cells, each cell can easily attain all the necessary nutrients directly from its surroundings. However, as cells divide and multiply, the growing embryo requires a more complex system to circulate oxygen, nutrients and signalling proteins throughout the developing tissue. Similarly, in engineered tissue, once a critical size is reached, nutrients can no longer rely on diffusion to sustain the comprising cells. In vitro, the use of bioreactors can help overcome many of these scaling-up issues. However, if such tissues are to be implanted into the body, the development of a perfusable vascular-like network is believed to be key to their clinical success.

This review will focus on the approaches which tissue engineers are employing today to vascularise engineered constructs and overcome the diffusion limits associated with scaling-up to thick viable tissues. In particular, strategies employing recent developments in 3D bioprinting and biofabrication will be discussed. This review will then highlight how these approaches may advance the field of bone tissue engineering.

2.2 The Process of Angiogenesis, Vasculogenesis and Remodelling

There are two processes by which a vascular network develops in vivo: vasculogenesis and angiogenesis (Figure 2.1). Vasculogenesis is the de novo development of blood vessels from precursor cells, while angiogenesis involves the formation of new
blood vessels from pre-existing vasculature (Charnock-Jones, Kaufmann and Mayhew, 2004).

**Figure 2.1** Scheme illustrating vasculogenesis, angiogenesis and the assembly of the vascular wall. Angioblasts, initially expressing vascular endothelial growth factor receptor (VEGFR) are stimulated by vascular endothelial growth factor (VEGF), secreted by the surrounding mesenchyme to form the primary capillary plexus by the process of vasculogenesis. Under additional stimulation by growth factors, competent endothelial cells of the primary capillary plexus form vascular sprouts in the earliest stages of angiogenesis. This is followed by the recruitment of surrounding mesenchymal cells to form the cellular elements of the vascular wall. PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; TGF-β, transforming growth factor-β. Adapted from (Carlson, 2013).

Vasculogenesis takes place primarily during gastrulation in early embryonic development but it can also occur in postnatal life during tumour growth or during the revascularisation of extensively damaged or ischemic tissue (Balaji et al., 2013). During vasculogenesis, endothelial precursor cells, known as angioblasts, are induced by fibroblast growth factors (FGFs) to differentiate from the mesoderm to form the primary vascular plexus. This primitive vascular network consists of small uniformly sized blood vessels which are lined with a single layer of endothelial cells. This primitive network is then remodelled into a mature vascular system through a series of angiogenic events.

Angiogenesis can be achieved through a variety of means such as sprouting, intussusception, elongation and intercalation (Table 2.1). Sprouting angiogenesis was the first identified form of angiogenesis and its mechanisms are therefore the most defined.
(Ausprunk and Folkman, 1977). The first step of sprouting angiogenesis is the enzymatic degradation of the capillary basement membrane. In a normal blood vessel, this basement membrane is approximately 100-200 μm thick and is composed mainly of laminins, type-IV collagen, type-VIII collagen and proteoglycans. Once the degradation of the basement membrane has occurred, the endothelial cells in the vessel walls are activated in response to angiogenic growth factors. Active endothelial cells are distinguished from their quiescent counterpart by a high mitotic index and an increased capacity for migration and matrix proteolysis (Ucuzian et al., 2010). Activated endothelial cells break free from the capillary intima, undergo proliferation and begin to migrate toward angiogenic stimuli. Sprouting angiogenesis is commonly initiated through hypoxia which is a deficiency in oxygen concentrations reaching tissues (Krock, Skuli and Simon, 2011). A common growth factor, secreted by parenchymal cells in response to these deficiencies, is vascular endothelial growth factor (VEGF) which is expressed as several splice variants, known as isoforms. The four most common forms of VEGF found in humans contain 121 amino acids, 165 amino acids, 189 amino acids and 206 amino acids and are known as VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub> respectively. The two shorter isoforms are secreted whilst the two longer isoforms are mainly cell-associated (Ferrara et al., 1991).

Table 2.1: Definitions of vasculogenic and angiogenic terminology.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>References</th>
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<tr>
<td>Vasculogenesis</td>
<td>The de novo formation of new blood vessels from non-vascular precursor cells (haemangioblasts)</td>
<td>(Risau and Flamme, 1995; Rohban, Prietl and Pieber, 2017)</td>
</tr>
<tr>
<td>Angiogenesis Type</td>
<td>Description</td>
<td>References</td>
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<tr>
<td>Sprouting angiogenesis</td>
<td>The formation of new blood vessels via lateral sprouting from an existing vessel.</td>
<td>(Ausprunk and Folkman, 1977; Risau, 1997; Carmeliet, 2003)</td>
</tr>
<tr>
<td>Intussusceptive angiogenesis</td>
<td>The formation of new vessels from an existing vessel by the formation of a transluminal pillar.</td>
<td>(Burri and Tarek, 1990; Kurz, Burri and Djonov, 2003)</td>
</tr>
<tr>
<td>Elongation-driven angiogenesis</td>
<td>The lengthening of existing blood vessel through the apical proliferation of endothelial cells.</td>
<td>(Gambino et al., 2002)</td>
</tr>
<tr>
<td>Intercalative angiogenesis</td>
<td>The lengthening of vessels through the incorporation of new endothelial cells into the endothelium of an existing vessel at multiple sites along the vessel rather than apically.</td>
<td>(Bentley et al., 2014)</td>
</tr>
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A subset of endothelial cells, known as tip cells, express many VEGF receptors thereby allowing them to sense increases in VEGF from their surrounding environment subsequently enabling them to align with the VEGF gradient. Tip cells drive the sprouting process by secreting large amounts of proteolytic enzymes, known as matrix metalloproteinases (MMPs), which digest a pathway through the ECM into which they can extend motile filopodia (Ghajar et al., 2006). Once these filopodia are securely anchored into the
surrounding ECM, contraction of actin filaments within the filopodia pulls the cell along the growth factor gradient.

Tip cells are followed by morphologically different endothelial cells which are known as stalk cells. These cells undergo rapid proliferation to form the trunk of the neovessel and to drive the extension of the sprouting further into this avascular space. Stalk cells form a luminised tube, through the fusion of vacuoles, which enables these new vessels to connect with other vessels to form a continuous network through which blood can flow. It was previously proposed that leading tip cells and the following stalk cells were fixed. However, it has now been shown that cells within a sprouting vessel are continually interchanging and phenotypes switching, resulting in stalk cells becoming new tip cells (Bentley et al., 2009, 2014). When the oxygen needs of the newly vascularised region are met by the influx of oxygenated blood, the VEGF levels return to normal and the endothelial cells return to their quiescent phenotype. The neocapillary is then stabilised through pericyte recruitment and the deposition of ECM to ensure that the vessel can withstand the mechanical shear stress experienced through blood flow (Adams and Eichmann, 2010).

2.3 Current Tissue Engineering Strategies for the Induction of Vascularisation

Attaining sufficient vascularisation in engineered tissue is deemed to be one of the greatest challenges in tissue engineering today. Numerous research strategies have been undertaken in the field of tissue engineering in an attempt to overcome this problem. These strategies include the encouragement of vessel infiltration from the external in vivo environment using growth factors or other stimuli, the spontaneous establishment of a primitive network by embedded endothelial cells and the more controlled establishment of vascular networks through direct bio-fabrication techniques.
2.3.1 Encouraging Vessel Infiltration from the Outside In

2.3.1.1 Growth Factors

When a tissue engineered construct is implanted in vivo, spontaneous vascularisation, driven by both the innate wound healing response and by the hypoxic state which the implanted cells are experiencing, will typically occur following the implantation surgery. This in turn stimulates the release of angiogenic factors leading to vessel infiltration. However, this spontaneous vessel ingrowth is often slow and fails to provide adequate oxygen and nutrients to maintain the viability of the implanted tissue. Proangiogenic growth factors, such as VEGF, FGF and platelet derived growth factor (PDGF), can be introduced into the scaffold to enhance and accelerate this spontaneous vascularisation. The most common method employed in the field to incorporate growth factors is to encapsulate them using micro- or nanoparticles (Perets et al., 2003; Ennett, Kaigler and Mooney, 2006; Rocha et al., 2008; Rui et al., 2012; Yan et al., 2019). This versatile method protects the growth factor from degradation and can enable a more controlled release profile as compared to other methods such as adsorption. For example, PLGA microspheres containing VEGF were shown to initiate the vascularisation of a tissue engineered neointestine. Small intestine organoid units and VEGF microspheres were loaded into polyglycolic acid (PGA) tubular scaffolds and implanted into the omentums of rats (Rocha et al., 2008). The release of VEGF resulted in the development of a significantly higher number of vessels, compared to the empty microsphere control group and enhanced epithelial cellular proliferation therefore resulting in larger intestinal constructs. Microspheres have also been used to control the local delivery of PDGF-BB with a view to enhancing neovascularisation (Figure 2.2). The growth factor was loaded into multiscale microspheres which are comprised of inorganic porous silicon particles encased in a biodegradable PLGA outer shell. The microspheres displayed a reduced initial burst release and a controlled localised release of the loaded PDGF for over 2
weeks *in vivo*. This resulted in the formation of functional neovasculature (Minardi *et al.*, 2017).

The synergistic effect of multiple growth factors on vessel formation has also been studied. When ovarian tissue was encapsulated within a fibrin hydrogel containing growth factors and implanted subcutaneously into mice, it was shown that a combination of FGF and VEGF resulted in significantly higher vascular density compared to hydrogels containing either VEGF or FGF2 alone. This result in turn led to lower levels of apoptotic cells and in higher levels of follicle survival in the combination group (Gao *et al.*, 2015). This finding has also been shown in acellular scaffolds implanted subcutaneously into rats. The addition of FGF2 and VEGF to a collagen–heparin scaffold led to mature vascular formation within 7 days which could support the oxygen demands of invading cells as indicated by the absence of any hypoxia inducible factor 1-α accumulation within the nuclei of the infiltrated cells (Nillesen *et al.*, 2007).
**Figure 2.2 (A)** Schematic of multiscale microspheres, composed by a nanostructured silicon multistage vector (MSV) core and a poly(dl-lactide-co-glycolide) acid (PLGA) forming outer shell (PLGA-MSV), for sustained release of functional platelet-derived growth factor-BB (PDGF-BB) to induce angiogenesis in vivo. **(B)** SEM micrographs of PLGA-MSV microspheres and individual discoidal MSV respectively. **(C)** Intravital microscopy images at 14 days after PLGA-MSV and PLGA-MSV/PDGF-BB were injected subcutaneously in mice. PLGA-MSV are in green, whereas vessels are in red. Adapted from (Minardi et al., 2017).

**Two Stage Vascularisation Approaches**

Studies into hepatocyte transplantation incorporated VEGF into biodegradable PLGA microspheres which were then incorporated into porous alginate scaffolds. These scaffolds were implanted into the liver lobes of rats and, 7 days later, hepatocytes were transplanted to the implantation site. The pre-implantation of these VEGF-releasing scaffolds significantly increased the vessel density at the implantation site and consequently improved hepatocyte engraftment when compared to control scaffolds without VEGF (Kedem et al., 2005).

Another effective two step vascularisation approach which has had much success in the bone tissue engineering field is the *in vivo* bioreactor (IVB) technique (Holt *et al.*, 2005). This approach involves leveraging the innate regenerative capacity of the body by implanting a construct into an ectopic site where it undergoes vascularisation, cellular colonisation and tissue maturation. This construct can then be explanted and implanted into the defect site. Multiple implant sites have been studied such as subcutaneous pockets and muscular flaps. To further enhance the efficacy of these approaches the implanted scaffold can be surgically embedded adjacent to an axial vascular bundle or arteriovenous loop. These vessels can undergo angiogenesis and lead to a more rapid vascularisation of the implanted construct.

The IVB approach has shown success in many small (Buehrer *et al.*, 2015; Ersoy *et al.*, 2015; Patel, Flanagan and Hollister, 2015) and large animal models (Eweida *et al.*, 2011;
Spalthoff et al., 2015; Tatara et al., 2015) and has been employed in multiple human trials (Cheng et al., 2006; Mesimäki et al., 2009; Horch et al., 2014). In 2006, Warnke et al. reported the treatment of an extended mandibular discontinuity defect using a custom scaffold which was pre-implanted into a muscle flap (Warnke et al., 2006). A titanium mesh was loaded with HA blocks that were coated with recombinant human BMP-7 and bone marrow-derived MSCs. This scaffold was then implanted into the patient’s latissimus dorsi muscle to allow for the generation of heterotopic bone and for the ingrowth of vessels from the thoracodorsal artery. After 7 weeks in vivo, this bone graft was transplanted to the large mandibular defect, which had been resected eight years previously due to cancer. The graft was a success and the patient regained full masticatory function (Warnke et al., 2006). A similar approach in humans was able to circumvent the need for exogenous hormones by inserting an axial vascular bundle within β-TCP cylinders before intramuscular implantation (Kokemueller et al., 2010). Bioartificial bone grafts were prefabricated in the latissimus dorsi muscle of a 57-year-old man. Each construct was first surgically supplied with axial perfusion through the insertion of a vascular bundle within the core of the implant. Next the implants were packed with autologous morselised bone and bone marrow. After 6 months of growth in the muscle, highly vascularised bone grafts were produced without the need for additional growth factors to enhance bone formation. These bone grafts were excised and used as bone grafts for mandibular reconstruction (Kokemueller et al., 2010).

2.3.1.2 Scaffold Architecture and Topography

The physical architecture of a scaffold can also greatly influence vascularisation (Rücker et al., 2006; Choi et al., 2013; Joshi et al., 2013). An early study into membrane pore sizes showed that architecture can have a stronger influence on vascularisation than chemical composition can. Neovascularisation of an implanted synthetic membrane was shown to be highest when membrane pore sizes were between 0.8 and 8.0 μm regardless of the material used (Brauker et al., 1995). Altering the pore size of a chitosan scaffold by varying the
processing temperature during cryogenic prototyping resulted in two scaffolds with a pore size of ∼90 μm and ∼35 μm. When implanted subcutaneously into rats, only the constructs with a pore size of ∼90 μm were invaded with host blood vessels (Lim et al., 2008). Although this effect of pore size is consistent throughout the literature, it should be noted that the method employed to determine pore size (e.g., using a histological technique with polymer resin-embedded samples (O’Brien et al., 2004), using image software such as ImageJ on SEM images (Madhavan et al., 2018), using a selective partitioning of fluorescent proteins within the pore structure followed by confocal microscopy (Chiu et al., 2010) can greatly influence pore size values making it difficult to compare across the literature. It has also been shown that the interconnectivity between pores has an even greater influence on neovascularisation determining not only the vessel density but also the vessel diameter (Klenke et al., 2008; Bai et al., 2010; Chiu et al., 2011; Feng et al., 2011).

Surface roughness can also influence neovascularisation. Although changes in pore size can influence surface roughness, it can be viewed as an independent material property. Studies into surface topography have shown this can affect the spatio-temporal pattern of neovascularisation. Significantly higher levels of peri-implant blood vessel density were seen with the rough implant compared to the smooth surface of the same material (Khosravi et al., 2018). Another example of how surface topography influences vascularisation is seen in a bio-inspired hybrid nanosack developed to stimulate host vessel infiltration to implanted tissue engineered scaffolds (Hwang et al., 2016). By combining an ECM-mimicking peptide amphiphile nanomatrix gel with an electrospun Polycaprolactone (PCL) nanofiber sheet with porous crater-like surface topography, the mechanical stability of the inner gel is protected. The crater-like surface topography enhanced blood vessel formation compared to a smooth control when implanted into the rat omentum. Furthermore, the delivery of FGF-2 along with the crater-like structures synergistically benefited the neovascularisation of the implant. The
combination of growth factor and surface topography resulted in higher vascularisation compared to either factor alone (Hwang et al., 2016).

2.3.2 Prevascularising Tissue Engineered Constructs

One of the major drawbacks to the vascularisation of tissue engineered implants through host angiogenesis is the time required for the host vessels to reach the inner core of the construct. Although this process can be accelerated with the use of growth factors, the formation of blood vessels involves a complex set of cell-cell interactions. Cells must proliferate, elongate, undergo lumenogenesis and tubulogenesis and finally, undergo stabilisation. This process is relatively slow with the average physiological growth rate of newly developing microvessels being only ~5 μm/h (Utzinger et al., 2015). This has led to the emergence of “prevascularisation” as a promising approach within the field of tissue engineering to overcome this problem. The basic concept of this approach is to establish preformed microvasculature within a construct which can either undergo anastomosis with host vessels at the periphery of the construct which leads to a more rapidly perfused engineered tissue or can be perfused immediately after implantation via surgical anastomosis with an arterio-venous (AV) loop (Beier et al., 2010; Laschke et al., 2011).

The incorporation of endothelial cells into tissue engineered constructs has been shown to support the development of primitive vascular networks through a process akin to vasculogenesis (Chen et al., 2012; Lee et al., 2014). This primitive network can be subsequently organised through angiogenic processes to create a more stable network. A supporting cell type is necessary for the formation of a stable perfusable network. Multiple cell types have been shown to carry out this supportive role and to stabilise endothelial networks, including fibroblasts (Chen et al., 2009), 10T1/2 mesenchymal precursor cells (Koike et al., 2004), and tri-cultures with myoblasts and embryonic fibroblasts (Levenberg et al., 2005). The inclusion of these perivascular-like cells supports the endothelial cell
capillary plexus which is vital for the formation of a mature vascular network (Melero-Martin et al., 2008).

Chen et al. showed that human blood-derived endothelial colony forming cells (ECFCs) were capable of forming capillary-like networks in a photopolymerisable gelatin methacrylate (GelMA) hydrogel in the presence of bone marrow-derived mesenchymal stem/stromal cells (MSCs) (Chen et al., 2012). Distinct lumens are formed through the fusion of ECFC intracellular vacuoles, while the MSCs act as stabilising cells by differentiating into perivascular cells occupying abluminal positions within the network. To investigate if these networks could form in vivo, ECFCs and MSCs suspended as single cells in GelMA were subcutaneous implanted into immunodeficient mice and formed extensive capillary-like networks that underwent functional anastomoses with the existing vasculature (Chen et al., 2012). In a similar study, HUVECs and 10T1/2 mesenchymal precursor cells were seeded in fibronectin-type I collagen gels and implanted into mice bearing chronic dorsal window chambers and formed stable vessels. These chambers allowed for the continuous observation of vessel integrity over time and showed that the implanted vessels remained functional for up to one year post-implantation (Koike et al., 2004). Furthermore, it has been shown that prevascularising fibrin scaffolds prior to implantation accelerates anastomosis with the host vasculature (Chen et al., 2009).

A particular advantage of using MSCs in co-cultures with angiogenic cells to produce vessel-like structures is that they can carry out both a pericyte-like role as well as acting as a precursor cell type for tissue regeneration for example for bone (Grayson et al., 2010; Zomorodian and Baghaban Eslaminejad, 2012; Daly et al., 2018) or adipose tissues (Choi, Park and Suh, 2005; Mauney et al., 2007; Vashi et al., 2008). MSCs were first isolated from bone marrow (Friedenstein, Gorskaja and Kulagina, 1976) but have since been isolated from multiple sources throughout the body including adipose tissue (Zuk et al., 2001), umbilical cord (Zeddou et al., 2010) and peripheral blood (Zvaifler et al., 2000). These different sources
each provide unique microenvironments which has been shown to affect MSCs’ potential. For example, MSCs derived from bone marrow aspirates have been shown to possess stronger osteogenic and lower adipogenic differentiation potentials compared to adipose tissue-derived MSCs (L. Xu et al., 2017). In terms of angiogenic potential, a comparison of bone marrow-derived MSCs and adipose tissue-derived MSCs showed bone cell types had equal angiogenic capacity both in vitro and in vivo (Ma et al., 2014). As previously described, during physiological angiogenesis, pericyte recruitment occurs during the later stages after ECs have first proliferated and developed chord-like structures (Adams and Eichmann, 2010). To recapitulate this, studies into EC co-cultures have delayed the addition of MSCs until after endothelial cells has first sprouted and formed a primitive vascular network within a collagen–glycosaminoglycan scaffolds (McFadden et al., 2013). A delay in MSC addition after 3 days resulted in enhanced vascular network density compared to simultaneous addition of both cell types. However, addition of MSCs after 6 days of culturing did not display this enhancement. Furthermore, without the addition of MSCs a regression in the vessels was observed through the disengagement of cells from tubule structure (McFadden et al., 2013). This study highlights the cellular crosstalk occurring between MSCs and ECs during neo-vascularisation.

It should be noted that, for cell-based prevascularisation approaches, there is a favourable ratio between endothelial cells and tissue specific cells within the engineered tissue. Studies into fat graft retention show that the inclusion of vascular fraction cells, consisting of endothelial and endothelial progenitor cells, can increase vascularisation in fat substitutes (Paik et al., 2015). However, the relationship between cell number and vascularity is not linear. Higher amounts of endothelial cells do not equate to higher levels of vascularisation but can in fact induce vascular regression. This may be due to the increase in metabolic load which initiates cell competition. Cell competition is a phenomenon which determines an organ’s final size during embryonic development and which maintains
homeostasis throughout later life (Johnston, 2009). Although the mechanisms involved are not fully elucidated, it is believed that cells from different populations can perceive differences in their discrete growth rates and metabolic burdens. Cells which are identified as the weaker population either cease proliferation or undergo apoptosis. This leaves the stronger population to continue to thrive unconstrained (Johnston, 2009; Penzo-Méndez and Stanger, 2014).

### 2.3.3 Direct Patterning of Vascular Cells Using Emerging Biofabrication Techniques

Allowing the *de novo* formation of a vascular network through cell seeding produces networks which are heterogeneous and unpredictable. For some tissue engineered applications, control over vascular architecture is desirable. To achieve this, it is possible to directly pre-fabricate microchannels into tissue engineered constructs. This approach enables a faster and more regulated establishment of a vascular network and the possibility of designing locations for either natural or surgical vascular anastomosis.

The ideal vascular network must ensure that all the cells are within the 200 µm distance of a blood source (Lovett *et al.*, 2009). To achieve this, a very fine distribution of vessels is needed which can dissipate the pressures experienced during blood flow. *In vivo*, this has been achieved through the organisation of the vascular network into a vascular tree; large vessels branching into smaller vessels which in turn diverge into capillaries which are distributed throughout the tissue. Many approaches have been investigated to create these desired patterns of perfusable channels within tissue engineered constructs to mimic *in vivo* vasculature. One powerful tool which can be utilised to facilitate the fabrication of these networks is 3D bioprinting. 3D bioprinting is an additive manufacturing technique which involves the sequential layer-by-layer binding of biocompatible materials to create a 3D construct (Burdis and Daniel J. Kelly, 2019). When that biomaterial contains a biologic (a cell, nucleic acid or biomolecule) it is referred to as a bioink (Nulty *et al.*, 2019). Commonly, bioinks lack mechanical strength and often have poor printing resolution which results in
many 3D bioprinting approaches to combine multiple materials in the same print such as
thermopolymers and sacrificial materials. The following section will review different
biofabrication techniques, including 3D bioprinting, which have been used to pre-vascularise
tissue engineered constructs.

2.3.3.1 The Incorporation of Seeded and Unseeded Perfusable Channels

In the pursuit of incorporating hollow microchannels within tissue engineered
constructs, many studies focussed on simple moulding and templating techniques. For
example, casting a hydrogel scaffold around a needle which can later be removed post-
fabrication which leaves behind a vacant channel (Sakaguchi et al., 2013; W. Zhang et al.,
2015). Although these techniques are straightforward, they are restricted to simple
architectures. Using more sophisticated techniques currently being developed in the rapidly
developing field of biofabrication, more complex channels can be created at a higher
resolution.

One method to fabricate a vascular network template is to use a sacrificial material
which can be embedded within a hydrogel and later removed creating hollow channels
within. Bellan et al. created microchannels within a biocompatible gelatin hydrogel construct,
using a sacrificial melt-spun shellac microfiber network. They exploited the pH-sensitive
solubility of shellac by changing the pH of the composite construct by submerging in a warm
ammonia bath. This enabled the dissolution of the sacrificial network without the use of
organic solvents or extreme temperatures (Bellan et al., 2012).

Miller et al. generated a cytocompatible means of creating densely populated tissue
constructs containing perfusable vascular channels (Miller et al., 2012). Sacrificial
carbohydrate glass was structured using a 3D printer to create a filamentous carbohydrate
glass lattice. This lattice was then protectively coated using poly (D-lactide-co-glycolide)
(PDLGA) and encapsulated within a cell-laden hydrogel. The carbohydrate glass was then
dissolved away in cell culture medium to create embedded channels. Miller et al. demonstrated this method’s versatility by using a wide range of both natural and synthetic ECM materials: agarose, alginate, PEG, fibrin and Matrigel which exhibit a variety of crosslinking approaches including chain entanglements, ionic interactions, photopolymerisation, enzymatic activity and protein precipitation respectively. Although this is a major step forward in the field, only relatively simple construct architectures and geometries can be produced using such a hybrid printed/moulded technique.

Kolesky et al. (Kolesky et al., 2014) endeavoured to overcome these shortcomings by employing a 3D bioprinting method to produce complex vascularised tissue containing multiple cell types. They developed a fugitive ink containing Pluronic F127 which is biologically inert and undergoes thermally reversible gelation (Wu, DeConinck and Lewis, 2011). Between 4-22 ºC, the fugitive ink is stiff and exhibits a solid-like response and strong shear thinning behaviour. Below 4°C, the ink liquefies and can be removed from the construct. GelMA was utilised as the cell carrier and bulk matrix. By exploiting the windows of contrasting gelation temperatures between the two inks, vascular networks could be embedded within GelMA, photo-polymerised and then cooled to allow the extraction of the pluronic under a gentle vacuum leaving behind perfusable interconnecting vessels. The embedded vasculature was shown to be easily endothelialised by flushing the vessels with a HUVEC cell suspension. Pluronic F-127 was similarly used to fabricate cell laden tissues exceeding 1cm in thickness containing embedded vasculature using a gelatin-fibrin matrix which is crosslinked by a dual enzymatic, thrombin and transglutaminase (TG) strategy (Kolesky et al., 2016) (Figure 2.3). There have been numerous other materials to date which have been used to create hollow vessel-like channels within tissue engineered constructs such as gelatin (Lee et al., 2016), alginate (Hammer et al., 2014) and agarose (Bertassoni et al., 2014).
Figure 2.3 Three-dimensional vascularised tissue fabrication. (A) Schematic illustration of the tissue manufacturing process. (i) Fugitive (vascular) ink, which contains pluronic and thrombin, and cell-laden inks, which contain gelatin, fibrinogen, and cells, are printed within a 3D perfusion chip. (ii) ECM material, which contains gelatin, fibrinogen, cells, thrombin, and TG, is then cast over the printed inks. After casting, thrombin induces fibrinogen cleavage and rapid polymerisation into fibrin in both the cast matrix, and through diffusion, in the printed cell ink. Similarly, TG diffuses from the molten casting matrix and slowly cross-links the gelatin and fibrin. (iii) Upon cooling, the fugitive ink liquefies and is evacuated, leaving behind a pervasive vascular network, which is (iv) endothelialised and perfused via an external pump. (B) HUVECs growing on top of the matrix in 2D, (C) HNDFs growing inside the matrix in 3D, and (D) hMSCs growing on top of the matrix in 2D; scale bar = 50 µm. (E) Images of printed hMSCs-laden ink after 3 days in the 3D printed filament where actin (green) and nuclei (blue) are stained. (F) Photographs of interpenetrated sacrificial (red) and cell inks (green) as printed on chip; scale bar = 2 mm.) (G) Top-down bright-field image of sacrificial and cell inks; scale bar = 50 µm.). (H-J) Photograph of a printed tissue construct housed within a perfusion chamber (H) and corresponding cross-sections (I and J); scale bars = 5 mm. Adapted from (Kolesky et al., 2016).

Despite the many successes of these methods, most have been limited by the resolution attainable from extrusion-based fabrication technology used. This restricts the vessel diameter size and fails to recapitulate the microvascular size and structure of native capillary beds. Advances in laser-assisted techniques have resulted in microchannels being fabricated with diameters as low as 10µm (Brandenberg and Lutolf, 2016; Arakawa et al.,...
2017; Lim et al., 2018). Photoablation can be used to directly produce microchannels within soft hydrogels. This method uses focalised pulsed lasers with sufficient energy to break down covalent bonds allowing for channels to be formed in any location within the gel and at any time. This method is compatible with various hydrogels including collagen (Ilina et al., 2011; Brandenberg and Lutolf, 2016), PEGDA (Heintz et al., 2016), PEGylated fibrinogen (Sarig-Nadir et al., 2009) and silk protein hydrogel (Applegate et al., 2015). However, due to the very high-energy light, many of these methods are not cytocompatible. To overcome this, multiphoton lithography can be utilised to photo-degrade channels. Using this approach, endothelialised microchannels with varying inner diameters, were created within a cell-laden synthetic peptide-polymer-based hydrogel containing a photolabile oNB moiety (Arakawa et al., 2017). Because this type of approach enables the modification of a 3D cell-laden hydrogel, this would in theory enable the modification of a tissue engineered construct which had been previously culturing for several days or weeks. This method, however, is only as powerful as the microscope being used. The depth of penetration into the sample and the fabrication speed is limited by the working distance and spot size of the objective lenses used.

Another laser-assisted technique gaining momentum in the field of tissue engineering is the technology of digital light processing (DLP). This technology involves the use of a digital mirror device (DMD) to control a laser beam within a curable polymer (Wu and Hsu, 2015). DMDs comprise of an array of several hundred thousand independent micro-mirrors which can project a patterned mask of light. Once one layer is cured, masks of light can be projected on top of that layer resulting in rapid layer-by-layer photo-polymerisation and the fabrication of a 3D object. This process has been used to produce cell-laden scaffolds with resolutions of 25–50 µm (Lim et al., 2018). Due to the resolution that is achievable with this technique, it has been shown that microarchitectures can be designed within GelMA scaffolds with which were capable of manipulating HUVEC morphology and guiding multicellular organisation (Zhang et al., 2012).
2.3.3.2 Printing within a support bath

One of the challenges, involved in trying to bioprint vascular networks, is recapitulating their complex architecture. Due to their hollow structure and the necessity to interconnect diverging diameters on multiple planes, they often collapse during the printing process. This issue can be overcome by printing directly into a support bath. The support bath can hold the printed bioink in place, resisting gravity-induced deformation, until the network can be stabilised by crosslinking. Early work in this field used the fugitive ink Pluronic F127 to print omnidirectional networks within a modified photocrosslinkable Pluronic F127 diacrylate solution bath with a less viscous fluid filler above (Figure 2.4 A) (Wu, DeConinck and Lewis, 2011; Wu et al., 2016). The purpose of the fluid filler was to fill the crevasses that were left behind after the needle passed through the bath. This fluid could then be photocured after the printing was complete. Other approaches overcame this problem of needle deformation by using shear-thinning and self-healing support baths (Highley, Rodell and Burdick, 2015; Song et al., 2018). A seminal paper in the field, used gelatin microparticles in the support bath in a method termed FRESH (freeform reversible embedding of suspended hydrogels) (Hinton et al., 2015). This microparticle slurry acted as Bingham plastic during the printing process. It behaved as a rigid body at low shear stresses but at higher shear stresses it flowed as a viscous fluid. An added benefit of these gelatin microparticles was that they could be easily washed away at 37°C. This method was very adaptable and could be used to print biomaterials with a range of crosslinking mechanisms such as ionic, enzymatic, pH/thermally driven mechanisms. For example, by incorporating thrombin within the support slurry, fibrinogen could be printed and polymerised into stable fibrin within the bath. The greatest benefit of this method is the ability to print collagen. The FRESH method has recently been refined (FRESH v2.0) and can now achieve consistent printing of collagen filaments ~250 µm in diameter (Lee et al., 2019). Collagen I is one of the most abundant proteins in the ECM of many tissues and organs and as such is an attractive
biomaterial for tissue engineering applications. However, due to its pH-driven fibrillation, it is unsuitable for many biofabrication approaches. With FRESH v2.0, high concentration, acidic collagen can be printed, and its pH rapidly neutralised within the bath. The resulting construct can be easily removed from the bath and can be glutaraldehyde-fixed for added stability. Although this method can enable printing of large-scale constructs (Figure 2.4 B), its acidic nature prevents cells being directly incorporated into the collagen bioink. Cells must be added to a separate bioink that can be printed alongside the collagen. Another method used a supporting hydrogel bath, as opposed to a microparticle slurry bath. In this approach, a sacrificial ink was extruded within a support bath in a helical shape (Song et al., 2018). The support bath was covalently crosslinked using UV light. This printed ink could then be removed, leaving a helix channel within the support ink. These channels were then seeded with HUVECs leading to endothelialised vessels which underwent angiogenic sprouting (Figure 2.4 C-E).
**Figure 2.4** (A) Schematics of omnidirectional printing of 3D microvascular networks within a hydrogel reservoir. (i) Deposition of a fugitive ink into a physical gel reservoir allows hierarchical, branching networks to be patterned. (ii) Voids induced by nozzle translation are filled with liquid that migrates from the fluid capping layer. (iii) Subsequent photopolymerisation of the reservoir yields a chemically cross-linked, hydrogel matrix. (iv/v) The ink is liquified and removed under a modest vacuum to expose the microvascular channels. (vi) Fluorescent image of a 3D microvascular network fabricated via omnidirectional printing of a fugitive ink (dyed red) within a photopolymerised Pluronic F127-diacylate matrix; scale bar = 10 mm. (B) Organ-scale FRESH 3D bioprinting of neonatal-scale heart. (C) Microchannel fabrication process where i) a PDMS holder is placed on a coverslip, ii) the PDMS reservoir is filled with a support hydrogel (grey) and an ink hydrogel (yellow) is printed within, iii) the hydrogel is exposed to light in the presence of a photoinitiator to stabilise the support hydrogel, and (iv) the fugitive ink hydrogel is washed with flow and the introduction of excess β-cyclodextrin (β-CD) in solution. Representative time lapse images of the hydrostatic perfusion of fluorescent beads (red,
individual bead in yellow circle tracked across images) through the microchannels. (D) Fluorescent images of endothelial cell-seeded spiral microchannels i) fluorescent beads (green) flowing through the microchannels and ii–v) endothelial cell-seeded channels stained for CD31 (red) and nucleus (blue) 2 d after seeding, including ii) maximum projection, iii) cross-section, and 3D reconstruction at iv) low and v) high magnification. A yellow dotted line and a white box in (ii) indicate acquisition position of (iii) and (v) images, respectively. (D) Representative maximum projection images of EC sprouting from the left channel into the support hydrogel, labelled with cell tracker (red) and Hoechst (nuclei, blue) on days 0, 2, and 3 and with controls of nondegradable crosslinker (DTT) or protease inhibitor (Marimastat) at day 3. Adapted from (Wu, DeConinck and Lewis, 2011; Song et al., 2018; Lee et al., 2019).

2.3.3.3 Droplet-based bioprinting

Droplet-based bioprinting, also known as inkjet or drop-on-demand printing, involves the precise ejection of bioink droplets which are formed via thermal, piezoelectric or electrostatic means (Burdis and Daniel J. Kelly, 2019). Droplet-based bioprinting has been used to engineer blood vessels in vitro (Schöneberg et al., 2018). Three different cell types embedded in different hydrogels were deposited dropwise in a defined spatial arrangement to mimic the different layers of a natural vessel. Firstly, gelatin containing a high concentration of HUVECs was printed to create a sacrificial core. A second solution containing thrombin was then deposited onto of this layer and finally the third bioink, containing fibrinogen with smooth muscle cells, was printed on the outside. These final two steps were repeated to create a thin fibrin layer around the core material. When the process was completed, the construct was embedded in a collagen/fibrin hydrogel containing fibroblasts and then the gelatin inner core was washed away to obtain the open channel. This approach resulted in the three layers of a blood vessel being recapitulated: the tunica intima (endothelium), the tunica media (elastic SMCs) and the tunica adventitia (matrix of fibroblast) (Figure 2.5 A-C). This approach allows for the formation of a confluent and continuous endothelium within the channel without the need for a post-seeding step.

In another example of droplet-based bioprinting, branched microvasculature was fabricated using alginate droplets printed onto a gelatin substrate containing CaCl₂. The layers of alginate droplets were crosslinked by the diffusion of calcium ions from the gelatin
substrate, through the bottom alginate layers. The vessels were printed transversally and were closed off by depositing droplets at the edges in incremental steps resulting in a “gothic arch”-style lumen (Pataky et al., 2012).

Xu et al. reported the production of 3D zigzag cellular tubes that were fabricated using a Z-shape platform-assisted inkjet printing system (Xu et al., 2012)(Figure 2.5 D). This method, which the authors have coined scaffold-free bioprinting, mixed 3T3 fibroblast cells in 1% sodium alginate solution which was deposited dropwise into a crosslinker pool. These sodium alginate droplets in turn fused to form tubular structures. This method however required relatively long printing times compared to other approaches.
Figure 2.5 (A) Schematic of vascular channel design: a gelatin core printed with a single printer head used at 37 °C. For the surrounding fibrin layer, the printing pattern is calculated by slicing the channel lengthwise and defining droplet positions in angular steps around the channel outline of each cross section. Two printer heads are used, filled with fibrinogen and SMCs alternatingly, as well as thrombin as a crosslinker. (B) i. Schematic cross section of a reactor shows a single channel. ii. close-up shows a single layer of endothelial cells (EC) iii. and an additional smooth muscle cell (SMC) layer. (C) i. Confluent layer of ECs at the channel wall after 14 days of flow in cross section (CD31 in green, Actin-phalloidin in red, DAPI in blue) ii. Combination of SMC layer and the EC layer showing the distribution of SMCs close to the intact channel as a cross section after 7
days of flow (CD31 in green, prelabelled fibroblasts in red, prelabelled SMCs in yellow, DAPI in blue). iii. A representative fluorescence microscopy image in cross-section highlights the whole architecture of a multi-layered vessel model after 4 days of dynamic cultivation; EC are homogenously distributed in the inner part of the lumen, surrounded by fibroblasts and SMCs. (Phalloidin in green, prelabelled fibroblasts in red, prelabelled SMCs in yellow, DAPI in blue). (D) Schematic of the Xu et al platform-assisted 3D inkjet bioprinting system. Adapted from (Xu et al., 2012; Schöneberg et al., 2018).

### 2.4 Vascularisation and Bone Tissue Engineering

Bone tissue engineering is one of the oldest and most established research areas in all of organ replacement. Despite the significant progress achieved over the last 30 years, there has been only a limited success in the clinical implementation of bone tissue engineering. One of the biggest hurdles to overcome is vascularisation. Creating a functioning vascular system is crucial to producing healthy and functional bone tissue.

In healthy bone, not only is vasculature necessary to supply the requisite oxygen and nutrients and to eliminate waste products but it is also essential for bone remodelling and repair. During bone fracture repair, intact vasculature is crucial for successful bone healing. The presence of vascular injuries during bone repair significantly delays healing, increasing the incidence of delayed and non-union repair from 10-20 % in the overall population (Aspenberg et al., 2010) to almost 50 % (Brownlow, Reed and Simpson, 2002). Along with supplying oxygen and nutrients during repair, blood vessels also supply the necessary constituents of mineralisation, calcium and phosphate. Recent evidence suggests that the perivascular cells surrounding vessels are the source of osteoprogenitor cells during repair. Cell lineage tracing in mice has shown that perivascular cells positive for αSMA could differentiate into chondrocytes and osteoblasts during bone remodelling (Grcevic et al., 2012). This has been further validated by studies into bone fracture healing which showed that the majority of callus cells were derived from αSMA-positive cells (Zhang et al., 2005). Endothelial cell involvement has also been linked to the degradation of the cartilage matrix by secreting the matrix-degrading protease MMP-9 (Colnot et al., 2003; Wang et al., 2013).
It has been suggested that they may also play a role in enabling mineralisation of the cartilage matrix by secreting BMPs (Yu et al., 2010; Matsubara et al., 2012; Bahney et al., 2014) and in regulating the deposition of collagen I (Ben Shoham et al., 2016).

2.4.1 Bone Development

The skeletal system provides essential structural support to the body. It comprises of bones, cartilage, tendons and ligaments and enables movement and protects delicate organs. It is a highly dynamic system which undergoes constant adsorption and rebuilding throughout its lifetime. Its main component, bone, contains multiple cell types, including osteoclasts, osteoblasts and osteocytes. There are two types of bones in the mammalian skeletal system; long bones and flat bones, formed by two distinct processes: endochondral ossification and intramembranous ossification (Kronenberg, 2003). Both of these processes start with the aggregation of mesenchymal cells to form mesenchymal condensations. For the majority of our bones this process continues down the endochondral ossification pathway, during which these MSC condensations differentiate into chondrocytes, the main cell type of cartilage (Figure 2.6 A). Cells surrounding these condensations form a perichondrium, a dense membrane that envelops the condensations. Chondrocytes secrete an extracellular matrix rich in type II collagen, which creates an avascular template. After the completion of this template, chondrocytes halt proliferation, undergo hypertrophy and begin to produce type X collagen. Hypertrophic chondrocytes drive bone growth through their secretion of factors such as the potent pro-angiogenic growth factor VEGF, multiple proteolytic enzymes such a MMP-13 and the TNF-family cytokine RANKL (Johansson et al., 1997; Rabie and Hägg, 2002; Xiong et al., 2011). VEGF results in the recruitment of surrounding vasculature to the cartilage template; MMP-13 is involved in early matrix remodelling whilst RANKL is involved in the differentiation of osteoclasts. Osteoclasts are multinucleated giant cells derived from hematopoietic progenitors of the monocyte-macrophage lineage. They are unique in their function to digest calcified matrix. During endochondral ossification, osteoclasts dissolve
and resorb the hypertrophic cartilage. The classically accepted model of endochondral ossification proclaims that the hypertrophic chondrocytes, having completed their role, undergo programmed cell death leaving room for the osteoblast progenitors cells, to differentiate into osteoblasts and, using the calcified cartilage as a scaffold, begin to secrete bone matrix known as osteoid (Maes et al., 2010). However, recent studies have proposed an alternative model for endochondral ossification in which chondrocyte programmed cell death may not occur; rather some hypertrophic chondrocytes regain some stem cell-like functionality facilitating a chondrocyte-to-osteoblast transformation (Hu et al., 2017).

Flat bones are formed through the process of intramembranous ossification (Figure 2.6 B). This process is initiated by mesenchymal stem cells within mesenchyme, or the medullary cavity of a bone fracture. A small group of mesenchymal stem cells begin to rapidly proliferate forming a dense cluster of cells, known as a nidus. Upon formation of the nidus, the stem cells within it stop replicating and start to enlarge and differentiate into osteoblasts. The containing Golgi apparatus and rough endoplasmic reticulum increase in size, allowing the efficient production of a type-I collagen-rich extracellular matrix called osteoid. Some of the osteoblasts become incorporated within the osteoid to become osteocytes. Next, the osteoid undergoes mineralisation forming primitive bone tissue known as spicules. These spicules further develop, firstly fusing together to form trabeculae and later becoming interconnected and forming woven bone. The periosteum is formed around the trabeculae (Percival and Richtsmeier, 2013).
Figure 2.6 Bone Formation and Development. (A) Endochondral Ossification. Endochondral ossification follows five steps. (a) Mesenchymal cells differentiate into chondrocytes. (b) The cartilage model of the future bony skeleton and the perichondrium form. (c) Capillaries penetrate cartilage. Perichondrium transforms into periosteum. Periosteal collar develops. Primary ossification centre develops. (d) Cartilage and chondrocytes continue to grow at ends of the bone. (e) Secondary ossification centres develop. (f) Cartilage remains at epiphyseal (growth) plate and at joint surface as articular cartilage. (B). Intramembranous Ossification. Intramembranous ossification follows four steps. (a) Mesenchymal cells group into clusters, and ossification centres form. (b) Secreted osteoid traps osteoblasts, which then become osteocytes. (c) Trabecular matrix and periosteum form. (d) Compact bone develops superficial to the trabecular bone, and crowded blood vessels condense into red marrow. Adapted from (Biga et al., 2013).

2.4.2 Bone Tissue Engineering Strategies

Although bone has an innate capacity to self-heal, this healing process is limited by the size of defect. When large bone defects occur, due to severe trauma or tumour resection, for example, the bone’s regenerative ability is depleted, and clinical intervention
is required. Autologous bone grafts are still regarded as the gold standard for treating such cases. This treatment involves harvesting bone from elsewhere in the patient’s body, most usually the iliac crest. Although these grafts have excellent osteoconductivity and desirable mechanical properties, the procedure can lead to donor site morbidity and is limited to the bone volume available (Pape, Evans and Kobbe, 2010). These drawbacks have led to the emergence of bone tissue engineering as a potential new therapy for bone regeneration.

Early bone tissue engineering strategies involved using osteoconductive 3D scaffolds to support cells which are osteogenically primed to produce bone matrix in a process akin to intramembranous ossification (Casabona et al., 1998; Yoshikawa et al., 1998). However, this process often resulted in engineered constructs that were dense and inhibited sufficient vascularisation when implanted in vivo. This leads to poor cell survival and in some cases core necrosis of the engineered tissue (Lyons et al., 2010). In more recent years, tissue engineers have started to move away from these more traditional strategies towards an approach which aims to recapitulate embryonic bone formation. This fusion of engineering principles with developmental biology concepts has been termed “developmental engineering” (Lenas, Moos and Luyten, 2009). Developmental bone tissue engineering aims to engineer hypertrophic cartilage templates which will later calcify to produce bone in a process akin to endochondral ossification. Both strategies have made an important impact on the field and are described in more detail below.

2.4.2.1 Intramembranous Bone Tissue Engineering Strategies

Many of the early bone tissue engineering strategies attempted to mimic the process of intramembranous ossification, focusing on the use of ceramics, particularly hydroxyapatite, to promote the direct osteoblastic differentiation of skeletal stem/progenitor cells. Hydroxyapatite (HA) is composed of calcium phosphate and is the major mineral component of bone. Since the 1980s, HA ceramics have been commercially available and successfully used in many dental and craniofacial surgeries (Heise, Osborn and Duwe, 1990;
Yoshikawa et al., 1998). Early studies demonstrated the importance of porosity in such ceramics. Dense HA and porous HA ceramic granules were implanted subcutaneously in a canine model and showed that bone had formed in the porous ceramics via intramembranous ossification but no bone had formed in the dense HA ceramic by 3 months (Yamasaki and Sakai, 1992). Additional studies have shown that the osteogenic capacity of porous ceramic scaffolds can be enhanced with β-tricalcium phosphate (β-TCP) or with autologous MSCs to drive bone formation both ectopically and orthotopically (Bruder et al., 1998; Le Nihouannen et al., 2005). In the early 2000s, a human clinical trial was undertaken using autologous bone marrow stromal cells on macroporous HA scaffolds. The trial was carried out on three patients, aged between 16 and 41 years old with bone defects ranging between 4 and 7cm long. The patients’ cells were isolated from their bone marrow and expanded *ex vivo* before being placed on macroporous HA scaffolds. The size and shape of these scaffolds were tailored to each patient and implanted at the lesion sites. External fixation devices were necessary for the first 6-13 months to provide mechanical stability before their removal when all three patients recovered the function of their affected limbs (Quarto et al., 2001). Despite these promising results, ceramics have seen only modest success in the field overall partly due to their brittle nature and slow degradation characteristics (O’Brien, 2011).

To find an alternative scaffold biomaterial with improved mechanical properties and faster degradation rates, many researchers have turned towards biodegradable polymers. Many different types of polymers have been used to date which include natural polymers, such as collagen and chitosan, and synthetic polymers such as poly(ε-caprolactone) (PCL) and Poly (lactic-co-glycolic acid) (PLGA).

In contrast to ceramics, most polymeric materials are biologically inert and require exogenous growth factors to drive osteogenesis. Although osteogenic media can be utilised to induce mineralisation *in vitro* prior to implantation, this treatment can reduce the porosity of the implant and prevent adequate vascularisation *in vivo* (Lyons et al., 2010). To overcome
this issue, growth factors can be directly incorporated into the implant. The direct delivery of growth factors in situ involves their association to scaffolds by one of several methods: physical entrapment with the scaffold material, covalent binding to the scaffold, or the use of nano- and microparticles as reservoirs within the scaffold (De Witte et al., 2018). One of the simplest methods for growth factor delivery, and the one which is most often used commercially, is protein adsorption (Draenert et al., 2013). In this process, scaffolds are simply immersed in a protein solution to incorporate growth factors into the scaffold. The success of this process is strongly influenced by varying scaffold properties such as surface roughness, charge and wettability (Chang et al., 2007; King and Krebsbach, 2012). However, this method is limited by poor release kinetics that can result in an initial burst release of growth factor which is then followed by a slower, uncontrolled release that is dependent on diffusion and the degradation rate of the scaffold biomaterial (Huang and Brazel, 2001; Cheng et al., 2018; Wang et al., 2019). To reduce the burst release of bioactive proteins and to allow for a more prolonged, controlled release profile, growth factors can be chemically bound to the polymer matrix of the scaffold through covalent bonds. Not only does this approach lead to prolonged release profile but it can also provide other advantages. For example, when comparing tethered epidermal growth factor (EGF) with free EGF it has been shown that the tethered presentation of the growth factor increases osteogenic colony formation from human bone marrow aspirates (Marcantonio et al., 2009). Another method for growth factor delivery is the use of nano- or microparticles. Microparticles can be produced from a number of different polymers such as gelatin, chitosan, alginate and silk fibroin (Bessa et al., 2010; Zhang, Wang and Wang, 2010; Cao et al., 2014; Ozkizilcik and Tuzlakoglu, 2014). Poly(N-isopropylacrylamide) (PNIPAM) is a synthetic temperature-responsive polymer which has gained considerable attention recently for its use as a growth factor delivery system (Garbern, Hoffman and Stayton, 2010; Ertan et al., 2013). In vitro studies investigating PNIPAM nanoparticles as growth factor delivery vehicles have
demonstrated that VEGF, incorporated into collagen hydrogels, initiated the osteogenic and angiogenic differentiation of human bone marrow-derived MSCs (Adibfar et al., 2018).

Porosity and pore interconnectivity remain an important factor in the success of biodegradable scaffolds in terms of bone formation (Ratanavarapor et al., 2011; Gupte et al., 2018; Freeman et al., 2019). It has been shown that by altering the pore size of nanofibrous PLLA scaffolds, the processes of chondrogenesis and endochondral ossification could be controlled. Smaller pore sizes enhanced chondrogenesis whilst the larger pore size enhanced endochondral ossification both in vitro and in vivo (Gupte et al., 2018).

2.4.2.2 Endochondral Ossification Strategies

Bone tissue engineering has undergone a paradigm shift in recent years, from the top-down approach of traditional tissue engineering to a bottom-up approach of developmental engineering. This bottom-up approach utilises chondrogenic cells to fabricate a cartilaginous template which initiates a semi-autonomous cascade of events to recapitulate endochondral ossification which occurs during skeletogenesis and the spontaneous healing of long bone fractures (Scotti et al., 2010; Gawlitta et al., 2015; Sheehy, Mesallati, Vinardell, et al., 2015; Daly et al., 2016; Freeman and McNamara, 2017; Liu et al., 2019). Mesenchymal stem cells are an attractive cell source for this approach and have been widely utilised in the field due to their capacity for chondrogenic differentiation and their ability to undergo hypertrophy both in vitro and in vivo (Mueller and Tuan, 2008; Hellingman et al., 2010; Scotti et al., 2010). Once MSCs have differentiated into chondrocytes, they become more adapted to the low oxygen and low nutrient microenvironment of the bone defect environment. Upon undergoing hypertrophy, chondrocytes secrete angiogenic factors which can promote vascular ingrowth and bone formation (Thompson et al., 2015). It has been shown that the type of tissue, from where the MSCs have been isolated, can affect the MSCs’ tendency to proceed down the endochondral route. Chondrogenically primed MSCs isolated from bone marrow, demonstrated increased type X collagen expression and higher levels of
mineralisation upon *in vivo* implantation compared to MSCs isolated from fat-pad and synovial membrane which appeared to undergo fibrous dedifferentiation (Vinardell *et al.*, 2012).

A number of other cell sources have been investigated within the field of endochondral bone tissue engineering such as chondrocytes, embryonic stem cells and induced pluripotent stem cells (iPSCs) with varying success (Jukes *et al.*, 2008; Pippenger *et al.*, 2015; Limraksasin *et al.*, 2020). Both human and porcine articular chondrocytes have been shown to undergo hypertrophy *in vitro*. However, neither type of chondrocyte has been shown to produce sufficient bone volume when implanted *in vivo* (Jeong, Zhang and Hollister, 2012; Narcisi *et al.*, 2012). More recently, chondrocytes isolated from the nasal septum have been investigated as a potential source of hypertrophic chondrocytes as nasal cartilage is widely accepted as the pacemaker for the growth of the tissues of the midface and the anterior portion of the skull (Hall and Precious, 2013). Hypertrophic cartilage templates engineered using nasal chondrocytes isolated from both rats and humans have been shown to form bone *in vivo* (Pippenger *et al.*, 2015; Bardsley *et al.*, 2017). A major limitation of chondrocytes for endochondral bone tissue engineering is their slow *in vitro* expansion and their tendency to de-differentiate during adherent monolayer culturing causing the loss of their chondrocyte-specific gene-expression profile leading to a fibroblastic cell morphology (Stokes *et al.*, 2001; Diaz-Romero *et al.*, 2005).

Embryonic stem cells have the capacity for indefinite undifferentiated proliferation *in vitro* (Levenberg *et al.*, 2003). These cells are isolated from the inner totipotent (capable of giving rise to any cell type) mass of an early mammalian embryo (Thomson, 1998) and they represent a promising cell source for many tissue engineering applications. Murine embryonic stem cells have been shown to produce a cartilage template following seeding onto ceramic particles and subsequent chondrogenic differentiation for 21 days. When implanted subcutaneously into immunodeficient mice, bone formed in and around the
mineralised cartilage matrix (Jukes et al., 2008). However, despite their exciting potential, the labour-intensive procedures necessary for expansion, along with associated ethical and moral objections, have hindered the use of embryonic stem cells in the field thus far.

The formation of iPSCs back in 2006 signified a major scientific breakthrough for biomedical research (Takahashi and Yamanaka, 2006). Terminally differentiated adult cells can be reprogrammed into cells which closely resembled embryonic stem cells through the transfer of four nuclear transcriptional factors (Oct4, Sox2, Klf4, and c-myc) (Okita and Yamanaka, 2010). iPSCs represent an ideal cell source to generated immunologically matched donor cells for tissue engineered tissues without any immune rejection concerns associated with the transplant process (Hirschi, Li and Roy, 2014). To date, iPSCs have been incorporated into multiple tissue engineering strategies for numerous target tissues such as retinal tissue (Zhong et al., 2014), cardiac tissue (Shiba et al., 2016), skin (Abaci et al., 2016) and hepatic tissue (Ma et al., 2016). In terms of endochondral bone, iPSCs have been used to fabricate bone/cartilage hybrid spheroids using osteogenic and chondrogenic media in combination with mechanical stimulation (Limraksasin et al., 2020). During the growth stage, these osteo-chondrogenic constructs exhibited an increased expression of Ncam along with an upregulation of Sox9, similar to the expression pattern of these genes in the mesenchymal condensation process during endochondral bone formation. When maintained in osteogenic medium culture these spheroids underwent osteogenic induction and mineralisation (Limraksasin et al., 2020).

2.4.2.3 Vascularisation Strategies in Bone Tissue Engineering

Scaffold Stiffness and Geometric Design

The mechanical properties of a scaffold is an important feature to be considered for bone tissue engineering. The mechanical cues a cell experiences can have a major effect on its phenotype (Mao, Shin and Mooney, 2016). For example, MSCs grown on stiffer substrates
have been shown to differentiate down an osteogenic pathway, whereas stem cells grown on softer substrates differentiate along an adipogenic pathway (Engler et al., 2006; Mao, Shin and Mooney, 2016). In relation to vascularisation, the extracellular stiffness that endothelial cells experience can affect their angiogenic capacity and directionality of sprouting. It has been found that softer gels improve vessel formation whilst stiffer environments can have the opposite effect (Vailhé et al., 1997, 1998). This makes the design of the scaffold for bone tissue engineering particularly challenging, as they are required to be mechanically robust and provide a matrix stiffness supportive of osteogenesis (stiff substrate), whilst simultaneously providing a substrate supportive of angiogenesis (softer substrate). One example of an approach to address this design challenge was to combine nano-scale and micro-scale starch/PCL fibres to mimic the protein fibril network architecture of ECM whilst giving mechanical stability to the overall scaffold (Tuzlakoglu et al., 2005). This scaffold supported the formation of microvessels without compromising the structural integrity necessary for a bone tissue engineering scaffold (Santos et al., 2007). PCL has also been used to mechanically reinforce composite scaffolds comprising mineralised collagen and glycosaminoglycans (CGCaP). The inclusion of a PCL framework not only provided 6000-fold increase to the bulk modulus of the CGCaP without having to sacrifice porosity, it also provided a foundation for standard bone plates to attach and secure the implant in place (Weisgerber et al., 2016).

As previously mentioned, the introduction of hollow channels within a tissue engineered scaffold can greatly influence vessel ingrowth in vivo (W. Zhang et al., 2015; Daly et al., 2018). In terms of bone tissue engineering, this method has been shown to be a powerful tool for improving the vascularisation and mineralisation of implants. Inspired by the architecture of naturally occurring channels in the root of the lotus plant, microchannels were introduced into 3D printed silicate-based bioceramic akermanite and, when implanted intramuscularly into rats, they were shown to act as conduits for invading host vasculature.
In a rabbit calvarial defect model, higher densities of microchannels led to higher levels of bone formation throughout the scaffold at 12 weeks. The absence of microchannels correlated with only modest bone formation limited to the periphery of the construct (Feng et al., 2017). In a recent study, hollow channels were combined with osteogenic ions to promote vascularisation and large bone regeneration in bioceramic scaffolds (W. Zhang et al., 2017). The unique hollow-pipe-stacked structure was created using a modified printer nozzle with a core/shell design. This enabled the extrusion of a hollow fibre which could be assembled layer by layer into a porous scaffold (Figure 2.7). When implanted into rabbit radius segmental defects, the hollow structures enhanced vascularisation compared to the solid fibre control and resulted in complete bone regeneration including bone marrow cavity reconnection and new bone marrow formation (W. Zhang et al., 2017).

![Figure 2.7](image)

**Figure 2.7** The fabrication of the 3D-printed silicate bioceramic scaffolds with hollow struts. (A) The modification of printer nozzle with a core/shell structure. (B) The structure of the BRT-H scaffolds with about 500 μm inner channels. BRT denotes bredigite bioceramic; BRT-H denotes hollow bredigite bioceramic. Adapted from (W. Zhang et al., 2017).

**Angiogenic Growth factors**

Angiogenic growth factors such as VEGF are commonly added to regenerative scaffold to enhance their capacity to support vascularisation. The benefits of including VEGF within a bone tissue engineering scaffold is twofold. Not only is VEGF a powerful angiogenic
agent (Tammela et al., 2005; Silva and Mooney, 2010), it is also believed to play a role in osteogenesis (Street et al., 2002; Clark et al., 2015; W.-L. Xu et al., 2017). For example, it has been demonstrated that the localised and sustained delivery of VEGF from macroporous biomineralised PLGA scaffolds leads to the rapid ingrowth of vessels, which in turn enhances osteogenesis within a critical-sized cranium bone defect (Murphy et al., 2004). VEGF is often delivered in combination with BMP-2 for bone tissue engineering applications to further enhance bone regeneration (Eğri and Eczacıoğlu, 2017; Ren et al., 2017; Wang et al., 2017). It has been shown that the temporal control over their delivery can enhance ectopic bone formation (Kempen et al., 2009; Geuze et al., 2012; Zhang et al., 2019). During normal fracture healing, VEGF expression is temporal, peaking between day 5 and day 10 of the healing process before subsiding (Pufe et al., 2002; Uchida et al., 2003). Kempen et al. reported a growth factor-delivering, composite implant composed of BMP-2-loaded PLGA microspheres incorporated into a solid poly (propylene fumarate) (PPF) rod which was surrounded by a cylindrical gelatin hydrogel containing VEGF. The outer gelatin layer released its growth factor load at a more rapid rate than the inner rod. This resulted in 2 distinct release profiles, with VEGF being released within 3 days to initiate angiogenesis and BMP-2 having a more sustained release profile over 56 days. In an ectopic model, the dual delivery of the growth factors led to significantly more bone formation than either growth factor individually. This synergistic effect was not observed when the construct was implanted into an orthotopic defect model (Kempen et al., 2009).

It should be noted that careful consideration is necessary for the use of angiogenic growth factors in bone tissue engineering, especially for cases where bone loss is due to the resection of a carcinoma. In such cases, angiogenic growth factors can shift from having a therapeutic effect to a detrimental effect.

Other angiogenic molecules have been studied for their ability to enhance vascularisation and bone healing. Sphingosine 1-phosphate (S1P) is a bioactive phospholipid
which has been shown to enhance cell migration, proliferation and survival in multiple cell
types including endothelial cells and osteoblast-like cells which makes it an attractive
molecule for bone tissue engineering (Maceyka et al., 2012). The effects of sustained release
of S1P from poly(lactic-co-glycolic acid) (PLAGA) films on microvessel growth has been
investigated in a dorsal skinfold window chamber model in adult mice (Sefcik et al., 2008).
The presence of S1P resulted in enhanced vessel formation due to a stimulation of mural cell
proliferation. When this approach was applied to bone healing in a rat cranial defect model,
the presence of S1P resulted in enhanced bone repair (Sefcik et al., 2008). This is
hypothesised to be due to a combination of the angiogenic effect of S1P and its ability to
stimulate migration and proliferation of osteoblast precursor cells (Ryu et al., 2006).

**Cell-Based Approaches**

There has been increased interest in the use of cell co-cultures and cell spheroids for
numerous tissue engineering applications (Mironov et al., 2009), including as a strategy to
pre-vascularise regenerative implants. Cell spheroids enable intensive cell-cell contact in a
3D environment. Cell-cell interactions between endothelial cells and osteoblasts have
previously been shown to stimulate osteoblast differentiation, upregulate proangiogenic
growth factors and promote the formation of a microvascular network (Herzog et al., 2014;
Honda and Aizawa, 2017). For example, it has been shown that primary human osteoblasts
(hOB) and human dermal microvascular endothelial cells (HDMEC) aggregate into co-
culture spheroids, and when implanted in vivo promote functional microvascular network
formation within two weeks. Similarly, spheroidal co-culture systems of human bone
marrow-derived mesenchymal stem cell (BMSC) and HUVECs have been shown to produce
primitive vascular networks in vitro. Furthermore, it was shown that the presence of HUVECs
enhanced activation of endogenous Wnt and BMP signalling and increased ALP expression
in hBMSCs (Saleh, Whyte and Genever, 2011). This intercellular crosstalk between
endothelial cells and osteoblasts/MSCs occurs via diffusible factors as well as cell-cell
contact and can be seen in 3D scaffold-based systems. When human dermal microvascular endothelial cells (HDMECs) were cultured with hOBs on starch PCL fibre-mess scaffolds, the presence of the endothelial cells resulted in an increased secretion of VEGF and Collagen I (Santos et al., 2009). VEGF stimulates endothelial sprouting whilst type I collagen, a known driver of endothelial migration, provides support for the organisation of a primitive vascular network (Davis and Senger, 2005; Santos et al., 2009). These co-culture systems essentially create an angiogenic microenvironment without the need for a supply of exogenous angiogenic stimuli.

This type of co-culture approach was utilised to prevascularise calcium phosphate cement (CPC) scaffolds prior to implantation into cranial defects in nude mice. Human-induced pluripotent stem cell-derived mesenchymal stem cells (hiPSC-MSCs) were co-cultured with HUVECs on CPC scaffolds, where they self-assembled into microvessels and deposited granular-like bone nodules over 21 days in vitro in the absence of any osteogenic supplements. These prevascularised, mineralised scaffolds resulted in significantly higher levels of new bone compared to monoculture and cell-free controls when implanted in vivo (Liu et al., 2017). These studies point to the significant potential of pre-vascularising implants intended to treat large bone defects.

**Bioprinting Vasculature within Bone**

Native bone is a complex tissue with a hierarchical architecture. Traditional bone tissue engineering approaches fail to replicate the complexity of this hierarchical organisation. 3D bioprinting has been employed to try meet this need for controlled biofabrication of both organised calcified regions and interpenetrated vasculature. Byambaa et al. used 3D bioprinting to fabricate bone substitutes with different physiological niches (Byambaa et al., 2017). By optimising the functionality of GelMA bioinks, along with introducing chemically conjugated VEGF gradients and osteogenic silicate nanoplatelets,
constructs were fabricated containing a well-defined inner vasculogenic niche and outer osteogenic niche. Firstly, an inner core of 5 % VEGF-loaded GelMA with HUVECs and hMSCs was bioprinted. Next 3 layers of 10 % GelMA loaded with silicate nanoplatelets, VEGF and hMSCs were bioprinted around the central vasculogenic fibre. The fast degradation of the inner GelMA resulted in the formation of a perfusible lumen with an endothelial lining at the core of the construct which enhanced cell survival of the mature bone niche in the outer layer during long-term culture (Byambaa et al., 2017). Another form of bioprinting being utilised to fabricate hierarchically tissue engineered bone constructs with multiphasic characteristics is stereolithography (SLA). Stereolithography based bioprinting systems use digital micromirror arrays to control the light intensity of each pixel to polymerise light-sensitive polymer materials (Wang et al., 2015). In combination with fused deposition modelling, Cui et al. used SLA to immobilise gradients of BMP2 and VEGF within GelMA to produce highly-vascularised bone tissue (Cui et al., 2016). Using laser-assisted bioprinting endothelial cells have been micropatterned to fabricate microvascular networks within bone constructs (Kérourédan et al., 2019). Endothelial progenitor cells (EPCs) and stem cells from the apical papilla (SCAPs) were deposited onto MSC-laden collagen hydrogels in bone calvaria defects in mice. This innovative in situ approach resulted in the formation of capillary networks following the printed pattern which significantly increase in vivo vascularisation and promote bone regeneration (Kérourédan et al., 2019).

Although bioprinting appears to be poised to transform the field of tissue engineering, there is still a long way to go before organ-level complexity can be recapitulated. Advancements in bioprinter technologies and bioprinting processes along with new cell sources and novel bioinks must be made for the goal of whole organ printing to be realised.
Chapter 3
The Development of Bioinks for the 3D Bioprinting of Prevascularised Constructs

3.1 Introduction

One of the biggest challenges facing the field of tissue engineering is the production of vascularised tissues and organs of a clinically relevant size. Although the emergence of 3D bioprinting has enabled the fabrication of many larger-scale tissue engineered constructs, vascularisation remains a challenge. In vivo, tissues rely on blood vessels to supply resident cells with the nutrients and oxygen necessary for healthy metabolism. It is estimated that cells must be within a 200µm distance of a blood supply to survive (Carmeliet, 2003). For tissue engineered constructs to successfully fulfil their role of replacing or repairing damaged or diseased tissues and organs, vascularisation must occur when implanted in vivo. The failure to establish a vascular network can lead to necrosis of the engineered tissue. The maximum time interval that a tissue can tolerate and still remain viable is defined as the critical ischemia time (Chafin et al., 1999). Critical ischemia time can vary between tissue type. Skeletal muscle tissue, for example, has a stated critical ischemia time of 4 hours (Gillani et al., 2012). If reperfusion were to occur after this time, ischemia reperfusion (IR) injury occurs. IR has a multifaceted pathophysiology involving the inflammatory response along with apoptosis in response to reactive oxygen species (Gillani et al., 2012).

There are two processes by which a vascular network develops in vivo: vasculogenesis and angiogenesis. Vasculogenesis is the de novo development of blood vessels from early endothelial cells known as preangioblasts. Once vasculogenesis
establishes basic vessels, angiogenesis can take place forming new blood vessels from pre-existing vasculature via sprouting and intussusception. Vascularisation is a slow process, with the average growth rate of a newly developing capillary only ~ 5 μm/h (Utzinger et al., 2015). This has led tissue engineers to employ several different methods in order to speed up the process of vascularisation within implanted tissue constructs. Some approaches involve the use of growth factors, such as VEGF and FGF-2, or other biological cues to encourage the ingrowth of vessels from surrounding tissue (Perets et al., 2003; Ennett, Kaigler and Mooney, 2006; Sefcik et al., 2008; Rui et al., 2012). Other approaches involve the incorporation of hollow channels within the construct to act as conduits to accelerate the ingrowth of host vasculature into the construct after implantation (Miller et al., 2012; Sakaguchi et al., 2013; Daly et al., 2018) Although these methods show promise and can reduce vascularisation times, both processes rely on the growth of host vessels from the periphery into the constructs. This ingrowth of host vessels may be too slow for larger implants.

An alternative method, currently being developed within the field of Tissue Engineering, is to prevascularise a scaffold with a capillary-like network prior to implantation. It is proposed that this network will anastomose with the host vasculature after implantation to give rise to rapid perfusion of the engineered tissue. Early studies into the assembly of microvascular networks in vitro focussed on creating such networks to create models for studying angiogenesis. The majority of these studies have created capillary-like vessels within soft 3D hydrogels such as fibrin (Morin and Tranquillo, 2013; Morin et al., 2013; Thomson et al., 2013), collagen (Deroanne, Lapiere and Nusgens, 2001; Raghavan et al., 2010) and fibronectin (Fournier and Doillon, 1994; Waters et al., 2013). This opens up the possibility of using such hydrogels as bioinks to 3D bioprint prevascularised constructs, assuming they can be appropriately modified to be made compatible with modern biofabrication technologies used in the field of tissue engineering today.
The overall aim of this study is to establish a 3D bioprinting strategy to generate prevascularised tissues of predefined size and shape. To this end, this study sought to identify a suitable bioink, with high print fidelity, which can support the formation of a microvascular network \textit{in vitro}. This study also aims to ascertain the appropriate cell combinations and \textit{in vitro} culturing regime to produce bioprinted constructs which can promote rapid vascularisation upon implantation \textit{in vivo}. To achieve this, this thesis will first compare the printing fidelity of three hydrogel bioinks (gelatin methacrylamide, fibrin and alginate). The capacity of these bioinks to permit endothelial sprouting using human umbilical vein endothelial cells (HUVECs) will then be assessed. The final goal of this study was to evaluate different cell combinations (HUVECs and MSCs) and culture conditions to produce stable microvessels both \textit{in vitro} and \textit{in vivo} following their subcutaneous implantation into a nude mouse model.

3.2 Materials and Methods

3.2.1 Bioink Fabrication

\textit{Fibrin bioink:} To enable the use of fibrin as a printable bioink, a gelatin carrier method was adapted from Kang et al. (Kang \textit{et al.}, 2016). Briefly, hyaluronic acid (Sigma) was added to Dulbecco's Modified Eagle Medium (DMEM; Gibco, Biosciences) at a concentration of 3 mg/mL and stirred overnight at 37°C. 10% (v/v) glycerol (Sigma) was then added and the solution was stirred for 1 h at room temperature. Gelatin type A (175 g bloom; Sigma) was added at a concentration of 40 mg/mL and stirred for 2 h at 37°C until fully dissolved. Before use fibrinogen (Sigma) was added to this carrier gel at a concentration of 30 mg/mL and stirred for 2 h at 37°C. To enhance the angiogenicity of the gel, D-erythro-sphingosine-1-phosphate (Avanti Polar lipids, AL, USA) was added at a concentration of 125 \textmu M/mL for all cell studies.
**γ-irradiated RGD-modified alginate bioink:** γ-irradiated RGD-modified alginate was kindly provided by Case Western Reserve University. Briefly, low molecular weight sodium alginate (γ alginate, 58 000 g mol⁻¹) was prepared by irradiating sodium alginate (MVG, 259 000 g mol⁻¹, Pronova Biopolymers, Oslo, Norway) at a gamma dose of 5 Mrad, as previously described (Alsberg et al., 2003). RGD-modified alginates were prepared by coupling the GGGGRGDSP to the alginate using standard carbodiimide chemistry. Briefly, 10 g alginate was dissolved at 1 % (w/v) in MES Buffer (0.1 M MES, 0.3 M NaCl, and pH 6.5). 274 mg sulfo-NHS (Pierce, Rockford, IL), 484 mg EDC (Sigma), and 100 mg GGGGRGDSP peptide (AlBioTech, Richmond, VA) were then added to the alginate solution. The reaction was stopped and the solution was purified and lyophilised as previously described. (Jeon et al., 2010) This lyophilised solution was prepared for use by combining with a gelatin carrier gel similar to that employed with the fibrinogen described previously with the substitution of gelatin type A (75 g bloom, Sigma). To enhance the angiogenicity of the gel, D-erythro-sphingosine-1-phosphate (Avanti Polar lipids) was added at a concentration of 125 μM/mL during all cell studies.

**Gelatin methacrylate (GelMA) bioink:** GelMA was synthesised by reaction of porcine type A gelatin (Sigma; gel strength ~175 g bloom) with methacrylic anhydride (Sigma Aldrich) at 50 °C for 4 h. Methacrylic anhydride was added to a 10 % solution of gelatin in PBS under constant stirring. To achieve a high degree of functionalisation, 0.6 g of methacrylic anhydride was added per gram of gelatin. The functionalised polymer was dialysed against distilled water for 7 days at 40 °C to remove methacrylic acid and anhydride, lyophilised and stored at −20 °C until use. This protocol has been shown to support a degree of methacrylation of approximately 75 % (Shin, Olsen and Khademhosseini, 2012; Lee et al., 2015) although no confirmation of the degree of methacrylation was undertaken. To enhance the angiogenicity of the gel, D-erythro-sphingosine-1-phosphate (Avanti Polar lipids) was added at a concentration of 125 μM/mL during all cell studies.
3.2.2 3D Bioprinting System

Bioink scaffolds were produced using the 3D Discovery multi-head bioprinting system (Regen Hu, Switzerland). The bioinks were printed using a pneumatic driven syringe with a 25 Gauge needle under pressures of between 0.05 MPa and 0.2 MPa. Prior to printing the γ-RGD alginate bioink was pre-crosslinked thoroughly with 60 mM CaCl₂. A luer lock dual syringe system was used to thoroughly mix the alginate and calcium solutions in a 7:3 ratio as previously described (Freeman and Kelly, 2017). To ensure homogeneity, the suspension was mixed between syringes 25 times. The solution (2.45 wt % RGD-γ alginate final) was then combined with HUVECs. Next the pre-crosslinked cell-laden alginate solution was loaded into the pressure driven piston system for printing. Post-printing, the constructs were immersed in a 100 mM CaCl₂ solution for 15 min to fully crosslink the alginate bioink. Before printing, 10 % (w/v) GelMA solution was mixed with Irgacure photoinitiator 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropionic; 0.05 %, Sigma) prior to loading with HUVECs enabling post crosslinking after printing through the application of UV light (Uvitec, Cambridge UK) for 30 min (365 × 10⁻⁹ m, 180 mW cm⁻²). Cells were added to this GelMA in a 1:1 ratio resulting in a final polymer concentration of 5 % GelMA. No further modifications were made to the fibrin bioink (from what was described in §3.2.1) prior to printing other than the addition of HUVECs. After printing, the construct was immersed in a thrombin (Sigma) bath (20 U/mL) for 15 min at RT to allow the thrombin-catalysed polymerisation of fibrinogen to fibrin. The 3D Discovery bioprinter was encased in a laminar flow hood to ensure sterility throughout the biofabrication process. All three bioinks were loaded with HUVECs between P4- P6 (10 million cells mL⁻¹ and 3 million cells mL⁻¹ for cell viability and sprouting studies respectively). In addition, fibrin bioinks were loaded with a co-culture of HUVECs and hBMSCs in a 1:1 ratio (3 million HUVECs mL⁻¹ and 3 million hBMSCs mL⁻¹) and a 2:1 ratio (3 million HUVECs cells mL⁻¹ and 1.5 million hBMSCs mL⁻¹) for stabilisation studies.
For constructs implanted subcutaneously into nude mice (further details below), bioinks were deposited into a cylindrical 3D printed polycaprolactone (PCL; CAPA Ingevity, SC, USA) sheath (wall thickness = 0.25 mm; height = 4 mm and diameter = 4 mm).

### 3.2.3 Cell Culture

Human Umbilical Vein Endothelial cells (HUVECs; Lonza, Walkersville, MD) were used in this study. HUVECs are a primary cell line and were routinely used between passage 4 and passage 8. HUVECs were cultured in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza). HUVECs were typically grown in T-175 flasks (Fisher) with 20 mL of media per flask and incubated at 37°C in a humidified atmosphere with 5 % pCO₂. The media was changed every two days until cells were approximately 85 % confluent. Trypsin Ethylenediaminetetraacetic acid (EDTA) was used to remove adhered cells at 37 °C for (3 - 5) min. The harvested cells were centrifuged at 350 x g for 4 min. For the sprouting studies, HUVECs were used between P4 - P6 (3 million cells mL⁻¹) and grown in EGM-2 supplemented with VEGF (50 ng/mL).

Human BMSCs (hBMSCs) were isolated from unprocessed human bone marrow (Lonza) on the basis of plastic adherence. Briefly, unprocessed bone marrow was plated at 2.5 X 10⁵ cells/cm² (estimated approx. 4000-5000 hBMSCs/cm²) in high glucose Dulbecco’s modified eagle’s medium (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under hypoxic conditions (37°C in a humidified atmosphere with 5 % pCO₂ and 5 % pO₂) for improved chondrogenic differentiation. Following colony formation, hBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic acid (EDTA) and tripotentiality was confirmed as previously described (Vinardell et al., 2009). hBMSCs used were seeded at 5000 cells/cm² expanded in DMEM supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/ml streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under
normoxic conditions (37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂) and used at P4.

3.2.4 Live/Dead Cell Assay

Cell viability was established using a live/dead assay. Printed cell-laden constructs were incubated in EGM-2 media for 30 min prior to the assay. All constructs were rinsed in PBS and incubated for 1 h in a solution containing 2 μM calcein and 4 μM of ethidium homodimer-1 (Cambridge Biosciences). After incubation, the constructs were rinsed again and imaged with Olympus FV-1000 Point-Scanning Confocal Microscope (488 nm and 543 nm channels). Cell viability was quantified using Image-J software.

3.2.5 Confirmation of Gelatin Elimination

To investigate whether or not the additional gelatin had been removed following incubation at 37 °C, an hydroxyproline biochemical analysis was carried out for both the fibrin- and alginate-based bioinks as previously described (Cunniffe et al., 2017). Gelatin was then quantified by its hydroxyproline content, after hydrolysis (110 °C; 18 h) in HCL (38 %), and assayed using chloramine-T assay assuming 13 % hydroxyproline per gelatin molecule (Ignat’eva et al., 2007).

3.2.6 Scanning Electron Microscopy (SEM) of Fibrin Gels.

Samples were prepared for SEM by dehydrating samples in decreasing ethanol series and immersed in hexamethyldisilazane (HMDS; 2 × 30 min). Fibrin samples were allowed to air dry for 2 h before being mounted on SEM stubs and left overnight to ensure complete removal of solvent. Samples were then coated with gold/palladium for 120s at a current of 40 mA using a Cressington 208HR sputter coater. SEM imaging was conducted at 5 kV in a Zeiss ULTRA plus. Although SEM sample preparation was carried out by the author, the SEM imaging was not.
3.2.7 *In vitro Microvessel Assessment:*

For assessing microvessel establishment, all GelMA and fibrin-based samples were fixed in 4 % paraformaldehyde overnight. Alginate samples were fixed in 4 % paraformaldehyde supplemented with barium chloride overnight to permanently crosslink the gels. Samples were then washed in PBS, permeabilised in 0.5 % Triton-X (Sigma) and incubated with Rhodamine Phalloidin (5 U/mL; 165nM; Thermo Fisher) for 40 min at 37°C followed by DAPI (4’,6-diamidino-2-phenylindole;1 µg/mL; Sigma) incubation for 20 min at room temperature. After incubation, the constructs were rinsed again, cut in half and imaged with Olympus FV-1000 Point-Scanning Confocal Microscope at 565 nm and 461 nm channels. Vessel lengths were calculated on projected stacks using either ImageJ or Angiotool (Zudaire *et al*., 2011) which is a program capable of detecting, marking and scoring the average vessel length.

3.2.8 *In vivo Experimental Design and Subcutaneous Implantation*  

All animal experiments were performed in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Four groups were bioprinted: HUVECs alone (3 million cells/mL), hBMSCs alone (1.5 million cells/mL), a 2:1 ratio of HUVECs: hBMSCs (3 million:1.5 million/mL) and a cell-free control. These 4 groups were exposed to one of two conditions; either grown in EGM-2 (Lonza) supplemented with 50 ng of recombinant human VEGF (Gibco) at 37 °C in a humidified atmosphere for 7 days or implanted into the mice without any further culturing or exposure to VEGF. Constructs were implanted subcutaneously into the back of BALB/cOlaHsd-Foxn1nu female nude mice (Envigo). Briefly, two subcutaneous pockets were made along the central line of the spine, one at the shoulders and the other at the hips. Three constructs were inserted into each pocket (randomised between top and bottom pockets). Nine constructs were implanted per group and constructs were harvested 7 days and 14 days post-implantation. Mice were anaesthetized using an intraperitoneal injection of xylazine hydrochloride and ketamine
hydrochloride, Carprofen was added to water for 48 h post-surgery, and mice were sacrificed by CO₂ inhalation. This protocol and study were approved by the animal welfare committee of Trinity College Dublin and the Health Products Regulatory Agency (HPRA, approval number AE19136/P069). Post-implantation samples were fixed in 4 % PFA and dehydrated in graded series of ethanol solutions (70-100 %), cleared in xylene, and embedded in paraffin wax (all Sigma-Aldrich). Sections (5 μm) were stained with haematoxylin and eosin (Sigma-Aldrich), imaged using an Aperio ScanScope slide scanner at 20X and evaluated for vessel infiltration by counting vessels visible across an entire section using Aperio ImageScope and ImageJ.

3.2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 6) software. To analyse variance between groups, two-way ANOVA was used with Tukey post-hoc test. Numerical and graphical results are displayed as mean ±standard deviation unless otherwise stated. Significance was accepted at a level of p<0.05.

3.3 Results

3.3.1 Comparison of Bioink Printability

To evaluate the printability of each hydrogel bioink, spreading ratios were compared for each formulation. The filament spreading ratio is defined as the width of the printed filament divided by the needle diameter. Lower spreading ratios, approaching the ideal ratio of 1, are desirable to allow the fabrication of cell laden hydrogel structures with high precision. A detailed description of the hydrogel printing properties can be found in Table 3.1, including the printing parameters adopted for each hydrogel formulation. Without a supporting gel, fibrinogen (the precursor to fibrin) is unprintable. The inclusion of the gelatin-based support gel enabled its printing and resulted in the fibrin-based bioink (herein termed FibGel) having the lowest spreading ratio (1.761 ± 0.09) (Figure 3.1 A&B). Without the use
of a carrier gel method, the γ- RGD alginate had a very high spreading ratio (6.828 ± 1.12), with inclusion of the gelatin-based support (herein termed AlgGel) reducing this by 64.8% (2.404 ± 0.21). GelMA had the second lowest spreading ratio (2.278 ± 0.1884) and produced a more consistent extrusion (as evident by the lowest standard deviation) compared to all other hydrogels (Figure 3.1 A&B).
**Table 3.1: Bioprinting processing parameters for each bioink.**

<table>
<thead>
<tr>
<th></th>
<th>FibGel</th>
<th>GelMA</th>
<th>Alginate only</th>
<th>Alginate (gelatin)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Printing temperature</strong></td>
<td>30°C</td>
<td>28°C</td>
<td>22°C</td>
<td>30°C</td>
</tr>
<tr>
<td><strong>Polymer concentration</strong></td>
<td>Fibrinogen 30 mg/mL, gelatin 30 mg/mL, HA 3 mg/mL, 10 % glycerol</td>
<td>5 % (w/v)</td>
<td>2.45 % (w/v) RGD-γ alginate and 18 mM CaCl₂</td>
<td>2.45 % (w/v) RGD-γ alginate in gelatin 21 mg/mL, HA 2.1 mg/mL, 7 % glycerol and 18 mM CaCl₂</td>
</tr>
<tr>
<td></td>
<td>GelMA, 0.05 % Irgacure</td>
<td>UV light (15 min)</td>
<td>Calcium chloride 100 mM bath (30 min)</td>
<td>Calcium chloride 100 mM bath (30 min)</td>
</tr>
<tr>
<td><strong>Post-crosslinking mechanism</strong></td>
<td>Thrombin bath 20 U/mL (30 min)</td>
<td>0.05 MPa</td>
<td>0.1 MPa</td>
<td>0.2 MPa</td>
</tr>
</tbody>
</table>
3.3.2 Evaluation of Cell Viability Post-Printing

Cell viability during and after 3D bioprinting is dependent on the shear stress experienced during extrusion, which in turn is dependent on the viscosity of the solution, the applied pressure and the needle diameter (Malda et al., 2013). In addition, the post-printing bioink crosslinking mechanisms can further influence cell viability. Since the solution viscosity, applied pressure and post-crosslinking mechanism varied across the three hydrogels, cell viability was evaluated post-printing for each bioink. All bioinks supported high levels of cell viability (>70%) (Figure 3.1 C). Cell viability appeared slightly lower in the GelMA bioink (Figure 3.1 C), possibly due to the UV crosslinking mechanism, although this difference was not statistically significant. This indicates that 3D structures containing viable endothelial cells could be 3D bioprinted using each bioink. The levels of cell viability
presented here are in agreement with the literature for similar printing systems (Nair et al., 2009; Billiet et al., 2014; Daly et al., 2015).

3.3.3 **SEM analysis of FibGel and a fibrin control**

SEM analysis revealed that the inclusion and subsequent elimination of gelatin within the fibrin-based bioink resulted in a more porous architecture. The fibrin fibres in the bioink were sparser and less densely packed than a fibrin gel at the same fibrinogen and thrombin concentration (Figure 3.2 A&B).

3.3.4 **Confirmation of Gelatin Elimination**

The inclusion of gelatin within the fibrin and alginate bioink is to facilitate their extrusion from the print head and to retain the structural integrity of the hydrogel until post-printing crosslinking can be carried out to stabilise the printed constructs. Once crosslinked, the presence of gelatin is no longer required. The melting point of gelatin is 37°C, so theoretically it should wash out of the printed constructs during culture in an incubator kept at a constant temperature of 37°C. To test this hypothesis, constructs were incubated for 24 h at 37°C and a hydroxyproline assay was carried out to quantify the amount of gelatin remaining on the constructs, assuming 13 % hydroxyproline per gelatin molecule (Ignat’eva et al., 2007). Hydroxyproline levels showed that within 24 h, gelatin was being washed out of the fibrin construct. Low hydroxyproline levels at the time 0 timepoint indicate that gelatin had begun to wash out in the thrombin bath during the 30-minute crosslinking step which took place before the time 0 samples were taken. In contrast, very little gelatin was being washed out from the alginate based bioinks during the 24-hour incubation period (Figure 3.2 C). It is hypothesised that the different hydrogel crosslinking mechanisms and/or hydrogel structures affects how gelatin is retained within the gel. It has been shown that gelatin and alginate can form intermolecular electrostatic interactions between the negatively charged carboxylate (COO−) groups of alginate and the positively charged ammonia (NH3+) groups of gelatin. Additionally, alginate and gelatin can form hydrogen bonds between hydroxyl,
carbonyl and amino groups on their biopolymer chains (Derkach et al., 2019). These interactions may be slowing the passive diffusion of gelatin from the gels, resulting in higher hydroxyproline levels at this timepoint. No further studies were carried out to investigate whether hydroxyproline levels further decreased over time.

Figure 3.2 SEM analysis of Fibrin bioink and Confirmation of gelatin elimination. (A) SEM micrograph of fibrin gel (without gelatin); scale bar = 200µm (B) SEM micrograph of fibrin bioink; scale bar = 200µm (C) Quantification of gelatin content per mg of construct. (Values are the mean ± SD, n = 3).

3.3.5 Comparison of Endothelial Sprouting in GelMA, Alginate and Fibrin Based Bioinks

To evaluate the potential of each bioink to facilitate endothelial sprouting, HUVECs were incorporated into each bioink and simple cylindrical constructs were bioprinted, cultured for up to 7 days in the presence of VEGF (50 ng/mL) and then evaluated for microvessel formation using confocal microscopy. By day 3, no endothelial sprouting was
seen in either the Alginate-based bioink or in the GelMA. In the Fibrin-based bioink, however, the endothelial cells had migrated towards each other and began to elongate and align unidirectionally by day 3. By day 5, these HUVECs had formed microvessels which began to sprout forming multidirectional vessels. By day 7, a microvessel network was observed (Figure 3.3 A). In the GelMA constructs, no evidence of endothelial sprouting was seen until day 7, at which point there was evidence of rapid proliferation and inconsistent vessel formation. This is hypothesised to be due to the cell-independent hydrogel degradation which provided the space for endothelial cells to rapidly proliferate. These rapidly proliferating cells appeared at times to form a monolayer architecture across the newly formed hollows as opposed to elongated microvessels. Over the course of 7 days, there was no endothelial proliferation or sprouting observed in the alginate-based bioink, presumably due to inability of HUVECs to produce the necessary MMPs to degrade the surrounding alginate gel, which is vital for vasculogenesis and angiogenesis to take place. The average vessel length of all three bioink was quantified using ImageJ, showing that the fibrin-based bioink had the most consistent growth of microvessels over time. (Figure 3.3 B). The percentage area endothelialised was calculated for each bioink by dividing the sum cell area by the total area. (Figure 3.3 C). A significant decrease in the percentage of endothelialised hydrogel in the alginate-based bioink suggested cell death over time. Overall a more controlled, consistent establishment of a microvessel network was observed with the fibrin-based bioink. Between day 3 and day 5 the area of endothelialisation did not increase, whilst the vessel length did, suggesting plexus remodelling during this time.
Figure 3.3 Comparison of bioink potential for facilitating endothelial sprouting. (A) Representative projected confocal stack of microvessel establishment over time, phallodin labelled F-actin (red) and DAPI nuclear staining (blue), scale bar = 100µm. White arrows denote endothelial cell alignment. (B) Average vessel length. a denotes significance compared to GelMA at corresponding timepoint, b, denotes significance compared to AlgGel at corresponding timepoint (P<0.001) two-way ANOVA, Bonferroni post-test. (C) % area endothelialised. a denotes significance compared to GelMA at corresponding timepoint, b, denotes significance compared to Alginate at corresponding timepoint, (P<0.001) two-way ANOVA, Bonferroni post-test. ^ denotes significance compared to day 7 GelMA, * denotes significance compared to day 0 AlgGel, (P<0.05), one-way ANOVA, Tukey post-test.
3.3.6 Stabilisation of Encapsulated Microvessel Network

Given the consistency and the superiority of the fibrin-based bioink to produce a premature vascular network in vitro, it was chosen as the optimum bioink for all future studies. However, it was observed that the established microvessels subsequently regressed after 10 days of culture in vitro, which presumably was due to the absence of a pericyte-like cell population to stabilise the network. (Figure 3.4 A).

Due to the recurrent concept that mesenchymal stem/stromal cells (MSCs) are pericytes or at least can play a pericyte role (Caplan, 2008) and have been shown to differentiate into a pericyte-like phenotype when in direct cell-cell contact with endothelial progenitor cells (Loibl et al., 2014), human bone marrow-derived stem/stromal cells (hBMSCs) were chosen as a companion cell to the HUVECs in the fibrin-based bioink system. This thesis investigated whether or not the addition of hBMSCs would result in a stabilisation of the established vascular network.

When comparing 2 different ratios of HUVEC:hBMSC, 1:1 and 2:1, both resulted in the stabilisation of the microvessel network beyond 10 days in culture (Figure 3.4 B). Using confocal imaging in conjunction with software for quantitative analysis of angiogenesis (Angiotool (Zudaire et al., 2011)), the vessel density and average vessel length was calculated for both co-culture ratios. (Figure 3.4 C & D). When comparing both co-cultures, there was a trend toward both higher vessel density and average vessel length in the 2:1 HUVEC:hBMSC co-culture, although no significance was detected.
Figure 3.4 Microvessel regression and stabilisation. (A) Representative confocal images of microvessel regression over time, phalloidin labelled F-actin (red) and DAPI nuclear staining (blue), scale bar = 100µm. (B) Representative confocal images of hBMSC stabilisation of microvessels at day 10, 1:1 and 2:1 HUVECs:hBMSCs respectively, phalloidin labelled F-actin (red) and DAPI nuclear staining (blue), scale bar = 100µm. (C) Vessel density and (D) average vessel length (Values are the mean ± SD, n = 3). E. Representative image of 2:1 HUVECs:hBMSCs, GFP labelled HUVECs (green) phalloidin labelled F-actin (red) and DAPI nuclear staining (blue), scale bar = 100µm.

3.3.7 In vivo Assessment of Prevascularised 3D Bioprinted Constructs

To determine whether the establishment of a microvessel network prior to implantation would result in a more rapid vascularisation of a bioprinted construct in vivo, these bioprinted constructs were next implanted subcutaneously into nude mice. A polycaprolactone sheath was incorporated into the bioprinted construct design to encourage a unidirectional in-growth of host vessels (Figure 3.5 A inset). One of following four cell populations was bioprinted into this sheath using the fibrin based bioink:

1. HUVECs alone

2. hBMSCs alone
3. A 2:1 co-culture of HUVEC: hBMSCs

4. A cell free control

These 4 groups were exposed to one of two conditions; the first involved an additional week of culturing in endothelial growth media supplemented with 50 ng of VEGF to establish a micro-vascular network prior to implantation (herein termed Prevascularised implants); the second involved the immediate implantation of the cell laden constructs (herein termed Cellularised implants) into the mice without any further culturing or exposure to VEGF (Figure 3.5).
Figure 3.5 (A) Schematic diagram of the experimental design and steps for the in vivo study. FibGel loaded with either HUVECs alone, hBMSCs alone, a 2:1 ratio of HUVECs:hBMSCs or left cell free was printed into PCL scaffolds. Half were implanted subcutaneously after a 12 h settling period, the other half were cultured for 7 days in VEGF supplemented EGM-2 media prior to implantation. (A Inset), diagram depicting the polycaprolactone.
(PCL) sheath was incorporated into the bioprinted construct design to encourage a unidirectional in growth of host vessels. (B) Study timeline highlighting fabrication, implantation and analysis timings. Constructs were harvested 7- and 14-days post-implantation and analysed for vessel density. See Materials and Methods for details.

To facilitate construct implantation, two independent skin incisions were made in the back of each mouse and two pockets were made in the subcutaneous space. Three bioprinted constructs were implanted into each pocket. Throughout the study, there was no evidence of mice exhibiting any pain behaviour and none of the mice displayed any visible inflammation or infection. The implants were resected at either 1 week or 2 weeks post-implantation. Macroscopically, the groups which had been cultured for 7 days prior to implantation appeared to be more vascularised, having a deeper red colour compared to all other groups. (Figure 3.6).
Figure 3.6 Macroscopic images of fibrin-based constructs after 7 and 14 days in vivo. Prevascularised refers to groups which underwent 7 days of vasculogenic priming prior to implantation. Cellularised refers to groups which did not undergo any in vitro priming prior to implantation; scale bar = 4 mm.

Microscopically, however, these apparent differences in vascular invasion did not always translate to the presence of blood vessels inside the printed implants. Often there was evidence of red blood cells without the presence of a vessel wall. For all vessel analysis, a vessel was defined by the presence of red blood cells surrounded by dense nuclei. At day 7, the only group which contained any vessels was the prevascularised construct produced using a co-culture of HUVECs and hBMSCs (Figure 3.7). By day 14, some constructs in the cultured HUVEC only group and the cell free group had become vascularised, but the prevascularised constructs persisted as the group supporting the highest level of vascularisation in vivo.
**Figure 3.7** (A) Haematoxylin and eosin (H&E) staining of constructs. Perfused blood vessels donated by black arrows, scale bar = 100 µm and 1 mm for images and inset images, respectively. (B) Vessels per construct at day 7 and (C) day 14, (n = 9 mean ± SEM).

### 3.4 Discussion

There are multiple studies in the literature demonstrating the capacity of hydrogels to facilitate angiogenesis (Liu *et al.*, 2007; Bayless, Kwak and Su, 2009; Morin and Tranquillo, 2013). Many of these hydrogels are not directly compatible with bioprinting technologies. In this study we demonstrate that three hydrogels (alginate, fibrin and GelMA) commonly used in tissue engineering can be successfully bioprinted, but that this often necessitated the inclusion of a temporary agent (gelatin) to increase the viscosity of the ink. Endothelial cells remained viable within these constructs post-printing. However further analysis revealed that only the fibrin-based bioink was capable of facilitating the controlled establishment of an encapsulated microvessel network *in vitro* over 7 days of culture. A microvascular network was rapidly established in the GelMA bioink between day 5 and 7 of culture, which correlated with a more inconsistent vascular architecture. No obvious microvessel network was established in the alginate based bioink.

Sphinosine-1-phosphate (S1P) was added to all bioinks to initiate endothelial sprouting. S1P is a sphingolipid metabolite which stimulates numerous diverse cellular responses such as proliferation, migration and cytoskeletal changes in numerous cell types such as vascular and immune cells (Yang *et al.*, 1999; Ancellin *et al.*, 2002; Olivera *et al.*, 2007; Venkataraman *et al.*, 2008). S1P has been shown to promote endothelial sprouting in numerous angiogenic *in vitro* assays (Bayless and Davis, 2003; Kang, Bayless and Kaunas, 2008; Su and Bayless, 2012). Studies investigating vasculogenesis in the mouse embryo showed that S1P contributes to the *de novo* assembly of blood vessels (Argraves *et al.*, 2004). Interestingly, these studies further showed that VEGF alone was not capable of mimicking this initiation of vasculogenesis. In this chapter, S1P was incorporated to initiate the *de novo*
formation of primitive vessels. Once this vascular plexus was established, the presence of VEGF in the culture medium could exert its angiogenic effects further enhancing the microvessel network. Without the addition of S1P a consistent formation of a network was not achieved (data not shown). It should be noted that the addition of S1P was for the in vitro prevascularisation benefit. Little to no S1P is expected to remain in the gels prior to in vivo implantation. If any S1P were to remain, it would be rapidly cleared by degradative enzymes. The half-life of S1P injected intravenously is reported to be between 1-15 min (Venkataraman et al., 2008; Salous et al., 2013).

Although alginate is widely used for tissue engineering applications, the finding that the HUVECs were unable to establish a microvessel network within the alginate bioink was not unexpected. It has been shown that the production of proteolytic enzymes, known as Matrix Metalloproteinases (MMPs), is a vital step in angiogenesis that facilitates the local remodelling of the ECM and endothelial cell invasion (Speirs, 2000). Alginate is the main structural component of marine brown algae and, although highly biocompatible, is impervious to cleavage by mammalian enzymes. Modifications have been made to the alginate used in this study (Alsberg et al., 2003), involving gamma irradiation, which reduces the molecular weight. This therefore accelerates passive degradation of the hydrogel, as well as the immobilisation of the cell adhesion peptide sequence arginine–glycine–aspartate (RGD). However, the encapsulated cells were unable to actively degrade their surrounding environment. This resulted in the lack of sprouting observed in response to the VEGF mediated endothelial activation. For alginate to be suitable for prevascularisation biofabrication techniques, further alginate modifications must be undertaken such as the inclusion of the MMP-sensitive domains (e.g. the peptide Pro-Val-Gly-Leu-Iso-Gly (Fonseca et al., 2014). From the results of this study, GelMA bioinks show some promise for the inclusion in angiogenic tissue engineered constructs. During pilot studies, polymer concentrations were varied to investigate optimum sprouting conditions. A higher percentage
of 10 % GelMA was found to inhibit endothelial sprouting, whereas lower percentages of 3 % were too low to crosslink (data not shown). However, although the protocol undertaken in this study has been shown to support a degree of methacrylation of approximately 75 % (Lee et al., 2015) (Shin, Olsen and Khademhosseini, 2012), there was no further investigation into the optimal degree of methacrylation for supporting the establishment of micro-vascular networks. It has been shown that the degree of functionalisation of GelMA can have a profound effect on cell spreading, with higher functionalisation resulting in a less spread cell phenotype (Klotz et al., 2018). Considering this, future studies should explore whether lowering the degree of methacrylation would improve vessel formation within GelMA based bioinks.

Based on the results of this study, the fibrin-based bioink was deemed the optimal bioink for the 3D bioprinting of prevascularised constructs. The addition of the gelatin blend enabled superior printability and its consistent ability to support microvessel formation over 7 days in vitro made it highly attractive for tissue engineering strategies requiring a prevascularised construct. The SEM analysis further reveals that the inclusion of the gelatin base leads to a more porous gel following fibrin polymerisation and subsequent gelatin elimination. This may further enhance the innate ability of fibrin to support endothelial sprouting.

Over an extended culture period, it became apparent the microvessel network had begun to regress, which was attributed to the absence of a supporting cell type. This is a phenomenon seen during innate vessel formation where pericytes are vital to vessel function. Pericytes not only serve as scaffolding to support the vascular network architecture, but also communicate with endothelial cells by direct physical contact and paracrine signalling pathways (Bergers and Song, 2005). In vitro, the importance of a supporting cell type has been shown in multiple studies to be necessary for the successful formation of a perfusable network. Multiple cell types have been shown to stabilise endothelial networks, including
fibroblasts (Chen et al., 2009), 10T1/2 mesenchymal precursor cells (Koike et al., 2004), and tri-cultures of myoblasts and embryonic fibroblasts (Levenberg et al., 2005). The inclusion of these supporting cells ensures vessel integrity. In this study, the decision to include hBMSCs as the supporting cell type was inspired by the emerging argument that all MSCs are pericytes (Caplan, 2008) and have been shown in literature to be able to mediate vascularisation by secreting angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoietin 1 (Ang-1), and epidermal growth factors (EGF) (Gallina, Turinetto and Giachino, 2015). The inclusion of hBMSCs into the fibrin-based gels enabled extended microvessel stability for up to 2 weeks for both a 1:1 and a 2:1 ratio of HUVECs:hBMSCs. The 2:1 co-culture was selected for further in vivo analysis as it had shown a trend towards supporting the development of a denser vascular network in vitro.

In vivo analysis revealed that the pre-vascularisation of bioprinted constructs, through the establishment of microvessels in vitro, led to a higher number of perfused vessels within the core of the construct. The inclusion of the same concentration of cells without this prevascularisation step did not lead to any enhancement in vessel formation, leading to the conclusion that the formation of bloods vessels within this construct in vivo is dependent on the preculturing of HUVECs and hBMSCs in the presence of the angiogenic growth factor VEGF to establish a stable microvascular network prior to implantation.

3.5 Conclusion

To conclude, the results from this study show that fibrin is the optimum bioink to support HUVEC sprouting. This study further shows that a HUVEC:hBMSC co-culture of 2:1 within 3D bioprinted constructs facilitates the development of a stable primitive vascular network in vitro. 7 days of culture of this HUVEC:hBMSC co-culture in the presence of VEGF prior to implantation led to enhanced vascularisation once implanted in vivo. The
following chapters of this thesis will utilise this novel combination of cells, bioink and preculture conditions to prevascularise tissue engineered constructs designed to support the regeneration of large bone defects.
Chapter 4

3D Bioprinting of Prevascularised Cartilage Templates for Endochondral Bone Tissue Engineering

4.1 Introduction

Although bone possesses an intrinsic ability to regenerate, in many clinical situations tissue loss and damage is so extensive that clinical intervention is necessary. This may arise due to severe trauma, infections, tumour resection or bone irradiation. The current gold standard to treat these large bone defects is autologous bone grafting, where bone is harvested from the patient’s own body, most commonly the iliac crest, and transferred to the site of injury (Laurencin, Khan and El-Amin, 2006). In many clinical cases, however, this treatment is not possible due to inadequate bone quantity or concerns around donor site morbidity (Kim et al., 2009). In these cases, allogeneic bone grafts obtained from human cadavers or living donors can be utilised. However, allogeneic bone grafts carry inherent limitations including immunogenicity and pathogen transmission risks (Donati et al., 2005). Therefore, there is an urgent need for alternatives to autologous and allogeneic grafts in order to promote bone repair.

Tissue engineering is emerging as a promising alternative approach for regenerating large bone defects. Bone tissue engineering is a multidisciplinary field which combines materials science, cell biology and engineering to generate a microenvironment which can
stimulate bone regeneration. To date many bone tissue engineering strategies have employed the classical tissue engineering triad of 3D scaffolds, cells and signalling models to direct bone formation, commonly attempting to mimic the process of intramembranous ossification by promoting the direct osteogenic differentiation of mesenchymal stem/stromal cells (MSCs). This approach can however lead to the development of an engineered construct with a dense calcified outer matrix, which fails upon implantation in vivo due to limited vascular invasion and associated cell death, particularly in the core of the construct (Lyons et al., 2010). This has hindered the successful translation of many bone tissue engineering strategies into clinical applications.

In more recent years, many bone tissue engineers have moved away from this traditional approach and turned towards a more developmentally inspired approach to bone regeneration. During embryonic development our long bones are formed through a process known as endochondral ossification (Kronenberg, 2003). This process involves the development of a cartilage template which is later vascularised and becomes calcified. The initial cartilage template is formed when MSCs condense and undergo chondrogenic differentiation and secrete a type II collagen rich matrix. These chondrocytes then undergo hypertrophy and begin to secrete type X collagen along with multiple factors, such as pro-angiogenic growth factors like VEGF and the pro-osteoclast ligand RANKL, which promotes the invasion of new vasculature and osteoblasts and results in the turnover of the cartilage template into bone. By aspiring to recapitulate the endochondral ossification pathway in bone tissue engineering, many of the hurdles associated with traditional tissue engineering can potentially be overcome. Indeed, it has been shown that MSCs can be used to engineer a cartilage construct in vitro that can undergo the endochondral ossification process in vivo to generate bone both ectopically and orthotopically (Scotti et al., 2010; Farrell et al., 2011; Cunniffe et al., 2015; Sheehy, Mesallati, Vinardell, et al., 2015; McDermott et al., 2019). As chondrocytes function at low oxygen levels in their native avascular environment, a tissue
engineered cartilage template is better equipped to survive upon \textit{in vivo} implantation until host vasculature can infiltrate and oxygenate the construct, at least for relatively small implants. Although promising, several challenges must be addressed before this approach can be translated to the clinic. One of the major hurdles to be overcome is the scaling up of such engineered grafts to clinically relevant sizes. This scaling up in turn amplifies the need for a means to rapidly vascularise these larger constructs once they are implanted \textit{in vivo}.

The emergence of 3D bioprinting has provided a powerful tool for the scaling up of anatomically accurate tissue engineered constructs. This chapter combines the prevascularising bioink described in Chapter 3 with a chondrogenically primed 3D bioprinted construct and asks whether prevascularising such a bioprinted cartilage template results in more rapid ectopic bone formation \textit{in vivo} following subcutaneous implantation into nude mice.

\section*{4.2 Material and Methods}

\subsection*{4.2.1 Isolation and Expansion of Human BMSCs}

Two sources of MSCs were used in this study. For the \textit{in vitro} studying comparing cell conc. and scaffold architecture, and for all PV bioinks, human bone marrow-derived stem cells (hBMSCs) were isolated from unprocessed human bone marrow (Lonza) on the basis of plastic adherence. Briefly, unprocessed bone marrow was plated at $2.5 \times 10^5$ cells/cm$^2$ (estimated approx. 4000-5000 hBMSCs/cm$^2$) in high glucose Dulbecco’s modified eagle’s medium (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under hypoxic conditions (37°C in a humidified atmosphere with 5 % pCO$_2$ and 5 % pO$_2$) for improved chondrogenic capacity. Following colony formation, hBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic acid (EDTA) and tripotentiality was confirmed as previously described (Vinardell \textit{et al.},}
hBMSCs used for the fibrin bioink were seeded at 5000 cells/cm² expanded in DMEM supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under normoxic conditions (37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂) and used at P4. hBMSCs used for bioprinting the cartilaginous template were expanded in the same media but under hypoxic conditions (5 % pO₂) and used for printing at P3. hBMSCs used for the cartilage template engineered for the in vitro study comparing the effect of EGM2 and the in vivo study were obtained from RoosterBio (RoosterBio Inc. MD, USA) and expanded using hMSC high performance media kit XF (RoosterBio Inc.) at 37°C in a humidified atmosphere with 5 % pCO₂ and 5 % pO₂.

4.2.2 Endothelial Expansion

Human Umbilical Vein Endothelial cells (HUVECs; Lonza, Walkersville, MD) were cultured at 2500 cells/cm² in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza) at 37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂). HUVECs were used at P4.

4.2.3 Bioink Fabrication

To enable the use of fibrin as a printable bioink, a gelatin carrier method was adapted from Kang et al (Kang et al., 2016). Briefly, hyaluronic acid (Sigma) was added to high glucose Dulbecco's Modified Eagle Medium (hgDMEM; Gibco, Biosciences) at a concentration of 3 mg/mL and stirred overnight at 37°C. 10 % (v/v) glycerol (Sigma) was then added and the solution was stirred for 1 h at room temperature. Gelatin type A (175 g bloom; Sigma) was added at a concentration of 40 mg/mL and stirred for 2 h at 37°C until fully dissolved. Before use, Fibrinogen (Sigma) was added to this thawed carrier gel at a concentration of 30 mg/mL and stirred for 2 h at 37°C. To enhance the angiogenicity of the bioink for use as PV bioink, D-erythro-sphingosine-1-phosphate (S1P; Avanti Polar lipids,
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AL, USA) was added at a concentration of 125 µM/mL. Fibrin bioink for use with cartilage template did not contain S1P.

### 4.2.4 3D Bioprinting System

Scaffolds were produced using the 3D Discovery multi-head bioprinting system (Regen Hu, Switzerland). Polycaprolactone (PCL; CAPA Ingevity, SC, USA) scaffolds were printed at 80°C at 0.5 MPa using a 27G needle. Two different architectures were designed and fabricated. Both designs were cylindrical (height = 4 mm and diameter = 4 mm) but one had solid walls and the other had porous walls. This porosity was achieved by printing 4 arcs which shifted every 5 layers by ~45°. Scaffolds were sterilised using ethylene oxide (ETO) gas prior to the addition of cell-laden bioinks. The bioinks were printed using a pneumatic driven syringe with a 25 Gauge needle at temperatures between 10-30°C under pressures of between 0.05-0.2 mPa. For cartilage template printing, hBMSCs were incorporated into the fibrin bioink at 5-, 10- or 20 × 10⁶ cells/mL. For PV bioink, HUVECs and hBMSCs were incorporated into the fibrin bioink at 3x10⁶ and 1.5 × 10⁶ cells/mL, respectively. To assist the accuracy of the printing process, 4 % (w/v) agarose (Sigma) well inserts were fabricated using custom-designed moulds printed with Form2 SLA printer (FormLabs, MA, USA). For cartilage template printing, these agarose moulds were soaked in thrombin (20 U/mL) prior to printing. After printing, all constructs were further immersed in a thrombin bath (20 U/mL) for 30 min at RT to allow the thrombin-catalysed polymerisation of fibrinogen to fibrin. For the bioprinting of the PV bioink, agarose moulds (without thrombin) were used to help printing accuracy. Hollow channels were fabricated using 40 % (w/v) pluronic F127 (MW 12.6 kDa; Sigma) extruded through a pneumatic driven syringe with a long 25 Gauge needle at 18°C and an extrusion pressure of 0.5 MPa. The 3D Discovery was encased in a laminar flow hood to ensure sterility throughout the biofabrication process.
4.2.5 *In vitro Culture Conditions*

For chondrogenic priming, bioprinted scaffolds were cultured in a chondrogenic medium consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin, 100 μg/ml streptomycin (both Gibco), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 4.7 μg/ml linoleic acid, 1.5 mg/ml bovine serum albumin, 1X insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma), 2.5 μg/ml amphotericin B and 10 ng/ml of human transforming growth factor-β3 (TGF-β3; Prospec-Tany TechnoGene Ltd., Israel) at 37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂ for 3 weeks before being switched to 20 % pO₂ for a further week of culture. Media was changed every 48 hours. For the final week of culture, and for the induction of endothelial sprouting samples were cultured in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza) with additional 50 ng/mL recombinant human vascular endothelial cell growth factor (VEGF; 165 amino acid residues/subunit; Gibco).

4.2.6 *Quantitative Biochemical analysis*

Samples from *in vitro* studies and pre-implantation constructs were retrieved from culture, washed in PBS and frozen for subsequent assessment. Samples were then digested in 3.88 U/mL papain digest in 100 mM Sodium Phosphate Buffer/5 mM Na2EDTA/10 mM L-cysteine, pH 6.5 (all from Sigma–Aldrich) at 60 °C under constant rotation for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay, with a calf thymus DNA as standard read using a Synergy HT multi-detection micro-plate reader (BioTek Instruments, Inc) at 360 nm excitation and 460 nm emission with sensitivity set to 120. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard read using the Synergy HT multi-detection micro-plate reader (BioTek Instruments, Inc) with a wavelength set to 656nm. sGAG was normalised to DNA
to determine the chondrogenic influence at a cellular level. Total collagen content was determined using a chloramine-T assay (Kafienah and Sims, 2004) to measure the hydroxyproline content and calculated collagen content using a hydroxyproline-to-collagen ratio of 1:7.69. Briefly, samples were mixed with 38 % hydrochloric acid (Sigma) and incubated at 110°C under constant rotation for 18 hours to allow hydrolysis to occur. Samples were subsequently dried in a fume hood overnight and the sediment resuspended in ultra-pure H₂O. 2.82 % (w/v) Chloramine T and 0.05 % (w/v) 4-(Dimethylamino) benzaldehyde (both Sigma) were added and the hydroxyproline content quantified with a trans-4-Hydroxy-L-proline (Fluka analytical) standard using a Synergy HT multi-detection micro-plate reader at a wavelength of 570 nm (BioTek Instruments, Inc).

For in vitro study comparing pre- and post-incubation in EGM-2, the “Pre-” samples were taken after bioprinting in the PV bioink but before any further culturing had occurred.

4.2.7 In vivo Study Design

To investigate the effect of prevascularisation on a cartilage template in vivo, three groups were compared: A solid cartilage template; a cartilage template with a 1.2 mm diameter empty channel at its core; and a cartilage template with a 1.2 mm diameter channel filled with the PV bioink described in Chapter 3. Firstly, 2 architectures were printed: solid and channelled. These were chondrogenically primed for 3 weeks under hypoxic conditions and 1 week under normoxic conditions. A set of empty channelled constructs were then filled with the PV bioink. All constructs were then cultured in EGM-2 media supplemented with 50 ng/mL of VEGF for 7 days under normoxic conditions to allow for the formation of
microvessels. Constructs were then implanted subcutaneously into nude mice (See §4.2.8) and explanted at 4 and 8 weeks for analyses.

![Figure 4.1 Schematic outlining the study design](image)

**4.2.8 In vivo Subcutaneous Implantation**

Constructs were implanted subcutaneously into the back of Balb/c female nude mice (Harlan, UK). Briefly, two subcutaneous pockets were made along the central line of the spine, one at the shoulders and the other at the hips. Two constructs were inserted into each pocket. Eight constructs were implanted per group and constructs were harvested 4 weeks and 8 weeks post-implantation. Mice were anaesthetised using an intraperitoneal injection of xylazine hydrochloride and ketamine hydrochloride, Carprofen was added to water for 24 h post-surgery, and mice were sacrificed by CO₂ inhalation. This protocol and study were reviewed and approved by the ethics committee of Trinity College Dublin. Post-implantation samples were fixed in 4% PFA for 24 h.

**4.2.9 Micro-computed Tomography**

Micro-computed tomography (µCT) scans were performed using a Scanco Medical 40 µCT system (Scanco Medical, Bassersdorf, Switzerland) in order to visualise and quantify mineral content and to assess mineral distribution within 3D bioprinted constructs.
Constructs were scanned in 50 % EtOH, at a voxel resolution of 12μm, a voltage of 70 kVp, a current of 114 μA. Reconstructed 3D images were generated to visualise the mineral content and distribution throughout the constructs. A Gaussian filter (sigma = 1.2, support = 2) was used to suppress noise site and a global threshold of 482.

4.2.10 Histological Analysis

Fixed samples were dehydrated in graded series of ethanol solutions (70-100 %), cleared in xylene, and embedded in paraffin wax (all Sigma-Aldrich). Sections (5 μm) were rehydrated in graded series and stained with haematoxylin and eosin, 1 % (w/v) alcian blue 8GX in 0.1 M HCL to assess sulphated glycosaminoglycan (sGAG) content with a counter stain of 0.1 % (w/v) nuclear fast red to assess cellular distribution, 0.1 % (w/v) picrosirius red to assess collagen distribution, 0.2 % (w/v) Safranin O to assess sGAG content post-implantation and Goldner’s trichrome (Groat's iron haematoxylin, Fuchsine, Orange G, Fast Green) for visualising bone (all from Sigma). Slides were then imaged using an Aperio ScanScope slide scanner and evaluated for vessel infiltration by counting vessels visible across an entire section using Aperio ImageScope and ImageJ software. It should be noted that PCL is cleared during the tissue processing and leaves empty spaces in constructs as a result.

4.2.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, CA, USA). To analyse significant differences between two groups at one timepoint, a standard two-tailed t-test was performed. To analyse variance between >2 groups at one timepoint, a one-way analyses of variance (ANOVA) was performed. To analyse variance between >2 groups at multiple timepoints, two-way ANOVA was used with Tukey post-hoc test. Numerical and graphical results are displayed as mean ± standard deviation unless otherwise stated. Significance was accepted at a level of p<0.05.
4.3 Results

4.3.1 Chondrogenesis of Human BMSCs in PCL-fibrin Composites

To realise the objectives of this study, a reinforcing PCL frame had to be first
developed that facilitated implantation (and later retrieval) of the 3D bioprinted cartilage
templates in vivo. To this end, two geometrical designs were chosen for comparison, a
cylindrical solid sheath (described in chapter 3) and a porous PCL sheath, designed to allow
for improved nutrient diffusion during in vitro culture in addition to facilitating
omnidirectional vascularisation of the constructs in vivo. Comparing different PCL scaffold
designs and different cell seeding concentrations revealed that scaffold architecture did not
have a significant effect on matrix deposition at lower cell concentrations. At the higher cell
concentration of $20 \times 10^6$ cells/mL, the lack of side porosity hindered both sulphated
glycosaminoglycan (sGAG) deposition and collagen production, with significantly lower
levels of total sGAG and collagen (Figure 4.2 C&E) deposition observed in the constructs
surrounded by the solid sheath. Doubling cell concentration from $10 \times 10^6$ cells/mL to $20 \times
10^6$ cells/mL did not result in a doubling of matrix production. Total DNA levels indicate that
the higher cell concentration led to higher nutrient demands and associated reductions in
matrix synthesis (Figure 4.2). Following this experiment the porous PCL architecture and a
cell concentration of $10 \times 10^6$ cells/mL were selected for future studies.
Figure 4.2 Biochemical analysis comparing PCL scaffold designs and cell concentrations. (A) Schematic of scaffold designs. (B) Total DNA content (µg). (C) Total GAG content (µg). (D) sGAG content normalised to DNA content. (E) Total collagen content (µg). (F) Collagen content normalised to DNA: after 21 days of chondrogenic priming. * denotes significance one-way ANOVA, p<0.05 (n = 4, Mean ± SD).

4.3.2 Elucidating the Effect of Vasculogenic Culture on sGAG Retention Within Engineered Cartilage Templates

To assess whether prevascularising an engineered cartilage template enhances its capacity to generate endochondral bone, three template designs were compared. The first consisted of solid cylinders of cartilage, the second contained an empty central channel (termed empty channel), while in the third design the central channel was filled with the vascular bioink (termed PV channel) developed in Chapter 3 (Figure 4.1). These three constructs were engineered in chondrogenic culture conditions for 4 weeks, followed by a further week in vasculogenic media to support the development of a prevascularised central channel in the PV channel group (Figure 4.3 A). To determine whether the further week of culturing in VEGF+EGM-2 media following the formation of the cartilage template would result in a loss of sGAG from the deposited matrix, biochemical analysis was carried out on constructs prior to vasculogenic culture and post vasculogenic culture (Figure 4.3 A). There were no significant differences in total sGAG levels between the pre- and post-EGM samples.
of the solid and PV channel groups (Figure 4.3 B). There was a significant decrease in total sGAG levels in the empty channel groups following a week of EGM-2 culture (Figure 4.3 B), however this significance was lost when sGAG levels were normalised to DNA content.

**Figure 4.3** Biochemical analysis of sGAG content and DNA content of all three groups before (Pre-EGM2) and after (Post EGM2) vasculogenic culturing. (A) Schematic explaining culturing regimes of the two groups. (B) Total sGAG content (µg). (C) Total DNA content (µg). (D) sGAG normalised to DNA content. * denotes significance two-way ANOVA, p<0.05 (n = 4, Mean ± SD).

### 4.3.3 Biochemical and Histological Analysis of Cartilage Templates Prior to Implantation

Following 3 weeks of chondrogenic priming in hypoxia, 1-week chondrogenic priming at normoxia and a further week in vasculogenic media, cartilage-like tissue formation was observed macroscopically in all groups prior to implantation (Figure 4.4 A). There were no significant differences in sGAG content of the solid, empty channel and PV channel constructs at the end of the in vitro culture period (Figure 4.4 B&C). Histologically, all groups displayed positive alcian blue staining for sGAG deposition and positive picrosirius red staining for collagen deposition. Staining appeared more intense in the empty channel group, possibly due to improved nutrient diffusion during in vitro priming (Figure 4.4 C).
Figure 4.4 Evaluation of implants prior to implantation. (A) Macroscopic images of constructs; Scale bar = 2 mm. (B) Total DNA content (µg). (C) Total sGAG content (µg). (D) sGAG normalised to DNA content. * denotes significance one-way ANOVA, p<0.05 (n = 4, Mean ± SD). (E) Histological analysis of all three groups following in vitro culturing; scale bar of inset image = 1 mm, scale bar for all high-resolution images = 200µm.
4.3.4 Histological Evaluation of Implant Composition and Vascularisation After 4 And 8 Weeks in vivo

After 4 weeks in vivo, safranin O staining demonstrated near complete loss of sGAG from the engineered tissue (Figure 4.5 A), confirming the temporary nature of the engineered cartilage in vivo. At this same time-point, both the empty channel group and the PV channel group showed evidence of perfused vessels at the core of the construct. Although vessels were present in the solid group, these were primarily present at the periphery of the construct, with minimal invasion into the centre of the cartilage template (Figure 4.5 A). There were no significant differences in the overall number of vessels between all three groups (Figure 4.5 B), however there were significance differences in the area of engineered tissue that was vascularised in vivo. Comparing total vessel area, the PV channel constructs were significantly more vascularised than both the solid and empty channel group (Figure 4.5 C). When normalising vessel area to total tissue area, both the empty channel and the PV channel had a significantly higher vascularised area compared to the solid group (Figure 4.5 D). After 8 weeks in vivo, there was no significant difference in the extent of vascularisation between the three groups.
Figure 4.5 Histological analysis and vessel analysis of constructs post-implantation. (A) Safranin O staining (Saf O), Haematoxylin and eosin staining (H&E) and Goldner’s Trichome staining (GT) at 4 week and 8-week post-implantation. Scale bar of inset image = 1 mm, scale bar for all high-resolution images = 200µm. (B)
quantification of no. of vessels per mm². (C) Vessel area and (D) percentage vascularised area, * denotes significance p<0.5 one-way ANOVA (n = 8, Mean ± SD).

4.3.5 Micro-computed Tomography Analyses of Bone Volume

Micro-computed tomography (μCT) analysis was used to assess the level of mineralisation that occurred within all three groups following in vivo implantation (Figure 4.6). No significant differences in overall levels of mineralisation was observed between the three groups. 3D reconstructions indicate that mineralisation for all groups was generally limited to the periphery of the constructs.
Figure 4.6. MicroCT analysis of bone volume of constructs post-implantation. (A) 3D reconstruction of constructs at 4- and 8-weeks post-implantation; scale bar = 1 mm. (B) Quantification of bone volume and (C)
bone density calculated from microCT scans of constructs at 4- and 8-weeks post-implantation. \( n = 8, \text{Mean} \pm SD \).

### 4.4 Discussion

To date, the scaling-up of engineered tissues and organs has been limited by the inability to pre-vascularise these constructs prior to their implantation into the defect environment. This study demonstrates both the adaptability of the PV bioink described in Chapter 3 and how it can be used as a platform technology to enhance the \textit{in vivo} vascularisation of 3D bioprinted tissues. This vascularisation strategy was compared with another commonly used approach, namely the introduction of a hollow channel, for their efficiency at enhancing vascularisation \textit{in vivo}. Superficially these two strategies were applied to the engineering of developmentally inspired cartilage templates for bone tissue engineering, using a solid cartilage template as a control. All three groups were engineered using a multiple-tool biofabrication strategy, with a specific culture regime to allow the formation of both a cartilage template and a microvascular network \textit{in vitro}. The aim was to better mimic developmental skeletogenesis, where cartilaginous bone-precursors are vascularised leading to ossification and bone formation. Following subcutaneous implantation, these 3D bioprinted cartilaginous templates were assessed for vascular infiltration (histologically) and for mineralisation (\( \mu \text{CT} \)).

Although there was no significant difference in the overall number of vessels between the groups, there were significantly larger vessels, endothelialising a larger area, in the PV groups compared to the solid group and empty channel groups at 4 weeks. This significance was lost at the later timepoints, as all groups became progressively more vascularised with time. This suggests that the benefits of pre-vascularising a tissue are most clearly evident in the first weeks after implantation, with less clear benefits at later timepoints, especially in well vascularised regions such as subcutaneous environment in the back of mice. Implant
vascularisation was also improved by simply introducing an empty channel into the centre of the implant. It has previously been shown in the literature that the presence of hollow channels can improve cell infiltration and host vessel invasion (W. Zhang et al., 2015; Feng et al., 2017; Daly et al., 2018). However, this approach relies on the growth of host vessels from the periphery of the construct. The growth of blood vessels is a relatively slow process, the average physiological growth rate of newly developing microvessels is only ~ 5 μm/h (Utzinger et al., 2015). This approach to rapid vascularisation therefore may not be suitable when scaling up tissue engineered constructs to clinically relevant sizes. By firstly forming an interconnected, primitive microvessel network in vitro, host vessels around the periphery of the construct, once implanted in vivo, can undergo anastomosis leading to a more rapidly perfused engineered tissue. Further studies are required to elucidate whether the benefits of pre-vascularisation become even more evident as the size of an engineered tissue increases.

Despite the significant differences seen in the levels of vascularisation between the groups, this did not translate to an enhancement in ectopic bone formation within the prevascularised implants. Overall, bone volumes were modest through all three groups. One possible explanation is the lack of mechanical stimulation of the constructs. Mechanical cues are critical for efficient endochondral ossification. For examples, studies into skeletal development in the chick embryo showed that foetal paralysis significantly decreases bone mass (Hosseini and Hogg, 1991). Similarly, in human, it has been shown that foetal movements are vital for normal skeletal developments (Verbruggen et al., 2018). In this study, cartilage templates were enveloped within a porous PCL scaffold. This may have acted as a barrier, preventing the constructs from experiencing any of the limited mechanical stimuli generated from the mouse movement in this subcutaneous model. It is also possible that the 1 week in vasculogenic culture conditions, while not significantly reducing sGAG levels, did suppress the endochondral potential of the engineered cartilage templates. Finally,
it is possible that extended culture conditions are required to generate a more hypertrophic cartilage template prior to its subcutaneous implantation.

4.5 Conclusion

In conclusion, the results of this study show that the inclusion of HUVECs and hBMSCs into a vasculogenic bioink can be used to prevascularise 3D bioprinted tissue engineered cartilage templates leading to enhanced vascularisation in vivo. While this did not lead to significantly enhanced bone formation in this model, it represents a powerful tool to enhance in vivo vascularisation which is compatible with 3D bioprinting techniques.
Chapter 5

Prevascularising Hypertrophic Microtissues for use in Bioprinted Constructs for Bone Tissue Engineering

5.1 Introduction

Clinically, large bone defects often present a challenging recipient site for regenerative implants due to decreased vascularisation after irradiation, trauma, or infection. For this reason, vascularised bone transplants are required. At present, autologous bone transplants, such as vascularised fibular grafts or iliac crest bone grafts, still represent the gold standard for treating such large bone defects. However, the volume of bone available is limited and harvesting the tissue necessitates an additional operation which risks donor site morbidity. A study investigating donor site morbidity following iliac crest bone grafting found up to 70% of patients reported post-operative complications (Palmer, Crawford-Skkes and Rose, 2008). This has motivated the field of tissue engineering to develop novel bone substitutes that overcome the limitations associated with conventional treatments.

The field of bone tissue engineering has made substantial progress in recent years, particularly in identifying suitable cell sources (Caplan, 1991; Li et al., 2007; Kahle et al., 2010; Csoibonyeiova et al., 2017) and growth factors (Zhang et al., 2019), as well as developing osteoinductive biomaterials to promote bone formation (Yamasaki and Sakai, 1992; Deng et al., 2017; Walsh et al., 2019). Despite these many advances, there are limited
examples of cell-based bone tissue engineering strategies successfully translating into the clinic. The reasons for this are multi-faceted but include technical limitations such as insufficient blood supply to the engineered tissue upon implantation into the body. Therefore, there is an urgent need for novel approaches to enhance the vascularisation of tissue engineered constructs and thereby overcome one of the main hurdles hindering the clinical success of many bone tissue engineering approaches.

3D cell spheroids have been utilised by cell biologists and tissue engineers over the last 20 years to study cell-cell interactions, cell differentiation, and as a model system for drug screening (Johnstone et al., 1998; Korff and Augustin, 1998; Friedrich et al., 2009; Huang et al., 2012; Walser et al., 2013; Wenzel et al., 2014). In recent years there has been an increased interest in using similar spheroidal systems as the building blocks for organ printing. The concept is to produce tissue spheroids or micro-tissues (μTissues), which are then fused together to form a larger engineered tissue. Multiple methods have been described in the literature to produce multicellular spheroids, including hanging drop (Yoon et al., 2012), spinner culture (Nyberg et al., 2005) and micro-moulds (Lopa et al., 2015), enabling the development of tissue engineered models of multiple tissues such as cartilage (Huang et al., 2013), skin (Furukawa et al., 2001), myocardium (Chimenti et al., 2011) and bone (Nilsson Hall et al., 2019). There are many advantages to using spheroids over traditional tissue engineering approaches, which typically use a population of (physically separated) cells suspended in biomaterials. Firstly, in the absence of a substrate biomaterial, cells are forced to interact with one another. This process mimics the cell aggregation that occurs during embryonic development and drives self-organisation (Laschke and Menger, 2017). It has been shown that the differentiation potential of MSCs increases during spheroidal culturing compared to 2D monolayer culture (Kapur et al., 2012; Yoon et al., 2012; S. Zhang et al., 2015). Another advantage of μTissues for tissue engineering applications is that it is relatively simple to combine multiple μTissues to produce scaled-up implants suitable for
treating larger defects. However, as such constructs increase in size, the overall nutritional demands of the engineered tissue will increase, necessitating novel approaches to vascularise such constructs prior to their implantation into the body.

The goal of this chapter is to prevascularise a tissue engineered construct that consists of multiple hypertrophic cartilage µTissues primed for endochondral bone formation, and to then examine whether such in vitro pre-vascularisation will enhance vessel formation and osteogenesis in vivo. Hypertrophic µTissues were fabricated in vitro using a non-adhesive micro-moulding technique designed in our lab (Burdis and D.J. Kelly, 2019). These µTissues were then embedded within a fibrin-based bioink containing HUVECs and hBMSCs. This implant was then cultured for 7 days in the presence of VEGF, which induced the formation of a microvascular network encompassing the µTissues. These constructs were then implanted into a subcutaneous mouse model to investigate whether this prevascularisation approach can lead to accelerated vascularisation and mineralisation of the engineered construct compared to a non-prevascularised control.

5.2 Materials and Methods

5.2.1 Isolation and Expansion of Human BMSCs

Human BMSCs (hBMSCs) were isolated from unprocessed human bone marrow (Lonza) on the basis of plastic adherence. Briefly, unprocessed bone marrow was plated at $2.5 \times 10^5$ cells/cm$^2$ (estimated approx. 4000-5000 MSCs/cm$^2$) in high glucose Dulbecco’s modified eagle’s medium (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under hypoxic conditions (37°C in a humidified atmosphere with 5 % pCO$_2$ and 5 % pO$_2$) for improved chondrogenic differentiation. Following colony formation, hBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic acid (EDTA) and tripotentiality was confirmed as previously
described (Vinardell et al., 2009). hBMSCs used for the fibrin bioink were seeded at 5000 cells/cm$^2$ expanded in DMEM supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under normoxic conditions (37°C in a humidified atmosphere with 5 % pCO$_2$ and 20 % pO$_2$) and used at P4. hBMSCs used for hypertrophic spheroids were expanded in the same media but under hypoxic conditions (5 % pO2) and aggregated into pellets at P3.

5.2.2 Endothelial Expansion

Human Umbilical Vein Endothelial cells (HUVECs; Lonza, Walkersville, MD) were cultured at 2500 cells/cm$^2$ in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza) at 37°C in a humidified atmosphere with 5 % pCO$_2$ and 20 % pO$_2$). HUVECs were used at P4.

5.2.3 Hypertrophic Cartilage µTissues Production

Hypertrophic µTissues were fabricated as previously described (Burdis and D.J. Kelly, 2019). An overview of the process is provided below:

Moulding Assembly Design and Fabrication: Positive moulds were first designed using Solidworks CAD software. Briefly, moulds were designed as a stamp intended to impression molten agarose with the desired µwell design. Each of the 401 µwells measured 1 mm in diameter (ø), had a total well depth of 1.5 mm – comprised of a 1 mm deep cylindrical section and a domed end which was 0.5 mm deep. The top of each µwell was a chamfered asymmetrically, 0.5 mm x 1 mm (width x depth) ensuring there were no flat sections of the mould between adjacent µwells. An STL file for the part was then prepared for printing using Preform 2.16.0 software (Formlabs, Massachusetts, United States), a 50 μm layer height was used for every mould. Positive moulds were fabricated using a Form 2 stereolithography printer (Formlabs, Massachusetts, United States). Completed parts were
processed post-printing in accordance with the manufacturer’s guidelines. Briefly, parts were washed in propan-2-ol (Sigma Aldrich) to clear any uncured resin, following which they were exposed to UV light (405 nm, 9.1 W) (Form cure, Formlabs, Massachusetts, United States) for 30 minutes at 60 °C to ensure complete crosslinking. Moulds were cleaned, and gas sterilised using ethylene oxide (EtO) prior to use.

**Agarose Microwell Inserts:** Microwells were inserted into individual wells of a 6 well plate (Nunc 6-Well Plate, Round, ThermoFisher Scientific, Massachusetts, United States). Agarose microwells were fabricated under sterile conditions, within a class II biosafety cabinet. Firstly, agarose (Sigma Aldrich) was dissolved in phosphate buffered saline (PBS; Sigma Aldrich) at a concentration of 4 % (w/v) and autoclaved to ensure sterility. The moulding procedure is outlined and discussed in the results section as part of figure 2C. Briefly, the molten agarose solution was pipetted into a well. The aforementioned sterile 3D printed positive moulds, were then inserted into the agarose ensuring no bubbles became trapped underneath the mould. A holder was then placed over mould and a M2 screw tightened to secure the height and position of the mould. This process was repeated for each well. Once cooled, excess solidified agarose above the mould was removed and the positive mould was pulled from the agarose, leaving the patterned agarose, imprinted with the microwells, within the well. All agarose micro-wells were soaked overnight in DMEM prior to cell seeding.

**Cell Seeding and Hypertrophic Cartilage Priming:** Cells were seeded into the microwells by pipetting an appropriate concentration (1 x 10³ cells, 2 x 10³ cells or 4 x 10³ cells/microwell) into each well. After seeding, plates were centrifuged at 700 x g for 5 minutes to collect cells at the bottom of each well. Plates were then incubated in high glucose Dulbecco’s modified eagle’s medium (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL bFGF2 (Prospect Bio) overnight to allow condensation to occur before
switching to chondrogenic media consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (both Gibco), 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid-2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1 X insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma), 2.5 μg/mL amphotericin B and 10 ng ml⁻¹ of human transforming growth factor-b3 (TGF-β3; Prospec-Tany TechnoGene Ltd., Israel) at 37 °C in a humidified atmosphere with 5 % pCO₂ and 5 % pO₂ for 3 weeks. μTissues were then moved to 20 % pO₂ and switched to hypertrophic media consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (both Gibco), 1 X insulin–transferrin–selenium, 4.7 μg/mL linoleic acid, 50 nM thyroxine, 100 nM dexamethasone, 250 μM ascorbic acid, 7 mM β-glycerophosphate and 2.5 μg/mL amphotericin B (all from Sigma) for a further 2 weeks. μTissues containing 2 x 10⁵ cells were chosen for further studies as 4 x 10⁵ cells/μTissues were too large for bioprinting.

**Harvesting Hypertrophic Cartilage μTissues:** After 5 weeks, μTissues were liberated from the μwells by first flushing media over the surface of the agarose using a pipette. The culture media was then removed from each well, and the agarose removed and inverted into a new 6 well plate. This plate was centrifuged at 700 x g for 5 min to collect the μTissues in the bottom of the well plate. Fresh culture media was washed over the surface of the wells of the plate to collect the μTissues. The suspension of μTissues was then passed through an appropriately sized pluriStrainer® (pluriSelect®, Leipzig, Germany) to capture any agarose fragments. The density of this purified μTissue suspension could then be quantified and combined with the bioink. *In vitro* histology samples were prepared by combining hypertrophic cartilage μTissues with the cell-free bioink, fixing in 4 % PFA overnight at 4 °C, and evaluating histologically.
5.2.4 Bioink Fabrication

To enable the use of fibrin as a printable bioink, a gelatin carrier method was adapted from (Kang et al., 2016). Briefly, hyaluronic acid (Sigma) was added to high glucose Dulbecco's Modified Eagle Medium (hgDMEM; Gibco, Biosciences) at a concentration of 3 mg/mL and stirred overnight at 37°C. 10% (v/v) glycerol (Sigma) was then added and the solution was stirred for 1 h at room temperature. Gelatin type A (175 g bloom; Sigma) was added at a concentration of 40 mg/mL and stirred for 2 h at 37°C until fully dissolved. Before use, Fibrinogen (Sigma) was added to this thawed carrier gel at a concentration of 30 mg/mL and stirred for 2 h at 37°C. To enhance the angiogenicity of the bioink for use as PV bioink, D-erythro-sphingosine-1-phosphate (S1P; Avanti Polar lipids, AL, USA) was added at a concentration of 125 µM/mL. For both cast constructs and printed constructs this fibrin bioink formulation was used.

5.2.5 Micro-computed Tomography Scan Conversion to G-code

A micro-computed tomography (µCT) scan of a rat femur was first segmented and converted to STL file format using 3D Slicer free software (www.slicer.org). This STL was then converted to G code using BioCAM™ and BioCAD™ software packages (RegenHU, Switzerland).

5.2.6 3D Bioprinting System

Scaffolds were designed using BioCAD™ software package and produced using the 3D Discovery multi-head bioprinting system (Regen Hu, Switzerland). Porous polycaprolactone (PCL; CAPA Ingevity, SC, USA,) disc scaffolds were printed at 80 °C at 0.5 MPa using a 27G needle (height = 1 mm and diameter = 4 mm). Scaffolds were sterilised using ethylene oxide (EtO) gas prior to the addition of cell-laden bioinks. The bioinks were printed using a pneumatic driven syringe with a 25G needle at temperatures between (10 – 30) °C under pressures of between 0.05 MPa - 0.2 MPa. Hypertrophic µTissues were
incorporated into the fibrin bioink at $17 \times 10^3$ spheroids/mL (680/Construct). For the PV bioink, HUVECs and hBMSCs were incorporated into the fibrin bioink at $3 \times 10^6$ cells/mL and $1.5 \times 10^6$ cells/mL, respectively. To assist the accuracy of the printing process, 4 % (w/v) agarose (Sigma) well inserts were fabricated using custom-designed moulds printed with Form2 SLA printer (FormLabs, MA, USA). These agarose moulds were soaked in thrombin (20 U/mL) prior to printing. After printing, all constructs were further immersed in a thrombin bath (20 U/mL) for 30 min at RT to allow the thrombin-catalysed polymerisation of fibrinogen to fibrin. The 3D Discovery was encased in a laminar flow hood to ensure sterility throughout the biofabrication process.

5.2.7 In vivo Study Design

To investigate the effect of prevascularisation on a cartilage template in vivo, three groups were compared: A prevascularised fibrin disc (PV only); a fibrin disc containing hypertrophic µTissues (µTissues only); and a prevascularised fibrin disc containing hypertrophic µTissues (PV µTissues). All disc constructs were cast seeded apart from the bioprinted, rat femoral segment which was printed as described in §5.2.6. All constructs were then cultured in EGM-2 media supplemented with 50 ng/mL of VEGF for 7 days under normoxic conditions to allow for the formation of microvessels. Constructs were then implanted subcutaneously into nude mice (See §5.2.8) and explanted at 2, 4 and 8 weeks for analyses.

5.2.8 In vivo Subcutaneous Implantation

Constructs were implanted subcutaneously into the back of Balb/c female nude mice (Harlan, UK). Briefly, two subcutaneous pockets were made along the central line of the spine, one at the shoulders and the other at the hips. Two constructs were inserted into each pocket. Six constructs were implanted per group and constructs were harvested 4 weeks and 8 weeks post-implantation. Mice were anaesthetised using an intraperitoneal injection of xylazine hydrochloride and ketamine hydrochloride, Carprofen was added to water for 24 h
post-surgery, and mice were sacrificed by CO₂ inhalation. This protocol and study were reviewed and approved by the ethics committee of Trinity College Dublin. Post-implantation samples were fixed in 4 % PFA for 24 h.

5.2.9 Micro-computed Tomography

Micro-computed tomography (μCT) scans were performed using a Scanco Medical 40 μCT system (Scanco Medical, Bassersdorf, Switzerland) in order to visualise and quantify mineral content and to assess mineral distribution within all constructs.

Constructs were scanned in 50 % EtOH, at a voxel resolution of 12μm, a voltage of 70 kVp, a current of 114 μA. Reconstructed 3D images were generated to visualise the mineral content and distribution throughout the constructs. A Gaussian filter (sigma = 1.2, support = 2) was used to suppress noise site and a global threshold of 482.

5.2.10 Histological Analysis

_in vivo_ samples were decalcified using ‘Decalcifying Solution-Lite’ (Sigma) for approximately 1 week. Samples were frequently x-rayed to determine if any mineral content remined. When no mineral was visible the sample was considered decalcified. All samples (in vitro and decalcified in vivo) were then dehydrated in graded series of ethanol solutions (70 % - 100 %), cleared in xylene, and embedded in paraffin wax (all Sigma-Aldrich). Sections (5 μm) were rehydrated in graded series and stained with haematoxylin and eosin, 1 % (w/v) alcian blue 8GX in 0.1 M HCL to assess sulphated glycosaminoglycan (sGAG) content with a counter stain of 0.1 % (w/v) nuclear fast red to assess cellular distribution, 0.1 % (w/v) picrosirius red to assess collagen distribution, 0.2 % (w/v) Safranin O to assess sGAG content post-implantation and Goldner’s trichrome (Groat's iron haematoxylin, Fuchsine, Orange G, Fast Green) for visualising bone (all from Sigma). Immunohistochemistry was performed for collagen type I (col I; Abcam ab90395 1:400), collagen type II (col II; Santa Cruz- sc52658 1:400), and collagen type X (col X; Abcam
Slides were then imaged using an Aperio ScanScope slide scanner. Samples stained for picrosirius red were imaged using polarised light microscopy (PLM) to determine collagen fibre orientation. *In vivo* sections were evaluated for vessel infiltration by counting vessels visible across an entire section using Aperio ImageScope and ImageJ software. Bone fraction of sections taken of the entire implant was determined using Goldner’s trichrome staining and MIPAR image analysis. It should be noted that PCL is cleared during the tissue processing and leaves empty spaces in constructs as a result.

### 5.2.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, CA, USA). To analyse significant differences between two groups at one timepoint a standard two-tailed t-test was performed. To analyse variance between >2 groups at one timepoint a one-way analyses of variance (ANOVA) was performed. To analyse variance between >2 groups at multiple timepoints, two-way ANOVA was used with Tukey post-hoc test. Numerical and graphical results are displayed as mean ± standard deviation unless otherwise stated. Significance was accepted at a level of p<0.05.

### 5.3 Results

#### 5.3.1 Hypertrophic Microtissue Fabrication

In this study, custom-made agarose microwell mould inserts were used to form human BMSCs μTissues (Figure 5.1 A). Positive 3D printed moulds were inserted into agarose filled wells of a 6 well plate. This created 401 micro-wells per well of a 6 well plate. Cell pellets formed through self-assembly. The design and non-adhesive nature of the agarose well directs cells to aggregate at the bottom of each well (Figure 5.1 A). Centrifugation further enhances this process. 24 hours post-seeding, cells had consistently formed pellets at the bottom of the wells. The diameters of μTissues containing 1000, 2000 and 4000 cells (per μTissue) were highly uniform after 24 hours (SD<0.025; Figure 5.1 C). The μTissues
increased in diameter with time in culture (Figure 5.1 C&D), and following the 5-week culture period, the 1000, 2000 and 4000 cells per μTissues had a mean diameter of 220μm, 320μm and 360μm respectively (Figure 5.1 D). The μTissues stained positive for the canonical cartilaginous extracellular matrix markers, sulphated glycosaminoglycan (sGAG), collagen, whilst calcium deposition (Figure 5.1 E), indicated that the \textit{in vitro} culture protocol successfully facilitated the production of hypertrophic cartilage μtissues. The presence of collagen type I was detected using IHC, as were high levels of collagen type II. Somewhat surprisingly, there was a distinct absence of collagen type X expression after the 5 weeks of \textit{in vitro} culture (Figure 5.1 E).
Figure 5.1 Hypertrophic microtissue fabrication and analysis. (A) schematic of mould design and fabrication process. 3D printed moulds (i/ii) were inserted into agarose containing wells (iii). Agarose was allowed to cool around the mould (iv) leaving behind agarose microwells (v). Cells were seeded on top and aggregated overtime to form pellets. (B) Culture regime for hypertrophic μTissues formation. (C) Visual comparison of μTissues diameter of 1000, 2000 and 4000 cells/μTissues at 24 hours and 28 days. (D) Growth curve of μTissues over 28 days (n = 48, Mean ± SD). (E) Histological evaluation of μTissues after 5 weeks hypertrophic priming. (row 1 from left to right) haematoxylin and eosin (H & E) staining, alcian blue staining, picrosirius red staining and alizarin red staining. (row 2) Polarised light microscopy, immunohistochemistry staining for Collagen type I, type II and Type X; scale bar of inset image = 1 mm and high mag image = 100µm.
5.3.2 Micro-computed Tomography Analysis of Bone Formation in vivo

Following 5 weeks of in vitro priming, µTissues were cast into 4 mm (ø) x 1 mm 3D printed polycaprolactone (PCL) discs in either a fibrin-based bioink, or in the PV bioink, described in Chapter 3, containing HUVECs and hBMSCs at a concentration of $3 \times 10^6$ and $1.5 \times 10^6$ cells/mL respectively. These two groups, along with a PV bioink control without any µTissues, were cultured for 7 days in EGM-2 media containing VEGF (50 ng/mL) to allow for the formation of a microvascular network. Micro-computed tomography (µCT) analysis performed on the constructs following 6 weeks of in vitro culture showed no detectable mineral deposition (data not shown). All groups were subsequently implanted subcutaneously into nude mice. Following 4 and 8 weeks in vivo, negligible mineral was observed in the PV bioink controls. µCT analysis revealed that prevascularising the µTissues prior to implantation led to significantly higher levels of bone formation after 4 weeks in vivo (Figure 5.2 A&B). No significant differences in construct mineralisation between the µTissues only group and the prevascularised µTissue group was observed at the 8-week timepoint (Figure 5.2 C).
Figure 5.2 MicroCT analysis of constructs post-implantation. (A) 3D reconstructions of microCT scans of the μTissues only and PV μTissues groups at 4 weeks and 8 weeks post-implantation. (B) Quantification of bone volume (mm$^3$) at 4 weeks and (C) 8 weeks * denotes significance one-way ANOVA with Tukey post-hoc test, p<0.05 (mean ± SD, n = 6). (D) Macro images of all three groups at 4 weeks and (E) 8 weeks; scale bar = 1 mm for all images.

5.3.3 Histological Analysis

Histological analysis was carried out on the μTissue constructs to investigate vascularisation and tissue formation after subcutaneous implantation. Safranin O staining demonstrated that no cartilage remained in the implants containing μTissues after 4 and 8 weeks in vivo (Figure 5.3 A & Figure 5.4 A). Goldner’s trichome staining showed that the
hypertrophic μTissues had mineralised at all timepoints in all μTissues groups (Figure 5.3 A & Figure 5.4 A). Blood vessels (yellow arrows) were present in all groups at both time points (Figure 5.3 A & Figure 5.4 A). There was a trend towards increased vascularisation (quantified by a higher number of vessels and larger vessels in the prevascularised groups) at the 4 week time point (Figure 5.3 C-E), although these differences were not statistically significant, and no longer discernible at the 8 week time point (Figures 5.4 C-E). Fusion between adjacent hypertrophic cartilage spheroids was noted in both μTissue groups at both time points (Black arrows; Figure 5.3A & Figure 5.4 A). Histological quantification of bone area indicated no significant differences at either timepoint (Figure 5.3 B & 5.4 B). Furthermore, there was no histological evidence of marrow cavity development throughout any groups.
Figure 5.3 Histological analysis of constructs 4 weeks post-implantation. (A) Haematoxylin and eosin staining (H&E), Safranin O staining (Saf O) and Goldner’s Trichome staining (GT) at 4 weeks; scale bar = 500µm for low resolution images; scale bar = 150µm for high resolution images. (B) Quantification of bone area (C)
quantification of vessel no. (D) Vessel area and (E) percentage vascularised area, * denotes significance two-way ANOVA with Tukey post-hoc test, $p < 0.05$ ($n = 6$, Mean ± SD).
Figure 5.4 Histological analysis of constructs 8 weeks post-implantation. (A) Haematoxylin and eosin staining (H&E), Safranin O staining (Saf O) and Goldner’s Trichome staining (GT) at 8 weeks; scale bar = 500µm for low resolution images; scale bar = 150µm for high resolution images. (B) Quantification of bone area (C)
quantification of vessel no. (D) Vessel area and (E) percentage vascularised area, * denotes significance two-way ANOVA with Tukey post-hoc test, \( p < 0.05 \) (\( n = 6 \), Mean ± SD).

### 5.3.4 3D Bioprinting to Scale up the Development of Prevascularised Tissues: A Proof of Principle

This thesis next sought to demonstrate that this approach could be combined with 3D bioprinting technology to produce scaled-up and anatomically defined pre-vascularised tissues. To this end, hypertrophic μTissues were bioprinted into a 3D printed polycaprolactone (PCL) scaffold that mimicked the geometry of a rat femoral bone defect model commonly used for studying long bone regeneration. Firstly, a μCT scan was taken of a rat femur. This scan was then segmented and converted to STL file format using 3D Slicer software (www.slicer.org). This STL was then converted to G code using BioCAM™ and BioCAD™ software packages and printed with PCL. Hypertrophic μTissues, in a PV bioink containing HUVECs and hBMSCs, was then bioprinted into this scaffold. This bioprinted scaffold was cultured in vasculogenic media for 7 days and then implanted subcutaneously into a nude mouse. After 4 weeks *in vivo* there was evidence of construct mineralisation showing, as proof of principle, that this approach was compatible with 3D printing technology (Figure 5.5).
Figure 5.5 Bioprinting proof of principle. (A) Design of an anatomically accurate scaffold of a segment of a rat femur. i microCT scan of a rat femur. ii & iii rat femoral bone segment STL file. iv & v 3D representation of printing design. (B) Macroscopic image of scaffold prior to implantation. (C) Macroscopic image of scaffold 4-week post-implantation. (D) microCT 3D reconstruction of bone volume after 4 weeks in vivo; all scale bars = 1 mm.

5.4 Discussion

Bone tissue engineering aims to overcome the shortcomings and limitations of current treatments for regenerating large bone defects by generating *in vitro* bone graft substitutes. A sufficient supply of nutrients and oxygen is essential for the success of these engineered tissues. This study demonstrates that hypertrophic µTissues, which can be fabricated *in vitro* and are inherently primed to generate endochondral bone, will calcify upon
in vivo implantation. The in vivo mineralisation of these constructs can be further enhanced by prevascularising the hydrogel that surrounds the µTissues prior to implantation. Additionally, this study shows, as a proof of concept, that this approach can be combined with emerging 3D bioprinting technologies to produce prevascularised tissues mimicking the geometry of a target defect site.

To date, cartilage rudiments for bone tissue engineering have been fabricated in vitro using pellets containing large amounts of cells (>2 × 10^5 cells/pellet) or cells encapsulated within a biomaterial (Scotti et al., 2010; Bahney et al., 2014; van der Stok et al., 2014; Cunniffe et al., 2015; Sheehy, Mesallati, Vinardell, et al., 2015). However, the use of large cell numbers within pellets can lead to core cell death or undifferentiated cells within the core due to diffusion-related challenges (Scotti et al., 2013), whilst the use of biomaterials can hinder vascularisation and bone formation in vivo due to slow degradation rates (Daly et al., 2018). Here µTissues, with diameters ranging between 220-360 µm, depending on the initial cell number, were fabricated using hBMSCs and custom-made agarose micro-moulds. As a result of their size, no diffusion-related challenges were observed, and cartilage tissue formed uniformly throughout the entire µTissues. Using a fibrin based bioink to integrate multiple µTissues into a single engineered construct did not impede bone formation in vivo. However, there was limited evidence of fusion between µTissues following subcutaneous implantation. This may be due to a lack of direct contact between the µTissues within the fibrin hydrogel. It has been shown that the ability of cell aggregates to fuse to one another can diminish over time (Bhumiratana et al., 2014). Decreasing the hypertrophic culture regime may improve the ability of the µTissues to fuse in vivo, as the peripheral cells would be less embedded within a dense calcified cartilage matrix. Furthermore, implantation of µTissue constructs into a load bearing environment (and not subcutaneously into nude mice) might lead to greater remodelling of the µTissues in vivo and better support homogenous bone development.
During developmental bone formation, the process of hypertrophic cartilage turnover and replacement by bone is initiated by vascular invasion. In this study, prevascularising the hypertrophic µTissues prior to implantation resulted in higher levels of mineralisation and neo-osseous tissue formation at 4 weeks compared to implantation of µTissues alone. This result is consistent with previous studies that have attempted to prevascularise bone tissue engineering constructs prior to implantation. For example, prevascularising a calcium phosphate cement scaffold with HUVECs and human-induced pluripotent stem cell-derived MSCs has been shown to promote vascularisation and mineralisation in a rat cranial defect model (Liu et al., 2017). The accelerated levels of bone formation in vivo did not correlate with significantly higher levels of implant vascularisation. It may be that prevascularising the cartilage µTissues in vitro further primed them for endochondral bone formation, leading to accelerated mineralisation of the constructs in the absence of higher levels of vascularisation in vivo. Previous studies have demonstrated that HUVECs enhance the activation of endogenous Wnt and BMP signalling and increased ALP expression in hBMSCs (Saleh, Whyte and Genever, 2011). It has also been shown that the addition of HUVECs to a cartilage pellet, enhances the mineralisation potential of MSCs compared to chondrogenic priming alone (Freeman, Haugh and McNamara, 2015).

As the hypertrophic chondrocytes that reside within the µTissues are programmed to release angiogenic factors that support vascularisation, it is perhaps unsurprising that prevascularisation of the µTissues did not lead to enhanced vascularisation in vivo. During the later stages of endochondral ossification, hypertrophic chondrocytes express Osterix which is a potent inducer of VEGF expression (Tang et al., 2012). This leads to high levels of VEGF within the hypertrophic cartilage which stimulate vessel infiltration (Carlevaro et al., 2000). It should be noted that there was a non-significant trend towards enhanced vascularisation after 4 weeks in vivo in the prevascularised µTissues, suggesting that increasing the power of the study might have demonstrated a benefit to prevascularisation in
terms of enhancing the vascularisation of engineered cartilage in vivo. It would also be interesting to examine vessel infiltration at an earlier timepoint prior to the innate upregulation of VEGF by the hypertrophic µTissues.

The concept of using spheroids and µTissues as intermediate building blocks to fabricate larger tissues and organs, although still in its infancy, shows great promise. By employing a modular design when engineering tissues, highly complex systems can be divided into more manageable parts which can then be optimised individually. To this end, 3D bioprinting offers a means of spatially organising various individual µTissues into a single construct. As such, the methodology for forming hypertrophic cartilage µTissues described in this chapter can be integrated with a common 3D bioprinting technology, specifically pneumatic extrusion. This, proof of concept, bioprinted extrusion of µTissues into an anatomically accurate implant offers an insight into the feasibility of the use of similar µTissues and bioprinting approaches for more complex, multicellular/tissue systems. Despite this promising outlook, further optimisation is needed to achieve comparable levels of µTissue density within the construct when 3D bioprinting compared to manual deposition.

5.5 Conclusion

To conclude, this study demonstrated a novel method of producing hypertrophic µTissues which undergo rapid, and complete mineralisation upon subcutaneous implantation into nude mice. Prevascularising these µTissues with the PV bioink described in Chapter 3 further enhanced their ability to undergo mineralisation in an ectopic model.
Chapter 6

Bioprinting of Prevascularised Constructs for the Repair of Critical-Sized Bone Defects

6.1 Introduction

Bone possesses an intrinsic capacity for self-repair and regeneration. However, when large bone defects occur, this innate healing process can be insufficient and medical intervention is required to ensure successful regeneration. The current gold standard for treating such cases is an autologous bone graft, where bone is harvested from a donor site within the patient's own body, usually the iliac crest, and transferred to the defect site. This treatment is limited by the volume of available bone to graft and can lead to donor site morbidity. This has motivated the field of tissue engineering to produce implantable bone substitutes to promote bone regeneration. Although much progress has been made in the field to date, ensuring adequate vascularisation of large bone defects remains a significant challenge impeding the clinical success of regenerative therapies.

Multiple strategies have been proposed to address the challenge of vascularisation, from the incorporation of potent angiogenic growth factors to the incorporation of hollow channels within tissue engineered constructs to stimulate endogenous host angiogenic responses (Murphy et al., 2004; Kempen et al., 2009; Zhang et al., 2014; W. Zhang et al., 2015; Feng et al., 2017; Daly et al., 2018). However, these approaches rely on the growth of host vessels from the periphery of the defect site into a biomaterial or tissue engineered
construct. This ingrowth of host vessels is relatively slow and often inadequate to meet the nutrient and oxygen demands of implanted tissues or endogenous cells that migrate into the defect site to mount a regenerative response. Furthermore, the use of exogenous growth factors carries the risk of off-target effects and may be unsuitable in cases such as bone resection due to osteosarcomas. This has led to the concept of ‘prevascularising’ a biomaterial construct as a means to improve vascularisation of such engineered tissues and the environment into which they are implanted. The basic concept of this approach is to establish a microvasculature within a construct which can undergo anastomosis with host vessels at the periphery of the construct leading to a more rapidly perfused engineered tissue (Laschke et al., 2011; Liu et al., 2017).

Prevascularising an engineered tissue typically requires the appropriate structuring of angiogenic cells within a three-dimensional (3D) biomaterial. Endothelial cells (ECs) form the lining of blood vessels and, as such, are critical for the formation of a vascular network. They have also been shown to promote osteoblast differentiation (Saleh, Whyte and Genever, 2011; Honda and Aizawa, 2017). Endothelial cells can form luminised microvessels in vitro, however without the presence of a supporting cell type these vessels remain immature and lack long term stability (Koike et al., 2004). Multiple co-culture systems have been shown to produce stable microvessels networks in vitro such as fibroblasts (Chen et al., 2009), 10T1/2 mesenchymal precursor cells (Koike et al., 2004), and tri-cultures of myoblasts and embryonic fibroblasts (Levenberg et al., 2005). The inclusion of these perivascular-like cells supports the endothelial cell capillary plexus which is vital for the formation of a mature vascular network (Melero-Martin et al., 2008). In recent years, the emergence of novel biofabrication strategies such as 3D bioprinting has enabled the spatial patterning of vascular cells within hydrogels to form lumen-like structures in vitro (Barbaschi et al., 2015). Despite the promise of such strategies in the field of tissue engineering, it remains to be elucidated
whether bioprinted prevascularised constructs will enhance the regeneration of critical-sized bone defects.

The objective of this chapter was to 3D bioprint prevascularised biomaterials and to assess the capacity of these constructs to accelerate the regeneration of critical-sized rat femoral bone defects. To this end, human bone marrow stem cells (hBMSC) and human umbilical vein endothelial cells (HUVECs) were incorporated into a vasculogenic bioink and deposited into a 3D printed polycaprolactone (PCL) scaffold that was surface functionalised with nano-hydroxyapatite (nHA). The in vivo osteogenic and angiogenic potential of these novel prevascularised constructs was determined using a critical-size femoral defect model in immunosuppressed rats. De novo bone formation was investigated using micro computed tomography (µCT) to identify and quantify mineralisation. A commercially available silicone rubber compound was used to perfuse, cure and form a three-dimensional cast of the vasculature to evaluate vessel formation in the defect sites. Histological analysis was carried out to evaluate the quality of repair.

6.2 Materials and Methods

6.2.1 Isolation and Expansion of Human BMSCs

Human bone marrow derived stem cells (hBMSCs) were isolated from unprocessed human bone marrow (Lonza) on the basis of plastic adherence. Briefly, unprocessed bone marrow was plated at $2.5 \times 10^5$ cells/cm$^2$ (estimated approx. 4000-5000 hBMSCs/cm$^2$) in high glucose Dulbecco’s modified eagle’s medium (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under hypoxic conditions (37°C in a humidified atmosphere with 5 % pCO$_2$ and 5 % pO$_2$). Following colony formation, hBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic acid (EDTA) and tripotentiality was confirmed as previously described (Vinardell et al., 2009).
hBMSCs were seeded at 5000 cells/cm² expanded in DMEM supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF-2 (Prospect Bio) and expanded under normoxic conditions (37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂) and used at P4.

6.2.2 Endothelial Expansion

Human Umbilical Vein Endothelial cells (HUVECs; Lonza, Walkersville, MD) were cultured at 2500 cells/cm² in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza) at 37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂). HUVECs were used at P4.

6.2.3 Bioink Fabrication

To enable the use of fibrin as a printable bioink, a gelatin carrier method was adapted from Kang et al. (Kang et al., 2016). Briefly, hyaluronic acid (Sigma) was added to high glucose Dulbecco's Modified Eagle Medium (hgDMEM; Gibco, Biosciences) at a concentration of 3 mg/mL and stirred overnight at 37°C. 10 % (v/v) glycerol (Sigma) was then added and the solution was stirred for 1 h at room temperature. Gelatin type A (175 g bloom; Sigma) was added at a concentration of 40 mg/mL and stirred for 2 h at 37°C until fully dissolved. Before use, Fibrinogen (Sigma) was added to this thawed carrier gel at a concentration of 30 mg/mL and stirred for 2 h at 37°C. To enhance the angiogenicity of the bioink for use as PV bioink, D-erythro-sphingosine-1-phosphate (S1P; Avanti Polar lipids, AL, USA) was added at a concentration of 125 μM/mL. For both cast constructs and printed constructs this fibrin bioink formulation was used.

6.2.4 3D Bioprinting System

Scaffolds were designed using BioCAD™ software package and produced using the 3D Discovery multi-head bioprinting system (Regen Hu, Switzerland). Porous polycaprolactone (PCL; CAPA Ingevity, SC, USA) scaffolds were printed at 80°C at 0.5
MPa using a 27G needle (height = 5 mm and diameter = 4 mm). Scaffolds were coated in nano hydroxyapatite (nHA; See §6.2.5) and sterilised using ethylene oxide (ETO) gas prior to the addition of cell-laden bioinks. For the PV bioink, HUVECs and hBMSCs were incorporated into the fibrin bioink at $3 \times 10^6$ and $1.5 \times 10^6$ cells/mL respectively and were printed using a pneumatic driven syringe with a 25G needle at temperatures between 10-30°C under pressures of between 0.05-0.2 mPa. To assist the accuracy of the printing process, 4 % (w/v) agarose (Sigma) well inserts were fabricated using custom-designed moulds printed with Form2 SLA printer (FormLabs, MA, USA). These agarose moulds were soaked in thrombin (20 U/mL) prior to printing. After printing, all constructs were further immersed in a thrombin bath (20 U/mL) for 30 min at RT to allow the thrombin-catalysed polymerisation of fibrinogen to fibrin. The 3D Discovery was encased in a laminar flow hood to ensure sterility throughout the biofabrication process.

6.2.5 Nano Hydroxyapatite (nHA) Coating of PCL Scaffolds

PCL scaffold were coated according to a protocol previously published (Eichholz and Hoey, 2019). To coat PCL scaffolds with nano hydroxyapatite (nHA) needles approximately 100nm in length and 37nm in diameter, scaffolds were washed in 70% (v/v) ethanol for 15 min under vacuum (VWR). Scaffolds were then immersed in 2 M NaOH at 37°C for 45 min. Scaffolds were then washed in milliQ water before being immersed in 0.05 M calcium solution (Sigma). Equal parts 0.03 M phosphate solution was added dropwise, and scaffolds were incubated for 30 min at 37°C. this process was repeated twice more resulting in a total of three coatings. Scaffolds were then immersed in 0.5 M NaOH and incubated for 30 min before being washed in milliQ water and left to dry.

6.2.6 Scanning Electron Microscopy (SEM) of PCL Scaffolds

Samples were prepared for SEM by dehydrating samples in graded ethanol series 50 % (2 x 10 min), 70 % (2 x 10 min) 90 % (2 x 10 min), 100 % (2 x 15 min) and immersed in hexamethyldisilazane (HMDS; 2 x 30 min). Samples were allowed to dry for 2 h before being
mounted on SEM stubs and left overnight to ensure complete removal of solvent. Samples were then coated with gold/palladium for 60s at a current of 40 mA using a Cressington 208HR sputter coater. SEM imaging was conducted at 5 kV in a Zeiss ULTRA plus. Although SEM sample preparation was carried out by the author, the SEM imaging was not.

**6.2.7 Surgical Procedure**

Male Wistar Han rats were bred in the Comparative Medicine Unit of the Trinity Biomedical Sciences Institute (TBSI). For the rat segmental surgery, 12-week old rats were anesthetised using 2-4 % (v/v) isoflurane in balanced oxygen and administered pre-operative analgesia of buprenorphine (0.5 mg/kg). Surgical access to the femur was achieved via an anterolateral longitudinal skin incision and separation of the hind limb muscles, the vastus lateralis and biceps femoris. The femoral diaphysis was exposed by circumferential elevation of attached muscles and the periosteum removed. Before the creation of the defect, a weight-bearing polyetheretherketone (PEEK) internal fixation plate was secured to the anterolateral femur. Holes were created in the femur with a surgical drill using the plate as a template. Screws were then inserted into the drill holes in the femur to maintain the fixation plate in position. A 5 mm mid-diaphyseal defect was created using an oscillating surgical saw under constant irrigation with sterile saline solution. Scaffolds were press-fit into the defect. Soft tissue was accurately readapted with absorbable suture material. Closure of the skin wound was achieved using suture material and tissue glue. Animals received daily systemic administration of immunosuppression for 3 weeks post-operation (See §6.2.8). This animal procedure and study was approved by the ethics committee in Trinity College Dublin and the Health Products Regulatory Authority (HPRA) in Ireland.

One defect was created per animal, \( n = 9 \) for both groups at both timepoints. 18 rats received a prevascularised nHA coated PCL scaffold (PV Scaffold) and 18 rats received a nHA coated PCL control scaffold (Scaffold only). Two weeks post-operation, \( \mu \)CT angiography was performed (see §6.2.9). Live \( \mu \)CT analysis was performed on remaining
rats at 6- and 12-weeks post-operation (see. §6.2.10). At 12 weeks rats were sacrificed by CO₂ asphyxiation and the affected femur, with the PEEK plate fixator intact, was excised for further analysis. One rat from the Scaffold only group had to be excluded from analysis.

**6.2.8 Immunosuppression Regime**

To prepare the immunosuppressive drugs, 6.67 mg/mL FK506 (tacrolimus monohydrate; Sigma) was dissolved in 10 % (v/v) ethanol, 2 % (v/v) Tween 80 88 % (v/v) saline (all Sigma). 3.33 mg/mL SEW2871 (Axon MedChem, Netherlands) was dissolved in 27 % (v/v) ethanol 2 % (v/v) Tween 80 and 71 % (v/v) Saline. These two solutions were mixed in a 1:1 ratio to provide a solution containing 3.33 mg/mL FK506 and 1.66 mg/mL SEW2871. Each rat received a daily100 μL subcutaneous injection of this mixture during the period of immunosuppression.

**6.2.9 Vascular μCT Analysis**

Contrast enhanced angiography was performed at week 2 post-operative to assess vascularisation within the bone defects. First, the rat was sacrificed using CO₂ asphyxiation. Next, the vasculature was immediately perfused through the ascending aorta with sequential solutions of heparin (25 U/mL), formalin (10 %), PBS (All Sigma), and a radiopaque contrast agent Microfil (Flow Tech, Carver, MA, USA). Microfil was prepared freshly prior to each procedure using 28 % (v/v) pigmented compound 69 % (v/v) diluent 3 % (v/v) curing agent. All solutions were thoroughly mixed before perfusion and maintained at 37°C for the duration of the perfusion procedure. A straight 20G intravenous catheter was used to deliver the perfusion solutions into the rats left ventricle. The right atrium was cut, and a peristatic pump was used to deliver the heparin, formalin, and PBS solutions at a rate of 6 mL/min. The contrast agent was delivered at a rate of 3 mL/min. After perfusion, animals were left overnight at 4°C to allow the Microfil to cure. The limbs were excised and scanned using μCT (as described in §6.2.10) with both bone and contrast agent present. To facilitate visualisation and quantification of the vascular volume alone, the excised limbs were next
decalcified in EDTA (15 % (w/v), pH 7.4) for 2 weeks to remove the bone mineral content. µCT scans were performed after decalcification. By comparing the two scans (pre and post decalcification) it was possible to define the defect VOI by using the relative position of the fixation screws present in both scans. Next, 3D evaluations were carried out on the segmented images to determine vascular volume and to reconstruct a 3D image. The vascular volume in the defect was quantified by measuring the total quantity of mineral in the centre of the defect. The vessel thickness, degree of anisotropy, and connectivity were analysed using trabecular thickness analysis scripts provided by SCANCO. *n* = 6 was selected from each group for quantification.

6.2.10 Micro-computed Tomography (µCT)

Micro-computed tomography (µCT) scans for vessel analysis were performed using a Scanco Medical 40 µCT system (Scanco Medical, Bassersdorf, Switzerland). Samples were scanned at a voxel resolution of 10 μm, a voltage of 70 kVp, and a current of 113 μA. Reconstructed 3D images were generated using A Gaussian filter (sigma = 3, support = 5) was used to suppress noise site and a global threshold of 363.

*In vivo* µCT scans were performed on constructs using a Scanco Medical vivaCT 80 system (Scanco Medical, Bassersdorf, Switzerland). Rats (*n* = 9) were scanned at 2 weeks before angiography was performed. All other rats (*n* = 9) received scans at 6--weeks post-surgery to assess bone formation within the defect. Animals were anesthetised using 2-4 % (v/v) isoflurane in balanced oxygen throughout the scan. Next, a radiographic scan of the whole animal was used to isolate the rat femur. The animal’s femur was aligned parallel to the scanning field-of-view to simplify the bone volume assessments. Scans were performed using a voltage of 70 kVp, and a current of 114 μA. A Gaussian filter (sigma = 0.8, support = 1) was used to suppress noise and a global threshold of 210 corresponding to a density of 399.5 mg hydroxyapatite/cm³ was applied. A voxel resolution of 35 μm was used throughout. 3D evaluation was carried out on the segmented images to determine bone volume and
density and to reconstruct a 3D image. Bone volume and bone density in the defects was quantified by measuring the total quantity of mineral in the central 5 mm of the defect.

6.2.11 Colony-Forming Unit (CFU) Analysis of Individual Rat Bone Marrow

To assess differences in the number of colony-forming units (CFUs) present in the bone marrow of individual rats, the contralateral femurs were excised. Under sterile conditions, the bone ends were cut and the bone marrow was flushed out using an 18 Gauge needle and hGdMEM GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco, Biosciences, Dublin, Ireland) into a 50 mL falcon tube. The volume was made up to 20 mL with media and triturred to remove any marrow clumps. Samples were then centrifuged at 650G for 5 min. The supernatant was discarded, and the pellet was transferred to a fresh falcon tube. More Media was added, the pellet was triturated, and the sample centrifuged again at 650G for 5 min. Once again, the supernatant was discarded. The pellet was resuspended and passed through a 40µm nylon mesh. A cell count was performed using 3 % (v/v) acetic acid (to lyse red blood cells) and trypan blue exclusion. Cells were seeded at a density of 133,333 cells/cm² and cultured under normoxic conditions (37°C in a humidified atmosphere with 5 % pCO₂ and 20 % pO₂). After 10 days of culture media was removed and flasks were washed twice with 10 mL of PBS. Cells were then fixed with 2 % (w/v) paraformaldehyde (PFA) for 15 min at RT. The cells were washed again in PBS and incubated with 1 % (w/v) crystal violet (Sigma) for 2 min at RT. Crystal violet was removed and flasks were washed under running tap water for 10 min. Flasks were left to fully dry overnight before imaging. Stained colonies were then counted using ImageJ software.

6.2.12 Histological Analysis

The samples were fixed in 10 % formalin overnight and decalcified using ‘Decalcifying Solution-Lite’ (Sigma) for approximately 1 week. Samples were frequently X-rayed to determine if any mineral content remined. When no mineral was visible the sample was considered decalcified. Samples were then dehydrated in graded series of ethanol
solutions (70-100 %), cleared in xylene, and embedded in paraffin wax (all Sigma-Aldrich). Sections (5 μm) were rehydrated in graded series and stained with haematoxylin and eosin, 1 % (w/v) alcian blue 8GX in 0.1 M HCL to assess sulphated glycosaminoglycan (sGAG) content with a counter stain of 0.1 % (w/v) nuclear fast red to assess cellular distribution, 0.1 % (w/v) picrosirius red to assess collagen distribution, 0.2 % (w/v) Safranin O to assess sGAG content post-implantation and Goldner’s trichrome (Groat's iron haematoxylin, Fuchsine, Orange G, Fast Green) for visualising bone (all from Sigma). Slides were then imaged using an Aperio ScanScope slide scanner and evaluated for vessel infiltration by counting vessels visible across an entire section using Aperio ImageScope and ImageJ software. Bone formation was assessed using ImageJ software. It should be noted that PCL is cleared during the tissue processing and leaves empty spaces in constructs as a result.

6.2.13 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, CA, USA). To analyse significant differences between two groups at one timepoint, a standard two-tailed t-test was performed. To analyse variance between >2 groups at one timepoint, a one-way analyses of variance (ANOVA) was performed. To analyse variance between >2 groups at multiple timepoints, two-way ANOVA was used with Tukey post-hoc test. Numerical and graphical results are displayed as mean ± standard deviation unless otherwise stated. Significance was accepted at a level of p<0.05.

6.3 Results

6.3.1 3D Bioprinting of Prevascularised Constructs.

Porous PCL scaffolds were first designed and 3D printed using fused deposition modelling (FDM; Figure 6.1 A). Scaffold dimensions were selected to ensure an accurate press-fit during surgical implantation. The PCL scaffolds were then surface coated with nano hydroxyapatite (nHA). Scanning electron microscopy (SEM) imaging confirmed the
presence of the nHA coating, revealing a rod-like architecture. Each nano-needle was approximately 100nm in length and 37nm in diameter (Figure 6.1 B). For the prevascularised (PV) scaffold group, a vasculogenic fibrin-based bioink, containing HUVECs and hBMSCs at $3 \times 10^6$ and $1.5 \times 10^6$ cells/mL respectively, were deposited into the nHA coated PCL scaffolds using a ‘Z-printing’ technique (Daly et al., 2018). These scaffolds were cultured for 7 days in Endothelial Growth Medium (EGM-2) which had been supplemented with EGM-2 BulletKit® (Lonza) and further supplemented with recombinant VEGF (50 ng/mL; Gibco) to allow for the formation of a microvascular network within the scaffold (Figure 6.1 B.iv). These constructs were then implanted into critical-sized (5 mm) defects created in the femurs of Wistar Han rats (Figure 6.1 C, D). To enable the implantation of human cells, rats were administered daily subcutaneous injections of an immunosuppressing drug cocktail containing FK506 and SEW2871.
Figure 6.1 Study Schematic. (A) Porous PCL scaffold design. 2 PCL scaffolds were bioprinted; both were 4 mm in diameter and 5 mm high and coated with nHA. One scaffold was left empty whilst the other was filled with PV bioink. (B) SEM images depicting nHA coating and confocal image of microvessels within scaffolds; scale bar in images i = 100µm, scale bar in image ii = 2µm, scale bar in image iii = 200nm and scale bar in image iv = 50µm. (C) Images highlighting rat femoral defect location and x-ray and microCT images showing defect dimensions. (D) Schematic outlining surgical procedure.

6.3.2 CT Angiography to Assess Vascular Network Formation in vivo

2 weeks post-implantation, µCT angiography was used to quantify and visualise vascular network formation in the defects. The PV Scaffold group had significantly higher total vascular volume and vessel connectivity compared to the Scaffold alone (Figure 6.2 B&C). 3D reconstructions of µCT angiography scans revealed extensive vascular networks had formed within the PV scaffold group, with vessels diameters of up to 0.350 mm detected (Figure 6.2 A). Additionally, the vascular network which formed in the PV scaffold group
trended toward vessels forming in dense bunches at the periphery of the defect site. This may indicate inosculation of host vasculature with the implanted vasculature.

**Figure 6.2** \(\mu\)CT angiography following 2 weeks of in vivo implantation. (A) Representative 3D morphometric reconstructions of vessel diameters within the defects. (B) Quantification of total vessel volume and (C) degree of vessel connectivity for both groups. * denotes significance student’s t-test, \(p<0.05\) (\(n = 6\), Mean ± SD).

### 6.3.3 \(\mu\)CT and Histological Analysis of Bone Formation at 2 Weeks Post-Implantation

\(\mu\)CT analysis of animals at 2 weeks post-implantation was carried out to assess early bone formation (Figure 6.3 A). The PV scaffold supported significantly higher levels of new bone formation compared to the Scaffold only group at this early timepoint (Figure 6.3 A.iii).
H&E staining revealed high cell infiltration in both groups. Positive Safranin O staining indicated that neoosseus tissue was, at least in part, being formed through endochondral ossification. Goldner’s trichrome staining positively stained new bone predominantly at the periphery of the defect area in both groups.
Figure 6.3 μCT and histological analysis at 2 weeks post-implantation. (A) i Reconstructed in vivo μCT analysis of bone formation in the defects at post-operative week 2; scale bar = 1 mm. ii Schematic highlighting the region of interest (ROI) utilised to quantify bone volume, iii Quantification of bone volume (mm$^3$) of Scaffold only and PV Scaffold group at 2 weeks. * denotes significance one-way ANOVA with Tukey post-hoc test, p<0.05 (n = 9, Mean ± SD). (B) Haematoxylin and eosin staining (H&E), Safranin O staining and Goldner’s Trichome staining at 2-week post-implantation, scale bar = 1 mm for low resolution image; scale bar = 200µm for high resolution images.

6.3.4 In Vivo μCT Analysis of Bone Formation at 6- And 12-Weeks Post-Implantation

μCT analysis was performed on animals 6- and 12-weeks post-implantation to visualise and quantify bone formation within the defects. (Figure 6.4 A). There were no significant differences in total bone volume between the two groups at either timepoint, although there was a trend toward higher levels of bone volume in the PV Scaffold group compared to the Scaffold Only group (mean 11.45, SEM 2.65 versus mean 24.94 SEM 5.97).

Examining bone volume at an individual level, two animals from the PV scaffold group had notably higher bone volumes after 12 weeks than the other animals (Figure 6.4 D). Performing a colony forming unit (CFU) assay on bone marrow harvested from the contralateral femur of animals revealed that those animals displaying superior healing had higher numbers of CFUs than their poorer healing counterparts (Figure 6.4 E). In spite of this finding, there was no significant correlation between bone volume and CFU number, with a number of animals with very high numbers of CFUs undergoing low levels of bone regeneration. High variation between CFU number further highlights the substantial variation between animals.
Figure 6.4 (A) Reconstructed in vivo µCT analysis of bone formation in the defects at post-operative week 6 and 12; scale bar = 1 mm. (B) Schematic highlighting the region of interest (ROI) utilised to quantify bone volume. (C) Quantification of bone volume (mm$^3$) in Scaffold only and PV Scaffold group at 6 and 12 weeks; * denotes significance two-way ANOVA with Tukey post-hoc test, p<0.05 ($n = 9$, Mean ± SD. (D) Bone Volume (mm$^3$) for each animal at 6 weeks and 12 weeks post-implantation. (E) Correlation graph of CFU counts versus bone volume.

6.3.5 Histological Analysis of Bone Repair at 12 weeks Post-Implantation

Histological evaluation of the defect sites was carried out to evaluate the neotissue which had formed within the implanted scaffolds after 12 weeks (Figure 6.5). H&E staining after 12 weeks revealed bone formation within all groups. Interestingly, positive Safranin O
staining was found at the centre of the defect in the PV Scaffold group, indicating bone formation was still ongoing in these defects and was proceeding through an endochondral ossification pathway. Goldner’s trichome staining indicated a sharp interface between the defect, where the scaffold was implanted and surrounding bone tissue in the Scaffold only group. In the PV Scaffold group, there was substantial new bone formation and evidence of integration between the host bone and the neotissue that formed within the scaffold, with no apparent interface between the two. This may further indicate that bone formation was still in progress in the PV Scaffold group whereas regeneration had halted in the Scaffold only group resulting in the capping of bone ends.
Figure 6.5 Histological analysis at 12 weeks post-implantation. Haematoxylin and eosin staining (H&E), Safranin O staining and Goldner’s Trichome staining at 2-week post-implantation; scale bar = 1 mm for low resolution image; scale bar = 100µm for high resolution images.

6.4 Discussion

The overall goal of this study was to investigate whether using a 3D bioprinting technique to prevascularise a PCL based scaffold would enhance vascularisation and bone regeneration following the implantation into critical-sized defects created in the femurs of immunosuppressed rats. The implantation of these PV scaffolds led to significantly higher levels of vascularisation within the defect site compared to the scaffold only control. This increase in vascularisation correlated with significant increases in early bone formation in defects treated with the PV constructs. While no significant difference in bone formation was detected at later timepoints using micro-CT, histological analysis of the defect site 12 weeks post-implantation demonstrated an ongoing regenerative response in defects treated with the PV scaffolds. These findings demonstrate how bioprinting can be used to enhance vascularisation of a target defect site, and the therapeutic potential of such tissue engineering strategies for accelerating the regeneration of critical-sized bone defects.

µCT scans of the radiopaque 3D cast of the vasculature within the defect site revealed that the vessel architecture in the PV scaffold treated animals was denser than in animals with the scaffold only. This was particularly obvious at the interface between host bone and scaffold. This result may indicate inosculation between the implanted microvasculature and host vessels, whereby host vessels begin to fuse with the implanted microvasculature. However, this dense vasculature did not continue throughout the length of the scaffold. This may be due to a number of reasons. Firstly, it is possible the microvessel network was partially damaged during implantation, and/or parts of the network that were established in vitro regressed upon in vivo implantation. Another possible explanation for this finding relates to the limitations of the µCT angiography technique. In this study, few vessels below
a diameter of 170µm were detected using µCT analysis. The average diameter of the implanted microvessels is below 50 µm, which although perfusable may be below the resolution of the X-ray contrast agent selected. As such, further optimisation may be needed to modify the Microfil perfusion mass in order to increase its working time and/or decrease its viscosity by altering the ratio of curing agent included in order to fully image the complete vessel network architecture.

In recent years there has been considerable interest in prevascularising tissue engineering constructs using endothelial cells. Typically, studies focus on in vitro microvessel formation (Unger et al., 2007; Morin et al., 2013; Freiman et al., 2016) and the do novo formation of vessels in vivo (Koike et al., 2004; Melero-Martin et al., 2007, 2008; Au et al., 2008; Traktuev et al., 2009). Investigation into the in vivo function of engineered capillary networks has been primarily undertaken in subcutaneous implantation models. These have shown that in vitro prevascularisation can lead to a more rapid vascularisation in vivo (Chen et al., 2009). It has been proposed that this may be advantageous when combined with established bone tissue engineering strategies such as osteoinductive scaffolds (Barabaschi et al., 2015). However, limited studies have been carried out to investigate the effects of prevascularisation on in vivo bone formation. One study implanted preformed vascular networks into critical-sized calvarial defects in nude rats and showed that the prevascularisation led to no significant differences in bone formation by 4 weeks (Roux et al., 2018). In this chapter, the effect of prevascularisation on bone regeneration was studied over 12 weeks in a critical-sized femoral defect model in immunosuppressed rats. At the 2-week time-point, the PV scaffold promoted significantly higher levels of bone formation (as assessed by µCT analysis) compared to the scaffold only group. These differences were no longer statistically at the later timepoints, however mean levels of bone formation were still approximately twice that of scaffold only controls, with significant animal-to-animal variability observed. Although vascularisation of the defect site is a key component of tissue
regeneration and was significantly improved by the implantation of a prevascularised scaffold, successful bone healing relies on a number of factors, including the delivery of osteoinductive cues. Although the presence of nHA on the PCL scaffolds has been shown to enhance osteogenesis (Yaghoobi et al., 2016), perhaps the prolonged benefits of prevascularisation would be more evident when included in a more potent osteogenic tissue engineered scaffold. Other factors, such as migration of the scaffold with the defect site, may have also contributed to the observed animal-to-animal variability in bone regeneration and therefore reduced the power of the study.

This study chose to study bone formation in a critical-sized femoral defect. This type of defect is defined as one which will not heal during the lifetime of the animal without surgical intervention (Schemitsch, 2017). No bridging was seen in either group after 12 weeks, however, the presence of cartilage in a subset of the PV Scaffold group at 12 weeks may indicate that the healing process was continuing for those animals. Future studies should explore the use of extended timepoints to evaluate the long-term effect of implanting prevascularised scaffolds into critical-sized bone defects.

Athymic rats are frequently used in studies involving human cells to investigate their regenerative potential. Using immunosuppression on Wistar han rats bred in-house enabled the implantation of human cells in a more cost-effective and efficient manner. FK506 and SEW2871 are commonly used as immunosuppressive drugs and prevent tissue rejection through the inhibition of T-lymphocyte activation and T-lymphocyte recirculation respectively (Thomson, Bonham and Zeevi, 1995; Dong et al., 2014). Specifically, FK506 inhibits T-cell activation by binding to FK506 binding protein (FKBP) which inhibits calcineurin (Thomson, Bonham and Zeevi, 1995). Calcineurin dephosphorylates, and thereby activates, the nuclear factor of activated T-cells (NFAT) transcription factor. By inhibiting calcineurin, FK506 inhibits T-lymphocyte signal transduction. SEW2871 has been shown to reduce circulating leukocyte count by inhibiting the lymphocyte egress from the thymus.
The mechanism of action by which SEW2871 inhibits lymphocyte egress is not fully understood. SEW2871 is a sphingosine-1-phosphate receptor 1 (S1PR1) agonist (Marciniak et al., 2018). It is thought that S1PR1 agonists, such as SEW2871, cause rapid internalization of S1P on lymphocytes. This is hypothesized to then prevent lymphocytes from responding to endogenous S1P (Ley and Morris, 2005). It should be noted that S1PR1 is associated with a wide variety of physiological processes including vascular development. The application of SEW2871 for immunosuppressive purposes may have increased vessel development in the control group. The effect of prevascularisation may have been more evident if athymic nude rats were used in this study. The application of FK506 and SEW2871 has also been suggested to enhance bone healing. FK506 was found to stimulate osteoblastic differentiation in vitro (Tang et al., 2002). However, studies in male Lewis rats indicated that systemic application of FK506 has no biomechanical or histological effect on fracture healing (Voggenreiter et al., 2005). Furthermore, studies investigating the osteogenic properties of genetically modified xenografted muscle, reported similar levels of bone formation in athymic rats compared to rats treated with a systemic dual delivery of FK506 and SEW2871 (Liu et al., 2015).

6.5 Conclusion

In conclusion, this study demonstrates for the first time that 3D bioprinting can be used produce prevascularised scaffolds that enhance vascularisation of critical-sized bone defects. While applied here for bone regeneration, this technique could easily be adapted to prevascularise scaffolds used in the regeneration of any vascularised tissue or organ. It may be particularly useful for the delivery of cells that are particularly sensitive to diminished oxygen or nutrient availability, such as pancreatic islet cells, or for the regeneration of ischemic tissues.
Chapter 7

Discussion

7.1 Summary

The overall goal of this thesis was to develop a 3D bioprinting strategy for prevascularising scaffolds or engineered tissues. The utility of this approach was assessed by attempting to prevascularise in vitro different bone tissue engineering constructs and then assessing what impact this would have on vascularisation and bone formation in vivo following their implantation into the body. This firstly required the identification of an endothelial cell-compatible bioink which could support the formation of a primitive microvascular network in vitro. In Chapter 3, alginate, GelMA and a fibrin were compared for their printability and for their ability to facilitate the sprouting of HUVECs in vitro. Both alginate and fibrin had to be modified using a temporary gelatin support gel to enable accurate 3D bioprinting. When investigating their ability to support a microvascular network in vitro, alginate failed to facilitate endothelial sprouting. GelMA supported sprouting after an initial delay, but it failed to produce a consistent network. The fibrin based bioink consistently produced an interconnected microvascular network over 7 days and was therefore chosen for all future investigations.

When in vitro culture periods were extended over periods greater than 7 days, it was observed that microvessels began to regress within the printed constructs. This problem was overcome through the addition of hBMSCs into the HUVEC laden bioink, which stabilised the primitive network and enabled longer culturing periods. To investigate the effect that this co-culture had on vessel formation in vivo, and to elucidate whether preforming vessels prior
to implantation affected construct vascularisation, a subcutaneous implantation model was employed. Cellularised scaffolds containing either HUVECs alone, hBMSCs alone or a combination of the two were compared to constructs which were firstly cultured for 7 days in the presence of the angiogenic growth factor VEGF to produce prevascularised implants. It was found that prevascularised implants best supported the development of vascularised tissues in vivo, with a period of in vitro preculture required following the bioprinting of HUVECs and hBMSCs to establish functional blood vessels in vivo.

To investigate the versatility of the prevascularising bioink that was developed in Chapter 3, the next phase of this thesis sought to utilise this 3D bioprinting strategy to prevascularise several bone tissue engineering constructs. In Chapter 4, this vasculogenic bioink was shown to enhance in vivo vascularisation when incorporated into 3D bioprinted cartilage templates designed to support endochondral bone formation. Three different groups, a solid cartilage template, a template containing an empty inner channel and a cartilage template which had been prevascularised prior to implantation were implanted subcutaneously into nude mice. All groups underwent endochondral ossification in vivo and produced bone. The prevascularised group supported significantly higher levels of vascularisation in vivo than the other two groups. This highlighted how a prevascularising bioink could be spatially deposited into an engineered tissue as a method to enhance vascularisation of the construct following its implantation into the body. In Chapter 5, it was demonstrated that hypertrophic cartilage µTissues could be fabricated using a relatively high-throughput approach involving custom-made micro-moulds. These hypertrophic µTissues underwent endochondral ossification in vivo to form mineralised tissue. It was further shown that prevascularising these hypertrophic pellets prior to implantation resulted in significantly higher levels of mineralisation at 4 weeks. Finally, in Chapter 6 it was demonstrated that prevascularising 3D printed PCL scaffolds results in higher levels of vascularisation within large bone defects in rats, with correlated with increased levels of bone formation.
7.2 Prevascularisation and its Impact for Tissue Engineering

This thesis introduces a novel approach for prevascularising tissue engineered constructs which is amenable with modern biofabrication techniques. The future goal of tissue engineering is to produce whole organs which contain a functional vascular system. 3D bioprinting appears to have the potential to fulfil this goal. A technique which can improve vascularisation and that is compatible with such technology may prove a powerful asset for future tissue engineering applications.

Comparing this work with other studies in the literature, which have investigated prevascularisation, is difficult. Numerous cell sources, co-culture systems and biomaterials have been used. A key advantage that the technique proposed in this thesis has over other techniques in the literature is its compatibility with micro-extrusion based bioprinting technology. The majority of work investigating the use of cellular co-cultures to produce prevascularised tissues have focused on biomaterials which are traditionally unsuited to bioprinting technologies such as collagen (Koike et al., 2004; Deng et al., 2010; Duffy et al., 2011; Perng et al., 2011), Matrigel (Melero-Martin et al., 2008) and unmodified fibrin (Chen et al., 2009; Morin et al., 2013; Samal et al., 2015). This thesis proposes an approach that represents a way to integrate these methods with 3D biofabrication technologies. Huge strides forward are being made in the field using sophisticated bioprinting techniques to improve vascularisation which are described in detail in Chapter 2 of this thesis. Strategies involving creating channels and embedded interconnected networks within printed constructs are limited by the resolution of the biofabrication tools currently being used (Wu, DeConinck and Lewis, 2011; Bertassoni et al., 2014; Kolesky et al., 2014, 2016; Zhang et al., 2016). These strategies may further benefit from incorporating microvessels with their fabricated macro-vessels. Combining these two approaches may enable surgical anastomosis during
implantation and lead to a fully perfused tissue with a vascular network with dimensions comparable to those of a native vascular bed.

Throughout this thesis, the implantation of prevascularised constructs has resulted in significantly higher levels of vascularisation *in vivo*, particularly at earlier timepoints. This effect is often lost at later timepoints when spontaneous vascularisation, driven by the hypoxic state of the implant and by the innate wound healing response following implantation surgery, has had sufficient time to drive vascularisation of the implant site. This difference in vascularisations times could mean the difference between a tissue engineered implant succeeding or it failing. The first few weeks after implantation are crucial to the success of any tissue engineering strategy. If vascularisation does not occur in the early stages, larger and densely seeded constructs may undergo ischemic necrosis. This necrosis not only causes the failure of an implant, but it can also have downstream effects on other tissues in the body. A mouse femoral defect model study showed that the addition of endothelial progenitor cells (EPC) to polycaprolactone-hydroxyapatite (PCL-HA) scaffolds that were seeded with osteoblasts shortened the time course of vessel infiltration (Yu *et al.*, 2008). This faster neovascularisation protected the implant from this ischemic necrosis which was present in the control (Yu *et al.*, 2008). In a similar study, poor vessel infiltration led to both necrotic tissue and deficient bone formation within the scaffold leading to weakened mechanical strength. The addition of endothelial cells improved vascularisation which in turn prevented necrosis and led to enhanced bone formation with higher mechanical properties (Yu *et al.*, 2009). One paper, which published a failed study on large segmental bone defects in rabbits observed critical amounts of necrotic tissue within their implant (Kaempfen *et al.*, 2015). Upon further investigation it was found that there was a significant inverse correlation between the necrotic area and the density of blood vessels present (Kaempfen *et al.*, 2015). The bone tissue engineering strategies that are used in this thesis which focussed on directing endochondral ossification (Chapter 4 and 5), aimed to prevascularised a cartilage template.
This type of approach resists necrosis as the chondrocytes that reside in the cartilage template, and in the μTissues, are adapted to survive in hypoxic environments are therefore relatively resistant to ischemia.

### 7.3 Developmentally Inspired Approaches for Bone Tissue Engineering

In recent years, the field of bone tissue engineering has moved away from traditional strategies, which aim to produce bone tissue directly by mimicking the process of intramembranous ossification, towards an approach that aims to recapitulate aspects of the development process of endochondral ossification. This new approach, often called developmental engineering, aims to replicate developmental processes by regenerating bone using engineered cartilage templates that undergo endochondral ossification *in vivo*. Multiple studies have sought to replicate this process for bone tissue engineering, with positive results reported in small animal studies. These studies typically use bone marrow-derived MSCs to first engineer cartilage templates. These templates can undergo endochondral ossification *in vivo* (Huang *et al.*, 2006; Scotti *et al.*, 2010, 2013; Visser *et al.*, 2015; Thompson *et al.*, 2016) and have been shown to repair large bone defects in rodent models (van der Stok *et al.*, 2014; Cunniffe *et al.*, 2015; Bernhard *et al.*, 2017; Daly *et al.*, 2018). Vascularisation into the cartilage template is vital for endochondral ossification to occur. Studies have shown that in larger-scaled cartilage templates, vascularisation often fails to reach the core (Mesallati *et al.*, 2015; Sheehy, Mesallati, Kelly, *et al.*, 2015). The approach described in Chapter 4, may represent a possible strategy to overcome this limitation and improve vascularisation in larger-scaled developmentally inspired bone tissue engineering approaches.
7.4 Fabrication of μTissues- a Modular Approach to Bone

Tissue Engineering

3D cell spheroid models have proven to be a powerful tool over the last 20 years for cell biologists to study cell-cell interactions and mechanisms of development and disease (Johnstone et al., 1998; Korff and Augustin, 1998; Friedrich et al., 2009; Huang et al., 2012; Walser et al., 2013; Wenzel et al., 2014). In more recent years, tissue engineers have begun to recognise them as potential building blocks for fabricating larger tissues. This concept typically involves producing large numbers of tissue spheroids or micro-tissues (μTissues) \textit{in vitro} and later combining them so that they fuse together to form an engineered tissue suitable for implantation into the body. Aggregation is a fundamental event during embryonic development and can be mimicked in μTissues which drives cells towards self-assembly (Laschke and Menger, 2017) and increases their differentiation potential (Kapur et al., 2012; Yoon et al., 2012; S. Zhang et al., 2015). The use of μTissues to fabricate larger-scaled tissues requires large numbers of cell aggregations. There are numerous methods which have been described in the literature to produce multicellular spheroids. These include hanging drop (Yoon et al., 2012), spinner culture (Nyberg et al., 2005) and micro-moulds (Lopa et al., 2015). In Chapter 5 of this thesis, μTissues were fabricated using hBMSCs and a custom-made micro-moulding technique. hBMSC aggregates were cultured in chondrogenic and hypertrophic media to differentiate them into chondrocytes and stimulate them to secrete extracellular matrix proteins, thus forming hypertrophic cartilage μTissues. These μTissues subsequently mineralised \textit{in vivo}. The ability to bioprint these μTissues suggests that this approach may be successfully integrated with a large-scale organ printing strategy, which can be prevascularised using the strategy developed in Chapter 3.
7.5 Limitations and Future Directions

HUVECs were used in this work to study the efficacy of this approach but they are not a clinically viable EC source. For this approach to be translated into the clinic, an autologous EC source is required. A possible candidate, which has recently gained a lot of interest for use in vascularising tissue engineered tissues, is the endothelial progenitor cell (EPC). EPCs can be easily isolated from peripheral blood and have the potential to differentiate into all capillary niches (Peters, 2018). EPCs can form microvascular networks, both \textit{in vitro} and \textit{in vivo}, and have been shown to surpass vascular-derived ECs in forming microvascular networks (Fuchs, Hofmann and Kirkpatrick, 2007; Melero-Martin \textit{et al.}, 2007, 2008; Peters \textit{et al.}, 2016; Rouwkema and Khademhosseini, 2016).

Urine-derived stem cells (USCs) have also been proposed as a possible source for autologous ECs. UPCs can be easily isolated from urine using a non-invasive, low-cost procedure. UPCs originate from the parietal cells in kidney glomeruli (Bharadwaj \textit{et al.}, 2013) and can be differentiated into endothelial-like cells which express endothelial markers, undergo network formation and secrete angiogenic factors (Liu \textit{et al.}, 2018). UPCs can generate large numbers of cells from a single clone (Bodin \textit{et al.}, 2010) and EC-induced USCs have been shown to give rise to ECs \textit{in vivo} making them a potential cell source for vascular tissue engineering purposes (Liu \textit{et al.}, 2018).

Another potential cell source for autologous endothelial cells are induced pluripotent stem (iPS). iPS cells are somatic cells which have been genetically reprogrammed to revert back to a pluripotent stem cell state, which highly resembles that of embryonic stem cells (Takahashi and Yamanaka, 2006). One benefit for using this cell source with a prevascularisation technique is that you could differentiate these cells into both endothelial cells but also into associated mural cells. iPS-derived endothelial cells have been shown to successfully form capillary-like structures \textit{in vitro} (Choi \textit{et al.}, 2009).
The major hypothesised advantage of prevascularisation is that it can lead to the survival of large-scale tissue engineered constructs. Although rodent species remain the most widely used animal model for biomedical research, they are relatively small in size and because of this, the studies carried out in this thesis were restricted to smaller scaled engineered scaffolds. To test the limits of this type of vascularisation approach, larger constructs are needed. The fabrication of larger engineered tissues brings with it the need for larger animal models to test their performance in vivo. There are numerous large animal models used in the field to test tissue engineered constructs including caprine (goat), porcine (pig) and ovine (sheep) (Brehm et al., 2006; Lewis et al., 2009; Loai et al., 2010; Jeng, Hsu and Spector, 2013; Lansdowne et al., 2014; Ohno et al., 2017; Gürer et al., 2018; Yang et al., 2018). However, translating this research to a large animal model would involve the isolation of ECs and MSCs from the large animal species being investigated.

In this thesis, Polycaprolactone (PCL) was using in all studies as a reinforcing scaffold material. PCL has a high print fidelity and enabled the accurate 3D printing of the anatomically accurate femoral segment fabricated in Chapter 5. The PCL also helped define the boundaries of the fibrin scaffolds implanted subcutaneously in Chapters 3, 4 and 5 and aided the scaffold’s retrieval. However, the PCLs inclusion may have hindered nutrient diffusion and at times impeded vessel infiltration. Including PCL in the scaffolds designed for large bone defect regeneration, in Chapter 6, provided a surface that could be coated with nano hydroxyapatite to impart osteoinductivity and allowed for a consistency in the scaffold dimensions which are necessary for their press fit implantation in the defect sites. However, PCL has a relatively slow degradation rate, which depends on fibre diameter and the porosity of a PCL printed scaffold. The polymer can take between 2 and 3 years to degrade (Sun et al., 2006; Woodruff and Hutmacher, 2010). This may impede the growth of neotissue within the defect area. The ideal scaffold material for tissue engineering is one with a degradation rate that equals the rate at which new tissue is forming (O’Brien, 2011). To improve the
degradation rate of the scaffold designed in Chapter 6, another polymer which is compatible with fused deposition modelling, such as PLGA or polymer blends such as PCL/PLGA could be incorporated into the proposed scaffolds (Shim et al., 2012, 2017; B. Zhang et al., 2017).

### 7.6 Conclusions

- A fibrin-based bioink containing a co-culture of human umbilical vein endothelial cells (HUVECs) and human bone marrow-derived stem cells (hBMSC) can support the formation of a primitive microvessel network *in vitro*. This vasculogenic bioink has a broad range of potential applications in the field of 3D bioprinting.

- The establishment of a microvascular network within a 3D bioprinted tissue prior to implantation enhances its vascularisation *in vivo* and represent a powerful tool that could potentially be used to prevascularise any engineered tissue or organ prior to its transplantation into the body.

- A bioprinted cartilage template can undergo endochondral ossification *in vivo*. Prevascularising such a template prior to implantation can significantly enhance vascularisation of the engineered tissue.

- Hypertrophic µTissues can be fabricated in a relatively high-throughput manner *in vitro*. These µTissues can undergo endochondral ossification *in vivo*, producing mineralised bone tissue. Prevascularising these µTissue constructs accelerates the mineralisation of these µTissues *in vivo*.

- Prevascularisation of 3D bioprinted scaffolds leads to higher levels of vascularisation and accelerated bone regeneration in large bone defects created in the femur of rats.
• A novel, vasculogenic bioink, which can integrate with numerous biofabricated tissue engineered strategies, can prevascularise bioprinted constructs leading to higher levels of vascularisation *in vivo*.
Chapter 8

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